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Dottorato di Ricerca in Biodiversità ed Evoluzione
XIX CICLO
Settore Scientifico Disciplinare BIO-05

A Multidisciplinary Approach to Taxonomy and Phylogeny of Australian Isoptera

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Chapter 1

INTRODUCTION

Isoptera are an ancient order of insects whose origin date back to more than 120 million years in the Cretaceous period. They are mainly distributed through tropical and subtropical regions. Termites have attracted interest due to their position as social organisms separately evolved from the social Hymenoptera. In fact, they are much closer to cockroaches and mantids. Often the three group are clumped into a super order called Dictioptera. Social organization in some species has reached the same level of sophistication as in the more advanced Hymenoptera, with colonies where each individual acts as part of a group governed by the demands of the colony and ultimately by the royal couple represented by the Queen and the King.

Termites have biting mouthparts and their soft bodies are small, rarely over 1 cm length. They typically inhabit dark nests and tunnels, only venturing out when the winged alates emerge to leave their parental colony, or when constructing shelter or when harvesting their food. Termite queens can produce from a few hundred to more than 10 million eggs per year in some species, resulting in very large colonies. These are highly organized, relying on chemical and sensory messages for communication and defence; these enable them to exist in total darkness. Termites are the dominant invertebrates in tropical ecosystem and usually prefer to feed on dead plant material, generally in the form of wood, leaf litter or soil, playing an important role in plant decomposition and in soil composition and structure. Because of their wood-eating habits, termites sometimes greatly damage buildings and other wooden structures, having therefore an important place in economic entomology. Their habit of

remaining concealed often results in their presence being undetected until the timbers are severely damaged and exhibit surface changes.

1.1 – BIOLOGY .

1.1.1 – CASTES.

Termites live in colonies comprising from several hundreds to several millions of individuals. They have gradual metamorphosis (emimetaboulos) and different developmental pathways leading to three or more different terminal castes, hence are polymorphic (Fig.1).

A typical termite colony comprises nymphs (semi-mature, young individuals), workers, soldiers, and reproductive individuals of both sexes, sometimes with several egg-laying queens.

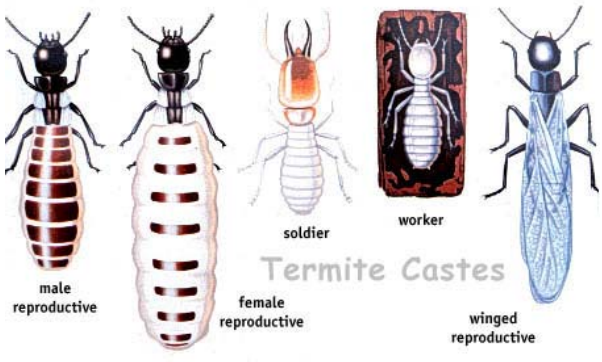

Fig. 1 – Termite castes.

WORKERS

Workers are small, generally white, wingless, and sexually immature individuals (Fig.2); they are also devoid of composite eye and ocelli. As for other social insects, termite workers have fundamental roles in the nest. They undertake foraging, food storage, brood care, building and maintenance of the nests and some of the defense efforts in certain species. Workers are the main caste in the colony for the cellulose digestion (see Chapter 1.1.2). Other worker roles include taking care and feeding the young and other non-wood feeding castes such as soldiers and the royal pair (see Chapter..). It is also proved that workers can groom and clean other castes (Lys and Leuthold, 1991).


Fig. 2 – Group of workers.

Workers are the most populous caste: they may represent the 90 per cent over and over of all colony members. Despite the apparent limitation due to the undeveloped eyes, workers are able to create elaborate nests and tunnel systems using a combination of soil, chewed wood-cellulose, saliva and faeces. Some African and Australian species have mounds more than 5 meters high. These are created and maintained by workers, taking care also for water collection through condensation and air conditioning. In few species workers, even practice agriculture, collecting plant matter to feed fungal gardens, upon which the colony then feed.

SOLDIERS

The soldier caste has anatomical and behavioural specializations, primarily useful against ant attack. Their larger head is longer and wider than the workers one and contains more muscles or an enlarged frontal gland (Pearce, 1997). Often soldier head is coloured yellow to brown with enlarged mandibles of various shape and size. Many soldiers have jaws so enlarged that they cannot feed themselves, but , like juveniles, they need to be fed by workers. The possibility to defend is due either to these big mandibles or to the frontal gland excretes. Most members of the pan-tropical sub family Nasutitermitinae have soldiers with reduced mandibles: the head is modified into a

Fig. 3 – *Nasutitermes* soldiers.

horn-like nozzle (nasus) (Fig. 3) from which a toxic glandular secretion is fired; in Rhinotermitidae the noxius liquid is excrete through a simple hole in the head (fontanelle).

The proportion of soldiers within a colony varies both within and between species. In some termite genera soldiers may be of different sexes, but generally they are not sexually mature. They're usually blind, but in some families, soldiers developing from the reproductive line have at least partially functional eyes. Many species are readily identified using the characteristics of the soldiers' heads, mandibles or nasus.

REPRODUCTIVES

Reproductives (Fig. 4) are the biggest members of a colony. They are dark colored and with two pairs of wings. The four wings are very long and when in repose are laid flat along the back, extending far beyond the tip of the abdomen. They are membranous and most of the veins are longitudinal. Frontal and hind wings show the same shape and size: this led to the name "Isoptera". Near the base of each wing a line of weakness, along which the wing breaks off after the so-called nuptial flight, is found. Termites may be characterized as monogamous. Males can be distinguished from females by the presence of styles on the 9th sternal segment. The females have an enlarged 7^{th} sternal segment and 8^{th} and 9^{th} segments are fused. A certain size of the colony must be reached before alates are produced. Alates leave the colony all together (Fig. 5) and their swarmings contain a few hundred individuals in lower termites or many thousands in higher termites. Before flight, alates congregate away from the main colony. They then leave from holes or slits in the ground, mound or wood or from special flight turrets. Slits cause damage to living wood, but

Fig. 4 – Soldiers and alates of *R. lucifugus*.

they can be repaired by the tree with new tissue (callus). The number of exit holes can vary from a few to several hundreds, especially in the mound builder. Before emergence, workers prepare the exit holes or build the flight turrets, while soldier stand guard. Rainfall may be the trigger for both the initial release and for other subsequent flights. Males and females emerge together. In some species there may be more males ensuring that each female will find a mate. In lower termites, and in temperate regions, flights may occur over several months or several times in a year. In higher termites, and especially in dry regions, there is a particular time of the year and moment of the day for flights and these are synchronized with other flights in the same region. Most termites are poor fliers and therefore do not reach great distances. Lower termites usually fly better than higher ones.

When alates land, they lose their wings immediately or following contact with the opposite sex. This is achieved by raising and twisting the abdomen, or by rubbing against an ostacle which breaks each wing off at the weak sutures. Then, in most species, individuals climb up grasses to form “tandems”. Alates have sternal and tergal glands on their abdomen (Ampion and Quennedy, 1981).

Fig. 5 – Swarming alates.

In females, tergal glands produce a volatile compound responsible for males attraction. Sternal glands of both sexes are used to lay down a scent trail so that the pairs doesn’t lose contact while looking for a place to burrow. Millions of alates may be killed by predators, especially ants, lizard and birds.

Dampened soil allows alates of soil-nesting species to burrow quickly, with both sexes taking part to the work. Wood-dwelling termites look for crevices and cracks in the wood. Mating may not take place straight away but even after a few weeks (Pearce, 1997). After digging the

soil or wood, the entrance is sealed. In drywood termite, a lattice is first constructed across the opening using mouthparts, and it is then filled with an abdominal secretion. Once under the ground or inside the wood, a royal room is constructed. The alate pairs do not feed but rely on their fat store and protein breakdown from wing muscles. In some species the royal pair can eat some of their first born.

The copularium is the starting point for the growth of the termite family and the expansion of a new gallery system of the colony. Pairing ensure lifelong monogamy and mate fidelity.

Monogamy and inbreeding are extremely important: they ensure that the growing population comprises full siblings, so that an exact equivalence of relatedness exists among all individuals in the colony. At maturity, a primary queen can lay several thousands of eggs a day.

Figura 6 - Queens, workers and eggs (left); physogastric queen, king and workers (right).

In physogastric species (Fig. 6), the queen adds an extra set of ovarioles at each moult, resulting in a greatly extended abdomen and increased fecundity. The distended abdomen increases its size in some species even to 10 centimetres, i.e., hundreds of times its original size, effectively immobilizing the queen. When these huge queens must be moved to a new chamber, hundreds of workers are required to push her. The queen is widely believed to be a primary source of pheromones useful in colony integration. As a reward for attending workers a juice is secreted from the queen's abdomen for the workers to drink. The king remains only slightly bigger than an average termite and continues to mate with the queen for all his life.

In certain species of termites, immature alates going through incomplete metamorphosis form a sub-caste. They behave as workers ('pseudergates'), but also as potential supplementary reproductives. Supplementaries (Fig. 7) have the ability to replace primary reproductives dead and, at least in some species, several of them are recruited once a primary queen is lost.

Replacement reproductives are not only important for the survival of a colony, but they also allow the formation of closely related satellite colonies, separated from the main colony. The supplementaries are usually more mobile and play a major role as pest. Supplementary reproductives with wing pads are very common (brachypterous neotenics). They often have shorter life than primary pair and their egg-laying capacity is lower, but with many supplementaries the overall number of termites produced can be large. Another kind of supplementaries (apterous) can be produced from wingless workers (Myles, 1999).

Fig. 7 – Supplementaries of *Reticulitermes lucifugus grassei* (left) and *Kalotermes flavicollis* (right).

In Kalotermitidae, workers (pseudergates) can develop into soldier, nymphs, supplementaries or alates. Thus demonstrating that a great developmental flexibility. In meso-eusocial termites (see chapter 1.1.4), neotenic development is usually restricted to either the nymphal or worker line. In fact, in Rhinotermitidae, workers are though to remain immature for their entire life, changing to soldier or reproductives when required. In the Termitidae, where meta-eusociality is the rule, this individual reproductive flexibility is further greatly restricted or completely lost. For example, termites in the subfamilies Macrotermitinae and Apicotermitinae do not have neotenics, and replacement is only possible by unflown alates.

Caste changes may be regulated with chemical messages distributed by termites passing or receiving secretions from other termites or by volatile secretions (pheromones) such as the Juvenile hormone (responsible for caste changes within colony).

1.1.2 – FEEDING BEHAVIOUR

CELLULOSE FEEDING

All termites eat cellulose in its various forms as plant fiber (Fig. 8). Thus, dead wood, withered leaves and grass are mostly composed of plant cell-wall material, which is primarily made up of two types of plant carbohydrate polymers, cellulose and lignin. This biomass is high in caloric value, but low in nutritive value. Lignocellulosic matter is the most abundant material produced annually in the biosphere by photosynthesis. Fungi are the main organisms that consume it, and they are able to do it because their threadlike hyphae have appropriate enzymes, and because the microscopic threads that make up woodrotting fungi are extremely economical in their nutrient requirements (Abe et al., 2000).

Fig. 8 – Nymphs and workers on wood.

Among invertebrates, a few groups of insects have evolved as successful detritivores, in all cases by coevolving symbiotic relationships with microbial organisms such as bacteria, protozoa, or fungi, these more primitive organisms having very diverse and useful metabolic machinery.

Besides termites other detritivores are cockroaches, crickets, flies, and beetles. Termites are the only social detritivores. Their way of life, leads to a higher level of coordinated and increased foraging, and therefore to an extraordinary level of lignocellulosic processing power.

Furthermore, they have ecologically radiated so that specialized genera exist for virtually every type of plant detritus, wood, humus, and dung, in every stage of decay, in virtually every type of temperate, subtropical, and tropical habitat (Pearce, 1997).

In termites, three main patterns of endosymbiotic relationships may be outlined. All lower termite families have a complex community of flagellate protozoa that assist cellulose digestion. Termites lose their intestinal simbionts every time they moult and have to reinoculate themselves from anal secretion of another member of the nest (see below: Trophallaxis). This group comprehends the families Mastotermitidae, Termopsidae, Kalotermitidae and Rhinotermitidae. However, in the Termitidae, which are the so called higher termites and account for approximatively the 60% of all termite species have lost the flagellate protozoans (although some of them harbour amoebae or ciliates) and have instead evolved a completely modified hindgut with various new segments, chambers, and diverticula in which various bacterial community are involved in digestion. Some researchers think that these prokaryotes do not play a role in cellulose digestion as important as the one of Protozoa in lower termites. The hypothesis that Termitidae can secrete cellulase (the enzyme that break down cellulose) has been put forward (personal communication, Dawes-Gromadzki, 2005).

Among higher termites, a peculiar situation is found in the fungus-growing Macrotermitinae: these cultivate a basidiomycete fungus, *Termitomyces*; they eat wood and litter which are deposited as pellets in a comb-like structure inside the nest (Wood and Thomas, 1989). *Termitomyces* fungi break the comb down to simpler materials which can be reingested by termites.

Asexual spores of the fungus form an important food store, as do the fungal hyhpae in the combs.

Figura 9 - *N. triodiae* workers arvesting grass.

Termites are usually grouped according to their feeding behaviour in several general categories: subterranean, soil-feeding, drywood, dampwood and grass eating (Fig. 9) termites. In particular, subterraneans and drywoods are primarily responsible for damage to human structures. But, while most primitive termites are effectively xylophagous, and feed on dead wood, the more advanced termites forage on a much wider array of foods, including fine woody debris, plant litter, leaf litter, dead grass, organic layers of soil, humus, highly decomposed wood, live wood, live herbaceous plants and grass, dung, fungi, fungus gardens, and lichens.

Termites feed in groups, often in row or circles (Pearce, 1997). There is a definite organized pattern of feeding which ensures that work is carried out at the same place and with the maximum use of all workers available (Collins, 1979).

TROPHALLAXIS

Trophallaxis is the direct transfer of alimentary liquid, including suspended particulats, and derivates, from one nestmate to another via regurgitation (stomodeal trophallaxis; Fig. 10) or anal feeding (proctodeal trophallaxis) (Suarez & Thorne, 2000). This is especially important when there is a shortage of food and moisture. It is also important for the transmission of

chemical messages to other termites, throughout the colony and finally back to the queen. Throphallaxis therefore enables feeding of dependent castes and instars, transfer of symbiont among nestmates, transfer of caste regulatory pheromones (Suarez & Thorne, 2000). The degree of trophallaxis within a colony depends on its size, age, and seasonal variation of food supply.

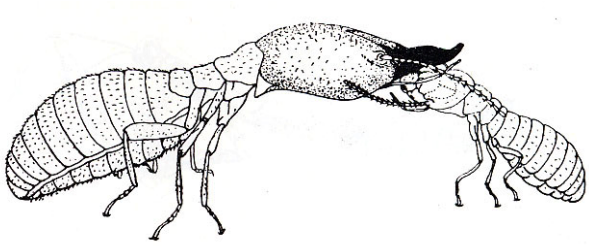

Fig. 10 – Stomodeal trophallaxis: a worker is feeding a soldier.

CANNIBALISM

Cannibalism is an important source of nitrogen, which is usually low in a cellulose-diet. Appendages are often bitten off and the alates that do not leave the nest may be eaten by the workers (Raina et al., 2004). The elimination of excessive supplementary reproductives (Fig. 11) involves detection by smell (Pearce, 1997). The presence of fatty acid originated from a leak of haemolymph can causes cannibalism (Raina et al., 2004). The other main sources of nitrogen are fungi, uric acid stored in the fat bodies, and anaerobic fixation of nitrogen gas by bacteria present in the gut.

Fig. 11 – Cannibalism.

1.1.3 – COMUNICATION

Comunication among colony members is the main mechanism of social integration. In a termite society, modes of comunication are mainly based on tactile and chemical stimulation, while visual signal do not appear to have a role in the everyday life of the colony. Termites can distinguish between odours using smell peg-like sensilla that have numerous holes on their walls allowing air to enter via diffusion. These are on antennae, palps and other mouthparts and are used in the detection of chemicals such as sex and trail pheromones. Other chemoreceptors are on antennae, mouthparts, labrum and other part of the body. In some termites the number of chemoreceptors can differ in different castes (Springhetti, 1972). Termites are known to perform vibratory movements (Grassè, 1986, Maistrello & Sbrenna, 1996), sometimes associated with head or abdomen banging, and this behaviour has been interpreted as a way to communicate alarm and excitement. Mechanoreceptors which rely on cuticole distortion are found in areas of the body where movements and contacts are more common (Grassè, 1986). Groups of receptors may be arranged to detect signals from different directions. Two groups of sensilla (chordotonal) are located in the second antennal segment (Kirchner et al., 1994): one is termed “Johnston organ” and respond to movement, while the other may respond to gravity. Pheromones probably play the most important role in the transmission of information among individuals; several compounds isolated from soldiers are involved in alerting the colony and stimulating aggressiveness in case of danger (Roisin et al., 1990). Other releaser pheromones are involved in trail leading to food sources, in recruitment and mate attraction and probably in mutual recognition. The morphology of esocrine glands that produce pheromones is covered in details by Quennedy (1975). Repeated inter-individual contacts such as grooming and trophallaxis between members of different castes are probably the way through which the pheromones that influence castes differentiation are distributed among colony members (Springhetti, 1985).

1.1.4 – SOCIALITY EVOLUTION

Two families of pro-social termites possess pseudergates or false workers, instead of true worker. These are the rotten wood termites (family Termopsidae) and the dampwood and drywood termites (family Kalotermitidae, Fig. 12). In these families, instead of an early developmental bifurcation between neuter and nymphal lines, all individuals follow the nymphal line. To prevent too many offspring from maturing and leaving the colony as alates, the king and the queen physically manipulate some of them by nibbling on the wings pad or hind legs. These minor physical injures trigger regressive molts, causing in these individuals the reduction of their wing pads and thus the delay of development for at least one season. In this condition, they are known as pseudergates . They effectively act as workers, but this developmental condition may not be permanent: they can later resume nymphal development and eventually become alates. Colonies of pro-social termites are confined to individual pieces of wood,; therefore, they often comprises only a few thousands of individuals, rarely exceedings 10,000 units.

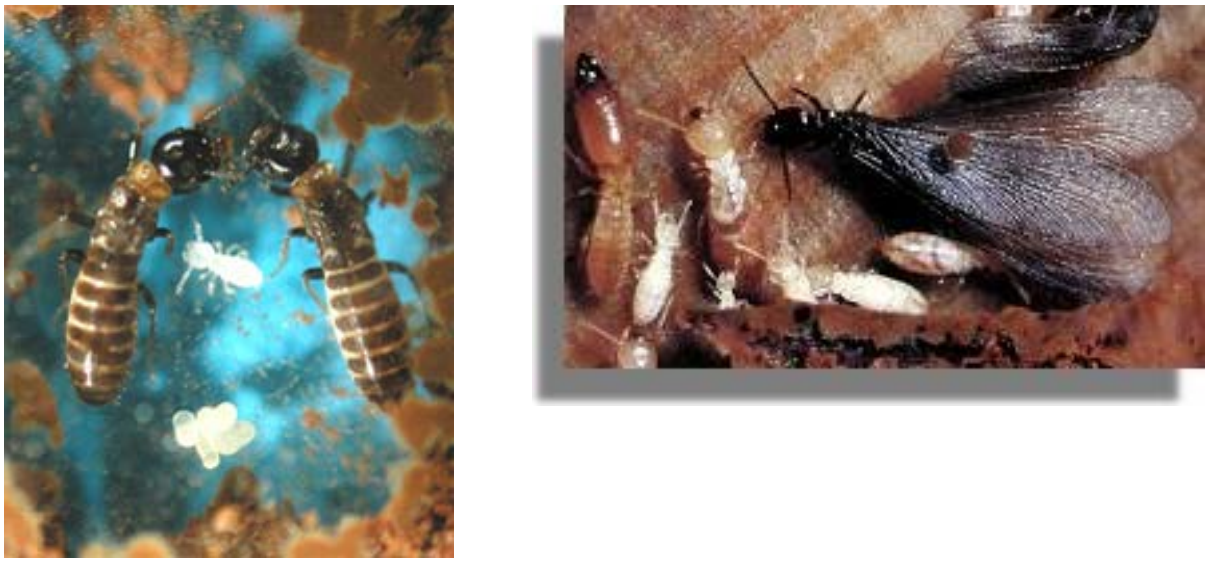

Fig. 12-13 – Reproductives, eggs and a young of *K. flavicollis* (left) and winged adults, workers, young and soldier of *Reticulitermes* (right).

Their nests are constituted of a simple system of multiple galleries inside undecayed pieces of wood. They do not require any contact with the soil in order to live; thus, they can seriously damage movable woodden objects such as furniture, structural timbers and woodworks in

buildings. Excreta and other debris are stored in unused chambers or casted out through small openings in the wood.

The next level of sociality may be termed meso-eusocial. This occurs in the subterranean Mastotermitidae, Rhinotermitidae (Fig. 13) and Serritermitidae families, where a true worker caste is present. These colonies attain population number from tens of thousands to millions having access to a larger quantity of food resources.

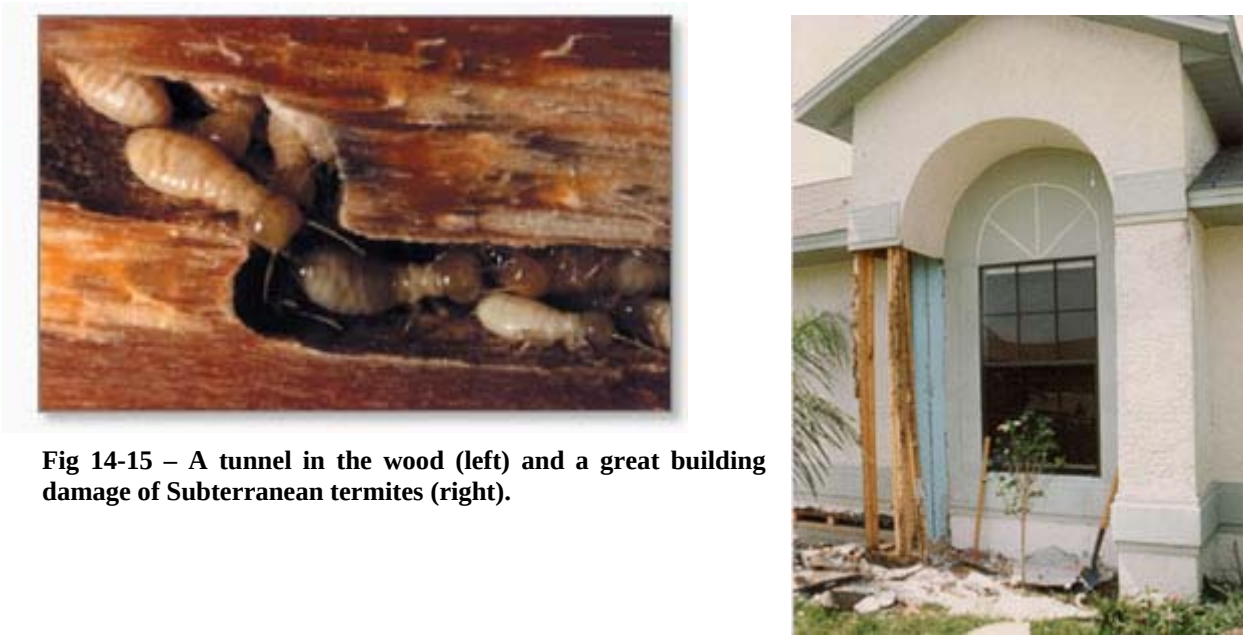

Fig 14-15 – A tunnel in the wood (left) and a great building damage of Subterranean termites (right).

However, their nests remain a primitive and poorly defined collection of galleries and cells in the soil or inside a log, or a tree trunk (Fig. 14). So, they can easily attack any wood in contact with the soil or construct covered galleries to reach wood several metres above the ground. They do not push wood particles or pellets outside the nest, but rather use them in the construction of the tunnel. Subterranean termites are the most destructive and frequently encountered kind of termites (Fig. 15).

The next stage in termite social evolution, meta-eusociality, is characterized by the construction of an organized nest. Two families of termites, the Hodotermitidae and Termitidae, have achieved meta-eusociality. The nest is usually in the soil and it is separate from any feeding source. It may be entirely below the surface (hypogeal) or on and above the

surface (epigeal). With the development of an organized nest, the social life of the colony is transformed. Both workers and soldiers present a further subdivision of functions between nest tasks and foraging tasks. In these case, for example, younger stages are occupied within nest, while older workers assume the more perilous tasks associated with foraging at greater distances from the nests.

In desert environments, hypogeal nests are often found below large rocks. Nests may also be attached to the trees trunks or branches (arboreal). In wood- or cellulose-feeding species, nests are generally composed of a hardened, pulpy, fecal plaster material called carton, which varies in color from light tan to dark brown. In humus and soil-feeding species, nests are made of a darker fecal material, presumably with a high content in lignins and polyphenols. Other nests are essentially excavations of tiers of galleries in the soil, containing chambers lined with fecal plastering.

The population size of mature nest-building termites is sometimes quite modest and it tends to overlap, rather than exceed, the population ranges of meso-eusocial termites, mainly reflecting ecosystem differences in the resource productivity that can be harvested by a population with a central nest. Some meta-eusocial species have evolved interconnected multiple-nest systems. These systems are analogous to those of meso-eusocial termites whose colonies spread by budding, and allow meta-eusocial species to achieve populations of several millions of individuals.

The final stage in termite sociality is ultra-eusociality. Ultra-eusocial termites usually have multiple worker and soldier subcastes and very large nests called mounds. Among mound-builders I can recall the Australian *Coptotermes acinaciformis*, the fungus-growing genera *Odontotermes* and *Macrotermes* of Africa and Asia, the South American Nasutitermitinae genera *Syntermes* and *Cornitermes* of, some *Nasutitermes spp.*, including the cathedral-mound building termite (*Nasutitermes triodiae,* Fig. 16), and some Northern Australian Amitermitinae, such as *Amitermes laurensis*, *A. meridionalis* (Fig. 17), and *A. vitosus.*

Figura 16-17 - *Nasutitermes triodiae* cathedral mound (left) and *Amitermes meridionalis* magnetic mound (right). (Photos: Silvia bergamaschi).

Mounds differ from nests not only in size, but also in having a massive outer wall of soil, clay, sand, fecal material, and saliva, and termites invest much more in fortifying them for defense. Sometimes the larger size also involves the addition of ventilation shafts and chimneys. In addition, mound builders often store large quantities of food in peripheral storage pits or in storage chambers in the mound itself (Fig. 18-19). Mounds are always long-lived structures, lasting for many decades, if not for centuries.

Figura 18-19 – Nests of *N. triodiae* with evident storage chambers filled with dry vegetables. (Photos: Silvia Bergamaschi).

1.1.5 – ISOPTERA-OTHER ANIMAL RELATIONSHIPS

With so much energy and so many nutrients flowing through colonies, termites are an extremely important food source. Their numbers is only one reason for that. The other reason is that termites are available to predators such as lizards, birds, reptiles, and mammals, throughout the year, unlike many other potential food sources (Fig. 20).

Termites often house both vertebrates and other arthropods. Some of these "inquilines" are so specialized that they can complete their life-cycle only in association with termites. Examples include species of isopoda, aracnida, mites, thysanura, blattoidea and coleoptera (Andersen et al., 2005). To become a successful inquiline a species must adopt particular strategies that allow them to be accepted by the host. These strategies include the sharing of the host colony odor on their bodies and an apterous condition, so that they cannot be easily picked up and carried out of the nest. Termite mounds may also become important nest sites for a wide range of reptiles, mammals and birds, in particular in the tropics. Termites themselves can also be inquilines in other termite species nests and non-aggressive ants can successfully exist in termite nests. Cohabitation with another termite species often occur when the soldier caste is low in number or when parts of the nest are no longer used and a different termites species can colonize them.

Fig. 20 – Termite mound are perfect for frilled lizard to bask in the sun and to feed.

1.2 – DISTRIBUTION

1.2.1 – GENERAL DISTRIBUTION

The great majority of genera are restricted to one or two continents. The best explanation of this cosmopolitan distribution is that these genera existed prior to the Pangea breakup and that the other genera evolved after the tectonic events forming the modern continents. If this is true, we would also expect these cosmopolitan genera to be the most species rich. This is, in fact, the case. Only 13 out of the 275 recognized genera have a cosmopolitan distribution. Among them I can recall the following: *Kalotermes, Glyptotermes, Cryptotermes, Coptotermes, Heterotermes, Microcerotermes, Amitermes, Termes and Nasutitermes*. The Palearctic distribution of Reticulitermes genus suggests it originated in Laurasia-Gondwana splitting.

There are 2.800 species of termites nowadays described. Termites are predominantly tropical and subtropical insects. The number of the species decreases sharply in temperate regions, and they are absent altogether in boreal and arctic regions. Termite distribution can be related to temperature and rainfall. These change with latitude, and the limit of survival are between latitudes 45° and 50° north and south. Termites tend to be most conspicuous in savannas (tropical grasslands), where their mounds often predominate in the landscape. They are more numerous on continents than on islands, and species number usually diminishes the more distant the islands are from continents. They are frequently quite numerous in deserts, but in this habitat they do not build large epigeal nests.

Mastotermitidae family is now found in Australia and Papua New Guinea with the only representative species, *Mastotermes darwiniensis*. Rhinotermitidae is spread in all the continents, as major pest. Termitidae occur mainly in tropical latitudes, with Macrotermitinae subfamily covering an important economic role as pest in Africa and Asia. The latter

subfamily is absent in Australia and Americas and its role being partially taken over by Nasutitermitinae and Termitinae.

Given that my PhD work is centered onto the Isoptera of Australian Northern Territory, in the next chapter I will detail Isoptera features of this area.

1.2.2 – AUSTRALIAN NORTHERN TERRITORY ISOPTERA

Termites are found throughout Australia and are usually detected in natural habitats by their conspicuous earthen mounds. Like other southern continents at comparable latitudes, Australia has a very diverse termite fauna (Watson & Gray, 1991). Australian termite taxonomy is far from being complete, and several taxa need to be revisioned (Watson & Gray, 1991; Miller, 1997; Bergamaschi et al., submitted).

Australia comprehend nearly 350 termite species, with more than 150 taxa located in the Northern Territory region. Most of them are poorly known, with many undetermined taxa. Termite mounds are a distinctive part of Northern Australian landscape. Indeed, northern Australia presents the most diverse range of termite mounds found anywhere in the world.

Fig. 21-22 – *Coptotermes acinaciformis* (left) and *Nasutitermes longipennis* (right) nests. (Photos: Silvia Bergamaschi).

These earthern structures range from small cones and domes hidden underground, to giant monoliths 5m height that stand in the open savannas creating spectacular scenarios. The considerable structural diversity of termite nests is associated with differences in social evolution, colony size and feeding habits, as well as the establishment of a suitable microclimate (Ozeki et al., 2006).

Fig. 23 – Termite mounds characterize savanna landscapes. (Photo: Silvia Bergamaschi).

Termites are the essential part of Australian savannas ecosystems (Fig. 23). The exceptionally infertile soil in addition to a highly seasonal rainfall means that northern Australia can not support the vast population of mammals so characteristic of some African savannas. Tropical landscapes of northern Australia often do not receive rain for up to 8 months per year. The length of the wet season is a primary driver of plant productivity, so it is critical that soils are able to capture and hold water from the first rain. Termites play a key role in this process, by creating large macropores in the soil, that help to maximize the infiltration of rainfall and therefore the amount of water stored in the soil (Andersen et al., 2005).

Termites are also the main decomposer insects in Northern Australia. They play a pivotal role in unlocking from dead plant material the energy and nutrients, which can then circulate throughout the ecosystem for plants and animals.

Considering the relationship between soil macroinvertebrates and land condition, several studies showed that as land condition declines, so too does the diversity and abundance of soil macroinvertebrates, but termites are particularly resilien. The soil macroinvertebrates in sites with poor land conditions, consist almost entirely of termites (Gromadzki, 2004). Moreover,

it seems that termites can help the restoration process in degraded savanna landscapes influencing soil turnover, porosity and decomposition of plant material (Jones, 1990). A diverse range of other animals are then attracted into the area because of the healthier soil, the subsequent plant growth and termites as food source.

Usually, Northern Territory termite species are divided into 4 groups following their feeding behaviour: wood-feeders, soil-feeders, debris-feeders and grass-harvester (Andersen et al., 2005).

The wood-feeders include species that eat dead wood inside living trees, such as *Coptotermes acinaciformis* and *Mastotermes darwiniensis*, as well as termites feeding on fallen logs and other pieces of dead wood, such as the species ascribed to the genera *Heterotermes, Microcerotermes* and *Schedorinotermes*. Only *Coptotermes* and *Microcerotermes* termites build mounds, so the wood-eating termites are often inconspicuous in the bush. Soil-feeding termites (*Lophotermes* and *Macrognathotermes* genera) subsist on the fine particles of organic matter contained in the soil and they have entirely subterranean nests. Debris-feeders are generally scavenger of litter and other plant material, wheras grass-harvesters feed predominantly on dry grass.

Fig. 24 – Drepanotermes nest. (Photo: Silvia Bergamaschi).

However, the distinction between them is not always clear, as most harvester feed on plant litter as well as on dry grass. Similarly, dry grass appears to be an important part of the diet of some debris-feeders, such as the magnetic and floodplain termites, *Amitermes* spp. The dry

grass collected by harvesters is cut into small pieces and usually stored inside mounds. These mounds range from the flat "slab-like pavements" of *Drepanotermes spp* (Fig. 24), through the relatively small, columnar or conical structures, such as those of *Tumulitermes spp.*, to the massive monoliths of *Nasutitermes triodiae*. Debris-feeders and harvesters build most of the termite mounds adorning the Northern Australian lanscapes.

1.3 – TAXONOMY AND SYSTEMATICS

1.3.1 –ABOUT THE ORIGIN OF ISOPTERA

Termites are among the most primitive insects of the terrestrial Neoptera. Isoptera together with Blattodea (cockroaches) and Mantodea (mantids) are often embodied in a group named Dictioptera. Recent phylogenetic studies have suggested that mantids were the earliest offshoot of the Dictyoptera, leaving cockroaches and termites as sister groups (Lo et al., 2003). The most primitive cockroaches (Cryptocercidae) comprises the single living Holarctic genus *Cryptocercus*. These cockroaches are highly related to termites: they eat wood, live in tunnel systems inside logs, have symbiotic flagellates in their hindgut, and live in small subsocial family groups in which parents share their burrows with one offspring brood they feed with proctodeal fluids. The most primitive termites (family Mastotermitidae) are at present represented only by *Mastotermes darwiniensis*. These Isoptera are most similar to cockroaches: f.i. they are the only termites that still lay eggs in an ootheca. From a morphological point of view, they have five-segmented tarsi and an anal lobe in the hind wing, just like cockroaches. Owing to these similarities, it has been suggested that termites evolved from social cockroaches (Lo et al., 2003). However, termites lack the many distinctive features of cockroaches: a wide flattened body with the pronotum expanded over the head as a shield, shortened and thickened anterior wings that barely project beyond the tip of the abdomen, and very spiny legs. Therefore termites seem morphologically simpler and

more primitive than cockroaches. Cockroaches developed a more robust body shape suited for a freeliving, detrivorous lifestyle; termites pursued a life of tunneling inside wood or soil substrates and developed a more advanced level of family integration.

However, some studies suggested that Cryptocercids may not be basal in the phylogeny of the Blattaria order, and therefore, if termites shared ancestry with Cryptocercidae, then the Isoptera order is cladistically encompassed within the Blattodea, rather than being its sister group. Consequently, termites should be considered a derivative of social cockroach. The conventional view, reflected by current taxonomy, is that the three orders branched from common ancestors now long extinct, and form distinct and valid independent clades of equal ordinal rank.

1.3.2 – INTRA-ORDER RELATIONSHIPS

MORPHOLOGICAL DATA

The generally accepted systematics of Isoptera (Fig. 25) relies heavily on soldier external morphology and, to a lesser extent, on mandibles morphology of alates and workers. Others morphological character may be the number of cerci segments and wing microsculpturing.

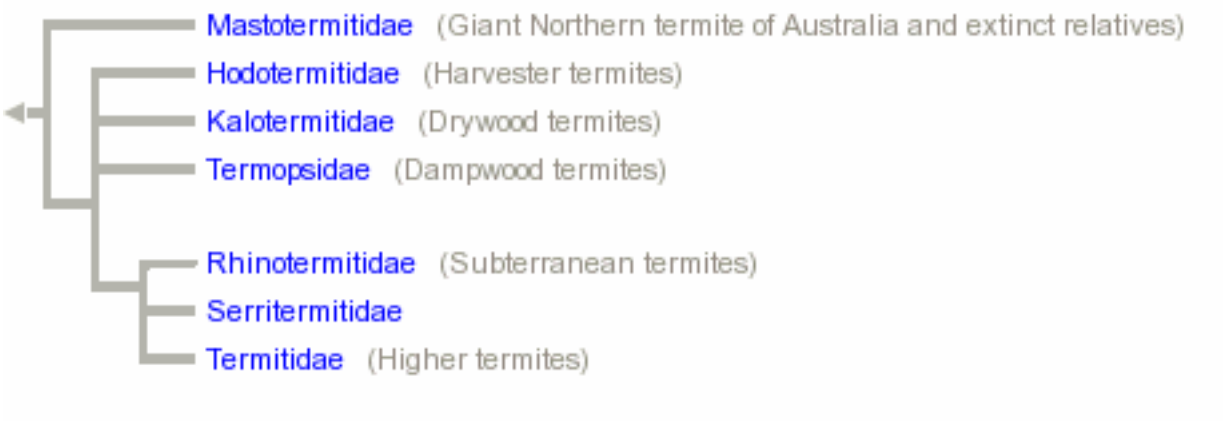

Fig. 25 – Isoptera family relationships tree based on Donovan et al (2000) morphological review.

Moreover, also the digestive tract provides many useful taxonomic characters (Noirot & Noirot-Timothee, 1969; Sands, 1972; Miller, 1986, 1991). Miller (1984) combined digestive

tract characters with external morphological ones to highlight the relationships between Australian species.

Many phylogenetic reconstructions of the relationship among or within Isoptera families are based on subsets of characters, such as worker-imago mandibles (Ahmad, 1950), the worker gut (Johnson, 1979; Noirot, 1995; Miller, 1997), or general morphology (Donovan et al., 2000).

In different studies, the Mastotermitidae family has been widely thought to be the most basal group, followed by the Hodotermitidae. The relative positions of the Kalotermitidae, Termopsidae, Rhinotermitidae and Serritermitidae are less certain, and they appear differently related in the different studies. For example, in Noirot (1995) the Termopsidae is the most basal family, while the Kalotermitidae appear to be a sister group of (Rhinotermitidae + Serrotermitidae + Termitidae). On the contrary, in the trees obtained by Thorne & Carpenter (1992) and Krishna (1961) Kalotermitidae and Mastotermitidae families appear as sister groups. It is unclear if Rhinotermitidae termites constitute a monophiletic or more probably a paraphyletic group, but they are generally considered as sister group of Termitidae at the apex of Isoptera tree. The Serritermitidae are of uncertain taxonomic position, and are placed either within Rhinotermitidae (Donovan et al., 2000) or Termitidae (Ahmad, 1950). This latter family always results a monophyletic group (Donovan et al., 2000) and comprises several subfamilies: Macrotermitinae (fungus-growing termites), Termitinae, Apicotermitinae, and Nasutitermitinae, that result polyphyletics.

A few other morphological studies have examined at a smaller taxonomic level, phylogenetic pattern, generally as part of a taxonomic revision: Miller (1991; 1997) for the Australian *Termes*-group and Australian Nasutitermitinae; Constantino (1995) for the *Syntermes* genus; and Roisin et al (1996) for Antillean Nasutitermitinae.

Generally, cladistic analysis based on morphological characters present low statistical support for the high incongruence between employed dataset. These studies highlight the particular problems of coding morphological characters in social insects with multiple castes.

In the next two chapters I will discuss about karyological and molecular data available for Isoptera, because karyology and mitochondrial DNA analysis are the main approaches utilized in this thesis.

KARYOLOGICAL DATA

The study of chromosomes has occupied and occupies still now an important place in comparative biology and phylogenetic studies.

Mitotic chromosomes metaphase can be measured and evaluated. These quantitative data can be used to classify each chromosome morphology. Then, they can be arranged and displayed in a standard format: in pairs, ordered by size and position of centromere for chromosomes of the same size. Karyotypes may be used to determine macroscopically visible aspects of an individual's genotype, such as sex. Chromosomes are also visible and stainable during I and II meiotic metaphase, providing further information on chromosome structure and relationships.

General informations on the karyology of Isoptera are still limited. The large number and the small size of their chromosomes made termites a difficult subject for cytogenetic studies. The discovery in 1977 by Syren and Luykx of multiple chromosome associations in male meiocytes, implied that all further investigations were mainly centered on this unusual sex chromosome system and its possible roles.

The most extensive surveys on chromosome numbers in termites are those of Vinqke and Tilquin (1978) on 22 species of higher termites (family Termitidae), of Luykx and Syren (1979) on 9 species of lower termites (family Kalotermitidae) and of Luykx (1989) on 24 species of lower termites (family, Kalotermitidae and Hodotermitidae). Moreover, Fontana and Goldoni (1980, 1982, 1985 1991) studied the chromosome complements of European

populations of *Reticulitermes lucifugus* Rossi and *Kalotermes flavicollis* Fabr., also presenting the so far unique G-banding and NORs localization. The general outcome gained from these studies is that higher termites are very uniform karyotypically, with most species having a diploid number of 42 and single sex-linked translocation resulting in a ring of four chromosomes in male meiosis. Lower termites appeared more variable, with diploid number ranging from 28 to 56 and sex-linked translocations ranging from none to eight, resulting in a range of sex chromosomes in the male from a simple XY system to one with 9 Xs and 9Ys.

Interchange heterozigosity is a widespread feature of the genetic system of many termite species. Chains or ring of chromosomes have been observed in male meiosis and are maintained by linkage to the sex-determining system, males being heterozigous for the exchanges, which are lacking in female meiosis.

In *Reticulitermes lucifugus,* the heterochromosomes are XY in the male and XX in the female (Fontana, 1991), as in some Jamaican colonies of *Incisitermes schwarzi* (Luykx, 1987). High variability occur within the Kalotermitidae family: *Kalotermes flavicollis* (Goldoni and Fontana, 1991), *Cryptotermes spp*., *Procryptotermes australiensis* (Luykx, 1990) have a multiple system X_1X_2Y in males and $X_1X_2X_1X_2$ in female, while *Incisitermes schwarzi, Kalotermes approximatus* and *Neotermes insularis* present from six to nine-membered rings. Higher termites often present an $X_1X_2Y_1Y_2$ male : $X_1X_xX_1X_2$ female system (Vincke and Tilquin, 1978). These multiple systems may be originated from a series of reciprocal translocations or Robertsonian changes (centric fusion or fission) between an ancestral XO or XY system and several autosomes probably bearing genes which determined some sex characteristics.

The number of chromosomes involved in these karyotype repatterning, may vary considerably even between conspecific (Syren and Luykx, 1981; Luykx, 1987). It should be noted that multiple sex-chromosome occur also in animals without highly organized societies, for example in the copepod *Mesocyclops edax* (Chinappa and Victor, 1979) and in some species

of the centipede *Otocryptos* (Ogawa, 1961), as well as in plants like *Viscum* and *Oenothera* (Cleland, 1972; Barlow et al., 1978), in invertebrates like *Delena cancerides* (huntsman spider; Rowell, 1985) and in monotrems such as platypus and echidna (Bick and Sharman, 1985; Wrigley and Graves, 1988; Grutzner et al., 2004).

For these and other reasons (Crozier and Luykx, 1985; Luykx, 1986) it seems unlikely that the multiple sex chromosomes system played a causal role in the evolution of termite eusociality. Multiple sex chromosome systems, rather than being a condition for eusocial evolution, probably arose sporadically and relatively recently in the evolution of termites, perhaps as a result of certain reproductive pattern accompanying eusocial behaviour (Bartz, 1979).

The reason for the wide occurrence of this otherwise rare phenomenon in the Isoptera order is still unclear. A possible explanation include heterozygote advantage in the face of the high ratio of inbreeding and an advantage to eusocial species associated with pattern of relatedness or uniformity engendered by sex-linked translocation heterozigosity (Rowell, 1985, 1986).

MOLECULAR DATA

Mitochondria play a central role in metabolism, apoptosis, disease and aging. They are the site of oxidative phosphorilation, essential for the production of ATP, as well as of a variety of other biochemical functions. Within these subcellular organelles a circular DNA (mtDNA) occurs. It is very commonly used in studies of molecular phylogenetics (Moritz et al., 1987). In animals, mtDNA is generally a small (15-20 Kb) genome containing 37 genes. Although much larger mitochondrial genomes have occasionally been found, these are the product mainly of duplications of the mtDNA rather than a variation in gene content.

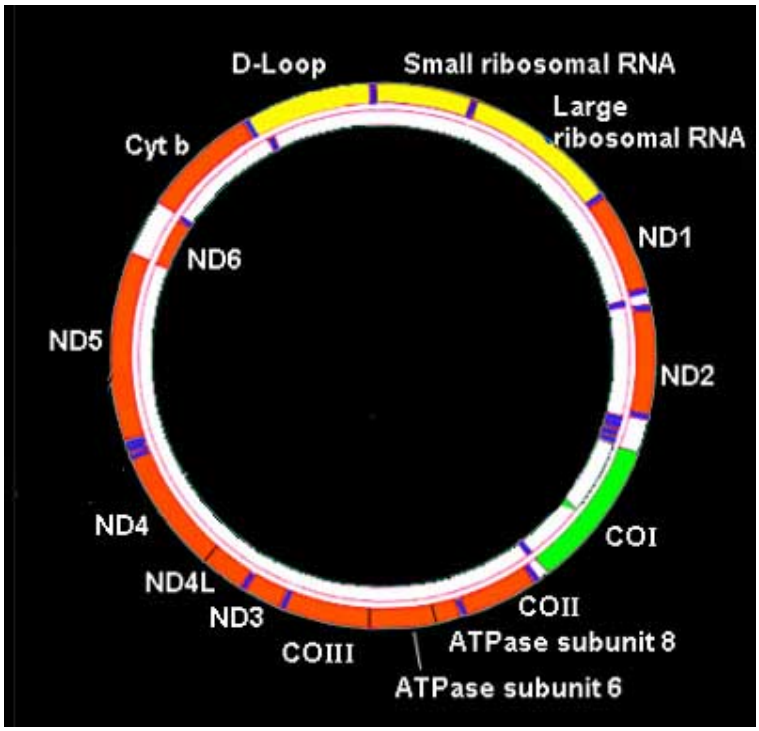

Fig. 26 - Scheme of insect mitochondrial genome

The most common gene complement (Fig. 26) encodes 13 protein subunits of the enzymes of oxidative phosphorylation (COI, COII, COIII, Cytb, ND1-6, ND4L, ATP6, ATP8), the two rRNAs of the mitochondrial ribosome (large, rrnL; small, rrnS), the 22 tRNAs (trnX) necessary for the translation of the protein encoded by mtDNA, and possess a large non-coding region known as "D-loop" in vertebrates and "A-T rich" region in Arthropods; these regions contains elements controlling replication and transcription.

Unlike nuclear DNA, in which genes are rearranged by ~50% each generation, mtDNA is maternally inherited (with the exception of some Bivalves) and has a rapid mutation rate easily measured and higher than nuclear DNA one. It is a powerful tool for phylogenetic studies (Avise, 2000). Biologists sequence a few selected genes across different species, and can build evolutionary trees depending on how conserved or divergent the sequences happen to be.

Mitochondrial DNA is by far the most widely used molecule for insects as well as for animals in general (Hillis et al., 1996). The popularity of mtDNA markers derives in large part from

its relative ease of isolation and amplification, even from marginally preserved specimens, and for the presence of universal primers (Simon et al., 1994).

For mitochondrial DNA, the most frequently sequenced genes are cytochrome oxidase I (COI), COII and 16S rDNA, with 12S rDNA not far behind. Of these, COII has been sequenced over the widest variety of taxa, with homologous sequences available for nearly all orders. COI has been sequenced in as many different groups. However, due to its length, the specific region chosen varies from study to study. COIII, NADH dehydrogenase 5 (ND5) and *cyt*b have been sequenced to a lesser extent. In recent years, the possibilities to sequence the whole mitochondrial genome and to use these data for phylogenetic studies may result in significant insights into the evolution both of organisms and of genomes.

The first molecular study on Isoptera interfamilies relationships was performed by Kambhampati et al. (1996) on a portion of the mitochondrial large ribosomal subunit gene (16S rRNA), in ten genera of five families. Two subsequent studies dealt with a full complement of families: Kambhampati and Eggleton (2000), on NADH 5 dehydrogenase gene; and Thompson et al. (2000) on 16S rRNA and COII genes. Although these studies considered different molecular markers, they gave broadly similar results (Fig. 27).

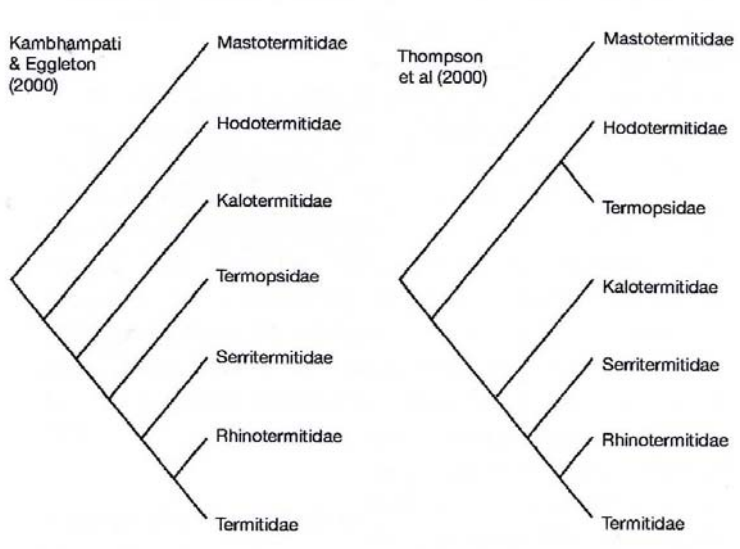

Fig. 27 – Family level phylogenies following Kambhampati & Eggleton (2000) and Thompson et al. (2000) studies.

Miura et al (1998) analyzed the COII sequences in 3 Rhinotermitidae and 12 Termitidae genera. Main result of this study is that the former family appears paraphyletic with respect to the latter that resulted monophyletic.

Subsequently, the relationships within the Rhinotermitidae family together with their correlation to Termitidae and Serritermitidae were investigated by Lo et al (2004) and Ohkuma et al., (2004), through the analysis of different mitochondrial markers. They found a substantial conflict with many previous hypotheses relative to the Rhinotermitidae family and its taxa, including the idea that it is monophyletic (Emerson, 1971). They suggested Rhinotermitidae as paraphyletic to Termitidae (in agreement with Miura (1998) hypotheses), with Serritermitidae being its sister group; this study also supported the wide accepted monophyly of the Termitidae.

Austin et al., (2004) examined the COII gene of 38 Rhinotermitidae species representing 10 genera; they suggested that the Rhinotermitidae should be polyphyletic and that Serritermitidae could be a subfamily of Rhinotermitidae.

Moreover, several studies took into account single genus or limited group of genera. Miura et al. (2000) presented a phylogenetic analysis of 17 Pacific Tropical species of the highly species-rich genus *Nasutitermes* using COII and 16S rRNA sequences. They concluded that the genus is clearly polyphyletic, with Neotropical and Australian species forming a single clade together with some of the Asian taxa; a subset of Asian taxa appears highly separated from congeneric species in the phylogenetic tree.

Other works detailed on Australian taxa: Thompson et al (2000) considered the relationships among 25 species from 7 genera of Australian lineages of drywood termites (Kalotermitidae) based on COII and Cytb sequences. They proved the monophyly of the genera; Lo et al. (2006) studied the Australian *Coptotermes* species using COII sequences and found that *C. acinaciformis* may be a species complex rather than a single entity. Ozeki et al. (2006)

considered the phylogeography of *Amitermes laurensis* on COII sequences, observing a correlation with mound shapes and environmental conditions.

The taxonomy and phylogeography of *Reticulitermes* taxa in Europe, were the object of several studies (Marini and Mantovani, 2002; Uva et al., 2004; Luchetti et al, 2004; Austin et al., 2006) based on different molecular markers. Nine main entities, either of specific or subspecific rank, are recognized in these works and their phylogenetic relationships are there highlighted.

1.4 – AIM OF THE RESEARCH

Several reasons stimulate biologists to study termites. The importance of this order is due to an extraordinary non-hymenopteran complex social system, to the ecological role of distribution, protection and stabilisation of organic matter in tropical ecosystem and to their part as pests for buildings, crops and trees. In fact, the economical relevance of termites is now well-known, and it is estimated that termites can cause yearly over $700 million worth of damage only in Australia.

Moreover, the hypothesis that termites could be utilized for the decomposition of lignocellulosic wastes produced by humans, has been put forward. In the future, landfills litter, and animal waste could be replaced by soil with the use of termites as waste recyclers (Pearce, 1997). Possible fields of interest include agronomy/agriculture, biogeography, ecology, forestry, metabolism, microbiology, physiology, soil-science, ingestion studies, isolation effects, but at the base of all, taxonomical and phylogenetical researches are needed. Some data, however, are very difficult to collect. Termite population samplings have traditionally been difficult for biologists; improved methods of population sampling are continually being developed.

The concentration of Isoptera species in Australia is particularly high, especially in the Northern Territory region (see chapter 1.2.2). Taxonomy and phylogenetic relationships among these taxa are very complex and far from being settled.

A wide experience in termite phylogeny and taxonomy characterize our research group (Marini and Mantovani, 2002; Luchetti et al., 2004; Luchetti, 2005). This facilitated and encouraged the beginning of several collaborations to share reciprocal competences and to create an internationally agreed set of data for analysis. Since 2003 a profitable collaboration with CSIRO (Sustainable Ecosystem Research Center in Darwin) has been started, with the possibility to enrich their morphological and ecological data with our karyological and molecular competences, allowing the construction of a more complete and detailed phylogeny, to highlight the intricate relationships among Northern Territory species.

Actually, it is now clear that a multidisciplinary approach is essential to better understand and resolve the taxonomic and phylogenetic problems.

In this study, a comprehensive karyological approach has been joined with an extended molecular one based on mitochondrial genes. A morphologic approach has been considered to support molecular taxonomic incongruences, using Scanning Electron Microscope (SEM).

Main results of my thesis are below presented as:

- Karyotype analysis and molecular phylogeny of Australian Isoptera taxa;
- Molecular Taxonomy and Phylogenetic Relationships among Australian *Nasutitermes* and *Tumulitermes* genera (Isoptera, Nasutitermitinae) inferred from mitochondrial COII and 16S sequences;
- Morphological analysis of *Nasutitermes* and *Tumulitermes* samples from the Northern Territory, based on Scanning Electron Microscope (SEM) images

My thesis will therefore presents a material and methods chapter where the employed techniques are pervasively illustrated, followed by three chapters corresponding to the three papers sent for publications. A final paragraph of conclusions will complete the thesis.

Chapter 2

MATERIAL AND METHODS

All the specimens considered in this study were collected in two samplings during 2003 and 2005 in the Australian Northern Territory. The first one included the North-West area of the Top-End, while the second one ranged in most of the Northern Territory, including the deserted Australian outback (Fig. 28). European samples were collected both in France and Italy during the period 2004-2006. Samples were employed in karyological, morphological and molecular analysis following different methodologies of collection and preservation.

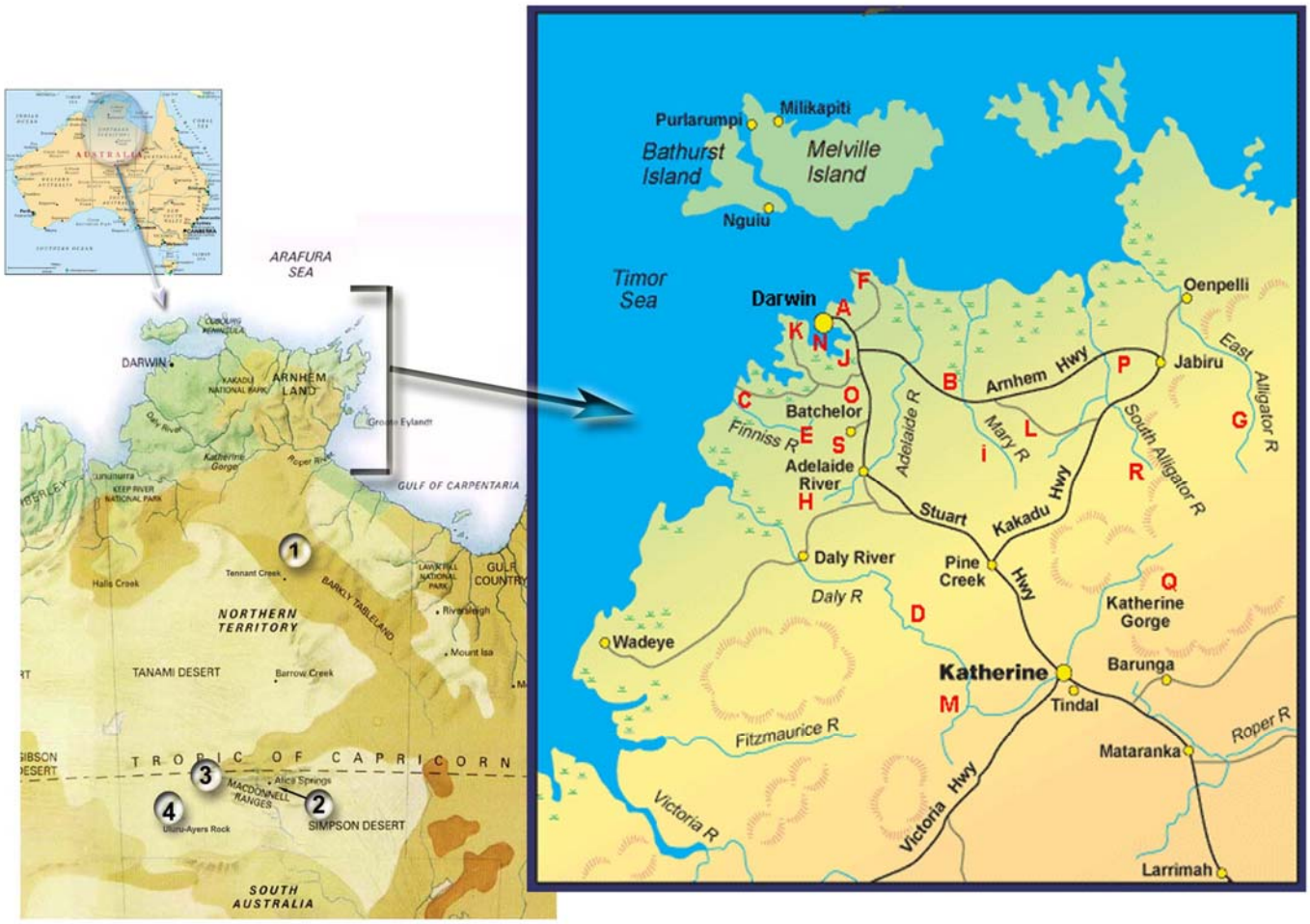

Fig. 28 - Collecting sites: **1** – Tennant Creek; **2** – Alice Spring; **3 –** MdDonnels Range; **4** – King's Kanyon; **a –** Darwin; **b** – Fogg Dam; **c** – Finnis River; **d** – Douglas Daly; **e** – Litchfield; **f** – Gunn Point; **g** –Kakadu Nat.Park; **h** – Adelaide River; **I** – Mary River Nat.Park; **j** – Palmerston; **k** – Mandorah; **l** – Mary River-Kakadu; **m** – Territory of Wild Life Park; **n** – Casuarina Beach; **o** – Katherine Gorge.

2.1 – MORPHOLOGICAL ANALYSIS.

The samples considered for this analysis are reported in Table 1. They are all utilized in the analysis reported in Chapter 5.

Tab. 1 – Species, collecting sites and collectiong dates of the samples considered in morphological analysis.

Species	Sample	Collecting site	Collecting date
Nasutitermes longipennis	1	Adelaide river	26/04/2003
	2	Adelaide river	26/04/2003
	3	Adelaide river	26/04/2003
	4	Douglas Daly	24/04/2003
	5	Mary river Nat. Park	19/10/2005
	6	Mary river Nat. Park	19/10/2005
	7	C. Darwin Nat.Park-Darwin	27/10/2005
	8	C. Darwin Nat.Park-Darwin	27/10/2005
	9	CSIRO-Darwin	25/11/2005
Nasutitermes eucalypti	1	Kakadu Nat. Park	07/05/2003
	2	Gunn point	25/11/2005
	3	Gunn point	25/11/2005
Tumulitermes hastilis	1	Kakadu-Mary river	31/10/2005
	2	Mandorah	02/10/2005
Tumulitermes pastinator	1	Palmerston	11/04/2003
	2	Douglas Daly	24/04/2003
	3	Douglas Daly	24/04/2003
Tumulitermes tumuli	1	King's canyon	11/11/2005
	2	King's canyon	11/11/2005

The scanning electron microscope (SEM) is a type of electron microscope capable of producing high resolution images of a sample surface with a characteristic three-dimensional appearance. SEM presents the ability to image not just thin films or foils but also bulk materials; it moreover, presents a variety of analytical modes available for measuring the composition and nature of the specimen. Depending on the instrument, the resolution can fall between less than 1 nm and 20 nm.

2.1.2 – PROTOCOLS:

(a) - Samples preparation

- Dehydration with a progressive scale of ethanol (70 – 80 – 90 – 95 – 100%) for 15 min each, if the sample is fresh. In any case the sample may have conserved in final absolute ethanol.
- Transfer the sample in 1:1, Hexamethyldisilazane(HMDS): absolute ethanol, solution for 20 min, under extractor fan; then put the samples in pure HMDS overnight or till complete evaporation. HMDS is useful for deactivating and coating several organic supports, since surface moisture is eliminated because this reagent dehydrates the surface.
- Mount samples on SEM stubs.
- Gold coating samples with BIO-RAD SC 502

(b) – Observation

Samples were observed under SEM JEOL – 5200, with a magnification range from 15X to 200,000X in 25 steps. The images were acquired with ImageSlave for Windows 2.14.

2.2 – KARYOLOGICAL ANALYSIS.

Samples karyologically analyzed are reported in Tab2. The results of this study are given in chapter 3. Sample numbers enclosed in parenthesis correspond to samples numbers repored in Tab 3.

Tab. 2 – Species, collecting sites and collecting dates of the samples considered in karyological analysis. Numbers within parenthesis correspond to samples number reported in Tab. 3.

Species	Samle	Collecting site	Collecting date
Nasutitermes graveolus	*1 (4)*	Fogg Dam	13/10/2005
	2 (5)	Fogg Dam	13/10/2005
Nasutitermes triodiae	*1*	Finnis River	23/04/2005
Nasutitermes longipennis	*1 (5)*	Mary River	19/10/2005
	2 (6)	Mary River	19/10/2005
	3 (9)	CSIRO-Darwin	25/11/2005
Tumulitermes pastinator	*1 (7)*	Gunn Point	25/11/2005
Microcerotermes nervosus	*1 (3)*	Territory of Wild Life Park	19/09/2005
	2 (4)	Adelaide river	13/10/2005
Microcerotermes boreus	*1*	Territory of Wild Life Park	27/09/2005
	2	Fogg Dam	13/10/2005
Amitermes germanus	*1*	CSIRO-Darwin	23/10/2005
Amitermes eucalypti	*1*	Home jungle-Darwin	01/10/2005
Amitermes darwini	*1*	CSIRO-Darwin	23/10/2005
	2	Mc Donnel Range-Alice Spring	12/11/2005
Amitermes parvus	*1*	CSIRO-Darwin	25/11/2005
Drepanotermes septentrionalis	*1*	Gunn Point	25/11/2005
	2	Gunn Point	26/11/2005
Macrognathotermes sunteri	*1*	Gunn point-Howard spring	25/11/2005
Lophotermes septentrionalis	*1*	Territory of Wild Life Park	03/10/2005
Ephelotermes taylori	*1*	Fogg Dam	13/10/2005
Ephelotermes melachoma	*1*	Hayes Creek - Kakadu	31/10/2005
Heterotermes vagus	*1*	Mango farm-Darwin	01/10/2005
	2	CSIRO-Darwin	02/10/2005
Coptotermes acinaciformis	*1*	Gunn point-Howard spring	25/11/2005
	2	Mandorah	02/10/2005
Mastotermes darwiniensis	*1*	CSIRO-Darwin	11/10/2005
Retuculitermes urbis	*1*	Italy – lab reared	
Reticulitermes lucifugus grassei	*1*	France – lab reared	
	2		
Kalotermes flavicollis	*1*	Palermo-Italy – lab reared	
	2	Croatia – lab reared	

The preparation of useful spreads of mitotic and meiotic metaphase chromosomes involves 5 steps: (1) selection of tissues with a high mitotic activity, (2) in vivo or in vitro treatment with a mitotic arresting reagent, (3) hypotonic treatment of tissues or cells, (4) fixing tissues or cells, and (5) making permanent chromosome preparations on slides. Several protocols exist to obtain mitotic spreads. Protocols followed in this study are reported below (2.2.1.b).

Chromosomes are chemically labelled with a dye ("stained"). The pattern of individual chromosomes is called chromosome banding. Giemsa stain is a conventional staining techniques used to uniformly stain chromosomes leaving the centromeres constricted, thus enabling the measurement of chromosome length, centromeric position, and arm ratio. The so call Romanovsky dyes (which include Giemsa, Leishman's, and Wright's stains) are now recommended for conventional staining, because the slides can be easily destained and banded by most banding procedures. Giemsa stain (2.2.1.a) is now the most popular stain for chromosome analysis (Gustashaw, 1991).

After staining, slides can be observed under a light microscope (2.2.1.c) coverslipping if required.

2.2.1 – PROTOCOLS:

(a) – Preparation of Mitotic and Meiotic Metaphase Chromosome

.

Solutions

- 0.05% colchicines in Ringer solution (NaCl 110mM; KCl 3mM; $CaCl_2$ 1mM; $NaHCO_3$ 2mM)
- 1% Sodium Citrate or 0.5% KCl
- Carnoy (solution 1:3, methyl alcohol: glacial acetic acid)
- 60% glacial acetic acid

Procedure

1. Dissect males or females reproductives or nymphs in a 0.05% colchicines solution in Ringer, drawing ovaries and testes. Leave gonads in this solution for 2-4h.
2. transfer gonads in 1% Sodium Citrate for hypotonic treatment.

3. fix in Carnoy for at least 1h.
4. transfer gonads on a slide cleaned with absolute ethanol and cover with 2-3 drops of 60% acetic acid. Disperse cells beating on slide boarder till necessary, then remove tissues.
5. dry slides on a hot plate.

(b) – Conventional Giemsa stain

Solutions

- Giemsa stain
- pH 6.8 phosphate buffer or distilled water
- Working stain: 4 mL Giemsa; 96 mL pH 6.8 buffer or distillate water

Procedure

1. Place slides in a Coplin jar or staining dish.
2. Prepare the working stain and pour it over the slides.
3. Stain for 7-15 minutes.
4. Rinse slides in two changes of distilled water.
5. Air dry slides; mount them with a cover slip if desired. (If sequential banding procedures are to follow, coverslipping is not recommended.)

(c) – Observation and karyotyping

A Zeiss light microscope was used to detect mitotic and meiotic spreads. Then they were photographed under a 100X immersion objective with a 100 ASA NeoPan (FujiFilm) film. This was developed in Rodinal S, diluted 1: 10 in distilled water. An IlFord multigrade IV RC deluxe photographic paper, then developed in IlFord HyPam fixer diluted 1: 4 in distilled

water, was used to capture imagines. Chromosomes from printed photographs were manually cut and paired or aligned checking size and morphology.

2.3 – MOLECULAR ANALYSIS.

All the samples considered in the molecular analysis are reported in Tab. 3. Actually, different subsets of these samples were used in different analysis as reported in chapter 3 and 4.

Tab. 3 – Collecting sites and dates, and Accession Numbers of the specimens analysed.

Species	Sample	Collecting site	Collecting date	Accession number	
				16S	COII
Nasutitermes graveolus	1	C. Darwin Nat.Park-Darwin	09/04/2003	EF078991	EF079010
	2	Mango farm-Darwin	25/04/2003	EF078991	EF079010
	3	Mango farm-Darwin	25/04/2003	EF078992	EF079010
	4	Fogg Dam	13/10/2005	EF078993	EF079010
	5	Fogg Dam	13/10/2005	x	EF079010
Nasutitermes triodae	1	Finnis river	23/04/2003	AY957642	EF079011
	2	Finnis river	23/04/2003	AY957643	EF079011
	3	Finnis river	23/04/2003	AY957644	EF079011
	4	Douglas Daly	24/04/2003	AY957639	EF079012
	5	Litchfield Nat Park	21/04/2003	EF078994	EF079013
Nasutitermes eucalypti	1	Kakadu Nat. Park	07/05/2003	AY957645	x
	2	Gunn point	25/11/2005	EF078995	EF079014
	3	Gunn point	25/11/2005	EF078996	EF079014
Nasutitermes longipennis	1	Adelaide river	26/04/2003	AY957646	EF079015
	2	Adelaide river	26/04/2003	AY957647	EF079016
	3	Adelaide river	26/04/2003	AY957648	EF079015
	4	Douglas Daly	24/04/2003	EF078998	EF079017
	5	Mary river Nat. Park	19/10/2005	EF078997	EF079018
	6	Mary river Nat. Park	19/10/2005	EF078997	EF079019
	7	C. Darwin Nat.Park-Darwin	27/10/2005	EF078999	EF079018
	8	C. Darwin Nat.Park-Darwin	27/10/2005	EF078999	x
	9	CSIRO-Darwin	25/11/2005	EF079000	EF079020
Tumulitermes pastinator	1	Palmerston	11/04/2003	EF079001	EF079021
	2	Douglas Daly	24/04/2003	EF079002	EF079022
	3	Douglas Daly	24/04/2003	EF079002	EF079023
	4	Adelaide river	13/10/2005	EF079003	EF079021
	5	C. Darwin Nat.Park-Darwin	27/10/2005	x	EF079024
	6	Mandorah	02/10/2005	EF079004	EF079025
	7	Gunn point	25/11/2005	EF079005	x
	8	Gunn point	25/11/2005	EF079005	EF079026
	9	Gunn point	25/11/2005	EF079002	EF079021
	10	Gunn point	25/11/2005	EF079002	EF079021

Tumulitermes hastilis	1	Kakadu-Mary river	31/10/2005	EF079006	EF079027
	2	Mandorah	02/10/2005	EF079007	EF079028
	3	Tennant Creek	14/11/2005	EF079008	EF079029
Tumulitermes tumuli	1	King's canyon	11/11/2005	EF079009	EF079030
	2	King's canyon	11/11/2005	EF079009	EF079030
microcerotermes nervosus	*1*	Kakadu Nat.Park.	24/03/2003	AY957652	EF442695
	2	Reynolds River	07/05/2003	AY957653	EF079031
	3	Territory of Wild Life Park	19/09/2005	x	x
	4	Adelaide river	13/10/2005	x	x
	5	CSIRO-Darwin	18/10/2005	x	x
	6	Casuarina beach - Darwin	23/10/2005	x	x
	7	Hayes Creek - Kakadu	31/10/2005	x	x
microcerotermes boreus	*1*	Territory of Wild Life Park	27/09/2005	x	EF442696
	2	Fogg Dam	13/10/2005	x	EF442697
	3	Katherine Gorge	15/11/2005	x	x
amitermes darwini	*1*	CSIRO-Darwin	23/10/2005	x	EF442698
	2	Mc Donnel Range-Alice Spring	12/11/2005	x	EF442699
	3	Alice springs	14/11/2005	x	x
	4	Alice springs	14/11/2005	x	x
	5	Tennant Creek	16/11/2005	x	x
amitermes germanus	*1*	CSIRO-Darwin	23/10/2005	x	EF442702
	2	Gunlom-Kakadu	07/11/2005	x	EF442703
	3	C. Darwin Nat.Park-Darwin	23/10/2005	x	x
amitermes eucalypti	*1*	Home jungle-Darwin	01/10/2005	x	EF442700
	2	COX peninsula	02/10/2005	x	EF442701
amitermes parvus	*1*	CSIRO-Darwin	25/11/2005	x	EF442704
Drepanotermes septentrionalis	*1*	Gunn Point	25/11/2005	x	EF442705
	2	Gunn Point	26/11/2005	x	EF442706
Macrognathotermes sunteri	*1*	CSIRO-Darwin	25/11/2005	x	Ef442707
Lophotermes septentrionalis	*1*	Territory of Wild Life Park	03/10/2005	x	EF442708
Ephelotermes taylori	*1*	Fogg Dam	13/10/2005	x	EF442709
Ephelotermes melachoma	*1*	Hayes Creek - Kakadu	31/10/2005	x	EF442710
Heterotermes vagus	*1*	Mango farm-Darwin	01/10/2005	x	EF442711
	2	CSIRO-Darwin	02/10/2005	x	EF442711
Coptotermes acinaciformis	*1*	Gunn point-Howard spring	25/11/2005	AY957654	EF442712
	2	Mandorah	02/10/2005	AY957655	EF442713
	3	CSIRO-Darwin	11/10/2005	x	x
Schedorinotermes actuosus	*1*	Territory of Wild Life Park	27/09/1995	x	x
	2	Hayes Creek - Kakadu	31/10/2005	x	x
Cryptotermes secundus	*1*	Mangrove forest-Batchelor	03/05/2003	x	EF442718
	2	Mangrove forest-Batchelor	03/05/2003	x	EF442718
Cryptotermes domesticus	*1*	Mandorah	12/10/2005	x	EF442719
	2	Mandorah	03/12/2005	x	EF442720
Mastotermes darwiniensis	*1*	Finnis river	11/03/2003	AY957656	AB014071
	2	Home jungle-Darwin	23/04/2003	AY957657	x
	3	CSIRO-Darwin	11/10/2005	AY957658	x
Retuculitermes urbis	*1*	Italy – lab reared		x	EF442714
Reticulitermes lucifugus grassei	*1*	France – lab reared		x	EF442715
	2			x	x
Kalotermes flavicollis	*1*	Palermo-Italy – lab reared		x	EF442716
	2	Croatia – lab reared		x	EF442717

2.3.1 – PROTOCOLS

(a) DNA isolation with method CTAB

Solutions:

- 2XCTAB Isolation Buffer

100mM Tris-HCl, pH 8.0

1.4 M NaCl

20mM EDTA

2% CTAB (Hexadecyltrimethylammonium bromide)

Procedure:

1. Grind a single termite head in a 1.5 ml eppendorf tube with 300µl of 2XCTAB buffer.
2. Incubate in 60°C water bath for 1hr.
3. Add 300µl of chloroform and shake for 2 min.
4. Centrifuge at 15.000g for 10 min.
5. Take aqueous and repeat chloroform extraction.
6. Label fresh eppendorfs and add 600µl cold 70% EtOH 25µl 3M NaOAc, and the aqueous from the chloroform extraction. Mix by inversion.
7. Centrifuge at 15.000 for 10 min.
8. Pour off EtOH and rinse pellet in 100 µl 70% EtOH.
9. Centrifuge at 15.000 for 5 min.
10. Pour off EtOH. Dry pellet at room temperature under extractor fan.
11. Re-dissolve the pellet in 30 µl of 1XTE.
12. Verify quality and quantity using 1-1.5% agarose gel.

(a) Amplification

PCR is used to amplify specific regions of a DNA strand, corresponding in this case to mitochondrial COII and 16S genes. PCR, as currently practiced, requires several basic components:

- *DNA template* that contains the region of the DNA fragment to be amplified
- One or more *primers,* which are complementary to the DNA regions at the 5' and 3' ends of the DNA region that is to be amplified
- *Taq polymerase* or another DNA polymerase with a temperature optimum around 70°C
- *Deoxynucleotide triphosphates,* (dNTPs) from which the DNA polymerase builds the new DNA
- *Buffer solution,* which provides a suitable chemical environment
- *Divalent cation*s as magnesium

PCR amplification was performed in 50µl mixture using the Taq polymerase Recombinant Kit (Invitrogen) and following the kit protocol. Thermal cycling was done in a Gene Amp PCR System 2400 (Applied Biosystem) programmable cyclic reactor. The conditions for amplification were as follows:

16S – initial denaturation at 94° for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing at 48°C for 1min, extension at 72°C for 2min, and final extension at 72° for 7 min.

COII – initial denaturation at 94° for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing 48/52°C for 30s, extension 72°C for 2min, and final extension at 72° for 7 min.

Primers for PCR amplification and sequencing were:

COII gene:

forward: 5'-CAG ATA AGT GCA TTG GAT TT-3' (modified A-tLeu, 3022-3042 in *D.Yakuba*)

reverse: 5'-GTT TAA GAG ACC AGT ACT TG-3' (named B-tLys [Liu and Beckenbach, 1992; Simon et al., 1994]; 3804-3784 in *D.yakuba*);

16S gene:

forward: 5'-TA CGC TGT TAT CCC TAA-3' (16S-F1, Kambhampati and Smith, 1995; 13000-13017 in *D.yakuba*)

reverse: 5'TCT GGT TTT TCA AGA AAT GA-3' (16S-R4, specific for termites, 13758-13733 in *D.yakuba*).

(b) Amplicons purifying.

Amplicons were purified using the Wizard SV Gel and PCR clear-up System (Promega) with the follow protocol:

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10 µl Membrane Binding solution per 10mg of gel slice. Vortex and incubate at 50-65°C until gel slice is completely dissolved.
3. Insert SV Minicolumn into collection tube.
4. Trsansfer dissolved gel mixture to the Minicolumn assembly. Incubate at room temperature for 1 min.
5. Centrifugate at 16,000 x g for 1 min. Discard flowthrough and reinsert minicolumn into collection tube.
6. Add 700µl Membrane Wash Solution. Centirfugate at 16,000x g for 1 min and discard flowthrough.

7. Repeat step 6 with 500µl membrane wash solution and centrifugate at 16,000 x g for 5 min.
8. Transfer minicolumn to a clean 1.5ml microcentrifuge tube.
9. Add 50 µl of nuclease-free water. Incubate at room temp for 1 min. Centrifuge at 16,000 x g for 1 min
10. Discard minicolumn and store DNA at 4°C or -20°C.

(c) Sequencing

Both strands were here sequenced with the DNA sequencing kit (BigDye terminator cycle sequencing, Applied Biosystem) in an ABI Prism 310 Genetic Analyzer.

The dideoxy method for DNA sequencing, developed in the Seventies by Sanger, takes advantage of the ability of DNA polymerase to incorporate analogues of nucleotide bases by using 2′,3′-dideoxynucleotides (ddNTPs) as substrates. When a dideoxynucleotide is incorporated at the 3′ end of the growing chain, chain elongation is terminated selectively at A, C, G, or T because the ddNTP lacks a 3′-hydroxyl group. Since its discovery, the method has undergone many improvements regarding labeling technology, chemistry and instrumentation, nevertheless, the basic protocol remains essentially unchanged.

The BigDye® Terminator kit from Applied Biosystems uses four different fluorescent dyes to label ddNTPs, which are added sequentially to the primer through a cycle sequencing reaction. The kit provides all required reagents for the sequencing procedure in a reaction-ready, pre-mixed format. These reagents are suitable for sequencing of single-stranded or double-stranded DNA templates, PCR products, and large templates. Buffer composition is designed to allow a single tube reaction for each primer, producing a series of molecules of different length, each one terminating and labeled at a different base. The ratio of dNTPs/ddNTPs is calculated so that termination is obtained at least once for every position of

the template. Reaction products can then be run in an automated sequencer to obtain the final sequence.

(d) Sequence elaborations

Alignments performed with the Clustal algorithm of the Sequence Navigator program (ver 1.0.1, Applied Biosystem Inc.) were also checked by sight. Nucleotide substitutions matrices were determined using MEGA3 (Kumar et al., 2004).

Phylogenetic relationships were inferred by a Maximum Parsimony (MP) method and a Maximum Likelihood (ML) method using PAUP 4.0b (Swofford,2001). The best substitution model for ML was selected using the program Modeltest 3.06 (Posada and Crandall, 1998).

Chapter 3

Karyotype analysis and molecular characterization of Australian Isoptera taxa (Bergamaschi *et al*., submitted).

ABSTRACT

A comprehensive karyological characterization of twenty Australian and three European species of Isoptera, together with a mitochondrial analysis is here presented.

Higher termites appear karyotypically very uniform, while lower termites are highly variable. The differences in chromosome number are explained through Robertsonian changes or multiple translocation events. An ancestral acrocentric karyotype can be suggested as the most primitive one. In Kalotermitidae chromosomal repatterning has repeatedly arisen with the XO-male type possibly representing a XY derived condition. This argues against a simple derivation of termites from cockroaches. The fixed chromosome number of Rhinotermitidae and Termitidae (2n = 42, XY male type) may be explained with the non-random nature of chromosomal evolution. . Of particular interest is the presence of a sex-linked multivalent with a ring or a chain aspect, in the majority of the species here considered. Phylogenetic analyses on COII sequences recognise Mastotermitidae as the basal lineage and the Rhinotermitidae + Termitidae cluster with a good bootstrap support. Kalotermitidae taxa fail to be joined in a single cluster in agreement with the chromosomal variability detected. On the other hand, the karyotypic conservation of the Termitidae family contrast with the polytomy evidenced at the subfamily level.

INTRODUCTION

Termites are an ancient order of social insects with origins that date back more than 120 million years ago to the early Cretaceous period (Rohr et al. 1986).

It has long been accepted that termites appeared closely related to cockroaches and mantis, and classified in the same monophyletic superorder (Dictyoptera). New researches have shed light on the details of termite evolution (Thorne, 1992, Lo et al.,2000, 2003, Kjer, 2004). There is now strong evidence suggesting that termites have actually evolved from wood-feeding cockroaches. *Cryptocercus* spp. should be the closest living relatives of termites, sharing similar morphology, social features and endosymbiotic bacteria with the primitive termite *Mastotermes darwiniensis* (fam. Mastotermitidae). On the other hand, cytological investigations have shown that *Cryptocercus spp.* chromosome numbers range from 2n=17-21 (Palearctic species) to 2n=37-47 (North American and North Pacific species), suggesting a possible genome duplication or reduction within this genus (Lo et al., 2006), while *M. darwiniensis* has a chromosome number of 2n=98 (Bedo, 1986). These quite different karyological situations do not allow to suggest any pattern of chromosomal evolution.

Molecular phylogenetic relationships among termite families were first highlighted in Kambhampati et al. (1996). These authors considered the mitochondrial 16SRNA gene fragment to demonstrate that the most ancient taxon is represented by Mastotermitidae and that Kalotermitidae family is possibly basal to a Rhinotermitidae-Termitidae group. Subsequent molecular works based on mitochondrial COII or 16S genes tried to clarify the relationships within Rhinotermitidae, Termitidae (Miura et al., 1998, 2000; Austin et al., 2004; Ohkuma et al., 2004) and Kalotermitidae families (Thompson et al., 2000).

Data on chromosome numbers in Isoptera (Vincke & Tilquin, 1978, Luykx and Syren, 1979, Luykx, 1989) pointed out that higher termites (Rhinotermitidae, Termitidae) are karyotypically very uniform, with most species sharing a diploid number of 42. Lower termites (Kalotermitidae, Termopsidae) appear more variable, with diploid numbers ranging

from 28 to 56. Nevertheless, a detailed karyotype analysis describing chromosome pairs, has been achieved only for few species (such as *Reticulitermes lucifugus* Rossi and *Kalotermes flavicollis* Fabr.; Fontana, 1982, 1991), mainly because termite chromosomes are small or very small sized, relatively numerous and with few distinguishing features.

A karyological peculiarity of termites is displayed by the wide occurrence of meiotic multiples in the male sex.

A segmental interchange complex of 11-17 chromosomes was first observed in *Incisitermes schwarzi* and *Kalotermes approximatus* males (Syren and Luykx, 1977) followed by the report of a sex-linked ring of four chromosomes in the male meiosis of 21 African species of Termitidae (Vincke and Tilquin, 1978). Subsequently, Luykx & Syren (1979) observed sex-linked translocations in 9 species of lower termites, while Fontana (1980) in the males of five populations of *Reticulitermes lucifugus* found comparable interchange multiples of four chromosomes. Since these complexes are restricted to males, they may imply the existence of a multiple sex chromosome system with males heterozygous for a specific series of translocations, involving a different number of chromosomes. These interchange complexes seems to have arisen independently many times and may take the form of ring or chains of chromosomes (Luykx, 1990). They vary within and between Isoptera species.

On the origin of the chromosome interchange system, there are good reasons to assume that the chains were initiated by a rearrangement event involving an original X chromosome and an autosome. Further translocations have resulted in what is effectively a multiple XY sex-determining mechanism (Rowell, 1985). It seems that all chromosomes behave like Xs and Ys in their segregation in male meiosis, but it is not clear how many of them are involved in sex determination (Santos and Luykx, 1985). Sex-determining genes might be located in only one of the chromosomes involved in the complex, the other chromosomes containing genes that are sex-linked only by virtue of the translocation. The fact that part of the genome has become sex-linked by means of such translocations suggests that there may be many loci

(such as Acp-1 and Est-3 in *Incisitermes*, see Santos and Luykx, 1985), widely distributed over the whole genome, upon which male-female differential selection is acting.

Meiotic multiples of four or more elements resulting from chromosome translocations are uncommon, but are found with different feature, in plants like *Viscum* and *Oenothera* (Cleland, 1972; Barlow et al., 1978), in invertebrates like *Otocryptos spp.* (centipede; Ogawa, 1954) and *Delena cancerides* (huntsman spider; Rowell, 1985) and in monotremes such as platypus and echidna (Bick and Sharman, 1985; Wrigley and Graves, 1988; Grutzner et al., 2004).

The reason for the wide occurrence of this otherwise rare phenomenon in the Isoptera order is still unclear. A possible explanation includes heterozygote advantage in the face of the high ratio of inbreeding, an advantage for eusocial species associated with patterns of relatedness or uniformity engendered by sex-linked translocation heterozygosity (Rowell, 1985, 1986). On the other hand, it seems unlikely that the multiple sex chromosomes of termites played a main role in the evolution of their sociality (Luykx, 1989, Thorne, 2003). This system probably arose sporadically and relatively recently, perhaps as a result of the reproductive pattern accompanying eusocial behaviour (Bartz, 1979).

The assumption that these chromosome interchanges are lacking in female is only partially supported by direct evidence, because only a low number of studies have taken into account female meiosis. In particular, Vincke and Tilquin (1978) first, and Luykx and Syren (1979), later claimed that translocation complexes were absent in females of analyzed American and African species, respectively. On the other hand, if females are also translocation heterozygotes, one would expect male offspring with different translocated chromosome (Syren and Luykx, 1981). But all the males from the same colony always show the same meiotic chromosome configuration, with a typical chain or ring. It seems likely, therefore, that only the males are translocation heterozygotes (Gruetzner et al., 2006). Only Fontana

(1980) observed a polymorphic situation in an Italian colony, with some males showing regular bivalents and others with a chain of four or six multiples.

This paper presents a comprehensive karyological characterization of twenty Australian and three European species of Isoptera. It aims to develop detailed karyotypes or chromosome numbers of species not yet studied and to evaluate if, in particular, Australian taxa exhibit sex-linked complexes in male and female meiosis and with which features. Further, a mitochondrial analysis was performed to add to the knowledge of evolutionary relationships within this insect order by comparing karyological and molecular data.

MATERIAL AND METHODS

Samplings

Samples were field collected during 2005 in North Australia and in Central-South Europe (Table 1). Australian termites were identified through the morphological keys of Hill (1942) and Miller (1991), and also through comparison with the CSIRO termite reference collection (CSIRO Entomology, Canberra).

Samplings include the only member of the family Mastotermitidae (*Mastotermes darwiniensis*), 3 species of the family Kalotermitidae, 4 species of the family Rhinotermitidae and 15 species of the family Termitidae. Twenty out of the 23 taxa molecularly analyzed here are karyologically analyzed for the first time. For each sample, alate, secondary reproductives and nymphs were kept alive for the karyological investigation, while workers and soldiers were immediately preserved in absolute ethanol for molecular analyses.

Cytological investigation

Mitotic and meiotic chromosome spreads were obtained from males and females gonads of alates (imagos) or from the gonads of secondary reproductives or nymphs. From each sample, separate preparations were obtained from 2 to 20 individuals.

Testes and ovaries were placed in a colchicine insect saline solution for two hours. A hypotonic shock was performed with sodium citrate 1% for 20 min and the tissue fixed for 1h in 3:1 methanol:glacial acetic acid. Following fixation tissues were dispersed in 60% acetic acid and the cells spread by placing the slides on a hotplate. Finally the slides were stained with Giemsa solution (5% in phosphate buffer). Photomicrographs of metaphase were taken on Neopan Fujifilm (100 ASA) and developed in Hypam.

Testes generally provided cells at every stage of meiosis and mitosis. For six species meiotic figures were also obtained from females. Interpretation of sex multivalents in the first meiotic division were confirmed by examining chromosome numbers in the second meiotic division and in mitotic cells and by determining the diploid number of the female in the mitotically dividing follicle cells of the ovary. In each species the diploid chromosome number was confirmed by examination of 15 up to 30 male and female mitotic figures. The karyotype was reconstructed for 15 species by considering at least the best three mitotic plates obtained. Chromosome nomenclature followed Levan et al. (1964), the symbols m, sm, st and t designating metacentric, submetacentric, subtelocentric and telocentric chromosome, respectively. This classification system allows the "fundamental number" (NF, the total number of major chromosome arms in the haploid set) to be simply determined from the total haploid chromosome number, *n,* and the haploid number of metacentrics (m) or sub-metacentrics (s*m)*: NF= *n* + *m*-*sm*.

Molecular analyses

Samples were conserved in absolute ethanol and then used for DNA extraction and amplification of the COII mitochondrial genes through polymerase chain reaction (PCR).

Total DNA was extracted following the Doyle & Doyle method (1987) from one up to four samples per species. To avoid symbiont DNA contamination, only the head was used as source tissue. PCR amplification was performed in 50µl reactions using the Invitrogen kit,

with recombinant Taq DNA polymerase. Thermal cycling was done in a GeneAmp PCR System 2400 (Applied Biosystem) programmable cyclic reactor. The conditions for amplification were as follows: initial denaturation at 94° for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing 48/52°C for 30s, extension 72°C for 2min, and final extension at 72° for 7 min. Primers for PCR amplification and sequencing were: 5'-CAG ATA AGT GCA TTG GAT TT-3' (modified A-tLeu, 3022-3042 in *D.Yakuba*)/ 5'-GTT TAA GAG ACC AGT ACT TG-3' (named B-tLys [Liu and Beckenbach, 1992; Simon et al., 1994]; 3804-3784 in *D.yakuba*. Amplicons were purified using the Wizard PCR prep DNA clear-up System (Promega), and both strands were sequenced with the DNA sequencing kit (BigDye terminator cycle sequencing, Applied Biosystem) in an ABI Prism 310 Genetic Analyzer. The nucleotide sequences of the newly analysed specimens have been submitted to the GenBank under following accession numbers: **EF442695**, **EF442696**, **EF442697**, **EF442698**, **EF442699**, **EF442700**, **EF442701**, **EF442702**, **EF442703**, **EF442704**, **EF442705**, **EF442706**, **EF442707**, **EF442708**, **EF442709**, **EF442710**, **EF442711**, **EF442712**, **EF442713**, **EF442714**, **EF442715**, **EF442716**, **EF442717**, **EF442718**, **EF442719**, **EF442720**. Alignments performed with the Clustal algorithm of the Sequence Navigator program (ver 1.0.1, Applied Biosystem Inc.) were also checked by sight. Nucleotide substitutions matrices were determined using MEGA3 (Kumar et al., 2004).

Phylogenetic relationships were inferred through a Maximum Parsimony (MP) method and a Maximum Likelihood (ML) method using PAUP 4.0b (Swofford,2001) with 2000 and 100 bootstrap replicates, respectively. The best substitution model (Tamura-Nei + I + G) for ML was selected using the program Modeltest 3.06 (Posada and Crandall, 1998).

European samples of *Reticuliterems spp.* and *Kalotermes flavicollis* and 3 species of the woodroaches *Cryptocercus* (Accession No: **DQ007645**, **DQ007643**, **DQ007642**), considered the closest living relatives of the eusocial termites, were included to complete the

phylogenetic analyses. In addition, *Periplaneta americana* sequences (Accession No: **DQ181546**) were employed as outgroup.

RESULTS

Karyological analysis

Standard karyotypes were not easy to reconstruct and to analyse from an evolutionary point of view owing to a high homogeneity in size and morphology of almost all the analysed chromosomes. However, chromosome numbers and size, number, position and type of secondary constrictions, meiosis characteristics and meiotic translocation complex traits, are here described for 23 species.

CHROMOSOME NUMBERS

Mastotermitidae. *Mastotermes darwiniensis.* Eight individuals pertaining to two different colonies were considered, all showing a diploid number of 2n=96 and NF=50 in each of the mitotic figures observed (Fig.1). In these karyotypes, it was only possible to pair with certainty a few chromosomes with some clear distinguishing features, the others arranged just according to decreasing size; most chromosomes are actually small and very homogeneous in size and traits. Analyzed karyotypes had a predominance of telocentric (t) chromosomes and two pairs (93-94, 95-96) of metacentric (m) or sub-metacentric (sm) chromosomes. No satellites bearing chromosomes were observed.

Kalotermitidae. *Kalotermes flavicollis.* The four studied colonies, two from Sicily (Italy) and two from Croatia, apparently showed a diploid chromosome complement that could be arranged in pairs (Fig. 2-3). Males karyotypes presented a complement of 2n=67 (NF= 37), composed of at least 4 pairs of m and the remainder t and st chromosomes. The uneven one was t. All female complements analyzed were 2n=68 (NF= 38) with 4 pairs of m. Thus, the

number agreed with previous data presented by Fontana (1982). All the specimens analyzed presented satellites on the short arms of the st pairs 1, 2, 3, 4, 11 and 12, in either homozygous or heterozygous condition. Their presence blurs the real localization of the centromere, and could easily produce mismatchings during attempts to arrange the chromosomes in pairs.

Rhinotermitidae. The diploid complement of all the analyzed members of this family was 2n=42 with a FN=25. More specifically:

Coptotermes acinaciformis. The low quality of the mitotic figures obtained allows to observe that the exact chromosomes numbers is 2n=42, but does not permit to reconstruct any karyotype.

Heterotermes vagus. No evidence of odd chromosomes was found in males or females of this species. Four pairs of m could be observed (19-21), while the other pairs were sm to t (Fig.4). For both male and female complements, one satellite was detected on the short arm of the telocentric chromosome pair 12.

Reticulitermes urbis – Reticulitermes lucifugus grassei . No odd chromosomes and 4 pairs of m were also observed in the mitotic complements of males and females of these taxa: 4 pairs of m chromosomes and one satellite on the short arms of the chromosomes 21-22 were observed (Fig.5).

Termitidae. A constant diploid complement of 2n=42 and the presence of numerous satellites characterise all the member of this family. The secondary constrictions can cause chromosome misclassifications, by making unclear the real position of the centromeres. Moreover, in both sexes most chromosomes have similar size and few distinguishing features. For these reasons, unclassifiable chromosomes were not arranged in pairs but just according to decreasing size. The following groups were analysed:

Nasutitermitinae subfamily. The karyotypes of *N. graveolus* (Fig.6), *N. longipennis* (Fig.7) and *T. pastinator* showed at least 4 pairs of m chromosomes. Moreover, the karyotypes

obtained from males and females of *N. longipennis*, showed a peculiar satellite on the short arms of chromosome 8, in addition to, at least, another 10 smaller satellites.

Termitinae subfamily. The karyotypes of the *Amitermes*-group (Fig. 8-9-10-11) showed the highest amount of secondary constrictions of all Isoptera taxa analysed: almost all chromosomes appear to bear satellites, making the description of the karyotype difficult. While it was possible to establish that the majority of the chromosomes were t to sm, it was impossible to define the exact number of the m ones. Karyotypes obtained from *Drepanotermes* samples (Fig. 10) showed much contracted chromosomes, and it was not always possible to distinguish primary from secondary constrictions.

In the *Termes*-group, at least 3 pairs of m chromosomes could be recognised. The number of satellites was lower in *Macrognathotermes* (Fig.12) than in *Ephelotermes* (Fig.13), where at least 10 chromosomes bore satellites on the short arms.

MEIOTIC CHROMOSOMES

Mastotermitidae. Many metaphase I divisions were found both in males and females, showing that all chromosomes form regular bivalents with a haploid number of n=48 (Fig.14.a). This agrees with the scoring of 96 mitotic chromosomes. Even if some bivalents were contracted or relatively small, it was possible to make out the two homologous in most instances. These suggested the presence of a high number of t and st chromosomes in which chromatid separation and terminal paring was being realized. The lack of any chain or ring of chromosomes showed that no sex-linked translocation complex occurs.

Kalotermitidae. Thirty-three regular bivalent and one trivalent were observed in each early metaphase I examined for males of *Kalotermes flavicollis*, confirming the diploid number of 67 (Fig. 14.b). No female meiosis could be obtained.

Rhinotermitidae. While *R. urbis* always presented 21 regular bivalents (Fig. 14.c), *R. lucifugus grassei* showed different numbers among colonies. In particular, in 15 out of 20

colonies, plates with 19 bivalents and a chain of 4 chromosomes were observed (Fig. 14.d), while the other 5 populations showed 21 regular bivalents. Similar situation was observed in *Coptotermes acinaciformis*, where only one of the two analyzed colonies presented the translocation complex.

The same segmental interchange complex of four chromosomes in a ring besides 19 bivalents, have been observed in all male meiosis of *H. vagus* (Fig. 14.e).

Termitidae. All male members of this family showed in early metaphase I the sex-linked ring quadrivalent in addition to 19 pairs, and 21 chromosomes in metaphase II (Fig. 14.f – 14.q). Female meiosis of *Amitermes darwini* e *Microcerotermes nervosus* showed 21 regular bivalents.

Molecular analyses

647 bp of the COII gene, encoding for 215 amino acids, were sequenced in 38 specimens representing the 20 termite species here karyologically analysed for the first time. Nucleotide variation for this gene showed an adenine-thymine bias (A+T= 63.9%). 413 out of 448 variable sites were parsimony informative. The majority of the variation occurred at the third codon position (61 %) followed by position 1 (24 %) and 2 (15 %). Each one of the 29 haplotypes scored for this dataset (Tab. 1) were sample-specific with the exception of a few haplotypes that corresponded to different samples of the same species: H1 for *N. graveolus*, H2 for *N. triodiae*, H3 for *N. longipennis*, H5 for *T. pastinator*, H23 for *H. vagus* and H30 for *C. secundus*.

Tamura-Nei distances (Gamma distribution shape parameter= 0.4906, Tab.2) for different Australian haplotypes of the same species ranged from 0.004 (*Amitermes darwini* 1 vs 2) to 0.120 (*Drepanotermes septentrionalis* 1 vs 2).

Interspecies distances within family, were quite conserved for Kalotermitidae (0.319-0.388) and Rhinotermitidae (0.141-0.145). A huge range of variation was observed in Termitidae

(0.031-0.303). Comparing the Australian haplotypes with European taxa haplotypes from GeneBank, we observed a lower differentiation within Rhinotermitidae (0.228-0.294), than within Kalotermitidae (0.376-0.408).

Distances between families ranged from 0.216 (*Heterotermes vagus vs Nasutitermes longipennis 3*) to 0.885 (*Mastotermes darwiniensis vs Microcerotermes boreus 2*).

The total number of substitutions presented the same ranges and trends with respect to Tamura-Nei distances (available from the authors).

In both MP and ML elaborations the haplotypes of Termitidae (Fig. 15; group I) and Rhinotermitidae (Fig. 15; group II) families, constitute two well defined clusters, with a further subdivision of Termitidae in subfamilies, even if polytomically related.

Kalotermes flavicollis and *Cryptotermes spp.* sequences never join in a single Kalotermitidae cluster (Fig. 15; group III). Moreover, in the ML tree they are polytomically related to *Mastotermes darwiniensis* (group IV), which appear basal to the Isoptera taxa here considered only in the MP tree.

DISCUSSION

At variance of previous data (Bedo, 1987) where *Mastotermes darwiniensis* showed a diploid number of 98 chromosomes, all the here analyzed mitotic plates presented 96 chromosomes and 48 bivalents were recognizable in meiotic figures. This difference could due to the higher quality of the metaphase figures here obtained that allowed accurate counting. As far as sex chromosome translocations are concerned, our observations on *M. darwiniensis* confirm the absence of these complexes both in male and female meiosis; on the other hand, observations on *Kalotermes flavicollis* agreed with previous data on the existence of a sex determining mechanism of the X_1X_2Y type in males (Fontana, 1982).

The trivalent formed in male meiosis may be composed of a medium-sized acrocentric chromosome (X1) one side, and of a small acrocentric (X2) the other, paired with two limbs of the intermediate Y of the same length of X1 (Fontana, 1982). The hypothesis of a possible origin by polyploidization of *K. flavicollis* (Clèment, 1977) must be rejected by the chromosome pairing here recognized. For this reason, the particular XXY sex-determining mechanism in *K.flavicollis* males, may probably originate from an initial X0/XX system. Afterwards, a reciprocal translocation between the original X and a pair of autosomes, may have brought to the trivalent complex formation at meiosis, such as in *Cryptotermes spp.* (Luykx, 1990).

It should be noted that the Kalotermitidae family shows different types of sex-linked complexes: *K. approximatus* and *Incisitermes schwarzi* presented reciprocal translocations involving from 11 to 19 chromosomes (Syren and Luykx, 1977, 1981) of the male chromosome set (2n=32); in *Neotermes insularis* (2n=52) a six-membered ring was found (Luykx, 1990); and 5 species of *Cryptotermes* on the 13 studied by Luykx (1990) presented an XXY trivalent at meiosis.

Different situation emerged also in Rhinotermitidae, even between populations of the same taxon. *R. lucifugus grassei* and *C. acinaciformis* presented some populations with a chain of four chromosomes and other with regular bivalents. On the other hand, *H. vagus* showed a multiple ring quadrivalent in each analyzed population, while *R. urbis* never presented this complex. Notably, Fontana (1980) found male meiosis with different features in individuals of the same population of *R. lucifugus* Rossi. In the same study Fontana evidenced the presence of chains involving 4-8 chromosomes.

The chain conformation of this complex in *Reticulitermes* suggested that two of the four chromosomes involved in the interchange are uniarmed and the other two probably sub-metacentric (Fontana, 1980). On the contrary, in all presently analyzed taxa of the Termitidae

family a ring was observed: this may be the product of a heterozygous reciprocal translocation between four pairs of sub-metacentric or sub-telocentric chromosomes.

The presence of a chain or a ring in most part of Rhinotermitidae males and as a constant in all the Termitidae, respectively, together with literature data (Vincke and Tilquin, 1978), suggests that these multiples may derive from a translocation between the Y chromosomes of an initial XY male type and one autosome. This would lead up to a system carrying 2 Y and 2 X chromosomes from a segregational point of view (Vincke and Tilquin, 1978).

In all the species considered, heteromorphic chromosomes could not be distinguished. However, the male sex appears as the only one involved in this translocation, suggesting that this may be the heterogametic sex, while females, forming regular bivalents, would be the homogametic one.

While sex multivalents are common in male meiosis of termites, they are not a general feature and do not appear to be essentially concomitant with termite eusociality (Crozier and Luykx, 1985). Interchange complexes, which vary within and between species, have arisen independently many times (Gruetzner et al., 2006) for still unclear causes; probably many factors may have acted: f.e. heterozygote advantage versus inbreeding, a cooperation to maintain the sociality, an increase of uniformity among relatives or an increased gene linkage in the male.

The here presented data further agree with literature ones and shows that higher termites are very uniform karyotypically, with most species having a diploid number of 42 and a single sex-linked translocation resulting in a ring of four chromosomes in male meiosis. The lower termites are highly variable, with diploid numbers ranging from 28 to 56 and with sex-linked translocations ranging from none to eight, resulting in sex chromosomes ranging from a simple XY pair to 9 XY pairs (present data; Luykx, 1990).

The large differences in chromosome numbers, observed between lower and higher termites, could be explained by several Robertsonian changes (centric fusions or fissions) or multiple

translocation events (Luykx, 1990), frequently involving sex chromosomes. The ancestral karyotype organization is not clear, but our observations on Mastotermitidae and Kalotermitidae suggest a set of acrocentric chromosomes as the most primitive, in agreement with Luykx's data on other lower termites (1990). At present, it is impossible to determine if a haploid complement of n=48, such as in *Mastotermes darwiniensis*, can be considered as the ancestral one, or if polyploidization events have occurred in this order. Robertsonian changes are recognized as a common theme of karyotype evolution in lower termites (Luykx, 1990) involving more centric fusions than centric fissions among autosomes as well as between sex chromosomes and autosomes. The Kalotermitidae situation appears puzzling and complex, suggesting that mutual interchanges and translocations have arisen independently many times within this family.

The XO-males type may represent the final stage of differentiation following an initial XY system. The XO-male type is generally considered highly evolved (Bull, 1983), originating from an ancestral XY type where the evolutionary loss of the Y chromosome may have occurred. This system was also found in the Kalotermitidae *Stolotermes victoriensis* (Luykx, 1990) and in cockroaches. This argues against a simple derivation of termites from cockroaches, but, in agreement with Grassè (1986) and Luykx (1990) hypotheses, a common ancestor with an XY/XX sex determination may be postulated. Consequently, the cockroache lineages may have rapidly lost the Y chromosomes. Moreover, length variation within Kalotermitidae could indicate that the chains are not the result of one or a few original rearrangements in common ancestors, but their development has been ongoing in the evolution of the group. On the other hand, Rhinotermitidae and Termitidae show a fixation of the XY-male type (always involved in a reciprocal translocation with two autosomes), as the unique and stable stage of differentiation.

An apparent reduction in chromosome number seems to be involved in Isoptera karyotypes evolution, but the chromosomal repatterning leading to a constant number of 2n=42 is still

unknown. Actually, the fixed chromosome number observed here and in the literature in all higher termites, indicates that numerical changes have not occurred during the divergence of Rhinotermitidae and Termitidae. On the other hand, species clustering in groups that share major karyotype characteristics may indicate that if the mechanism of speciation within each group involved chromosome rearrangements, these may have been small or cryptic structural mutations, that did not modify the karyotypic morphology (f.e., paracentric inversion or reciprocal translocations with segments of equal size). King (1993) explained the chromosome complement stability through the non-random nature of chromosomal evolution. The model considers that structural characteristics of the genome restrict the position and number of both possible breaks and type of rearrangements. Molecular and chromosome data support that chromosomal mutations are non-random and are also constrained by the chromosome structure in the allowed kind of change (Peters, 1982; Narayan, 1988; King, 1993). Our data suggest that species ascribed to Rhinotermitidae and Termitidae families likely evolved in a concerted way, maintaining karyotype morphology and chromosome structures.

The molecular analyses here performed, beside being in agreement with literature ones (see below), well match the karyological data: the incongruence between MP and ML elaborations are mainly linked to the Kalotermitidae family, which represents the most variable taxon also at the chromosome level.

From a strictly molecular point of view, a substantial agreement with current taxonomy (following Donovan et al., 2000; Kambhampati & Eggleton, 2000 and Engel & Krishna, 2004) was found in all the analyses, with a proper subdivision in the main families of the order. Moreover, also families relationships in parsimony and likelihood analysis mainly agree with other studies based on morphological or molecular approaches (Kambhampati et al., 1996, Kambhampati & Eggleton, 2000, Thompson et al., 2000). Mastotermitidae is the basal lineage among the 4 families included in this study with the exception of the ML

analysis where a polytomy occurred between *M. darwiniensis* and Kalotermitidae taxa. The uncertain position of Kalotermitidae in our study may be due to the absence of members of Hodotermitidae and Termopsidae, usually considered as basal to this family. However, Thorne and Carpenter (1992) found Mastotermitidae and Kalotermitidae to be sister families on the basis of previously published morphological, developmental and anatomical characters. The position of Kalotermitidae was found by Kambhampati et al.(1996) to be relatively apical among lower termites, as in our MP topology, and relatively basal in the morphological analyses (Ahmad, 1950, Krishna, 1970, Emerson & Krishna, 1975, Thorne and Carpenter, 1992). All this suggests the need for further investigation on the Kalotermitidae family, which appears the most diversified also at the chromosome level. On the other hand, in our analysis Kalotermitidae family was always related to the Rhinotermitidae + Termitidae group, in agreement with Kambhampati (1996) and, on the base of gut anatomy, with Noirot (1995).

Within Rhinotermitidae, a clear cut differentiation occurred between *Reticulitermes* and a group containing both *Coptotermes* and *Heterotermes* haplotypes. This splitting was also observed in Austin et al. (2004) study, on the COII gene sequences.

Termitidae monophyletism is here also strongly supported (82%ML, 79%MP), as well as subfamilies relationships (Donovan et al., 2000; Miura et al., 1998; Ohkuma et al., 2004). Termitinae subfamily is the less clearly defined entity: in present dendrograms the relationships between the so-called *Amitermes*-group and *Termes*-group was in fact depicted by a polytomy. This suggests that the monophyly of Termitinae should be doubtful. Nasutitermitinae constitute a group strongly supported by boostrap values, but the monophyly of genera within this subfamily is still discussed and uncertain (Bergamaschi et al., submitted).

On the whole, both molecular and karyological data showed a wide variability within the Kalotermitidae family; in fact, the phylogenetic relations among its genera were resolved in a polytomy and the karyotype features appeared extremely diversified. At variance,

Rhinotermitidae and Termitidae families show a high molecular and karyological affinity among genera and species, suggesting an ongoing concerted evolution.

Karyotype features may be utilized only to discriminate among families; these taxonomic characters include: chromosome number and size (lower *vs* higher termites), and the amount and position of secondary constrictions (Rhinotermitidae *vs* Termitidae). On the other hand, karyological data do not appear useful in rooting phylogenetic relations among genera or species owing to the high stability of karyotype features within higher families. The karyotypic evolution of the order could be explained through the occurrence of Robertsonian changes and reciprocal translocations. These genomic repatternings appear to develop in a concerted way within Rhinotermitidae and Termitidae families, while a puzzling situation characterizes the Kalotermitidae family. Of particular interest is the presence of a sex-linked multivalent with a ring or a chain aspect, in 18 out of 20 species analysed. The involvement of these complexes in the evolution of termite eusociality is now rejected considering that observed sex chromosomes complexes have evolved much more recently than the origin of termites in the Cretaceous (Wilson, 1971 cited by Crozier & Luykx, 1985), the presence of active genes being demonstrated on Y chromosomes as well as on the Xs (Santos and Luykx, 1985). Moreover, the Mastotermitidae family, considered as the basal lineage of Isoptera, doesn't exhibit this complex, but it shows a complete social organization. Only a mix of causes could give an explanation to this mechanism diffused in Isoptera.

Tab. 1 – Termite species, collecting sites, COII haplotypes, diploid mitotic and haploid meiotic chromosome numbers in 23 species of Isoptera. O= multivalent ring, C= multivalent chain.

Taxon	Collecting place	Sample	COII halpotypes	Chromosome number		
				2n	n males	
Nasutitermes graveolus	C. Darwin NP – Darwin	*1*	H1	42	19+OIV	
	Mango farm – Darwin	*2*	H1			
		3	H1			
Nasutitermes triodiae	Finnis River	*1*	H2	42	/	
		2	H2			
	Douglas Daly	*3*	H2			
Nasutitermes longipennis	Mary River	*1*	H3	42	19+OIV	
		2	H3			
	CSIRO – Darwin	*3*	H4			
Tumulitermes pastinator	Palmerston	*1*	H5	42	19+OIV	
	Douglas Daly	*2*	H5			
	Gunn Point	*3*	H5			
Microcerotermes nervosus	Kakadu Nat.Park.	*1*	H6	42	19+OIV	
	Reynolds River	*2*	H7			
Microcerotermes boreus	Territory of Wild Life Park	*1*	H8	42	19+OIV	
		2	H9			
Amitermes darwini	CSIRO – Darwin	*1*	H10	42	19+OIV	
	Alice Spring	*2*	H11			
Amitermes germanus	CSIRO – Darwin	*1*	H12	42	/	
	Gunlom – Kakadu	*2*	H13			
Amitermes eucalypti	Home jungle – Darwin	*1*	H14	42	/	
	COX peninsula	*2*	H15			
Amitermes parvus	CSIRO – Darwin	*1*	H16	42	19+OIV	
Drepanotermes septentrionalis	Gunn Point	*1*	H17	42	19+OIV	
		2	H18			
Macrognathotermes sunteri	CSIRO – Darwin	*1*	H19	42	19+OIV	
Lophotermes septentrionalis	Territory of Wild Life Park	*1*	H20	42	19+OIV	
Ephelotermes taylori	Fogg Dam	*1*	H21	42	19+OIV	
Ephelotermes melachoma	C. Darwin NP-Darwin	*1*	H22	42	19+OIV	
Heterotermes vagus	CSIRO – Darwin	*1*	H23	42	19+OIV	
		2	H23			
Coptotermes acinaciformis	Adelaide River	*1*	H24	42	19+OIV	
	Reynolds River	*2*	H25		21	
Cryptotermes secundus	Mangrove forest-Batchelor	*1*	H26	(40)	(20)	(1)
		2	H26			
Cryptotermes domesticus	Mandorah	*1*	H27	(29/30)	(13+XXY)	(1)
		2	H28			
Mastotermes darwiniensis	CSIRO-Darwin	*1*	H29	96	48	
Retuculitermes urbis	Italy – lab. Reared			42	21	
Reticulitermes lucifugus grassei	France – lab. Reared			42	19+CIV 21	
Kalotermes flavicollis	Palermo-Italy			67/68	(33+XXY)	(2)
	Croatia – lab. Reared			67/68	33+XXY	
Cryptocercus relictus	Russia			(17)	/	(3)
Cryptocercus primarius	China			(19)	/	(3)
Cryptocercus punctulatus	North America			(37,47)	/	(3)

References for karyological data: (1) Lo et al., 2006;(2) Fontana, 1982; (3) Luykx, 1989)

Tab. 2 – Tamura-Nei distances among haplotypes scored for COII gene. Gamma distribution shape parameter = 0.4906 (for haplotype numbers see Table 1). Colours correspond to: red: Termitidae haplotypes; Pink: Rhinotermitidae haplotypes; Yellow: Kalotermitidae haplotypes; Green: Mastotermitidae haplotype.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1																																	
2	0.067																																
3	0.093	0.075																															
4	0.075	0.066	0.019																														
5	0.080	0.067	0.032	0.031																													
6	0.267	0.243	0.234	0.224	0.225																												
7	0.268	0.244	0.223	0.225	0.238	0.028																											
8	0.253	0.250	0.234	0.213	0.224	0.083	0.080																										
9	0.256	0.246	0.237	0.216	0.228	0.089	0.082	0.026																									
10	0.274	0.277	0.236	0.220	0.260	0.212	0.220	0.242	0.245																								
11	0.266	0.269	0.229	0.214	0.252	0.205	0.213	0.241	0.244	0.004																							
12	0.304	0.293	0.243	0.249	0.247	0.220	0.205	0.231	0.239	0.074	0.069																						
13	0.292	0.290	0.260	0.239	0.253	0.229	0.218	0.221	0.210	0.082	0.077	0.051																					
14	0.303	0.276	0.260	0.261	0.278	0.212	0.186	0.230	0.245	0.079	0.074	0.045	0.054																				
15	0.285	0.252	0.230	0.230	0.245	0.210	0.203	0.228	0.244	0.075	0.070	0.041	0.047	0.015																			
16	0.270	0.284	0.252	0.253	0.228	0.203	0.193	0.197	0.203	0.128	0.123	0.116	0.115	0.100	0.107																		
17	0.238	0.259	0.207	0.216	0.222	0.261	0.237	0.262	0.262	0.186	0.179	0.136	0.151	0.148	0.141	0.118																	
18	0.284	0.249	0.241	0.251	0.236	0.256	0.241	0.258	0.260	0.155	0.149	0.100	0.124	0.094	0.095	0.110	0.120																
19	0.248	0.266	0.214	0.199	0.226	0.266	0.242	0.250	0.252	0.216	0.209	0.211	0.212	0.248	0.251	0.252	0.226	0.249															
20	0.288	0.284	0.238	0.215	0.250	0.200	0.189	0.208	0.212	0.219	0.212	0.225	0.201	0.245	0.235	0.251	0.237	0.262	0.119														
21	0.268	0.259	0.262	0.235	0.265	0.233	0.219	0.243	0.263	0.240	0.233	0.265	0.208	0.273	0.265	0.287	0.290	0.298	0.123	0.113													
22	0.289	0.281	0.239	0.233	0.252	0.245	0.239	0.241	0.246	0.213	0.211	0.216	0.184	0.215	0.195	0.213	0.226	0.236	0.154	0.139	0.139												
23	0.219	0.257	0.231	0.216	0.239	0.285	0.273	0.324	0.293	0.297	0.288	0.310	0.302	0.339	0.330	0.306	0.288	0.328	0.269	0.283	0.270	0.328											
24	0.277	0.287	0.282	0.265	0.284	0.332	0.334	0.317	0.296	0.292	0.284	0.322	0.311	0.317	0.297	0.315	0.313	0.303	0.307	0.279	0.331	0.330	0.141										
25	0.277	0.282	0.288	0.270	0.290	0.348	0.334	0.317	0.296	0.313	0.304	0.315	0.304	0.310	0.290	0.322	0.294	0.296	0.329	0.284	0.338	0.337	0.145	0.006									
26	0.345	0.339	0.365	0.351	0.342	0.356	0.340	0.313	0.334	0.427	0.427	0.380	0.333	0.396	0.376	0.338	0.368	0.361	0.373	0.329	0.354	0.365	0.234	0.294	0.272								
27	0.357	0.379	0.399	0.370	0.374	0.357	0.339	0.321	0.329	0.381	0.380	0.368	0.325	0.395	0.376	0.348	0.410	0.375	0.354	0.376	0.358	0.382	0.228	0.293	0.286	0.107							
28	0.609	0.613	0.572	0.580	0.587	0.596	0.617	0.752	0.783	0.557	0.542	0.604	0.582	0.564	0.598	0.570	0.644	0.550	0.556	0.634	0.498	0.661	0.546	0.520	0.544	0.585	0.635						
29	0.624	0.599	0.559	0.566	0.574	0.581	0.603	0.732	0.761	0.544	0.530	0.591	0.569	0.551	0.584	0.558	0.661	0.538	0.568	0.651	0.508	0.677	0.558	0.532	0.557	0.573	0.621	0.002					
30	0.644	0.554	0.556	0.559	0.524	0.657	0.697	0.629	0.586	0.686	0.685	0.694	0.690	0.798	0.758	0.669	0.768	0.637	0.698	0.805	0.848	0.785	0.502	0.542	0.554	0.521	0.511	0.391	0.382				
31	0.699	0.625	0.689	0.683	0.675	0.559	0.636	0.682	0.690	0.671	0.654	0.603	0.622	0.634	0.668	0.613	0.741	0.625	0.654	0.563	0.599	0.694	0.561	0.683	0.665	0.530	0.625	0.385	0.376	0.319			
32	0.693	0.666	0.737	0.733	0.724	0.529	0.588	0.663	0.671	0.680	0.662	0.667	0.704	0.696	0.726	0.608	0.748	0.639	0.633	0.596	0.668	0.705	0.544	0.659	0.642	0.475	0.553	0.408	0.398	0.388	0.082		
33	0.752	0.751	0.744	0.768	0.650	0.776	0.834	0.855	0.885	0.869	0.845	0.696	0.735	0.707	0.725	0.798	0.711	0.576	0.631	0.711	0.694	0.719	0.703	0.690	0.689	0.691	0.739	0.461	0.471	0.515	0.467	0.482	

Fig. 1 – 13. Giemsa stained karyotypes of: (1) *Mastotermes darwiniensis*, female (2) *Kalotermes flavicollis*, female. (3) *Kalotermes flavicollis*, male. (4) *Heterotermes vagus*, male. (5) *Reticulitermes urbis*, male. (6) *Nasutitermes graveolus*, male. (7) *Nasutitermes longipennis*, male. (8) *Microcerotermes nervosus*, male. (9) *Microcerotermes boreus*, male (10) *Amitermes darwini*, female. (11) *Drepanotermes septentrionalis*, male. (12) *Macrognathotermes sunteri*, male. (13) *Ephelotermes melachoma*, male. Bar = 10 μm in all the figures.

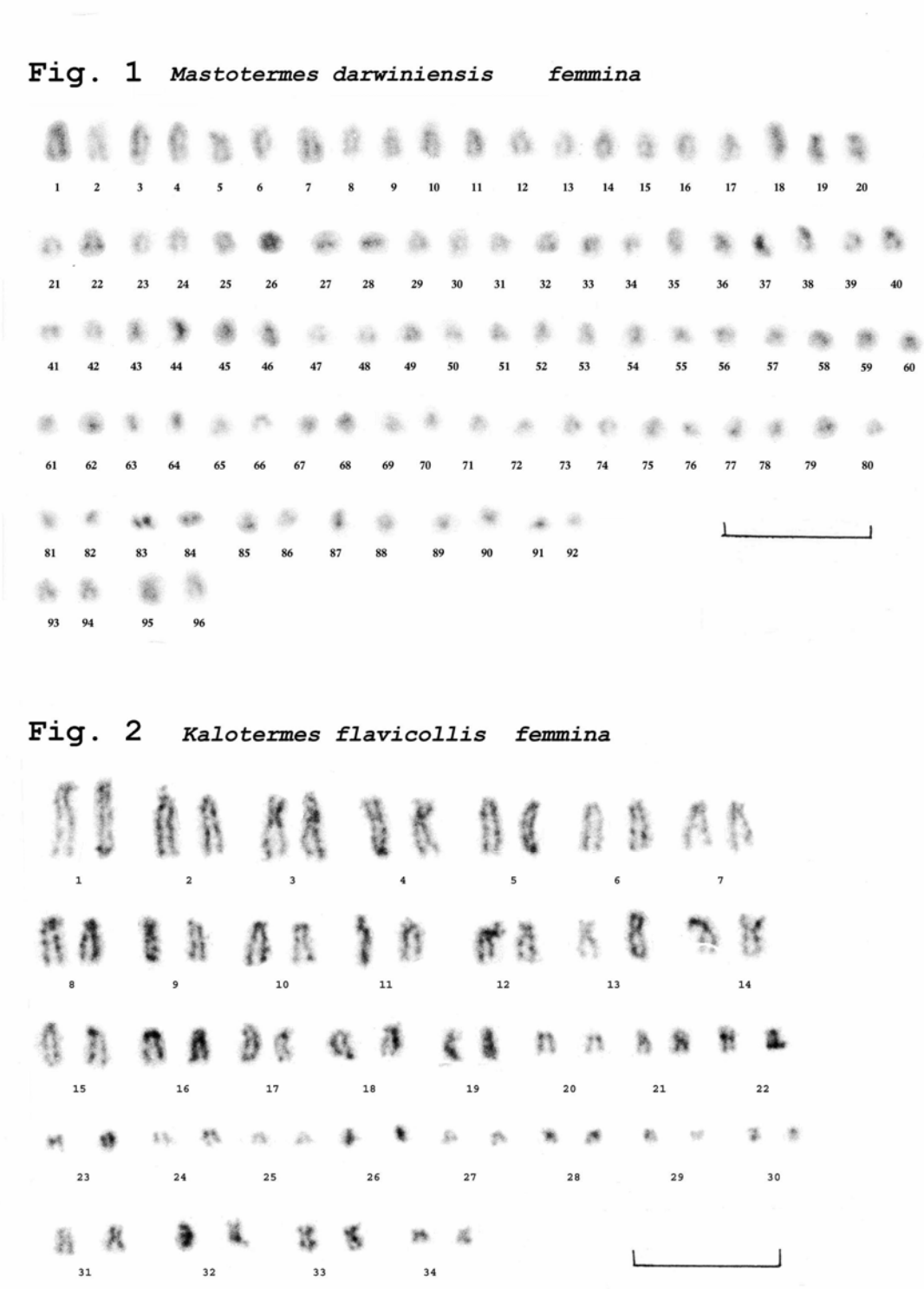

Fig. 3 *Kalotermes flavicollis maschio*

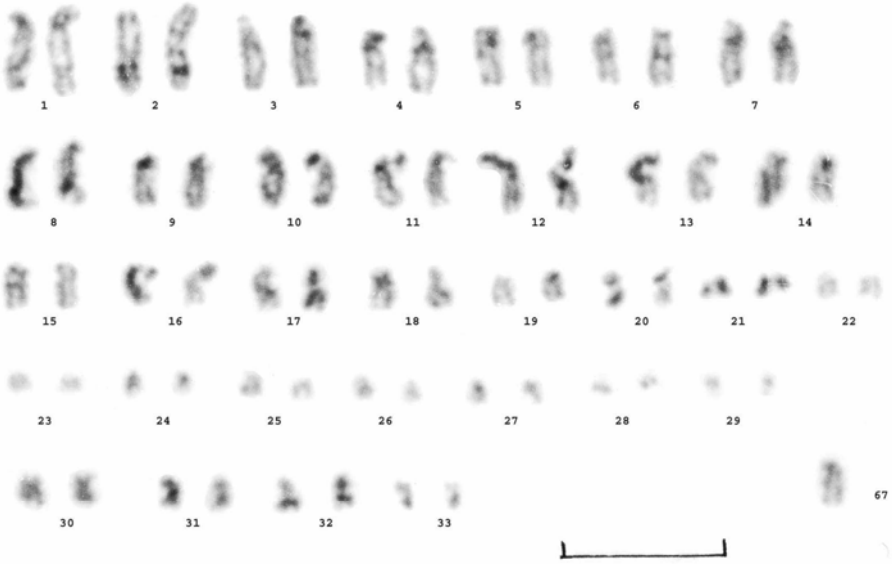

Fig. 4 *Heterotermes vagus maschio*

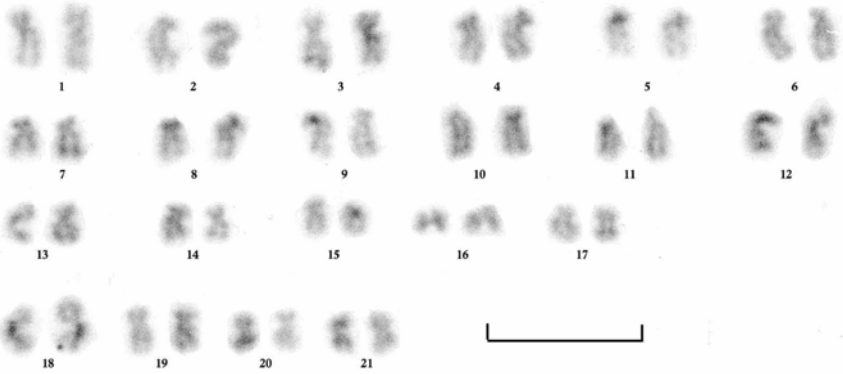

Fig. 5 *Reticulitermes urbis maschio*

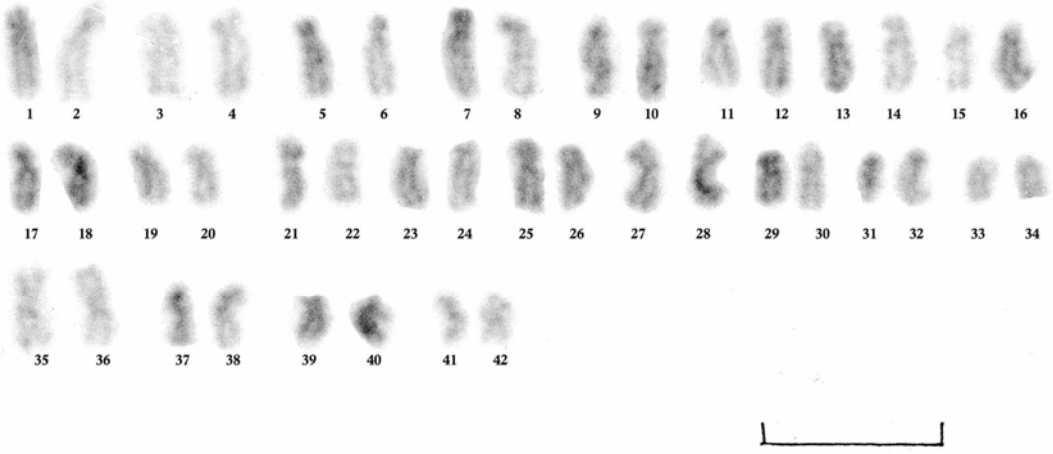

Fig. 6 *Nasutitermes graveolus* *maschio*

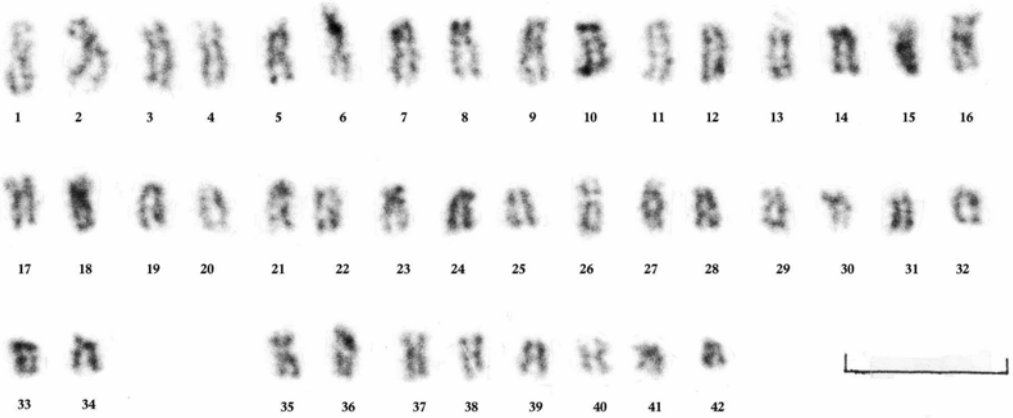

Fig. 7 *Nasutitermes longipennis* *maschio*

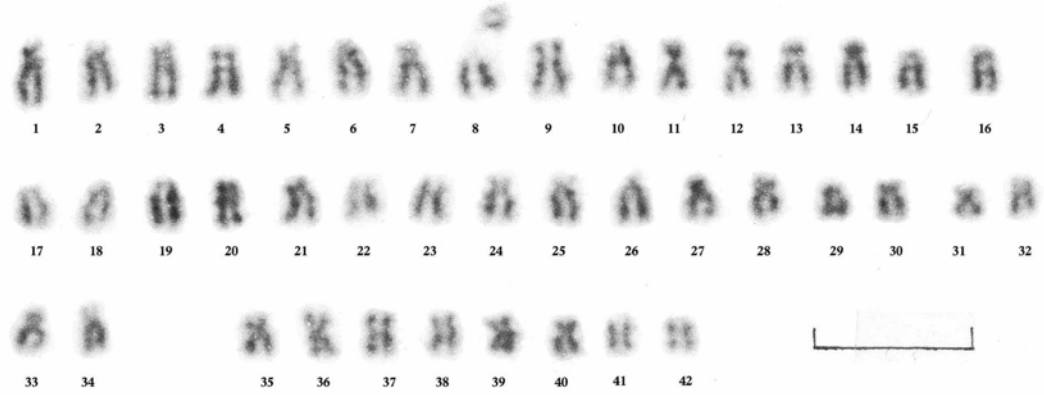

Fig. 8 *Microcerotermes nervosus* *maschio*

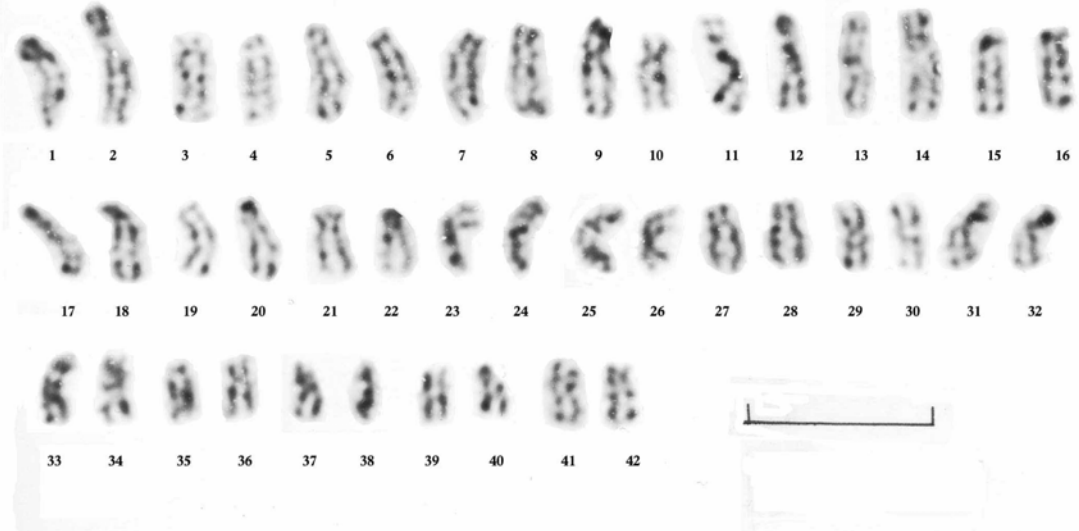

Fig. 9 *Microcerotermes boreus maschio*

Fig. 10 *Drepanotermes septentrionalis maschio*

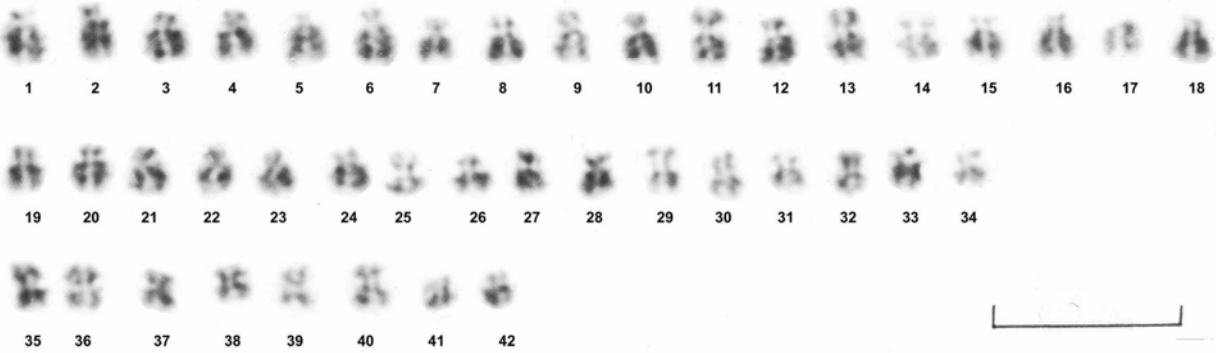

Fig. 11 *Amitermes darwini femmina*

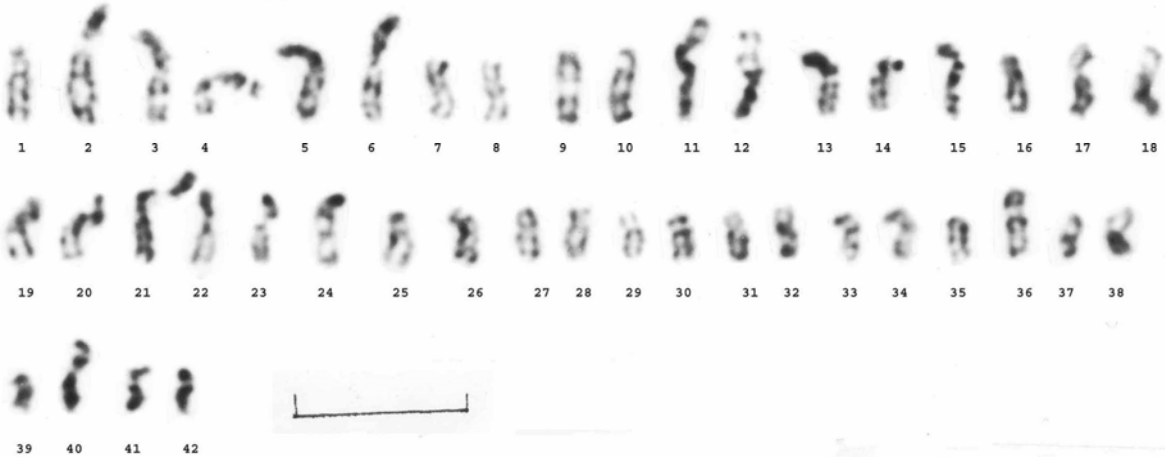

Fig. 12 *Macrognathotermes sunteri maschio*

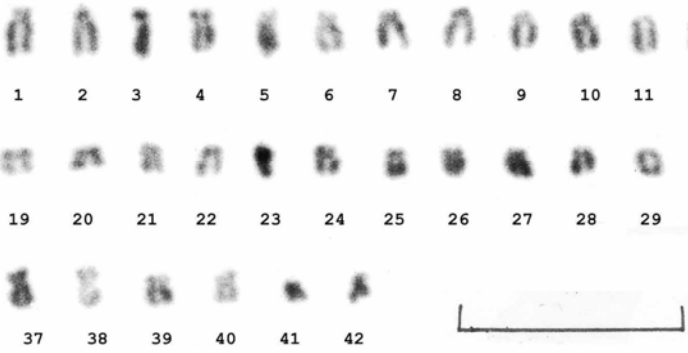

Fig.13 *Ephelotermes melachoma maschio*

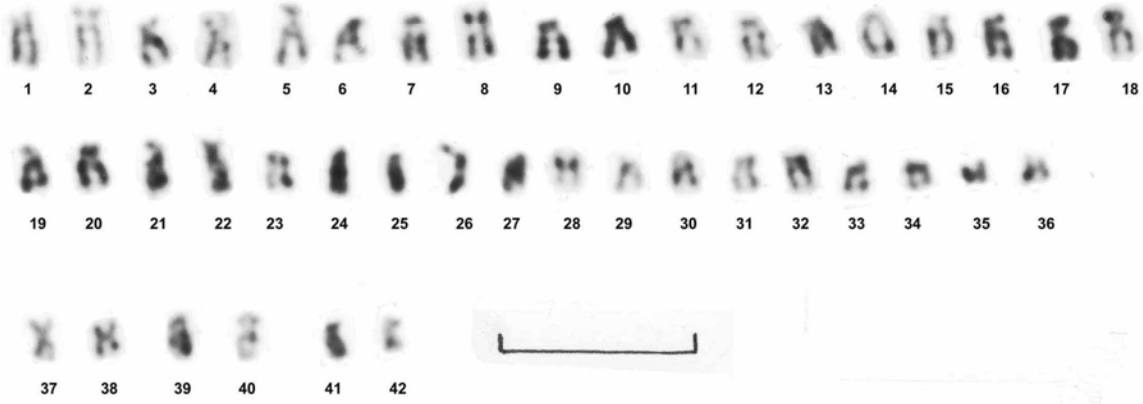

Fig. 14 (a – o). Meiotic cells from several termite species. (a) *Mastoermes darwiniensis.* Male, metaphase I, 48 bivalents. (b) *Kalotermes flavicollis.* Male, metaphase I, 33 bivalnet and 1 trivalent (arrow). (c) *Reticulitermes urbis*. Male, metaphase I with 21 bivalents and II with 21 chromosomes. (d) *Reticulitermes lucifugus grassei.* Male, 19bivalents + CIV. (e) *Heterotermes vagus.* 19 bivalents and a multivalent ring of 4 chromosomes, OIV (arrow). (f) *Ephelotermes taylori.* 19 bivalents + OIV. (g) *Macrognathotermes sunteri.* 19 bivalents + OIV. (h) *Microcerotermes boreus.* 19 bivalents + OIV. (i – j) *Microcerotermes nervosus.* 19 bivalents + OIV and metaphase II with 21 chromosomes. (k) *Amitermes darwini.* 19 bivalents + OIV. (l – m) *Nasutitermes longipennis.* Metaphase I with 19 bivalents + OIV and metaphase II with 21 chromosomes. (n – o) *Nasutitermes graveolus.* Metaphase I with 19 bivalents + OIV and metaphase II with 21 chromosomes. Bar = 10 μm in all the figures.

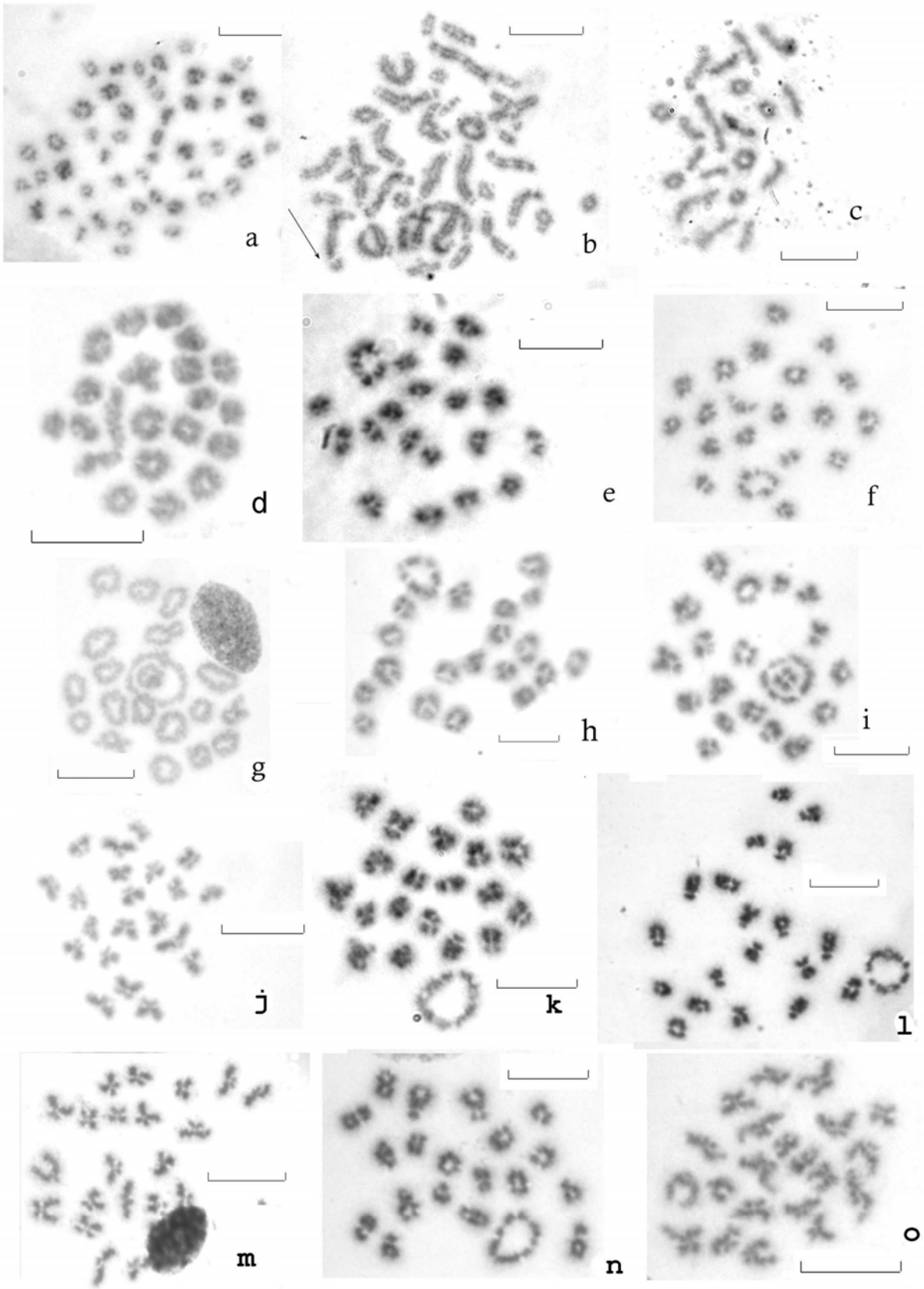
a
b
c
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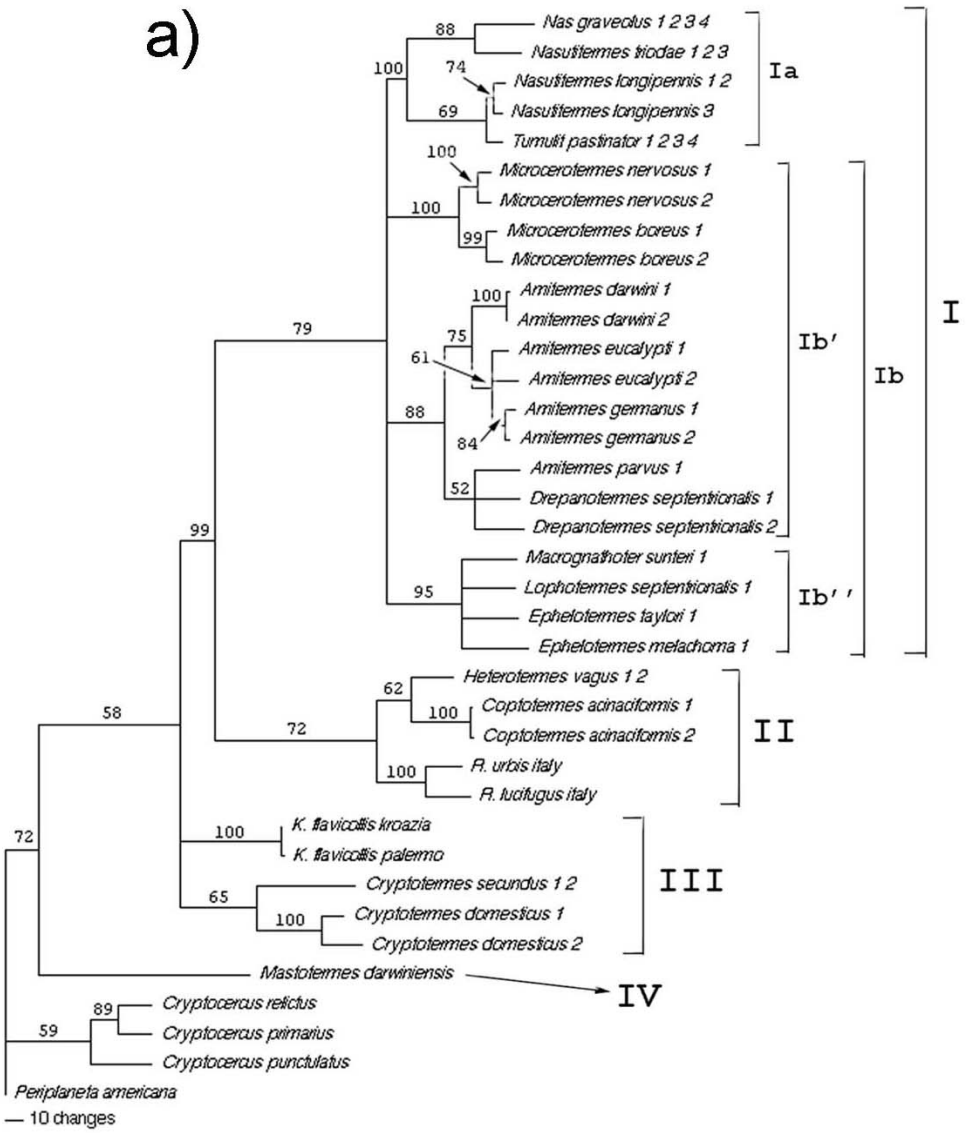
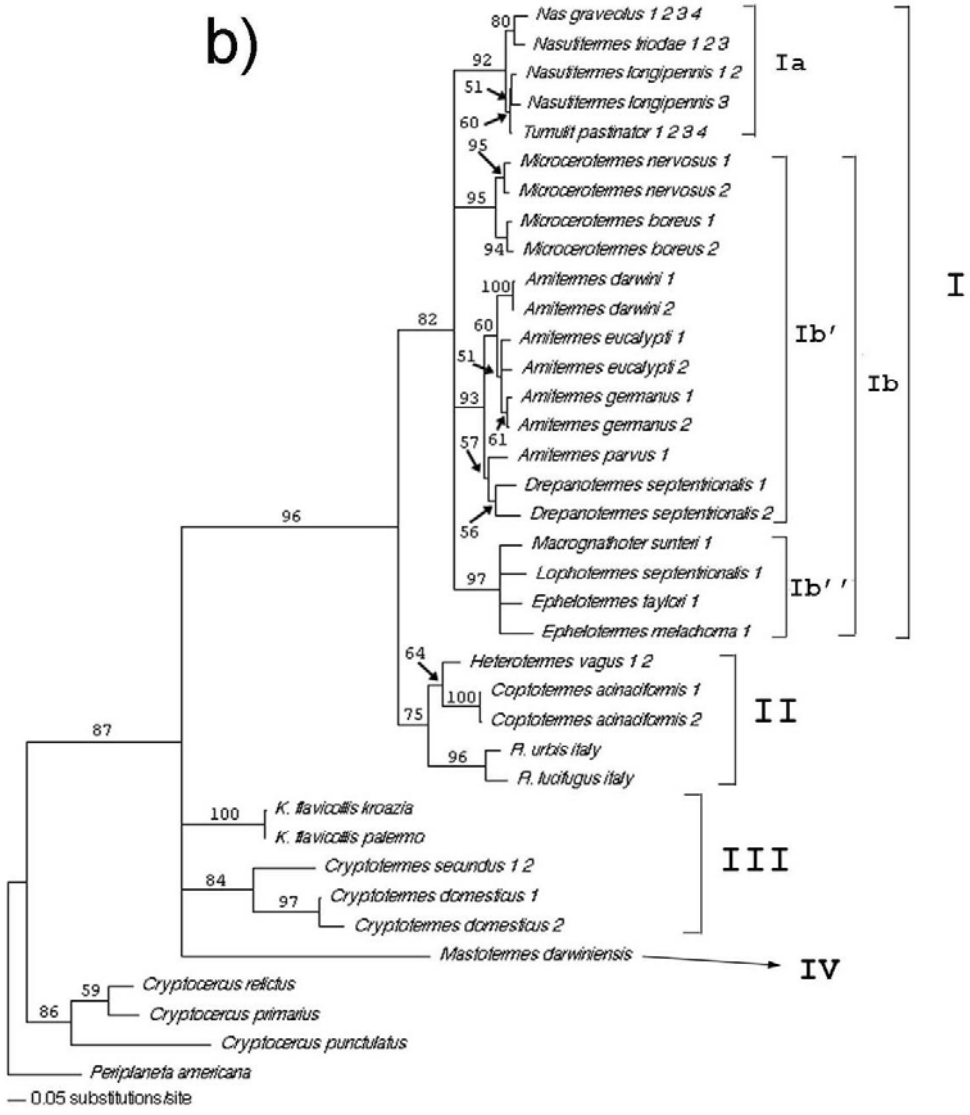
Fig. 15 – (a) Maximum Parsimony tree based on COII sequences (647 bp), TL: 1481, CI: 0.390, RI: 0.646, RC: 0.252, HI: 0.610. Values above branches indicate bootstrap support as percentage of 1000 trials. Vertical bars and numbers on the right side of the tree represent families and subfamilies according to the generally accepted classification of termites (Kambhampati and Eggleton, 2000). Numbers correspond to: I, Termitidae family; II, Rhinotermitidae family; III, Kalotermitidae family; IV, Mastotermitidae family; Ia, Nasutitermitinae subfamily; Ib, Termitinae subfamily; Ib', Amitermes – group; Ib'', Termes – group. Nodes with less than 50% support were collapsed to form polytomies. The scale bar indicates the number of changes. **(b)** Maximum likelihood tree based on COII gene (647 bp), -lnL= 6823, 78177). Values above branches indicate bootstrap support as percentage of 100 trials. Vertical bars and roman numbers represent families and subfamilies as described in the MP tree. The scale bar indicates expected nucleotide substitutions per site.




b)
Nas graveolus 1 2 3 4
Nasutitermes triodae 1 2 3
Nasutitermes longipennis 1 2
Nasutitermes longipennis 3
Tumulit pastinator 1 2 3 4
Microcerotermes nervosus 1
Microcerotermes nervosus 2
Microcerotermes boreus 1
Microcerotermes boreus 2
Amitermes darwini 1
Amitermes darwini 2
Amitermes eucalypti 1
Amitermes eucalypti 2
Amitermes germanus 1
Amitermes germanus 2
Amitermes parvus 1
Drepanotermes septentrionalis 1
Drepanotermes septentrionalis 2
Macrognathoter sunteri 1
Lophotermes septentrionalis 1
Ephelotermes taylori 1
Ephelotermes melachorna 1
Heterotermes vagus 1 2
Coptotermes acinaciformis 1
Coptotermes acinaciformis 2
R. urbis italy
R. lucifugus italy
K. flavicollis kroazia
K. flavicollis palermo
Cryptotermes secundus 1 2
Cryptotermes domesticus 1
Cryptotermes domesticus 2
Mastotermes darwiniensis
Cryptocercus relictus
Cryptocercus primarius
Cryptocercus punctulatus
Periplaneta americana
0.05 substitutions/site
Ia
Ib′
Ib
Ib′′
I
II
III
IV

Chapter 4

Molecular Taxonomy and Phylogenetic Relationships among Australian *Nasutitermes* and *Tumulitermes* genera (Isoptera, Nasutitermitinae) inferred from mitochondrial COII and 16S sequences. (Bergamaschi *et al.*, submitted)

ABSTRACT

The Nasutitermitinae Hare (1937) subfamily is a tropical and subtropical group, generally considered as the most specialised subfamily of Termitidae. To highlight some taxonomic incongruences, a study of the phylogenetic relationships among seven Australian species, morphologically ascribed to *Nasutitermes* and *Tumulitermes* genera, was performed through the analyses of the mitochondrial markers Cytochrome Oxidase II and 16S ribosomal RNA genes.

In our trees the *N. longipennis* samples clearly pertain to two different specific entities with an apparently parapatric distribution. Through a comparison with literature data, only one lineage should correspond to *N. longipennis* as considered by current taxonomy, while the other should pertain to a different species. The phylogenetic analysis performed on separated and combined data sets clearly shows the placement of *Tumulitermes* species within a clade grouping *Nasutitermes* species, and viceversa. Further, tests for alternative topologies support that *Nasutitermes* and *Tumulitermes* genera are not monophyletic. Our results confirm the hypothesis that the morphological features used to establish relationships among these species are not phylogenetically decisive.

INTRODUCTION

Termitidae is the Isoptera family with the largest number of species. These are mainly distributed in the tropics and very few species are found in the temperate regions. Nasutitermitinae, with 93 genera, is the second largest subfamily of Termitidae. It is thought to be a sister group of Termitinae, at the apex of the Isoptera phylogenetic tree inferred from mitochondrial COII sequences (Miura et al., 1998). The Nasutitermitinae genera are traditionally divided into two sections. The first comprises a Neotropical group whose soldiers have mandibles with a biting function. The second section embodies termites in which the soldier mandibles are greatly reduced and the frontal area is always elongated into a sharply-pointed nasus or rostrum, through which the secretion of the frontal gland can be squirted at a considerable distance. All the Australian genera belong to the latter section which is on the whole represented by 42 described species in six genera (Miller, 1997). Among these genera, *Nasutitermes* (the type-genus of Nasutitermitinae) and *Tumulitermes* are of particular interest owing to their dubious taxonomy and mutual phylogenetic relationships. It could be noted that the current distinction between *Nasutitermes* and *Tumulitermes* was not present in Hill's work (1942), where all the species presently distributed between the two genera were all ascribed to the genus *Eutermes* Heer.

At present, the genus *Nasutitermes* seems to represent a heterogeneous assemblage of full nasute termites (Miura, 2000), a puzzling group in need of a revision. *Nasutitermes* is the largest genus of wood-feeding Isoptera in term of number of species (more than 240); it has a huge distribution, being ecologically dominant in the tropics throughout the world. Australian *Nasutitermes* taxa comprises termites that construct strikingly different nest structures and feed on different forms of cellulosic material: there are wood-feeding arboreal and epigeous nester species and a large number grass feeders, some of these building typical huge mounds in open woodland and savannas. The nomenclature of this genus is very tangled and well depicted in a recent review made by Constantino (2002). Many species now placed in

Nasutitermes were originally included in *Eutermes* Heer, 1849. In 1890 Dudley described a new genus *Nasutitermes* ("soldier with beak"), without indicating any nominal species. Holmgren, in 1912, and later Banks (1918) included in *Eutermes=Nasutitermes* several species, and among them, Emerson (1925) designated *Nasutitermes* (*Eutermes) costalis* Holmgren as the type-species. This constitutes a valid designation following Constantino (2002). Recently, Krishna and Engel (2005) proposed to the Bullettin of Zoological Nomenclature the suppression of *Eutermes* Heer and the conservation of the generic names *Nasutitermes* Dudley (1890) and *Microcerotermes* Silvestri (1901). Adverse comments were made about this proposal (Roisin, 2005) and the question remains open.

On the other hand *Tumulitermes* is endemic of the Australian region, with 17 accepted species. Several surveys, particularly in Western and Central Australia, have disclosed a substantial undescribed fauna, primarily composed by soil-dwelling detritivores or harvesters (Watson and Abbey, 1993). It now appears that *Tumulitermes* may be a generic complex including not 17 but 50 or more species, of major ecological significance in the arid and semi-arid parts of Australia (Watson and Abbey, 1993). The name *Tumulitermes* was used for the first time by Holmgren (1912), as a subgenus of *Eutermes*. The same Author introduced *Eutermes tumuli* Froggatt (1898) as the nominal species. Later, Snyder (1949) raised the Holmgren 's subgenera to a generic rank.

As far as the relationships between *Nasutitermes* and *Tumulitermes* have been investigated, the CSIRO's Report of Research (1995-97) about "Biology and Taxonomy of termites" stated that "the examination of the phylogeny of the Australian Nasutitermitinae shows that criteria currently used to define genera are inadequate. These criteria allow species to be assigned to more than one genus when characters of different castes are used. Further morphological analysis shows that some species currently assigned to *Nasutitermes* and *Tumulitermes* belong to different undescribed genera". The characters upon which the Australian genera were established are inadequate from a taxonomic point of view and this had obviously an impact

on the clarification of their phyletic relationships. Most of the apparently anomalous placement of species lies within genera *Nasutitermes* and *Tumulitermes*. Other works highlight this point: the phylogeny of Nasutitermitinae based on morphological characters (Miller, 1997) supports the placement of *T. pastinator* within the *Nasutitermes* genus. Moreover, Bulmer and Crozier (2002) showed that the relationship among *T. pastinator* and several Australian *Nasutitermes* species, cannot be resolved by the phylogeny constructed on Termicine (defensive-like peptide) mRNA sequences. They observed that *T. pastinator* samples pertained to different clusters of *Nasutitermes,* pointing toward the absence of monophyly in these genera. As far as the genus *Nasutitermes* is concerned, Miller's morphological analysis (1997) highlighted that Australian *Nasutitermes* should be separated from other neotropical congeners and placed in a distinct genus. A different position emerged in Miura phylogenetic analysis inferred from sequences of COII and 16S mitochondrial genes (2002): this study revealed a strongly supported clade including species from south-eastern Asia, New Guinea and Australian/American tropics. To complete the picture, it should be recalled that Emerson (1941) suggested, on the basis morphological characters, the evolution of fully-nasute soldiers from different mandibulate stems, i.e. a diphyletism in the Nasutitermitinae lineage. Morphological evidence about frontal gland secretion (Prestwich & Collins, 1981) and differences in gut structure (Miller, 1986) do not correlate with the diphyletic theory of the subfamily, and led Miller (1986) to suggest a monophyletic phylogeny.

In the present work, we give the results of the phylogenetic analysis performed on samples from the Northern Territory region in Australia. These specimens have been morphologically attributed to seven species. The Indonesian long-legged species *Longipeditermes longipes* and *Hospitalitermes medioflavus* were included as representative of other full nasute, even more specialized than *Nasutitermes* (Miura et al., 2000). In order to better understand the

phylogenetic relationship among these groups, other *Nasutitermes* species from Neotropical and Indonesian regions were drawn from GenBank and included in the analysis.

Two mitochondrial genes encoding for cytochrome oxidase II (COII) and large ribosomal subunit (16S) have been considered to probe taxonomy and phylogenetic relationships among the analyzed samples. Previous works (Marini and Mantovani, 2002; Luchetti et al., 2004) showed a higher resolution power of the COII gene in comparison with the 16S gene, but also that the analyses of the combined dataset gave a better supported picture.

MATERIAL AND METHODS

The nasute termites used in this study were collected in the Australian Northern Territory Region, in March-May 2003 and September-December 2005 (Fig. 1). Specimens were preserved in absolute ethanol until DNA extraction. Table 1 gives all information on analysed samples. Sequences of *N. exitiosus* (Australia, GenBank accession no: 16S, **AB037350**; COII, **AB037333**), *N. magnus* (Australia, GenBank accession no: 16S, **AB037348**; COII, **AB037331**), *N. princes* (Australia, GenBank accession no: COII, **AB037334**) and *N. walkeri* (Australia, GenBank accession no: 16S, **AB037349**) were drawn from GenBank for genetic comparison. Six non-Australian taxa were included in the analysis: *N. nigriceps* (Central America, GenBank accession no:16S, **AB037346**; COII, **AB037329**), *N. ephrate* (Central-South America, GenBank accession no: 16S, **AB037345**; COII, **AB037328**), *N. .longinasus* (Indonesia, GenBank accession no:16S, **AB037356**; COII, **AB037339**), *N. regularis* (Indonesia, GenBank accession no: 16S, **AB037355**; COII, **AB037338**), *Longipeditermes longipes* (Indonesia, GenBank accession no: 16S, **AB037357**; COII, **AB109498**), *Hospitalitermes medioflavus* (Indonesia, GenBank accession no: 16S, **AB037358**; COII, **AB005574**). *Microcerotermes nervosus* (GenBank accession no: 16S, **AY957652**; COII, **EF079031**) was chosen as outgroup because this mandibulate termite pertains to the

Termitinae, a sister taxon of the Nasutitermitinae (Miura et al. 1998). Two - ten colonies were included for each species, to assess the degree of intraspecific variability.

Termites were identified using the morphological keys by Hill (1942) and Miller (1991), and also using the CSIRO termite reference collection, based at CSIRO Entomology, Canberra.

Total DNA was extracted following Doyle & Doyle method (1987). To avoid symbionts DNA contamination, only the head was used as source tissue.

PCR amplification was performed in 50µl mixture using the Taq polymerase Recombinant Kit (Invitrogen) and following the kit protocol. Thermal cycling was done in a Gene Amp PCR System 2400 (Applied Biosystem) programmable cyclic reactor. The conditions for amplification were as follows: 16S – initial denaturation at 94° for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing at 48°C for 1min, extension at 72°C for 2min, and final extension at 72° for 7 min. COII – initial denaturation at 94° for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing 48/52°C for 30s, extension 72°C for 2min, and final extension at 72° for 7 min. Primers for PCR amplification and sequencing were for the COII gene: 5'-CAG ATA AGT GCA TTG GAT TT-3' (modified A-tLeu, 3022-3042 in D.Yakuba)/ 5'-GTT TAA GAG ACC AGT ACT TG-3' (named B-tLys [Liu and Beckenbach, 1992; Simon et al., 1994]; 3804-3784 in D.yakuba); and for the 16S gene 5'-TA CGC TGT TAT CCC TAA-3' (16S-F1, Kambhampati and Smith, 1995; 13000-13017 in D.yakuba) / 5'TCT GGT TTT TCA AGA AAT GA-3' (16S-R4, specific for termites, 13758-13733 in D.yakuba). Amplicons were purified using the Wizard PCR prep DNA clear-up System (Promega), and both strand were sequenced with the DNA sequencing kit (BigDye terminator cycle sequencing, Applied Biosystem) in an ABI Prism 310 Genetic Analyzer. The nucleotide sequences of the newly analysed specimens have been submitted to the GenBank (GenBank accession nos: **EF079010**, **EF079011**, **EF079012**, **EF079013**, **EF079014**, **EF079015**, **EF079016**, **EF079017**, **EF079018**, **EF079019**, **EF079020**, **EF079021**, **EF079022**, **EF079023**, **EF079024**, **EF079025**, **EF079026**, **EF079027**, **EF079028**, **EF079029**,

EF079030, **EF079031,** for COII, and **EF078991**, **EF078992**, **EF078993**, **EF078994**, **EF078995, EF078996**, **EF078997**, **EF078998**, **EF078999**, **EF079000**, **EF079001**, **EF079002, EF079003**, **EF079004**, **EF079005**, **EF079006**, **EF079007**, **EF079008**, **EF079009**, for 16S).

Alignments performed with the Clustal algorithm of the Sequence Navigator program (ver 1.0.1, Applied Biosystem Inc.) were also checked by sight. Nucleotide substitutions matrices were determined using MEGA3 (Kumar et al., 2004).

Phylogenetic relationships were inferred by a Maximum Parsimony (MP) method and a Maximum Likelihood (ML) method using PAUP 4.0b (Swofford,2001) with 2000 and 100 bootstrap replicates, respectively. The best substitution model (Tamura-Nei + I + G for 16S and COII+16S combined dataset; Tamura-Nei + G for COII dataset) for ML was selected using the program Modeltest 3.06 (Posada and Crandall, 1998).

To evaluate possible mutational saturation, the number of transitions and transversions scored between 16S, COII sequences and at the COII 3rd codon position were plotted against pairwise genetic distances. The Partition homogeneity Test (also named Incongruence Length Difference test-ILDtest, Farris et al., 1995) was performed for the combined dataset of COII and 16S genes, to assess combinability of partitions from the two dataset. This test was calculated with PAUP 4.0b (Swofford, 1998) after 100 random replicates.

To test whether the mtDNA data were consistent with the hypothesis of monophyly of *Nasutitermes* and *Tumulitermes* genera, we employed the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) implemented in PAUP 4.0b, which compare the fit of the data for *a priori* hypotheses (monophyly) to the fit of the data for ML and MP trees (an *a posteriori* hypothesis). We tested whether trees that forced these two genera to be monophyletic were significantly worse than the best tree. Moreover, we applied the Kishino-Hasegawa (KH) topology test (Kishino and Hasegawa, 1989) to the same dataset. This test is

analogous to the SH test, but it is used to distinguish among *a priori* hypotheses rather than to compare *a priori* with *a posteriori* ones.

RESULTS

Average sequence size was 687 bp for the 16S gene, and 647 bp for the COII gene, encoding for 215 amino acids.

Nucleotide variation for both genes showed an adenine-thymine bias in their nucleotide composition (A+T= 71.1% for 16S and A+T= 63.3% for COII) that is consistent with data on other insect mitochondrial genes (Crozier and Crozier, 1993; Kim et al., 2006; Podsiadlowski et al., 2006). In the aligned 16S gene sequences, 215 variable sites were scored. For the 233 variable sites of the COII gene, 75.3% occurred at the third codon position, followed by first position (16.6%) and second position (8.1%). Amino acid sequences showed 39 variable sites (18.1%). The combined dataset presented 449 variable sites out of 1333 bp.

The 16S and COII genes showed respectively 23 and 21 different haplotypes, while the combined dataset showed 27 mitotypes (Tab.1). All the observed haplotypes appeared species-specific.

Regression analysis of transitions and transvertions against genetic distances indicate the lack of saturation in both genes and at the 3rd codon position of the COII ($P<0.001$, Fig. 2).

Fourteen out of the 23 16S haplotypes pertained to the *Nasutitermes* genus and 9 to the *Tumulitermes* one. The great majority of colony samples showed the co-occurrence of different haplotypes with the exception of *N. longipennis* from Mary river Nat. Park (H12) and from Charles Darwin Nat. Park (H13), *T. pastinator* from Douglas Daly (H16) and *T. tumuli* from King's Canyon (H23), where a single haplotype was scored. Further, the same haplotype occurred for the same species in different collection sites (see f.e. haplotypes H1, H4, H5, H9, H16).

Within species, inter-haplotype substitutions ranges from 0 to 2 showing low level of variability, with the exception of *N. eucalypti*, *N longipennis* and *T. hastilis* haplotypes, where a significantly higher divergence was observed for some haplotypes: i.e. *N. longipennis* H12/13/14 *vs*. H9/10/11 showed 42-48 substitutions, *N. eucalypti* H6 *vs*. H7/8 presented 34-35 mutations and were scored 11-12 substitutions comparing *T. hastilis* H22 with H20/21.

Interspecific comparisons showed higher variability among species of the same genera than between species of different genera: i.e. *N. triodiae* haplotypes differed from *N. eucalypti* group for 46-54 mutations, while *N. eucalypti* haplotypes vs. *T. hastilis* presented only 14-35 substitutions. Moreover, the same range of variability occurred comparing *N. triodiae* with *N. longipennis* H12/13/14 or *N. triodiae* with *T. pastinator* (33-37 and 32-34 respectively). Further, *N. longipennis* H9/10/11 showed fewer mutations in comparison with *T. hastilis* haplotypes, than with *N. graveolus* group (3-13 and 48-51 respectively).

It appears quite evident that some supposed intraspecific comparisons overlap with interspecific ones.

Obviously when the two genera were compared, a huge range of variation emerged with three to 61 substitutions differentiating *Nasutitermes* and *Tumulitermes* haplotypes. The lower values are due to the comparison between *T. hastilis* (H20) and *N. longipennis* (H10), while the higher was obtained comparing *N. graveolus* (H3) with *T. tumuli* (H23).

Twenty-one haplotypes were scored for the COII sequences (Table 1). Among them 11 pertained to the *Nasutitermes* genus and 10 to the *Tumulitermes* one; only sample-specific haplotypes have been observed.

As for the 16S gene, the majority of colony samples showed the co-occurrence of different haplotypes with the exception of *N graveolus* (H1), *N. triodiae* from Finnis River (H2), *N eucalypti* from Gunn Point (H5) and *T. tumuli* (H23), where a single haplotype was scored. Further, a lower number of haplotypes shared by different colonies is observed (H1, H9, H12).

Similarly to 16S data, substitutions range within species showed low variability (1-13) with the exception of *N. longipennis* and *T. hastilis* haplotypes: comparing *N. longipennis* H6/7/8 with H9/10/11, 77-84 substitutions have been scored, while 25-29 mutations discriminated *T. hastilis* H18/19 with H20.

Interspecific comparisons showed the same trend occurred for 16S haplotypes: *i.e. N. eucalypti* H5 presented less variation in comparison with *T. hastilis* haplotypes than with *N. triodiae-graveolus* group (25-30 and 73-74 , respectively) and identical results have been observed comparing *N. longipennis* H6/7/8 with the same haplotypes mentioned above (2-28 and 77-83, respectively). Moreover, *N. longipennis* H9/10/11 differed for 18-26 and 38-75 mutations with respect to *T. pastinator* group and *N. triodiae-graveolus* haplotypes.

Comparing the two genera we observed that 2-85 substitutions differentiate *Nasutitermes* and *Tumulitermes*, considering the lower as the results of the comparison between *N. longipennis* H7 and *T. hastilis* H18, and the higher as the difference between *N. longipennis* H6 and *T. pastinator* H13/14.

Tamura-Nei distances showed the same range and trends with respect to the total number of substitution (Tables 3-4)

Phylogenetic analysis

The Maximum Parsimony (MP) and Maximum Likelihood (ML) trees were computed on the haplotypes of COII and 16S genes (Table 1), either separated or combined, because the ILD test gave a non-significant P value. All the topologies obtained either for MP or ML analyses presented the same terminal branching pattern (Fig 3), with some differences in the deep branching topology.

Four terminal groups occurred in all dendrograms. In group I, high bootstrap values supported the close relationship between *T. pastinator* haplotypes and Northern *N. longipennis* clade. Group II and III embodied the haplotypes scored for *N. triodiae* and *N. graveolus* samples,

respectively. In group IV a cluster of the Southern samples of *N. longipennis* with *T. hastilis* from Kakadu-Mary River and Mandorah can be observed, with *T. hastilis* from Tennant Creek and *N. eucalypti* haplotypes polytomically distributed.

Further, in all elaboration but the ML tree on 16S sequences (Fig. 3B), groups I-III were usually combined in a single cluster with bootstrap support percentages varying from 59 (in the ML tree on COII sequences ; fig. 3A) to 88 (in the ML tree on the combined data set; (Fig. 3D).

As far as Australian *Nasutitermes* were concerned, *N. exitiosus* and *N. magnus* always clustered with group I and group II, respectively with the only exception of *N. magnus* in the highly polytomic ML tree obtained on 16S sequences.

A polytomic distribution of the Indonesian samples was observed in all the analyses, with *Hospitalitermes medioflavus* and *Longipeditermes longipes* grouped in a single clade.

The South American taxa *N. nigriceps* and *N. ephrate* were always recognized as a single cluster, with a higher affinity for groups I-III in the ML tree on COII sequences and in the MP tree of the combined data set (Fig. 3C).

On the whole, *N. longipennis* samples pertained to two different groups in all dendrograms and neither *Nasutitermes* nor *Tumulitermes* genera appeared as a monophyletic clade. Alternative phylogenetic hypotheses and results of SH and KH tests are summarized in Table 2. For both *Nasutitermes* and *Tumulitermes* genera monophyly was rejected comparing the tree constrained under different monophyly hypotheses to the unconstrained tree. The tests showed for each genera the unconstrained topology as the best one ($P<0.01$).

DISCUSSION

The aim of the present work was to gain objective molecular data to better define the current accepted taxonomy/phylogeny of this termite group. Scored data evidence some

inconsistencies with the current accepted taxonomy. The *N. longipennis* samples analysed clearly pertain to different specific entities with an apparently parapatric distribution: a *N. longipennis* lineage with a higher affinity for *T. pastinator, N. triodiae* and *N. graveolus* and a second lineage poorly differentiated from northern *T. hastilis* samples, which on the other hand show a certain degree of diverge from southern *T.hastilis* sample**.** As far as the two *N. longipennis* lineages are concerned, they share many ecological and behavioural features. Both were found eating sound and rotten wood, grass and vegetable surface debris. Nests also were almost identical: conical in shape, circular at the base and sloping symmetrically on the sides, with rounded apex. The only clear difference was the location of the nests: sandy well drained open forest and grassland, the former, gravely and stony grounds often near small streams, the latter. Through a comparison with literature data and considering their placement in the phylogenetic trees, the first lineage should correspond to Miller's *N. longipennis* while the haplotypes clustering in the *Tumulitermes* clade should pertain to a different species. We are currently investigating their morphology using a scanning electron microscope and preliminary data suggest differences in head capsule, rostrum length, hair number and distribution and in the shape of the secretion pore (Bergamaschi et al., in preparation).

The phylogenetic analysis performed on separated and combined data sets clearly shows that the two genera are not monophyletic. This work complements other studies evaluating the phylogenetic relationships among Nasutitermitinae genera. Miller (1997) considered that the characters upon which the Australian genera were established were inadequate to reflect the true relationships among species, in particular among those ascribed to *Nasutitermes* and *Tumulitermes* genera. In his studies, characters of the digestive tract combined with "traditional" characters of external morphology (head capsule and rostrum shapes) of soldier and winged adult were used to determine whether the present divisions were natural, or Hill's (1942) comments on the anomalous placement of some species (f.e. *N. eucalypti* and *T.*

pastinator) were justified. For these anomalies Hill's (1942) chose to place all the species in the *Eutermes* Heer genus without distinction between *Nasutitermes* and *Tumulitermes*.

Even if mismatches occur at a few nodes, all the topologies obtained in this study were identical for the reciprocal relations among taxa of *Tumulitermes* and *Nasutitermes* genera, and most of the inferred relationships had strong support as indicated by bootstrap analysis.

At first glance, our molecular data support the hypothesis put forward by Miller (1997), i.e. the placement of *T. pastinator* within a clade grouping *Nasutitermes* species, and of *N. eucalypti* within a *Tumulitermes* cluster. The hypothesis that the morphological features used to establish relationships among these species are not phylogenetically decisive is therefore also supported. In this view, group IV should be fully supported as *Tumulitermes* genus and groups I-III as *Nasutitermes.* On the other hand, the lack of clear cut morphological data distinguishing the two genera and the ongoing confusion in determining taxa does not exclude the possibility that we are dealing with a single genus. If this corresponds to *Eutermes* sensu Hill (1942) or to a new different entity must be verified.

This possibility can be discussed also considering some molecular-biogeographical data. In Miller's opinion (1997) in fact the Australian *Nasutitermes* should simply reflect an endemic speciation event and could be separated from their neotropical congeners with the formation of a distinct genus. On the other hand, Miura's study (2002) demonstrated the existence of a strongly supported clade including species from south-eastern Asia, New Guinea, Australia and American tropics. In our study a link between Australian and South American *Nasutitermes* is supported in COII and combined data set analyses, the other elaborations with their deep polytomies leaving the question open or better agreeing with the existence of a single genus. Only further analyses with other molecular markers will help in understanding this aspect.

Whatever the outcome of further analyses will be, it is obvious that considering *Nasutitermes* distribution, we are dealing with very ancient taxa, which, as suggested by Miura and other

authors (Weston and Crisp, 1996 and Keast, 1996), may have a Gondwana origin. The evolution of the Australian flora and fauna during the Tertiary Period (65 to 1.8 million years before present) determined the differentiation of the original ancient biota of Gondwana in response to conditions of increasing geographical isolation and climate change. This would also support the possibility of an endemic speciation of Australian *Nasutitermes*, but at present molecular data do not demonstrate it in a clear manner.

On the whole, further molecular analyses are needed, but we are also involved in an in-deep cytogenetic study with the purpose of investigate the modification probably occurred in the chromosome arrangement during the evolution of the Isoptera order. This could add more information in order to better understand the complex relationships noticed in this group of insects.

Tab. 1 –Collecting sites and haplotypes-mitotypes of the specimens analysed for 16S and COII genes (ND: haplotype not determined).

Species	Sample		Collecting site	Collecting date	16S haplotype	COII haplotype	Mitotype
Nasutitermes graveolus	1	a	C. Darwin Nat.Park-Darwin	9-4-2003	H1	H1	1
	2	a	Mango farm-Darwin	25-4-2003	H1	H1	1
	3	a	Mango farm-Darwin	25-4-2003	H2	H1	2
	4	b	Fogg Dam	13-10-2005	H3	H1	3
	5	b	Fogg Dam	13-10-2005	ND	H1	ND
Nasutitermes triodae	1	c	Finnis river	23-4-2003	H4	H2	4
	2	c	Finnis river	23-4-2003	H5	H2	5
	3	c	Finnis river	23-4-2003	H5	H2	5
	4	d	Douglas Daly	24-4-2003	H4	H3	6
	5	e	Litchfield Nat Park	21-4-2003	H5	H4	7
Nasutitermes eucalypti	1	g	Kakadu Nat. Park	7-5-2003	H6	ND	ND
	2	f	Gunn point	25-11-2005	H7	H5	8
	3	f	Gunn point	25-11-2005	H8	H5	9
Nasutitermes longipennis	1	h	Adelaide river	26-4-2003	H9	H6	10
	2	h	Adelaide river	26-4-2003	H10	H7	11
	3	h	Adelaide river	26-4-2003	H9	H6	10
	4	d	Douglas Daly	24-4-2003	H11	H8	12
	5	i	Mary river Nat. Park	19-10-2005	H12	H9	13
	6	i	Mary river Nat. Park	19-10-2005	H12	H10	14
	7	a	C. Darwin Nat.Park-Darwin	27-10-2005	H13	H9	15
	8	a	C. Darwin Nat.Park-Darwin	27-10-2005	H13	ND	ND
	9	a	CSIRO-Darwin	25-11-2005	H14	H11	16
Tumulitermes pastinator	1	j	Palmerston	11-4-2003	H15	H12	17
	2	d	Douglas Daly	24-4-2003	H16	H13	18
	3	d	Douglas Daly	24-4-2003	H16	H14	19
	4	h	Adelaide river	13-10-2005	H17	H12	20
	5	a	C. Darwin Nat.Park-Darwin	27-10-2005	ND	H15	ND
	6	k	Mandorah	2-10-2005	H18	H16	21
	7	f	Gunn point	25-11-2005	H19	ND	ND
	8	f	Gunn point	25-11-2005	H19	H17	22
	9	f	Gunn point	25-11-2005	H16	H12	23
	10	f	Gunn point	25-11-2005	H16	H12	23
Tumulitermes hastilis	1	l	Kakadu-Mary river	31-10-2005	H20	H18	24
	2	k	Mandorah	2-10-2005	H21	H19	25
	3	m	Tennant Creek	14-11-2005	H22	H20	26
Tumulitermes tumuli	1	n	King's canyon	11-11-2005	H23	H21	27
	2	n	King's canyon	11-11-2005	H23	H21	27

Tab. 2 – Shimodaira and Hasegawa (1999) and Kishino and Hasegawa (1989) tests results for comparisons of alternative hypotheses.

Alternative hypotheses	MP (-ln L)	ML (-ln L)	Topology tests (MP / ML)	
			KH	SH
(Nasutitermes, Tumulitermes)	7283.51	7282.26	best	best
((Nasutitermes), (Tumulitermes))	7612.02	7585.96	**/**	**/**
(Nasutitermes, (Tumulitermes))	7554.15	7538.05	**/**	**/**
((Nasutitermes), Tumulitermes)	7551.49	7547.95	**/**	**/**

Table 3 – Tamura-Nei distances among haplotypes scored for 16S gene. Gamma distribution shape parameter = 0.6206. (for haplotypes numbers and colours see Table 1).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1																							
2	0.002																						
3	0.002	0.003																					
4	0.032	0.034	0.030																				
5	0.034	0.036	0.032	0.002																			
6	0.034	0.036	0.032	0.002	0.000																		
7	0.100	0.102	0.102	0.105	0.108	0.108																	
8	0.100	0.103	0.103	0.092	0.094	0.094	0.067																
9	0.101	0.104	0.104	0.093	0.096	0.096	0.065	0.005															
10	0.100	0.103	0.100	0.089	0.091	0.091	0.073	0.027	0.025														
11	0.097	0.100	0.097	0.087	0.089	0.089	0.071	0.025	0.023	0.002													
12	0.095	0.098	0.095	0.084	0.087	0.087	0.073	0.029	0.027	0.005	0.003												
13	0.058	0.060	0.058	0.069	0.071	0.071	0.095	0.102	0.100	0.095	0.093	0.095											
14	0.058	0.060	0.058	0.069	0.071	0.071	0.095	0.102	0.100	0.095	0.093	0.095	0.002										
15	0.051	0.053	0.053	0.062	0.065	0.065	0.086	0.086	0.084	0.083	0.081	0.081	0.028	0.026									
16	0.047	0.049	0.049	0.060	0.062	0.062	0.075	0.088	0.089	0.090	0.088	0.083	0.044	0.041	0.034								
17	0.047	0.049	0.049	0.060	0.062	0.062	0.075	0.088	0.089	0.090	0.088	0.083	0.044	0.041	0.034	0.000							
18	0.049	0.051	0.051	0.062	0.064	0.064	0.077	0.090	0.091	0.092	0.090	0.085	0.045	0.043	0.035	0.002	0.002						
19	0.047	0.049	0.049	0.060	0.062	0.062	0.075	0.088	0.089	0.090	0.088	0.083	0.044	0.041	0.034	0.000	0.000	0.002					
20	0.049	0.051	0.051	0.062	0.064	0.064	0.075	0.088	0.089	0.090	0.088	0.083	0.044	0.041	0.034	0.002	0.002	0.003	0.002				
21	0.095	0.098	0.093	0.082	0.084	0.084	0.067	0.029	0.028	0.007	0.005	0.007	0.092	0.092	0.079	0.085	0.085	0.088	0.085	0.085			
22	0.097	0.100	0.095	0.084	0.086	0.086	0.069	0.031	0.030	0.008	0.007	0.008	0.094	0.094	0.081	0.087	0.087	0.090	0.087	0.087	0.002		
23	0.103	0.106	0.106	0.095	0.098	0.098	0.073	0.027	0.025	0.023	0.021	0.021	0.100	0.100	0.089	0.091	0.091	0.093	0.091	0.091	0.019	0.021	

Table 4 – Tamura-Nei distances among haplotypes scored for COII gene. Gamma distribution shape parameter = 0.2061 (for haplotype numbers and colours see Table 1).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1																					
2	0.078																				
3	0.081	0.002																			
4	0.081	0.002	0.003																		
5	0.294	0.294	0.298	0.298																	
6	0.355	0.364	0.369	0.369	0.064																
7	0.355	0.364	0.369	0.369	0.056	0.005															
8	0.322	0.302	0.307	0.307	0.058	0.011	0.009														
9	0.129	0.099	0.102	0.102	0.344	0.341	0.327	0.308													
10	0.126	0.096	0.099	0.099	0.339	0.336	0.322	0.304	0.005												
11	0.101	0.087	0.089	0.089	0.377	0.419	0.401	0.376	0.022	0.02											
12	0.104	0.09	0.093	0.093	0.413	0.396	0.379	0.326	0.038	0.036	0.037										
13	0.117	0.102	0.105	0.105	0.417	0.439	0.419	0.36	0.046	0.044	0.045	0.005									
14	0.101	0.089	0.092	0.092	0.393	0.417	0.399	0.344	0.043	0.041	0.042	0.007	0.012								
15	0.093	0.081	0.083	0.083	0.377	0.399	0.381	0.328	0.041	0.039	0.040	0.005	0.01	0.005							
16	0.116	0.102	0.105	0.105	0.419	0.435	0.427	0.361	0.053	0.055	0.056	0.017	0.023	0.017	0.016						
17	0.096	0.083	0.086	0.086	0.381	0.404	0.386	0.333	0.039	0.037	0.038	0.003	0.009	0.003	0.002	0.014					
18	0.335	0.342	0.347	0.347	0.055	0.005	0.003	0.005	0.321	0.316	0.393	0.371	0.411	0.391	0.373	0.409	0.378				
19	0.356	0.335	0.340	0.340	0.065	0.012	0.01	0.012	0.341	0.336	0.422	0.399	0.442	0.419	0.402	0.437	0.407	0.007			
20	0.284	0.292	0.297	0.297	0.055	0.059	0.052	0.054	0.305	0.300	0.341	0.309	0.314	0.313	0.297	0.337	0.302	0.051	0.061		
21	0.234	0.249	0.252	0.252	0.152	0.175	0.159	0.197	0.304	0.300	0.347	0.328	0.365	0.314	0.315	0.352	0.319	0.17	0.167	0.173	

Fig. 1 – Collecting sites: **a**-Darwin; **b**-Fogg Dam; **c**-Finnis River; **d**-Douglas Daly; **e**-Litchfield; **f**-Gunn Point; **g**-Kakadu Nat.Park; **h**-Adelaide River; **i**-Mary River Nat.Park; **j**-Palmerston; **k**-Mandorah; **l**-Mary River-Kakadu; **m**-Tennant Creek; **n**-King's Canyon.

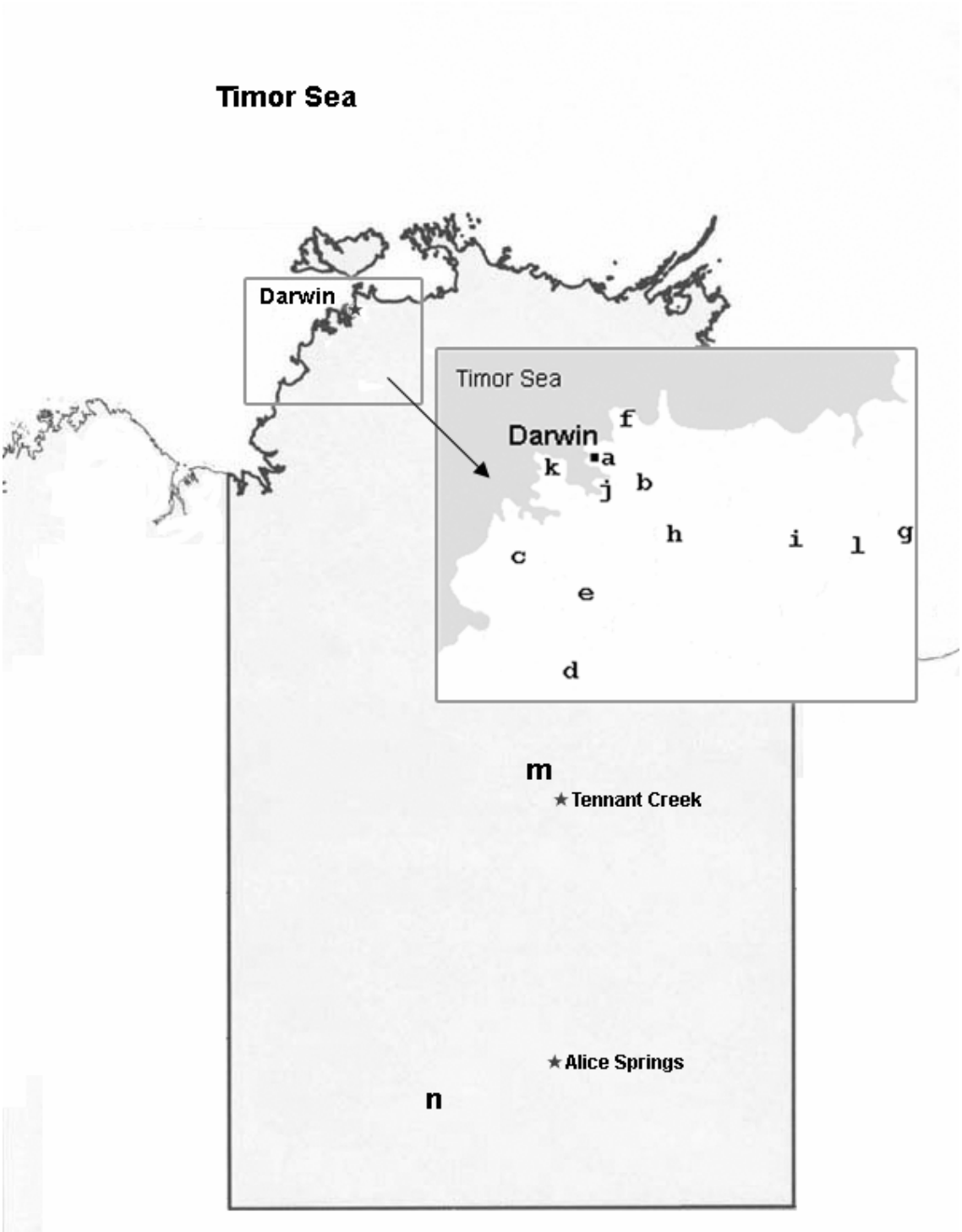


Fig. 2 – Regression analysis of the number of transitions (ts, triangles) and transversion (tv, circles) versus Tamura-Nei distances among each pair of taxa. A) COII gene B) COII gene third codon position C) 16S gene.

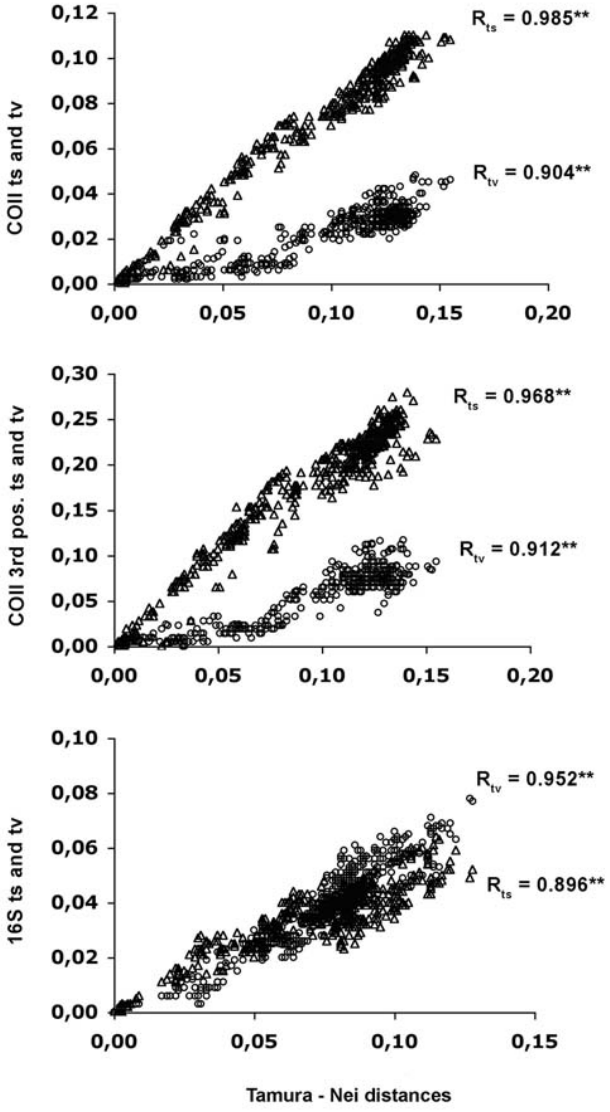
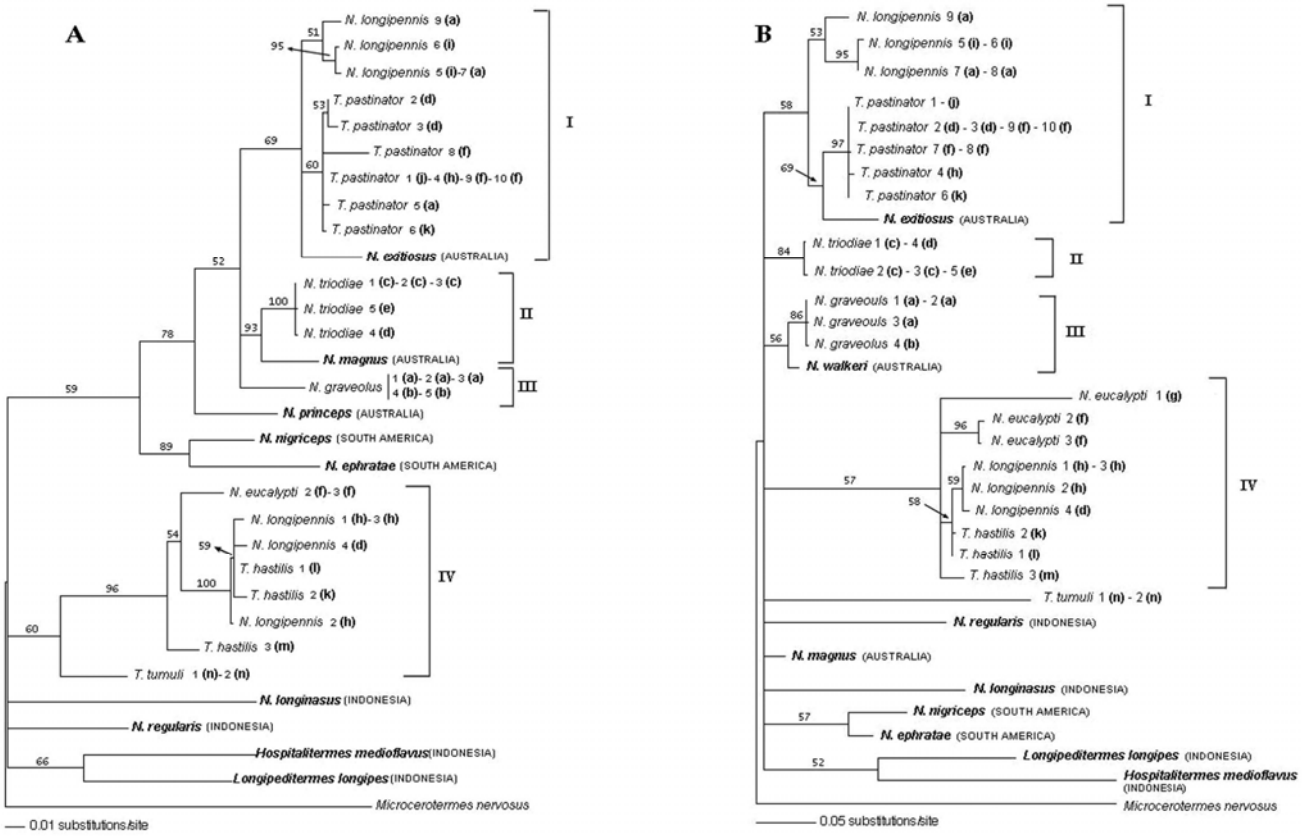
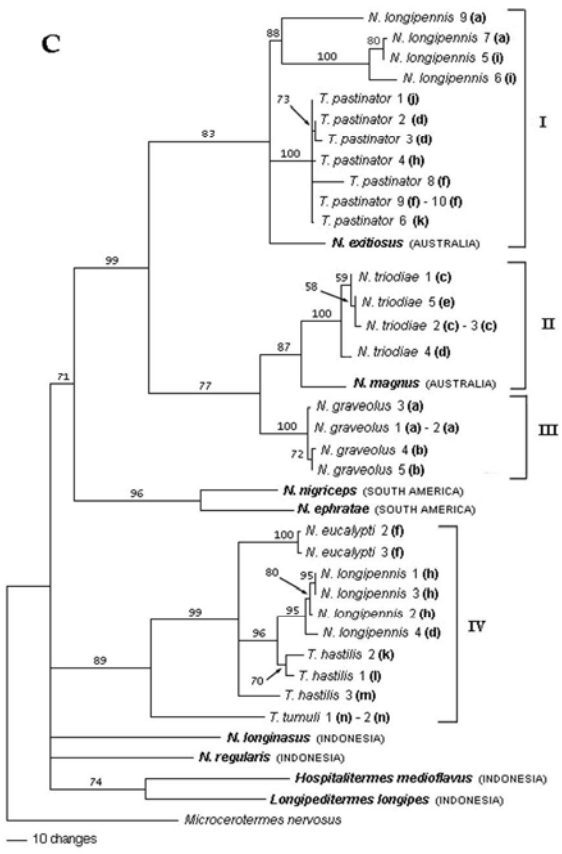
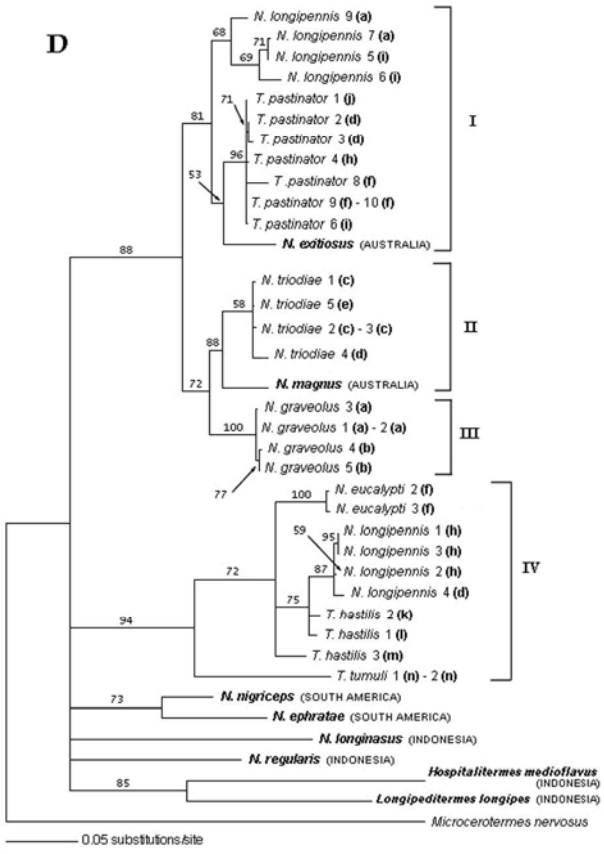


Fig. 3 – Maximum likelihood (A, B, D) and Maximum Parsimony (C) trees obtained on COII (A), 16S (B) and combined sequences (C, D). Samples numbers are as in Table 1. The scale indicates expected nucleotide substitution per site (A, B, D) or number of changes (C). Values above branches represent bootstrap percentages supported at ≥50%. Roman numerals refers to the clades discussed in the text.
A, -Ln L =3677,729; B, -Ln L = 3476,213; C, Tree length 1215, CI 0.497, RI 0.756; D, -Ln (Likelihood) = 7423. 352.




C
N. longipennis 9 (a)
N. longipennis 7 (a)
N. longipennis 5 (i)
N. longipennis 6 (i)
T. pastinator 1 (j)
T. pastinator 2 (d)
T. pastinator 3 (d)
T. pastinator 4 (h)
T. pastinator 8 (f)
T. pastinator 9 (f) - 10 (f)
T. pastinator 6 (k)
N. exitiosus (AUSTRALIA)
I
N. triodiae 1 (c)
N. triodiae 5 (e)
N. triodiae 2 (c) - 3 (c)
N. triodiae 4 (d)
N. magnus (AUSTRALIA)
II
N. graveolus 3 (a)
N. graveolus 1 (a) - 2 (a)
N. graveolus 4 (b)
N. graveolus 5 (b)
III
N. nigriceps (SOUTH AMERICA)
N. ephratae (SOUTH AMERICA)
N. eucalypti 2 (f)
N. eucalypti 3 (f)
N. longipennis 1 (h)
N. longipennis 3 (h)
N. longipennis 2 (h)
N. longipennis 4 (d)
T. hastilis 2 (k)
T. hastilis 1 (l)
T. hastilis 3 (m)
T. tumuli 1 (n) - 2 (n)
IV
N. longinasus (INDONESIA)
N. regularis (INDONESIA)
Hospitalitermes medioflavus (INDONESIA)
Longipeditermes longipes (INDONESIA)
Microcerotermes nervosus
10 changes


D
N. longipennis 9 (a)
N. longipennis 7 (a)
N. longipennis 5 (i)
N. longipennis 6 (i)
T. pastinator 1 (j)
T. pastinator 2 (d)
T. pastinator 3 (d)
T. pastinator 4 (h)
T .pastinator 8 (f)
T. pastinator 9 (f) - 10 (f)
T. pastinator 6 (i)
N. exitiosus (AUSTRALIA)
I
N. triodiae 1 (c)
N. triodiae 5 (e)
N. triodiae 2 (c) - 3 (c)
N. triodiae 4 (d)
N. magnus (AUSTRALIA)
II
N. graveolus 3 (a)
N. graveolus 1 (a) - 2 (a)
N. graveolus 4 (b)
N. graveolus 5 (b)
III
N. eucalypti 2 (f)
N. eucalypti 3 (f)
N. longipennis 1 (h)
N. longipennis 3 (h)
N. longipennis 2 (h)
N. longipennis 4 (d)
T. hastilis 2 (k)
T. hastilis 1 (l)
T. hastilis 3 (m)
T. tumuli 1 (n) - 2 (n)
IV
N. nigriceps (SOUTH AMERICA)
N. ephratae (SOUTH AMERICA)
N. longinasus (INDONESIA)
N. regularis (INDONESIA)
Hospitalitermes medioflavus (INDONESIA)
Longipeditermes longipes (INDONESIA)
Microcerotermes nervosus
0.05 substitutions/site

Chapter 5

Morphological analysis of *Nasutitermes* and *Tumulitermes* samples from the Northern Territory, based on Scanning Electron Microscope (SEM) images. (Bergamaschi et al., submitted)

INTRODUCTION

Termite classification has relied heavily on characters of external morphology of the soldier, being the most morphologically differentiated caste in termites (Hill, 1942) or, to a lesser extent, on mandible dentition of the winged adult and worker, and on the structure of the digestive tract (Noirot and Kovoor, 1958; Noirot and Noirot-Timothèe, 1969; Sands, 1972; Miller, 1991; Donovan et al., 2000). Also a combination of all mentioned characters has been considered (Miller, 1997).

The Nasutitermitinae (Hare) subfamily, with 93 described species, appears the most specialized subfamily of the Termitidae (Krishna, 1970). Much taxonomic incongruence involves the genera *Nasutitermes* and *Tumulitermes* (Miller, 1997; Bulmer and Crozier, 2002; Bergamaschi et al., 2006 submitted).

Nasutitermes soldiers generally have a rounded head capsule with a relatively short rostrum and well developed points on the mandibles; *Tumulitermes* soldiers usually show a constricted head capsule, with a relatively slender rostrum (Gay, 1970) and vestigial or absent mandibular point (Miller, 1997). In addition, the imago left mandible of *Nasutitermes* has a straight or evenly curved cutting edge between the fused first plus second marginal tooth and the third marginal tooth; in *Tumulitermes* the cutting edge is sinuate with a notch anterior to the third marginal tooth (Miller, 1997). However, some species have a constrained soldier head capsule and "*Nasutitermes* type" mandibles, whereas others posses a rounded head capsule and a short stout rostrum combined with "*Tumulitermes* type" mandibles (Miller, 1997). As also observed

by Hill (1942), dimorphic species may have soldiers with both rounded and constricted head capsule.

Hill (1942) (Appendix I) noted that the placement of some species was difficult, particularly when the winged adult is unknown, but as pointed out by Miller (1997), the discovery of adults which were unknown to Hill has added further confusion.

The taxonomy and systematics of *N. eucalypti* and *T. pastinator* are in particularly discussed. The former species presents a general shape of the head capsule and rostrum more similar to other Australian *Nasutitermes*, such as *N. longipennis* (Hill, 1942). However, examination of the winged adult showed that it is morphologically very similar to *T. tumuli*. On the basis of colors, pilosity, wing membrane, and mandibular definition, the adults of *N. eucalypti* would intuitively be placed with *T. tumuli* ones (Miller, 1997). In addition, studies that consider associated inquilines to describe species (Watson, 1973) found a supposed *Nasutitermes* species with a high affinity to *N. eucalypti* associated with an extremely specialized staphylinid beetle; but this *Nasutitermes* taxon was subsequently recognized as a new species of *Tumulitermes* (Miller, 1997). *T. pastinator* soldiers show markedly constricted head capsules, even more than *T. tumuli*. For this character, the species was assigned to *Tumulitermes* by Snyder (1949), but, again winged adult morphology (Miller, 1997), resulted almost indistinguishable from that of *N. longipennis* for several features. Overall, *T. pastinator* appears closer to the *Nasutitermes* group comprising the species "*tridoae-graveolus-longipennis"*, than to the *Tumulitermes* genus, with the soldier constricted head capsule being the single character linking it to that genus.

Besides *N. eucalypti* and *T. pastinator* definition, we had the chance to verify incongruency also for *N. longipennis*: in a phylogenetic study based on COII and 16S mitochondrial genes (Bergamaschi et al., submitted) samples morphologically ascribed to this species were splitted in two phylogenetically distant groups, clustering with different *Tumulitermes* species, either *T. pastinator* or *T. hastilis*.

Therefore, a comprehensive morphological analysis has been undertaken to obtain more details on *N. longipennis, N. eucalypti, T. hastilis, T. tumuli* and *T. pastinator* samples.

Given that misclassification may be also due to the use of a stereo microscope on samples not well preserved, we considered the possibilities of a deeper morphological analysis with more detailed images, through scanning electron microscopy. The goal of this work was therefore to search morphological details using a Scanning Electron Microscope (SEM) to gather clear morphological characters of the above mentioned species and in particular to discriminate between the two groups "*N. longipennis"* obtained with the molecular approach which one could be effectively ascribed to Miller (1991) *N. longipennis* species (formerly, *Eutermes longipennis* by Hill (1942)).

MATERIAL AND METHODS

Samples were collected in the Australian Northern Territory and conserved in 70 % or absolut ethanol.

Termites were identified through the morphological keys of Hill (1942) and Miller (1991), and also through comparison with the CSIRO termite reference collection (CSIRO Entomology, Canberra)..

The group of *N. longipennis* samples clustering with *T. pastinator* + *N. triodiae* + *N. graveolus* haplotypes in Bergamaschi et al., (2006), was called "group A"; while the other group of *N. longipennis* samples was named as "group B" (compare with Fig. 1).

Five samples for group "A" and four samples for group "B" were considered in this analysis (Tab. 1). Three up to five individuals per sample were analyzed.

Moreover, three samples ascribed to the species *N. eucalypti*, two samples recognized as *T. hastilis,* and two ascribed to *T. tumuli*, were considered for morphological comparison with *N. longipennis* "B", because they clustered together in molecular analysis. For the same reason, three individuals of *T. pastinator* were compared with *N. longipennis* "A" morphology.

Samples were dehydrated with a progressive scale of ethanol (70 – 80 – 90 – 95 – 100%) for 15 min each and then transferred in 1:1, Hexamethyldisilazane (HMDS): absolute ethanol, solution for 20 min. Samples were then transferred in pure HMDS overnight or till complete evaporation and mounted on SEM sterile stubs.

The subsequent gold coatings were done with BIO-RAD SC 502.

Samples were observed under a SEM JEOL – 5200, with a magnification range from 15X to 200,000X in 25 steps. The images were acquired with ImageSlave for Windows 2.14.

Observations dealt mainly on head capsule, mandibles and rostrum of the soldiers, as these features resulted useful to discriminate among the taxa of interest. Images were captured with the same orientation and enlargement for comparison.

RESULTS

N. longipennis. Group "A" (individuals 5, 6, 7, 8, 9) showed differences in shape and size of soldier head capsule, hairs distribution and number in the rostrum, with respect to group "B" (individuals 1, 2, 3, 4), while the body colors appeared completely identical between the two groups.

Group "A" individuals were characterized by the follow measurements (Fig. 5.2-8): Total length, 3.00-4.00 mm; head, to apex of rostrum, long, 1.500-2.000 mm; head, wide, 1.750-1.125 mm; pronotum, wide, 0.50-0.65 mm. The dorsal view of the head capsule was broadly pyriform, and the rostrum approximately one-third the entire length of the head. Typically, all individuals presented a stress convexity in the dorsal margin of the head in profile, just above the insertion of the antennae. Antennae were often of 14 segments, rarely of 13, with 3rd and 4th segments very close. 1st segment was always the longer one. The rostrum presented only few short hairs with the exception of 4 main long sensorial hairs at the apex while the rest of the head, torax and abdomen, were covered with rather numerous hairs. The hole at the apex of the nasus, measured 0.10-0.12 mm.

Group “B” specimens presented these measurements (Fig 5.2-8): Total length, 2.80-3.10 mm; head, to apex of rostrum, long, 1.350-1.650 mm; head, wide, 0.800-0,900 mm; pronotum, wide 0.30-0.50 mm. Dorsal view of the head was rather oval sharply constricted at the anterior third, and strongly narrowed at the beginning of the rostrum, which was a two-fifth the total length of the head. The dorsal face resulted rather straight in profile view, without any convexity. Antennae were always of 14 segments, with the 1st as the longer and the 2nd the shortest. Numerous hairs were observed on the head with particular regard to the rostrum surface, where several long and stout hairs covered mainly the apical part. The end of the nasus presented a narrowing 0.15-0.20 mm long, that determined a terminal hole of 0.5-0.8 mm of diameter.

The main morphological comparisons between species are as follows:

N. longipennis “A” *vs Tumulitermes pastinator* (Fig 5.8). *T. pastinator* had markedly constricted head capsule, even more than all the other *Tumulitermes* observed, in front of the rather rounded head of *N. longipennis* “A”. Despite that, a great affinity between the two taxa are given by numerous features, such as rostrum length, number and distribution of the hairs on the nasus, size of rostrum hole, and mainly, the convexity of the dorsal margin of head in profile.

N. longipennis “B” *vs Nasutitermes eucalypti* (Fig 5.10). The main differentiation is given by the shortest rostrum of *N. eucalypti*, about one-half the head capsule. The two taxa showed similar total body length, head capsule shape with rather straight profile, the same narrowing at the apex of the rostrum, similar distribution of the hairs with a higher amount of them in the rostrum of *N. eucalypti*, and the same structure of antennae articles.

N. longipennis “B” *vs T. hastilis* (Fig 5.11). The comparison evidenced a really high affinity for all the features taken into account, with the exception of the body colors with particular regard to the head, reddish-brown in the former taxon, orange-dark, ferruginous in the second.

N. longipennis "B" *vs T. tumuli* (Fig 5.11). The second species is characterised with respect to the former by the dark colour of head and body, the high number of hairs on the rostrum, the low number of hairs in the rest of the head and body, and a longer and narrower rostrum.

DISCUSSION

It is now clear that only a multi-disciplinary approach can help in resolving the phylogenetic relationships among whatever taxa (Eggleton, 2001). Morphological analysis in this and other case (Hill, 1942; Miller, 1991; Miller, 1997; Donovan et al., 2000) highlighted the clear difficult in recognizing and classifying some species. On the other hand, molecular analysis alone could not always resolve all taxonomic incongruences.

On the basis of our molecular (Bergamaschi et al., submitted) and morphological results, and and taking into account Hill (1942) and Miller (1997), the group "A" individuals of *N. longipennis* samples can be considered effectively as pertaining to *N. longipennis* species, while group "B" should be ascribed to the *Tumulitermes* genus. Possible differences in size observed with respect to Hill's studies, may be due to the different sample conservation and treatment before observation, but mainly to the different techniques of observation.

Moreover, considering that *N. eucalypti* shares more features with *Tumulitermes* species than *Nasutitermes* ones, and that the molecular analysis confirms this preferential affinity, we could suggest the placement of *N. eucalypti* within the *Tumulitemes* genus. Similarly, all *T. pastinator* samples showed several characters in common with *N. longipennis* and *N. triodiae-N. graveolus* (not shown) groups, despite the presence of a head capsule constriction that at present looses its phylogenetically informative role. This support the placement of this species within the *Nasutitermes* genus.

The high morphological divergence of *T. tumuli*, with respect to all the other *Tumulitermes* species here observed, found a support in Miller's study (1997); here a higher number of *Tumulitermes* species were considered and *T. tumuli* was also molecularly (Bergamaschi et

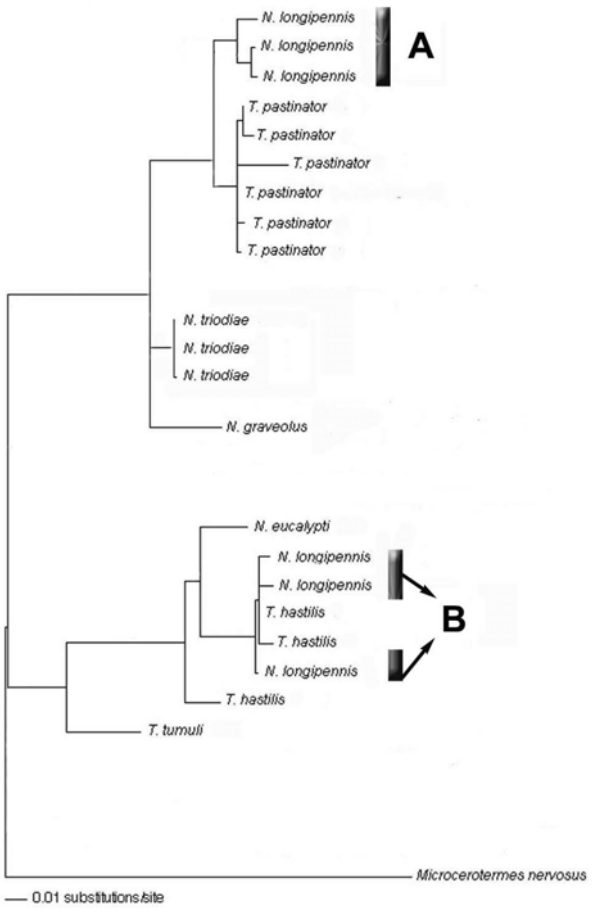
al., submitted) placed in a cluster separated from the one embodying *T. hastilis* and *N. (T.) eucalypti*.

An alternative taxonomic hypothesis may be to suppress present genera and to recognize that we are dealing with a unique genus that contain all the species analyzed in present and Miller's studies. Only a wider analysis comprising the other *Eutermes* species (Hill, 1942) will define whether this unique genus corresponds to the more widespread *Nasutitermes* genus, rather than to *Eutermes* as indicated by Hill (1942), or it constitutes a new Australian entity.

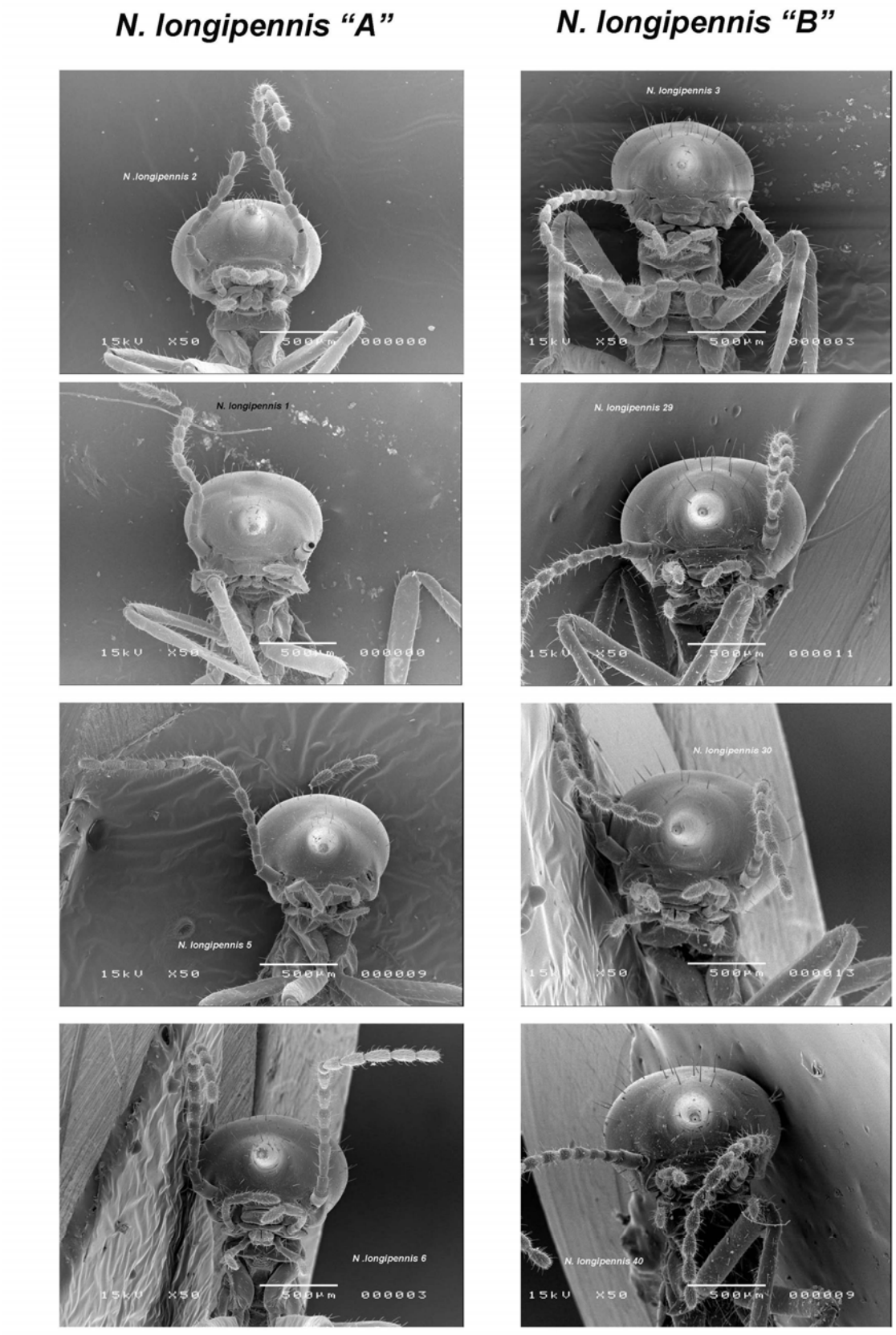
Tab 1 – Samples numbers, collecting site and dates of the species analyzed.

Species	Sample	Collecting site	Collecting date
Nasutitermes longipennis	1	Adelaide river	26/04/2003
	2	Adelaide river	26/04/2003
	3	Adelaide river	26/04/2003
	4	Douglas Daly	24/04/2003
	5	Mary river Nat. Park	19/10/2005
	6	Mary river Nat. Park	19/10/2005
	7	C. Darwin Nat.Park-Darwin	27/10/2005
	8	C. Darwin Nat.Park-Darwin	27/10/2005
	9	CSIRO-Darwin	25/11/2005
Nasutitermes eucalypti	1	Kakadu Nat. Park	07/05/2003
	2	Gunn point	25/11/2005
	3	Gunn point	25/11/2005
Tumulitermes hastilis	1	Kakadu-Mary river	31/10/2005
	2	Mandorah	02/10/2005
Tumulitermes pastinator	1	Palmerston	11/04/2003
	2	Douglas Daly	24/04/2003
	3	Douglas Daly	24/04/2003
Tumulitermes tumuli	1	King's canyon	11/11/2005
	2	King's canyon	11/11/2005

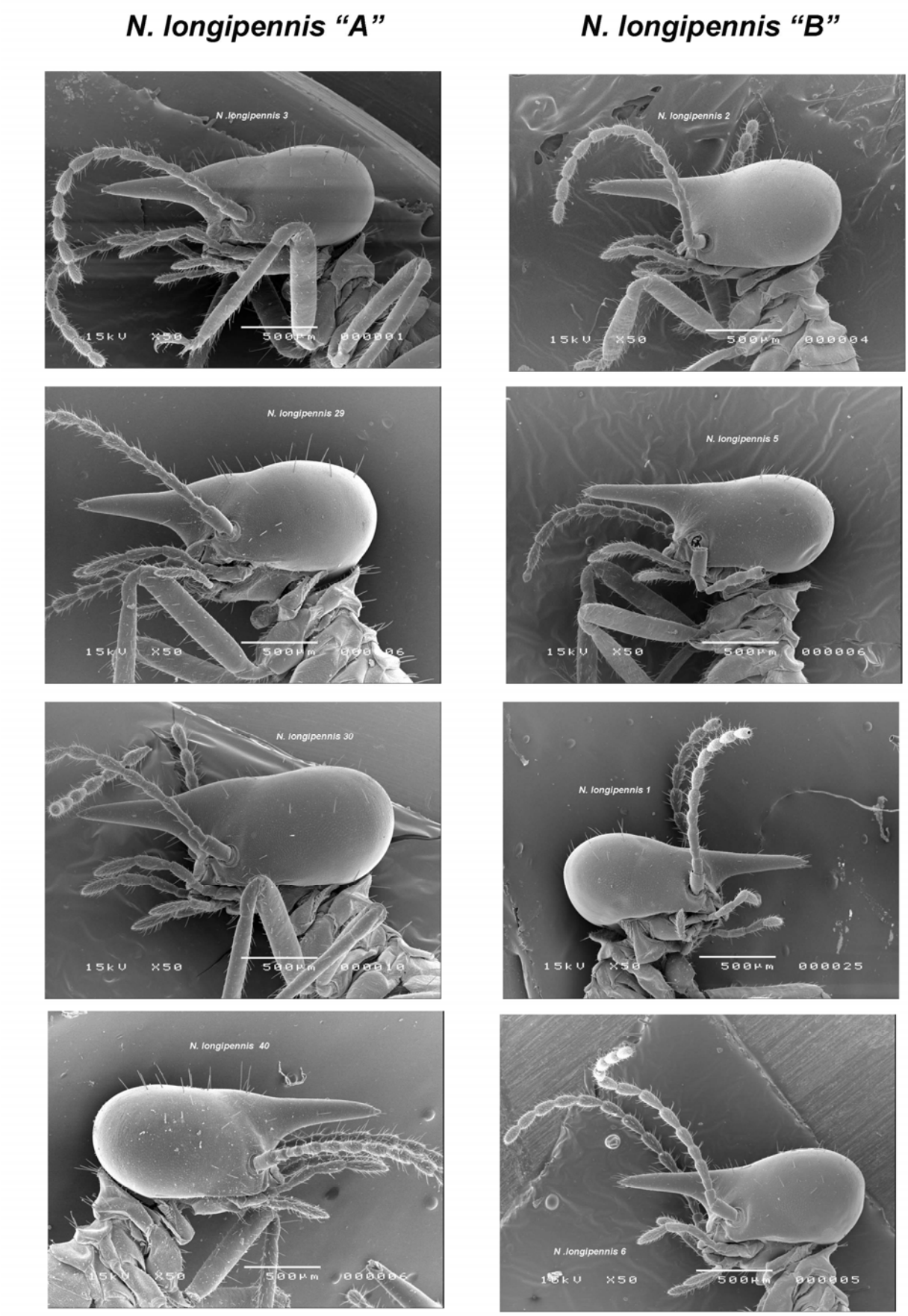
Fig. 1 – ML Tree modified from Bergamaschi et al. (submitted) based on COII sequences. The two groups of *N. longipennis* are named "A" and "B".



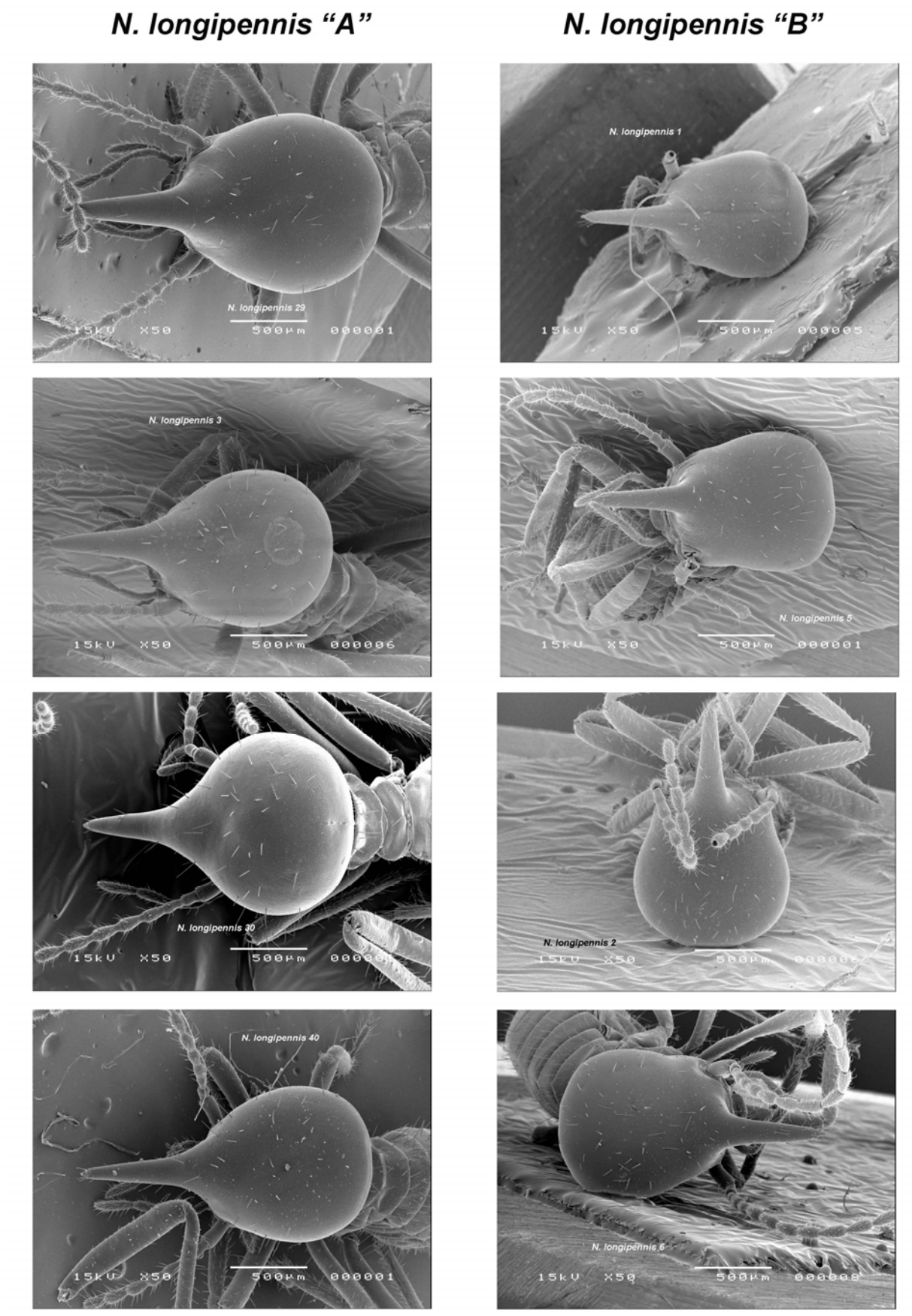
<u>Fig. 2</u> – Comparison between the frontal head capsule of *N. longipennis* "A" and "B" samples.



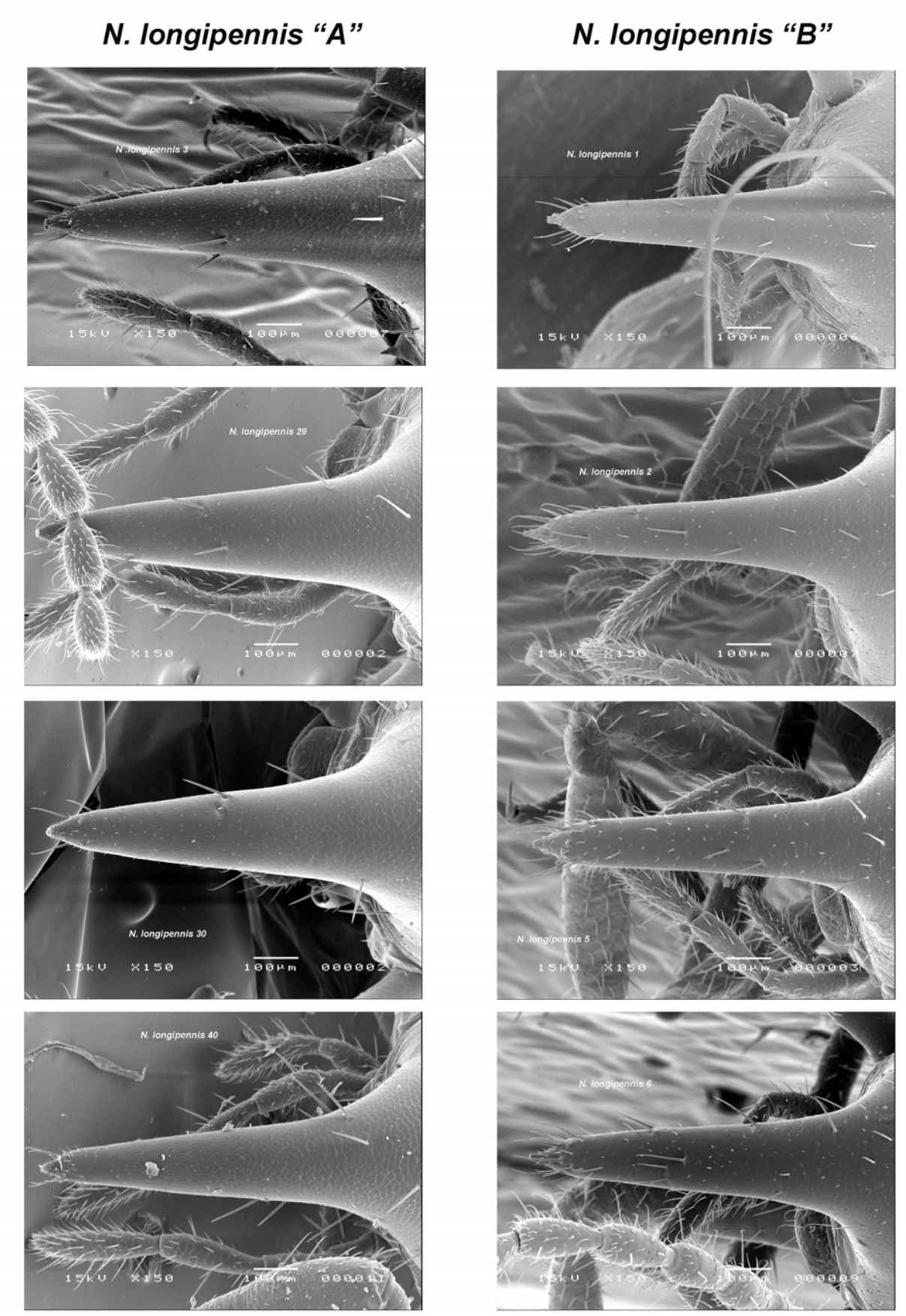
<u>Fig. 3</u> – Comparison between lateral head capsule of *N. longipennis* "A" and "B" samples.



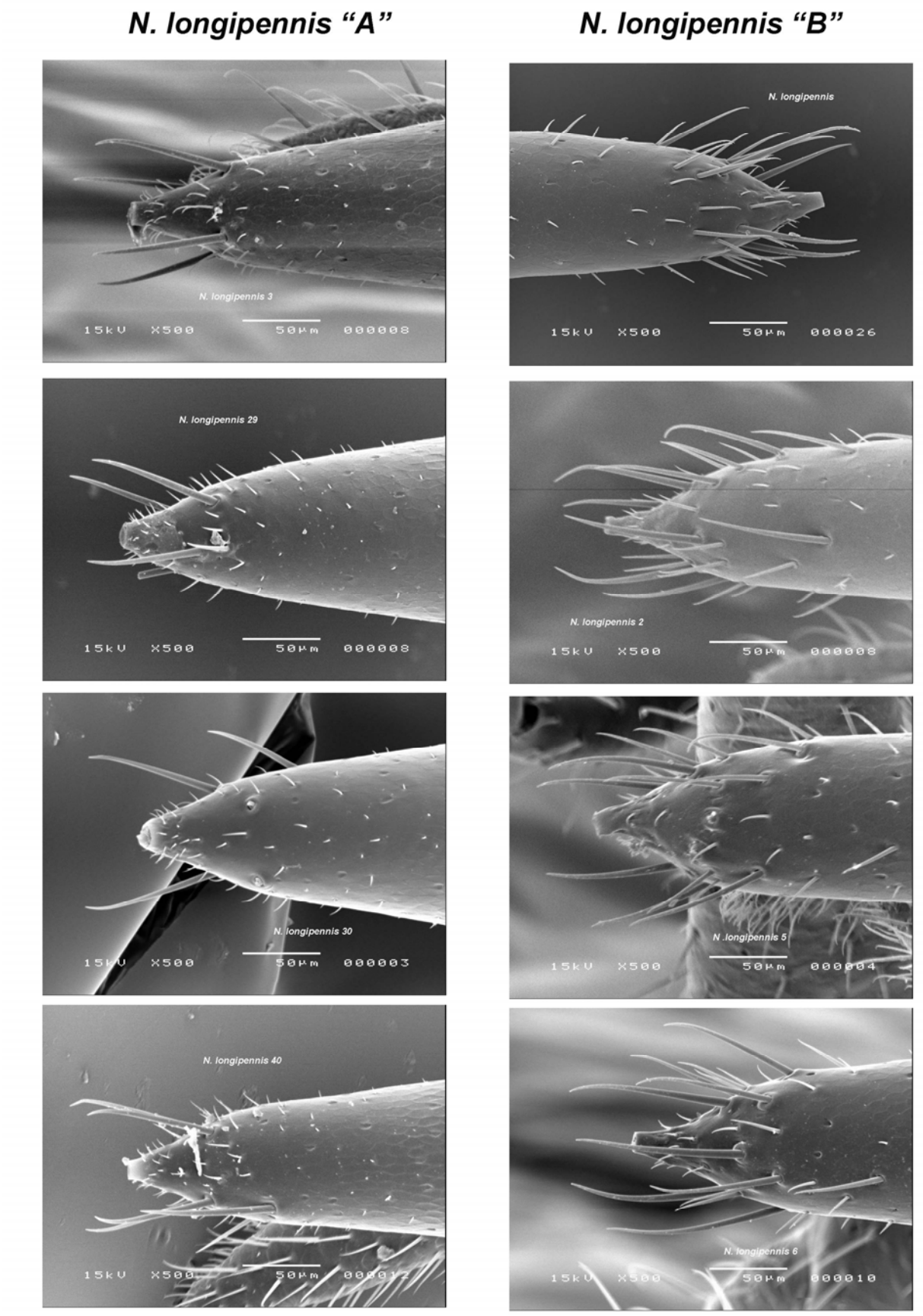
<u>Fig. 4</u> – Comparison between dorsal head capsule view of *N.longipennis* "A" and "B" samples.



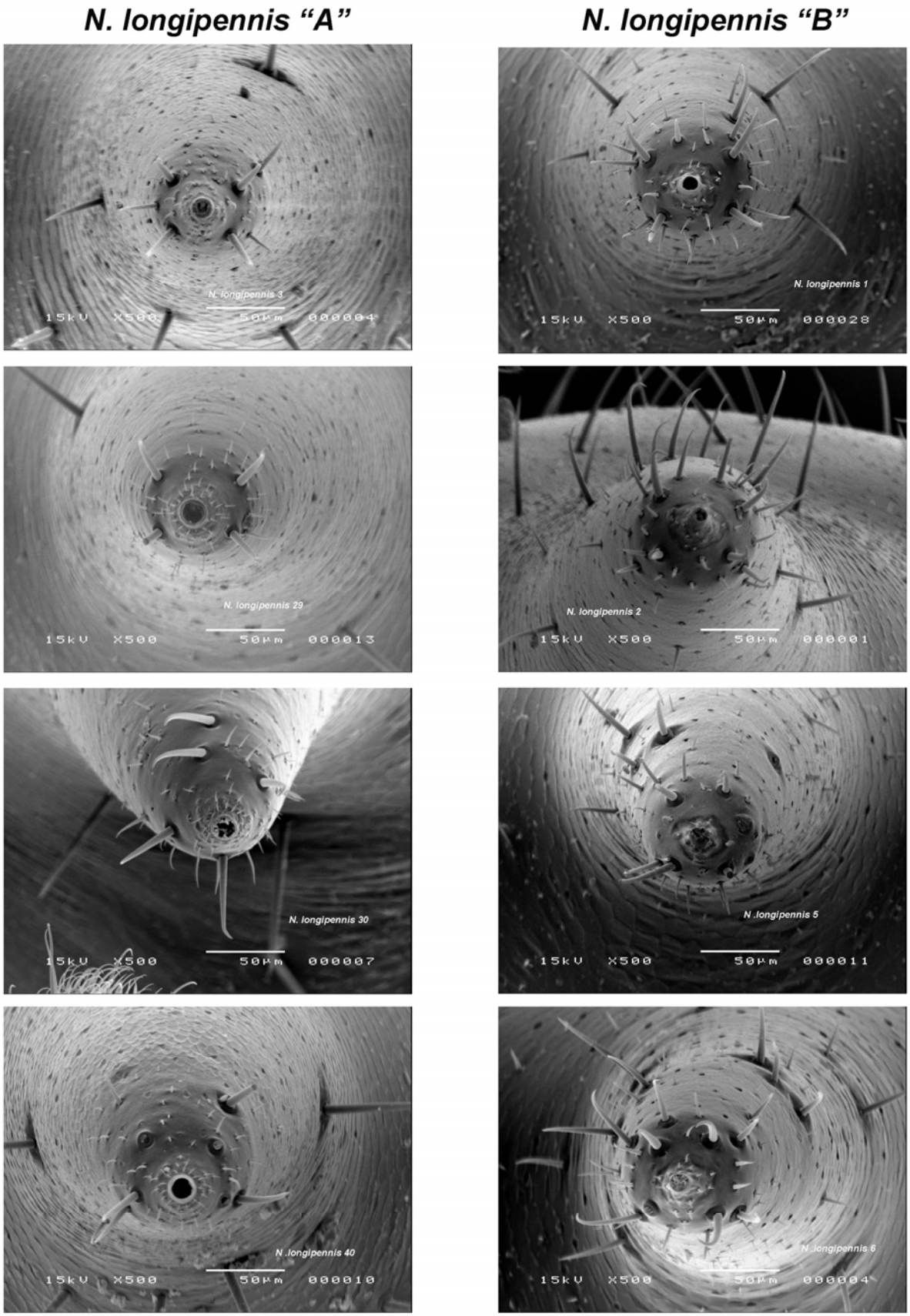
<u>Fig. 5</u> – Comparison between rostrum dorsal view of *N. longipennis* "A" and "B" samples.



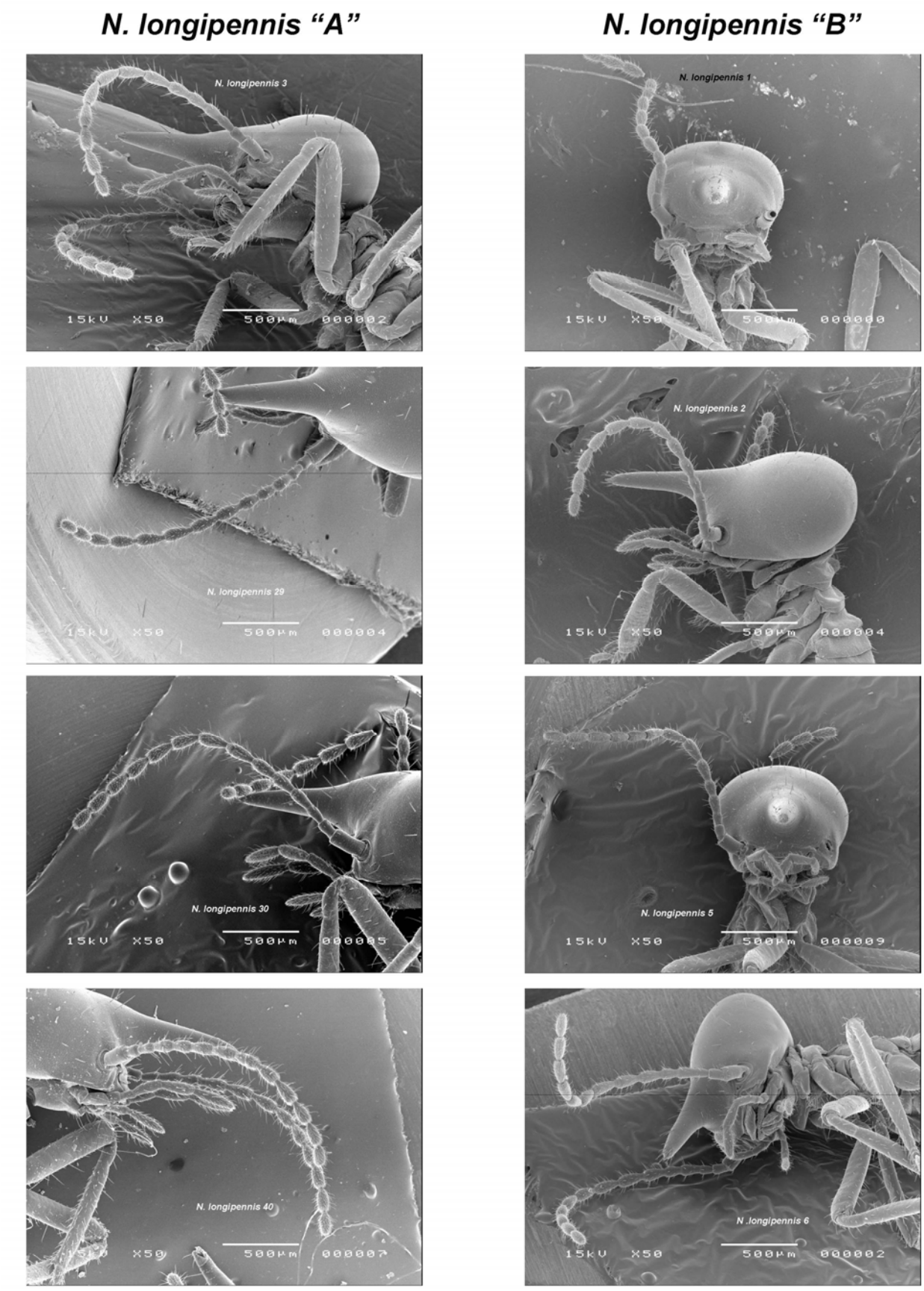
<u>Fig. 6</u> – Comparison between the apex of rostrum of *N. longipennis* "A" and "B" samples



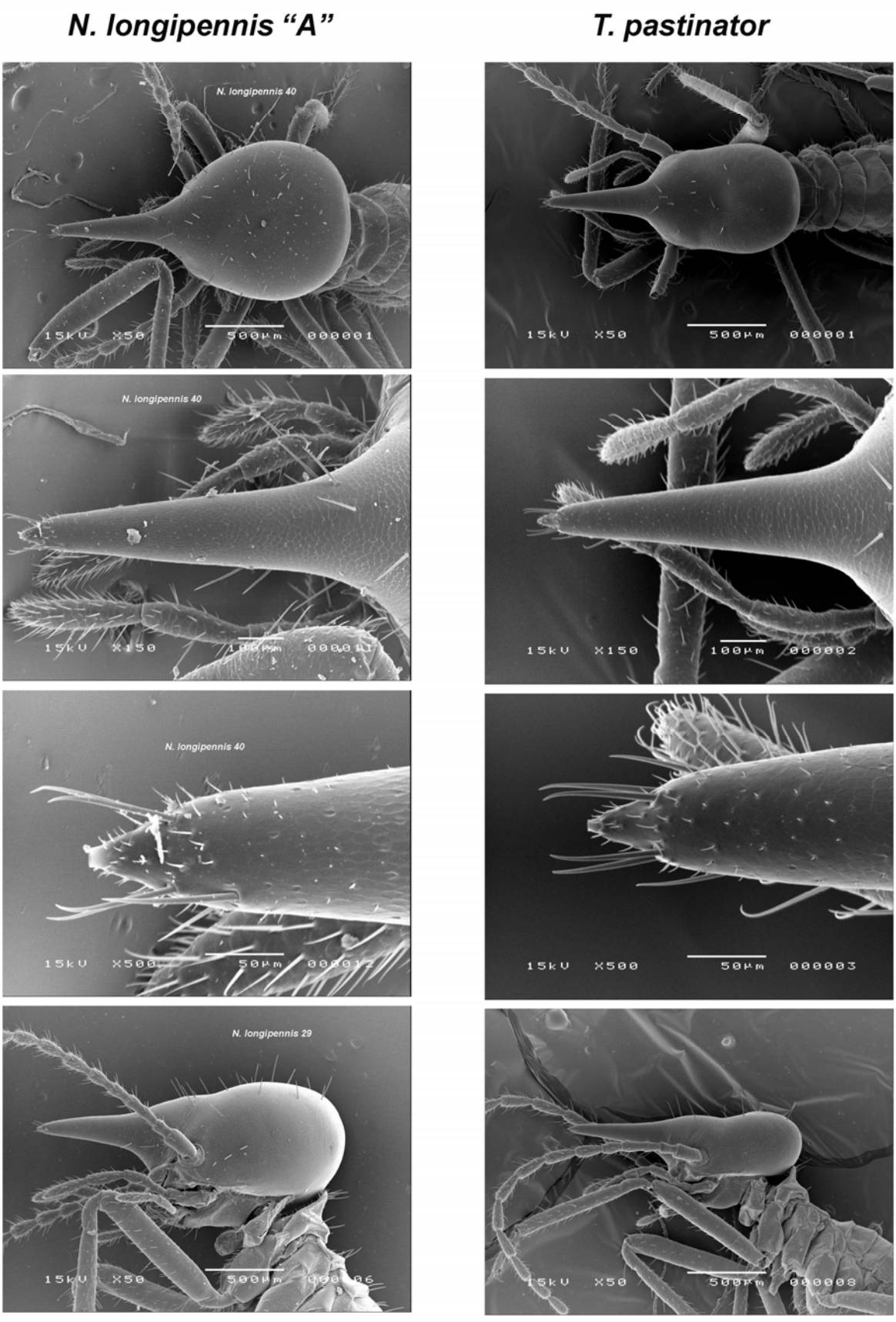
<u>Fig. 7</u> – Comparison between the rostrum hole of *N. longipennis* "A" and "B" samples.



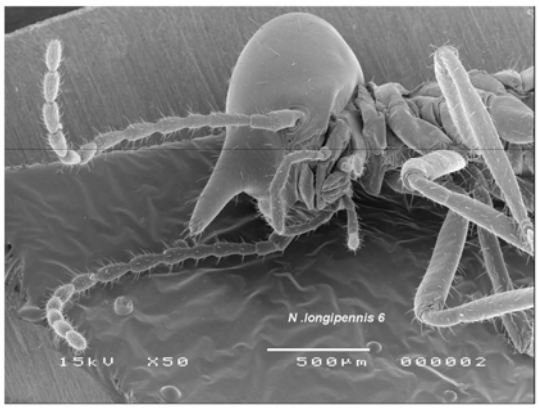
<u>Fig. 8</u> – Comparison between the antennae of *N. longipennis* "A" and "B" samples.

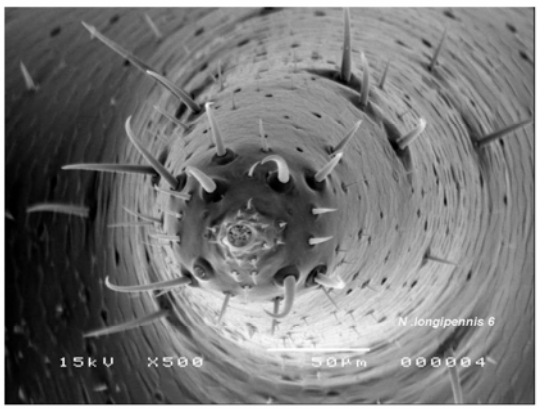


<u>Fig. 9</u> – Comparison between *N. longipennis* "A" and *T.pastinator* samples.

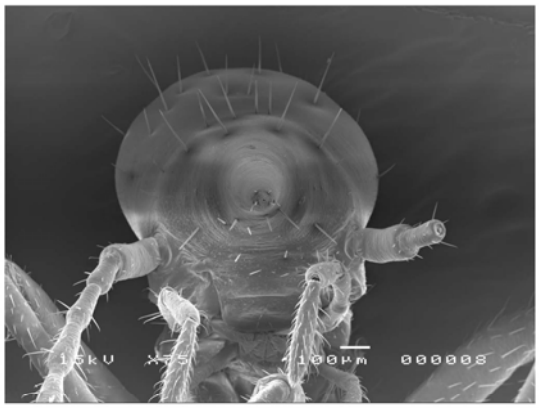


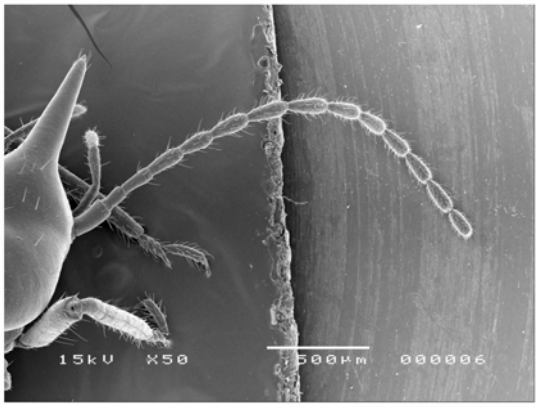
N. longipennis "A"

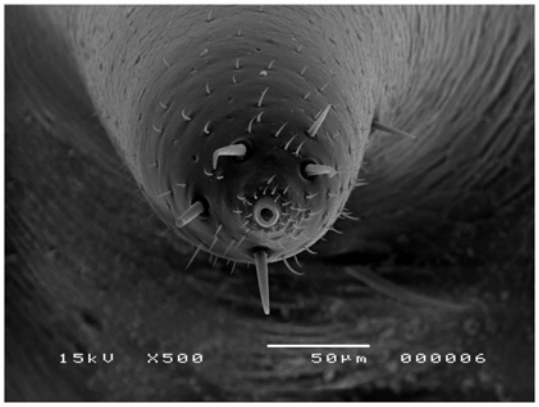




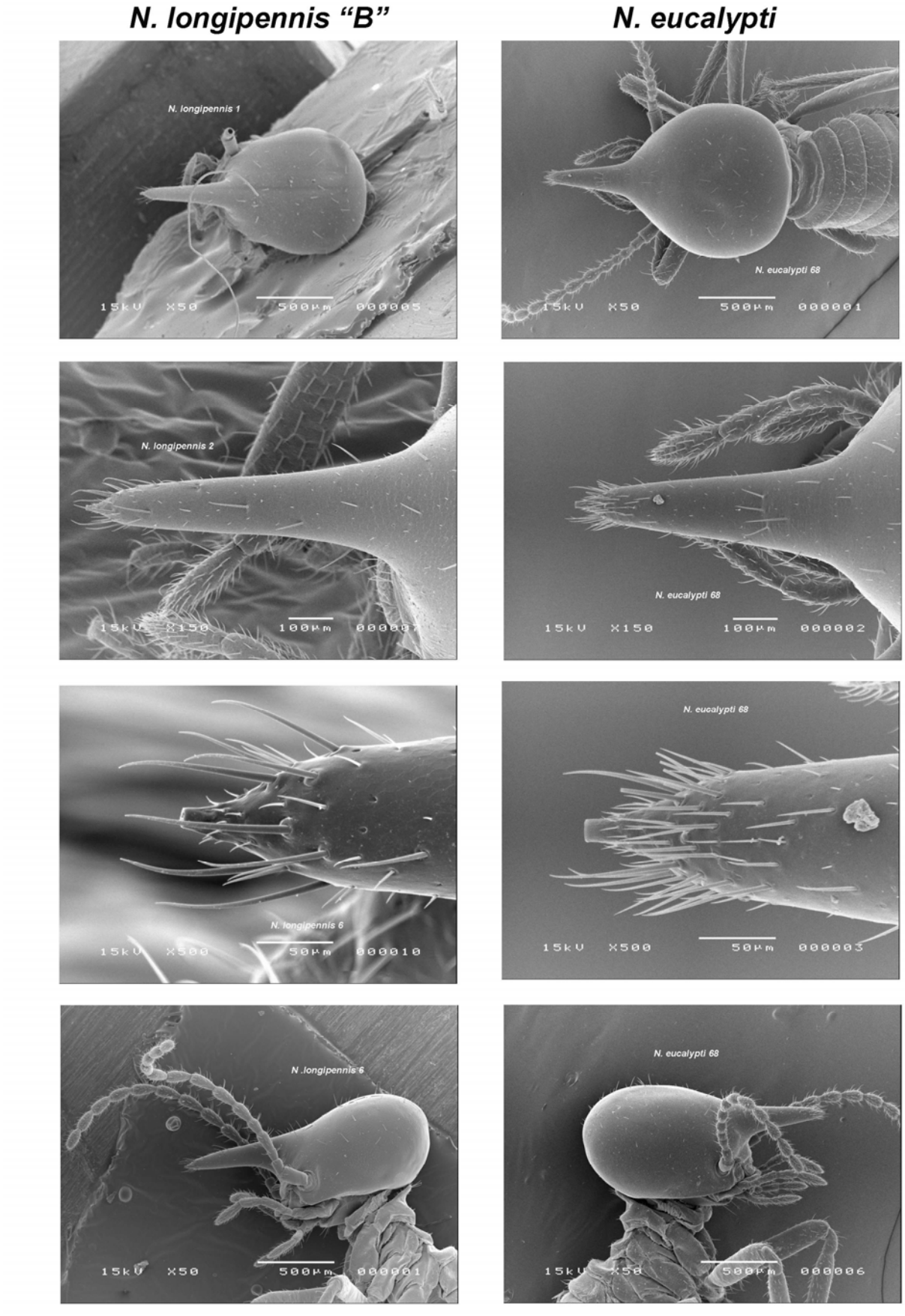
T. pastinator



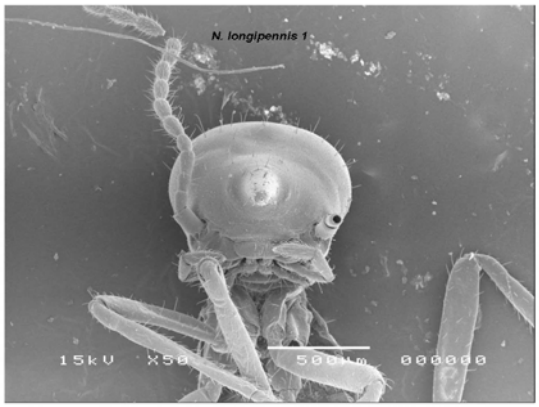


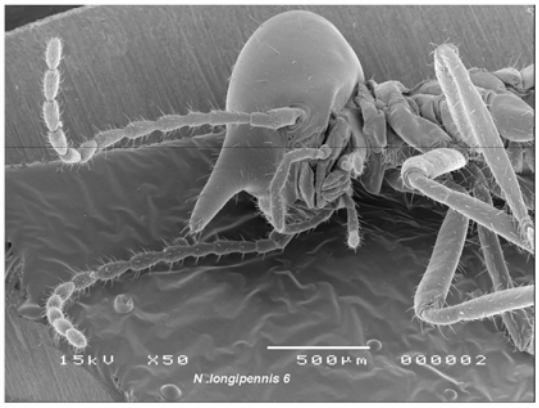


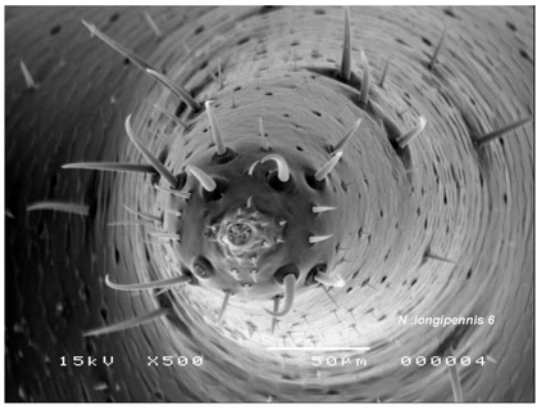
<u>Fig. 10</u> – Comparison between *N. longipennis* "B" and *N. eucalypti* samples.



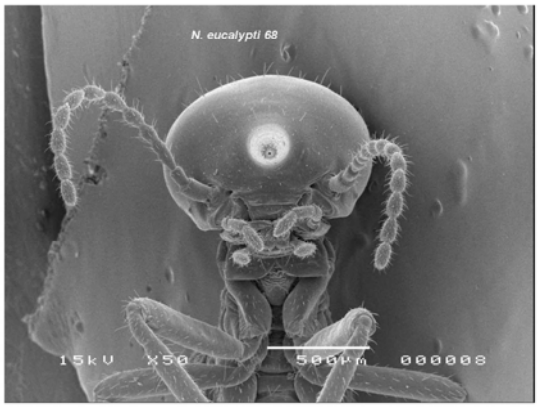
N. longipennis "B"

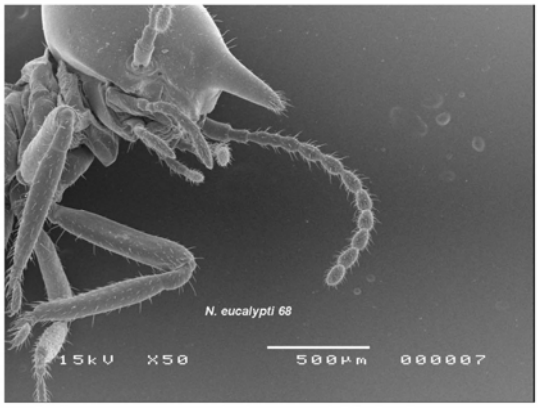






N. eucalypti





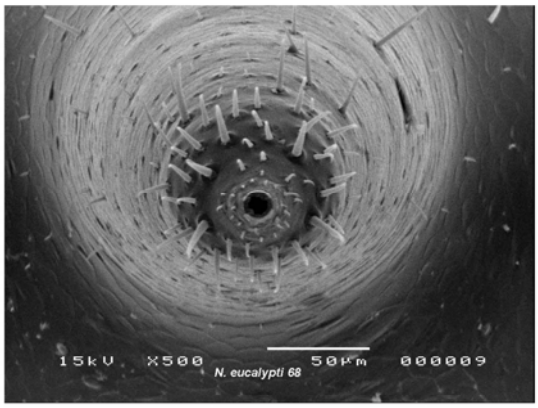
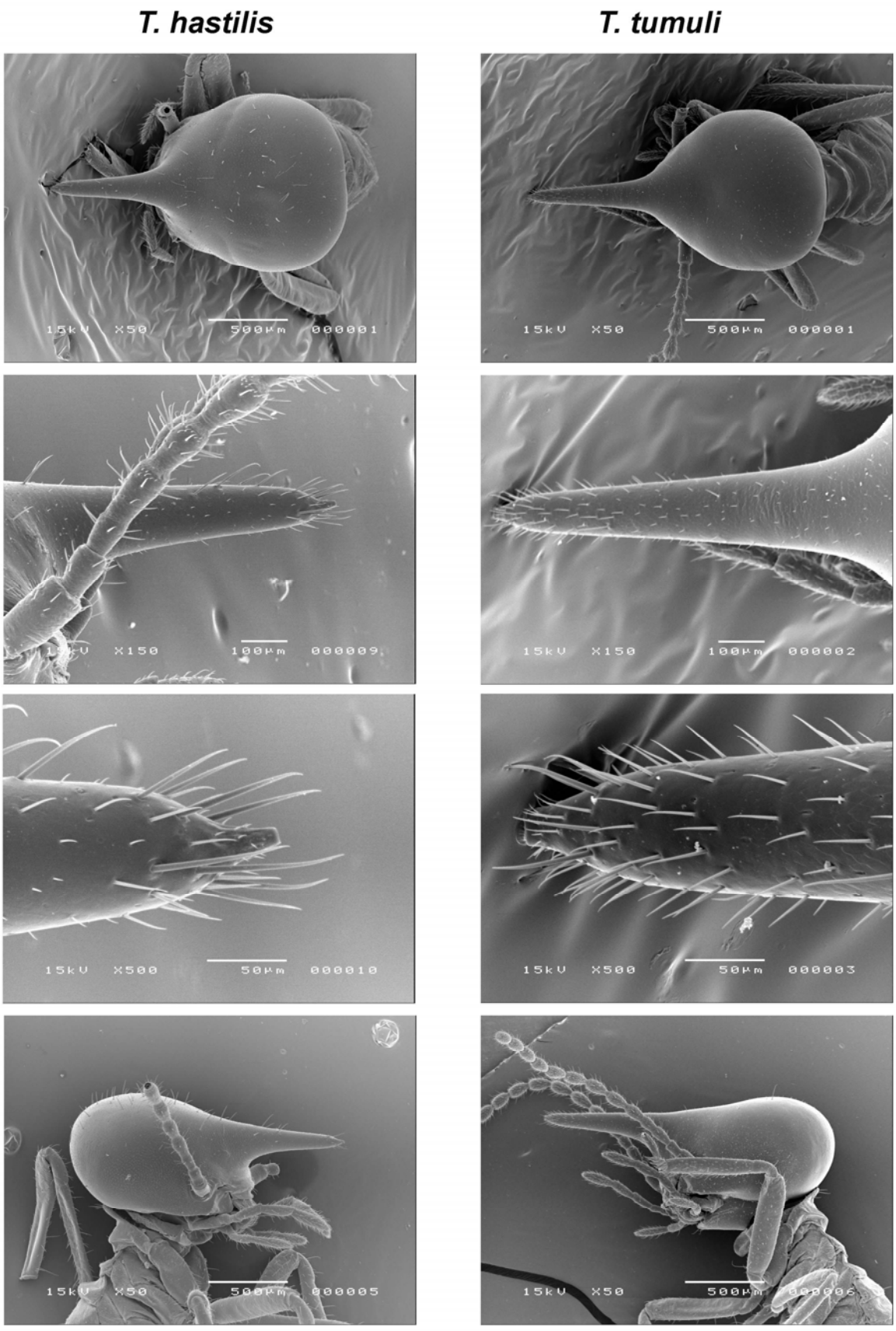
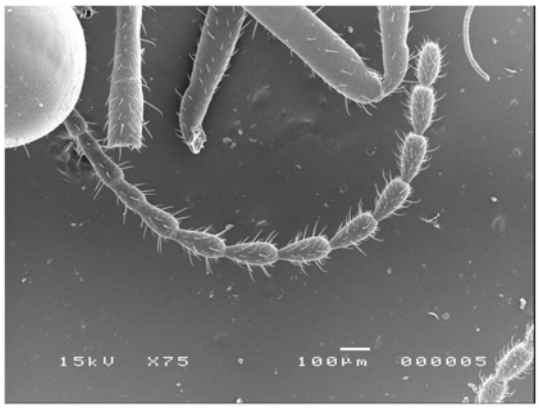
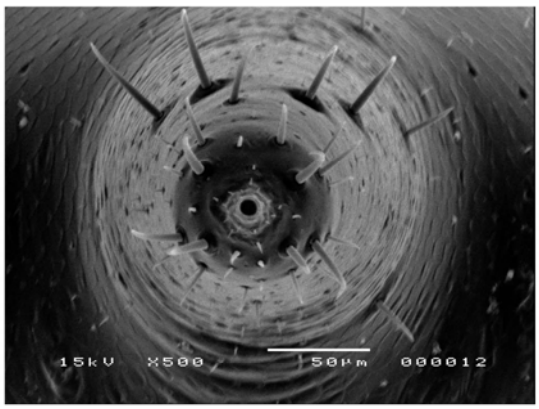


Fig. 11 – Comparison between *T. hastilis* and *T. tumuli* samples.

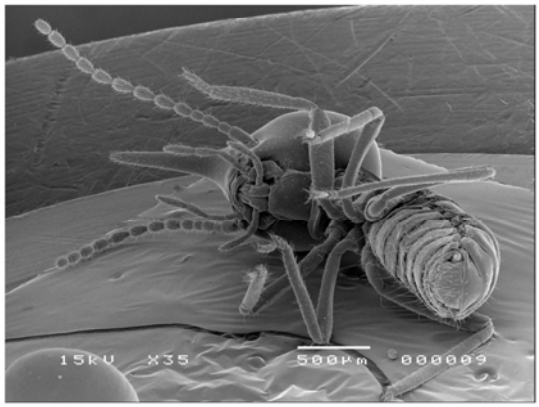


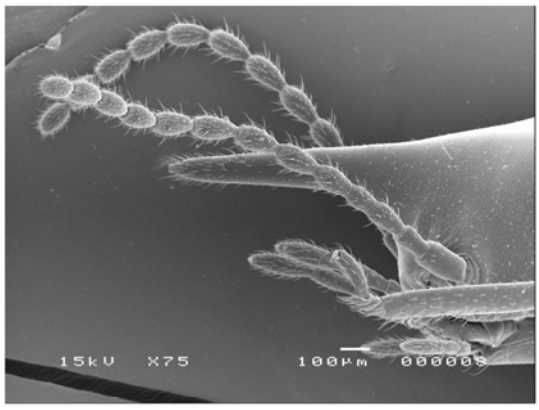
T. hastilis

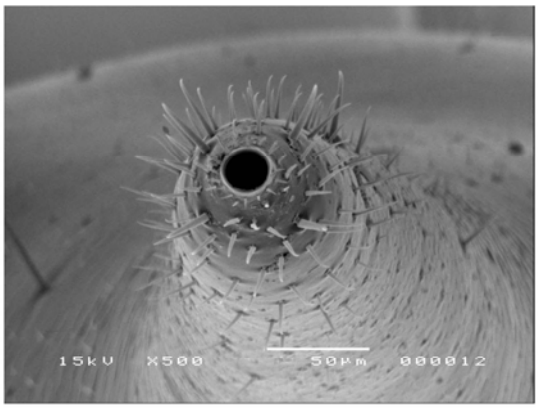




T. tumuli







Chapter 6

General remarks

In the last fifteen years, a remarkable increase in the number of termite phylogenetic papers took place. Many hypotheses of relationships are now much more clearly established. These include: the relationships between termites and cockroaches, the position of the relictual and monotypic family Mastotermitidae, and the monophyly of the [Rhinotermitidae+Termitidae] group and of the Termitidae family. In particular, presented molecular results confirm that Rhinotermitidae and Termitidae are sister lineages that occupy the most apical branch of the isopteran tree. My study showed a high genealogical affinity between the two families under both a karyological and molecular point of view. In particular, within Rhinotermitidae and Termitidae a high karyological affinity was observed among genera and species, suggesting an ongoing concerted evolution.

On the other hand, the data showed a wide variability within Kalotermitidae family; in fact, the phylogenetic relations among its genera were resolved in a polytomy and their karyotype features appeared extremely diversified.

Termitidae are referred to as “higher termites” primarly because they harbour only bacteria in their hindgut; Rhinotermitidae are considered either as “lower termites” having cellulotyc protozoa as well as bacteria in their hindgut, or “higher termite” considering the advanced eusociality of their colony. My findings well support Thompson et al.’s view (2000): the subjective terms “higher” and “lower” in reference to termite systematics must be left aside, because evolutionary taxonomy should represent genealogical and cladistic relationships and “lower termites” are now well established to be a polyphyletic group.

Moreover, my study shows that within the Termitidae, the phylogenetic relationships among subfamilies are still unclear. While karyological data demonstrate a high uniformity of the

karyotype features between Nasutitermitinae and Termitinae subfamilies, even if with different "satellites" composition (see below), molecular analysis clearly highlight the polyphyly of these groups. The groups that should be more deeply analyzed are the *Amitermes*-group and *Termes*-group within Termitinae and *Nasutitermes*-*Tumulitermes*-group for Nasutitermitinae.

Within the latter, molecular data evidence some inconsistencies with the current accepted taxonomy. Present analysis clearly shows that the two genera are not monophyletic and their species always intermingle. The *N. longipennis* samples clearly pertain to two different entities; a comprehensive morphological approach using the ScanningElectronMicroscope solved this incongruence, identifying one of this entities as the right one and the other as pertaining to the *Tumulitermes* genus.

Many problems in Isoptera taxonomy and phylogeny derive from the fact that more often morphological, karyological and molecular studies are not integrated. Actually, there is a pressing need for studies that employ a wide range of approaches. As shown in the present study, the addition and critical analyses of karyotype and chromosomes evolution data, to morphological and molecular ones, led to a more complete view on phylogenetic taxa relationships.

Moreover, during my PhD a wider range of taxa, not here reported, were karyologically and molecularly characterized. In particular, a comprehensive cytologic study, to detect Nucleolar Organizer Regions both with Ag-staining and in situ hybridizations, has been recently started. NORs contain rDNA cistrons and can be usually identified as secondary constrictions on metaphase chromosomes. The high presence of secondary constrictions founded in most of the analyzed species, motivated to delve into this aspect to highlight the correlation between rDNA cistrons and cytological satellites.

At the same time, a molecular study involving a higher number of taxa ascribed to all Isoptera families is carried out. The collaboration with CSIRO in Darwin and other research centers will be profitably for samples and data retrieval. It should be reminded that all studies of termite phylogenetics have a major taxon-sampling problem. It is highly difficult to obtain a fully taxon sampling across the whole group, and this problem is most pronounced for molecular studies requiring material stored in particular way..

Probably, the developing of truly international collaborative projects, ranging from morphology and karyology-cytogenetic to various molecular approaches, will be essential to overcome these limitations.

APPENDIX I

Hill's (1942) species description

- ***Eutermes (Nasutitermes) longipennis***, soldier:

"Of medium size, variable in size, with reddish brown head, light brown thorax and abdomen, and somewhat darker antennae. Head, thorax and abdomen with rather numerous stout, reddish hair. Head in dorsal aspect: almost spherical to broadly pyriform, rostrum short and rather narrow, about three-eights the length of the entire head. Dorsal margin of head in profile markedly convex above the insertion of the antennae. Antennae of 13-14 segments. In antennae with 13 the 3rd and 4th are closely or completely fused, thus forming the longest segment of all excepting the 1st …… In 14 segmented antennae the 3rd usually is shortest of all; 2nd and 4th usually equal…… segmentation not always alike in the antennae of the same individual. Either 13 or 14 segmented antennae may predominate in nest series, or all may have antennae of either 13 or 14 segments. Mandibles with long stout points.

Measurements: total length 3.50-4.50 mm; head, to apex of rostrum, long 1.43-1.83 mm; head wide 0.84-1.17 mm; pronotum, wide 0.47-0.60 mm.

Distribution: Northern Territory, Queensland.

Biology: the nests are generally conical in shape (Fig. I), circular at the base and sloping symmetrically on the side to the bluntly pointed or rounded apex. They average about 15 inches (38 cm) in diameter and 18-20 inches (51 cm) in high. They may be located on open grassland, on sandy well drained open forest or scrub country……in forest and scrub country they often are constructed at the base of dead trees or fens posts, or against logs……"

Figura I- *N. longipennis* nest in a gravelly soil (left) and on a *Pandanus* trunk (right). (Photo: Silvia Bergamaschi).

- ***Eutermes (Nasutitermes) eucalypti*** , soldier:

"A very small species with rufous head and short, stout rostrum. Head broadly oval, slightly narrowed anteriorly, dorsal surface nearly straight when viewed in profile, glabrous with very few hairs; rostrum about one-half as long as head capsule. Mandibles without points. Antennae of 13 segments, proximal segments variable; 3rd segment usually distinctly longer, but often shorter, then 2nd; 4th shorter of all, or 4th very rarely longer then 3rd. (Usually the brain can be seen as a broad, dark band across the middle of the head, and the salivary duct as a narrow, pale, medium line).

Measurement: Total length, 2.50-3.00 mm; head, to apex of rostrum, long 1.06-1.39 mm; head, wide, 0.64-0.80 mm; pronotum, wide, 0.40-0.53 mm.

Distribution: Northern Territory, Western Australia.

Biology: ……. Samples were taken under the following circumstances: 1- soldiers and immature workers in the clayey wall of a nest of *Coptptermes acinaciformis*, 2- as above,

Amitermes perelegans found in adjacent galleries, 3- as above, with soldiers and workers of *E. longipennis*, in occupied nest of *Microcerotermes* sp., 4- as above, with soldiers and workers of *E. longipennis* in base of occupied nest of *E. hastilis*…….

Notes of synonymy: the winged adult is the same as *E. hastilis*."

- ***Eutermes (Tumulitermes) hastilis***, soldier

"Head variable in size, or various shades of orange to light ferruginous, the base and apical one-third of head capsule palest, rostrum light to dark ferruginous, thorax. Legs and tergites of abdomen light yellowish brown. Head and abdomen with scanty, fine hairs. Head in dorsal aspect wide broadly rounded, or almost truncate posteriorly, sharply constricted at the anterior third; rostrum very variable usually about three-fifths the length of the remainder of the head, and very narrow, sometimes distinctly longer and rather wide at base. Antennae of 14 segments; 3rd segment usually shortest of all; 4th long, longer than 5th; sometimes 2nd, 3rd and 5th of equal length, shorter than 4th; or 2nd shortest of all, and 3rd very little shorter than 4th. Mandibles usually without points.

Measurements: total length, 4.75-5.20; head, to apex of rostrum, long 1.46-1.65; head, wide 0.84-0.91; pronotum, wide 0.44-0.55.

Distribution: Queensland, Northern Territory, Western Australia.

Biology: nest of this termite may be described as tall, narrow, intensely hard, earthy columns up to 6 feet (ca 183 cm) in height and 18 inches (ca 46 cm) wide at ground level (Fig. II)……These aggregations invariably are on gravelly rises and stony ridges in worthless open forest country……

Notes of synonymy: the soldier is the same as *E. eucalypti*."

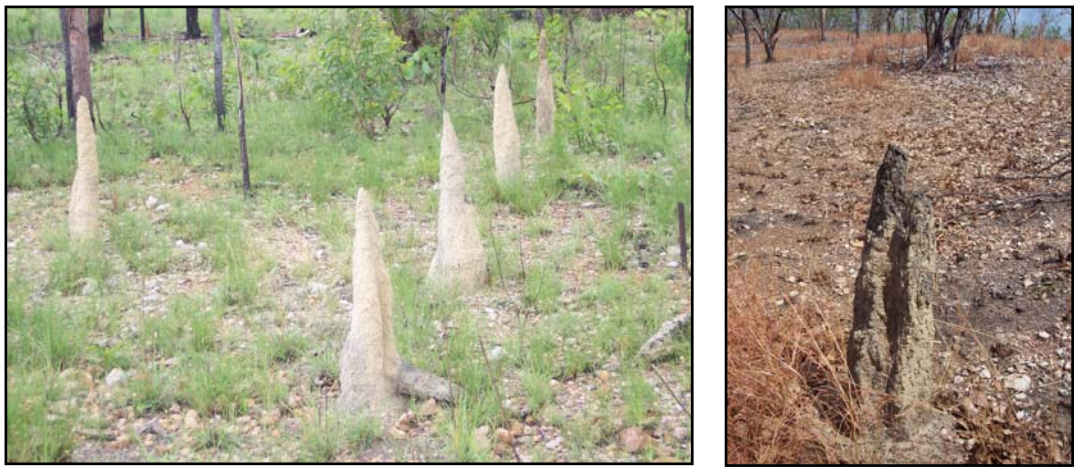


Figura II – *T. hastilis* nests. (Photo: Silvia Bergamaschi).

- ***Eutermes (Tumulitermes) pastinator***, soldier.

"A small species of variable size and colour, head usually light to rather dark brown, posterior part palest, base of rostrum dark brown, apical part of rostrum ferruginous……Head with very scanty long, stout hairs, as on thorax and abdomen, widest at the posterior third, constricated at the anterior third, broadly rounded o almost truncate posteriorly; rostrum long, about two-thirds the length of the remaninder of the head……head in profile strongly concave about the posterior third. Antennae of 13-14 segments, rarely 13……

Measurement: total length, 3.50-3.75; head, to apex of rostrum, long, 1.38-1.68; head, wide, 0.55-0.77; pronotum, wide, 0.40-0.53.

Distribution: Northern territory, Queensland, Western Australia.

Biology: ……The nests are generally low dame-shaped structures with a very thin outer wall varying in hardness according to the nature of the soil, and with an interior of somewhat similar, or often softer material (Fig. III). Short pieces of grass, similar to those stored in masses within the nest, usually are built into the walls to bind the earthy material used in their construction……as far as is known E. pastinator occur only on inferior or worthless well drained, sandy, gravelly, or stony country, where it appears to subsist exclusively on grass.

Affinities: see *E. longipennis*

Figura III - *T. pastinator* nests. (Photo: Silvia Bergamaschi).

- ***Eutermes (Tumulitermes) tumuli***, soldier.

"Large form: head mainly dark reddish orange, rostrum dark ferruginous……Posterior two-thirds of head in dorsal aspect hemispherical rostrum long, rather stout at base, about two-thirds as long as the remainder of the head……Apical half of rostrum, the other part of the head, thorax and abdomen almost hairless. Head in profile slightly concave in the middle. Antennae of 12-14 segments, usually 13……mandibles usually without points……

Small form: similar in color to large form, head proportionately narrower, rostrum longer……

Measurement: total length, 4,90 large form, 3,96 small form; head, to apex of rostrum, long, 1.79 large form, 1.50 small form; head, wide, 0.97 large form, 0.73 small form; ; pronotum, wide, 0.55 large form, 0.44 small form.

Distribution: Western Australia, South Australia, Central Australia.

Biology: nest is being up to 10 inches (25.40 cm) high and up to 12 inches (38,48 cm) in diameter at the base, and always situated under low scrubby bushes (Fig. IV). The galleries contained great quantities of small grey globules which appear to consist of chewed up grass……"

Figura IV - *T. tumuli* nest on *Eucalyptus* tree. (Photo: Silvia Bergamaschi).

APPENDIX II – PUBLISHED PAPERS

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