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PHYLOGENY AND POPULATION DYNAMICS IN EUROPEAN *RETICULITERMES* AND *KALOTERMES* GENERA (INSECTA, ISOPTERA)

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CHAPTER 1

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ISOPTERA

1.1 MORPHOLOGY

Given that the order Isoptera embodies around 2,600 species, each of them subdivided in different castes (figure 1.1), it's difficult to give a unique description of the morphology of these organisms. Despite this, some general remarks can be done.

Termites are small insects with a body length rarely exceeding 2 cm. The tegument is thin and whitish in workers, while it is light brown and usually black in soldiers and reproductives, respectively. The head is dorso-ventrally depressed and orthognathus, with a chewing mouthpart apparatus, allowing the wood alimentation. In the soldier caste the jaws are highly modified for defence purposes.

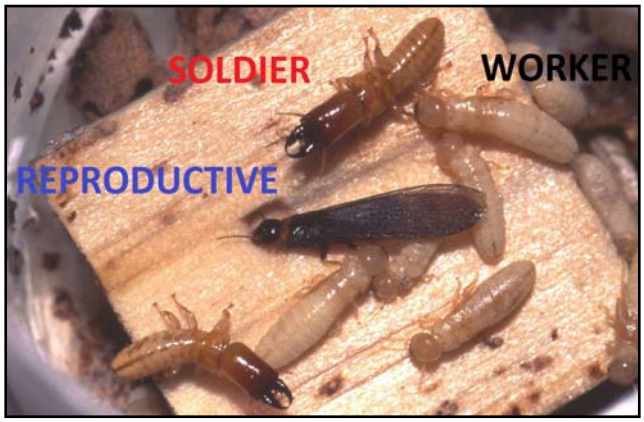

Figure 1.1 - Example of individuals of *Kalotermes flavicollis*. The three main castes are shown.

The compound eyes are well developed in reproductives but strongly reduced or totally absent in workers and soldiers. The moniliform antennae have a variable number of segments, depending on species, caste and age.

Workers and soldiers are apterous, while reproductives have two pairs of wings. These are equal in length and shape - so the name of the order is Isoptera - and consist of a transparent membrane in which the veins are visible. At the basis of the wings there is a breaking line called “basal suture” that allows the separation of the wings from the thorax after the nuptial flight.

The abdomen is formed by ten segments with two posterior caudal cerci. The ventral portion of the abdomen is morphologically different between female and male, the former having the 7th segment highly developed, forming a large ventral plaque that protects the genital chamber.

1.2 PHYLOGENY AND SYSTEMATICS

1.2.1 Origin of the order

Termites are believed to be the earliest-evolving social insects. They date back to the Cretaceous period, around 130 millions years ago, but some recent fossil records suggest an even more ancient origin, in the upper Jurassic (Korb, 2007). The evolution of isopterans is strictly associated with the two orders Blattodea (cockroaches) and Mantodea (mantids), together forming a natural assemblage known as Dictyoptera. Moreover, the high affinity between Isoptera and Blattodea has suggested that termites arose from a single “social cockroach” species that diversified into the different termite families.

There are ~ 2,600 species allocated in 280 genera and in seven families (Engel & Krishna, 2004; Inward *et al*., 2007). These are: Mastotermitidae, Kalotermitidae,

Termopsidae, Hodotermitidae, Rhinotermitidae, Serritermitidae and Termitidae. There is a strong asymmetric repartition of the species among the above mentioned families; for example, Kalotermitidae and Rhinotermitidae embody around 400 and 300 species, respectively, while the Mastotermitidae and Serritermitidae families comprise only one species each (*Mastotermes darwiniensis* and *Serritermes serrifer* respectively). Termitidae, the largest family, encompasses around 1,800 species (the 70% of the described taxa) mainly from the tropical regions of Australia, Africa, Asia and South America.

1.2.2 Intra-order relationships

Despite recent advances, the phylogenetic relationships among the seven families of Isoptera are far from being resolved. In two recent works, the authors inferred the intra-order evolution, using either the results of a combination of both molecular (COII, 12S and 28S) and morphological approaches (40 characters; Inward *et al*., 2007) or only molecular data (12S, 16S, 18S, 28S, COI, COII and cyt b; Legendre *et al*., 2008). Both analyses show a similar trend: the order Isoptera results monophyletic and strictly associated with the order Blattodea underling a strong relationship of these two taxa.

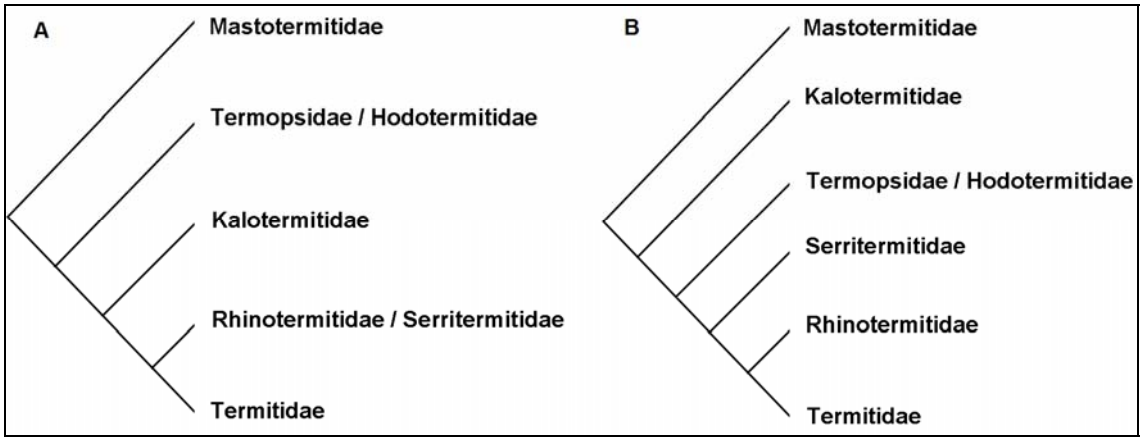

Figure 1.2. Phylogenetic trees allocating the seven termite families obtained by (A) Inward *et al*., (2007) and (B) Legendre *et al*., (2008).

As shown in figure 1.2, two main differences are present; first of all, in the phylogeny by Legendre *et al*. (2008) the position of the Kalotermitidae family is basal to the Termopsidae/Hodotermitidae clade, while in Inward *et al*. (2007) the two clusters are inverted. Moreover, also the position of Serritermitidae is problematic, since in Legendre *et al*. (2008) it represents a separate cluster that lies basal to Rhinotermitidae, while in Inward *et al*. (2007) it falls into the Rhinotermitidae family.

It also should be noted that in both papers Mastotermitidae and Termitidae are the most basal and the most apical families respectively, and the Hodotermitidae family falls into the Termopsidae one.

As described above, it is clear that the deep phylogeny of the order is not well resolved, underling the necessity of further analyses.

1.3 SOCIAL ORGANIZATION

Termites are characterized by a social organization in which the basal structure is represented by the colony. Individuals are grouped in castes that perform different roles, and are also characterized by specific biological traits in anatomy, physiology and behaviour. Three main castes can be found in a colony: reproductives, soldiers and workers.

In the so-called “higher termites” (i.e. Termitidae and Rhinotermitidae), castes are well separated among them, while lower termites exhibit a greater plasticity, allowing caste changing for the individuals. The caste determination follows different mechanisms among families. In fact, environmental factors, such as nutritional and pheromonal

signals, seems to be the most important ones in lower termites (Grassé, 1949), while, recently, some authors evidenced that in higher termites a strong genetic basis is involved (Hayashi *et al*., 2007; Lo *et al*., 2009).

1.3.1 Reproductives

Reproductives are the only individuals with functional gonads and through reproduction they can build new colonies and they can maintain the established ones.

Figure 1.3. A reproductive alate of the species *Reticulitermes grassei*.

Two main categories of reproductives are actually recognized: the imagoes and the neotenics. The imagoes are also called alates or primary reproductives (figure 1.3). They have wings and compound eyes: these features allow the swarming (see below) and the subsequent colony establishment. The neotenics are called secondary reproductives and are wingless or with reduced wings. For this reason, their principal role is to maintain established colonies. Neotenics may derive from workers – and in this case they are called "ergatoids" – or from nymphs – the so called "nymphoids".

Reproductive males (kings) and females (queens) can be distinguished from the external morphology: males have styles on the 9^{th} sternal segment, while females have an enlarged 7^{th} sternal segment and the 8^{th} and the 9^{th} are fused.

Termites are diplo-diploid species: it means that males and females are diploid and contribute equally to the progeny. The reproductive system is gonochoric despite there are some exceptions. For example, *Reticulitermes speratus* is the first isopteran species

in which a thelytokous automictic parthenogenesis was described. This reproductive strategy allows the unmated primary queens to establish anyhow new colonies (Matsuura *et al.*, 2004; 2009).

1.3.2 Soldiers

The soldier caste is devoted to colony defence. Anatomical differentiations include mainly a brown pigmented body, a pair of enlarged jaws and a large head (figure 1.4). Jaws are so strongly modified that soldiers are unable to chew and are fed by workers following the trophallaxis modality (see below). The proportion of soldier within a colony is linked to different factors, such as colony size and season. Following Hawerty (1977), it is generally comprised between 1% and 10%.

Figure 1.4. Soldier of *Kalotermes flavicollis.*

The defending roles that soldiers perform are mainly to contrast the attack of other arthropods species (especially ants), but also of other animals, such as mammals (echidnas, pangolins and apes).

Soldiers of different species follow four main defending strategies:

- in the majority of the species, soldiers use their tough jaws to attack heterospecific individuals invading the colony;
- in few species, soldiers clutch the enemy and spread their body with salivary secretions;
- in some Kalotermitidae taxa, soldiers have an expanded head (called “fragmotic”) that is used to occlude nest’s galleries and keep out the intruders;

- in many species of the Rhinotermitidae and Termitidae families, soldiers do not have expanded jaws, but they spray defensive compounds produced by a frontal gland against enemies.

1.3.3 Workers

In terms of abundance, workers constitute the main caste in a termite colony since they can represent over the 90% of individuals. In the great majority of the species, workers are small, white, wingless and eyeless (figure 1.5). The high density of workers within a nest is obviously due to their many functional roles. They execute in fact all the logistic functions that are necessary for the maintenance of the nest: workers build and repair galleries, they look for food and feed reproductives, soldier and juveniles and, if necessary, they can also help soldier in colony defence. In few species of the Termitidae family, workers even practice agriculture: they collect plant matter to produce fungal gardens, upon which they feed.

Figure 1.5. *R. urbis* workers.

1.4 COLONY ESTABLISHMENT

As above reported, the colony is the basal structure for termite's social life. At present, three main mechanisms have been described to explain foundation modalities. These are: swarming, budding and sociotomy.

1.4.1 Swarming

This modality is generally considered the most common one. It takes place when the winged individuals (i.e. reproductives) swarm from their original nest and perform the so-called “nuptial flight”. Depending on species biology, swarming takes place one or more times in a year and it can last even few days. Since termites are not good flyer, individuals do not go very far away from the nest of origin: the flying ability in fact allows reproductives to cover only some dozen of metres. After this, they land and lose their wings at the basal suture line and try to find opposite sex individuals to reproduce. The couple proceeds in tandem, with male following the female, searching for a good site to form the new nest. The royal pair takes care of the first brood of eggs and juveniles.

To better allow the encounter among reproductives, it’s very important that the time of swarming is synchronized for the members of the same population. In many species the swarming happens in particular weather conditions, for example immediately after the rain.

1.4.2 Budding

Budding is an alternative modality to found new colonies. It happens when a group of individuals separates from the colony of origin. Generally, these individuals live at the periphery of big and subterranean colonies, where the transmission of pheromones and other stimuli is less strong and so, neotenics begin to reproduce. Budding can be also the result of strong weather events like floods or soil disruption.

In some species, alates are unable to found new colonies, and budding represents the only way to do it (Campedelli, 1987).

1.4.3 Sociotomy

Sociotomy occurs when a group of individuals of each caste, including the royal couple, leaves the nest and found a new colony. In the old nest reproduction is carried on by neotenics.

Sociotomy is rare in termites: until today, it was observed only in *Anoplotermes* and *Trinervitermes* species (Grassé, 1949).

1.5 COLONY STRUCTURE

Despite the colony is considered as the basal social structure in termites, these can be further subdivided on the basis of their breeding system and family structure. Three types are actually known: simple family, extended family and mixed family.

In a simple family, all the individuals are the offspring of a single pair of reproductives, that represents the founding couple. This is the simplest colony structure, and it is generally developed in the early stages of the colony establishment.

In the extended family, the offspring is produced by multiple neotenics, originated from a single pair of primary reproductives. Neotenics reproduce among themselves, or together with one or both of the primary reproductives. This structure is typical in old and well established colonies, and allows colony persistence after the death of the royal couple. Moreover, with this kind of colony structure, a spatial expansion can take place.

Finally, colonies are considered as mixed families when the offspring derives by more than two unrelated reproductives. It can happen, for example, after a fusion process between two or more colonies or when an established colony adopts unrelated

reproductives born in other colonies.

As explained above, the colony reproductive structure can be considered as a good index in detecting the level of development of the colony.

1.6 ECOLOGY

1.6.1 Feeding behaviour

Termites have a predominantly tropical distribution, with the highest species diversity found in tropical forest, where they are considered the most important invertebrate decomposers (Eggleton & Tayasu 2001). It was estimated that, in this ecosystem, termites ingest from 50 to 100% of the dead plant biomass, contributing significantly to the recycling of the elements (Abe *et al*., 2000). The main food resource for these organisms is in fact the cellulose, which is obtained from dead wood, leaf litter, dry grass and soil.

The specific ecological niche that termites occupy is allowed by the symbiosis with prokaryotes and protozoans that are present in their gut. Three main patterns of symbiotic relationships can be described. In Mastotermitidae, Termopsidae, Kalotermitidae and Rhinotermitidae families, a complex community of flagellate protozoa occurs, while in the great majority of the Termitidae family only bacteria communities can be found in the chambers and diverticula in which their gut differentiate. Finally, among higher termites, a particular feeding behaviour is performed by the subfamily Macrotermitinae: these termites cultivate the basidiomycetes *Termitomices* supplying it with plant-derived materials. The fungi can

therefore grow and degrade these materials, allowing termites to eat them, with spores and hyphae also constituting an important food source for the termites.

Trophallaxis is a particular feeding modality. The transfer of organic material, from one individual to another, can take place through regurgitation (stomodeal trophallaxis, figure 1.6) or through anal secretion (proctodeal trophallaxis). The latter modality allows to transfer, other than food, the gut endosymbionts, that are important for juveniles or individuals that lost symbionts after the moult. Moreover, it has also been demonstrated that trophallaxis plays a key role in the transfer of caste regulatory pheromones (Suárez & Thorne, 2000).

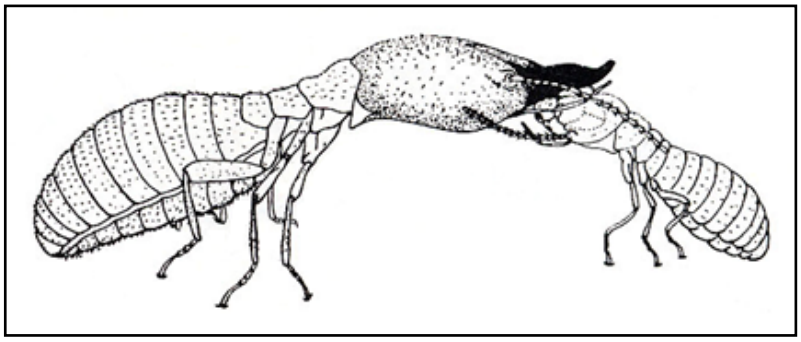

Figure 1.6. Stomodeal trophallaxis: a worker is feeding a soldier. From Bergamaschi (2007).

1.6.2 Communication

One of the most important feature of termite's biology is that individuals are grouped in colonies where, for the good functioning of the colony itself, the different tasks are performed by the assigned caste. In this way, a strict system of communication among individuals of the same caste and of different ones is necessary. At the moment, the two main recognized modes are based on tactile and chemical stimulations, while, given that the great majority of individuals lack eyes, visual communications don't seem to play a fundamental role.

Termites are known to perform vibratory movements associated with the head or the

abdomen, banging in order to communicate an alarm situation. The mechanoreceptors, essential for the reception of the vibratory stimuli, are arranged to detect signals from different directions and are placed predominantly in the second antennal segment. Two groups of sensilla are actually known: the first is named “Johnston organ” and allows to perceive vibratory movements, while the second is devoted to the detection of the gravity force.

The most important role in the transmission of information among individuals is played by pheromones. Several compounds, isolated from soldiers, are essential for the transmission of the alert signals and to stimulate a defensive behaviour among workers (Roisin *et al*., 1990). Other pheromones are devoted to inform about the availability of new food resources, in mate attraction, inter-individuals recognition and, in basal termites species, caste differentiation. Probably, as argued by Springhetti (1985), the repeated contacts among individuals, such as the trophallaxis, is the principal way for the diffusion of pheromones among colony members.

1.6.3 Termite lifetypes

Following the classification proposed by Abe (1987), termites can be grouped in three distinct groups on the basis of their ecology and feeding behaviour. These are: “single-piece” nesters, “intermediate-piece” nesters and “separate-piece” nesters. In the single-piece nester (sometimes referred to as “one-piece” nester) species, individuals feed and nest in the same specific substrate; all the wood feeding termites belong to this group. Intermediate-piece nester species build the nest in their feeding substrate, but they can also forage outside. Finally, in the separate-piece nesters, nest and feeding areas do not overlap: so termites actively forage away from the nest. The most important ecological

feature of this classification is that it separates species according to the degree to which their nesting and feeding substrates overlap temporally and spatially.

Following this classification, the two genera I have analyzed are either single-piece nesters (genus *Kalotermes*, Kalotermitidae, "dry-wood termites") or intermediate-pieces nesters (genus *Reticulitermes*, Rhinotermitidae, "subterranean termites").

It is to be noted that this kind of ecological classification does not have taxonomic or phylogenetic implications, since there is no complete correlations among them. For example, the Kalotermitidae *Paraneotermes simplicornis* is known to be an intermediate-piece nester, while the three Rhinotermitidae genera *Coptotermes*, *Psammotermes* and *Schedorhinotermes* have developed, probably independently, a separate-piece nesting habitus (Eggleton & Tayasu, 2001).

1.6.4 Distribution

As reported above, there are over 2,600 termite species nowadays described. Their distribution is not homogeneous in the world, since the species biodiversity declines when the latitude increases, with the 50° northern and southern parallels representing the maximum limits. The tropical and subtropical regions share the highest biodiversity that decreases in the temperate regions, while in the boreal and arctic regions isopterans are totally absent. This typical pattern of distribution evidences a tropical origin of the order with a progressive colonization of colder ecosystems. Thirteen genera out of the 275 described ones have a cosmopolitan distribution: the genus *Kalotermes* is one of these, suggesting an origin prior to the Laurasia-Gondwana splitting, while the *Reticulitermes* one, having a Palearctic distribution, was probably originated after this geological event.

These two genera are the only one present in the wild in Europe, with a distribution from Portugal to the Turkish coasts, comprising the major and minor islands of the Mediterranean sea.

CHAPTER 2

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MOLECULAR MARKERS

In relationship to the different kind of topics dealt, I had to utilize markers derived from the mitochondrial and nuclear genomes. In particular from the former, I've collected sequences from *cytochrome oxydase* I (COI), 16S, *control region* (CR) and COI/tRNA^{Leu}/COII genes, while from the nuclear genome I've genotyped both microsatellite and Inter-SINE loci. Some very general aspects on the utility of these markers for phylogenetic and genetic variability purposes are below reported.

2.1 MITOCHONDRIAL DNA

The animal mitochondrial genome is a relatively small circular molecule of 15-20 Kb that typically codes for 37 gene products (figure 2.1). In particular these are: 13 protein subunits of the enzymes for oxidative phosphorylation (COI, COI, COIII, cyt b, ND1-6, ND4L, ATP6, ATP8), two ribosomal RNAs (rrnL and rrnS), and 22 tRNAs (trnX). It also includes a non-coding region known as D-Loop in Vertebrata or control region (CR) in Arthropoda.

Several features make mtDNA often a good tool for phylogenetic studies. In general terms, the mitochondrial genome is haploid, it is not processed by recombination, the genes are orthologous - allowing the comparison among distant taxa - and it has mainly

a maternal inheritance, enabling the reconstruction of the species life history also when reproductive strategies differing from gonochorism takes place.

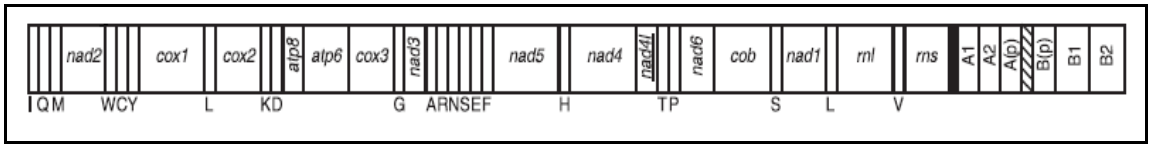

Figure 2.1. Linearized mitochondrial genome of *Reticulitermes* spp. The tRNA genes are indicated by the single letter IUPAC-IUB abbreviation for their corresponding amino acid. Modified from Cameron & Whiting (2007).

Moreover, mitochondrial loci have different mutation rates (generally higher with respect to the nuclear genome), ensuring their utility at different levels of phylogeny. Finally, from a technical point of view, it is easy to amplify and to obtain mitochondrial sequences in insects, giving the existence of universal primers (Simon *et al*., 1994; 2006).

In termites, the most sequenced genes for phylogenetic analyses are the COII and 16S genes (Inward *et al*., 2007; Nobre *et al*., 2006; Kutnik *et al*., 2004); in fact their combination in a unique dataset allow authors to obtain a good resolution power (Marini & Mantovani, 2002; Lo *et al*., 2004; Luchetti *et al*., 2004a; 2007). Details on these papers will be showed in the chapter 3.1.

2.2 MICROSATELLITES

Microsatellites, also known as Short Tandem Repeats (STRs), are small portions of DNA, generally from two to six nucleotides (i.e. the motif), that are repeated consecutively for a variable number of times. Microsatellites are present in the nuclear

genome of eukaryotes, mainly in portions of non-coding DNA. For this reason, STRs are not processed by selection, or are processed by a weak selective pressure; this allows microsatellites to mutate very quickly, with an estimated mutation rate of 10^{-3} per locus per generation (Goldstein & Schlötterer, 1999).

The most important mechanism in the microsatellite mutation process seems to be the "*polymerase slippage*" during DNA replication. It consists in the separation of the two DNA strands followed by a re-union in a different position: in this way, one of the two filaments creates a loop. If the loop is formed in the newly synthesized strand, the STR will be increased in the number of repetitions, while it will be reduced if the loop is formed in the template strand.

Given the presence of microsatellites in non-coding DNA and their high mutation rate, STRs are considered an optimal molecular marker for the analysis of genetic variability, especially at the population level. Microsatellites are therefore widely used in the study of population genetics, conservation genetics, migratory fluxes and colonization histories, in both animals and plants. In termites, they resulted particularly useful in the study of colony structure and fusion processes (see for example DeHeer & Vargo, 2004; Dronnet *et al*., 2005; Nobre *et al*., 2008; Leniaud *et al*., 2009; Perdereau *et al*., 2010). As for the previous paragraph, more details on these papers will be shown in chapter 3.2.

2.3 INTER-SINE

The inter-SINE methodology has been only recently developed and it is well suited for

the study of the genetic variability at the population level.

Short INterspersed Elements (SINEs) are non-autonomous nuclear retrotransposons, usually present with more than 10^5 copies (Kostia *et al*., 2000; Ohshima & Okada, 2005). The power of SINEs in phylogenetics is that their insertion is random and unidirectional, so that the absence of a copy is always the ancestral condition (Nishihara & Okada, 2008).

The methodology that I've used was based on the amplification of the regions comprised between two copies of the same SINE, followed by an electrophoretic run. In this way a binary data matrix can be produced and data can be used as for other most known dominant markers such as RAPD, ISSR and AFLP.

From a technical point of view, with respect to these markers, the I-SINE methodology uses specific primers: these anneal within the SINE, avoiding the problem of unspecific amplification, that occurs very frequently when using RAPD or ISSR markers. Moreover, I-SINE are cheaper and faster with respect to AFLP, because the use of restriction enzymes is not required.

I-SINE have been tested mainly for phylogenetic studies in mammals in combination with mitochondrial data (Shafer & Stewart, 2007), or with other nuclear dominant markers (Kostia *et al*., 2000), but they have been never used in invertebrates so far.

CHAPTER 3

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STATE OF THE ART AND RESEARCH AIMS

3.1 EUROPEAN TERMITES PHYLOGENY

As reported in paragraph 1.6.4, two termite genera are native in Europe: *Reticulitermes* and *Kalotermes*; while the phylogeny of the former has been widely approached (Marini & Mantovani 2002; Kutnik *et al*., 2004; Luchetti *et al*., 2004a; Luchetti *et al*., 2005; Nobre *et al*., 2006; Luchetti *et al*., 2007), the evolutionary history of the latter was analyzed in only one paper (Luchetti *et al*., 2004b).

In 2002, Marini & Mantovani's paper was one of the first work analyzing European *Reticulitermes* phylogeny. The authors sequenced the mitochondrial COII and 16S genes in 21 Italian and French populations. Their results showed a clear cluster of *R. lucifugus lucifugus* populations from peninsular Italy and a trans-Tyrrhenian distribution of *R. lucifugus corsicus*, which is present in Corse, Sardinia and along the Tuscany coast. Moreover, a new lineage was discovered in north-eastern Italy. The same paper highlighted that the French taxon *R. santonensis* is genetically indistinguishable from North American *R. flavipes.*

Kutnik and co-authors (2004) combined molecular (ITS2, COII and a sequence comprising fragments of $tRNA^{Leu}$, NADH and 16S) and chemical markers (cuticular hydrocarbons) to study samples from the Iberian peninsula. Two lineages were

identified on the basis of both markers, i.e. *R. grassei* and *R. banyulensis*, but while cuticular hydrocarbons showed their complete separation, the DNA analysis evidenced a close kinship of the two species in southern Spain. The authors explained the discrepancy with a possible derivation from a polymorphic ancestor in this ice age refugium.

The first analysis that tried to infer the timing of the cladogenetic events in European termites was performed by Luchetti *et al*. (2005) on specimens from the Iberian peninsula, Italy and Turkey. Two methodologies for time estimates were applied to the COII dataset. These were an independent molecular clock calculated on a geological event (i.e. the separation between Sicily and mainland, around 12,000 years ago, during the Würmian period) and the substitution rate commonly applied for insect mtDNA (~2.3%/Mya). The first approach resulted the best one to explain *Reticulitermes* cladogenetic events, that were placed during the last cold period, assigning to the glacial refugia a fundamental role.

Finally, in a more recent paper (Luchetti *et al*., 2007), the authors focused their attention on the eastern area of the Mediterranean basin. Using both COII and 16S mitochondrial markers, the resulting phylogram showed two main lineages. The first one embodied all the samples from the Balkanic peninsula and western Greece. These were assigned to the species *R. urbis*, distinguishable in a Balkanic and a Peloponnesus clade. The second lineage embodied samples from eastern Greece, Turkey and Aegean islands and it comprises the species *R. balkanensis* and two not yet described taxa defined as *R. lucifugus* – northern Turkey and *R. lucifugus* – southern Turkey following Austin *et al*., (2002).

In the unique analysis on *K. flavicollis* (Luchetti *et al*., 2004b), the authors sequenced a

portion of mitochondrial DNA comprising the 3' region of the COI, the $tRNA^{Leu}$ and the 5' region of the COII genes for a total of 910 bp, in 10 populations from Greece, Balkans and Italy. Results showed an overall high genetic affinity, with the presence of only four haplotypes differing for 6 substitutions. Authors justified this pattern of low variability with a more recent evolution of the *Kalotermes* taxon in the European area with respect to *Reticulitermes* ones.

On the whole, the current status of the art on the taxonomy and distribution of the native European termites is reported in figures 3.1 and 3.2.

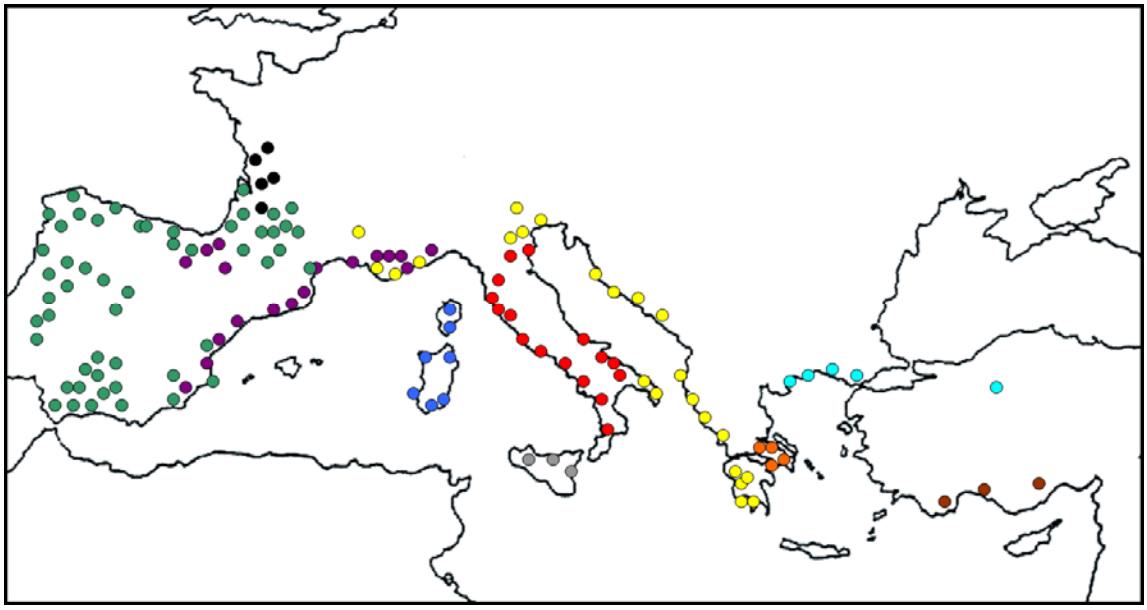

Figure 3.1. Distribution of the *Reticulitermes* species in Europe. Green = *R. grassei* (Clément), violet = *R. banyulensis* (Clément), red = *R. lucifugus lucifugus* (Rossi), blue = *R. lucifugus corsicus*, grey = *R. lucifugus* "Sicily", yellow = *R. urbis* (Bagnères *et al*.), orange = *R. balkanensis* (Plateau & Clément), light blue = *R. lucifugus* – northern Turkey, brown = *R. lucifugus* southern Turkey. In black the distribution of the introduced *R. santonensis* (i.e. *R. flavipes*). For the new sampling localities of the Crete island see chapter 4.

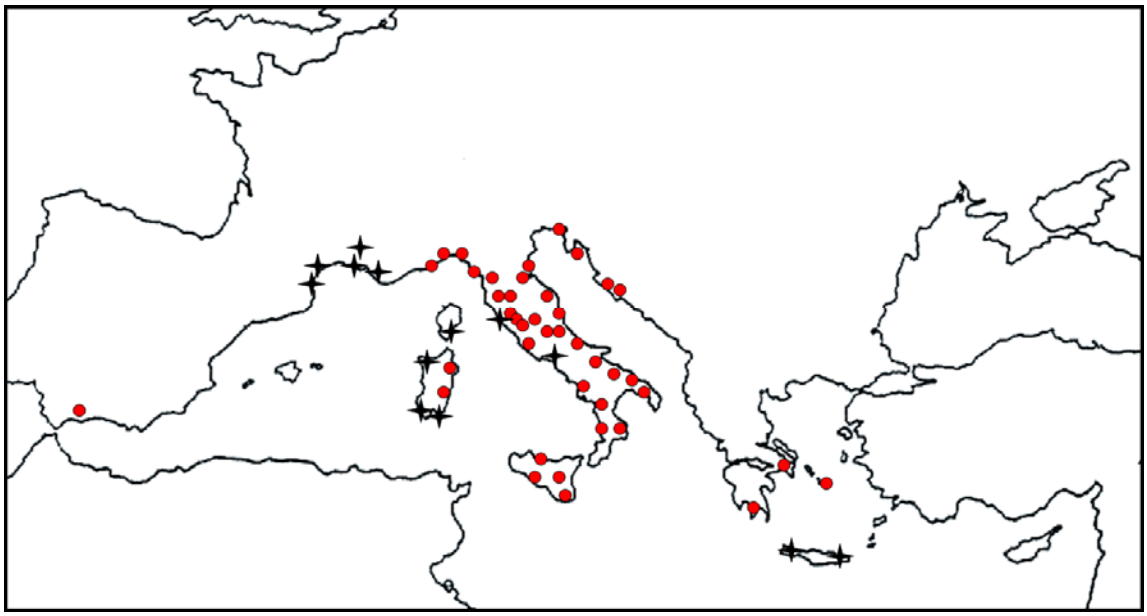

Figure 3.2. Distribution of the *Kalotermes flavicollis* populations in Europe. Black stars represents newly sampled populations used in this thesis.

3.2 COLONY GENETIC STRUCTURE

As for phylogenetic analysis, colony genetic structure was in depth studied in subterranean termites, while no papers were centred on drywood termites. This is probably linked to the greater infestation capacity and damages that subterranean termites can cause to human manufactures.

Three main papers analyzed the colony genetic structure in European populations of subterranean termites (Dronnet *et al*., 2005; Leniaud *et al*., 2009; Perdereau *et al*., 2010).

For *R. santonensis* (i.e. *R. flavipes*), Dronnet and co-authors (2005) analyzed the genetic structure of a natural population from Oléron island and of an urban one from Paris by the use of nine microsatellite markers. Genotypes distribution, *F*-statistics and

relatedness agree with an extended family condition. Moreover, the urban population showed a lower level of variability with respect to the natural one, as it can be expected for a recent human mediated introduction.

Leniaud *et al*. (2009) used a combined approach consisting of aggressive tests, estimates of population density and molecular genetics to detect the relationships among 29 collection points of the Domène *R. urbis* population. The genetic analysis showed a limited number of alleles at each locus ($\overline{n} = 4$) and a number of genotypes again in accordance with an extended family condition. Finally, the authors suggested that, in Domène, *R. urbis* is present with an unicolonial population: this may have descended from closely related females or even from a single pair of reproductives, and during its expansion, it has evolved different reproductive centres.

Perdereau *et al*. (2010) investigated the colony genetic structure of a French population derived from the American subterranean termites *R. flavipes* using both microsatellites loci and mtDNA. They showed that all colonies contained numerous related secondary reproductives and a consistent percentage of colonies derived from more than two unrelated reproductives. They concluded that the observed pattern is the result of colony fusion processes; moreover, the high occurrence of secondary reproductives and the greater ability to merge is justified by authors as a way to increase the invasive success.

3.3 RESEARCH AIMS

As suggested by previous paragraphs, my main interests during the PhD research period were to investigate both phylogenetic and colony organization aspects in the two

European native genera *Reticulitermes* and *Kalotermes*.

My studies, performed using different types of molecular markers in relation to the specific problem under consideration, had the following main goals:

- an in-depth phylogenetic analysis of the native European *Reticulitermes* species; this aspect was analyzed through the study of COII and 16S mitochondrial markers and I-SINE nuclear markers;
- a more detailed resolution of the biodiversity of European populations of *Kalotermes flavicollis* using an increased dataset with respect to Luchetti *et al*., (2004b) both in terms of populations analyzed (18) and molecular markers (CR, COI/tRNA/COII, microsatellites and I-SINE) used;
- the study of the colony genetic structure of the introduced *R. urbis* population in the town of Bagnacavallo (RA, Italy);
- the first analysis of the colony genetic structure in a drywood termite, *K. flavicollis*, from the Italian population of Duna di Feniglia (Tuscany).

The above reported topics will be presented in chapters 4-7, while in chapter 8 I will discuss the obtained results in a comparative view.

While chapters 4 and 5 correspond to either printed or DOI available papers, chapters 6 and 7 are given as papers to be submitted.

CHAPTER 4

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STARTING FROM CRETE, A PHYLOGENETIC RE-ANALYSIS OF THE GENUS *RETICULITERMES* IN THE MEDITERRANEAN AREA

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

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ABSTRACT

Owing to its peculiar paleogeographic history, Crete island is one of the most interesting biodiversity hotspots within the Aegean area. We here analyze the lineage diversity of Cretan *Reticulitermes* termites obtained on mitochondrial genes (COII and 16S) and nuclear Inter-SINE loci. The evolutionary pattern here detected shows a high correlation between clade divergence and geological events of the specific geographical area. The new haplotypes identified in Crete converge with those of specimens collected in northern Turkey, Thrace and Macedonia/Calcydia: this allows to suggest a unique genetic lineage for the Aegean area. A taxonomic and phylogenetic re-analysis of the *Reticulitermes* genus in Mediterranean Europe agrees with the species rank suggested for *Reticulitermes balkanensis* and *Reticulitermes urbis*, as well as for *Reticulitermes banyulensis* and *Reticulitermes grassei* from France and the Iberian peninsula. A level of divergence compatible with a specific rank of differentiation is scored also among the three *Reticulitermes lucifugus* subspecies from Italy and Corse, with the Sardo-Corsican entity basal to the other taxa. In the eastern area, the "Aegean" entity, including the Cretan lineages, results the most apical clade while *R. urbis,* distributed along the East Adriatic shores and Peloponnesus, lays as the most basal one.

1. Introduction

Termites are wood-feeding, diplo-diploid eusocial insects. They are of great interest for their unique ecological role and biological traits, which have caused a peculiar evolutionary history.

In the northern Mediterranean region – from the Iberian peninsula to Turkey – only two genera are known as autochthonous: *Kalotermes* (Kalotermitidae) and *Reticulitermes* (Rhinotermitidae). Despite their comparable distribution, the number of entities (either of specific or subspecific level) is significantly different. *Kalotermes* is in fact present only as *Kalotermes flavicollis*, even though recent data suggest the presence of more lineages (Velonà et al., unpublished). On the other hand, the genus *Reticulitermes* lists around 10 taxa of specific/subspecific rank (Marini and Mantovani, 2002; Kutnik et al., 2004; Luchetti et al., 2004a,b, 2007; Lefebvre et al., 2008).

One possible explanation of this difference involves the colonization history. *Reticulitermes* ancestors could have colonized Europe before *Kalotermes* ones and in this way they may have been influenced for a longer time by the geological events that have characterized the region, generating the high genetic variation presently known. A second possible explanation is given by the different ecological features of these termites. *Kalotermes* individuals live in dead trees, and colonies may be transported passively, for example by human activities, more easily than *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 the cause of the higher genetic homogeneity. Actually, it is 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,b; Velonà et al., unpublished).

Many phylogenetic and phylogeographical investigations exploited the above mentioned high level of diversification of the *Reticulitermes* genus (Marini and Mantovani, 2002; Kutnik et al., 2004; Luchetti et al., 2007, 2005; Nobre et al., 2008), but despite the suggestion proposed by Vargo and Husseneder (2009), the taxonomy and phylogeny of Mediterranean *Reticulitermes* entities are far from being settled.

In our former papers, we also focused on *Reticulitermes* populations from the eastern Mediterranean area and many divergent lineages were scored (Luchetti et al., 2004a,b, 2007, 2005): (i) *Reticulitermes urbis*, widely distributed in the south-western

Abbreviations: SINE, short interspersed element; I-SINE, Inter-SINE; MYA, million years ago; CP, calibration point.

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Peloponnesus, northern Greece and Croatia, showing a certain degree of differentiation between northern and southern populations; (ii) *Reticulitermes balkanensis* restricted to the Attican region; (iii) a highly structured oriental clade divided into a northern lineage comprising Ankara samples together with Thracian and Macedonian/Calcydic peninsula ones (the *Reticulitermes lucifugus* – Turkey – northern clade), and a southern lineage encompassing highly differentiated Turkish colonies sampled south of the Taurus-Antitaurus mountains, as well as the Israelian *Reticulitermes clypeatus* population (the *R. lucifugus* – Turkey – southern clade). The latter clade appeared phylogenetically related to *R. balkanensis*, even if this relationship was not bootstrap-supported in all elaborations. It is to be noted that the Turkish lineage was defined as *R. lucifugus* by Snyder (1949). This specific name was utilized by Austin et al. (2002) in their study on Anatolian *Reticulitermes* colonies, even if in the same paper the authors recognized that they were dealing with a new taxon. We prefer to maintain this designation until a formal description of the Turkish lineage is released.

The peri-Aegean region represents one of the richest area in terms of biodiversity with taxa showing quite complicated phylogeographical histories (Kasapidis et al., 2005; Parmakelis et al., 2005, 2006; Poulakakis et al., 2008; Papadopoulou et al., 2009). This pattern is mainly linked to the highly structured geological history of the area, due to the periodical interchange of sea level expansions and regressions (Creutzburg, 1963; Anastasakis and Dermitzakis, 1990; Dermitzakis, 1990; Lambeck, 1996). Twelve million years ago (MYA), a unique landmass called Agäis spanned from Greece to present-day Turkish shores. The progressive opening of the so-called "Mid-Aegean trench" led to the fragmentation of the Agäis, until the formation of the Aegean sea about 9 MYA. During the Messinian salinity crisis (5.95–5.33 MYA) (Krijgsman et al., 1999) the drying up of the Mediterranean sea allowed migration across the Aegean area; at the end of Messinian period, lands were definitively separated. Early Pliocenic flooding partially submerged Crete and the island was subdivided into smaller islands (the so-called "Cretan paleoislands") (Meulenkamp, 1985).

These paleogeographic events seem to explain why Crete island hosts a high number of endemic species and is considered a biodiversity hotspot (Parmakelis et al., 2006; Legakis and Kypriotakis, 1994; Sfenthourakis and Legakis, 2001).

In this paper we present the molecular analysis of Cretan *Reticulitermes* samples in order to describe the lineage diversity across the island and to determine if the observed pattern was influenced by the paleogeographic events that shaped this region. Moreover, the study of Cretan lineages allowed a re-analysis of the time frame in which cladogenetic events of all European *Reticulitermes* taxa took place.

2. Materials and methods

Specimens were field caught in Crete and preserved in absolute ethanol until molecular investigation. All pertinent information on samples analyzed here is given in Table 1 and Fig. 1A. For total DNA extraction, single termite heads were homogenized in a quick extraction buffer (PCR buffer 0,1×, SDS 0,1×), mixed with proteinase K, then frozen at 80 °C, incubated at 65 °C for 1 h and at 95 °C for 15 min. Two workers for each colony were used for both mitochondrial and nuclear DNA analyses. For Inter-SINE analysis, the following specimens of available colonies were also 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 Marathon and Penteli populations in the Attica region and 4 individuals of *R. lucifugus lucifugus* from Feniglia and Castellaneta populations in peninsular Italy.

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	Kokkinos 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

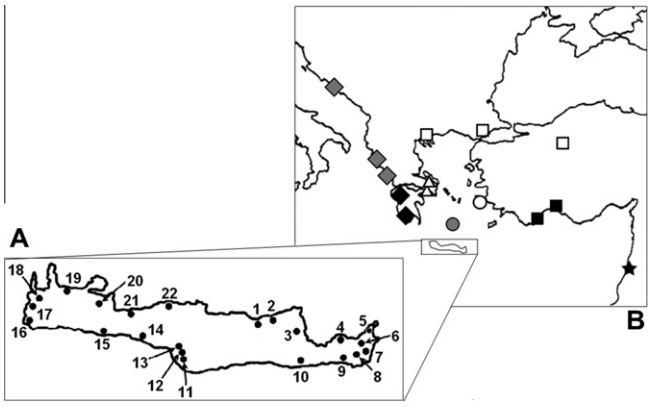

Fig. 1. (A) Sampling sites of the 22 Cretan colonies given in Table 1 and (B) geographic distribution of the other eastern Mediterranean samples drawn from GenBank (detailed in Supplementary Table S1) for the phylogenetic analysis (gray diamonds = *R. urbis* – northern clade, black diamonds = *R. urbis* – southern clade, white squares = *R. lucifugus* – Turkey – northern clade, black squares = *R. lucifugus* – Turkey – southern clade, white circle = *Reticulitermes* sp. [Samos], gray circle = *Reticulitermes* sp. [Amorgos], blackstar = *R. clypeatus*).

2.1. Mitochondrial markers

PCR amplification was performed in a 50 µl mixture with *Taq* polymerase (Invitrogen), following manufacturer protocol. Thermal cycling was as follows: an initial denaturation step at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s, extension at 72 °C for 30 s, a final elongation step at 72 °C for 7 min. Both strands were directly sequenced at Macrogen Inc. (Korea). COII and 16S primers for PCR amplification and sequencing were as described in Luchetti et al. (2004a,b). PCR products were purified before sequencing using the Wizard SV PCR and Gel cleaning kit (Promega). Sequences were aligned with CLUSTAL algorithm of the Sequence Navigator software (v. 1.0.1, Applied Biosystems) and alignments were edited by eye. Newly scored haplotypes were deposited into GenBank (Table 1).

2.2. Inter-SINE markers

Inter-SINE fingerprinting methodology is based on the PCR amplification of the region between a pair of copies of the same Short INterspersed Element retrotransposon. Here, two SINEs isolated in *R. lucifugus* were tested: *Talua* and *Talub* (Luchetti and Mantovani, 2009, and unpublished).

The PCR amplification of the Inter-SINE loci 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′) for the *Talua* or *Talub* elements, 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 s, 42 °C for 30 s and 72 °C for 30 s, 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 10× and 1 U of *Taq* polymerase (Invitrogen). The PCR products were resolved in 2% agarose gels using TAE 1× buffer, and bands were used to create a presence (1)/absence (0) matrix.

2.3. Statistical analyses

Modeltest v. 3.7 (Posada and Crandall, 1998) was run to determine the best substitution models for mitochondrial dataset, according to the hLRT criterion (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 5th base) and Maximum Likelihood analyses were performed with PAUP* v 4.0b (Swofford, 2001); node supports were calculated with 1000 and 100 bootstrap replicates, respectively. Bayesian analysis was done with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), which allows the two substitution models for the two gene partitions to be used simultaneously, setting 5×10^5 generations (when the two runs converged onto stationary distribution; variance of split frequencies <0.01). Trees were sampled every 100 generations, for a total of 5001 trees; the improvement of $-\ln L$ was graphically analyzed and the "steady state" was determined to have occurred by the 10,000th generation. Thus, first 10,000 generations were discarded (burnin = 100) and a consensus tree was computed on the remaining 4901 trees.

For a first phylogenetic analysis, a representative subset of the available COII and 16S sequences of eastern Mediterranean taxa were drawn from Genbank (Fig. 1B; Table S1). For this analysis, limited to the eastern taxa, we utilized samples of *R. lucifugus lucifugus* from the Italian colonies of Bologna and Chieti (AF291723, AF291738/AF29202) as outgroups.

A second phylogenetic analysis was done in order to estimate divergence time. We then added all the European *Reticulitermes* samples for which the same region of COII and 16S genes were available (Marini and Mantovani, 2002; Luchetti et al., 2004a,b, 2007; Austin et al., 2002) (see Table S2 in Supplementary materials for GenBank A.N.) to the first data set. *Coptotermes formosanus* and *Reticulitermes flavipes* were used as outgroup/ingroup (GenBank A.N.: COII AF107488, AF107484; 16S U17778, U17824). A new analysis on this second more comprehensive data set was performed with both Modeltest 3.7 and ILD test. The Partition Homogeneity test resulted not significant ($P = 1$), allowing us to analyze the combined dataset. According to hLRT criterion, Modeltest highlighted the HKY + Γ + I ($\Gamma = 1.2215$, $I = 0.5771$) 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 results allowed us to reject the molecular clock hypothesis (LRT = 108.83; df = 89; $P < 0.05$). Estimation of clade divergence time was therefore 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 method used by Kasapidis et al. (2005), two calibration points (CPs) were set: the first, at the Agäis fragmentation about 10 MYA and the second at the end of the Messinian salinity crisis about 5.3 MYA.

A genetic pairwise distance matrix has been calculated on the whole data set with PAUP* using the HKY + Γ + I substitution model parameters; following Hebert et al. (2004) frequencies of intra-clade and inter-clade divergence values are either reported in histograms (Fig. 4) or as range (Table S3 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.

3. Results

3.1. Mitochondrial DNA sequence variation and phylogeny

The sequencing of the mitochondrial markers in 44 individuals from Crete (22 colonies) revealed 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; newly scored haplotypes differ by 1–12 substitutions. The 16S rDNA fragment showed 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 *Reticulitermes* taxa used 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 were widely different, with Bayesian posterior probabilities being on average higher than the corresponding bootstrap percentages.

In the 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; the samples from central Crete are divided in a central-eastern group (2) and a central-western group (3), partially overlapping in southern Crete. Finally, the 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 shown in Fig. 2B.

Tree topology further confirms the existence of 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 in the southern clade from Peloponnesus; the *R. lucifugus* – Turkey – southern clade, linked to *R. balkanensis*, and *R. clypeatus*, for which the unsupported dichotomies do not allow the detection of their evolutionary relationships; the *R. lucifugus* – northern clade from northern Turkey, Thrace/Macedonia (also comprising colonies from the Calcydic peninsula) and the Amorgos colony which is basal to the Cretan cluster. The

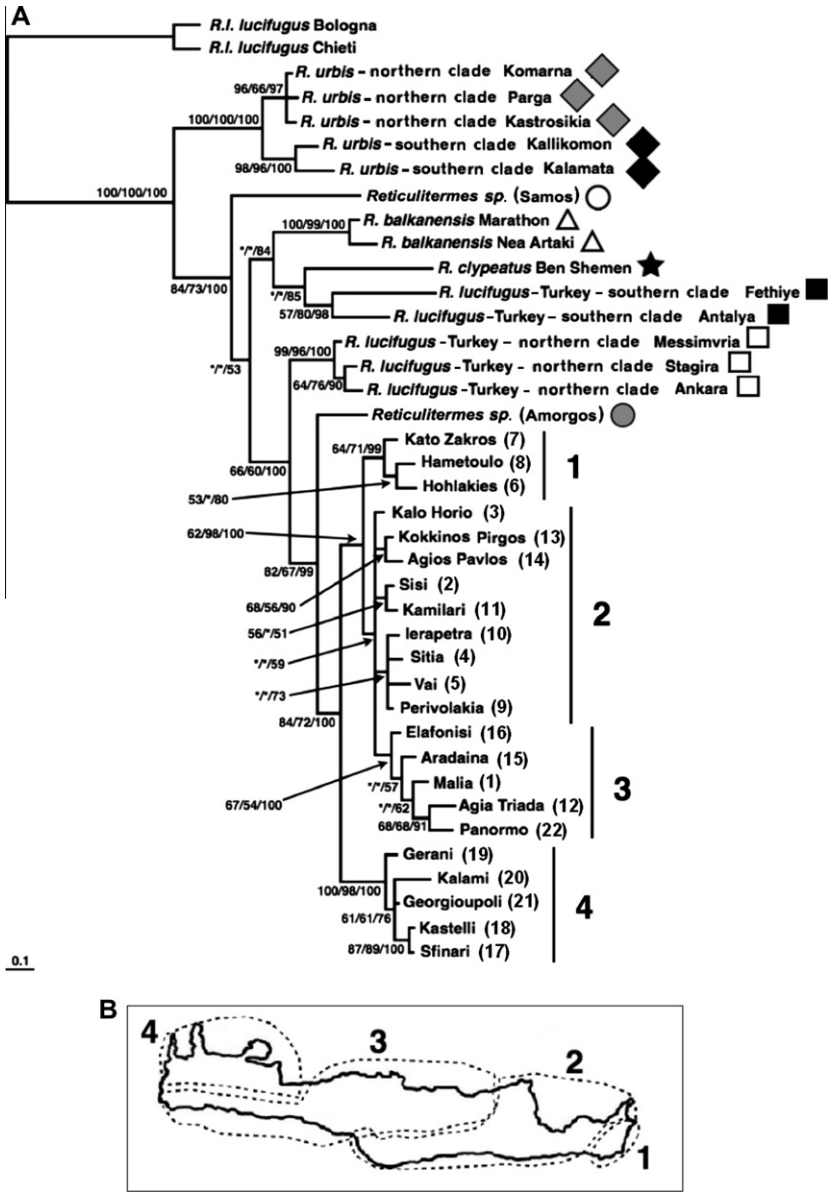

Fig. 2. Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI) trees calculated on COII + 16S mitochondrial DNA sequences (A) and geographic distribution of the four Cretan lineages (B). In (A), numbers at nodes indicate bootstrap values for MP and ML analyses and posterior probability expressed as percentage for BI analysis. Numbers in parentheses and symbols refer to Fig. 1, Table 1 and Supplementary Table S1.

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 study (Luchetti et al., 2007).

3.2. 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, and the *Talub* marker 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, with values ranging from 0.086 (clades 1–3; NS) to 0.129 (clades 2–3; $P < 0.05$). On the other hand, the four Cretan groups were highly divergent from the other analyzed *Reticulitermes* taxa, with values ranging from 0.256 (clade 2 *vs R. lucifugus lucifugus*; $P < 0.01$) to 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 themselves, the only exception being the two clades of *R. urbis* (0.186, NS).

3.3. Setting the time frame of divergence

The 10 MYA CP indicates that the main dichotomy between western and eastern lineages took place 27.19 MYA. Within the latter lineage, *R. urbis* diverged from the other taxa 20.87 MYA and its northern and southern sub-clades 8.46 MYA. The cladogenetic events of the other eastern taxa occurred between 17.11 and 10.00 MYA. Within the western lineage (*Reticulitermes banyulensis*/*Reticulitermes grassei*/*R. lucifugus* complex), taxa diverged between 16.88 and 7.58 MYA.

In the chronogram obtained using the 5.33 MYA CP (Fig. 3), the separation between western and eastern lineages dates back to 23.61 MYA. *R. urbis* originated 15.73 MYA and its two sub-clades

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	–

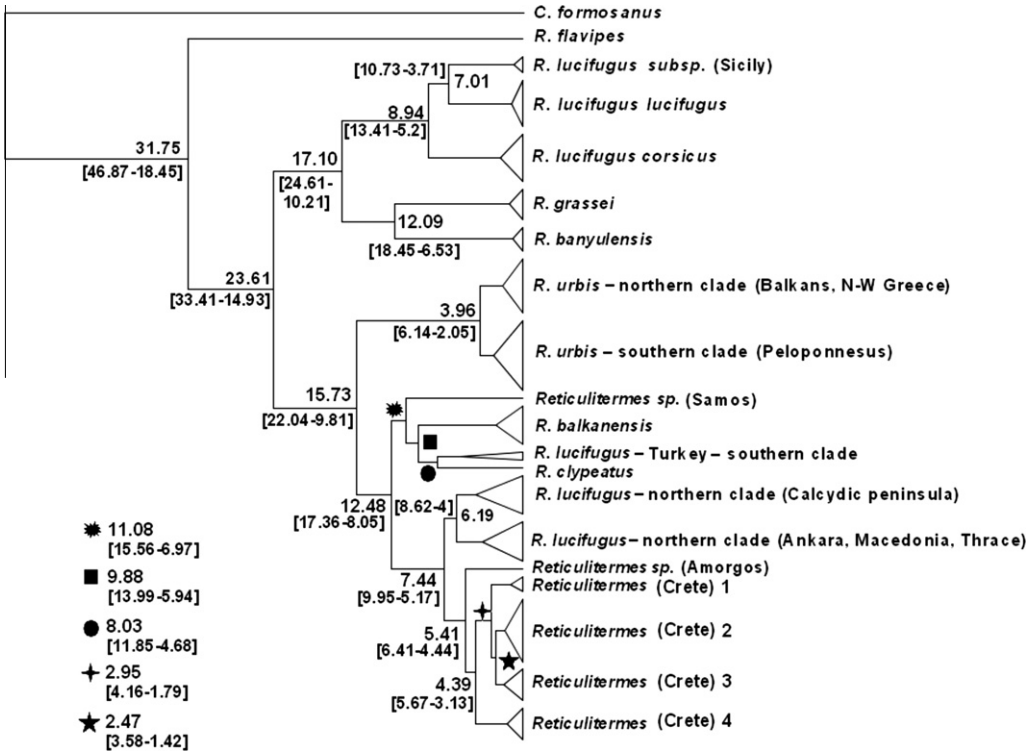

Fig. 3. Chronogram tree representing the divergence time estimations and, in parentheses, the relative 95% confidence intervals for each dichotomy. Numbers are expressed in million years ago (MYA).

diverged 3.96 MYA; within the Aegean area, the diversification of the other taxa spans from 12.48 to 5.41 MYA.

As far as Cretan lineages are concerned, the 10 MYA CP place their diversification between 7.68 and 4.93 MYA, while the 5.3 MYA CP indicates that their divergence occurred between 4.39 and 2.47 MYA.

On the whole, the comparison between the estimated time frames and the geological history of the Aegean area (Creutzburg, 1963; Anastasakis and Dermitzakis, 1990; Lambeck, 1996; Krijgsman et al., 1999; Meulenkamp, 1985) indicates that using the 10 MYA calibration point produces a level of high discrepancy regarding the evolution of both Cretan and other peri-Aegean lineages (see Section 4); therefore, this time calibration will not be further considered.

3.4. 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 (Fig. 4, Table S3).

The four Cretan lineages showed an overlapping distribution (Fig. 4A). A comparable situation is observed within the *R. lucifugus* – Turkey – northern clade when the sequences from the Calcydic peninsula are compared to the Ankaran, Thracian and Macedonian ones (Fig. 4B). The same applies when these two groups are paired together against Cretan populations, so that the whole group (*R. lucifugus* – Turkey – northern clade + Cretan populations) appears as a unique entity (Fig. 4C).

A frequency overlapped distribution also emerged in the *R. urbis* northern and southern clade comparison (Fig. 4D).

On the other hand, all pairwise comparisons involving *R. balkanensis*, *R. urbis* and the *R. lucifugus* – Turkey – northern clade/Cretan populations group showed completely separated frequency distributions (Table S3).

All comparisons dealing with the western lineages also showed clearly non-overlapping distributions (Fig. 4E and F, Table S3).

4. Discussion

Mitochondrial and nuclear marker analysis of the Cretan samples allowed us to detect four endemic lineages. Their geographic

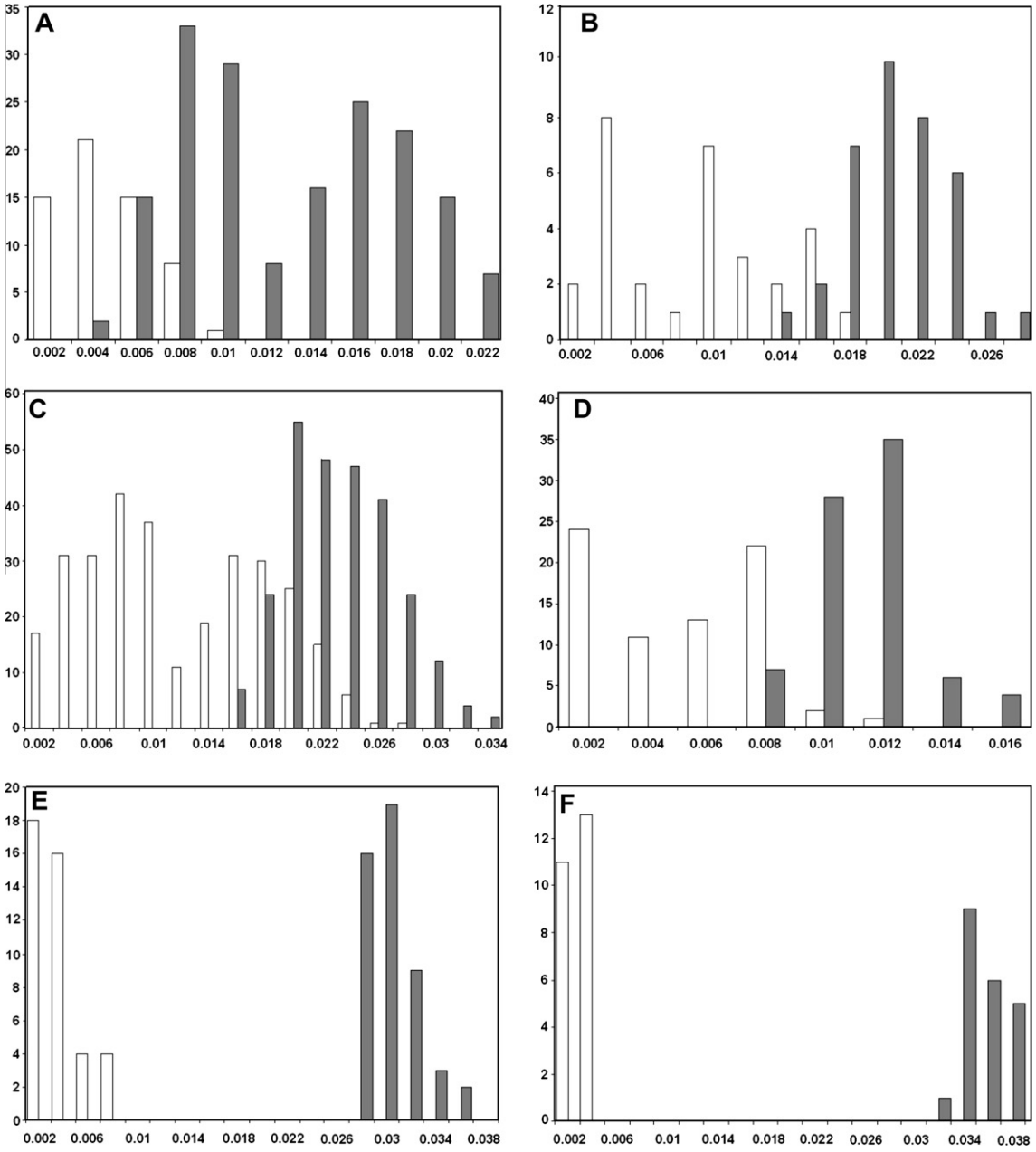

Fig. 4. Histograms showing the frequencies of intra-clade divergence values (white bars) and inter-clade divergence values (gray bars). (A) *Reticulitermes* from Crete, groups 1–2–3–4; (B) *R. lucifugus* from northern Turkey and Thrace *vs R. lucifugus* from Calcydia; (C) *Reticulitermes* from Crete, 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* and (F) *R. lucifugus lucifugus vs R. lucifugus* subsp. (Sicily).

distribution nicely fits with the Early Pliocene Cretan landscapes represented by the Cretan paleoislands (Meulenkamp, 1985). The high similarity between the surface of these paleoislands and the distribution of the four Cretan lineages, strongly suggests the influence of geological history in Cretan *Reticulitermes* population diversification. Present results are congruent with several lineage breaks and endemisms observed for a number of organisms distributed across Crete (Parmakelis et al., 2005, 2006; Legakis and Kypriotakis, 1994; Douris et al., 1998; Poulakakis et al., 2003). The analysis of mitochondrial DNA variability as well as the F_{ST} values determined by I-SINE markers are in line with an intra-specific level of divergence; this is particularly evident in F_{ST} pairwise values of Cretan lineages: they are statistically significant, but fairly below those here observed between known good species.

On the other hand, the phylogenetic analysis also evidenced the monophyly of the Cretan cluster with respect to other Aegean taxa, reflecting the complete isolation that Crete has experienced since 5.33 MYA and the limited ability of subterranean termite to disperse overseas.

The Cyclades Amorgos sample shows a sister relationship with the Cretan clade – as also reported for other taxa such as gastropods (Parmakelis et al., 2005) or coleopterans (Chatzimanolis et al., 2003) – and with *R. lucifugus* from northern Turkey, Thrace and Macedonia/Calcydia. In a previous analysis, this clade was found to extend to eastern Peloponnesus (Aria colony) (Luchetti et al., 2007). It is therefore arguable that these phylogroups might derive from a single entity that once spread from the northern Aegean to the eastern Greek shores, Cyclades and Crete, and progres-

sively diversified following the east–west fragmentation of the Agäis to give the present-day taxon distribution. According to the estimated cladogenetic time frames, these divergences took place between 7.44 and 5.33 MYA, just after the formation of the mid-Aegean trench and the final flooding of the land bridge between Peloponnesus and Crete; this partially reflects the evolutionary pattern hypothesized for some Aegean *Dendarus* species (Chatzimanolis et al., 2003).

The relationship between *R. balkanensis* and *R. lucifugus* – Turkey – southern clade + *R. clypeatus* is here maintained even if the deep node is still quite unstable (Luchetti et al., 2007). On the other hand, their relationship is confirmed by further mitochondrial and nuclear data (Uva et al., 2004). The range of cladogenesis dates seems to be in line with the northward opening of the mid-Aegean trench: this fragmented the Agäis, the unique mainland present in this area until 9.5 MYA, and became a permanent barrier until the Messinian salinity crisis. The geological separation caused by the trench has already been proposed as a key factor in determining the speciation pattern of different animal taxa, both vertebrate and invertebrate (Parmakelis et al., 2006; Poulakakis et al., 2008; Douris et al., 2007; Poulakakis and Sfenthourakis, 2008).

As a general remark, the nearly overlapping distribution of divergent lineages across the present Aegean Sea well depicts the complex geological history of the basin, which is supposed to be one of the main reasons of the taxonomic diversity observed in this area.

As far as the westernmost taxa are concerned, *R. urbis* appears a monophyletic clade with a clear dichotomy between Balkans/N-W Greece populations and Peloponnesus ones as already observed in Luchetti et al. (2007). 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) that placed the event around 9000 years ago. Despite a relatively ancient separation, present taxonomic analysis (see below) does not allow to consider the two *R. urbis* entities as different species but at most as two subspecies. This could be explained by further aspects on the geology of the region: after the formation of the Corinth gulf, around 3.5 MYA, in fact, 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 final emergence of the Corinth gulf around 9000 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 have 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.

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

In order to attempt to contribute to the clarification of the taxonomic status of European *Reticulitermes* taxa, we also analyzed the genetic diversity using what has been called the "barcoding gap" (Hebert et al., 2004). In this kind of approach, two opposite situations can be produced with intra-clade and inter-clade frequency distributions either overlapped or completely separated: in the latter instance the occurrence of a "barcoding gap" should highlight a specific level of divergence. In the eastern area, only three entities of specific level could be unequivocally recognized; the first one is *R. urbis*, whose significantly earlier divergence from the common eastern ancestor may support a subspecific differentiation of its northern and southern lineages. *R. urbis* appears basal to the other two taxa: *R. balkanensis*, with a limited distribution in the Attica and Peloponnesus, and a new "Aegeum" entity embodying samples from northern Turkey, Thrace, Macedonia/Calcydia, the Cycladic Amorgos and Crete island. *R. balkanensis* and the "Aegeum" clade represent two divergent genetic lineages, the former being related to southern Turkey populations/*R. clypeatus*. The Dodecanese Samos lineage appears basal to both groups. The unsupported dichotomies shown by *R. balkanensis*, *R. lucifugus* – Turkey – southern clade and *R. clypeatus* do not allow to discuss a clear evolutionary pattern but, excluding the basal Samos sample and the *R. clypeatus* lineage, low but significant bootstrap values for the remaining nodes can be obtained (available from the authors), clearly evidencing 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/Iberian peninsula and a clearly divergent central one present in Italy and Corse. The first lineage leads to *R. banyulensis* and *R. grassei* for which our analysis seems to support the species status already evidenced mainly through both chemical (cuticular hydrocarbons) and molecular (nuclear sequences) studies (Kutnik et al., 2004). The same analysis on mitochondrial markers – only partially overlapping with present ones – evidenced that the two entities share ancestral haplotypes. Obviously the presence of ancestral haplotypes could obscure the barcoding gap, thus defining a possible limit of the approach.

Regarding the peri-Thyrrenian populations, Uva et al. (2004) analyzed the colonial divergence of *R. lucifugus lucifugus* from Tuscany region and *R. lucifugus corsicus* from Corse, and discovered a high level of divergence (~5%), as well as 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, according to the "barcoding gap", the genetic differentiation of *R. lucifugus corsicus* and *R. lucifugus lucifugus* appears of specific level. It should be noted that *R. lucifugus corsicus* and *R. lucifugus lucifugus* are known to hybridize in nature (Lefebvre et al., 2008), yet this does not seems to contradict their suggested specific status of differentiation, given the number of examples of interspecific hybridization events occurring in nature either in vertebrates or invertebrates (Feldhaar et al., 2008; Petrusek et al., 2008; Trigo et al., 2008). From our data, a specific level of divergence seems to apply also to the other so far suggested *R. lucifugus* subspecies from Sicily (Luchetti et al., 2004a,b).

Although the barcoding gap approach can be envisaged as a simpler and more objective method to establish species boundaries, it must be used in tandem with traditional taxonomical tools, because DNA variation can be influenced by many ecological and demographic factors (Tautz et al., 2003). For this reason, our results should simply help in better resolving the taxonomic picture.

Finally, 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., unpublished), we believe that this marker is a promising candidate

as a good tool for molecular analysis, also considering its quickness and cost effectiveness.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.04.037.

References

Anastasakis, G.C., Dermitzakis, D.M., 1990. Post-Middle-Miocene palaeogeographic evolution of the Central Aegean Sea and detailed Quaternary reconstruction of the region. It's possible influence on the distribution of the Quaternary mammals of the Cyclades Islands. Neue Jahrb. Geol. Paläontol. 1, 1–16.

Austin, J.W., Szalanski, A.L., Uva, P., Bagnères, A.G., Kence, A., 2002. A comparative genetic analysis of the subterranean termite genus *Reticulitermes* (Isoptera: Rhinotermitidae). Ann. Entomol. Soc. Am. 95, 753–760.

Chatzimanolis, S., Trichas, A., Giokas, S., Mylonas, M., 2003. Phylogenetic analysis and biogeography of Aegean taxa of the genus *Dendarus* (Coleoptera: Tenebrionidae). Insect Syst. Evol. 34, 295–312.

Creutzburg, N., 1963. Palaeogeographic evolution of Crete from Miocene till our days. Cretan Annals 15/16, 336–342.

Dermitzakis, D.M., 1990. Paleogeography, geodynamic processes and event stratigraphy during the Late Cenozoic of the Aegean area, International Symposium on: Biogeographical Aspects of Insularity, Roma 1987. Accad. Naz. Lincei 85, 263–288.

Douris, V., Cameron, R.A.D., Rodakis, G.C., Lecanidou, R., 1998. Mitochondrial phylogeography of the land snail Albinaria in Crete: long-term geological and short-term vicariance effects. Evolution 52, 116–125.

Douris, V., Giokas, S., Thomas, D., Lecanidou, R., Rodakis, G.C., 2007. Inference of evolutionary patterns of the land snail *Albinaria* in the Aegean archipelago: is vicariance enough? Mol. Phylogenet. Evol. 44, 1224–1236.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1995. Testing significance of incongruence. Cladistics 10, 315–319.

Feldhaar, H., Foitzik, S., Heinze, J., 2008. Lifelong commitment to the wrong partner: hybridization in ants. Phil. Trans. R. Soc. B 363, 2891–2899.

Hebert, P.D., Stoekle, M.Y., Zemlak, T.S., Francis, C.M., 2004. Identification of birds through DNA barcodes. PLoS Biol. 10, e312.

Huelsenbeck, J.P., Crandall, K.A., 1997. Phylogeny estimation and hypothesis testing using maximum likelihood. Ann. Rev. Ecol. Syst. 28, 437–466.

Huelsenbeck, J.P., Ronquist, F., 2001. F. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Kasapidis, P., Magoulas, A., Mylonas, M., Zouros, E., 2005. The phylogeography of the gecko *Cyrtopodion kotschyi* (Reptilia: Gekkonidae) in the Aegean Archipelago. Mol. Phylogenet. Evol. 35, 612–623.

Ketmaier, V., Giusti, F., Caccone, A., 2006. Molecular phylogeny and historical biogeography of the land snail genus *Solatopupa* (Pulmonata) in peri-Tyrrenian area. Mol. Phylogenet. Evol. 39, 439–451.

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400, 652–655.

Kutnik, M., Uva, P., Brinkworth, L., Bagnères, A.G., 2004. Phylogeography of two European *Reticulitermes* species: the Iberian refugium. Mol. Ecol. 13, 3099–3113.

Lambeck, K., 1996. Sea-level change and shore-line evolution in Aegean Greece since upper palaeolithic time. Antiquity 70, 588–611.

Lefebvre, T., Châline, N., Limousin, D., Dupont, S., Bagnères, A.G., 2008. From speciation to introgressive hybridization: the phylogeographic structure on an island subspecies of termite, *Reticulitermes lucifugus corsicus*. BMC Evol. Biol. 8, 38.

Legakis, A., Kypriotakis, Z., 1994. A biogeographical analysis of the island of Crete, Greece. J. Biogeogr. 21, 441–445.

Luchetti, A., Mantovani, B., 2009. *Talua* SINE biology in the genome of the *Reticulitermes* subterranean termites (Isoptera, Rhinotermitidae). J. Mol. Evol. 69, 589–600.

Luchetti, A., Trenta, A., Mantovani, B., Marini, M., 2004a. Taxonomy and phylogeny of north mediterranean *Reticulitermes* termites (Isoptera, Rhinotermitidae): a new insight. Ins. Soc. 51, 117–122.

Luchetti, A., Bergamaschi, S., Marini, M., Mantovani, B., 2004b. Mitochondrial DNA analysis of native European Isoptera: a comparison between *Reticulitermes* (Rhinotermitidae) and *Kalotermes* (Kalotermitidae) colonies from Italy and Balkans. REDIA LXXXVII, 149–153.

Luchetti, A., Marini, M., Mantovani, B., 2005. Mitochondrial evolutionary rate and speciation in termites: data on European *Reticulitermes* taxa (Isoptera, Rhinotermitidae). Ins. Soc. 52, 218–221.

Luchetti, A., Marini, M., Mantovani, B., 2007. Filling the European gap: biosystematics of the eusocial system *Reticulitermes* (Isoptera, Rhinotermitidae) in the Balkanic peninsula and Aegean area. Mol. Phylogenet. Evol. 45, 377–383.

Marini, M., Mantovani, B., 2002. Molecular relationship among European samples of *Reticulitermes* (Isoptera, Rhinotermitidae). Mol. Phylogenet. Evol. 22, 454–459.

Meulenkamp, J.E., 1985. Aspects of the Late Cenozoic Evolution of the Aegean Region. In: Stanley, D.J., Wezel, F.C. (Eds.), Geological Evolution of the Mediterranean Basin. Springer, New York, pp. 307–321.

Nobre, T., Nunes, L., Bignell, D.E., 2008. Colony interactions in *Reticulitermes grassei* population assessed by molecular genetics methods. Ins. Soc. 55, 66–73.

Papadopoulou, A., Anastasiou, J., Keskin, B., Vogler, A., 2009. Comparative phylogeography of tenebrionid beetles in the Aegean archipelago: the effect of dispersal ability and habitat preference. Mol. Ecol. 18, 2503–2517.

Parmakelis, A., Pfenninger, I., Spanos, L., Papagiannakis, G., Louis, C., Mylonas, M., 2005. Inference of a radiation in *Mastus* (Gastropoda, Pulmonata, Enidae) on the island of Crete. Evolution 59, 991–1005.

Parmakelis, A., Stathi, I., Chatzaki, M., Simaiakis, S., Spanos, L., Louis, C., 2006. Evolution of *Mesobuthus gibbosus* (Brullé, 1832) (Scorpiones: Buthidae) in the northeastern Mediterranean region. Mol. Ecol. 15, 2883–2894.

Peakall, R., Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetics software for teaching and research. Mol. Ecol. Notes 6, 288–295.

Petrusek, A., Seda, J., Machácek, J., Ruthová, S., Smilauer, P., 2008. *Daphnia* hybridization along ecological gradients in pelagic environments: the potential for the presence of hybrid zones in plankton. Phil. Trans. R. Soc. B 363, 2931–2941.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Poulakakis, N., Sfenthourakis, S., 2008. Molecular phylogeny and phylogeography of the Greek populations of the genus *Orthometopon* (Isopoda, Oniscidea) based on mitochondrial DNA sequences. Zool. J. Linn. Soc. 152, 707–715.

Poulakakis, N., Lymberakis, P., Antoniou, A., Chalkia, D., Zouros, E., Mylonas, M., Valakos, E., 2003. Molecular phylogeny and biogeography of the wall-lizard *Podarcis erhardii* (Squamata: Lacertidae). Mol. Phylogenet. Evol. 28, 38–46.

Poulakakis, N., Pakaki, V., Mylonas, M., Lymberakis, P., 2008. Molecular phylogeny of the Greek legless skink *Ophiomorus punctatissimus* (Squamata: Scincidae): the impact of the Mid-Aegean trench in its phylogeography. Mol. Phylogenet. Evol. 47, 396–402.

Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. Available from http://tree.bio.ed.ac.uk/software/tracer/.

Sfenthourakis, S., Legakis, A., 2001. Hotspots of endemic terrestrial invertebrates in southern Greece. Biodiv. Conserv. 10, 1387–1417.

Simaiakis, S., Mylonas, M., 2008. The *Scolopendra* species (Chilopoda: Scolopendromorpha: Scolopendridae) of Greece (E-Mediterranean): a theoretical approach on the effect of geography and paleogeography on their distribution. Zootaxa 1792, 39–53.

Snyder, T.E., 1949. Catalog of the Termites (Isoptera) of the World, Smithsonian Miscellaneous Collections, vol. 112.

Swofford, D.L., 2001. PAUP* -Phylogenetic Analysis Using Parsimony (*and Other Methods), Ver 4b. Sinauer Associates, Sunderland, MA.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H., Vogler, A.P., 2003. A plea for DNA taxonomy. Trends Ecol. Evol. 18, 70–74.

Trigo, T.C., Freitas, T.R.O., Kunzler, G., Cardoso, L., Silva, J.C.R., Johnson, W.E., O'Brien, S.J., Bonatto, S.L., Eizirik, E., 2008. Inter-species hybridization among Neotropical cats of the genus *Leopardus*, and evidence for an introgressive hybrid zone between *L. geoffroyi* and *L. tigrinus* in southern Brazil. Mol. Ecol. 17, 4317–4333.

Uva, P., Clément, J.L., Austin, J.W., Aubert, J., Zaffagnini, V., Quintana, A., Bagnères, A.G., 2004. Origin of a new *Reticulitermes* termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data. Mol. Phylogenet. Evol. 30, 344–353.

Uva, P., Clément, J.L., Bagnères, A.G., 2004. Colonial and geographic variations in agonistic behaviour, cuticular hydrocarbons and mtDNA of Italian populations of *Reticulitermes lucifugus* (Isoptera, Rhinotermitidae). Ins. Soc. 51, 163–170.

Vargo, E.L., Husseneder, C., 2009. Biology of subterranean termites: insights from molecular studies of *Reticulitermes* and *Coptotermes*. Ann. Rev. Entomol. 54, 379–403.

S1. GenBank accession numbers for all the COII and 16S sequences added for phylogenetic analysis. Symbols are as in Fig. 1 and 2.

Species	Locality	Accession numbers	
		COII	16S
R. urbis	Komarna (Dalmatia, Croatia) ◇	DQ487822	DQ487838
	Parga (Epirus, Greece) ◇	DQ487824	DQ487836
	Kastrosikia (Epirus, Greece) ◇	DQ487825	DQ487836
	Kallikomon (Peloponnese, Greece) ◆	DQ487830	DQ487837
	Kalamatas (Peloponnes, Greece) ◆	DQ487832	DQ487837
R. balkanensis	Marathon (Attica, Greece) △	DQ487835	DQ487849
	Nea Artaki (Attica, Greece) △	AY954667	DQ487850
R. lucifugus - Turkey	Messimvria (Thrace, Greece) □	AY954666	DQ866987
	Stagira (Macedonia, Greece) □	DQ866978	DQ866986
	Ankara (North Turkey) □	AF525333	AF525330
	Antalya (South Turkey) ■	AF525338	DQ431056
	Fethiye (South Turkey) ■	AF525334	DQ431055
Reticulitermes sp.	Amorgos (Cyclades, Greece) ●	AY954664	DQ487852
Reticulitermes sp.	Samos (Dodecanes, Greece) ○	AY954669	DQ487851
R. clypeatus	Ben Shemen (Israel) ★	AF525320	DQ431058

S2. GenBank accession numbers for all 16 and COII sequences added for chronogram and taxonomic analyses.

List of taxa	Samples localities	GenBank A.N.	
		COII	16S
R. urbis - northern clade	Katoki	DQ487827	DQ487841
	Lefkada	DQ487826	DQ487840
	Igoumenitsa	DQ866972	DQ866980
	Zivogosce	DQ487822	DQ487836
	Klec	DQ487822	DQ487836
R. urbis - southern clade	Kalavrita	DQ487828	DQ487837
	Kato Ackaia	DQ487829	DQ487842
	Kiparissia	DQ487823	DQ487837
	Pylos	DQ487831	DQ487843
	Areopolis	AY267867	AY268356
	Neapolis	AY954668	DQ487837
	Gradac	DQ487823	DQ487837
	Sivota	DQ487823	DQ487839
R. balkanensis	Epidaurus	DQ487834	DQ487845
	Kiffisia	AY954662	DQ487847
	Schinias	AY954663	DQ487848
	Penteli	DQ487835	DQ487846
R. lucifugus - Turkey - northern clade	Komotini	AY954665	DQ487854
	Xanthi	DQ866979	DQ866987
	Lussino	AY954660	DQ487855
	Aria	DQ487833	DQ487844
	Jeroussi	DQ866973	DQ866981
	Sarti	DQ866977	DQ866985
	Nea Fokea	DQ866975	DQ866983
	Nea Marmaras	DQ866974	DQ866982
	Paliouri	DQ866976	DQ866984
R. lucifugus lucifugus	Castel di Decima	AF291724	AF292007
	Firenze	AF291726	AF292009
	Castel Porziano	AF291739	AF292009
	Forlì	AF291725	AF292008
	Roma	AF291739	AF292022
	Napoli	AF291740	AF292023
R. lucifugus corsicus	Alghero	AF291729	AF292012
	Capitana di Quartu	AY267865	AF292011
	Flumini di Quartu	AF291728	AF292011

	Parco dell'Uccellina	AF291727	AF292010
	Pulas is Molas	AY267861	AY268362
	Saccheddu	AF291730	AF292013
	Corsicus 1	AY267858	AY268360
R. lucifugus subsp. (Sicily)	Agrigento	AY267864	AY268365
	S. Stefano Quisquinia	AY267864	AY268366
	Palermo	AY267857	AY268361
R. grassei	Bilbao	AF291744	AF292027
	Mimizan	AF291732	AF292015
	Mimizan	AF291733	AF292016
	Foret	AF291731	AF292014
R. banyulensis	Beziers	AF525319	-
	Tafalla	AY510599	-
	Banyulensis 1	AY267859	AY268369

S3. Range values and mean ± standard deviations of intra-lineage and inter-lineage variability for the comparisons not presented in Fig. 4. In parentheses, the number of sequences used.

Lineage pairs	**Intra-lineage variability**	**Inter-lineage variability**
Rcr+RluT+RluC (34) *vs* Rbk (6)	0-0.0262 0.010 ± 0.006	0.0264-0.049 0.037 ± 0.005
Rcr+RluT+RluC (34) *vs* Rur (18)	0-0.0262 0.010 ± 0.006	0.034 - 0.067 0.0482 ± 0.005
Rur (18) *vs* Rbk (6)	0-0.015 0.007 ± 0.004	0.039-0.058 0.046 ± 0.004
Rlc (7) *vs* Rls (3)	0-0.007 0.003 ± 0.002	0.027-0.032 0.029 ± 0.002
Rur (18) *vs* Rll (7)	0-0.015 0.007 ± 0.004	0.080-0.098 0.087 ± 0.004
Rur (18) *vs* Rlc (7)	0-0.015 0.007 ± 0.004	0.069-0.094 0.077 ± 0.005
Rur (18) *vs* Rls (7)	0-0.015 0.007 ± 0.004	0.065-0.090 0.074 ± 0.005
Rbk (6) *vs* Rll (7)	0-0.010 0.004 ± 0.002	0.086-0.097 0.090 ± 0.003
Rbk (6) *vs* Rlc (7)	0-0.010 0.005 ± 0.003	0.067-0.088 0.076 ± 0.005
Rbk (6) *vs* Rls (3)	0-0.010 0.006 ± 0.003	0.075-0.096 0.085 ± 0.006
Rbn (4) *vs* Rgr (5)	0-0.003 0.001 ± 0.001	0.042-0.060 0.052 ± 0.006
Rbn (4) *vs* Rll (7)	0-0.035 0.008 ± 0.013	0.064-0.080 0.071 ± 0.005
Rbn (4) *vs* Rlc (7)	0-0.007 0.003 ± 0.002	0.052-0.071 0.061 ± 0.006
Rbn (4) *vs* Rls (3)	0-0.003 0.002 ± 0.001	0.060-0.080 0.071 ± 0.007
Rgr (5) *vs* Rll (7)	0-0.003 0.002 ± 0.001	0.057-0.061 0.058 ± 0.001

Rgr (5) *vs* Rlc (7)	0-0.007 0.003 ± 0.002	0.040-0.047 0.042 ± 0.002
Rgr (5) *vs* Rls (3)	0-0.003 0.001 ± 0.002	0.050-0.055 0.052 ± 0.002

RluT=*R. lucifugus* from northern Turkey and Thrace, RluC=*R. lucifugus* from Calcydia, Rcr=*Reticulitermes* from Crete, Rbk=*R. balkanensis,* Rur=*R. urbis*, Rbn=*R. banyulensis*, Rgr=*R. grassei*, Rll=*R. lucifugus lucifugus*, Rlc=*R. lucifugus corsicus*, Rls=*R. lucifugus* subsp. (Sicily).

CHAPTER 5

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MITOCHONDRIAL AND NUCLEAR MARKERS HIGHLIGHT THE BIODIVERSITY OF *KALOTERMES FLAVICOLLIS* (FABRICIUS, 1793) (INSECTA, ISOPTERA, KALOTERMITIDAE) IN THE MEDITERRANEAN AREA.

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Mitochondrial and nuclear markers highlight the biodiversity of *Kalotermes flavicollis* (Fabricius, 1793) (Insecta, Isoptera, Kalotermitidae) in the Mediterranean area

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Abstract

The biodiversity of the European termite *Kalotermes flavicollis* is here studied through the analysis of mitochondrial (303 bp of control region and 912 bp of COI/tRNA^{Leu}/COII) and nuclear (five microsatellite and 20 Inter-SINE loci) markers on 18 colonies collected in Southern France, Corsica, Sardinia, peninsular Italy, the Balkans and Greece. Different statistical analyses (Bayesian phylogenetic analysis, parsimony network, *F*-statistics, PCA) were performed. Mitochondrial sequences produced an unresolved polytomy including samples from peninsular Italy, Balkans and Greece, and three main clades: southern France, Corsica-Sardinia and Portoscuso (SW Sardinia). Nuclear markers confirm these data, further highlighting a more significant divergence at the regional scale. The results obtained for the peri-Tyrrhenian area agree with major paleogeographic and paleoclimatic events that shaped the biodiversity of the local fauna. *K. flavicollis* biodiversity and its phylogeographic pattern are also evaluated in the light of the data available for the other native European termite taxon (genus *Reticulitermes*), in order to produce a more complete scenario of the Mediterranean. In the area comprised between southern France and Italy, the degree of diversity is similar; however, in the eastern area, while *K. flavicollis* is differentiated only at the population level, the genus *Reticulitermes* comprises at least six entities of specific and/or subspecific level. This discrepancy may be explained by taking into account the different evolutionary histories of the two taxa.

Keywords: European area, evolutionary histories, molecular markers, phylogeography, termites

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Introduction

The order Isoptera arose around 150 Myr ago in the late Jurassic/Early Cretaceous period (Engel *et al.*, 2009). At present, seven families are recognized; they comprise around 280 genera for a total of over 2600 diplo-diploid species (Engel & Krishna, 2004). Isopterans are particularly interesting for their behavior, reproductive biology and ecological roles (Inward *et al.*, 2007). The typical population structure of

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these insects consists of colonies, each composed of various castes. Colony foundation is often linked to a single pair of reproducing individuals. Over time, secondary males and females may be produced within the colony, increasing the reproductive output, but this closed system clearly lacks panmixia. Colony defense is performed by soldiers, while foraging and nursing are carried out by workers. In basal termite species (the so-called 'lower termites', as for example Kalotermitidae), workers are actually nymphs (named 'pseudoergates') and are characterized by the ability to differentiate either into soldiers or reproductives (Korb, 2007, 2008).

From an ecological point of view, termites represent one of the most important degraders in tropical and temperate forest ecosystems, their diet being principally soil and wood based. Termites digest plant material (cellulose) with the help of symbiotic bacteria and protozoans in their guts.

Two genera are native in Europe: *Reticulitermes* and *Kalotermes*. They show differences in ecology, habitat preference and colony dynamics; while *Reticulitermes* species are subterranean and build colonies of thousands of individuals with different reproductive centers in the soil, *Kalotermes* spp. produce smaller colonies, generally formed by few hundred individuals that do not have a strictly subterranean reproduction (Prota, 1962). The two genera also belong to different lifetypes. *Kalotermes* species (and more generally Kalotermitidae) are classified as 'one-piece' nesters; individuals nest and feed in the same substrate, i.e. dry wood. A different lifetype is proposed for *Reticulitermes* species, in which the nesting and the feeding areas are separated, forcing the individuals to forage away from the nest (Eggleton & Tayasu, 2001).

Hitherto, biodiversity and evolutionary researches have mainly focused on the *Reticulitermes* genus, which, according to recent data, consists of eight taxa of species level in Europe (Clément *et al.*, 2001; Austin *et al.*, 2002; Marini & Mantovani, 2002; Luchetti *et al.*, 2004a,b, 2007; Uva *et al.*, 2004; Velonà *et al.*, 2010). In contrast, the genus *Kalotermes* has been poorly studied and is believed to comprise only one species in Europe, *K. flavicollis* (Fabricius, 1793) (Clément *et al.*, 2001; Luchetti *et al.*, 2004a). Yet, physiological, morphometric and reproductive biology data suggest that there may be some degree of geographic differentiation, e.g. between French and Sardinian colonies on one side, and colonies from the Italian peninsula on the other (Luscher, 1956; Springhetti, 1967; Rossi & Springhetti, 1983). Currently available mtDNA sequence data (910 bp fragment of COI/tRNALeu/COII) suggest that there is no genetic structuring in *K. flavicollis* across Italy, the Balkans and Greece (Luchetti *et al.*, 2004a). In contrast, within the same area, the genus *Reticulitermes* shows at least six lineages (Luchetti *et al.*, 2007; Velonà *et al.*, 2010).

In this paper, we present a molecular analysis of *K. flavicollis* colonies sampled from France, Italy, the Balkans and Greece. Both mitochondrial (part of the control region and the COI/tRNA^{Leu}/COII fragment) and nuclear (microsatellites and Inter-SINE loci) markers are used. In particular, five new polymorphic microsatellite loci are here characterized for *K. flavicollis* and the use of Inter-SINEs fingerprinting (Shafer & Stewart, 2007) is explored for the first time in Kalotermitidae.

SINEs (Short INterspersed Elements) are non-autonomous nuclear retrotransposons, usually present with more than 10^5 copies (Kostia *et al.*, 2000; Ohshima & Okada, 2005). The power of SINEs in phylogenetics is that their insertion is random and unidirectional, so that the absence of a SINE is always an ancestral condition (Nishihara & Okada, 2008). In this analysis, we used a primer designed on the SINE *Talua*, and we amplified the region between two copies of the SINE (Luchetti, 2005; Luchetti & Mantovani, 2009) to produce a presence/absence matrix.

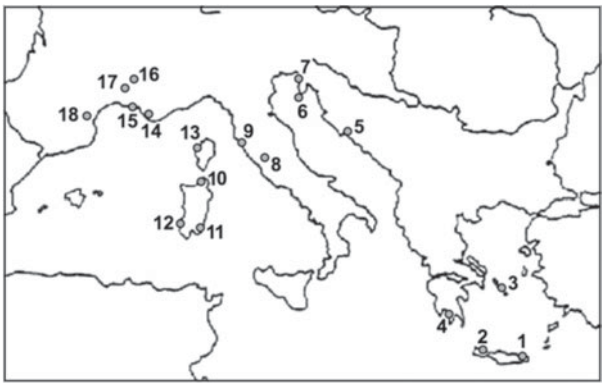

Fig. 1. Sampling localities of *Kalotermes flavicollis* populations. Numbers refer to table 1.

The aim of the present analysis is to infer genetic relationships between Mediterranean populations of *K. flavicollis* and to correlate these with the history of the basin. Results are also compared with those previously obtained for *Reticulitermes* spp. (Luchetti *et al.*, 2005, 2007; Velonà *et al.*, 2010) in order to evaluate the contribution of the historical events that have shaped the present-day biodiversity in this geographic area.

Methods

Sampling

Present work was carried out on 18 colonies collected in Greece, the Balkans, Italy and France (fig. 1, table 1). Five of these samples (Amorgos, Areopolis, Vransko, Brijoni and Porto Rose) were already analyzed for the COI/tRNA^{Leu}/COII fragment in Luchetti *et al.* (2004a) (table 1).

At each sampling site, two individuals were sequenced at two mitochondrial loci, and 8–10 specimens were genotyped for both microsatellites and I-SINE markers (with the exception of the Estagel population, for which only four individuals were available). Total DNA was extracted from the head of each termite using the CTAB protocol (Doyle & Doyle, 1987).

Mitochondrial markers

A portion of 303 bp of the control region (CR), homologous to the region surveyed by Jenkins *et al.* (1999) for *Reticulitermes* taxa, and a 912 bp fragment, spanning from the 3′ end of the cytochrome oxydase sub. I (COI) to the cytochrome oxydase sub. II (COII) gene, including the tRNA^{Leu} in between, were PCR amplified with the primer pairs AT-KR (5′-GTG GCT ATA CCC ACT ATA AA-3′)/TM-N-193 (5′-TGG GGT ATG AAC CAG TAG C-3′), and C1-J-2797 (5′-CCT CGA CGT TAT TCA GAT TAC C-3′)/TK-N-3785 (5′-GTT TAA GAG ACC AGT ACT TG-3′), respectively.

PCR amplifications were performed in a 50 µl reaction using *Taq* DNA polymerase PCR kit (Invitrogen), following

Table 1. Sampling sites, scored haplotypes and GenBank accession numbers for each mitochondrial sequence.

Localities		Haplotype			GenBank A.N.	
		CR	COI/tRNA^{Leu}/COII	Combined	CR	COI/tRNA^{Leu}/COII
GREECE						
Crete						
	1 Toplou a	A1	B1	H1	FJ750486	AY954678
	Toplou b	A2	B1	H2	GU931795	
	2 Kastelli a	A1	B2	H3		FJ750507
	Kastelli b	A3	B1	H4	FJ750493	
Cyclades						
	3 Amorgos a	A1	B1*	H1		
	Amorgos b	A1	B1	H1		
Peloponnesus						
	4 Areopolis a	A1	B3*	H5		AY954674
	Areopolis b	A1	B1*	H1		
BALKANS						
Croatia						
	5 Vransko a	A4	B1*	H6	FJ750490	
	Vransko b	A1	B1	H1		
	6 Brijoni a	A1	B1*	H1		
	Brijoni b	A2	B1	H2		
Slovenia						
	7 Porto Rose a	A5	B1*	H7	FJ750488	
	Porto Rose b	A1	B1	H1		
ITALY						
Peninsula						
	8 Viterbo a	A1	B4	H8		FJ750513
	Viterbo b	A1	B5	H9		GU931797
	9 Feniglia a	A1	B1	H1		
	Feniglia b	A6	B1	H10	GU931793	
Sardinia						
	10 Rena Majore a	A7	B6	H11	FJ750495	FJ750510
	Rena Majore b	A1	B7	H12		GU931798
	11 Geremeas a	A8	B8	H13	FJ750491	FJ750506
	Geremeas b	A9	B6	H14	GU931796	
	12 Portoscuso a	A7	B9	H15		FJ750511
	Portoscuso b	A10	B9	H16	FJ750497	
FRANCE						
Corsica						
	13 Calvi a	A11	B10	H17	FJ750494	FJ750509
	Calvi b	A1	B6	H18		
Continental						
	14 Marsiglia a	A12	B11	H19	FJ750483	FJ750504
	Marsiglia b	A13	B11	H20	GU931794	
	15 Port de Bouc a	A14	B12	H21	FJ750500	FJ750515
	Port de Bouc b	A13	B13	H22		FJ750502
	16 Avignone a	A14	B14	H23		FJ750503
	Avignone b	A14	B15	H24		GU931799
	17 Boulbon a	A12	B13	H25		
	Boulbon b	A13	B16	H26		GU931800
	18 Estagel a	A12	B13	H25		
	Estagel b	A13	B13	H22		

Numbers before each population are the same as for fig. 1. Asterisks indicate sequences drawn from Luchetti *et al.* (2004a).

the kit protocol. The amplification conditions were: 30 cycles of denaturation at 94°C for 30″, annealing at 48°C for 30″ and extension at 72°C for 30″, plus an initial denaturation at 94°C for 5′ and a final extension at 72°C for 7′. The amplified products were purified with the Wizard SV Gel and PCR Clean-up System kit (Promega, Milan, Italy) and both strands were sequenced at Macrogen Inc. (Korea). GenBank accession numbers are reported in table 1.

Microsatellites markers

A microsatellite-enriched genomic library was obtained using the F.I.A.S.CO. protocol (Zane *et al.*, 2002; Velonà *et al.*, 2009).

Microsatellite loci were identified using the TANDEM REPEAT FINDER software (Benson, 1999). PCR primers were then designed using the PRIMER3 online software (Rozen &

Table 2. List of primers for the five microsatellite loci here isolated.

Locus	Primer Sequence	Motif	T_m	GenBank A. N.
C22	F: 5′-TCCCAAAAATCCTTGCGTAT-3′ R: 5′-CGATGTCAGTGAAGGAAGCA-3′	$(TG)_{10}$	54°C	FJ750516
C24	F: 5′-TAGCATCGGGAATGGACTTT-3′ R: 5′-GAGGTACACTTGGCCGTCTT-3′	$(AC)_2AG(AC)_6$	54°C	FJ750517
D17	F: 5′-GAGTTGGAAGACCCAAGCTG-3′ R: 5′-GGCCCCTTTATGAATGTTTTC-3′	$(AC)_6$	54°C	FJ750518
D30	F: 5′-CTGTGGGAAAAAGGATGACG-3′ R: 5′-AGCTGACTGGATCTGCCACT-3′	$(CA)_2GA(CA)_6$	56°C	FJ750519
D52	F: 5′-TGAGAGCATGGAGTGGTGAG-3′ R: 5′- CCGGAGGCAACAGACTAAAT-3′	$(TG)_{10}$	54°C	FJ750520

Motifs, annealing temperatures and Genbank accession numbers of sequenced alleles are also given.

Skaletsky, 2000). Each primer pair was optimized for PCR amplification by testing over a range of annealing temperatures. The amplification conditions were 94°C for 5′ for the initial denaturation, 35 cycles at 94°C for 30″, annealing for 30″ (see in table 2 the temperature utilized for each locus) and 72°C for 30″, plus a final extension at 72°C for 7′.

The 10 µl PCR reactions included 8 ng of genomic DNA, 10 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 mM of buffer 10× (Invitrogen kit), 1 µl BSA 0.2% and 1 U of *Taq* polymerase (Invitrogen). Genotyping was performed in a Beckman CEQ8000, using 5′-labelled forward primers (Sigma).

Inter-SINE markers

This method is based on the PCR amplification of sequences comprised between two copies of a SINE retrotransposon. The SINE used here was isolated in the subterranean termite species *Reticulitermes lucifugus*, and its presence was also confirmed within the Kalotermitidae family, in both *Cryptotermes secundus* and *K. flavicollis* (Luchetti, 2005; Luchetti & Mantovani, 2009). Two primers were tested, *Ta*F (5′ - AGT GGC CGT GCG GTC TAA G - 3′) and *Ta*R (5′- CGT CGC AGA GAC CTC TAC CTG - 3′). The first anneals at the 5′ end of the SINE, priming upstream, the second anneals within the SINE, priming downstream. Only the *Ta*F primer generated a repeatable fingerprinting pattern, using the following amplification conditions: 94°C for 5′ for the initial denaturation, 35 cycles at 94°C for 30″, 42°C for 30″ and 72°C for 30″, plus a final extension at 72°C for 7′. 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 10× and 1 U of *Taq* polymerase (Invitrogen). PCR products were resolved in 2% agarose gels in a TAE buffer 1 × and bands were used to create a presence (1)/absence (0) matrix.

Statistical analyses

Sequences were aligned with CLUSTALW algorithm implemented in MEGA4 (Tamura *et al.*, 2007); this software was also used to calculate the number of variable sites.

The Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999) was performed with PAUP* 4.0b (Swofford, 2001) to determine if the two isolated mitochondrial fragments could be analyzed in a combined dataset; significance of *P* was computed after 1000 replicates. The test resulted significant ($P < 0.05$), impeding the use of the combined dataset for the phylogenetic analysis. So, through the software MODELTEST 3.06 (Posada & Crandall, 1998), we determined the best substitution model for both the control region (TrN + Γ, Γ = 1.6368) and the COI/tRNA^{Leu}/COII (TIM + I, I = 0.7794).

The Bayesian phylogenetic analysis was carried out using MRBAYES 3.01 (Huelsenbeck & Ronquist, 2001), partitioning the total dataset and setting the ML parameters (lset) as follows: for the control region nst = 6, rates = gamma and for the COI/tRNA^{Leu}/COII nst = 6, rates = propinv. The Markov Chain Monte Carlo process was set so that two runs of four chains ran simultaneously for 10^6 generations, when the two runs converged onto stationary distribution (standard deviation of split frequencies < 0.01); trees were sampled every 100 generations, for a total of 10,001 trees. Burnin was set to 25% of sampled tree, so that a consensus tree was calculated on 7501 trees. A parsimony network was inferred using TCS 1.21 (Clement *et al.*, 2000), with gaps considered as 5th state characters. A pairwise divergence matrix was calculated using PAUP* (Swofford, 2001).

For microsatellite markers, the presence of null alleles was tested with MICROCHECKER 2.2.1 (Van Oosterhout *et al.*, 2004). The allele number, allelic richness and Weir and Cockerham *F*-statistics were calculated with FSTAT 2.9.3.2 (Goudet, 2002). Expected and observed heterozygosities were computed with GENETIX 4.05 (Belkhir *et al.*, 2003). The software GENEPOP 1.2 (Raymond & Rousset, 1995) was utilized for linkage disequilibrium analysis and to calculate the probability to fit to the Hardy-Weinberg equilibrium (HWE).

For the analysis of Inter-SINE (I-SINE) markers, we calculated the Shannon Index using FAMD 1.108b (Schlüter & Harris, 2006) and the genetic diversity using HICKORY 1.1 (Holsinger *et al.*, 2002) as measures of variability within populations. HICKORY uses a Bayesian approach without assuming Hardy-Weinberg equilibrium. The analysis was performed first by testing all the models implemented in the software. To select the best model, we used the Deviance Information Criterion (DIC: Spiegelhalter *et al.*, 2002), an analogue of the Akaike Information Criterion. The model with the lowest DIC value was chosen as the best one. The same analysis evaluated inbreeding (f) and differentiation (θ) coefficients.

For both microsatellite and I-SINE markers, the multivariate dataset was reduced into a two-dimensional space through a principal components analysis (PCA) performed with PAST 1.76 (Hammer *et al.*, 2001).

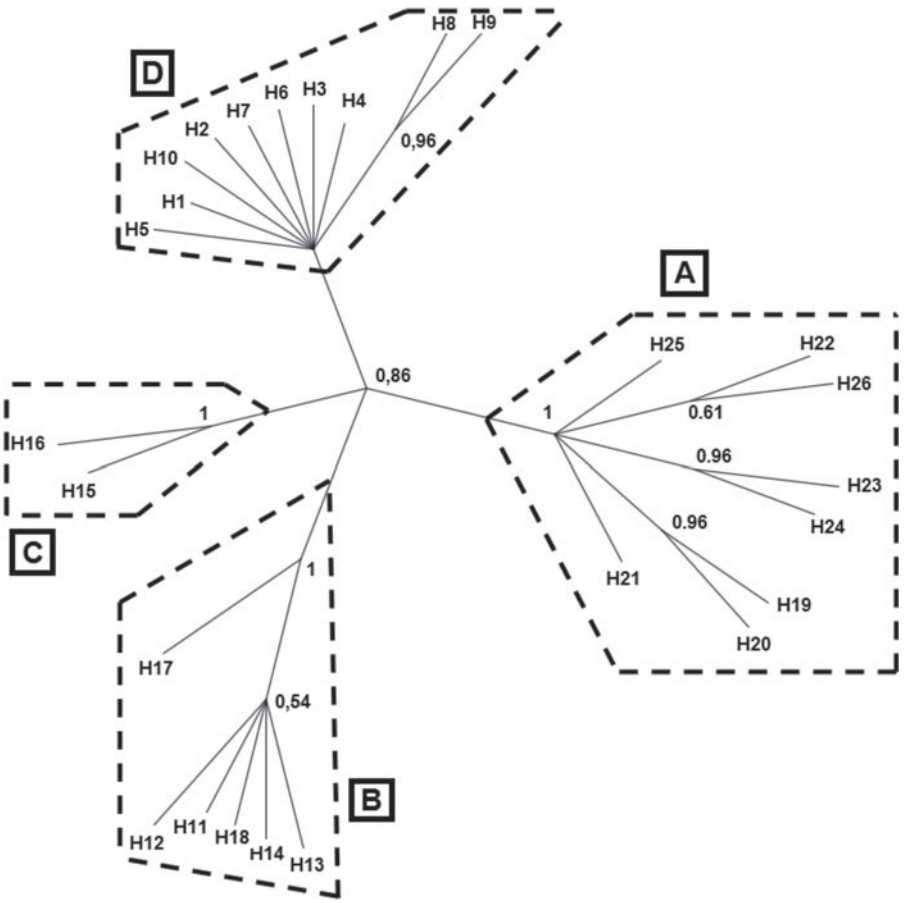

Fig. 2. Bayesian analysis performed on mitochondrial markers. Numbers indicate bayesian posterior probability values. Capital letters indicate the three lineages identified (A–B–C) and the unresolved polytomy (D).

Results

Mitochondrial DNA

The CR amplified fragment showed 15 variable sites out of 303, leading to 14 haplotypes. In the COI/tRNA^{Leu}/COII fragment, 16 out of the COI 178 bp were variable (six at the first, three at the second and seven at the third codon position), while the COII sequence showed 32 variable sites out of 662 bp (six at the first, five at the second and 21 at the third codon position); the tRNA^{Leu}, 72 bp long, showed only one variable site across its length. The total number of haplotypes for the COI/tRNA^{Leu}/COII fragment is 16.

The alignment (CR + COI/tRNA^{Leu}/COII) showed 64 variable sites in 1215 bp, leading to 26 haplotypes. Only the two individuals of the Amorgos sample shared the same haplotype H1, which is also the most common combined haplotype present in six other populations, spanning from Greece to Croatia and peninsular Italy (table 1).

The Bayesian phylogenetic analysis (fig. 2) showed three distinct clades: the first (A) includes all the specimens from continental France, the second clade (B) embodies the specimens from Corsica and Sardinia, except Portoscuso (Sardinia), which constitutes the third clade (C). All the specimens from the area spanning from Crete to the peninsular Italy constitute an unresolved polytomic group (D).

The haplotype parsimony network, built only on the more variable COI/tRNA^{Leu}/COII fragment, partially agrees with the phylogenetic inference (fig. 3). The analysis splits the haplotypes into two networks; the first includes all the continental French samples (B11–B16), and the second includes all the other colonies. The polytomy seen in the phylogenetic tree (D) is a star-like network connected, albeit with a certain number of missing haplotypes, both to the Portoscuso lineage (B9) and to the much more differentiated Corsica-Sardinian clade (B6–B8, B10).

The genetic diversity within each of the four identified groups is up to 0.7% (average = 0.2%) and does not overlap the range of between-group distances (fig. 4). The highest values of divergence are found in the comparison between the continental France lineage and the Corsica-Sardinia clade but Portoscuso (from 2.5% to 3.2%). Portoscuso haplotypes are less differentiated from the peninsular Italy, the Balkans and the Greece populations (0.5%; square in fig. 4) than from the Corsica/Sardinian (1.3%; circle in fig. 4) and the continental France ones (1.8%; diamond in fig. 4; see also table S1 in supplementary materials).

Microsatellites markers

The enriched library screening produced a total of 70 positive clones, but only nine of them allowed primer

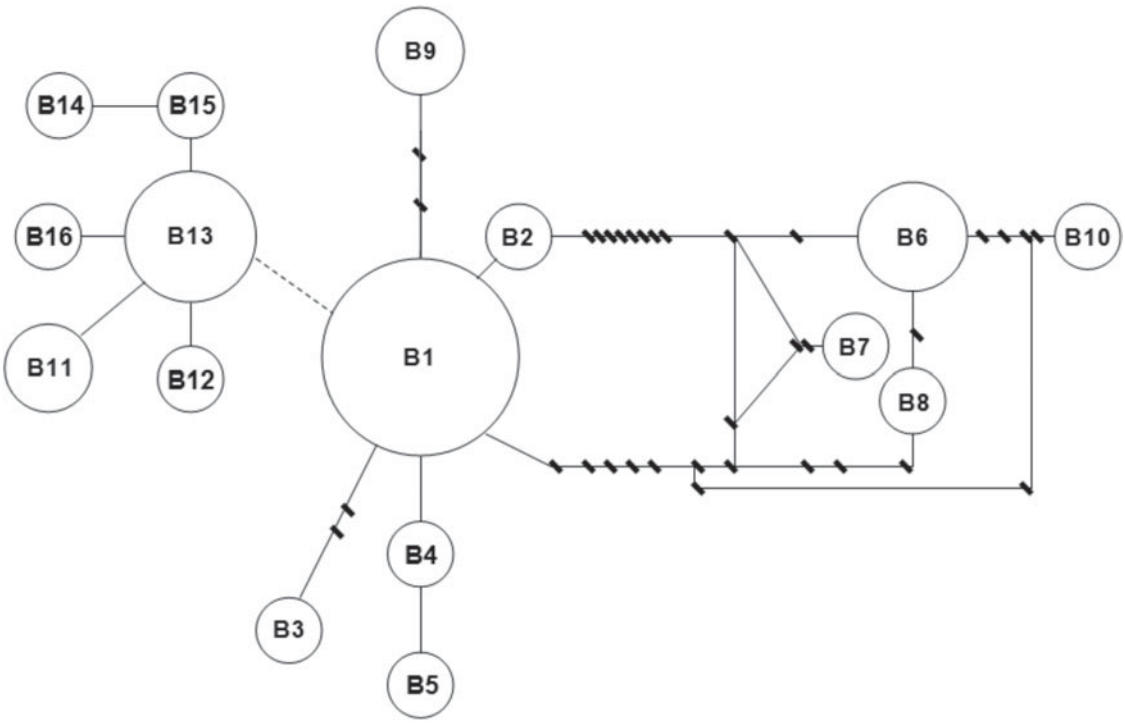

Fig. 3. Parsimony network built on COI/tRNA^{Leu}/COII sequences. Haplotype designation is as in table 1; the small lines across branch connections represent possible missing/ideal haplotypes. Dotted branch connection is not supported by parsimony 95% limit. Circle magnitudes are proportional to scored haplotype frequency.

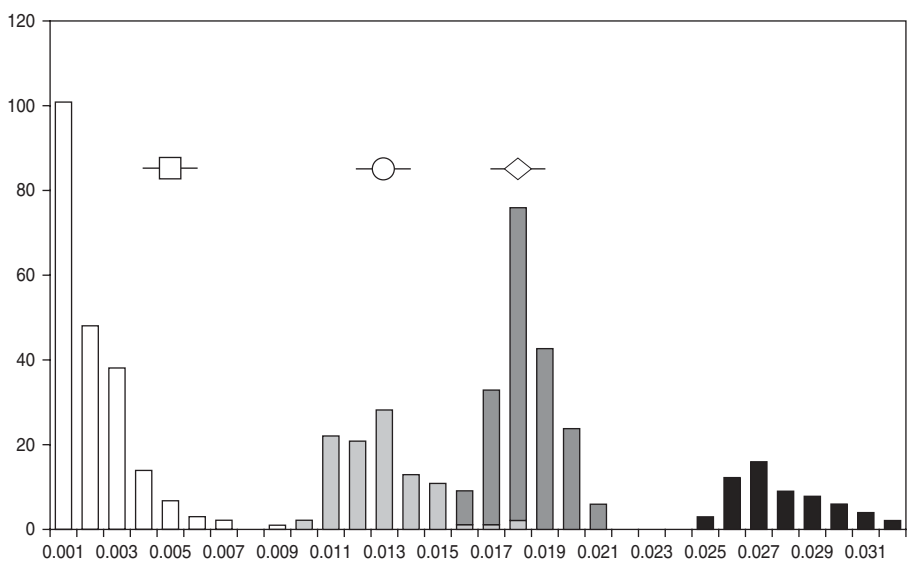

Fig. 4. Histogram representing the frequency of pairwise sequences divergence among analyzed samples. Each bin represents the 0.1% of sequence divergence. Within group variability – white bars. Inter-group variability – light grey: peninsular Italy/Balkans/Greece polytomy (D) vs Corsica/Sardinia (clade B); dark grey: peninsular Italy/Balkans/Greece polytomy (D) vs Continental France (clade A); black: continental France (clade A) vs Corsica/Sardinia (clade B).
Indicators represent average divergence values ± standard deviation (horizontal bars) between Portoscuso (clade C) and □, peninsular Italy/Balkans/Greece polytomy; ○, Corsica/Sardinia; ◊, Continental France.

design, and five were found to be polymorphic after population PCR assay. In particular, locus C22 was polymorphic in all populations, while C24 in only half of them (table 3). The linkage-disequilibrium analysis did not show any significant association between loci (see table S2 in supplementary materials).

Table 3. Number of alleles (A), allelic richness (A_C), number of individuals (N), observed (H_O)/expected (H_E) heterozygosity per locus and per population over all loci are given for the five microsatellite loci.

	C22	C24	D17	D30	D52	ALL LOCI	S.I. ±S.D.	H_S ±S.D.
	A/A_C H_O/H_E	A/A_C H_O/H_E	A/A_C H_O/H_E	A/A_C H_O/H_E	A/A_C H_O/H_E	N/A_C H_O/H_E		
1 Toplou	4/2.624	1/1.000	2/1.222	1/1.000	2/1.883	10/1.546	1.160	0.318
	0.333/0.653*	–	0.111/0.105	–	0.400/0.480	0.169/0.247	0.01	0.02
2 Kastelli	3/1.776	3/2.145	1/1.000	2/1.368	4/2.602	10/1.778	0.974	0.223
	0.222/0.364	0.286/0.541	–	0.200/0.180	0.500/0.675*	0.242/0.352	0.01	0.02
3 Amorgos	2/1.908	2/1.924	1/1.000	2/1.857	4/2.493	10/1.836	0.793	0.174
	0/0.494**§	0/0.480*	–	0/0.444*	0.300/0.650*§	0.060/0.414***	0.03	0.03
4 Areopolis	4/2.460	1/1.000	1/1.000	2/1.833	2/1.624	10/1.583	0.280	0.113
	0/0.617***§	–	–	0/0.444**§	0.400/0.320	0.080/0.276***	0.02	0.03
5 Vransko	3/2.444	2/1.883	1/1.000	3/1.627	3/2.409	10/1.873	0.850	0.172
	0.6/0.645	0/0.480**§	–	0.333/0.290	0.600/0.635	0.307/0.410	0.02	0.02
6 Brijoni	2/1.222	1/1.000	2/1.906	2/1.876	4/2.210	10/1.643	0.816	0.191
	0.111/0.105	–	0.900/0.495*	0/0.469**§	0.600/0.530	0.322/0.320	0.02	0.02
7 Porto Rose	2/1.908	3/2.072	3/2.182	1/1.000	4/2.697	10/1.972	0.300	0.122
	0.667/0.494	0.200/0.535*§	0.833/0.542	–	1.000/0.680	0.540/0.450	0.001	0.03
8 Viterbo	2/1.368	1/1.000	1/1.000	2/1.509	2/1.789	10/1.333	0.835	0.182
	0.200/0.180	–	–	0.300/0.255	0.571/0.408	0.214/0.169	0.03	0.02
9 Feniglia	3/1.915	2/1.833	2/1.845	3/1.990	8/3.431	10/2.213	0.783	0.187
	0.400/0.455	0/0.480**§	0.300/0.455	0.200/0.460*	0.800/0.855	0.340/0.539**	0.04	0.02
10 Rena Majore	2/1.368	1/1.000	2/1.881	2/1.368	3/2.351	10/1.594	0.544	0.110
	0.200/0.180	–	0.778/0.475	0.200/0.180	1.000/0.611*	0.436/0.289	0.03	0.02
11 Geremeas	2/1.673	3/2.557	2/2.000	5/2.134	3/2.000	10/2.073	0.790	0.157
	0.444/0.346	0/0.625*	0/0.500	0.300/0.485*	0.250/0.406	0.199/0.472*	0.03	0.02
12 Portoscuso	2/1.222	2/1.505	2/1.876	3/2.170	2/1.576	10/1.670	0.600	0.127
	0.111/0.105	0/0.245	0.250/0.469	0.500/0.531	0.333/0.279	0.239/0.325	0.001	0.02
13 Calvi	3/2.147	1/1.000	2/1.450	3/2.089	6/3.104	10/1.958	0.288	0.095
	0.500/0.540	–	0/0.219	0.300/0.545	0.750/0.781	0.310/0.417	0.01	0.03
14 Marsiglia	2/1.765	2/1.923	2/1.405	2/1.200	2/1.816	10/1.622	0.854	0.184
	0.556/0.401	0.750/0.500	0/0.197	0.100/0.095	0.625/0.430	0.406/0.324	0.03	0.02
15 Port de Bouc	2/1.912	1/1.000	2/1.929	1/1.000	2/1.786	8/1.524	0.682	0.146
	0.875/0.492	–	0.750/0.469	–	0.500/0.375	0.425/0.267	0.01	0.02
16 Avignone	3/2.442	1/1.000	2/1.286	1/1.000	1/1.000	9/1.300	0.477	0.104
	0.889/0.642	–	0.143/0.134	–	–	0.206/0.155	0.00	0.02
17 Boulbon	2/1.368	4/2.590	4/2.580	2/1.368	5/2.809	10/2.143	0.575	0.151
	0/0.180	0/0.656***§	0/0.667***§	0/0.180	0.125/0.711***§	0.025/0.478***	0.01	0.03
18 Estagel	2/2.000	1/1.000	1/1.000	1/1.000	1/1.000	4/1.200	0.000	0.115
	1.000/0.500	–	–	–	–	0.200/0.100	0.00	0.03

§, possible presence of null alleles; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Shannon Index (S.I.), genetic diversity (H_S) and their standard deviations are computed on I-SINE marker.

Five populations (Feniglia, Geremeas, Portoscuso, Marsiglia and Boulbon) were polymorphic at all loci, while Estagel and Avignone samples were polymorphic only at one (C22) or two (C22, D17) loci, respectively (table 3). Allelic richness values per population ranged from 1.000 to 3.431; the range was 1.200–2.213 over all loci per population. On the whole, the number of alleles per locus ranged from eight (C24 and D17) to 13 (D52), with an average allele number per locus of 9.8 (not shown). Considering the total number of loci analyzed over all populations, 29% of the polymorphic loci (20 out of 68) showed significant deviations from Hardy-Weinberg equilibrium (HWE); null alleles may explain 55% (11 out of 20) of these deviations. Considering all loci, five populations (Amorgos, Areopolis, Feniglia, Geremeas and Boulbon) did not match the HWE (table 3).

The Weir and Cockerham *F*-statistic showed a relatively low F_{IS} (0.285; $P<0.001$; 95% confidence interval = 0.123–0.536) and a high F_{ST} (0.376; $P<0.001$; 95% confidence interval = 0.259–0.461); this is reflected by significant F_{ST} in the majority of pairwise population comparisons (see table S3 in supplementary materials). In the PCA analysis (axis 1 + axis 2 = 88.16% of the total variance), colonies from peninsular Italy, the Balkans and Greece cluster together, as well as Portoscuso sample with the other colonies from Sardinia and Corsica; the continental France samples were the most differentiated (fig. 5a).

Inter-SINE markers

The 20 loci detected were polymorphic throughout the entire dataset. The model choice analysis indicated the 'full model' as the best one to describe our dataset (DIC = 563.944).

Shannon index ranged between 0 (Estagel) and 1.160 (Toplou), while the genetic diversity range is 0.095 (Calvi)–0.318 (Toplou; table 3). The inbreeding coefficient analysis showed a low value ($f=0.168$; 95% confidence interval = 0.006–0.541), while the inter-population divergences were higher ($\theta=0.418$; 95% confidence interval = 0.373–0.463). The PCA analysis (axis 1 + axis 2 = 87.01% of the total variance) depicts a picture highly congruent with the one obtained from the microsatellite data. Samples from Greece, the Balkans and peninsular Italy form one group, and the samples from Portoscuso and Corsica-Sardinia form another one; the continental France colonies are the most divergent (fig. 5b).

Discussion

The very first mtDNA analysis of *K. flavicollis* (Luchetti *et al.*, 2004a) did not reveal any structuring of COI/tRNA^{Leu}/COII haplotypes over a geographic area comprising peninsular Italy, Sicily, the Balkans, the Peloponnesus and the Cyclades. The results here presented, obtained from the same mitochondrial marker with the addition of the CR fragment, are in line with this previous analysis. Yet, the wider geographic area surveyed showed a more defined structuring due to the divergent and well-defined lineages present in Corsica/Sardinia, Portoscuso and continental France.

The range of within-group variability is, in all comparisons, separated by a large gap from the between-group divergence range; this recalls a 'barcoding' gap (Hebert *et al.*, 2004). Even considering the limits that the evolutionary history of each taxon imposes on these threshold estimates, the mean within- vs between-group divergences here observed are well above the conventional DNA barcode gap (Hebert *et al.*, 2004). With respect to the Hebert *et al.* (2004) dataset, our estimates are based on fewer samples and on different mtDNA genes; nevertheless, present values suggest that the here scored mtDNA variability could not represent a mere population-level divergence. With respect to our analysis, comparable results can also be found in other insect groups, such as Hemiptera (Žurovcová *et al.*, 2010) and Lepidoptera (Hajibabaei *et al.*, 2006).

A high level of differentiation is also supported by the microsatellite and I-SINE data, which suggest a clear-cut differentiation at the regional scale, with the French continental colonies being the most divergent. The existence of different entities in France and Italy was already hypothesized on physiological grounds (Luscher, 1956) and present molecular data support this possibility. Still other geographic taxa were suggested based on soldier morphometry, reproductive biology, and crossing experiments (Springhetti, 1967; Rossi & Springhetti, 1983); these data evidenced a significant divergence of Sardinian and Corsican colonies. Accordingly, the data presented here indicate that the Corsican and Sardinian colonies constitute a separate lineage that is differentiated from the continental France and peninsular Italy colonies. A more complex situation emerges when considering the Portoscuso sample. In our mitochondrial phylogeny, it seems to represent a separated lineage, but the level of divergence in the Portoscuso vs peninsular Italy, Balkans and Greece population comparison is low and falls in the within-species range. On the other hand, both nuclear markers define a strict relationship between Portoscuso and the Corsica-Sardinia lineage. On the whole, the data could be explained by a recent introduction of this population from peninsular Italy, followed by outbreeding events with local demes.

The Mediterranean basin's fauna shows a high level of diversity in several hotspots, as well as a number of endemic taxa, owing to various paleogeographic/paleoclimatic events. The range of sequence divergence observed among *K. flavicollis* clades points to roughly contemporary cladogenetic events. Quaternary climatic oscillations have been one of the major forces driving taxa evolution and distribution (Hewitt, 2004; Schmitt, 2007); during the glacial periods, Mediterranean peninsulas provided refugia for a number of organisms, while successive climate warming allowed northward recolonization. From present results, *K. flavicollis* colonies from the Balkans and peninsular Italy do not show the expected divergence, as populations lack mitochondrial lineage break across the entire area; only nuclear data suggest some degree of divergence. This agrees with the pattern observed, for example, in *Maniola jurtina* where the eastern European lineage spans from Italy to Balkans (Schmitt, 2007). Therefore, during the last glacial phase, an extensive gene flow within the Adriatic-Pontic area is suggested to have occurred, while the most western taxa remained isolated (Schmitt *et al.*, 2005a). Data here presented may reflect a comparable situation.

The continental French clade may represent the leading edge of a northward recolonization from a glacial refugium. Since the scored genetic divergence (average ~1.8%) excludes a close affinity with the Italian peninsular clade, the recolonization route may have initiated from an Iberian refugium. For example, this route has been followed by another European termite species, *Reticulitermes banyulensis* (Kutnik *et al.*, 2004) and by other strictly Mediterranean insects, such as the stick-insect *Leptynia hispanica* (Ghiselli *et al.*, 2007) or the

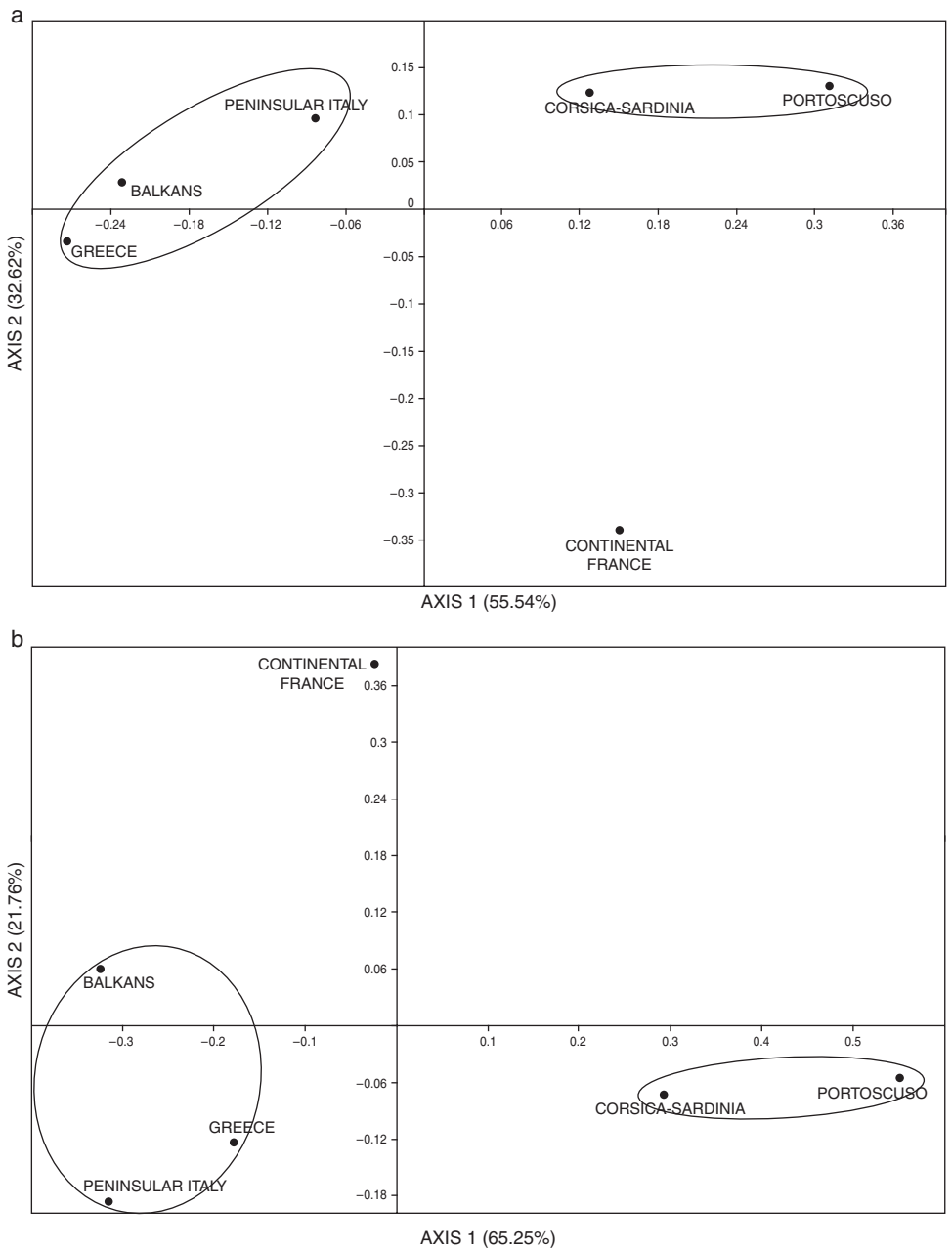

Fig. 5. Principal component analysis (PCA) built on (a) microsatellite dataset and (b) I-SINE dataset. In parenthesis the percentage of variance for each axis is reported.

butterfly *Polyommatus hispana* (Schmitt *et al.*, 2005b). However, the possibility of a refugial area in southern France, as hypothesized for other insects (Vasconcelos *et al.*, 2006; Wahlberg & Saccheri, 2007), cannot be ruled out. Further sampling in north-western Italy and, especially, in the Iberian peninsula will obviously highlight this point.

As for the origin of the Corsica-Sardinian colonies, except Portoscuso, the genetic divergence observed between this clade and the other eastern populations (~1.3%) well correlates with the Pliocene-Quaternary transgression, which, as argued for *Solatopupa* spp. (Ketmaier *et al.*, 2006), may have caused the speciation by vicariance.

As proposed by Arbogast & Kenagy (2001), the comparative phylogeography approach is a good tool in detecting historical biogeography. The comparison of present results with those from the sympatric *Reticulitermes* spp. termites is of interest since, despite their largely overlapped distribution, these two groups show differences in present-day biodiversity. Taking into account the area considered in this paper, *Reticulitermes* spp. comprises eight species (Clément *et al.*, 2001; Luchetti *et al.*, 2004b, 2007; Uva *et al.*, 2004; Velonà *et al.*, 2010), while *K. flavicollis* shows only a half of lineages.

In the western part of the examined area, the biodiversity level of the two termite genera is similar. This does not

necessarily mean that they share the same biogeographic history, but geological and paleoclimatic events might have similarly affected their diversity. *K. flavicollis* populations from Sardinia and Corsica are differentiated from the peninsular Italy ones, but with a level of divergence that could be associated with a relatively recent event. Differently, a speciation event may have originated in the same area, in a vicariance context, *Reticulitermes lucifugus corsicus* from *R. lucifugus lucifugus* around 9 million years ago (Velonà *et al.*, 2010).

On the other hand, east of Italy, *K. flavicollis* is genetically more homogeneous than *Reticulitermes*. The Aegean area is known as a hotspot of high biodiversity (Sfenthourakis & Legakis, 2001), mainly because of a complex paleogeographic history (Perissoratis & Conispoliatis, 2003); this also has been demonstrated in a number of animal systems (see for example Parmakelis *et al.*, 2006 and references therein). Accordingly, *Reticulitermes* spp. show a consistent degree of diversification; in particular, known diversity hotspots, such as Peloponnesus, Cyclades Islands and Crete (Sfenthourakis & Legakis, 2001), show their own subterranean termite lineages (Luchetti *et al.*, 2007; Velonà *et al.*, 2010). At the same localities, *K. flavicollis* diversity is limited to a population level of differentiation. Therefore, in the eastern Mediterranean range, the two termite taxa do not share the same taxon radiation. The explanation is probably linked with the different colonization histories. In fact, *K. flavicollis* species could have colonized this area later with respect to *Reticulitermes* spp.; in this way, it was not processed by the geological/paleoclimatic events shaping eastern biodiversity (see Velonà *et al.*, 2010 and references therein).

Considering our results and those obtained in a previous paper (Velonà *et al.*, 2010), it is possible to hypothesize that *K. flavicollis* species colonized recently the European area, and this event was followed by a diversification in at least three lineages.

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Supplementary Material

The online tables can be viewed at http://journals.cambridge.org/ber

References

Arbogast, B.S. & Kenagy, G.J. (2001) Comparative phylogeography as an integrative approach to historical biogeography. *Journal of Biogeography* **28**, 819–825.

Austin, J.W., Szalanski, A.L., Uva, P., Bagnères, A.G. & Kence, A. (2002) A comparative genetic analysis of the subterranean termite genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Annals of the Entomological Society of America* **95**, 753–760.

Belkhir, K., Borsa, P., Chikki, L., Raufaste, N. & Bonhomme, F. (2003) GENETIX, logiciel sous Windows TM pour la génétique des populations. Available online at http://www.genetix.univ-montp2.fr/genetix/genetix.htm.

Benson, G. (1999) Tandem Repeat Finder: a program to analyze DNA sequences. *Nucleic Acids Research* **27**, 573–580.

Clement, M., Posada, D. & Crandall, K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657–1660.

Clément, J.L., Bagnères, A.G., Uva, P., Wilfert, L., Quintana, A., Reinhard, J. & Dronnet, S. (2001) Biosystematics of *Reticulitermes* termites in Europe: morphological, chemical and molecular data. *Insectes Sociaux* **48**, 202–215.

Doyle, J.J. & Doyle, J.L. (1987) A rapid DNA isolation procedure for small amounts of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11–15.

Eggleton, P. & Tayasu, I. (2001) Feeding groups, lifetype and the global ecology of termites. *Ecological Research* **16**, 941–960.

Engel, M.S. & Krishna, K. (2004) Family-group names for termites (Isoptera). *American Museum Novitates* **2570**, 1–31.

Engel, M.S., Grimaldi, D.A. & Krishna, K. (2009) Termites (Isoptera): their phylogeny, classification, and rise to ecological dominance. *American Museum Novitates* **3650**, 1–27.

Ghiselli, F., Milani, L., Scali, V. & Passamonti, M. (2007) The *Leptynia hispanica* species complex (Insecta Phasmida): polyploidy, parthenogenesis, hybridization and more. *Molecular Ecology* **16**, 4256–4268.

Goudet, J. (2002) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3.2). Available online at http://www2.unil.ch/popgen/softwares/fstat.htm.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W. & Hebert, P.D.N. (2006) DNA barcodes distinguish species of tropical Lepidoptera. *PNAS* **103**, 968–971.

Hammer, Ø., Harper, D. & Ryan, P. (2001) PAST – PAleontological STatisitics software package for education and data analysis. *Palaeontologia Electronica* **4**, 9 pp.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S. & Francis, C.M. (2004) Identification of birds through DNA barcodes. *PLoS Biology* **10**, e312.

Hewitt, G.M. (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London, Series B* **359**, 183–195.

Holsinger, K.E., Lewis, P.O. & Dey, D.K. (2002) A Bayesian approach to inferring population structure from dominant markers. *Molecular Ecology* **11**, 1157–1164.

Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755.

Inward, D., Vogler, A. & Eggleton, P. (2007) A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. *Molecular Phylogenetics and Evolution* **44**, 953–967.

Jenkins, T.M., Basten, C.J., Dean, R., Mitchell, S.E., Kresovich, S. & Forschler, B.T. (1999) Matriarchal genetic structure of *Reticulitermes* (Isoptera: Rhinotermitidae) populations. *Sociobiology* **33**, 239–263.

Ketmaier, V., Giusti, F. & Caccone, A. (2006) Molecular phylogeny and historical biogeography of the land snail genus *Solatopupa* (Pulmonata) in the peri-Tyrrhenian area. *Molecular Phylogenetics and Evolution* **39**, 439–451.

Korb, J. (2007) Termites. *Current Biology* **17**, R995–999.

Korb, J. (2008) Termites, hemimetabolous diploid white ants? *Frontiers in Zoology* **2008**, 5–15.

Kostia, S., Ruohonen-Lehto, M., Väinölä, R. & Varvio, S.L. (2000) Phylogenetic information in inter-SINE and inter-SSR fingerprints of the artiodactyla and evolution of the bov-tA SINE. *Heredity* **84**, 37–45.

Kutnik, M., Uva, P., Brinkworth, L. & Bagnères, A.G. (2004) Phylogeography of two European *Reticulitermes* (Isoptera) species: the Iberian refugium. *Molecular Ecology* **13**, 3099–3113.

Luchetti, A. (2005) Identification of a short interspersed repeat in the *Reticulitermes lucifugus* (Isoptera Rinothermitidae) genome. *DNA Sequence* **16**, 304–307.

Luchetti, A. & Mantovani, B. (2009) *Talua* SINE Biology in the Genome of the *Reticulitermes* Subterranean Termites (Isoptera, Rhinotermitidae). *Journal of Molecular Evolution* **69**, 589–600.

Luchetti, A., Bergamaschi, S., Marini, M. & Mantovani, B. (2004a) Mitochondrial DNA analysis of native European Isoptera: a comparison between *Reticulitermes* (Rhinotermitidae) and *Kalotermes* (Kalotermitidae) colonies from Italy and Balkans. *Redia* **87**, 149–153.

Luchetti, A., Trenta, M., Mantovani, B. & Marini, M. (2004b) Taxonomy and phylogeny of north mediterranean *Reticulitermes* termites (Isoptera, Rhinotermitidae): a new insight. *Insectes Sociaux* **51**, 117–122.

Luchetti, A., Marini, M. & Mantovani, M. (2005) Mitochondrial evolutionary rate and speciation in termites: data on European *Reticulitermes* taxa (Isoptera, Rhinotermitidae). *Insectes Sociaux* **52**, 218–221.

Luchetti, A., Marini, M. & Mantovani, B. (2007) Filling the European gap: Biosystematics of the eusocial system *Reticulitermes* (Isoptera, Rhinotermitidae) in the Balkanic Peninsula and Aegean area. *Molecular Phylogenetics and Evolution* **45**, 377–383.

Luscher, M. (1956) Hemmende und fordernde Faktoren bei der Entstehung der Ersatzgeschlechtstiere bei der Termite *Kalotermes flavicollis* Fabr. *Revue Suisse Zoologie* **63**, 261–267.

Marini, M. & Mantovani, B. (2002) Molecular Relationship among European Samples of *Reticulitermes* (Isoptera Rhinotermitidae). *Molecular Phylogenetics and Evolution* **22**, 454–459.

Nishihara, H. & Okada, N. (2008) Retrotransposons: genetic footprints on the evolutionary paths of life. pp. 201–225 *in* Murphy, W.J. (*Ed*.) *Methods in Molecular Biology: Phylogenomics*. Totowa, NJ, USA, Humana Press.

Ohshima, K. & Okada, N. (2005) SINEs and LINEs: symbionts of eukaryotic genomes with a common tail. *Cytogenetic and Genome Research* **110**, 475–490.

Parmakelis, A., Stathi, I., Chatzaki, M., Simaiakis, S., Spanos, L., Louis, C. & Mylonas, M. (2006) Evolution of *Mesobuthus gibbosus* (Brullé, 1832) (Scorpiones: Buthidae) in the northeastern Mediterranean region. *Molecular Ecology* **15**, 2883–2894.

Perissoratis, C. & Conispoliatis, N. (2003) The impacts of sea-level changes during latest Pleistocene and Holocene times on the morphology of the Ionian and Aegean seas (SE Alpine Europe). *Marine Geology* **196**, 145–156.

Posada, D. & Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Prota, R. (1962) L'infestazione termitica in Sardegna. *Bollettino dell'Istituto di Patologia del Libro* **XXI**, 1–35.

Raymond, M. & Rousset, F. (1995) GENEPOP (version 1.2): a population genetics software for exact test and ecumenicism. *Journal of Heredity* **86**, 248–249.

Rossi, R. & Springhetti, A. (1983) Morphometric research on soldiers of *Kalotermes flavicollis* Fabr. from Italy. *Annali dell'Università di Ferrara - Biologia* **3**, 41–50.

Rozen, S. & Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. pp. 365–386 *in* Misener, S., Kravetz, S.A. (*Eds*) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Totowa, NJ, USA, Humana Press.

Schlüter, P.M. & Harris, S.A. (2006) Analysis of multilocus fingerprinting data sets containing missing data. *Molecular Ecology Notes* **6**, 569–572.

Schmitt, T. (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology* **2007**, 4–11.

Schmitt, T., Rober, S. & Seitz, A. (2005a) Is the last glaciation the only relevant event for the present genetic population structure of the meadow brown butterfly *Maniola jurtina* (Lepidoptera: Nymphalidae)? *Biological Journal of the Linnean Society* **85**, 419–431.

Schmitt, T., Varga, Z. & Seitz, A. (2005b) Are *Polyommatus hispana* and *Polyommatus slovacus* bivoltine *Polyommatus coridon* (Lepidoptera: Lycaenidae)? The discriminatory value of genetics in taxonomy. *Organisms Diversity and Evolution* **5**, 297–307.

Sfenthourakis, S. & Legakis, A. (2001) Hotspots of endemic terrestrial invertebrates in southern Greece. *Biodiversity and Conservation* **10**, 1387–1417.

Shafer, A.B.A. & Stewart, D.T. (2007) Phylogenetic relationships among Nearctic shrews of the genus *Sorex* (Insectivora, Soricidae) inferred from combined cytochrome *b* and inter-SINE fingerprint data using Bayesian analysis. *Molecular Phylogenetics and Evolution* **44**, 192–203.

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

Spiegelhalter, D.J., Best, N.G., Carlin, B.P. & van der Linde, A. (2002) Bayesian measures of model complexity and fit. *Journal of the Royal Statistical Society Series Series B* **64**, 483–689.

Springhetti, A. (1967) Incroci tra reali di alcune popolazioni italiane di *Kalotermes flavicollis* Fabr. *Annali dell'Università di Ferrara - Biologia* **III**, 11–17.

Swofford, D.L. (2001) PAUP*-Phylogenetic Analysis Using Parsimony (* and Other Methods), Ver 4b. Sunderland, MA, USA, Sinauer Associates.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.

Uva, P., Clément, J.L., Austin, J.W., Aubert, J., Zaffagnini, V., Quintana, A. & Bagnères, A.G. (2004) Origin of a new *Reticulitermes* termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data. *Molecular Phylogenetics and Evolution* **30**, 344–353.

Van Oosterhout, C., Hutchinson, W., Wills, D. & Shipley, P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**, 535–538.

Vasconcelos, T., Horn, A., Lieutier, F., Branco, M. & Kerdelhué, C. (2006) Distribution and population genetic structure of the Mediterranean pine shoot beetle *Tomicus destruens* in the Iberian Peninsula and Southern France. *Agricultural and Forest Entomology* **8**, 103–111.

Velonà, A., Luchetti, A., Scanabissi, F. & Mantovani, B. (2009) Genetic variability and reproductive modalities in European populations of *Triops cancriformis* (Crustacea, Branchiopoda, Notostraca). *Italian Journal of Zoology* **76**, 366–375.

Velonà, A., Ghesini, S., Luchetti, A., Marini, M. & Mantovani, B. (2010) Starting from Crete, a phylogenetic re-analysis of the genus *Reticulitermes* in the Mediterranean area. *Molecular Phylogenetics and Evolution* **56**, 1051–1058.

Wahlberg, N. & Saccheri, I. (2007) The effects of Pleistocene glaciations on the phylogeography of *Melitaea cinxia* (Lepidoptera: Nymphalidae). *European Journal of Entomology* **104**, 675–684.

Zane, L., Bargelloni, L. & Patarnello, T. (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* **11**, 1–16.

Žurovcová, M., Havelca, J., Stary, P., Věchtová, P., Chundelová, D., Jarošová, A. & Kučerová, L. (2010) 'DNA barcoding' is of limited value for identifying adelgids (Hemiptera: Adelgidae) but supports traditional morphological taxonomy. *European Journal of Entomology* **107**, 147–156.

SUPPLEMENTARY MATERIALS

Table S1. Pairwise genetic distance matrix used to calculate the frequencies of intra- and inter-group distances.

	1	2	3	4	5	6	7	8	9
1 Toplou a	-								
2 Toplou b	0.00176	-							
3 Kastelli a	0.00083	0.00264	-						
4 Kastelli b	0	0.0018	0.00083	-					
5 Amorgous a	0	0.00179	0.00083	0	-				
6 Amorgous b	0	0.00177	0.00083	0	0	-			
7 Areopolis a	0.00251	0.00448	0.00335	0.00252	0.00251	0.00251	-		
8 Areopolis b	0	0.00179	0.00083	0	0	0	0.00251	-	
9 Vransko a	0.00084	0.00269	0.00167	0.00084	0.00084	0.00084	0.00335	0.00084	-
10 Vransko b	0	0.00177	0.00083	0	0	0	0.00252	0	0.00084
11 Brijoni a	0	0.00179	0.00083	0	0	0	0.00251	0	0.00084
12 Brijoni b	0.0025	0.00089	0.00333	0.00255	0.00254	0.0025	0.00506	0.00254	0.00338
13 Porto Rose a	0.00084	0.00269	0.00168	0.00085	0.00084	0.00084	0.00335	0.00084	0.00168
14 Proto Rose b	0	0.00176	0.00083	0	0	0	0.00251	0	0.00084
15 Viterbo a	0.00084	0.00269	0.00168	0.00086	0.00086	0.00084	0.0034	0.00086	0.00171
16 Viterbo b	0.00174	0.00371	0.00261	0.00178	0.00177	0.00174	0.00441	0.00177	0.00264
17 Feniglia a	0	0.0018	0.00092	0	0	0	0.0028	0	0.00093
18 Feniglia b	0.00169	0.0027	0.00254	0.00172	0.00171	0.00169	0.00429	0.00171	0.00257
19 Rena Majore a	0.01018	0.01267	0.00933	0.01035	0.01031	0.01021	0.01289	0.01031	0.01117
20 Rena Majore b	0.01441	0.01247	0.01355	0.01465	0.01459	0.01444	0.01719	0.01459	0.01545
21 Geremeas a	0.01256	0.01427	0.01172	0.01276	0.01271	0.01259	0.01527	0.01271	0.01356
22 Geremeas b	0.01127	0.01295	0.0104	0.01127	0.01127	0.01129	0.01389	0.01127	0.01214
23 Portoscuso a	0.00421	0.00537	0.00506	0.00426	0.00425	0.00421	0.00682	0.00425	0.00511
24 Portoscuso b	0.00592	0.00718	0.00678	0.00598	0.00597	0.00592	0.00855	0.00597	0.00597
25 Calvi a	0.01277	0.01361	0.01192	0.01298	0.01293	0.0128	0.01553	0.01293	0.01379
26 Calvi b	0.01027	0.01277	0.0094	0.01044	0.01039	0.01025	0.01301	0.01039	0.01126
27 Marsiglia a	0.01714	0.01912	0.01803	0.01735	0.01731	0.01717	0.01995	0.01731	0.01819
28 Marsiglia b	0.01712	0.01828	0.01805	0.01712	0.01712	0.01709	0.01986	0.01712	0.01803
29 Port de Bouc a	0.01708	0.01911	0.01797	0.0173	0.01726	0.01709	0.01988	0.01726	0.01813
30 Port de Bouc b	0.01702	0.01701	0.01801	0.01726	0.01721	0.01706	0.0201	0.01721	0.01816
31 Avignone a	0.01798	0.02099	0.01887	0.01819	0.01815	0.01801	0.02079	0.01815	0.01903
32 Avignone b	0.01689	0.01978	0.01776	0.01709	0.01705	0.01689	0.01964	0.01705	0.01791
33 Boulbon a	0.01622	0.01912	0.01711	0.01642	0.01638	0.01623	0.01901	0.01638	0.01726
34 Boulbon b	0.01771	0.01885	0.01858	0.01794	0.01789	0.01772	0.02048	0.01789	0.01875
35 Estagel a	0.01622	0.01912	0.01711	0.01642	0.01638	0.01626	0.01901	0.01638	0.01726
36 Estagel b	0.01686	0.01795	0.01774	0.01708	0.01703	0.01687	0.01962	0.01703	0.0179

(continue)

	10	11	12	13	14	15	16	17	18
10 Vransko b	-								
11 Brijoni a	0	-							
12 Brijoni b	0.0025	0.00254	-						
13 Porto Rose a	0.00084	0.00084	0.00339	-					
14 Proto Rose b	0	0	0.0025	0.00084	-				
15 Viterbo a	0.00084	0.00086	0.00339	0.00086	0.00084	-			
16 Viterbo b	0.00174	0.00177	0.00348	0.00266	0.00174	0.00088	-		
17 Feniglia a	0	0	0.0018	0.00092	0	0.00092	0.00186	-	
18 Feniglia b	0.00169	0.00171	0.00337	0.00257	0.00169	0.00258	0.00347	0.00092	-
19 Rena Majore a	0.01021	0.01031	0.01283	0.01031	0.01018	0.01105	0.01161	0.01028	0.01215
20 Rena Majore b	0.01446	0.01459	0.01709	0.01546	0.01441	0.01551	0.01606	0.01011	0.01559
21 Geremeas a	0.01261	0.01271	0.01519	0.01357	0.01256	0.01361	0.01321	0.01103	0.01369
22 Geremeas b	0.0113	0.01127	0.01308	0.01215	0.01126	0.01233	0.01277	0.01147	0.01329
23 Portoscuso a	0.00422	0.00425	0.00678	0.00425	0.00421	0.00505	0.00621	0.0028	0.00604
24 Portoscuso b	0.00593	0.00597	0.00849	0.00597	0.00591	0.00676	0.00801	0.0047	0.00779
25 Calvi a	0.01281	0.01293	0.01546	0.01293	0.01277	0.01365	0.01435	0.01029	0.01482
26 Calvi b	0.01025	0.01039	0.01198	0.01127	0.01025	0.01128	0.01141	0.01013	0.01196
27 Marsiglia a	0.0172	0.01731	0.01984	0.01731	0.01714	0.01802	0.01898	0.01608	0.01934
28 Marsiglia b	0.01709	0.01712	0.01893	0.01802	0.01709	0.01825	0.01893	0.01594	0.01931
29 Port de Bouc a	0.01712	0.01726	0.01975	0.01726	0.01706	0.01793	0.01888	0.01608	0.01924
30 Port de Bouc b	0.01709	0.01721	0.01806	0.01816	0.01702	0.01823	0.01901	0.01749	0.01844
31 Avignone a	0.01804	0.01815	0.02068	0.01815	0.01798	0.01886	0.01986	0.01804	0.0202
32 Avignone b	0.01692	0.01705	0.01951	0.0179	0.01686	0.01794	0.01865	0.01682	0.01901
33 Boulbon a	0.01626	0.01638	0.01889	0.01638	0.0162	0.01707	0.01798	0.01608	0.01836
34 Boulbon b	0.01774	0.01789	0.01862	0.01875	0.01768	0.01878	0.01951	0.01769	0.01984
35 Estagel a	0.01628	0.01638	0.01891	0.01638	0.01622	0.0171	0.01801	0.01608	0.01839
36 Estagel b	0.0169	0.01703	0.01778	0.01789	0.01684	0.01792	0.01862	0.01677	0.01898

(continue)

	19	20	21	22	23	24	25	26	27
19 Rena Majore a	-								
20 Rena Majore b	0.00427	-							
21 Geremeas a	0.00252	0.00673	-						
22 Geremeas b	0.00087	0.00523	0.00343	-					
23 Portoscuso a	0.01102	0.01461	0.01359	0.0123	-				
24 Portoscuso b	0.01274	0.01637	0.01533	0.01408	0.00169	-			
25 Calvi a	0.00421	0.00856	0.00675	0.00524	0.01361	0.01534	-		
26 Calvi b	0	0.00431	0.0017	0.00087	0.01127	0.01304	0.00432	-	
27 Marsiglia a	0.0259	0.03056	0.02856	0.02681	0.01803	0.01983	0.02494	0.02657	-
28 Marsiglia b	0.0267	0.03129	0.02912	0.02719	0.01827	0.01922	0.02574	0.02686	0
29 Port de Bouc a	0.0258	0.02963	0.02844	0.02666	0.01623	0.01801	0.02667	0.02641	0.00168
30 Port de Bouc b	0.02806	0.02763	0.02958	0.0277	0.01728	0.01928	0.02804	0.02841	0.00093
31 Avignone a	0.02674	0.03146	0.02939	0.02766	0.01888	0.02068	0.0276	0.02742	0.00253
32 Avignone b	0.02584	0.03013	0.02812	0.02634	0.01796	0.01975	0.0267	0.02611	0.00168
33 Boulbon a	0.02495	0.02964	0.0276	0.0258	0.01708	0.01886	0.02582	0.02556	0.00084
34 Boulbon b	0.02667	0.03094	0.02892	0.0263	0.01879	0.02057	0.02755	0.02693	0.00252
35 Estagel a	0.02496	0.02964	0.0276	0.02583	0.01711	0.01889	0.02582	0.02559	0.00084
36 Estagel b	0.02583	0.0301	0.02809	0.02543	0.01794	0.01971	0.02669	0.02608	0.00168

(continue)

	28	29	30	31	32	33	34	35	36
28 Marsiglia b	-								
29 Port de Bouc a	0.00179	-							
30 Port de Bouc b	0	0.00093	-						
31 Avignone a	0.00269	0.00252	0.00283	-					
32 Avignone b	0.00177	0.00168	0.00185	0.00084	-				
33 Boulbon a	0.00089	0.00084	0.00093	0.00168	0.00084	-			
34 Boulbon b	0.00177	0.00252	0	0.00336	0.00248	0.00168	-		
35 Estagel a	0.00089	0.00084	0.00093	0.00168	0.00084	0	0.00168	-	
36 Estagel b	0.00088	0.00168	0	0.00252	0.00165	0.00084	0.00083	0.00084	-

Table S2. Pairwise linkage disequilibrium analysis among the five microsatellite loci.

Locus pair	Chi-square	d.f.	P-value
C22 & C24	16.607	16	0.411
C22 & D17	14.976	16	0.526
C24 & D17	13.797	8	0.087
C22 & D30	18.786	24	0.763
C24 & D30	7.448	12	0.827
D17 & D30	5.436	14	0.979
C22 & D52	16.209	20	0.704
C24 & D52	22.292	14	0.073
D17 & D52	13.236	14	0.508
D30 & D52	14.294	20	0.815

Table S3. Pairwise F_{ST} values below the diagonal and their P-values above the diagonal. * P<0.05, ** P<0.01, *** P<0.001. NS=not significant, NA=computation not possible.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	**Toplou**	-	NS	*	**	**	*	**	**	**	**	NA	**	**	**	*	**	**	*
2	**Kastelli**	0.112	-	*	***	***	***	**	***	*	***	NA	***	**	***	**	***	***	*
3	**Amorgous**	0.362	0.209	-	**	**	**	*	*	*	*	NA	*	NS	*	*	***	**	NS
4	**Areopolis**	0.253	0.271	0.298	-	***	***	**	***	**	***	NA	**	**	***	**	***	***	*
5	**Vransko**	0.268	0.214	0.216	0.316	-	***	***	***	***	***	NA	***	***	***	**	***	***	**
6	**Brijoni**	0.202	0.215	0.322	0.310	0.377	-	***	***	*	***	NA	***	***	***	**	***	***	**
7	**Porto Rose**	0.265	0.198	0.207	0.271	0.196	0.282	-	***	NS	***	NA	**	***	***	**	**	***	*
8	**Viterbo**	0.605	0.473	0.405	0.634	0.319	0.631	0.320	-	***	***	NA	**	***	***	**	***	***	*
9	**Feniglia**	0.146	0.039	0.086	0.140	0.173	0.094	0.035	0.351	-	***	NA	***	**	***	***	***	***	*
10	**Rena Majore**	0.557	0.440	0.306	0.581	0.348	0.570	0.213	0.320	0.271	-	NA	***	***	***	**	***	***	*
11	**Geremeas**	0.439	0.348	0.284	0.472	0.331	0.426	0.182	0.426	0.174	0.292	-	NA	NA	NA	NA	NA	NA	NA
12	**Portscuso**	0.551	0.439	0.214	0.545	0.306	0.569	0.321	0.373	0.295	0.212	0.294	-	**	***	**	***	***	*
13	**Calvi**	0.442	0.253	0.021	0.420	0.286	0.425	0.234	0.359	0.141	0.254	0.301	0.196	-	***	**	**	***	**
14	**Marsiglia**	0.522	0.369	0.427	0.537	0.372	0.573	0.386	0.532	0.328	0.488	0.406	0.488	0.378	-	**	**	***	**
15	**Port de Bouc**	0.605	0.405	0.444	0.616	0.350	0.610	0.383	0.552	0.316	0.461	0.415	0.477	0.341	0.339	-	**	*	*
16	**Avignone**	0.640	0.444	0.500	0.646	0.449	0.660	0.476	0.618	0.380	0.533	0.547	0.558	0.393	0.259	0.282	-	***	*
17	**Boulbon**	0.464	0.355	0.357	0.471	0.227	0.488	0.293	0.422	0.280	0.389	0.257	0.356	0.312	0.338	0.176	0.430	-	*
18	**Estagel**	0.639	0.425	0.442	0.614	0.371	0.654	0.464	0.646	0.341	0.550	0.506	0.546	0.353	0.378	0.142	0.231	0.301	-

CHAPTER 6

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COLONY GENETIC STRUCTURE OF THE INVASIVE FORM *R. URBIS* (ISOPTERA, RHINOTERMITIDAE) AT BAGNACAVALLO (RAVENNA, ITALY)

This part of the thesis was mainly developed during my research term at the Institut de Recherche sur la Biologie de l'Insecte (University of Tours, France) in collaboration with Prof. Anne-Geneviève Bagnères. Preliminary results were presented as a poster communication at the XIII° AISASP congress. The full reference is:

Velonà A., Dupont S., Bagnères A.G., Mantovani B. (2010). Utilizzo di marcatori microsatelliti per l'analisi della struttura coloniale della specie invasiva *Reticulitermes urbis* (Insecta; Isoptera; Rhinotermitidae) a Bagnacavallo (RA). XIII° Congresso Associazione Italiana per lo Studio degli Artropodi Sociali e Presociali (AISASP), Reggio Calabria, Italy, 3-6 May, p. 62 (poster).

ABSTRACT

Reticulitermes urbis is considered an invasive termite species that, starting from the Balkanic peninsula, colonized some cities in Italy and southern France. In this paper the dynamics of the introduced population of Bagnacavallo (RA, Italy) was investigated through the genotyping of nine microsatellite loci. Different statistical analyses (allelic richness, expected/observed heterozygosity, *F*-statistics, AMOVA, PCA) were performed to detect the genetic structure and the breeding system of this population. The results evidenced a low variability, and a distribution of *F*-statistics and relatedness in line with a reproductive system headed by monogamous reproductive pairs. Moreover individuals resulted highly related, suggesting that the entire population is formed by an unique macrocolony. Results are compared with those obtained for other *Reticulitermes* spp. to better understand the biology of this species.

Keywords: breeding system, colony structure, invasive species, relatedness, termites.

INTRODUCTION

Reticulitermes urbis Bagnères, Uva, Clément (2003) is a recently described isopteran species belonging to the Rhinotermitidae family. The taxon is supposed to have originated in the Balkanic peninsula and, except for one population located in a forest close to Sophia Antipolis in southern France (Uva *et al*., 2004), it has never been collected in other natural environments outside the Balkans. On the contrary, some

populations were identified in urban areas of Italy and France (Luchetti *et al*., 2004, 2007; Uva *et al*., 2004). In fact, as the specific name suggests, *R. urbis* is an invasive species, with a high infestation capacity in cities; this feature is probably linked with the high production of alates that allows to colonize large areas very rapidly.

In their natural habitat, termites are useful recyclers of organic compounds (i.e. cellulose), but this feature makes them harmful in human environments since, feeding on wooden components, they cause damages with resulting strong economic consequences.

Two genera of termites are of particular interest for their ability in attacking man-made structures: *Coptotermes* and *Reticulitermes*. From the first genus, the species *Coptotermes formosanus* is considered the most infesting termite and one of the 100 worst invasive species in the world (Global Invasive Species Database, 2010). It's native of China mainland and at present it's known to have colonized South Africa, Sri Lanka, Japan and U.S.A. (Su, 2003).

Given the economic impact that termites produce in human environments, the analysis of their reproductive biology/colony organization is necessary to better understand, and maybe control, their spatial expansion. For this reason, in the Mediterranean area, the colony genetic structure of the two invasive *Reticulitermes* species, *R. santonensis* and *R. urbis* has been deeply investigated (Dronnet *et al*., 2005; Leniaud *et al*., 2009; Perdereau *et al*., 2010).

Three types of colony breeding systems have been so far described in termites. In a simple family, all the individuals are the offspring of a single pair of reproductives, representing the founding couple; in an extended family, the offspring is produced by multiple neotenics, originated from a single pair of primary reproductives. Finally,

colonies are considered as mixed families when the offspring descends by more than two unrelated reproductives.

Dronnet and co-authors (2005) analyzed in a comparative view two *R. santonensis* populations, one deriving from the natural area of Oléron and the other from the urban area of Paris. *R. santonensis* was demonstrated to correspond to the North American taxon *R. flavipes*, some specimens of which having been introduced in Europe, through naval commercial routes, in the harbour of La Rochelle (France) during the 19th century (Dronnet *et al*., 2005). By means of railway transports of wooden materials, they then spread in different cities of north-western France (Lohou *et al*., 1997).

On the basis of nine microsatellite loci, Dronnet and co-authors (2005) evidenced that data on genotype distribution, *F*-statistics and relatedness were in agreement with an extended family condition in both populations. Moreover, as it could be expected, the Oléron population showed a higher level of variability with respect to the Paris one.

As far as *R. urbis* is concerned, the colony genetic structure of an introduced French population was already analyzed by Leniaud *et al*. (2009). The authors used a combined approach of behavioural tests, colony densities and spatial extension and genetic analysis to detect the relationships among 29 sampling points. Their main finding was that the entire population was shaped as an unique macrocolony that, during its expansion, developed different reproductive centres. The population also showed a low variability; this feature was explained as due to an introduction event carried out by a limited number of reproductives.

In this paper, I will present the analysis of the colony genetic structure and of the breeding system of the introduced population of *R. urbis* at Bagnacavallo (RA, Italy) through the genotyping of nine microsatellites loci in 200 individuals. Results are

analyzed and compared with those obtained in previous analyses of invasive *Reticulitermes* termites (Dronnet *et al*., 2005; Leniaud *et al*., 2009) to better understand the biology of *R. urbis*.

MATERIALS AND METHODS

Sampling and DNA extraction

Specimens were sampled in the city of Bagnacavallo (Ravenna, Italy) as reported in figure 1. Twenty workers at each of the ten sampling points were genotyped at nine microsatellites loci. Six samples (1-6) were collected in 2001 and four (1-3, 7) in 2002 and preserved in absolute ethanol until use. DNA extraction was performed with Promega DNA extraction kit following the manufacture protocol.

DNA amplification

The PCR amplification was performed in 10 µL reaction using Qiagen PCR reaction kit. For each PCR reaction, two microsatellite loci were amplified. Details on microsatellite loci and primers are given in table 1.

Each tube of the PCR reaction contained 3 µL of the master mix solution, 3 µL of MilliQ water, 1 µL of Q solution, 1 µL of primer solution and 2 µL (8 ng) of genomic DNA.

The PCR was performed in a Biometra thermocycler using an initial DNA denaturation step of 15 min at 95°C, followed by 40 cycles at the following T°/time: 94°C/30 s, 58°C/1.5 min for primers annealing and 72°C/1 min for extension. Finally, a step of 30

min at 60°C for the final elongation was performed.

Electrophoresis and allele checking

The electrophoresis reactions were performed in 6% polyacrilamide gels. Each gel was made by: 20 mL of polyacrillamide, 15 μL of TEMED solution and 150 μL of PSA 10%.

The electrophoretic run was performed at 1,500V and 45°C in a Li-Cor 4000L DNA automated sequencer and sized using 50-350 bp IRDye800 standard.

After each run, the allele sizes were checked using the software GENEPROFILER v. 4.03 (Scanalytics Inc.).

Statistical analyses

The mean number of alleles, allelic richness, *F*-statistics (Weir & Cockerham, 1984) and relatedness (Queller & Goodnight, 1989) were calculated using FSTAT v. 2.9.3.2 (Goudet, 2001). It must be noted that in social insects, the three component of variation of the *F*-statistics have different meanings with respect to classical ones. "I" is the individual level, "C" is the colony level and "T" is the total population level. In this way, F_{IT} is the coefficient of inbreeding for individuals relative to the total population; F_{CT} estimates the amount of genetic differentiation among colonies; F_{IC} is the coefficient of inbreeding for individuals within colonies. To detect if the *F*-statistics and relatedness were significantly different from zero, the 99% confidence intervals were used. Results were compared with termite breeding structure models proposed by Thorne *et al*. (1999) and Bulmer *et al.* (2001).

Observed and expected heterozygosities were evaluated with GENETIX v. 4.05 (Belkhir,

2003), while probability to fit to the Hardy-Weinberg equilibrium was calculated with GENEPOP v. 1.2 (Raymond & Rousset, 1995). Two analyses of molecular variance (AMOVA) were performed: the 2-levels AMOVA without sub-structuring of samples, and the 3-levels AMOVA calculated grouping specimens by the year of sampling. Both analyses were done using 999 permutations through GENALEX v. 6.1 (Peakall & Smouse, 2006). The same software was used for the Principal Coordinate Analysis (PCA).

RESULTS

All loci resulted polymorphic with a number of alleles per locus ranging from 2 (Rf6-1, Rf21-1, Rs2, RS33) to 5 (RS15). On the whole, the 9 microsatellites loci evidenced 26 alleles, with a mean number of 2.88 alleles at each locus. At the sample level, site 5 (2001) had the lowest value of allelic richness (1.443), while site 3 (2002) showed the highest value (2.084) (table 2). The mean value of allelic richness on all sample is 1.834.

Considering each locus at each sampling site, 64 loci resulted polymorphic, while 26 were monomorphic. The 29% (19) of the polymorphic loci did not match the Hardy-Weinberg equilibrium (HWE); only in one of these 19 loci, the absence of the HWE can be explained by the presence of null alleles, since the great majority (17 out of 19) showed a higher observed heterozygosity with respect to the expected one.

Each locus in all samples, but the 5 (2001) one, is not in HWE: all deviations are caused by a higher frequency of observed heterozygosity (table 2).

The *F*-statistics analysis evidenced a F_{IT} not divergent from zero, a low but significant F_{CT} (0.260), a negative F_{IC} (-0.367), and a high relatedness (0.526). These results are in accordance with the model of a breeding system showing a prevalence of monogamous reproductive pairs (figure 2) (Thorne *et al*.,1999; Bulmer *et al*., 2001).

The 2-levels analysis of molecular variance (AMOVA) highlighted that ~ 1/3 of the variation is among sites and 2/3 within sites (table 3). The 3-levels AMOVA evidenced an absence of variation between years of sampling (0%), and, similarly to the 2-levels results, the 38% of variation is among sites within year, and the 62% of variation is within sites, evidencing an absence of diversification during the sampling time (table 3).

Finally, the PCA (axis 1 + axis 2 = 86.74% of the total variance) embodied in a unique group the great majority of the sampling sites (in green), while two different groups where formed by sample 5 (2001) (in red) and 6 (2001) and 7 (2002) (in blue), respectively (figure 3).

DISCUSSION

The colony genetic structure of the *R. urbis* population in Bagnacavallo (Ravenna, Italy) was here inferred by molecular analyses of the genetic variability at nine microsatellite loci.

First of all, results indicate that all sampling points, and therefore the entire population, show a very low variability. This level of variability, comparable with those obtained in recent works (Dronnet *et al*., 2005; Leniaud *et al*., 2009), suggests a strong founder effect, possibly due to the introduction of a reduced number of alates. Moreover, given

the significantly low level of divergence detected among sampling sites, it is also probable that the infestation took place recently.

Comparing the results of *F*-statistics and relatedness with those obtained from numerical simulations by Thorne *et al*. (1999) and Bulmer *et al*. (2001), it's evident that the breeding system model better describing the condition of the *R. urbis* population in Bagnacavallo is the one in which the entire population is headed by monogamous reproductive pairs, indicating a simpler condition if compared with those obtained for other *Reticulitermes* species (Dronnet *et al*., 2005; Perdereau *et al*., 2010).

For example, Dronnet *et al*., (2005) described - in both a natural and an anthropic habitat - a reproductive breeding system in which *R. santonensis* colonies are maintained by a large number of neotenic reproductives derived from a pair of primary reproductives. Neotenics further underwent inbreeding within colonies for at least three generations. Considering that also *R. santonensis* is an invasive species, and comparing these results with the extended family breeding systems obtained for *R. urbis* (Leniaud *et al*., 2009), it is possible to hypothesize that the population from Bagnacavallo is actually in an early stage of infestation. This is also in line with the low level of diversification evidenced (F_{CT} coefficient result).

The high value of relatedness and the results of the PCA analysis evidenced a high level of homogeneity among samples (but samples 5 (2001), 6 (2001), 7 (2002), see below), suggesting that the entire population is a unique macrocolony. The presence of a unique colony find also support in the analysis by Ferrari *et al*., (1998): the authors, using worker samples from the same population, evidenced the absence of aggressive behaviour, allowing the coexistence of workers. This is not surprising in *R. urbis*, since in this species a very similar condition was described in the city of Domène (France) by

Leniaud *et al*., (2009) both from a genetic and from a behavioural point of view.

It should be noted that, following PCA data, three samples (5 (2001), 6 (2001), 7 (2002)) constitute two groups highly distant from the main cluster given by the other 7 samples. A similar situation is present in the Domène population and also in a *Coptotermes formosanus* population analyzed by Husseneder *et al*., (2005). In both cases, authors suggested that there were spatially separated reproductive centres among which exchange of individuals was limited. In Bagnacavallo, this fact could be viewed as the starting point for the differentiation in three divergent colonies.

On the whole, the *R. urbis* population at Bagnacavallo shows a behaviour comparable to the one retrieved for the population of Domène: the low variability, the absence of aggressive behaviour previously described, and the high relatedness lead to a unique genetic entity defined as a macrocolony. Despite this, the breeding system discovered is quite different, and it is probably linked with a possible more recent introduction.

REFERENCES

Belkhir K., Borsa P., Chikki L., Raufaste N., Bonhomme F. (2003) GENETIX, lociciel sous windows TM pour la genetique des populations.
Available online at: http://www.genetix.univmontp2.fr/genetix/genetix.htm.

Bulmer M.S., Adams E.S., Traniello J.F.A. (2001) Variation in colony structure in the subterranean termite *Reticulitermes flavipes*. *Behavioral Ecology and Sociobiology* 49: 236–243.

Dronnet S., Bagnères A.G., Juba T.R., Vargo E.L. (2004) Polymorphic microsatellite loci in the European subterranean termite, *Reticulitermes santonensis* Feytaud. *Molecular Ecology Notes* 4: 127-129.

Dronnet S., Chapuisat M., Vargo E.L., Lohou C., Bagnères A.G. (2005) Genetic analysis of the breeding system of an invasive subterranean termite, *Reticulitermes santonensis*, in urban and natural habitats. *Molecular Ecology* 14: 1311–1320.

Ferrari R., Marini M., Tiglié I., Zaffagnini V. (1998) Indagine sulle popolazioni di termiti *Reticulitermes lucifugus* Rossi (Isoptera: Rhinotermitidae) con metodiche di tripla marcatura e ricattura. *Disinfestazione & igiene ambientale* 15(1), 14–20.

Global Invasive Species database (2010) *Coptotermes formosanus* (insect). Available from: http://www.issg.org/database/species/ecology.asp?si=61&fr=1&sts=&lang=EN.

Goudet J. (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3.2).
Available online at: http://www2.unil.ch/popgen/softwares/fstat.htm.

Hayashi Y., Kitade O., Kojima J.I. (2002) Microsatellite loci in the Japanese subterranean termite, *Reticulitermes speratus*. *Molecular Ecology Notes* 2: 518-520.

Husseneder C., Messenger M.T., Su N.Y., Grace J.K., Vargo, E.L. (2005) Colony social

organization and population genetic structure of an introduced population of Formosan subterranean termite from New Orleans, Louisiana. *Journal of Economic Entomology* 98: 1421–1434.

Leniaud L., Pichon A., Uva P., Bagnères A.G. (2009) Unicoloniality in *Reticulitermes urbis*: a novel feature in a potentially invasive termite species. *Bulletin of Entomological Research* 99: 1-10.

Lohou C., Burban G., Clément J.L., Jecquel M., Leca J.L. (1997) Protection des arbres d'alignement contre les termites souterrains; l'expérience menée à Paris. *Phytoma* 492: 42–44.

Luchetti A., Trenta M., Mantovani B., Marini M. (2004)Taxonomy and phylogeny of north mediterranean *Reticulitermes* (Isoptera, Rhinotermitidae): a new insight. *Insectes Sociaux* 51: 117–122.

Luchetti A., Marini M., Mantovani, B. (2007) Filling the gap: biosystematics of the eusocial system *Reticulitermes* (Isoptera, Rhinotermitidae) in the Balkanic Peninsula and Aegean area. *Molecular Phylogenetics and Evolution* 45: 377–383.

Peakall R., Smouse P.E. (2006) Genalex 6: genetic analyses in Excel. *Molecular Ecology Notes* 6: 288-295.

Perdereau E., Bagnères A.G., Dupont S., Dedeine F. (2010) High occurrence of colony

fusion in a European population of the American termite *Reticulitermes flavipes*. *Insectes Sociaux* 57: 393–402.

Queller D.C., Goodnight K.F. (1989) Estimating relatedness using genetic markers. *Evolution* 43: 258–275.

Raymond M., Rousset F. (1995) GENEPOP (version 1.2): a population genetics software for exact test and ecumenicism. *Journal of Heredity* 86: 248–249.

Su N.Y. (2003) Overview of the global distribution and control of the Formosan subterranean termite. *Sociobiology* 41: 7-16.

Thorne B.L., Traniello J.F.A., Adams E.S., Bulmer M. (1999) Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera Rhinotermitidae), a review of the evidence from behavioural, ecological, and genetic studies. *Ethology Ecology and Evolution* 11: 149–169.

Uva P., Clément J.L., Austin J.W., Aubert J., Zaffagnini V., Quintana A., Bagnères A.G. (2004) Origin of a new *Reticulitermes* termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data. *Molecular Phylogenetics and Evolution* 30: 344–353.

Vargo E.L. (2000) Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*. *Molecular Ecology* 9: 817-829.

Weir B.S., Cockerham C.C. (1984) Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358-1370.

FIGURE LEGENDS

Figure 1. Collecting sites of the *R. urbis* samples in the city of Bagnacavallo. Sites were sampled in 2001 (green) and/or in 2002 (red).

Figure 2. *F*-statistics and relatedness analyses results (in blue) and corresponding values of the simulation analysis (in red) performed by Thorne *et al*. (1999) and Bulmer *et al*., (2001). Stars indicate the significant divergence from zero of the experimental results, using 99% confidence intervals.

Figure 3. Principal Coordinate Analysis results. For each axis the corresponding percentage of variance is reported. Colours evidence the main group (green) and the two divergent ones (blue and red).

Table 1. Primer sequences, repeat unit, number of alleles (Na), primer concentration, GenBank accession numbers and reference for each microsatellite locus used in this study.

Locus	Primer sequence 5'-3'	Repeat unit	Na	Primer [ ]	GenBank A.N.	Reference
Rf 6-1	F: AGACTTGGAGTGCACTGTTGTT R: GCCATCAGTCATCTCAGCAA	$(GTT)_5(GAT)_8$	2	1 µM	AF195933	Vargo, 2000
Rf21-1	F: CACACACGTTCGTTGTTTTG R: CAAGAGGCGTGGGGTACTAA	$(CTA)_{21}$	2	0.5 µM	AF195937	Vargo, 2000
Rs2	F:CCGCCGTACCTTCTCAGAT R:AAATGCGACCAACTTTGACA	$(CG)_5\ldots(CA)_{13}$	2	0.5 µM	AB086247	Hayashi *et al*., 2002
RS10	F: TCCGGCTGACAAATGACATA R: TATTACTGCTGTTGGCGCTG	$(TAG)_3TAA(TAG)_9$	4	0.5 µM	AY423583	Dronnet *et al*., 2004
RS15	F: GGTCGTTGTGGAGGTAGCTG R: ACAAAGGAGCGCCTTACAAA	$(TAG)_6$	5	0.5 µM	AY423585	Dronnet *et al*., 2004
RS16	F: CCATGACCCGAATACGGAC R: TTCCACACGAGATGAAGCTG	$(GA)_2GC(GA)_8$	3	0.5 µM	AY423586	Dronnet *et al*., 2004
RS33	F: GCTTGTAGGCATCGCAAGTT R: GGAAGTATTTGCCACGAGGA	$(TTCA)_5$	2	0.5 µM	AY423587	Dronnet *et al*., 2004
RS43	F: CGGACAGACAGGAAGGTAGG R: ACCTCACAAAAGCACCTTGC	$(CAGA)_6$	3	0.5 µM	AY423588	Dronnet *et al*., 2004
RS62	F: GTAGCGCATTGTCTCAACCA R: GAATCCCCAGCCAATATTCA	$(GTTT)_5$	3	0.7 µM	AY423590	Dronnet *et al*., 2004

Table 2. Number of alleles (A), allelic richness (A_c), observed (H_o) and expected (H_e) heterozygosity for each locus and on all loci in each sampling sites. § possible presence of null alleles. * P<0.05, ** P<0.01, ***P<0.001.

		1 (2001)	2 (2001)	3 (2001)	4 (2001)	5 (2001)	6 (2001)	1 (2002)	2 (2002)	3 (2002)	7 (2002)
Rf 6-1	**A/A_c**	1/1.000	2/1.550	2/2.000	2/2.000	2/2.000	2/2.000	2/2.000	2/2.000	2/2.000	2/2.000
	H_o/H_e	0/0	0.05/0.048	1/0.500***	0.571/0.408	0.357/0.497	1/0.500***	0.500/0.500	0.764/0.493	0.600/0.480	0.450/0.498
Rf21-1	**A/A_c**	1/1.000	1/1.000	1/1.000	1/1.000	1/1.000	1/1.000	1/1.000	1/1.000	1/1.000	1/1.000
	H_o/H_e	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Rs2	**A/A_c**	2/2.000	2/2.000	2/2.000	2/2.000	1/1.000	2/2.000	2/2.000	2/2.000	2/2.000	2/2.000
	H_o/H_e	0.812/0.482*	0.647/0.437	0.800/0.500*	0.900/0.495***	0/0	0.777/0.475*	0.944/0.498***	0.555/0.493	0.736/0.465*	0.615/0.426
RS10	**A/A_c**	2/1.804	1/1.000	1/1.000	1/1.000	1/1.000	2/1.967	1/1.000	2/1.551	2/1.550	2/1.917
	H_o/H_e	0.100/0.095	0/0	0/0	0/0	0/0	0.200/0.180	0/0	0.050/0.048	0.050/0.048	0.150/0.138
RS15	**A/A_c**	2/1.995	2/2.000	2/2.000	2/1.856	2/2.000	2/1.999	2/2.000	3/2.550	3/2.545	2/1.987
	H_o/H_e	0.277/0.239	0.466/0.357	0.529/0.389	0.111/0.104	0.357/0.293	0.400/0.320	0.384/0.310	0.350/0.411	0.350/0.296	0.250/0.218
RS16	**A/A_c**	3/2.850	3/3.000	3/2.934	3/2.995	3/2.994	3/2.545	3/2.856	3/2.802	3/2.721	3/2.917
	H_o/H_e	0.388/0.328	0.437/0.585*	0.736/0.511	0.650/0.605	0.214/0.482**§	0.350/0.296	0.611/0.452	0.450/0.366	0.250/0.226	1/0.563***
RS33	**A/A_c**	1/1.000	1/1.000	1/1.000	1/1.000	1/1.000	2/1.989	1/1.000	1/1.000	2/1.987	2/2.000
	H_o/H_e	0/0	0/0	0/0	0/0	0/0	0.235/0.207	0/0	0/0	0.250/0.218	0.550/0.398
RS43	**A/A_c**	2/2.000	3/2.687	3/2.829	2/2.000	1/1.000	2/1.975	2/2.000	2/1.996	2/1.963	2/1.999
	H_o/H_e	0.411/0.327	0.500/0.388	0.736/0.498	0.736/0.465*	0/0	0.210/0.188	0.411/0.389	0.166/0.152	0.176/0.160	0.400/0.320
RS62	**A/A_c**	2/2.000	3/2.829	2/2.000	2/2.000	1/1.000	2/2.000	2/2.000	2/2.000	3/2.998	2/2.000
	H_o/H_e	0.947/0.498***	0.736/0.498	0.850/0.488**	1/0.500***	0/0	0.777/0.475*	0.888/0.493**	0.611/0.424	0.850/0.576*	0.842/0.487**
ALL LOCI	**A/A_c**	1.777/1.738	2/1.896	1.888/1.862	1.777/1.761	1.444/1.443	2/1.941	1.777/1.761	2/1.877	2.222/2.084	2/1.980
	H_o/H_e	0.326/0.219***	0.315/0.257*	0.517/0.320***	0.441/0.286***	0.103/0.141	0.439/0.293***	0.415/0.293***	0.327/0.265*	0.362/0.274***	0.473/0.339***

Table 3. Two and three levels AMOVA analysis. Degrees of freedom (df), sum of squares (SS), mean of squares (MS), and percentage of variation (%) are shown for each source of variation.

Source of variation	df	SS	MS	%
Among sites	9	357.26	39.70	36%
Within sites	190	613.95	3.23	64%
Between years	1	18.16	18.16	0%
Among sites, within years	8	339.10	42.39	38%
Within sites	190	613.95	3.23	62%

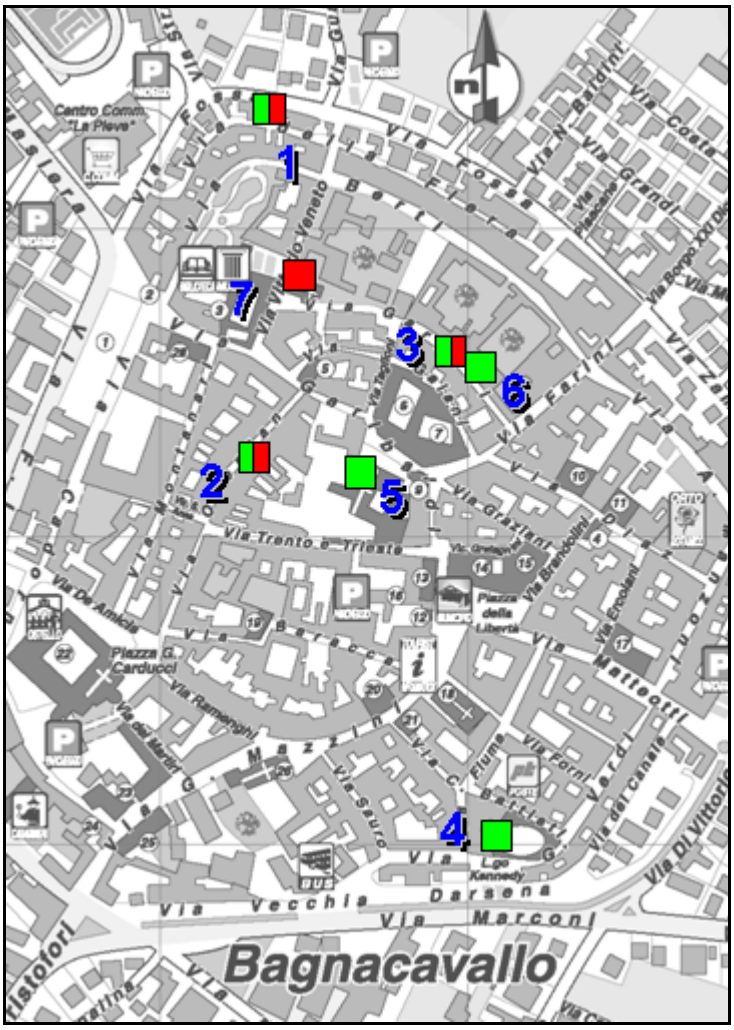

Figure 1.

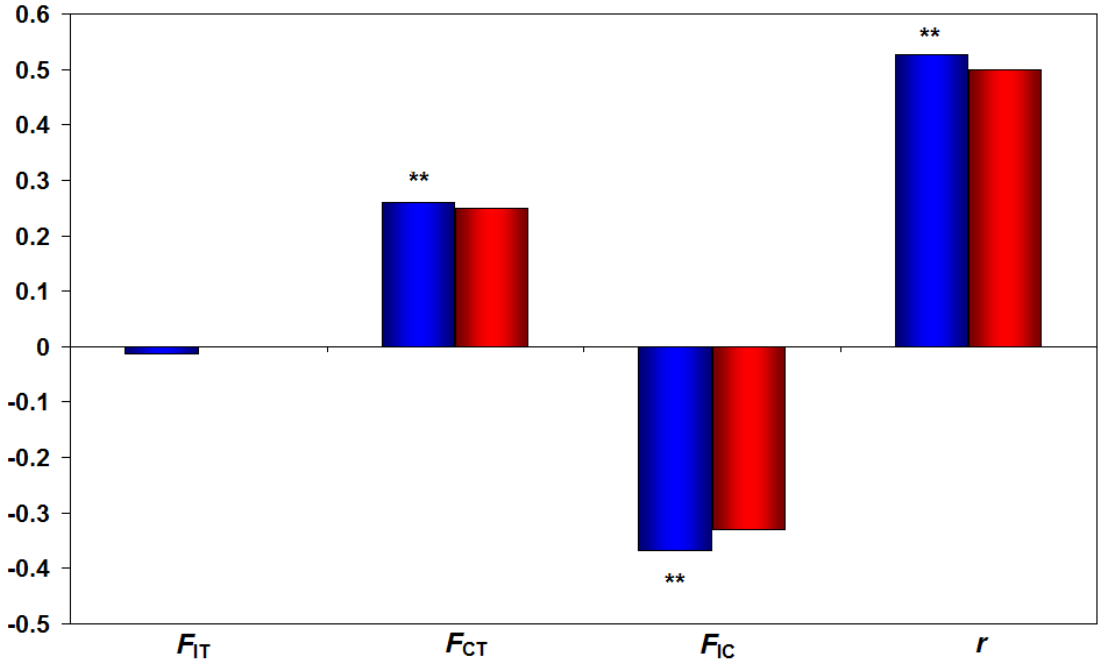

Figure 2.

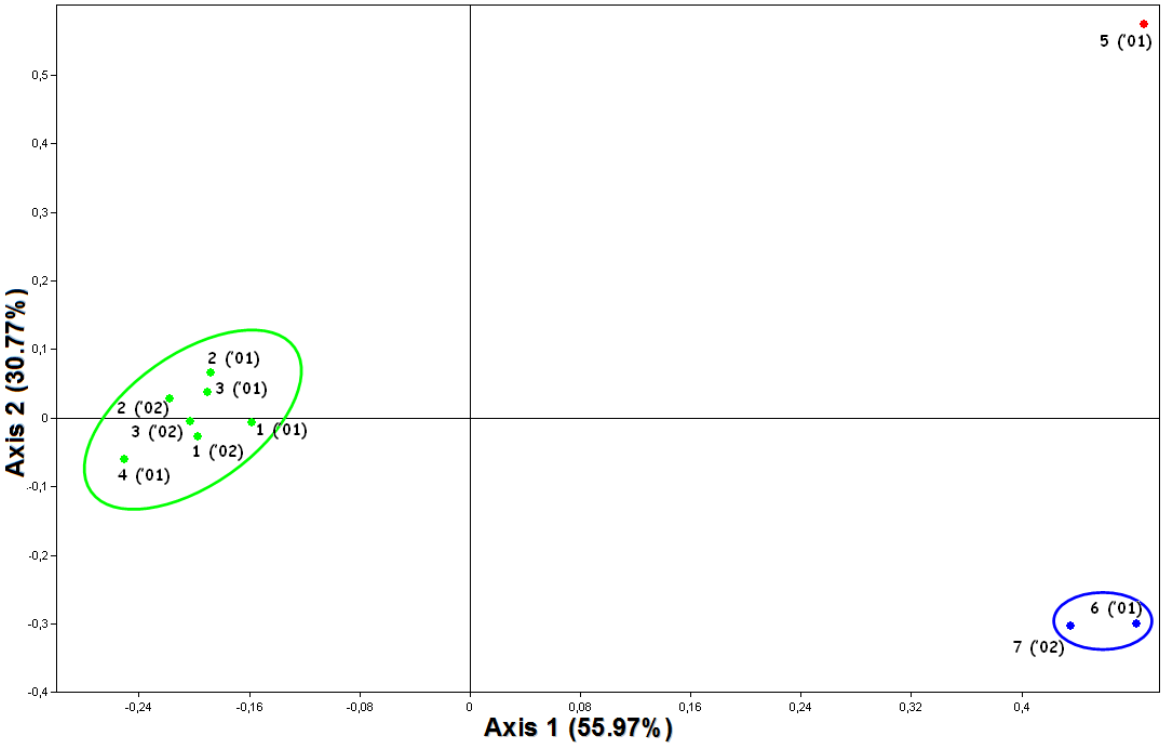

Figure 3.

CHAPTER 7

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COLONY GENETIC STRUCTURE OF THE *KALOTERMES FLAVICOLLIS* POPULATION FROM THE NATURAL RESERVE "DUNA DI FENIGLIA" (GROSSETO, ITALY)

This chapter of the thesis was preliminarily presented as a poster communication at the XVI° IUSSI congress. The full reference is:

Velonà A., Luchetti A., Mantovani B., (2010). Colony structure of *Kalotermes flavicolli*s (Isoptera, Kalotermitidae): preliminary results from two molecular markers. XVI° World Congress of the International Union for the Study of Social Insects (IUSSI), Copenhagen, Denmark, 8th-14th August, p.143 (poster).

ABSTRACT

Kalotermes flavicollis (Isoptera, Kalotermitidae) is a social termite that lives in the Mediterranean regions of Europe, from the Iberic peninsula to Turkey. The social life style is allowed by the formation of colonies with castes devoted to perform specific tasks. Like all the other members of the Kalotermitidae family (drywood termites), this species is classified as a single-piece nester because individuals nest and feed in the same specific substrate, predominantly the dead wood, with an apparently lack of interactions among individuals of different colonies. In this paper, the colony genetic structure of the population Duna di Feniglia is inferred through mitochondrial (control region) and nuclear (Inter-SINE and microsatellites) molecular markers. Different statistical analyses (haplotype parsimony network, genetic distance, F_{ST}, population structure) were performed to detect the genetic structure and dynamics of the nine colonies sampled. Results clearly indicate the coexistence of two lineages, named A and B: the former belong to *K. flavicollis sensu stricto*, while the latter at the moment doesn't have a clear taxonomic position. A high haplotype richness is found in each colony, as a results of adoption and colony fusion processes. Finally, results here obtained are compared with the ones observed for termites of the genus *Reticulitermes* to better understand the different dynamics at work.

Keywords: colony structure, lineages, parsimony network, single-piece nester, termites.

INTRODUCTION

Kalotermes flavicollis is a termite species belonging to Kalotermitidae (drywood termites), a basal family in the evolution of the order Isoptera (Inward *et al*., 2007; Legendre *et al*., 2008). The species was known to occur along the Mediterranean coasts of Europe, from the Iberic peninsula to Turkey as a unique entity, but it has been recently demonstrated to embody at least four genetic lineages (Velonà *et al*., 2011).

As all isopterans, *K. flavicollis* has a social life style. Colonies are established in dry wood of dead plants pertaining to the genera *Populus*, *Pinus*, *Quercus*, *Platanus*, *Tilia*, *Castaneus*, but a lot of attacks in living plants of the genera *Vitis*, *Olea* and *Ficus* are also well documented (Sbrenna & Micciarelli-Sbrenna, 2008 and references therein; Maistrello *et al*., 2010).

K. flavicollis builds relatively small colonies in comparison with other termites, its societies comprising only some hundreds of individuals. Three castes are known in *K. flavicollis*: reproductives, soldiers and pseudoergates. While reproductives and soldiers have well established roles (the former being able to found new colonies and to maintain established ones through mating, the latter defending colonies from intruders), pseudoergates are totipotent individuals. They perform logistic roles such as food recruitment, feeding of other castes, galleries building. They also help soldiers in the defence of the colony, and are even able to differentiate into reproductives, increasing the reproductive output.

On the basis of their ecology, termites can be classified following Abe (1987) in three categories: single-, intermediate- and separate-piece nester. In the single-piece (or one-piece) nester species, individuals feed and nest in the same specific substrate; all the

wood feeding termites belong to this group. Intermediate-piece nesters build the nest in their feeding substrate, but can also look for food outside the nest. The great majority of the Rhinotermitidae species, including the genus *Reticulitermes*, belong to this group. Finally, in the separate-piece nesters, nesting and feeding areas do not overlap; so termites are forced to forage away from the colony. Following this classification, *K. flavicollis* is a single-piece nester. A major consequence of the single piece nester habitus is that, except for the nuptial flight, individuals reside within the nest, so that colonies of the same population are isolated among them, apparently lacking interactions.

In this paper, the colony genetic structure of the population from the natural reserve Duna di Feniglia (Tuscany, Italy) is analyzed through the sequencing of a portion of the mitochondrial control region and the genotyping of nuclear Inter-SINE and microsatellite markers. Results are discussed in the light of the current knowledge concerning the ecology and the evolutionary histories of this species, and finally compared with those obtained for *Reticulitermes urbis* to better understand the different population dynamics. Owing to the peculiar pattern of mitochondrial divergence scored in the Duna di Feniglia samples, a wider analysis of the mitochondrial marker was also performed taking into account more specimens from all European samples available.

MATERIALS AND METHODS

Sampling and DNA extraction

Specimens were sampled in the natural reserve Duna di Feniglia (Grosseto, Italy) in

September 2009 in an area of ~ 500 m^2. Nine samples (KF1-9) were collected for a total number of 160 termites and preserved in absolute ethanol until laboratory analyses. Further 135 individuals from the 18 European populations available (Velonà *et al*., 2011) were utilized in the present study, but only for mitochondrial analyses (see below; table 1).

Total DNA was extracted using the CTAB protocol (Doyle & Doyle, 1987) from the head of each termite to avoid contamination from protozoan gut fauna.

Mitochondrial marker

A portion of 303 bp of the control region (CR; Velonà *et al*., 2011) was amplified using Polymerase Chain Reaction (PCR). Amplifications were performed using the primers AT-KR (5'-GTG GCT ATA CCC ACT ATA AA-3′) and TM-N-193 (5-TGG GGT ATG AAC CAG TAG C-3'), in a reaction mixture of 50 μL using *Taq* DNA polymerase PCR kit (Promega) following the kit protocol. The amplification conditions were: 30 cycles of DNA denaturation at 94°C for 30″, annealing at 48°C for 30″ and extension at 72°C for 30″, plus an initial denaturation at 94°C for 5′ and a final extension at 72°C for 7′. The amplified products were purified with the Wizard SV Gel and PCR Clean-up System kit (Promega) and both strands were sequenced at Macrogen Inc. (Korea).

Inter-SINE markers

This method is based on the PCR amplification of sequences comprised between two copies of a SINE retrotransposon. The primer *Ta*F (5′-AGT GGC CGT GCG GTC TAA G-3′), designed on the SINE *Talua* (Luchetti, 2005), was used following these

amplification conditions: 94°C for 5′ as initial denaturation, 35 cycles at 94°C for 30″, 42°C for 30″ and 72°C for 30″, plus a final extension at 72°C for 7'. The reactions were performed in 10 μL of solution comprising: 8ng of genomic DNA, 10μM of primer, 1.5mM $MgCl_2$, 200μM of dNTPs, 10mM of buffer 10× and 1U of *Taq* polymerase (Invitrogen). PCR products were resolved in 2% agarose gels in a TAE buffer 1× and bands were used to create a presence (1)/absence (0) matrix. This marker was applied to individuals of all the colonies sampled from the Duna di Feniglia reserve.

Microsatellite markers

The same microsatellite loci isolated by Velonà *et al*. (2011) were used. Following the mitochondrial results (see below), microsatellite loci were genotyped in individuals of four representative Duna di Feniglia colonies (table 1). The amplification conditions were 94°C for 5' for the initial denaturation, 35 cycles at 94°C for 30″, 30″ of annealing (see table 2 in Velonà *et al*. (2011) for the temperatures used for each locus) and 72°C for 30″, plus a final extension of 7′ at 72°C. The 10μl PCR reactions included 8ng of genomic DNA, 10μM of each primer, 1.5mM $MgCl_2$, 200μM dNTPs, 10mM of buffer 10× (Invitrogen kit), 1μl BSA 0.2% and 1U of *Taq* polymerase (Invitrogen). Genotyping was performed in a Beckman CEQ8000, using 5'-labelled forward primers (Sigma).

Statistical analyses

For the mitochondrial dataset of the Duna di Feniglia population, the sequences were aligned with CLUSTAL-W algorithm implemented in MEGA4 (Tamura *et al*., 2007); this software was also used to calculate the number of variable sites and nucleotide

composition.

The software MODELTEST v. 3.06 (Posada & Crandall, 1998) was used to determine the best substitution model (F81+Γ, Γ=0.1535).

The parsimony haplotype network was built utilizing TCS v. 1.21 (Clement *et al*., 2000) with gaps considered as 5th state character. A pairwise sequence divergence matrix, on F81+Γ substitution model, was calculated through PAUP* v. 4.0b (Swofford, 2001), and results were showed as histogram.

On the basis of the mitochondrial results on the Duna di Feniglia samples, and with the aim to obtain a clearer scenario of the taxonomic position of the population here analyzed, a Neighbor-Joining tree was calculated with the software MEGA4 (Tamura *et al*., 2007) using 500 bootstrap replicates. For this analysis, all the sequences obtained in this work and those presented in Velonà *et al*., (2011) (GenBank A.N: GU931793-GU931796, FJ750(483, 486, 488, 490, 491, 493, 494, 495, 497, 500)) were used, for a total dataset of 267 samples.

For microsatellites and I-SINE markers, two pairwise F_{ST} analyses were performed with GENALEX v. 6.1 (Peakall & Smouse, 2006), grouping the colonies on the basis of mitochondrial results. The statistical significance of the results was assessed using 999 permutations.

For both the microsatellites and the I-SINE datasets the software STRUCTURE v. 2.3.3 (Prichard *et al*., 2000) was used to assign each individual to a specific cluster (*K*), set by the user, on the basis of the percentage of membership (*q*); giving the results obtained with the mitochondrial marker, the analysis was performed with *K*=2. For both datasets the run was set with 10,000 burn-in periods and 10,000 MCMC repetitions after burn-in, using the implemented admixture model.

RESULTS

Mitochondrial marker

The control region amplified fragment on the Duna di Feniglia samples showed 33 variable sites out of 303 nucleotidic positions, leading to 15 haplotypes. The nucleotide composition was: 26.1% (T), 15.6% (C), 47.6% (A), 10.7% (G). The number of haplotypes ranges from 1 (KF7/KF8) to 9 (KF4) with a mean value of 4.22 in each colony (figure 1).

The haplotype parsimony network clearly shows two lineages (named A and B), with a similar number of haplotypes (7 and 8, respectively). Both lineages have a typical star-like network with one highly represented haplotype each (figure 1). From a colony point of view, all the Duna di Feniglia colonies have a mix of haplotypes of both lineages, except colonies KF7/KF8 showing only the most common haplotype of lineage A, and colony KF9 comprising only three haplotypes of the B lineage.

The divergence values distribution showed a clear separation between intra-lineage and inter-lineages comparisons, with a level of divergence even ten-fold higher (figure 2).

In order to clarify the affinity of the two genetic lineages retrieved in the population of Duna di Feniglia, we widened the analyses taking into account other European populations (table 1; Velonà *et al.*, 2011), to verify the possible existence of further areas of sintopy. The phylogenetic analyses performed on the whole data set (figure 3), clearly demonstrate that all lineage A individuals form a unique cluster together with all European populations of *K. flavicollis*, while the lineage B individuals constitute a separated and well supported cluster.

Inter-SINE markers

All the Duna di Feniglia samples were analyzed for inter-SINE loci (table 1). The fingerprinting pattern obtained using the SINE *Talua* allowed to characterize 15 loci on the entire dataset.

The F_{ST} pairwise analysis showed the highest value when comparing colonies KF7/KF8 (mtDNA lineage A) to KF9 (mtDNA lineage B) (F_{ST}=0.616). The lowest value (F_{ST}=0.173) is obtained in the comparison between colonies KF7/KF8 and KF1-6 (mixed) (figure 4a). The level of divergence between KF9 and KF1-6 (F_{ST}=0.394) is in an intermediate position.

In the population structure analysis, the coefficient of membership (q) clearly indicates individuals of colonies KF7/KF8 as belonging to mitochondrial lineage A, while individuals of colony KF9 to mitochondrial lineage B. Mitochondrial mixed colonies (KF1-6) showed individuals with mixed membership values (figure 5a).

Microsatellite markers

On the basis of mitochondrial results, microsatellite analyses were performed on colonies KF7/KF8 (lineage A), KF9 (lineage B) and KF6 (mixed). All loci resulted polymorphic, with the number of alleles ranging from two (C24 and D17) to six (D52). On the whole, 18 alleles were scored, with a mean number per locus of 3.6 alleles.

The F_{ST} pairwise analysis showed very similar values when comparing colony KF9 with KF7/KF8 and with KF6 (0.385 and 0.363, respectively), while a low divergence in the comparison between KF7/KF8 and KF6 (figure 4b).

In the analysis of the population structure, all the individuals of colony KF9 showed a high percentage of membership with the lineage B, while in both KF7/KF8 and KF6 the

individuals showed membership with lineages A and B (figure 5b).

DISCUSSION

Unlike subterranean termites, the species *Kalotermes flavicollis*, and in general the drywood termites, have received no attention regarding their colony genetic structure, (Vargo, 2003; DeHeer & Vargo 2004; Dronnet *et al*., 2005; Leniaud *et al*., 2009; Perdereau *et al*., 2010). This probably reflects the greater capacity of rhinotermitids with respect to kalotermitids to colonize and damage human manufactures.

In this paper, we analyzed the colony genetic structure of a population of *K. flavicollis* from the natural reserve Duna di Feniglia.

The analyses performed clearly evidenced the presence in the same population of two highly divergent mitochondrial lineages. On the basis of the comparison performed on available data, while the lineage A appear to belong to *K. flavicollis sensu stricto*, the lineage B doesn't have a clear taxonomic position. Moreover, the level of divergence detected is well above the expected one for a population level of differentiation, highlighting the necessity of deeper taxonomic and biogeographical analyses. It must also be considered that no haplotypes of the lineage B were found in the area comprised between southern France and Crete (Velonà *et al*., 2011; present analyses), suggesting the Iberic peninsula as a possible reservoir.

Apart from their phylogenetic and taxonomic relationships, both mitochondrial lineages were either the unique ones (pure colonies, KF7-KF8-KF9) or were found together in the same colony (mixed colonies; KF1-6), suggesting that cross-breeding is allowed.

This possibility is well supported by nuclear markers that, in all analyses performed, clearly confirm it.

Yet, the nuclear markers behave in a different way: while I-SINE were in accordance with mitochondrial DNA results, indicating a clear separation of lineage A (KF7/KF8), lineage B (KF9) and mixed (KF1-6) colonies, microsatellites seem to recognize only colony KF9 as pertaining to a pure lineage (B), while the other colonies are retrieved as mixed ones.

Considering both the F_{ST} and the population structure analyses, it's clear that colony KF9 (lineage B) is the most divergent with respect to the others; this, in combination to the lower quantity of the lineage B component in mixed colonies, suggest that this lineage is more recent and it's introducing its alleles in the established population.

At the colony level, a high number of haplotypes were detected. The mean value of 4.22 haplotypes per colony is the results of two possible mechanisms: colony fusion and adoption (Vargo & Husseneder, 2011). The first one happens, for example, when two colonies, living in the same piece of wood, enter in communication and mix. As a result of this process, the new colony encompasses the genotypes of both original colonies. This mechanism was well described in another Kalotermitidae species (*Cryptotermes secundus*, Korb & Schneider, 2007).

The adoption is the process in which an established colony accepts alates native of neighbour colonies, allowing them to mate within it. This obviously leads to an increased genetic variability. These two mechanisms are not mutually exclusive and maybe both of them could have generated the pattern of variability here described.

Apart from which of these two processes has been the most determinant in generating this level of variability, it is clear that the colonies of this single-piece nester species

complex are not separate entities as previously supposed, but they communicate, producing a complex network of interactions.

The comparison between *K. flavicollis* and *R. urbis* population dynamics indicates that the two species behave in different ways and show different strategies. In fact, while *R. urbis* populations are established by single large colonies with different reproductive centres (Leinaud *et al*., 2009; chapter 6 of this thesis), *K. flavicollis* produces a high number of small colonies that are able to mix and/or to adopt alates from other colonies. It's possible to suppose that while the *R. urbis* strategy is highly advantageous to colonize new areas in a short time, the *K. flavicollis* one can be genetically more prone in maintaining an established population ensuring it's high genetic flow.

REFERENCES

Abe T. (1987) Evolution of life types in termites. In: *Evolution and Coadaptation in Biotic Communities.* (Eds) Kawano S., Connell J.H., Hidaka T., pp.126–148. University of Tokyo Press, Tokyo.

Clement M., Posada D., Crandall K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1660.

DeHeer C.J., Vargo E.L. (2004) Colony genetic organization and colony fusion in the termite *Reticulitermes flavipes* as revealed by foraging patterns over time and space *Molecular Ecology* 13: 431–441.

Doyle J.J., Doyle J.L. (1987) A rapid DNA isolation procedure for small amounts of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Dronnet S., Chapuisat M., Vargo E.L., Lohou C., Bagnères A.G. (2005) Genetic analysis of the breeding system of an invasive subterranean termite, *Reticulitermes santonensis*, in urban and natural habitats. *Molecular Ecology* 14: 1311–1320.

Inward D., Vogler A., Eggleton P. (2007) A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. *Molecular Phylogenetics and Evolution* 44: 953–967.

Korb J., Schneider K. (2007) Does kin structure explain the occurrence of workers in a lower termite? *Evolutionary Ecology* 21: 817-828.

Legendre F., Whiting M.F., Bordereau C., Cancello E.M., Evans T.A., Grandcolas P. (2008) The phylogeny of termites (Dictyoptera: Isoptera) based on mitochondrial and nuclear markers: Implications for the evolution of the worker and pseudergate castes, and foraging behaviours. *Molecular Phylogenetics and Evolution* 48: 615–627.

Leniaud L., Pichon A., Uva P., Bagnères A.G. (2009) Unicoloniality in *Reticulitermes urbis*: a novel feature in a potentially invasive termite species *Bulletin of Entomological Research* 99: 1-10.

Luchetti A. (2005) Identification of a short interspersed repeat in the *Reticulitermes lucifugus* (Isoptera Rhinotermitidae) genome. *DNA Sequence* 16: 304–307.

Maistrello L., Ocete R., Ángeles López M. (2010) Seasonal Trends in the Social composition and inside-trunk distribution of *Kalotermes flavicollis* (Isoptera: Kalotermitidae) colonizing grapevines. *Environmental Entomology* 39: 295-302.

Peakall R., Smouse P.E. (2006) Genalex 6: genetic analyses in Excel. *Molecular Ecology Notes* 6: 288-295.

Perdereau E., Bagnères A.G., Dupont S., Dedeine F. (2010) High occurrence of colony fusion in a European population of the American termite *Reticulitermes flavipes*. *Insectes Sociaux* 57: 393–402.

Posada D., Crandall K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

Pritchard J.K., Stephens M., Donnelly P. (2000) Inference of Population Structure Using Multilocus Genotype Data. *Genetics* 155: 945–959.

Sbrenna G., Micciarelli-Sbrenna A. (2008) Le termiti italiane. Catalogo topografico e considerazioni zoogeografiche (Isoptera). *Memorie della Società Entomologica Italiana* 87: 33-60.

Swofford D.L. (2001) PAUP*-Phylogenetic Analysis Using Parsimony (* and Other Methods), Ver 4b. Sunderland, MA, USA, Sinauer Associates.

Tamura K., Dudley J., Nei M., Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596–1599.

Vargo E.L. (2003) Genetic structure of *Reticulitermes flavipes* and *R. virginicus* (Isoptera: Rhinotermitidae) colonies in an urban habitat and tracking of colonies following treatment with hexaflumuron bait. *Environmental Entomology* 32: 1271-1282.

Vargo E.L., Husseneder C. (2011) Genetic structure of termites colonies and population. In: *Biology of termites: a modern synthesis*. (Eds) Bignell D.E., Roisin Y., Lo N., pp 321-347. Springer, New York.

Velonà A., Luchetti A., Ghesini A., Marini M., Mantovani B. (2011) Mitochondrial and nuclear markers highlight the biodiversity of *Kalotermes flavicollis* (Fabricius, 1793) (Insecta, Isoptera, Kalotermitidae) in the Mediterranean area. *Bulletin of Entomological Research*. doi:10.1017/S000748531000060X.

FIGURE LEGENDS

Figure 1. Haplotype parsimony network. Circles are proportional to the scored haplotype frequencies, while pieces represent the frequency of individuals harboring the specific haplotype for each colony. Black dots represent missing/ideal haplotypes.

Figure 2. Intra- and inter-lineages (A and B) distribution of divergence values calculated using the F81+Γ evolutionary model.

Figure 3. Neighbor-Joining tree calculated on all European populations of *K. flavicollis*. In green: Duna di Feniglia, lineage A sequences; in red: Duna di Feniglia, lineage B sequences; in blue: other European *K. flavicollis* populations sequences. Numbers indicate bootstrap values obtained after 500 replicates.

Figure 4. Pairwise F_{ST} analyses performed on I-SINE (a) and microsatellite (b) markers grouping the colonies on the basis of the mitochondrial results. **P<0.01, ***P<0.001.

Figure 5. Population genetic structure analysis performed on I-SINE (a) and microsatellite (b) markers. Vertical bars represent single individuals that are divided into the two clusters (*K*) on the basis of the results of the coefficient of membership (*q*). In green, lineage A and in red, lineage B.

Table 1. Localities and sample sizes for the here analyzed *Kalotermes* populations.

Localities		**CR**	**I-SINE**	**Microsatellites**
Duna di Feniglia				
	KF1	20	7	
	KF2	13	11	
	KF3	20	15	
	KF4	19	15	
	KF5	20	20	
	KF6	20	15	20
	KF7	10	10	10
	KF8	11	12	12
	KF9	18	17	14
Greece				
	Toplou	8		
	Kastelli	8		
	Amorgos	8		
	Areopolis	8		
Balkans				
	Vransko	8		
	Brijoni	8		
	Porto Rose	8		
Italy				
	Viterbo	8		
	Duna di Feniglia	8		
	Rena Majore	8		
	Geremeas	8		
	Portoscuso	8		
France				
	Calvi	8		
	Marsiglia	8		
	Port de Bouc	6		
	Avignone	7		
	Boulbon	8		
	Estagel	2		

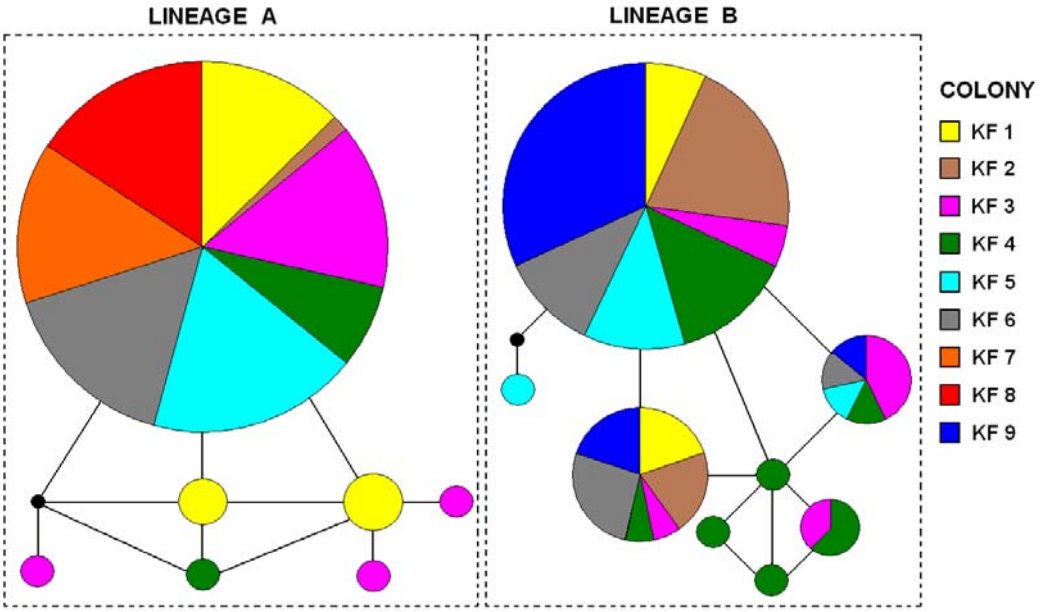

Figure 1

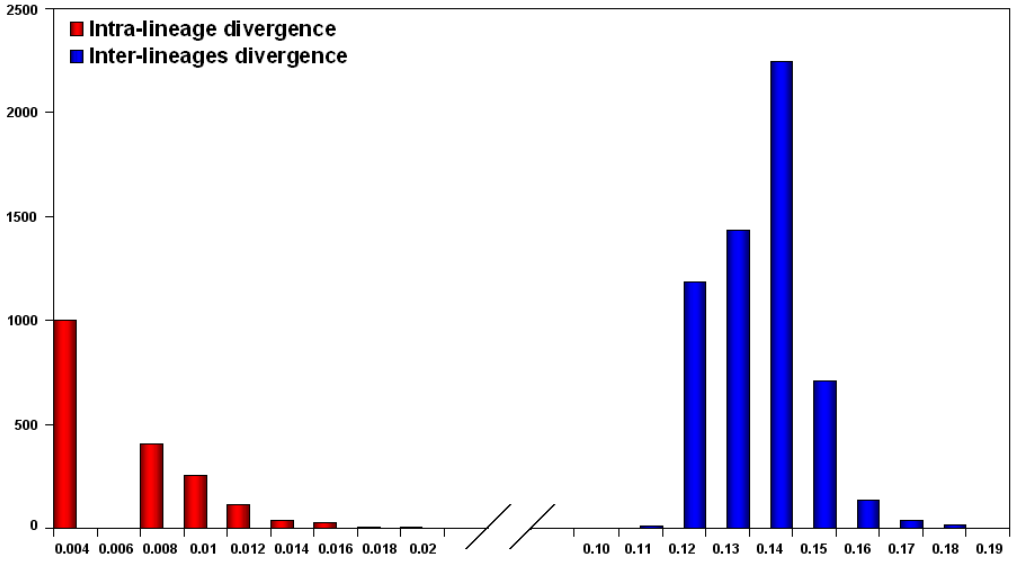

Figure 2

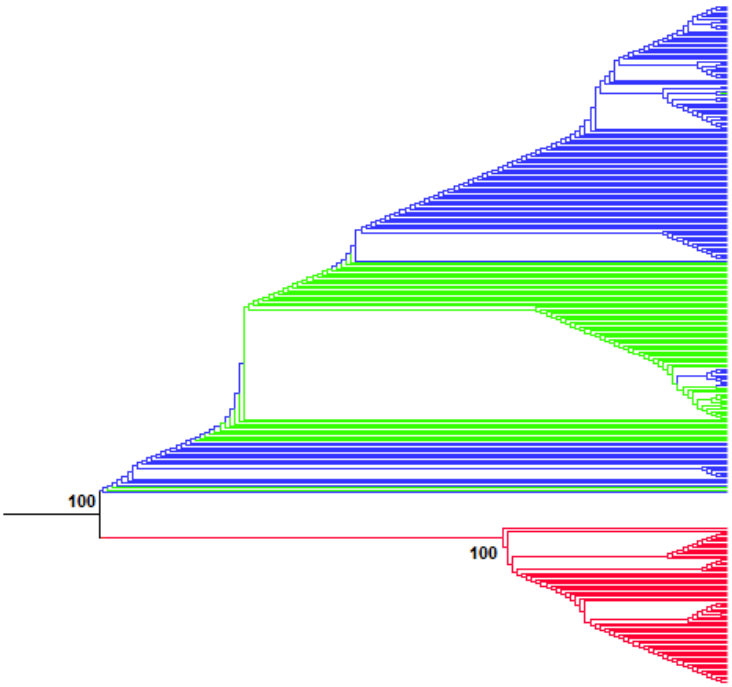

Figure 3

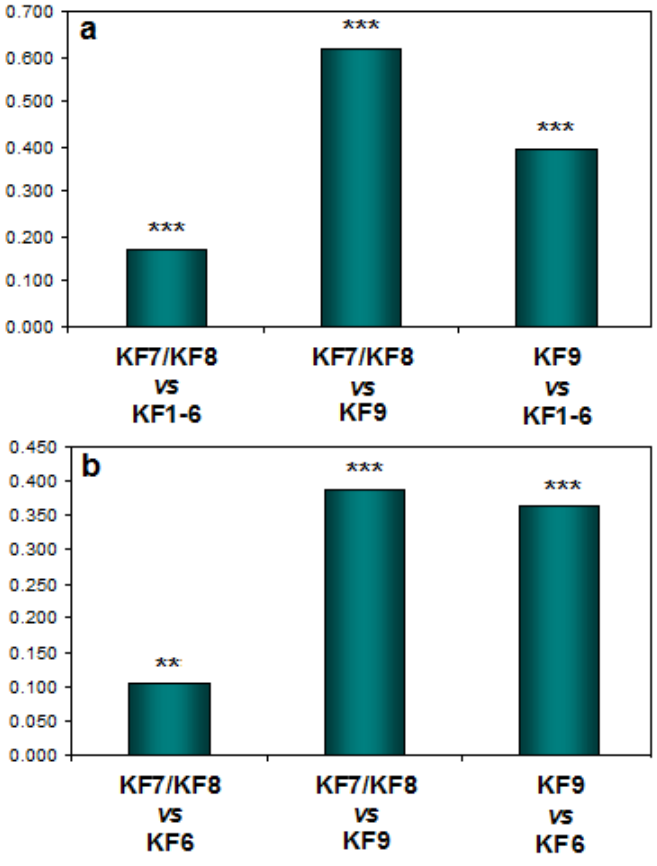

Figure 4

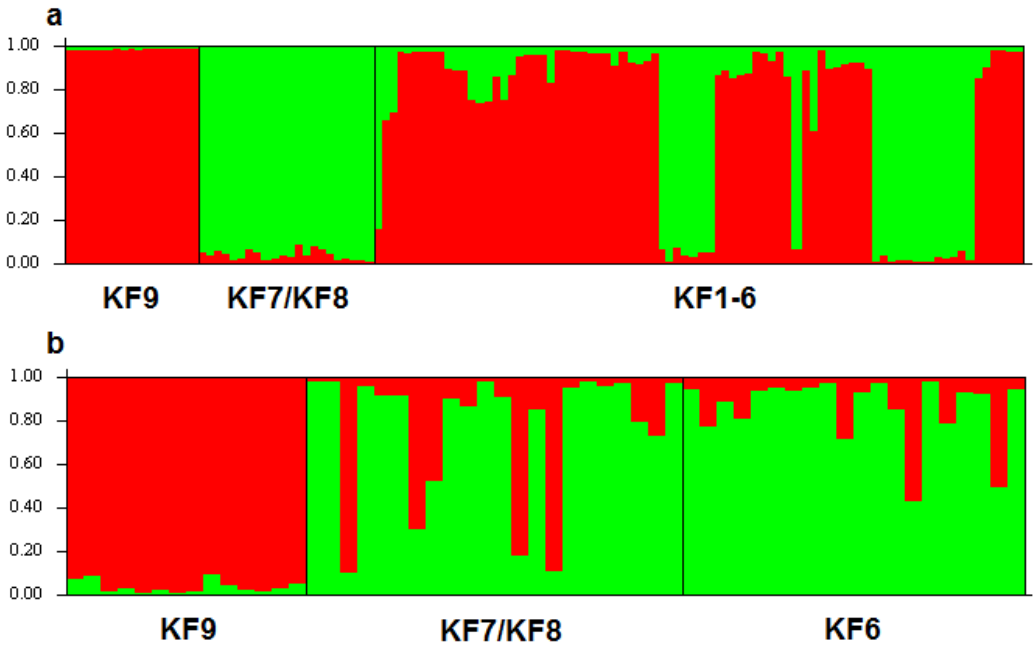
a
1.00
0.80
0.60
0.40
0.20
0.00
KF9
KF7/KF8
KF1-6
b
1.00
0.80
0.60
0.40
0.20
0.00
KF9
KF7/KF8
KF6

Figure 5

CHAPTER 8

***

CONCLUSIONS

Termites represent an interesting field in biodiversity and evolution studies, given their social life style and their peculiar reproductive dynamics. During my PhD thesis I've investigated two main themes: the phylogeny and the colony genetic structure of the European species belonging to the genera *Reticulitermes* and *Kalotermes*.

8.1 PHYLOGENETIC CONSIDERATIONS

In the *Reticulitermes* genus, my work has first demonstrated that the evolutionary history is more ancient with respect to previous analyses (Luchetti *et al*., 2005; Lefebvre *et al*., 2008). The main dichotomy, that split the western and the eastern taxa in fact, took place in the upper Miocene, ~ 23 MYA, while the most recent ones at the end of the Pliocene, ~ 2.5 MYA.

From a taxonomic point of view, my data highlighted different biodiversity levels with respect to previous analyses. For example, the *R. urbis* taxon shows a clear divergence of subspecific level between the two clades of Balkans/N-W Greece and west Peloponnesus, while *R. lucifugus lucifugus* and *R. lucifugus corsicus* appear to be differentiated at the specific level. Further, in the Aegean area, other than *R. balkanensis*, a unique species, which has to be described, was found along the coasts of

N-E Greece, northern Turkey, Cyclades and Crete.

As far as *Kalotermes flavicollis* biodiversity is concerned, the use of an increased dataset, both in terms of sampled area and number of molecular markers, allowed me to detect a greater population structuring in the European area. In fact, while populations from peninsular Italy to Greece appear highly homogeneous, three lineages are detected in western Europe; these lineages correspond to continental France, Corse/Sardinia and Portoscuso populations.

The different level of biodiversity detected between the two genera is probably the result of a different colonization history. In fact, following the results here reported, the *Reticulitermes* spp. complex has colonized Europe before *Kalotermes* one, and in this way it was processed by more geological events that generated the extant greater biodiversity.

8.2 POPULATION DYNAMICS CONSIDERATIONS

Concerning the analysis of population dynamics, the structure of the *R. urbis* introduced population at Bagnacavallo confirmed its invasive capacity. The presence of an unique expanded macrocolony well confirmed its ability in colonizing human environments, while the breeding system detected and the low genetic variability clearly indicate that the infestation took place recently.

In the Duna di Feniglia population of *Kalotermes flavicollis*, a high level of variability was detected, with an average number of more than four mitochondrial haplotypes in each colony, suggesting colony fusion and/or adoption as main processes of

interactions. The coexistence of two sympatric lineages evidenced the necessity of in depth taxonomic and biogeographical analyses, to better understand the evolutionary relationships among them.

On the whole, the population dynamics of *R. urbis* and *K. flavicollis* resulted highly different. In fact, *R. urbis* populations are established by single large colonies with different reproductive centres, while *K. flavicollis* produces a high number of small colonies that are able to mix and/or to adopt alates of other colonies.

These two features are possibly the result of two different strategies: in fact, while in *R. urbis* the rapid colonization of new areas is privileged, *K. flavicollis* can be genetically more prone in maintaining an established population ensuring a high genetic flow.

8.3 PERSPECTIVES

On the basis of the results obtained during my PhD course, it's possible to delineate some future projects.

First of all, a taxonomic description of the *Reticulitermes* spp. of the Aegean area, including the *R. lucifugus* – northern Turkey and *R. lucifugus* – southern Turkey (*sensu* Austin *et al.*, (2002)) is needed.

Second, giving the extremely poor knowledge about the biodiversity and the evolution of *K. flavicollis*, deeper phylogenetic and phylogeographic analyses will be done, further increasing the sampling area by taking into account other populations, especially from Spain, Portugal, Turkey and from the other major islands of the Mediterranean sea, as for example Sicily, Cyprus, the Balearics and Malta.

For the colony genetic structure, having already analyzed the population dynamics and the breeding system in the infesting *R. urbis* population from Bagnacavallo, future works should be focused on populations from its native area of Balkans, to detect possible differences in the reproductive biology and ecology.

Finally, the perspectives for the *K. flavicollis* complex will concern the taxonomic characterization of the genetic lineage B, its native range and the interbreeding level with *K. flavicollis sensu stricto*.

REFERENCES

Abe T. (1987) Evolution of life types in termites. In: *Evolution and Coadaptation in Biotic Communities.* (Eds) Kawano S., Connell J.H., Hidaka T., pp.126–148. University of Tokyo Press, Tokyo.

Abe T., Bignell D.E., Higashi M. (2000) *Termites: Evolution, Sociality, Symbiosis, Ecology*. Kluwer Academic, Dordrecht, The Netherlands.

Austin J.W., Szalanski A.L., Uva P., Bagnères A.G., Kence A. (2002) A comparative genetic analysis of the subterranean termite genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Annals of the Entomological Society of America* 95: 753–760.

Bergamaschi S. (2007) A multidisciplinary approach to taxonomy and phylogeny of Australian Isoptera. PhD thesis, University of Bologna.

Cameron S.L., Whiting M.F. (2007) Mitochondrial genomic comparisons of the subterranean termites from the genus *Reticulitermes* (Insecta: Isoptera: Rhinotermitidae). *Genome* 50: 188-202.

Campadelli G. (1987) Prima segnalazione di *Reticulitermes lucifugus* Rossi per la Romagna. *Bollettino dell'Istituto di Entomologia "Guido Grandi", Università degli Studi di Bologna,* 42: 175-178.

DeHeer C., Vargo E.L. (2004) Colony genetic organization and colony fusion in the termite *Reticulitermes flavipes* as revealed by foraging patterns over time and space. *Molecular Ecology* 13: 431-441.

Dronnet S., Chapuisat M., Vargo E.L., Lohou C., Bagnères A.-G. (2005) Genetic analysis of the breeding system of an invasive subterranean termite, *Reticulitermes santonensis*, in urban and natural habitats. *Molecular Ecology* 14: 1311-1320.

Eggleton P., Tayasu I. (2001) Feeding groups, lifetypes and the global ecology of termites. *Ecological research* 16: 941-960.

Engel M.S., Krishna K. (2004) Family-group names for termites. *American Museum Novitates* 3432: 1-9.

Goldstein D., Schlötterer C. (1999) Microsatellites: evolution and applications. Oxford University Press, Oxford, UK.

Grassé P.P. (1949) Ordre des Isoptères ou Termites. In: *Traité de Zoologie: Anatomie, Systématique, Biologie*. Vol. IX. Masson, Paris.

Haverty M. I. (1977) The proportion of soldiers in termite colonies: a list and a bibliography. *Sociobiology*, 2 (3): 199-216.

Hayashi Y., Lo N., Miyata H., Kitade O. (2007) Sex-linked genetic influence on caste

determination in a termite. *Science*, 318: 985-987.

Inward D., Vogler A., Eggleton P. (2007) A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. *Molecular Phylogenetics and Evolution* 44: 953–967.

Korb J. (2007) Termites. *Current Biology* 17: R995–999.

Kostia S., Ruohonen-Lehto M., Väinölä R., Varvio S.L. (2000) Phylogenetic information in inter-SINE and inter-SSR fingerprints of the artiodactyla and evolution of the bov-tA SINE. *Heredity* 84: 37-45.

Kutnik M., Uva P., Brinkworth L., Bagnères A.G., 2004. Phylogeography of two European *Reticulitermes* species: the Iberian refugium. *Molecular Ecology* 13: 3099–3113.

Legendre F., Whiting M.F., Bordereau C., Cancello E.M., Evans T.A., Grandcolas P. (2008) The phylogeny of termites (Dictyoptera: Isoptera) based on mitochondrial and nuclear markers: Implications for the evolution of the worker and pseudergate castes, and foraging behaviours. *Molecular Phylogenetics and Evolution* 48: 615–627.

Leniaud L., Pichon A., Uva P., Bagnères A.-G. (2009) Unicoloniality in *Reticulitermes urbis*: a novel feature in a potentially invasive termite species. *Bulletin of Entomological Research* 99: 1-10.

Lo N., Kitade O., Miura T., Constantino R., Matsumoto T. (2004) Molecular phylogeny of the Rhinotermitidae. Insectes Sociaux 51: 365-371.

Lo N., Hayashi Y., Kitade O. (2009) Should environmental caste determination be assumed for termites? *American Naturalist*, 173 (6): 848-53.

Luchetti A., Trenta A., Mantovani B., Marini M. (2004a) Taxonomy and phylogeny of north mediterranean *Reticulitermes* termites (Isoptera, Rhinotermitidae): a new insight. *Insectes Sociaux* 51: 117–122.

Luchetti A., Bergamaschi S., Marini M., Mantovani B. (2004b) Mitochondrial DNA analysis of native European Isoptera: a comparison between *Reticulitermes* (Rhinotermitidae) and *Kalotermes* (Kalotermitidae) colonies from Italy and Balkans. *REDIA* 87: 149–153.

Luchetti A., Marini M., Mantovani B. (2005) Mitochondrial evolutionary rate and speciation in termites: data on European *Reticulitermes* taxa (Isoptera, Rhinotermitidae). *Insectes Sociaux* 52: 218–221.

Luchetti A., Marini M., Mantovani B. (2007) Filling the European gap: biosystematics of the eusocial system *Reticulitermes* (Isoptera, Rhinotermitidae) in the Balkanic peninsula and Aegean area. *Molecular Phylogenetics and Evolution* 45: 377–383.

Marini M., Mantovani B., (2002) Molecular relationship among European samples of

Reticulitermes (Isoptera, Rhinotermitidae). *Molecular Phylogenetics and Evolution* 22: 454–459

Matsuura K., Fujimoto M., Goka K. (2004) Sexual and asexual colony foundation and the mechanism of facultative parthenogenesis in the termite *Reticulitermes speratus* (Isoptera, Rhinotermitidae). Insectes Sociaux 51: 325-332.

Matsuura K., Vargo E.L., Kawatsu K., Labadie P.E., Nakano H., Yashiro T., Tsuji K. (2009). Queen succession through asexual reproduction in termites. *Science* 323: 1687.

Nishihara H., Okada N. (2008) Retrotransposons: genetic footprints on the evolutionary paths of life. In: *Methods in Molecular Biology: Phylogenomics.* (Ed.) Murphy W.J., pp 201-225. Humana Press, Totowa.

Nobre T., Nunes L., Eggleton P., Bignell D.E. (2006) Distribution and genetic variation in *Reticulitermes* (Isoptera: Rhinotermitidae) in Portugal. *Heredity* 96: 403-409.

Nobre T., Nunes L., Bignell D.E., (2008) Colony interactions in *Reticulitermes grassei* population assessed by molecular genetic methods. *Insectes Sociaux* 55: 66-73.

Ohshima K., Okada N. (2005) SINEs and LINEs: symbionts of eukaryotic genomes with a common tail. *Cytogenetic and Genome Research* 110: 475–490.

Perdereau E., Bagnères A.G., Dupont S., Dedeine F. (2010) High occurrence of colony

fusion in a European population of the American termite *Reticulitermes flavipes*. *Insectes Sociaux* 57: 393-402.

Roisin Y., Everaerts C., Pasteels J.M., Bonnard O. (1990) Caste-dependent reactions to soldier defensive secretion and chiral alarm/recruitment pheromone. *Journal of Chemical Ecology* 16: 2865-2875.

Shafer A.B.A., Stewart D.T. (2007) Phylogenetic relationships among Nearctic shrews of the genus *Sorex* (Insectivora, Soricidae) inferred from combined cytochrome *b* and inter-SINE fingerprint data using Bayesian analysis. *Molecular Phylogenetics and Evolution* 44: 192-203.

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

Simon C., Buckley T. R., Frati F., Stewart J. B., Beckenbach A.T. (2006) Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics* 37: 545-579.

Springhetti A., (1985) The function of the royal pair in the society of *Kalotermes flavicollis* (Fabr.) (Isoptera, Kalotermitidae). In: *Caste differentiation in social insects*,

pp. 165-175. Oxford pergamon press.

Suárez M. E., Thorne B.L. (2000) Rate, amount, and distribution pattern of alimentary fluid transfer via trophallaxis in three species of termites (Isoptera: Rhinotermitidae, Thermopsidae). *Annales of the Entomological Society of America* 93: 145–155.

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Alessandro

APPENDIX

Scientific production during the PhD course

In extenso papers:

- Velonà A., Luchetti A., Ghesini A., Marini M., Mantovani B. (2011) Mitochondrial and nuclear markers highlight the biodiversity of *Kalotermes flavicollis* (Fabricius, 1793) (Insecta, Isoptera, Kalotermitidae) in the Mediterranean area. *Bulletin of Entomological Research*. doi:10.1017/S000748531000060X.

- Velonà A., Luchetti A., Ghesini S., Marini M., Mantovani B. (2010) Starting from Crete, a phylogenetic re-analysis of the genus *Reticulitermes* in the Mediterranean area. *Molecular Phylogenetics and Evolution* 56: 1051-1058.

- Velonà A., Luchetti A., Scanabissi F., Mantovani B. (2009) Genetic variability and reproductive modalities in European populations of *Triops cancriformis* (Crustacea, Branchiopoda, Notostraca). *Italian Journal of Zoology* 76 (4): 366-375.

Congresses:

- Luchetti A., Mingazzini V., Ghesini S., Velonà A., Marini M., Mantovani B. (2010) Evoluzione del genoma: elementi mobili e strategie riproduttive. LXXI° Congress of the Italian Zoological Union (UZI), Palermo, Italy, 20th-23rd September, p.35-36 (oral communication).

- Velonà A., Luchetti A., Mantovani B. (2010) Colony structure of *Kalotermes flavicolli*s (Isoptera, Kalotermitidae): preliminary results from two molecular markers. XVI° World Congress of the International Union for the Study of Social Insects (IUSSI), Copenhagen, Denmark, 8th-14th August, p.143 (poster).

- Velonà A., Luchetti A., Ghesini S., Marini M., Mantovani B. (2010) Il genere *Reticulitermes* (Insecta; Isoptera; Rhinotermitidae): rianalisi del genere nell'Europa mediterranea. XIII° Congresso Associazione Italiana per lo Studio degli Artropodi Sociali e Presociali (AISASP), Reggio Calabria, Italy, 3rd-6th May, p. 34 (oral communication).

- Velonà A., Dupont S., Bagnères A.-G., Mantovani B. (2010) Utilizzo di marcatori microsatelliti per l'analisi della struttura coloniale della specie invasiva *Reticulitermes urbis* (Insecta; Isoptera; Rhinotermitidae) a Bagnacavallo (RA). XIII° Congresso Associazione Italiana per lo Studio degli Artropodi Sociali e Presociali (AISASP), Reggio Calabria, Italy, 3rd-6th May, p. 62 (poster).

- Luchetti A., Ghesini S., Velonà A., Marini M., Mantovani B. (2008) Biosystematics and evolution of Central-East European termites: mitochondrial and nuclear molecular analyses. IV° European Meeting of International Union for the Study of Social Insects (IUSSI), La Roche-en-Ardenne, Belgium, 30th August – 4th September, p.105 (oral communication).