HONEY BEE BEHAVIOURAL EVOLUTION AND *ITPR* GENE STRUCTURE STUDIES

Thesis submitted by

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Rika Raffiudin

ABSTRACT

Honey bees (genus *Apis*) display a rich variety of fascinating traits, which can reveal considerable information about their evolution by means of analysis and investigation. Therefore, my main objective is to investigate some of these traits in order to determine food recruitment and nesting behaviour ancestral traits in *Apis*.

In my first experiment, I focused on foraging behaviour performed by the forager honey bee. Its ability to conduct food recruitment through elegant dance behaviour is facilitated by memory formation in the brain. In the first experimental study described in Chapter 2, I conducted an *itpr* gene characterisation, a highly expressed gene present in honey bee brain (Kamikouchi et al. 1998). A partial sequence of the *A. mellifera itpr* gene was obtained which comprised of 2, 091 bp and showed 62%, 60%, 33%, 56%, and 56% similarities respectively to those in *Panulurius argus* (lobster), *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis*, and *Mus musculus itpr*-1. A phylogenetic analysis using *itpr* demonstrated that *D. melanogaster itpr* is closest to that of *A. mellifera itpr*. It has two introns showing the same positions as those of *D. melanogaster itpr* introns (Sinha and Hasan 1999) and there are four *Apis itpr* introns which appear absent in *D. melanogaster*. In my research, I investigated several conserved putative sites in *A. mellifera* IP_3R protein namely protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites. These particular phosphorylation sites are considered to be important in honey bee memory formation (Menzel 2001).

Certain parts of *itpr* characterised in Chapter 2 were used as molecular markers for honey bee molecular phylogenetic reconstruction, concatenated with *COII* and *lsRNA* genes.

In further analysis based on the more complex model of DNA evolution, another hypothesis of *Apis* evolution was revealed. According to this model, the monophyletic *Apis* genus is split into two lines; those are the *A. mellifera* group line and the *A. dorsata* and *A.*

florea groups line. Another outcome based on *COII* molecular phylogenetics combined with previous data, indicated the ambiguity detected in *A. koschevnikovi* by Willis et al. (1992) resulted from a possible error.

A. florea and *A. andreniformis* were confirmed as basal species in *Apis* phylogeny followed by the more derived species: *A. dorsata*, *A. laboriosa*, *A. d. binghamii*, *A. mellifera*, *A. koschevnikovi*, *A. cerana*, *A. nuluensis*, and *A. nigrocincta*. My findings suggest that *A. nuluensis* and *A. nigrocincta* are the most derived species and that they have recently speciated from *A. cerana*.

By mapping dance behaviour characteristics onto the weighted Maximum Likelihood (ML) consensus tree, an interesting result was produced. It was found that unordered trait analysis did not answer the question whether horizontal dancing or vertical dancing was the ancestral trait because species with vertical dance behaviour are monophyletic and so are those with horizontal dance behaviour. However, given that horizontal dancing behaviour is less complex, an ordered dance character state seems justified. Based on these considerations, horizontal dance behaviour seems most likely to be ancestral. Another possibility of dance behaviour evolution hypothesis revealed by applying another DNA evolution model in ML analysis, mentioned that the vertical dancer honey bees (*A. mellifera* and *A. dorsata* group) are not monophyletic; they are clustered in different clade. The latter are in the same clade with the other horizontal dancers, *A. florea* group. Hence, it turns another possibility that vertical dance could be the ancestral to that of horizontal dance.

Inferring honey bee nest behaviour by mapping onto the molecular phylogenetics tree led me to the conclusion that there were two alternative evolutionary histories accounting well for this behaviour. One scenario has the ancestral state for *Apis* being open nesting with cavity nesting being a derived state. In the other, equally parsimonious scenario, cavity nesting in *Apis* is ancestral and apparent derived directly from cavity nesting in the

Bombini and Meliponini, and open-nesting has been evolved twice (or once) in the *A. florea* and *A. dorsata* groups.

Only open nesters that construct platform at the top of their nests perform horizontal dancing. Other open nesters such as the *A. dorsata* group do not construct such platform and so it is possible that their dance behaviour has evolved into a vertical dance. Similar features namely no platform and vertical dance behaviour exists in the *A. mellifera* group. Hence, there is an adaptation of dance behaviour to the nest structure, which shows a correlation between these two behavioural character traits.

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DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

March 27, 2002

Rika Raffiudin

Chapter 1. General Introduction: Honey bee biology and neural system

INTRODUCTION

E.O. Wilson wrote in his review paper for Karl von Frisch book 'The Dance Language and Orientation of Bees' that "The life of bees is like a magic well, the more you draw from it, the more there is to draw" (Wilson 1968).

This chapter provides some essential background to honey bee biology. In particular, it will cover honey bee current taxonomy, biogeography and the fossil record. This general information is important to introduce specific areas of interest in my project, which are nesting and dance behaviour in honey bees, and how this behaviour relates to gene expressed in the brain, specifically the inositol-1,4,5 trisphosphate receptor (*itpr*) gene.

It is the aim of my study to explore the behavioural evolution of this social insect, in terms of dance and nesting behaviour. Descriptions of these two behavioural traits will follow the second section of this introductory chapter. Behavioural evolutionary traits will be discussed by using molecular phylogenetic approach in Chapter 3.

Honey bee dance communication can be described as special interesting behaviour, due to the elaborate way of advertising a food source to the nestmates in the colony. Honey bee brain facilitates their ability to perform such advanced communication. Neurophysiological studies have referred certain parts of the brain, which shape learning and memory ability in this social insect. In an attempt to trace the molecular basis of dance behaviour, this topic will be discussed towards the end of this chapter which will conclude with a reference to particular gene members expressed in the honey bee brain.

Honey bee biology

Honey bees live in a colony containing thousands of individuals. As an eusocial insect the drone (male) and the queen are the reproductive members in the colony, and there

are overlapping generations of parent and offspring. Brood provisioning is occurring with live overlap times between parent and offspring generations (Wilson 1971). Only a single queen can exist in the colony (monogyny) (Winston 1987), but she can mate with more than ten drones (polyandry) (Palmer and Oldroyd 2001). In contrast, worker honey bees are non-reproductive caste members, although they also have limited reproductive capabilities (Ratnieks 1995; Oldroyd and Ratnieks 2000; Halling et al. 2001). Honey bees have male haploid mechanism of sex determination where fertilised eggs produce a female (queen or worker) while unfertilized eggs produce a male (drone) (Wilson 1971; Winston 1987).

Honey bee colonies also exhibit highly developed division of labour. Different tasks within the colony are carried out by different worker castes. The tasks performed are primarily age-related. In the first three weeks after pupation, workers spend their time inside the nest where tasks involve cleaning, brood rearing, constructing the nest, and guarding (Lindauer 1961). Following this, in the final stages of her life time (3-6 weeks), a worker perform outside foraging tasks (Seeley 1991).

Honey bee taxonomy

Honey bees (genus *Apis*) belong to the order Hymenoptera, suborder Apocrita, infraorder Acuelata, superfamily Apoidea, family Apidae, subfamily Apinae, tribe Apini. The Apidae are characterised by long tongues and recent classifications describe this family as comprising the three subfamilies Xylocopinae, Nomadinae and Apinae (which contain the honey bee). Within Apinae are nineteen tribes, which are classified as either corbiculate (including the honey bees) or non-corbiculate bees (Michener 2000). Corbiculate group bees has a “corbicula” or pollen basket (Michener et al. 1978) in the worker’s hind leg. Corbiculate bees include the tribes Euglossini, Bombini, Meliponini and Apini (which include *Apis*) (Michener 2000).

Genus Apis

The genus *Apis* is a small group of bees, having an apiform (apine-like) body type. Worker body size ranges from small to large (7-19 mm) with hairy-erected eyes. The hind tibia lack spurs and the hind wing has both jugal and vannal lobes, with a shallow

incision. The male genitalia are reduced in *Apis*, but the endophallus is highly developed (Michener 2000).

Traditionally, the *Apis* genus was known consisted of four species, those are *A. mellifera*, *A. cerana*, *A. dorsata* and *A. florea* (Koeniger 1976). Current studies mentioned that there are five more *Apis* species, namely, *A. andreniformis* (Wu and Kuang 1987), *A. koschevnikovi* (Tingek et al. 1988), *A. laboriosa* (Sakagami and Matsumura 1980; Underwood 1990b), *A. nigrocincta* (Hadisoesilo et al. 1995) and *A. nuluensis* (Tingek et al. 1996).

Honey bee species are clustered into three species groups based on body and wing size. *A. florea* species group consists of dwarf honey bees with worker forewing length of 5-7 mm. The drone has an inner lobe process at the hind tibia (Maa 1953). Medium sized honey bees are clustered in the *A. mellifera* group, with a forewing length of 7-10 mm. The giant honey bees are classified in the *A. dorsata* group with a forewing length of 12-15 mm. The two latter drone honey bee groups lack of inner lobe in the hind leg (Ruttner 1975). The following is a description of each species.

A. florea Fabricius

This species is a dwarf honey bee, 7-10 mm for workers body length. The queen is almost twice as long as the workers (13-15 mm) and drone is 11-13 mm long (Wu and Kuang 1987). Two distinctive worker characteristics are white basitarsal hairs and bright red-brown colours on the first and second abdominal tergites. The cubital index (that is length ratio of two cubital veins) is 2.78 (Wongsiri et al. 1990) - 2.864 (Rinderer et al. 1995). Worker head width is 2.60 $\pm$ 0.03 mm and wing length is 6.26 $\pm$ 0.10 mm (Seeley 1982). Drones have distinct characteristics of a long inner lobe on the hind tibia (Maa 1953; Wu and Kuang 1987) and an endophallus which is thin and curved at the terminal end and a double coiled of the bursal cornua (Wongsiri et al. 1990). *A. florea* drone mating flight is from 14.00 h - 16.45 h (Rinderer et al. 1993).

A. andreniformis Smith

A. andreniformis is another dwarf honey bee (Wu and Kuang 1987). Worker body length is 8-9 mm. Cubital index of the front wing is 6.282-6.378 which is much longer than that of *A. florea* (Rinderer et al. 1995; de Guzman et al. 1992). Drones have an inner lobe on the hind tibia, which is half the size of *A. florea*. Workers have black hairs on the hindleg and the chestnut-brown color of the first and second abdominal tergites. Drone endophallus has a thick and straight terminal end (Wongsiri et al. 1990). Drone mating flight takes place from 12.15 h - 13.45 h (Rinderer et al. 1993).

A. mellifera Linneaus

A. mellifera is a medium size honey bee, with a forewing length of 7.64-9.70 mm and a forewing cubital index of 2.30. Mean value of hind wing hook (hamuli) is 21.30. Workers head width is 3.77 $\pm$ 0.04 mm (Seeley 1982). Drones have an endophallus with one pair of cornua bulbs and chitinuous plates are present. The upper cornua of the drone endophallus are rudimentary and *A. mellifera* drone cells cap lack of pores (Ruttner 1988).

A. cerana Fabricius

A. cerana, another medium size honey bee, has specific brownish yellow mouthparts, scutellum, coxae II and trochanter II-III. The wing is almost clear hyaline (Maa 1953) while the femur II and tibia II are black. Workers head width is 3.38 $\pm$ 0.06 mm and wing length is 7.54 $\pm$ 0.14 mm (Tingek et al. 1996). Cubital index measurement of the forewing is 4.40 (Seeley 1982). *A. cerana* endophallus lacks of the chitinuous plate, which occurs in the *A. mellifera* endophallus. It has three pairs of upper cornua and perforated drone cap cells (Ruttner 1988).

A. nigrocincta Smith

A. nigrocincta was described by Smith (1861), based on the A.R. Wallace collection. Workers are characterised by a pale or rusty color of the antennae scape, clypeus, labrum, mandibles, scutellum, legs and abdomen (Smith 1861; Hadisoesilo et al. 1995) with reddish-tan hair over most of the body. Forewings are slightly darker hyaline (Maa, 1953). Morphometric results show significant differences between sympatric *A.*

cerana in hind femur, tibia, metatarsus length, tergite III and IV and forewing cubital index which is 3.84 ± 0.228 (Hadisoesilo et al. 1995). Reproductive isolation between sympatric *A. cerana* and *A. nigrocincta* has been confirmed based on drone mating flight (12.30 h – 14.30 h in *A. nigrocincta* and 15.20 h - 17.30 h in *A. cerana*) and on differences in male genitalia (Hadisoesilo and Otis 1996). *A. nigrocincta* drone cell cap composed of thin wax without pore, unlike that of *A. cerana* drone cap cell which is hard and porous (Hadisoesilo and Otis 1998).

A. nuluensis Tingek, Koeniger, Koeniger

A. nuluensis is a montane medium sized honey bee characterised by light-brown femur II and femur III. There are four whitish tomenta on the tergite and long abdominal hairs on workers (0.23 ± 0.016 mm). Drone pigmentation is entirely black on the thorax and abdomens (Tingek et al. 1996). Morphometric analysis reveals that *A. nuluensis* is similar in body size measurements to *A. nigrocincta* but wing venation measurements are closer to those of *A. cerana* (Fuchs et al. 1996). Drone mating flight is at 10.44 h-13.12 h (Koeniger et al. 1996), which clearly distinguishes from *A. cerana* (16.15 h-17.15 h) (Koeniger and Wijayagunasekera 1976).

A. koschevnikovi Enderlein

A. koschevnikovi workers have their distinctive yellow-orange colour in most body parts while the drone is mostly brown (Woyke 1997). Workers are medium sized (body length approximately 10-11 mm), forewing length of 8.46 ± 0.11 mm, with cubital index 7.64 ± 1.40 (Tingek et al. 1996). Overall, it is larger than *A. cerana* (Ruttner et al. 1989). *A. koschevnikovi* drone mating flight is at 16.15 h - 18.15 h, which clearly separate it from sympatric *A. cerana* at 13.45 h - 15.30 h (Koeniger et al. 1988).

A. dorsata Fabricius

A. dorsata has a rusty brown pubescence and dark tinge on the workers wings (Ruttner 1988). Workers have a bicolor integument - yellow and black on the abdomen tergites, and a raised ocular (Sakagami and Matsumura 1980). Workers head width is 4.71 ± 0.09 mm, wing length is 12.34 ± 0.34 mm (Seeley 1982). Drone endophallus is unique in form having three long, thin bursal cornua that are bent ventrally (Ruttner 1988).

Drone flight mating time is 18.00 h - 18.45 h (Koeniger and Wijayagunasekera 1976; Rinderer et al. 1993).

A. laboriosa Smith

This giant honey bee is restricted to high elevations (Maa 1953). However, several studies have classified *A. laboriosa* as a subspecies of *A. dorsata* (Koeniger 1976; Engel 1999). Several morphometric characteristics significantly distinguish it from sympatric *A. dorsata*. Different drone mating flights distinguish *A. laboriosa* from *A. dorsata* being 12.20 h - 14.20 h (Underwood, 1990b) and 18.00 h - 18.45 h (Koeniger and Wijayagunasekera 1976), respectively. Overall body size is larger in *A. laboriosa* than *A. dorsata*, in forewing (length and width), head width and malar ratio. Abdomen coloration integument is uniformly black in *A. laboriosa* in contrast to the yellow and black bicolor of *A. dorsata*. The other striking characteristic is the ocellar area, which is flat in *A. laboriosa* and distinctly raised in *A. dorsata* (Maa 1953; Sakagami and Matsumura 1980). However, there are no apparent endophallus characteristics distinctions between *A. laboriosa* and *A. dorsata* (McEvoy and Underwood 1988).

Honey bee distribution

The genus *Apis* is native to the Old World: Asia, Europe and all of Africa. *A. cerana* and *A. mellifera* are the most widely distributed of all honeybees. *A. cerana* mostly occupies the eastern world while *A. mellifera* spread throughout Europe and Africa, before being introduced as an agricultural commodity to the New World (Ruttner 1988). General descriptions of *Apis* distribution are provided in the following paragraphs.

A. florea is restricted to the South East (SE) Asian mainland from Thailand, Vietnam and India, but it has recently reported dispersed further west to Iraq and Oman (Otis 1996). While *A. florea* is usually found in lowland areas below 500 m (Wu and Kuang 1987), it has also been found in high elevations such as 1600 m in Thailand (Wongsiri et al. 1997) and up to 1900 m in Iran (Ruttner et al. 1995).

A. andreniformis is distributed throughout Indochina and Sundaland. On the mainland it is distributed throughout Thailand and the Malay Peninsula (Otis 1996). *A.*

andreniformis is also found in the SE Asian archipelago: Sumatra (Salmah et al. 1990), Borneo (Raffiudin, R personal observation; Koeniger et al. 2000) and Palawan Island (de Guzman et al. 1992; Otis 1996). *A. andreniformis* occurs in both lowland areas 0 - 500 m elevation (Salmah et al. 1990) and in highland areas up to 1600 m in Thailand (Wongsiri et al. 1997).

A. mellifera is the most widely distributed honey bee species, and is found in desert and cold temperate regions. However, this species is not native to most Asian regions. Based on morphometric analysis, *A. mellifera* subspecies is classified into three lineages, those are the A, M, and C lineage (Ruttner et al. 1978). Further, the C lineage is differentiated into two lineage, C and O lineage (Ruttner 1988); Arias and Sheppard 1996; Franck et al. 2000). South and Central African subspecies covered by the A lineage *A. mellifera* subspecies: *A. m. lamarckii, A. m. litorea, A. m. scutellata, A. m. yemenitica, A. m. monticola, A. m. adansonii, A. m. unicolor,* and *A. m. capansis* (Ruttner 1988). The M lineage covers the North European subspecies (*A. m. mellifera*), Spain and Portugal (*A. m. iberica*) and the North African (*A. m. saharensis*, *A. m. intermissa*). The C lineage includes subspecies from Italy (*A. m. ligustica*), Austria and Yugoslavia (*A. m. carnica*), Northern Greece and Bulgaria (*A. m. macedonica*), Southern Greece (*A. m. cecropia*) and Sicily (*A. m. sicula*). The O lineage includes subspecies from the Caucasus Mountain (*A. m. caucasica*), Armenia (*A. m. armenica*), Turkey (*A. m. anatoliaca*), Syria (*A. m. syriaca*), Cyprus (*A. m. cypria*) and Crete (*A. m. adami*) and *A. m. meda* (Ruttner 1988).

Molecular studies on *A. mellifera* subspecies variations have been done in the last ten years. Overall, morphometric and molecular approaches concur in supporting the subspecies lineages of *A. mellifera* (Smith 1991; Garnery et al. 1992), except for several subspecies, such as the North African subspecies (Franck et al. 2001). This subspecies is grouped in the O lineage based on mitochondrial data (Franck et al. 2001), but in the M lineage on the basis of morphometrics (Ruttner 1988). Moreover, in Ruttner's O morphometric lineage, only *A. m. syriaca* has the O mitochondrial haplotype (Franck et al. 2000). Another difference between the two kinds of data is in the classification of *A. m. anatolica*, the Turkish subspecies. It is grouped in the O morphometric lineage

(Ruttner 1988), whereas it has the C mitochondrial haplotype (Smith et al. 1997). Further, the A mitochondrial haplotype is also detected in the M morphometric lineage *A. m. iberica*, and in the C morphometric lineage *A. m. sicula*.

A. cerana is another honey bee species, which occupies a vast area, ranging from West Afghanistan to the Philippines. This species also has several subspecies. Western and Northeast Asian countries (Afghanistan, Pakistan, Kashmir, China, and Korea) are occupied by *A. c. cerana* (Ruttner 1988). *A. cerana* also exists in the Himalayan region (*A. cerana himalayana*). In the South and SE Asia, *A. c. indica* (Maa 1953) is the main subspecies which covers mainland India, Srilanka, Myanmar, Thailand, Malaysia, Sumatra, Java, Borneo, Lombok, Bali, Flores and most of Sulawesi, Timor, and Sabah (Damus and Otis 1997). The Philippine archipelago exhibits another subspecies namely *A. c. philippina* (Maa 1953) and Japan has *A. c. japonica*, except for Hokkaido Island (Hepburn et al. 2001).

A. nuluensis is a montane species found at elevations 1 524 - 3 400 m in two mountainous regions of Sabah, namely Mount Kinibalu and the Crocker Range (Gunung Emas) (Tingek et al. 1996).

A. koschevnikovi that is sympatric with *A. cerana*, is distributed in several regions Sumatra, the Malay Peninsula, Borneo and Java. Sadly, recent observations suggest that *A. koschevnikovi* is now rarely seen, except in Borneo, due to the destruction of forest habitat (Otis 1991). This species is usually found mostly at sea level, but has also been discovered at elevations up to 1000 m (Otis 1996).

A. nigrocincta is endemic to Sulawesi and adjacent islands (Selayar, Buton) and Sangihe (Damus and Otis 1997). In Sulawesi, this species exists sympatrically with *A. cerana*. *A. nigrocincta* tend to nest in forest areas, whereas *A. cerana* is predominant in urban area (Otis 1996).

A. dorsata has a large distribution area in Asia and is found as far west as India. It is distributed throughout Vietnam and the entire Asian mainland (Thailand, the Malay

Peninsula), and the South East Asian islands (Sumatra, Borneo, Java, Timor, Sulawesi, and the Philipinnes). Two subspecies of *A. dorsata* occur in Sulawesi namely *A. d. binghamii* and in the Philipinnes namely *A. d. breviligula* (Maa 1953). *A. dorsata* is also found in lowland areas and up to 1 300 m elevation (Himalayan region) (Sakagami and Matsumura 1980).

A. laboriosa is another montane honey bee which is restricted to elevations of 1200 – 4000 m, in the Himalayan region and distributed throughout India, Nepal, Bhutan, China including Tibet and Yunnan (Sakagami and Matsumura 1980; Roubik et al. 1985; Underwood 1990a) and Vietnam (Trung et al. 1996).

Bee fossil records

The corbiculate tribe Apinae originated in the late Cretaceous period. The oldest bee fossil is *Trigona* (Tribe Meliponini) in New Jersey amber 96-74 million years ago (Michener and Grimaldi 1988). Another Meliponini fossil was found in Miocene Dominican amber (Michener 1982), and two Meliponini fossils (*Kelneriapis eocenica* [Kelner-Pillault] and *Liotrigonopsis rozeni* Engel) from Baltic amber (Engel 2001a). The true Bombini fossil has not been found in amber as yet, although many fossils from Tribe Bombini (bumble bee) exist (Engel 2001a). Two species of Euglosini fossil have been found in Miocene amber from the Dominican Republic (Engel 1999). The fossil Electrapini (genus *Electrapis*) from Eocene Baltic amber is of special interest due to its close to resemblance to Bombini and Apini. It resembles Bombini by having the hind tibial spur and is related to Apini because of its jugal lobe in the hind wing (Cockerell 1908).

Honey bee fossils have been found in Baltic Amber (Zeuner and Manning 1976) and in China (Zhang 1990) all of which are from Tertiary strata. Recent compilation of *Apis* fossil data has cited seven accepted species of *Apis* fossils (Engel 1999), four of which are from the upper Miocene period namely *A. ambrusteri* (Germany) (Zeuner and Manning 1976), *A. longtibia* (China), *A. miocenica* (China) and *A. petrefacta* (Bohemia) (Engel 1998). Three of the fossils are from the Oligocene period namely *A. vetustus* (Germany), *A. henshawi* (Europe), and *A. cuenoti* (France) (Cockerell 1907; Engel 2001a).

Examination of wing venation in the three fossils in the genus *Electrapis*, fossil of *A. armbursteri*, fossil of *A. henshawi* and the extant *A. mellifera* have revealed no significant differences among them (Ruttner 1988; Engel 1998). Honey bee fossil wings have the same venations that exist in *A. mellifera*. The only differences are due to the venation wing lengths that affect wing cell size. The *A. armbursteri* fossil resembles the current *A. mellifera* in size and in the wax mirror on the abdomen (Zeuner and Manning 1976).

It is widely accepted that eusocial insects dominate terrestrial ecosystems, and this success has been attributes to their eusociality (Wilson, 1990). Engel (2001b), however, notes that numerous probably eusocial bee genera seen in fossil assemblages have died out leaving a depauperate bee fauna today, and questions the importance of eusociality in achieving ecological dominance. However, as acknowledged by Engel (2001b), the loss of diversity has probably resulted from competitive pressure by the most successful lineages, which in the case of bees is the genus *Apis*.

Honey bee behaviour: nest structure and dance behaviour

Eusociality developed in three tribes of corbiculate bees; Bombini, Meliponini and Apini (Michener 1974). Among them, Apini appears to exhibit the most advanced behaviour shown by their ability to communicate (Lindauer 1961). The colony members work as a unit with highly integrated and decentralised decision-making (Seeley 1998).

Honey bees use a variety of signals to communicate. These signals can be classified as modulators and specific signals. Hölldobler (1984, p. 366) mentions that in a modulatory communication system "…in it, signals do not release specific behaviour patterns but rather modulate the probability of reactions to other stimuli by influencing the motivational state of the receiver". Modulatory signals are transmitted by honey bees by means of tremble dances, and shaking signals (Schneider et al. 1986; Seeley 1992; Anderson and Ratnieks 1999). The most sophisticated and complex signals are conveyed by specialised ritualised signals such as the waggle dance (Lindauer and Kerr 1960; Hölldobler 1984).

Honey bee dance communication

Karl von Frisch and his group pioneered the research into honey bee dance communication (von Frisch 1967). When a forager returns from a food source, she unloads the nectar to the young receiver honey bees (Hart and Ratnieks 2001). Subsequently, she communicates the food source position (Seeley 1994).

Three types of dance communication for food source distance recruitment are displayed according to the food distance (von Frisch 1967). The round dance conveys an adjacent food source by a series of round runs (or round dances). Intermediate food distance information is conveyed by sickle dance. For long distances, a waggle dance is performed where the forager performs a series of wagging runs (Tautz and Rohrseitz 1996) followed by loop walking and repeating the waggle run. The waggle run appears to be the most important component in advertising the distance of the food source (Michelsen et al. 1992). Dance attenders extract considerable information from this behaviour, include the distance and the direction of the food source from the nest, together with the richness of the food (Lindauer 1961; Judd 1995). The distance of the food source is indicated by the duration of the waggle dance while the food source direction is shown by the angle of the waggle dance based on the position of the sun as the compass (Gould 1980). The richer the food sources the more lively the dance (Lindauer, 1961; Seeley et al. 2000).

Among honey bee species, dance behaviour is performed in two distinct orientations according to nest construction. The first type is the vertical dance which is performed by *A. mellifera*, *A. cerana*, *A. dorsata* (Koeniger and Koeniger 1980; Dyer 1985b; Punchihewa et al. 1985), *A. laboriosa* (Kirchner et al. 1996) and *A. nigrocincta* (Otis personal communication). The second type is the horizontal dance which is performed by *A. florea* (Koeniger et al. 1982; Dyer 1985a) and *A. andreniformis* (Rinderer et al. 1992).

In the vertical dance, the direction of the food source is indicated with respect to the gravity direction on the comb (Edrich 1977). The vertical dancer waggles upward if the food source is towards the sun and downwards if the food source is in the opposite

direction to the sun (von Frisch 1967). In contrast, the horizontal dance is performed by the forager on the flat platform at the top of the comb (Dyer 1985a). Here the waggle run points directly to the food source. Both vertical and horizontal dances use the sun as a compass (Lindauer 1956; Gould 1980). The horizontal dancers cannot compensate for gravity; they are only able dance on a horizontal and slope nest (Dyer 1991).

The evolution of these sophisticated behaviour traits has been studied for some time. According to Lindauer (1961), the horizontal dance is the ancestral trait. Others suggest that the vertical dance is the ancestral characteristic (Koeniger 1976). In Chapter 3, I will focus on the polarity of this such behaviour, by mapping and reconstructing dance characteristics on the phylogenetic tree.

Nest sites

Nest structure is an example of another sophisticated architectural behaviour performed by honey bees. The nests comprise of compact hexagonal cells attached to a thin sheet of wax as a living area, which are use to rear the brood and for food storage (Michener 1974). Unlike other corbiculate bees, honey bee brood and food storage cells are mostly uniform in shape and size, except in *A. dorsata*, differ in their cell depth (Thakar, 1961). Male's cells are slightly larger because of their greater body size. The queen or the gyne cell formed a tube, large cell (Winston 1987). Brood cells are located in the middle part of the comb while pollen and nectar surround at the outer layers of the brood area (Camazine 1991). Reproductive cells (drone and queen cells) are located at the lower part of the comb.

Several differences in honey bee nest: size and comb numbers are apparent in each nest. The open-air nests consist of single comb but there is great size variation (Sakagami and Matsumura 1980). The giant bees nest can extend up to one metre in length whereas the dwarf honey bee nest covers only 20-30 cm (Rinderer et al. 1996).

Honey bee nests are found in two niches namely open air and cavity nests (Seeley 1983). The cavity nesters, *A. mellifera*, *A. cerana*, *A. nigrocincta*, *A. koschevnikovi* and *A. nuluensis*, construct several vertical parallel combs in hollow tree trunks. While these

are the habitual nesting sites of honey bee species, the fact that *A. mellifera* can occasionally nest in the open, as when combs are constructed hanging from a branch, indicates that there is a degree of flexibility in this behavior.

Open air nests can occupy a big branch of a tall tree (*A. dorsata*) (Wongsiri et al. 1996), hang on cliffs (*A. laboriosa*) (Underwood 1990a) and attached to small twigs (*A. andreniformis* and *A. florea*). Figure 1-4 illustrated the variety of honey bee nest morphologies: the cavity nesting *A. cerana*, and the open nesting *A. florea*, *A. andreniformis* and *A. dorsata*.

Figure 1. The cavity nesting of *A. cerana* (photograph taken from Seeley (1983))

Figure 2. *A. dorsata* open nesting

Figure 3. *A. florea* open nesting

Figure 4. *A. andreniformis* open nesting.

The *A. andreniformis* nest has two midribs, one below and one above the supporting branch. The upper midrib functions as the "nest crown", providing for honey storage. The nest crown does not exist in *A. florea* nest, hence it has only the lower midrib. It is located under the supporting branch, close to the brood area (Rinderer et al. 1996). Overall, the depth and width of worker and drone cells are smaller in *A. andreniformis* compared to those of *A. florea* (Rinderer et al. 1996). In both dwarf honey bees, pollen is stored at the top of the brood nest location (Wongsiri et al. 1997).

The single combed *A. florea* group and the *A. dorsata* group reveals architectural differences. *A. dorsata* nests (Figure 2) are attached to the substrate (branch, wall, building and cliff), and so there is no free space above the nest (Starr et al. 1987; Underwood 1990a). On the other hand, *A. florea* and *A. andreniformis* nests (Figure 3 and 4) are attached around the twig, thus allowing free space at the top of the nest (Lindauer 1961; Raffiudin, R. personal observation). The freely attached nest has a flat platform of nectar cells. This flat platform at the top of *A. andreniformis* and *A. florea* nests serves as horizontal dance platform.

Evolution of the honey bee nest type is a behavioural trait that will be studied in detail in my study. Along with dance behaviour, nesting behaviour will be mapped in the molecular phylogenetic tree in Chapter 3.

Honey bee: behaviour and brain

Foraging bees obtain a variety of signals from celestial and terrestrial cues which guide them back to the nest and are subsequently transcribed when advertising their findings (Gould et al. 1970; Dyer and Gould 1983). The signals are received through honey bee chemosensory receptors, antennae and eyes (Collet 2000). Signals received by the antennae are relayed to antennal lobes while signals from the eyes are relayed to optical lobes. Finally, signals from the two lobes are relayed to specific parts of the honey bee brain namely corpora pedunculata or mushroom bodies (Mobbs 1982). These signals can be linked together to achieve associated learning (Srinivasan 1998). Hence, the ability of the honey bee to integrate many informational cues into the waggle dance is

facilitated by advanced memory ability in the brain (Menzel 2001; Menzel and Giurfa 2001).

General anatomy of A. mellifera central nervous system

The insect brain comprises three paired sections namely the protocerebrum (forebrain), deutocerebrum (midbrain) and tritocerebrum (hindbrain). The protocerebrum consists of the corpus pedunculata or mushroom bodies and two optical lobes. The antennal lobes comprise the deuterocerebrum region and the tritocerebrum region corresponds with the labrum and visceral nervous systems (Burrows 1996).

The mushroom bodies are paired organs in the insects forebrain which serve as a higher centre for signal integration (Menzel et al. 1994). They consist of calyx and neuron (Kenyon) cells, and a pedunculus. The calyx consists of dendrite arborisation derived from Kenyon cells and is divided into three parts (the lip, collar and the basal ring) according to the input signal source. The lip at the tip of the calyx receives signals from antennal lobes, the collar region receives signals from optical lobes and the basal ring region receives signals from both areas as well as the esophageal (Mobbs 1984; Gronenberg 1986). Axons of Kenyon cells extend to the peduncles and are further structured as α and β lobes in the output region (Grunewald 1999).

Kenyon cells are located in the centre of the calyx, contain of small and large types (Kenyon 1896). The large type cells are located on the inner side, whereas the small types are layered on the outer side (Farris et al. 1999). The *A. mellifera* brain contains 340 000 cells of this type (Witthorft 1967), whereas *Drosophila* have 2 500 cells in each body. Such great variations in cell numbers lead to the hypothesis that this organ is associated with complex behaviour in social insects (Ehmer and Hoy 2000; Gronenberg 2001). The great difference in cell numbers are reflected from the number of calyx in each mushroom body; a pair of calyx in *A. mellifera* (Mobbs 1982), and one calyx in *Drosophila*.

Mushroom body neuropils show an increasing growth size from nurse to forager bee stage and even in different forager ages. Differences apparent in the extended

mushroom body neuropil region (Withers et al. 1993) and in calyx subcompartments (lip and collar region) (Durst et al. 1994). The increase in mushroom body size is mainly due to dendritic growth (Durst et al. 1994), and not to cell proliferation since there is no neurogenisis in the adult honey bee (Fahrbach et al. 1995).

Honey bee task age-related variations have been reported in molecular neurobiological studies (Wagener-Hulme et al. 1999). Multiple genes have been identified as expressed in the brain of *A. mellifera*. Those are eight biogenic amine receptor genes (Ebert et al. 1998; Blenau et al. 2000), a neurotransmitter glutamate transporter (Kucharski et al. 2000), and a group of second messenger genes (Kamikouchi et al. 1998; Kamikouchi et al. 2000; Eisenhardt et al. 2001).

Biogenic amines such as dopamine, serotinin and octopamine are the neuromodulators in the nervous system (Erber et al. 1993). It has been demonstrated that octopamine receptor genes expression is significantly increased in the forager honey bees compared to nursing honey bees, which maybe means the gene is age-related in the division of labour (Wagener-Hulme et al. 1999).

The second messenger genes group is of particularly interest to intracellular communication due to their role in first messenger transmission that received to antenna or eyes. Chapter 2 will deal with this topic and present the findings of *A. mellifera itpr* characterization study, which is one of the gene expressed in the Kenyon cells.

Chapter 2. *A. mellifera* Inositol 1,4,5-Trisphosphate Receptor (*Itpr*) Gene Structure

Abstract

The inositol 1,4,5 - trisphosphate receptor (*itpr*) gene codes for the IP_3 Receptor (IP_3R) protein, a calcium release channel protein in the endoplasmic reticulum membrane. This gene is highly expressed in *A. mellifera* mushroom bodies, which are the essential parts of the honey bee brain for memory formation. In this study I attempt to characterise the *itpr* gene based on putative A1 clone gene sequences (Kamikouchi et al. 1998) through *A. mellifera itpr* genomic and cDNA inverse PCR (IPCR) and DNA sequencing.

The sequence obtained was found to comprise 2, 091 bp of a partial sequence of the *A. mellifera itpr* gene encoding 697 inferred amino acids. *A. mellifera* IP_3R amino acids show 62%, 60%, 33%, 56%, and 56% similarities to those of *Panulurius argus* (lobster), *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis*, and *Mus musculus itpr*-1, respectively. A phylogenetic analysis using *itpr* sequence demonstrated that *D. melanogaster itpr* has the closest sequence to that of *A. mellifera* of those known. Seven exons and six introns interrupt this partial *itpr* gene sequence. The *A. mellifera itpr* exons of this study aligned to the *D. melanogaster itpr* embryonic variant (Genbank: AJ238949) at nucleotide 3616–5784. Exon-intron organisation comparison of *A. mellifera itpr* gene with that of *D. melanogaster* showed that two introns were at conservative positions and that *A. mellifera itpr* has several additional introns.

The inferred *A. mellifera* IP_3R amino acid sequence aligned with the modulatory domain of *D. melanogaster*. Several conserved motifs were observed in *A. mellifera* putative IP_3R namely an IP_3R signature and four protein kinase phosphorylation sites, comprising cAMP dependent protein kinase (PKA), protein kinase C (PKC), tyrosine kinase and casein kinase II. PKA and PKC are two known protein kinases needed for *A. mellifera* signal transduction in short and long term memory formation, respectively. Further study is needed to confirm PKA and PKC phosphorylation sites in *A. mellifera* IP_3R in order to determine the role of *itpr* among other kinases signalling in honey bee mushroom bodies.

INTRODUCTION

Intracellular second messengers and their genes target in *A. mellifera* brain

Coordination of biochemical reactions enables living cells to react to stimuli, integrate the signal information, and then respond to the environment. Such signal mechanisms are present in all cells, but the highest complexity is found in nervous system cells (Levitan and Kaczmarek 1997).

Extracellular signals are recognised by neurons cells through specific membrane receptors that are coupled to different kinds of transduction mechanisms via intracellular second messengers (Hall 1992). Nervous cells contain several major types of intracellular second messengers, namely cyclic adenosine monophosphate (cAMP), cyclic guanyl monophosphate (cGMP), and membrane phospholipid turnover phosphatidylinositol (Nicholls 1994). Each second messenger has its specific target to be activated. cAMP target molecule is cAMP-dependent protein kinase A (PKA), cGMP is the substrate of cyclic guanyl monophosphate dependent protein kinase (cGMP). Phosphatidylinositol turnovers produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 target protein is inositol 1,4,5-trisphosphate receptor (IP_3R), while DAG and Ca^{2+} are the substrates of Ca^{2+}/phospholipid-dependent protein kinase C (PKC) (Levitan and Kaczmarek 1997).

Over the past few years, extensive researches in *A. mellifera* molecular neurobiology have resulted in the discovery of several neurotransmitter receptors (Ebert et al. 1998; Blenau et al. 2000) as well as glutamate transporters (Kucharski et al. 2000) and complete PKA cDNA (complementary DNA) in honey bee brain (Eisenhardt et al. 2001). The remaining second messenger receptors have been partially characterised as PKC, Ca^{2+}/calmoduline-dependent protein kinase II (CaMkinaseII) (Kamikouchi et al. 2000), and putative clone A1, predicted to be the IP_3R homologue (Kamikouchi et al. 1998).

IP_3R is encoded by the inositol 1,4,5-trisphosphate receptor gene (*itpr*), which has been completely characterised in several arthropods, nematode, and mammals. This gene is of special interested because complete cDNA sequences are available from those

organisms; hence, it is useful for comparisons and phylogenetic analysis involving the gene from *A. mellifera* from this study. All the intron positions are known for the embryonic *D. melanogaster itpr* gene (Sinha and Hasan 1999) and this information is useful for comparing its exon-intron organisation with that of *A. mellifera*. In *Drosophila* the protein in the adult is shorter than that seen in embryos, and is a splice variant of the latter. I therefore refer to the embryonic form in comparisons below.

Inositol 1,4,5-trisphosphate (IP_3): a second messenger pathway

Inositol is a six-carbon alcohol molecule, a glucose derivative called myo-inositol in its active state. Within the cells inositol exists in several phosphorylation stages namely phosphatydylinositol 1,4-biphosphate (PlP_2), IP_3, inositol 1,3,4,5-tetrakisphosphate (IP_4) and inositol 1,3,4,5,6-pentakisphosphate (IP_5) (Berridge and Irvine 1989).

Phosphatydylinositol 1,4-biphosphate (PlP_2), abundant in the plasma membrane, is hydrolysed by a neurotransmitter received by receptors in cell membrane. Many receptors are coupled to the membrane enzyme phospholipase C (PLC) through the G protein. PLC acts as a phosphodiesterase to split PlP_2 into two products, the water soluble IP_3 and diacylglycerol (DAG) that remains in the membrane. IP_3 diffuses in the cytoplasm and binds to a specific Ca^{2+} receptor (IP_3R) channel on the endoplasmic reticulum. The binding triggers calcium release from intracellular storage (Berridge 1993).

The Ca^{2+} release from IP_3R channel is the intermediate of many cellular pathways (Berridge 1993). Several studies have shown that Ca^{2+} and IP_3R are important in the *D. melanogaster* olfactory system (Deshpande et al. 2000). An experiment using IP_3R mutant *D. melanogaster* shows that this protein is important to maintain the *D. melanogaster* olfactory system (Deshpande et al. 2000). Studies on rat IP_3R suggest a role in circadian rhythms (Hamada et al. 1999).

***Itpr* gene and the splicing sites**

The complete *itpr* cDNA has been reported for *Mus musculus* (mouse) (Furuichi et al. 1989), *Rattus norvegicus* (rat) (Mignery et al. 1990), *D. melanogaster* (fruit fly)

(Yoshikawa et al. 1992), *Xenopus laevis* (frog) (Kume et al. 1993), human (Yamada et al. 1994), *Caenarhabditis elegans* (nematode) (Baylis et al. 1999), *Panulurius argus* (lobster) (Munger et al. 2000), *Bos taurus* (bovine) (unpublished data: Genbank AF157625) and *Gallus gallus* (chicken) (partial gene: unpublished data: Genbank AF312710). These studies have established that the gene has approximately 8 600 base pairs (bp) and that IP_3R consists of approximately 2700 amino acids.

Two splicing sites are apparent in mouse (Danoff et al. 1991) and rat (Mignery et al. 1990) *itpr*, namely the SI and SII sites. The mouse SI splicing site occurs between *itpr* gene nucleotides 952-996, correspond to IP_3R residues 317-331 (fifteen amino acids) which are located at IP_3 binding site in N-terminal region (Mignery and Sudhof 1990). The existence of the SI splicing region is presumably due to the exons splicing, since there is a donor site (GTNNN) in the SI domain (Breitbart et al. 1987). The SII splicing site is found between mouse *itpr* nucleotide 5074-5193 or IP_3R residues number 1692-1731 (fourty amino acids), falling between two PKA phosphorylation sites (Nakagawa et al. 1991).

Those two splicing sites yielded three protein transcriptions (Nakagawa et al. 1991) which are IP_3R-1, IP_3R-2 and IP_3R-3. Mammals have those three types of IP_3R (Danoff et al. 1991). IP_3R-1 is mostly expressed in mouse cerebellum (Wojcikiewicz 1995), whereas IP_3R-2 and IP_3R-3 are mostly expressed in the non neural regions (Ross et al. 1992; Blondel et al. 1994). Three types of IP_3R were found in rat brain, IP_3R-1 is highly expressed in Purkinje cells, IP_3R-2 in glial cells and IP_3R-3 in neuronal cells (Sharp et al. 1999). Only IP_3R-1 was expressed in *D. melanogaster* (Yoshikawa et al. 1992) and *C. elegans* (Baylis et al. 1999) and it is encoded by the *itpr*-1 gene (Venkatesh and Hasan 1997). In *D. melanogaster*, IP_3R is highly expressed in the antenna (Yoshikawa et al. 1992) while in *A. mellifera*, partial IP_3R is strongly expressed in the mushroom bodies of the brain (Kamikouchi et al. 1998).

Inositol 1,4,5-trisphosphate receptor structure

The IP_3R protein has three domains, namely N-terminal, modulatory and C-terminal domains. The N-terminal domain has a 750 residue ligand binding site that binds the IP_3 molecule. The modulatory domain comprises most of IP_3R region lying between the

ligand binding and the transmembrane calcium channel sites [approximately at IP_3R residue number 700-2250 (Maeda et al. 1991)]. This domain regulates activation of Ca^{2+} channel and it has several functional phosphorylation sites such as PKA (Danoff et al. 1991), casein kinase (Sinha and Hasan 1999) and ATP phosphorylation (Maeda et al. 1991a). The third domain is the C terminal domain, which occupies only a small region of IP_3R (Furuichi et al. 1989). A calcium channel is located in this domain performed six to eight transmembrane spanning regions (Michikawa et al. 1994).

Conserved and variable regions within IP_3R

IP_3R has 72 % similarity between human and mouse IP_3R-1 (Yamada et al. 1994) and 62 % similarity between rat IP_3R-1 and IP_3R-3 (Mignery et al. 1990). Conserved regions are mostly found in the N-terminal domain particularly in ligand binding sites and in the amino acids region within the multiple transmembrane spanning region (Baylis et al. 1999).

Despite of IP_3R conserved region, it has three variable regions (area I, II, and III) (Yamada et al. 1994) based on human, mouse, rat, *X. laevis*, *D. melanogaster* amino acids alignment. Area I consists of approximately twenty residues at *D. melanogaster* residue number 1245-1265, whereas area II is at residue number 1799-1845. Both are in the modulatory domain. Area III is at residues 2577-2602, inserted between the last two transmembrane spanning regions of the calcium channel. Of the thirty two amino acid replacements between human and mouse IP_3R-1, seventeen replacements were concentrated in area I, six replacements are in area II and seven in area III (Yamada et al. 1994).

IP_3R displays functional differences among several organisms as well. Mouse (Furuichi et al. 1989), rat (Mignery et al. 1990), human (Yamada et al. 1994) and lobster IP_3R-1 has two PKA phosphorylation sites, however these two PKA sites are not found in the *D. melanogaster* (Yoshikawa et al. 1992) and *X. laevis* IP_3R-1 (Kume et al. 1997). On the other hand, adult and embryonic IP_3R *D. melanogaster* has several casein kinase binding sites in the modulatory domain (Sinha and Hasan 1999).

The other difference in IP_3R structure functions is that there are two ATP binding sites both of which are present in mouse, rat and human IP_3R at residue numbers 1720-1725 and 2094-2099, respectively (Mignery et. al 1990; Yamada et. al. 1994). However, *D. melanogaster* IP_3R contains only the second ATP binding site (Hasan and Rosbash 1992).

Inositol 1,4,5-trisphosphate receptor: homo- and heterotetramer protein

Homotetrameric IP_3R is found in cells containing only one IP_3R type, such as IP_3R in mouse cerebellum Purkinje cells (Maeda et al. 1991a). The protein is composed of four non-covalently bound identical subunits (Miyawaki et al, 1991) and electron microscopy revealed that it is a homotetramer protein with four fold symmetry and four radial arms (Chadwick et al. 1990). However, heterotetrameric IP_3R formation was found in cells that contain all IP_3R types such as in liver cells (Monkawa et al. 1995) and in human neuroblastoma cell (Wojcikiewicz 1995).

Exon-intron organisation

Types of introns

Protein coding genes are transcribed into mRNA and are subsequently translated into protein. Genes comprise of coding (exon) regions but they are often interrupted by non-coding (intron) regions. Exons are represented in mRNA whereas introns are cleavaged from mRNA prior to the protein translation (Lewin 1993).

Based on cleavage mechanisms, there are three known types of introns, namely nuclear spliceosomal intron, intron type I and intron type II. The former intron is spliced from RNA by a protein complex called spliceosome that occurs in most eukaryotes (Padgett et al. 1986). The other two intron types are self-splicing and most abundant in fungal, plant mitochondrial and chloroplast ribosomal genes (Lambowitz and Belfort 1993). Nuclear intron splice site is recognised by spliceosome at conserved dinucleotides, namely GT at the 5' intron and AG at the 3' intron, hence known as GT/AG rule. This splice site occurred in 99 % of intron splice region in many organisms (Deutsch and Long 1999).

Intron evolution theory

The origin of nuclear introns has become source of debate since they were discovered. Two theories of nuclear intron evolution have emerged, namely "introns early" and "introns late". The "introns early" theory has introns arising at the origin of life (Gilbert 1978). According to this view, the first minigene composed of exons while spacers of the minigenes are thought of as the intron origin. This theory developed into the exon shuffling theory since the author postulates that intron insertion plays a role in gene assembly (Gilbert 1985). Hence, exon shuffling promoted the earliest gene formation (Blake 1978).

However, other authors have suggested that the exon shuffling theory is not identical to the intron early theory, due to a lack of any evidence suggesting that introns are derived from the spacer (Li 1997). Palmer and Logsdon (1991) suggest that although exon shuffling has played an important role in vertebrate gene evolution, there is little or no basis for ascribing a role to it in the assembly of primordial proteins.

Mapping nuclear intron distribution on a tree of life reconstructed based on rRNA data shows that introns are inserted in the eukaryotic lineages while they are lost in the bacterial and ancient eukaryote lineages (Palmer and Logsdon 1991). "Introns early" explained the intron loss in bacterial genome and ancient eukaryotes due to selection of streamlined genomes (Doolittle 1978).

The "introns late" theory views this region is inserted at the late stages of eukaryotic evolution (Rogers 1989; Cavalier-Smith 1991; Palmer and Logsdon 1991). Mounting evidence of intron late theory include for example, intron insertion in dasyurid marsupials intronless *SRY* gene (O'Neill et al. 1998), in human odor protein (Lacazette et al. 2000), and the *A. mellifera* elongation factor 1α (EF-1α) gene has more introns than that of *D. melanogaster* (Waldorf and Hovemann 1990). On the other hand, insect triosephosphate isomerase (TPI) gene is consistent with both views (Tyshenko and Walker 1997).

Intron distribution in itpr gene

Of all *itpr* genes that have been published, only those of *D. melanogaster* and *C. elegans* are known with respect the intron position. Intron distributions in the *D. melanogaster itpr* (Sinha and Hasan 1999) are different to those in *C. elegans itpr* gene (Baylis et al. 1999). *D. melanogaster itpr* has a total of eleven introns, with six of them clustered in the modulatory domain. However, *C. elegans* has 32 introns, which are more dispersed: eleven, twelve and nine at C-terminal, modulatory and N-terminal domain, respectively (Baylis et al. 1999).

OBJECTIVES

The aims of my study were to characterise the *A. mellifera itpr* gene and to elucidate putative IP_3R protein structure. *Itpr* gene structure is determined by exon-intron organisation. Comparisons of *itpr* gene intron positions between *A. mellifera* and that of *D. melanogaster* are evaluated in order to investigate intron present and absent in this gene. The similarity of the *A. mellifera itpr* gene with respect to those in other organisms is ascertained and subsequently used to infer phylogenetic relationships with other organisms. IP_3R structural function is examined by searching the amino acid motifs within the Prosite protein database and used to infer the role of IP_3R in *A. mellifera* neuron signalling.

MATERIALS AND METHOD

DNA: extraction, sequencing and genomic walking

Genomic DNA was extracted from bee thoraces (mesosomes) using the standard CTAB method. The first genomic region was amplified based on cDNA clone A1 of putative IP_3R gene (Kamikouchi et al. 1998) using oligonucleotide primers 1 and 2 (Table 1) designed by the author. In order to extend the region, several genomic walk steps were subsequently conducted by performing inverse PCR (IPCR) using primers designed to face the unknown regions (Triglia et al. 1988).

For IPCR, genomic DNA was first cut with *HincII* and then the resulting fragments were circularized using T4 ligase. The walk began using primers 3 and 4 (Table 1). Amplifications were performed in 50 µl volumes containing 5x Buffer B (Gibco), 200 µM dNTP, 1 µM of each primer, and DNA template. Prior to IPCR, the PCR mixture was heated at 94^0C for three minutes, then 1 unit of Elongase polymerase (Gibco) was added. IPCR was performed in 2 min at 94^0C for initial denaturing, 35 cycles of 30 s at 94^0C, 30 s at 50-58^0C and 2-6 min at 68^0C for DNA elongation, followed by 10 minutes at 72^0C for the final extension. PCR products were purified with Qiagen kit prior to cloning them into pGEM-T vector (Promega). The cloned products were amplified either by using the previous primers or primers number 8 and 9 (Table 1, which are pUC/M13 universal primers also present in pGEM-T vector). DNA sequencing was carried out by ABI Prism BigDye automated sequencing using the manufacturer's protocol. *Bfa1* digest was used at the next genomic walking using primers number 2 and 5. Eight other restriction enzymes and several other primers (not shown in Table 1) were applied in genomic walking but were yielded no further results because of the unspecific PCR products. Therefore, I used complementary DNA (cDNA) walking for the second technique.

Table 1. Oligonucleotide primers used in *A. mellifera itpr* gene characterisation; sense primers are coded by "F" or "For", and antisense primers are coded by "R" or "Rev"

No	Primers	5' – 3' Sequence
1	IP3For	5' GAATATCCTCTGGTGATGGATACA 3'
2	IP3Rev	5' CCATGTTCTTCTGATGCTTTAGA 3'
3	IP3W3F	5' GGTTTCCGATAGTGATGTTGAAT 3'
4	IP3W4R	5' CCCTCCGCTTGTGTACCTATT 3'
5	IP3F1658	5' GACGGGAAAGAAGATGTTAGA 3'
6	*ITPR*-F2280	5' TTAGCTTGTCGTACAATGGGC 3'
7	IP3W2R	5' AATTTTGTATCCATCACCAGAGG 3'
8	F23	5' CCCAGTCACGACGTTGTAAAACG 3'
9	R24	5' AGCGGATAACAATTTCACACAGGA 3'
10	5'RACE= SMART II	5'AAGCAGTGGTAACAACGCAGAGTACGCGGG 3'
11	3'RACE= CDS PRIMER	5' AAGCAGTGGTAACAACGCAGAGTAC(T)30VN 3'
12	DSCUP="PCR PRIMER"	5' AAGCAGTGGTAACAACGCAGAGT 3'
13	*Itpr*Rev1781	5' AGGATTAAGAAATAAATCTAA 3'
14	*Itpr*For1658	5' GACGGGAAAGAAGATGTTAGA 3'
15	*Itpr*Rev2284	5' TGCCCATTGTACGACAAGC 3'

RNA: extraction, single and double stranded cDNA amplification, and cDNA walking

Total RNA was extracted from three heads of *A. mellifera* foragers. Three heads (20 mg) were excised and preserved in 500 µl of RNA*later* RNA stabilisation Reagent from RNeasy protect Kits (Qiagen). RNA was extracted using the RNAeasy mini Kit (Qiagen). Extraction procedure was followed the instructions in the kit manual.

For first strand cDNA synthesis, I used SMART PCR cDNA Synthesis Kit (Clontech). A total volume of 3 µl of RNA template, 10 µM 3' RACE primer and 10 µM 5'RACE primer (Table 1) was incubated in 72^0C (2 min). To the mixture was added 5x First Strand Buffer, 20 mM DTT, 50x dNTP mix and 1 µl of Superscript II enzyme (200 U/µl) (Clontech). First Strand cDNA synthesis was carried out by incubating the mixture in 42^0 C (1 hr). Forty µl of TE buffer (10mM Tris, 1mM EDTA) was added for dilution.

For second strand cDNA synthesis, the same kit was used. The PCR mix contained 10x Advantage 2 PCR Buffer, 50x dNTP mix, DSCUP primer (Table 1), 50x Advantage 2 Polymerase Mix, 2 µl single strand (ss) cDNA template and ddH_2O adjusted to 25 µl. PCR was performed in a Perkin Elmer 9700 for 95^0C (5 sec), 65^0C (5 sec), and 68^0C (6 min) in 18 cycles.

cDNA polishing was carried out in order to blunt ds cDNA ends by adding T4 DNA polymerase (Huang et al. 1990). The 25 µl amplified double strand (ds) cDNA was combined with 2 µl of proteinase K after which it was heated at 90^0C in order to inactivate proteinase K. The tube was chilled in ice for 2 min in order to stop the reaction and 15 units of T4 DNA polymerase were subsequently added and incubated at 16^0C (30 min) and heated at 72^0C (10 min). To extract the cDNA, 27.5 µl of 4 M ammonium acetate were added and ethanol precipitation was conducted by adding ~210 µl of 95% ethanol. The mixture was spun at 13 000 rpm (20 min) and pellets were washed with 80% ethanol and subsequently spun at 13 000 rpm (10 min). Then, 100 µl dH_2O were added to the air-dried pellet.

The blunt end ds cDNA was circulated with the T4 DNA ligase (Huang et al. 1993). The 100 µl ds blunt end cDNA were combined with 25 µl of 10x Ligation Buffer, 2 µl of T4 DNA ligase (3U/µl) (Promega). The mixture was incubated at 18^0C for 16 h and purified with GeneClean Kit.

cDNA IPCR is performed using the same method as in genomic walking IPCR, based on the last genomic walking result. IPCR used primer combination primer number 2, 4, 5, 6 and 7 (Table 1). Intron splice sites were confirmed based on the cDNA region that were amplified using primers combination number 1+2, 3+13 and 14+15 (Table 1).

Nucleotide, amino acids and motif protein analyses

For each genomic walking step, DNA was translated into three reading frames using MacVector™ and ANGIS (BioNavigator by Entigen Corporation) amino acid translation programs. Each translated amino acid strand between two stop codons was investigated for its homology within the protein database. The strand that returned as an

IP_3R homolog was chosen and established as *A. mellifera itpr* exon. Introns were recognised by the presence of stop codon and denotion from the sequence required the yield known IP_3R sequences, further introns were delineated using the known intron splicing site. Prosite (Hofmann et al. 1999), PRINTS (Attwood and Beck 1994) and pfam (Bateman et al. 1999) databases were used to determine protein domain, motifs, protein fingerprint and protein family, respectively, in *A. mellifera* putative IP_3R.

***Itpr* phylogenetic analysis**

Genbank sequences for IP_3R from various organisms (*D. melanogaster* adult type: D90403, *D. melanogaster* embryo type: AJ238949*, C. elegans*: AJ243179, *X. laevis*: D14400, *P. argus*: AF055079, *M. musculus* IP_3R type-1: X15373) and *A. mellifera* IP_3R (this study) were aligned using the CLUSTALX program (Thompson et al. 1997). Tree construction was performed using the Maximum Likelihood (ML) method implemented in the TREEPUZZLE 5.0 program using quartet puzzling as the algorithm with 1000 replicates (Strimmer and von Haeseler 1996). ML rather than Maximum Parsimony (MP) was used because basic MP counts every replacement as a unitary change, whereas some replacements imply a larger change in protein chemistry than others and should therefore be counted more. Ad hoc methods exist for changing these weights, but then mean that MP converges on ML, while the latter also correctly takes account of amino acid frequencies in a natural determination of weights. The amino acids substitution models tested were the Dayhoff (Dayhoff et al. 1978), VT (Müller and Vingron. 2000) and WAG (Whelan and Goldman 2001) models and the model giving the highest likelihood score was used. These models use sets of proteins in known phylogenies to determine empirically the probability of one amino acid being replaced by another.

RESULTS

Itpr gene structure: exon intron organisation

A. mellifera itpr gene characterisation in this study began by using the published putative A1 clone *itpr* gene sequences (Kamikouchi et al. 1998). The honey bee *itpr* gene sequence of this study was extended through genomic and cDNA walking to cover a total of 2,091 bp exons and 810 bp introns of partial gene. Amino acids deduced from *itpr* gene DNA sequence showed the similarity of 62%, 60%, 33%, 56%, and 56% to those of *P. argus* (lobster) (Munger et al. 2000), *D. melanogaster* (Yoshikawa et al. 1992; Sinha and Hasan 1999), *C. elegans* (Baylis et al. 1999), *X. laevis* (Kume et al. 1993), *M. musculus itpr*-1 (Furuichi et al. 1989), respectively. However, searching *A. mellifera* intron gene sequences on an Intron Searching Intron Database (ISIS-http://isis.bit.uq.edu.au) did not yield any homologous regions.

Exon regions (2,091 bp) of *A. mellifera* partial *itpr* gene were aligned with the nucleotide *D. melanogaster* embryonic *itpr* gene (Genbank: AJ238949) at nucleotide numbers 3616 – 5784. There are seven exons and six introns in this partial gene. Hence, in this study, I stated exon 1 as starting at nucleotide number 3616 based on the *D. melanogaster* embryonic *itpr* gene. The first intron inserted in this partial gene was regarded as the first intron as well.

Exon-intron organisation in *itpr* was examined through both genomic and cDNA sequences. Six introns were distributed at nucleotide position numbers 312 – 1775 based on *A. mellifera itpr* cDNA positions. However, this study has not explored intron existence in two regions, namely nucleotide between numbers 1-310 and 1776-2091 (Figure 1). Moreover, the first intron inserted in this gene has not been fully sequenced as well. The *A. mellifera itpr* nucleotide and predicted amino acid sequence are shown in Figure 1.

```
ATGAGTATTAATGATTATGATGCAAACAGGATGCGGGATCAAAATAAAGAAGCTGTACGTGCTAAATTTA   70
 M  S  I  N  D  Y  D  A  N  R  M  R  D  Q  N  K  E  A  V  R  A  K  F     23

GCGCAACTATAATGTTTGTAGAAGATTATTTGTGTAATGTTGTAGCAAAAATGTGGTCCTTTGCTGATCA  140
S  A  T  I  M  F  V  E  D  Y  L  C  N  V  V  A  K  M  W  S  F  A  D  Q     47

AGAACAAAATAAACTTACATTTGAAGTCGTTAAATTGGCACGTGATTTAATTTATTTTGGATTCTATAGT  210
  E  Q  N  K  L  T  F  E  V  V  K  L  A  R  D  L  I  Y  F  G  F  Y  S     70

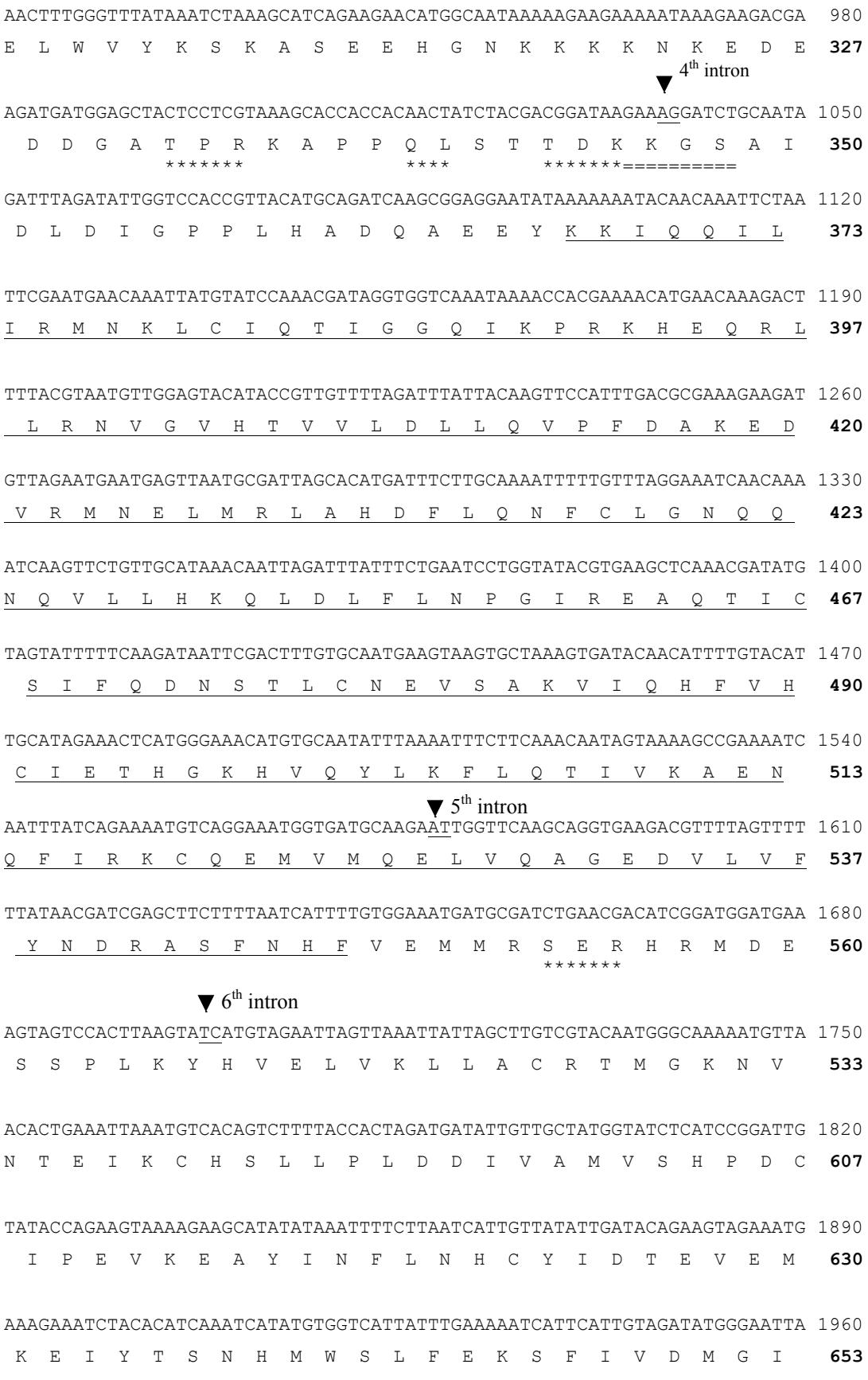
TTCAGTGATCTTTTGAGATTAACGAAAACATTACTCAGTATTTTGGATTGTATTTCAGAAAATGATGTAG  280
F  S  D  L  L  R  L  T  K  T  L  L  S  I  L  D  C  I  S  E  N  D  V     93
                          ▼ 1st intron
CCGATGGAAAAATTCCAACTGGTGAAATTGATGCGGAAGGTGGAGTATTAAGATGTATTGGAGACATGGG  350
A  D  G  K  I  P  T  G  E  I  D  A  E  G  G  V  L  R  C  I  G  D  M  G    117

TGCAGTAATGACGAGCTTAACACTGGGACCAGCAGGACAAGTATTAGCAGGAAGTTCTTCTCCAAGACCA  420
  A  V  M  T  S  L  T  L  G  P  A  G  Q  V  L  A  G  S  S  S  P  R  P    140
                                                      *******
AAACCACTTTTAAAGAAAGAATATCCTCTGGTGATGGATACAAAATTGAAAATAATCGAAATTTTACAAT  490
 K  P  L  L  K  K  E  Y  P  L  V  M  D  T  K  L  K  I  I  E  I  L  Q    163

TTATACTTGATGTTCGATTGGATTATAGAATTTCTTGTTTATTGAGTATTTTCAAACAAGAATTTGATGA  560
F  I  L  D  V  R  L  D  Y  R  I  S  C  L  L  S  I  F  K  Q  E  F  D  E    187

AACTGAAAGAGCTTCTGGTGATTTGAGTCTCGGCCAGAAAACTATTGATTTAGAATTAATAGGTACACAA  630
  T  E  R  A  S  G  D  L  S  L  G  Q  K  T  I  D  L  E  L  I  G  T  Q    210
                ▼ 2nd intron
GCGGAGGGTATATTTGGTAGCAGCGAGGAATGTGTGGCGTTAGATTTAGATGGACAAGGTGGTAGAACAT  700
 A  E  G  I  F  G  S  S  E  E  C  V  A  L  D  L  D  G  Q  G  G  R  T    233

TTCTGCGTGTTTTACTCCATTTGGCAATGCATGACTATCCTCCACTAGTTTCCGGAGCATTACATTTGCT  770
F  L  R  V  L  L  H  L  A  M  H  D  Y  P  P  L  V  S  G  A  L  H  L  L    257
                                      ▼ 3rd intron
TTTTAGGCATTTTAGTCAAAGACAAGAAGTCTTACAAGCATTTAAACAAGTTCAACTTTTGGTTTCCGAT  840
  F  R  H  F  S  Q  R  Q  E  V  L  Q  A  F  K  Q  V  Q  L  L  V  S  D    280
               *******
AGTGATGTTGAATCTTACAAACAAATAAAGTCAGATTTGGACGTTTTAAGACAATCGGTTGAAAAATCGG  910
 S  D  V  E  S  Y  K  Q  I  K  S  D  L  D  V  L  R  Q  S  V  E  K  S    303
               *******
```


AACTTTGGGTTTATAAATCTAAAGCATCAGAAGAACATGGCAATAAAAAGAAGAAAAATAAAGAAGACGA 980
E L W V Y K S K A S E E H G N K K K K N K E D E 327
4th intron
AGATGATGGAGCTACTCCTCGTAAAGCACCACCACAACTATCTACGACGGATAAGAAAGGATCTGCAATA 1050
D D G A T P R K A P P Q L S T T D K K G S A I 350
GATTTAGATATTGGTCCACCGTTACATGCAGATCAAGCGGAGGAATATAAAAAAATACAACAAATTCTAA 1120
D L D I G P P L H A D Q A E E Y K K I Q Q I L 373
TTCGAATGAACAAATTATGTATCCAAACGATAGGTGGTCAAATAAAACCACGAAAACATGAACAAAGACT 1190
I R M N K L C I Q T I G G Q I K P R K H E Q R L 397
TTTACGTAATGTTGGAGTACATACCGTTGTTTTAGATTTATTACAAGTTCCATTTGACGCGAAAGAAGAT 1260
L R N V G V H T V V L D L L Q V P F D A K E D 420
GTTAGAATGAATGAGTTAATGCGATTAGCACATGATTTCTTGCAAAATTTTTGTTTAGGAAATCAACAAA 1330
V R M N E L M R L A H D F L Q N F C L G N Q Q 423
ATCAAGTTCTGTTGCATAAACAATTAGATTTATTTCTGAATCCTGGTATACGTGAAGCTCAAACGATATG 1400
N Q V L L H K Q L D L F L N P G I R E A Q T I C 467
TAGTATTTTTCAAGATAATTCGACTTTGTGCAATGAAGTAAGTGCTAAAGTGATACAACATTTTGTACAT 1470
S I F Q D N S T L C N E V S A K V I Q H F V H 490
TGCATAGAAACTCATGGGAAACATGTGCAATATTTAAAATTTCTTCAAACAATAGTAAAAGCCGAAAATC 1540
C I E T H G K H V Q Y L K F L Q T I V K A E N 513
5th intron
AATTTATCAGAAAATGTCAGGAAATGGTGATGCAAGAATTGGTTCAAGCAGGTGAAGACGTTTTAGTTTT 1610
Q F I R K C Q E M V M Q E L V Q A G E D V L V F 537
TTATAACGATCGAGCTTCTTTTAATCATTTTGTGGAAATGATGCGATCTGAACGACATCGGATGGATGAA 1680
Y N D R A S F N H F V E M M R S E R H R M D E 560
6th intron
AGTAGTCCACTTAAGTATCATGTAGAATTAGTTAAATTATTAGCTTGTCGTACAATGGGCAAAAATGTTA 1750
S S P L K Y H V E L V K L L A C R T M G K N V 533
ACACTGAAATTAAATGTCACAGTCTTTTACCACTAGATGATATTGTTGCTATGGTATCTCATCCGGATTG 1820
N T E I K C H S L L P L D D I V A M V S H P D C 607
TATACCAGAAGTAAAAGAAGCATATATAAATTTTCTTAATCATTGTTATATTGATACAGAAGTAGAAATG 1890
I P E V K E A Y I N F L N H C Y I D T E V E M 630
AAAGAAATCTACACATCAAATCATATGTGGTCATTATTTGAAAAATCATTCATTGTAGATATGGGAATTA 1960
K E I Y T S N H M W S L F E K S F I V D M G I 653

```
TAGCAACAGCTACACATGATCGCGAACATGCTGATATATCTCTGGAAAATTATGTGACAGGTTGCCTAAT 2030
I  A  T  A  T  H  D  R  E  H  A  D  I  S  L  E  N  Y  V  T  G  C  L  M  677

GAATATCATTACAACGTTCTTTAGCAGTCCATTTTCAGATCAAAGTACCACAGTACAGAAA          2091
  N  I  I  T  T  F  F  S  S  P  F  S  D  Q  S  T  T  V  Q  K           697
```

Figure 1. Nucleotide and predicted amino acid sequence of partial *A. mellifera itpr* based on genomic and cDNA. *First line*, nucleotide sequence; *second line*, deduced amino acid sequence. Plain and bold numbers at the right indicated nucleotide and amino acids, respectively. IP_3R signature was boxed, arrow between the underlined nucleotide showed intron insertion position, cAMP phosphorylation residues were double underlined, PKC phosphorylation site residues shown by * and ryanodine-*itpr* homolog (RIH) residues indicated by single underline (residues 368-547).

Intron lengths in *A. mellifera itpr* range from 70-410 bp, five being less than 100 bp (Table 2). Intron insertions are in all possible phases (Patthy 1987). All introns inserted at the conserved intron insertion (GT/AG) (Table 2).

Although there are high levels of similarity between IP_3R sequences among different organisms, the *A. mellifera* partial *itpr* gene cloned from a single bee contained two alleles that showed five nucleotide differences between them (Table 3). One nucleotide substitution was at the first codon position, resulting in an amino acid change whereas four other occurred at the third codon position and were synonymous.

Table 2. Partial *A. mellifera itpr* gene intron length, conserved and non-conserved introns splice-sequences; unexplored 5' splice site shown with *; the underlined nucleotides are the conserved splicing sites

Intron number	Intron length	5' Splice Site (exon/intron)	3'splice site (intron/exon)	Intron phase
1	410 ?	*	gtttataatatttaata/GCG	?
2	84	AGC,AG/gtaatacattattcat	tattttattttatttag/C,GAG	2
3	75	CAA/gtatgttacattatttata	taaattattgtaaatag/GTT	0
4	94	AAA,G/gttttgttaataatttt	attatttataattatag/GA,TCT	1
5	70	GAA/gtaagtgataactatttga	taattgctttttttttag/TTG	0
6	76	CTT,AA/gtatgcatttaagata	atatatattttag/G,TAT,CAT	2

Table 3. *A. mellifera itpr* gene nucleotide variants

No	*A. mellifera* partial *itpr* cDNA nucleotide position	1st allele			2nd allele		
		Nucleo - tide	Codon-amino acid	Found in	Nucleo - tide	Amino acid	Found in
1	574	T	TCT- S	clone 1,3,4	CCT	CCT- P	clone 2
2	669	G	GCG- A	clone 1,3,4	GCA	GCA- A	clone 3
3	696	A	AGA- R	clone 1,2,4	AGG	AGG- R	clone 3
4	717	C	CTC- L	clone 1,3,4	CTT	CTT- L	clone 2
5	895	G	TCG- S	clone 1,3,4	TCA	TCA- S	clone 2

Intron position comparison between A. mellifera and D. melanogaster embryonic itpr gene

The *D. melanogaster* embryonic *itpr* gene comprises eleven introns, six of which inserted in the IP_3R modulatory domain (Sinha and Hasan 1999). Of the six introns, three found in the region aligning with the partial *A. mellifera itpr* gene. In this study, two introns are at identical position ie. between *A. mellifera* 1st intron and *D. melanogaster* embryonic type 8th intron at position 3926 (Figure 2). Second similarity is the *A. mellifera* 3rd intron and *D. melanogaster* embryonic type 8th intron at *D. melanogaster* nucleotide number 4569. Besides those conserved introns, *A. mellifera itpr* gene exhibits three more introns and one intron loss at the aligning region with that of *D. melanogaster*.

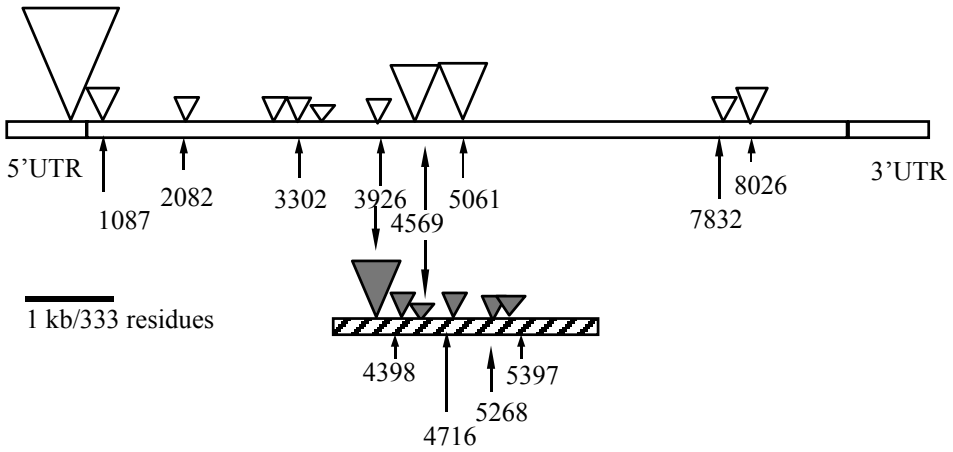


Figure 2. *A. mellifera* and *D. melanogaster itpr* gene (Sinha and Hasan, 1999) and the intron positions comparisons; *A. mellifera itpr* gene (▨) and its introns (▼); *D. melanogaster itpr* gene (▭), and its introns (▽)

Itpr gene phylogenetic analysis

Arthropod, nematode and mammals *itpr* phylogentic tree is shown in Figure 3 based on approximately 697 amino acids deduced from the *itpr* gene. Alignments was employed with CLUSTAL X and *C. elegans itpr* was used as the outgroup. Tree topology was revealed the same by using three kinds of amino acid substitution model (WAG, VT and Dayhoff) implemented in TREEPUZZLE 5.0 program. The WAG model giving the highest likelihood score was chosen as the best model (Table 4).

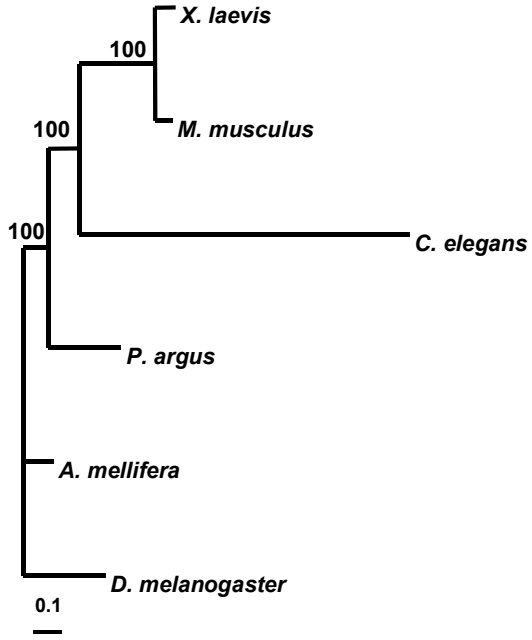


Figure 3. The quartet puzzling tree with its support values constructed from amino acids *itpr* gene of *A. mellifera*, *D. melanogaster* (Genbank: D90403), *P. argus* (AF055079), *C. elegans* (AJ243179), *X. laevis* (D14400), *M. musculus* (X15373). Tree reconstruction uses TREEPUZZLE 5.0. with WAG model. Numbers above the node are the quartet puzzling support values with 1000 replicates.

Table 4. Likelihood value of each *itpr* tree using different amino acid models

Model	Ln likelihood
WAG	-7254.70
VT	-7334.73
Dayhoff	-7300.30

IP_3R: motifs structure and function

Characterisation of 2,091 bp exons of *A. mellifera* partial *itpr* gene inferred 697 residues for IP_3R with a mass of 79.31 kDa (BioNavigator by Entigen Corporation). The *A. mellifera* partial IP_3R was aligned at *D. melanogaster* IP_3R from amino acid positions 877-1583 which are situated in the modulatory domain of this protein (Yoshikawa et al. 1992).

An *A. mellifera* IP_3R motif search using Prosite revealed one of the IP_3R signatures (PR00779) (Hofmann et al. 1999) (APPENDIX 1). Putative *A. mellifera* IP_3R signature consisted of twenty-six conserved amino acids (Figure 1). Other motifs included the conserved regions of phosphorylation sites, namely one cAMP- or cGMP-dependent protein kinase site, seven PKC phosphorylation sites, fourteen casein kinase II phosphorylation sites and one tyrosine kinase phosphorylation site. Determined by PRINTS, a protein motif finger-print database (Attwood and Beck 1994), *A. mellifera* partial IP_3R protein is a homologue of a Ryanodine receptor (PF01365) and they belongs to RYR-IP_3R Homologue (RIH) protein family. The homologous region is shown in residues number 368-574 of *A. mellifera* partial *itpr* gene (Figure 1).

DISCUSSION

The molecular and structural properties of *A. mellifera itpr* gene are described in this section. The exon-intron organisation and variations of intron positions in this gene are compared with other organisms. Overall, it was found that, although amino acid similarity is highly conserved among nematode and *Drosophila*, several variations were observed to have occurred in gene structure such as intron splice variant and intron position.

Itpr gene structure

Exon-intron organisation in A. mellifera partial itpr gene: common and unique features

Exon-intron organisation of partial *itpr* gene exhibited certain features in common with other genes such as the number of introns for every kilobase (kb) of exon. There were six introns in 2,091 bp exons of partial *itpr* gene that represented an average of three introns per one kb of exon. These results coincided with results from a compilation of intron-exon structures in an overall database whereby 3.7 introns exist in 1 kb exons, while 2.7 introns per kb exist in *D. melanogaster* genome (Deutsch and Long 1999).

Other features commons to all itpr gene introns were located at the phase 2, 0, 1. Introns were classified according to their relative positions in gene reading frame. The phase 0 intron is the intron which lies between two codons, while those falling between first and second nucleotides are known as Phase 1 introns, and those interrupting codons between second and third nucleotides are known as Phase 2 introns (Sharp 1981). In the "intron early" theory Gilbert (1985) claimed that most introns are inserted in Phase 0 (Tomita et al. 1996), which was used as the basis for exon shuffling theory, an extended view of intron early theory.

However, *A. mellifera itpr* gene introns displayed common features, such as the characteristics at their splicing sites. All introns started with GT and ended with AG. Pre-mRNA splicing sites take place in a large spliceosomes. In the conserved GT/AG splice site, spliceosomes contain four small nuclear ribonucleoproteins (snRNPs) namely the U1, U2, U4/U6 and U5 snRNPs (Newman and Norman 1992) and a

complex non-snRNPs protein. The latter protein belongs to an SR family (U2AF) with RNA recognition motifs (RRM), which are needed to enhance U1 and U2 recruitment to the binding region (Valcarcel et al. 1996). U1 snRNP binds to the 5'GA splice site, U2 snRNP binds to the branchpoint of 3' AG splice sites, and U4/U6.U5 assembly required for intron lariat connection (Lopez 1998).

Conservation and divergence of intron insertion sites in itpr gene

Two introns are at conserved positions between *A. mellifera* and *D. melanogaster* which are *A. mellifera* 2nd intron and *D. melanogaster* embryonic type 8th intron at *D. melanogaster itpr* gene (Figure 2).

In addition to conserved intron positions relative to *Drosophila*, the *A. mellifera itpr* sequence shows that there are three introns not seen in *Drosophila* and one that occurs in *Drosophila* but absent in *Apis*. The addition of introns at different positions in the *A. mellifera* and *D. melanogaster itpr* genes may be the result of a more recent insertion event as shown in intron gain of intron late view (Cavalier-Smith 1991). Insertional theory accounts for introns as being the result of a dynamic process of occasional insertion and deletion (Palmer and Logsdon 1991). Recent studies have produced evidence that intron insertions in the different organismal gene families are more likely to take place than intron loss. Evidence for intron gain also has been observed in intron-bearing dasyurid marsupials in the usually intronless *SRY* gene (O'Neill et. al 1998) and in *Chironomus thummi* which has a single intron in the usually intronless globin genes (Gruhl et al. 1997). Supporters of the intron gain theory have postulated that the sources of nuclear intron insertions were derived from mobile group II introns from yeast and mitochondria because these intron types have their own reverse transcriptase for making double stranded DNA from a single RNA strand (Eickbush 2000). Other authors have postulated that there *P element* insertion (Nouaud et al. 1999) or intron duplication (Tarrio et al. 1998) became the intron insertional source.

Variable itpr region

Despite high level conservation of *itpr* gene, several variants occur in this gene. Five nucleotide differences between two alleles of the *A. mellifera itpr* gene are due to both

purin and pyrimidine changes. Nucleotide variations are also evident in clones of rat *itpr-1* gene, all of which had purine changes only (Mignery et al. 1990). Another variable region shown in *P. argus* IP_3R deduced from the *itpr* gene. It has twenty highly hydrophobic amino acids which are absent in IP_3R from other organisms. However, some of these hydrophobic regions exist in the *A. mellifera* putative IP_3R (APPENDIX 1, dotted line). This hydrophobic region is presumably due to the existence of *P. argus* IP_3R in plasma membrane, which is a different location for this receptor (Munger et al. 2000).

Putative *A. mellifera* partial IP_3 Receptor

My study has revealed a total of 697 residues of putative IP_3R protein. Complete IP_3R from several organisms exhibit nine conserved amino acids or protein signatures, one of which was identified in putative *A. melifera* IP_3R, that represents a significantly reliable amino acid in this partial *itpr* gene (APPENDIX 1).

IP_3R: similarity, phylogeny and protein family

A. mellifera IP_3R showed high similarity (more than fifty percent) to this protein from other organisms, except for those of *C. elegans*. High gene conservation is also evident in other genes expressed in *A. mellifera* brain such as gene encoded for partial PKC reveals high degree of similarity, namely 86% and 83% to that of *D. melanogaster* and rat-α PKC (Kamikouchi et al. 2000). *A. mellifera* brain biogenic amine receptors (ie. serotinin, tyramine/octopamine, and dopamine) reveal high degrees of similarity, namely 84%, 72% and 65%, respectively to those of *D. melanogaster* homologue (Ebert et al. 1998).

Based on partial IP_3R amino acid similarities, *P. argus* shows the highest degree of similarity to *A. mellifera*, exceeding that of *D. melanogaster*. However, a phylogenetic analysis based on ML was carried out and revealed that *D. melanogaster* IP_3R is the sister to *A. mellifera*. This phylogenetic tree reflects *itpr* evolution in agreement with current views on animal phylogeny. Current metazoan phylogeny based on 18srRNA revealed two monophyletic groups across bilateria metazoans, deuterostomes (including chordates) and protostomes (including nematodes and arthropods) (Adoutte et al. 1999).

A. mellifera partial IP_3R has been shown to be homologous with Ryanodine (*ryr*), a calcium transport receptor found in muscle (Tunwell et al. 1996). Three subtypes of *ryr* are expressed preferentially in muscular tissue (Tunwell et al. 1996), cardiac tissue (Gorza et al. 1997) and in Purkinje cells (McPherson and Campbell 1993) of sarcoplasmic reticulum. *D. melanogaster* thorax tubular muscle also expresses this gene (Hasan and Rosbash 1992). There has been no study of *ryr* expression in the honey bee, while *A. mellifera* muscle regions (thorax and legs) show slightly IP_3R expression (Kamikouchi et al. 1998b). *Ryr* and *itpr* phylogenetic analyses show that both are paralogous genes. Gene duplication resulted in *ryr* and *itpr* occurring in invertebrate lineage while the three subtype receptors in vertebrates resulted from gene duplication (Saier et al. 1999).

Motifs in A. mellifera IP_3R

In this study, seven putative protein kinase phosphorylation sites were detected in the putative *A. mellifera* IP_3R which are also found in IP_3R of other organisms. Phosphorylation is a post-translational modification process involving a reaction in which phosphate groups are added to hydroxyl groups on protein (Stryer 1995). This process is usually catalysed by a kinase (an enzyme that catalyses phosphate transfer from ATP to a second substrate) using ATP as the phosphate donor (Hunter 1995). Activation of phosphorylation sites by kinase enzymes in the IP_3R modulatory domain regulates the opening of calcium channels (Furuichi et al. 1989).

Of all the phosphorylation sites detected in *A. mellifera* partial IP_3R, PKA is the most interesting one due to the absence of this site in *D. melanogaster* IP_3R (Yoshikawa et al. 1992). PKA catalyses phosphorylation of a number of different enzyme and non-enzyme proteins and hence it plays an important role in metabolic regulation (Taylor et al. 1990). PKA phosphorylation conserved amino acids are R/K-R/K-X-S where R, K, X and S represent arginine, lysin, any amino acid and serin; and the last residue is the phosphorylation site (Feremisco et al. 1980). *A. mellifera* has one putative PKA phosphorylation sites (KKDS), two PKA phosphorylation sites in *P. argus* (KKES and RKPS) (Munger et al. 2000), and one site in rat (RRES) (Danoff et al. 1991). *A.*

mellifera putative PKA phosphorylation site position is at S_{348} that is located at the variable area 1 region (APPENDIX 1).

The occurrence of putative PKA phosphorylation sites in *A. mellifera* but not in *D. melanogaster* suggests that there is a different mechanism and more complex second messenger signal transductions in *A. mellifera* compared to those of *D. melanogaster*. In *D. melanogaster*, signalling is only activated by a single second messenger (IP_3), whereas in *A. mellifera* two second messenger are possibly needed in signal transduction.

Protein kinase C (PKC) phosphorylation sites also exist in the putative *A. mellifera* IP_3R. PKC is the major kinase occurring in neuronal tissue (Huang 1989). Conserved substrate specificity for PKC phosphorylation amino acids are (S/T) or (R/K) with S (serine) as the phosphorylation site (Kishimoto et al. 1985). PKC phosphorylation sites also exist in most IP_3R in other organisms (Munger et al. 2000).

Does the *itpr* gene mediate honey bee memory formation?

Two regions in the honey bee brain act as memory formation areas, namely the olfactory glomerulus which acts as the first memory storage area and mushroom bodies which act as the highest integration modulation signal (Hammer and Menzel 1995). Memory formation in the honey bee has been confirmed as developing in five stages, namely early short term memory (eSTM), late short term memory (lSTM), middle term memory (MTM), early long term memory (eLTM) and late long term memory (lLTM) (Menzel and Giurfa 2001). STM enables honey bee to retain information from several seconds to minutes. MTM can last for several hours while LTM can even last for days (Menzel 2001). In STM, the honey bee uses PKA as protein kinase to phosphorylated signals (Menzel 1999). PKC appears to be the basic protein kinase in later memory or in the lLTM stage (Grunbaum and Muller 1998) which is associated with NO (Muller 1996).

Immunohistochemistry studies of honey bee brains have revealed that PKA and PKC expression occurs in different distributions, i.e. PKA occurs in most neuronal brains

whereas PKC is highly expressed in mushroom bodies (Muller 1997; Muller 1999). My study highlights the occurrence of putative PKA and PKC phosphorylation sites in IP_3R. It is possible then that *A. mellifera* IP_3R can play role in mediating signal pathways at certain stages of memory formation by means of the PKA and PKC phosphorylation sites in the protein modulatory domain. Moreover, Muller's Figure 1 diagram (1999) shows the scheme of second messenger-regulated phosphorylating cascades including PKA and PKC and also the occurrence of intracellular organelle contributes to the production of Ca^{2+}.

Complete honey bee PKA cDNA (Eisenhardt et al. 2001), partial PKC and Ca^{2+}/CaMKII cDNAs (Kamikouchi et al. 2000) have been characterised. Research studies which assess the contribution of IP_3R to brain signalling pathways can be conducted by means of experiments using *A. mellifera* PKA and (or) PKC mutant. *D. melanogaster dunce* mutant for cAMP phosphodiesterase (Dudai et al. 1976; Chen et al. 1986) and rutubaga mutant for Ca^{2+}/Calmoduline-responsive adenylyl cyclase (Levin et al. 1992) show that these are defective in learning. Another study of the role of the *itpr* gene in relation to PKA has already been demonstrated in *D. melanogaster* molting development (Venkatesh and Hasan 1997; Venkatesh et al. 2001), it is therefore possible to study the role of this gene in *A. mellifera* signalling neurons, either directly using generated mutants or, perhaps more feasibly, using antisense RNA under RNAi as already attempted in honey bees (Beye et al., 2002)**.**

FUTURE STUDY

Further studies are necessary in order to complete the entire *A. mellifera itpr* sequence. As a result of this current study, all introns are known to be inserted at conserved splicing sites. Complete *A. mellifera itpr* sequence can be done by using cDNA walking combined with the use of conserved primers to amplify *A. mellifera* cDNA and DNA. *A. mellifera itpr* introns can be explored at the genomic level using the sequence difference between cDNA and DNA sequences.

The *A. mellifera itpr* promoter region needs to be characterised and as well as the IP_3R transmembrane spanning and IP_3 ligand binding sites. *A. mellifera* putative

phosphorylation sites (PKA, cGMP, PKC, tyrosine, glucosamine and casein kinase) should be confirmed and the results expanded to the entire *A. mellifera* IP_3R. In the context of honey bee brain calcium signalling, it should also be possible to characterise *A. mellifera RYR*. This receptor is expressed in *the D. melanogaster* tubular muscle and has a low expression in neuronal tissue (Hasan and Rosbash 1992).

APPENDIX 1. Partial *itpr* gene alignment; the IP_3R signature is boxed; arrows indicate the conserved intron position between *A. mellifera* and *D. melanogaster itpr* gene embryonic type; bold amino acids are the *D. melanogaster* IP_3R adult type insertional region; the thick line is the IP_3R variable area I (Yamada et. al 1994); the *A. mellifera* PKA phosphorylation site (S_{348}) is underlined; dotted spots covered the similar hydrophobic regions in *P. argus* (Munger et. al 2000) and *A. mellifera*; stars denote the identical amino acids of all taxa.

```
A.mellife       ---------------------------------------------MSINDYDANRMR--- 12
D.mel_embryo    DETMPYELRASFCRLMLHLHVDR-DPQEPVTPVKYARLWSEIPSKMSIQDYDGKNQQP-- 889
D.mel_adult     DETMPYELRASFCRLMLHLHVDR-DPQEPVTPVKYARLWSEIPSKMSIQDYDGKNQQP-- 889
P.argus         EETLAYDLRAAFCRLMLHMHVDC-EPQEMVTPVKYARLWSEIQPHMSIADYDKHAAM--- 824
C.elegans       DNRLPYDLRGSFTRLMLHLHVVRGSP---MSAIRHARLWWSIPENVNVSTYESVSVEAYS 883
X.laevis        DENLPFDLRASFCRLMLHMHVDR-DPQEQVTPVKYARLWSEIPSEIAIDDYDS------S 807
M.musculu       DENLPYDLRASFCRLMLHMHVDR-DPQEQVTPVKYARLWSEIPSEIAIDDYDSSGTS--- 825
                                                              *

A.mellife       DQNKEAVRAKFSA-TIMFVEDYLCNVVAKMWSFAD-QEQNKLTFEVVKLARDLIYFGFYS 70
D.mel_embryo    DQNKQACRAKFNT-TIAFVENYLCNVATKVWLFTD-QEQNKLTFEVVKLARDLIYFGFYS 947
D.mel_adult     DQNKQACRAKFNT-TIAFVENYLCNVATKVWLFTD-QEQNKLTFEVVKLARDLIYFGFYS 947
P.argus         -HSTEAAETTFKD-VIVFVEEYLCNVVDKMWSFSD-CEQNKLTFEVVKLARYLIYFGFYS 881
C.elegans       DGSRMRIGEGIAHKVLATVETYLMGLRNQSMEERQSVNSSKLTYEIVNLAKALAQFNFYS 943
X.laevis        GTSRDDIKERFAQ-TMEFVEEYLRDVVGQRFPFAD-KEKNKLTFEVVNLARNLIYFGFYN 865
M.musculu       ---KDEIKERFAQ-TMEFVEEYLRDVVCQRFPFSD-KEKNKLTFEVVNLARNLIYFGFYN 880
                           ** **                ***  * * **  *  * **

A.mellife       FSDLLRLTKTLLSILDCISENDVADGKIPTGEID---------AEGGVLRCIGDMGAVMT 121
D.mel_embryo    FSDLLRLTKTLLSILDCVS--DTSSGEFASTDID---------SEGGVLRSIGDINTVMT 996
D.mel_adult     FSDLLRLTKTLLSILDCVS--DTSSGEFASTDID**SVEEETNAE**AEGGVLRSIGDINTVMT 1005
P.argus         FNDLLRLTKTLLSILDYSF--DTDSKYFSNSIPQG-----TASAKGGVIKSLGDMGAVVT 934
C.elegans       FNDLLQLTQNLLAIINEGP---ATEQVPS------------HRAMVNAIRN-------MS 981
X.laevis        FSDLLRLTKILLAIVDCVH---LIANFPSGKLGKG--E--ESKGGSNVMRSIHGVGELMT 918
M.musculu       FSDLLRLTKILLAILDCVH---VTTIFPISKMTKG-----EENKGSNVMRSIHGVGELMT 932
                   *** **  ** *

A.mellife       SLTLGP--AGQVLAGSSSPRPK----PLLKKEYPLVMDTKLKIIEILQFILDVRLDYRIS 175
D.mel_embryo    SLALGS--VGQAIAAPTISLQQRKSVSQLMKEYPLVMDTKLKIIEILQFILDVRLDYRIS 1054
D.mel_adult     SLALGS--VGQAIAAPTISLQQRKSVSQLMKEYPLVMDTKLKIIEILQFILDVRLDYRIS 1063
P.argus         NLALGTTRMGPRLGGGSSPKKK---VGATEKEDTLVMDTKLKIIEILEFILNVRLDYRIS 991
C.elegans       KSMMRGGNKENSKDLAKTPSVTAEEAGRTKEGRALNVKTKLIVAEILQFVMDVRRDYRIT 1041
X.laevis        QVVLRGGGFLPMTPMAAPEGTIKAQREPEKED-ILVMDTKLKIIEILQFILNVRLDYRIS 977
M.musculu       QVVLRGGGFLPMTPMAAAPEGN-VKQAEPEKEDIMVMDTKLKIIEILQFILNVRLDYRIS 991
                                                ***   *** *   ** ****

A.mellife       CLLSIFKQEFDETER-----------------------------------------ASGD 194
D.mel_embryo    CLLSIFKREFDESEVPLRPLAMRQVSSSRNNRNRRRLAAPMRLIPSTVPSLWPPRCAAAA 1114
D.mel_adult     CLLSIFKREFDESEVPLRPLAMRQVSSSRNNRNRRRLAAPMRLIPSTVPSLWPPRCAAAA 1123
P.argus         CLLSIFKKESDENPSS---LTGEGISQGLKNKN--------------VENIW----AQAQ 1030
C.elegans       MALSWFKNVFPCD-------------------------------------------EDGS-- 1058
X.laevis        CLLCIFKSEFDESNAQ-----------------------------------SVEGSTE 1000
M.musculu       CLLCIFKREFDESNSQ---------SSETSSGN----------------------SSQE 1019
                  *  **

A.mellife       LSLGQKT-IDLELIGTQAEGIFGSSE--ECVALDLDGQGGRTFLRVLLHLAMHDYPPLVS 251
D.mel_embryo    TTARQKN-IDLESIGVQAEGIFDCERS-DAANLDLDGQGGRTFLRVLLHLIMHDYAPLVS 1172
D.mel_adult     TTARQKN-IDLESIGVQAEGIFDCER--TPANLDLDGQGGRTFLRVLLHLIMHDYAPLVS 1180
P.argus         SIFDETSGLGLVWSSSQRKPSLQTSSCEENSSLDLDGEGGKKFLRVLLHLTMHEYPPLVS 1090
C.elegans       --LMHSASINERMASELYDAIYRSS----GHELHLDGRDGQLLLAILLQMTMSDYPPLTS 1112
X.laevis        AITVVPGTLDFEHIEEQAEGIFGGSE--ENTPLDLDDDGGRTFLRVLLHLTMHDYPPLVS 1058
M.musculu       GPSNVPGALDFEHIEEQAEGIFGGSE--ENTPLDLDDHGGRTFLRVLLHLTMHDYPPLVS 1077
                                           * **   *   *  **   *  * ** *
A.mellife       GALHLLFRHFSQRQEVLQAFKQVQLLVSDSDVESYKQIKSDLDVLRQSVEKSELWVYK-- 309
D.mel_embryo    GALHLLFRHFSQRQEVLQAFRQVQLLVSDSDVESYKQIKSDLDILRQSVEKSELWVYK-- 1230
D.mel_adult     GALHLLFRHFSQRQEVLQAFRQVQLLVSDSDVESYKQIKSDLDILRQSVEKSELWVYK-- 1238
P.argus         RSLQLLFRHFSQRQEVLQNFKQVQLLVQDGDVESYKQIKEDSDDLRNLVEKSELWVYK-- 1148
C.elegans       IALKVFFRHFTQYQELLEDLKQVQLLVSNNDVENYRQIDRDLFILKNLTEKSELWVHGDR 1172
X.laevis        GALHLLFRHFSQRQEVLQAFKQVQLLVTSQDVDNYKQIKQDLDQLRSIVEKSELWVY--- 1115
M.musculu       GALQLLFRHFSQRQEVLQAFKQVQLLVTSQDVDNYKQIKQDLDQLRSIVEKSELWVYK-- 1135
                  *   **** * ** *    ******   **  * **  *   *    *******
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                                           ............................
A.mellife         ------------SKASEEHGN----KKKKNKEDED---------DGATPRKAPP---QLS 341
D.mel_embryo      ------------AKATDELG---------------------------------------- 1238
D.mel_adult       ------------AKATDELG---------------------------------------- 1246
P.argus           ------------SRSTDEDGGGTKKKKKKNKDDDE---------DDALSKPKKPPAPKLT 1187
C.elegans         HHSIDTKEVDEKERTTEHDLLDHDLKSPRAFDSGDSMEALMAVLNEHYPSIRNECLQLLN 1232
X.laevis          ---KGS---GPEEVTAAQAGGADKGETP-----G-------------------------- 1138
M.musculu         ------------GQGPDEPMDG--------------------------------------A 1146

                            Area I
                  ___________________
A.mellife         TTDKKGSAIDLDIGPPLHADQAE--EYKKIQQILIRMNKLCIQTIG---GQIKPRKHEQR 396
D.mel_embryo      ATDAGGDAVSLEYNAALSQEQRN--EYRKVKEILIRMNKFCVTASGP-GSVVKPRKHEQR 1295
D.mel_adult       ATDAGGDAVSLEYNAALSQEQRN--EYRKVKEILIRMNKFCVTASGP-GSVVKPRKHEQR 1303
P.argus           AQDKQESAIDLGLGPPLEPEQAD--NYKRIQQILVRMNKLCVTQSS--HGNLSPKRNEQR 1243
C.elegans         RLLIKDDRNDAAVALQELSDKAPLIAYPLIRQMLVRLTGMCYRKGDP-----KPDTMNQQ 1287
X.laevis          ----KAKK----------SESTSSYNYRVVKEILLRLSKLCVQENTTG--R-RNRKQQQR 1181
M.musculu         SGENEHKKTEEGTSKPLKHESTSSYNYRVVKEILIRLSKLCVQESA---SVRKSRKQQQR 1203
                                            *      * *                      *

A.mellife         LLRNVGVHTVVLDLLQVPFDAKEDVRMNELMRLAHDFLQNFCLGNQQNQVLLHKQLD--- 453
D.mel_embryo      LLRNVGVHTVVLDLLQNPYDEKDDELMKELMCLAHEFLQNFCLGNQQNQVLLHNHLD--- 1352
D.mel_adult       LLRNVGVHTVVLDLLQNPYDEKDDELMKELMCLAHEFLQNFCLGNQQNQVLLHNHLD--- 1360
P.argus           LLRNMGIHSVVLELLQIPYDRKEDKRMNELIELAHQFLQNFCLGDRANQALLYKSID--- 1300
C.elegans         LLKNMRVYEVVLEFISVPHDKKHDHDMMKLITLSHEFLRSFCKTNKENQSRLYKFISYEK 1347
X.laevis          LLRNMGAHSVVLELLQIPYEKTEDTRMQEIMKIAHEFLQNFCAGNQQNQALLHKHIN--- 1238
M.musculu         LLRNMGAHAVVLELLQIPYEKAEDTKMQEIMRLAHEFLQNFCAGNQQNQALLHKHIN--- 1260
                  ** *     ***     *     *  *       * **  **     **  *

A.mellife         -----LFLNPGIREAQTICSIFQDNSTLCNEVSAKV--IQHFVHCIETHGKHVQYLKFLQ 506
D.mel_embryo      -----LFLNPGILEAKTVCAIFKDNLALCNEVTDKVGVGHSVVHCIEIHGRHVAYLQFLQ 1407
D.mel_adult       -----LFLNPGILEAKTVCAIFKDNLALCNEVTDKV---VHFVHCIEIHGRHVAYLQFLQ 1412
P.argus           -----LFLNPGLLEAKTVCAVFKDNSHLCSEVSERV--IQHFIHCIETHGRHVQYLKFLQ 1353
C.elegans         DAKEGMLRVETIEEVGTLVAIFRNNRELASNVPEEL--IAHIVGLIEHNSRNPIFLELLQ 1405
X.laevis          -----LFLTPGILEAVTMQHIFMNNFQLCSEINERV--VQHFAHCIETHGRNVQYIKFLQ 1291
M.musculu         -----LFLKPGILEAVTMQHIFMNNFQLCSEINERV--VQHFVHCIETHGRNVQYIKFLQ 1313
                               *  *    *  *  *                 **           **

A.mellife         TIVKAENQFIRKCQEMVMQELVQAGEDVLVFYNDRASFNHFVEMMRSERH-RMDESS--- 562
D.mel_embryo      TVVAAENQFIRRCQDMVMQELINSGEDVLVFYNDKGSFNHFVQMMQQQML-GMEKLSDDS 1466
D.mel_adult       TVVAAENQFIRRCQDMVMQELINSGEDVLVFYNDKGSFNHFVQMMQQQML-GMEKLSDDS 1471
P.argus           TIVKAEGQFLRRSQDIVMQELVNAGEDVLVFYNERASFNMFIEMMKADRN-RMDFDDS-S 1411
C.elegans         ALVCVYDKEIESGQEKVANEICAASDEVRQLYVDNASFEELEAMMKDEKESKGRSSDSRR 1465
X.laevis          TIVKAEGRYIKKCQDIVMAELVNSGEDVLVFYNDRASFQTLVQMMRSERE---R-MDENS 1347
M.musculu         TIVKAEGKFIKKCQDMVMAELVNSGEDVLVFYNDRASFQTLIQMMRSERD-RMDENS--- 1369
                    *          *  *  *       *   *    **     **
                                                                        ▼
A.mellife         PLKYHVELVKLLACRTMGKNVNTEIKCHSLLPLDDIVAMVSHPDCIPEVKEAYINFLNHC 622
D.mel_embryo      PLKYHVELVKLLACCTMGKNVYTEIKCNNLLSLDDIVTIICHPLCMPEVKEAYVDFLNHC 1526
D.mel_adult       PLKYHVELVKLLACCTMGKNVYTEIKCNNLLSLDDIVTIICHPLCMPEVKEAYVDFLNHC 1531
P.argus           PLRYHIELVKLLACCTEGKNASTEIKCHSLLPLDDIVAMVEHKDCIPEVKEAYINFLNHC 1471
C.elegans         KLKYHIELVRLLAMCTRGKNGNTELKCASQIPMDHIVRVVTAKQCLVEVKTVYLQLLLHC 1525
X.laevis          PLMYHIHLVELLAVCTEGKNVYTEIKCNSLLPLDDIVRVVTHEDCVPEVKIAYINFLNHC 1407
M.musculu         PLMYHIHLVELLAVCTEGKNVYTEIKCNSLLPLDDIVRVVTHEDCIPEVKIAYINFLNHC 1429
                   * ** ** ***  * ***  ** **      * **       *  ***  *    * **
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```
A.mellife       YIDTEVEMKEIYTSNHMWSLFEKSFIVDMGIIATATHDREHADISLENYVTGCLMNIITT 682
D.mel_embryo    YIDTEVEMKEIYASGHMWSLFEKSFLVDINQLITN--PAAASNKTLQAYVLNGVTNLLGS 1584
D.mel_adult     YIDTEVEMKEIYASGHMWSLFEKSFLVDINQLITN--PAAASNKTLQAYVLNGVTNLLGS 1589
P.argus         YIDTEVEMKEIYNSHHIWSLFEKSFLVDMGRVATAPPDRRHADKALENYVINSLMTIITT 1531
C.elegans       YIDTDAEMKDAYKTEYVDHILNN-LLEDI-RSLRVEKLTGAETATLEHYICHTVTEVLIK 1583
X.laevis        YVDTEVEMKEIYTSNHMWKLFEN-FLVDICRVCNSTSDRKHADIVLERYVTEIVMSIVNT 1466
M.musculu       YVDTEVEMKEIYTSNHMWKLFEN-FLVDICRACNNTSDRKHADSILEKYVTEIVMSIVTT 1488
                * **  ***  *              *                *  *

A.mellife       FFSSPFSD-QSTTVQK 697
D.mel_embryo    FFASPFSD-QSAIVQS 1599
D.mel_adult     FFASPFSD-QSAIVQS 1604
P.argus         FFNSPFSD-QSQTIQT 1546
C.elegans       FFEAPYSALQQAKVDV 1599
X.laevis        FFSSPFSD-QSTTLQA 1481
M.musculu       FFSSPFSD-QSTTLQT 1503
                **  * *  *
```

APPENDIX 2. *A. mellifera* partial *itpr* gene: intron (lowercase) and exon (uppercase) sequences

tttatatatatacgcacattttgatatatttttaagtgattaactaactaaatattga
aaacttctaaagctgattctgtggattctcaggacataggtaatatattattgagaat
taattaggtagcatgttactttgtaatatttcttgcacttctatttgtttcacgtaac
atcttcctttgatactttccttcttaattttattttaagtcagttttgtgttaaaaaa
atataaatattttattaaaaacagcaatgtcaaataatgtaactatattcaatcttaa
ttcaatttttaaagcctgaagttatttttttttaaatttataataaaaaatatttaa
acataaaatcattctatttaataaattaagaacgcatttaaaaatgtttataatattt
aataGCGGAAGGTGGAGTATTAAGATGTATTGGAGACATGGGTGCAGTAATGACGAGC
TTAACACTGGGACCAGCAGGACAAGTATTAGCAGGAAGTTCTTCTCCAAGACCAAAAC
CACTTTTAAAGAAAGAATATCCTCTGGTGATGGATACAAAATTGAAAATAATCGAAAT
TTTACAATTTATACTTGATGTTCGATTGGATTATAGAATTTCTTGTTTATTGAGTATT
TTCAAACAAGAATTTGATGAAACTGAAAGAGCTTCTGGTGATTTGAGTCTCGGCCAGA
AAACTATTGATTTAGAATTAATAGGTACACAAGCGGAGGGTATATTTGGTAGCAGgta
atacattattcatttttgtaatctttttttttatttatttatattaaataaatatttc
ataaattattttatttttatttagCGAGGAATGTGTGGCGTTAGATTTAGATGGACAAG
GTGGTAGAACATTTCTGCGTGTTTTACTCCATTTGGCAATGCATGACTATCCTCCACT
AGTTTCCGGAGCATTACATTTGCTTTTTAGGCATTTTAGTCAAAGACAAGAAGTCTTA
CAAGCATTTAAACAAgtatgttacattatttataatattatcgtgtaatataaatgaa
aaaatataaatttattaaattattgtaaatagGTTCAACTTTTGGTTTCCGATAGTGA
TGTTGAATCTTACAAACAAATAAAGTCAGATTTGGACGTTTTAAGACAATCAGTTGAA
AAATCGGAACTTTGGGTTTATAAATCTAAAGCATCAGAAGAACATGGCAATAAAAAGA
AGAAAAATAAAGAAGACGAAGATGATGGAGCTACTCCTCGTAAAGCACCACCACAACT
ATCTACGACGGATAAGAAAGgttttgttaataatttttttttaaataaaatattgaaat
aaattgaaaaatcaaaggcaaaattttcctaatacaaatattatttataattatagGA
TCTGCAATAGATTTAGATATTGGTCCACCGTTACATGCAGATCAAGCGGAGGAATATA
AAAAAATACAACAAATTCTAATTCGAATGAACAAATTATGTATCCAAACGATAGGTGG
TCAAATAAAACCACGAAAACATGAACAAAGACTTTTACGTAATGTTGGAGTACATACC
GTTGTTTTAGATTTATTACAAGTTCCATTTGACGCGAAAGAAGATGTTAGAATGAATG
AGTTAATGCGATTAGCACATGATTTCTTGCAAAATTTTTGTTTAGGAAATCAACAAAA
TCAAGTTCTGTTGCATAAACAATTAGATTTATTTCTGAATCCTGGTATACGTGAAGCT

CAAACGATATGTAGTATTTTTCAAGATAATTCGACTTTGTGCAATGAAGTAAGTGCTA
AAGTGATACAACATTTTGTACATTGCATAGAAACTCATGGGAAACATGTGCAATATTT
AAAATTTCTTCAAACAATAGTAAAAGCCGAAAATCAATTTATCAGAAAATGTCAGGAA
ATGGTGATGCAAGAAgtaagtgataactatttgacaaaataaattcatttatatttaa
aaattattaataattgctttttttagTTGGTTCAAGCAGGTGAAGACGTTTTTAGTT
TTTTATACGATCGAGCTTCTTTTAATCATTTTGTGGAAATGATGCGATCTGAACGACA
TCGGATGGATGAAAGTAGTCCACTTAAgtatgcatttaagatatataccaatagttat
aattataataattatgtatacataaatatataatatatatttagGTATCATGTAGAA
TTAGTTAAATTATTAGCTTGTCGTACAATGGGCAAAAATGTTAACACTGAAATTAAAT
GTCACAGTCCTTTACCA

Chapter 3. Honey bee behavioural evolution: insights from molecular phylogenetic analysis

Abstract

My study deals with honey bee behavioural evolution in terms of ancestral states for food recruitment and nesting behaviour. It also seeks to resolve questions about honey bee phylogeny mainly the relationship of two newly recognised species.

Phylogenetic reconstruction was performed under a Maximum Likelihood (ML) framework using data from two mitochondrial genes (*COII* and *lsRNA*) and a nuclear gene, *itpr*. ML analysis under GTR+G+I model of substitution revealed that *A. dorsata* and *A. florea* groups are clustered in the same clade, separate to that of *A. mellifera* group. Another possible honey bee evolutionary scenario from a weighted consensus ML tree revealed the following topology: ((((*Apis mellifera*, (((*A. nuluensis*, *A. nigrocincta*), *A. cerana*), *A. koschevnikovi*)), ((*A. laboriosa*, *A. d. binghamii*), *A. dorsata*)), (*A. florea*, *A. andreniformis*)), *Trigona fimbriata*, *Bombus terrestris*). This topology is in agreement with previous morphological and molecular results. In addition, my study discovered a discrepancy in the *COII* sequence of *A. koschevnikovi COII* (Willis et al. 1992) and thereby explains some puzzling results.

Dance behaviour was mapped onto the consensus phylogenetic tree. Species with vertical dance behaviour are monophyletic, as are those with horizontal dance behaviour. An unordered trait analysis does not resolve which type of dance behaviour (horizontal or vertical) is the ancestral. However, given that horizontal dance behaviour is less sophisticated with respect to translating foraging information for potential recruits, an ordered dance character state seems justified, and yields simple results. From these considerations, horizontal dance seems most likely to be the ancestral condition.

Whether cavity- or open nesting was ancestral cannot be determined because the two possibilities are equally parsimonious (i.e. both require two changes). Either ancestral *Apis* evolved open nesting from a common ancestor with the (cavity nesting) Bombini or Meliponini, or cavity nesting was ancestral with two or one origin(s) of open nesting.

The flexibility has shown by the cavity nesters. *A. mellifera* (which can nest in the open for extended periods) suggests that it may stem from open nesting ancestor, thereby indicating that open-nesting was ancestral.

INTRODUCTION

Eusociality in Apidae occurs in the corbiculate bees, namely the group of bees which have a corbicula (pollen basket) on the hindleg (Michener et al. 1978). The corbiculate group consists of four tribes: the Euglossini, Bombini, Meliponini, and Apini (Michener 2000). The tribe Euglossini is non-eusocial bees (most species are solitary, but some are communal or quasisocial), whereas the other groups (Bombini, Meliponini and Apini) live in eusocial colonies (Michener 1944; Prentice 1991).

The Apini consists of one genus, *Apis* that contains nine species. In this study, I seek to elucidate the behavioural history of *Apis*, with respect to nesting and dance behaviour. In inferring honey bee phylogeny, I used Bombini and Meliponini as the outgroups. The following sections describe behavioural characteristics reported in Bombini and Meliponini. Details of *Apis* nesting and dance behaviour were given in Chapter 1.

Nest construction

The nests made by bees placed in the Bombini, Meliponini and Apini, in which young are cared for communally and two generations of adult females live in the nest, reflect their eusociality (Michener 1974). Here I follow the original meaning of 'eusocial' (Crozier and Pamilo, 1996 page 4) as denoting species with continued and cooperative care of the young, a reproductive division of labour, and colonies with two adult generations, with 'advanced eusociality' pertaining to cases in which the egg-layers are morphologically differentiated from other individuals. Crespi and Yanega (1995) restrict 'eusociality' to species usually defined as 'advanced eusocial' and Sherman et al. (1995) suggest describing no species as 'eusocial' but rather giving them a score on a eusociality index. Neither of these suggestions is as useful as the original definition, at least for the work reported here.

Bombini or bumble bees, live mostly in the soil and often in an abandoned rodent nest burrow covered with dry grass and leaves (Michener 1974). Several species build their nests above the soil surface, mostly beneath vegetation. The bumble bee nest consists of brood cells and food storage pots which are made from mixed pollen and wax. Sometimes they make food storage pots from old cocoons (Michener 1964).

The Meliponini or stingless bees, usually build their nests in cavities inside hollow trees, although some species nest in soil, and others live in the open air (Roubik 1983). The fossorial species sometimes form an association with termite or ant nests such as *T. moorei* in *Crematogaster* (Salmah et al. 1990). Meliponini nests are made of wax, and some parts are made of cerumen (a mixture of resin and wax). In several cavity stingless bee species, the nest is encircled by batumen (thick cerumen plates), which seals most of the nest. The stingless bee nests in partially-exposed areas are providing with thick batumen which provides protection from heavy winds blows and temperature fluctuations (Wille and Michener 1973). Most stingless bees have an entrance tunnel to the nest. Their nests consist of brood cells and food storage pot, both made of wax and resin. Some brood cells are arranged horizontally, and others are in spirals with vertical openings. There is one species which builds vertical cells which are double-sided, back-to-back cells as seen in the honey bee (Michener 1974).

Outgroup data are essential for identifying character state homologies and determining polarity of evolutionary events. Table 1 (below) describes the nest structure features in Bombini, Meliponini, and Apini, which will be used here for comparative analyses.

Table 1
Nest structure comparisons in Bombini, Meliponi, and Apini (Michener 1974; Roubik 1983)

NEST DESCRIPTION	BOMBINI	MELIPONINI	APINI
Location			
Underground	yes	yes	no
Cavity (i.e. tree trunk)	no	yes	yes
Open area	yes	yes	yes
Nest material	Mostly wax and resin	Mostly wax and resin	Wax
Nest component			
Brood cells	Vertical opening cells	Vertical opening cells only one species with horizontal opening cells	Two-sided horizontal opening cells
Brood and food cells	Differ	Differ	the same form
Food storage	pot	pot	cells

Dance behaviour

Dance behaviour in honey bee is a repetitive behaviour that gives information about the distance and the direction of a certain place (von Frisch 1967). Dance behaviour is performed by forager, scout and drone honey bees. The forager honey bee gives food source information whereas the scout bee communicates the location of the new nest (Lindauer 1961), while at least in *A. andreniformis* the drone dances to coordinate departure by the colony's males (Rinderer et al. 1992). The focus of my study will be dance behaviour as performed by the forager honey bee.

Dance behaviour basically is food recruitment behaviour; it is a form of communication that brings nestmates to point in space where work is required. In the foraging context of food recruitment, dance behaviour is a communication that brings nestmates to the food source (Wilson 1971).

One example of food recruitment in Bombini is that of *B. terrestris* (Dornhaus and Chittka 2001), where the returning forager stimulates nestmates to search for food by a repetitive irregular running while fanning the wings. However, no information about distance or direction of the food location is provided (Dornhaus and Chittka 2001). While performing the runs, the bumble bee unloads the nectar into the nectar pot, then continues the excited running for several minutes before flying back to the food source. Recruitment mechanisms involve distributing the pheromone from the dancer to the recruits. A considerably higher amount of honey is produced after the dancer has recruited nestmates (Dornhaus and Chittka 2001).

Among the Meliponini, wide ranges of food communication mechanisms have evolved. The first of these is the alerting signal, which indicates that food is available by means using of a scent trail (Esch et al. 1965). In *Scaptotrigona postica* route to the food source from the nest is marked with a scent trail (Lindauer and Kerr 1960). "Jostling running" is performed by *Melipona scutellaris and M. quadrifasciata* foragers (Hrncir et al. 2000; Jarau et al. 2000). *M. panamica* shows a recruitment behaviour by performing clock and anticlockwise dance as the honey bee round dance (Nieh 1998). The polarity of dance behaviour will be discussed as well.

Previous studies of morphology and molecular phylogenetics in *Apis*

In order to understand the evolution of nesting and dancing behaviours we require a comprehensive phylogeny of this genus. Previous phylogenetic analyses were based on morphology (Alexander 1991) and mitochondrial DNA sequences using cytochrome oxidase II (*COII*) (Garnery et al. 1991; Willis et al. 1992) and large unit of ribosomal (*lsRNA*) (Cameron et al. 1992) regions for five species of honey bee. Most phylogenetic results have found that *A. florea* is the most basal species in the tree, with *A. dorsata* and *A. mellifera* as more derived groups (Alexander 1991; Garnery et al. 1991; Engel and Schultz 1997).

Controversy surrounds the position of *A. koschevnikovi*. One study found a close relationship of *A. koschevnikovi* to *A. florea* and *A. andreniformis* (Willis et al. 1992). Using the *COII* data of Willis et al. (1992), Engel, and Schultz (1997) confirmed that they show a close relationship of *A. koschevnikovi* to *A. florea* and *A. andreniformis*, whereas morphology and the other gene support the traditional place of *A. koschevnikovi* close to *A. cerana*. Tanaka et al. (2001) confirmed these findings from *COII* and concurred with Engel and Schultz (1997) that *COII* cannot be used for *Apis* phylogenetic studies. I have reexamined this condition by obtaining new or independent *COII* sequence for all the *Apis* species.

In addition to using the phylogenetic tree for tracing the evolution of honey bee behaviour, I will address the relationships of new honey bee species, *A. nuluensis* and *A. nigrocincta*, to the established species.

The previous honey bee phylogenetics used *Bombus* and *Xylocopa* as outgroups (Garnery et al. 1991). The latter was also used by Cameron et al. (1992) while Tanaka et al. (2001) used wasp as the outgroup. Apid molecular phylogenetics based on cytochrome b (Koulianos et al. 1999) showed that the Apini form a sister tribe of the Euglossini; and the Bombini are the sister tribe to the Meliponini. Data from several genes (*opsin*, 28srDNA, *lsRNA* and *cytochrome b* genes) also yielded the same results (Cameron and Mardulyn 2001; Lockhart and Cameron 2001). On the other hand, Ascher et al. (2001) found that *opsin* sequences failed to resolve the higher qualification

of the corbiculate tribes; but that adding morphological and behavioral data yielded the same, traditional result as do the behavioural and morphological data alone. Schultz et al. (2001) note that some molecular data sets are equivocal and argue that therefore, all molecular results should be set aside in favour of the morphological, paleontological and behavioral data sets. For the purpose of this study, I have selected Bombini and Meliponini as the outgroups because they perform the type of social behaviour which is the focus of interest here.

Nuclear and mitochondrial gene combined data

Previous molecular phylogenetic analyses have used mitochondrial DNA for analysing honey bee evolution. In this study I will contribute further to the inference of *Apis* phylogeny by including a nuclear gene, the inositol 1,4,5-trisphosphate receptor (*itpr*) from the study described in Chapter 2. The molecular phylogeny combines the *itpr* gene with the two mitocondrial DNA genes: cytochrome oxidase II (*COII*) and the large unit of ribosomal (*lsRNA*).

OBJECTIVE

The main objectives here are to elucidate behavioural evolution of honey bee in the areas of nesting and dance behaviour and to determine ancestral characteristic states of such behaviour traits. I have used molecular phylogenetics as my approach in tracing behavioural evolution. In addition to map the behavioural characteristics into the inferred molecular tree, I would resolve honey bee molecular phylogenies and include the newly recognised honey bee species, in my new phylogeny.

MATERIALS AND METHOD

DNA: extraction, sequencing and genomic walking

Genomic DNA was extracted from the thoraces of single bees using phenol-chloroform extraction and ethanol precipitation. DNA target regions were amplified using the Polymerase Chain Reactions (PCRs). PCRs were carried out in 50 µl reactions. Cycle sequencing conditions varied depending on the DNA target length. The basic amplification protocol was 2 min at 94^0C for initial denaturing, 35 cycles of 30 s at 94^0C, 30 s at 40-58^0C and 1 min at 72^0C for DNA elongation, followed by 10 minutes

for the final extension. DNA Sequencing was carried out according to the ABI BigDye protocol automated sequencing instruction of the supplier.

Taxon analysis

The ingroup honey bees comprise nine species of *Apis* and one subspecies, *A. d. binghamii*. *T. fimbriata* (Meliponini) and *B. terrestris* (Bombini) comprise the outgroups in the phylogenetic analysis (Table 2).

Table 2.
Bee sample locations and collectors

Bees species	Location	Collector
A. mellifera	Sydney, Australia	BPO
A. dorsata	Sabah, Malaysia	RR
A. d. binghamii	Sulawesi, Indonesia	GWO
A. laboriosa	Nepal	JP
A. florea	Chiangmai, Thailand	RR
A. andreniformis	Sumatra, Indonesia	RR
A. cerana	Sabah, Malaysia	RR
A. koschevnikovi	Sabah, Malaysia	RR
A. nigrocincta	Sulawesi, Indonesia	SH
A. nuluensis	Sabah, Malaysia	RR
T. fimbriata	Chiangmai, Thailand	RR
B. terrestris	Switzerland	RS

Collector abbreviations: BPO: Ben P. Oldroyd; RR: Rika Raffiudin; GWO: Gard W. Otis; JP: Jurgen Paar; SH: Soesilowati Hadisoesilo; RS: Regula Schimdt-Hempel

Molecular markers

Apis itpr and *COII* were analysed for all honey bees and outgroup bees. Sequence data from several *lsRNA* honey bees (Cameron et al. 1992) were used and combined with the new mitochondrial sequence data from this study.

Itpr gene

Three exons (starting 2nd exon) and two introns of *itpr*, derived from the *itpr* characterisation study (Chapter 2), were used for reconstructing honey bee phylogeny. The total region comprised of approximately 1.2 kb. In order to amplify these regions, two oligonucleotide primer sets were designed. The first set was the set of IP3For 5'-

GAATATCCTCTGGTGATGGATACA-3' and IP3Rev 5'-CCATGTTCTTCTGATG CTTTAGA 3'. The second set was the IP3W3F 5' GGTTTCCGATAGTGATGTTGA AT 3' and IP3R1781 5' AGGATTAAGAAATAAATCTAA 3'.

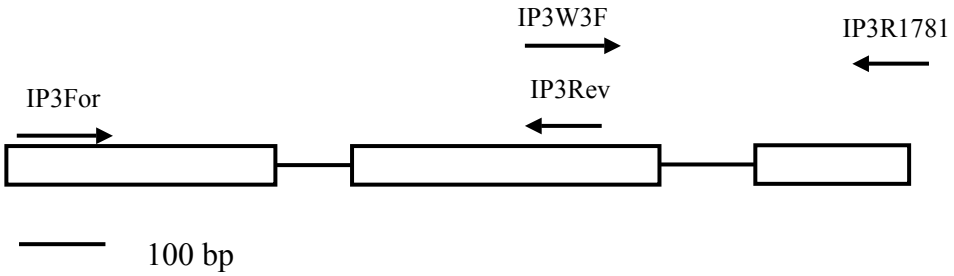


Figure 1. *Itpr* exons (boxed) and intron (lined) regions amplified. The arrows indicate primer positions in this study

Itpr gene sequences resulted in double peaks with the same intensity, is considered to denote heterozygots; therefore, PCR products were cloned. Four clones were sequenced, and the commonest nucleotide was used in the data analysis.

COII gene

Honey bee *COII* whole regions were amplified by using two sets of primers (Figure 2, Table 3). The first region (A) was amplified using E2 (Garnery et al. 1991) and H1 (Estoup et al. 1996) while the second region was amplified by using A298 (Simon et al. 1994) and tRNA aspartic acid (Willis et al. 1992). As *T. fimbriata COII* failed to be amplified using these primers pairs, I substituted with E3 and H1(Estoup et al. 1996) and reamplified using E2 and H1 as the internal primers. The B region was amplified using A298 (Simon et al. 1994) and ATP-8 (Andrew Beckenbach, personal communication) (Table 3).

Large Subunit Ribosomal RNA Gene (lsRNA)

Half of the 5'end of *lsRNA* region was amplified using primers 875-16SmF and 874-16SlR (Cameron et al. 1992).

Table 3.
PCR and sequencing primers of honey bee *COII* and *lsRNA* genes. Primer positions are based on the *A. mellifera* complete mitochondrial genome (Crozier and Crozier 1993)

Primers	**Sequence 5'-3**	**Position in *A. mellifera***	**References**
	***Cytochrome oxidase II* gene**		
E2	GGCAGAATAAGTGCATTG	3363-3380	(Garnery et al. 1991)
A298	ATTGGACACCAATGGTATTGA	3915-3935	(Simon et al. 1994)
tRNA aspartic acid	GGCCGTCTGACAAACTAATGTTAT	4312–4335	(Willis et al. 1992)
E3	ATACCACGACGT TAT TCAGA	3093-3112	(Estoup et al. 1996)
H1	GTTCATGAATGAATTACATCT G	4082-4103	(Estoup et al. 1996)
ATP8	ATTGGTGCTATTTGTGGAAT	4444-4463	Andrew Beckenbach (pers.communication)
	***lsRNA* gene**		
875-16SmF	TTATTCACCTGTTTATCAAAACAT	13924-13948	Cameron et al. (1992)
874-16SlR	TATAGATAGAAACCAATCTG	13367-13386	Cameron et al. (1992)

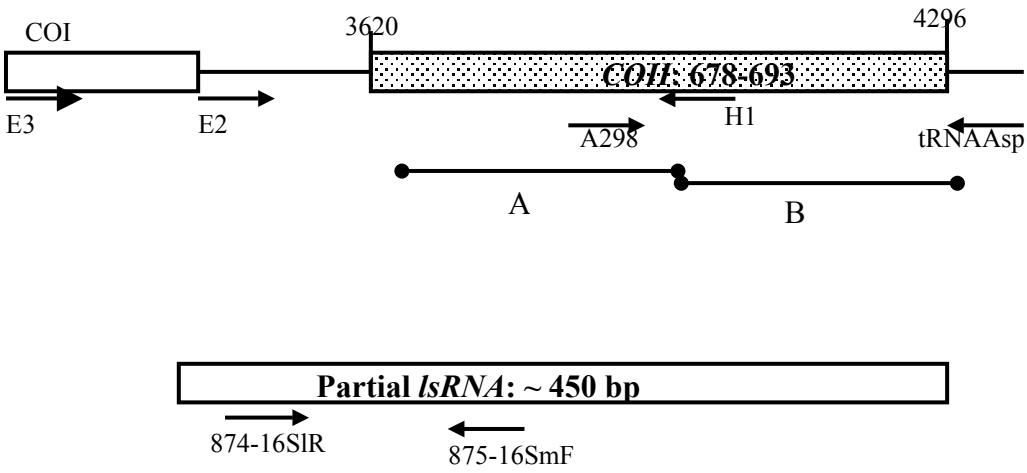


Figure 2. Honey bee *COII* (upper) and *lsRNA* (lower) primers that are used in this study. References of the primers above are referred to the Table 3.

Phylogenetic analysis

Multiple alignments of honey bee *COII* (693 bp), *lsRNA* (450 bp) and *itpr* (1.2 kb) were carried out using ClustalX (Thompson et al. 1997) and improved by eye using Se-Al (http://evolve.zoo.ox .ac.uk/Se-Al/Se-Al.html). The *MEGA* version 2.0 program (Kumar et al. 2001) was used to analyse genetic distance of each codon position of the protein coding gene (*COII* and *itpr* exons) and the transition and transversion value in *lsRNA*.

Homogeneity of the sequence data

The homogeneity of base composition between sequences was tested using the TREEPUZZLE 5.0 program (Strimmer and von Haeseler 1996).

Tree construction

Maximum Likelihood (ML) approach. ML trees (Felsenstein, 1981) for each gene and the concatenated datasets were constructed firstly by using PAUP* 4.0b10 program (Swofford 2002), applying model of DNA substitutions determined from MODELTEST 5.0. program (Posada and Crandall 1998). Bootstrap analysis (Felsenstein 1985) used 1000 replicates with random-addition sequence searches and TBR branch swapping for the each gene and the concatenation.

To take account of suboptimal trees which are not significantly different from the best, I use MOLPHY vers. 2.3. (Adachi and Hasegawa 1996), and TREEPUZZLE 5.0 (Strimmer and von Haeseler 1996) as the second analysis. The most complex model available in those programs was HKY+G+I and was used in both analysis.

By using MOLPHY program, trees were constructed firstly on the basis of each gene. Secondly, protein coding genes (*itpr* and *COII*) were partitioned for each codon position using MOLCODON as implemented in MOLPHY program. Seven partitions resulted, that are three for each protein coding gene and one for *lsRNA*. Trees were inferred for each partition and for all three genes concatenated. One substitution model was used for the concatenated data set in some major papers recently (Murphy et al 2001, Madsen et al 2001), although all their sequences were nuclear. A recent paper shows that ML (Suzuki et al., 2002) is robust in treating concatenating data in that it will correctly indicate no significance when there are different models in the partitions with different topologies. Hence, it is robust to using a simpler model that can be justified by the data. Paralleling the finding that ML treats concatenations robustly (Suzuki et al., 2002), Cunningham (1997) found that their concatenation usually yielded an improved analysis even when partitions presented different evolutionary histories. Transition and transversion ratio (α/β) of each partition and each gene were optimized using the NUCML command in MOLPHY program. Support for the nodes were obtained from

local branch probability (LBP) values using local rearrangement generated through RELL (Hasegawa and Kishino 1994).

Next, using the concatenated sequence data, the topologies derived from the trees referred to in previous paragraph were tested using the Kishino Hasegawa (KH) test (Kishino and Hasegawa 1989) implemented in MOLPHY program. The KH test has received some criticism (Goldman et al., 2000; Shimodaira and Hasegawa 1999), but further analysis has shown that it is in fact satisfactory and the best of the tests available (Strimmer and Rambaut 2002).

The test compared the likelihood of each topology to the best tree topology. For each tree topology, the KH test produces a likelihood score, and the difference in likelihood for each tree is then compared to the best (ML) tree (Δ ln), and standard error (S.E.). In addition, the KH test determines which tree topologies are significantly different from the best tree. The various trees found not to be significantly different when run against the concatenated datasets were used to infer a ML consensus tree using the program TREECONS (Jermiin et al. 1997). TREECONS weights the likelihood values against the highest (ML) result, in my case using the recommended option of exponential weighting. The data set of trees was then, as recommended by Jermiin et al. (1997), fed into the PHYLIP program CONSENSE (Felsenstein 1993), yielding a consensus tree with the support for each branch reflecting the likelihoods of those trees in which it appeared.

The tree topologies derived under the GTR+G+I and HKY+G+I substitution models were compared using the KH test implemented in PAUP* 4.0b10 (Swofford 2002). The KH test compares the ln likelihood between those two topologies with 1000 replicates under RELL bootstrapping. The comparative analyses were done under HKY+G+I and GTR+G+I model of substitution.

Maximum Parsimony approach. Maximum Parsimony (MP) unweighted based tree constructions were analysed using PAUP* 4.0b10 (Swofford 2002) for each gene and the concatenated datasets. The heuristic search and TBR branch rearrangement options

were used. Bootstrap analysis (Felsenstein 1985) used 1000 replicates with random-addition sequence searches and TBR branch swapping for each gene and the concatenation. The numbers of sites determined to be constant, variable uninformative and informative parsimony were recorded for both the single gene and partitions of each codon position.

COII comparisons

Previous studies produced anomalies results using honey bee *COII* sequences. Therefore, I reamplified the gene from all species. I combined all the sequences in order to investigate the discrepancy among *COII* honey bee sequences. *COII* sequence lists used for comparison with this present study are shown in Table 4.

Table 4.
Honey bee *COII* sequence used in data comparison

Species	Willis et al. (1992)	Tanaka et al. (2001)
A. andreniformis	M77208	-
A. dorsata	M77209	AF153126
A. cerana	M77210	AF153116
A. florea	M77211	
A. koschevnikovi	M77212	AF153124
A. mellifera	(Crozier and Crozier 1993)	AF214670
A. nuluensis	-	AF153115

Behaviour characters mapping

Given the phylogeny inferred from the above analyses, I used the parsimony approach to reconstruct the character evolution. Nesting and dance behaviour traits were mapped using MacClade version 3.07 (Maddison and Maddison 1992).

Nest behaviour data was mapped by using two hypotheses; firstly the cavity nesting in Bombini is non-homologous with that of Apini and secondly that those characters are homologous. In the first hypothesis, data were assumed to be both unordered and ordered (in different analyses) and were entered as 0, 1, 2 respectively for food pot storages and brood cells and cavity nesting, food and brood cells and open nesting and food and brood cells and cavity nesting nest. In the ordered analysis I took the order to

be 0 – 1 – 2. Secondly, I explored nesting behavioural evolution by using the hypothesis of nesting behaviour that the Bombini, Meliponini and Apini cavities are homologous. Data were assumed to be unordered and were entered as 0 and 1, respectively for cavity nesting and open.

Dance behaviour was coded as 0, 1, 2, 3 for food alert, scent trail and non-directional dance, directional horizontal dance and directional vertical dance and were assumed to be both unordered and ordered. I ordered the food recruitment behaviour started from food alert, non-directional dance behaviour, then the horizontal directional dance moving towards to the directional vertical dance.

RESULTS

Characteristics of the sequence data

Itpr sequence

Honey bee molecular phylogenetic reconstruction used a total of 1 216 bp of exon numbers 2, 3 and 4 (*A. mellifera itpr* cDNA nucleotide 322-1 287) and intron numbers 2, 3, 4 interrupted in those above exons, based on *A. mellifera itpr* characterisation in Chapter 2. The exons revealed a deletion of one amino acid in *A. cerana* and *A. nuluensis*. Consequently, both species had only 963 bp of *itpr* exons, whereas other species had 966 bp.

Intron length ranged from 70–163 bp (Table 5) reflecting insertions and deletions. The *A. koschevnikovi* 3rd intron contains 68 bp of TA repeat insertion and *B. terrestris* 4th intron has 70 bp of insertion (Table 5). These two large insertions were excluded prior to phylogenetic analysis. All honey bee and outgroup introns were inserted in the same intron phase, namely 2, 0, 1, respectively and all was commenced and ended according to the standard "GT/AG" rule.

Itpr exons and introns were AT biased, and the two highest numbers were found at the 3rd codon position and in intron region at 78.6 % and 87.4 %, respectively (Table 6). However, there were no significant variations in base composition across honey bee exon and intron data based on analysis from the TREEPUZZLE 5.0 program (Strimmer and von Haeseler 1996) (Table 7).

Itpr exons 1st and 2nd codon positions contribute five and two parsimony informative sites, respectively as compared to 70 at the 3rd codon position (Table 8). Figure 3 also shows 3rd codon substitution (ts and tv) contribution across all species. However, most 3rd codon position substitutions do not result in amino acids substitutions, leading to *itpr* amino acid conservation through all honey bee species.

Introns produced 56 informative sites (Table 8). Along with the introns, only the 3rd codon position can contribute significantly to honey bee *itpr* molecular phylogenetic reconstruction (Figure 6).

Table 5.
Itpr exon - intron length and intron deletion parts

***Itpr* partitions**	**Sequence length (basic/aligned)**	**Deletion**
Exons	963-966 bp	-
2nd intron	70-84/84	-
3rd intron	72-163/70	*A. koschevnikovi*: 68 bp
4th intron	93-163/96	*B. terrestris*: 70 bp

Table 6.
Nucleotide sequence data for each codon position and or each gene

Gene	**Base frequency average**				**% AT**
	T	**C**	**A**	**G**	
***Itpr* exons**					
1st position	20.4	19.7	27.4	32.5	47.8
2nd position	31.6	17.5	34.9	16.0	66.5
3rd position	33.2	7.4	45.3	14.0	78.6
1+2+3	28.3	14.9	35.9	20.9	64.2
***Itpr* introns**	46.0	4.6	41.4	8.1	87.4
COII					
1st position	33.2	12.3	39.4	15.1	72.5
2nd position	43.1	17.6	29.2	10.1	73.3
3rd position	49.0	3.6	46.8	0.6	95.8
1+2+3	41.8	11.2	38.5	8.6	80.3
LsRNA	38.7	7.6	39.4	14.3	77.1
***Itpr* + *COII*+16s**	34.4	14.1	36.3	15.2	70.7

COII

COII lengths ranged from 678 bp and 681 bp, whereas *B. terrestris* has 693 bp (APPENDIX 2). The 3rd codon position shows the highest AT biases (Table 6), however there is no significant difference in base composition between species (Table 7). While there is little difference in 1st and 2nd *COII* codon position rate, the 3rd *COII* codon position shows slight saturation (Figure 4).

Table 7.
The homogeneity of base composition between sequences was tested using TREEPUZZLE 5.0 program (Strimmer and von Haeseler 1996); ns = non-significant in 5 % significant level

Species	*itpr*	*COII*	*lsRNA*	*Itpr+ COII+ lsRNA*
A.mellifera	ns	ns	ns	ns
A.nuluensis	ns	ns	ns	ns
A.cerana	ns	ns	ns	ns
A.nigrocincta	ns	ns	ns	ns
A.koschevnikovi	ns	ns	ns	ns
A.laboriosa	ns	ns	ns	ns
A.d.binghamii	ns	ns	ns	ns
A.dorsata	ns	ns	ns	ns
A.florea	ns	ns	ns	ns
A.andreniformis	ns	ns	ns	ns
T.fimbriata	< 0.05	ns	ns	< 0.05
B.terrestris	< 0.05	ns	< 0.05	< 0.05

Table 8.
Numbers of constant, variable and informative sites of unweighted Maximum Parsimony analyses of *itpr*, *COII,* and *lsRNA* genes

Genes and partitions	Total characters	Constant characters	Variable uninformative Characters	Parsimony informative characters
COII (all codon positions)	693	426	112	155
1st codon position		151	39	41
2nd codon postion		194	19	18
3rd codon position		81	54	96
Itpr (exons and introns)	1216	898	185	133
Itpr exons only	966	786	103	77
1st codon position		305	12	5
2nd codon postion		318	2	2
3rd codon position		163	89	70
Itpr introns only	250	112	83	56
LsRNA	503	137	231	135
COII + 16s	1194	562	342	290
Itpr + *COII* + 16s	2410	1460	423	527

lsRNA

In *lsRNA*, transversions predominate over transitions (Figure 5) and thus high transition/transversion ratio is obtained, which is 4.

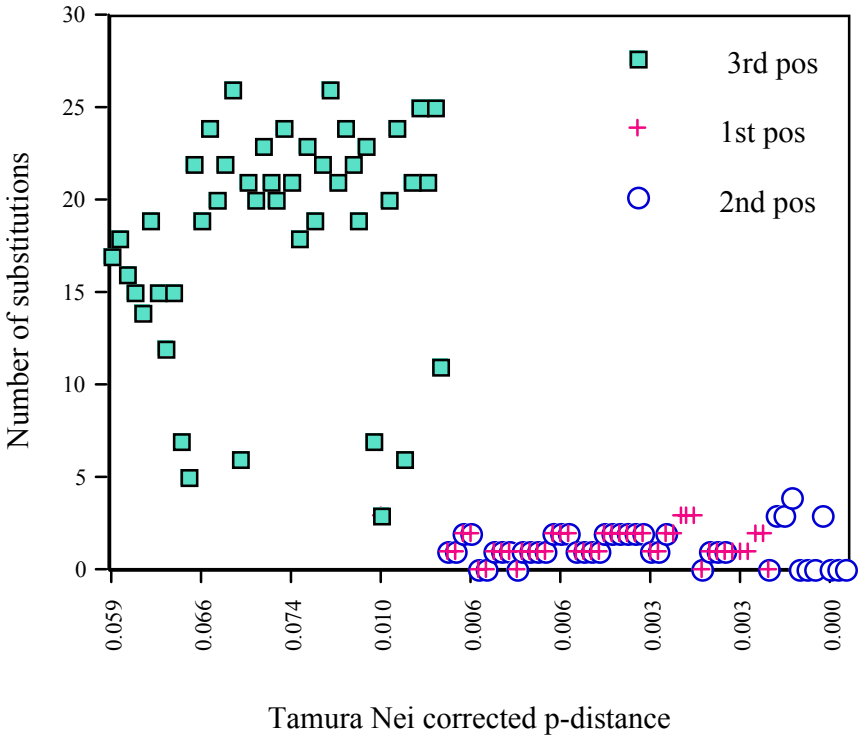


Figure 3a. *Itpr* exons substitution numbers and distances for each codon positions. Each point denotes a comparison between two sequences

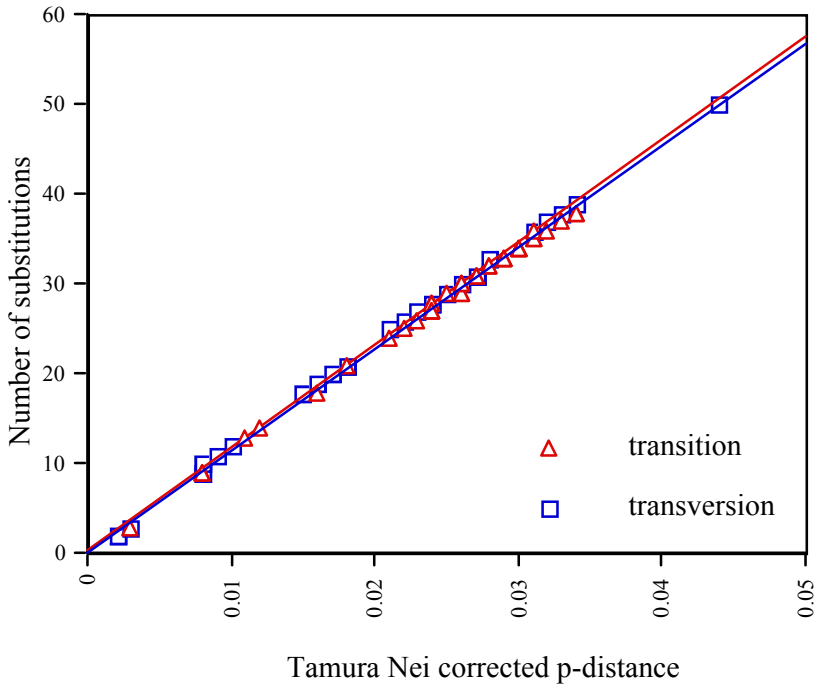


Figure 3b. *Itpr* transition and transversion relative rates. Tamura-Nei corrected distance versus substitution number of each pair of taxa

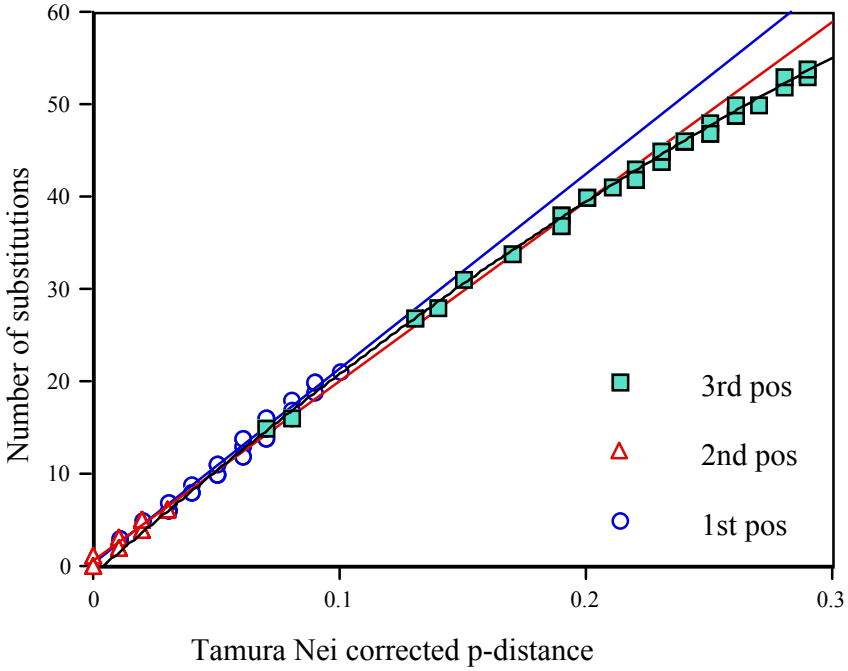


Figure 4. *COII* relative rate. Substitution numbers of each pair of species and Tamura Nei corrected distances for each codon position

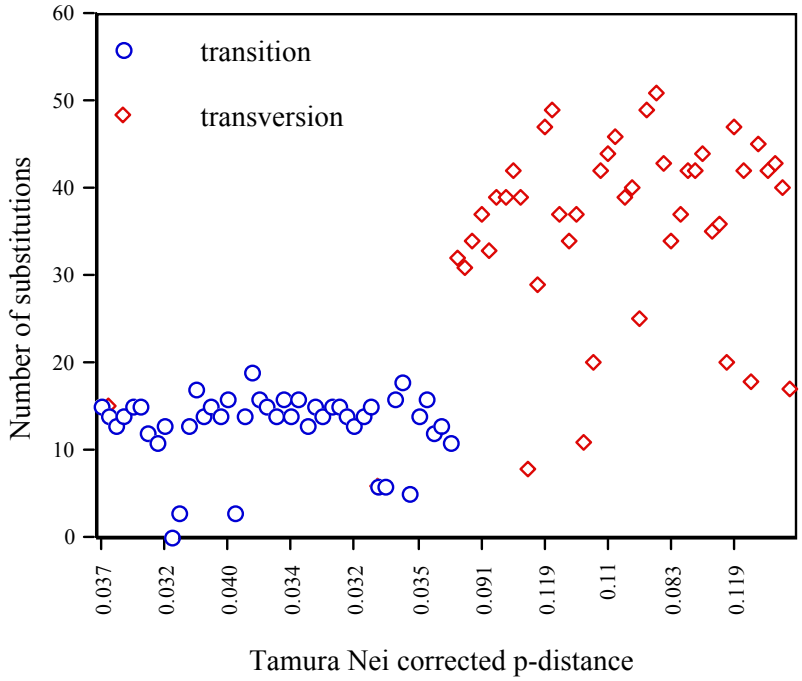


Figure 5. *lsRNA* substitution numbers of each pair of species and Tamura Nei corrected distances. Transition are replaced by transversion on higher levels of divergence

Homogeneity test of base composition between sequences

The homogeneity of base composition between sequences was tested using the TREEPUZZLE 5.0 program (Strimmer and von Haeseler 1996). The analysis was done firstly for each gene, and for three concatenated genes examined. The χ^2 test results, shown in Table 7, revealed no significant differences in all ingroup honey bee sequence data both for each gene and for the three concatenated genes but importantly show significant deviation for all data sets except that for *COII*.

Trees construction

I have used MODELTEST program to obtain model of substitution appropriate for each gene and the concatenated datasets (Table 9). ML tree for concatenated datasets applying those determined model of evolution was constructed through PAUP* 4.0b10 program as shown in Figure 6.

Table 9. List of model of substitutions determined by MODELTEST program (Posada and Crandall 1998) for each gene and the concatenated datasets.

Gene(s)	Model of substitution
Itpr	F81+G
COII	GTR+G
lsRNA	F81+G
Concatenated datasets	GTR+G+I

MOLPHY version 2.3. (Adachi and Hasegawa 1996), and TREEPUZZLE 5.0 (Strimmer and von Haeseler 1996) programs were used to construct ML trees of the different genes and applying HKY+G+I model of substitution are shown in Figure 7a, 8a, and 9a. Unweighted MP trees for each genes are presented in Figures 7b, 8b, and 9b.

The results are generally very similar, although for *COII* both MP and HKY (Figure 8a, 8b) lead to a clearly incorrect rooting of the *Apis* tree, while GTR does not (Figure 8c). *Itpr* tree reconstructed both in ML based on HKY+G+I substitution model and unweighted MP framework produced the same topologies which favoured *A. mellifera* as basal in honey bee phylogeny (Figure 7a, 7b). On the other hand, the *lsRNA* ML revealed similar topology to that of Cameron (1992), but the unweighted
MP did not (Figure 9a, 9b).

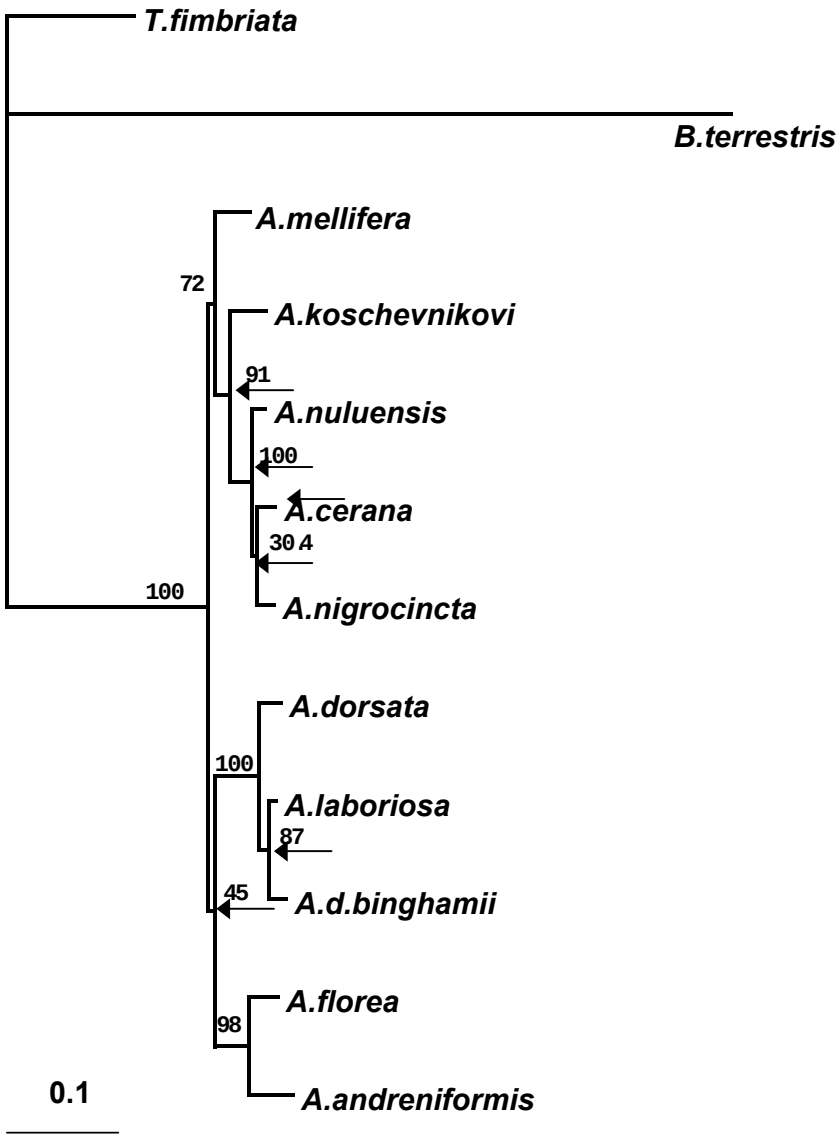


Figure 6. Honey bee most likely tree based on *itpr, COII* and *lsRNA* genes by using GTR+G+I model of substitution (ln = -9649.26840); numbers above the nodes are support for internal branches generated from 1000 bootstrap replicates.

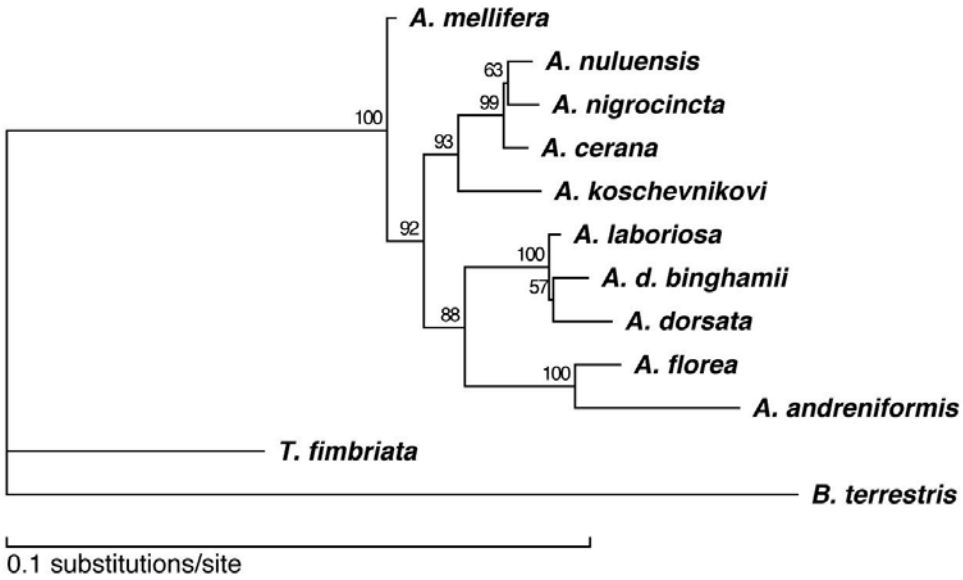


Figure 7a. Honey bee *itpr* ML tree based on HKY+G+ I substitution model; numbers above the nodes are support for internal branches generated from RELL.

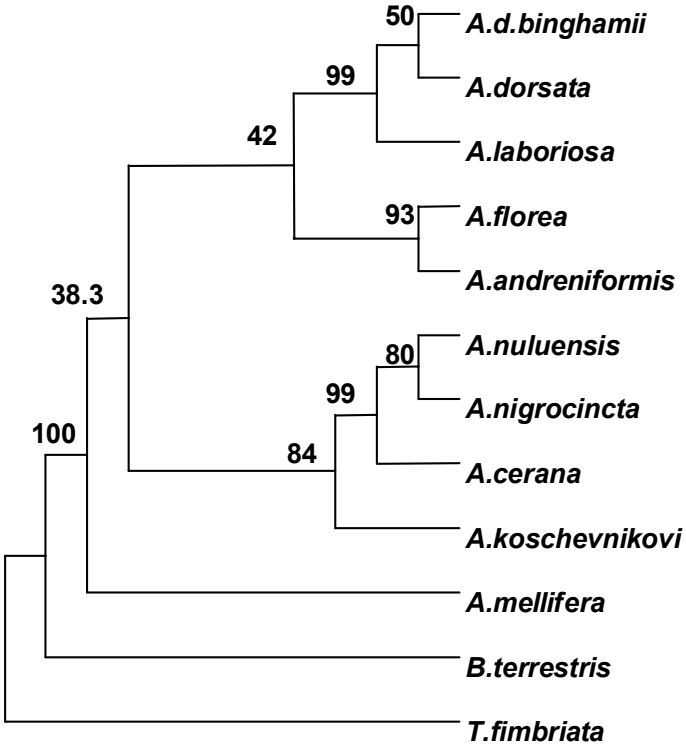


Figure 7b. Honey bee *itpr* unweighted MP tree; numbers above the nodes are support from 1000 bootstrap replicates.

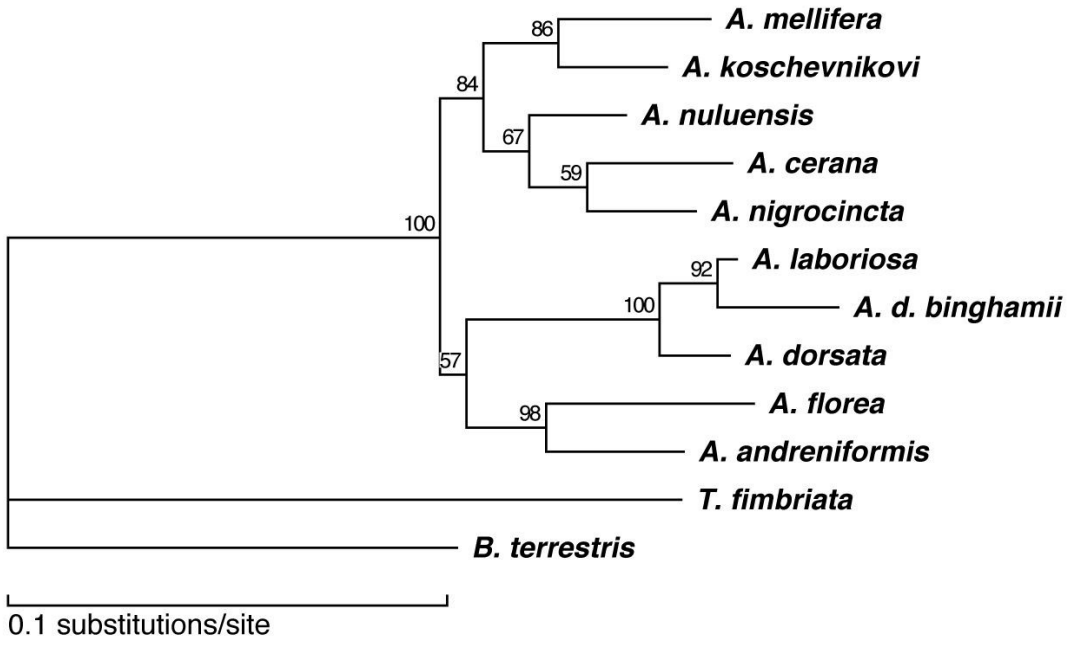


Figure 8a. Honey bee *COII* ML tree based on HKY+G +I substitution model; numbers above the nodes are support for internal branches generated from RELL.

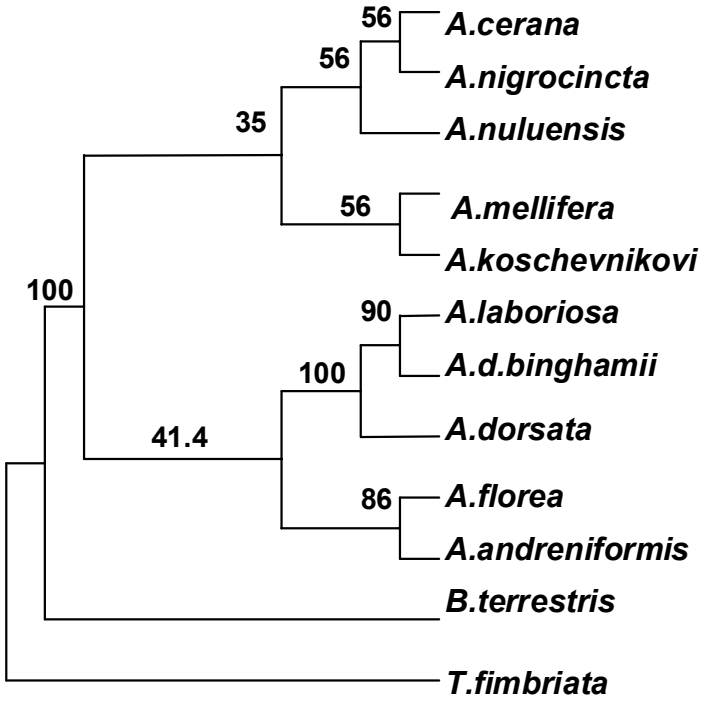


Figure 8b. Honey bee *COII* unweighted MP tree; numbers above the nodes are support from 1000 bootstrap replicates.

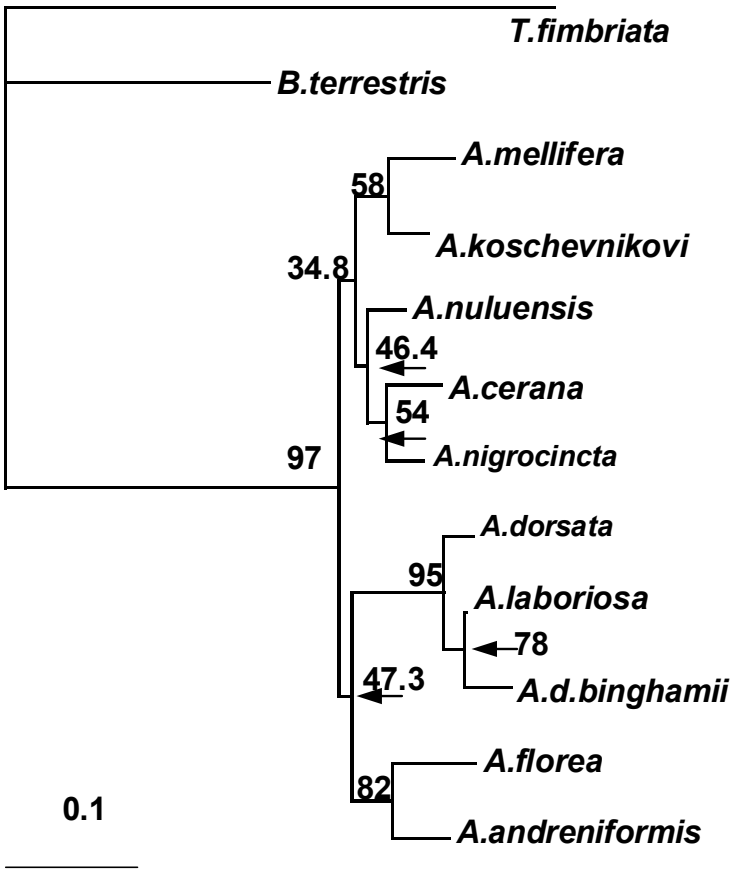


Figure 8c. Honey bee *COII* ML tree based on GTR+G substitution model; numbers above the nodes are support from 1000 bootstrap replicates.

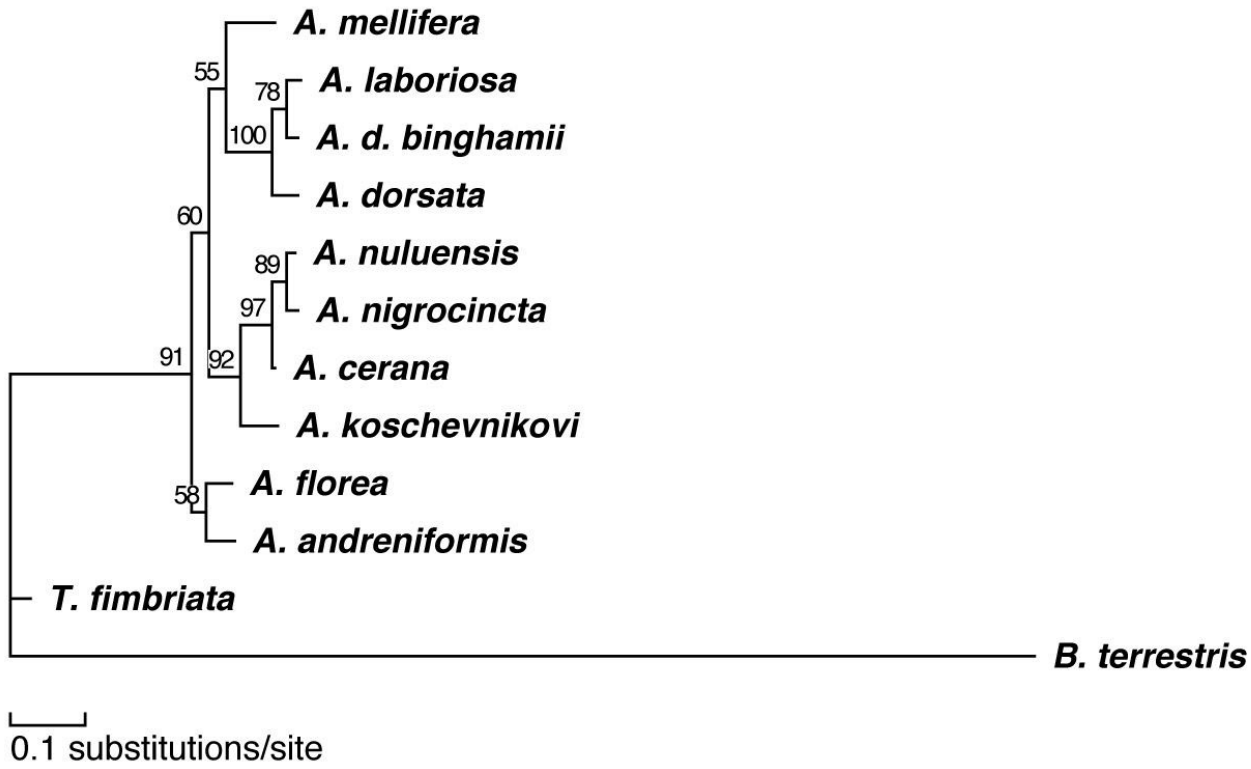


Figure 9a. Honey bee *lsRNA* ML tree based on HKY+G+I substitution model; numbers above the nodes are support for internal branches generated from RELL.

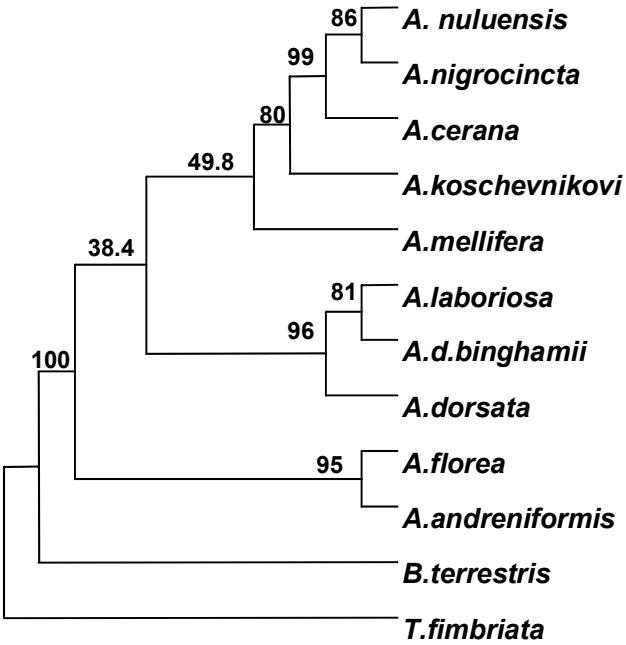


Figure 9b. Honey bee *lsRNA* unweighted MP tree; numbers above the nodes are support from 1000 bootstrap replicates.

Tree topology comparisons

By using NUCML as implemented in the MOLPHY program, tree topologies were derived from each gene as well as each codon position of the gene and the concatenated datasets. All topologies were obtained from the MOLPHY program using the HKY+G+I substitution model (Table 10).

Table 10.
Tree topology comparisons using the concatenated datasets and the HKY+G+I model. The datasets and models that produced the topologies are shown. Significance values pertain to the Kishino-Hasegawa test: M = *A. mellifera*, N = *A. nuluensis*, Ni = *A. nigrocincta*, C = *A. cerana*, K = *A. koschevnikovi*, L = *A. laboriosa*, B = *A. dorsata binghamii*, D = *A. dorsata*, F = *A. florea*, A = *A. andreniformis*, T= *Trigona fimbriata*, B = *Bombus terrestris*; ΔLn L= the difference of likelihood of a particular tree to the ML

No	Topology	Gene(s) or gene partititons tree-HKY+G+I model.	ΔLn L± S.E.	P value
1	((((M,(((N,Ni),C),K)),((L,B),D)),(F,A)),T,Bo)	3 genes-Concatenation	-10088.3	-
2	((((M,((L,B),D)),(((N,Ni),C),K)),(F,A)),T,Bo)	16s gene	- 10.6 ± 11.9	NS
3	((((M,K),(N,(C,Ni))),(((L,B),D),(F,A))),T,Bo)	*COII* gene	- 24.5 ± 23.0	NS
4	((M,((((N,Ni),C),K),((L,(B,D)),(F,A)))),T,Bo)	*Itpr* exon and intron	- 20.5 ± 16.4	NS
5	(((((M,((N,(C,Ni)),K)),((L,B),D)),A),F),T,Bo)	*COII* 1st codon position	- 30.4 ± 19.4	NS
6	((((M,(((N,C),Ni),K)),(F,A)),((L,D),B)),T,Bo)	*COII* 2nd codon position	- 13.6 ± 19.2	NS
7	((M,((((N,C),Ni),(((L,B),D),(F,A))),K)),T,Bo)	*COII* 3rd codon position	- 31.4 ± 24.0	NS
8	((((M,((N,C),(B,D))),(((Ni,F),A),T)),L),K,Bo)	*Itpr* 1st codon position	- 908.4± 70.0	< 0.05
9	((M,T),(((N,C),(Ni,((L,D),(B,(F,A))))),K),Bo)	*Itpr* 2nd codon position	-457.9 ±47.5	< 0.05
10	((M,(((((N,Ni),C),K),(L,(B,D))),(F,A))),T,Bo)	*Itpr* 3rd codon position	-41.9 ± 17.1	NS

The tree topology resulting from three concatenated genes yielded the highest likelihood score. Topologies from each gene (*lsRNA*, *COII*, and *itpr*) were not significantly worse and also the tree revealed from each codon position of *COII*. KH test only rejected the tree topologies resulted from 1st and 2nd *itpr* codon position.

The next procedure was to combine all trees that did not differ significantly from the ML tree. A consensus tree was generated using weighting scheme V in the Treecons program (Jermiin et al. 1997) at a 5% significance level. Branch confidence values were obtained from the CONSENSUS command in the PHYLIP package and the result is shown in Figure 9a. Branch length was obtained from the TREEPUZZLE 5.0 program, by using HKY+G+I substitution model and by applying tree topology results from the CONSENSUS command as the user-defined topology.

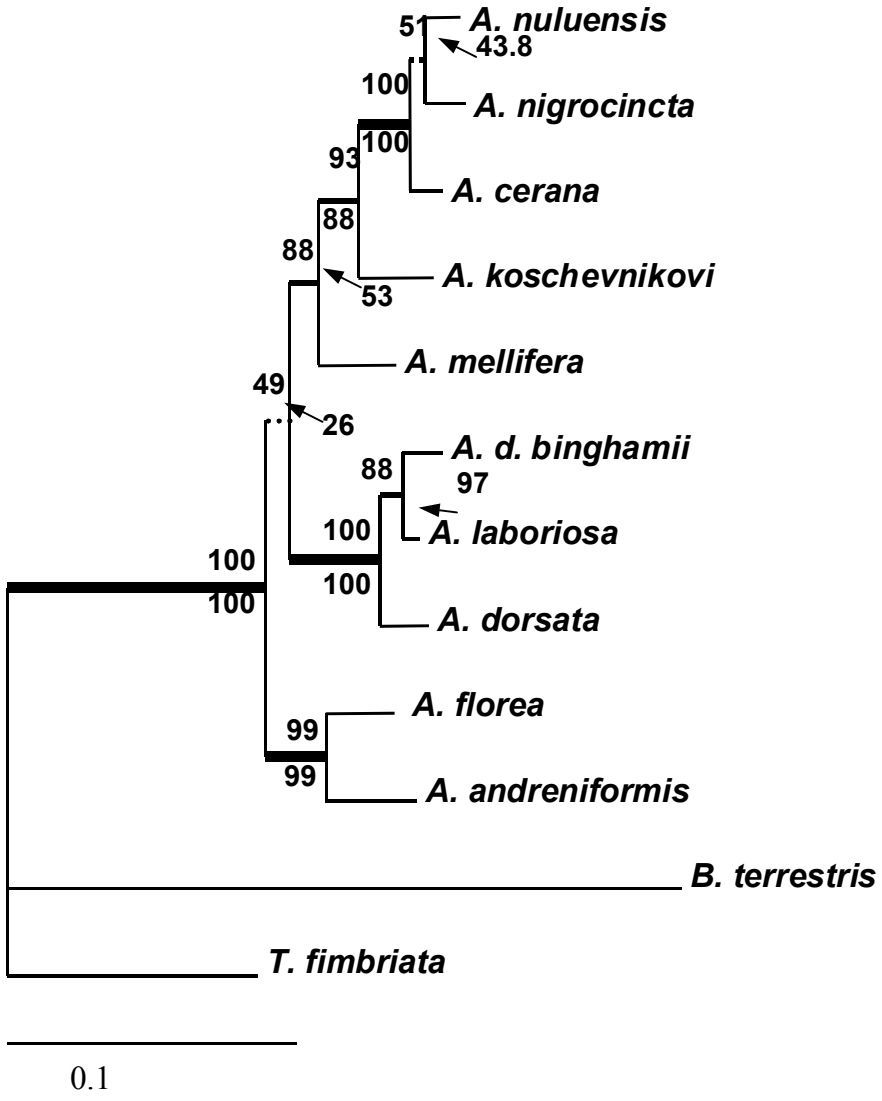


Figure 10a. ML-rule consensus of honey bee phylogenetic tree by using HKY+G+I substitution model (ln likelihood = -10088.3); numbers above the nodes are support for internal branches generated from RELL; numbers below the nodes are support from 1000 bootstrap replicates of unweighted MP tree.

Besides ML weighted consensus tree as described above (Figure 10a), trees for the concatenated datasets were constructed based the unweighted MP tree (Figure 10b).

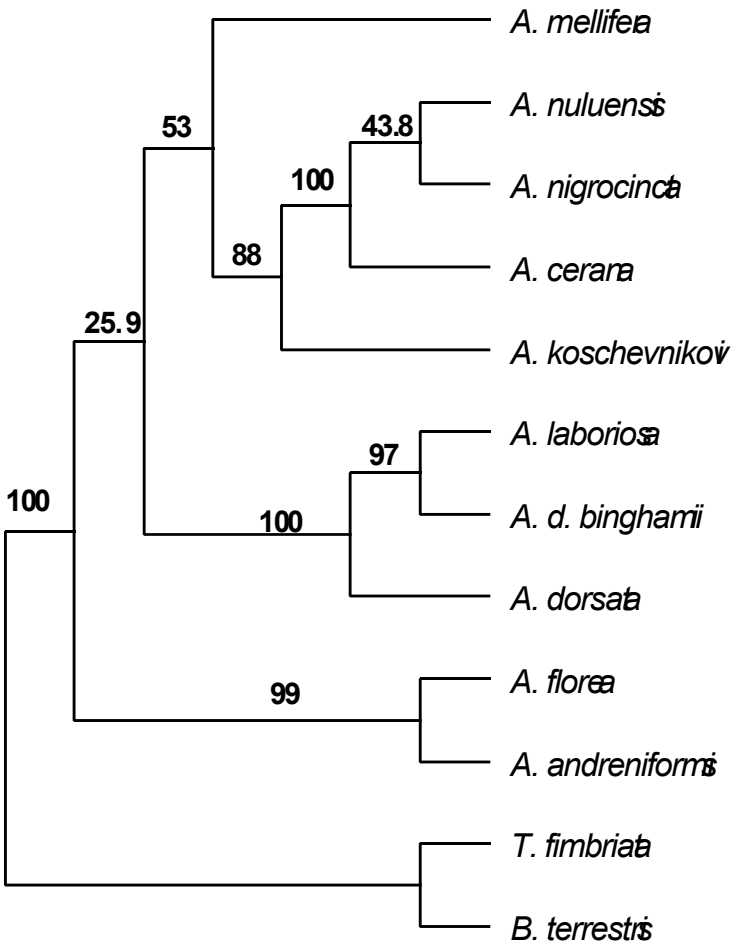


Figure 10b. The most parsimony honey bee phylogenetic tree (unweighted MP); numbers above the nodes are support for internal branches generated from 1000 bootstrap replicates. Tree length = 1482; CI = 0.748; HI = 0.252, RI = 0.586

By applying GTR+G+I model of substitution revealed that the genus *Apis* was split into two lines, the *A. mellifera* group line and the other line consists of *A. dorsata* and *A. florea* group (Figure 6). Hence, the dwarf bees group adds to the giant honey bees.

A more traditional tree was obtained from ML analysis by using HKY+G+I model of substitution and unweighted MP (Figure 10a, 10b); that is, the first split is the dwarf honeybees group (*A. florea* and *A. andreniformis*). The next split occurred in the giant honey bee *A. dorsata* group and the medium *A. mellifera* group occupied the apical tree. Hence, the dwarf honey bees are the sister group to all other honey bees.

The next step is to test whether HKY+G+I tree topology is significantly worse than that of GTR+G+I topology, and topology comparisons showed that the HKY+G+I tree topology is not significantly worse than that of the GTR topology, both under GTR and HKY models of substitution analysis (Table 11 and 12).

Table 11.
Tree topology comparisons between honey bee GTR+G+I and HKY+G+I topology under GTR+G+I model of substitution; Significance values pertain to the Kishino-Hasegawa test; ΔLn L= the difference of likelihood of a particular tree to the ML (best).

No	Tree	-ln L	ΔLn L	P
1	GTR+G+I	9649.26840	(best)	
2	HKY+G+I	9652.98495	3.71655	0.623

Table 12.
Tree topology comparisons between honey bee HKY+G+I and GTR+G+I tree topology under HKY+G+I model of substitution; Significance values pertain to the Kishino-Hasegawa test; ΔLn L= the difference of likelihood of a particular tree to the ML (best).

No	Tree	-ln L	ΔLn L	P
1	HKY+G+I	10086.49102	(best)	
2	GTR+G+I	10088.57827	2.08725	0.893

Further examination shows that the topologies differ in where the outgroup (*Trigona* and *Bombus*) joins the *Apis* tree. In HKY+G+I tree topology the outgroup joins the dwarf honey bees and in the GTR+G+I tree it joins the mellifera group. This result

indicates that rooting the *Apis* tree is not certain yet. To test this finding, using the GTR+G+I tree, I shifted the outgroup branch to that of the dwarf honey bees. Then, tree topology comparisons was done between the GTR+G+I and the GTR+G+I outgroup shifted to dwarf honey bee tree. The result is shown in Table 13; the GTR+G+I tree with the outgroup shifted is not significantly worse than that of the original GTR+G+I topology.

Table 13.
Tree topology comparisons between honey bee GTR+G+I and GTR+G+I outgroup shifted to dwarf honey bees tree topology under GTR+G+I model of substitution; Significance values pertain to the Kishino-Hasegawa test; ΔLn L= the difference of likelihood of a particular tree to the ML (best).

No	Tree	-ln L	ΔLn L	P
1	GTR+G+I	9649.26841	(best)	0.532
2	GTR+G+I outgroup shifted to dwarf honey bees	9650.65625	1.38785	

The newly-recognised *A. nuluensis* shows the closest relationship to the new *A. nigrocincta* (Figure 9a - under HKY+G+I model of evolution) as it supported with 51 % bootstrap value. Another topology for those species revealed from GTR+G+I model of substitution (Figure 6). It shows an ambiguity relationship of *A. nuluensis* to the *A. nigrocincta*. The topology shows that the most derived species are *A. cerana* and *A. nigrocincta*, but is supported by a lower confidence value (30.4%)

***COII* tree topology comparisons**

Honey bee *COII* sequences have been analysed by several authors who have described different topologies, particularly concerning the sequence of *A. koschevnikovi*. Firstly, Willis et al. (1992) found that *A. koschevnikovi* position is closely related to *A. florea*. The same *COII* tree topology was obtained by Engel and Schultz (1997) using the Willis et al. (1992) data. More recent studies, Tanaka et al. (2001) yielded the same results, using the *A. koschevnikovi* sequence from Willis et al. (1992). Engel & Schultz (1997) and Tanaka et al. (2001) concluded that the *COII* sequence is unsuitable for inferring molecular phylogenetics in *Apis.*

This present study reports *COII* sequences derived by me from all honey bee species. A phylogenetic tree derived from *COII* sequence according to Willis et al. (1992), Tanaka et al. (2001) and my present study is shown in Figure 10. The analysis was carried out using the ML approach implemented in the MOLPHY program with the HKY substitution model for estimating ts/tv ratios. The *COII* tree in Figure 11 demonstrate that the current study and those Tanaka et al. (2001), and Willis et al. (1992) are in agreement, except for placing the *A. koschevnikovi* sequence from the Willis et al. (1992) data. This finding shows that the *A. koschevnikovi* sequence reported by Willis et al. (1992) is anomalous and probably in error. Furthermore, it removes one of the three examples of Lockhart et al (1994) indicating the importance of accounting for non-stationary in phylogenetic inference.

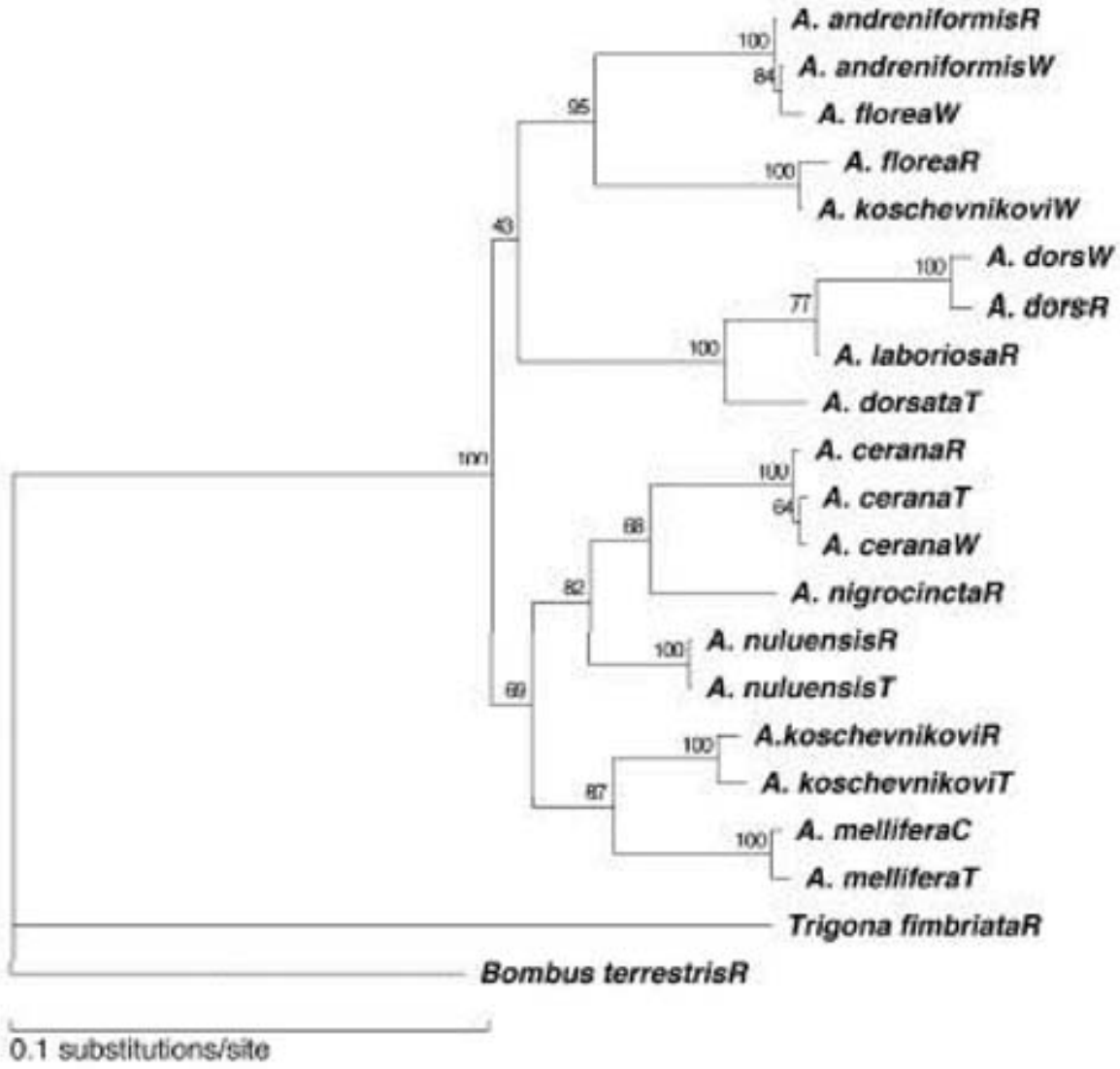


Figure 11. Honey bee *COII* phylogenetic ML tree based on HKY substitution model, compiled from previous studies. A capitol letter at the end of bee species referred to the author name, **C**: Crozier and Crozier (1993), **W**: Willis et al. (1992), **T**: Tanaka et al. (2001) and **R**: present study.

Dance behaviour characteristics map

Using honey bee ML consensus molecular phylogenetic tree and unweighted MP as my basis, I inferred the evolutionary ontogeny of dance and nesting behaviour. In order to determine the polarity of such behaviour trait evolution, I conducted a comparative analyses of those behaviour traits between the ingroup (Apini) and the outgroup (Bombini and Meliponini) bees to determine their homology.

The returning *B. terrestris* forager performs alert food running behaviour to convey information and recruit the nestmate to the food source (Dornhaus and Chittka 2001). In Meliponini, most foragers recruit nestmates to the food source by marking the pathway with a scent trail (Lindauer and Kerr 1960), although one species, *M. panamica* performs a non-directional dance (Nieh 1998). Excited food-alert running in Bombini and Meliponini produces irregular movements instead of repetitive or patterned movements as described for honey bee dance behaviour (Dornhaus and Chittka 2001). Based on that comparative analyses, food recruitment behaviour in Bombini and Meliponini bees do not indicate a common ancestor with that in Apini. Therefore, food recruitment behaviour among the bee tribes is non-homologous.

Food recruitment behaviour was mapped onto the ML (HKY+G+I model of nucleotide substitution) and unweighted MP trees, applying unordered (Figure 12a) and ordered assumption (Figure 12b). The same behaviour trait was mapped based on ML analysis under GTR+G+I evolutionary model as shown in Figure 13a (unordered) and Figure 13b (ordered assumption). I ordered the food recruitment behaviour started from food alert; scent trail or non-directional dance behaviour, horizontal directional dance and move towards to the directional vertical dance behaviour.

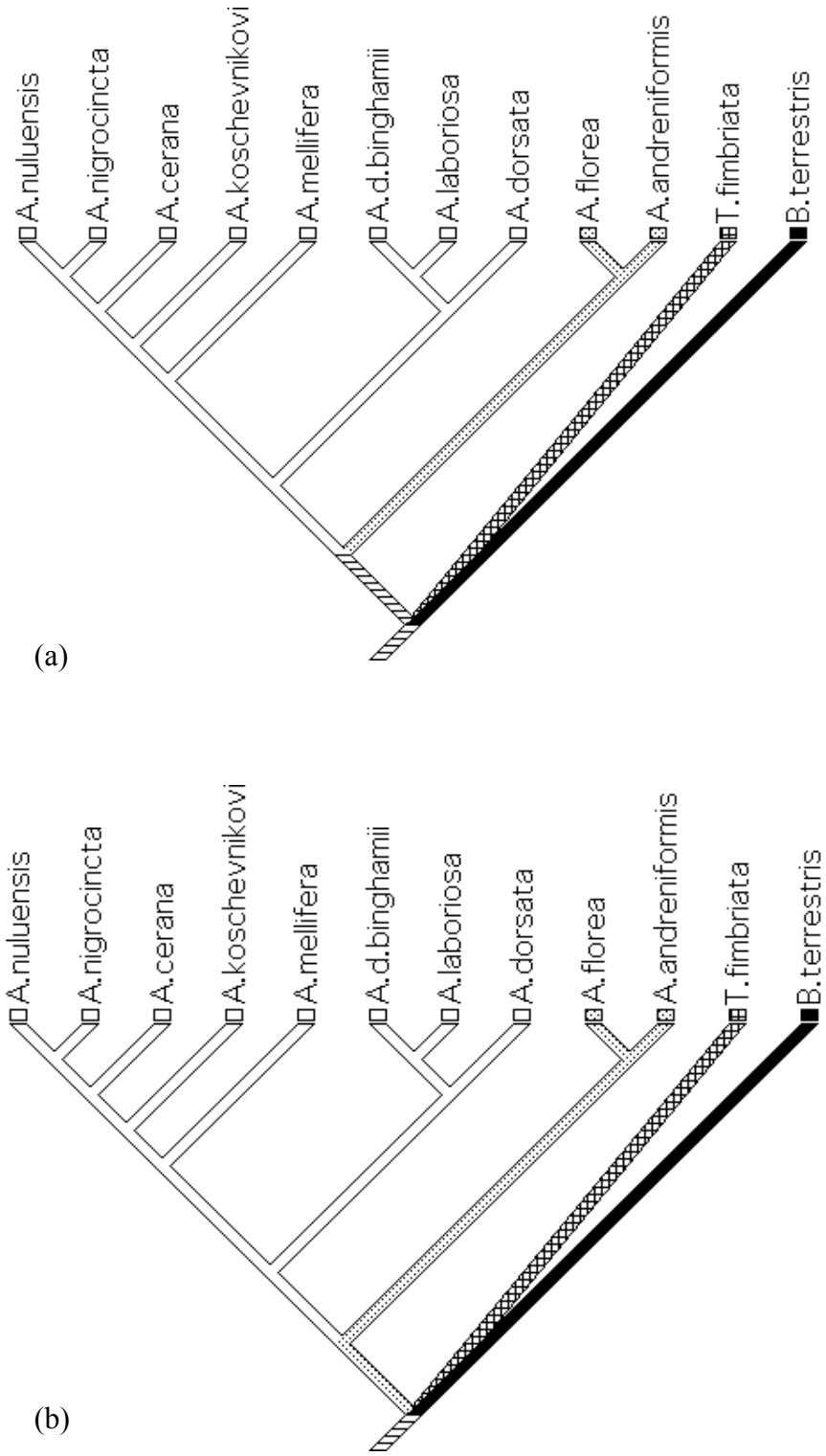
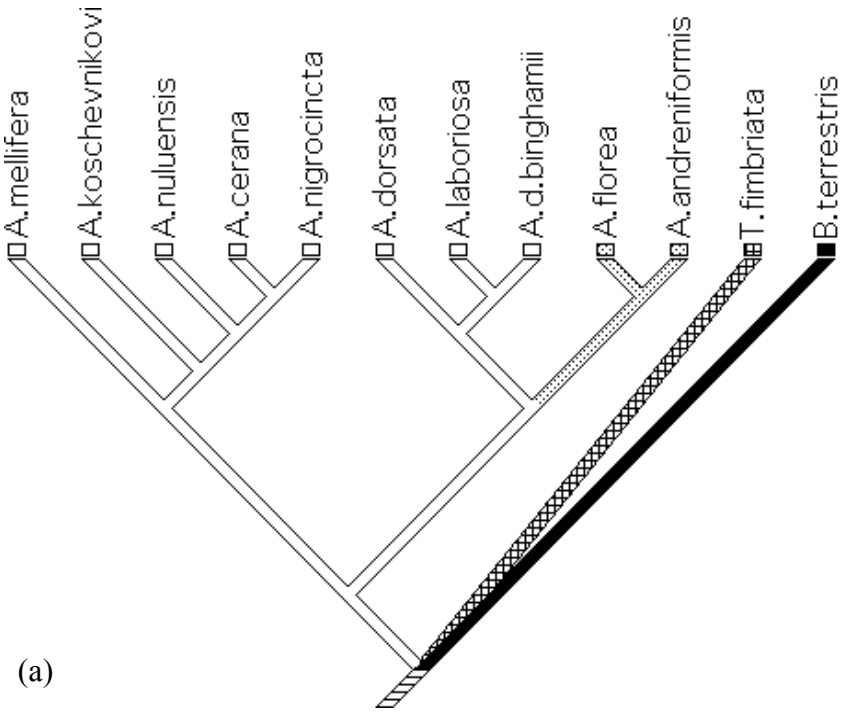
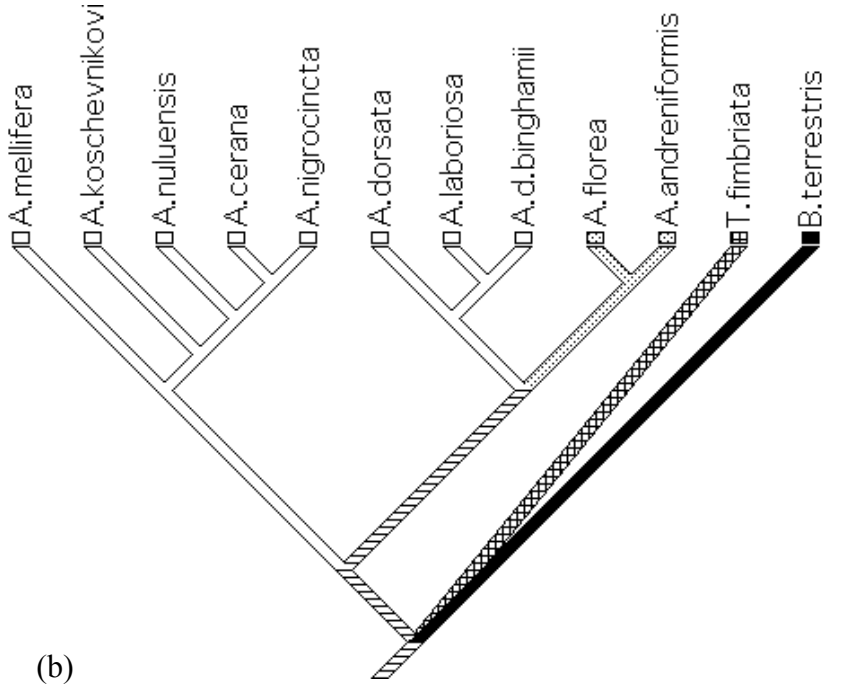


Figure 12. Mapping of honey bee **dance** behaviour onto **ML** (**HKY+G+I** model of substitution) & unweighted **MP** tree, by using (a) unordered and (b) ordered assumptions.

■ = food alert; ▦ = scent trail or non-directional dance;
▨ = horizontal directional dance; ▭ = vertical directional dance;
▤ = equivocal



(a)



(b)

Figure 13. Mapping of honey bee **dance** behaviour onto **ML** tree (**GTR+G+I** model of substitution), by using (a) unordered and (b) ordered assumptions.
■ = food alert; ▦ = scent trail or non-directional dance;
▨ = horizontal directional dance; ▭ = vertical directional dance;
▬ = equivocal

Nest behaviour characteristics map

I conducted a comparative analyses of Bombini, Meliponini and Apini nest construction behaviour. While both Bombini and Meliponini mostly nest in cavities, they build distinct types of cells for holding food and brood. Food is stored in a pot whereas broods are reared in a cell (Michener 1974). These traits do not occur in honey bee where identical cells provide both storage for food and brood. Another trait was observed, that is cavity and open-nesting. I mapped the nesting behaviour by applying homologous and non-homologous hypotheses for this trait.

Based on those comparative behaviour data, two kinds of character traits are observed: firstly pot versus non-pot cells trait, secondly cavity versus open nest trait. I explored bees nest behaviour evolution by using both traits; pot (vs non-pot cells) and cavity (vs open nest). I traced the evolution reconstruction of nesting behaviour onto the ML tree (under HKY+G+I model of DNA substitution) and the unweighted MP, by using non-homologous of nesting trait approach, and applying unordered (Figure 14a) and ordered (Figure 14b) assumption. The same behaviour was mapped onto ML tree by using GTR+G+I evolutionary model as shown in Figure 15a (unordered assumption) and Figure 15b (ordered assumption), applying non-homologous of nesting trait approach as well.

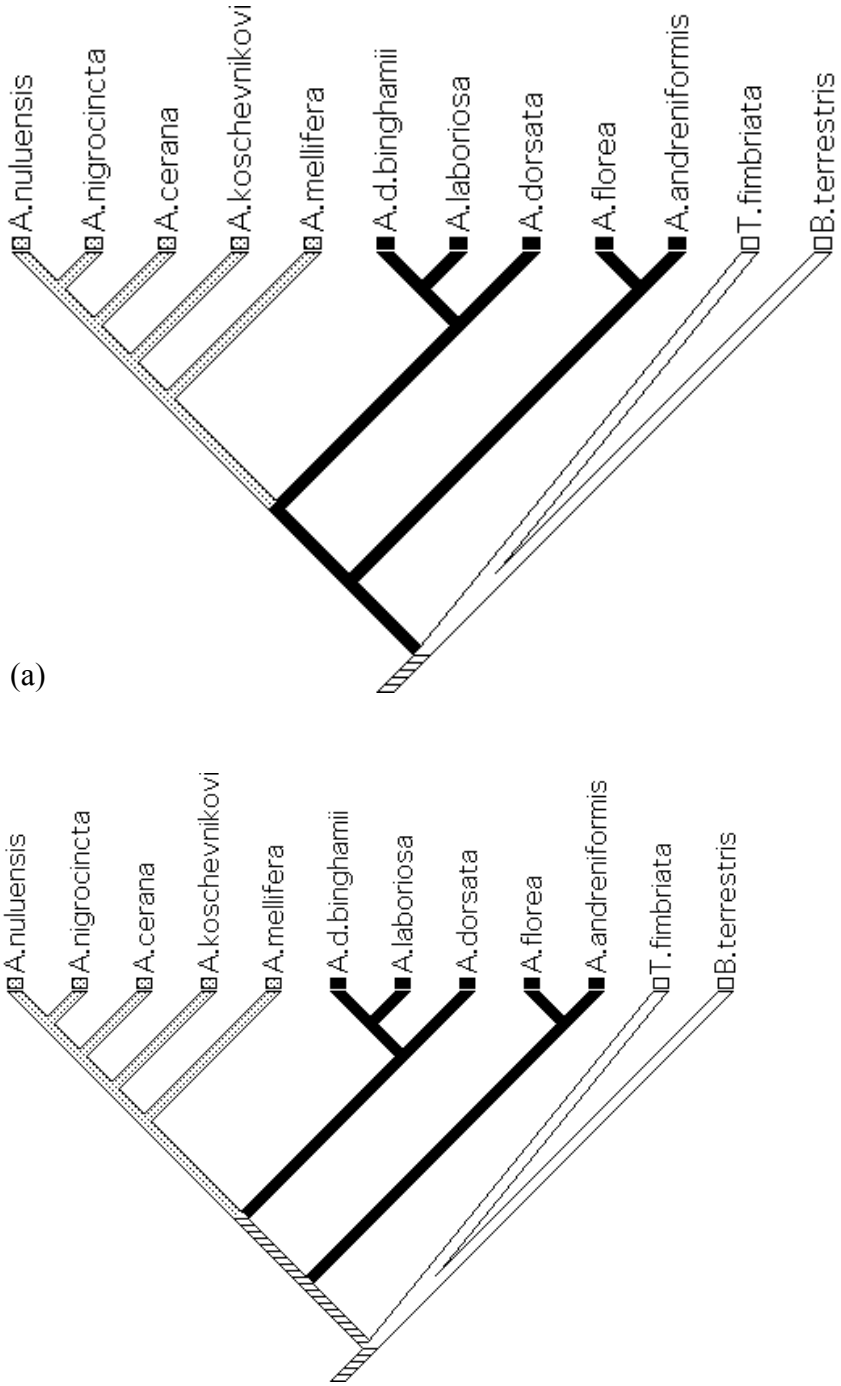


Figure 14. Mapping of honey bee **nesting** behaviour onto **ML** (**HKY+G+I** model of substitution) & unweighted **MP** tree, by using (a) unordered and (b) ordered assumptions, applying **non-homologous** assumption of nesting trait

□ = food pot storage and brood cells cavity nesting;
▒ = food and brood cells open nesting;
■ = food and brood cells cavity nesting;
▤ = equivocal

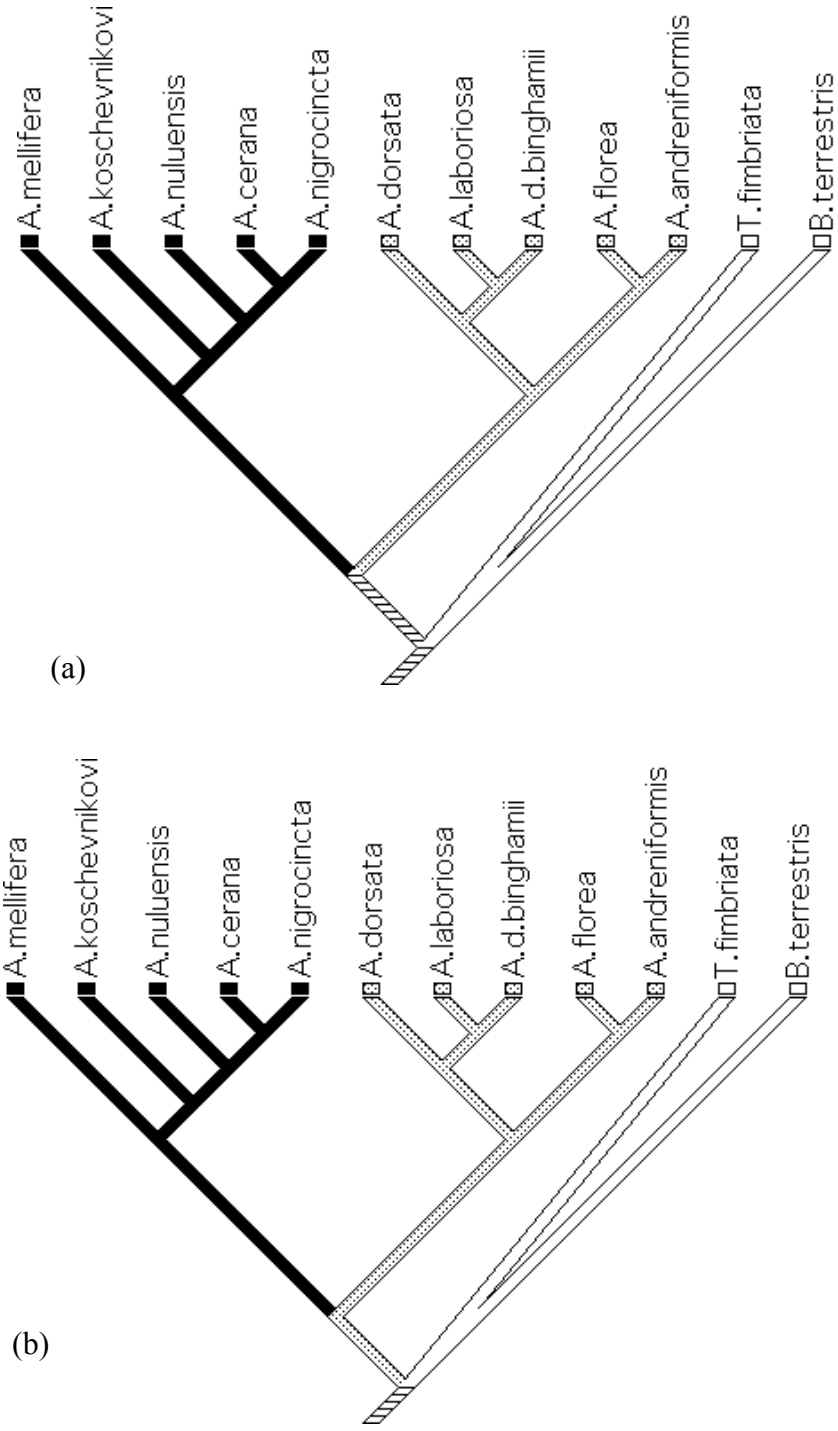


Figure 15. Mapping of honey bee **nesting** behaviour onto **ML** tree under **GTR+G+I** model of substitution, by using (a) unordered and (b) ordered assumptions, applying **non-homologous** assumption of nesting trait.
□ = food pot storage and brood cells cavity nesting,
▒ = food and brood cells open nesting;
■ = food and brood cells cavity nesting;
▤ = equivocal

Secondly, I explored nesting behavioural evolution by using hypothesis of cavity nesting behaviour of Bombini, Meliponini and Apini are homologous. That is, this hypothesis assumes honey bee (Apini) cavity nesting come from the same common of Bombini and Meliponini nests (Figure 16a and 16b).

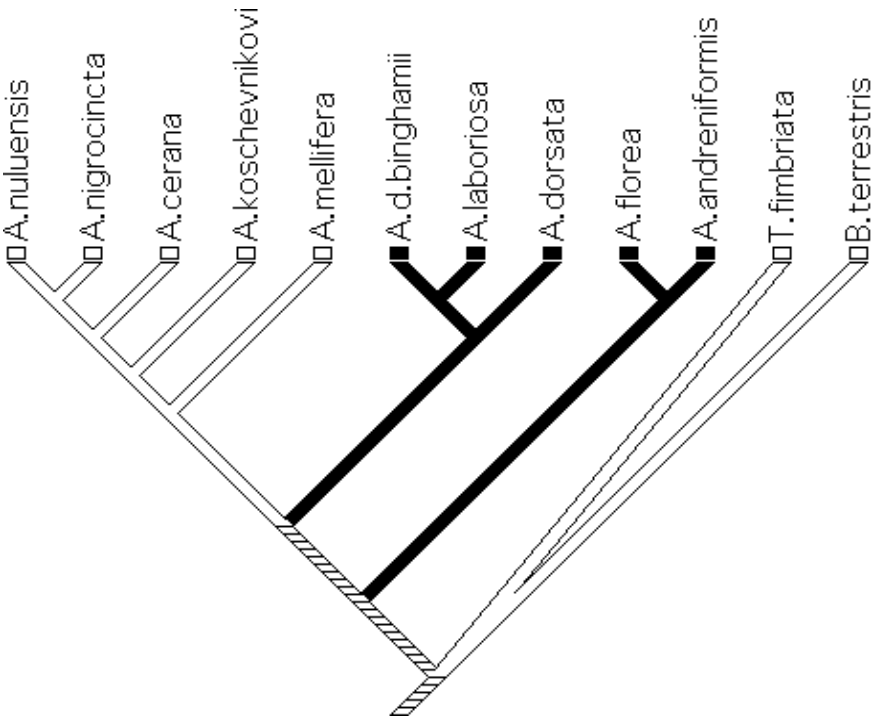


Figure 16a. Mapping of honey bee nesting behaviour onto **ML** (**HKY+G+I** model of substitution) & unweighted **MP** tree, by using unordered and ordered assumptions, and applying Bombini, Meliponini and Apini **homologous** cavity nesting behavioural trait
□= cavity nesting; ■ = open nesting; ▭= equivocal

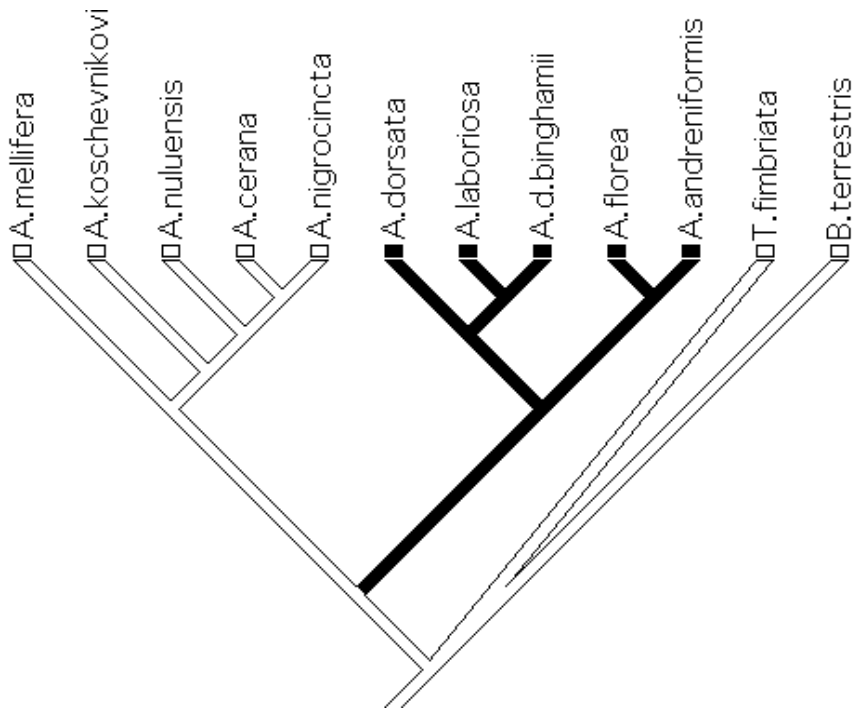


Figure 16b. Mapping of honey bee nesting behaviour onto **ML** tree under **GTR+G+I** model of substitution by using unordered and ordered assumptions, and applying Bombini, Meliponini and Apini **homologous** cavity nesting behavioural trait

□= cavity nesting; ■ = open nesting; ▤= equivocal

DISCUSSION

Honey bee tree topology

This study represents the first honey bee molecular phylogenetic study of all presently known species. ML analysis by using GTR+G+I model of evolution shows that *A. dorsata* and *A. florea* groups formed a single clade separate to that of *A. mellifera* group.

Consensus tree topologies derived from ML and unweighted MP based on three concatenated genes (*itpr*, *COII,* and *lsRNA*) produce traditional tree topology that is a monophyletic honey bee tree. My ML analysis applying HKY+G+I model of substitution and unweighted MP topology agree with that obtained by Alexander (1991) based on morphology for the species that are in common. In addition, previous unresolved relationships in morphological data for the *A. dorsata* groups have been resolved in the current study.

Considering the root of *Api*s, it may be that this has been affected by violation of stationarity involving *Trigona* and *Bombus* DNA sequences. Significant deviation from sequence homogeneity was found for the three genes concatenated dataset including *Trigona* and *Bombus* (Table 7, p. 66). Hence, at least from the molecular data of this study, the root for *Apis* is uncertain as yet.

A. nuluensis and *A. nigrocincta* are two recently described species of honey bee discussed in this study, with a very short branch connecting them with *A. cerana* based on HKY+G+I model of substitution and MP approaches. This finding is very interesting because both species are restricted in their distribution. Therefore, I have assumed that both species are the result of recent speciation from *A. cerana*.

Current distribution status for *A. nuluensis* is in the Kinibalu and Crocker mountainous regions of Sabah, Borneo, where *A. cerana* and *A. nuluensis* lives sympatrically (Tingek et al. 1996). However, *A. nuluensis* occupies a higher elevation level (up to 2000 m), whereas *A. cerana* is commonly found up to 1500 m above sea level. It is possible that the mountainous region of Sabah acts as a barrier to isolate *A. nuluensis* and *A. cerana.*

Both species differ in their mating times yielding a pre-mating barrier (Koeniger et al. 1996), justifying their species status under the Biological Species Concept.

A.nigrocincta distribution is restricted to the Sulawesi where *A. nigrocincta* and *A. cerana* live sympatrically as well (Hadisoesilo et al. 1995). They also differ in their mating times which serve as pre-mating behaviour barriers between the two species (Hadisoesilo and Otis 1996).

An alternative scenario regarding to *A. nuluensis* and *A. nigrocincta* evolution was shown in the ML tree under GTR+G+I model of substitution. This topology mentioned that *A. cerana* is the sister spesies to *A. nigrocincta,* however it is supported by low bootstrap value (30.4 %). Hence based on this topology both *A. cerana* and *A. nigrocincta* are derived from *A. nuluensis*. Further studies are needed to confirm the relationship of those three honey bee species.

Separate analyses of *itpr* and mtDNA sequence data also revealed different results concerning of *A. d. binghamii* relationship among other giant honey bees. Based on *itpr* gene, *A. d. binghamii* is apparently derived from *A. laboriosa* and not from *A. dorsata*. This finding might suggest that *A. d. binghamii* is a new species, or else that all three taxa are in fact conspecific. It would be interesting to investigate drone mating times occurring between *A. dorsata* and *A. d. binghamii*.

Is horizontal dance an ancestral characteristic state?

In studies of character evolution, the basic questions to be inferred from the molecular tree involve to reconstructing the character's history, in order to ascertain character changes might have occurred and in what lineages such changes took place (Ridley 1983).

Dance behaviour refers to behaviour performed by the forager honey bee in order to indicate a certain flower patch location. This type of behaviour does not exist in Bombini and Meliponini bees. Therefore, different food recruitment behaviour has evolved in each tribal lineage. If this assumption is correct, it can be reasonably claimed that dance behaviour has evolved only in the *Apis* species. From the molecular phylogeny based on ML under HKY+G+I model of evolution and unweighted MP, the earliest split is between the dwarf honey bee and the rest. The dwarf honey bees perform a horizontal dance whereas others in that clade are vertical dancers. It is more parsimonious for the ancestral honey bee to have used the dance then for the horizontal and vertical dances to have evolved independently. Hence, either horizontal or vertical dance was ancestral for the other. Which scenario is correct cannot be determined from the phylogeny.

Horizontal dance behaviour in *A. florea* has been well-documented in several studies. (Dyer 1985a; Koeniger et al. 1982). It has been established that *A. florea* can dance in the slope area of the nest platform under natural conditions as well as *A. andreniformis*. The ability of the dwarf honey bees to dance in this way represents a transition towards vertical dance behaviour. Because the horizontal dance is less versatile than the vertical one (being disarranged by darkness as in a cavity), it is mere reasonable to regard horizontal as evolving into vertical dancing than the converse; Lindauer (1961) also suggested that the horizontal dance was ancestral.

Another hypothesis of dance behaviour evolution arised by using ML analysis applying the GTR+G+I model of nucleotide evolution. Based on this model, the genus *Apis* was split into two lines, the first line consists of *A. mellifera* group and the second line consists of *A. dorsata* and *A. florea* group. Both *A. mellifera* and *A. dorsata* groups

performs vertical dance, hence there is an indication that vertical dance is the ancestral to that of horizontal.

Is open nesting an ancestral characteristic state?

My comparative nest behaviour analyses of outgroup bees and honey bees comprise two approaches, the first approach uses two different traits (pot versus non-pot and cavity versus open nest) and the second approach uses cavity nest versus open nest. Homology means when the same state is derived from a common ancestor without an intervening different state. The use of the integrated life pattern and hence compound characters precluded determining homology.

In my attempt to investigate nesting behaviour evolution, I inferred nest behaviour to be according to the second approach, namely cavity versus open nest. By making ordered and unordered assumptions, from both ML analyses (HKY and GTR model of evolution), it became evident that open nesting behaviour is an intervening state. The other possibility was that *Apis* open nest behaviour was an independently - derived behavioural character state. Consequently, I suggest that cavity nesting in Bombini and Meliponini bees is homologous with that in Apini. Therefore, honey bee cavity nest behaviour seems likely to have occurred as a reversal evolutionary trait, which originated in Bombini and Meliponini bees.

Although *A. mellifera* is well-known for cavity living, there is considerable evidence that it can also nest in the open (Ben Oldroyd, personal communication; Butler 1958). This open-nesting behaviour in *A. mellifera* could indicate a swarming nest. It is for swarming cavity nesting honey bees to choose a temporary place such as on a tree branch before determining the final cavity nesting site. Scouts honey bee performs dance behaviour which advertise the future nest place. If the colony cannot decide on a destination, it will continue to nest in the open. In this particular situation, *A. mellifera* displays ancestral characteristics of open area nest behaviour. However, no other honey bee species information data concerning their ability to live in open nests appears to be available.

Given the above data, I have formed the opinion that the ancestral condition of honey bee was either open or cavity nesting. Therefore, nest behaviour ancestral states could not be completely identified as a result of my present study. Bee nesting characteristics require further investigation in order to improve our understanding of this evolutionary behavioural trait.

Finally, further sequence information is still required in order to test some of the nodes with low support, hopefully increasing these supports.

Potential areas for further research

Honey bees are rich in interesting traits, which can be analysed in order to discover how they evolved. Examples of such traits include those of multi comb cavity behaviour versus the single comb open nesting (Ben Oldroyd, personal communication); silent waggle dance behaviour versus buzzing waggle dance behaviour (Kirchner et al. 1996), and porous versus non-porous honey bee drone cap cell construction (Ruttner 1988; Hadisoesilo and Otis 1998). These examples provide potential areas of study for future behavioural research.

Besides honey bee behavioural evolution, evolutionary studies of corbiculate bees have proved to be interesting areas for discussion. The areas of disagreement which exists between corbiculate molecular (Cameron and Mardulyn 2001) and morphological-paleontological (Schultz et al. 2001) phylogenetic trees can be analysed by using tree topology comparisons as provided in the ML analysis described in this present study. Furthermore, it is then possible to examine and map the behaviour of corbiculate bees.

APPENDIX 1. *Itpr* DNA sequence alignment

Species abbreviation: *A.mellifer*: *Apis mellifera*, *A.nuluensi*: *A. nuluensis*, *A.cerana*: *A. cerana*, *A.nigrocin*: *A. nigrocincta*, *A.koschevn*: *A. koschevnikovi*, *A.laborios*: *A. laboriosa*, *A.d.bingha*: *A. d. binghamii*, *A.dorsata* : *A. dorsata*, *A.florea*: *A.florea*, *A.andrenif* : *A. andreniformis*, *T.fimbriat*: *Trigona fimbriata*, *B.terrestr*: *Bombus terrestris*.

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A.mellifer GGA GTA TTA AGA TGT ATT GGA GAC ATG GGT GCA GTA ATG ACG AGC TTA  [  48]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  48]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  48]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  48]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  48]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  48]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ...  [  48]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ...  [  48]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  48]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  48]
T.fimbriat ... ... ... ... ... ..C ... ..T ... ..C ... ... ... ... ... ...  [  48]
B.terrestr ... ... ... ... ... ... ... ..T ... ..C ... ... ... ... ... ...  [  48]

A.mellifer ACA CTG GGA CCA GCA GGA CAA GTA TTA GCA GGA AGT TCT TCT CCA AGA  [  96]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
A.nigrocin ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..?  [  96]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
A.florea   ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
A.andrenif ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [  96]
T.fimbriat ... T.. ..T ... ..T ..C ... ... ..G ... ... ... ..A ..A ... ...  [  96]
B.terrestr ... ... ..T ... ..T ..T ... ... ..G ... ..C ... ..A ... ..T ...  [  96]

A.mellifer CCA AAA CCA CTT TTA AAG AAA GAA TAT CCT CTG GTG ATG GAT ACA AAA  [ 144]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.koschevn ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
A.andrenif ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 144]
T.fimbriat ..G ... ... ... ..G ..A ... ... ... ... ..A ..A ... ..C ... ...  [ 144]
B.terrestr ... ..G ... ... ..G ..A ... ... ... ..C ..A ..A ... ..C ..G ..G  [ 144]

A.mellifer TTG AAA ATA ATC GAA ATT TTA CAA TTT ATA CTT GAT GTT CGA TTG GAT  [ 192]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ...  [ 192]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 192]
T.fimbriat C.. ... ... ... ... ... ..G ... ..C ... ..C ..C ... ... C.. ..C  [ 192]
B.terrestr C.. ... ... ..A ... ... ... ... ..C ... ..C ... ... A.. ... ..C  [ 192]
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A.mellifer TAT AGA ATT TCT TGT TTA TTG AGT ATT TTC AAA CAA GAA TTT GAT GAA  [ 240]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 240]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 240]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 240]
A.koschevn ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ...  [ 240]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 240]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 240]
A.dorsata  ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...  [ 240]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 240]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 240]
T.fimbriat ... ..G ... ... ... ... C.. ..C ..C ... ..G ... ..G ..C ..C ...  [ 240]
B.terrestr ..C ..G ... ... ... ..G C.. ..C ..C ... ..G ... ..G ... ... ...  [ 240]

A.mellifer ACT GAA AGA GCT TCT GGT GAT TTG AGT CTC GGC CAG AAA ACT ATT GAT  [ 288]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 288]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 288]
A.nigrocin ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ...  [ 288]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 288]
A.laborios ... ... ... ..G ... ... ... ... ... ..T ... ... ... ... ... ...  [ 288]
A.d.bingha ... ... ... ..G ... ... ... ... ... ..T ... ... ... ... ... ...  [ 288]
A.dorsata  ... ... ... ..G ... ... ... ... ... ..T ... ... ... ... ... ...  [ 288]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 288]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 288]
T.fimbriat ..G ... ... ..C ... ... ... C.. ... ..T ..T ... ... ..C ..C ...  [ 288]
B.terrestr ... ... ... ... ..C ... ..C ... ... ..T ..T ... ... ... ..C ...  [ 288]

A.mellifer TTA GAA TTA ATA GGT ACA CAA GCG GAG GGT ATA TTT GGT AGC AGG TAA  [ 336]
A.nuluensi ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ...  [ 336]
A.cerana   ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ...  [ 336]
A.nigrocin ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ...  [ 336]
A.koschevn ... ... ... ... ... ... ... ... ..A ... ... ... ... ..C ... ...  [ 336]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 336]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 336]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 336]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 336]
A.andrenif ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ...  [ 336]
T.fimbriat ... ... ... ... ..C ... ... ..A ... ... ... ... ... ... ... ...  [ 336]
B.terrestr ... ... ... ... ..C ... ... ..A ... ... ... ... ... ... ... ...  [ 336]

A.mellifer TAC ATT ATT CAT TTT TTG TAA TCT TTT TTT TAT TTA TT- --- -TA TAT  [ 384]
A.nuluensi ... ... ... ... ... ..A ... .T. -.. ... ... ... ..- --- -.. ...  [ 384]
A.cerana   ..- --- -.. ... ... ..A ... .T. ... .-- ... ... ..- --- -.. ...  [ 384]
A.nigrocin ... ... ... ... ... ..A ... .T. .-- --- .-. ... ..T A-T T.. ...  [ 384]
A.koschevn ... ... ... ... ... ..A ... .T. .-- --. ... ... ..T A-T T.. ...  [ 384]
A.laborios ..T ... ... ... ... ..A ... .A. .-. ... .TA ..T A.T TA- --- ...  [ 384]
A.d.bingha ..T ... ... ... ... ..A ... .A. .-. ... .TA ..T A.T TA- --- ...  [ 384]
A.dorsata  ..T ... ... ... ... ..A ... .A. .-. ... .TA ..T A.T TAT A.T A.A  [ 384]
A.florea   ... ... ... ... ... ..A ... .A. AA. C.. .T. --T .CT ACT T-- A..  [ 384]
A.andrenif ... ... ... ... ... ..A ... .A. AA. C.. .T. ..T ACT TAT T.. ...  [ 384]
T.fimbriat .T. .C. ... TT. ..A ..T .T. CAA .-- --- --- --- .CA TTA A.T AGA  [ 384]
B.terrestr .TT ... ... T.. G.. ..T C.G .-- --- --- --- --- .CT TTC ACT ACA  [ 384]

A.mellifer TAA ATA AAT ATT TCA TAA ATT ATT TTA TTT AGC GAG GAA TGT GTG GCG  [ 432]
A.nuluensi ... ... ... ... .T. .G. ... ... ... ... ... ... ... ... ... ...  [ 432]
A.cerana   ... ... ... ... ... .G. ... ... ... ... ... ... ... ... .C. ...  [ 432]
A.nigrocin ... ... ... ... ... .G. ... ... ... ... ... ... ... ... ... ...  [ 432]
A.koschevn ... ... ... ... ... .G. ... ... ... ... ... ... ... ... ... ..A  [ 432]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 432]
A.d.bingha ... ... ... ... ... .G. G.. ... ... ... ... ... ... ... ... ...  [ 432]
A.dorsata  ... ... TT. CA. ... AT. ... ... ... ... ... ... ... ... ... ...  [ 432]
A.florea   ... ... ... ... ... .G. ... ... ... ... ... ... ... ... ... ..A  [ 432]
A.andrenif ... ... ... ... ... .G. ... ... ... ... ... ... ... ..C ... ..A  [ 432]
T.fimbriat ..T TA. ... ... ..G .G. ... G.C CC. C.. ... ... ... ... ..A ...  [ 432]
B.terrestr ..T TAG ... ... ... .G. ... ... C.. ... ... ... ... ... ... ...  [ 432]
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A.mellifer TTA GAT TTA GAT GGA CAA GGT GGT AGA ACA TTT CTG CGT GTT TTA CTC  [ 480]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 480]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 480]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 480]
A.koschevn ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ...  [ 480]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 480]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 480]
A.dorsata  C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 480]
A.florea   ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ...  [ 480]
A.andrenif ... ..C ... ..C ... ... ... ... .A. ... ... ... ... ... ... ...  [ 480]
T.fimbriat ..G ... ... ..C ..C ... ... ..C ... ... ... T.. ... ... ... ..T  [ 480]
B.terrestr ..G ... C.. ... ..C ... ... ..C C.. ... ... ..A ... ... ..G T.A  [ 480]

A.mellifer CAT TTG GCA ATG CAT GAC TAT CCT CCA CTA GTT TCC GGA GCA TTA CAT  [ 528]
A.nuluensi ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ..C  [ 528]
A.cerana   ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ...  [ 528]
A.nigrocin ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ...  [ 528]
A.koschevn ... ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ...  [ 528]
A.laborios ... ... ... ... ... ... ... ... ... ?.G ... ... ... ... ... ...  [ 528]
A.d.bingha ... ... ... ... ..C ... ... ... ... ..G ... ..T ... ... ... ...  [ 528]
A.dorsata  ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ...  [ 528]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 528]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 528]
T.fimbriat ..C ... ..T ... ..C ..T ... ..G ..G ..G ... ... ... ..G ... ...  [ 528]
B.terrestr ... ... ..T ... ..C ..T ... ..A ..G T.. ... ... ... ..G ... ...  [ 528]

A.mellifer TTG CTT TTT AGG CAT TTT AGT CAA AGA CAA GAA GTC TTA CAA GCA TTT  [ 576]
A.nuluensi ... ... ... ... ... ... ... ..G ... ... ... ..T ... ... ... ...  [ 576]
A.cerana   ... ... ... ... ... ... ... ..G ... ... ... ..T ... ... ... ...  [ 576]
A.nigrocin ... ... ... ... ... ... ... ..G ... ... ... ..T ... ... ... ...  [ 576]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...  [ 576]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...  [ 576]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...  [ 576]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...  [ 576]
A.florea   ... ... ... ... ... ... ..C ... ... ... ... ..T ... ... ... ...  [ 576]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...  [ 576]
T.fimbriat ... ... ... ..A ..C ..C ..C ... ... ... ... ... C.. ..G ..G ...  [ 576]
B.terrestr ... ... ..C ..A ..C ... ..C ... ... ... ... ..T ... ... ... ...  [ 576]

A.mellifer AAA CAA GTA TGT TAT TTA TAA TAT TAT CGT GTA ATA TAA ATG AAA AAA  [ 624]
A.nuluensi ... ... ... ... ... ... ... ... .G. .A. A.. ... ... ... ... ...  [ 624]
A.cerana   ... ... ... ... ... ... ... ... ... ... .A. A.. ... ... ... ...  [ 624]
A.nigrocin ... ... ... ... ... ... ... ... ... .G. .A. A.. ... ... ... ...  [ 624]
A.koschevn ... ... ... ... ... ... ... ... ..A ... ... A.. ... ... ..A ...  [ 624]
A.laborios ... ... ... ... .T. .AT ... .G. ... .A. A.. ... ... ... ... ...  [ 624]
A.d.bingha ... ... ... ... .T. .AT ... .G. ... .A. A.. ... ... ... ... ...  [ 624]
A.dorsata  ... ... ... ... .T. .AT ... .G. ... .A. A.. ... ... ... ... ...  [ 624]
A.florea   ... ... ... ... .T. .GT A.T AT. --- -.. A.. ... ... ... ... ...  [ 624]
A.andrenif ... ... ... ... .T. .GT A.T AT. ATC GTG TA. TA. A.. ... ... ...  [ 624]
T.fimbriat ... ... ... C.. ... A.. .CG C.G AG. .A. TA. TAG ..G G.A ..C GGG  [ 624]
B.terrestr ... ... ... C.. ... AG. -.T C.. ..A .A- .G. GG. G.. .A. ... C..  [ 624]

A.mellifer TAT AAA TTT ATT AAA TTA TTG TAA ATA GGT TCA ACT TTT GGT TTC CGA  [ 672]
A.nuluensi ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 672]
A.cerana   ... ... C.. ... ... ... ... ... ... ... ... ... ... A.. ... ...  [ 672]
A.nigrocin ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 672]
A.koschevn ... ... C.. ... ... ... ... ..A ... ... ... ... ... ... ... ...  [ 672]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 672]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 672]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 672]
A.florea   ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 672]
A.andrenif ..C ... C.. ... ... ... ... ... ... ... G.. ... ... ... ... ...  [ 672]
T.fimbriat G.A .G. ... ... ... ... ... .G. .C. ... G.. ... ... ... ... ...  [ 672]
B.terrestr G.A .G. ... ... ... ... ... CG. T.. ... G.. ... ... ... ... ...  [ 672]
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A.mellifer TAG TGA TGT TGA ATC TTA CAA ACA AAT AAA GTC AGA TTT GGA CGT TTT  [ 720]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ...  [ 720]
A.cerana   ... ... ... ... ... ... T.. ... ... ... A.. ... ... ... T.. ...  [ 720]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ...  [ 720]
A.koschevn ... ... ... ... ... ... ... ... ... ... A.. ... ... ... T.. ...  [ 720]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ...  [ 720]
A.d.bingha ... ... ... ... ... ... ... ... ... ... A.. ... ... ... T.. ...  [ 720]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ...  [ 720]
A.florea   ... ... ... ... ... ... T.. ... ... ... A.. ... ... ... ... ...  [ 720]
A.andrenif ... ... ... ... ... ... T.. ... ... ... A.. ... ... ... ... ...  [ 720]
T.fimbriat ... ... C.. A.. ... ... ... G.. ... ... ... ... ... ... ... ...  [ 720]
B.terrestr ... C.. C.. ... ... C.. T.. ... ... ... A.. C.. C.. A.. T.. ...  [ 720]

A.mellifer AAG ACA ATC AGT TGA AAA ATC GGA ACT TTG GGT TTA TAA ATC TAA AGC  [ 768]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 768]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 768]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 768]
A.koschevn ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ... ...  [ 768]
A.laborios ... ... ... ... ... ... G.. ... ... ... ... ... ... ... ... ...  [ 768]
A.d.bingha ... ... ... ... ... ... G.. A.. ... ... ... ... ... ... ... ...  [ 768]
A.dorsata  ... ... ... ... ... ... G.. ... ... ... ... ... ... ... ... ...  [ 768]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 768]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 768]
T.fimbriat ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ... ...  [ 768]
B.terrestr .C. ... G.. G.. ... G.. ... A.. ... ... ... ... C.. ... ... ...  [ 768]

A.mellifer ATC AGA AGA ACA TGG CAA TAA AAA GAA GAA AAA TAA AGA AGA CGA AGA  [ 816]
A.nuluensi ... ... ... ... ... ... ... ... ... A.. ... ..G ... ... ... G..  [ 816]
A.cerana   ... ... ... ... ... ... ... ... ... A.. ... ..G ... ... ... G..  [ 816]
A.nigrocin ... ... ... ... ... G.. ... ... A.. ... ... ... ... ... ... G..  [ 816]
A.koschevn ... ... ... ... ... ... C.. ... ... ... ... ..G ... ... ... ...  [ 816]
A.laborios ... ... ... ... ... ... C.. ... A.. ... ... ... ... ... ... ...  [ 816]
A.d.bingha ... ... ... ... ... ... C.. ... A.. ... ... ... ... ... ... ...  [ 816]
A.dorsata  ... ... ... ... ... ... C.. ... A.. ... ... ... ... ... ... ...  [ 816]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ...  [ 816]
A.andrenif ... ... ... ... ... ... ... ... ... ... R.. ... ... ... G.. ...  [ 816]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 816]
B.terrestr ... G.. G.. ... ... T.. ... ... ... ... G.G ... ... G.. A.. ...  [ 816]

A.mellifer TGA TGG AGC TAC TCC TCG TAA AGC ACC ACC ACA ACT ATC TAC GAC GGA  [ 864]
A.nuluensi ... ?.- --. ... ... ... ... G.. ... ... ... ... ... ... A.. A..  [ 864]
A.cerana   ... ..- --. ... ... ... ... G.. ... ... ... ... ... ... ... A..  [ 864]
A.nigrocin ... ... ... ... ... ... ... G.. ... ... ... ... ... ... ... A..  [ 864]
A.koschevn ... ... ... ... ... ... ... G.. ... ... ... ... ... ... ... ...  [ 864]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 864]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 864]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 864]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ...  [ 864]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ...  [ 864]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 864]
B.terrestr ... ... ... ... ... ... A.. ... T.. G.. T.. .T. G.. ... AT. ...  [ 864]

A.mellifer TAA GAA AGG TTT TGT TAA TAA TTT TTT TTA AAT AAA ATA TTG AAA TAA  [ 912]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...  [ 912]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...  [ 912]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..G ...  [ 912]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...  [ 912]
A.laborios ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 912]
A.d.bingha ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 912]
A.dorsata  ... A.. ... ... ... ... ... ... ... ... C.. ... ... ... ... ...  [ 912]
A.florea   ... ... ... ... ... ... ... AA. ... ... ... ... ..G .A. ... ...  [ 912]
A.andrenif ... ... ... ... ... ... ... ... ... ... ..A T.. .AT G.A G.. -.T  [ 912]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [ 912]
B.terrestr ... A.. ... .A. .CC .G. ... .C. CAC A.. TGG .GT .AT .AA ... ..G  [ 912]
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A.mellifer ATT GAA AAA TCA AAG GCA AAA --T TTT CCT AAT ACA AAT ATT ATT TAT  [ 960]
A.nuluensi G.. ... ... ... ... ... ... --. ... ... ... ... T.. ... ... ...  [ 960]
A.cerana   G.. ... ... ... ... ... ... --. ... ... ... ... ... ... ... ...  [ 960]
A.nigrocin G.. ... ... ... ... ... ... --. ... ... ... .T. ... ... ... ...  [ 960]
A.koschevn G.. ... ... ... ... ... ... --. ... ... ... .A. ... ... ... ...  [ 960]
A.laborios G.. ... ... ... ... ... ... --. ... T.. ... ... ... ... ... ...  [ 960]
A.d.bingha G.. ... ... ... ... ... ... --. ... ... ... ... ... ... ... ...  [ 960]
A.dorsata  G.. ... ... ... ... ... ... --. ... T.. ... ... ... ... ... ...  [ 960]
A.florea   G.- -.. ... ... ... A.. ... AA. ... T.. ... .T. ... ... ... ...  [ 960]
A.andrenif .AG T.. ... ... ... A.. C.. --. ... T.. ... .T. ... ... ... ...  [ 960]
T.fimbriat ... ... ... ... ... ... ... --. ... ... ... ... ... ... ... ...  [ 960]
B.terrestr GAA T.T .GG AAT ..A AT. T.- -T. C.. T.. G.C GT. G.A ... ... ...  [ 960]

A.mellifer AAT TAT AGG ATC TGC AAT AGA TTT AGA TAT TGG TCC ACC GTT ACA TGC  [1008]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...  [1008]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ...  [1008]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1008]
B.terrestr ... .G. ... G.. ... G.. ... ... ... C.. ... ... G.. A.. G.. C..  [1008]

A.mellifer AGA TCA AGC GGA GGA ATA TAA AAA AAT ACA ACA AAT TCT AAT TCG AAT  [1056]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ...  [1056]
A.cerana   ... ... ... ... ... ... ... G.. ... ... ... ... .T. ... ... ...  [1056]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ...  [1056]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ...  [1056]
A.laborios ... ... ... ... ... ... C.. ... ... T.. ... ... ... ... ... ...  [1056]
A.d.bingha ... ... ... ... ..R ... C.. ... ... ... G.. ... ... ... ... ...  [1056]
A.dorsata  ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ...  [1056]
A.florea   ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ... ...  [1056]
A.andrenif ... ..T ... ... ... ... ... ... ... ... G.. ... ... ... ... ...  [1056]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1056]
B.terrestr ... C.. ... ... ... ... ... ... G.. ... G.. G.. ... ... ... ...  [1056]

A.mellifer GAA CAA ATT ATG TAT CCA AAC GAT AGG TGG TCA AAT AAA ACC ACG AAA  [1104]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ...  [1104]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ...  [1104]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ...  [1104]
A.koschevn ... T.. ... ... ... ... ... ... ... ... ... G.. ... ... ... ...  [1104]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1104]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1104]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1104]
A.florea   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1104]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1104]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1104]
B.terrestr ... ... G.. ... C.. T.. ... ... ... ... ... ... ... ... ... ...  [1104]

A.mellifer ACA TGA ACA AAG ACT TTT ACG TAA TGT TGG AGT ACA TAC CGT TGT TTT  [1152]
A.nuluensi ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ... ...  [1152]
A.laborios ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
A.d.bingha ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
A.dorsata  ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
A.florea   ... C.. ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
A.andrenif ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...  [1152]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1152]
B.terrestr ... C.. G.. ... ... ... ... ... C.. C.. ... ... ... G.. C.. C..  [1152]
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A.mellifer AGA TTT ATT ACA AGT TCC ATT TGA CGC GAA AGA AGA TGT TAG AAT GAA  [1200]
A.nuluensi ... ... ... ... ... ... ... ... ?.. ... ... ... ... ... ... ...  [1200]
A.cerana   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1200]
A.nigrocin ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1200]
A.koschevn ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1200]
A.laborios ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ... ...  [1200]
A.d.bingha ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ... ...  [1200]
A.dorsata  ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ... ...  [1200]
A.florea   ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ... ...  [1200]
A.andrenif ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ... ...  [1200]
T.fimbriat ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [1200]
B.terrestr ... C.. G.. G.. ... ... G.. ... ... ... G.. ... C.. C.. ... ...  [1200]

A.mellifer TGA GTT AAT GCG ATT A [1216]
A.nuluensi C.. A.. ... ... ... G [1216]
A.cerana   ... A.. ... ... ... G [1216]
A.nigrocin ... A.. ... ... ... G [1216]
A.koschevn ... ... ... ... ... . [1216]
A.laborios ... A.. ... ... ... . [1216]
A.d.bingha ... A.. ... ... ... . [1216]
A.dorsata  ... A.. ... ... ... . [1216]
A.florea   ... ... ... ... ... . [1216]
A.andrenif ... ... ... ... ... . [1216]
T.fimbriat ... ... ... ... ... . [1216]
B.terrestr C.. ... G.. ... ... G [1216]
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APPENDIX 2. *COII* sequence alignment

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A.mellifera     ATT TCC ACA TGA TTT ATA TTT ATA TTT CAA GAA TCA AAT TCA TAT TAT  [ 48]
A.nuluensis     ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ...  [ 48]
A.cerana        ... ..T ... ... ..C ... ... ..G ... ... ... ... ... ... ... ...  [ 48]
A.nigrocincta   ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ..C ...  [ 48]
A.koschevnikovi ... ..A ... ... ... ... ... ... ... ... ... ..T ... ... ..C ...  [ 48]
A.laboriosa     ... ..A ..G ... A.A ... C.A ... ... ... ... ... ... ... .TC ...  [ 48]
A.d.binghamii   ... ..A ..G ... A.A ... C.A ... ... ... ... ... ... ... .TC ...  [ 48]
A.dorsata       ... ..A ... ... A.A ... ..A ... ... ... ... ... ... ... .T. ...  [ 48]
A.florea        ... ..T ... ... A.A ... ... ... ... ... ... ... ... ..C ... .T.  [ 48]
A.andreniformis ... ..T ... ... A.A ... ... ... ... ... ... ..G ... ... ... .TA  [ 48]
T.fimbriata     ..G ..A ..T ... AA. ... .A. TC. ... ... ... ..T ... ... ..T .TA  [ 48]
B.terrestris    ... ..T ... ... AAC ... ... T.. ... ... ... A.T ... ... ..T .TC  [ 48]

A.mellifera     GCT GAT AAT TTA ATT TCA TTT CAT AAT ATA GTT ATA ATA ATT ATT ATT  [ 96]
A.nuluensis     ... ... ... ... ..C ... ... ... ... ... ..A ... ... ... ... ...  [ 96]
A.cerana        ... ... ... ... ..C ... ... ... ... ... ..A ... ... ... ... ...  [ 96]
A.nigrocincta   ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ...  [ 96]
A.koschevnikovi ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C  [ 96]
A.laboriosa     ..A ... ... ... ..C ..T ... ... ... ... ..A ... .C. ..A ... ...  [ 96]
A.d.binghamii   ..A ... ... ... ... ..T ... ... ... ... ..A ... .C. ..A ... ...  [ 96]
A.dorsata       ..A ... ... ... ... ... ... ... ... ... ..A ... .C. ..A ... G.A  [ 96]
A.florea        ... ... ... ... G.G ... ... ..C ... T.. ..A ... ... ... ... ...  [ 96]
A.andreniformis ... ... ... ... G.A ..T ... ... ..C ... ..A ... ..G ... G.A ...  [ 96]
T.fimbriata     T.C ... ... ... ... ... ... T.C ... T.T ..A ... ..G T.. ... G.A  [ 96]
B.terrestris    T.. ... ... C.T ... ... ... ... ... T.. ACA ... ... ..A ..A ..A  [ 96]

A.mellifera     ATA ATT TCA ACA TTA ACT GTA TAT ATT ATT TTA GAT TTA TTT ATA AAC  [144]
A.nuluensis     ... ... ... ..T ... ..A ..T ..C ... ... .C. ... C.T ... T.. ..T  [144]
A.cerana        ... ... ..T ..T ... ..A ... ... ... ... A.. ... ... ... T.. ..T  [144]
A.nigrocincta   ... ... ..T ..T ... ..A ..T ... ... ... ... ... C.. ... T.. ..T  [144]
A.koschevnikovi ... ... ... ... ... ..A ..T ... ... ... A.. ... ... ... T.. ...  [144]
A.laboriosa     ... ... ... ... ... ..A A.T ... ... ... A.. ..C ... ... T.. ..T  [144]
A.d.binghamii   ... ... ... ... ... ..A A.T ... ... ... A.. ..C ..G ... T.G ..T  [144]
A.dorsata       ... ... ... ... ... ..A A.T ... ..C ... A.. ... C.. ..C ... ..T  [144]
A.florea        ... ... ... ... C.T ..A A.T ... ... ... ..T ... ... ... ... ..T  [144]
A.andreniformis ... ... ... ... ... ..A ..T ... ... ... ..T ... ... ... T.. ..T  [144]
T.fimbriata     ... ... A.T T.. ... ... T.. .T. T.. ... A.T ... ..T A.. ... ..T  [144]
B.terrestris    ... ... ATT ..T ... ..C A.. .T. T.C ... A.. ... ..T .CA ... ..T  [144]

A.mellifera     AAA TTC TCA AAT TTA TTT TTA TTA AAA AAT CAT AAT ATT GAA ATT ATT  [192]
A.nuluensis     ... ..T ... ... ... ..C ... ... ... ... ... ... ... ... ... ...  [192]
A.cerana        ... ..T ... ... C.. ... ... ... ... ... ... ... ... ... ..C ...  [192]
A.nigrocinsta   ... ..T ... ... ... ..C ... ... ... ... ... ... ... ... ... ..C  [192]
A.koschevnikovi ... ..T ..T ... ... ... ... ... ... ... ... ... ... ... ... ...  [192]
A.laboriosa     ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [192]
A.d.binghamii   ... ..T ... ... ... ... ... ... ... ... A.. ... ... ... ... ...  [192]
A.dorsata       ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [192]
A.florea        ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [192]
A.andreniformis ... ..T ... ... ... .A. ... C.T ... ... ... ... ... ... ... ..C  [192]
T.fimbriata     ... .AT ATT ..C ... AA. ... ... ... ... ... .CA ... ... ... G.A  [192]
B.terrestris    ..T ... .T. ... ... AA. C.. ... ... ... ... .C. ... ... ... ...  [192]
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A.mellifera     TGA ACA ATT ATT CCA ATT ATT ATT CTA TTA ATT ATT TGT TTT CCA TCA  [240]
A.nuluensis     ... ... G.. ..C ... ... ... ... T.. ... ... ... ... ... ... ...  [240]
A.cerana        ... ... G.A ... ... ... ... ... T.. ... ... ... ... ... ... ...  [240]
A.nigrocincta   ... ... G.. ... ... ... ... ... T.. ... ... ... ... ... ..T ...  [240]
A.koschevnikovi ... ... G.A ... ... ... ... ... ..T ... ... ... ... ... ... ..T  [240]
A.laboriosa     ... ... ... ... ... ... T.. G.. ..T ... ... ... ... ... ... ...  [240]
A.d.binghamii   ... ... ... ... ... ... T.. G.. ..T ... ... ... ... ... ... ...  [240]
A.dorsata       ... ... ... ... ..T ... T.. G.. ..T ... ..A ... ... ... ... ...  [240]
A.florea        ... ... ... G.. ..T ... G.A ... T.. ... ... ... ... ... ... ...  [240]
A.andreniformis ... ... ... G.. ..T ... G.. ... T.. ... ... ... ... ... ... ...  [240]
T.fimbriata     ... ... ... ..A ..T ..A ... G.A T.. ... G.A ... ..C .A. ... ...  [240]
B.terrestris    ... ..T T.A .C. ..T ..A ... ... T.G A.. ... ... ... ..C ... ..T  [240]

A.mellifera     TTA AAA ATT TTA TAT TTA ATT GAT GAA ATT GTA AAT CCT TTT TTT TCA  [288]
A.nuluensis     ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ..T  [288]
A.cerana        ... ... ... ... ... ... ... ... ... ... ... ... ..A ..C ... ..T  [288]
A.nigrocincta   ... ... ... ... ... ... ... ... ... ... ... ... ..G ..C ... ..T  [288]
A.koschevnikovi ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ..T  [288]
A.laboriosa     ... ... ... C.. ... ... ... ... ... ... ... ... ..A ... ... ...  [288]
A.d.binghamii   ... ... ... C.. ... ... ... ... ... ... ... ... ..A ... ... ...  [288]
A.dorsata       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [288]
A.florea        ... ... ... ... ... ... ... ... ... ... A.T ... ..A ..C ... ..T  [288]
A.andreniformis ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ..T  [288]
T.fimbriata     C.T ... ... ... ..C ..C ... ... ... ..A ..T ... ... .A. A.A ...  [288]
B.terrestris    ... ... ... ... ... .AT ... ... ... ... A.. ... ... .A. ... ...  [288]

A.mellifera     ATT AAA TCA ATT GGT CAT CAA TGA TAT TGA TCA TAT GAA TAT CCA GAA  [336]
A.nuluensis     ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ...  [336]
A.cerana        G.A ... ... ... ... ... ... ... ... ... ..C ... ... ... ..T ...  [336]
A.nigrocincta   G.A ... ..T ... ... ... ... ... ... ... ..T ... ... ... ..T ...  [336]
A.koschevnikovi ... ... ... ... ... ... ..G ... ... ... ..C ... ... ... ... ...  [336]
A.laboriosa     ... ... ..T ... ... ... ... ... ..C ... ... ... ... ... ..T ...  [336]
A.d.binghamii   ... ... ..T ... ... ... ... ... ..C ... ... ... ... ... ..T ...  [336]
A.dorsata       ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ..T ...  [336]
A.florea        ... ... ... ... ..A ..C ... ... ..C ... ... ... ... ..C ..T ...  [336]
A.andreniformis ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..T ...  [336]
T.fimbriata     ... ..G G.. ... ... ..C ..G ... ... ... ... ... ... ..C ..T ...  [336]
B.terrestris    ... ... ..T ... ... ... ... ... ... ... ..T ... ... ..C ... ...  [336]

A.mellifera     TTT AAT AAT ATT GAA TTT GAT TCA TAT ATA CTA AAT TAT AAT AAT TTA  [384]
A.nuluensis     ... ... ... ... ... ... ... ..T ... ... T.. ... ... .GA ... ...  [384]
A.cerana        ... ... ... ... ... ... ... ..T ... ... T.. ... ... .GA ... ...  [384]
A.nigrocincta   ... ... ... ... ... ... ... ... ... ... T.. ... ... .GA ..C ...  [384]
A.koschevnikovi ... ... ... ... ... ... ... ... ... ... T.. ... ... .GA ..C ...  [384]
A.laboriosa     ..Y ... ... ... ... ..Y ... ... ... ... T.. ... ... .CA ... ...  [384]
A.d.binghamii   ... ... ... ... ... ... ... ... ... ... T.. ... ... .TA ... ...  [384]
A.dorsata       ..C ... ... ... ... ... ..C ... ... ... T.. ... ... .CA ... ...  [384]
A.florea        ... ... ... ... ... ... ... ... ... ... T.. ... ... .GA ... ...  [384]
A.andreniformis ... ... ... ... ... ... ... ... ... ... T.. ... ... .GA G.. ...  [384]
T.fimbriata     ... ... ... ... ... A.C A.C .GT ..C ... AA. G.G ... .TA GTA G.T  [384]
B.terrestris    ... ... ... TA. ... ... ... ... ... ... T.. ... ... G.A TCA A..  [384]

A.mellifera     AAC CAA TTT CGT TTA CTA GAA ACT GAT AAT CGA ATA GTA ATT CCA ATA  [432]
A.nuluensis     ..T ... ... ..A ... T.. ... ... ... ... ... ... A.T ..C ..T ...  [432]
A.cerana        ..T ... ... ..A ... T.. ... ... ... ... ... ... A.T ..C ..T ...  [432]
A.nigrocincta   ..T ... ... ..A ... T.. ... ... ... ... ..T ... ... ... ..T ...  [432]
A.koschevnikovi ..T ... ... ..A ... T.. ... ... ... ... ... ... ... ..C ... ...  [432]
A.laboriosa     ..T ... ... ..A ... T.. ... ..A ... ... ... ... ... ... ..C ...  [432]
A.d.binghamii   ..T ... ... ..A ... T.. ... ..A ... ... ..T ... ... ..A ..T ...  [432]
A.dorsata       ..T ... ... ..A ... T.. ... ..A ... ... ... ... ... ... ..T ...  [432]
A.florea        ..T ... ... ..A ... T.. ... ... ..C ... ... ... A.T ..C ... ...  [432]
A.andreniformis ..T ... ... ... ... T.. ... ... ... ... ... ... A.T ... ..T ...  [432]
T.fimbriata     ..T ... ... ..A C.T T.. ... ... ..C ... ... ... ... ... ..T T.T  [432]
B.terrestris    ..T ... ... ..A C.T T.. ... ... ... ... ... T.. A.T ..C ... T.T  [432]
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A.mellifera     AAA ATC CCA CTA CGT TTA ATT ACA ACA TCA ACA GAT GTA ATT CAT TCA  [480]
A.nuluensis     ..T ..T ... T.. ..A ... ... ... ..T ..T ... ... ... ... ... ...  [480]
A.cerana        ..T ..T ... T.. ..A ... ... ... ..T ..T ... ... ... ... ... ...  [480]
A.nigrocincta   ..T ..T ... T.. ..A ... ... ... ..C ... ..T ... ... ... ... ...  [480]
A.koschevnikovi ..T ... ... T.. ... ... ... ... ... ... ... ... ... ... ... ...  [480]
A.laboriosa     .G. ..A ..T A.. ... ... ... ..T ... ... ... ... ... ... ... ...  [480]
A.d.binghamii   ..T ..A ..T T.. ... ... ... ..T ..T ... ..T ... ..T ... ..C ...  [480]
A.dorsata       .G. ..A ..T T.. ... ... ... ..T ... ... ... ... ... ... ... ...  [480]
A.florea        ... ..T ..T A.. ..A ... ... ..T ... ... ..T ... ... ... ... ...  [480]
A.andreniformis ... ..T ..T T.. ..A ... ... ..T ... ... ..T ... ... ... ... ...  [480]
T.fimbriata     ... ..T ... A.. ..A ... ... GT. T.. ..T TT. ... ..T ... ... ...  [480]
B.terrestris    ... ..T T.. A.. ... A.. ... GT. T.T ..T T.. ... ... ... ..C ...  [480]

A.mellifera     TGA ACA GTT CCA TCC TTA GGT ATT AAA GTT GAT GCA GTT CCA GGA CGA  [528]
A.nuluensis     ... ..T ... ... ..T ... ..A ... ... ... ... ... ... ... ... ..T  [528]
A.cerana        ... ..T ... ... ..A C.T ..A ... ... ... ... ... ... ... ... ...  [528]
A.nigrocincta   ... ..T ... ... ..T ... ... ... ... ... ... ..T ... ... ... ...  [528]
A.koschevnikovi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [528]
A.laboriosa     ... ..T ... ... ..T ... ..A ... ... ..A ... ..T ... ... ... ...  [528]
A.d.binghamii   ... ..T ... ... ..T ... ..A ..C ... ..A ... ..T ... ... ... ...  [528]
A.dorsata       ... ..T ... ... ..T ... ..A ... ... ..A ... ..T ... ... ... ...  [528]
A.florea        ... ..T ..C ..T ..A ... ... ... ... ... ... ... ... ... ..T ...  [528]
A.andreniformis ... ..T ... ... ..T ... ..A ... ... ... ... ... ... ... ... ...  [528]
T.fimbriata     ... ... A.C .A. ..T ... ..A ... ... ..A ... ... ..C ..G ..T ...  [528]
B.terrestris    ... ... A.. ..T ..A ... ..A ... ... ... ... ... A.. ..T ... ...  [528]

A.mellifera     ATT AAT CAA TTA AAT TTA ATT AGA AAA CGT CCA GGA ATT TTT TTT GGT  [576]
A.nuluensis     ... ... ... ... ... ... ... ... ... ..A ..T ... ... ..C ... ...  [576]
A.cerana        ... ... ... ... ... ... ... ... ... ..A ..T ... ..C ... ... ...  [576]
A.nigrocincta   ... ... ... ... ... ... ... ..G ... ..A ... ... ..C ... ... ...  [576]
A.koschevnikovi ... ... ... ... ... ... ... ... ... ..C ... ... ..C ... ... ...  [576]
A.laboriosa     ... ... ... ... ... ... ... ... ... ..A ... ... ... ..C ..C ...  [576]
A.d.binghamii   ... ... ... ... ... ... ... ... ... ..A ... ... ... ..C ... ...  [576]
A.dorsata       ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ..C ...  [576]
A.florea        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [576]
A.andreniformis ... ... ... ..G ... ... ... ... ... ..A ..T ... ... ... ... ...  [576]
T.fimbriata     ... ... ... ... ... ... T.. TCG .TT ..A ..T ..T C.A .AC ... ..A  [576]
B.terrestris    ... ... ... ... ... ... T.. T.T .TT ... ... ... ... .A. ... ...  [576]

A.mellifera     CAA TGT TCA GAA ATT TGT GGT ATA AAT CAT AGA TTT ATA CCA ATT ATA  [624]
A.nuluensis     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [624]
A.cerana        ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...  [624]
A.nigrocincta   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [624]
A.koschevnikovi ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  [624]
A.laboriosa     ... ... ..T ... ..C ... ..A ... ... ... ... ... ... ... ... ...  [624]
A.d.binghamii   ... ... ... ... ..C ... ... ... ... ... ... ..C ... ... ... ...  [624]
A.dorsata       ... ... ..T ... ..C ... ..A ... ... ... ... ... ... ... ... ...  [624]
A.florea        ... ... ... ... ... ... ..A ... ... ... ... ..C ... ..T ... ...  [624]
A.andreniformis ... ... ..T ... ... ... ..A ... ... ... ... ... ... ... ... ...  [624]
T.fimbriata     ... ... ... ..G ... ... ... G.. ... ... ... ... ... ..T ... ...  [624]
B.terrestris    ... ... ..T ... ... ... ..A ... ... ... ..T ... ... ... ... ...  [624]

A.mellifera     ATT GAA TCA ACT TCA TTT CAA TAT TTT TTA AAT TGA GTA AAT AAA CAA  [672]
A.nuluensis     ... ... ... ..A ... ... A.. ... ..C ... ... ... ..T ... ... ...  [672]
A.cerana        G.A ... ..T ..A ... ... A.. ... ... C.T ... ... ... ... ... ...  [672]
A.nigrocincta   G.. ... ..T ..A ... ... A.. ..C ... ... ... ... ... ... ... ...  [672]
A.koschevnikovi ... ... ... ..A ... ... A.. ... ... ... ..C ... A.T ... ... ...  [672]
A.laboriosa     ... ... ... ... ... ... A.T ... ... ... ... ... ..T ... ... ...  [672]
A.d.binghamii   ... ... ... ... ... ... G.T ... ... ... ... ... ..T ... ... ...  [672]
A.dorsata       ... ... ... ... ... ... A.T ... ... ... ... ... ..T ... ... ...  [672]
A.florea        G.. ... ... ..A ... ... A.. .T. ..C ... ... ... A.T ... ... AT.  [672]
A.andreniformis G.. ... ... ..A ... ... A.. ... ... A.. ... ... A.T T.. ... AT.  [672]
T.fimbriata     T.A ... AGT ..A AG. .A. A.. A.. ... A.. G.. ... ..T ..C ... A..  [672]
B.terrestris    T.A ... AG. ..C ..T .A. G.. ATA ... ... ... ... A.T ..A ... A..  [672]
```

```
A.mellifera     ATC --- --- --- --- --- TAA  [693]
A.nuluensis     .AT AAC --- --- --- --- ...  [693]
A.cerana        .AT AAT --- --- --- --- ...  [693]
A.nigrocincta   .AT AAC --- --- --- --- ...  [693]
A.koschevnikovi .AT --- --- --- --- --- ...  [693]
A.laboriosa     TCT --- --- --- --- --- ...  [693]
A.d.binghamii   TCT --- --- --- --- --- ...  [693]
A.dorsata       TCT --- --- --- --- --- ...  [693]
A.florea        .A. --- --- --- --- --- ...  [693]
A.andreniformis .AT --- --- --- --- --- ...  [693]
T.fimbriata     ..T GAA --- --- --- --- ...  [693]
B.terrestris    ..A AT? TGA AAA ATT AGT ...  [693]
```

APPENDIX 3. *lsRNA* sequence alignment

Species abbreviation: *A.mellifer*: *Apis mellifera*, *A.nuluensi*: *A. nuluensis*, *A.cerana*: *A. cerana*, *A.nigrocin*: *A. nigrocincta*, *A.koschevn*: *A. koschevnikovi*, *A.laborios*: *A. laboriosa*, *A.d.bingha*: *A. d. binghamii*, *A.dorsata* : *A. dorsata*, *A.florea*: *A.florea*, *A.andrenif* : *A. andreniformis*, *T.fimbriat*: *Trigona fimbriata*, *B.terrestr*: *Bombus terrestris*.

```
A.mellifer --GGTCGATC TGCTCCATGA AATTT----T TTAAATAGCT GCAGTATGT-  [ 50]
A.nuluensi -A........ .......... ....AA---. .......... .......A.-  [ 50]
A.cerana   --........ .......... ...AAA---. .......... .......A.-  [ 50]
A.nigrocin -A........ .......... ...A.A---. .......... .......A.-  [ 50]
A.koschevn TA........ .......... .G.AAA--A. .......... .......A.-  [ 50]
A.laborios --........ .....A.... ...A----T. ..T....... .......A.-  [ 50]
A.d.bingha --........ .....A.... ...A.-TTT. ..T....... .......A.-  [ 50]
A.dorsata  --........ .......... .....----. ..T....... .......A.-  [ 50]
A.florea   TA........ .......... .TAAAATTT. .......... .......A.-  [ 50]
A.andrenif --A....... .......... .T.AAC---. .......... ..G....T.-  [ 50]
T.fimbriat -AA....... ...C.A.... ...A.----. .G..TGGCTG CAGTATAAC-  [ 50]
B.terrestr -CA.A.TTAA .TA.TA.ATT TC..CATTTA AA.TT..T.. TA.T.CAACA  [ 50]

A.mellifer TGACTGTACA AAGGTAGCAT AATAAATTG- TCTTTTAATT GAAGAATTGT  [100]
A.nuluensi .......... .......... .........- .......... .........A  [100]
A.cerana   .......... .......... .........- .......... .........A  [100]
A.nigrocin .......... .......... .........- .......... .........A  [100]
A.koschevn .......... .......... .........- .......... .........A  [100]
A.laborios .......... .......... .........- .......... .........A  [100]
A.d.bingha .......... .......... .........- .......... .........A  [100]
A.dorsata  .......... .......... .........- .......... .........A  [100]
A.florea   .......... .......... .........- .......... .........A  [100]
A.andrenif .......... .......... .........- .......... .......A.A  [100]
T.fimbriat .......... .......... ...C.....A .T...A...- ...ATC.G.A  [100]
B.terrestr .CGAG..CGC ..TCATCTT. TTC..TAA.A .....A..AA ..TATTAC.C  [100]

A.mellifer ATGAAAGAAT TAATGAAATA ATAACTGTCT CTAAATTATT AAATGAATTT  [150]
A.nuluensi .....T.... .G........ .C........ ...T...... T.........  [150]
A.cerana   .....T.... .G........ .C........ ...TT..... ..........  [150]
A.nigrocin .....T.... .G........ .C........ ...TT..... ..........  [150]
A.koschevn .....C.... .G........ .C........ ...TT..... G.........  [150]
A.laborios .....C.... .......... .......... ..G....... ..........  [150]
A.d.bingha .....C.... .......... .......... ...G...... TT........  [150]
A.dorsata  .....C.... .......... .......... .......... T.........  [150]
A.florea   .....T.... .......... ..T....... .......... T.........  [150]
A.andrenif .....C.... .G........ ..T....... .........A .......C..  [150]
T.fimbriat .......G.. .......... TA........ .AC.TA.... TT........  [150]
B.terrestr -..TT.TCCC ...G.T...T TACT..T.T- .ATTT...C. ...A..TCAA  [150]

A.mellifer AAATTTTTA- ----------G TTAAAATTCT AAAATTAATT -ATGGGACGA  [200]
A.nuluensi .........- ----------. .......... G.....T.A. T.........  [200]
A.cerana   .........- ----------. .A........ G.....T.A. T.........  [200]
A.nigrocin .........- ----------. .A........ ......T.A. T.........  [200]
A.koschevn .........- ----------. .......G.. ......T.A. T.........  [200]
A.laborios .........- ----------. .......... ......T.A. T.........  [200]
A.d.bingha .........- ----------. .......... ......T.A. T.........  [200]
A.dorsata  ........G- ----------. .......... ......T.A. T.........  [200]
A.florea   .........- ----------. .......... ......T.A. T.........  [200]
A.andrenif .........- ----------. ...C...... ......T.A. T.........  [200]
T.fimbriat ...A.....A TAAAAAT--. ........T. TTT..GGGAC G..AA..---  [200]
B.terrestr ..TA.A..CT TTAATTAAA. ..G.T...TA ...TAA.G.. T.ATAA.TTT  [200]
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```
A.mellifer TAAGA----C CCTATAGAAT TTAATTTAAT TTTTAAATTT ATTTATAATT  [250]
A.nuluensi .....----. .......... ..T....... A.AATT.... .....A.T.A  [250]
A.cerana   .....----. .......... ..T....... A.AATT.... .....ATT.A  [250]
A.nigrocin .....----. .......... ..T....... A.AGTT.... .....A.T.A  [250]
A.koschevn .....----. .......... ..T......A .AA.T.T..A T..A.ATTA.  [250]
A.laborios .....----. .......... ..T....... A...T.T..A T.AA......  [250]
A.d.bingha .....----. .......... ..T....... A...T.T..A T.AA......  [250]
A.dorsata  .....----. .......... ..T....... A...T.T..G T.AA......  [250]
A.florea   .....----. .......... ..T....... ..A.-T...- TAA.T...A.  [250]
A.andrenif .....----. .......... ..T......A ..A.-T...- TAA.T...A.  [250]
T.fimbriat ---------. .......... ..T..A---C .AA.TT.... TAA..CTTAA  [250]
B.terrestr ACCT.TCCT. ..A.C.A... A.T.A.ATTC AA.A.T.AAA T.A..ATTAA  [250]

A.mellifer T--TAAT-AA ATATATTT-A AGAT--TAAA TTTGATTGGG AGGATTGGTA  [300]
A.nuluensi .--.TG.G.. TATATAA.-T .AT.--.... .......... ..........  [300]
A.cerana   .---TTGG.. ......AATT .AT.--.... .......... ..........  [300]
A.nigrocin .--.CT.G.. TATATAA.-T .AT.--.... .......... ..........  [300]
A.koschevn .----TGT.. ...A..AATT .AT.--.... .......... ..........  [300]
A.laborios A--.T.ATTT G..A..AA-G .TG.--.... .......... .......A..  [300]
A.d.bingha A--.TTATTT G..A..AA-G GT..--.... .......... .......A..  [300]
A.dorsata  .--.TT.ATT TATA.A.AAT .T..--.... .......... .......A..  [300]
A.florea   .AT--T.A.. .ATA.A-.A. ------.... .......... ......AA..  [300]
A.andrenif .TTATT.TT. .ATA.AA.A. TA.ATT.... .......... ......AA..  [300]
T.fimbriat .ATA..ATTT ------AAAT TAG.A----- .......... ......AT..  [300]
B.terrestr AATATTAAT. TA.A...CT. TAGGGTCTT. .CGTCCCATA .ATTAATT.T  [300]

A.mellifer AATTTAATAA ACTTTATTTA -AGATTTAAT TTTGATATAA AGATTAATTT  [350]
A.nuluensi ...AA..AT. .......C.. -.A.A..... ......GA.. .....TT...  [350]
A.cerana   ...AA..AT. .......C.- -.A.A..... ......GA.. .....T....  [350]
A.nigrocin ...AA..GT. .......C.. -.A.A..... ......GA.. ....AT....  [350]
A.koschevn ...A...AT. .......C.T -.T.AA.... ...A..TAG. .A..ATT...  [350]
A.laborios ....A.T... .......... ATAT--.T.A ...TGAT.T. .AGA..GA.A  [350]
A.d.bingha ......T... ........A- -.ATA..T.A ...TGATAT. .AGA...A.A  [350]
A.dorsata  ....A..A.. ........A- ---TA.-T.- --.TTG.... .AGA..TA.A  [350]
A.florea   ....-T.AT. .........T T.A....... ......TAG. ....AT.GAA  [350]
A.andrenif .......... .........T T.A..A.... ......TA.. .....T.A.A  [350]
T.fimbriat ......TA.. .....T.AA. -TT..G...A C..T.AT.T. ...AATTA.A  [350]
B.terrestr .GCA------ --...T---- --------.A C.AA.A..TT .A........  [350]

A.mellifer TTGAATTATA AATTGTAATT AAAAGAATAA ATTACCTTAG G-ATAACA--  [400]
A.nuluensi ....C..... .T..A.G... .......... .......... .G......--  [400]
A.cerana   ....C..... .T..A.G... .......... .......... .-......--  [400]
A.nigrocin ....C..... ....A.G... .......... .......... .G......--  [400]
A.koschevn .-..TC.... ....A..... .......... .......... .G......--  [400]
A.laborios .......... .T..A..... ..T...G... .......... .G......--  [400]
A.d.bingha .......... .T..A..... ..T....... .......... .G......--  [400]
A.dorsata  .......... .T..A..... ..T....... .......... .-......--  [400]
A.florea   A...T..... .T..A...A. .T........ .......... .-......--  [400]
A.andrenif ....TC.... .T..A..... .......... .......... .G......--  [400]
T.fimbriat A...TC.T.. .T..AA...A TTT....... .......... .G......--  [400]
B.terrestr -----AATAT .TA..AG.CA ----.TG..T ..CT.A...A CTC.TT..TT  [400]
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A.mellifer GCGTAATATC TTTTGATAGA CCATATAGAT AAAGATG-TT TGCGACCTCG  [450]
A.nuluensi .......... .......... ...C..T... .......-.. ..........  [450]
A.cerana   .......... .......... ...C..T... .......-.. ..........  [450]
A.nigrocin .......... .......... ...C..T... .......-.. ..........  [450]
A.koschevn .......... .......... ......T... ........-A. ..........  [450]
A.laborios .......... .......... .......... .......A.. ..........  [450]
A.d.bingha .......... .......... ...C...... ........-A. ..........  [450]
A.dorsata  .......... .......... ..T....... ........-A. ..........  [450]
A.florea   .......... .........G T.....T... ........-A. ..........  [450]
A.andrenif .......... .......... ..T...T... ........-A. ..........  [450]
T.fimbriat ........CT ....T..... ...C.....A ...AG..-A. ..........  [450]
B.terrestr C.AGTT.T.A A..AA.A.AC AAT.GATT-A TGCT.CCT.. GTAC.GTCAA  [450]

A.mellifer ATGTTGAATT AAGATGAAAA TTAGGCGCAG TAGTTTAAGT TTTTAAGTCT  [500]
A.nuluensi .......... ....GA...G ...AA..... G.......AA ..........  [500]
A.cerana   .......... ....GA...G ...AA..... G.......AA ..........  [500]
A.nigrocin .......... ....GA...G ...AA..... G.......AA ..........  [500]
A.koschevn .......... .....A.... ....A..... A.......AA A.........  [500]
A.laborios .......... .....T.... ...AA..... A.......A- ....G.....  [500]
A.d.bingha .......... .....T.... ...AAT.... A.......A- ....G.....  [500]
A.dorsata  .......... .....T...G ...AAT.... A.......A- ....G.....  [500]
A.florea   .......... .....A.... ...AAT.... G.......T. A.........  [500]
A.andrenif .......... ...G.A.... ...AAT.... G.......T. A.........  [500]
T.fimbriat .......... .....A..TT ...AA..... G.......TA A-........  [500]
B.terrestr T.A.ACCGCA --.CCATTT. A.TATTTA.A ATCA..GG.C AGAATT.A..  [500]

A.mellifer G [501]
A.nuluensi . [501]
A.cerana   . [501]
A.nigrocin . [501]
A.koschevn . [501]
A.laborios . [501]
A.d.bingha . [501]
A.dorsata  . [501]
A.florea   . [501]
A.andrenif . [501]
T.fimbriat . [501]
B.terrestr T [501]
```

CHAPTER 4. General Discussion

My research has focussed on honey bee molecular research. In Chapter 2, I added to the characterization of *itpr* gene expressed in the *Apis mellifera* brain. Then, using *itpr* and two other mitochondrial genes, I extended the resolution of honey bee molecular phylogenetics, as described in Chapter 3. In this chapter, I discussed aspects of honey bee behavioural evolution in the light of the molecular phylogeny, and some implications of this work for current questions in molecular evolution.

Putative PKA and PKC phosphorylation sites in *itpr*

My study extended the *itpr* sequence derived by Kamikouchi et al. (1998) by exploring both genomic and cDNA sequences. I investigated several functional motif sites in the IP_3 Receptor (IP_3R) protein, which is encoded by the gene *itpr*. Putative PKA and PKC phosphorylation sites were included in these investigations as well.

Protein phosphorylation plays an important role in regulating many cellular processes particularly those of signal transduction. One major phosphorylation requirement is the protein kinase (Hunter 1995). PKA or cAMP-dependent protein kinase is the major mediator cAMP reaction (Taylor et al. 1990). cAMP signalling pathways contribute to learning and memory processes as reported in *Drosophila* (Davis 1993), honey bee (Hammer 1997), *Aplysia* (Kandel and Schwartz 1982) and mammals (Abel et al. 1997). While PKA is important in honey bee short term memory, PKC is important for its role in the long-term memory of this insect (Menzel and Giurfa 2001).

Of the two PKA isoforms in existence, only isoform II is the type that has been characterised in the honey bee (Muller 1997). However, because PKA and PKC phosphorylation sites observed in honey bee *itpr* were based on motif searches from protein family databases further biochemical analyses are needed in order to determine the isoform type. In addition, the position of PKA and PKC phosphorylation sites observed in modulatory domains implicated their function as regulating calcium channel opening. I propose that research in this area would clarify the role of these two *itpr* kinases in calcium release from endoplasmic reticulum storage.

Itpr amino acid sequences showed high conservation between honey bee and other vertebrates and invertebrates. Exon evolution is constrained because exons encode amino acid sequences, hence there is little potential for change (Lewin 1993). Changes mostly involve substitution in the third base position, and thus many changes do not affect codon meanings (Nei and Kumar 2000). This phenomenon is well supported by the current study.

On the other hand, intron divergence patterns involve both size difference (due to deletion and insertion) and base substitution. This situation exists because introns evolve much more rapidly than exons (Li 1997) due to the lack of the functional constraints of the latter. In the honey bee *itpr* shows large changes in the case of introns divergence in *A. koschevnikovi* 3rd and *B. terrestris* 4th introns, where both introns are approximately 60–78 bp longer than other homologous introns from other species.

High intron divergence is due to the absence of constraints imposed of coding functions and therefore intron is able to accumulate point substitution and other changes quite freely. Such changes imply that introns have few in any sequence–specific functions (Lewin 1993). A review of nuclear gene evolution found that the highest average rate substitution is for pseudogenes, with introns second in substitution rates (Li 1997).

Comparative analysis of intron position

In my study of *A. mellifera itpr* intron evolution, I conducted a comparative study with that of *Drosophila* introns (Sinha and Hasan 1999). It was found that of the two introns shared the same position along aligned sequences. *A. mellifera* revealed four unique introns that were absent in *D. melanogaster*. However, intron positions in *Drosophila* were not obtained from genomic walking sequence data. Rather, introns positions were obtained by using a series of oligonucleotide primers (Sinha and Hasan 1999). Intron size was determined according to the difference between genomic and cDNA PCR product size. Three of the predicted introns (those at position 2082, 3302 and 3783 were completely sequenced while the other three were not (Sinha and Hasan 1999).

Hence, comparative data of *Apis* and *Drosophila* introns could not be very precise because the imperfect knowledge of the latter. Information on *C. elegans* (nematode)

intron positions is available as well, but without size information (Baylis et al. 1999). *C. elegans* has two introns at the *itpr* alignments among *A. mellifera*, *D. melanogaster* and *C. elegans*. However, none of those two *C. elegans* introns are shared at the same position any of either *A. mellifera* or *Drosophila*.

Ideally, to understand *itpr* intron insertion or intron gain we need intron data from other organisms such as *P. argus* (lobster), rat, and humans. As these are obtained, we can infer intron ancestral positions. According to the "intron late" theory, intron insertion takes place during eukaryote evolution (Palmer and Logsdon 1991), thus implying that intron insertion is a derived trait. On the other hand according to the "introns early" theory, most introns are lost from ancestral genes (Gilbert 1997). If this is the case, intron absence is a secondary, but in the "introns late" theory, intron absence is an inferred primitive trait.

Honey bee molecular phylogenetics

A molecular phylogenetic tree of *Apis* was constructed using a nuclear (*itpr*) and two mitochondrial DNA (*COII* and *lsRNA*) genes. Several features differentiate mtDNA and nuclear genes, for example mtDNA evolves at a rate tenfold faster than nuclear DNA (Page and Holmes 1998). Because differences such as mtDNA are maternally inherited, there is a lack of recombinant in this gene, compared to nuclear genes such as biparentally inherited. *COII* is a region in mitochondria that evolve quickly (Simon et al. 1994). It has been used in many studies, and has proved itself to be appropriate for inferred evolution of closely-related species (Thompson et al. 2000). *lsRNA* in mitochondrial genes has also been widely used in several studies (Dowton et al. 1998).

If well supported molecular phylogenies have been established for specific taxa, the taxonomic distribution of behavioural aspects can elucidate evolutionary origins and directional change of such characteristics (Avise 1994). This theory also implies that mapping characteristics for the purpose of tracing evolutionary patterns of a certain trait must be conducted from independently of the data to be mapped, such as molecular data (Avise 1994). Thus, I inferred honey bee behavioural evolution onto ML and MP consensus tree based on the three concatenated genes.

The phylogenetic tree from my study extended the knowledge of the relationship among to the known of nine *Apis* species. It was concurred that that the monophyletic *Apis* genus are split further into two lines that is the cavity and the open honey bee nesters. However, another tree topology revealed from my study was in agreement with the morphology results of Alexander (1991) for the common species. Further, the *COII* phylogenetic tree analysis from this study resolved the problem with respect to the Willis et al. (1992) *A. koschevnikovi* sequence.

Adapted honey bee behavioural traits

"Adaptation" refers to "design" in life-those properties of living things that enable them to survive and reproduce in nature" (Ridley 1996)

In Chapter 3, I concluded that neither of the two theories of the evolution of *Apis* nesting behaviour could be ruled out - ancestrally, nesting could have been either in the open or cavities because these alternate scenarios are equally parsimonious. In considering the evolution of dance behaviour, it is apparent that, among the open nesters, only *A. florea* and *A. andreniformis* perform horizontal dance. The other open nesters, *A. dorsata* group honey bees perform a vertical dance. Open nest dwarf honey bees (*A. florea* and *A. andreniformis*) have an open space at the top of the nest, which is used for building a platform nest region, facilitates their horizontal dance. A different nest construction occurs in *A. dorsata* group; their open nest has no open space at the top of nest. *A. dorsata* group nests are directly attached to a substrate (i.e. branch, roof, and cave). Therefore, it is possible that due to being no possibility of a horizontal platform, *A. dorsata* evolved a vertical dance at the nest curtain. Hence, it is most likely that the vertical dance evolved because of constraint facing bees such as giant honey bee and was then shown in the ancestral to the *A. mellifera* group. Given those phenomena, honey bee dance behaviour shows its adaptation to nest structure and demonstrates a correlation between those two behaviour traits.

It is tempting to suggest that the common ancestor of the *A. dorsata* and *A. mellifera* groups evolved vertical dancing because of the constraint facing the giant honey bee today. The horizontal dance of *A. florea* can proceed when the sky is not visible from

the dance arena or during the cloudy days, due to the ability of *A. florea* to use landmarks as their compass (Koeniger et al. 1982; Dyer 1984). However, in Dyer (1984) in the same experiments as before, he observed that several foragers does not dance pointed to the direction of the food source. The disoriented dance of some open nesters implied that celestial cues are needed in some stage as the open nesters compass. A more detailed study of a horizontal dance behavioural data in a completely dark cavity (as in the tree trunk) is sought-after. This information is crucial, in order to test the idea that the vertical dance is necessary before honey bees (specifically the *A. mellifera* group) could become cavity nesters.

Multi-comb nesting is another trait associated with cavity nesters while single comb nesting occur in open nests (Ben Oldroyd, personal communication). Single comb nests are covered with several curtain layers whereas in multi comb nests a single layer is formed to cover each comb. In an attempt to study the advantages of cavity nests, Dyer and Seeley (1991) measured honey bee worker tempo, which includes body temperature, flight speed, wing loading and mass specific metabolic rates. These authors found that the cavity nesters have higher tempos (high body temperature, flight speed, wing loading and mass specific metabolic rates) compared to the open nesting honey bees. Although a higher tempo brings a cost (a shorter life span, resulting in fewer workers available for the nest protection), it is relatively unimportant for honey bees, because cavity walls are provided to reduce the need for protection. Hence, it appears that *Apis* evolution involved reversal of nesting behaviour because of the more efficient life style allowed cavity nesters.

Evolution of open nesting and cavity nesting is influenced by honey bee ecology and distribution (Seeley 1983). Thermoregulatory ability exists in both open and cavity nesting behaviour as the result of environmental adaptation. *A. florea* and *A. andreniformis* compensate for high nest temperatures by using water drops and fanning behaviour to cool the nest. At high temperatures, honey bees within the colony will spread instead of remaining in tight cluster (Wilson 1971). Others, such as *A. mellifera* acquired sophisticated thermoregulation techniques for survival in temperate regions.

To heat the colony, the foragers contract their muscle to produce heat while large amount of honey is stored during the winter season (Winston 1987).

A phylogenetic hypothesis underlies conclusions made about comparative evolution (Harvey and Pagel 1991). In addition, in phylogenetic mapping, the general goal is to distinguish whether shared features arose through common ancestry or through convergent evolution from unrelated ancestors (Avise 1994). Hence, for my future research project, I propose to study the behaviour of stingless bees, because these corbiculate bee type displays advanced eusociality. Stingless bees show various open-, cavity- and ground-nesting, and it would be interesting to determine using molecular phylogeny whether groups with the same behaviours evolved independently in each lineage thus producing a convergent evolution. Comprehensive stingless bee molecular phylogeny is required in order to infer such behaviour.

"Phylogeny is in large part the history of branching or speciation and extinction"

(Maddison and Maddison 1992)

My study has added further information to the area of honey bee molecular phylogenetics. On the basis of the phylogenetics constructed in my study, it can be inferred that *A. nigrocincta* is the result of recent speciation from *A. cerana*. It is possible that sympatric speciation occurred because both species are found in regions only 12 km apart (Hadisoesilo et al. 1995). On the other hand, it is likely for allopatric speciation (Page and Holmes 1998) took place yielding *A. nuluensis*, the montane Sabah honey bee, from *A. cerana* because they are separated by altitude (Tingek et al. 1996). Further population genetic work may explore the biogeography of *A. nigrocincta* and *A. nuluensis* in relation to *A. cerana*.

A. nigrocincta in Sulawesi and in adjacent islands (Sangihe) revealed differences in mitochondrial DNA (mtDNA) haplotypes (Smith et al. 2000). However, there do not appear to be any studies involving distribution of *A. koschevnikovi*, the Borneo honey bee. Because Borneo was connected to Palawan during the mid or late Pleistocene (Heaney 1986), I assumed that *A. koschevnikovi* was also distributed in Palawan, as similar distribution patterns to *A. andreniformis* were evident in both regions (de

Guzman et al. 1992). Further *A. koschevnikovi* exploration needs to be undertaken in order to investigate its distribution and also the genetic population structure of this species.

REFERENCES

Abel T, Nguyen PV, Barad M, Deuel TAS, Kandel ER, Bourtchuladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocamus-based long-term memory. Cell 88:615-626

Adachi J, Hasegawa M (1996) MOLPHY: Programs for Molecular Phylogenetics Based on Maximum Likelihood. Version 2.3. Institute of Mathematics, Tokyo.

Adoutte A, Balavoine G, and Lartillot N, De Rosa R (1999) Animal evolution: the end of the intermediate taxa ? [Review]. Trends in Genetics 15:104-108

Alexander BA (1991) Phylogenetic analysis of the genus *Apis* (Hymenoptera: Apidae). Annual Entomology Society of America 84:137-149

Anderson C, Ratnieks FLW (1999) Worker allocation in insect societies: coordination of nectar foragers and nectar receivers in honey bee (*Apis mellifera*) colonies. Behavioural Ecology and Sociobiology 46:73-81

Ascher JS, Danforth BN, Ji SQ (2001) Phylogenetic utility of the major opsin in bees (Hymenoptera: apoidea): A reassessment. Molecular Phylogenetics and Evolution 19:76-93

Ashmead WH (1904) Remarks on honey bees. Proceedings of the Entomological Society of Washington 6:120-122

Attwood TK, Beck ME (1994) PRINTS - A protein motif finger-print database. Protein Engineering 7:841-848

Avise JC (1994) Molecular Markers, Natural History and Evolution. Chapman and Hall, New York

Bateman A, Birney E, Durbin R, Eddy SR, Finn RD, Sonnhammer ELL (1999) Pfam 3.1: 1313 multiple alignments match the majority of proteins. Nucleic Acids Research 27:260-262

Baylis HA, Furuichi T, Yoshikawa F, Mikoshiba K, Sattelle DB (1999) Inositol 1,4,5-trisphosphate receptors are strongly expressed in the nervous system, pharynx, intestine, gonad and excretory cell of *Caenorhabditis elegans* and are encoded by a single gene (*itr*-1). Journal of Molecular Biology 294:467-476

Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361:315-325

Berridge MJ, Irvine RF (1989) Inositol phosphate and cell signalling. Nature 341:197-205

Beye M, Härtel S, Hagen A, Hasselmann M, Omholt SW (2002) Specific developmental gene silencing in the honey bee using a homeobox motif. Insect Molecular Biology 11:527–532.

Blake CCF (1978) Do genes-in-pieces imply proteins-in-pieces? Nature 273:267-268

Blenau W, Balfanz S, Baumann A (2000) Amtyr1: Characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. Journal of Neurochemistry 74:900-908

Blondel O, Moody MM, Depaoli AM, Sharp AH, Ross CA, Swift H, Bell GI (1994) Localization of inositol trisphosphate receptor subtype 3 to insulin and somatostatin secretory granules and regulation of expression in islets and insulinoma cells. Proceedings of the National Academy of Sciences of the United States of America 91:7777-7781

Breitbart RE, Andreadis A, Natal-Ginard B (1987) Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Annual Review of Biochemistry 56:467-495

Burrows M (1996) The Neurobiology of an Insect Brain. Oxford University Press, Oxford

Butler CG (1958) The World of the Honeybee. St. James Place, London

Camazine S (1991) Self-organizing pattern formation on the combs of honey bee colonies. Behavioural Ecology and Sociobiology 28:61-76

Cameron SA, Derr JN, Austin AD, Woolley JB, Wharton RA (1992) The application of nucleotide sequence data to phylogeny of the Hymenoptera: a review. Journal of Hymenoptera Research 1:63-79

Cameron SA, Mardulyn P (2001) Multiple molecular data sets suggest independent origins of highly eusocial behavior in bees (Hymenoptera : Apinae). Systematic Biology 50:194-214

Cavalier-Smith T (1991) Intron phylogeny: a new hypothesis. Trends in Genetics 7:145-148

Chadwick CC, Saito A, Fleischer S (1990) Isolation and characterization of the inositol trisphosphate receptor from smooth muscle. Proceedings of the National Academy of Sciences, USA 87:2132-2136

Chen CN, Denome S, Davis RL (1986) Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the *Drosophila* dunce^{+} gene, the structural gene for cAMP phosphodiesterase. Proceedings of the National Academy of Sciences of the United States of America 83:9313-9317

Cockerell TDA (1907) A fossil Honey bee. Entomologist 40:227-229

Cockerell TDA (1908) Descriptions and records of bees-XX. Annals and Magazines of Natural History. ser. 8, 2:323-334

Collet T (2000) Measuring beelines to food. Science 297:817-818

Crespi BJ, Yanega D (1995) The definition of eusociality. Behavioral Ecology 6:108-115.

Crozier RH, Crozier YC (1993) The mitochondrial genome of the honeybee *Apis mellifera*: Complete sequences and genome organization. Genetics 133:97-117

Crozier RH, Pamilo, P (1996) Evolution of Social Insect Colonies: Sex allocation and kin selection. Oxford University Press, Oxford.

Cunningham CW (1997) Can three incongruence tests predict when data should be combined? Molecular Biology and Evolution 14(7):733-740.

Damus MS, Otis GW (1997) A morphometric analysis of *Apis cerana* F and *Apis nigrocincta* Smith populations from Southeast Asia. Apidologie 28:309-323

Danoff SK, Ferris CD, Donath C, Fischer GA, Munemitsu S, Ullrich A, Synder SH, Ross CA (1991) Inositol 1,4,5-trisphosphate receptors:distinct neuronal and nonneuronal forms derived by alternative splicing differ in phosphorylation. Proceedings of the National Academy of Sciences of the United States of America 88:2951-2955

Davis RL (1993) Mushroom bodies and *Drosophila* learning. Neuron 11:1-14

Dayhoff MO, Schwartz JH, Orcutt BC (1978) A model of evolutionary change in protein. In: Dayhoff MO (ed) Atlas of Protein Sequence and Structure, vol 5 Supplement 3. National Biomedical Research Foundastion, Washington, DC., pp 345-352

de Guzman LI, Forbes M, Cervancia CR, Rinderer TE, Somera S (1992) *Apis andreniformis* Smith in Palawan, Philippines. Journal Apicultural Research 31:111

Deshpande M, Venkatesh K, Rodrigues V, Hasan G (2000) The inositol 1,4,5-trisphosphate receptor is required for maintenance of olfactory adaptation in *Drosophila* antennae. Journal of Neurobiology 43:282-288

Deutsch M, Long M (1999) Intron-exon structures of eukaryotic model organisms. Nucleid Acids Research 27:3219-3228

Doolittle WF (1978) Genes in pieces: were they ever together? Nature 272:581-582

Dornhaus A, Chittka L (2001) Food alert in bumblebees (*Bombus terrestris*): possible mechanisms and evolutionary implications. Behavioral Ecology & Sociobiology 50:570-576

Dowton M, Austin AD, Antolin MF (1998) Evolutionary relationships among the Braconidae (Hymenoptera, Ichneumonoidea) inferred from partial 16s rDNA gene sequences. Insect Molecular Biology 7:129-150

Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S (1976) *Dunce*, a mutant of *Drosophila* deficient in learning. Proceedings of the National Academy of Sciences of the United States of America 73:1684-1688

Durst C, Eichmuller S, Menzel R (1994) Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. Behavioral and Neural Biology 62:259-263

Dyer FC (1984) Comparative studies of dance language and orientation of four species of honey bees. Thesis. Princeton University

Dyer FC (1985a) Mechanism of dance orientation in the Asian honey bee *Apis florea* L. Journal of Comparative Physiology A. 157:183-198

Dyer FC (1985b) Nocturnal orientation by the Asian honey bee, *Apis dorsata*. Animal Behaviour 33:769-774

Dyer FC (1991) Comparative studies of dance communication: analysis of phylogeny and function. In: Smith DR (ed) Diversity in the Genus *Apis*. Westview Press., Oxford., pp 177-198

Dyer FC, Gould JL (1983) Honey bee navigation. American Scientist 71:587-597

Dyer FC, Seeley TD (1991) Nesting behavior and the evolution of worker tempo in four honey bee species. Ecology 72:156-170

Ebert PR, Rowland JE, Toma DP (1998) Isolation of seven unique biogenic amine receptor clones from the honey bee by library scanning. Insect Molecular Biology 7:151-162

Edrich W (1977) Interaction of light and gravity in the orientation of the waggle dance of honey bees. Animal Behaviour 25:342-363

Ehmer B, Hoy R (2000) Mushroom bodies of vespid wasps. Journal of Comparative Neurology 416:93-100

Eickbush TH (2000) Introns gain ground. Nature 404:940-943

Eisenhardt D, Fiala A, Braun P, Rosenboom H, Kress H, Ebert PR, Menzel R (2001) Cloning of a catalytic subunit of cAMP-dependent protein kinase from the honeybee (*Apis mellifera*) and its localization in the brain. Insect Molecular Biology 10:173-181

Engel MS (1998) Fossil honey bees and evolution in the genus *Apis* (Hymenoptera, Apidae). Apidologie 29:265-281

Engel MS (1999) The taxonomy of recent and fossil honey bees (Hymenoptera: Apidae; *Apis*). Journal of Hymenoptera Research 8:165-196

Engel MS (2001a) A monograph of the Baltic amber bees and evolution of the Apoidea (Hymenoptera) [Review]. Bulletin of the American Museum of Natural History:5-192

Engel MS (2001b) Monophyly and extensive extinction of advanced eusocial bees: Insights from an unexpected Eocene diversity. Proceedings of the National Academy of Sciences, USA 98(4):1661-1664.

Engel MS, Schultz TR (1997) Phylogeny and behavior in honey bees (Hymenoptera, Apidae). Annals of the Entomological Society of America 90:43-53

Erber J, Kloppenburg P, Scheidler A (1993) Neuromodulation by serotonin and octopamine in the honeybee- behaviour, neuroanatomy and electrophysiology [Review]. Experientia 49:1073-1083

Esch H, Esch I, Kerr WE (1965) Sound: an element common to communication of stingless bees and to dances of the honey bee. Science 149:320-321

Estoup A, Solignac M, Cornuet JM, Goudet J, Scholl A (1996) Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera, Apidae) in Europe. Molecular Ecology 5:19-31

Fahrbach SE, Strande JL, Robinson GE (1995) Neurogenesis is absent in the brains of adult honey bees and does not explain behavioral neuroplasticity. Neuroscience Letters 197:145-148

Farris SM, Robinson GE, Davis RL, Fahrbach SE (1999) Larval and pupal development of the mushroom bodies in the honey bee, *Apis mellifera*. Journal of Comparative Neurology 414:97-113

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, Univ. of Washington, Seattle

Feremisco JR, Glass DB, Krebs EG (1980) Optimal spatial requirement for the location of basic residues in peptide sucstrate for the cyclic AMP-ependent protein kinase. The Journal of Biological Chemistry 255:4240-4245

Fuchs S, Koeniger N, Tingek S (1996) The morphometric position of *Apis nuluensis* Tingek, Koeniger and Koeniger, 1996 within cavity-nesting honey bee. Apidologie 27:387-405

Furuichi T, Yoshikawa S, Miyawaki A, Wada K, Maeda N, Mikoshiba K (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P 400. Nature 342:32-38

Garnery L, Vautrin D, Cornuet JM, Solignac M (1991) Phylogenetic relationship in the genus *Apis* inferred from mitochondrial DNA sequence data. Apidologie 22:87-92

Gerstacker CEA (1863) On the geographical distribution and varieties of the honey bees, with remarks upon the exotic honey-bees of the Old World. Annals and Magazines of Natural History, series 3, 11:270-283, 333-347

Gilbert W (1978) Why genes in pieces? Nature 271:501

Gilbert W (1985) Genes-in-pieces revisited. Science 228:823-824

Gilbert W (1997) Origin of genes. Proceeding of National Academic of Science of United States of America 94:7698-7703

Goldman N, Anderson J, Rodrigo AG (2000) Likelihood-based tests of topologies in phylogenetics. Systematics Biology 49:652–670.

Gorza L, Vettore S, Tessaro A, Sorrentino V, Vitadello M (1997) Regional and age-related differences in mRNA composition of intracellular Ca^{2+}-release channels of rat cardiac myocites. Journal of Molecular and Cellular Cardiology 29:1023-1036

Gould JL (1980) Sun compensation by bees. Science 207:545-547

Gould JL, Henerey M, Macleod M, C. (1970) Communication of direction by the honey bee. Science 169:544-554

Gronenberg W (1986) Physiological and anatomical properties of optical input fibers to the mushroom body in the bee brain. Journal of Insect Physiology 32:695-704

Gronenberg W (2001) Subdivisions of hymenopteran mushroom body calyces by their afferent supply. Journal of Comparative Neurology 435:474-489

Gruhl M, Kao WY, Bergtrom G (1997) Evolution of Orthologous intronless and intron-bearing globin genes in two insect species. Journal of Molecular and Evolution 45:499-508

Grunbaum L, Muller U (1998) Induction of a specific olfactory memory leads to a long-lasting activation of protein kinase C in the antennal lobe of the honeybee. Journal of Neuroscience 18:4384-4392

Grunewald B (1999) Morphology of feedback neurons in the mushroom body of the honeybee, *Apis mellifera*. Journal of Comparative Neurology 404:114-126

Hadisoesilo S, Otis GW (1996) Drone flight times confirm the species status of *Apis nigrocincta* Smith, 1861 to be a species distinct from *Apis cerana* F, 1793, in Sulawesi, Indonesia. Apidologie 27:361-369

Hadisoesilo S, Otis GW (1998) Differences in drone cappings of *Apis cerana* and *Apis nigrocincta*. Journal of Apicultural Research 37:11-15

Hadisoesilo S, Otis GW, Meixner M (1995) Two distinct populations of cavity-nesting honey bees (Hymenoptera, Apidae) in South Sulawesi, Indonesia. Journal of the Kansas Entomological Society 68:399-407

Hall ZW (1992) An Introduction to Molecular Neurobiology. Sinauer Associates, Sunderland

Halling LA, Oldroyd BP, Wattanachaiyingcharoen W, Barron AB, Nanork P, Wongsiri S (2001) Worker policing in the bee *Apis florea*. Behavioral Ecology and Sociobiology 49:509-513

Hamada T, Liou S, Fukoshima T, Maruyama T, Watanabe S, Mikoshiba K, Ishida N (1999) The role of inositol trisphosphate-induced Ca^{2+} release from IP_3-receptor in the rat suprachiasmatic nucleus on circadian entrainment mechanism. Neuroscience letter 263:125-128

Hammer M (1997) The neural basis of associative reward learning in honeybees. Trends in Neuroscience 20:245-252

Hammer M, Menzel R (1995) Learning and memory in the honeybee [Review]. Journal of Neuroscience 15:1617-1630

Hart AG, Ratnieks FLW (2001) Why do honey-bee (*Apis mellifera*) foragers transfer nectar to several receivers? Information improvement through multiple sampling in a biological system (vol 49, pg 330, 2001). Behavioral Ecology and Sociobiology 49:330

Harvey PH, Pagel MD (1991) The Comparative Method in Evolutionary Biology. Oxford University Press, Oxford

Hasan G, Rosbash M (1992) *Drosophila* homologs of two mammalian intracellular Ca2+-release channels: identification and expression patterns of the inositol 1,4,5-triphosphate and the ryanodine receptor genes. Development 116:967-975

Hasegawa M, Kishino H (1994) Accuracies of the simple methods for estimating the bootstrap probability of a maximum-likelihood tree. Molecular Biology & Evolution 11:142-145

Hasegawa M, Kishino H, Yano TA (1985) Dating the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160-174

Heaney LR (1986) Biogeography of mammalsin SE Asia: estimates of rates of colonization, extinction and speciation. Biological Journal of the Linnean Society. 28:127-165

Hepburn HR, Smith DR, Radloff SE, Otis GW (2001) Infraspecific categories of *Apis cerana*: morphometric, allozymal and mtDNA diversity [Review]. Apidologie 32:3-23

Hofmann K, Bucher P, Falquet L, Bairoch A, Tachida H (1999) The PROSITE database, its status in 1999. Nucleic Acids Research 27:215-219

Holldobler B (1984) Evolution of Insect Communication. In: Lewis T (ed) Insect Communication. Academic Press, London, pp 349-377

Hrncir M, Jarau S, Zucchi R, Barth FG (2000) Recruitment behavior in stingless bees, Melipona scutellaris and M-quadrifasciata. II. Possible mechanisms of communication. Apidologie 31:93-113

Huang K (1989) The mechanism of protein kinase C activation. Trends in Neurosciences 12:425-432

Huang SH, Hu YY, Wu CH, Holcenberg J (1990) A simple method for direct cloning cDNA sequence thath flanks a region of known sequence from toral RNA by applying the inverse polymerase chain reaction. Nucleic Acid Research 18:1922

Huang SH, Wu CH, Cai B, Holcenberg J (1993) cDNA cloning by inverse polymerase chain reaction. Methods Molecular Biology 15:349-356

Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling [Review]. Cell 80:225-236

Jarau S, Hrncir M, Zucchi R, Barth FG (2000) Recruitment behavior in stingless bees, *Melipona scutellaris* and *M-quadrifasciata*. I. Foraging at food sources differing in direction and distance. Apidologie 31:81-91

Jermiin LS, Olsen GJ, Mengersen KL, Easteal S (1997) Majority-rule consensus of phylogenetic trees obtained by maximum-likelihood analysis. Molecular Biology and Evolution 14:1296-1302

Judd TM (1995) The waggle dance of the honey bee: which bees following a dancer succesfully acquire the information? Journal of Insect Behaviour 8:343-354

Kamikouchi A, Takeuchi H, Sawata M, Natori S, Kubo T (2000) Concentrated expression of Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C in the mushroom bodies of the brain of the honeybee *Apis mellifera* L. Journal of Comparative Neurology 417:501-510

Kamikouchi A, Takeuchi H, Sawata M, Ohashi K, Natori S, Kubo T (1998) Preferential expression of the gene for the putative inositol 1,4,5-trisphosphate receptor homologue in the mushroom bodies of the brain of the worker honeybee Apis mellifera. Biochemical and Biophysical Research Communication 242:181-186

Kandel ER, Schwartz JH (1982) Molecular biology of learning: modulation of transmitter release. Science 218:433-443

Kenyon FC (1896) The brain of the bee. A preliminary contribution to the morphology of the nervous system of the Arthropoda. Journal of Comparative Neurology 6

Kirchner WH, Dreller C, Grasser A, Baidya D (1996) The silent dances of the Himalayan honeybee, *Apis laboriosa*. Apidologie 27:331-339

Kishimoto A, Nishiyama K, Nakanishi H, Uratsuji Y, Nomura H, Takeyama Y, Nishizuka Y (1985) Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3':5' monophosphate-dependent protein kinase. The Journal of Biological Chemistry 260:12492-12499

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. Journal of Molecular Evolution 29:170-179

Koeniger G, Koeniger N, Tingek S, Kelitu A (2000) Mating flights and sperm transfer in the dwarf honeybee *Apis andreniformis* (Smith, 1858). Apidologie 31:301-311

Koeniger N (1976) Neue aspekte der phylogenie innerhalb der gattung Apis. Apidologie 7:357-366

Koeniger N, Koeniger G (1980) Observations and experiments of migration and dance communication of *Apis dorsata* in Srilanka. Journal of Apicultural Research 19:21-34

Koeniger N, Koeniger G, Gries M, Tingek S, Kelitu A (1996) Reproductive isolation of *Apis nuluensis* Tingek, Koeniger and Koeniger, 1996 by species-specific mating time. Apidologie 27:353-359

Koeniger N, Koeniger G, Punchihewa RWK, Fabritius M, Fabritius M (1982) Observations and experiments on dance communication in *Apis florea* in Sri Lanka. Journal of Apicultural Research 21:45-52

Koeniger N, Koeniger G, Tingek S, Mardan M, Rinderer T (1988) Reproductive isolation by different time of drone flight between *Apis cerana* Fabricius, 1793 and *Apis vechti* (Maa, 1953). Apidologie 19:103-106

Koeniger N, Wijayagunasekera HNP (1976) Time of drone floght in the three Asiatic honeybee species (*Apis cerana, Apis florea, Apis dorsata*). Journal of Apicultural Research 15:67-71

Koulianos S, Schmid-Hempel R, Roubik DW, Schmid-Hempel P (1999) Phylogenetic relationships within the corbiculate Apinae (Hymenoptera) and the evolution of eusociality. Journal of Evolutionary Biology 12:380-384

Kucharski R, Ball EE, Hayward DC, Maleszka R (2000) Molecular cloning and expression analysis of a cDNA encoding a glutamate transporter in the honeybee brain. Gene 242:399-405

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) *MEGA2: Molecular Evolutionary Genetics Analysis* software, Bioinformatics (submitted).

Kume S, Muto A, Aruga J, Nakagawa T, Michikawa T, Furuichi T, Nakade S, Okano H, Mikoshiba K (1993) The *Xenopus* IP_3receptor: structure, function, and localization in oocytes and eggs. Cell 73:555-570

Kume S, Yamamoto A, Inoue T, Muto A, Okano H, Mikoshiba K (1997) Developmental expression of the inositol 1,4,5-trisphosphate receptor and structural changes in the endoplasmic reticulum during oogenesis and meiotic maturation of *Xenopus laevis*. Developmental Biology 182:228-239

Lacazette E, Gachon AM, Pitiot G (2000) A novel human odorant-binding protein gene family resulting from genomic duplicons at 9q34: differential expression in the oral and genital spheres. Human Molecular Genetics 9:289-301

Lambowitz AM, Belfort M (1993) Introns as mobile genetic elements. Annual Review of Biochemistry 62:587-622

Levin LR, Han PL, Hwang PM, Feinstein PG, Davis RL, Reed RR (1992) The *Drosophila* learning and memory gene *rutabaga* encodes a Ca^{2+}/calmodulin-responsive adenylyl cylase. Cell 68:479-489

Levitan IB, Kaczmarek LK (1997) The Neuron: Cell and Moleular Biology, 2nd. ed edn. Oxford University Press, New York

Lewin B (1993) Genes V. Oxford, Cambridge

Li WH (1997) Molecular Evolution. Sinauer Associates, Inc. Publishers, Massachusetts

Lindauer M (1956) Uber die verstandigung bei indischen bienen. Zeitschrift fur vergleichende physiologie 38:521-557

Lindauer M (1961) Communication among Social Bees. Harvard University Press, Cambridge

Lindauer M, Kerr WE (1960) Communication between the workers of the stingless bees. Bee World 41:29-64, 65-71

Lockhart PJ, Cameron SA (2001) Trees for bees. Trends in Ecology & Evolution 16:84-88

Lockhart PJ, Steel MA, Hendy MD, Penny D (1994) Recovering evolutionary trees under a more realistic model of sequence evolution. Molecular Biology and Evolution 11:605-612

Lopez JA (1998) Alternative spicing of pre-mRNA: developmental consequences and mechanisms regulation [Review]. Annual Review of Genetics 32:279-305

Maa T (1953) An inquiry into the systematics of the tribus Apidini or honeybees (Hym.). Treubia 21: 525-640

Maddison WP, Maddison DR (1992) MacClade. Analysis of phylogeny and character evolution. Version 3. Sinauer Associates, Sunderland, MA

Madsen O, Scally M, Douady CJ, Kao DJ, DeBry RW, Adkins R, Amrine HM, Stanhope MJ, de Jong WW, Springer MS (2001) Parallel adaptive radiations in two major clades of placental mammals. Nature 409(6820):610-614.

Maeda N, Kawasaki T, Nakade S, Yokota N, Taguchi T, Kasai M, Mikoshiba K (1991) Structural and functional characterization of inositol 1,4,5-triphosphate receptor channel from mouse cerebellum. The Journal of Biological Chemistry 266:1109-1116

McEvoy MV, Underwood BA (1988) The drone and species status of the Himalaya honey bee, *Apis laboriosa* (Hymenoptera, Apidae). 61:246-249

McPherson PS, Campbell KP (1993) Characterization of the major brain form of the ryanodine receptor/Ca^{2+} release channel. The Journal of Biological Chemistry 268:19785-19790

Menzel R (1999) Memory dynamics in the honeybee [Review]. Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology 185:323-340

Menzel R (2001) Searching for the memory trace in a mini-brain, the honeybee [Review]. Learning and Memory 8:53-62

Menzel R, Durst C, Erber J, Eichmuller S, Hammer M, Hildebrant H, Mauelshagen J, Muller U, Rosenboom H, Rybak J, Schafer S, Scheidler A (1994) The mushroom bodies in the honeybee: From molecules to behaviour. In: K. S, Elsner N (eds) Neural basis of behavioral adaptation. Fortschritte der Zoologie 39. Gustav Fischer Verlag, Stutgart, pp 81-102

Menzel R, Giurfa M (2001) Cognitive architecture of a mini-brain: the honeybee [Review]. Trends in Cognitive Sciences 5:62-71

Michelsen A, Andersen BB, Storm J, Kirchner WH, Lindauer M (1992) How honey bees perceive communication dances, studied by means of a mechanical model. Behavioural Ecology and Sociobiology 30:143-150

Michener CD (1944) Comparative external morphology, phylogeny, and a classification of the bees (Hymenoptera). Bulletin of the American Museum of Natural History 82:151-326

Michener CD (1964) Evolution of the nests of bees. American Zoologist 4:227-239

Michener CD (1974) The Social Behavior of the Bees. Belknap Press of Harvard University Press, Cambridge, Massachusetts

Michener CD (1982) A new interpretation of fossil social bes from the Dominican Republic. Sociobiology 7:37-45

Michener CD (2000) The Bees of the World. The John Hopkins University Press, Baltimore and London

Michener CD, Grimaldi DA (1988) The oldest fossil bee: Apoid history, evolutionary stasis, and antiquity of social behavior. Proceedings of the National Academy of Sciences of the United States of America 85:6424-6426

Michener CD, Winston ML, Jander R (1978) Pollen manipulation and related activities and structures in bees of the Family Apidae. The University of Kansas Science Bulletin 51:575-601

Michikawa T, Hamanaka H, Otsu H, Yamamoto A, Miyawaki A, Furuichi T, Tashiro Y, Mikoshiba K (1994) Transmembrane topology and sites of N-glycosylation of inositol 1,4,5-trisphosphate receptor. The Journal of Biological Chemistry 269:9184-9189

Mignery GA, Newton CL, Archer BT, Sudhof TC (1990) Structure and expression of the rat inositol 1,4,5-trisphosphate receptor 1. The Journal of Biological Chemistry 265:12679-12685

Mignery GA, Sudhof TC (1990) The ligand binding site and transduction mechanism in the inositol 1,4,5-trisphosphate receptor. The EMBO Journal 9:3893-3898

Mobbs PG (1982) The brain of the honey bee Apis mellifera.1. The connections and spatial organization of the mushroom bodies. Philos. Trans. R. Soc. Lond. Ser. B, 298:309-354

Mobbs PG (1984) Neural network in the mushroom bodies of of the honey bee. Journal of Insect Physiology 30:43-58

Monkawa T, Miyawaki A, Sugiyama T, Yoneshima H, Yamamotohino M, Furuichi T, Saruta T, Hasegawa M, Mikoshiba K (1995) Heterotetrameric Complex Formation of Inositol 1,4,5-Trisphosphate Receptor Subunits. Journal of Biological Chemistry 270:14700-14704

Müller T, Vingron. M (2000) Modeling Amino Acid Replacement. Journal of Computational Biology 7:761-776

Muller U (1996) Inhibition of nitric oxide synthase impairs a distinct form of long-term memory in the honeybee, *Apis mellifera*. Neuron 16:541-549

Muller U (1997) Neuronal cAMP-dependent protein kinase type II is concentrated in mushroom bodies of *Drosophila melanogaster* and the honeybee *Apis mellifera*. Journal of Neurobiology 33:33-44

Muller U (1999) Second messenger pathways in the honeybee brain: Immunohistochemistry of protein kinase A and protein kinase C. Microscopy Research and Technique 45:165-173

Munger SD, Gleeson RA, Aldrich HC, Rust NC, Ache BW, Greenberg RM (2000) Characterization of a phosphoinositide-mediated odor transduction pathway reveals plasma membrane localization of an inositol 1,4,5-trisphosphate receptor in lobster olfactory receptor neurons. Journal of Biological Chemistry 275:20450-20457

Murphy WJ, Eizirik E, O'Brien SJ, Madsen O, Scally M, Douady CJ, Teeling E, Ryder OA, Stanhope MJ, de Jong WW and others. 2001. Resolution of the early

placental mammal radiation using Bayesian phylogenetics. Science 294:2348-2351.

Nakagawa T, Okano H, Furuichi T, Aruga J, Mikoshiba K (1991) The subtypes of the mouse inositol 1,4,5-trisphosphate receptor are expressed in a tissue-specific and developmentally specific manner. Proceedings of the National Academy of Sciences of the United States of America 88:6244-6248.

Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University Press, Oxford ; Melbourne

Newman AJ, Norman C (1992) U5 snRNA interacts with exon sequences at 5' and 3' splice sites. Cell 68:743-754

Nieh JC (1998) The food recruitment dance of the stingless bee, *Melipona panamica*. Behavioral Ecology and Sociobiology 43:133-145

Nouaud D, Boeda B, Levy L, Anxolabehere D (1999) A P element has induced intron formation in *Drosophila*. Molecular Biology and Evolution 16:1503-1510

Oldroyd BP, Ratnieks FLW (2000) Evolution of worker sterility in honey-bees (*Apis mellifera*): how anarchistic workers evade policing by laying eggs that have low removal rates. Behavioral Ecology and Sociobiology 47:268-273

O'Neill RJW, Brennan FE, Delbridge ML, Crozier RH, Graves JAM (1998) De novo insertion of an intron into the mammalian sex determining gene, sry. Proceedings of the National Academy of Sciences of the United States of America 95:1653-1657

Otis GW (1991) A Review of the Diversity of Species within *Apis*. In: Smith DR (ed) Diversity in the Genus *Apis*. Westview Press, Oxford, pp 29-50

Otis GW (1996) Distributions of recently recognized species of honey bees (Hymenoptera, Apidae - *Apis*) in Asia. Journal of the Kansas Entomological Society 69:311-333

Padgett RA, Grabowski PJ, Konarska MM, Seiler S, Sharp PA (1986) Splicing of messenger RNA precursors. Annual Review of Biochemistry 55:1119-1150

Page RDM, Holmes EC (1998) Molecular Evolution: A Phylogenetic Approach. Blackwell Science, Oxford

Palmer JD, Logsdon JMJ (1991) The recent origins of introns. Current Opinion in Genetics and Development 1:470-477

Palmer KA, Oldroyd BP (2001) Mating frequency in *Apis florea* revisited (Hymenoptera, Apidae). Insectes Sociaux 48:40-43

Patthy L (1987) Intron-dependent evolution: preferred types of exons and introns. FEBS Letters 214:1-7

Posada K, Crandall KA (1998) Modeltest: testing the moodel of DNA substitution. Bioinformatics 14:817-818.

Prentice M (1991) Morphological analysis of the tribes of Apidae. In: Smith DR (ed) Diversity in the Genus *Apis*. Westview Press, Oxford

Punchihewa RWK, Koeniger N, Kevan PG, Gadawski RM (1985) Observations on the dance communication and natural foraging ranges of *Apis cerana*, *Apis dorsata* and *Apis florea* in Sri Langka. Journal of Apicultural Research 24:168-175

Ratnieks FLW (1995) Evidence for a queen-produced egg-marking pheromone and its use in worker policing in the honey bee. Journal of Apicultural Research 34:31-37

Ridley M (1983) The explanation of organic diversity : the comparative method and adaptations for mating. Clarendon, Oxford

Ridley M (1996) Evolution, 2nd edn. Blackwell Science, Oxford

Rinderer TE, Oldroyd BP, Sylvester HA (1992) Evolution of bee dances. Nature 360:305

Rinderer TE, Oldroyd BP, Wongsiri S, Sylvester HA, Deguzman LI, Potichot S, Sheppard WS, Buchmann SL (1993) Time of drone flight in 4 honey bee species in South-Eastern Thailand. Journal of Apicultural Research 32:27-33

Rinderer TE, Oldroyd BP, Wongsiri S, Sylvester HA, Deguzman LI, Stelzer JA, Riggio RM (1995) A morphological comparison of the dwarf honey bees of Southeastern Thailand and Palawan, Philippines. Apidologie 26:387-394

Rinderer TE, Wongsiri S, Kuang B, Liu J, Oldroyd BP, Sylvester HA, De Guzman LI, Kuang H, Dong X, Zhai W (1996) Comparative nest architecture of the dwarf honey bees. Journal Apicultural Research 35:19-26

Rogers JH (1989) How were introns inserted into nuclear genes? Trends in Genetics 5:213-216

Ross CA, Danoff SK, Schell MJ, Synder SH, Ullrich A (1992) Three additional inositol 1,4,5-trisphosphate receptors: molecular cloning and differential localization in

brain and peripheral tissues. Proceedings of the National Academy of Sciences of the United States of America 89:4265-4269

Roubik DW (1983) Nest and colony characteristics of stingless bees from Panama (Hymenoptera: Apidae). Journal of the Kansas Entomological Society 56:327-355

Roubik DW, Sakagami SF, Kudo I (1985) A note of distribution and nesting of the Himalayan honeybee *Apis laboriosa* Smith (Hymenoptera, Apidae). Journal of Kansas Entomological Society 58:746-749

Ruttner F (1975) Ein metatarsaler haftapparat bei den drohnen der gattung Apis (Hymenoptera: Apidae). Entomologica germanica 2:022-029

Ruttner F (1988) Biogeography and Taxonomy of Honeybees. Springer-Verlag, Berlin

Ruttner F, Kauhausen D, Koeniger N (1989) Position of the red honey bee, *Apis koschevnikovi* (Buttel-Reepen 1906), within the Genus *Apis*. Apidologie 20:395-404

Ruttner F, Mossadegh MS, Kauhausenkeller D (1995) Distribution and variation of size of *Apis florea* F in Iran. Apidologie 26:477-486

Saier MHJ, Eng BH, Fard S, Garg J, Haggerty DA, Hutchinson WJ, Jack DL, Lai EC, Liu HJ, Nusinew DP, Omar AM, Pao SS, Paulsen IT, Quan JA, Sliwinski M, Tseng TT, Wachi S, Young GB (1999) Phylogenetic characterization of novel transport protein families revealed by genome analyses. Biochimica et Biophysica Acta 1422:1-56

Sakagami S, Matsumura T (1980) *Apis laboriosa* in Himalaya, the little known world largest honeybee (Hymenoptera, Apidae). Insect Matsumurana 19:47-77

Salmah S, Inoue T, Sakagami SF (1990) An analysis of apid bee richness (Apidae) in Central Sumatra. In: Natural History of Social Wasps and Bees in Equatorial Sumatra. Hokkaido University Press, pp 139-174

Schneider SS, Stamps JA, Gary NE (1986) The vibration dance of the honeybee I. Communication regulating foraging on two time scales. Animal Behavior 34:377-385

Schultz TR, Engel MS, Ascher JS (2001) Evidence for the origin of eusociality in the Corbiculate bees (Hymenoptera:Apidae). Jounal of Kansas Entomological Society 74:10-16

Seeley TD (1982) How honeybees find a home. Scientific American 247:158-168

Seeley TD (1983) The ecology of temperate and tropical honeybee societies. American Scientist 71:264-271

Seeley TD (1991) Age polyethism for the hive duties in honey bees-illution or reality. Ethology 87:284-297

Seeley TD (1992) The tremble dance of the honey bee: message and meanings. Behavioral Ecology and Sociobiology 31:375-383

Seeley TD (1994) Honey bee foragers as sensory units of their colonies. Behavioral Ecology and Sociobiology

Seeley TD (1998) Thoughts on information and integration in honey bee colonies. Apidologie 29:67-80

Seeley TD, Mikheyev AS, Pagano GJ (2000) Dancing bees tune both duration and rate of waggle-run production in relation to nectar-source profitability. Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology 186:813-819

Sharp AH, Nucifora FC, Blondel O, Sheppard CA, Zhang CY, Snyder SH, Russell JT, Ryugo DK, Ross CA (1999) Differential cellular expression of isoforms of inositol 1,4,5-trisphosphate receptors in neurons and glia in brain. Journal of Comparative Neurology 406:207-220

Sharp PA (1981) Speculations on RNA splicing. Cell 23:643-646

Sherman PW, Lacey EA, Reeve HK, Keller L. 1995. The eusociality continuum. Behavioral Ecology 6:102-108.

Shimodaira H, Hasegawa M (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Molecular Biology and Evolution 16(8):1114-1116.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers [Review]. Annals of the Entomological Society of America 87:651-701

Sinha M, Hasan G (1999) Sequencing and exon mapping of the inositol 1,4,5-trisphosphate receptor cDNA from *Drosophila* embryos suggests the presence of differentially regulated forms of RNA and protein. Gene 233:271-276

Smith DR, Villafuerte L, Otis G, Palmer MR (2000) Biogeography of *Apis cerana* F. and *A. nigrocincta* Smith: insights from mtDNA studies. Apidologie 31:265-279

Smith F (1861) Descriptions of new species of hymenopterous insects collected by Mr. A.R. Wallace at Celebes. Proceeding of the Linnean Society, London 5:57-93

Snodgrass RE (1935) Principles of Insect Morphology. McGraw-Hill Book Company, London

Srinivasan MV (1998) Honeybee link sights to smells. Nature 396:637-638

Starr CK, Schmidt PJ, Schmidt JO (1987) Nest-site preferences of the giant honey bee, *Apis dorsata* (Hymenoptera: Apidae), in Borneo. Pan-Pacific Entomologist 63:37-42

Strimmer K, Rambaut A (2002) Inferring confidence sets of possibly misspecified gene trees. Proceeding of the Royal Society London B 269:137-142.

Strimmer K, von Haeseler A (1996) Quartet puzzling - a quartet maximum-likelihood method for reconstructing tree topologies. Molecular Biology and Evolution 13:964-969

Stryer L (1995) Biochemistry, 4th ed. edn. Freeman and Company, New York

Suzuki Y, Glazko GV, Nei M (2002) Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. Proceedings of the National Academy of Sciences of the United States of America 99:16138–16143.

Swofford DL (2002) PAUP* 4.0b10. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Tanaka H, Roubik DW, Kato M, Liew F, Gunsalam G (2001) Phylogenetic position of *Apis nuluensis* of northern Borneo and phylogeography of *A. cerana* as inferred from mitochondrial DNA sequences. Insectes Sociaux 48:44-51

Tarrio R, Rodriquez-Trelles F, Ayala FJ (1998) New *Drosophila* introns originate by duplication. Proceedings of the National Academy of Sciences of the United States of America 95:1658-1662

Tautz J, Rohrseitz K (1996) One-strided waggle dance in bees. Nature 382:32

Taylor SS, Buechler JA, Yonemoto W (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annual Review of Biochemistry 59:971-1005

Thakar CV, Tonapi KV (1962) Nesting behaviour of Indian honeybees. II Nesting habits and comb cell differentiation in *Apis florea* Fab. Indian Bee Journal 24:27-31

Thompson GJ, Miller LR, Lenz M, Crozier RH (2000) Phylogenetic analysis and trait evolution in Australian lineages of drywood termites (Isoptera, Kalotermitidae). Molecular Phylogenetics & Evolution 17:419-429

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Research 25:4876-4882

Tingek S, Koeniger G, Koeniger N (1996) Description of a new cavity nesting species of *Apis* (*Apis nuluensis* n. sp.) from Sabah, Borneo, with notes on its occurrence and reproductive biology. Senckenbergiana Biologica 76:115-119

Tingek S, Mardan M, Rinderer TE, Koeniger N, Koeniger G (1988) The rediscovery of *Apis vechti* Maa 1953: the Sabah honeybee. Apidologie 19:97-102

Tomita M, Shimizu N, Brutlag DL (1996) Introns and reading frames - correlation between splicing sites and their codon positions. Molecular Biology and Evolution 13:1219-1223

Triglia T, Peterson GM, Kemp DJ (1988) A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acid Research 16:8186

Trung LQ, Dung PX, Ngan TX (1996) A scientific note on first report of *Apis laboriosa* F. Smith, 1871 in Vietnam. Apidologie 27:487-488

Tunwell REA, Wickenden C, Bertrand BMA, Shevchenko VI, Walsh MB, Allen PD, Lai FA (1996) The human cardiac muscle ryanodine receptor-calcium release channel: identification, primary structure and topological analysis. Biochemical Journal 318:477-487

Tyshenko MG, Walker VK (1997) Towards a reconciliation of the introns early or late views - triosephosphate isomerase genes from insects. Biochimica et Biophysica Acta - Gene Structure and Expression 1353:131-136

Underwood BA (1990a) Seasonal nesting cycle and migration patterns of the Himalayan honey bee *Apis laboriosa*. National Geographic Research 6:276-290

Underwood BA (1990b) Time and drone flight of *Apis laboriosa* Smith in Nepal. Apidologie 21:501-504

Valcarcel J, Gaur RK, Singh R, Green MR (1996) Interaction of $U2AF^{65}$ RS region with pre-mRNA of branch point and promotion base pairing with U2 snRNA. Science 273:1706-1709

Venkatesh K, Hasan G (1997) Disruption of the IP3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. Current Biology 7:500-509.

Venkatesh K, Siddhartha G, Joshi R, Patel S, Hasan G (2001) Interactions between the inositol 1,4,5-trisphosphate and cyclic AMP signaling pathways regulate larval molting in *Drosophila*. Genetics 158:309-318

von Frisch K (1967) The Dance Language and Orientation of Bees. Harvard University Press, Cambridge, Massachusetts

Wagener-Hulme C, Kuehn JC, Schulz DJ, Robinson GE (1999) Biogenic amines and division of labor in honey bee colonies. Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology 184:471-479

Waldorf U, Hovemann BT (1990) *Apis mellifera* cytoplasmic elongation factor 1α (EF-1α) is closely related to *Drosophila melanogaster* EF-1α. FEBS Letters 267:245-249

Whelan S, Goldman N (2001) A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Molecular Biology and Evolution 18:691-699

Wille A, Michener CD (1973) The nest architecture of stingless bees with special reference to those of Costa Rica (Hymenoptera: Apidae). Revista de Biologia Tropical 21:9-278

Willis GL, Winston ML, Honda BM (1992) Phylogenetic relationships in the honeybees (Genus *Apis*) as determined by the sequence of the *cytochrome oxidase II* region of mitochondrial DNA. Molecular Phylogenetics and Evolution 1:169-178

Wilson EO (1968) Karl von Frisch and the magic well. Science 159:864-865

Wilson EO (1971) The Insects Societies. The Belknap Press of Harvard University Press, Massachusetts

Wilson EO. 1990. Success and dominance in ecosystems: the case of the social insects. Kinne O, editor. Oldendorf: Ecology Institute. 104 p.

Winston ML (1987) The Biology of the Honey Bee. Harvard University Press, Cambridge

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238-240

Witthorft W (1967) Absolute anzahl und verteilung der zellen im hirn der honigsbiene. Z. Morph. Tiere 61:160-184

Wojcikiewicz RJH (1995) Type I, II, and III inositol-trisphosphate receptor are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. The Journal of Biological Chemistry 270:11678-11683

Wongsiri S, Lekprayoon C, Thapa R, Thirakupt K, Rinderer TE, Sylvester HA, Oldroyd BP, Booncham U (1997) Comparative biology of *Apis andreniformis* and *Apis florea* in Thailand. Bee World 78:23-35

Wongsiri S, Limbipichai K, Tangkanasing P, Mardan M, Rinderer T, Sylvester HA, Koeniger G, Otis G (1990) Evidence of reproductive isolation confirms that *Apis andreniformis* (Smith, 1858) is a separate species from sympatric *Apis florea* (Fabricius, 1787). Apidologie 21:47-52

Wongsiri S, Thapa T, Oldroyd B, Burgett DM (1996) A magic bee tree - home to *Apis dorsata*. American Bee Journal 136:796-799

Woyke J (1997) Expression of body and hair color in three adult castes of the red honeybee *Apis koschevnikovi* Von-Buttel-Reepen, 1906 in Sabah, Borneo. Apidologie 28:275-286

Wu Y, Kuang B (1987) Two species of small honeybee-a study of the genus *Micrapis*. Bee World 68:153-155

Yamada N, Makino Y, Clark RA, Pearson DW, Mattel MG, Guenet JL, Ohama E, Fujino I, Miyawaki A, Furuichi T, Mikoshiba K (1994b) Human inositol 1,4,5-trisphosphate type-1 receptor, $InsP_3R1$: structure, function, regulation of expression and choromosomal localization. Biochemical Journal 302:781-790

Yoshikawa S, Tanimura T, Miyawaki A, Nakamura M, Yuzaki M, Furuichi T, Mikoshiba K (1992) Molecular cloning and characterization of the inositol 1,4,5-trisphosphate receptor in *Drosophila melanogaster*. The Journal of Biological Chemistry 267:16613-16619

Zeuner FE, Manning FJ (1976) A monograph of fossil bees (Hymenoptera: Apoidea). Bulletin of the British Museum of Natural History 27:155-254

Zhang JF (1990) New fossil species of Apoidea (Insect:Hymenoptera). Acta Zootaxon Sinica 15:83-91