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MOLECULAR VARIABILITY IN ISOPTERA

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PREFACE

This work aims to investigate some aspects of termite molecular variability. In particular, it concerns the isolation and characterization of the transposable element R2 in some species of European termites, and the analysis of the phylogeny of termites from Cyprus, based on mitochondrial markers.

Chapter 1 introduces termite biology, ecology and systematics. The final paragraphs are devoted more specifically to European termites.

Chapter 2 contains an introduction to transposable elements and, particularly, to the retrotransposon R2 and to the aspects of its structure and evolution that are most relevant for this study.

Chapter 3 is a brief note on the use of mitochondrial DNA as a phylogenetic marker.

Chapter 4 presents in more detail the aims of this work.

Chapter 5 contains an account of the materials and methods used in this study. The methods used for the phylogenetic analysis of Cyprian termites are for the most part the same that have been used for some aspects of the analysis of R2, so they are presented together, with two introductive paragraphs indicating which methods apply to each of the two analyses.

Chapter 6 presents the results obtained in this study, and is followed by a discussion in Chapter 7.

CHAPTER 1: ISOPTERA

Isoptera, commonly known as termites, are hemimetabolous insects that live in perennial societies composed by reproductives, soldiers and workers. Mature colonies can number from some hundred to several million individuals.

The first termite fossils date back to the Early Cretaceous (Engel et al., 2007). It is estimated that Isoptera diverged from cryptocercid cockroaches in the Late Jurassic. This would make them the oldest group of eusocial animals (Engel et al., 2009).

The main food sources for termites are wood and other materials containing cellulose. In tropical and subtropical ecosystems, termites play a fundamental role in the recycling of dead plant biomass. In built-in areas, some species are considered pests, because they can damage wooden structures, furniture and other objects containing cellulose, such as cardboard and paper.

There are over 2600 described species of termites (Abe et al., 2000), traditionally classified in seven families (§ 1.8). Termites belonging to the family Termitidae are commonly known as "higher termites".

Termite abundance and diversity are maximal in tropical and subtropical regions. A small number of species are adapted to temperate regions. Termites of some genera, such as *Psammotermes* and *Anacanthotermes*, can live in semi-arid conditions. The greatest altitude is reached by termites of the genus *Archotermopsis*, that live at 3000 m above sea-level, in the Himalayas (Pearce, 1997). Outside their natural range of distribution, some populations of termites can be found in built-in areas, where they survive thanks to the favourable microclimatic conditions that human settlements provide.

Human activities can be responsible of the introduction of termites to localities far from their place of origin. Termites can be unintentionally transported inside the soil, for example in pots of ornamental plants, or with a great variety of wooden objects and structures (timber, furniture, ornamental objects, railway sleepers, wooden boats, etc.).

1.1 MORPHOLOGY

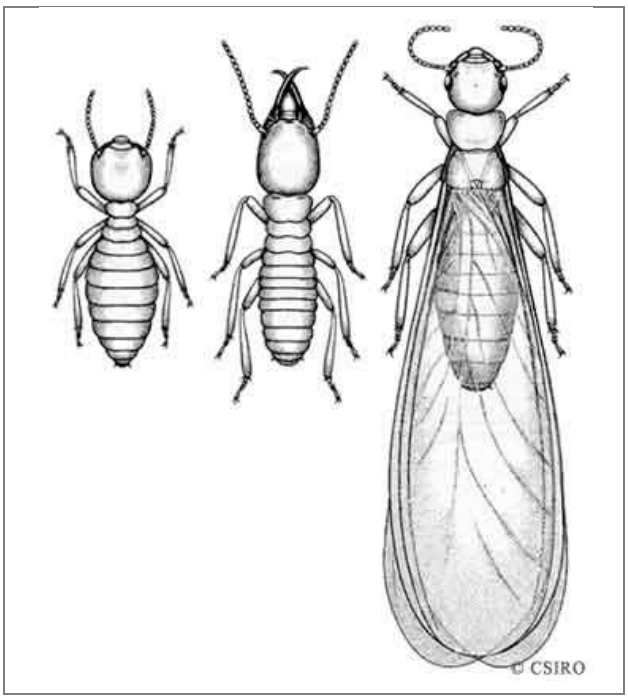

Fig. 1 - A worker, a soldier and a winged reproductive of *Coptotermes acinaciformis* (www.ento_csiro.au).

Many aspects of termite morphology and anatomy differ not only among species, but also among castes of the same species (Fig. 1).

Body length normally ranges from 2.5 to 20 mm, but physogastric queens can exceed 9 cm in length.

Workers have a thin integument, whitish or nearly transparent, so that the white fatty tissue and the dark content of the digestive tube can often be seen through it. Imagoes are usually brown or blackish. Soldiers have a well sclerotized brown head.

The head is dorsoventrally depressed and orthognathus. Mouthparts are of chewing type, with mandibles fit for tearing and chewing wood. Soldiers have modified mandibles, unfit for eating.

Compound eyes are placed laterally on the head. They are well developed in primary reproductives, reduced or absent in soldiers and workers. An interesting exception are soldiers and workers of the family Hodotermitidae (harvester termites), whose eyes are quite developed. One pair of ocelli is present in most species.

Antennae are moniliform. The number of antennal segments varies depending on species, caste and age.

The thorax is rather primitive, with large membranous zones connecting sclerites. The pronotum is big. It can be flat or saddle-shaped.

The three pairs of legs are similar to one another. They are usually quite short, since they are fit for walking inside nest galleries.

Alates have two pairs of membranous wings, similar in shape and length. Wings are

transparent or slightly blackened and often show iridescent reflexes. Longitudinal veins near the costal margin are strongly chitinized. Transversal veins form an irregular network. At the base of each wing there is a breaking line, called a basal suture, that, when the dispersal flight has taken place, favours the detachment of the main portion of the wing, leaving a short wing scale.

The abdomen, nearly cylindrical, is formed by ten recognisable segments and bears two short cerci.
Female reproductives can be distinguished by the presence of a large 7^{th} sternite, forming a plaque that covers the genital chamber. *Mastotermes darwiniensis* females have a short ovipositor, which is vestigial or absent in all the other termite species. In males, the extension of abdominal sternites decreases progressively towards the end of the abdomen, and in some species the 9^{th} sternite has styles (Grassé, 1949).

1.2 ANATOMY

The integument of termites is scarcely sclerotized. In particular, larvae, workers and soldiers (the latter limitedly to their abdomen) have a very thin integument.
Termites have many integumental glands. Salivary glands are very developed in workers, where they occupy a large part of the thorax. The saliva they produce is collected in reservoirs and mixed with food when the termite is eating. It is also used for feeding juveniles, soldiers and reproductives, and as a sort of mortar for sticking together particles during the construction and the maintenance of the nest. Soldiers have small salivary glands, with the exception of soldiers of the genus *Pseudacanthotermes*, that have big salivary glands and huge reservoirs, and use their viscous saliva as a defence against intruders.
A frontal gland is characteristic of the families Rhinotermitidae and Termitidae. It produces defensive compounds, whose composition differs with the species (Deligne et al., 1981; Prestwich, 1983). It is particularly developed in soldiers, where it occupies most of the head, and in some species can extend to a wide portion of the thorax and of the abdomen.
In the abdomen, one or more sternal glands are present in correspondence of sternites

3, 4 or 5 (their number and location vary depending on the family). They secrete trail pheromones in neuter castes and a sex-pheromone in some adults (Quennedey et al., 2008).

The nervous system is formed by the cerebron, by subesophageal ganglia and by a ventral chain, with three pairs of thoracic ganglia and six abdominal ganglia.
Compound eyes are well developed and fully functional in imagoes, reduced or absent in workers and soldiers. In primary reproductives that live inside the nest, where no light can be seen, compound eyes degenerate progressively.
A tympanic organ is situated on the tibia of each leg.
Mechanosensory organs include Johnston's organ, situated in the second antennal segment.

The respiratory system, formed as in all the pterygote insects by interconnected tracheae and tracheoles, communicates with the exterior through ten pairs of spiracles: two on the thorax and eight on the abdomen.

The structure of the alimentary canal varies among species and among castes of the same species. The foregut begins with a short pharynx, followed by the oesophagus and the crop, whose posterior region has thick walls and a strong musculature for triturating food. The midgut is bent in a U shape and is followed by the hindgut, which is quite long and expanded in its anterior portion to form a paunch where symbiont microorganisms are situated. The hindgut ends with the colon and the rectum.

Ovaries are situated dorsolaterally in the abdomen and are composed by panoistic ovarioles. The number of ovarioles varies among species and usually increases with age (Grassé, 1949). The more the ovaries grow, the more the abdomen becomes larger, thanks to the extension of intersegmental membranes (physogastry). Each ovary communicates with an oviduct, and the two oviducts come together to form a short vagina, opening between the 7^{th} and the 8^{th} sternites. Female reproductive system also includes a spermatheca and colleterial glands.
Eggs have an elongated, slightly arched shape. Their length ranges from 0.4 up to 1.8 mm (Grassé, 1949). Micropylar openings vary in number among species and even

among eggs of the same species (Costa-Leonardo, 2006). Eggs are laid separately, except in the species *Mastotermes darwiniensis*, where they are grouped together in oothecae.

Testes are situated dorsolaterally in the hind portion of the abdomen. They are each composed by groups of tubules, that open in a vas deferens. Seminal vesicles can be independent structures, or formed by a simple enlargement of the vas deferens. Vasa deferentia connect to the ejaculatory duct, that opens to the outside between the 9^{th} and the 10^{th} abdominal sternites.

Spermatozoa can be conical (Mastotermitidae and Kalotermitidae), pin-like (Hodotermitidae) or spheroidal (Rhinotermitidae and Termitidae). They are aflagellate, except those of *Mastotermes darwiniensis*, that have about 100 feebly motile flagella (Baccetti & Dallai, 1978; Jamieson, 1987).

1.3 CASTES

Termite societies are characterized by the presence of castes, that is groups of individuals that differ from a morphological, anatomical, physiological and behavioural point of view. Three main castes can be found in a termite colony: reproductives, workers and soldiers.

Higher termites have castes that are well separated from one another, while lower termites show a greater plasticity, so that individuals can change caste more or less easily. In lower termites, worker duties are carried out by immature individuals. Inter-castes, that is individuals with intermediate characteristics between those of two castes, are quite common in some species. For example, fertile soldiers can be found in colonies of the genus *Zootermopsis* (Grassé, 1949).

Contrary to what happens in hymenopterans, where sterile castes are formed exclusively by females, in termites workers and soldiers can be male or female, even though the proportion of individuals of the two sexes can vary with species and caste (Roisin, 2001).

Caste determination mechanisms have not been fully clarified yet. Traditionally, by most authors, caste determination was thought to be controlled by environmental factors, such as pheromonal and nutritional signals from other colony members

(Grassé, 1949). Recent studies reveal that a genetic basis is likely to be involved too (Hayashi et al, 2007; Lo et al., 2009).

Reproductives

Reproductives are the only individuals that have functional gonads. There are many kinds of reproductives, that can be classified in several ways (Myles, 1999), but two main typologies can be distinguished: imagoes, also known as alates or primary reproductives, and reproductives that are wingless or with underdeveloped wings, also known as neotenics (Fig. 2). Not all kinds of reproductives can be found in all the species.

Fig. 2 - Primary reproductives (left), a neotenic deriving from a worker (middle) and a neotenic deriving from a nymph (right) of *Reticulitermes urbis*.

Alates can be distinguished by the presence of fully developed wings and compound eyes, and by the colour of their body, which is normally blackish or brown. These characters enable them to live outside the nest in occasion of the short dispersal flight.

Reproductives that are wingless or with wing pads, deriving respectively from workers (ergatoids) and from nymphs (nymphoids), show in a lesser degree characteristics that are typical of alates, such as some pigmentation and some degree of development of compound eyes. They can differentiate when one or both primary reproductives die, but also when primary reproductives are still alive. In the first case, they are called replacement reproductives, because they substitute the original reproductives, while in the second case they are called supplementary reproductives.

Workers

The majority of a termite colony is composed by workers, that usually make up more than 90% of the individuals. Workers have a very thin integument and are whitish, wingless and eyeless. An exception are workers of the family Hodotermitidae, that have a light brown pigmentation and small compound eyes (Fig. 3).
Workers carry out most of the activities that are necessary for the survival of the colony: they build, enlarge and repair the nest, procure food and water, feed juveniles, reproductives and soldiers, take care of eggs and juveniles, and help soldiers in defending the colony against enemies.

Fig. 3 - On the left, workers of *Kalotermes flavicollis*. On the right, a worker of *Anacanthotermes* sp.

The duties a worker performs can vary depending on its age and sex. Foraging is usually carried out by old workers. In some species the queen is attended by female workers, while foragers are male workers (Pearce, 1997).

Soldiers

Soldiers are specialized for the defence of the colony against predators. They are more sclerotized and more pigmented than workers, particularly in the head and the pronotum (Fig. 4). Soldier heads present a great variety of shapes, corresponding to different defence strategies (§ 1.7).

Fig. 4 - A soldier of *Kalotermes flavicollis*.

Soldiers have modified mandibles, unfit for chewing food, so that they have to be fed by workers.

The transformation of a termite in a soldier requires two moults; after the first one, the individual is called a white soldier, because its appearance is already similar to a soldier's, but it is still not pigmented.

The proportion of soldiers in a termite colony depends on many factors, including species, colony size and season. In most cases, the proportion of soldiers in field colonies varies from 1% to 10%, even though in some occasions higher proportions have been recorded (Haverty, 1977; Deligne et al., 1981).

In some termite species, mostly belonging to the genus *Anoplotermes*, the soldier caste is absent.

1.4 FORMATION OF NEW COLONIES

New termite colonies can be established either by winged reproductives after swarming (foundation) or by a group of individuals of different castes separating from a pre-existing colony (budding and sociotomy).

Swarming takes place one or more times a year. In many species its duration is limited to one or few days, but there are also species where flights occur over several months. Especially when the duration of swarming is short, it is essential that alates of different colonies of the same species come out from their nests at the same time. This synchronization is obtained by timing flights with particular weather conditions. Many species swarm after the rain, so that the new couples can easily burrow their new nest in softened soil.

A few days before swarming, alates assemble in peripheral regions of the nest, where they wait for a favourable moment to begin the flight. When that moment comes, they leave from holes or turrets prepared by workers and guarded by soldiers.

Alates of most species are poor flyers. They can reach distances of a few hundred meters if they start from an elevated position and are supported by propitious air currents (Pearce, 1997).

After landing, alates shed their wings, breaking them off at the basal suture. Timing and mechanisms of dealation vary with species (Myles, 1988). A timely wing shedding reduces the predation risk during the period spent running on the ground (Matsuura & Nishida, 2002).

The formation of pairs occurs after landing, except in *Pseudacanthotermes* spp., where the male grasps the female while she is still flying. Once formed, the new pair runs on the ground or on the wood, with the male following the female in what is called a tandem (Fig. 5). When they find a suitable site for digging in, they start burrowing a nuptial chamber. The royal pair takes care of the first brood of eggs and juveniles. When juveniles have grown enough to take charge of workers duties, the royal pair starts doing nothing except reproduce.

Fig. 5 - A couple of *Reticulitermes grassei* walking in tandem.

Contrary to what happens in social hymenopterans, where males die short after mating, termite male reproductives are long lived. The members of a termite royal pair can live together many years, mating from time to time.

In some species, females can reproduce parthenogenetically, so a new colony can be founded by a couple of females or by a single female. These colonies have a lower fitness than those that are founded by a female-male couple, so it can be assumed that facultative parthenogenesis in termites is an opportunity for females that fail to find a male (Matsuura & Nishida, 2001).

Budding takes place when a group of termites becomes independent and separates from its mother colony. Budding often happens at the periphery of big colonies, where connections with the core of the colony are progressively reduced and neotenics begin to reproduce, but can also be caused by external events, that determine a sudden separation of a group of termites from the main body of the colony.

In some species, alates are unable to found new colonies, and budding is the only way in which a new colony can be formed (Campadelli, 1987).

Sociotomy consists in the departure from the nest of a big group of termites of different castes, including the royal couple. This group looks for a new site where to settle and build a new nest, while in the old nest reproduction is taken over by neotenics.
Formation of new colonies by sociotomy is not very common in termites. It was observed, for example, in the genera *Anoplotermes* and *Trinervitermes* (Grassé, 1949).

1.5 TERMITE NESTS

Termites of most species spend all their lives inside their nest and the galleries departing from it, where they find protection from predators and a suitable microclimate. Any break in the walls of the nest is promptly repaired by workers, and guarded by soldiers.

Termite nests can be built inside the wood, in the ground, or above the ground (mounds or arboreal nests).
Wood, soil, clay, and sand, cemented with saliva or liquid faeces, are the most common building materials. In spite of their simple composition, the walls of the nest and in particular, when present, of the royal chamber, can be very hard and strong.
Some species, such as *Nasutitermes* spp. and *Coptotermes* spp., use wood and saliva for the production of carton nests.

Termite nests come in a great variety of shapes and sizes. The appearance and kind of nest is fairly constant for many species, but it can vary with environmental conditions (Pearce, 1997). For example, the nests of *Cubitermes* spp. are mushroom-shaped when built in rainy areas, where a convex roof helps protecting the nest from water, while in dry areas the cap is absent.
The simplest kind of nest is a system of cavities and galleries burrowed in the soil

and/or in the wood. Such nests are common for dry-wood, damp-wood and subterranean termites.
In several species, mostly belonging to the family Termitidae, the nest is initially subterranean, then it becomes progressively bigger, emerging from the ground and forming a mound. Some termite mounds reach considerable sizes, up to about eight meters, so that, in some areas, they are one of the most characterizing elements of natural landscapes. The internal structure of termite mounds is often quite complex, including shafts and galleries for ventilation and temperature regulation, and systems of chambers reserved to specific uses, such as food storage or fungus cultivation.

In the nest, temperature and humidity are kept as much as possible constant. Their regulation is less important for termites that live underground, where environmental conditions are more stable, while termites that live in epigeous mounds often have to adopt specific strategies in order to control the microclimatic conditions of the nest.
When the external temperature is lower than optimal levels, termite mounds are built with thick walls, that insulate the nest and reduce the loss of internal heat, which is produced by the metabolism of termites and, in the case of fungus-growing species, of fungi. For big colonies, this solution, implying a scarce ventilation, can lead to high concentrations of CO_2 inside the nest, so that a trade-off between gas exchange and thermoregulation may arise (Korb, 2003).
When the external temperature is high, termites can limit the exposure of the nest to sunlight and favour the dissipation of internal heat by increasing the ventilation and by building nests with a high surface/volume ratio. For instance, in order to expose the smallest possible area to the sun, Australian *Amitermes* spp. build thin mounds, north-south oriented, a feature which has owned them the denomination of "magnetic termites".

The maintenance of a high humidity rate is of particular importance because termites have a poorly sclerotized integument, that does not protect them efficiently from the loss of water. In dry environments, termites build nests in relatively moist places, for instance under the stones, where condensed water can be found. The biggest colonies can even dig vertical shafts that reach the water table.
The location of termite mounds has been traditionally used by man as a sign of the presence of ground water (Rao, 1971) and is at present considered as a valid

hydrologic indicator (Badrinarayanan, 2009).

1.6 FEEDING

The main food source for termites is cellulose, that can be obtained from different kinds of vegetable matter: wood, grass, leaf litter, humus, or soil.
It is estimated that, in tropical ecosystems, termites ingest from 50% to 100% of the dead plant biomass (Abe et al., 2000), thus contributing significantly to its recycling.

In higher termites, the degradation of cellulose is performed by enzymes that are produced by the salivary glands and midgut, while in lower termites some steps of the degradation are carried out by gut symbionts: flagellates and bacteria (Radek, 1999).
Wood-eating termites often prefer wood that has been attacked by fungi or bacteria, probably because its nutritional content is richer and more varied than that of sound wood.
Fungus-growing termites (Termitidae, subfamily Macrotermitinae) cultivate fungi of the genus *Termitomyces* inside their nests, in special chambers where they accumulate plant-derived material. They feed on the mycelia of the fungi that grow on this substrate and on the substrate itself, once it has been degraded by fungi.
Termites can occasionally eat exuviae and dead members of the colony. Sick or mutilated individuals, as well as reproductives that are not essential for the colony, are normally killed and eaten (Pearce, 1997).

Termites often exchange partially digested food with other members of the colony, a behaviour known as trophallaxis. This food can be emitted either through the mouth or through the anus. In the first case, it can be composed mostly by saliva. In the second case it contains gut symbionts, so that its intake is particularly important for juveniles, that are born without symbionts, and for individuals that, having moulted, have lost them. Trophallaxis also serves the purpose of spreading chemical messages through the colony.

1.7 DEFENCE MECHANISMS

Termites can be preyed upon by many animal species. Most of these predators feed only occasionally on termites, taking advantage of individuals that they find outside the nest. Swarming alates are particularly vulnerable to predation, and constitute an episodic food source for many animals that do not usually eat termites.
Ants are among the most dangerous enemies of termites. Ants of many species eat termites occasionally, but there are also several ant species that are specialized in preying termites. Some ants live near or inside termite nests, in separate cells, and they steel part of the brood. Some other species conduct raids inside termite nests. Their action can be highly invasive; for instance, ants of the genus *Dorylus* can destroy entire colonies.
Several mammals feed nearly exclusively on termites and ants. This is the case of echidnas, numbats, anteaters, pangolins, and of the aardvark. Despite belonging to different families, most of these species have evolved similar features: strong claws for breaking the walls of termite nests and long sticky tongues for catching termites. Even a few carnivores, for example the earth wolf *Proteles cristatus*, feed mainly on termites (Deligne et al., 1981).

The nest and the galleries departing from it represent an effective protection against unspecialized predators. In many higher termites, the royal pair is further protected by a royal cell with very robust walls.
When the nest is under attack, workers and soldiers work together for its defence. Workers can block the openings of the nest with building materials, thus impeding the access to small predators. In many species of soldierless termites, workers can stop or slow down small predators by making their own abdomen explode, spreading about its contents, that irritate and entangle predators.

Defence strategies adopted by soldiers can be classified in four main groups (Deligne et al., 1981):

- In the majority of termite species, soldiers have strong mandibles, that can have different shapes and functions. Serrated mandibles, bigger but morphologically not much different from those of workers, are used to bite. Elongated, sabre-shaped

mandibles that can cross far over one another are used to bite and cut. Long, thin mandibles, either symmetrical or asymmetrical, with a flat plate near their tip are compressed against each other until they snap, delivering violent blows.

- The soldiers of certain Kalotermitidae have blunt and heavily sclerotized heads that they use to occlude the galleries and keep out the intruders (phragmotic soldiers). The heads of non-phragmotic soldiers can, at least to some extent, serve the same function, in fact the diameter of the galleries of termite nests are usually calibrated on the width of the soldier heads.
- In a few species, soldiers seize the enemy with their mandibles and then pour over its body their salivary secretions.
- The soldiers of many Rhinotermitidae and Termitidae have a frontal gland that produces defensive compounds and opens to the exterior through a frontal pore. The direction of flow of the secretions can be controlled by a frontal gutter or a frontal tube, that can be so developed as to form a nasus (Termitidae, subfamily Nasutitermitinae).

The presence of particular defensive mechanisms in soldiers is correlated with termite phylogeny only to some extent, in fact there are many instances of convergent evolution (Scholtz et al., 2008).

1.8 PHYLOGENY AND SYSTEMATICS

There are over 2600 described species of termites (Abe et al., 2000), traditionally classified in seven families: Mastotermitidae, Termopsidae, Hodotermitidae, Kalotermitidae, Serritermitidae, Rhinotermitidae and Termitidae.

The family Mastotermitidae includes a single living species, the Australian *Mastotermes darwiniensis*. It is the most basal termite family, showing many ancestral features.
The frontal gland is absent. Antennae are composed by many articles (up to 32). Primary reproductives have two ocelli. The pronotum is wide. Forewings are longer and narrower than hindwings, the latter having a distinct anal lobe. Tibiae have

spines. Tarsi are formed by five articles. Pulvilli are present. Adult females have an ovipositor and eggs are grouped in oothecae.
The colonies of *Mastotermes darwiniensis* are usually small, but when food is abundant can increase rapidly over a million individuals. The species is economically very important (Gillott, 1995)

The family Kalotermitidae includes about 400 species, living in tropical and in temperate regions.
The frontal gland is absent. Primary reproductives have two ocelli. The pronotum is flat, wider than the head. Tarsi are formed by four articles and in some species have pulvilli.
They live in simple nests constituted by a net of galleries and cavities burrowed in the wood. They are commonly known as dry-wood termites, owing to their ability to tolerate relatively low humidity rates. The colonies of Kalotermitidae can contain several thousand individuals. Some species are of major economic importance.

The family Termopsidae includes about 20 species, distributed mainly in warm temperate regions.
They are among the largest termites, measuring up to 25 mm in length. The frontal gland is absent. Ocelli are absent. The pronotum is flat, narrower than the head. Tarsi are formed by four or five articles.
Termopsidae are commonly known as damp-wood termites, because they build their nests in wet and rotting wood. They can be found occasionally in damp structural timbers (Gillott, 1995). Colony size is moderate, up to approximately 10,000 individuals.

The family Hodotermitidae includes 15 species, that can be found in desert and steppe regions of Africa and Asia.
The frontal gland is absent. Antennae are composed by many articles (up to 34). Ocelli are absent. The pronotum is saddle-shaped, and larger than the head. Workers and soldiers show some degree of pigmentation, and have compound eyes and long legs.
Hodotermitidae are commonly known as harvester termites, since they forage above ground for grass and leaves. The nest is built underground.

The family Rhinotermitidae includes more than 300 species, that occur in most continents.
The frontal gland is often present. Antennae are formed by 14-22 articles. Primary reproductives often have ocelli. The pronotum is flat. Tarsi are formed by four articles.
The Rhinotermitidae are commonly known as subterranean termites, since their nests are formed by nets of galleries and cavities burrowed in the ground or in pieces of wood situated inside or near the ground. Colony size can reach some millions of individuals. Some species, such as the North-American *Reticulitermes flavipes* and *R. hesperus*, are of major economic importance.

The Serritermitidae are represented by a single species, the South American *Serritermes serrifer*, whose appearance and ecology is similar to those of the Rhinotermitidae.
It is one of the smaller termite species, with alates attaining about 4 mm of length. Soldiers have serrated teeth along the inner margin of their mandibles.

The Termitidae constitute the largest family of termites, counting more than 1800 species distributed mainly in the tropical regions of Africa, Asia, South America and Australia.
The frontal gland is present. The pronotum is flat in primary reproductives and saddle-shaped in soldiers.
The Termitidae are commonly known as higher termites. A true worker caste is present. Colonies can attain huge sizes, thanks to the high prolificacy of their queens.

Studies based on molecular and morphological characters seem to indicate that the Termopsidae are paraphyletic and include the Hodotermitidae as a monophyletic nested group. The Rhinotermitidae seem to be paraphyletic, with the Serritermitidae included among them (Inward et al., 2007b).
A recent study (Engel et al., 2009), based on the analysis of a set of morphological characters in living and fossil species, suggests a revision of the higher classification of termites, with the definition of new taxa. With regard to living termites, this would imply the attribution of Termopsidae species to the new families Archotermopsidae

and Stolotermitidae, and the preservation of the distinction between Serritermitidae and Rhinotermitidae.
The very advisability of maintaining the denomination Isoptera has been debated, since phylogenetic analyses based on morphological characters (Klass & Meier, 2006) and molecular markers (Inward et al., 2007a) indicate that termites are a nested group within Blattodea. Some authors suggest to include termites in a single family (named Termitidae) in the order Blattodea (Inward et al., 2007a), while others suggest to rank termites as a suborder, infraorder, superfamily or epifamily, avoiding drastic nomenclature revolutions and maintaining existing names (Lo et al., 2007).

1.9 EUROPEAN TERMITES

In Europe, termites are represented by the genera *Kalotermes* (Kalotermitidae) and *Reticulitermes* (Rhinotermitidae). The genus *Kalotermes* is represented by the single species *K. flavicollis* (Fabricius), while the genus *Reticulitermes* includes several species and subspecies.
In the last decade, taxonomy and phylogeny of European termites of the genus *Reticulitermes* have been the object of many studies, based on morphological and molecular data (Clément et al., 2001; Jenkins et al., 2001; Marini & Mantovani, 2002; Uva et al., 2004; Austin et al., 2002; 2004 and 2006; Luchetti et al., 2004, 2005, and 2007; Nobre et al., 2006), that have permitted to clarify the distribution of known species and to distinguish new taxa of specific and subspecific level.

The classified European species are:
- *R. lucifugus* (Rossi), distributed in Italy, Corse and Provence. In peninsular Italy, it is represented by the subspecies *R. lucifugus lucifugus*; in Sardinia, Tuscany and Corse by *R. lucifugus corsicus*; and in Sicily by a third form, whose definition is still in progress.
- *R. grassei* Clément, distributed in the south-west of France, in Portugal and in the west of Spain;
- *R. banyulensis* Clément, distributed in the east of Spain and in the south of France;
- *R. urbis* Bagnères et. al, distributed in the east of Greece, on the coasts of Croatia

and in some localities in Italy and France;

- *R. balkanensis* Plateau & Clément, distributed in the west of Peloponnesus and in Attica;
- *R. flavipes* (Kollar), introduced in Europe from the North America, now present in some localities in France, Germany, and Italy (Ghesini et al, submitted).

For North Mediterranean termites, including European, Turkish and Israeli taxa, the current state of phylogenetic reconstruction is summarized in Fig. 6 (Velonà et al., submitted). Two main clades can be identified: the first includes species from western Europe (*R. lucifugus*, *R. grassei* and R. *banyulensis*), while the second includes taxa from eastern Europe, Turkey and Israel (*R. urbis*, *R. balkanensis*, a subclade including *R. clypeatus* and populations from southern Turkey, and a subclade including populations from the Chalcidice Peninsula, Thrace, Northern Turkey and Crete).

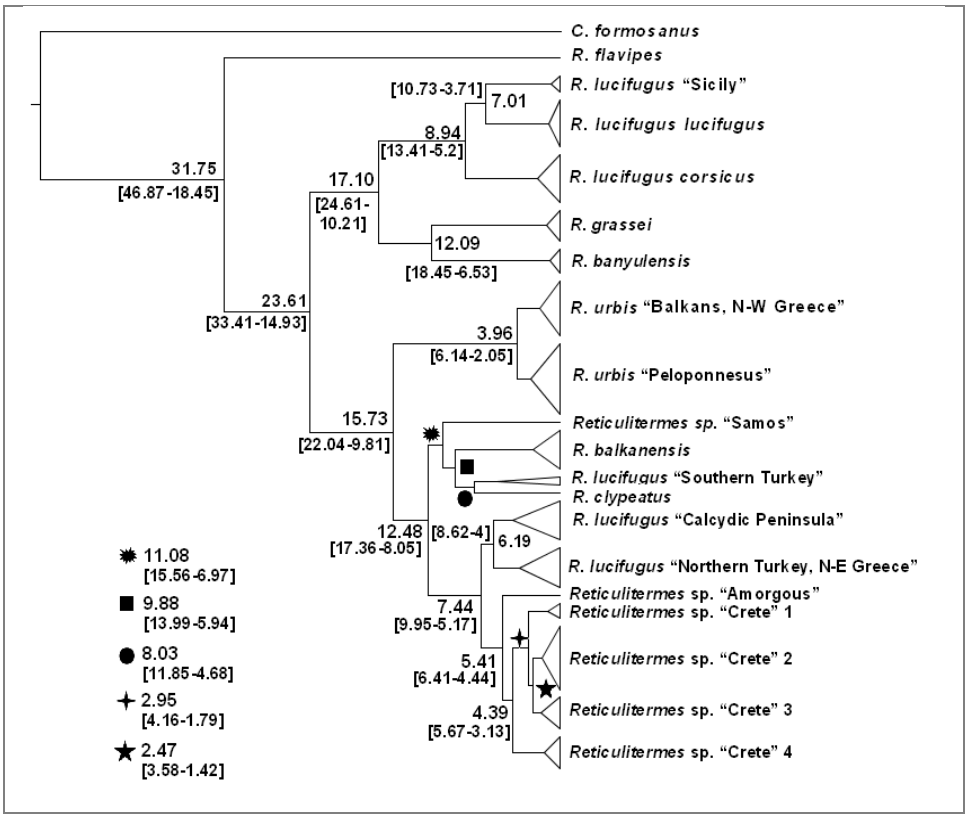

Fig. 6 - Chronogram tree of the North-Mediterranean *Reticulitermes* taxa. (from Velonà et al., submitted)

1.10 TERMITES FROM CYPRUS

Despite being the third largest Mediterranean island after Sicily and Sardinia, Cyprus has received very scarce attention by termitologists. To date, a single report of a *Reticulitermes* sample collected in Cyprus, analyzed from a morphological point of view together with Turkish samples, has appeared (Weidner, 1972).

Cyprus: Notes on geography and geology

Cyprus is situated in the eastern part of the Mediterranean, about 75 km from Turkey to the north, and about 100 km from Syria and Lebanon to the east.
In the central and western part of the island is the Troodos Massif, whose maximum elevation is 1953 m. Along the northeast margin of the island is the Kyrenia Range. The two ranges are separated by the plain of Mesaoria (Fig. 7).

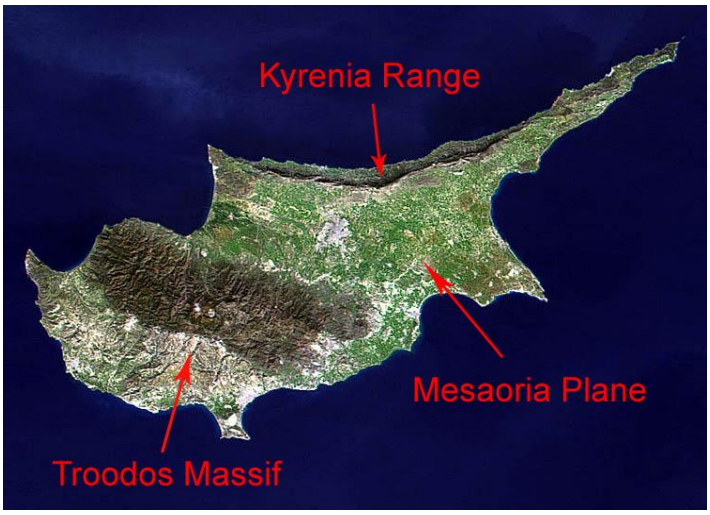

Fig. 7 - The island of Cyprus. (NASA, courtesy of nasaimages.org; modified)

The island of Cyprus is what is called an oceanic island, i.e. it does not sit on the continental shelf. The genesis of Cyprus took place through a series of tectonic episodes, caused by the subduction of the African plate beneath the Eurasian plate. The first portion of what was later to become the island of Cyprus arose from the sea about 20 million years ago, and was constituted by the tip of what is now the Troodos Massif. The uplift of the island to almost its present position, accompanied by the placement of the Pentadaktylos Range in the northern part of the Troodos zone took place 10-15 million years ago. The final tectonic episode occurred 1-2 million years ago: Troodos and Pentadaktylos Ranges were uplifted in elevations higher than today's. The abrupt uplift combined with heavy rainfall, resulted in extensive erosion of the ranges, with the transportation

of large quantities of erosion material, that were deposited in large valleys and in the Mesaoria region (Cyprus Geological Survey, 2002).

CHAPTER 2: TRANSPOSABLE ELEMENTS

Transposable elements (TEs) are DNA sequences that have the ability to move from one site to another in the genome. They form a major fraction of many eukaryote genomes, even more than 70% in some plants and amphibians (Biémont & Vieira, 2006). Nearly half of human genome is derived from TEs (Lander et al., 2001; Cordaux & Batzer, 2009) (Fig. 8).

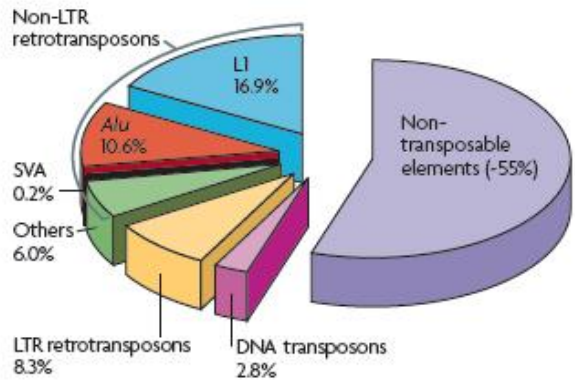

Fig. 8 - The transposable element content of the humam genome (from Cordaux & Batzer, 2009).

The first TEs were discovered in the 1940s by geneticist Barbara McClintock, who observed in maize what she called "controlling elements", i.e. elements that could move from a location to another, regulating the expression of genes and resulting in different chromosome morphologies and different phenotypes (McClintock, 1950).

The importance of TEs has been underestimated for a long time, until the discovery of a great variety of elements and the realization that they constitute a big fraction of many genomes have emphasized the necessity to understand their properties, their interactions with host genomes, and the roles they have played and are still playing in evolutionary processes.

TEs have been found in all living kingdoms, and in most of the species where they have been looked for, with very few exceptions. They have not been found in some unicellular eukaryotes, such as the red alga *Cyanidioschyzon merolae*, some apicomplexans, including *Plasmodium falciparum*, and the unikont *Encephalitozoon cuniculi*. Most of these organisms are distantly related to the majority of eukaryotic genome sequences available in the databases, so the lack of reported TEs in some cases might reflect the inability to identify them based on sequence homology to known TEs types. On the other hand, the very small size of their genomes could indicate that natural selection is effectively removing TEs, perhaps due to a selective

pressure to maintain cell size, that in unicellular eukaryotes is strongly correlated with genome size (Pritham, 2009).
TE content varies from species to species, with regards to the kinds of elements that are present, their copy number, the proportion of genome that they occupy, and their activity level. TE content also differs among chromosomes of the same organism and among different regions of the same chromosome (Hua-Van et al., 2005; Wessler, 2006).

2.1 CLASSIFICATION OF TRANSPOSABLE ELEMENTS

Since the proposal of the first classification system of transposable elements (Finnegan, 1989), a vast amount of new data have become available, including the discovery of new kinds of TEs. Moreover, the need to conduct comparative studies across living kingdoms has emerged. For these reasons, the original classification has been integrated and modified, and a unified hierarchical classification system has been proposed (Wicker et al., 2007). In this system, TEs are classified based on their mechanisms of transposition, structural organization, and sequence similarities. Where not differently stated, the following is based on Wicker et al. (2007).

Two main classes are identified:

- Class I elements, also known as retrotransposons, transpose via an RNA intermediate.
  Their transposition takes place thanks to a "copy-and-paste" mechanism: The RNA intermediate is transcribed from a genomic copy, then reverse transcribed into DNA by a reverse transcriptase. The original copy remains in its site, so each transposition event generates a new copy.
- Class II elements, also known as DNA transposons or transposons *in sensu strictu*, transpose via a DNA intermediate.
  The elements included in Subclass I use a "cut-and-paste" mechanism: They are excised from a genomic site by a transposase and then integrated into a new site. The number of copies does not normally increase with transposition, unless particular conditions are satisfied, for example if transposition takes place during

chromosome replication, from a position that has already been replicated to one that has not been replicated yet.

The elements included in Subclass II use a "copy and paste" mechanism, involving the displacement of a single DNA strand.

Both Class I and Class II include autonomous and non-autonomous elements. The first encode all the domains needed for their transposition, while the second lack some or all of those domains and can transpose thanks to the products encoded by other elements (Fig. 9).

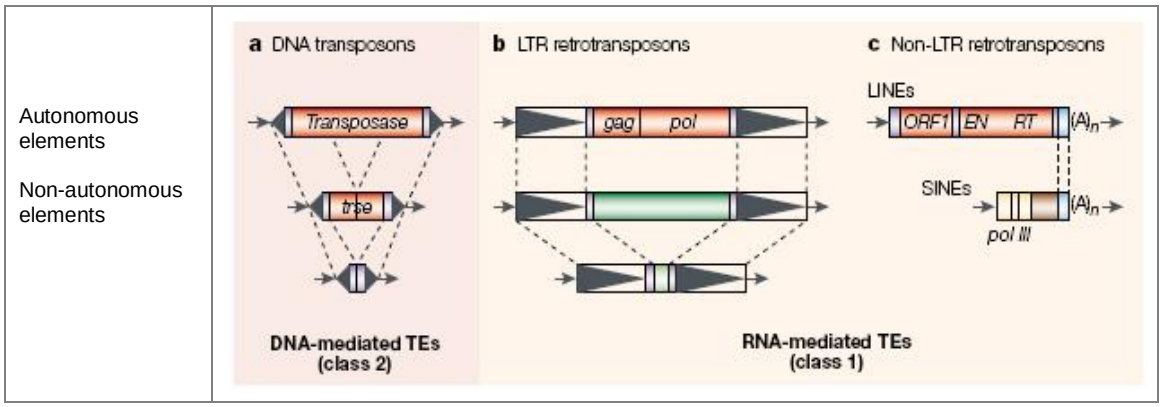

Fig. 9 - The main kinds of transposable elements (from Feschotte et al., 2002)

Class I is divided in five orders: long terminal repeat (LTR) retrotransposons, *DIRS*-like elements, *Penelope*-like elements, long interspersed elements (LINEs), and short interspersed elements (SINEs).

- LTR retrotransposons owe their name to the presence of flanking sequences ranging from a few hundred base pairs to more than 5 kb. They typically contain ORFs for the capsid protein GAG, an aspartic proteinase, a reverse transcriptase, an RNase H, and an integrase. Their integration generates target site duplications of 4-6 bp. LTR retrotransposons are closely related to retroviruses, whose sequences contain in addition portions encoding an envelope protein and a set of additional proteins and regulatory sequences.
- *DIRS*-like elements are similar to LTR retrotransposons, but they encode a tyrosine recombinase gene instead of an integrase and their terminal sequences are not long terminal repeats. Their integration does not generate target site duplications.
- *Penelope*-like elements encode a reverse transcriptase and a an endonuclease. They

have LTR-like sequences, that can be in a direct or an inverse orientation.

- LINEs lack long terminal repeats. Their structure can be quite variable. For this reason their classification is somewhat complex, entailing five superfamilies, comprising each many families. They contain at least a reverse transcriptase and a nuclease. Their insertion normally generates target site duplications. At their 3' end, they can display either a poly(A) tail, a tandem repeat or an A-rich region.
- SINEs are short non-autonomous elements that transpose thanks to LINEs products. They do not derive from autonomous Class I elements, but from the accidental retrotransposition of Polymerase III transcripts. Their insertion generates target site duplications of 5-15 bp. SINEs are widely dispersed in the host genome and their insertions are irreversible. These features make them a useful tool for phylogeny reconstruction (Shedlock & Okada, 2000).

Class II is formed by two subclasses. Subclass I includes the elements that transpose through a "cut-and-paste" mechanism and is constituted mainly by terminal inverted repeat (TIR) elements. Subclass II includes "copy-and-paste" elements and is formed by two orders: *Helitron*-like elements and *Maverick*-like elements.

Class II also includes insertion sequences (IS), i.e. simple TEs that are found in prokaryotes.

- TIRs are characterized by the presence of terminal inverted repeats of variable length. They encode for a transposase, that recognizes the terminal repeats and cuts both strands at each end. Their insertion generates target site duplications.
- *Helitron*-like elements appear to be related with viruses that replicate via a rolling-circle mechanism, with only one strand cut. They do not generate target site duplications.
- *Maverick*-like elements, also known as polintons, are long elements that encode an integrase and a set of proteins that are homologous with some virus proteins (Pritham et al., 2007).

2.2 TRANSPOSABLE ELEMENTS AND HOST GENOMES

Transposable elements are a source of genomic variation, both directly, through their insertion or excision, and indirectly, through chromosomal rearrangements favoured by their presence.

Mutations induced by TEs can range from point mutations (substitution, deletion or insertion of a single nucleotide) to modifications in size and arrangement of whole genomes (Kidwell & Lisch, 2001; Wessler, 2006; Oliver & Greene, 2009; Venner et al., 2009).

If a TE inserts in a gene or in its regulatory sequence, it is likely to make the gene non-functional. Similar consequences can result from the imprecise excision of a TE. Moreover, imprecise transposition events can mobilize flanking sequences.

The presence of multiple copies of a TE in the host genome favours the occurrence of non-homologous recombination, that leads to chromosomal rearrangements: duplications, deficiencies, translations, and inversions. The repetitive nature of TEs makes them a target for molecular drive (Dover, 1982, 2002).

Like every other mutation, those caused by TEs are in most cases harmful for the host, but they produce novelties, that in a few cases can result beneficial and contribute to the evolution of the genome of the host species.

TE activity has been shown to increase under conditions of stress, thus providing potentially useful genetic variability. Episodic bursts of TE activity, such as those occurring under stressful conditions or when a new or modified element appears in a genome, may be correlated with periods of fast evolution, followed by periods of stasis when cellular control mechanisms become effective (Oliver & Greene, 2009; Zeh et al., 2009).

Interactions between TEs and hosts can be imagined as ranging from parasitism at one extreme to mutualism at the other extreme. They produce complex patterns of coevolution, also dependent on the length of the association.

Coevolution can produce three main kinds of consequences (Kidwell & Lisch, 1997; Kidwell & Lisch, 2001):

- TEs can evolve mechanisms that minimize their negative effects on the host, and thus reduce negative selective pressure against their presence. Some TEs regulate

their own rate of transposition, or limit their transposition to the germline. In some cases TEs insert specifically in regions where their presence is less likely to produce damages, for example in non-coding regions or into preexisting TEs.

- The host can evolve defence mechanisms, for example cytosine methylation, that represses transcription, and RNA interference (RNAi), that results in a post-transcriptional silencing (Slotkin & Martienssen, 2007).
- TEs can be "domesticated", i.e. the host can recruit some of their functions. In fact, TEs encode many proteins, such as transposases, integrases, reverse transcriptases, structural and envelope proteins, that can become useful for the host. Many TE-derived genes have been discovered in eukaryotes, some of them fulfilling essential functions (Miller et al., 1997; Volff, 2006; Feschotte & Pritham, 2007).

Reproductive modalities of the host are strictly correlated with TE dynamics.
In an asexual host, only those TEs that are not harmful will go to fixation, while in a host that reproduces sexually, given at least a transposition event per generation, fixation will occur if fitness is not reduced by more than one half. Therefore, outcrossing sexual reproduction favours the evolution of aggressive transposons, which in turn favours the evolution of defensive mechanisms by the host (Hickey, 1982; Bestor, 1999; Arkhipova, 2005).
It was suggested that TEs may have triggered the origin of sexual reproduction for their own dispersal (Hickey, 1982). Besides, sexual reproduction, through recombination and segregation, can be a way to eliminate deleterious mutations, such as those caused by TEs. It is possible that, once sexual reproduction was established, its maintenance was favoured by the presence of TEs (Arkhipova, 2005).

Genomes are dynamic entities, where complex interactions among different components, including TEs, take place. To some extent, these processes can find an analogous in those that occur in ecological communities, so that in some cases it can be useful to view genomes as ecosystems and TE families as species taking part in them (Kidwell & Lisch, 1997; Brookfield, 2005; Venner et al., 2009).
The genomic environment of a TE is constituted not only by host genes, but also by the other TEs that share the same host. TEs can compete for common limited resources, such as components needed for transposition and space in the genome. Parasitic relationships exist between autonomous and non-autonomous elements, with

the seconds taking advantage of the proteins that the firsts encode and could use for their own transposition. In some cases, cooperative relationships between different TEs may exist (Leonardo & Nuzhdin, 2002).

2.3 LONG INTERSPERSED ELEMENTS

Long interspersed elements (LINEs) are highly abundant in eukaryotes. They can be referred to also as non-LTR elements, because one of their distinctive features is the absence of long terminal repeats. Instead, they end frequently with a poly(A) tail.

There are several distinct lineages of non-LTR retrotransposons, that differ for their structure and coding capacity (Malik et al., 1999). Two main kinds of structures can be identified.
The first one is characterized by the presence of a single ORF, with a reverse transcriptase (RT) domain situated about its central region. C-terminal to the RT is a conserved domain that appears to be an endonuclease (Eickbush & Jamburuthugoda, 2008).
The second one presents two ORFs, that can be either in different reading frames, often overlapping for a short distance, or in the same reading frame, separated by termination codons (Eickbush, 1992). The first ORF may have functional similarity to the *gag* gene of retroviruses, while the second encodes the RT domain and, at its N-terminal end, an endonuclease domain of the apurinic-apyrimidinic type. Some of these elements have a RNase H domain downstream of the RT domain (Malik et al., 1999; Eickbush & Jamburuthugoda, 2008).
In elements of both kinds, the coding region can be flanked by untranslated regions (UTR).

Unlike LTR elements, that show little or no site-specificity, several LINEs insert into specific sequences (Eickbush, 1992).
Their mechanism of transposition is based on the cleavage of the target DNA by the endonuclease they encode, followed by the reverse transcription of the cDNA copy directly in the target site. Details of this mechanism have been determined only in the

case of the R2 element (§ 2.4, Transposition mechanism).

Non-LTR elements are monophyletic with respect to the other retroelements (Eickbush & Jamburuthugoda, 2008). Phylogenetic analyses of these elements are conducted based on the RT domain, as it is the only portion that they all share (Fig 10).

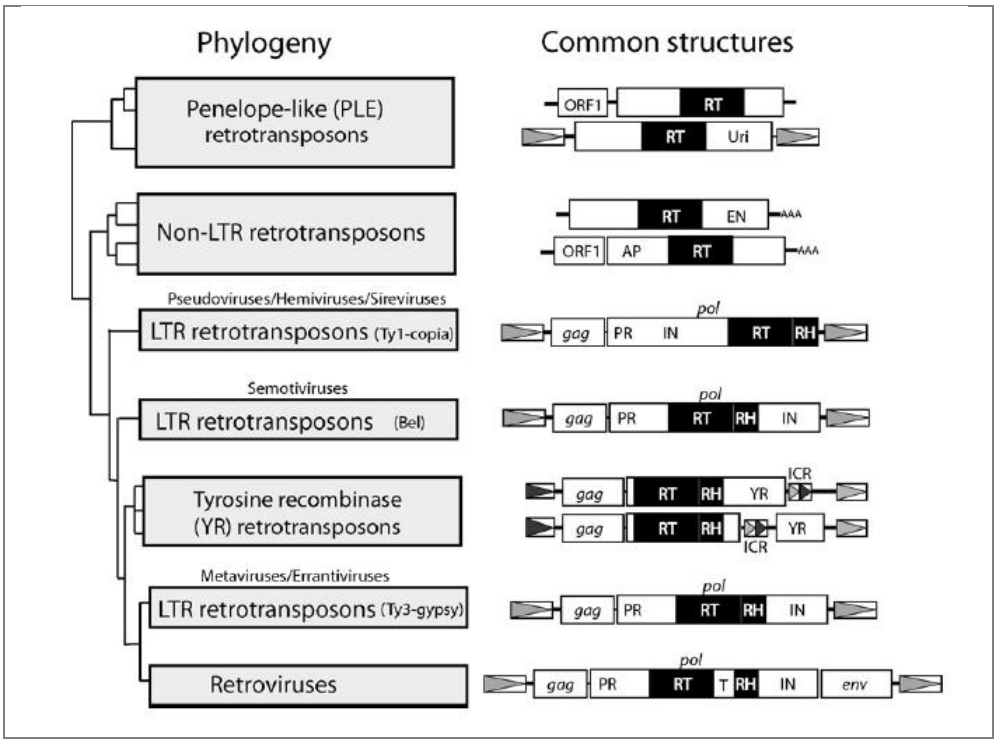

Fig. 10 - Position of non-LTR elements in the phylogeny of retrotransposons (from Eickbush & Jamburuthugoda, 2008).

Phylogenetic relationships of non-LTR elements are often incongruent with those based on non-mobile genes of their hosts (Eickbush, 1992). This could be due to horizontal transfer, but also to species hybridization or to the presence of ancient polymorphisms. The latter explanation seems to be the most probable, because no evidence of horizontal transfer for non-LTR elements has been detected yet. Assuming a vertical descent, the phylogeny of non-LTR retrotransposons suggests that they are as old as eukaryotes (Malik et al., 1999).

2.4 THE ELEMENT R2

R2 is a family of LINEs that insert specifically into the 28S ribosomal gene. Six other families of elements inserting into the 28S gene have been identified: R1, R4, R5, R6, R9, and RT (Jakubczac et al., 1991; Besansky et al., 1992; Burke et al., 1995; Burke et al., 2003; Kojima & Fujiwara, 2003; Gladyshev & Arkhipova, 2009) (Fig. 11).
R7 and R8 insert into the 18S gene (Kojima & Fujiwara, 2003; Kojima et al., 2006). R8 structure and sequence denounce its belonging to one of the clades of R2, suggesting that it changed its target from the 28S to the 18S gene (Kojima et al., 2006).

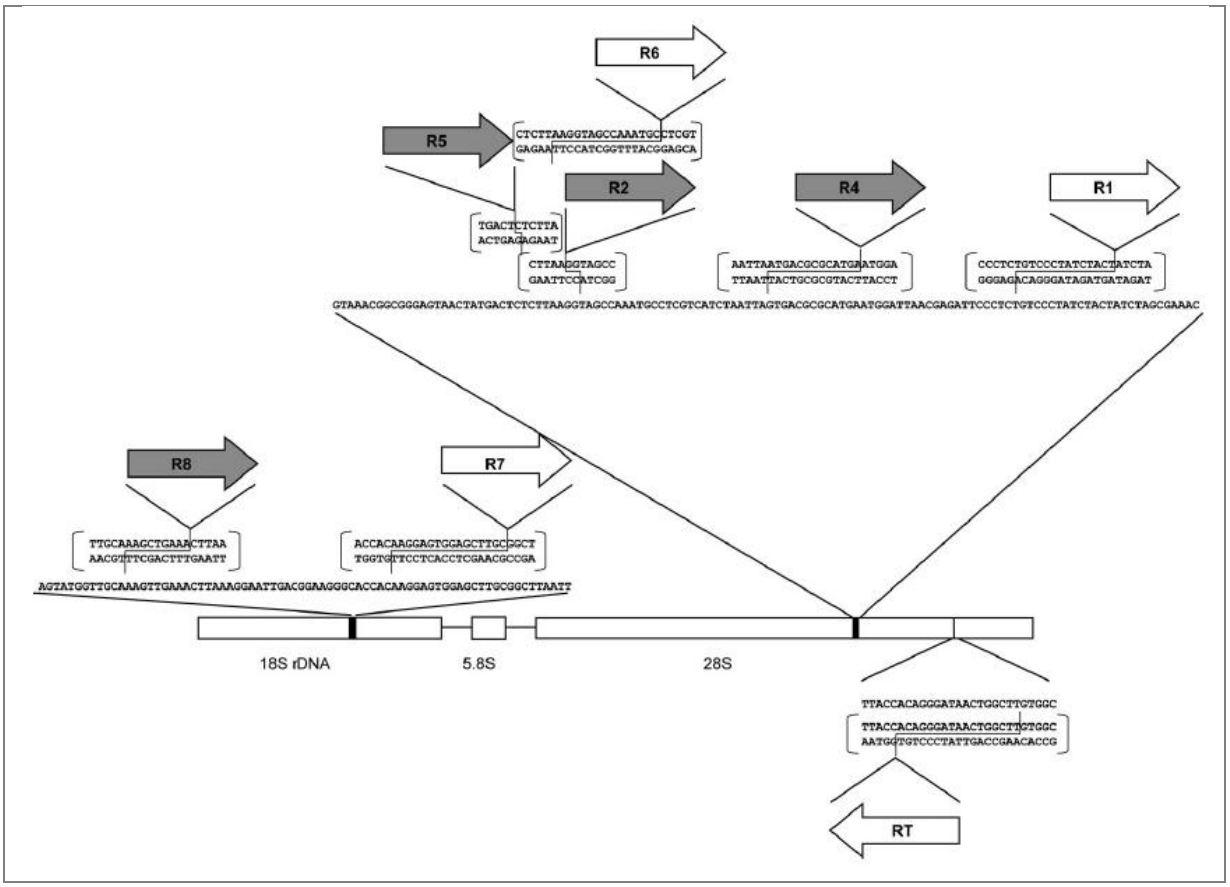

Fig. 11 - Insertion sites of rDNA-specific LINEs (from Kojima et al., 2006).

R2 has been found in many lineages of arthropods (Jakubczak et al., 1991; Burke et al., 1998), but also in other animal phyla, such as Cnidaria, Plathyhelmintes, Echinodermata, and Chordata (Kojima & Fujiwara, 2004, 2005; Kojima et al., 2006).

Ribosomal DNA

Ribosomes are formed by two subunits, a large one and a small one, composed each by an RNA-protein complex. In eukaryotes, ribosomal RNA is typically encoded by four genes: 5S, 18S, 5.8S, and 28S. While 5S gene can be situated in different positions, the other rDNA genes are organized in tandem repeated units, each one containing the three genes, separated by internal transcribed spacers (ITS), and an external transcribed spacer (ETS). Units are separated from one another by a non transcribed region (NTS) (Fig. 12).

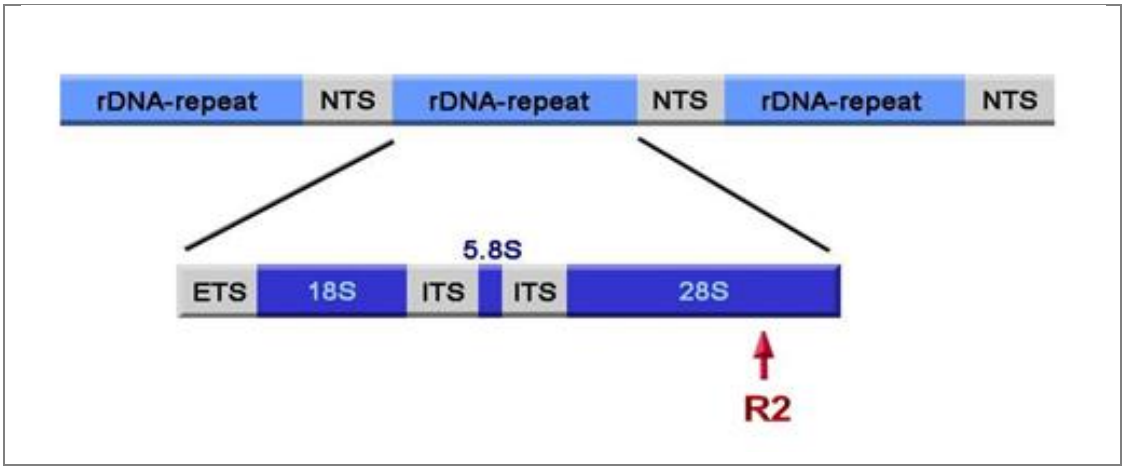

Fig. 12 - Structure of rDNA units. The red arrow indicates the point of insertion of R2.

18S gene codes for the RNA component of the small ribosomal subunit, while 5.8S and 28S genes, together with 5S gene, code for the RNA component of the large ribosomal subunit.

Eukaryotes typically encode hundreds of copies of the rDNA unit, on one or few chromosomes. Owing to concerted evolution, the sequences of the different units of each species are highly uniform, while they are comparatively divergent among species (Eickbush & Eickbush, 2007).

All known R2 elements insert in the same site, between the bases G and T of the 28S sequence 5'-AAGGTAGC-3' (Fig. 11), with the possible exception of the element of the cnidarian *Nematostella vectensis* (Kojima et al., 2006).

The region of the 28S gene near the R2 insertion site is highly conserved among animal species, both in those where R2 insertions are present and in those where they are absent. A remarkable exception are some ticks, that have substitutions in correspondence of R2 insertion site (Bunikis & Barbour, 2005).
The copies of the 28S gene that contain an R2 insertion are not functional.

The proportion of inserted 28S genes can vary greatly among species, among different strains of the same species, and, for the same species or strain, with time. For instance, the percentage of inserted unities was found to be from 2% to 28% in *Drosophila melanogaster* (Jakubczak et al., 1992), from 19% to 35% and from 14% to 44% in *Drosophila simulans* (Pérez-Gonzales & Eickbush, 2001; Zhang & Eickbush, 2005), from 38% to 44% in *Rhynchosciara americana* (Madalena et al., 2008), 30% in *Popillia japonica* (Burke et al., 1993), less than 1% in *Ambylomma americanum* (Bunikis & Barbour, 2005), about 7% in *Hydra magnipapillata* (estimation based on data from Kojima et al., 2006).

R2 structure

R2 elements encode a single ORF, varying in length approximately from 1000 to 1200 amino acids, and flanked by untranslated sequences of variable extension.
Besides the RT domain, present about the centre of the ORF, other highly conserved regions exist, in connection with DNA-binding motifs and the endonuclease domain. Outside these regions, there is essentially no sequence conservation between R2 elements.
The N-terminal domain of the protein can contain one, two, or three cysteine-histidine (zinc-finger) motifs, their number varying depending on R2 lineage. When there are three of them, they are respectively of the CCHH, CCHC, and CCHH kind. Downstream of the zinc-finger motifs is a conserved domain similar to the DNA-binding motifs of the oncoptrotein c-myb (Burke et al., 1999).
The C-terminal end of the R2 protein includes a CCHC zinc-finger motif and the endonuclease domain (Yang et al., 1999).
Some R2 sequences end with short repeat sequences, due to the capability of the R2 reverse transcriptase of adding nontemplated nucleotides before engaging the RNA in the reverse transcription reaction (George et al., 1996).

Transposition mechanism

R2 transcript can serve as a template both for the synthesis of the R2 protein and for reverse transcription. There is no evidence for the presence of a promoter in the 5' UTR of R2 or in the 28S sequences upstream of R2 insertion. It is probable that R2 elements are co-transcribed with the 28S gene (George & Eickbush, 1999).

R2 protein can bind R2 RNA sequences either near the 5' end or near the 3' end of the full length R2 transcript. Thus, a transcript is bound by a homodimeric R2 protein.

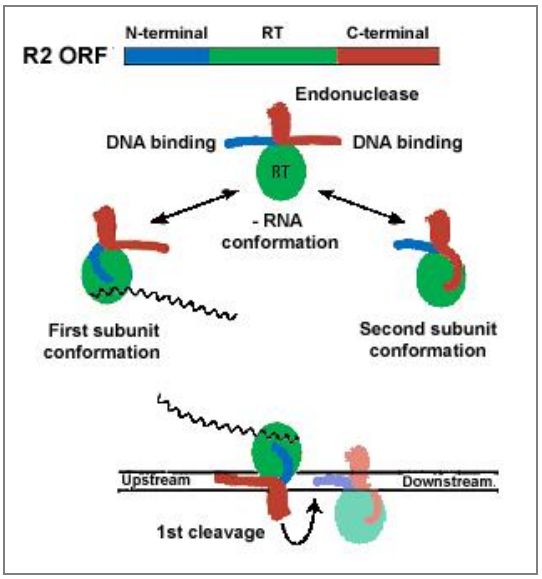

Fig. 13 - R2 protein and its relations with R2 RNA and 28S (from Christensen & Eickbush, 2005, modified).

The subunit bound to the 3' end of R2 RNA binds the 28S gene a short distance upstream of the insertion site, while the subunit bound to R2 5' end binds the 28S gene a short distance downstream of the insertion site (Fig. 13). The upstream subunit cleaves the first strand of 28S DNA thanks to its endonuclease, then its RT starts the reverse transcription reaction using the 3' hydroxyl group released by the cleavage as a primer (a process called target primed reverse transcription). The downstream subunit then cleaves the second strand of 28S DNA and uses the released 3' end to prime the transcription of the second strand of R2 DNA, based on the complementary strand already existing. R2 RNA is displaced during this synthesis (Christensen et al., 2006; Kurzynska-Kokorniak et al., 2007; Eickbush & Jamburuthugoda, 2008) (Fig. 14).

When the RNA template is cleaved by cellular RNases or the reverse transcriptase dissociates before reaching the 5' end of the transcript, a R2 truncated copy can be produced. When the length of the truncation is so extended to cause the deletion of portions that are necessary for transposition, the resulting copy is not active.

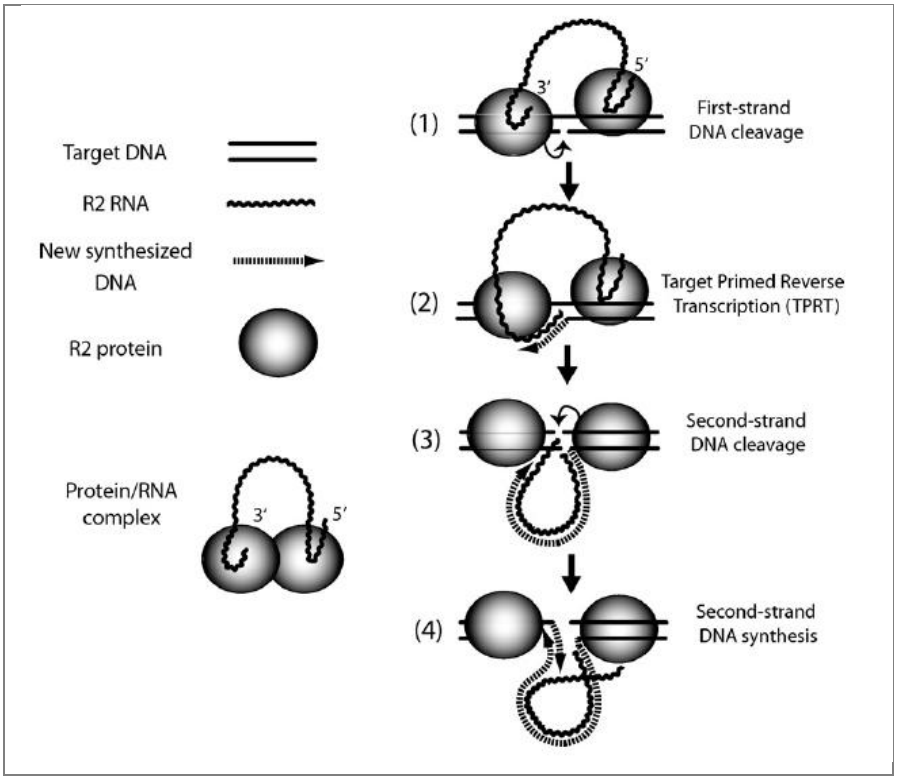

Fig. 14 - Mechanism of retrotransposition of R2 (from Eickbush & Jamburuthugoda, 2008).

The location of the first-strand nick in the target DNA is conserved throughout arthropods, while the second-strand cleavage can vary in location. This generates either short deletions, or duplications of a portion of the 28S gene (Burke et al., 1999).

R2 phylogeny

The reconstruction of R2 phylogeny based on the C-terminal half of reverse transcriptase shows the presence of 11 subclades, whose internal topology reflects the phylogeny of the hosts (Kojima and Fujiwara, 2005) (Fig. 13).

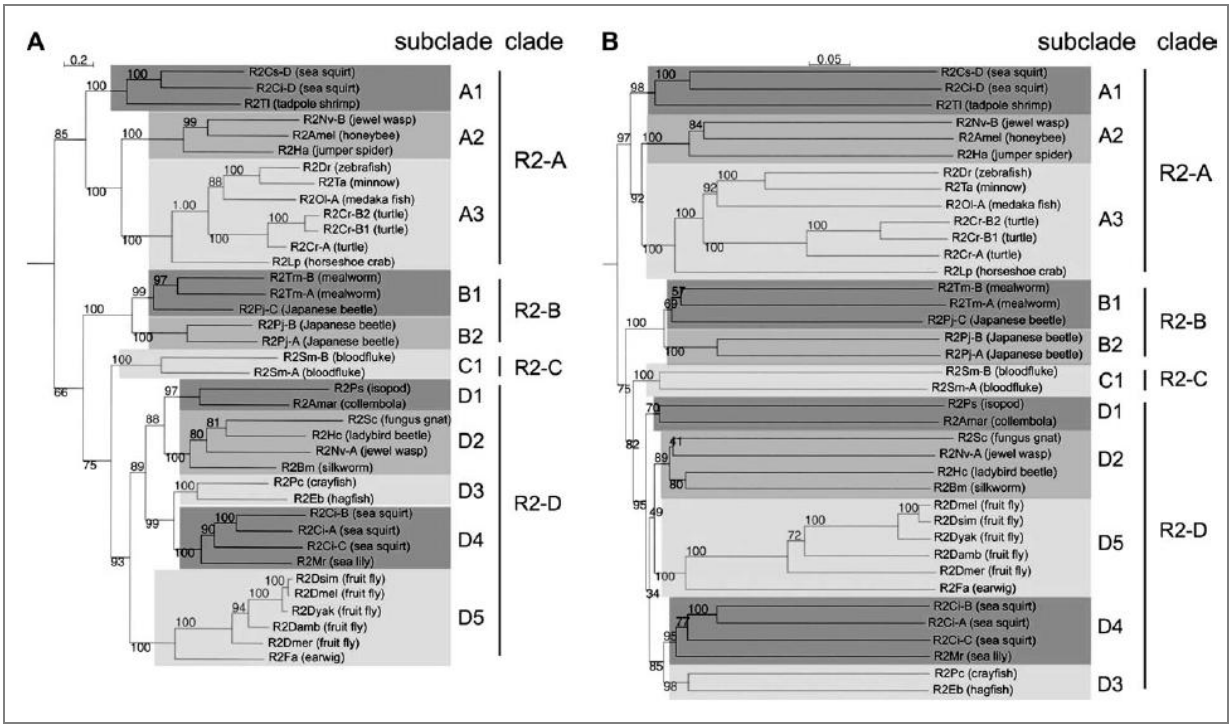

Fig. 13 - R2 phylogeny (bayesian tree on the left, NJ tree on the right), showing the 11 subclades (from Kojima & Fujiwara, 2005).

These 11 subclades are grouped in four clades, that are consistent with the number of zinc-finger motifs in the N-terminal end of the ORF (Burke et al., 1998; Kojima and Fujiwara, 2004, 2005) (Fig. 14).

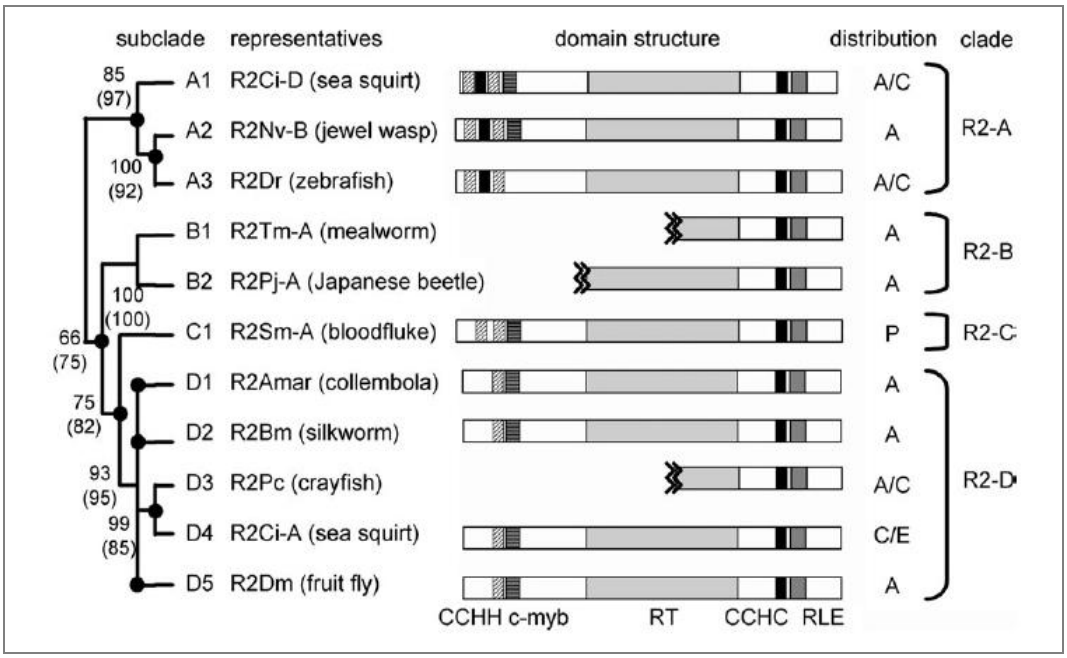

Fig. 14 - R2 phylogeny, showing the four main clades and their corresponding R2 structures (from Kojima & Fujiwara, 2005).

R2 phylogeny shows striking discrepancies with the phylogeny of the hosts. In many cases elements from distant species are found in the same subclade, while elements from species that are more tightly related end up in different subclades.
Some species host multiple lineages of R2. This is, for instance, the case of the beetle *Popillia japonica* (Burke et al., 1993) and of the sea squirt *Ciona intestinalis*, whose lineages form two very distant groups in R2 phylogeny.
Inconsistencies between R2 and hosts phylogenies can be explained by horizontal transfer or by the presence of paralogous R2 lineages. Divergence-versus-age analysis shows no evidence for the horizontal transfer, suggesting for R2 an origin at least as ancient as the divergence of protostomes and deuterostomes (Kojima and Fujiwara, 2005).

R2 and rDNA dynamics

The truncation variants generated during transcription can be used to monitor R2 activity (Pérez-Gonzales & Eickbush, 2001). In fact, the appearance of a new variant shows that a new transposition event has occurred.
Full-length elements can also display a small degree of length variability, owing to the presence of short deletions, or insertion of non-templated nucleotides at their 5' junction (George et al., 1996), so that, in some cases, it is possible to detect new full-length element insertions.

The study of R2 transposition in isofemale lines *Drosophila simulans* has shown that the level of activity of the element can be very variable, with lines in which new insertions are frequent and lines in which no activity is detected (Zhang & Eickbush, 2005).
Retrotransposition activity was found to be uncorrelated both with the size of the rDNA locus and with the number of uninserted units (Zhang & Eickbush, 2005). Instead, R2 transcript levels, and consequently its levels of activity, appear to depend upon the distribution within the rDNA locus of units inserted with R2 full-length copies. Animals typically transcribe contiguous blocks of rDNA units, so, if inserted units were recognized by the host cell and inactivated through modifications of their chromatin structure, transcription would be likely to occur mostly in regions of the rDNA locus that contain low frequencies of R2-inserted units. In this way, large

numbers of R2 elements could be prevented from transcription. Recombination events can rearrange rDNA units, allowing the transcription of R2 copies that were previously inactive (Eickbush et al., 2008; Zhou & Eickbush, 2009).

Studies in *Drosophila* spp. have found a rapid turnover of R2 elements (George et al., 1996; Averbeck & Eickbush, 2005). In *D. melanogaster*, the estimated R2 retrotransposition rate, measured over a period of 353 generations, is 0.7×10^{-4} insertion events per copy per generation, while its rate of elimination is 2.3×10^{-5} insertion events per copy per generation (Pérez-Gonzales & Eickbush, 2002).

High rates of R2 retrotransposition lead to the rapid loss of preexisting R2 elements (Zhang et al., 2008). The elimination of R2 copies might be due either to gene conversion or to unequal crossovers. In the first case, a single R2 copy would be eliminated with each event, while, in the second case, multiple copies could be simultaneously removed. Actually, the elimination of R2 copies appears to involve the loss of large blocks of R2-inserted rDNA units. Such deletions are probably the result of recombination occurring between two units on the same DNA strand, and of the consequent formation of an extrachromosomal circle, that is lost from the cell (Pérez-Gonzales et al., 2003; Zhang et al., 2008).

The rate of elimination is not the same for all R2 copies. Elements with the highest rates of elimination might be located near the middle of the rDNA loci, where recombination is more frequent (Pérez-Gonzales & Eickbush, 2002).

The length of the rDNA array, reduced by the elimination of blocks of inserted units, can be restored if interchromosomal or sister-chromatid unequal crossovers do occur, and are followed by the positive selection of longer variants. If these crossovers were more frequent in regions where insertions are rare, the number of units could increase without increasing the proportion of inserted units (Pérez-Gonzales et al., 2003).

CHAPTER 3:
MITOCHONDRIAL DNA AS A PHYLOGENETIC MARKER

In animals, mitochondrial DNA typically encodes for 37 genes: 13 protein subunits of the enzymes for oxidative phosphorylation (COI, COII, COIII, Cytb, ND1-6, ND4L, ATP6, ATP8), two ribosomal rRNAs (rrnL and rrnS), and 22 t-RNAs (trnX). It includes also a non coding region known as D-loop or A-T rich region.

Mitochondrial DNA is regarded as the marker of choice for the reconstruction of phylogenetic relationships at several taxonomic levels, from populations to phyla (Gissi et al., 2001). The reasons underlying its wide use in phylogenetic reconstructions are manifold: Its isolation and amplification are comparatively straightforward, thanks also to the availability of universal primers (Simon et al., 1994; 2006); its inheritance is in most cases uniparental; it contains regions evolving at different rates, but generally faster than nuclear DNA; the genes it encodes are strictly orthologous, allowing comparisons among distant taxa.

For phylogenetic studies of insects, the most frequently sequenced mitochondrial genes are cytochrome oxidase I (COI), COII, 16S rDNA, and 12S rDNA. Of these, COII has been sequenced over the widest variety of taxa, so that homologous sequences are available for nearly all orders (Caterino et al., 2000).

The mitochondrial genes that have been most extensively used for phylogenetic analyses of European termites are COII and 16S. The analysis of their combined dataset allows to obtain well resolved topologies (Marini & Mantovani, 2001; Luchetti et al., 2004; 2007).

CHAPTER 4: AIMS OF THIS WORK

Termites are an interesting subject both from a practical (pest control) and from a theoretical point of view. What makes them a unique subject from a theoretical point of view are their biological peculiarities. In fact, they are an insect group entirely formed by eusocial species. Eusociality implies that only some specialized individuals reproduce, while the rest of the individuals, often forming the large majority of the colony, are sterile. This aspect likens them to some groups of hymenopterans, but there are many features that differentiate the two cases: Contrary to social hymenopterans, termites are hemimetabolous insects, their colonies are entirely constituted by diploid individuals, and parthenogenesis, when present, is accessory to amphigonic reproduction.

The peculiarities of termite reproductive biology are likely to affect their molecular evolution. In particular, the presence of sterile castes implies that the effective size of termite populations is smaller than their absolute size. The ensuing bottlenecks are likely to favour genetic drift, and consequently genetic diversification. The presence in termite colonies of related neotenic reproductives can lead to a high level of inbreeding, reducing the genetic variability of the colony.

This work aims to contribute to a better understanding of some aspects of termite biology. In particular, the objects of this study were to isolate and characterize the retrotransposable element R2 in some species of European termites and to analyze the phylogeny of *Reticulitermes* termites from the island of Cyprus.

R2 retrotransposon in termites

The preliminary aim of this study was to obtain the sequences of complete R2 elements in termites, in particular in the two European genera *Reticulitermes* and *Kalotermes*, in order to characterize their structures and to compare them with those of the other known elements.

The subsequent aim was to investigate the position of termite elements in R2 phylogeny, based on the 3' portion of the ORF of the complete elements already obtained and of partial elements sequenced for the purpose.

The last part of the work aimed to analyze R2 truncation variants in *R. urbis*. A portion of a mature colony, formed by workers and soldiers, was analyzed in order to

determine its truncation profile and to see whether any differences existed between the two castes. Three incipient colonies, formed each by a couple of reproductives and by their offspring, were analyzed in order to observe the transmission of truncated variants from the parental generation to the filial generation.

Reticulitermes termites from Cyprus

Taxonomy and phylogeny of Eastern Mediterranean termites still present some aspects of uncertainty, due mainly to the comparatively poor sampling of some areas. This work aimed to study the collocation of Cyprian *Reticulitermes* termites in the phylogeny of European termites, through the analysis of portions of the mitochondrial genes COII (cytochrome oxidase II) and 16S (mitochondrial large ribosomal subunit). These genes have proved to be good molecular markers for the reconstruction of phylogeny in termites, and their use in many studies makes them useful for making comparisons among termite taxa.

CHAPTER 5: MATERIALS AND METHODS

5.1 R2 METHODS OVERVIEW

R2 structure

The isolation and characterization of the element R2 was carried out on five species of European termites: four belonging to the genus *Reticulitermes* and the fifth, *Kalotermes flavicollis*, represented by two populations showing a strong divergence in mitochondrial DNA sequences (Tab. 1).

Species	Collecting sites
R. urbis	Bagnacavallo (Ravenna - Italy)
R. lucifugus	Castel Porziano (Roma - Italy)
R. grassei	Ychoux (France)
R. balkanensis	Marathon (Greece)
K. flavicollis 1	Sisi (Crete - Greece)
K. flavicollis 2	Riserva Feniglia (Livorno - Italy)

Tab. 1 - Termite samples used for the isolation of R2.

Total DNA was isolated as described in § 5.3.

In the case of *R. urbis* and *K. flavicollis* 2, the 3' end of R2 was amplified (§ 5.4.1) using a forward degenerate primer annealing to a comparatively conserved region inside R2 and a reverse primer annealing to the 28S gene downstream of R2 insertion. Four forward primers were tested: R2IF1, R2IF2, R2IIF1, and R2IIF2 (Kojima & Fujiwara, 2005). The best quality of amplification was obtained with R2IIF1 in the case of *R. urbis* and with R2IF1 in the case of *K. flavicollis* 2. The reverse primer was in both cases 28S-B-R (5'- CGT CTC CCA CTT ATG CTA CAC CTC -3').

The amplification products were purified from gel (§ 5.6) and cloned into a vector (§ 5.7). Positive clones were sequenced (§ 5.8). The presence in the clones of the 3' end of R2 sequence, containing a portion of the ORF, was checked using the ORF Finder tool at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Based on the R2 partial sequences obtained in this way, new primers were designed (§ 5.5), in order to fill sequencing gaps and to extend the sequencing towards R2 5' end. The 5' end of the element was amplified using the primer 28S-F, annealing to 28S gene upstream of R2 insertion, coupled with a primer annealing to a region inside R2, designed for each species based on the adjoining sequence. Some of the primers that were designed for a particular species proved to be effective also in other species of the same genus, so that it was not always necessary to design new specific primers.

For the amplification of R2 3' end in *R. lucifugus*, *R. grassei*, *R. balkanensis*, and *K. flavicollis* 1, no degenerate primers were used, because the primers designed for *R. urbis* and *K. flavicollis* 2, respectively, permitted to obtain good amplification products.

A list of the primers that were designed and used for the amplification of R2 portions is shown in § 5.4.1.

R2 complete sequences were obtained for each species combining in a consensus sequence partial sequences of different clones. The sequences were aligned and analyzed as described in § 5.9.

R2 phylogeny

The collocation of termite elements in R2 phylogeny was studied aligning their amino acid sequences with those of R2 elements isolated in other species and available in GenBank. The alignment was the same used by Mingazzini et al. (submitted), with the addition of the recently isolated element of *Rhynchosciara americana* (Madalena et al., 2008). The SLACS retrotransposon of *Trypanosoma brucei* (Aksoy et al., 1990) was considered as an outgroup.

The alignment was based on the C-terminal region of the ORF (440-530 aa, depending on the species), including a portion of the RT. This is the region that is usually considered for phylogenetic reconstructions, because it can be aligned quite precisely across different R2 lineages and, for many species, it is the only available portion. Phylogenetic analyses were carried out as described in § 5.9.

Truncated variants

For the analysis of truncated variants, four colonies of *R. urbis* were considered. The first was a mature colony, collected in Bagnacavallo (Ravenna, Northern Italy) in 2003 and maintained in laboratory conditions since then, composed by at least 1000 individuals, of which 14 were sampled for the analysis. The other three were selected from a group of 20 colonies bred in the laboratory with the special purpose of obtaining the offspring of known parents (§ 5.11). A total of 51 individuals were analyzed (Tab. 2).

Colony	Queen	King	Workers	Soldiers	Juveniles	Total
1	-	-	10	4	-	14
2	1	1	-	-	9	11
3	1	1	-	-	10	12
4	1	1	-	-	12	14
Total						51

Tab. 2 - Composition of the *R. urbis* colonies used for the analysis of truncate variants.

The different 5'-truncated R2 copies were identified based on PCR products obtained using a forward primer that binds to the 28S gene upstream of R2 insertion site and a series of reverse primers that bind to different regions of R2, a procedure described in Pérez-Gonzalez and Eickbush (2001). The primers were the same used for the amplification and sequencing of R2 portions: the forward primer 28S-F and the five reverse primers URBRIN1, ... URBRIN5 (§ 5.4.1). The PCR products were run overnight on a 1.5% agarose gel. The presence of bands corresponding to truncated variants was detected with Southern blots (§ 5.10).

5.2 MITOCHONDRIAL DNA METHODS OVERVIEW

Samples of termites from the island of Cyprus, belonging to the genus *Reticulitermes*, were collected by Prof. Marini in the year 2009, both in the Greek and in the Turkish portions of the island. Sampling localities are shown in Fig. 15. For locality 4 (Vasileia) two termite populations were collected. From now on, the corresponding samples will be referred to as sample 4A and sample 4B.

1 Polis
2 Kakopetria
3 Karpasei
4 Vasileia
5 Charkeia
6 Kaplica
7 Rizokarpaso
8 Trikomo
9 Sotira
10 Asgata
11 Pentakomo
12 Episkopi
13 Petra Tou Romiou

Fig. 15 - Collection sites of *Reticulitermes* sp. in the island of Cyprus.

Total DNA was isolated from two individuals for each sample (§ 5.3). For each individual, a 684 bp portion of the mitochondrial gene COII and a 504 bp portion of the mitochondrial gene 16S were amplified (§ 5.4.2). The amplicons were purified (§ 5.6) and sequenced (§ 5.8). The analysis of sequences and the construction of phylogenetic trees were carried out as described in § 5.9, aligning the sequences of Cyprus samples together with those of other European *Reticulitermes* spp. analyzed in Velonà et al. (submitted). Sequences from *Coptotermes formosanus* (accession numbers AF107488 and 16S U17778) were used as outgroup.

5.3 DNA ISOLATION

Total DNA was extracted from the cephalic portion of individual termites preserved in 100% ethanol, using the following protocol:

- Grind the tissue in 300 µl 2x CTAB buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB)
- Incubate at 65 °C for 1 hour
- Add 300 µl of chloroform
- Shake for 2 minutes
- Centrifuge for 10 minutes at 15000 rcf
- Transfer the supernatant in a new tube and add 300 µl of chloroform
- Shake for 2 minutes
- Centrifuge for 10 minutes at 15000 rcf
- Transfer the supernatant in a new tube and add 25 µl of 3M sodium acetate and 600 µl of cold 70% ethanol
- Centrifuge for 10 minutes at 15000 rcf
- Discard the supernatant and add 100 µl of cold 70% ethanol
- Discard the supernatant and dry the pellet under laminar flow hood
- Suspend in 30 µl 1x TE or alternatively in 30 µl PCR water
- Keep in the refrigerator (-20 °C for long periods) until further processing

5.4 AMPLIFICATION

Amplification by PCR was performed with different kits and different conditions depending on features of the expected product and of the primers.

5.4.1 AMPLIFICATION OF R2 FRAGMENTS

Amplification of R2 fragments was carried out with specifically designed primers. In some cases, a primer was found to be effective in more than one species. A list of the primers that were used for each species or population is shown in Tab. 3.

R. urbis			
Primer name	**Sequence**	**F/R**	**Annealing position**
URB DIN	5’- CCA AGA TTC TTT GGT TAG GGA AAT TA -3’	F	3885 - 3906
URB RIN1	5’- GTA ATA ACT TTG CGA GAG ATG TGG A -3’	R	4667 - 4691
URB RIN2	5’- AGA AGG GTA ATG AAG AAC CAT TGA G -3’	R	3472 - 3496
URB RIN3	5’- AAG TCA CCC TTC TAA TCC TCC TCT CTA -3’	R	2873 - 2899
URB RIN4	5’- GCA ACT CTT TAT CCT TAC TCA AGT CC -3’	R	2007 - 2032
URB RIN5	5’- GAA AAG TCA AAT CCA AGT TAT GGT G -3’	R	1315 - 1339
URB FWD	5’- AAA GTT CTT ACC ACG ACG TAC CC -3’	F	633 - 655
R. lucifugus			
Primer name	**Sequence**	**F/R**	**Annealing position**
URB DIN	5’- CCA AGA TTC TTT GGT TAG GGA AAT TA -3’	F	3889 - 3914
URB RIN	5’- GTA ATA ACT TTG CGA GAG ATG TGG A -3’	R	4671 - 4695
URB RIN2	5’- AGA AGG GTA ATG AAG AAC CAT TGA G -3’	R	3476 - 3500
URB RIN3	5’- AAG TCA CCC TTC TAA TCC TCC TCT CTA -3’	R	2877 - 2903
URB RIN4	5’- GCA ACT CTT TAT CCT TAC TCA AGT CC -3’	R	2011 - 2036
URB RIN5	5’- GAA AAG TCA AAT CCA AGT TAT GGT G -3’	R	1316 - 1340
CPF	5’- TGA ATA AAA ACA AGA CCT GGA CAA T -3’	F	3432 - 3456
CPR	5’- TAG ATA TCC TGC AAC GTT AGA GAC C -3’	R	3975 - 3999
CPOF	5’- TAA TAA TTC TGT TGA TCA TGC ACT TG -3’	F	1153 - 1178
CPOR	5’- ACT TAT ACC CCT CAA ATC TCT TGC TA -3’	R	1627 - 1652
R. grassei			
Primer name	**Sequence**	**F/R**	**Annealing position**
URB DIN	5’- CCA AGA TTC TTT GGT TAG GGA AAT TA -3’	F	1156 -1181
URB RIN2	5’- AGA AGG GTA ATG AAG AAC CAT TGA G -3’	R	743 -767
BALF	5’- TCC TTG CTA AAC TCT ACA ACT TGC T -3’	F	-25(*)
BALR	5’- CGT TTC AGT GCG GAC TTA AGT TTA T -3’	R	1266 - 1290
R. balkanensis			
Primer name	**Sequence**	**F/R**	**Annealing position**
URB DIN	5’- CCA AGA TTC TTT GGT TAG GGA AAT TA -3’	F	1156 -1181
URB RIN2	5’- AGA AGG GTA ATG AAG AAC CAT TGA G -3’	R	743 -767
BALF	5’- TCC TTG CTA AAC TCT ACA ACT TGC T -3’	F	-25(*)
BALR	5’- CGT TTC AGT GCG GAC TTA AGT TTA T -3’	R	1266 - 1290
MARC	5’- GTG TTC AAA CCA GAT ATA GTG ACG A -5’	F	1673 - 1697

K. flavicollis 1			
Primer name	**Sequence**	**F/R**	**Annealing position**
K DIN	5’- CAC GAG GTT TGG TCC TTA AAC -3’	F	3138 - 3158
KF RIN	5’-CTG GCA TAT AGT ACA TGG TCT GTT GTA-3’	R	3307 - 3333
KF RIN BIS	5’- AAA TTT ATT GCT GGC TCT CAC CAC -3’	R	3527 - 3550
KF RIN2	5’- GAT ATT TAA GTG CAG AGG AGG GTC T -3’	R	3035 - 3059
KF FWD	5’- CTC AAT ATG GAG CAT TGA TGA GAT T -3’	F	835 - 859
K RIN3	5’- AGA GAG GTC TTA TGT TCC TTC CAA C -3’	R	2289 - 2313
K. flavicollis 2			
Primer name	**Sequence**	**F/R**	**Annealing position**
K DIN	5’- CAC GAG GTT TGG TCC TTA AAC -3’	F	3135 - 3155
K RIN	5’- ACA AAG TAG TCA GCC AAA GCT ATA C -3’	R	4042 - 4068
K RIN2	5’-TAT TCA TGT AGC AAC CAA CAT GAT CTA C-3’	R	2886 - 2912
K RIN3	5’- AGA GAG GTC TTA TGT TCC TTC CAA C -3’	R	2289 - 2313
K RIN4	5’- GTC TCG ATC TTC AAG GCA TAG G -3’	R	1677 - 1698
K FWD	5’- AGG AGG GTC TGC TAT GGT TGT -3’	F	835 - 859

Tab. 3 - Primers used for the amplification of R2 fragments, with indication of their annealing positions (bp, counting from the beginning of the sequence). F=forward, R=reverse. (*)=The primer BALF anneals 25 bp before the beginning of the sequence.

When the expected length of the PCR product was longer than 1000 bp, PCR was performed in a 50 µl mixture using TaKaRa *LA Taq*™ (Lonza) and following the enclosed protocol. Reaction conditions were set as follows:

- Initial denaturation at 94 °C for 2 minutes
- 35 cycles composed by denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 70 °C for 3-10 minutes (depending on the length of the expected product, approximately 2 minutes per kbp)
- Final extension at 70 °C for 12 minutes.

When the expected length of the PCR product was shorter than 1000 bp, PCR was performed in the same conditions described for the amplification of mitochondrial genes (§ 5.4.2), with the exception that the annealing temperature was 50 °C instead of 48 °C.

5.4.2 AMPLIFICATION OF MITOCHONDRIAL GENES

Amplification of mitochondrial genes was obtained with the following primers:

Product	Primer name	Primer sequence
COII	TL2-J-3034 = mtD-13	5'- AAT ATG GCA GAT TAG TGC A -3'
	TK-N-3785 = mtD-20	5'- GTT TAA GAG ACC AGT ACT TG -3'
16S	LR-J-12887 = mtD32	5'- CCG GTC TGA ACT CAG ATC ACG T -3'
	LR-N-13398 = mtD34	5'- CGC CTG TTT AAC AAA AAC AT -3'

PCR was performed in a 50 µl mixture using Taq polymerase Recombinant Kit (Invitrogen), following the enclosed protocol. Reaction conditions were set as follows:

- Initial denaturation at 94 °C for 5 minutes
- 30 cycles composed by denaturation at 94 °C for 30 seconds, annealing at 48 °C for 30 second, extension at 72 °C for 30 seconds
- Final extension at 72 °C for 7 minutes.

5.5 PRIMER DESIGNING

Primers for amplifying and sequencing R2 portions in different termite species were designed with the software Primer3 (Rozen & Skaletsky, 2000) based on known flanking sequences (§ 5.1). Risks of misannealing or self-annealing were evaluated with the software Amplify 3 (available at http://engels.genetics.wisc.edu/amplify/).

5.6 PURIFICATION

Purification of amplified DNA was carried out either directly from PCR solution or after slicing the appropriate band from gel. In both cases, purification was carried out with Promega Wizard® SV Gel and PCR Clean-up System, following the enclosed protocol:

Dissolving gel slice:

- Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5 ml microcentrifuge tube
- Add 10 µl Membrane Binding solution per 10 mg of gel slice. Vortex and incubate at 50-65 °C until gel slice is completely dissolved

Processing PCR reactions:

- Add an equal volume of Membrane Binding solution to the PCR reaction

Binding DNA:

- Insert SV Minicolumn into collection tube
- Transfer dissolved gel mixture to the Minicolumn assembly. Incubate at room temperature for 1 minute
- Centrifuge at 16000 rcf for 1 minute. Discard flowthrough and reinsert Minicolumn into collection tube

Washing:

- Add 700 µl Membrane Wash Solution. Centrifuge at 16000 rcf for 1 minute and discard flowthrough
- Repeat the previous step with 500 µl Membrane Wash Solution and centrifuge at 16000 rcf for 5 minutes
- Empty the collection tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol

Elution:

- Transfer Minicolumn to a clean 1.5 ml microcentrifuge tube
- Add 50 µl of Nuclease-free Water. Incubate at room temperature for 1 minute. Centrifuge at 16000 rcf for 1 minute
- Discard Minicolumn and store DNA at 4 °C or -20 °C.

5.7 CLONING

Cloning procedure is used for the isolation and amplification of desired DNA fragments starting from amplicons of bad quality and/or containing also unwanted products. The procedure requires two main steps: ligation, consisting in the insertion of DNA fragments in a suitable vector (§ 5.7.1), and transformation, consisting in the

insertion of the vector in competent cells, that subsequently multiply forming colonies (§ 5.7.2).
The presence of the insertion in the cells can be preliminarily verified through a blue/white screening, based on the presence in the culture medium of X-gal (bromo-chloro-indolyl-galactopyranoside), which, when cleft by the β-galactosidase enzyme encoded by the lac-Z gene of the vector, generates a blue product. The insert, if present, is located in the lac-Z gene and disrupts its function, so the colony remains white. White colonies can then be selected for the amplification of the insert (§ 5.7.3).

5.7.1 CLONING SHORT FRAGMENTS

DNA fragments shorter than 1500 bp were cloned with pGEM®-T Easy Vector System I (Promega), following the enclosed protocol, with slight modifications:

Ligation

- Briefly centrifuge the pGEM®-T Easy Vector and vortex 2x Rapid Ligation Buffer vigorously
- Mix in a 0.5 ml tube 5 µl of 2x Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector (50 ng), 1 µl of T4 DNA Ligase and a quantity of PCR product calculated with the following formula:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio}$$

  where vector size is 3000 bp and the chosen insert:vector molar ratio was 3:1
- Add PCR water to a final volume of 10 µl
- Incubate for 16 hours at 4 °C.

Transformation

- Remove a tube of frozen High Efficiency Competent Cells from storage and place it in an ice bath until just thawed
- Carefully transfer 30 µl of cells into a tube and add 2 µl of ligation reaction product
- Gently flick the tube to mix and place it on ice for 45 minutes
- Heat-shock the cells for 30 seconds in a water bath at 42 °C

- Immediately return the tube to ice for 2 minutes
- Add 300 µl room-temperature S.O.C. medium
- Incubate for 1 hour at 37 °C with shaking (~150 rpm)
- Equilibrate two LB/ampicillin/IPTG/X-Gal plates at room temperature
- Plate 160 µl of the transformation culture on each plate
- Incubate the plates overnight at 37 °C.

5.7.2 CLONING LONG FRAGMENTS

DNA fragments longer than 1500 bp were cloned with TOPO TA Cloning® Kit (Invitrogen) and One Shot® TOP10 competent cells (Invitrogen), following the enclosed protocol with minor modifications.

Ligation

- Gently mix in a 0.5 ml tube 4 µl of PCR product, 1 µl of Salt Solution, 1 µl of TOPO® Vector, and water to a final volume of 6 µl
- Incubate at room temperature for 30 minutes
- Place the reaction product on ice or store it at -20 °C.

Transformation

- Thaw on ice 1 vial of One Shot® TOP10 cells
- Transfer the cells to a 15 ml tube, add 2 µl of the ligation reaction product and mix gently
- Incubate on ice for 45 minutes
- Heat-shock the cells for 30 seconds at 42 °C
- Immediately return the tube to ice for 2 minutes
- Transfer the cells to a 15 ml tube and add 250 µl of room temperature S.O.C. medium
- Shake the tube (~200 rpm) at 37 °C for 1 hour
- Spread 200 µl of transformation product on a prewarmed LB/ampicillin/X-Gal plate and incubate overnight at 37 °C.

5.7.3 AMPLIFICATION OF INSERTS

The insert of white bacterial colonies weas amplified with the primers M13 Forward (5'- GTA AAA CGA CGG CCA G -3') and M13 Reverse (5'- CAG GAA ACA GCT ATG AC -3').
PCR was performed in a 50 µl mixture using Taq polymerase Recombinant Kit (Invitrogen) and following the enclosed protocol. Reaction conditions were set as follows:

- Initial denaturation at 94 °C for 2 minutes
- 30 cycles composed by denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute
- Final extension at 72 °C for 7 minutes.

5.8 SEQUENCING

Amplicons were sequenced with the same primers that were used in amplification. Both strands were sequenced. Sequencing was performed either by ENEA-Casaccia Laboratories (Rome, Italy) or by Macrogen Inc. (Seoul, South Korea).

5.9 SEQUENCE ANALYSIS

The preliminary alignment and analysis of the sequences of R2 fragments, as well as the alignment of mitochondrial DNA sequences, were performed with MEGA version 4 (Tamura et al., 2007). Consensus sequences for the complete R2 elements were obtained with the software CLC Sequence Viewer 6 (CLC bio A/S, http://www.clcbio.com). Open reading frames were found with the ORF Finder tool at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Amino acid sequences were obtained applying the standard genetic code and aligned with Clustal X in MEGA version 4.
The parsimony network for COII haplotypes of Cyprian termites was obtained with the software TCS 1.21 (Clement et al., 2000).

Models of nucleotide substitution for the mitochondrial dataset were tested with Modeltest 3.06 (Posada & Crandall, 1998). Estimation of clade divergence time was obtained with the software package BEAST 1.4.8 (Drummond and Rambaut, 2007), as described in Velonà et al. (submitted).
Maximum parsimony trees were obtained with PAUP* 4.0b10 (Swofford, 2003), with gaps treated as informative characters and bootstrap values determined after 1000 replicates. Minimum evolution trees were obtained with MEGA version 4, with bootstrap values determined after 5000 replicates.
Bayesian trees were obtained with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Convergence was reached after 2 million generations. Trees were sampled every 1000 generations, and the first 200 trees were discarded as burn-in, after graphic visualization.

5.10 SOUTHERN BLOT

Southern blot is used for the detection of a specific DNA sequence. The procedure requires the transfer of DNA from an electrophoresis gel to a membrane, followed by the detection of the fragments containing the desired sequence by hybridization with a specifically designed probe.

Transferring DNA to a membrane

- Load DNA on a 1.5% agarose gel and run the electrophoresis until the bands are well separated
- Submerge and shake the gel for 20 minutes in Denaturation solution
- Repeat the previous step
- Submerge and shake the gel for 30 minutes in Transfer buffer
- Set up the blot assembly: Place a glass pane on a tray containing Transfer buffer, leaving a free space at the extremities. Place a piece of Whatman 3 mm paper on the glass, making sure that its ends are soaked and keep in contact with Transfer buffer. Lay the gel on the paper and carefully place on it a nitrocellulose membrane (Amersham Hybond™-N^+, GE Healthcare). Place on top 3 layers of Whatman 3 mm paper and a stack of blotting-paper. Put a weight on the assembly

- Let the blot assembly rest overnight
- Disassemble the blot pile and let the membrane dry
- In order to fix the DNA to the membrane, expose the membrane to UV light for 3 minutes.

Obtaining probes

In order to obtain probes for the detection of R2 truncated versions, new primers (called DIN primers) were designed to be used in couple with RIN primers already used for R2 amplification and sequencing, so that the length of each amplicon would range from 400 to 452 bp. DIN primers were designed with the software Primer3 and checked with Amplify 3 (§ 5.5). Their sequences and annealing positions on *R. urbis* R2 complete element are shown in Tab. 4.

Primer name	Sequence	Annealing position
URB DIN1	5'-AAA CGC AGG CTC ATG TTC TT-3'	4246 - 4265
URB DIN2	5'-TTT GAC ACA ATC CCC CAT TC-3'	3033 - 3052
URB DIN3	5'-TCT ACC GCG ATA AGG ACC TG-3'	2434 - 2453
URB DIN4	5'-GAC CGA AGA GGA GGT CCA AT-3'	1589 - 1608
URB DIN5	5'-TTG AGG TGT CTC CTC GGT CT-3'	867 - 886

Tab. 4 - Primers used for the amplification of probes for Southern blots.

Each DIN primer was used in couple with the corresponding RIN primer (URB DIN1 with URB RIN1 and so on) in PCR with the same conditions already described for the amplification of short R2 fragments (§ 5.4.1). Amplicons were purified and cloned into pGEM®-T Easy Vector (§ 5.7.1). Positive clones were amplified in PCR (§ 5.7.3) and sequenced. Sequences were controlled and aligned with the complete sequence of *R.urbis* element. Amplicons whose sequences were found not to match the expected R2 portion were discarded, while those whose sequences corresponded with the expected R2 portion were kept to be used as probes.

Probe labelling

Probes were labelled using DIG-High Prime kit (Roche), with the following steps:

- Add 5 µl template DNA and 11 µl PCR water to a reaction vial

- Denature the DNA by heating in a boiling water bath for 10 minutes and quickly chill in an ice bath
- Mix DIG-High Prime thoroughly and add 4 µl to the denatured DNA
- Incubate for 20 hours at 37 °C
- Stop the reaction by adding 2 µl 0.2 M EDTA pH 8 and heating to 65 °C for 10 minutes
- Add 2.5 µl LiCl and 75 µl cold ethanol
- Keep at -80 °C for 30 minutes
- Centrifuge at 13000 rcf at 4 °C for 15 minutes
- Discard the supernatant
- Add 50 µl cold 70% ethanol
- Empty the tube and dry the pellet under a laminar flow hood
- Suspend in 50 µl 1x TE.

The yield of the labelling reaction was estimated with the help of the graph enclosed in DIG-High Prime manual, showing final labelled probe concentration depending on initial probe concentration and incubation time.

Prehybridization and hybridization

- Place the membrane in a roller bottle with DNA side facing inward, add 50 ml of prewarmed Hybridization buffer and incubate for 2 hours at 65 °C with gentle agitation
- Denature DIG-labelled DNA probe (25 ng/ml of Hybridization buffer) by boiling for 10 minutes and rapidly cooling on ice
- Add probe to prewarmed Hybridization buffer
- Pour Hybridization buffer out of the bottle and add 25 ml of Hybridization buffer + probe
- Incubate overnight at 65 °C under gentle agitation.

Stringent washes

Two-stage stringent washes were performed in order to eliminate undesired hybrids, the first stage (low stringency) requiring high salt concentrations and room temperature, the second stage (high stringency) requiring low salt concentrations and

high temperature. The following protocol was observed:

- Wash the membrane for 5 minutes in 100 ml of a 2x SSC, 0.1% SDS solution, at room temperature
- Repeat the previous step
- Wash the membrane for 15 minutes in 25 ml of a 1x SSC, 0.1% SDS solution, at 65 °C
- Repeat the previous step
- Wash the membrane for 15 minutes in 25 ml of a 0.1x SSC, 0.1% SDS solution, at 65 °C.

Detection

Detection of DIG-labeled DNA was performed with CDP-S*tar* ready-to-use (Roche), with the following steps:

- After stringent washes, rinse the membrane for 1-2 minutes in Washing buffer
- Incubate for 30 minutes in 50 ml Blocking solution
- Incubate for 30 minutes in 25 ml Antibody solution
- Rinse with Buffer 1
- Wash for 15 minutes in 25 ml Washing buffer
- Repeat the previous step
- Equilibrate the membrane for 5 minutes in 100 ml Detection buffer
- Place the membrane with DNA side facing up on a plastic folder and apply drops of CDP-*Star* ready-to-use, until all the surface of the membrane is covered
- Cover the membrane with the folder to spread the substrate evenly and without air bubbles over the membrane
- Incubate for 5 minutes at room temperature
- Seal the membrane in a plastic wrap and incubate for 1-2 hours
- Expose the membrane to a photographic plate for 15-25 seconds
- Develop and fix.

Solutions

Denaturation solution:	0.5 M NaOH, 1.5 M NaCl
Transfer buffer:	1 M sodium acetate, 0.02 M NaOH
20x SSC:	0.3 M sodium citrate, 3 M NaCl; adjust to pH 7.0

Maleic acid buffer:	0.1 M maleic acid, 0.15 M NaCl; adjust to pH 7.5 with NaOH
Blocking solution:	10% Blocking reagent in Maleic acid buffer, autoclaved for 20 minutes at 120 °C
Hybridization buffer:	5x SSC, 0.1% N-Lauroylsarcosine, 0.02% SDS, 10% Blocking solution
Washing buffer:	0.3% Tween in Maleic acid buffer
Antibody solution:	Anti-dioxigenin-AP (vial 4 of DIG-High Prime kit) diluted 1:20000 in Blocking solution
Detection buffer:	0.1 M Tris-HCl, 0.1 M NaCl; adjust to pH 9.5 with HCl

5.11 TERMITE BREEDING

In order to study the transmission of R2 truncate versions from parents to offspring, 20 new *R. urbis* colonies were formed, starting from individual termites collected in Bagnacavallo and preserved in laboratory conditions.

The simplest way to obtain new termite colonies with easily identifiable parents would be to isolate a couple of alates. Unfortunately, *R. urbis* primary reproductives are not able to found new colonies (Springhetti, 1966) and reproduction is carried out entirely by neotenics that can differentiate either from nymphs or from workers (Campadelli, 1987). Reproductives deriving from nymphs were preferable in this study since, compared with reproductives deriving from workers, they differentiate more quickly (Ghesini and Marini, 2009) and are easier to tell from "real" workers. The sex of nymphs was assessed by checking the width of the 7^{th} sternite (§ 1.1). A couple of nymphs (female and male) was placed in each new colony.

In *R. urbis*, nymphs are not able to found new colonies without the help of workers and colonies containing less than 20 workers show very high mortality rates; but workers, especially when present in large numbers, often kill reproductives deriving from nymphs (Ghesini & Marini, 2009). In order to obtain a high survival rate for reproductives, the littlest number of workers compatible with colony survival was chosen, so 20 workers were placed in each new colony.

Each group of termites, formed by a female nymph, a male nymph and 20 workers,

was placed in a Petri dish (Ø 9 cm) containing a substrate of moistened sand and a piece of fir (*Picea abies*) wood. New pieces of wood and water were added when needed.

Two years after their formation, 11 colonies had survived. Surviving colonies were screened for excluding those that were not suitable for further study, that is those where no juveniles were born, those where one or both reproductives deriving from nymphs had died, and those where reproductives deriving from workers had developed, thus making uncertain the attribution of parent-offspring relationships. When two parents and their offspring could be found, they were separated from workers and preserved in 100% ethanol for further analysis.

CHAPTER 6: RESULTS

6.1 THE TRANSPOSABLE ELEMENT R2 IN TERMITES

The sequence of complete R2 elements was obtained for *Reticulitermes urbis*, *R. lucifugus*, and two populations of *Kalotermes flavicollis*. The sequence of the 3' portion of the element, extending over the last 2291 bp and containing the complete RT domain, was determined for *R. grassei* and *R. balkanensis*.
The sequences obtained in this study are deposited in GenBank under accession numbers GU949554 - GU949559.

6.1.1 R2 STRUCTURE IN TERMITES

The essential features of R2 structure in termites are in accordance with what is found in all the R2 elements that were sequenced so far. As expected, the element contains a single ORF, including a reverse transcriptase domain. The sequences of complete elements show that in the 5' portion of the ORF there are three zinc-finger motifs, as in R2 elements belonging to the A clade (Kojima & Fujiwara, 2005), and some conserved residues with similarities to the c-myb DNA-binding motif. In the 3' portion of the ORF there are a fourth zing-finger motif and the endonuclease (EN) domain. The ORF is flanked by untranslated regions (UTRs).
The complete elements of both *Reticulitermes* spp. and *K. flavicollis* begin with a short tandem repeat. In *R. urbis* and *R. lucifugus* all the clones that were sequenced contain a 1-bp deletion of the 28S gene at the 5' insertion of R2. In the case of the two populations of *K. flavicollis*, such a deletion is not apparent, except in a single clone of *K. flavicollis* 1.
In all the species that were considered, R2 ends with a short poly-A tail, formed by three As in *Reticulitermes* spp. and two in *K. flavicollis*. In correspondence of the 3'boundary between the end of R2 and 28S gene, the latter shows a 2-bp deletion, both in *Reticulitermes* spp. and in *K. flavicollis*.

The lengths of the complete element and of its main portions, as well as the relative positions of the main domains, are very similar in the different species of the genus *Reticulitermes*, when compared with other species. A high degree of similarity is

observed also between the two *K. flavicollis* populations (Tab. 5, Fig. 16).

In the case of *K. flavicollis*, the position of the beginning of the ORF is somewhat doubtful, because the amino acid sequence upstream of the zinc-finger motifs contains two methionine residues separated by 41 aa, so that the ORF could begin with either. From now on, it will be assumed that the ORF begins with the first one of these methionine residues.

Some evident differences exist between the elements of *Reticulitermes* spp. and those of *K. flavicollis*. *K. flavicollis* elements are shorter than those of *Reticulitermes* spp. Their ORFs are longer, but their UTRs, particularly the one at the 5' end of the element, are much shorter (Tab. 5, Fig. 16).

Tab. 5 shows the length of complete R2 elements, as well as the location (determined counting from the 5' end of the element) and the length of their main portions. The ORF length in bp includes the stop codon, which is not considered in the length in aa. This distinction does not exist in the case of the RT length, because no stop codon is found at the end of the RT.

The RT of *Reticulitermes* spp. elements, when compared with the RT of *K. flavicollis* elements, shows a 6-bp deletion and a 6-bp insertion, so that the total RT length turns out to be the same in the two genera.

	R2 length (bp)	**ORF location (bp)**	**ORF length (bp)**	**ORF length (aa)**
R. urbis	5020	1138 - 4734	3597	1198
R. lucifugus	5021	1139 - 4735	3597	1198
R. grassei	-	-	-	-
R. balkanensis	-	-	-	-
K. flavicollis 1	4482	335 - 4264	3930	1309
K. flavicollis 2	4474	335 - 4258	3924	1307

	RT location (bp)	**RT length (bp)**	**RT length (aa)**	**5' UTR (bp)**	**3' UTR (bp)**
R. urbis	2764 - 3537	774	258	1137	289
R. lucifugus	2768 - 3541	774	258	1138	289
R. grassei		774	258	-	289
R. balkanensis		774	258	-	289
K. flavicollis 1	2300 - 3073	774	258	334	221
K. flavicollis 2	2297 - 3070	774	258	334	219

Tab. 5 - Length of the complete R2 elements, and length and location of their main portions.

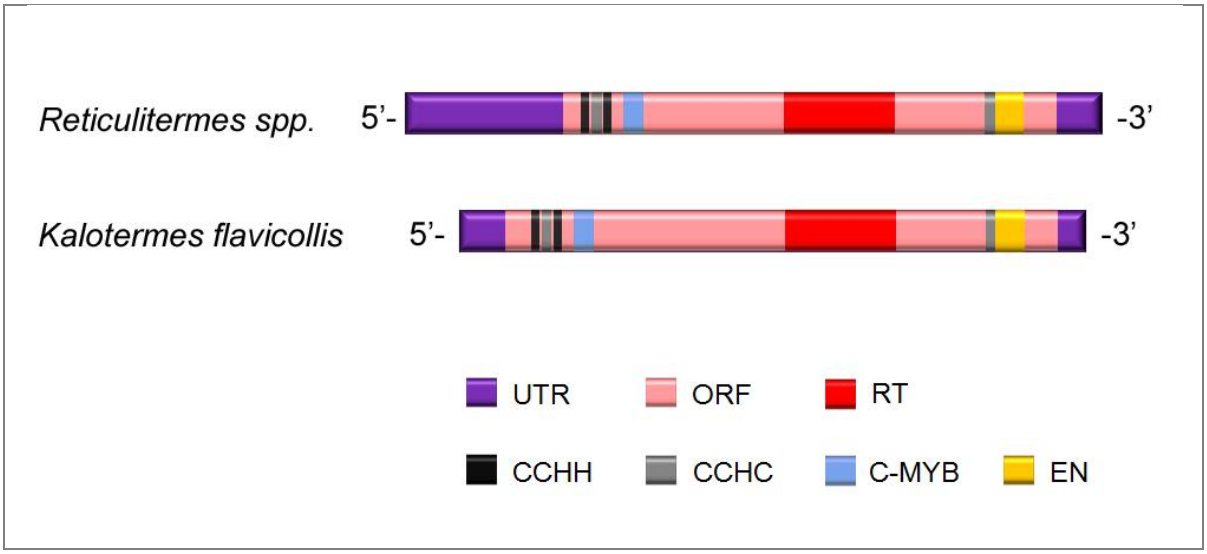

Fig. 16 - R2 structure in *Reticulitermes* spp. and *Kalotermes flavicollis*.

Table 6 shows the amino acid alignment of zinc-finger, c-myb and endonuclease domains in termite elements and, for comparison, in some elements from other species. Highly conserved residues are indicated in bold.

	1st zinc-finger motif	2nd zinc-finger motif
R. urbis	(38)**C**EL**C**GVLLTS-----LQGVRE**H**CHRS**H**	(8)**C**TK**C**DKGFSSYRGICC**H**FSK**C**
R. lucifugus	(38)**C**GL**C**GVLLTS-----LQGVRE**H**CHRA**H**	(8)**C**TK**C**DKGFSSYRGICC**H**FSK**C**
K. flavicollis 1	(59)**C**PG**C**RTLLTS-----EETISS**H**HRRV**H**	(8)**C**YG**C**DSPFMTYRAIKC**H**LPK**C**
K. flavicollis 2	(59)**C**PG**C**TTLLTS-----EETLVS**H**HRRV**H**	(8)**C**YG**C**DAPFMTYRAIKC**H**LPK**C**
N. vitripennis B	(114)**C**PK**C**LQGGTQLLCMGSWELSR**H**ISKE**H**	(10)**C**GA**C**QRRCTTLRSWSC**H**VPH**C**
D.melanogaster	-	-
S. mansoni	(35)**C**LI**C**FAIFPT-----HNILLS**H**ATAI**H**	-

	3rd zinc-finger motif	c-myb motif
R. urbis	(13)**C**SE**C**ERKFDSKRALST**H**ERHM**H**	(44) **W**TEEE (27)**K**TCKQIS
R. lucifugus	(13)**C**SE**C**EREFDSKRALST**H**ERHM**H**	(45) **W**TEEE (27)**K**TCKQIS
K. flavicollis 1	(11)**C**NG**C**TKRFESQRGLSL**H**KRRA**H**	(44)**W**SIDE (27)**K**TNKQVS
K. flavicollis 2	(11)**C**NG**C**TKRFESQRGLSL**H**KRRA**H**	(44)**W**SIDE (27)**K**TNKQVS
N. vitripennis B	(13)**C**EH**C**SLSFDSQIGLSQ**H**ERHV**H**	(20)**W**SDED (27)**R**TGRQVS
D. melanogaster	(27)**C**TV**C**GRSFNSKRGLGV**H**MRSR**H**	(19)**W**SDEE (29)**R**SVEAI**K**
S. mansoni	(17)**C**VL**C**AAAFSSNRGLTQ**H**IRHR**H**	(20)**W**SPFD (31)**R**TAEAV**K**

	4th zinc-finger motif	Endonuclease
R. urbis	**C**RF**C**KDIPETQA**H**VLGL**C**(9)**RHD**(20)**E**(11)**KPD**(12)**D**(17)**K**IRK**Y**	
R. lucifugus	**C**RF**C**KDIPETQA**H**VLGL**C**(9)**RHD**(20)**E**(11)**KPD**(12)**D**(17)**K**IRK**Y**	
R. grassei	**C**RF**C**KDIPETQA**H**VLGL**C**(9)**RHD**(20)**E**(11)**KPD**(12)**D**(17)**K**IRK**Y**	
R. balkanensis	**C**RF**C**KDIPETQA**H**VLGL**C**(9)**RHD**(20)**E**(11)**KPD**(12)**D**(17)**K**IRK**Y**	
K. flavicollis 1	**C**RR**C**HGKPETLG**H**VLGE**C**(9)**RHD**(19)**E**(10)**KPD**(12)**D**(17)**K**IEK**Y**	
K. flavicollis 2	**C**RR**C**HGKPETLG**H**VLGE**C**(8)**RH**E(19)**E**(10)**KPD**(12)**D**(17)**K**IEK**Y**	
N. vitripennis B	**C**RK**C**QGSSETLG**H**ILGR**C**(9)**RHN**(19)**E**(10)**KPD**(13)**D**(17)**K**IGK**Y**	
D.melanogaster	**C**RAG**C**DAPETTN**H**IMQK**C**(9)**RHN**(19)**E**(10)**KPD**(12)**D**(16)**K**INR**Y**	
S. mansoni	**C**RGG**C**AHHETIH**H**ILQH**C**(9)**RHN**(19)**E**(10)**KPD**(12)**D**(15)**K**ISK**Y**	

Tab. 6 - Amino acid alignment of zinc-finger motifs, c-myb motive and endonuclease domain of termite elements, compared with some elements isolated in other species.

The A+T content differs among elements and among different portions of the same element (Tab. 7, Fig. 17). It is always higher in *Reticulitermes* spp. than in *K. flavicollis*.

In *Reticulitermes* spp., the region with the lowest A+T content is UTR at the 5' end of the element, while the region with the highest A+T content is the UTR at the 3' end of the element. The coding region exhibits intermediate values. In *K. flavicollis*, A+T content values are more homogeneous across the different portions of the element. The region with the lowest A+T content the UTR at the 5' end of the element, while the region with the highest A+T content is the RT.

	R2	**ORF**	**RT**	**3' ORF**	**5' UTR**	**3' UTR**	**UTR**
R. urbis	53.4	55.9	54.7	55.0	42.8	63.7	47.0
R. lucifugus	52.9	55.3	54.2	54.7	42.5	63.3	46.7
R. grassei	-	-	55.7	55.6	-	63.7	-
R. balkanensis	-	-	54.8	54.7	-	63.7	-
K. flavicollis 1	46.3	47.1	49.6	47.8	40.3	46.6	40.9
K. flavicollis 2	46.8	47.5	49.6	48.6	38.7	47.0	41.9

Tab. 7 - A+T content (%) of the complete R2 elements and of their main portions. ORF 3' is the portion extending from the beginning of the RT to the end of the ORF. In the column UTR, the two untranslated regions (5' UTR and 3' UTR) are considered as one.

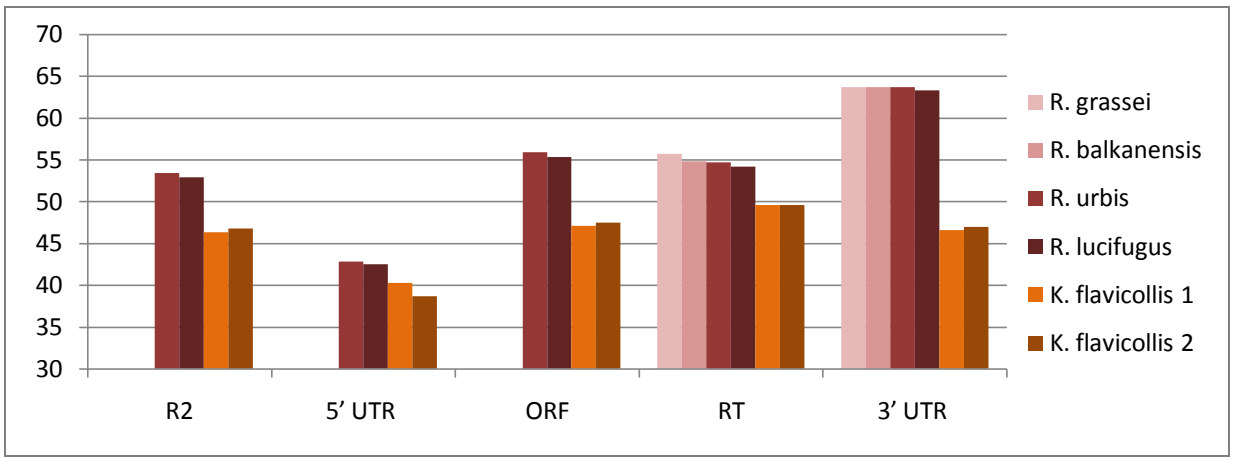

Fig. 17 - A+T content of some portions of termite R2 elements.

For comparison, in Tab. 8 is reported the A+T content of some complete R2 elements representative of three of the four main R2 clades (§ 2.4, R2 phylogeny) and of their main portions. For the other clade (clade B in Fig. 14) no complete sequence is available.

Clade		R2	ORF	RT	3'ORF	5'UTR	3'UTR	UTR
A	*N. vitripennis* B	47.5	46.7	48.4	48.0	46.5	54.5	49.4
A	*D. rerio*	42.8	43.3	46.2	44.3	45.4	41.1	41.5
C	*S. mansoni*	51.1	50.0	48.6	50.3	60.6	-	-
D	*C. intestinalis* A	45.2	44.9	47.9	46.1	44.1	48.1	46.7
D	*F. auricularia*	55.2	51.1	54.1	54.3	56.1	59.8	57.5
D	*D.melanogaster*	52.4	48.4	51.1	51.4	56.5	63.3	59.7

Tab. 8 - A+T content (%) of some complete R2 elements and of their main portions. For a description of the content of the columns, see the caption in Tab. 8.

The element of *R. lucifugus*, in comparison with the one of *R. urbis*, has a 1-bp insertion in the first UTR, a 3-bp insertion and a 3-bp deletion in the ORF. Nucleotide p-distance between *R. urbis* and *R. lucifugus* complete elements is 0.027 ± 0.002. Nucleotide p-distance between the two ORFs is 0.028 ± 0.003. There are 102 substitutions: 22 (21.6%) at the first codon position, 19 (18.6%) at the second, and 61 (59.8%) at the third codon position.

The element of *K. flavicollis* 1, in comparison with *K. flavicollis* 2 element, has two 3-bp insertions in the ORF and a 2-bp insertion in the second UTR. Nucleotide p-distance between the complete elements of the two *K. flavicollis* populations is 0.045

± 0.003. Nucleotide p-distance between the two ORFs is 0.044 ± 0.003. There are 173 substitutions: 54 (31.2%) at the first codon position, 44 (25.4%) at the second, and 75 (43.3%) at the third codon position.

Nucleotide p-distances among the reverse transcriptase domains of termite elements are quite low within the same genus (approximately 2-9%) and much higher (about 50%) between species of the two genera. Among the *Reticulitermes* species, the one that differs more markedly from the others is *R. grassei* (Tab. 9).

	R. urbis	*R.lucifugus*	*R. grassei*	*R. balkanensis*	*K. flavicollis*1	*K. flavicollis*2
R. urbis	-	0.006	0.010	0.005	0.018	0.018
R. lucifugus	0.030	-	0.009	0.006	0.018	0.018
R. grassei	0.083	0.074	-	0.010	0.018	0.018
R. balkanensis	0.021	0.030	0.084	-	0.018	0.018
K. flavicollis 1	0.500	0.495	0.503	0.501	-	0.005
K. flavicollis 2	0.505	0.496	0.503	0.507	0.023	-

Tab. 9 - Nucleotide p-distances among the reverse transcriptase domains of termite R2 elements (below diagonal). Standard errors are shown above diagonal.

The comparison between the amino acid sequences of the ORFs of *R. urbis* and *R. lucifugus* shows the presence of 44 differences, leading to a p-distance of 0.036 ± 0.005. Between the two populations of *K. flavicollis*, the sequences of the ORFs differ for 103 amino acids, corresponding to a p-distance of 0.079 ± 0.007.

Amino acid p-distances between the two genera are not shown for the whole ORF region, because the highly variable N-terminal ends make their alignment quite unreliable.

The amino acid p-distances among the RTs (Tab. 10) exhibit a pattern comparable to what observed in the case of nucleotide p-distances. Within the same genus, p-distances are lower than 7%, while distances between the two genera are about 56-59%. As already seen for nucleotide distances, also in the case of amino-acid distances *R. grassei* is the species that differs more considerably from the other *Reticulitermes* species.

Within the genus *Kalotermes* and between the two genera, amino acid distances are slightly higher than nucleotide distances, while within the genus *Reticulitermes* amino acid distances are usually slightly lower than nucleotide distances.

	R. urbis	*R.lucifugus*	*R. grassei*	*R. balkanensis*	*K. flavicollis*1	*K. flavicollis*2
R. urbis	-	0.009	0.015	0.010	0.031	0.031
R. lucifugus	0.019	-	0.015	0.009	0.031	0.031
R. grassei	0.066	0.062	-	0.015	0.031	0.031
R. balkanensis	0.027	0.023	0.066	-	0.031	0.031
K. flavicollis 1	0.574	0.566	0.570	0.566	-	0.012
K. flavicollis 2	0.590	0.582	0.586	0.582	0.039	-

Tab 10 - Amino acid p-distances (below diagonal) among the reverse transcriptase domains of termite R2 elements. Standard errors are shown above diagonal.

6.1.2 R2 PHYLOGENY

For the reconstruction of R2 phylogeny presented here, termite sequences were added to the alignment of Mingazzini et al. (submitted), with the further addition of the recently isolated sequence from the sciarid *Rhynchosciara americana* (Madalena et al., 2008).

The matrix of p-distances, an excerpt of which is presented in Tab. 11, shows in the comparisons between species belonging to different genera distances ranging approximately from 0.580 to 0.825. The lowest values correspond to comparisons within the *Drosophila* genus (distances from 0.030 to 0.388) and within termite genera (from 0.013 to 0.057 for *Reticulitermes* spp. and 0.128 for the two populations of *F. flavicollis*) .
The p-distances between the sequence of SLACS retrotransposon of *Trypanosoma brucei*, considered as an outgroup, and the other sequences ranges from 0.842 to 0.902.
The overall average of the distances is 0.738.

	1	2	3	4	5	6	7	8	9
1 - *R. urbis*	-								
2 - *R. lucifugus*	0.028	-							
3 - *R. grassei*	0.055	0.057	-						
4 - *R. balkanensis*	0.013	0.021	0.053	-					
5 - *K. flavicollis* 1	0.649	0.642	0.640	0.643	-				
6 - *K. flavicollis* 2	0.680	0.679	0.678	0.678	0.128	-			
7 - *N. vitripennis* B	0.669	0.666	0.662	0.667	0.658	0.657	-		
8 - *D. melanogaster*	0.775	0.770	0.768	0.777	0.786	0.799	0.760	-	
9 - *C. intestinalis* A	0.775	0.774	0.779	0.777	0.768	0.779	0.789	0.671	-
10 - *S. mansoni* A	0.760	0.762	0.764	0.760	0.786	0.795	0.761	0.726	0.713

Tab. 11 - Amino acid p-distances among the 3' portions of the ORF of R2 elements of termites and other animal species.

The dendrograms obtained with Minimum evolution method (Fig. 18) and Bayesian analysis (Fig. 19) show the same terminal branching pattern. The four main R2 clades identified by Kojima and Fujiwara (2005) are well recognizable, as well as their subclades. The addition of tick sequences produces a new subclade in clade D, as already evidenced in Mingazzini et al. (submitted).
Minor differences occur in the internal branching pattern of a few subclades, in correspondance of nodes with comparatively low support values. The main differences are found in the basal branching pattern, i.e. in the relationships among the main clades. The support values of several basal nodes are low.

The sequences of termite elements are included in subclade A2, together with those from the cockroach *Blattella germanica*, the hymenopterans *Apis mellifera* and *Nasonia vitripennis* (element B), and the spider *Hasarius adansoni*. Sequences from *Reticulitermes* spp. and from *Kalotermes flavicollis* form two distinct groups. In the Minimum evolution tree, the *Reticulitermes* group is basal to the A2 subclade, while in the bayesian dendrogram it is the sister clade of the group of *K. flavicollis* sequences.
Phylogenetic relationships among the R2 sequences of *Reticulitermes* spp. do not correspond to those existing among the host species (§ 1.9).

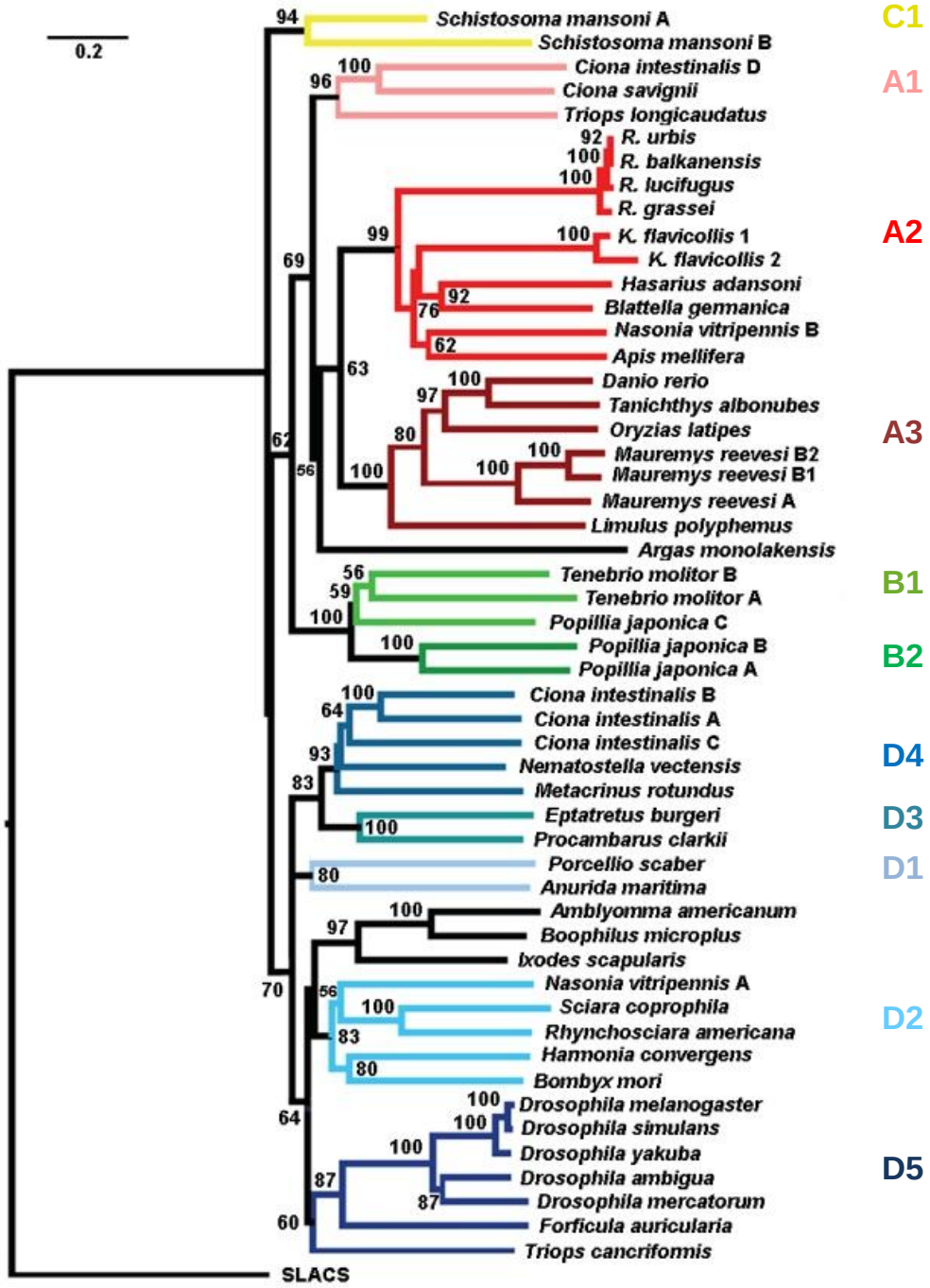

Fig. 18 - Minimum Evolution dendrogram (SBL=23.65). Bootstrap values >50 are shown near the corresponding nodes. The scale bar indicates genetic distance. Acronyms on the right refer to the subclades identified in Kojima & Fujiwara (2005).

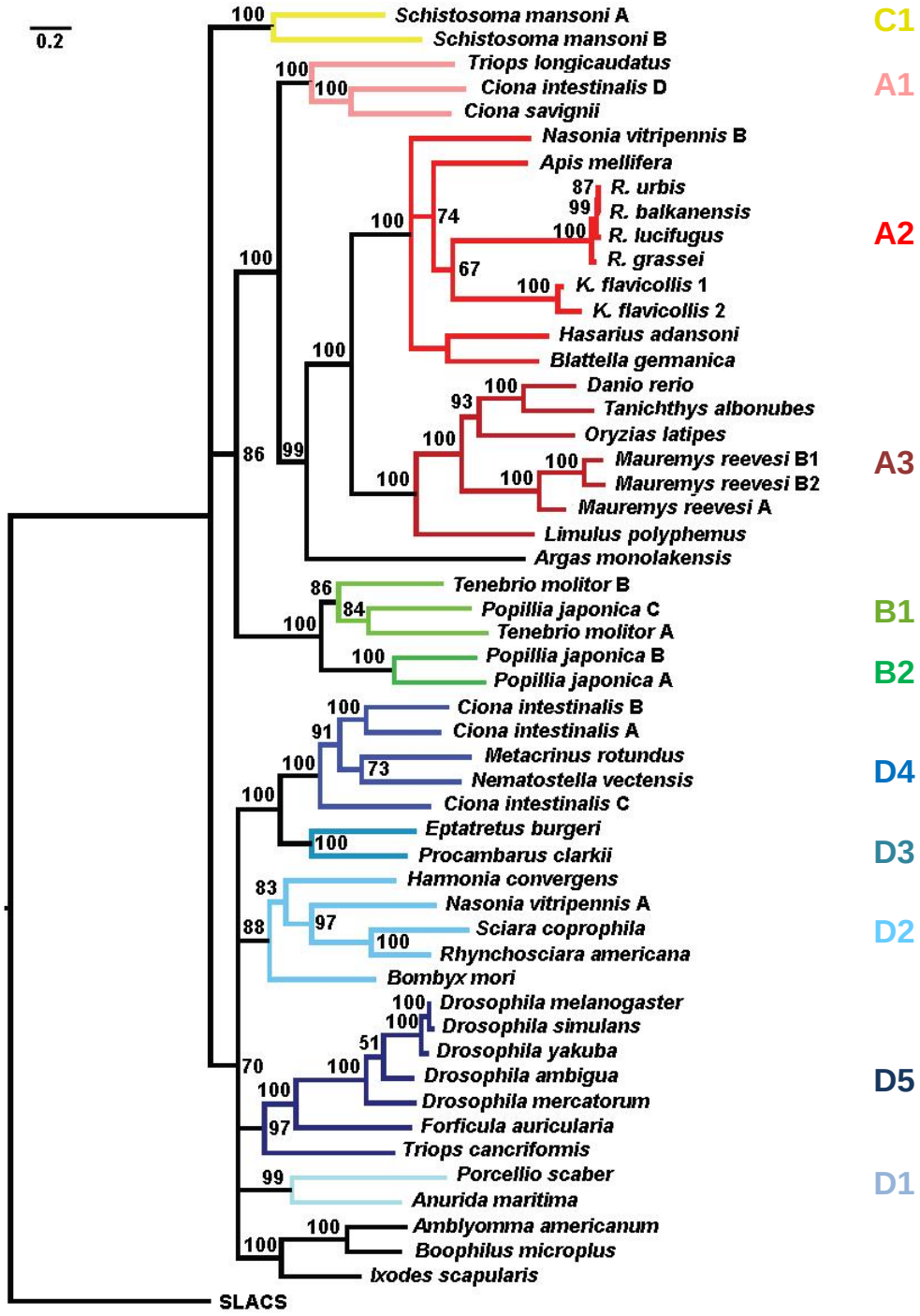

Fig. 19 - Bayesian dendrogram (-lnL=47107.91). Posterior probability values >50 are shown near the corresponding nodes. The scale bar indicates genetic distance. Acronyms on the right refer to the subclades identified in Kojima & Fujiwara (2005).

6.1.3 R2 TRUNCATED VARIANTS

The analysis of truncated variants was carried out on 51 individuals belonging to four *R. urbis* colonies. A total of 255 PCR reactions and 20 Southern blots were performed. The first colony was a mature colony, composed by at least 1000 individuals, 14 of which (10 workers and four soldiers) were considered for the analysis. The other three were specifically bred in order to analyze the progeny of known parents. For each one of them, both parents and their offspring (9-12 juveniles) were considered for the analysis (§ 5.1).

On the whole, 13 truncated variants were identified (Fig. 20), whose approximate lengths are shown in Tab. 12. The complete element and nine of the truncated variants (the eight shortest variants and the 4365 bp one) are present in all the individuals. The number of truncations that are found in each specimen ranges from nine to 13 (Fig. 21).

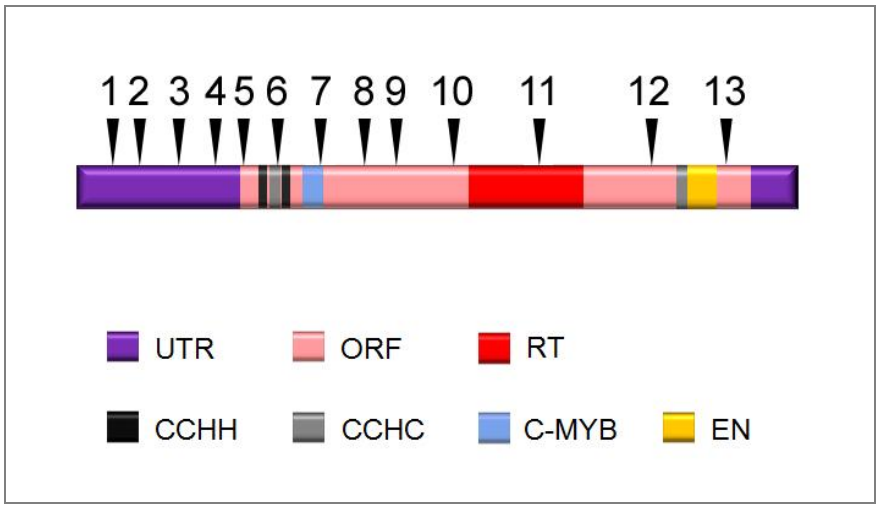

Fig. 20 - *R. urbis* element and its truncations. Each arrowhead indicates the position of a truncation. The numbers above the arrowheads refer to those in the first column of Tab. 12.

N.	Length of deleted portion (bp)	Length of truncated variant (bp)	Colonies
1	280	4740	3, 4
2	450	4570	4
3	655	4365	1, 2, 3, 4
4	930	4090	1, 2, 3, 4
5	1140	3880	1, 3, 4
6	1425	3595	1, 2, 3, 4
7	1765	3255	1, 2, 3, 4
8	2055	2965	1, 2, 3, 4
9	2240	2780	1, 2, 3, 4
10	2545	2475	1, 2, 3, 4
11	3260	1760	1, 2, 3, 4
12	3940	1080	1, 2, 3, 4
13	4500	520	1, 2, 3, 4

Tab. 12 - Average lengths of R2 deletions, average lengths of the corresponding truncation variants, and colonies where each variant is found. The numbers in the first column refer to Fig. 20.

All the individuals from the mature colony (colony 1 in Fig. 21) exhibit the same truncation profile. There are 11 truncated variants, with no difference between workers and soldiers.

In each of the three colonies formed by a parental couple and their offspring, the two parents share the same truncation profile. The progeny shows the same pattern found in the parents, except in colony 2, where two juveniles lack a truncation variant that is present in their parents.

The truncation profile shows some degree of differentiation among the colonies. Ten variants are shared by all the colonies, two variants are shared by two colonies, and one variant is present in a single colony. Compared with the mature colony, the parents of colony 2 lack a variant, while those of colonies 3 and 4 have one and two additional truncations, respectively.

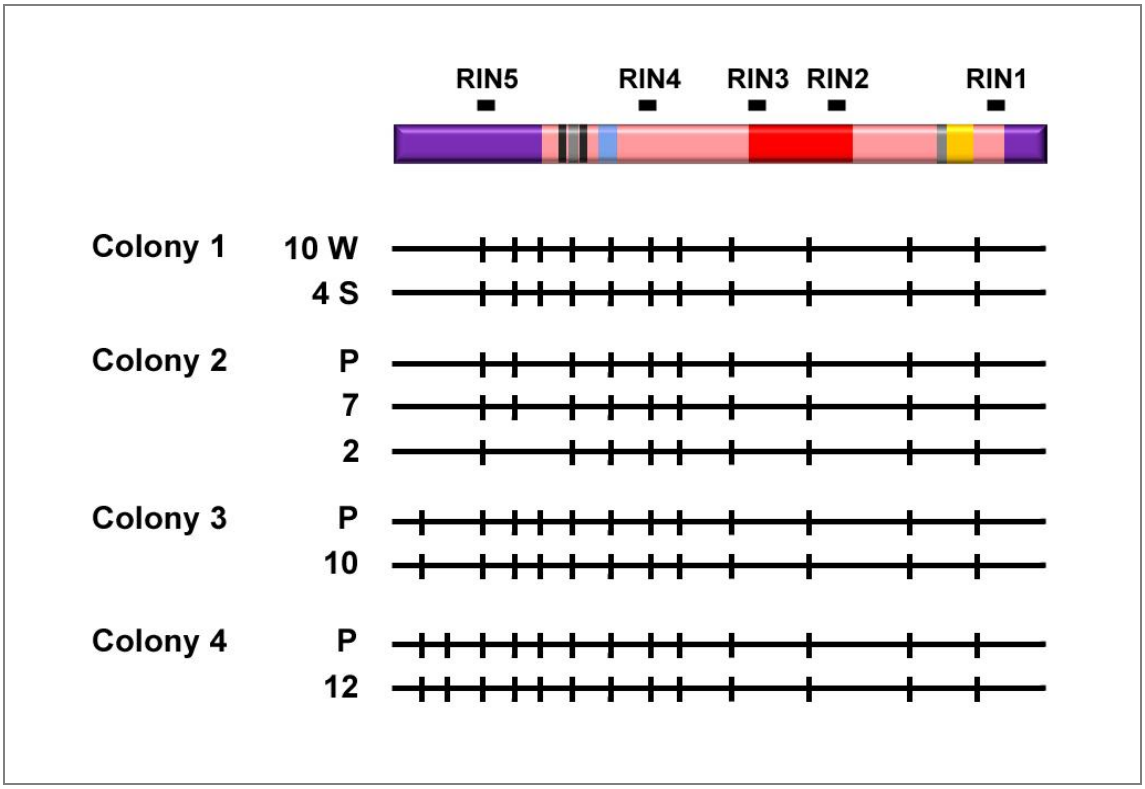

Fig. 21 - R2 truncation profile in *R.urbis* colonies (1-4). In the upper part of the image are shown the complete element and the positions of the probes used for Southern blots (RIN1, ... RIN5). In the lower part of the image are shown the truncation profiles of the colonies, each vertical bar indicating the position of a truncation. Numbers flanking each line represent the number of individuals sharing the corresponding profile. W = workers, S = soldiers, P = parents (2 individuals).

6.2 PHYLOGENY OF *RETICULITERMES* POPULATIONS FROM CYPRUS

A 684 bp portion of the mitochondrial gene COII, encoding for 228 amino acids, and a 504 bp portion of the mitochondrial gene 16S were sequenced in 14 populations of Cyprian termites belonging to the genus *Reticulitermes*. The sequences obtained in this study are deposited in GenBank under accession numbers GU993821-GU993840.

For the gene COII, 12 haplotypes are present. Samples 4B, 5, and 9 share the same haplotype (C5 in Fig. 22 and Tab. 13), while all the other samples are characterized

by a distinct haplotype, differing from the most common one for 1-5 substitutions (Fig. 22). There are 18 variable sites (23.4% at the first codon position and 76.6% at the third codon position), and the number of substitutions among populations ranges from zero to six. There are neither insertions nor deletions. The maximum p-distance between populations is 0.009 ± 0.004; the average p-distance among all the populations is 0.005 ± 0.003. The average A+T content is 62.6%.

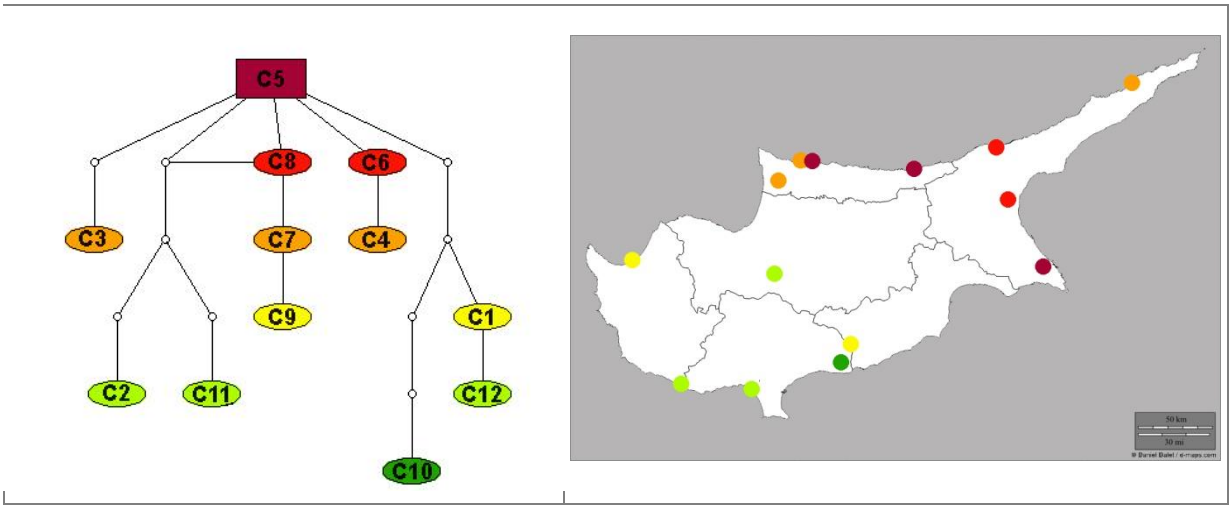

Fig. 22 - To the left, COII haplotype parsimony network (white dots represent hypothetical missing haplotypes). To the right, geographical distribution of the haplotypes.

For the gene 16S, seven haplotypes are present. Samples 1, 4A, 4B, 5, 6, 7, 8, 9, and 13 share the same haplotype (R1 in Tab. 13), while the other samples have each a distinct haplotype, that differs from the most common one by a single substitution. There are five variable sites, and the number of substitutions among haplotypes ranges from zero to two. There are neither insertions nor deletions. The maximum p-distance between populations is 0.004 ± 0.003, while the average p-distance among all the populations is 0.001 ± 0.001. The average A+T content is 62.9%.

Populations 4B and 9 have the same haplotype both for COII and 16S genes, while all the other populations have a distinct combined haplotype (Tab. 13).

N.	Locality	COII haplotypes	16S haplotypes	Combined mitotypes
1	Polis	C1	R1	M1
2	Kakopetria	C2	R2	M2
3	Karpasei	C3	R3	M3
4A	Vasileia A	C4	R1	M4
4B	Vasileia B	C5	R1	M5
5	Charkeia	C5	R4	M6
6	Kaplica	C6	R1	M7
7	Rizokarpaso	C7	R1	M8
8	Trikomo	C8	R1	M9
9	Sotira	C5	R1	M5
10	Asgata	C9	R5	M10
11	Pentakomo	C10	R6	M11
12	Episkopi	C11	R7	M12
13	Petra Tou Romiou	C12	R1	M13

Tab. 13 - Haplotypes of Cyprian populations: COII, 16S and combined (COII+16S). Numbers in the first column refer to Fig. 15 (Materials and Methods).

The reconstruction of phylogeny presented here is based on the alignment of the combined sequences (COII+16S) of Cyprian populations with the sequences considered in Velonà et al. (submitted).

The topology of the tree obtained with the Maximum Parsimony method (TL = 751, CI = 0, RI = 0.886, RC = 0.488, HI = 0.449, bootstrap values after 500 replicates), is in substantial accordance with what found in Velonà et al. (submitted) (Fig. 23).

All the populations from Cyprus end up in a single monophyletic clade, whose internal structure is not well supported.

Basal to the Cyprus clade is the population from Lussino (Croatia). The sister clade of the group Cyprus + Lussino includes the populations from Ankara (Turkey), Stagira, Xanthi, Messimvria, and Komotini (Thrace, Greece). Together with the populations from Crete, Amorgous, Chalcidice peninsula, and Aria (Peloponnesus), the group including the populations from Cyprus, Lussino, Ankara and Thrace form a well supported "Aegean clade".

The divergence time of the Cyprian clade from the group of populations from Thrace is estimated to be about 2 million years ago.

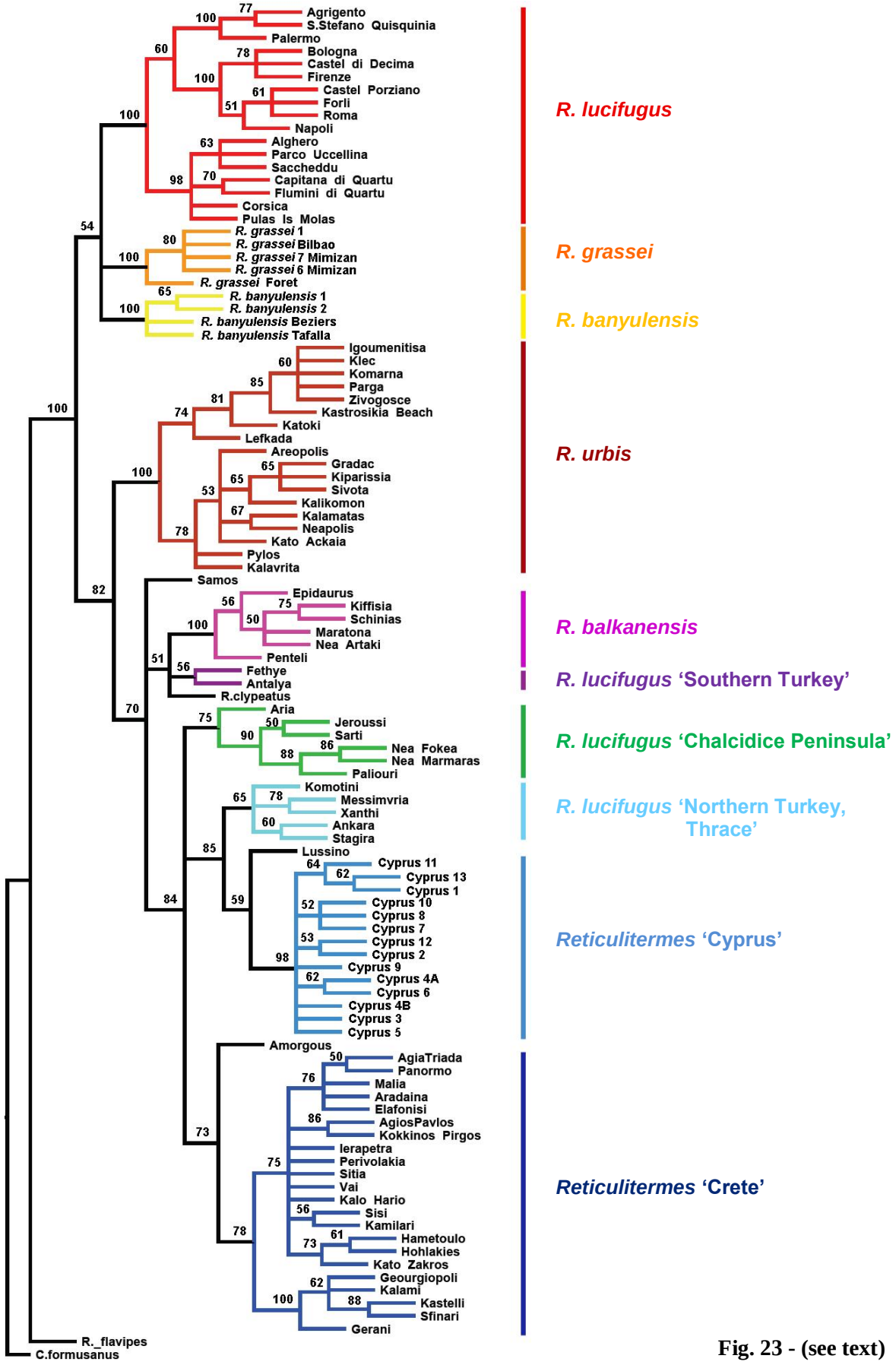

Fig. 23 - (see text)

CHAPTER 7: DISCUSSION

7.1 THE TRANSPOSABLE ELEMENT R2 IN TERMITES

This work aimed to isolate and characterize the retrotransposon R2 in some species of European termites. Complete R2 sequences were obtained for *Reticulitermes urbis*, *R. lucifugus*, and two populations of *Kalotermes flavicollis*, in order to determine the structure of the element in the two genera. For *R. grassei* and *R. balkanensis*, the sequencing was limited to the 3' portion of the element, including the C-terminal portion of the ORF. This region is the most useful for making comparisons among different R2 elements and for phylogenetic analysis.

R2 structure

Complete R2 elements isolated in *R. urbis* and *R. lucifugus* are 5020 and 5021 bp long, respectively. This places them among the longest complete R2 elements sequenced so far, their length being exceeded only by the elements of *Danio rerio* (5183 bp) and *Nasonia vitripennis* (5028 bp). The elements of the two populations of *K. flavicollis*, with 4482 and 4474 bp respectively, can be considered of average length.

The essential structural features are the same in all the elements that were obtained in this study and correspond to those that are found in all known R2 elements: there is a single ORF, flanked by untranslated regions, that contains a reverse transcriptase domain, an endonuclease domain, and DNA-binding motifs (Burke et al., 1999; Yang et al., 1999; Eickbush & Jamburuthugoda, 2008). In particular, termite elements share the presence of three zinc-finger motifs in the N-terminal portion of the ORF. This feature is a clue of their belonging to the R2 A clade (Kojima & Fujiwara, 2005), a hypothesis confirmed by phylogenetic analysis.

The ORFs of termite elements are quite long compared with those of other R2 elements. In particular, the elements of the two populations of *K. flavicollis* have the longest ORFs found so far, exceeding by 43 and 45 aa, respectively, the length of the ORF of *Nasonia vitripennis* B element. In *K. flavicollis* elements, ORFs might actually be 42 aa shorter: There are two methionine residues, separated by 41 aa,

upstream of the zinc-finger motifs, so that the ORF might begin with either. The location of the exact beginning of the ORF is not always straightforward in R2 elements. The presence of two or more methionine residues upstream of the zinc-fingers is quite common. For example, this is the case of the elements of *Drosophila mercatorum*, *Limulus polyphemus*, and *Danio rerio*. In some other instances, such as in the elements of some *Drosophila* species, no methionine can be found upstream of the zinc finger motifs. This suggests that the translation initiates upstream of the stop codon preceding the ORF, and that such stop codon is bypassed (George & Eickbush, 1999).

Termite elements have a 258 aa long RT. In the other complete R2 elements sequenced so far, the RT ranges in length between 258 (*Porcellio scaber*) and 268 aa (*Ciona intestinalis* B).

The complete elements of both *Reticulitermes* spp. and *K. flavicollis* begin with a short tandem repeat. Termite R2 elements end with a poly-A tail, formed by three As in *Reticulitermes* spp. and two in *K. flavicollis*. Poly-A tails are present in many R2 elements, owing to the capability of the RT of adding nontemplated nucleotides before the reverse transcription actually begins (George et al., 1996). In some cases, they are quite extended, for instance the element A of *Ciona intestinalis* ends with a sequence of 41 As.

The insertion site of termite elements is the same as in the majority of the other organisms studied so far, i.e. between the bases G and T of the 28S gene sequence 5'-AAGGTAGC-3'. In correspondence of R2 5' insertion, a 1-bp deletion of the 28S gene is observed in *Reticulitermes* spp., while in *K. flavicollis* no deletions are apparent, except a 1-bp deletion in a single clone. In correspondence of R2 3' insertion, there is a 2-bp deletion of the 28S gene. Such deletions are caused by R2 insertion when the cleavage of the top strand is shifted in relation to the cleavage of the bottom strand (George et al. 1996).

The nucleotide p-distance between *R. urbis* and *R. lucifugus* elements is similar in the different R2 portions (about 0.030), whereas the distance between the two *Kalotermes* elements is lower in the RT than in the whole element or in the ORF (about 0.045 and 0.023, respectively). Both in *Reticulitermes* spp. and in *K. flavicollis*, amino acid p-distances are higher than the corresponding nucleotide p-distances, owing to the high proportion of substitutions in the first and second codon

positions.
Amino acid p-distances are slightly higher than the corresponding nucleotide p-distances also in the comparison between *Drosophila melanogaster* and *D. mercatorum* elements, and between *Ciona intestinalis* elements A and B, both in the ORF and in the RT. Also in these cases, a comparatively high proportion of substitutions in the first and second codon position are present.
The level of amino acid sequence conservation varies in the different regions of the ORF (Burke et al., 1999), so that an high overall amino acid variability is compatible with the presence of highly conserved regions.

R2 phylogeny

Minimum evolution and bayesian trees obtained in this work identify the same R2 clades and subclades found by Kojima & Fujiwara (2995), with differences in support values probably due to the addition of new sequences and to the use of different methods and settings for the analysis.
Dendograms are concordant in placing termite elements in R2 clade A, in particular in subclade A2, together with the elements from *Blattella germanica, Apis mellifera*, *Nasonia vitripennis* (element B) and *Hasarius adansoni*.
The element from *Forficula auricularia*, the other heterometabolous insect in which R2 has been isolated, belongs to the D clade, so that in heterometabolous insects at least two R2 clades are present.

Contrary to what is observed in the genus *Drosophila* (Lathe et al., 1997), the phylogeny of R2 in *Reticulitermes* spp. does not correspond to the phylogeny of the host species.
Discrepancies between R2 and hosts phylogenies are a common occurrence. In transposable elements, similar situations can be explained either by horizontal transfer of by the existence of paralogous lineages. In the case of R2, the second explanation seems to be more adequate (Kojima and Fujiwara, 2005). It is currently believed that the discrepancies between R2 and hosts phylogeny are due to the ancient origin of the element, preceding the divergence of protostomes and deuterostomes, and to the differential extinction of R2 lineages in host lineages.

R2 truncated variants

The truncation variants generated during transcription can be used to monitor R2 activity (Pérez-Gonzales & Eickbush, 2001). In fact, the appearance of a new variant shows that a new transposition event has occurred. The disappearance of a preexisting variant indicates that the turnover mechanisms of ribosomal DNA have eliminated one or more inserted units. A limitation of this approach is that it does not score a new insertion if it has the same length as a preexisting variant. This happens more frequently for the insertions of complete elements, while the generation of truncated copies of the same length of preexisting ones is not a frequent event. Moreover, if a length variant is present in more than one copy, the elimination of a copy is not detected (Pérez-Gonzalez & Eickbush, 2003).

The analysis of truncated variants of *R. urbis* element was carried out on a portion of a mature colony, containing workers and soldiers, and on three incipient colonies, formed each by a parental couple and its offspring.
In the mature colony, all individuals shared the same truncation profile. No difference between workers and soldiers was found. This is not an unexpected result, because soldiers develop from the same line as workers.
In each of the three colonies formed by a parental couple and their offspring, the two parents shared the same truncation profile, which was found also in their progeny. The only exception were two juveniles of colony 2, that lacked a truncation variant present in their parents. The loss of the same variant in more than one individual of the filial generation can be explained assuming that such loss occurred at an early stage of gametogenesis. No new insertions were detected, so that there was no evidence of recent R2 activity.

In *Drosophila simulans*, several lines have been found in which virtually all individuals differed in their R2 insertion profiles (Pérez-Gonzalez & Eickbush, 2001; Zhang & Eickbush, 2005). A high variability was found also in *Triops cancriformis* (Mingazzini et al., submitted). On the other hand, there are also instances of scarce variability. In several lineages of *Drosophila simulans* all individuals showed the same collection of truncated variants (Zhang & Eickbush, 2005).
The high variability of truncation profiles in a line or in a population is considered as

a hallmark of active retrotansposition (Pérez-Gonzalesz & Eickbush, 2001), so that the absence of variability observed in the mature colony of *R. urbis* could be the consequence of the element being inactive. Another possible explanation of this homogeneity is that the peculiar reproductive biology of termites reduces the genetic variability inside each colony.

7.2 PHYLOGENY OF *RETICULITERMES* TERMITES

The first molecular data on termites of *Reticulitermes* termites from the island of Cyprus were obtained in this work. Sequences of portions of the mitochondrial genes COII and 16S were analyzed for 14 populations sampled all over the island.
The addition of Cyprian sequences to the alignment considered in Velonà et al. (submitted) does not change the topology of the dendrogram.
All the Cyprian populations form a single monophyletic clade. Contrary to what might be expected considering the geographic location of Cyprus, the *Reticulitermes* populations that show the highest affinity with Cyprian ones are not those from the south of Turkey, but those from Lussino (Croatia), Ankara (Northern Turkey), Stagira, Xanthi, Messimvria, and Komotini (Thrace, Greece). The population from Lussino, geographically distant from the others of its group, is probably the result of an introduction by man.
Together with the populations from Crete, Amorgous, Chalcidice peninsula, and Aria (Peloponnesus), the group including the populations from Cyprus, Lussino, Ankara and Thrace form what could be called an "Aegean clade".

Despite its proximity to the nearby mainland (75 km to the Turkish coast, about 100 km to the coasts of Syria and Lebanon), Cyprus is one of the most biogeographically isolated Mediterranean islands (Simmons, 1999). Whether it has ever been connected to the nearby mainland is still a debated question. The island is separated from Turkey, Syria, and Lebanon by deep waters (Adana, Cilicia and Latakia Basin), so that it is probable that, even in periods characterized by a low sea level, no land bridges existed between Cyprus and the mainland.

Zoogeographic evidence is controversial. The very oligotypic Quaternary fauna of the island seems to confirm the fact that Cyprus was characterized by a very low rate of faunistic immigration from the nearest mainland. Many reptile forms inhabiting the opposite coast of both south-eastern Turkey and Syria-Lebanon are absent from Cyprus (Corti et al., 1999). On the other hand, in the case of the amphibian genus *Rana* and of the reptilian genus *Ablepharus*, Cyprian populations have a strong affinity with Syrian populations (Poulakakis et al., 2005; Lymberakis et al., 2007), suggesting that a connection between the island and the mainland might have existed.

The colonization of an island by termites might occur through the transport of primary reproductives or portions of colonies. Termite primary reproductives are not strong flyers and usually do not autonomously fly more than a few hundred meters from their colony of origin, but they can be carried to longer distances by wind. Portions of termite colonies can travel inside logs or branches floating on water.
A possibility that has to be taken into account is the unintentional introduction of termites by man, through the transport of wooden material or soil.

The dating of the split of the Cyprian clade from the clade including populations from Thrace and Northern Turkey (2 million years ago), if confirmed, would indicate that the immigration of an ancestral termite population dates back to that period.
The possibility of an introduction by man after his settlement on the island (dating back to the 10^{th} millennium B.C.) appears less probable, because, in order to explain the diversity of Cyprian termites, multiple introduction events of related termite populations would have to be assumed.

The high level of similarity between the populations from Cyprus and those from Thrace and Northern Turkey, in opposition to the comparatively high level of divergence with the populations of the nearby lands (Southern Turkey and Syria-Lebanon), might be explained in at least two ways.
The first possibility is that the termites that colonized Cyprus came from the north of the Aegean Sea. If this is the case, it must be assumed that the waters separating the island from the mainland acted as a barrier that remained effective also when the sea level was low. The problem with this hypothesis is that it does not explain how the termites coming from the Aegean Sea succeeded to cross the deep waters that

separate the South-Aegean islands from Cyprus.

The second possibility is that Cyprus was colonized by termites coming from the nearby lands. In this case, it must be assumed that the Northern-Turkish taxon was once distributed also in the south of Turkey. Termite sampling in Turkey is still poor, so the possibility that populations with affinity with those of Cyprus be still present in the south of Turkey should not be completely ruled out.

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IMAGES

If not differently stated in the caption, images are by the author.

APPENDIX

I - Ghesini S. and Marini M. (2009) – Caste differentiation and growth of laboratory colonies of *Reticulitermes urbis* (Isoptera, Rhinotermitidae). *Insectes Sociaux*, 56: 309-318.

II - Ghesini S., Messenger M. T., Pilon N., and Marini M. – First report of *Reticulitermes flavipes* (Isoptera, Rhinotermitidae) in Italy. Submitted.

III - Velonà A., Ghesini S., Luchetti A., Marini M., and Mantovani B. – Starting from Crete, a phylogenetic re-analysis of the genus *Reticulitermes* in the Mediterranean area. Submitted.

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RESEARCH ARTICLE

Caste differentiation and growth of laboratory colonies of *Reticulitermes urbis* (Isoptera, Rhinotermitidae)

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Abstract A total of 280 laboratory colonies of *Reticulitermes urbis* were raised for 3 years, in order to observe caste differentiation and overall colony growth, and to investigate whether the initial composition (0, 2 or 4 nymphs; 0, 5, 10, 20 or 50 workers) affected colony survival, population growth and other colony dynamics. We also wanted to determine the minimum number of individuals needed to establish a vital colony. As a result, every colony beginning with less than 20 workers died within 60 weeks, while colonies beginning with at least 20 workers survived until the end of the tests, with varied survival rates dependent upon the initial size and the time of formation (summer or autumn). The number of nymphs did not affect colony survival and colony growth. Reproductives developed from nymphs within 2 weeks and later from workers. Many of the reproductives derived from nymphs were killed by workers. The majority of the colonies contained two reproductives 3 years after initiating the tests. In addition, a few individual workers were still alive at the end of the 3-year tests. At the end of the tests, the proportion of soldiers ranged anywhere from 5.23 to 7.69% of the total population. The number of viable "juveniles" from each colony was relatively low throughout the tests and the overall population growth was not sufficient enough to replace dead workers or increase the colony size considerably. These results indicate that the potential to establish viable and sustainable colonies for groups of *R. urbis* composed of 5–50 workers is very low.

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Introduction

Reticulitermes spp. (Isoptera, Rhinotermitidae) are subterranean termites that can be found both in natural and in urban areas of the Holarctic Region. In nature, termites play a crucial ecological role, breaking down and releasing energy stored in cellulose back into the environment. However, in urban areas, termites are considered pests since they are responsible for causing extensive damage to wooden structures, furniture and libraries (Bordereau et al., 2002).

Previous studies on *Reticulitermes* spp. biology have clarified many aspects concerning caste differentiation and caste ratios (Light and Weesner, 1955; Buchli, 1956; Howard and Haverty, 1980, 1981; Haverty and Howard, 1981), reproductive strategies (Howard et al., 1981; Matsuura and Nishida, 2001; Roisin, 2001; Matsuura et al., 2002, 2004; Hayashi et al., 2003), colony structure (Bulmer et al., 2001; DeHeer et al., 2005) and colony dynamics (Watanabe and Noda, 1991; Thorne et al., 1999; Long et al., 2003; Grube and Forschler, 2004; Long et al., 2007). The cryptic nature of subterranean termites prevents through biological investigation under field conditions. Therefore, behavioural and developmental studies need to be conducted and observed in the laboratory.

Reticulitermes urbis was recently identified (Clément et al., 2001) and described (Bagnères et al., 2003; Uva et al., 2004). Before any formal descriptions occurred, *R. urbis* was mistaken for *R. lucifugus* (Rossi) due to morphological similarities (Campadelli, 1987, 1988; Marini and Ferrari, 1998). *R. urbis* native distribution

includes the Balkan Peninsula (Marini and Mantovani, 2002; Luchetti et al., 2004, 2005, 2007; Austin et al., 2006). In Italy and southern France, it is primarily found in urban areas where it was most likely introduced by man.

The purpose of the current study was to investigate colony growth and caste differentiation in laboratory colonies of *R. urbis* initially composed of small groups of individual termites. Laboratory studies on reproduction, colony development and caste composition are often carried out with colonies founded by two or more primary reproductives (Thorne et al., 1999; Matsuura and Nishida, 2001; Long et al., 2003; Grube and Forschler, 2004; Long et al., 2007). This was not suitable in this study, since in peninsular Italy reproduction of *Reticulitermes* spp. is carried out entirely by neotenics that can differentiate either from nymphs or from workers, while the primary reproductives seem unable to found new colonies (Jucci, 1924; Springhetti, 1966). Therefore, nymphs, workers or a combination of both were used to initiate colonies in this study.

We performed three tests: Test 1 aimed to determine whether nymphs could found new colonies without the help of workers and whether the number of nymphs could influence colony survival and growth rates; Test 2 aimed to investigate whether, in the absence of nymphs, a small number of workers could differentiate reproductives and, if so, how the initial absence of nymphs could influence colony growth; Test 3 aimed to determine whether the initial number of workers could influence survival and growth rates.

When referring to different caste members that can be found in a termite colony, it is not always clear what terms should be used, even though valuable suggestions for the standardization of the terminology have been made (Thorne, 1996; Myles, 1999; Lainé and Wright, 2003). For the purposes of this study, it was convenient to distinguish between the workers that were placed in the laboratory colonies at the beginning of the tests by labelling them as "workers," and the individuals that were born after the initiation of each test by labelling them as "juveniles."

Materials and methods

Subsamples from *R. urbis* colonies were collected from infested wood in Bagnacavallo (Ravenna, northern Italy) in the summer of 2003 and maintained in the laboratory in plastic buckets until they were used for the tests.

Three tests were conducted, for a total of 280 laboratory colonies, as shown in Table 1. For each test, termites were extracted from the wood, taking care not to hurt them. Visibly healthy nymphs with long wing buds (pads) and workers were chosen for each replication. The sex of nymphs was not a priority to this study and was therefore not determined in order to reduce excessive handling. Each group of termites was placed in a Petri dish (ϕ 9 cm) containing a mixture of granite sand (granulometry 1 mm)/vermiculite (1 mm)/water (vol 1/1/1) and a block of poplar wood (*Populus alba*) and filter paper Whatman® No. 1 as food. The dishes were sealed along the edge with insulating tape to prevent desiccation. Colonies were kept at room temperature, varying anywhere from 20°C in the winter to 26°C in the summer, with relative humidity of 95–97%.

Table 1 Starting period, composition and number of replicates of the colonies considered in this study

	Start	Colony types	Replicates
Test 1	29 August 2003	4n + 20w	40
		2n + 20w	40
		4n	40
		2n	40
Test 2	06 November 2003	4n + 20w	20
		20w	20
Test 3	13 November 2003	4n + 50w	20
		4n + 20w	20
		4n + 10w	20
		4n + 5w	20

n nymphs, *w* workers

Each replication was inspected at least two times per month by looking through the walls of the dishes and counting individual caste members. Presoldiers were counted as soldiers. Colonies were inspected more frequently during the first few months, when individuals were differentiating between castes and during egg-laying periods. Additional water and food were added when needed. After 3 years, the dishes were opened, the colonies dismantled and the termites counted.

All the data presented in this paper, with the exception of colony survival rates, are computed considering only colonies with surviving members. Differences between the distributions of the number of individuals were evaluated using the Kolmogorov–Smirnov test (KS). Differences in the ratio between different variables were evaluated using the Mann–Whitney test (MW) when comparing two samples and the Kruskal–Wallis test (KW) when comparing more than two samples. Independence between variables was tested with Chi-square test (χ^2) or, whenever one or more expected values were smaller than 10, with Fisher's exact test (FE). *P* values of MW and FE were calculated as two-tailed. Statistical analysis of the data recorded after a year for 4n + 5w colonies of Test 3 and after 3 years for all the types of Tests 2 and 3 was limited because of the small sample sizes caused by low survival rates.

Results

A great variability in behaviour and colony dynamics was observed among colonies, even among those of the same test and of the same type.

Nearly all the nymphs (95.18%) moulted within the first 8 weeks, transforming into reproductives with light brown pigmentation and unsclerotized wings (Fig. 1). These non-functioning wings were quickly lost, being either shed or chewed off by workers. Workers often showed an aggressive behaviour towards the reproductives derived from nymphs, many of which they mutilated, nibbled and slowly ate, starting from the tip of the abdomen. Reproductives reacted very passively to these aggressive behaviours and tended to move away or aggregate with other reproductives. No aggression towards reproductives was observed after the first 12 weeks, when their survival rates were as shown in Fig. 2. Numerous colonies did not contain any reproductives that had derived from nymphs after the first 12 weeks (45.00% of 2n + 20w colonies and 15.00% of 4n + 20w colonies in Test 1, 15.00% of 4n + 20w colonies in Test 2, 60.00% of 4n + 50w colonies, 20.00% of 4n + 20w colonies, 10.00% of 4n + 10w colonies and 22.22% of 4n + 5w colonies in Test 3). Reproductives that were not killed in the first 12 weeks usually remained alive for the duration of the tests.

From 7 to 26 weeks after the beginning of the tests, reproductives derived from workers (ergatoids) began to appear (Table 2). The transition from a worker to a reproductive seemed to occur within a single instar and involved the progressive lengthening and lateral compression of the abdomen, the increasing pigmentation of the body and the appearance of four longitudinal brown stripes on the head. These reproductives could be distinguished by those derived from nymphs by the absence of wings and wing buds. Workers showed no aggressive behaviour towards ergatoids.

No worker–worker aggression was observed during this study, and cannibalism was rarely observed. Over time, the number of surviving workers declined in all colony types.

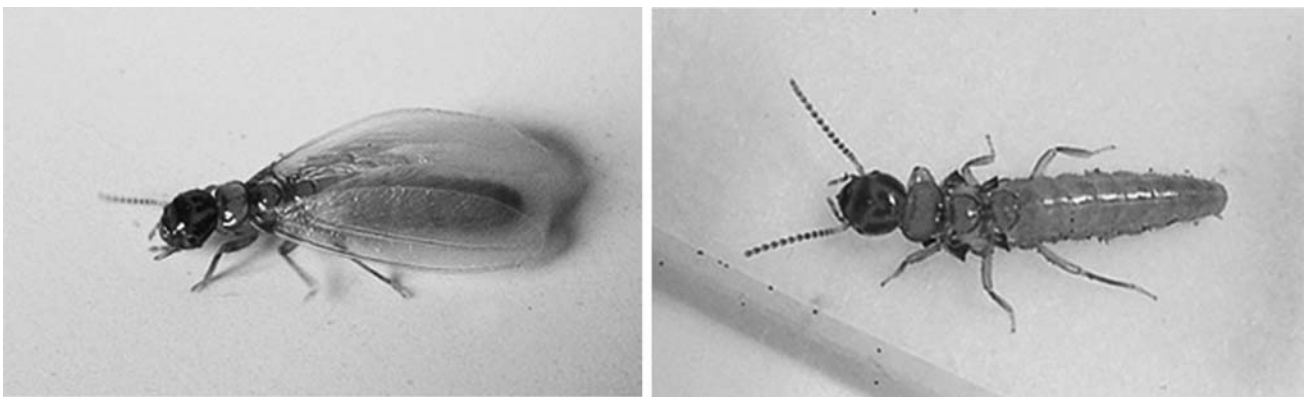

Fig. 1 Reproductives derived from nymphs. On the *left*, reproductive with floppy wings. On the *right*, the same kind of reproductive after dealation

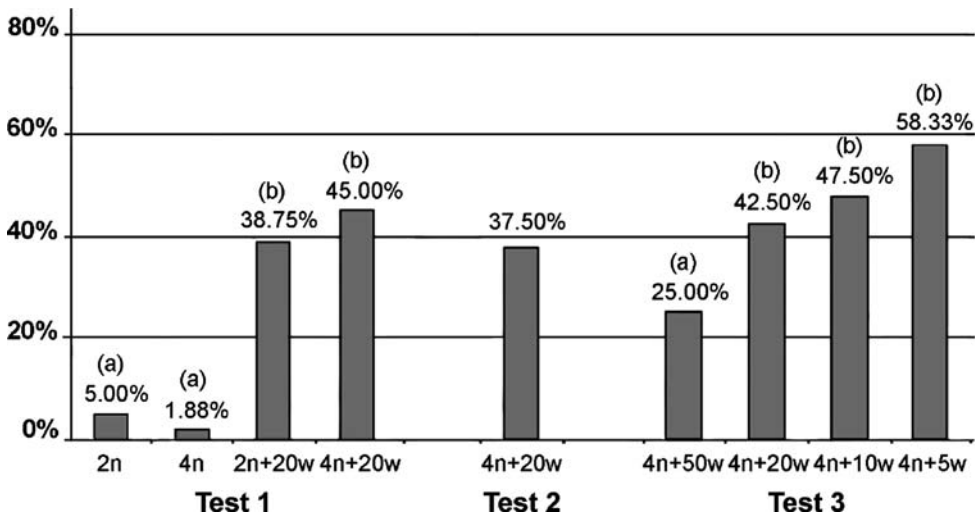

Fig. 2 Survival rates of reproductives derived from nymphs after 12 weeks. *Different letters* indicate significant differences among colony types of the same test ($P \leq 0.05$). No significant differences were observed among 4n + 20w colonies from different tests

Table 2 Time (weeks) needed for caste differentiation and oviposition

	Rep. N	Rep. W	Soldiers	Eggs	Juveniles
Test 1					
4n + 20w	1	7	3	2	9
2n + 20w	1	7	3	2	9
4n	1			3	
2n	1			3	
Test 2					
4n + 20w	1	16	13	11	17
20w		9	11	34	41
Test 3					
4n + 50w	2	13	6	10	18
4n + 20w	2	14	14	11	19
4n + 10w	2	20	13	10	20
4n + 5w	2	26		12	34

The number of weeks is not indicated, the correspondent category did not appear in that colony type

In a few colonies, this decline was particularly rapid and usually accompanied by considerable mould growth on wood and paper. Some of the workers that were initially placed in the colonies were alive after 3 years or developed into either reproductives or soldiers.

The first soldiers began to appear anywhere from 3 to 14 weeks after the beginning of the tests (Table 2). During the first few months, soldiers differentiated from some of the workers that were initially placed in the colonies. Later, in colonies where there was successful reproduction, soldiers differentiated from juveniles. Soldiers derived from juveniles could be distinguished from those derived from workers based on their smaller size.

Egg laying was well synchronized among colonies for the same and for different tests. Eggs could be found anytime from late January to the beginning of November. In each colony, egg laying was not continuous. In general, egg production was suspended for a few weeks after the first batch of eggs was deposited. Eggs were usually maintained as clusters on the bottom of the dish, under the wood or stuck to the lid. The exact number of eggs deposited in each colony was difficult to determine without excessive handling of each colony. However, the number of eggs observed through the Petri dish rarely exceeded 10. The time needed for egg eclosion was difficult to evaluate with precision, since we could not observe each egg separately, but was apparently very variable, ranging from 4 to 8 weeks.

Juvenile growth was relatively slow in this study. Each year their growth stopped or at least slowed down considerably during the cold months (November to January, when the room temperature was lower than 22°C). At the age of nearly 3 years, their size was still comparatively small, so that they were quite easy to tell from the workers that were initially placed in the colonies.

During the first months, colony sizes generally declined, owing to cannibalism towards reproductives and to the death of some of the workers. The birth of juveniles tended to increase colony sizes, but in many cases its effect was counterbalanced by the death of older workers. Colony sizes after 3 years remained small (Table 4). The maximum annual growth rate, recorded in a 4n + 20w colony of Test 1, was 13.39%.

Test 1

Nymphs were not able to establish viable colonies in the absence of workers. They moulted regularly and a few were able to deposit eggs; however, no eclosion occurred. Every colony lacking workers died within 18 weeks, with the exception of two colonies composed of two nymphs each, which lived for 26 and 48 weeks, respectively. Nearly all the colonies containing workers were still living at the end of the 1st year (Table 3). Survival rates of the two colony types did not differ significantly either after 1 year (FE, $P = 0.615$) or after 3 years (χ^2, $P = 0.478$; Tables 3, 4).

After the 1st year, the number of reproductives derived from nymphs was significantly higher in 4n + 20w colonies when compared to 2n + 20w colonies (KS, $P = 0.001$), but their survival rates did not differ significantly in the two colony types (χ^2, $P = 0.235$). After the first 3 years, there were no significant differences between the two colony types, in the number of reproductives derived from nymphs (KS, $P = 0.937$) and in their survival rates (χ^2, $P = 0.133$). Reproductives derived from workers started to appear in both colony types after 7 weeks.

The number of reproductives, considering both those derived from nymphs and those derived from workers, was not significantly different between 2n + 20w and 4n + 20w colonies, either after a year (KS, $P = 0.174$) or after 3 years (KS, $P = 0.834$). The modal number of reproductives that could be found in colonies of both types was 2, both after 1 year and after 3 years.

The first soldiers were visibly present after 3 weeks. Three years after the beginning of the test, they represented 6.55 and 5.50% of the total colony population size in 2n + 20w and in 4n + 20w colonies, respectively. At the same time, 36.84% of 2n + 20w colonies and 30.00% of 4n + 20w colonies contained no soldiers.

The first eggs were laid from 2 weeks after test initiation, and the first juveniles were observed 7 weeks later. Three years after the beginning of the test, juveniles were found in 78.57% of 2n + 20w colonies and in 88.00% of 4n + 20w colonies. There were no significant differences

Table 3 Colony survival rates, mean number of reproductives, workers, soldiers and juveniles per colony and mean relative colony size (final colony size/initial colony size rate) after a year

	Survival (%)	Rep. N	Rep. W	Rep. T	Workers	Soldiers	Juveniles	Size
Test 1								
4n + 20w	97.50 a	1.85 (1.09) a	0.74 (0.79)	2.59 (0.97)	9.64 (2.50)	0.79 (0.47)	7.41 (6.61)	78.74% (26.73%)
2n + 20w	92.50 a	0.76 (0.80) b	1.22 (0.79)	1.97 (0.69)	10.51 (3.04)	0.68 (0.58)	4.89 (5.87)	73.46% (29.68%)
4n	0.00 b							
2n	0.00 b							
Test 2								
4n + 20w	60.00	1.82 (0.75)	0.09 (0.30) a	1.91 (0.83)	11.36 (5.37)	0.64 (0.50)	4.55 (6.06)	73.86% (43.62%)
20w	55.00		1.83 (1.03) b	1.83 (1.03)	12.58 (3.73)	0.67 (0.65)	0.42 (1.44)	65.00% (20.78%)
Test 3								
4n + 50w	95.00 a	0.84 (1.17) a	1.74 (1.15) a	2.58 (0.96)	23.16 (9.01) a	1.74 (1.10) a	14.74 (13.40) a	74.95% (37.10%)
4n + 20w	75.00 ab	1.53 (0.74) b	0.60 (1.06) b	2.13 (0.64)	11.40 (5.07) b	0.80 (1.08) ab	3.93 (4.92) b	72.78% (33.40%)
4n + 10w	60.00 b	1.67 (0.78) b	0.67 (0.49) b	2.33 (0.78)	5.83 (1.95) c	0.33 (0.49) b	2.17 (2.44) b	73.81% (32.42%)
4n + 5w	40.00 b	2.75 (0.46)	0.25 (0.46)	3.00 (0.76)	2.25 (1.58)	0.00	2.50 (1.60)	86.11% (41.47%)

Standard errors are shown between brackets. Different letters indicate significant differences among colony types of the same test ($P \leq 0.05$)
Rep. N reproductives derived from nymphs, *Rep. W* reproductives derived from workers, *Rep. T* Rep. N + Rep. W

Table 4 Colony survival rates, mean number of reproductives, workers, soldiers and juveniles per colony and mean relative colony size (final colony size/initial colony size rate) after 3 years

	Survival (%)	Rep. N	Rep. W	Rep. T	Workers	Soldiers	Juveniles	Size
Test 1								
4n + 20w	62.50 a	1.04 (1.06)	0.56 (0.77)	1.60 (1.04)	2.68 (3.97)	0.84 (0.80)	11.56 (9.40)	63.67% (35.59%)
2n + 20w	70.00 a	0.75 (0.75)	1.00 (0.86)	1.75 (0.65)	3.04 (3.19)	0.96 (0.69)	10.93 (8.14)	66.88% (29.51%)
4n	0.00 b							
2n	0.00 b							
Test 2								
4n + 20w	30.00	1.33 (1.03)	0.33 (0.52)	1.67 (0.52)	0.50 (0.55)	1.33 (0.52)	21.17 (8.42)	97.22% (35.42%)
20w	10.00		2.50 (0.71)	2.50 (0.71)	3.00 (1.41)	1.00 (0.00)	10.00 (7.07)	65.00% (28.28%)
Test 3								
4n + 50w	45.00	0.56 (0.88)	1.00 (0.00)	1.56 (1.00)	1.78 (0.96)	2.44 (1.00)	44.44 (16.19)	85.63% (30.03%)
4n + 20w	15.00	1.67 (0.58)	1.00 (0.00)	2.67 (0.58)	1.33 (0.58)	1.00 (0.00)	10.67 (5.03)	55.56% (27.11%)
4n + 10w	0.00							
4n + 5w	0.00							

Standard errors are shown in brackets. Different letters indicate significant differences among colony types of the same test ($P \leq 0.05$)
Rep. N reproductives derived from nymphs, *Rep. W* reproductives derived from workers, *Rep. T* Rep. N + Rep. W

between 2n + 20w and 4n + 20w colonies, either in the number of juveniles that were born (KS, $P = 0.240$ after 1 year and $P = 0.857$ after 3 years) or in the rate between final and initial colony size (MW, $P = 0.441$ after 1 year and $P = 0.857$ after 3 years).

Test 2

Survival rates of the two colony types did not differ significantly either after a year (FE, $P = 1.000$) or after 3 years (FE, $P = 0.235$). Colonies of this test seemed from the beginning less fit than colonies from Test 1. Mould could be visibly observed in nearly all the colonies.

Reproductives derived from workers started to appear after 9 weeks in 20w colonies and 7 weeks later in 4n + 20w colonies (Table 2). After the first year, the number of reproductives that could be found in 20w colonies did not differ significantly from the total number of reproductives that could be found in 4n + 20w colonies (KS, $P = 0.993$), since the lack of reproductives derived from nymphs was compensated by a higher production of reproductives derived from workers (KS, $P = 0.019$). The

modal number of reproductives that could be found in colonies of both types was 2, both after a year and after 3 years.

Soldiers began to appear 11 weeks after the beginning of the test (Table 2). After 3 years, all the colonies contained at least a soldier. Soldiers represented 7.69 and 5.51% of the individuals, respectively, in 20w and in 4n + 20w colonies.

In colonies of the 4n + 20w type, the first eggs were deposited towards the end of January, 11 weeks after the beginning of the test, and the first juveniles were observed 6 weeks later. In colonies of the 20w type, the first eggs were observed towards the end of June, 34 weeks after the beginning of the test, and the first juveniles were born 7 weeks later (Table 2). Three years after the beginning of the test, 100% of the colonies of both types contained at least a juvenile.

After the first year, there were no significant differences in the ratio between final and initial colony sizes between the two colony types (MW, $P = 0.7718$).

Test 3

After the 1st year, survival rates differed significantly in the four colony types, being higher for colonies that initially contained more workers (FE, $P = 0.020$ between 4n + 50w and 4n + 10w colonies, $P = 0.054$ between 4n + 20w and 4n + 5w colonies; Table 3). Mould growth was not observed in 4n + 50w colonies, while it could be seen in all the other colony types. In 4n + 10w and 4n + 5w colonies, mould was often very widespread, covering not only wood and paper, but extending over the surface of the sand. Three years after the beginning of the test, surviving colonies were few. In particular, all the colonies that initially contained less than 20 workers died within 60 weeks. Caste differentiation and reproduction usually started earlier in colonies that initially contained more workers (Table 2).

Twelve weeks after the beginning of the test, the survival rate of reproductives was lower in colonies where the initial number of workers was higher (χ^2, $P = 0.019$ between 4n + 50w and 4n + 20w colonies; Fig. 1). One year after the beginning of the test, the same trend was still evident. The opposite trend could be observed for reproductives derived from workers, so that the total number of reproductives did not differ significantly among the different colony types (KW, $P = 0.137$; Table 3). One year after the beginning of the test, the modal number of reproductives that could be found in colonies was 2, except in 4n + 5w colonies, where it was 3. Three years after the beginning of the test, the modal number of reproductives was 1 in 4n + 50w colonies and 3 in 4n + 20w colonies.

Soldiers began to appear from 6 to 13 weeks after the beginning or the test, depending on colony type (Table 2). After the 1st year, soldiers were fewer in 4n + 10w colonies when compared with 4n + 50w colonies (KS, $P = 0.011$). Soldiers did not differentiate in 4n + 5w colonies. Three years after the beginning of the test all the colonies contained at least a soldier and soldiers represented 5.23 and 7.32% of the individuals, respectively, in 4n + 50w and in 4n + 20w colonies.

After 1 year, 4n + 50w colonies contained more juveniles than 4n + 20w colonies (KS, $P = 0.030$) and 4n + 10w colonies (KS, $P = 0.008$), while no such difference was found between 4n + 20w and 4n + 10w colonies (KS, $P = 0.658$). Three years after the beginning of the test, all the colonies contained at least a juvenile. One year after the beginning of the test, the ratio between final colony size and initial colony size did not differ significantly in different colony types (KW, $P = 0.713$).

Comparison between 4n + 20w colonies from different tests

Both after 1 year and 3 years, the survival rate of Test 1 colonies was significantly higher than the survival rates of Test 2 and Test 3 colonies (FE, $P < 0.001$ and $P = 0.013$, respectively, after 1 year, $P = 0.028$ and $P < 0.001$, respectively, after 3 years), while no significant differences were found between Test 2 and Test 3 colonies (FE, $P = 0.501$ after 1 year, $P = 0.451$ after 3 years).

After 1 year, there were fewer surviving workers in Test 1 when compared to both Test 2 and Test 3 (KS, $P = 0.030$ and 0.010, respectively). At the same time, there were no significant differences in the number of surviving workers between Test 2 and Test 3 (KS, $P = 0.985$). The number of juveniles was higher in Test 1 than in Test 3 (KS, $P = 0.054$). No significant differences in the number of juveniles were found between Test 1 and Test 2 (KS, $P = 0.160$), and between Test 2 and Test 3 (KS, $P = 0.217$).

No significant differences were found among 4n + 20w colonies of the three tests with respect to the number of reproductives, the number of soldiers and total colony size (KS, $P > 0.05$).

Discussion

The results of the present study show that nymphs are not able to found viable colonies without the help of workers. Even though they developed into reproductives and did not show any evident problems, they all died.

All the colonies containing five or ten workers died within 60 weeks. Their overall condition was considered

poor since the beginning of the test. In particular, a considerable amount of mould growth was observed on both the wood and paper. It can be doubted whether mould was the cause or the effect of the weakening of the colonies. However, since mould was absent in colonies with 50 workers of the same test, it seems likely that bigger colonies are capable of inhibiting mould growth. There appears to be a minimum colony size under which colonies cannot survive for very long in the laboratory. Such limits range anywhere from 10 to 20 workers. Similar results were obtained with *R. santonensis* (Feytaud) (=*R. flavipes* from western France) and *R. grassei*, where colonies composed of 30 workers could live for 32 months (Pichon et al., 2007).

The survival rate was higher for the colonies that were formed in summer than for those that were formed in autumn. This may be explained by the decrease in temperature slowing the overall activity of termites in each colony, thus making it difficult for them to adapt to new environments.

Within 8 weeks, nearly all the nymphs moulted and developed into reproductives with brown pigmentation and floppy wings. The presence of wings might induce to consider these individuals as primary reproductives, but since their overall appearance was more similar to that of secondary reproductives and their wings were not functional, we are inclined to consider them as a particular form of secondary reproductives. Individuals with a similar morphology, but lacking pigmentation, were described in *R. lucifugus* and named pseudoimagos (Grassé, 1949). The reproductive type that we observed in *R. urbis* is probably more similar to normal adultoids sensu Myles (1999).

It is likely that the nymphs used in our tests, if left inside their original field colony, may have become primary reproductives and dispersed the following spring. The early separation from the field colony may have accelerated their development towards sexual maturity and prevented their development into normal alates. The death of all the colonies that contained nymphs and no workers may be explained by the fact that *R. urbis* secondary reproductives normally differentiate inside their original colony. It is likely that isolation is an anomalous situation, which secondary reproductives are not able to stand.

In colonies containing workers, many of the reproductives derived from nymphs were killed during the first 12 weeks. The killing of newly differentiated reproductives has been reported for *R. speratus* Kolbe (Miyata et al., 2004) and for some species of Kalotermitidae (Ruppli, 1969; Lenz et al., 1982). It is possible that workers tend to eliminate unnecessary reproductives and allow only one pair. However, in several colonies of the current study, workers eliminated all reproductives derived from nymphs.

Contrary to the observations on *R. speratus* (Miyata et al., 2004), *R. urbis* workers were seen to attack only reproductives derived from nymphs and not nymphs or reproductives derived from workers. After 12 weeks, when cannibalism ended, the proportion of surviving reproductives was not significantly different in 2n + 20w colonies when compared with 4n + 20w colonies (Test 1). The proportion of surviving reproductives was higher in colony types with fewer workers (Test 3). Reproductives derived from workers began to differentiate 7–26 weeks after the beginning of the tests. Considering the number of reproductives after 1 and 3 years, it can be noticed that in colony types where reproductives derived from nymphs were fewer, the number of reproductives derived from workers was higher. After 3 years, the modal number of reproductives that could be found in each colony was 2, except in Test 3, where there were few surviving colonies. It seems likely that a single reproductive pair may be the ideal number of reproductives for small-sized colonies. Similar results were obtained for *R. speratus* (Matsuura et al., 2002) and *R. flavipes* (Kollar) (Grube and Forschler, 2004).

The number of surviving workers declined over the course of time, more or less rapidly, depending on overall colony health conditions. Workers exhibited no aggressive behaviour towards each other, and the rare cases of cannibalism were possibly directed towards the dead or sick workers. Some of the workers that were placed in the colonies at the beginning of the tests remained alive after 3 years. Workers of *R. lucifugus* and *R. flavipes* are reported to live 5 years or more; however, workers in small colonies may not live as long (Buchli, 1958).

Soldiers began to appear after 3 weeks in Test 1, and not before 6 weeks in tests that started in autumn. Colonies of *R. urbis* that initially contained only workers (20w type of Test 3) differentiated reproductives before differentiating soldiers, which is contrary to what Pawson and Gold (1996) observed in *R. virginicus* (Banks), *R. flavipes* and *R. hesperus* Banks. In the current study, soldiers did not develop in colonies that initially contained only five workers. In these colonies, workers were evidently too few to initiate the development into soldiers. After the first few months, in colonies where juveniles were born, soldiers differentiated from them. This is consistent with the suggestion that, in order to maximize their growth rate, young colonies should produce soldiers from early instars (Horiuchi et al., 2002).

After 3 years, the proportion of soldiers in the colonies considered in this study ranged from 5.23 to 7.69% of the total number of individuals. These values are quite high when compared with those reported in the literature for other species of the same genus: from 1.2 to 2.9% in colonies of *R. flavipes* collected on the field (Howard and

Haverty, 1981), from 1 to 2% in laboratory colonies of *R. flavipes* and *R. virginicus* (Pawson and Gold, 1996), and 2.6% in laboratory colonies of *R. flavipes* (Long et al., 2003). Higher values, varying from 4 and 10%, have been reported for laboratory colonies of *R. hageni* Banks (Pawson and Gold, 1996).

Eggs were observed from late January to the beginning of November. Egg laying was not continuous, but interspersed between periods of reproductive inactivity. The number of eggs that could be found at the same time within each colony was low, usually less than ten. The time needed for egg eclosion seemed to be variable, ranging from 4 to 8 weeks. For other *Reticulitermes* species, different hatching periods have been reported: from 45 to 55 days for *R. flavipes* (Buchli, 1958); from 30 to 90 days, depending on the temperature during incubation, for *R. hesperus* and *R. lucifugus* (Grassé, 1949); and an average of 34.95 ± 0.12 days for inseminated eggs of *R. speratus* (Matsuura et al., 2007).

Three years after the beginning of the tests, all Tests 2 and 3 colonies contained juveniles, while in some of the Test 1 colonies no reproduction had occurred. This result is apparently in contrast with the observation that overall colony conditions were worse in colonies formed during the autumn than in those formed during the summer, but it may be explained assuming that, in Tests 3 and 4, only the healthiest colonies could survive until the 3rd year, while in Test 1, where health conditions were better, weaker colonies could survive too.

The juveniles developed slowly in this study. Their growth seemed to slow considerably during cold months, which is similar to the observations of Buchli (1956) on *R. lucifugus* and *R. santonensis*. After 3 years, juveniles remained distinguishable from older workers because of their smaller size. Their slow growth might be due to small colony size, as Buchli (1958) observed in *R. flavipes*, where juveniles tend to develop more slowly in small colonies than in bigger colonies, rarely exceeding the eighth instar in 2 years. The overall number of juveniles in each colony was low.

In the first few months following colony formation, colony sizes tended to contract, owing to the death of some of their members. In many colonies, the birth of juveniles was not sufficient to replace dead workers and the overall colony size continued to shrink. Even in healthier colonies, size did not increase considerably, with the maximum annual growth rate being 13.4%. Similar results are reported for laboratory colonies of other species in the genus *Reticulitermes*; these works are not perfectly comparable, since they differ in many aspects, including initial colony composition and duration of the tests, but the main results concerning colony sizes are essentially concordant. In fact, the number of juveniles that were born in the first few months following colony formation was low in *R. speratus* (Watanabe and Noda, 1991), *R. flavipes*, *R. virginicus* and *R. hageni* (Pawson and Gold, 1996), never exceeding a few dozens. The biggest colony sizes have been recorded in *R. flavipes*, in colonies founded by primary reproductives. Some of these colonies were capable of reaching a few hundreds of individuals after 2 years (Thorne et al., 1999; Grube and Forschler, 2004); approximately 7,000 individuals after 8 years (Long et al., 2003); and approximately 11,000 individuals after 11 years (Long et al., 2007).

Population sizes attained in laboratory colonies are low when compared with colony sizes observed in the field, which can be in hundreds of thousands (Howard et al., 1982; Forschler and Townsend, 1996; Marini and Ferrari, 1998; Tsunoda et al., 1999; Leniaud et al., 2009). It can be assumed that, in the years immediately following colony foundation, colony growth is slow and that it begins to accelerate later, when supplemental reproductives start to differentiate and contribute to reproduction.

In our experiments with *R. urbis*, colonies formed by less than 20 workers did not survive for very long in a laboratory setting. Colonies containing 20 or 50 workers showed after 3 years, survival rates varying from 10 to 70%, and the total colony size did not increase considerably. This seems to indicate that 20 individuals is approximately the minimum size needed for a colony to survive. Based on our data, it can be assumed that when the initial colony size is small, the potential for growth is low.

Unintentional introductions of small numbers of termites to new localities could easily occur owing to the difficulty in successfully locating their activity, while large numbers of termites may be detected more easily and subsequently destroyed. Therefore, it is likely that the colonization within an urban area may begin with only a small number of termites. This could explain the low level of genetic diversity observed for *R. urbis* in Domène (Leniaud et al., 2009).

Field colonies of *R. urbis* were observed in certain localities of northern Italy about 60 years ago (Jucci and Springhetti, 1957). Recently, the same *R. urbis* colonies were observed occupying the same territory without significantly spreading to outlying urban areas (M. Marini, unpubl. data). This is what was observed in downtown Bagnacavallo, where the infestation was first identified in 1970 (Campadelli, 1987, 1988). The infestation was active until 2004 (Marini and Mantovani, 2002), when a pest control program was initiated (M. Marini and R. Ferrari, in preparation). In order to evaluate the true invasive potential of this species, it would be useful to study colonies from different regions of its distribution area. In addition, increasing the initial colony size will be considered in future work.

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FIRST REPORT OF *RETICULITERMES FLAVIPES* (ISOPTERA: RHINOTERMITIDAE) IN ITALY

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The first record of *Reticuliterms flavipes* (Kollar) (Isoptera: Rhinotermitidae) in Europe was in 1837, in Vienna, Austria, after infested plants imported from the U.S. were discovered in the greenhouse of the Schönbrunn Palace (Kollar 1837). In 1924, *R. flavipes* was found on the French Atlantic coast and initially described as a new species, *R. santonensis* (Feytaud 1924). The synonymy of *R. santonensis* with *R. flavipes* was eventually confirmed by mitochondrial DNA analyses (Austin et al. 2002). In France, *R. flavipes* is currently distributed in an area extending from the Gironde region up to Paris and Normandy, and causes structural damage to buildings and trees (Lohou et al. 1997). During the 1930s, *R. flavipes* was discovered in wooden forepoles of channel construction in the steam heating district of Hamburg, Germany, where populations were supported by the favourable microclimate (Weidner 1937). Today, popluations of *R. flavipes* remain established in Hamburg, and cause damage to buildings and trees (Hertel & Plarre 2006).

In October 2008, a subterranean termite infestation was discovered by a homeowner in a detached house and adjacent garden situated in a residential district built in the 1970s in the outskirts of Olgiate Olona (Varese), in northern Italy. Termite workers and soldiers were observed and collected during a structural inspection in November 2008. Specimens are maintained in the Marini termite collection at the University of Bologna.

Molecular analysis was used to determine the termite species, which were preserved in 100% ethanol prior to DNA extraction. A 684-bp region of the mitochondrial cytochrome oxidase subunit II gene and a 491-bp region of the

mitochondrial 16S ribosomal RNA gene were amplified by PCR. Sequencing was performed by Macrogen Inc. (Seoul, South Korea). Closely related sequences were identified from GenBank using the BLAST network service (Altschul et al. 1990) at NCBI.

For both genes, nucleotide sequences were identical in the two workers (GenBank Accession GU070788 and GU070789). Sequences from the Olgiate Olona house corresponded (97-100% coverage, 100% similarity for COII sequence; 92-100% coverage, 100% similarity for 16S sequence) to GenBank sequences of *R. flavipes* from North America and France and of *R. arenincola* Goellner (Table 1). This latter species appears to be identical to *R. flavipes* based on the DNA sequences obtained so far.

In North America, 47 16S haplotypes of *R. flavipes* have been described (Austin et al 2005a). *Reticulitermes flavipes* in France shows only four haplotypes (F, M and GG in Austin et al. 2005b, and another haplotype in Marini & Mantovani 2002), all of which can be found in North America, so it can be assumed that the European *R. flavipes* is allochthonous (non-native) (Austin et al. 2005a). French haplotypes can be found within or near the Mississippi River basin region of the U.S. once belonging to the French colonial empire in North America. In particular, three French haplotypes occur in Louisiana and Arkansas, and two French haplotypes can be found in Mississippi, Texas, Kansas, Oklahoma, Iowa, and Indiana (Austin et al. 2005a). Introduction of *R. flavipes* to France might date from the colonial period.

The Olgiate Olona sample shares the same 16S haplotype with French and USA populations (Table 1). Even though the possibility of an American origin for the Olgiate Olona population cannot be ruled out, it seems much more likely that *R. flavipes* was

introduced to Italy from France because of the geographical proximity and history of trade activity between the two countries.

The human-aided introduction of *R. flavipes* to non-endemic regions of the world, such as Olgiate Olona, should be carefully monitored in order to identify and treat new infestations to prevent future introductions, especially since *R. flavipes* is a major structural pest wherever it has been introduced.

SUMMARY

An existing infestation of subterranean termites in and around a home in northern Italy was genetically determined to be the Nearctic species, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). This is the first report of an established *R. flavipes* population in Italy. The source of the *R. flavipes* introduction is unknown.

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TABLE 1. GENBANK SEQUENCES MATCHING OLGIATE OLONA COII AND 16S SEQUENCES.

GenBank Accession No.	Species	Location	Sequence Type
AY168210	*R. flavipes*	W. Lafayette (Indiana - USA)	COII
AY536415	*R. flavipes*	W. Lafayette (Indiana - USA)	COII
AF291742	*R. santonensis*	Biscarrosse (France)	COII
AY512590	*R. santonensis*	Royan (France)	COII
EF206315	*R. santonensis*	Elevage Dijon (France)	COII
EU253889	*R. santonensis*	(*)	COII
AF525353	*R. arenincola*	Indiana - USA	COII
AY168209	*R. arenincola*	Dune Acres (Indiana - USA)	COII
AY453589	*R. arenincola*	Indiana - USA	COII
AY168228	*R. flavipes*	W. Lafayette (Indiana - USA)	16S
AF292025	*R. santonensis*	Biscarrosse (France)	16S
AY808127	*R. santonensis*	Tussac (France)	16S
EF206315	*R. santonensis*	Elevage Dijon (France)	16S
FJ806149	*R. santonensis*	(*)	16S
AY168226	*R. arenincola*	Dune Acres (Indiana - USA)	16S

* = locality not listed.

Starting from Crete, a phylogenetic re-analysis of the genus *Reticulitermes* in the Mediterranean area.

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Abstract

The molecular characterization of Cretan termites of the genus *Reticlitermes* through the sequencing of two mitochondrial genes (COII and 16S) and the amplification of the Inter-SINE loci is here proposed, followed by a phylogenetic and taxonomic re-analyses of the *Reticulitermes* genus in Mediterranean Europe.

Different statistical analyses were performed for both mitochondrial (Maximum Parsimony, Maximum Likelihood, Bayesian inference, parsimony network) and nuclear markers (pairwise F_{ST}). The evolutionary radiation of this species complex, resulted more ancient with respect to previous analyses, with a highly correlated pattern between clades divergence and geological events of the specific geographical area.

Our analysis underlined that the new haplotypes identified in Crete clearly converged with those of specimens collected in northern Turkey, Thrace and Macedonia/Calcydia: this allows to suggest a unique entity presently indicated as *R. aegei*. The species status is here confirmed for *R. balkanensis* and *R. urbi*s, as well as for *R. banyulensis* and *R. grassei* from France and the Iberian peninsula. On the other hand, the high divergence among the three *R. lucifugus* subspecies from Italy and Corse indicates a specific rank of differentiation. Phylogeny presents *R. aegei* as the most apical clade in the eastern area, while *R. urbis* the most basal one. In the western area, the evolutionary relationships among taxa points to *R. corsicus* as basal with respect *R. lucifugus* and *R. siciliae*.

Keywords: cytochrome oxidase II, chronogram, cladogenesis, 16S, inter-SINE, Isoptera.

Introduction

The fundamental aspect of termite biology is represented by their colony organization with diploid individuals morphologically distinguished in specific castes, performing different roles. This eusocial system is generally built by alates, soldiers and workers. Reproduction is performed by alate individuals, both in terms of colony maintenance and of new colonies foundation. The colony defense is given by soldiers, while workers provide foraging and nursing activities.

In the north Mediterranean region, from the Iberian peninsula to Turkey, only two genera are known as autoctonous: *Kalotermes* (Kalotermitidae) and *Reticulitermes* (Rhinotermitidae). Despite their overlapped distribution, the biodiversity at the species/subspecies level is significantly different. *Kalotermes* is in fact present only with *K. flavicollis*, even though recent data suggest the presence of more lineages (Velonà et al., work in progress). On the other hand, the genus *Reticulitermes* lists around 10 entities of specific/subspecific rank (Marini and Mantovani, 2002; Kutnik et al., 2004; Luchetti et al., 2004a, 2004b, 2007; Lefebvre et al., 2008).

A first possible explanation of this difference involves the colonization history. *Reticulitermes* ancestors could have colonized Europe before *Kalotermes* ones and in this way, they could have been influenced for a longer time by the geological events that have characterized the region, generating the high biodiversity known at present. A second possible explanation is given by the different ecological features of these termites; *Kalotermes* individuals live in dead trees and colonies could be transported passively, for example by human activities, more easily with respect to *Reticulitermes* ones that - as the definition “subterranean termites” indicates - build their nest below the ground level. This difference in nest allocation may allow a higher gene flow between *Kalotermes* populations and it could be at the basis of its higher genetic homogeneity. Actually, it’s hard to discriminate between the two hypotheses, and since they are not mutually exclusive, their

combination could represent the best one (Luchetti et al., 2004a; Velonà et al., work in progress).

Many phylogenetic and phylogeographical investigations exploited the above mentioned high species radiation of the *Reticulitermes* genus (see for example Marini and Mantovani, 2002; Kutnik et al., 2004; Luchetti et al., 2005a, 2007; Nobre et al., 2008), but at variance of Vargo and Husseneder (2009)'s suggestion, the taxonomy and phylogeny of Mediterranean *Reticulitermes* entities are, unfortunately, far from being settled.

In our former papers, we mainly focused on *Reticulitermes* populations from the eastern Mediterranean area and many divergent lineages were scored (Luchetti et al 2004a, 2005a, 2007): i) *R. urbis,* widely distributed in south-western Peloponnesus, northern Greece and Croatia showing a certain degree of differentiation between northern and southern populations; ii) *R. balkanensis* restricted to the Attican region; iii) a highly structured oriental clade distinguished in a northern lineage comprising Ankara samples together with Thracian and Macedonian/Calcydic peninsula ones (the *R. lucifugus* - Turkey - northern clade) and a southern lineage embodying highly differentiated Turkish colonies sampled southern of the Taurus-Antitaurus mountains together with the Israelian *R. clypeatus* population (the *R. lucifugus* - Turkey - southern clade). The latter clade appeared phylogenetically related to *R. balkanensis*, this relationship not being bootstrap supported in all elaborations. The indication of *R. lucifugus* for the Turkish lineages is *sensu* Austin et al. (2002).

The geographical area under study has been also at the basis of many phylogeographical analyses performed on different animals (Kasapidis et al., 2005; Parmakelis et al., 2005, 2006; Poulakakis et al., 2008; Papadopoulou et al., 2009) owing to its highly structured geological history, with the periodical interchange of sea level expansions and regressions (Creutzburg, 1963; Anastasakis and Dermitzakis 1990; Dermitzakis, 1990; Lambeck, 1996). Also Crete island is considered a biodiversity hotspot for its high number of

endemic species. Many authors consider its high biodiversity as the results of the geographical separation from the mainland and of its specific geological history (Legakis and Kypriotakis, 1994; Sfenthourakis and Legakis, 2001; Parmakelis et al., 2006).

In this paper we present the molecular analysis of Cretan populations followed by a phylogenetic re-analyses of the genus *Reticulitermes* in the Mediterranean area, also comprising the western entities, i.e. *R. banyulensis* and *R. grassei* from France and Iberian peninsula (initially considered subspecies of *R. lucifugus*; Plateaux and Clèment, 1984), and *R. lucifugus* from Italy and Corse, known to be differentiated at the subspecific level in *R. lucifugus lucifugus, R. lucifugus corsicus, R. lucifug*us *subsp*.(Sicily).

Given the resolution power obtained in previous and ongoing analyses (Luchetti et al., 2007; Velonà et al., work in progress), we utilized both mitochondrial (cytocrome oxydase sub.II and 16S genes) and nuclear markers (Inter-SINE).

SINEs (Short INterspersed Elements) are considered homoplasy-free markers and they are present in eukaryotic genomes with a high copy number (more than 10^5 copies following the estimates performed by Ohshima and Okada (2005) (Nishihara and Okada 2008; Li et al., 2009). Given these features, SINEs are considered as optimal molecular markers for phylogenetic purposes. Some recent studies have found that using the Inter-SINE fingerprinting methodology, based on the amplification of the regions between copies of the same element, good phylogenetic information can also be obtained (Kostia et al., 2000; Bannikova et al., 2005; Shafer and Stewart 2007). Considering this, since no information on I-SINE utility for taxonomic purpose has been provided so far in termites, we tested it using the two elements *Talua* and *Talub* (Luchetti and Mantovani 2009 and unpublished data).

Materials and Methods

Specimens were field caught in Crete and preserved in absolute alcohol until molecular investigation. All pertinent information on samples here analyzed is given in Table 1 and Fig. 1a. For total DNA extraction, single termite heads were ground in a quick extraction buffer (PCR buffer 0,1x, SDS 0,1x), added with proteinase K, then frozen at -80°, warmed at 65° for 1 hour and at 95° for 15 minutes. Two workers for each colony were used for both mitochondrial and nuclear DNA analyses. For Inter-SINE analysis, also the following specimens of available colonies were considered: 4 individuals belonging to *R. urbis* - northern clade from Komarna and Parga populations, 4 individuals of *R. urbis* - southern clade from Kalamata and Kallikomon populations, 4 individuals of *R. balkanensis* from Maratona and Penteli populations in the Attic region and 4 individuals of *R. lucifugus lucifugus* from Feniglia and Castellaneta populations in peninsular Italy.

Mitochondrial markers

PCR amplification was performed in a 50 µl mixture with *Taq* polymerase (Invitrogen), following manufacturer protocol. Thermal cycling was done using 30 of the following cycle: denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec, extension at 72°C for 30 sec. Both strands were directly sequenced at Macrogen Inc. (Korea). COII and 16S primers for PCR amplification and sequencing were as in Luchetti et al. (2004b). Sequences were aligned with CLUSTAL algorithm of the Sequence Navigator software (Version 1.0.1, Applied Biosystems) and alignments were edited by eye. Newly scored haplotypes were entered into GenBank (Table 1).

Inter-SINE markers

The PCR amplification of the loci comprised between two copies of the SINEs *Talua* or *Talub* was performed with the primers Ta-F (5'-AGT GGC CGT GCG GTC TAA G-3') and Tb-F (5'-ATG GCT CAG GCG GTT AGT C-3'), respectively.

PCR reactions were carried out with an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 42°C for 30 sec and 72°C for 30 sec; with a final extension at 72°C for 7 min. The 10 µL PCR reactions included 8 ng of genomic DNA, 10 µM of primer, 1.5 mM $MgCl_2$, 200 µM of dNTPs, 10 mM of buffer 10X and 1 U of *Taq* polymerase (Invitrogen). The PCR products were resolved in 2% agarose gels using TAE 1X buffer, and bands were used to create a presence (1) / absence (0) matrix.

Statistical analyses

Modeltest v. 3.06 (Posada and Crandall, 1998) was run to determine the best substitution models for mitochondrial dataset (COII: HKY + Γ; 16S: K81uf + Γ; combined data: HKY + Γ). Partition Homogeneity test (ILD test; Farris et al., 1995) was used to determine if the two genes could be analyzed in a combined dataset. Significance of *P* was computed after 100 replicates (*P*=0.99, allowed combined dataset).

Maximum Parsimony (TBR branch swapping, taxon stepwise addition with 500 random replicates; gaps coded as 5^{th} base) and Maximum Likelihood analyses were performed with PAUP* v 4.0b (Swofford, 2001); node supports were calculated with 1,000 and 100 bootstrap replicates, respectively. Bayesian analysis was done with MrBayes 3.01 (Huelsenbeck, 2001), that allows the two substitution models for the two gene partitions to be used simultaneously. The Markov Chain Monte Carlo (MCMC) process was set so that two runs of four chains ran simultaneously for 5 x 10^5 generations, until the two runs converged onto stationary distribution (variance of split frequencies < 0.01); trees were sampled every 100 generations, for a total of 5,001 trees. The improvement of –ln L was graphically analyzed, and the “steady state” was determined to have occurred by the 100^{th} tree. Thus, first 100 trees were discarded (burnin=100) and a consensus tree was computed on the remaining 4,901 trees.

For a first phylogenetic analysis, a representative subset of the available COII and 16S sequences of the following eastern Mediterranean taxa were drawn from Genbank (Fig. 1b):

R. urbis: Komarna, Parga, Kastrosikia (DQ487822, DQ487824, DQ487825 / DQ487836, DQ487838, *R. urbis* - northern clade); Kallikomon and Kalamatas (DQ487830, DQ487832 / DQ487837, *R. urbis* - southern clade).

i. *R. balkanensis*: Maratona, Nea Artaki (DQ487835, AY954667 / DQ487849, DQ487850).
ii. *R. lucifugus* - Turkey: Messimvria, Stagira, Ankara (AY954666, DQ866978, AF525333 / DQ866986, AF525330, *R. lucifugus* - Turkey - northern clade); Antalya, Fethiye (AF525338, AF525334 / DQ431056, DQ431055, *R. lucifugus* - Turkey - southern clade).
iii. *R. clypeatus*: Ben Shemen (AF525320; DQ431058).
iv. *Reticulitermes sp.* (Amourgous) (Cyclades) (AY954664 / DQ487852).
v. *Reticulitermes sp.* (Samos) (Dodecanese) (AY954669 / DQ487851).

Samples from Italian colonies of Bologna and Chieti pertaining to *R. lucifugus lucifugus* were utilized as outgroups (AF291723, AF291738 / AF29202).

A parsimony network was performed using TCS 1.21 (Clement et al., 2000) on this first-step data set, but the result showed the complete separation of all main clusters so that the more variable COII was excluded from the analysis and a network of the 16S gene is presented.

For divergence time estimation, we then included all the European *Reticulitermes* samples for which the same region of COII and 16S genes were available (Austin et al., 2002; Marini and Mantovani, 2002; Luchetti et al., 2004a, 2004b; 2007; see table S1 in supplementary materials for GenBank A.N.). Since our total dataset was then increased, we performed a new analysis with Modeltest 3.06 and a new ILD test. The Partition

Homogeneity test resulted not significant (*P*=1), allowing us to analyze the combined dataset. Modeltest highlighted the HKY+Γ+I (Γ=1.2215, I=0.5771) as the best substitution model for the total dataset.

The Likelihood ratio test (LRT; Huelsenbeck and Crandall, 1997) was used to verify the constancy of substitution rate across the branches of the *Reticulitermes* phylogeny. The result allowed to reject the molecular clock hypothesis (LRT = 108.83; df = 89; P<0.05), therefore an algorithm assuming a “relaxed” molecular clock was used to compute the timing of cladogenetic events.

Estimation of clade divergence time was done with BEAST v. 1.4.8 software package (Drummond and Rambaut, 2007), using relaxed molecular clock settings. The program ran for 10 million generation in order to generate reliable node age estimates and relative 95% confidence intervals. In order to verify the MCMC search process, two runs were performed with identical parameters and compared with Tracer v. 1.4 (Rambaut and Drummond, 2007) to verify the support of their convergence (variance of split frequencies < 0.01). Following the results obtained by Kasapidis et al., (2005) we set the calibration point at the last separation of Crete from the mainland, around 5.3 MYA, at the end of the Messinian salinity crisis.

Coptotermes formosanus and *R. flavipes* were used as outgroup/ingroup (GenBank A.N.: COII AF107488, AF107484; 16S U17778, U17824).

Following Hebert et al. (2004), a genetic pairwise distance matrix has been calculated on the whole data set with PAUP* using the HKY+Γ+I substitution model parameters; frequencies of intra-clade and inter-clade divergence values are either reported in histograms or as range (table S2 in supplementary materials).

For I-SINE markers a F_{ST} pairwise differentiation matrix was performed using the software Genalex v. 6.1 (Peakall and Smouse, 2006); Cretan samples were subdivided following mitochondrial phylogeny.

Results

Mitochondrial DNA sequence variation and phylogeny

The sequencing of the mitochondrial markers in 44 individuals from Crete (22 colonies) retrieved 19 and 15 new haplotypes for COII (683 bp) and 16S (502-505 bp) genes, respectively. In the COII gene, 35 nucleotide sites were variable: the 28.6% at the 1st codon position, the 2.8% at the 2nd and the 68.6% at the 3rd. Newly scored haplotypes differ for 1 - 12 substitutions. The 16S rDNA fragment shows a lower variability, with only 15 polymorphic sites; scored haplotypes differ for 1 - 8 mutations.

The number of base differences among Cretan combined haplotypes ranged from 1 to 18.

Including the other *Reticulitermes* taxa for the first step phylogenetic analysis (excluding outgroup) a total of 37 combined haplotypes were analyzed (Fig. 2a).

Maximum Parsimony (single trees' island with 575 equally parsimonious trees; tree length: 323 steps; consistency index: 0.681), Maximum Likelihood (-ln L = 3395.23) and Bayesian inference methods (Fig. 2a) produced largely congruent trees; on the other hand, support values are widely different, with Bayesian posterior probabilities being averagely higher than bootstrap percentages.

In the combined tree, the Cretan samples build a single well supported branch in which four main groups can be identified (Fig. 2a). Group 1, is composed by the easternmost colonies of Kato Zakros, Hametoulo and Hohlakies; it is followed by the samples from central Crete distinguished in a central-eastern (2) and a central-western (3) clade, partially overlapping at their edge in southern Crete. Finally, a fourth, most differentiated cluster includes the five colonies from the north-west part of the island. The geographical subdivision of the four Cretan lineages is highlighted in Fig. 2b.

Tree topology further confirms the three main groups scored in Luchetti et al. (2007). These are: *R. urbis,* basal to all other taxa, with samples distinguished in the northern

clade from Croatia and N-W Greece and the southern clade from Peloponnesus; the *R. lucifugus* - Turkey - southern clade, linked to *R. balkanensis*, and *R. clypeatus*, for which the not supported dichotomies do not allow to detect their evolutionary relationships; the *R. lucifugus* - northern clade from northern Turkey, Thrace/Macedonia (also comprising colonies from the Calcydic Peninsula) and the Amourgos colony which is basal to the Cretan cluster. The Samos sample is basal to the *R. lucifugus* - Turkey - southern clade, with a significant support, thus partially solving the polytomy of the *R. lucifugus* - Turkey - southern clade/*R. balkanensis*/*R. clypeatus*/Samos cluster observed in a previous paper (Luchetti et al., 2007).

The parsimony network basically agrees with phylogenetic inference. The Cretan samples are well differentiated from the other sequences; colonies from region 2 and 3 show the lower number of missing haplotypes with respect to *R. lucifugus* - Turkey - northern clade. Haplotypes from region 1 are directly connected to those from regions 2 and 3, while colonies from region 4 appear the most divergent from the others. Finally, the Amorgous population shows four and five missing haplotypes with respect to *R. lucifugus* - Turkey - northern clade and Crete populations, respectively (Fig. 3).

Inter-SINE markers

The fingerprinting pattern obtained with the use of the two SINE markers (*Talua* and *Talub*) allowed us to detect a total of 32 loci. In particular, the *Talua* marker evidenced 25 loci, while *Talub* 7 loci; all loci were polymorphic.

The F_{ST} pairwise analysis (Table 2) was performed subdividing the samples from Crete island following the results of mitochondrial analyses. The four resulting groups were poorly differentiated among them, with values ranging between 0.086 (clades 1-3; NS) and 0.129 (clades 2-3; $P<0.05$). On the other hand, the four Cretan groups were highly divergent with respect to the other analyzed *Reticulitermes* taxa, with values ranging

between 0.256 (clade 2 *vs R. lucifugus lucifugus*; P < 0.01) and 0.773 (clade 1 *vs R. balkanensis* or *vs R. urbis* - northern clade, P < 0.01). Also the other *Reticulitermes* taxa appeared significantly divergent among them, the only exception being the two clades of *R. urbis* (0.186, NS).

Timing cladogenetic events

In the chronogram comprising the complete data set (Fig. 4), all dichotomies fall from the Oligocene to the Pliocene periods (from 31 MYA to 2.47 MYA).

The main dichotomy, splitting the western European taxa (*R. lucifugus* complex, *R*. *grassei* and *R. banyulensis*) from the eastern ones, dates back to 23.61 MYA.

Within the eastern cluster, the divergence of the *R. urbis* clade from the other eastern taxa and the splitting between *Reticulitermes sp.* (Samos)/*R. balkanensis*/*R. lucifugus* - Turkey - southern clade/*R. clypeatus* from *R. lucifugus* - Turkey - northern clade/Cretan lineages are somehow coeval (15.73 MYA *vs* 12.48 MYA).

As far as more recent dichotomies are concerned, while among the western taxa they are comprised between 7.01 (*R. lucifugus lucifugus* / *R. lucifugus subsp.* (Sicily)) and 12.09 MYA (*R. grassei* / *R. banyulensis*), in the eastern Mediterranean area the majority of them are more recent, from 2.47 (Crete group 2 / group 3) to 9.88 MYA (*R. balkanensis* / *R. lucifugus* - Turkey - southern clade). Also the separation between *R. urbis* northern and southern clades appears quite recent (3.95 MYA).

Taxonomic analysis

The genetic divergence analysis was carried out to verify the distribution of intra-clade and inter-clade divergence frequencies in European *Reticulitermes* lineages. The analysis was performed for all possible comparisons if at least three sequences for each taxonomic unit were available.

In this kind of approach two opposite situations can be produced with intra-clade and inter-clade frequency distribution either overlapping or being completely separated. In the latter instance, as proposed by Hebert et al. (2004), the occurrence of a “barcoding gap” highlights a specific level of divergence (Fig. 5, Table S2). In our analyses, the four Cretan lineages showed an overlapped distribution (Fig. 5a). A comparable situation is observed within the *R. lucifugus* - Turkey - northern clade when the sequences from the Calcydic peninsula were compared to the Ankara, Thracian and Macedonian ones (Fig. 5b). The same applies when these two groups were paired together against Cretan populations, so that the whole group (*R. lucifugus* - Turkey - northern clade + Cretan populations) appears as a unique entity (Fig. 5c). A frequency overlapped distribution emerged also in *R. urbis* northern and southern clade comparison (Fig. 5d).

On the other hand, all pairwise comparisons involving *R. balkanensis*, *R. urbis* and the *R. lucifugus* - Turkey - northern clade/Cretan populations group showed a completely separated frequency distribution (Table S2).

Also all comparisons dealing with the western lineages suggested a specific level of differentiation of the entities considered. This confirms the specific status of *R. banyulensis* and *R. grassei*, but cast doubts on the subspecific level of differentiation of the *R. lucifugus* subspecies (*R. lucifugus lucifugus*, *R. lucifugus corsicus* and *R. lucifugus subsp.* (Sicily)) (Fig. 5e, 5f, Table S2).

Discussion

The molecular characterization of the Cretan populations allowed us to detect 21 new combined haplotypes. The phylogenetic analysis evidenced the monophyly of the Cretan cluster and its relationship with the Cycladic Amorgous population and *R. lucifugus* from northern Turkey, Thrace and Macedonia/Calcydia.

Since the topology of Amorgous and *R. lucifugus* - Turkey - northern clade lineages is better evidenced in comparison to a previous analysis (Luchetti et al., 2007), we suppose that the survey of the Cretan group could have produced a more stable and reliable node. It is also to be noted that – from a strictly technical point of view – in the phylogenetic analysis here proposed, Maximum Parsimony, Maximum Likelihood and Bayesian methods topologies are highly congruent, but nodal support values widely differ, with Bayesian posterior probabilities being averagely higher than bootstrap percentages. This fact confirms that posterior probabilities may contrast with Maximum Likelihood bootstrap values, overestimating the nodal support (Alfaro et al., 2003; Douady et al., 2003).

Within Cretan island four divergent lineages can be recognized; they date back from 4.39 to 2.47 MYA during the Pliocene period. In this period, due to the sea level expansion, Crete was partially submerged by sea water that subdivided the island into smaller islands (the so called “Cretan paleoislands”). The high similarity between the surface of these islands and the distribution of the four Cretan lineages, suggests a role of the geological history in this diversification. Similar results were also obtained in other organisms (Legakis and Kypriotakis, 1994; Parmakelis et al., 2005, 2006). The scored mitochondrial variability, as proposed by Hebert et al. (2004), is in line with an intra-specific level of divergence. Comparable conclusions are supported by I-SINE marker results: these evidenced low values of divergence among the four lineages, while their differentiation from the other taxa was significantly higher.

The timing of cladogenesis between *R. balkanensis* and *R*. *lucifugus* - Turkey - southern clade + *R. clypeatus* seems to be in line with the formation of the mid-Aegean trench between the Cyclades islands and the eastern Aegean sea, which originated after the separation of the Agäis, the unique mainland present in this area until 9.5 MYA. This trench became a permanent barrier until the end of Messinian salinity crisis (~ 5.5 MYA). This geological separation has already been proposed as a key factor determining the

speciation pattern of different animal taxa, both vertebrate and invertebrate (Parmakelis et al., 2006; Douris et al., 2007; Poulakakis et al., 2008; Poulakakis and Sfenthourakis 2008). As in Luchetti et al. (2007), *R. urbis* appears a monophyletic clade with a clear dichotomy between Balkans/N-W Greece populations and Peloponnesus ones. Our dating for this separation (3.96 MYA) is in line with the first formation of the Corinth gulf during the Pliocene period (around 3.5 MYA) and it is in contrast with a previous hypothesis (Luchetti et al., 2007) placing the event around 9,000 years ago, on the basis of a supposed recent European colonization by *Reticulitermes* taxon. Despite a relatively ancient separation though, our taxonomic analysis does not allow to consider the two *R. urbis* entities as different species but at most as two subspecies. To explain this fact, new considerations on the geology of the region are needed. After the formation of the Corinth gulf, around 3.5 MYA, the region was subjected to the regression of the sea level in the Pleistocene period (around 0.8 MYA) that closed the gulf, possibly allowing gene flow among *R. urbis* populations until the last emergence of the Corinth gulf around 9,000 years ago (Parmakelis et al., 2006; Douris et al., 2007; Simaiakis and Mylonas, 2008).

Another interesting point is represented by the complex *R. lucifugus lucifugus*/*R. lucifugus corsicus* from Italy and Corse. In our analyses, the cladogenetic event is suggested to be happened 8.94 MYA during the Tortonian, in the Late Miocene. This seems to be in line with the hypothesis proposed by Ketmaier et al., (2006): the Corse-Sardinia microplate during its anti-clock wise rotation remained connected with the border of Paleo-Europe through a land bridge that would have constituted the future Maritime Alps and the Ligurian Apennines. The separation of the microplate from the mainland took place from 8 to 5 MYA and interrupted the gene flow between the two lineages. In a previous work, Uva et al. (2004) analyzed the colony divergence of *R. lucifugus lucifugus* from Tuscany region and *R. lucifugus corsicus* from Corsica and also highlighted a good level of divergence (~ 5%) together with a low level of *R. lucifugus lucifugus* intrasubspecies divergence (~

0.5%). Our analysis, performed on a greater number of *R. lucifugus corsicus* colonies, shows a comparable level of divergence and following Hebert et al. (2004), the genetic differentiation of *R. lucifugus corsicus* and *R. lucifugus lucifugus* appears of specific level. From our data, a specific level of divergence applies also to the other so far suggested *R. lucifugus* subspecies from Sicily (Luchetti et al., 2004b).

Our analysis also confirms the specific status of *R. banyulensis* and *R. grassei* already evidenced through both chemical (cuticular hydrocarbons) and molecular (nuclear and mitochondrial sequences) studies (Kutnik et al., 2004).

Generally speaking, the use of the end of Messinian salinity crisis (5.3 MYA) as a calibration point for the separation of Cretan populations from the other *Reticulitermes* taxa highlighted different dating with respect to previous works (Luchetti et al. 2005a, 2007; Lefebvre et al., 2008). On the other hand, here, good geological/paleoclimatic correlations were found to explain the nowadays scored biodiversity. Moreover, all the cladogenetic events discussed happened in a vicariance context; this is in line with the ecology of these termites for which the dispersal ability, without some human effect, is low.

To conclude, following our results, a taxonomic and phylogenetic revision of the genus *Reticulitermes* in Europe is here proposed (see Fig. 6).

In the eastern area, only three entities of specific level can be univocally recognized; the first one is *R. urbis*, whose significantly old divergence from the common eastern ancestor may support a subspecific differentiation of its northern and southern lineages well evident in all analyses. *R. urbis* appears basal to the other two specific entities, i.e. *R. balkanensis* with a limited distribution in the Attic region and Peloponnesus, and the new species here suggested as *R. aegei* that with its wide distribution embodies samples from northern Turkey, Thrace, Macedonia/Calcydia and Crete island. *R. balkanensis* and *R. aegei* represent two divergent genetic lineages related to southern Turkey populations/*R. clypeatus*/ Dodecanese Samos deme and to Cycladic Amorgous sample, respectively. The

unsupported dichotomies showed by *R. balkanensis*, *R. lucifugus* - Turkey - southern clade and *R. clypeatus* do not allow us to discuss a clear evolutionary pattern but, excluding Samos sample and *R. clypeatus*, low but significant bootstrap values for the remaining nodes can be obtained (available from the authors), clearly supporting the need to widen samplings.

In the remaining part of the Mediterranean basin, our analysis shows a clear dichotomy between a more western lineage distributed in continental France and in the Iberian peninsula, clearly divergent from a central one present in Italy and Corse. The former leads to *R. banyulensis* and *R. grassei* for which the species status is confirmed as previously hypothesized (Kutnik et al., 2004). In the latter, the high level of divergence and the antiquity of the origin of the three *R. lucifugus lucifugus*, *R. lucifugus corsicus* and *R. lucifugus subsp.* (Sicily) speak in favour of a specific status of differentiation. Under this assumption, these central Mediterranean taxa should be revised as follows: *R. lucifugus* in peninsular Italy, *R. corsicus* from Sardinia and Corse - despite some colonies were found along the Italian Thyrrenian coast as a result of human mediated transport (Marini and Mantovani, 2002; Uva et al., 2004; Lefebvre et al., 2008) - and a new species from Sicily for which we propose the name *R. siciliae*. From a phylogenetic point of view, *R. siciliae* and *R. lucifugus* are far more strictly related, as also hypotizable on geological basis. In a previous analysis, Lefebvre et al. (2008) handled these three last taxa as subspecies, and the absence of supported dichotomies didn't allow to clarify their evolutionary pattern.

On the whole, we therefore propose the presence of the following eight autoctonous *Reticulitermes* species in the European Mediterranean area: *R. aegei*, *R. balkanensis*, *R. urbis*, *R. banyulensis*, *R. grassei*, *R. lucifugus*, *R. corsicus* and *R. siciliae*.

As a final remark, our results showed a good resolution power of Inter-SINE markers and, as mitochondrial DNA, they evidenced a clear divergence among *Reticulitermes sp*. from Crete and other *Reticulitermes* species analyzed. On the basis of these results and the

ones obtained in a parallel study on *Kalotermes* taxa (Velonà et al., work in progress), we believe that this marker could be a promising candidate as a good tool for molecular analysis purposes, also considering its quickness and cheapness,.

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Figure legends

Fig. 1. a) Collection sites of the 22 Cretan colonies (see also Table 1) and b) geographic distribution of the other eastern Mediterranean samples drawn from GenBank for the phylogenetic analysis (grey diamonds=*R. urbis* - northern clade, black diamonds=*R. urbis* - southern clade, white squares=*R. lucifugus* - Turkey - northern clade, black squares=*R.lucifugus* - Turkey - southern clade, white circle=*Reticulitermes sp.* (Samos), grey circle=*Reticulitermes sp.* (Amorgous)). For details on populations utilized see Materials and Methods section and Fig. 2a.

Fig. 2. Combined Maximum parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI) trees calculated on COII+16S mitochondrial DNA sequences (a) and geographic distribution of the four Cretan lineages (b). In a, numbers at nodes indicate bootstrap values for MP and ML analyses and posterior probability expressed as percentage for BI analysis.

Fig. 3. Parsimony network obtained from 16S haplotypes. Black dots represent missing haplotypes; circles are proportional to haplotype frequencies. Symbols as in Fig. 2a.

Fig. 4. Chronogram tree representing the divergence time estimations and, in parenthesis, the relative 95% confidence intervals for each dichotomy. Numbers are expressed in million years ago (MYA).

Fig. 5. Histograms showing the frequencies of intra-clade divergence values (white bars) and inter-clade divergence values (grey bars). a) *Reticulitermes* from Crete, groups 1-2-3-4; b) *R. lucifugus* from northern Turkey and Thrace *vs R. lucifugus* from Calcydia; c) *Reticulitermes* from Crete, groups 1-2-3-4 *vs R. lucifugus* from northern Turkey and Thrace + *R. lucifugus* from Calcydia; d) *R. urbis* - northern clade *vs R. urbis* - southern clade; e) *R. lucifugus lucifugus vs R. lucifugus corsicus*. f) *R. lucifugus lucifugus vs R. lucifugus subsp.* (Sicily).

Fig. 6. Diagram showing the autochtonous *Reticulitermes spp*. presently identified in the Mediterranean area; branches length is proportional to the dating of cladogenetic events. Broken lines represent taxa for which the low number of available sequences didn't allow the pairwise divergence analysis.

Figure 1

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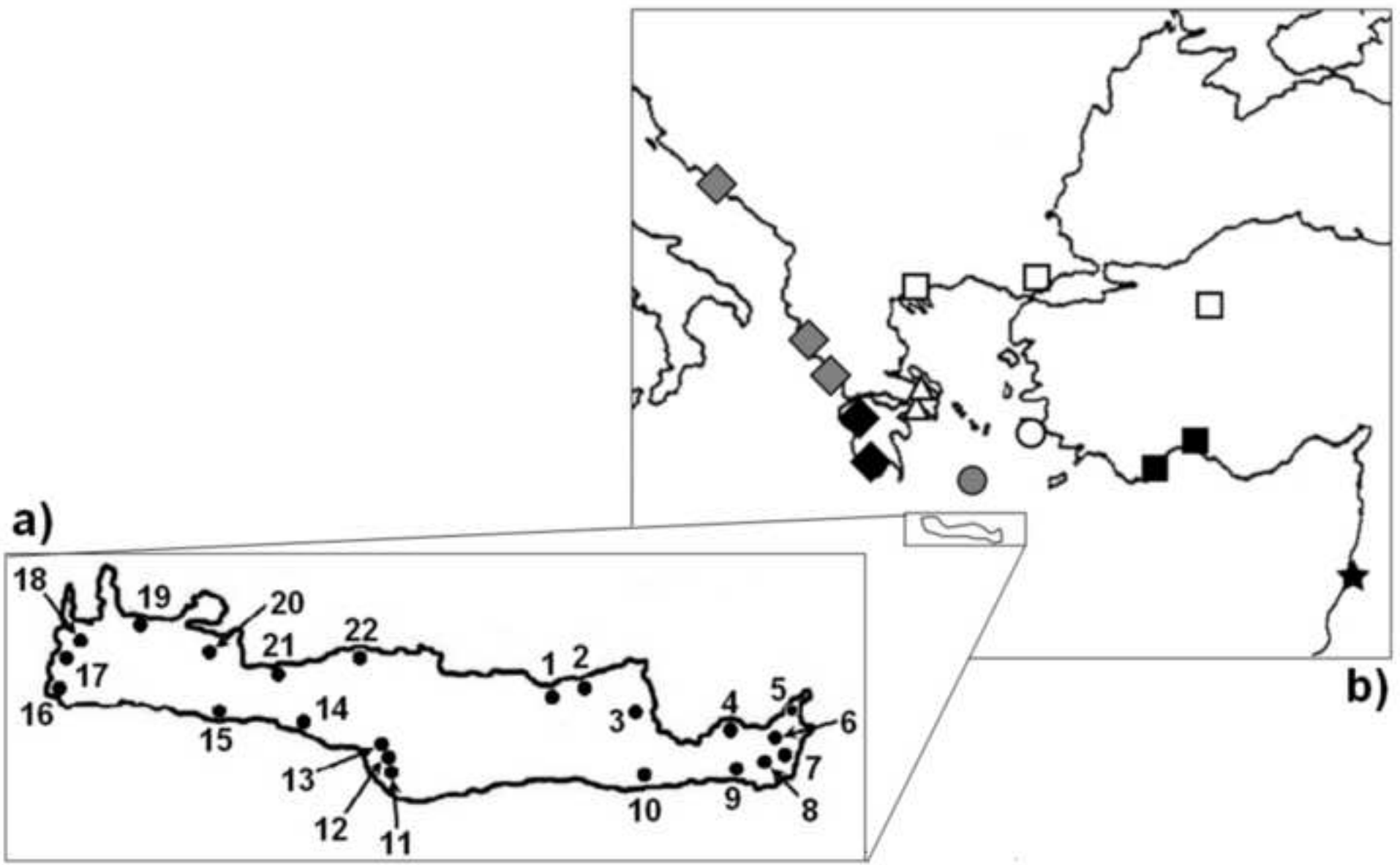

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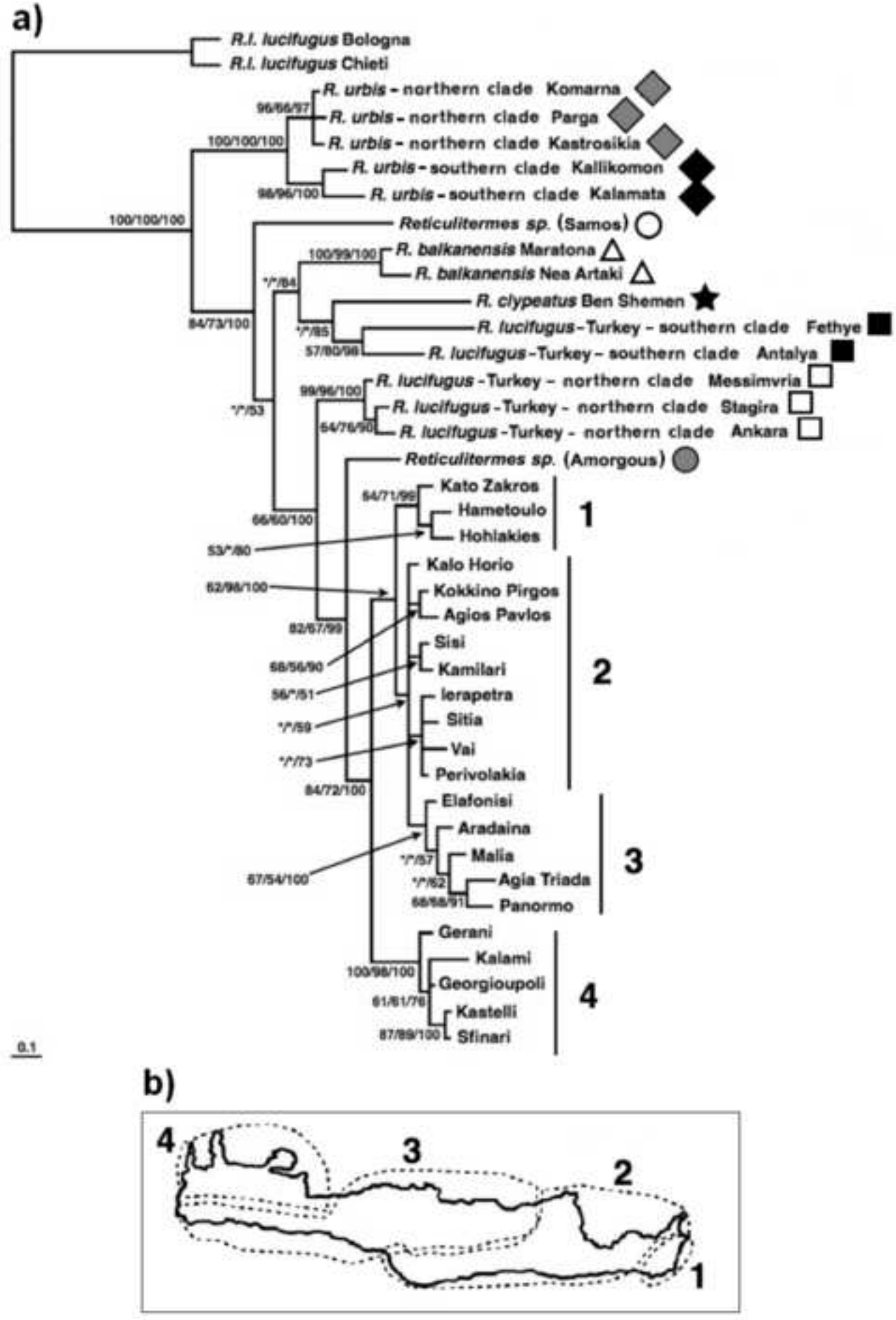

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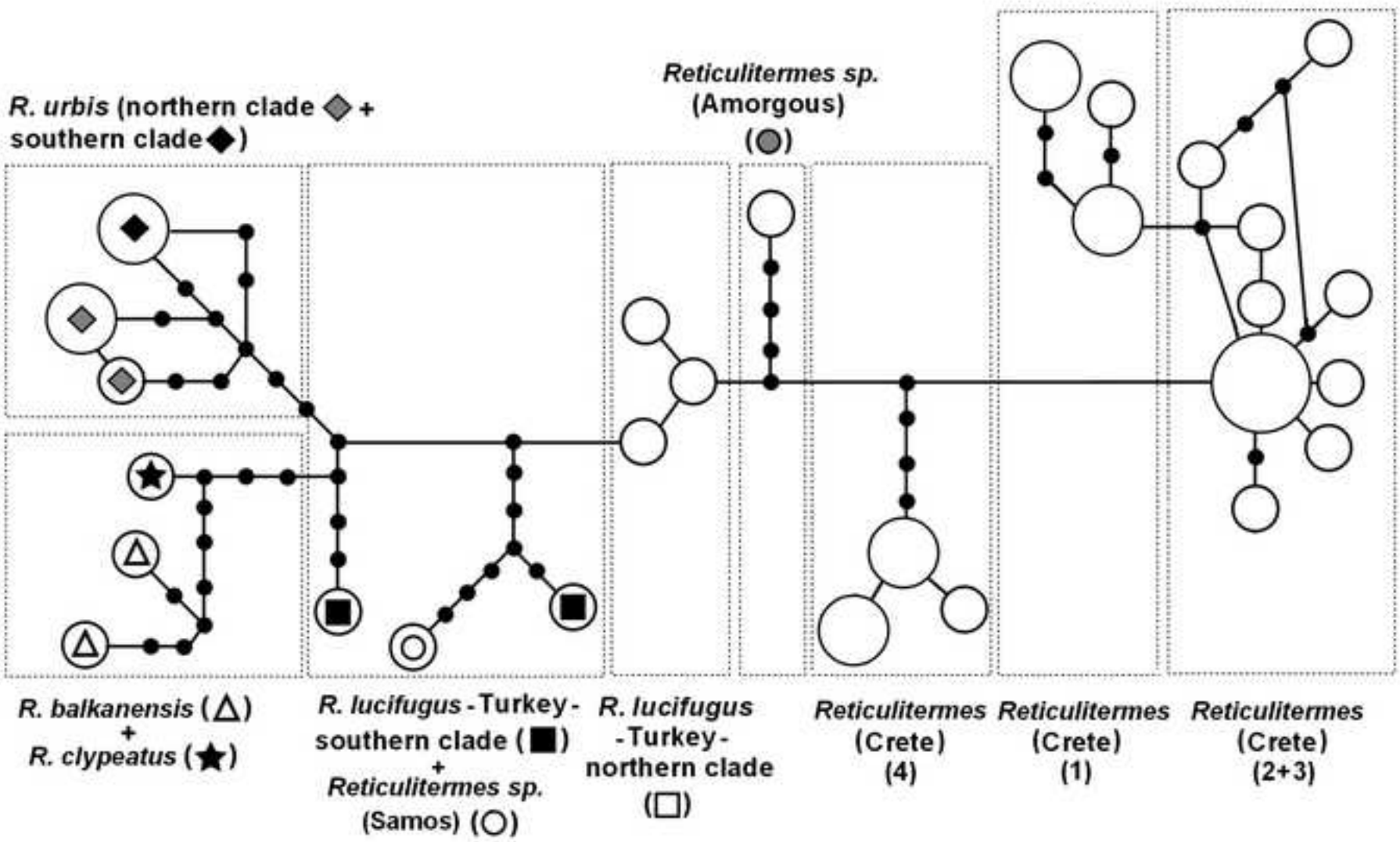

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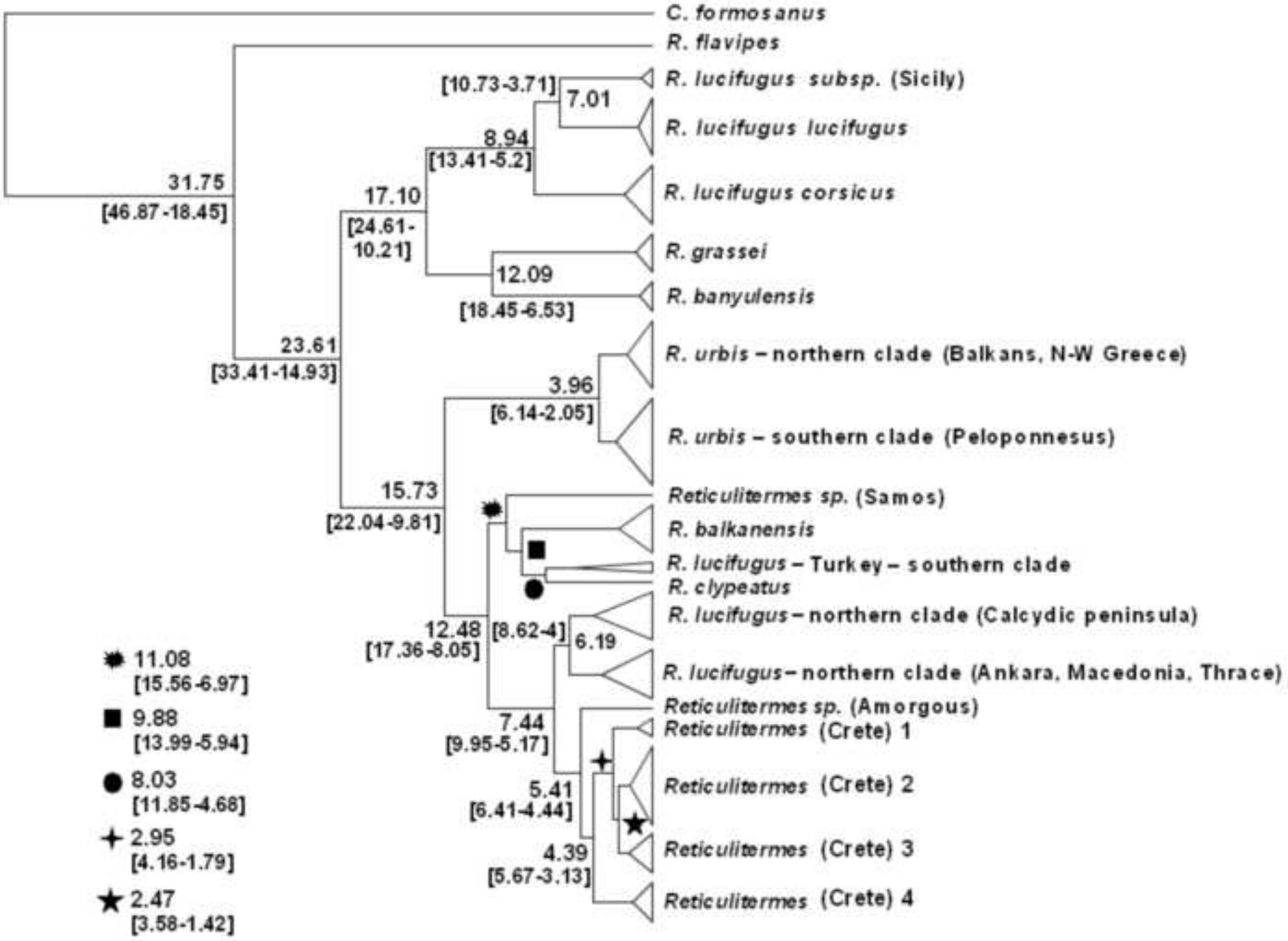

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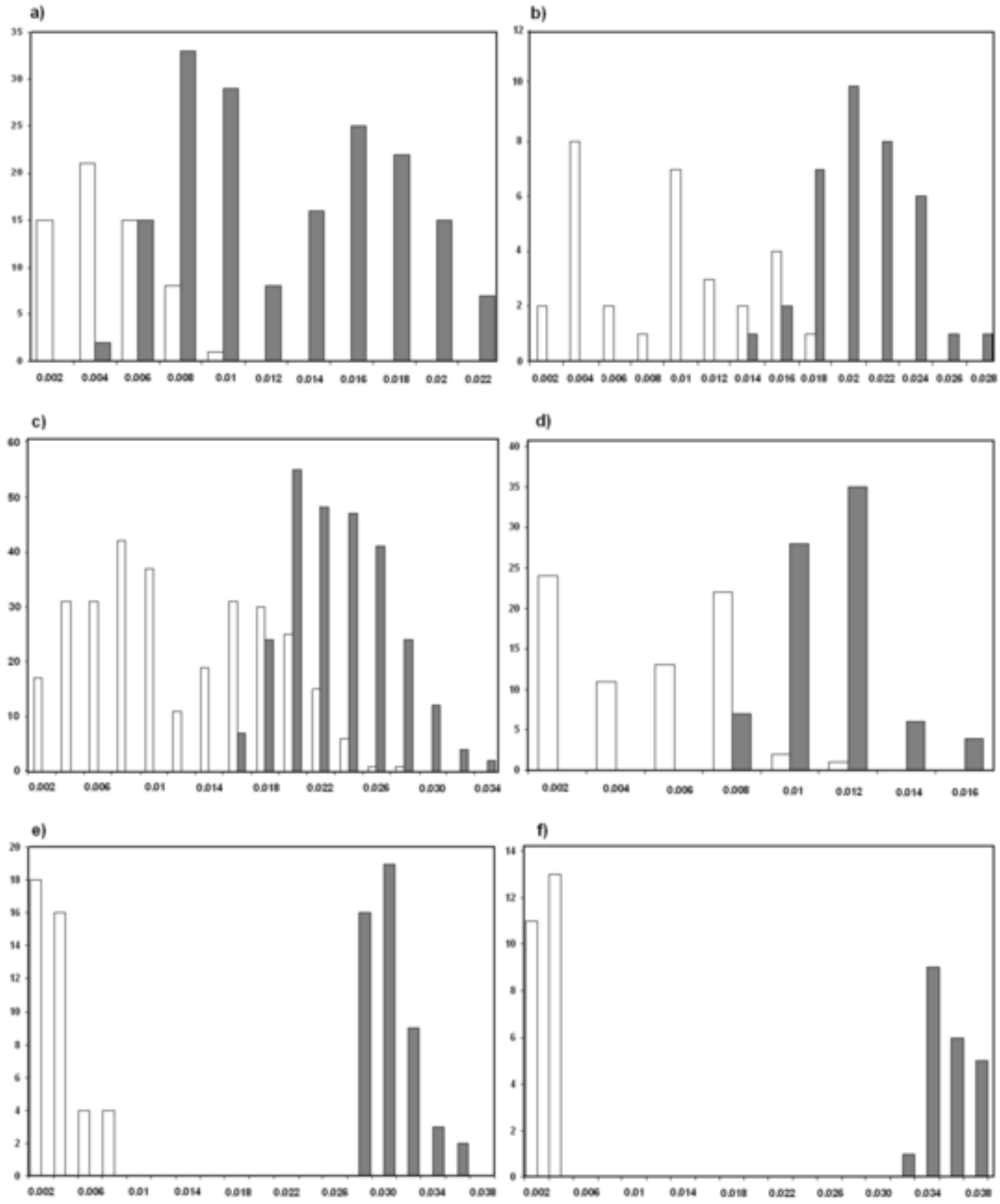

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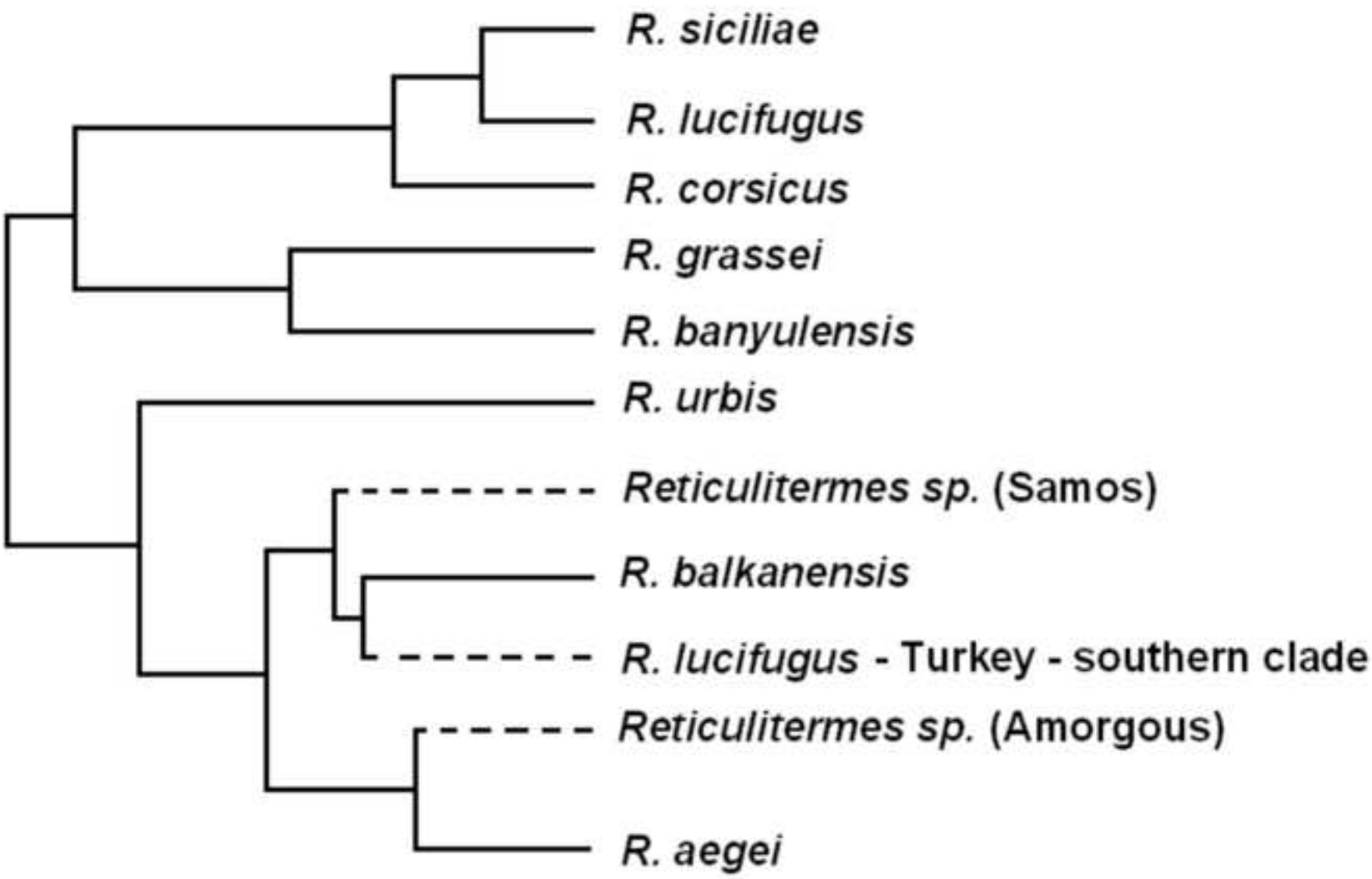

Tables

Table 1. Collecting sites, scored haplotypes and GenBank accession numbers for Cretan samples. Locality numbers refer to Fig. 1a.

Locality		Haplotype			GenBank A.N.	
		COII	16S	Combined	COII	16S
1	Malia	c1	r1	mt1	GU373606	GU373584
2	Sisi	c2	r2	mt2	GU373613	GU373591
3	Kalo Horio	c3	r3	mt3	GU373602	GU373580
4	Sitia	c4	r4	mt4	GU373598	GU373576
5	Vai	c5	r5	mt5	GU373599	GU373577
6	Hohlakies	c6	r6	mt6	GU373597	GU373575
7	Kato Zakros	c7	r4	mt7	GU373609	
8	Hametoulo	c8	r4	mt8	GU373593	
9	Perivolakia	c9	r4	mt9	GU373610	
10	Ierapetra	c9	r7	mt10		GU373572
11	Kamilari	c2	r8	mt11		GU373585
12	Agia Triada	c10	r1	mt12	GU373600	
13	Kokkino Pirgos	c11	r9	mt13	GU373592	GU373570
14	Agios Pavlos	c12	r10	mt14	GU373601	GU373579
15	Aradaina	c13	r11	mt15	GU373604	GU373582
16	Elafonisi	c14	r11	mt16	GU373605	
17	Sfinari	c15	r12	mt17	GU373612	GU373590
18	Kastelli	c15	r12	mt17		
19	Gerani	c16	r13	mt18	GU373595	GU373573
20	Kalami	c17	r14	mt19	GU373596	GU373574
21	Georgioupoli	c18	r13	mt20	GU373611	
22	Panormo	c19	r15	mt21	GU373603	GU373581

Table 2. Pairwise F_{ST} values (below the diagonal) and their statistical significance (above the diagonal) calculated on I-SINE data.

NS= not significant.

	Crete				***R. urbis* - northern clade**	***R. urbis* - southern clade**	***R. balkanensis***	***R. lucifugus lucifugus***
	Group 1	**Group 2**	**Group 3**	**Group 4**				
Crete - Group 1	-	P<0.05	NS	P<0.05	P<0.01	P<0.05	P<0.01	P<0.01
Crete - Group 2	0.119	-	P<0.01	P<0.05	P<0.001	P<0.01	P<0.01	P<0.01
Crete - Group 3	0.086	0.129	-	P<0.01	P<0.01	P<0.01	P<0.01	P<0.001
Crete - Group 4	0.099	0.102	0.127	-	P<0.01	P<0.01	P<0.01	P<0.01
***R. urbis* - northern clade**	0.773	0.369	0.698	0.499	-	NS	P<0.05	P<0.05
***R. urbis* - southern clade**	0.679	0.267	0.630	0.410	0.186	-	P<0.05	P<0.05
R. balkanensis	0.773	0.325	0.657	0.475	0.580	0.371	-	P<0.05
R. lucifugus lucifugus	0.699	0.256	0.626	0.432	0.302	0.243	0.381	-