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GENETIC DIVERSITY AND BREEDING SYSTEMS OF EUROPEAN TERMITES (BLATTODEA, TERMITOIDAE)

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Abstract

Termitoidae is an epifamily of the order Blattodea comprising hemimetabolous eusocial insects commonly known as termites. Native European termite species belong to two genera: *Reticulitermes* and *Kalotermes.* The former genus includes six subterranean-termite species. Among these, *R. urbis* is commonly found in the wild of the Balkans while it can be observed in urban habitats in Italy and France where it causes damages to structures and wooden artifacts. Therefore, it was suggested that this species is native from the Balkan Peninsula and introduced in Italy and France. On the other hand, a recent survey questioned about the invasive status of *R. urbis* in Italy and France, hypothesing a native origin of *R. urbis* in Italy and France on the basis of some similarities in the distribution areas of this species with those of the native *R. lucifugus*. On the whole, a deeper genetic and phylogeographic investigation is required to clarify the *R. urbis* distribution status. Considering the same genus, previous studies described the occurrence of the Asexual Queen Succession (AQS) reproductive strategy in the Italian *R. lucifugus* while no genetic investigations were performed so far on the French native *R. grassei* and the invasive *R.* flavipes in order to verify the presence\absence of this particular reproductive mode. Moreover, it has been suggested that AQS system could be the force shaping the inclusive fitness in social diploids organisms like termites but no genetic investigations supporting such theoretical prediction, to date, were described in the AQS species *R. lucifugus*. As far as *Kalotermes* is concerned, recent molecular studies showed that two dry-wood termite species are present in Europe: *K. flavicollis* and *K. italicus*. The first one comprises at least three main mitochondrial lineages: the lineage A (also termed *K. flavicollis sensu stricto*) includes all samples collected from the Aegean islands to the Italian Peninsula; the lineage SC comprises colonies found in Sardinia and Corsica; the lineage SF limited to Southern France. No data are available for the taxonomic and phylogenetic status of *Kalotermes* populations from the Iberia Peninsula. The other species, *K. italicus*, easily recognizable as adult alates, show a black or dark brown pronotum (as opposed to the yellow-necked *K. flavicollis*), was found only in Southern Tuscany and in a small area on the Italian mid-Adriatic coast. The taxonomy and the distribution pattern of *Kalotermes* taxa, thus, is far from being complete and many issues remain unresolved. Referring to same genus, a particular strategy influencing the breeding system was described in

Italian populations of *K. flavicollis, i.e.* the occurrence of extreme colony fusion events explained in the light of the 'Accelerated nest inheritance' theory. These colonies were found to fuse in the field, with instances of extreme fusion given by up to nine mitochondrial haplotypes even belonging to highly divergent genetic lineages. Taking into account that the recently described *K. italicus* shows a sympatric distribution area with *K. flavicollis,* it is not to be excluded, therefore, a possible occurrence of this phenomenon even at an interspecific level. Obviously, this hypothesis requires further investigation. Finally, it is poorly known if *Wolbachia,* a genus of parasitic endosymbiotic bacteria living in the termite germinal line cells, could affect the reproductive biology of these hosts and, thus, if this microbe could be involved in the onset of the AQS strategy and in the interspecific hybridization events between divergent *Kalotermes* taxa.

My PhD research project focused on the analysis of the genetic diversity and breeding systems in European *Reticulitermes* and *Kalotermes* taxa. Investigations were performed at the intrageneric level with comparisons also at the intergeneric one in order to highlight new insights on the evolution of eusociality. My surveys, performed through genetic and morphological approaches, had the following main goals: i) a deep phylogeographic investigation of Balkan, Italian, and French colonies of *R. urbis* in order to clarify the invasive status of this species and to identify the native source population(s); ii) a more detailed picture of *Kalotermes* biogeography and evolution in Europe, quantifying, in addition, the real extent of colony mixing in these termites; iii) a microsatellite survey of the French *R. grassei*, *R. flavipes* and Italian *R. lucifugus* populations in order to verify, providing genetic evidences, the reproductive strategies of the former two species and to describe new insights of the AQS strategy in Italian colonies of *R. lucifugus*; iiii) a preliminary molecular investigation, using the bacterial *FtsZ* marker, on *Reticulitermes* and *Kalotermes* termite species in order to identify *Wolbachia* infection and to characterize the relevant strains, paying particular attention to the AQS species *R. lucifugus* and to mixed colonies of the *Kalotermes* genus to verify whether *Wolbachia* presence can be related to particular breeding systems.

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CHAPTER 1

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THE EPIFAMILY TERMITOIDAE (INSECTA, BLATTODEA)

1.1 SYSTEMATICS, DISTRIBUTION AND PHYLOGENY

Termitoidae is an epifamily of the order Blattodea comprising hemimetabolous eusocial insects commonly known as termites. These insects are believed to be the earliest-evolving social insects: their origin dates back either to the Cretaceous period, around 130 million years ago, or even more ancient, the upper Jurassic, as highlighted by recent fossil records (Korb, 2007). Around 3.000 termite species are described so far (Krishna *et al.*, 2013) and they are allocated in 282 genera from seven different families: Mastotermitidae, Stolotermitidae, Hodotermitidae, Archotermopsidae, Kalotermitidae, Rhinotermitidae, Termitidae (Cameron *et al.*, 2012; Figure 1.1). Species distribution among the above-mentioned families is biased: Kalotermitidae and Rhinotermitidae, for example, embody around 456 and 315 species respectively, while Stolotermitidae and Mastotermitidae families comprise only two and one species, respectively. Termitidae is the largest family encompassing around 2.107 species (the 70% of the described taxa), mainly residing in tropical and sub-tropical habitats. As for other animals distribution, the highest termite biodiversity is in the tropical and subtropical regions and decreases in the temperate regions, but it is possible to find them even at extreme latitudes and altitudes (Scheffrahn *et al.*, 2015).

From a phylogenetic point of view, Mastotermitidae, Stolotermitidae, Hodotermitidae, Archotermopsidae and Kalotermitidae families are considered the most ancient taxa, with Mastotermitidae resulting as sister group of all other termites (Cameron *et al.*, 2012; Figure 1.1). Termite species belonging to these families are usually referred to as “lower termites”.

Rhinotermitidae and Termitidae families, on the other hand, include the most recently derived species (Cameron *et al.*, 2012; Figure 1.1); Termitidae are commonly called "higher termites", while Rhinotermitidae retain characters intermediate between lower and higher termites (Vargo & Husseneder, 2009) but usually included with the lower termites.

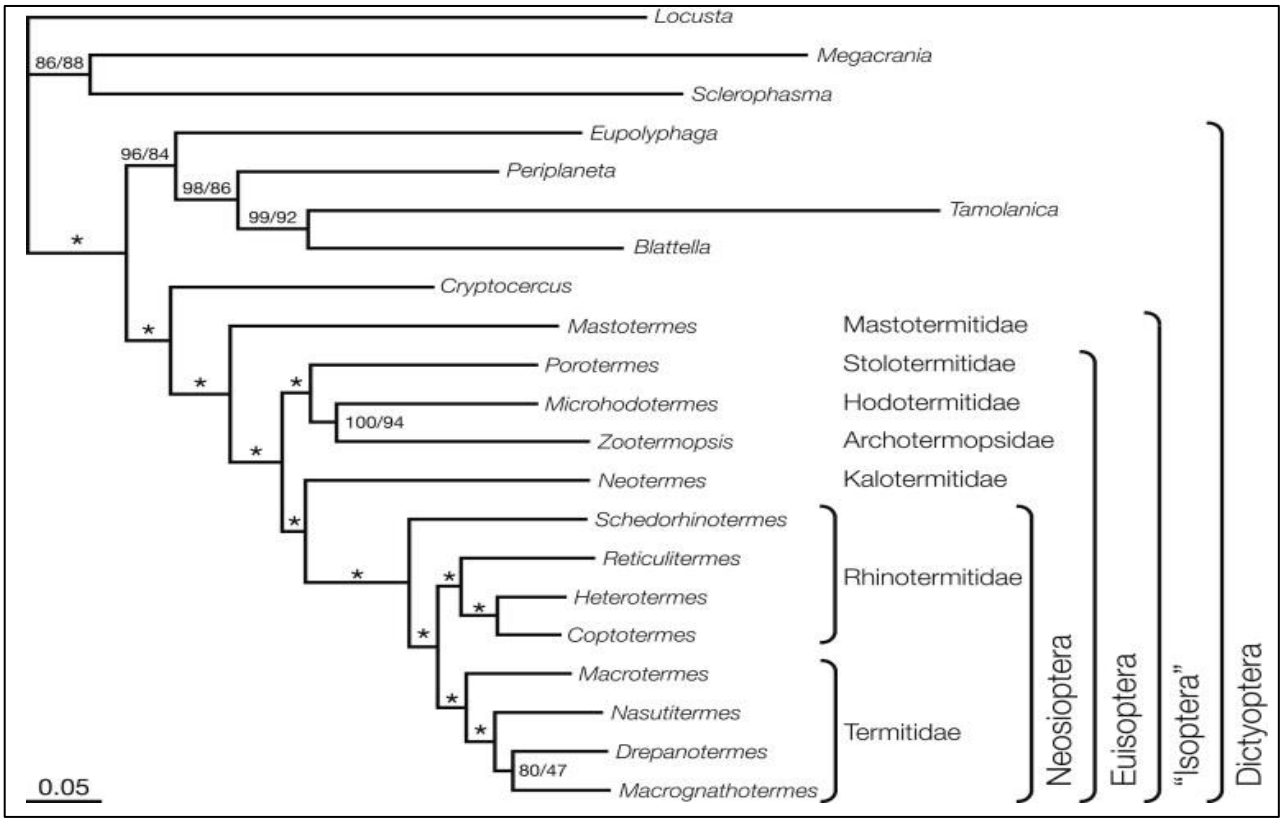

Figure 1.1 Phylogenetic tree of termites based on mitochondrial genomic data.
[Source: Cameron *et al.*, 2012]

For a long time, termite families have been classified in a single order called Isoptera (Brullé, 1832). This order, together with mantis and cockroaches (Mantodea and Blattodea orders, respectively) formed the so-called Dictyoptera superorder (Inward *et al*., 2007a; Legendre *et al*., 2008; Ware *et al*., 2008). However, subsequent phylogenetic investigations demonstrated that termite species are phylogenetically related to cockroaches of the family Blattidae, with the *Cryptocercus* genus as sister group; therefore, termites have been eventually placed within the order Blattodea (Inward *et al*., 2007b; Beccaloni & Eggleton, 2011; Wang *et al.*, 2017; Zongqing *et al.*, 2017). For this reason, the order level of classification was deemed to be unwarranted and termite taxa were classified as the

Termitoidae epifamily. Despite these findings, some Authors reject this idea for reasons of nomenclatural stability (Lo *et al.,* 2007).

1.2 BIOLOGY

Termites are social insects: their colonies are composed by several hundred to many thousands of individuals constituting different morpho-functional castes. In general terms, termites are very small insects with a worker body length ranging from 2.5 to 20 mm. The head is dorso-ventrally depressed with a chewing mouthpart apparatus and two moniliform antennae having a variable number of segments. The thorax shows a pronotum narrower than head, with sclerites joined together by large membranes, and three pairs of equally developed legs. The abdomen, consisting of ten segments, is cylindrical and ends with a couple of cerci. The seventh sternite (the ventral portion of a segment) is useful for sexes recognition because in females it forms a large plate under the genital chamber (Zimet & Stuart, 1982). The integument is thin, transparent and, therefore, it does not efficiently protect individuals from dehydration. For this reason, termites usually live in humid environments. In fact, these insects spend most of their time in the nest which provides the best microclimatic conditions for their survival as well as protection from predators. The nest consists of numerous tunnels and chambers and it can be built inside a piece of dead wood (as in the case of the "dry-wood" *Kalotermes* termite species), underground (as in "subterranean" termites of the *Reticulitermes* genus) or realized through epigean structures (as, for example, in several Termitidae species). Nest shape and size are therefore highly variable and the environmental conditions often can affect them (Pearce, 1997). The colony lives inside the nest with individuals performing different tasks depending on the caste to which they belong. Given the social organization, there is a highly sophisticated system of communication among individuals (Borderau & Pasteels, 2011). This

communication occurs through tactile and chemical signals. Tactile signals are generally played banging the head or the abdomen in order to communicate an alarm situation. These vibratory stimuli are detected by mechanoreceptors usually present in the second antennal segment Eggleton, 2014). Chemical communication, instead, is performed through pheromones. Several compounds and molecules are used for the transmission of different signals which induce response behaviours such as alert and defence, mate attraction, inter-individuals recognition, caste differentiation and information about the availability of new food resources (Borderau & Pasteels, 2011).

As far as food resources are concerned, termites feed on cellulose, which is obtained from the digestion of dead wood, leaf litter, dry grass and soil. For this reason, these organisms are considered the most important invertebrate decomposers (Eggleton & Tayasu, 2001). Cellulose is a polysaccharide consisting of a linear chain of several hundred to many thousands of β-1-4 linked glucose units. Termites can digest this organic compound thanks to the symbiosis with prokaryotes (bacteria and archaea) in higher termites and unicellular eukaryotes (flagellated protists) in lower termites, present in their gut, which are able to broke the β-1-4 glycosidic bond (Bignell, 2011). In some termite species, as in the case of those belonging to the subfamily Macrotermitinae, the basidiomycetes *Termitomices* is reared inside the nest and supplied with plant-derived materials (Nobre *et al.*, 2011). The fungi, therefore, degrade these materials allowing termites to eat them. In some instances, termites use a particular feeding modality, called trophallaxis. It consists in the transfer of organic material, from one individual to another, through regurgitation (stomodeal trophallaxis) or anal secretion (proctodeal trophallaxis). Trophallaxis plays a key role in the transfer of gut endosymbionts, and it is important for juveniles or individuals that lost symbionts after the moult (Nalepa, 2015).

1.3 SOCIAL ORGANIZATION

Termites show a society in which individuals are grouped in castes, performing different roles. These castes are characterized by specific biological traits in anatomy, physiology and behaviour. In very general terms, a colony is composed by three main castes: reproductives, workers and soldiers. The last two castes forego their own reproduction to help the reproductive caste to raise their offspring. Caste development change between higher termites (Figure 1.2) and lower termites (Figure 1.3). In the first ones, the developmental pathway is strictly determined and early splits in two divergent lines, leading to the differentiation of sterile and reproductive castes (Figure 1.2). Lower termites, on the contrary, have a linear developmental pathway and castes, accordingly, exhibit a greater plasticity, allowing caste changing for the individuals (Figure 1.3). Gene networks and genetic pathways but also nutritional and pheromonal signals are involved in the caste determination (Grassé 1949; Lo *et al.*, 2009; Osamu *et al.*, 2011; Cornette *et al.*, 2013).

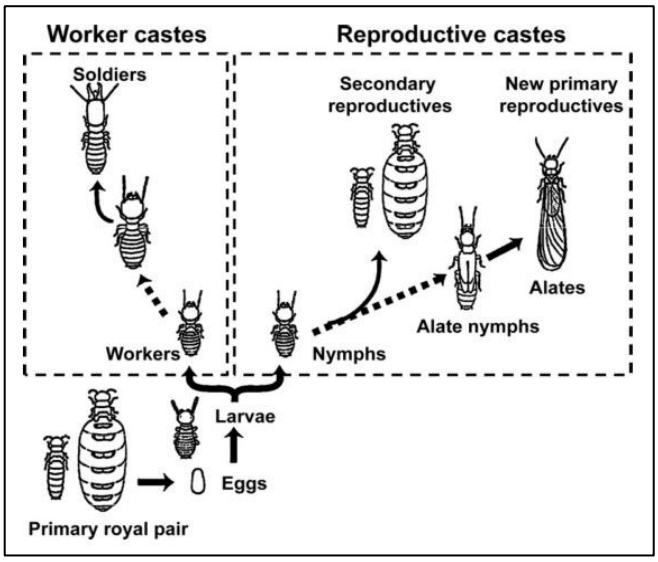

Figure 1.2 Caste differentiation in higher termites.
[source: Wenseleers & Van Oystaeyen, 2011]

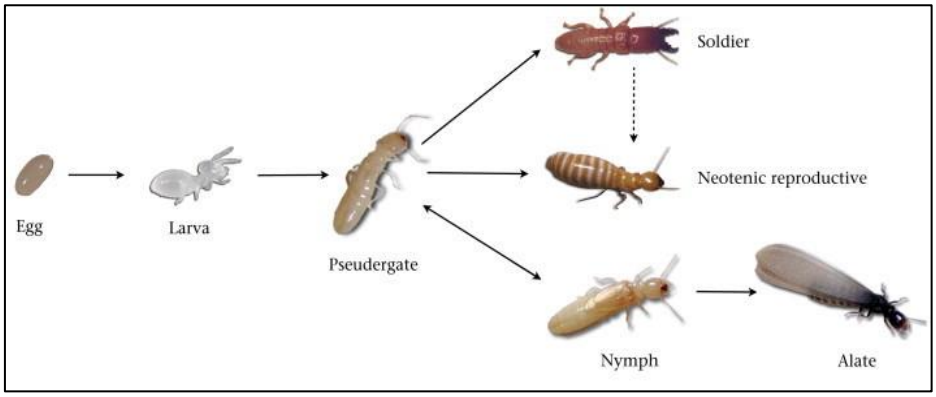

Figure 1.3 Caste differentation lower termites.
[source: Hartke & Baer, 2011]

1.3.1 Reproductives

The reproductive caste, as previously mentioned, includes individuals able to perform reproduction, being the only ones in the colony with functional gonads. Two types of reproductives are recognized: primary and secondary reproductives. Primary reproductives (Figure 1.4), also called "imagoes" or "alates" (and commonly referred as "swarming individuals"), are dark brown or black coloured, and have two pairs of fully developed wings and compound eyes: these features allow them to swarm outside the natal nest, looking for a partner to run the nuptial flight and found a new colony, through sexual reproduction. Secondary reproductives (Figure 1.5) are neotenics, as they reach sexual maturity without moulting into the adult stage (i.e., the imagoe). They are whitish, wingless or with wing buds. These individuals may derive from workers (and they are called "ergatoids") or from nymphs (the so called "nymphoids"). Neotenics are unable to leave the natal nest and, for this reason, they usually contribute to the offspring output or replace primary reproductives in the production task.

Figure 1.4 Primary reproductives of *Reticulitermes lucifugus*. [source: www.alexanderwild.com].

Figure 1.5 Secondary reproductive of *Cryptotermes cynocephalus*. [source: www.termiteweb.com].

1.3.2 Workers

In most termite species, workers are small, whitish, wingless and eyeless (Figure 1.6). They represent the 90% or more of the individuals in a colony and they perform all the logistic tasks for

the maintenance of the nest. Workers, in fact, build and repair galleries, look for food and, through trophallaxis, they feed reproductives, soldier and juveniles. When necessary, they can also help soldier in colony defense. In fungus-growing termites, workers are even able to practice agriculture collecting plant matter to produce fungal gardens. Despite being usually a neuter caste in most of the higher termites, in some species, workers can moult into ergatoids and start reproducing (Vargo & Husseneder, 2009; see also previous paragraph). Furthermore, in many lower termites (for examples in the Archotermopsidae and Kalotermitidae families) workers are referred to as "false workers" or "pseudergates": these individuals are in fact immatures, being under a developmental stationary state, but they are able to become reproductive whenever the conditions are favorable (Korb, 2007; Korb *et al.*, 2012).

Figure 1.6 Workers of *Reticulitermes lucifugus.* (source: www.bugguide.net)

1.3.3 Soldiers

The soldier caste comprises from 1% to 10% of the individuals belonging to the colony. The main task of these individuals is the colony defence. Soldiers show a brown pigmented body, a large and robust head (Eggleton, 2014). Here they may show a pair of modified jaws used as real weapons (Figure 1.7), or anatomical structures to spray defensive chemical compound or they may have a phragmotic

Figure 1.7 Soldier of *Kalotermes flavicollis.* (source: www.biodiversidadvirtual.org)

head for closing accesses to nest tunnels. Large-jawed soldiers are unable to feed themselves, thus they are fed by workers through trophallaxis (Eggleton & Tayasu, 2001).

1.4 THE COLONY: ESTABLISHMENT AND STRUCTURE

The colony is the basal unit of termite's social life. Considering castes and task subdivision, a colony can be referred to as a "superorganism" (Emerson, 1939) where castes represent specific organs, each unable to survive without the others. Colony performances are therefore strictly linked to the cooperation between individuals of the different castes. For this reason, natural selection does not act on the individual but on the group, that is the colony (Eggleton, 2011).

1.4.1 Colony establishment

Three main mechanisms have been described to explain foundation modalities. These are: dispersal flights, budding and sociotomy.

1.4.1.1 Dispersal flights

This is the most common modality to found a colony. It takes place when the winged individuals (i.e. primary reproductives, future kings and queens) fly out of their natal nest, looking for a partner to perform the nuptial flight and forming, eventually, heterosexual tandems. Depending on the species, swarming takes place one or more times in a year and in different seasons. Synchronized swarming within the same population increase the probability of encounter among non-nestmate reproductives. Moreover, this probability is, possibly, further limited by male and female alates covering different distances during the swarm (Vargo & Husseneder, 2011).

After the nuptial flight, individuals lose their wings at the basal suture line and start to search for a colonization site. After the new nest is built, the royal pair starts to produce the offspring sexually, taking care of the first brood of eggs and juveniles inside a cavity placed in the deep of the nest, called the “royal chamber” in Rhinotermitidae and higher termites.

1.4.1.2 Budding

In this alternative modality of colony foundation, a group of individuals, including neotenics, separate from the natal colony (Husseneder *et al.*, 1998). Generally, this occurs at the periphery of large and expanded colonies, where the transmission of pheromones and other suppressor stimuli secerned by royals is less strong. It can be also the result of accidental events such as dramatic weather impact, floods or soil disruption (Husseneder *et al.*, 1998). In any case, neotenics become active reproductives and found a new colony.

1.4.1.3 Sociotomy

Colony establishment through sociotomy occurs when the royal couple, together with some individuals from the other castes, leave the original nest and establish a new colony. In the old nest, the reproduction and, thus, the maintenance of the colony is continued by neotenics. Sociotomy is rare in termites: in fact, it was observed only in *Anoplotermes* and *Trinervitermes* species (Grassé, 1949).

1.4.2 Colony structure

During its lifetime, a colony grows and changes its family structure, also possibly influenced by the breeding system expressed. Three family types are recognized (Vargo & Husseneder, 2009): simple family, extended family and mixed family.

1.4.2.1 Simple family

A simple family represents the simplest colony structure where all the individuals are the offspring of a single pair of reproductives, that is the founding couple. This condition is usually observed during the early stages of the colony establishment.

1.4.2.2 Extended family

When secondary reproductives develop and start contributing to the offspring production, the family structure of the colony becomes extended. These neotenics are the offspring of the reigning queen and king and they reproduce among themselves or, in some instances, together with one or both of the primary reproductives. The extended family structure is typically found in mature/late stages of colony life, and allows colony persistence, growth and expansion after the death of the royal couple.

1.4.2.3 Mixed family

A mixed family structure emerges when in a colony three or more unrelated primary reproductives occur. Mixed family can be produced through several mechanisms such as fusion events between two or more colonies, colony founding by three or more primary queens and kings (also known as pleometrosis) or, in more rare instances, when an established colony adopts unrelated reproductives.

1.5 EVOLUTIONARY THEORIES AND EUSOCIALITY

As it can be deduced from what it has been described so far, eusociality is defined by cooperative behaviours (altruism) and subdivision of labour. This system is the most advanced form of social organization in animals and represents one of the major transition from simplicity to complexity, in the evolution of life (Szathmary & Maynard-Smith, 1995). The existence of altruism, in which individuals forego their own reproduction to increase the fitness of others, constitutes, nevertheless, an evolutionary paradox being in conflict with Darwin's concept of reproductive self-interest (Darwin, 1859). "Kin-selection" theory (Maynard-Smith, 1964), arose from Hamilton's theory of inclusive fitness (Hamilton, 1964), provides one of the best explanation resolving such a conflict. This theory, through the inequality $R > c/b$, explains how natural selection favours cooperation when the relatedness (R) between altruists and beneficiaries is higher than the ratio of costs (c; fitness lost by altruists) to benefits (b; fitness gained by beneficiaries). Individuals, thus, forego their reproduction and address their resource investment towards close relatives so as to maximize the transmission rate of their own genes to the next generation (Hamilton, 1964). Relatedness, therefore, seems to be a driving force for the emergence of eusociality. Among eusocial animals, social Hymenoptera (all ants, some bees and wasps) are the most successful demonstration of this theory because of their haplo-diploid genetic system for sex determination resulting in a high relatedness asymmetry between individuals. It was observed, in fact, that diploid workers invest more resources in their diploid sisters (queens) being more related to the latter than to their haploid brothers (drone bees; Trivers & Hare, 1976; Boomsma & Grafen, 1991; Sundström, 1994; Queller & Strassmann, 1998). Although kin selection appears as the best model for altruism in haplodiploid Hymenoptera, there are other instances in which this theory fails to explain the evolution of eusociality. Social behaviour, indeed, is also present in several diplo-diploid animals apparently lacking relatedness asymmetries between sexes and\or generations such as termites,

some shrimp species and naked mole rats (Korb & Heinze 2008). For these groups, the evolutionary game theory (Maynard-Smith & Price, 1973) seems to better explain the existence of altruistic behaviours, together with the Darwinian competition. Using the "Prisoner's dilemma" example game, which tests the payoffs of cooperating or in defecting from cooperation (Nowak *et al.*, 1995), the theory demonstrates that altruism among individuals, who interact repeatedly, can be equal or even more advantageous than competition. Mutual cooperation, thus, allows individuals to gain a benefit that will be greater than the cost paid by each of them for adopting altruistic behaviour. Moreover, each individual, after having implemented an altruistic behaviour at first, is able to adopt the most advantageous behaviour imitating, at each interaction, those adopted by the other ("tit for tat" game strategy; Maynard-Smith, 1982). Game theory, therefore, provides an alternative interpretation, independent from inclusive fitness, to understand the evolution of social behaviour also in diplo-diploid eusocial animals, like termites, and even between unrelated individuals, like in the case of several animals living in groups (e.g. herds, flocks etc.).

1.5.1 Accelerated nest inheritance

Termite life-history provides a good example for understanding the game theory and its dynamics influencing social behaviour. Damp-wood (Archotermopsidae) and dry-wood (Kalotermitidae) termites, often referred together as 'wood- dwelling termites', include species that forms small colonies in a single piece of wood. These termites spend their entire life inside the nest using it both as shelter and food (Abe 1987, 1990). Workers of these species are totipotent and show a very flexible development (pseudoergates; see Chapter 1.3). They are, in fact, kept under a developmental stationary state by the pheromones secreted by the reigning queen; once released from this chemical bound, they are able to develop into soldiers or in winged (alates) or secondary

reproductives (Roisin 2000; Roisin & Korb 2011). The chance to become reproductives gives these workers, beside the indirect fitness derived from assisting relatives, also a direct fitness deriving from the possibility to inherit the nest after the death of primary reproductives. According to this, experimental studies on laboratory colonies of the archotermopsid *Zootermopsis nevadensis* and the kalotermitid *Cryptotermes secundus* have described that interactions and eventual fusion between colonies lead to the death of reigning reproductives allowing workers to become reproductives themselves and to inherit the nest (Thorne *et al.,* 2003; Korb & Schneider 2007). These studies have also suggested that the opportunity for workers to inherit the nest could be frequent in natural populations where colonies interact and merge in mixed-family colonies. This phenomenon, called Accelerated Nest Inheritance (ANI; Thorne *et al.*, 2003), may have promoted the evolution of eusociality in lower termites by favouring cooperation between unrelated colonies (Thorne *et al*. 2003; Johns *et al*. 2009; Howard & Thorne 2011).

1.6 REPRODUCTION IN TERMITES

Being diplo-diploid organisms, the common reproductive system in termites is gonochorism, with males and females equally contributing to the progeny for the nuclear genome. Through sexual reproduction, reigning queen and king produce workers, soldiers, winged dispersing reproductives and neotenics. However, in addition to sexual reproduction, there are some instances in which female termites are able to reproduce through parthenogenesis (Matsuura, 2011; Kobayashi & Miyaguni, 2016). For example, this happens in *Reticulitermes speratus* when female alates fail to mate with males, during the swarming season. In these circumstances, some species form homosexual tandem in which two females cooperate and found together the colony, helping each other in the brood care (Matsuura & Nishida, 2001; Matsuura *et al.,* 2002).

1.6.1 Asexual Queen Succession (AQS)

Parthenogenetic (often referred to as “asexual”) reproduction can be a reproductive step characterizing the life cycle of a colony. In some termite species, in fact, the primary queen, after founding the colony through sexual reproduction with the primary king, produces female secondary

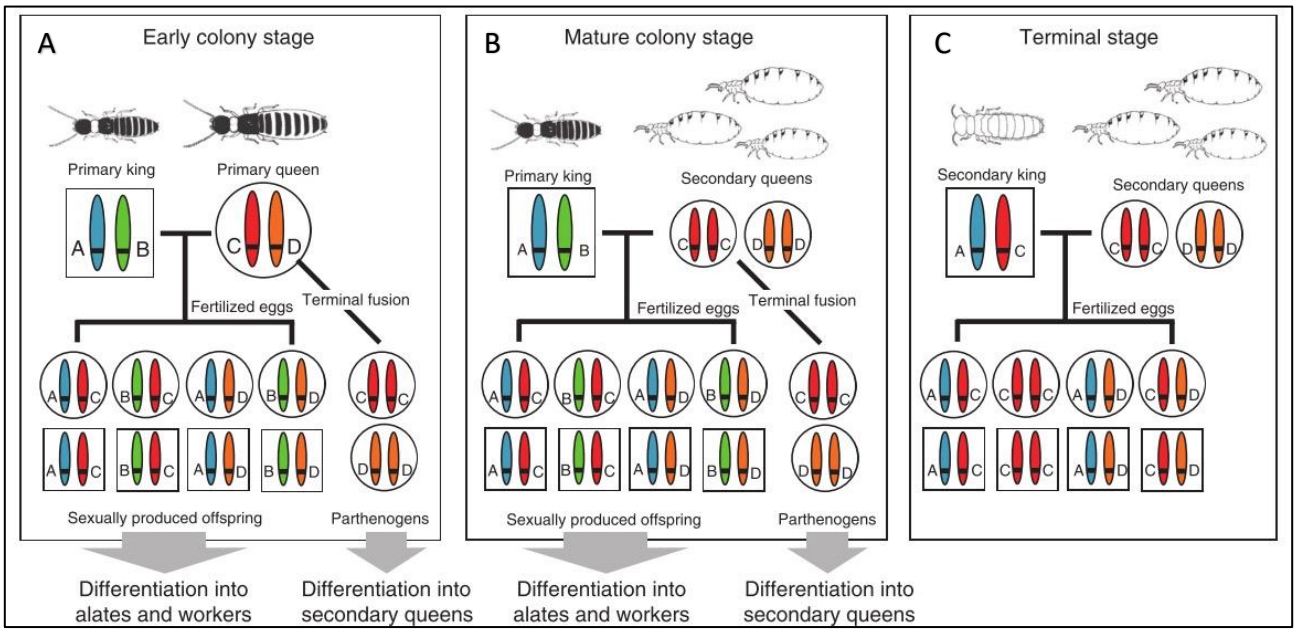

Figure 1.8 Schematic diagram of Asexual queen succession (AQS) strategy in long lived species of the *Reticuliterms* genus.
(source: Kobayashi *et al.*, 2013)

reproductives (secondary queen) through thelytokous parthenogenesis (Figure 1.8, A). These secondary queens, upon primary queen’s death, will mate with the primary king and contribute to the growth of the colony (Figure 1.8, B) extending the founder queen genetic input over the time (Asexual Queen Succession, AQS; Matsuura *et al.*, 2009; Matsuura 2011, 2017).

The AQS strategy seems to be an ideal compromise between sexual and asexual reproduction outcomes, allowing to maintain the original genetic variability of the colony that otherwise would be progressively reduced by parent-offspring mating or by sib-mating between secondary reproductives (Matsuura, 2011).

It has been suggested that under the AQS the diplo-diploid system of termites could fit into the Hamilton's inclusive fitness model (Kobayashi *et al*., 2013). At later stage of colony life, the primary king die and is replaced by a sexually-produced male neotenic. This secondary king carry, therefore, genes from the primary king and from the primary queen through its clones, secondary queens. When this secondary king mates with the parthenogenetically produced secondary queens (Figure 1.8, C), a mother-son inbreeding occurs generating a sex-asymmetric genetic inheritance which would increase, as a consequence, a female-biased allocation of dispersers (Kobayashi *et al*., 2013). This theoretical prediction was put forward on the basis of a female-biased sex-ratio of dispersing reproductives in the AQS species, but no genetic evidence supporting such hypothesis has been reported to date (Kobayashi *et al*., 2013).

Notwithstanding the envisaged advantage of this mating system, this strategy does not appear widespread among termite taxa. To date, in fact, AQS strategy has been described only in three *Reticulitermes* species (Rhinotermitidae) and in three neotropical termites belonging to the Termitidae family (Matsuura *et al.*, 2009; Vargo *et al.*, 2012; Luchetti *et al.*, 2013b; Fougeyrollas *et al.,* 2015; Fournier *et al.,* 2016; Fougeyrollas *et al*., 2017). Given that the occurrence of this reproductive mode appears patchy within the phylogeny of these taxa, a multiple independent origin of AQS has been suggested (Dedeine *et al.*, 2016).

This can be somehow confirmed by the finding of some differences in the AQS expression among *Reticulitermes* and Termitidae species. First, the cytological mechanism of ploidy restoration is different: it involves a terminal fusion in *Reticulitermes* species and a gamete duplication or a central fusion in Termitidae taxa (Matsuura, 2017). Second, while it is generally acknowledged that AQS may bring advantage to the colony on the long timespan, as in the case of the long-lived *Reticulitermes* species, in the termitid species *Silvestritermes minutus* the AQS seems to mediate a

faster colony growth and production of swarming alates within a very short colony lifespan (Fougeyrollas *et al*., 2017).

1.6.2 Reproduction driven by *Wolbachia* microbe

As introduced in Chapter 1.2, microbial symbionts play a crucial role in termite biology providing benefits through mutualism (Dedeine *et al.*, 2003). Termite gut microbiota is composed by both prokaryotes (bacteria and archaea) and unicellular eukaryotes (flagellated protists) which degrade lignin, cellulose, and hemicelluloses to fermentable carbohydrates, enabling termites to feed (Berlanga *et al.*, 2011; He *et al*., 2013). However, termites may also harbour symbionts which, in contrast, may carry disadvantages. These are endosymbiotic parasitic bacteria that live within the cytoplasm of their host gonads and can induce several effects on reproduction. The most renown of these intracellular symbionts are, undoubtedly, α-proteobacteria of the genus *Wolbachia*. It includes obligate intracellular bacteria that are cytoplasmically inherited in arthropods and filarial nematodes (Lo & Evans, 2007). Four out the sixteen molecularly-identified supergroups of *Wolbachia* (Glowska *et al.*, 2015) infect termites: supergroups A, B, and F infects the majority of termite species, including both derived (i.e., *Reticulitermes*) and more primitive taxa (i.e., *Kalotermes*), while supergroup H is only found in *Zootermopsis* species (Lo *et al*., 2002; Salunke *et. al*, 2010).

The different phenotypes of *Wolbachia* infection are feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (CI; Figure 1.9). CI is the most frequent *Wolbachia*-induced phenotype and it consists in an incompatibility between sperm from infected males and eggs of uninfected females or between individuals harbouring different *Wolbachia* strains (Lo & Evans,

2007). The incompatibility generated by this microbe is due to the disruption of the hosts cell cycle, which results in an asynchronous development of male and female pronuclei (Werren *et al.,* 2008).

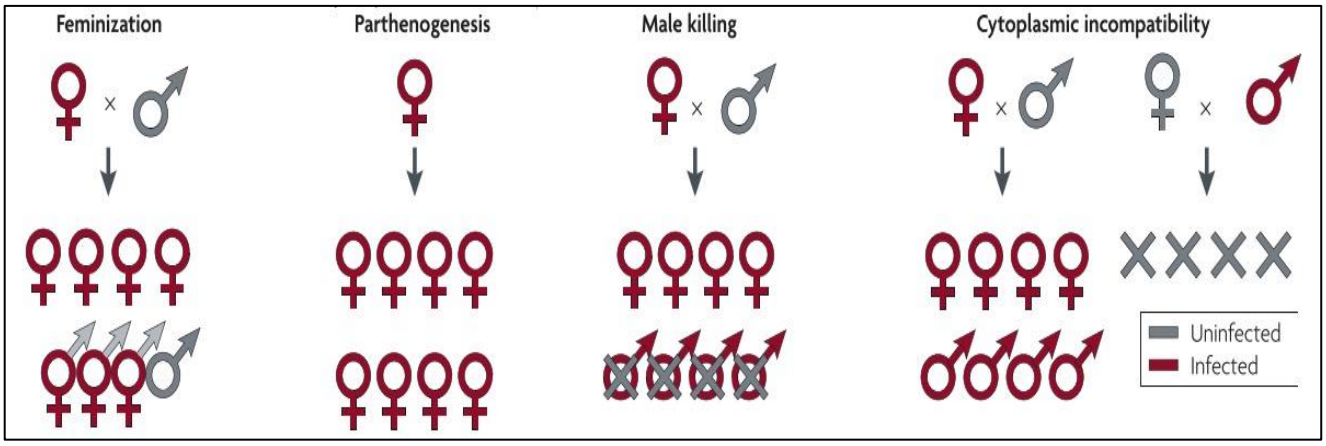

Figure 1.9 *Wolbachia*-induced phenotypes.
(source: Werren *et al.*, 2008)

CI induced by this microbe can results as a post-copulatory reproductive isolation and, for this reason, it was suggested that *Wolbachia* could be involved in speciation events (Brucker & Bordenstein, 2012). At the moment, however, the phenotype of *Wolbachia* infection in termites is currently unknown.

CHAPTER 2

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MOLECULAR MARKERS

Molecular markers are, at present, highly used tools for taxonomic, phylogenetic, phylogeographic and population genetics purposes. Their success is due to the fact that diagnostic morphological characters are often limited and difficult to identify, and because of a high intraspecific variability or peculiar adaptations. For termites, the existence of morphologically differentiated castes, often not available at the same time, adds to the difficulties in species identification. Molecular data, on the contrary, are available in large number and do not depend on intraspecific morphological variation or developmental stage. Another advantage of molecular data is that all known life forms are based on nucleic acids and, theoretically, each nucleotide position can be considered as a character and assumed to be independent.

Basically, a molecular marker is a particular segment of DNA that is representative of the variation at the genome level (Khan, 2015). An ideal molecular marker should be universal (present in all taxa), vertically inherited (inheritance can be monitored), ubiquitous and polymorphic. Moreover, it should be non-recombinant (but see below) and undergoing into neutral evolution. In fact, recombinant DNA sequences tend to be disregarded for phylogenetic purposes because their presence may hinder the correct signal, introducing instances of reticulate evolution. Furthermore, a marker under selective pressures will not vary or it will vary under constraints, therefore not providing useful information about taxa divergence time or returning genetic relationships biased by adaptation and/or convergent evolution (Ho, 2008).

In animals, a large number of different markers are available and each of them differs in their informational content, also depending on their origin (nuclear or mitochondrial DNA). Nuclear and mitochondrial genomes show, in fact, several differences. The former, usually, is bi-parentally inherited: it means that the genome, when bisexual reproduction takes place, undergoes meiosis and, therefore, may be subject to recombination events (e.g. crossing-over) and random homologues segregation followed by amphymixis. Overall, nuclear genes show lower levels of variability with respect to mitochondrial ones and, therefore, they are considered excellent markers for the phylogenetic analysis of distantly related species (Hirt & Horner, 2005). The nuclear genome embodies, though, also variable and hypervariable tracts. These can be so highly polymorphic to allow to differentiate among populations or even between individuals of the same population. For this reason, these hypervariable regions are the best option for population genetics and DNA fingerprinting analyses (Allendorf & Luikart, 2009). Examples of these markers are SNP, microsatellites, RFLP, AFLP and RADP. The first three markers are called *codominant* as they give the possibility to identify both alleles of any given locus, allowing to identify the heterozygote and homozygote profiles. AFLP and RADP, on the contrary, are defined as *dominant* markers: they only detect the presence\absence of a given allele, and cannot allow to discriminate heterozygote and homozygote profiles.

Mitochondrial DNA (mtDNA) is, in most of animals, uniparentally inherited from the parent female, exception being so far evidenced only in mollusks (Breton *et al.*, 2014; Gusman *et al.*, 2016). The mtDNA genome is haploid and do not experience crossing over. Animal mitochondrial genome is a relatively small circular molecule, typically comprising 37 genes, for a total of 15-20 Kb. Generally speaking, there are 13 genes encoding for protein subunits of the enzymes for the oxidative phosphorylation pathway (COI, COI, COIII, cyt-b, ND1-6, ND4L, ATP6, ATP8), two rRNA genes (12S and 16S), and 22 tRNA genes. It also includes a non-coding, hypervariable region

known as control region (CR). Notwithstanding its highly-conserved content, the gene order may vary in metazoan. mtDNA genes are usually more variable than the nuclear ones: in fact, the mutational rate of the mitochondrial genome is 5 - 10 times higher than in the nuclear genome (Lynch, 2007). This high mutation rate of the mitochondrial genome is caused by a low efficiency of DNA repair pathways and\or by a more mutagenic organellar environment. Yet, the mutational rate varies among its genes: protein coding genes, for example, show a low mutational rate while the control region is much more variable. The use of mtDNA as a marker is very popular in phylogenetic and population genetic studies since, being haploid, no allelic discrimination is required; hence, DNA sequences can be directly isolated and amplified (Hurst & Jiggins, 2005). Moreover, mtDNA can be easily amplified through PCR methodology thanks to the availability of universal primers, i.e. primers that allow to amplify the same DNA fragment in several different taxa (see, for example, Cheng *et al.*, 2012). Given all these features, mtDNA markers can be very powerful in resolving species-level phylogenies (Grechko, 2002). However, attention must be paid to the use of mitochondrial markers in specific instances. For example, being uniparentally inherited and, thus, haploid, mitochondrial markers do not allow to identify hybrid species. In this case, the use of codominant markers, such as microsatellites, is more appropriate.

During my PhD research, molecular analysis on termites have been performed using both nuclear (MS loci) and mitochondrial (COI/tRNALeu/COII portion, cytochrome oxydase II (COII) and 16S genes) markers, in order to achieve different goals. Moreover, the bacterial *FtsZ* gene has been used for the analyses dealing with *Wolbachia* microbe detection. In the following chapters, some general aspects of these markers are given.

2.1 MICROSATELLITES

Short Tandem Repeats (STRs), commonly called MicroSatellites (MS), are small portions of DNA composed by two up to six nucleotides (i.e. the motif) tandemly repeated for a variable number of times. Alleles are, thus, identified by the length of the tandem repeats array. Located in the nuclear genome of eukaryotes, microsatellites consist of non-coding DNA and, for this, they are considered neutral or, in some instances, experiencing only weak selective pressures. Microsatellites can mutate very quickly, with an estimated mutation rate of 10^{-3} each generation (Li *et al*., 2002). The most important mechanism generating mutation process is the "polymerase slippage" during DNA replication (Figure 2.1). This happens when one of the two DNA strands, after the separation and the following re-union, overlaps in a different position due to the repeated motif, forming a loop. When this loop is formed in the newly synthesized strand, the MS units will increase in number; on the contrary, the motif number will be reduced if the loop is formed in the template strand.

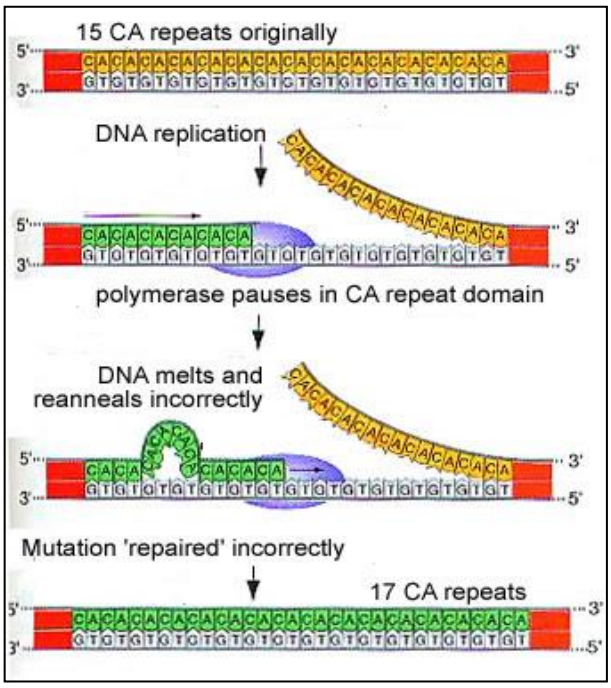

Figure 2.1 The *polymerase splippage* phenomenon [source: www. virtuallaboratory.colorado.edu]

MS genotyping at multiple loci allows to carry out population genetics studies, comparing the genetic variability and relationship among different populations. It is further useful to identify the parent-offspring relationship and the genetic relatedness among family members. In termites, therefore, in addition to population genetics studies, MS resulted particularly useful in the study of colony structure and reproductive strategies (Vargo & Husseneder, 2011).

2.2 CYTOCHROME OXIDASE I & II (COI & COII)

COI and COII genes encode for two of the seven polypeptide subunits in the cytochrome c oxidase complex, which is present in both bacteria and mitochondria. In *Reticulitermes* termites, the COI gene consists of approximately 1545 bp while COII is about 684 bp (Cameron & Whiting, 2007). COI and/or COII sequences have been applied to phylogenetic problems at a wide range of hierarchical levels in insects, from closely related species to genera and subfamilies, families, and even orders (Hebert *et al.*, 2003; Park *et al.*, 2012). The COI gene is slowly evolving if compared to other protein coding mitochondrial genes and it is the marker used for DNA barcoding in animals (Hebert *et al.*, 2003). The COII gene, although more variable than COI and not canonically used in DNA barcoding analyses, provides a good phylogenetic signal and its use in clarifying interspecific relationships is increasingly widespread (Roe & Sperling, 2007).

2.3 MICROBIAL *FTSZ* GENE

The bacteria-specific "filamenting temperature sensitive mutant Z" (*FtsZ*) is a nucleoid bacterial gene. It is about 750bp in length and belongs to the Fts gene complex encoding for cell division proteins. *FtsZ* protein plays a central role during bacterial cytokinesis. In fact, it assembles into the contractile Z-ring and coordinates more than a dozen other cell division proteins at the mid-cell site of the closing septum (Vollumer, 2008)

Considering its function, *FtsZ* protein is a prokaryotic homologue of the eukaryotic protein tubulin. *FtsZ* gene is one of the most used markers for detecting *Wolbachia* infection and for strains characterization, as demonstrated in several studies (e.g. Schulenburg *et al.,* 2000; Lo *et al.,* 2002; Bordenstein & Rosengaus, 2005; Casiraghi *et al.,* 2005*;* Simões *et al.*, 2011; Lefoulon *et al.*, 2016).

CHAPTER 3

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STATE OF THE ART AND RESEARCH AIMS

3.1 STATE OF THE ART

3.1.1 Genetic diversity and phylogeography of European termites

Native European termite species belong to two genera: *Reticulitermes* and *Kalotermes.* The former genus includes six subterranean-termite species (Austin *et al.*, 2002; Marini & Mantovani, 2002; Velona`*et al*., 2010; Dedeine *et al.,* 2016), with very different distribution ranges. *R. grassei* and *R. banyulensis* are distributed in the Iberian Peninsula (Kutnik *et al.* 2004), while *R. lucifugus*, the *R. grassei* sister species (Dedeine *et al*., 2016), occurs across Italy and in southern France. Two subspecies, *R.l.lucifugus* and *R.l.corsicus* are known from Penisular Italy-South France and from Corsica and Sardinia islands, with some population in Tuscany, respectively. A divergent mitochondrial lineage, probably a third subspecies, has been found in Sicily (Luchetti *et al.*, 2005, 2013c). In northern Italy, France and Germany is also present the invasive *R. flavipes*, native of North America (Ghesini *et al.* 2010; Perdereau *et al*. 2013). In the Balkans (from Croatian coasts down to Peloponnese and to Eastern Greece) two species are recognized: *R. urbis* (Croatia to Peloponnese) and *R. balkanensis* in Eastern Greece (Austin *et al.*, 2002; Uva *et al.,* 2004; Luchetti *et al.*, 2007; Dedeine *et al.*, 2016). *R. urbis*, recently described as new species by Bagnères *et al.*

(2003) shows a disjunct distribution in the Balkans, South and North-East Italy and South France, with two main mitochondrial lineages: these are geographically partitioned North and South to the Corinth strait, but were found mixed in Italian and French populations (Luchetti *et al.*, 2007; Leniaud *et al*., 2009a). In addition, this species is mainly observed in urban habitats in Italy and France, where it causes damages to structures and wooden artefacts (Leniaud *et al.*, 2009b; Ferrari *et al.*, 2011), while it can be commonly found in the wild within the Balkans range (Marini & Mantovani, 2002; Bagnères *et al.*, 2003; Uva *et al.*, 2004; Luchetti *et al.*, 2007; Perdereau *et al.*, 2013). Therefore, it was suggested that *R. urbis* is native from the Balkan Peninsula and was successively introduced in Italy and France (Luchetti *et al.*, 2007; Leniaud *et al.*, 2009a, b). On the other hand, a recent survey of Italian *Reticulitermes* species distribution questioned about the invasive status of *R. urbis* in Italy and France (Ghesini & Marini, 2012). Considering some similarities in the distribution areas of this species with those of the native *R. lucifugus*, basically consisting of the presence of both species only in urban habitats in the Italian northern regions, this study hypothesised a relict distribution of *R. urbis* in Italy and France rather than a secondary introduction (Ghesini & Marini, 2012). However, other studies suggested that also some *R. lucifugus* colonies could have been introduced in northern towns by human-mediated transports (Luchetti *et al.*, 2004; Luchetti *et al.*, 2013c), while its distribution follows a clear phylogeographic structure in agreement with a pattern of recent natural dispersion (Luchetti *et al.,* 2013c). On the whole, a deeper genetic and phylogeographic investigation is required to clarify the *R. urbis* distribution status. Very recently, *Reticulitermes* populations from the Aegean range and Cyprus have been described as a new species, *R. aegeus* (Ghesini & Marini, 2015).

As far as *Kalotermes* is concerned, recent molecular studies showed that two dry-wood termite species are present in Europe: *K. flavicolli*s and *K. italicus* (Velonà *et al.*, 2011; Ghesini & Marini, 2013; Luchetti *et al.,* 2013a). The first one comprises at least three main mitochondrial lineages

(Luchetti *et al.,* 2004, 2013a; Velonà *et al.*, 2011): the lineage A (also termed *K. flavicollis sensu stricto*; Velonà *et al.,* 2011; Luchetti *et al.,* 2013a) includes all samples collected from the Aegean islands (Crete and the Cyclades) to the Italian Peninsula; the lineage SC comprises colonies found in Sardinia and Corsica; the lineage SF is, actually, limited to Southern France. No data are available for the taxonomic and phylogenetic status of *Kalotermes* populations from the Iberia Peninsula (Maistrello *et al*., 2010). Furthermore, a fourth, highly divergent lineage, in sympatry with lineage A, was discovered in an Italian population and named lineage B. Interestingly, several colonies were found harboring mitochondrial DNA haplotypes of both lineages A and B and data on nuclear DNA markers suggested the possibility of interbreeding (Luchetti *et al.*, 2013a). The other species, *K. italicus*, easily recognizable as adult alates show a black or dark brown pronotum (as opposed to the yellow-necked *K. flavicollis*), was found only in Southern Tuscany and in a small area on the Italian mid-Adriatic coast (Ghesini & Marini, 2013). The taxonomy and the distribution pattern of *Kalotermes* taxa, thus, is far from being complete and many issues remain unresolved such as possible relationships among lineage B and *K. italicus*, and *Kalotermes* samples genetic characterization in the Iberian Peninsula.

3.1.2 Breeding systems and social organization in European termites

Luchetti *et al.* (2013b) described the occurrence of the AQS strategy (see Chapter 1.6.1) in Italian colonies of *R. lucifugus*. As explained in Chapter 1.6.1, the main diagnostic feature of this strategy is the facultative use of thelytokous parthenogenesis (Matsuura *et al*., 2009; Vargo *et al.*, 2012; Luchetti *et al.,* 2013b; Fougeyrollas *et al.,* 2015; Fournier *et al.,* 2016; Fougeyrollas *et al.,* 2017). In AQS termites, in fact, the primary queen, after founding the colony with the primary king, is replaced by multiple secondary queens, produced by thelytoky (Matsuura, 2017). In addition to

the main adaptive significance of the AQS strategy, e.g. the conservation of the founder queen genes even after her death and, thus, the maintenance of the initial level of genetic diversity in the colony over the time (Matsuura, 2017), it has been suggested that AQS system could provide the basis for a better understanding of the inclusive fitness theory (Hamilton, 1964) in social diploids organisms like termites (Kobayashi *et al*., 2013). Through a clear theoretical model, Kobayashi and co-workers (2013) showed, in fact, that the replacement of founders with parthenogenetic secondary queens and sexually produced secondary kings leads to a sex-asymmetric genetic inheritance increasing a female-biased allocation in the following offspring. However, this theoretical prediction was supposed only on the basis of a female-biased sex-ratio of dispersing reproductives in the other two congeneric AQS species, the Japanese *R. speratus* and American *R. virginicus* (Kobayashi *et al*., 2013), but no genetic investigations supporting such theoretical prediction, to date, were described. In *R. lucifugus*, further, data about sex-ratio of dispersers have not so far reported. It is therefore unclear whether the sex-biased resources allocation in swarming individuals could occur even at a morphological level, considering that a direct correlation between the expression of the AQS system and an increase in body size, as a consequence of a greater fertility, was described in secondary queens of *R. speratus* (Yamamoto & Matsuura, 2012). On the whole, deeper investigations for a better definition of the *R. lucifugus* AQS system, even at the morphological level, is required. As far as the other European *Reticulitermes* species are concerned, *R. urbis* does not display AQS strategy, as resulted from a genetic investigation performed in the Italian range (Luchetti *et al.,* 2013b). Even *R. grassei* and *R. flavipes*, on the basis of preliminary data (Matsuura, 2011; Dedeine *et al.*, 2016), do not seem to exhibit AQS strategy but no genetic investigations have been performed so far. In addition, the above mentioned preliminary investigations on *R. flavipes* were performed on native American populations but nothing is known about colonies in the invasive range. For this reason,

investigations about AQS occurrence in European *R. grassei* and *R. flavipes* (invasive range) samples appear to be needed.

Another particular strategy influencing the breeding system was described in the *Kalotermes* genus. In Italian populations of *K. flavicollis,* Luchetti *et al.* (2013a) demonstrated the occurrence of extreme colony fusion events explained in the light of the 'Accelerated Nest Inheritance' theory (Thorne *et al.*, 2003; see Chapter 1.5.1.). These colonies were found to fuse in the field, with instances of extreme fusion given by up to nine mitochondrial haplotypes even belonging to highly divergent genetic lineages (lineage A and Lineage B; see Chapter 3.1.1). Moreover, it was also observed that, after fusion, hybrid individuals emerged (Luchetti *et al.*, 2013a). Taking into account that the recently described *K. italicus* shows a sympatric distribution area with *K. flavicollis* (Ghesini & Marini, 2013) it is not to be excluded, therefore, a possible occurrence of this phenomenon even at an interspecific level. Obviously, this hypothesis requires further investigation.

3.1.3 *Wolbachia* and reproductive biology

As explained in Chapter 1.6.2, *Wolbachia* is an α-proteobacteria genus including parasitic endosymbiotic microbes which infect several termite species (Lo *et al*., 2002; Salunke *et. al*, 2010). Although the systematics and phylogeny of this termite symbionts as well as the *Wolbachia* lineages distribution among the hosts have been widely studied, it is poorly investigated if *Wolbachia* infection can affect the reproductive biology of termites (Matsuura *et al.*, 2004; Lo & Evans, 2007; Werren *et al.*, 2008). This microbe, in fact, can induce several effects on the host reproductive biology such as feminization, male killing, parthenogenesis and cytoplasmic incompatibility (Lo & Evans, 2007).

For native European *Reticulitermes* species, evidences about *Wolbachia* infection were described only in the soldier cast of *R. grassei* (Berlanga *et al*., 2011), the only taxon analyzed so far. Previous analyses revealed *Wolbachia* occurrence also in the American *R. flavipes* and the Japanese *R. speratus*, the latter being the first AQS species identified (Matsuura *et al.*, 2004; Matsuura *et al*., 2009). The presence of *Wolbachia* infection in both a non-AQS and an AQS species does not suggest an involvement of *Wolbachia* in the conditional use of parthenogenesis during AQS. Notwithstanding these previous results, the analysis of *Wolbachia* occurrence in the Italian AQS *R. lucifugus* (Luchetti *et al.*, 2013b) could be of interest.

As far as the *Kalotermes* genus is concerned, *Wolbachia* infection was detected only in *K. flavicollis,* at that time the only known species of this genus in Europe, infected by F strain (Lo *et al*., 2002; Casiraghi *et al.,* 2005). No investigations, thus, were performed so far on the newly described *K. italicus*. Moreover, as previously reported, hybridization and colony fusion events occur in *K. flavicollis* (Luchetti *et al.*, 2013a). It was supposed that the possibility of hybridization between different genetic lineages could facilitate the fusion of more than two colonies overcoming mechanisms of nest-mate recognition (Thorne *et al.*, 2003), but no investigations have been so far performed in order to clarify if *Wolbachia* may be involved in such hybridization and, therefore, colony fusion events occurring in this taxon.

On the whole, at least as a preliminary investigation, the analysis to detect *Wolbachia* occurrence in European termites appears of interest.

3.2 AIMS

Taking into account the above reported data, my PhD research project focused on the analysis of the genetic diversity and breeding systems in European *Reticulitermes* and *Kalotermes* taxa.

Investigations were performed at the intrageneric level with comparisons also at the intergeneric one in order to highlight new insights on the evolution of eusociality.

My surveys, performed through genetic and morphological approaches, had the following main goals:

- A deep phylogeographic investigation of Balkan, Italian, and French colonies of *R. urbis* in order to clarify the invasive status of this species and to identify the native source population(s). Analysis were conducted using mitochondrial DNA sequences of cytochrome oxidase II (COII) and 6 microsatellite loci.
- A more detailed picture of *Kalotermes* biogeography and evolution in Europe, using the highly informative cox1/trnL/cox2 mitochondrial DNA marker i) to analyze samples also from previously unsampled areas, ii) to define the relationships between lineage B and *K. Italicus*, iii) to quantify the real extent of colony mixing in these termites.
- A microsatellite survey of the French *R. grassei*, *R. flavipes* and Italian *R. lucifugus* populations in order to verify, providing genetic evidences, the reproductive strategies of the former two species and to describe, more in detail, the occurrence of the AQS strategy in Italian colonies of *R. lucifugus*.
- To test sex-biased resources allocation through sex ratio evaluation and morphometric analyses on swarming individuals belonging to *R. lucifugus* colonies, trying to understand the evolutionary and ecological forces behind the onset of this phenomenon.
- A preliminary molecular investigation, using the bacterial *FtsZ* marker, on *Reticulitermes* and *Kalotermes* termite species in order to identify *Wolbachia* infection and to characterize the relevant strains, paying particular attention to the AQS species *R. lucifugus* and to mixed colonies of the *Kalotermes* genus to verify whether *Wolbachia* presence can be related to parthenogenesis or hybridization events.

The above reported topics will be presented in Chapters 4-8. In Chapter 9, results obtained will be discussed in a comparative view.

While Chapters 4 and 5 correspond to either printed or DOI available papers, Chapters 6, 7 and 8 are attached as papers to be submitted.

CHAPTER 4

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GENETIC DIVERSITY AND INVASION HISTORY OF THE EUROPEAN SUBTERRANEAN TERMITE *RETICULITERMES URBIS* (BLATTODEA, TERMITOIDAE, RHINOTERMITIDAE)

Biol Invasions
DOI 10.1007/s10530-017-1510-5

ORIGINAL PAPER

Genetic diversity and invasion history of the European subterranean termite *Reticulitermes urbis* (Blattodea, Termitoidae, Rhinotermitidae)

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Abstract Biological invasions are among key factors of ecological changes, and social insects appear as highly successful invasive animals. Subterranean termites of the holarctic genus *Reticulitermes* are present in Europe with six native and one invasive (the nearctic *R. flavipes*) species. The species *R. urbis* shows a disjunct distribution in the Western Balkans, Eastern Italy and Southern France. Previous molecular and population genetics data suggested that the taxon originated from the Balkans, and that Italian and French populations are invasive, but it is still unknown how many introduction events occurred and from which Balkan source populations. To address these questions, a population genetics analysis was performed on a larger sampling than previous studies, using mitochondrial cytochrome oxidase II and 6 microsatellite markers on 47 colonies collected across the whole distribution area. Mitochondrial analysis confirmed the presence of two major lineages where colonies from Balkans, Italy, and France intermingle. Similarly, microsatellite loci analysis indicated the presence of two genetic clusters, though not corresponding to the two mitochondrial clades, each including colonies from the three sampled areas and with individuals showing mixed cluster membership. Overall, French and Italian populations showed indications of bottleneck (reduced genetic diversity and change of allele frequencies) and do not appear genetically differentiated from the Balkan population. Results presented here support a history of multiple introductions in Italy and France, in a scenario consistent with continuous exchanges between native and invasive areas, as expected along human trades routes.

Keywords Invasive species · Population genetics · mtDNA · Microsatellites · Social insects · Termite

Electronic supplementary material The online version of this article (doi:10.1007/s10530-017-1510-5) contains supplementary material, which is available to authorized users.

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Introduction

A species that spreads outside its native range, settling and expanding into a new introduced range, and impacting upon local biodiversity and resources is defined as invasive (EU Regulation No. 1143/2014; Kolar and Lodge 2001). Biological invasions often cause environmental changes such as ecosystem degradation and reduced biodiversity, as well as

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problems to agriculture and public health (Evans et al. 2013). In some instances, invasive species can cause extensive economic damage as pests in urban areas and anthropic settlements (Pejchar and Mooney 2009). Understanding the success of biological invasions is essential to develop effective prevention and management strategies against invasive species. A crucial piece of information to understand the invasion success is the determination of the geographical origin of these species (Caldera et al. 2008; Muirhead et al. 2008). Phylogenetic and population genetics studies can help to obtain information about the source population(s), invasion routes, and the pattern of colonization (Perdereau et al. 2011, 2013a). Social insects are among the most successful animal species at invading new environments, and their invasiveness can be favored by life-history traits such as social structure, breeding system, and mode of dispersal as well as the ability to resist to biotic aggression thanks to both individual- and colony-level responses (Moller 1996).

Most studies on invasive social insects are concerned with Hymenoptera (Buttermore 1997; Holway et al. 2002; Tsutsui and Suarez 2003; Ascunce et al. 2011; Lander et al. 2014; Sarnat et al. 2015), while much less is known about the invasive biology of termites, despite the evidence that they can provide an interesting framework for understanding processes of biological invasions (Vargo and Husseneder 2009; Evans et al. 2013; Perdereau et al. 2015; Buczkowski and Bertelsmeier 2016) and their impact on local ecosystems (Holt and Lepage 2000; Sugimoto et al. 2000). Termites can be also destructive pests causing extensive damage to cellulose-containing materials and wooden structures; therefore, they are among the most destructive global pest species with an estimated global economic impact higher than $40 billion (US) per year, with subterranean termites alone causing about 80% of damages (Su and Scheffrahn 2000; Su 2002; Rust and Su 2012).

Reticulitermes Holmgren, 1913 is a Holarctic genus of subterranean termites belonging to the Rhinotermitidae family (Blattodea, Termitoidae). *Reticulitermes* species are widespread in temperate regions where they play important ecological roles, especially in the recycling of organic matter (Bignell and Eggleton 2000). Furthermore, *Reticulitermes* termites frequently attack wooden structures and, consequently, cause significant economic damage in urban settings (Su 2002). According to recent time-scaled phylogenetic studies, this genus shows four main lineages distributed among four major geographical regions: north America, western Europe, eastern Europe + western Asia, and eastern Asia (Dedeine et al. 2016). Eight *Reticulitermes* species are found in the Mediterranean Basin (Austin et al. 2002; Marini and Mantovani 2002; Luchetti et al. 2004, 2007, 2013a; Uva et al. 2004; Velonà et al. 2010; Ghesini and Marini 2012). *R. grassei* and *R. banyulensis* are distributed in the Iberian Peninsula and in southern France (Kutnik et al. 2004). *R. lucifugus* occurs across Italy and in southern France with two known subspecies and, based on mitochondrial phylogeny, probably a new subspecies in Sicily (Luchetti et al. 2004, 2013a). In the eastern Mediterranean basin, three species are found: *R. balkanensis* in Greece, *R. clypeatus* in Israel, and the newly described species, *R. aegeus*, in the Aegean islands (Austin et al. 2002; Luchetti et al. 2007; Ghesini and Marini 2015). In addition, two invasive *Reticulitermes* species are found in the Mediterranean basin. The first one, the north American species, *R. flavipes*, was introduced to France from Louisiana (USA), and now can be found in France, Germany and Italy (Ghesini et al. 2010; Perdereau et al. 2013a, b). The second species, *R. urbis* (Bagnères et al. 2003), shows a disjunct distribution in the Balkans (Greece and Croatian coasts), Italy, and France, with two mitochondrial lineages distributed along the northern and the southern area of the Corinth strait, respectively, but mixed in Italian and French populations (Luchetti et al. 2007; Leniaud et al. 2009a). These phylogenetics and population genetics studies, then, suggested that *R. urbis* is native from the Balkan Peninsula and was successively introduced in Italy and France (Luchetti et al. 2007; Leniaud et al. 2009a). This is consistent with the fact that it is mainly observed in urban habitats in Italy and France whereas it can be found in the wild in the Balkans range (Marini and Mantovani 2002; Bagnères et al. 2003; Luchetti et al. 2007; Perdereau et al. 2013b). In its introduced ranges, large colonies colonized the oldest part of towns damaging structures and wooden artifacts. In the northeastern Italian town of Bagnacavallo, the management of termite infestation control took almost 15 years (Ferrari et al. 2011), and the eradication plan took six years with an economic impact of about € 1 million (Municipality of Bagnacavallo, press release of 12th

August 2006; http://www.comune.bagnacavallo.ra.it/). A similar situation occurred in the entire town of Domène in France, which was entirely infested by a single genetic colonial entity (Leniaud et al. 2009b).

On the other hand, a recent survey of Italian *Reticulitermes* species distribution questioned about the invasive status of Italian and French *R. urbis* populations based on some similarities in their distribution areas with those of the native *R. lucifugus* (Ghesini and Marini 2012). Basically, these similarities are that they are both present only in urban habitats in the Italian northern regions and this has been explained with possible microclimatic oscillations that may have allowed termite to thrive in the wild and then forced their retreat within towns as temperatures cooled (Ghesini and Marini 2012). This interpretation, though, cannot be conclusive because also *R. lucifugus* can be introduced in northern town by human-mediated transport (Luchetti et al. 2004; Luchetti et al. 2013a). Moreover, for *R. lucifugus* a clear phylogeographic structure is evident along its distribution, in agreement with a pattern of recent natural dispersion (Luchetti et al. 2013a). On the contrary, the pattern of natural dispersion does not hold for Italian *R. urbis* populations (Luchetti et al. 2007). As subterranean termites are particularly subject to human-mediated transport (Jenkins et al. 2001; Perdereau et al. 2013a, b), a correct phylogeographic signal may be drawn only from a wide sampling. Here, we present a genetic and phylogeographic analysis conducted on mitochondrial DNA sequences of cytochrome oxidase II (COII) and 6 microsatellite loci in Greek, Italian, and French *R. urbis* colonies. The sampling presented in this study, wider than previous ones, confirmed the invasive status of *R. urbis* in Italy and France. Moreover, this study also adds knowledge about possible routes of invasion and the minimum number of introduction events of this pest termite.

Materials and methods

Sample collection

A total of 47 sites were sampled from 29 European localities (Fig. 1; Table 1). Five sites were sampled within pine-woods across western Greece (from Peloponnesus to the Epirus coast), and three along the Croatian coast. Thirty-nine sites were further sampled: 15 in southern France, two in the northeast and 22 in the southeast of Italy. Overall, 94 workers, two for each site, have been analyzed. Specimens were preserved in 95% ethanol until DNA extraction.

Molecular techniques

Total DNA was isolated from termite heads, to avoid contamination with gut endosymbionts, using the CTAB method (Doyle and Doyle 1987). The mitochondrial cytochrome oxidase II (COII) gene was amplified with the primers: B-tLys (5′-GTT TAA GAG ACC ATT ACT TA-3′, Simon et al. 1994) and a modified A-tLeu (5′-CAG ATA AGT GCA TTG GAT TT-3′, Miura et al. 2000). PCR amplification was performed on 20 ng of template DNA in a 50 µl mixture with the GoTaq G2 Flexi DNA Polymerase kit (Promega, Madison, WI, USA), following the manufacturer protocol. Thermal cycling was as follows: an initial denaturation step at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, followed by annealing at 54 °C for 30 s, and extension at 72 °C for 30 s; and a final elongation step at 72 °C for 7 min. PCR products were purified using the Wizard SV PCR and Gel cleaning kit (Promega) and sequenced with the Sanger method at Macrogen Inc., European Laboratory. Obtained sequences have been checked with MEGA v. 6 (Tamura et al. 2013) and haplotypes have been submitted to GenBank, under accession numbers MF374825–MF374832.

All 94 individuals were also genotyped at six microsatellite loci (Rf6-1, Rf21-1, Rf5-10, Rs10, Rs15, Rs33) previously described by Vargo (2000) and Dronnet et al. (2004). PCR amplification was performed as for the COII gene but using modified thermal cycling conditions, with an annealing temperature of 58 °C. Forward primers were WellRED 5'dye-labeled oligo (Sigma), with dye-labeling as follow: D2-PA for Rf6-1 and Rf5-10; D3-PA for Rf21-1 and Rs10; D4-PA for Rs15 and Rs33. PCR products were then read on a Beckmann Coulter CEQ^{TM} 8000 Genetic Analysis System to determine alleles size.

Genetic diversity

Haplotype and nucleotide diversity (h_d and π) were estimated from mitochondrial data using DnaSP v5.10.1 (Librado and Rozas 2009). Allelic richness (Ar), the mean number of effective alleles (Ne), observed (H_O)

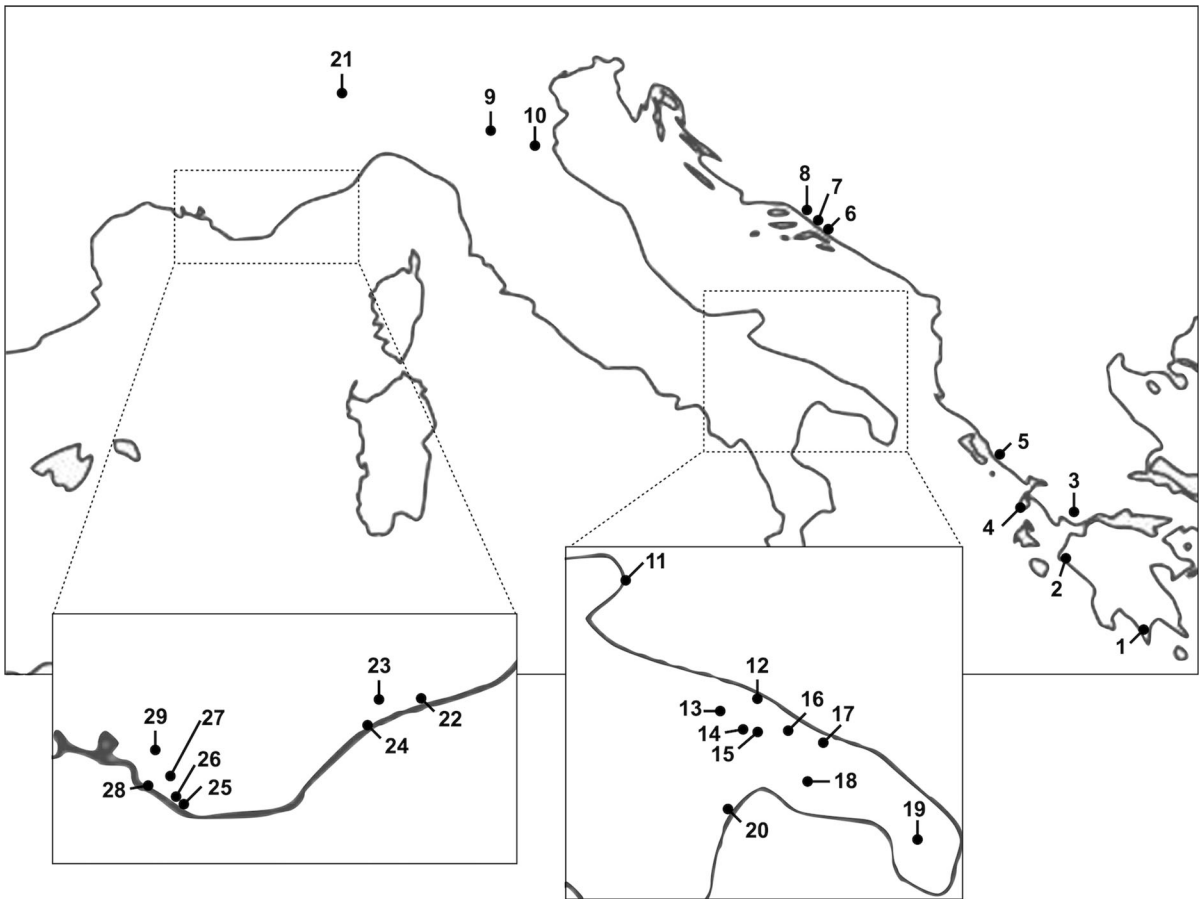

Fig. 1 Map of sampling localities. Numbers refer to Table 1

and expected heterozygosity (H_E) were calculated for microsatellite data using GenAlEx v. 6.502 (Peakall and Smouse 2012), GENEPOP v. 1.2 (Raymond and Rousset 1995) and FSTAT v. 2.9.3.2 (Goudet 1995). BOTTLENECK v. 1.2.02 software (Piry et al. 1999) with Infinite Allele Model (IAM), the Stepwise Mutation Model (SMM), and the Two-Phase Model (TPM), was used to check whether there was any evidence of recent bottlenecks in the introduced populations.

Phylogenetic and population structure analyses

The Maximum Likelihood phylogenetic tree, with 100 bootstrap replicates, and the estimate of the best substitution model (HKY) were determined using MEGA v. 6. The haplotype parsimony network was calculated using TCS v. 1.13 (Clement et al. 2000).

The analysis of molecular variance (AMOVA) and pairwise F_{st}, which measure the amount of genetic differentiation between populations, among the three geographic areas were performed with GenAlEx software, with 999 permutations. Bonferroni correction has been used for multiple comparisons. Genetic clusters were calculated by means of Bayesian analyses performed with STRUCTURE 2.3.4 (Pritchard et al. 2000), combining nuclear and mitochondrial data. The genetic membership q was inferred by considering probabilities through 1 million Markov Chain Monte Carlo simulations, after a burn-in period of 500,000 runs. The best value of K (=genetic groups) was then calculated using log likelihood (Pritchard et al. 2000) and delta K (Evanno et al. 2005), running all simulations with the admixture model of 100,000 repetitions after a burn-in of 50,000 repetitions, for each value of K between 1 and 20. The "admixture model" was enabled during the running and individuals were considered as having mixed genotype if membership proportion resulted >0.2. Finally, Nei's

Table 1 Sampling localities of analyzed colonies and COII haplotypes

Localities		Acronyms	COII haplotypes
Balkans			
1	Areopolis	ARE	H1
2	Kato Achaia	KAC	H2
3	Katoki	KAT	H3
4	Lefkada	LEF	H4
5	Sivota	SIV	H5
6	Klek	KLE	H3
7	Gradac	GRA	H5
8	Zivogosce	ZIV	H3
Italy			
9	Bagnacavallo	BGN	H6
10	Salsomaggiore	SAM	H3
11	Torre Calalunga	TCL	H3
12	Bitonto	BIT1	H7
		BIT2	H3
		BIT3	H5
		BIT4	H3
13	Bari	BAR	H3
14	Selva di Fasano	SEF	H6
15	Copertino	COP	H6
16	Bosco delle Pianelle	BOP	H5
17	Turi	TUR	H5
18	Cassano delle Murge	CMU	H5
19	Mercadante	MER1	H5
		MER2	H5
		MER3	H5
		MER4	H5
		MER5	H5
20	Castellaneta Marina	CAS1	H3
		CAS2	H5
		CAS3	H8
		CAS4	H5
		CAS5	H3
		CAS6	H3
France			
21	Domène	DOM1	H5
		DOM2	H5
		DOM3	H5
22	Allauch	ALL	H3
23	Ceyreste	CEY	H3
24	La Ciotat	LAC1	H3
		LAC2	H3
25	St Cyr Les Lecques	SCL	H3
26	St Cyr Sur Mer	SCM1	H3
		SCM2	H3
27	Cannes	CAN1	H3
		CAN2	H3
28	Nice	NIC	H3
29	Sofia Antipolis	SOA1	H3
		SOA2	H3

Numbers refer to Fig. 1

D pairwise genetic distances (Nei 1972) were used to compute a Principal Coordinates Analysis (PCoA), through GenAIEx, to further examine the genetic relationships among samples.

Results

Genetic diversity

Mitochondrial DNA (COII)

Ninety-four sequences were obtained from the analyzed samples and all individuals from the same colony showed identical nucleotide sequence. The sequences obtained across the collection sites ($n = 47$) showed 16 variable alignment positions. They allowed the characterization of eight haplotypes (Table 1) that differed each other by 1–11 nucleotide substitutions (Supplementary Table S1). In the Balkans five haplotypes were identified (H1, H2, H3, H4, and H5), while in Eastern Italy and Southern France five (H3, H5, H6, H7 and H8) and two (H3 and H5) haplotypes were detected, respectively (Table 1). Three out of the five haplotypes recorded in Italy (H6, H7, H8) were only present in this area. By contrast, the two Southern France haplotypes also occurred in the Balkans and in Italy (Table 1). Both COII haplotype and nucleotide diversities computed in the Balkans were slightly higher than those scored in French and Italian areas (Table 2).

Microsatellites

The six loci were all polymorphic within the sampled areas. Overall, the number of alleles per locus ranged

Table 2 Genetic diversity values (Mean ± S.E.) within Balkans, Italian and French ranges for mitochondrial COII (number of haplotypes, N_h; haplotype diversity, h_d; nucleotide diversity, π) and nuclear microsatellite (number of effective alleles, Ne; allelic richness, Ar; observed (H_O) and expected (H_E) heterozygosity) markers

	COII			Microsatellite			
	N_h	h_d	π	Ne	Ar	H_o	H_E
Balkans	5	0.857 ± 0.108	0.010 ± 0.009	3.279 ± 0.683	5.166 ± 0.872	0.240 ± 0.057	0.614 ± 0.082
Italy	5	0.688 ± 0.063	0.007 ± 0.001	2.017 ± 0.320	3.719 ± 0.602	0.233 ± 0.050	0.445 ± 0.080
France	2	0.448 ± 0.134	0.005 ± 0.019	1.977 ± 0.212	3.303 ± 0.375	0.194 ± 0.042	0.454 ± 0.077

Table 3 Allele frequency at six analyzed microsatellite loci in native and introduced ranges

Locus	Allele size	Balkans	Italy	France
Rf6-1	125	0.063		
	131	0.125		0.033
	134			0.017
	137		0.010	
	140	0.313	0.010	0.033
	143	0.125	0.594	0.500
	149	0.063	0.135	
	152	0.188	0.021	
	158	0.063		
	161	0.063	0.229	0.417
Rf21-1	178	0.656	0.229	0.367
	181	0.313	0.750	0.600
	184	0.031	0.021	0.033
Rs15	214		0.021	
	256	0.063	0.042	0.150
	259	0.125	0.010	
	262	0.250	0.302	0.033
	265	0.094	0.125	0.583
	268	0.156	0.052	
	271	0.313	0.427	0.167
	274		0.021	0.067
Rf5-10	136	0.063		0.033
	139	0.250	0.094	0.017
	142	0.094	0.010	
	145	0.125		
	148	0.031	0.125	
	151	0.406	0.771	0.950
	154	0.031		
Rs10	150	0.813	0.927	0.750
	153	0.031		0.067
	156	0.031		
	159	0.125	0.073	0.183
Rs33	252	0.375	0.052	0.050
	256	0.563	0.688	0.483
	260	0.063	0.073	
	272		0.010	
	276		0.177	0.467

from 3 (Rf21-1) to 10 (Rf6-1). Allele frequencies vary widely between Balkans and French/Italian populations at all loci (Table 3). The mean value of the effective alleles (±S.E.) ranges from 1.977 ± 0.212 (South France) to 3.279 ± 0.683 (Balkans), and the allelic richness (±S.E.) spans from 3.303 ± 0.375 (South France) to 5.166 ± 0.872 (Balkans) (Table 2). Observed heterozygosity (H_O) values were similar between the Balkans and the Italian populations, but lower in Southern France (Table 3). On the other hand, expected heterozygosity (H_E) resulted higher in the Balkan colonies than in French and Italian ones (Table 2). Overall, the Balkans populations appeared to be more variable than the others, although the difference is not statistically significant. Results from BOTTLENECK analysis indicated the evidence of bottleneck effect only in the Italian populations (SMM and TPM $p < 0.01$).

Phylogeny and population structure

The Maximum Likelihood tree constructed with mitochondrial COII sequences showed two well-supported clades where *R. urbis* sequences are partitioned. Sequences from the sites collected in the Balkans distribute in the two clades: LEF, KAT, ZIV and KLE, on one side, and SIV, GRA, KAC and ARE, on the other side (Fig. 2a). Consistently with previous

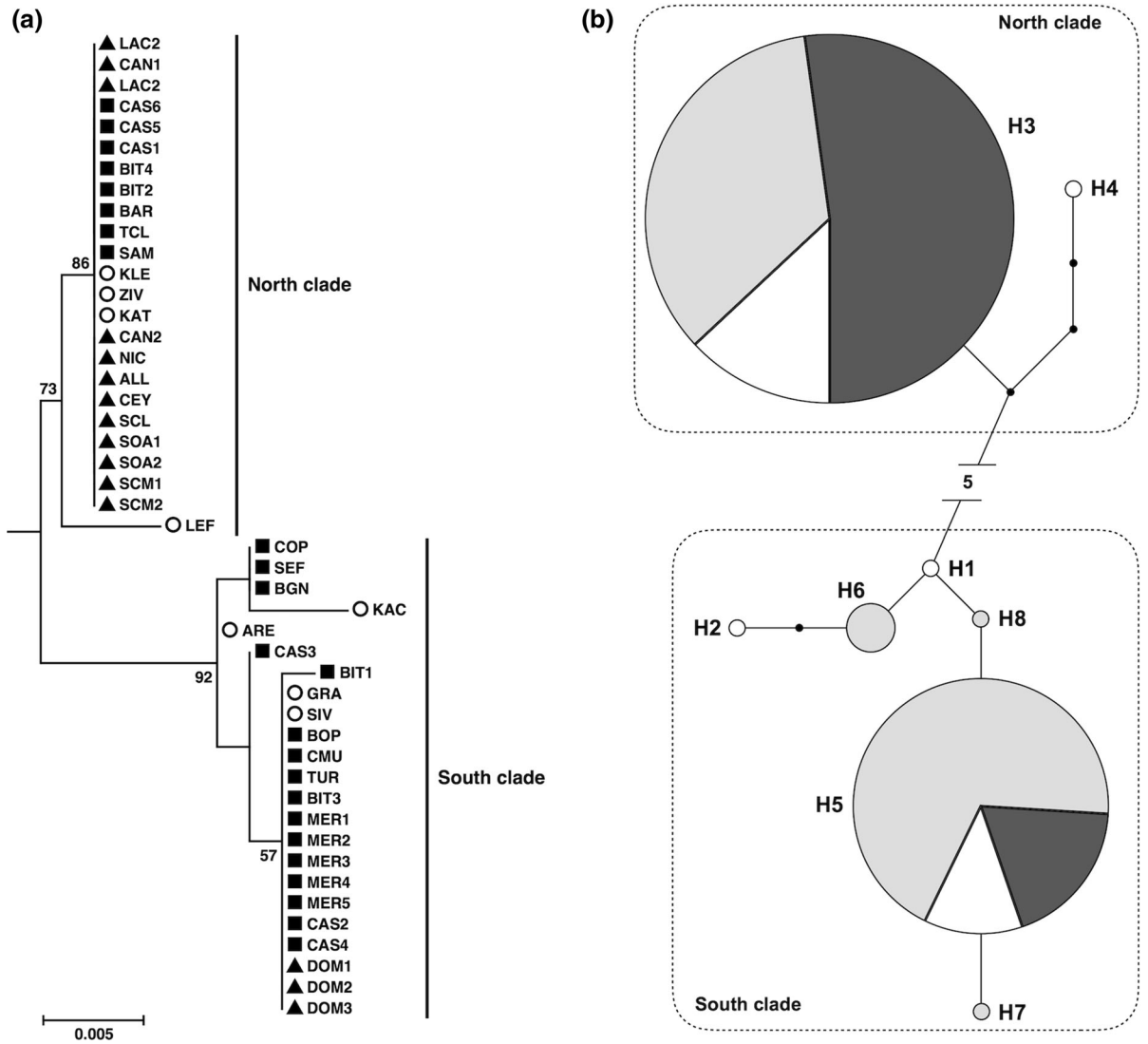

Fig. 2 Mitochondrial COII analyses. **a** Maximum Likelihood tree (−lnL = 1120.52). Numbers at nodes are bootstrap support >50%. *Open circles* indicate samples from Balkans; *filled squares* and *triangles* represent samples from Italy and France, respectively. **b** Parsimony network haplotype analysis. *Circles* widths are proportional to haplotype frequency; *white areas* represent proportion of Balkans samples; *grey areas* are Italian range colonies and *dark grey* areas indicate the French populations. Small, *black dots* indicate missing/ideal haplotypes; the number between subnetworks indicates the number of missing/ideal haplotypes needed to connect them

analyses, this pattern allowed us to identify the first clade as the North clade, and the second one as the South clade (Luchetti et al. 2007). On the other hand, sequences obtained from France and Italy were scattered across the two clades: eight out of 24 East Italian samples grouped in the North clade, while the remaining 16 samples were grouped in the South clade. The absence of any relationship between geographic distribution and clustering was particularly evident for Italian colonies collected in the same locality (for example, BIT and CAS) or in the same region (e.g., SAM and BGN). In both cases, haplotypes were scattered across the two clades (Fig. 2a). On the other hand, southern France samples were clustered in the North clade, with only the samples from Domène (DOM1-3) falling into the South clade (Fig. 2a).

The statistical parsimony network connected all the COII haplotypes, the two haplotype groups observed

in the phylogenetic analysis being connected by five mutational steps (Fig. 2b). The AMOVA analysis performed on microsatellite data revealed that the variation among colonies and within individuals explain the higher amount of the observed variation (35 and 40%, respectively). On the other hand, divergences between Balkans samples as well as French and Italian ones only accounted for 11% of the variation. Pairwise F_{st} values showed significant divergences between the three regions examined, although higher F_{st} values were observed between the native and introduced ranges (Table 4).

Table 4 Pairwise F_{st} and related probability

	F_{st}	p
Balkans vs Italy	0.141	<0.001
Balkans vs France	0.181	<0.001
Italy vs France	0.098	<0.001

The Bayesian clustering analysis performed with STRUCTURE suggested that samples can be grouped in 2 genetically distinct clusters supported by high values of LnP(K) and Delta K when K = 2 (Mean LnP(K) = −1277.6; Delta K = 31.4) (Fig. 3a). Balkans colonies appeared to belong to both clusters: although well-defined, the two clusters do not group colonies according to mitochondrial DNA-defined South and North clades. Colonies collected from Italian and French ranges showed either total membership to one of the two genetic clusters or, in seven instances, a mixed membership to both clusters (Fig. 3a). In agreement with AMOVA and STRUCTURE results, the PCoA analysis revealed that samples from the three areas cannot be distinguished (Fig. 3b).

Discussion

In the present study we analyzed the genetic diversity for both mitochondrial and nuclear markers of an

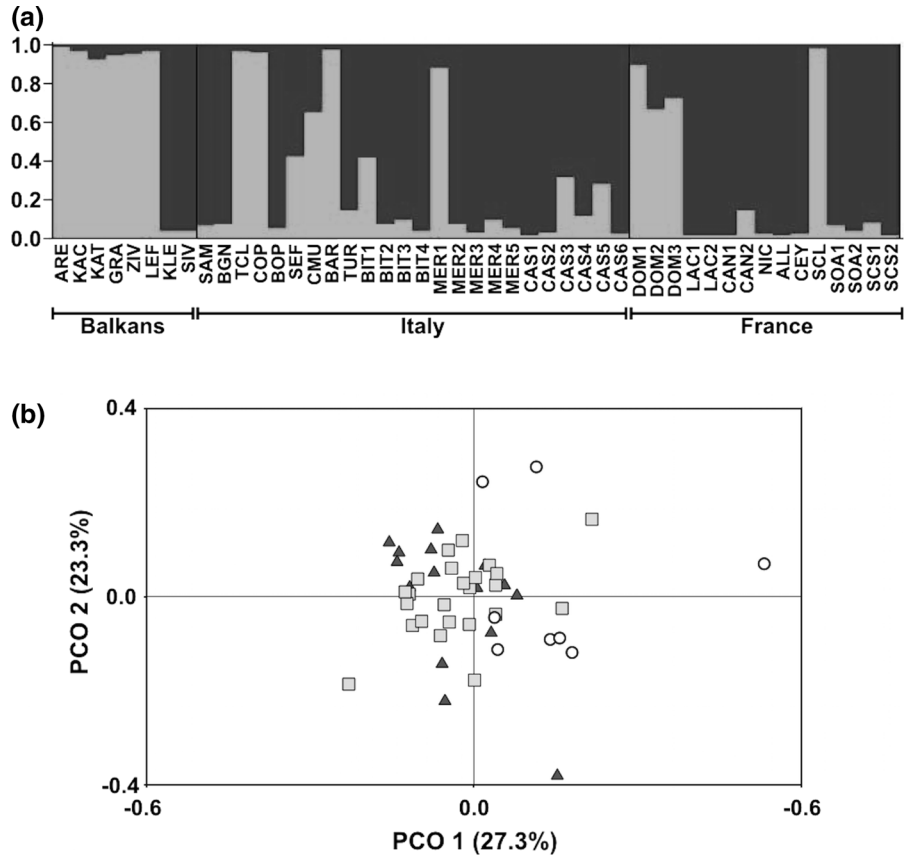

Fig. 3 Microsatellite loci analyses. **a** STRUCTURE plot. Each *vertical bar* represents a single individual that is attributed to one of the two genetic clusters (K) or that shows mixed membership. **b** Principal Coordinate Analysis (PCoA) performed on Nei's D distance. *Open circles* indicate samples from Balkans; *grey squares* and *dark grey triangles* represent colonies from Italy and France, respectively

European termite species, *R. urbis*. This work aimed to verify the invasive status of the French and Italian populations, as well as to identify the route of invasion and estimate the number of introduction events that led to the present-day distribution. All data presented here indicate the absence of a clear phylogeographic structure in the studied populations; this general pattern supports the hypothesis that this species was introduced in Italy and France from the Balkan Peninsula (Luchetti et al. 2007; Leniaud et al. 2009a).

Notwithstanding the overall low genetic diversity scored at both mitochondrial and nuclear markers, our data point out a lower diversity in the Italian and French populations with respect to the Balkans area. Although not statistically significant, the low genetic diversity of introduced populations is consistent with a previous analysis (Leniaud et al. 2009a). It is well-known that, after invasion, colonizers may show a reduction in genetic diversity due to the founder effect (Puillandre et al. 2008). Reduction in allelic diversity is a strong predictor of genetic bottleneck, although it may not appreciably affect observed heterozygosity. For example, the loss of rare alleles has little influence on the heterozygosity level (Allendorf 1986; Spencer et al. 2000; Dlugosh and Parker 2008). Although the allelic richness is lower among Italian and French colonies, the observed heterozygosity is comparable to that observed within the Balkans range. On the other hand, expected heterozygosity in Italian and French populations resulted lower than in the Balkan population. It has been shown the expected heterozygosity predicts possible bottlenecks better than the observed one (Spencer et al. 2000): this is in line with traces of a bottleneck effect found in the Italian sample.

Overall, mitochondrial DNA analyses clearly provided evidences for the distinction of two clusters, which regroup the North and the South samples. These two clades were already identified in the native area of *R. urbis* (Luchetti et al. 2007). Microsatellite data also identified two distinct genetic clusters in Balkans colonies, although these lineages do not coincide with the two mitochondrial clades. Contrasting patterns between mitochondrial and nuclear markers are often observed and may result from different factors such as different evolutionary histories of the two genomes, sex-biased dispersal, allele/haplotype introgression and/or secondary contacts between divergent populations (Toews and Brelsford 2012). One likely explanation in our case is that the introgression of alleles and/or haplotypes that followed secondary contacts has shaped the present pattern. North and South clades originally referred to the fact that they included colonies collected north or south of the Corinth Canal, respectively. However, one haplotype from the South clade was found in two samples collected north of Corinth Canal, Sivota and Gradac (included in the present analysis), suggesting that migration took place after clade divergence (Luchetti et al. 2007). According to divergence time estimates, the split between the two mitochondrial lineages dates back to 2–4 million years ago (Velonà et al. 2010; Dedeine et al. 2016), suggesting that Italian and French populations originated later. In fact, colonies collected in the invasive range exhibited scattered representation in the two mitochondrial clades and nuclear clusters. Two out of five haplotypes found in the Balkans were found in 79% of Italian colonies and 100% of French ones. Furthermore, 33% of Italian colonies and 80% of French ones fall in the North clade. As far as microsatellite data is concerned, 79% of Italian colonies and 80% of French ones showed full membership to one of the two genetic clusters identified by STRUCTURE. Therefore, as also supported by AMOVA and PCoA analyses, there is no substantial divergence between Balkans, Italian, and French populations. On the other hand, F_{st} analysis between the three areas gave significant values, indicating that populations became substantially isolated from each other after the invasion. This is likely the effect of a genetic bottleneck after the introduction, as it may cause changes in allele frequencies among invading individuals (Spencer et al. 2000; Dlugosh and Parker 2008; Kinziger et al. 2011). Such a hypothesis is consistent with the wide variation of allele frequencies observed among populations analyzed in the present study.

The data presented here confirm the invasive status of Italian and French *R. urbis* populations, and also suggest that multiple introduction events occurred from different populations of the native range. Mitochondrial DNA clearly indicates that northeastern and southeastern Italian colonies originated from native populations belonging to both North and South clades. On the other hand, in the southern French range, only the colony from the city of Domène appears to have originated from a South-clade population. *R. urbis* from Domène are known to live in a super-colony, a unique social organization so far documented in termites. Genetic data obtained in this colony are consistent with a

possible origin from a few, closely related individuals or even from a single pair of reproductives (Leniaud et al. 2009b). Present microsatellite data indicate a mixed membership of Domène samples, which would suggest the introduction from (at least) two genetically distinct colonies that crossed after coming into contact. The fact that all three Domène samples share the same haplotype is consistent with the already discussed discordance between mitochondrial DNA clades and the two nuclear genetic clusters.

While in the French area individuals with genotypes derived from the crossbreeding of the two genetic clusters are limited to Domène, mixed membership genotypes are well represented across the Italian range. Multiple invasions are generally thought to help the invasion phase, because the introduction of diverse gene pools may limit the reduction of genetic diversity and have a significant impact on the survival chance of invaders. Multiple invasions usually lead to higher genetic diversity, although exceptions have been reported (Dlugosh and Parker 2008; Hagenblad et al. 2015). Under these considerations, presently observed mixed-membership genotypes reflects post-invasion crosses: individuals introduced in multiple, independent events and from different source populations would have successively crossed leading to the presently-observed mixed genotypes. It is also worth noting that repeated introductions from multiple source populations could explain why the reduction of genetic diversity in introduced populations is not statistically significant. Based on scored allele frequencies, it appears that in many cases some alleles reached high frequency among invaders, while they were at low or intermediate frequency in the native genetic pool. This may be explained by post-invasion genetic drift or by selective pressures promoting some alleles over other ones.

It is interesting to speculate about the historical conditions that may have triggered *R. urbis* invasions in Italy and France. For example, between the 8th and 5th century B.C., Greek peoples colonized southern Italy and southern France: Taraes (now Taranto, Apulia) and Massalia (now Marseille) were among the first founded Greek colonies (Astour 1985). Notably, citizens from Taraes lately founded further colonies in southern Apulia, such as Hydruntum (now Otranto) and Callipolis (now Gallipoli) (Astour 1985). It is thus possible that human repeated migrations from Greece to overseas colonies would have mediated the introductions of *R. urbis*. Interestingly, *R. lucifugus* colonies in Southern France are also suspected of being secondarily introduced during this same time frame (Lefebvre et al. 2008). Moreover, later, the Byzantine Empire extended from the Balkans to Eastern Italy, with Ravenna (in northeastern Italy) as the main outpost (Jeffreys et al. 2008): this is consistent with the presence of *R. urbis* in Ravenna and surrounding towns (Luchetti et al. 2007).

This investigation provides further evidence about the origin and dispersion of *R. urbis* in the Mediterranean, and demonstrates multiple introductions. Other animal species having a trans-Adriatic distribution similar to the *R. urbis* range show an exclusive genetic composition consistent with their biogeography (Schmitt and Seitz 2001; Mattucci et al. 2016). The absence of such a phylogeographic pattern of distribution in this study suggests that *R. urbis* is an introduced species in Western Europe. Italian and French invasive populations came from the Eastern Balkans, the native range of this species, as a result of multiple introduction events. Our findings allow us to reconstruct the history of *R. urbis* in Western Europe, and provide a more detailed knowledge for future studies about reproductive strategies adopted during the invasions, such as the capacity of colonies to produce numerous functional secondary reproductives (neotenics) of both sexes (Luchetti et al. 2013b; Perdereau et al. 2013a, b). Reproductive strategies of *R. urbis* revealed that introduced colonies do not reproduce through parthenogenesis and have a balanced sex ratio of winged adults (Luchetti et al. 2013b). It will be interesting to study more in detail the reproductive biology and dispersal mode of *R. urbis* in its native range in order to verify if these characteristics could be among post-invasion adaptations facilitating the setting of a stable population (Luchetti et al. 2013b), as also suggested in other subterranean species (Perdereau et al. 2015).

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Table S1. Number of substitutions observed between scored COII haplotypes

	Haplotype	1	2	3	4	5	6	7
1	H1							
2	H2	4						
3	H3	7	9					
4	H4	9	11	4				
5	H5	2	6	9	11			
6	H6	1	3	8	10	3		
7	H7	3	5	8	10	1	4	
8	H8	1	5	8	10	1	2	2

CHAPTER 5

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MOLECULAR SYSTEMATICS, BIOGEOGRAPHY, AND COLONY FUSION IN THE EUROPEAN DRY-WOOD TERMITES *KALOTERMES* spp. (BLATTODEA, TERMITOIDAE, KALOTERMITIDAE)

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Molecular systematics, biogeography, and colony fusion in the European dry-wood termites *Kalotermes* spp. (Blattodea, Termitoidae, Kalotermitidae)

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Abstract

European dry-wood termites belong to the genus *Kalotermes* (Kalotermitidae), one of the two termite genera in Europe. Until the recent description of two new species, *Kalotermes italicus* in Italy and *Kalotermes phoenicae* in the eastern Mediterranean area, *Kalotermes flavicollis* was the only taxon known in this region. The presence of additional entities, suggested by morphological and physiological variation observed in *K. flavicollis*, was supported by molecular studies revealing four distinct genetic lineages: lineage A, *K. flavicollis sensu strictu*, from the Aegean area to Italy; lineage B, in Tuscany; lineage SC, in Sardinia and Corsica; lineage SF, in southern France. Lineages A and B may form mixed colonies, suggesting hybridization. To draw a more detailed picture of *Kalotermes* evolution and biogeography in Europe, we analyzed samples from previously unsampled areas, such as Spain and southern Italy, by means of the highly informative *cox1*/*trnL*/*cox2* mitochondrial DNA marker. Overall, phylogenetic analyses confirmed previously identified lineages and taxa, but widened the distribution of the lineage SC to the mainland and of the lineage SF to Spain and Portugal. Results further provided evidence for the synonymy between lineage B and *K. italicus*. Species delimitation analysis suggested that the three *K. flavicollis* lineages, as well as *K. italicus*, can be separate taxa. Data also suggest a possible interspecific hybridization between *K. italicus* and both *K. flavicollis* lineages A and SC.

Keywords: hybridization, molecular diversity, social insects, European species distribution, termites, colony structure

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Introduction

Termites are wood-feeding eusocial insects related to cockroaches; they are ecologically important due to their role in the decomposition of organic matter (Bignell & Eggleton, 2000). Two termite genera, *Reticulitermes* and *Kalotermes*, are distributed in Western Europe. The former is a genus of subterranean termites (Rhinotermitidae) occurring along the Mediterranean and Atlantic coasts, as well as in urban areas, with colonies often composed by diffuse nests and multiple feeding sites connected by underground tunnels (Vargo & Husseneder, 2009). Dry-wood termites of the genus *Kalotermes* (Kalotermitidae) are more restricted to Mediterranean coasts where they form small colonies in deadwood of various tree species.

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Until recently, only one species of *Kalotermes* was thought to be distributed across Europe, the yellow-necked *K. flavicollis*. Early studies, though, had already noticed morphometric and physiological variations between Italian, Sardinian and French populations (Luscher, 1956; Springhetti, 1967). More recently, molecular studies showed that the taxon *K. flavicollis* is, in fact, composed by at least three main lineages that could represent distinct taxa (Luchetti *et al.*, 2004, 2013a; Velonà *et al.*, 2011). Lineage A includes all samples collected from the Aegean islands (Crete and the Cyclades) to the Italian peninsula. This genetically homogenous lineage was previously termed *K. flavicollis sensu stricto* (Velonà *et al.*, 2011; Luchetti *et al.*, 2013a). Lineage SC includes colonies collected in Sardinia and Corsica, while lineage SF comprises those collected in southern France; this third lineage appeared significantly diverging from both lineages A and SC (Velonà *et al.*, 2011). Furthermore, a fourth, highly divergent lineage was found in sympatry with lineage A in an Italian population and termed lineage B. Interestingly, several colonies were found harbouring mitochondrial DNA haplotypes of both lineages A and B and data on nuclear DNA markers suggested the possibility of interbreeding (Luchetti *et al.*, 2013a).

Beside *K. flavicollis*, two new *Kalotermes* species were recently described (Ghesini & Marini, 2013, 2015). The first new species, *Kalotermes italicus*, is recognizable by a black (or dark brown) pronotum; it is found in central Italy on both sides of the peninsula (Ghesini & Marini, 2013). Interestingly, Becker (1955) described a *K. flavicollis* form with black pronotum, designated as 'var. *fuscicollis*', and demonstrated that the two color variants can interbreed giving offspring with dark or dark-yellow pronotum. The second new species, *Kalotermes phoenicae*, was found in Cyprus and along Lebanon and Israel coasts (Ghesini & Marini, 2015). Altogether, these studies shed new light on the biodiversity of European *Kalotermes* termites.

The taxonomy and the distribution pattern of *Kalotermes* taxa are far from being complete and many issues remain unresolved. For instance, *K. italicus* was found only in three localities in Central Italy (Ghesini & Marini, 2013), although its geographical distribution is probably more extensive. The same is true for the French lineage and nothing is known on the taxonomic and phylogenetic status of *Kalotermes* from the Iberia peninsula (Maistrello *et al.*, 2010). To increase the knowledge about *Kalotermes* diversity, taxonomy, and distribution in Europe, we sequenced 911 bp of the highly informative *cox1*/*trnL*/*cox2* mitochondrial DNA region for 43 colonies collected from 28 locations from Spain to southern Italy, including previously unsampled areas of Sicily and Sardinia. Data were then integrated with those provided from previous studies to get a more global picture.

Materials and methods

New collection points were chosen to cover previously unsampled or poorly sampled areas. Termites were collected in the field from logs or other pieces of dead wood; most of the specimens were pseudergates (i.e., false workers), which constitute the majority of the colony. For each collection point, pseudergates were carefully taken from the same tunnel and were considered to belong to the same colony. The only exception was the sample of Renzetto (REZ), where we caught swarming alates instead of pseudergates from tunnels. Therefore, we cannot exclude that REZ individuals belong to distinct colonies. All samples were conserved in 100% ethanol until molecular analyses. In total, 43 colonies from 28 localities were analyzed (table 1 and fig. 1a).

Total DNA was isolated using the CTAB method (Doyle & Doyle, 1987) from two pseudergates per colony, with the exception of five colonies in which a single individual was analyzed (table 1). A 911 bp mitochondrial fragment encompassing a part of the *cox1*, the entire length of *trnL*, and a part of the *cox2* regions was PCR amplified and sequenced using the primers C1-J-2797 (5′-CCT CGA CGT TAT TCA GAT TAC C-3′) and TK-N-3785 (5′-GTT TAA GAG ACC AGT ACT TG-3′). Amplification reactions were performed in 50 µl mixtures, using 20 ng of template DNA, with GoTaq DNA polymerase kit (Promega, Madison, WI, USA) following the manufacturer's protocol. The PCR amplification program includes: initial denaturation for 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 50°C, 30 s at 72°C; final extension for 7 min at 72°C. Sanger sequencing of both strands was performed at Macrogen Europe (The Netherlands). Sequences were submitted to Genbank, under accession numbers MF589135–MF589164.

The 81 sequences obtained in this study were analyzed together with sequences taken from previous studies (Luchetti *et al.*, 2004, 2013a; Velonà *et al.*, 2011; Ghesini & Marini, 2013), the *cox2* sequence from a Portuguese sample of *K. flavicollis* (GenBank accession number DQ442147; Inward *et al.*, 2007) and two *cox1*/*trnL*/*cox2* haplotypes of *K. italicus* from Grosseto and Portonovo samples (Ghesini & Marini, 2013). Moreover, two *cox2* sequences belonging to the two divergent lineages of *K. phoenicae* were added (samples Benouaiti and Kaplica, accession numbers KC914299 and KC914300; Ghesini & Marini, 2015). Finally, the *cox2* of the New Zealand species *Kalotermes brouni* (accession number AF189104; Thompson *et al.*, 2000) was used as outgroup.

Sequence alignment (with Clustal W algorithm), molecular divergence (uncorrected *p*-distance), and the best substitution model were calculated using MEGA v. 7 (Kumar *et al.*, 2016). The best substitution model was obtained for each gene individually (*cox1*: T92; *trnL*: JC; *cox2*: HKY + G) and for the entire region (HKY + G + I). Maximum Likelihood phylogenetic tree was calculated using MEGA v. 7, with nodal support based on 100 bootstrap replicates. As MEGA v. 7 does not allow to treat partitions separately, the substitution model HKY + G + I was used for the entire sequence. Bayesian Inference was calculated with MrBayes v. 3.2 (Ronquist *et al.*, 2012) on a gene-partitioned data set, running for 10^6 generations and sampling trees every 500 generations. Convergence was reached when the average divergence of split frequencies fell below 0.01. Maximum Likelihood and Bayesian Inference methods yielded a substantially identical topology and similar confidence levels; the Maximum Likelihood tree was therefore used for further analysis.

Haplotype (h_D) and nucleotide diversity (π), and Tajima's *D* analyses were computed with DnaSP v. 5.1 (Librado & Rozas, 2009). Species delimitation was estimated by using three different methods: single threshold GMYC (Generalized Mixed Yule Coalescent; Fujisawa & Barraclough, 2013), PTP (Poisson Tree Processes; Zhang *et al.*, 2013), and statistical parsimony network (Hart & Sunday, 2007). As GMYC results appeared to be strictly dependent on the method used for ultrametric tree calculation, we followed Tang *et al.*'s (2014) advice and used BEAST v. 1.8 (Drummond & Rambaut, 2007). Moreover, possible biases due to the molecular clock algorithm used

Table 1. List of colony sampling, with scored haplotypes per colony.

	Sampling locations	Colony ID	Haplotypes per colony
1	Portonovo	PTNa	H1
		PTNb	H1
2	Sirolo	SIRa	H2/H3
		SIRb	H1
3	Renzetto	REZ[1]	H3
4	Tremiti Islands	TRE	H4
5	Bari	BAR	H3
6	Davoli Marina	DVM	H3/H5
7	San Sostene	SST	H3
8	Sant'Andrea Apostolo dello Ionio	STA	H3
9	Agrigento	AGR	H3
10	Cinnisi	CNS	H3/H6
11	Firenze	FIRa	H7
		FIRb	H8
		FIRc	H1
		FIRd	H9
12	San Rossore Natural Reserve	ROSa	H1/H10
		ROSb	H7/H11
		ROSc	H7/H12
13	Feniglia Natural Reserve	FENa	H13/H14
		FENb	H15/H16
		FENc	H3/H17
14	Capalbio	CAPa	H18
		CAPb	H19
15	Pescia Marina	PEMa	H20
		PEMb	H21
16	Montalto di Castro	MOC	H3
17	Riva dei Tarquini	RTAa	H22
		RTAb	H3/H23
18	Fregene	FRE	H24
19	Ostia	OST	H3
20	Sabaudia	SAB	H3
21	Monterosso	MTR	H25
22	Nozarego	NOZa	H7
		NOZb	H26
		NOZc	H27
23	Siniscola	SIN	H7
24	Marseilles	MAR[2]	H1
25	Banyuls-sur-Mer	BAMa[2]	H28
		BAMb[2]	H29
26	Santa Cristina d'Aro	SCA[2]	H30
27	Logrono	LOG[2]	H30
28	Siviglia	SIV	H30

[1]Only swarming individuals.
[2]A single individual sequenced.

(Monaghan *et al.*, 2009) were overcome with the use of four ultrametric trees obtained with different settings: we built trees using both strict and lognormal relaxed clocks, each implementing either the Yule or the coalescent (with constant population size) tree priors. Calibration was arbitrarily set, imposing the age of the ingroup node to 1.0 and modelling a normal prior distribution with 0.1 of standard deviation; this was done to facilitate the convergence of runs. Each tree was, then, calculated after two runs set at 20×10^6 generations each, sampling every 1000, and the convergence was assessed by estimated sample size >200. The PTP analysis was performed on the web server http://species.h-its.org/, using 5×10^5 Markov chain Monte Carlo generations, *burnin* = 0.25 and removing the outgroup. Finally, the parsimony network was obtained through TCS v. 1.21 (Clement *et al.*, 2000), calculating the 95% connection limit between possible sub-networks: putative specific entities are discriminated based on the number of sub-networks.

Results

Thirty haplotypes, differing from 1 to 65 nucleotide substitutions, were identified (H1-H30; table 1) in the 81 sequences obtained in this study. The most common haplotype (H3) is distributed from Sicily (AGR) up to the Feniglia Reserve (FENc) (table 1).

Maximum Likelihood and Bayesian Inference trees were built on haplotypes from all data available (present data; Luchetti *et al.*, 2004, 2013a; Velonà *et al.*, 2011; Ghesini & Marini, 2015). Obtained trees gave overlapping topologies and split haplotypes in two main clusters, each further structured into well-supported sub-clusters. These clusters mirror known *K. flavicollis* lineages and *K. italicus* species (fig. 2).

The first main cluster is subdivided into two sub-clusters (fig. 2). The first one embodies haplotypes H3, H5, H13-15, H20-24, H27 and the samples known to belong to *K. flavicollis* lineage A. It also includes a further small cluster grouping haplotypes H6 and H7 together with the sequences of lineage SC. The second sub-cluster shows a sister relationship with the other one, and groups haplotypes H28-30 together with those of *K. flavicollis* lineage SF. The *cox2* of the Portuguese sample of *K. flavicollis* is also included in this sub-cluster, being identical to haplotype H30. Given the absence of sub-structures in this lineage, it will be henceforth referred to as the Ibero-French lineage (lineage IF). The second main cluster is also structured in two sub-clusters (fig. 2). The first one (I) includes haplotypes H16, H18, and H19, *K. flavicollis* lineage B from Feniglia, and *K. italicus* from Grosseto. The second sub-cluster (II) groups the remaining 10 haplotypes and the other two sequences of *K. flavicollis* lineage B (Rimigliano) and *K. italicus* (Portonovo). The two *K. phoenicae* samples form a single clade that has a sister relationship with the two main clusters (fig. 2).

The sequence divergence between clusters and sub-clusters varies widely, ranging from 1.2 to 6.1–6.7% (table S1). *K. flavicollis* lineage B + *K. italicus* cluster appeared the most variable based on both haplotype and nucleotide diversity (table 2). The *K. flavicollis* lineage IF showed a slightly higher haplotype diversity than *K. flavicollis* lineages A and SC, the latter appearing as the less variable one (table 2). Tajima's *D* values resulted negative for the four lineages, with only *K. flavicollis* lineages A and SC showing significant departures from 0 (table 2).

Overall, the three species delimitation methods are congruent in defining some entities and discordant in other instances (fig. 3). The GMYC method gave the higher number of putative entities, ranging from 6 to 9 depending on the ultrametric tree used. When using the relaxed clock with Yule prior, GMYC splits lineage A into four distinct taxa, while it indicated only two possible species when using the strict clock tree with coalescent prior. In comparison, PTP recognized a single entity. Although the parsimony network groups lineages A and SC in a single taxon, the latter lineage is always defined as a single, separate entity in the other analyses (fig. 3). Lineage IF is indicated as a distinct taxon by all methods, while variation in species delimitation can be observed across methods for the *K. flavicollis* lineage B + *K. italicus* clade (fig. 3). GMYC defined three or two taxa and, again, the use of relaxed clock with Yule prior gave more estimated species. On the other hand, PTP and parsimony analyses indicated this clade as a single entity.

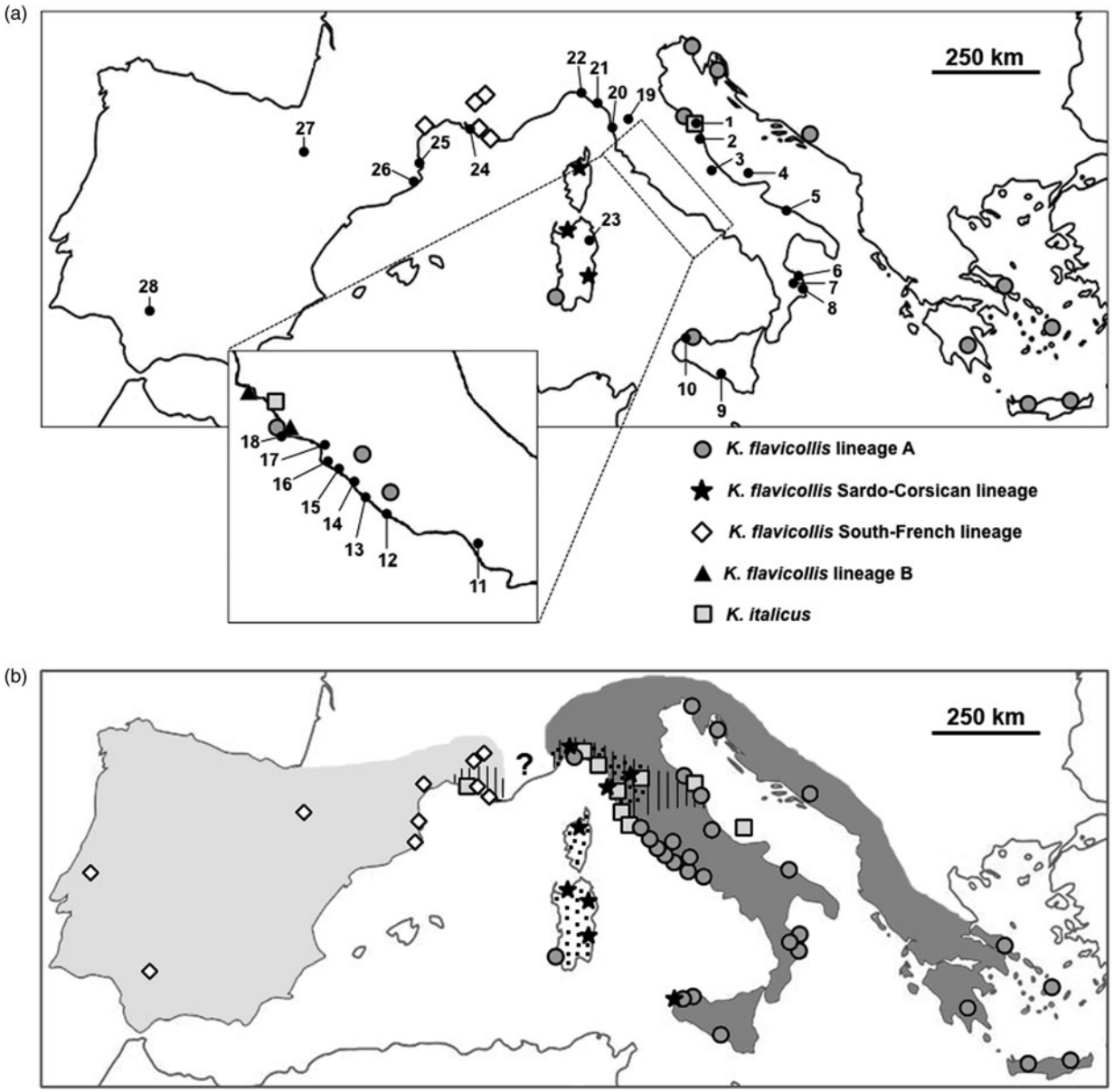

Fig. 1. (a) *Kalotermes* sampling locations and lineages distribution known so far. Numbers refer to table 1. (b) Summary of European *Kalotermes* taxa distribution as derived from the present analysis. Light gray area: *Kalotermes flavicollis* lineage IF; dark gray area: *K. flavicollis sensu strictu*; dotted area: *K. flavicollis* lineage SC; hatched area: *K.italicus*. The question mark indicates the lack of information about the distribution boundaries of *Kalotermes* taxa in that range.

Of the 38 colonies for which two individuals were sequenced, different haplotypes were found in ten (26.3%; table 1). In six instances, the two distinct haplotypes even belong to different clusters (table 1; fig. 2; summarized in table 3). The Sicilian sample from Cinnisi (CNS) exhibited haplotypes from *K. flavicollis* lineages A and SC, while two San Rossore colonies (ROSb and ROSc) carried haplotypes from *K. flavicollis* lineage SC and *K. flavicollis* lineage B + *K. italicus* clade. Finally, colonies FENb and FENc, from the Feniglia Natural Reserve, and SIRa, from Sirolo, contained haplotypes of both *K. flavicollis* lineage A and *K. flavicollis* lineage B + *K. italicus* clade.

Discussion

The evolutionary diversification pattern of the genus *Kalotermes* is poorly known in Europe, compared with the European *Reticulitermes*. In particular, the taxonomic level of divergence among lineages and the geographical range of taxa distribution still remain to be defined. The present survey provides additional knowledge on the systematics, evolutionary history, and biogeography of *Kalotermes* taxa across the Mediterranean area.

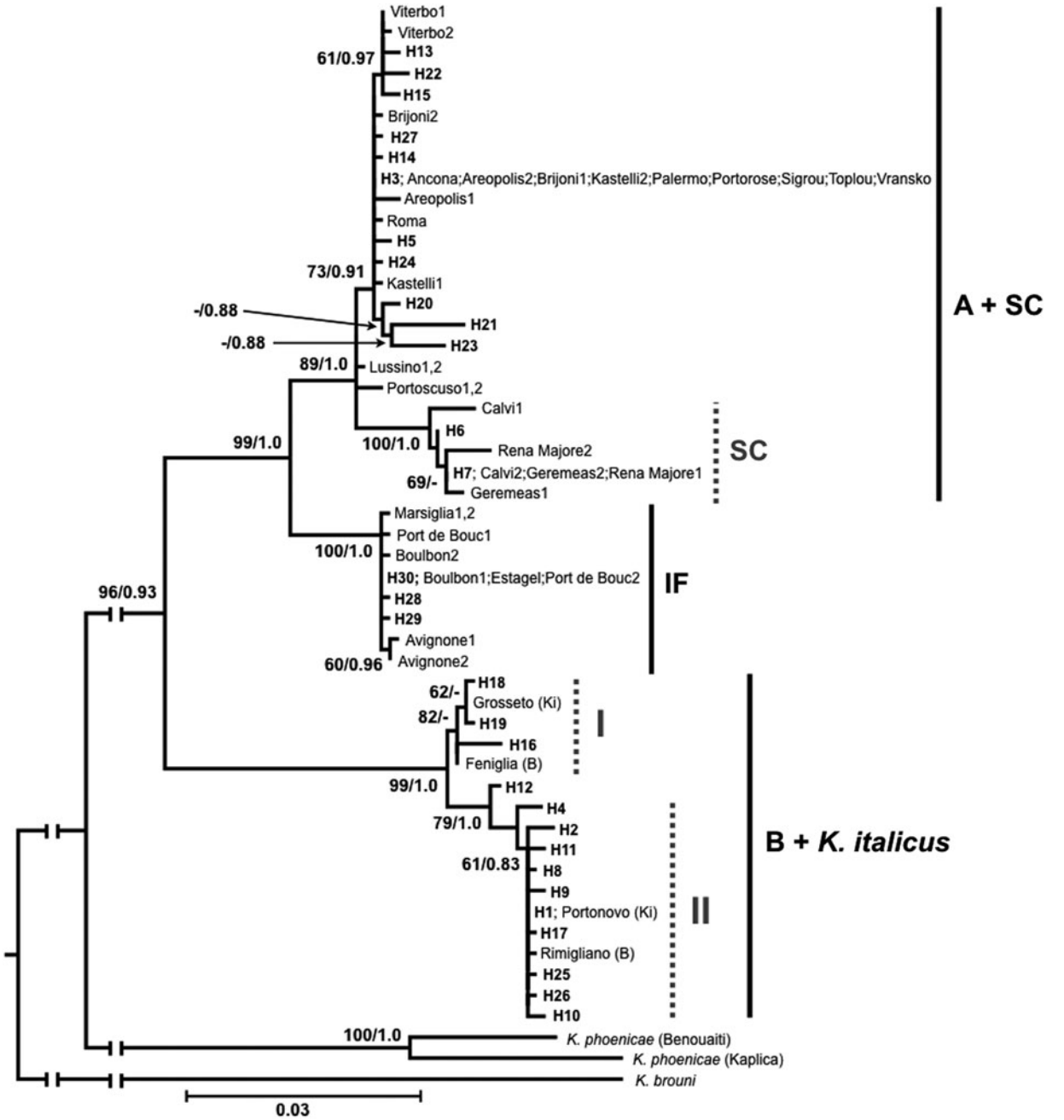

Fig. 2. Maximum Likelihood tree (−*lnL* = 2953.453) obtained from *cox1*/*trnL*/*cox2* haplotypes. Bayesian Inference analysis (−*lnL* = 2973.756) resulted in an overlapping topology. Haplotype codes as in table 1; previously identified haplotypes are reported with the name of the sampling location (consistently with Velonà *et al*., 2011). Lineages are indicated with vertical bars. In the B + *K. italicus* cluster, samples previously ascribed to lineage B are indicated with 'B' in brackets, while those described as *Kalotermes italicus* are indicated with 'Ki'. Numbers at nodes are bootstrap values >60%/Bayesian posterior probabilities >0.8. Abbreviations: A, lineage A; B, lineage B; SC, Sardo-Corsican lineage; IF, Ibero-French lineage.

Phylogenetic relationships among Kalotermes *lineages*

The present analysis is based on a single mitochondrial fragment that proved to be informative, especially to identify new phylogenetic lineages (Velonà *et al*., 2011; Ghesini & Marini, 2013, 2015; Luchetti *et al*., 2013a,b). The nucleotide variability scored reveals that the *Kalotermes* genus in Western Europe is structured in two well-supported clusters. The first one, including lineages A, SC, and IF, clearly shows a monophyletic origin, with lineage IF branching first. The relationship between lineages A and SC appears less clear, mostly due to Lussino and Portoscuso haplotypes, which clearly diverged from lineage A. The colony of Portoscuso was already

Table 2. Genetic diversity and Tajima's *D* test for scored *Kalotermes* lineages.

Lineage	N	h_N	h_D	S	π	*D*
K. flavicollis A	64	19	0.647	41	0.0027	−2.413**
K. flavicollis SC	15	5	0.476	13	0.0019	−2.227**
K. flavicollis IF	17	8	0.728	7	0.0011	-1.737^{ns}
K. flavicollis B + *K. italicus*	34	17	0.877	28	0.0055	-1.080^{ns}

N, number of sequences; h_N, number of haplotypes; h_D, haplotype diversity; *S*, number of segregating sites; π, nucleotide diversity; ns, not significant; $^*P < 0.05$; $^{**}P < 0.01$.

interpreted as a divergent haplotype within lineage A (Velonà *et al.*, 2011). The second cluster includes sequences of *K. flavicollis* lineage B (Luchetti *et al.*, 2013a) and the recently described species *K. italicus* (Ghesini & Marini, 2013). This cluster is partitioned in two sub-clusters, with a nucleotide divergence similar to the one scored between *K. flavicollis* lineages A and SC (1.2 vs. 1.5%; table S1). However, haplotype pairs belonging to lineage B and *K. italicus* samples cluster together, supporting the hypothesis that *K. flavicollis* lineage B and *K. italicus* are the same taxon. Therefore, all samples included into this cluster will be considered as *K. italicus*.

Species delimitation and taxonomic considerations

The three methods used to delimitate *Kalotermes* species gave slightly different results. The GMYC method, which is widely used for species delimitation on a single marker, is known to be strictly dependent on the algorithm used for ultrametric tree calculation (Monaghan *et al.*, 2009; Tang *et al.*, 2014). The analysis conducted with *Kalotermes* sequences confirmed this observation, with different results depending on the clock model and/or the tree prior used. The use of a strict clock with a coalescent prior gave the most conservative result and it is more consistent with PTP and parsimony analyses. Irrespective of the clock model and prior used, GMYC analyses always indicated that Portoscuso and Lussino haplotypes constitute a taxonomic entity that is separated from all other haplotypes grouped within lineage A. This is consistent with previous results (Velonà *et al.*, 2011). On the other hand, the PTP and parsimony analyses did not differentiate these two haplotypes from other clades within lineage A. It has been observed that the GMYC method may not perform well when dealing with poly- or paraphyletic lineages (Hendrich *et al.*, 2010): this could be the case of *K. flavicollis* lineage A, as the divergence of Portoscuso and Lussino haplotypes place them in an unresolved position (fig. 2).

On the whole, lineages A and SC most likely represent two distinct taxonomic entities within *K. flavicollis*, even if the parsimony analysis group them together. These results are in line with Springhetti's preliminary studies, which found differences of morphometric parameters and reproductive traits between Sardinian and Italian peninsular colonies (Springhetti, 1967). The *K. flavicollis* lineage IF is consistently recognized as a single, separate taxon; as previously found (Velonà *et al.*, 2011), molecular data mirror the physiological divergence observed by Luscher (1956) between Italian and French *Kalotermes* populations. This suggests that *K. flavicollis* lineage IF might represent a new *Kalotermes* species. Except

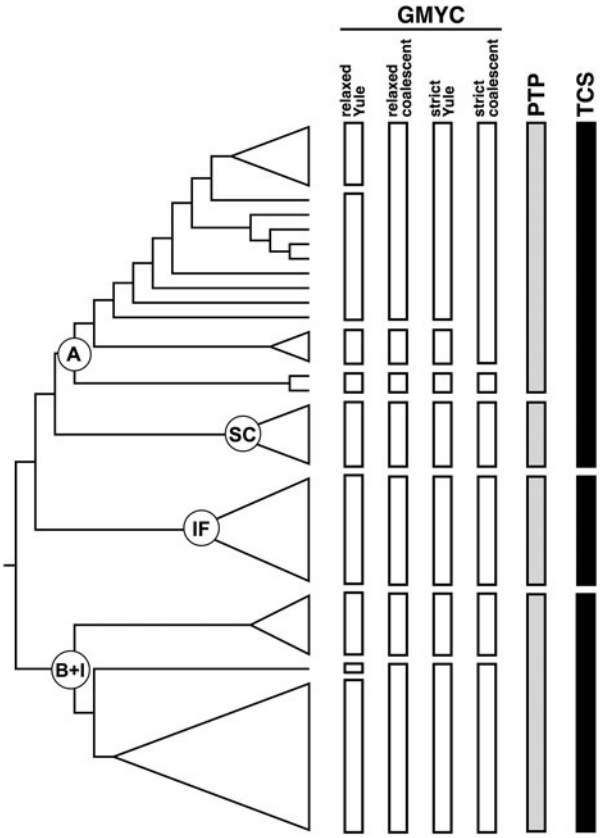

Fig. 3. Species delimitation analyses. Main clades are indicated by circles at their respective ancestral nodes. Outgroups have been omitted in the figure. Abbreviations: A, lineage A; SC, Sardo-Corsican lineage; IF, Ibero-French lineage; B + I: lineage B and *Kalotermes italicus*.

for GMYC analysis, the two other analyses indicated that *K. italicus* most likely constitute a single taxon, even if it is structured into two sub-clusters which might reflect some degree of intraspecific differentiation.

Biogeography and evolution of European Kalotermes *termites*

Data presented in this study revealed a significant phylogeographic structure of western European *Kalotermes* termites. The phylogenetic relationships among *K. flavicollis* lineages are indeed consistent with their geographic location (summarized in fig. 1b). Present study supports a wider distribution of *K. flavicollis sensu stricto* (lineage A), its range spanning from the Aegean coasts to the whole Italian Peninsula and Sicily. Our results also revealed that *K. flavicollis* lineage SC is not restricted to Sardinia and Corsica islands, as previously thought (Velonà *et al.*, 2011), but it is also present on the mainland, along Ligurian and Tuscanian coasts.

The phylogeographic pattern found in *Kalotermes* lineage SC nicely mirrors that observed in *Reticulitermes lucifugus* subspecies, with the Sardo-Corsican *R. lucifugus corsicus* observed also on the mainland (Luchetti *et al.*, 2013b). *R. lucifugus* diverged from the Iberian lineage and migrated to the Sardo-Corsican microplate after its detachment from the

Table 3. Colonies with mixed haplotype composition.

Sampling locations	Colony ID	Haplotypes	Lineages
Cinnisi	CNS	H3/H6	*K. flavicollis* A/*K. flavicollis* SC
Davoli Marina	DVM	H3/H5	*K. flavicollis* A
Feniglia Natural Reserve	FENa	H13/H14	*K. flavicollis* A
	FENb	H15/H16	*K. flavicollis* A/*K. italicus*
	FENc	H3/H17	*K. flavicollis* A/*K. italicus*
Riva dei Tarquini	RTAb	H3/H23	*K. flavicollis* A
San Rossore Natural Reserve	ROSa	H1/H10	*K. italicus*
	ROSb	H7/H11	*K. flavicollis* SC/*K. italicus*
	ROSc	H7/H12	*K. flavicollis* SC/*K. italicus*
Sirolo	SIRa	H2/H3	*K. flavicollis* A/*K. italicus*

Iberian Peninsula (~10 million years ago; Dedeine *et al.*, 2016). Although our analyses do not provide time estimates, *K. flavicollis sensu stricto* and lineage SC could have followed a similar path. In fact, its close relationship with the lineage IF cluster is reminiscent of the relationship between Iberian *Reticulitermes grassei-Reticulitermes banyulensis* and the *R. lucifugus corsicus* subspecies (Luchetti *et al.*, 2013b; Dedeine *et al.*, 2016). Although the dataset might be limited, it is interesting that *K. flavicollis sensu stricto* and lineage SC show signatures of a recent and rapid population growth (Tajima's *Ds* −2.227 and −2.413, $P < 0.01$), while lineage IF does not. This pattern possibly results from Pleistocenic glaciations, which could have imposed a southward contraction of the Italian population, followed by a recolonization after climate warming (Hewitt, 1996). On the contrary, lineage IF appears to have remained in equilibrium, suggesting the possibility that it was not affected by Quaternary climatic oscillations. Still, the Tajima's *D* value obtained with Ibero-French lineage was negative, suggesting that this lineage may have experienced a more limited population expansion.

The distribution of *K. italicus* is limited to certain areas along the northern Tyrrhenian coast, overlapping the northern edge of *K. flavicollis sensu stricto* distribution, and in two areas on the Adriatic side. This can be explained either by a naturally limited distribution or by a more recent colonization from an unknown area. Our analyses showed that *K. italicus* is genetically structured and does not exhibit any signature of population size changes. In fact, the Tajima's *D* value is not significantly different from 0, suggesting that *K. italicus* is at mutation-drift equilibrium. The high genetic diversity of this species might suggest that *K. italicus* geographical range is rather stable, although such an hypothesis remains to be tested. However, recent colonizations by this species seem rather unlikely since such events usually result in population bottlenecks. An alternative explanation is that *K. italicus* was introduced several times in the same places. Termites are indeed easily transported by means of human activities, for instance through lumber industry and/or wooden artifacts trade (Evans *et al.*, 2013; Scicchitano *et al.*, 2017), sometimes confounding the study of natural distributions. In order to precisely determine the natural distribution of these organisms, a large and detailed sampling is often required (Luchetti *et al.*, 2013b).

Interspecific colony fusion and implications for hybridization

Three types of colony breeding structure are known in termites: (i) simple families are composed of offspring from a primary couple; (ii) extended families possess offspring of primary and/or secondary reproductives; (iii) mixed families include offspring of more than two unrelated reproductives (Vargo & Husseneder, 2011). Nearly one-third of the presently analyzed colonies are mixed families exhibiting two distinct haplotypes, indicating that at least two females are involved in the reproduction (table 3). Mixed-family colonies are not rare in termites, especially in termopsid and kalotermitid species: in these taxa, several studies showed that independent colonies of the same taxon can fuse into a single social entity (Thorne *et al.*, 2003; Johns *et al.*, 2009; Velonà *et al.*, 2011; Korb & Roux, 2012; Howard *et al.*, 2013; Luchetti *et al.*, 2013a). We recently reported an extreme case of colony fusion in an Italian population of *K. flavicollis* (Feniglia Natural Reserve; Luchetti *et al.*, 2013a) with an exceptionally high frequency of mixed-family colonies, containing up to nine mitochondrial haplotypes. That study found also that some mixed-family colonies contained haplotypes belonging to the two divergent lineages A and B, which are here assigned to *K. flavicollis sensu stricto* (lineage A) and *K. italicus* (lineage B), respectively. In the present analysis, we found three further mixed-family colonies showing *K. flavicollis sensu stricto* and *K. italicus* haplotypes. For the first time, we also found two mixed-family colonies with *K. flavicollis* lineage SC and *K. italicus* haploypes and another one with *K. flavicollis sensu stricto* and *K. flavicollis* lineage SC haplotypes. These new results suggest that also interspecific colony fusion could be a widespread phenomenon in *Kalotermes* taxa.

It is interesting to consider possible outcomes of interspecific colony fusion. In the previous study, mixed-family colonies of *K. flavicollis sensu stricto*/*K. italicus* (at that time only indicated as lineages A and B, respectively; Luchetti *et al.*, 2013a), the analysis of nuclear microsatellite markers indicated that individuals with *K. flavicollis sensu stricto* mitochondrial haplotype showed nuclear genetic membership to *K. italicus* and *vice-versa*. This indicated that the two taxa are able to interbreed (Luchetti *et al.*, 2013a), thus suggesting that *K. flavicollis sensu stricto* and *K. italicus* may naturally hybridize. When Ghesini & Marini (2013) described *K. italicus* species they proposed that, based on morphological evaluations, the taxon *K. flavicollis* var. *fuscicollis* observed by Becker (1955) might be the result of *K. flavicollis sensu stricto* and *K. italicus* hybridization. Interestingly, Becker (1955) himself showed that *Kalotermes* individuals with black pronotum and *K. flavicollis sensu stricto* may interbreed, also giving viable offspring.

Species hybridization in social insects is not expected to occur at a high rate, but it was, nevertheless, evidenced in ants and termites (Feldhaar *et al.*, 2008). In termites, instances of natural hybridization and/or introgression were observed in lower termites, such as *Zootermopsis* and *Kalotermes*

(Aldrich & Kambhampati, 2007; Luchetti *et al.*, 2013a), and Rhinotermitidae (*Coptotermes* spp. and *Reticulitermes* spp.; Lefebvre *et al.*, 2008; Chouvenc *et al.*, 2015; Lefebvre et al., 2016). Moreover, laboratory colonies established by heterospecific mates in *Nasutitermes corniger* × *Nasutitermes ephratae* and *Coptotermes formosanus* × *Coptotermes gestroi* pairs were found to be more productive in term of offspring output (Hartke & Rosengaus, 2011; Chouvenc *et al.*, 2015). It is still not clear if the high frequency of colony fusion observed in *Kalotermes* might have facilitated the hybridization or if it is the reverse situation. Further studies along the sympatry area between *K. flavicollis* and *K. italicus* would likely provide interesting insight into reproductive boundaries and colony mate recognition in these social insects.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485317001080

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Table S1. Intra- and inter-clade divergence (uncorrected p-distances).

		1	2	3	4	5
1	*K.flavicollis* A	**0.003**				
2	*K.flavicollis* SC	0.015	**0.002**			
3	*K.flavicollis* IF	0.020	0.031	**0.001**		
4	*K.flavicollis* B+*K.italicus* (I)	0.054	0.061	0.057	**0.003**	
5	*K.flavicollis* B+*K.italicus* (II)	0.059	0.067	0.061	0.012	**0.002**

CHAPTER 6

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A SEXUAL QUEEN SUCCESSION AND GENETICS IN EUROPEAN *RETICULITERMES* TERMITES (BLATTODEA, TERMITOIDAE, RHINOTERMITIDAE): NEW INSIGHTS FROM TWO NATIVE AND ONE INVASIVE SPECIES

Asexual Queen Succession distribution and genetics in European *Reticulitermes* termites (Blattodea, Termitoidae, Rhinotermitidae): new insights from two native and one invasive species.

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Abstract

Sexual reproduction is the most prevalent reproductive strategy in animals but it is not the only mode of reproduction. In fact, conditional or obligatory use of asexual reproduction also occurs, as in the case of in social insects where the life cycle of several species of bees, wasps, ants, and termites is characterized by the co-occurrence of amphigony with different types of parthenogenesis. In some termite species, for example, founders are replaced by secondary kings and queens sexually and parthenogenetically produced, respectively. This particular reproductive mode is known as Asexual Queen Succession (AQS) and occurs in several species of the Rhinotermitidae and Termitidae families. From an evolutionary point of view, AQS strategy appears to be an ideal compromise between sexual and asexual reproductions, allowing to maintain the desirable genetic diversity in the offspring while extending the genetic contribution of the primary queen over the time and, thus, overcoming one of the major drawbacks of termite reproductive strategy, i.e. the dilution of genetic material in each sexually produced generation. Moreover, it has been theorized by Kobayashi and co-workers (2013) that the AQS system could promote the onset of kin selection in social diploids organisms like termites by generating a sex-biased investment.

Here, a microsatellite survey on three Rhinotermitidae species, i.e. the French *Reticulitermes grassei*, *R. flavipes* (invasive range), and the Italian AQS species *R. lucifugus,* was carried out. Main aims are to verify the reproductive strategies of *R. grassei* and *R. flavipes* and to perform a deep investigation on the AQS strategy in *R. lucifugus* termites.

For the first time, the occurrence of secondary kings in Italian AQS colonies of *R. lucifugus* is reported. Genetic and colony structure data indicated the presence of thelytokous secondary queens and sexually produced secondary kings in *R. lucifugus* while, in *R. grassei* and *R. flavipes*, secondary reproductives of both sexes were produced through gonochorism. Moreover, workers and winged reproductives resulted all produced by amphigony, as predicted by the AQS model, although the possibility of thelytokous parthenogesis for the production of some alates could be taken into account. Overall, these results confirmed the presence of AQS in *R. lucifugus* and highlighted its absence in the other two species. Moreover, data confirmed that *R. lucifugus* thelytokous parthenogenesis is carried out through a mechanism of terminal fusion. Finally, the analysis of genetic relatedness among actual and potential reproductives gives indication about the colony life-stage, also accordingly to the sex-asymmetry model of Kobayashi *et al.* (2013).

Keywords: AQS, kin selection, sex allocation, termites

Introduction

Sexual reproduction is the most prevalent reproductive strategy in animals. However, it is not the only mode of reproduction: in fact, among the others, conditional or obligatory use of asexual reproduction occurs (Bell, 1982; Schon *et al.*, 2009). A clear example is given in social insects: the life cycle of several species of bees, wasps, ants and termites is characterized by the co-occurrence of amphigony with different types of parthenogenesis (Heimpel & de Boer, 2008; Sumner & Keller, 2008; Wenseleers & Van Oystaeyen, 2011). Among ants, for example, queens produce workers from fertilized eggs by amphigonic reproduction, while new queens develop from unfertilized eggs through thelytokous parthenogenesis (Wenseleers & Van Oystaeyen 2011; Rabeling & Kronauer, 2013). This unusual reproductive system occurs, similarly, in termites and it is known as Asexual Queen Succession (AQS; Matsuura *et al.,* 2009). In AQS termites, primary queens are replaced by numerous female secondary reproductives (neotenic individuals, also called nymphoid) produced through thelytoky; on the other hand, workers, soldiers and adult alates (swarming individuals, future colony founders and primary reproductive) are produced through the mating of the primary king with the primary queen or with her parthenogenetic daughters, secondary queens (reviewed in Matsuura, 2017).

From an evolutionary point of view, the main adaptive significance of the AQS strategy appears to be an ideal compromise between amphigonic and parthenogenetic reproductions, allowing to maintain the desirable genetic diversity in the offspring while extending the genetic contribution of the primary queen over the time and, thus, overcoming one of the major drawbacks of termite sex, i.e. the dilution of genetic material in each sexually produced generation (Pearcy *et al.*, 2004; Matsuura, 2011). Queen replacement by thelytokous daughters leads, in fact, to the conservation of the founder queen genes even after her death (Matsuura, 2017). Moreover, it has been suggested that AQS system could provide the basis for a better understanding of the inclusive fitness theory

(Hamilton, 1964) in social diploids organisms like termites (Kobayashi *et al*., 2013). In a clear theoretical model, Kobayashi and co-workers (2013) showed that, in AQS species, after the replacement of the primary king with a secondary king, produced through the mating of the primary king with secondary queens, the genetic contribution of the primary queen rise to the 75%: in fact, the secondary king will carry half genome of the primary queen and secondary queens are all half-clone of the primary queen. Therefore, the mating between the secondary king and secondary queens will be, *de facto*, a mother-son inbreeding: this sex-asymmetric genetic contribution will lead to a higher relatedness between the queen and the offspring. Moreover, this sex-asymmetric genetic inheritance could increase a female-biased allocation in the following offspring (Kobayashi *et al.*, 2013; Matsuura, 2017).

AQS strategy was first described in three *Reticulitermes* termite species belonging to the Rhinotermitidae family: *R. speratus*, *R. virginicus* and *R. lucifugus* (Matsuura *et al.,* 2009; Vargo *et al.*, 2012; Luchetti *et al.,* 2013a). During the diversification of the *Reticulitermes* genus, AQS seems to have appeared independently multiple times (Dedeine *et al.,* 2016). In fact, although *R. speratus* exhibits the AQS strategy, this reproductive mode in absent in its congeneric *R. chinensis* (Huang *et al*., 2013); the same occurs between *R. virginicus* and the congeneric species *R. flavipes* (Vargo *et al.,* 2012; Matsuura, 2011). In this latter species, native in north America and invasive in France (Perdereau *et al.,* 2013), the absence of AQS was suggested based only on the winged adult sex ratio (Matsuura, 2011), but no genetic investigations have been performed so far. The AQS strategy was also found in three neotropical termite species belonging to the Termitidae family, suggesting that such a breeding system is more widespread than previously thought (Fougeyrollas *et al.,* 2015; Fournier *et al.,* 2016; Fougeyrollas *et al*., 2017; Matsuura, 2017). Though, there are differences between *Reticulitermes* AQS and Termitidae AQS. First, the cytological mechanism of ploidy restoration is different: terminal fusion in *Reticulitermes* and gamete duplication or central fusion

in Termitidae (reviewed in Matsuura, 2017). Second, while it is generally considered that AQS bring advantage to the colony on the long timespan, in the termitid species *Silvestritermes minutus* it seems that AQS mediates a faster colony growth and alates production within a very short colony lifespan (Fougeyrollas *et al*., 2017).

In this work, we present a microsatellite survey of four French *R. grassei* and *R. flavipes* populations, and of two additional populations of the Italian *R. lucifugus*. The main aims are to verify the reproductive strategies of *R. grassei* and *R. flavipes*, providing genetic evidence and further describe more in detail the occurrence of AQS in Italian colonies of *R. lucifugus*.

Materials and Methods

Sample collection

Tweny one termite colonies from six French and Italian localities were collected and preserved in 100% ethanol until DNA extraction; all sampling pertinent information are given in Table 1 and Suppl. Figure S1. Each sample was first screened to discriminate castes (workers, soldiers and reproductives) and to search for neotenics and adult alates (swarming reproductives and fifth instar nymphs). Sex in the reproductives' cast was determined based on the morphology of the last two abdominal sternites (Zimet & Stuart, 1982).

Molecular techniques

Total DNA was extracted from termites' head following the CTAB method (Doyle and Doyle, 1987) for Italian samples while from whole termites' body, using the Wizard® Genomic Purification Kit (Promega), for the French ones. Species were confirmed through the mitochondrial cytochrome oxidase 2 (COII) haplotype characterization following Luchetti *et al.* (2013b) and Dedeine *et al.*

(2016), using two workers per colony. Sequences obtained were compared with a set of reference sequences drawn from Genbank: *R. grassei* (AN: KM245780), *R. flavipes* (AN: KM245765-67) *R. lucifugus lucifugus* (AN: AF291738 - KC576871) and *R. lucifugus corsicus* (AN: AY267858 - KM245781). The analyses led to sample characterization as follows: colonies RG1 – 5 = *R. grassei;* colonies RF1 – 4 = *R. flavipes;* colonies RLS1-12 = *R. lucifugus* (subsp. *lucifugus*).

Overall, 802 individuals were genotyped, including workers and, when available, adult alates and neotenics of both sexes. To analyse the best-performing microsatellite loci, three different sets of loci were used for the three *Reticulitermes* species (Baudouin *et al.*, 2017; Luchetti *et al.*, 2013a); standard PCR cycling conditions were used, following reagents' manufacturer information (Suppl. Table S1).

Genetic analysis

Genetic variability parameters (effective number of alleles, *Ne*; expected, *He*, and observed, *Ho*, heterozygosity; allelic richness, *Ar*) per species were calculated on workers only, using GenAlex v. 6.502 (Peakall & Smouse, 2012), GENEPOP v. 1.2 (Raymond & Rousset 1995) and FSTAT v. 2.9.3.2 (Goudet 1995). The breeding structure was estimated by determining the family type from genetic data, as explained in Vargo & Husseneder (2011):

- *Simple family:* A colony headed by single royal pair and, accordingly, the genotypes of their offspring are expected to be consistent with Mendelian ratios.
- *Extended family:* simple families become extended when secondary reproductives develop, and more than four genotypes or three classes of homozygotes may occur in the offspring. Further, genotypes distribution and frequencies are not consistent with those of simple families: significance of deviations was assessed by a goodness-of-fit G test on observed vs. expected genotypic frequencies.

- *Mixed families*: headed by more than two unrelated reproductives, recognizable by the presence of five or more alleles at least at one locus.

The goodness-of-fit G test on observed vs. expected genotypic frequencies was also used for determining genotype frequency distortion due to AQS in neotenics and in adult alates.

For simple families showing secondary reproductives, as in the case of *R. lucifugus* colonies, genotypes of royal pairs were also reconstructed using GERUD v. 2.0 (Jones, 2005) considering the genotypes of parthenogenetic secondary queens and their nest-mate workers. In the same colonies, relatedness (*r*) among nest mates and between inferred royals was also calculated with Konovalov and Heg (2007) maximum likelihood estimator as implemented in Kingroup v. 2 (Konovalov *et al*., 2004). Statistical significance of relatedness (*r*) deviation from expectations - 0.75 mother-son inbreeding offspring; 0.5 for parent-offspring or full-sibling; 0.25 for half-sibling - was assessed by Wilcoxon one-sample test.

To determine the mode of thelytoky responsible for the origin of neotenic parthenogens in *Reticulitermes* colonies, the generational rate of transition to homozygosity for the loci heterozygous in the inferred mother was calculated and compared with those expected under different modes of thelytoky (Pearcy *et al.*, 2006).

Results

Workers genetic diversity and colonies family type

A total of 802 individuals belonging to *R. grassei*, *R. flavipes* and *R. lucifugus* were genotyped at 10, 8 and 6 microsatellite loci, respectively (Suppl. Table S2-S4). The percentage of polymorphic loci varied across the three species. In *R. grassei*, the 80% of the loci examined were polymorphic apart from loci Rg32 and Rg46. In *R. flavipes* and *R. lucifugus*, all loci resulted in polymorphism. Genetic

diversity indices have been calculated on workers only as, at best of our knowledge, they are produced by amphigonic reproduction only. The mean values of allelic richness (*Ar*) and number of effective alleles (*Ne*) ranged from 2.5 to 4.5 and from 1.85 to 2.97 in the three taxa analysed, with the lowest values observed in *R. lucifugus* and the highest one in *R. flavipes*. All the three taxa showed an observed heterozygosity lower than expected (Table 2).

Based on workers genotypes, the colony breeding structure was determined. All *R. grassei* colonies resulted extended families, the observed genotypes being inconsistent with those expected based on a simple Mendelian family (Figure 1; Suppl. Table S2). Two out four *R. flavipes* colonies resulted as extended families (RF3, RF4), one as simple family (RF2) and one as mixed family (RF1), the latter having more than 4 alleles at a single locus (Figure 1; Suppl. Table S3). On the other hand, all *R. lucifugus* colonies, but one, showed the structure of simple families. The colony RL6, in fact, showed a frequency of genotypes different from the expectation of a simple family (P_G<0.05; Figure 1; Suppl. Table S4).

Neotenics and adult alates genotyping

Neotenics of both sexes were found in different colonies of analyzed species; their distribution, though, does not appears equal. In *R. grassei* and *R. flavipes* all colonies showed both secondary queens and kings, although in one *R. grassei* colony, RG4, a higher proportion of females was observed (P_{χ^2}<0.001; Table 1). In *R. lucifugus*, on the other hand, five out of eight colonies hosted only secondary queens; in the remaining three, two had significantly more females (P_{χ^2}<0.05) and one showed 1:1 sex ratio (Table 1). Moreover, in two colonies (RL1, RL3), all females neotenics were physogastric indicating they were functional secondary queens (Suppl. Figure S1).

Heterozygosity of workers and neotenics was similar in both *R. grassei* and *R. flavipes*, even when different sexes are considered (Figure 2). On the contrary, in *R. lucifugus*, female neotenics

(secondary queens) were fully homozygous in five out eight colonies and in one colony (RL12) the proportion of homozygote females was 80%, where two females were heterozygote at one and two loci, respectively (Figure 2; Suppl. Table S3). In the colonies RL3 and RL11, five secondary queens were heterozygote at one or two loci (Figure 2; Suppl. Table S3). Interestingly, in the colony RL3, five secondary queens were heterozygote at the same locus, carrying the same genotype, and one of them resulted heterozygous at a second locus. In the colony RL11, though, three out five female neotenics were heterozygous at two loci; surprisingly, RL11 workers were heterozygous at a single locus (Figure 2; Suppl. Table S3).

Heterozygote *R. lucifugus* secondary kings, though, reached the 100% in two out three colonies; in the third one (RL12), only one male resulted heterozygote at two loci, while the others were heterozygote at a single locus (Figure 2; Suppl. Table S3).

Alate genotypes mostly followed what has been observed for neotenics in both *R. grassei* and *R. flavipes*, heterozygosity being significantly similar to the one observed in workers (P_{χ^2} = 0.911 and P_{χ^2} = 0.988, respectively; Figure 2). In *R. lucifugus*, alates heterozygosity is significantly lower than that expected based on worker heterozygosity ($P_{\chi^2} < 0.01$). Male and female adult alates of *R. grassei* and *R. flavipes* showed the same level of heterozygosity ($P_{Wilcoxon\text{-}paired}$ = 0.357 and $P_{Wilcoxon\text{-}paired}$ = 1, respectively). On the other hand, heterozygous female alates appeared to be significantly less than male alates from the same nest ($P_{Wilcoxon\text{-}paired} < 0.05$); notably, in the colony, RL5, all female alates were homozygote compared to the 30% of heterozygote males (Figure 2; Suppl. Table S3).

To highlight genotype distortion in female neotenics, we only analyzed those individuals that were fully homozygous and, therefore, produced by parthenogenesis (Matsuura et al., 2009). Considering the inferred primary queen genotypes at sequenced loci, we estimated that in all *R. lucifugus* colonies neotenics showed a significant distortion of genotype frequency ($P_G < 0.05$). *R. lucifugus* alates showed deviation from expected genotype frequencies in five out six analyzed colonies ($P_G <$

0.01). As a comparison, all *R. grassei* and *R. flavipes* adult alates showed genotype frequencies do not deviating from expectations ($P_G \geq 0.759$), apart from the *R. flavipes* colony RF1 ($P_G < 0.05$).

Parthenogenesis and restoration of ploidy in R. lucifugus

In both two populations, the parthenogenetic female neotenics were not all fully homozygous at all loci considered but some individuals were heterozygous for the loci that were heterozygous in the inferred maternal genotypes. This scenario was significantly different from that expected under apomixis or under automixis with gamete duplication (Pearcy *et al*., 2006). The rate of transition to homozygosity (R) ranged from 0.6 to 0.97 per locus: these rates were consistent with the values expected under automixis with terminal fusion as supported by χ^2-test results (Table 3).

Relatedness distribution among R. lucifugus colonies

In *R. lucifugus* colonies, based on the female neotenics and workers' genotypes, it is possible to infer the primary queen and primary king genotype. For the eight colonies where this is possible we always scored a single possible primary queen and primary king, apart of colony RL3 where two possible primary kings have been inferred (named PK1 and PK2; Suppl. Table S4). In average, primary queens and kings were related to each other, showing an average $r = 0.437 \pm 0.101$ (± standard error; $P_{0.5} = 0.445$). Female neotenics showed an average relatedness of 0.507 ± 0.018 with inferred primary queens ($P_{0.5} = 0.182$), while having an average $r = 0.279 \pm 0.028$ with primary kings ($P_{0.5} < 0.001$). On the other hand, male neotenics showed a relatedness of 0.478 ± 0.036 ($P_{0.5} = 0.654$) with the primary queen and of 0.654 ± 0.070 with the primary king ($P_{0.5} < 0.05$), but an average $r = 0.255 \pm 0.025$ with female neotenics ($P_{0.5} < 0.001$; $P_{0.25} = 0.662$). Workers showed an average relatedness of 0.515 ± 0.019 ($P_{0.5} = 0.180$) and 0.556 ± 0.023 ($P_{0.5} < 0.01$) with primary queen and king, respectively; moreover, they showed $r = 0.154 \pm 0.010$ ($P_{0.5} < 0.001$; $P_{0.25} = < 0.001$) and $r = 0.349 \pm$

0.031 ($P_{0.5}$ < 0.001; $P_{0.25}$ < 0.01) with female and male neotenics, respectively. In the colonies RL1 and RL3, adult alates showed r = 0.623 ± 0.028 ($P_{0.5}$ < 0.001) and r = 0.4237 ± 0.032 ($P_{0.5}$ = 0.071) with primary queen and king, respectively, without differences between sexes. The same can be observed about the relatedness with female neotenics which resulted, in average, r = 0.296 ± 0.020 ($P_{0.5}$ < 0.001; $P_{0.25}$ = 0.506).

Colony RL3 represent an interesting case as two primary kings have been inferred (Suppl. Table S4). The relatedness between the inferred primary kings, PK1 and PK2, and the primary queen was 0.250 and 0.670, respectively. Workers relatedness with inferred primary kings was r = 0.508 ± 0.061 and r = 0.602 ± 0.035, respectively; in the first case, PK1, values do not deviate significantly from parent-offspring expectation ($P_{0.5}$ = 0.686), while in the second one, PK2, workers showed higher relatedness ($P_{0.5}$ < 0.05). Adult alates of the RL3 colony showed relatedness value significantly deviating from the parent-offspring expectation with both PK1 and PK2: r = 0.264 ± 0.052 ($P_{0.5}$ < 0.01) and r = 0.599 ± 0.048 ($P_{0.5}$ < 0.05), respectively. This deviation held also when calculating the relatedness with the primary queen, r = 0.677 ± 0.055 ($P_{0.5}$ < 0.01). Interestingly, adult alates relatedness with the primary queen and the inferred PK1 king do not deviated from 0.75 ($P_{0.75}$ = 0.297) and 0.25 ($P_{0.25}$ = 0.879), respectively.

Discussion

The AQS reproductive strategy was first found in the Japanese *R. speratus*, and then described also in the North American *R. virginicus* and the Italian *R. lucifugus* (Matsuura *et al.*, 2009; Vargo *et al.*, 2012; Luchetti *et al.*, 2013). Interestingly, although the envisaged advantage of this mating system (Matsuura *et al.*, 2009, 2011) and the close phylogenetic relationship among *Reticulitermes* species (Dedeine *et al.*, 2016), AQS does not occur in all taxa. We here analyzed the European populations

of three species, *R. lucifugus*, *R. grassei* and *R. flavipes*, to confirm the presence/absence of AQS and to detail possible AQS-related feature in colony castes.

AQS and non-AQS species

In those colonies where neotenics have been found, sex distribution quite different resulted. In *R. grassei* and *R. flavipes* male and female neotenics occurred equally, apart from one *R. grassei* colony showing a females-biased ratio. In *R. lucifugus*, female neotenics were in the majority but, for the first time, some male neotenics occurred; interestingly, in one colony (RL12) a 1:1 sex ratio was observed. Moreover, *R. grassei* and *R. flavipes* neotenics (both females and males) showed a level of heterozygosity fully comparable to that of workers and adult alates, that are known to be produced by amphigony. On the contrary, *R. lucifugus* female neotenics were fully homozygotes, except for three colonies. Heterozygous secondary queens in an AQS system may occur because of recombination during the oogenesis or because of occasional bisexual production of females neotenics (Matsuura *et al.*, 2009; Vargo *et al.*, 2012). In our data, two colonies showed up to the 50% of genotyped female neotenics as heterozygotes at the same locus: it is difficult to discern whether observed heterozygosity was due to recombination to amphigonic reproduction, because genotypes of inferred primary reproducers mostly carry same alleles. The only difference was in the colony RL11, where the primary king carry an allele not found in heterozygous female neotenics. Though, the frequency of heterozygotes does not appear compatible with the rare event of recombination, we therefore consider that a proportion of female neotenics were produced by amphigony. Overall, *R. lucifugus* female neotenics showed genotypes consistent with thelytokous parthenogenesis; moreover, the test for ploidy restoration confirms the cytological mechanisms of automixis with terminal fusion. In line with this finding, *R. lucifugus* female neotenics showed an *r* value consistent with a parent-offspring relationship when compared to inferred primary queens,

while the same relatedness can be rejected with inferred primary kings. Genotyped *R. lucifugus* male neotenics resulted as heterozygous as workers and adult alates, in line with the prediction that they are produced by amphigony. Moreover, they all showed *r* values with both primary reproducers significantly consistent with a parent-offspring relationship. Full sibling relationship between males and females neotenics can be rejected, and the estimated value of *r* (0.233 ± 0.028) suggest a half-sibling relationship: this is expected in the AQS system between secondary queens and their offspring (Matsuura *et al.*, 2009; Matsuura, 2011). In the AQS system, all-females neotenics are expected except for colonies that are in the terminal phase of development, when the primary king is replaced by (a) secondary king(s) (Matsuura, 2017). On the other hand, the presence of many males neotenics, even up to a 1:1 sex ratio, is quite surprising as, in previous analyses, only one neotenic male per colony was found (Matsuura *et al.,* 2009). Male neotenics we collected in *R. lucifugus* colonies were not taken from the royal chamber, therefore we have not information about their possible functionality. However, female neotenics were found outside the royal chamber, and in two colonies we found functional secondary queens. This seems to suggest that primary reproducers replacement may occur elsewhere within the nest tunnels or even that new reproductive centres can be established. Overall, we confirm the presence of AQS in *R. lucifugus* and evidence its absence in *R. grassei* and *R. flavipes*. As for the latter species is concerned, the absence of AQS was already suggested in North American colonies (Matsuura, 2011); here we showed that also colonies in the invasive range lack of this peculiar reproductive strategy.

Colonies family type, breeding structure and life stage

It is interesting to note that, as already noted (Vargo *et al.,* 2012; Luchetti *et al.,* 2013a), the prediction of the family type within colonies can be affected by the presence/absence of AQS. By definition, in fact, colonies where secondary reproducers occur are extended families: this type of

mating structure can be inferred by the presence of genotypes that are not compatible with two parents mating (as in simple families) or whose frequency deviate significantly from the expected Mendelian segregation (Vargo & Husseneder, 2011). In *R. grassei*, all colonies have neotenics and, accordingly, genotypes distribution inspection indicated all are extended families. In *R. flavipes*, two out four colonies resulted extended families, one a mixed family and one a simple family. Mixed families are rare in *Reticulitermes*, but where found quite frequent in the *R. flavipes* invasive ranges (Perdereau *et al.*, 2010; 2015): they come out when two nests eventually fuse and lead to a mixed colony or when the colony is founded by multiple reproducers ($\geq$ 3; Vargo & Husseneder, 2011). Analyzed *R. flavipes* family types are mostly compatible with the presence of neotenics. *R. lucifugus* colonies with neotenics all resulted as simple families, apart from colony RL6 which appeared as an extended family. Since neotenics that are derived only from the primary queen and mate with the primary king, the effect on colony genotype is still that of a simple pair mating; therefore, this may hinder the genetic signal to infer the family type. An alternative explanation for our data is that female neotenics were not yet functional, although this can be excluded for colonies RL1 and RL3 where we collected physogastric secondary queens.

Another point we can draw from relatedness analysis concerned the life stage of *R. lucifugus* colonies. In line to the known life-cycle and the AQS model (Vargo & Husseneder 2009; Matsuura, 2017), a colony is founded by a bisexual pair of dispersing alates; then, during the colony development, the primary queen is replaced by multiple secondary queens, produced by parthenogenesis, that will mate with the primary king. During the terminal stage of the colony life, the primary king is replaced by a secondary king, produced through the mating of the primary king and secondary queens: from this moment onward, secondary king will mate with secondary queens. Following the model of sex-asymmetric inbreeding in AQS (Kobayashi *et al.,* 2013), the offspring at this late stage will be related by 0.75 to the primary queen and 0.25 to the primary king: this is

because the secondary king will bear half the genome of the primary queen and the mating with secondary queens (half-clones of the primary queen) will result in a mother-son inbreeding. Our data indicated, though, that workers, secondary kings, and alates have a relatedness with the primary pair of about 0.5, as expected from parent-offspring relationship; moreover, they showed a relatedness similar or lower than the half-sibling value (0.25) with female neotenics, as expected from the AQS model (Matsuura *et al.,* 2009; Matsuura, 2011). Notably, workers showed a relatedness with female neotenics significantly lower than the expected 0.25, while secondary kings and alates did not. This discrepancy is likely because while collected workers may belong to different generations, thus allelic frequency can be different, secondary kings and alates were collected from the same generation. For instance, alates have been all collected during the same swarming phase. As they showed parent-offspring relationship with primary pairs and half-sibling with secondary queens, we can conclude they were offspring of the primary king and secondary queens. We can, therefore, infer that assayed colonies were at a mature stage, but not yet in the terminal phase, that is when headed by a primary king and secondary queens. In this regard, however, the colony RL3 could represent a noticeable exception. Here two primary kings have been inferred, PK1 and PK2, the first being much less related with the primary queen: this raise the question whether the second inferred king PK2 could represent a secondary king. Following the expectation of the sex-asymmetry inbreeding model, alates (representing a single generation born at the field collection moment) showed relatedness significantly similar to 0.75 with the primary queen and significantly similar to 0.25 with the PK1 king (thus, being the true primary king). Therefore, we could suggest that RL3 colony is, actually, at the terminal stage of development.

The relatedness observed between inferred primary king and primary queen resulted quite high (0.437 ± 0.101), not significantly different from the parent-offspring relationship, suggesting colonies could be headed by a secondary king (Vargo *et al.*, 2012). On the other hand, based on the

sex-asymmetry inbreeding model expectations, we ruled out this possibility; the same relatedness value, in fact, can be that of full-sibling, suggesting that colony founders are related because they share parents. It is worth nothing that a genetic investigation indicated that the San Rossore *R. lucifugus* population is constituted of mostly interconnected nests originated by budding (Scicchitano *et al.*, unpublished): this might suggest that primaries may have had a close genetic relationship.

Genetics of alates

One of the evolutionary advantage of AQS is the possibility to perpetuate in the time the mating between primary reproducers, the genetic contribution of the queen being carried by thelytokous secondary queens: this would keep the heterozygosity of the new offspring at higher level, avoiding the king-daughter inbreeding and the possible sib-mating between male and female neotenics (Matsuura, 2017). It is important for workers, soldiers, and alates to have a high heterozygosity as they are more subject to environmental variables: for example, swarming adult alates are those that will leave the nest to found a new one. In *R. virginicus*, an AQS species living in North America, it was observed that female alates were significantly more inbred than male (DeHeer & Vargo, 2006); though, the discovery of AQS in this species led to the explanation that a part of these swarming females was produced by parthenogenesis. Our data on adult alates strongly confirm this suggestion, alate females being significantly less heterozygous than male alates. Moreover, in the colony RL5 the 100% of female alates were homozygous, compared to the 30% of male alates. Therefore, it is possible to hypothesize that AQS species may also produce female alates though parthenogenesis. In line with this, the two non-AQS species here analyzed, *R. grassei* and *R. flavipes*, did not show any heterozygosity decrease in female alates with respect to males alates.

It was observed that, during parthenogenesis, conflicts by selfish genetic elements (SGE) may occur causing a distortion in the genotypic frequencies. These SGE, thus, could influence the first meiotic division enhancing the transmission of one of the two possible maternal emi-genomes in the next thelytokous generation (Matsuura, 2011; 2017). In line with this, analyzed *R. lucifugus* neotenics showed significant distortion of genotype frequencies, indicating SGE had a role also in this species. We presently demonstrated that analyzed alates were the offspring of the primary king and secondary queens: as such, they showed a significant distortion in genotype frequencies as expected based on the SGE model (Matsuura, 2011) and observed in another AQS-species, *R. speratus* (Matsuura *et al.*, 2009). Notably, alates of the non-AQS species *R. grassei* and *R. flavipes* do not showed genotype frequencies distortions.

Several studies described sexual genetic conflicts among insects and mechanisms by which one gene can counteract a rival during meiosis (Cazemajor *et al.*, 2000; Pennisi, 2003). In the case of AQS, due to SGE, genetic conflicts are triggered at intra-sexual level between maternal emi-genomes (Matsuura, 2011). It is possible to hypothesize, alternatively to the parthenogenetic origin of adult female alates, that the loss of heterozygosity observed in swarmings alates could results from such maternal genetic conflicts.

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Tables & Figures

Table 1. Species, sampling locality, colony ID, and the number of genotyped individuals per colony.

Species/Locality	Colony ID	Workers	Adult alates		Neotenics	
			♀	♂	♀	♂
R. grassei						
La Tremblade	RG1	20	10	10	-	-
	RG2	20	10	10	-	-
Pissos	RG3	29	5	5	20	20
	RG4	18	1	4	21	1
	RG5	20	10	8	9	3
R. flavipes						
Paris	RF1	9	10	10	-	-
La Tremblade	RF2	19	10	10	9	10
	RF3	17	10	10	10	10
Saint-Georges-d'Oléron	RF4	18	10	10	10	10
R. lucifugus						
San Rossore Natural Reserve	RL1	10	10	10	8	-
	RL2	10	-	-	10	-
	RL3	10	10	10	10	-
	RL4	10	10	10	-	-
	RL5	10	10	10	-	-
	RL6	10	10	10	-	-
	RL7	10	10	10	-	-
	RL8	10	-	-	10	3
Roccelletta di Borgia	RL9	10	-	-	10	2
	RL10	9	-	-	5	-
	RL11	10	-	-	10	-
	RL12	9	-	-	10	10

Table 2. Workers genetic diversity, per locus (with mean and standard error), in analyzed species. [Ar: allelic richness; Ne: number of effective alleles; Ho: observed heterozygosity; He: expected heterozygosity]

Species	Locus	Ar	Ne	Ho	He
R. grassei	Rg3	2.00	1.20	0.11	0.17
	Rg9	2.00	1.47	0.35	0.32
	Rg15	3.00	1.35	0.18	0.26
	Rg23	3.00	2.34	0.26	0.57
	Rg32	1.00	1.00	0.00	0.00
	Rg35	2.00	2.00	0.37	0.50
	Rg39	2.00	1.53	0.39	0.35
	Rg44	8.00	5.39	0.56	0.81
	Rg46	1.00	1.00	0.00	0.00
	Rg48	2.00	1.72	0.26	0.42
	Rf21-1	6.00	4.15	0.33	0.76
	Mean	2.91	2.10	0.26	0.38
	SE	0.65	0.42	0.05	0.08
R. flavipes	Rf11-1	4.00	2.01	0.51	0.50
	Rf6-1	8.00	3.50	0.49	0.71
	Rs1	5.00	4.34	0.24	0.77
	Rf21-1	5.00	3.10	0.30	0.68
	Rs43	2.00	1.49	0.32	0.33
	Rs15	4.00	3.45	0.51	0.71
	Rf15-2	4.00	3.10	0.19	0.68
	Rf1-3	4.00	2.80	0.43	0.64
	Mean	4.50	2.97	0.37	0.63
	SE	0.60	0.31	0.04	0.05
R. lucifugus	Rf24-2	7.00	2.80	0.35	0.64
	Rf21-1	5.00	3.40	0.26	0.71
	Rs02	2.00	1.14	0.08	0.13
	Rf5-10	3.00	1.43	0.30	0.30
	Rs10	4.00	1.29	0.18	0.22
	Rf1-3	2.00	1.04	0.04	0.04
	Mean	3.83	1.85	0.20	0.34
	SE	0.79	0.41	0.05	0.11

Table 3. Test of ploidy restoration for *R. lucifugus* secondary queens (* P< 0.05; ** P<0.01; *** P<0.001; n.s., not significant)

Locus	PQ_{het}	SQ_{tot}	SQ_{homo}	*R*	Apomixis	Automixis			
					(r = 0)	central fusion (r = 0 - 0.33)	random fusion (r = 0.33)	terminal fusion (r = 0.33 - 1)	gamete duplication (r = 1)
Rf24-2	7	63	58	0.92	***	***	***	n.s.	***
Rf21-1	6	58	56	0.97	***	***	***	n.s.	***
Rs02	2	15	14	0.93	***	***	***	n.s.	***
Rf5-10	4	38	33	0.87	***	***	***	n.s.	***
Rs10	1	10	6	0.60	***	n.s.	n.s.	n.s.	***

Figure 1. Respective proportion of simple families (white), extended families (grey) and mixed families (black) among colonies of the three *Reticulitermes* species.

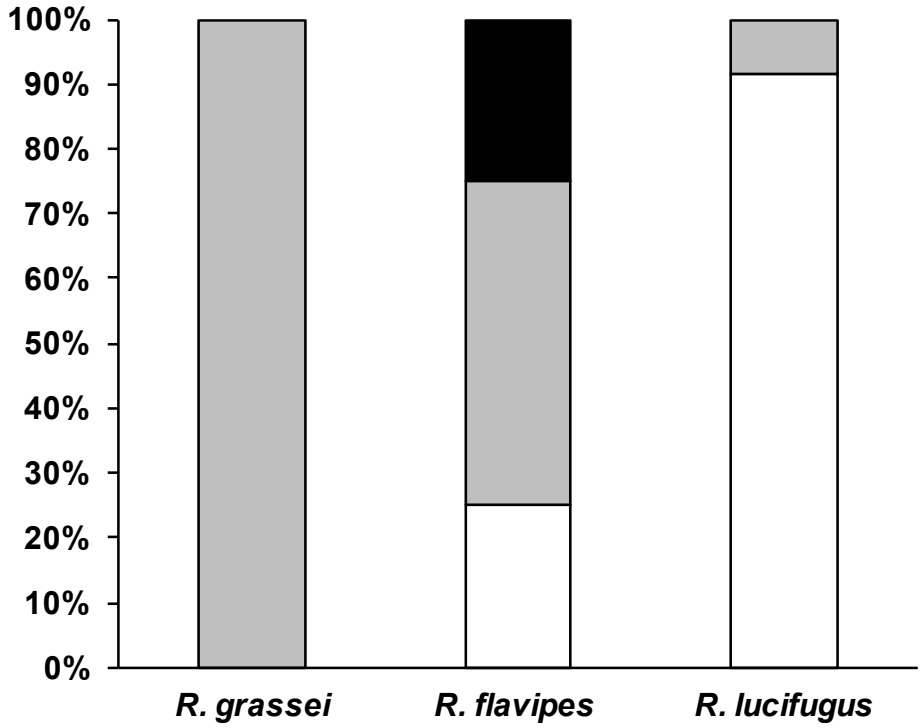
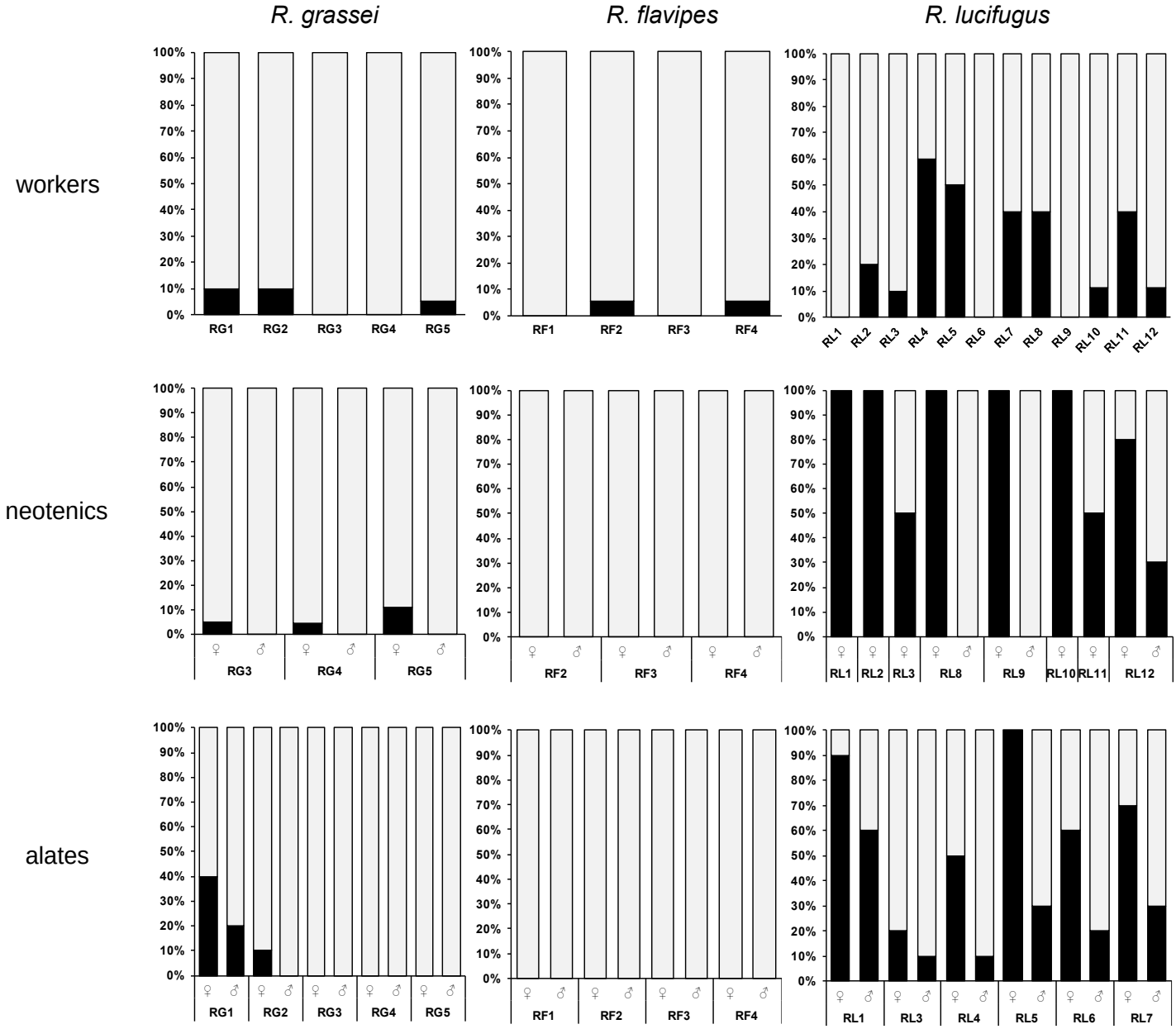


Figure 2. Respective proportion of homozygous (black) and heterozygous (white) individuals among castes, in each colony of the three *Reticulitermes* species.



Suppl. Table S1. Information on microsatellite loci chosen for genotyping.

Species	Microsatellite loci	References	Annealing Temp.
R. grassei	Rg3; Rg9; Rg15; Rg23; Rg32; Rg35; Rg39; Rg44; Rg46; Rg48; Rf21-1	this study; Dronnet *et al.*, 2004; Vargo, 2000;	55°C
R. flavipes	Rf11-1; Rf6-1; Rf21-1; Rf15-2; Rf1-3; Rs1; Rs43; Rs15	DeHeer *et al.*, 2005; Dronnet *et al.*, 2004; Vargo, 2000; Baudouin *et al.*, 2016	55°C
R. lucifugus	Rf24-2; Rf21-1; Rf5-10; Rf1-3; Rs02; Rs10	Dronnet *et al.*, 2004; Vargo, 2000;	56°C

Suppl. Table S2. Genotypes of *R. grassei* colonies (A_f/m: alate female/male; N_f/m: neotenic female/male; w: worker)

Colony ID/individuals	Microsatellite loci																					
	Rg15		Rg32		Rg35		Rg39		Rg46		Rg3		Rg9		Rg23		Rg44		Rg48		Rf21-1	
RG1																						
A_f1	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	190	211
A_f2	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	190	190
A_f3	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	211	211
A_f4	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	226
A_f5	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	226	226
A_f6	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	121	121	162	162	190	190
A_f7	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	211	226
A_f8	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	190	211
A_f9	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	190	190
A_f10	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	156	102	102	162	162	211	211
A_m1	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	226	226
A_m2	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	0	0
A_m3	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	211	211
A_m4	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	190	211
A_m5	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	211	211
A_m6	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	211	211
A_m7	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	156	102	121	162	162	190	211
A_m8	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	211	226
A_m9	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	211	211
A_m10	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	190	211
w1	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	211	211
w2	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	211	226
w3	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	211	211
w4	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	102	162	162	211	226
w5	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	211
w6	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	121	162	162	211	211
w7	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	211
w8	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	211
w9	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	190	211
w10	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	211
w11	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	102	121	162	162	211	226
w12	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	156	121	121	162	162	190	190
w13	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	211	226
w14	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	211
w15	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	211	226
w16	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	211	226
w17	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	121	162	162	211	211
w18	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	211	226
w19	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	211
w20	118	118	139	139	105	105	216	216	63	63	172	172	307	307	156	168	102	102	162	162	190	211
RG2																						
A_f1	118	118	139	139	105	112	216	216	63	63	170	172	307	307	159	159	127	127	162	162	190	226
A_f2	118	118	139	139	112	112	216	218	63	63	170	172	307	307	159	159	106	106	162	162	190	226
A_f3	118	118	139	139	112	112	216	216	63	63	172	172	307	307	159	168	102	102	162	162	226	226
A_f4	118	118	139	139	105	112	216	216	63	63	172	172	307	307	159	159	106	127	162	162	226	226
A_f5	118	118	139	139	105	112	216	218	63	63	170	170	307	307	159	159	102	106	162	162	226	226
A_f6	118	118	139	139	105	105	216	216	63	63	170	170	307	307	159	159	102	102	162	162	226	226
A_f7	118	118	139	139	105	112	216	216	63	63	170	170	307	307	159	168	102	106	162	162	226	226
A_f8	118	118	139	139	105	112	218	218	63	63	172	172	307	307	159	159	102	127	162	162	226	226
A_f9	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	106	127	162	162	190	226
A_f10	118	118	139	139	105	105	216	216	63	63	170	172	307	307	159	159	106	127	162	162	226	226
A_m1	118	118	139	139	105	112	216	216	63	63	170	170	307	307	159	159	102	127	162	162	226	226
A_m2	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	106	106	162	162	226	226

Colony ID/individuals	Microsatellite loci																					
	Rg15		Rg32		Rg35		Rg39		Rg46		Rg3		Rg9		Rg23		Rg44		Rg48		Rf21-1	
RG2																						
A_m3	118	118	139	139	105	105	216	216	63	63	170	172	307	307	159	159	106	106	162	162	226	226
A_m4	118	118	139	139	105	112	218	218	63	63	170	170	307	307	159	159	102	102	162	162	226	226
A_m5	118	118	139	139	105	112	216	218	63	63	170	170	307	307	159	159	106	127	162	162	226	226
A_m6	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	102	102	162	162	226	226
A_m7	118	118	139	139	112	112	216	218	63	63	170	172	307	307	159	159	106	127	162	162	226	226
A_m8	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	102	106	162	162	226	226
A_m9	118	118	139	139	105	112	218	218	63	63	170	172	307	307	159	159	102	127	162	162	226	226
A_m10	118	118	139	139	105	112	216	218	63	63	170	170	307	307	159	159	106	127	162	162	226	226
w1	118	118	139	139	105	112	216	216	63	63	170	172	307	307	159	159	106	127	162	162	190	226
w2	118	118	139	139	112	112	216	216	63	63	172	172	307	307	159	159	102	106	162	162	226	226
w3	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	102	127	162	162	226	226
w4	118	118	139	139	105	112	218	218	63	63	170	170	307	307	159	159	102	127	162	162	226	226
w5	118	118	139	139	105	105	216	216	63	63	172	172	307	307	159	159	106	106	162	162	226	226
w6	118	118	139	139	112	112	216	216	63	63	172	172	307	307	159	159	106	106	162	162	226	226
w7	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	102	106	162	162	226	226
w8	118	118	139	139	105	105	216	218	63	63	170	172	307	307	159	159	102	106	162	162	226	226
w9	118	118	139	139	105	105	216	216	63	63	170	172	307	307	159	159	127	127	162	162	226	226
w10	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	106	106	162	162	226	226
w11	118	118	139	139	105	105	216	218	63	63	170	172	307	307	159	159	102	106	162	162	226	226
w12	118	118	139	139	105	112	216	216	63	63	170	170	307	307	159	159	102	106	162	162	226	226
w13	118	118	139	139	105	105	216	218	63	63	170	172	307	307	159	159	102	127	162	162	190	226
w14	118	118	139	139	105	105	216	216	63	63	170	172	307	307	159	159	102	106	162	162	226	226
w15	118	118	139	139	105	112	216	216	63	63	170	172	307	307	159	168	106	127	162	162	226	226
w16	118	118	139	139	105	112	216	218	63	63	170	170	307	307	159	168	102	106	162	162	226	226
w17	118	118	139	139	112	112	216	216	63	63	170	172	307	307	159	159	106	127	162	162	226	226
w18	118	118	139	139	105	105	216	218	63	63	172	172	307	307	159	159	106	127	162	162	226	226
w19	118	118	139	139	105	105	216	216	63	63	170	170	307	307	159	159	106	127	162	162	226	226
w20	118	118	139	139	105	112	216	218	63	63	170	172	307	307	159	159	102	127	162	162	226	226
RG3																						
A_f1	115	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	102	123	162	168	229	229
A_f2	118	118	139	139	105	112	216	216	63	63	172	172	307	307	168	168	123	125	168	168	229	229
A_f3	115	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	123	123	162	168	229	229
A_f4	118	118	139	139	105	105	216	218	63	63	172	172	278	307	168	168	123	123	168	168	229	229
A_f5	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	123	168	168	229	229
A_m1	115	115	139	139	112	112	216	218	63	63	172	172	307	307	168	168	125	125	168	168	229	229
A_m2	115	115	139	139	105	105	216	218	63	63	172	172	278	278	168	168	102	125	162	168	229	229
A_m3	118	118	139	139	105	112	216	216	63	63	172	172	307	307	168	168	102	123	168	168	229	229
A_m4	118	118	139	139	112	112	216	216	63	63	172	172	307	307	168	168	102	125	168	168	229	229
A_m5	118	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	123	123	168	168	229	229
N_f1	115	115	139	139	105	112	216	216	63	63	172	172	307	307	168	168	102	123	168	168	229	229
N_f2	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	123	168	168	229	229
N_f3	118	118	139	139	105	112	218	218	63	63	172	172	307	307	168	168	102	123	162	162	229	229
N_f4	115	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	102	123	162	168	229	229
N_f5	115	115	139	139	105	105	216	216	63	63	172	172	278	307	168	168	123	123	168	168	229	229
N_f6	115	115	139	139	105	112	216	216	63	63	172	172	278	278	168	168	102	123	168	168	229	229
N_f7	115	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	102	123	168	168	229	229
N_f8	115	115	139	139	105	112	218	218	63	63	172	172	278	278	168	168	102	125	162	162	229	229
N_f9	115	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	102	123	162	168	229	229
N_f10	118	118	139	139	105	112	216	218	63	63	172	172	278	307	168	168	102	102	168	168	229	229
N_f11	118	118	139	139	105	112	216	216	63	63	172	172	307	307	168	168	102	125	168	168	229	229
N_f12	118	118	139	139	105	112	218	218	63	63	172	172	307	307	168	168	125	125	162	168	229	229
N_f13	118	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	123	123	168	168	229	229
N_f14	115	118	139	139	105	112	216	216	63	63	172	172	307	307	168	168	102	125	168	168	229	229
N_f15	115	115	139	139	112	112	216	218	63	63	172	172	278	307	168	168	123	125	162	168	229	229
N_f16	115	115	139	139	105	105	216	218	63	63	172	172	278	278	168	168	125	125	162	168	229	229
N_f17	115	115	139	139	112	112	216	216	63	63	172	172	278	307	168	168	102	125	162	168	229	229
N_f18	115	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	123	125	168	168	229	229
N_f19	115	115	139	139	105	112	216	216	63	63	172	172	307	307	168	168	123	125	162	168	229	229

Colony ID/individuals	Microsatellite loci																					
	Rg15		Rg32		Rg35		Rg39		Rg46		Rg3		Rg9		Rg23		Rg44		Rg48		Rf21-1	
RG3																						
N_f20	118	118	139	139	112	112	216	218	63	63	172	172	278	278	168	168	102	102	162	168	229	229
N_m1	115	115	139	139	112	112	216	218	63	63	172	172	307	307	168	168	123	123	168	168	229	229
N_m2	115	115	139	139	112	112	216	216	63	63	172	172	307	307	168	168	102	125	162	168	229	229
N_m3	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	123	123	162	168	229	229
N_m4	115	118	139	139	105	112	216	216	63	63	172	172	307	307	168	168	123	125	168	168	229	229
N_m5	118	118	139	139	105	112	216	218	63	63	172	172	278	307	168	168	102	123	168	168	229	229
N_m6	115	118	139	139	105	112	216	216	63	63	172	172	278	278	168	168	123	125	168	168	229	229
N_m7	115	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	125	168	168	229	229
N_m8	115	115	139	139	105	112	216	218	63	63	172	172	278	278	168	168	102	102	162	162	229	229
N_m9	115	115	139	139	105	112	216	216	63	63	172	172	307	307	168	168	123	125	162	168	229	229
N_m10	118	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	102	102	162	168	229	229
N_m11	115	118	139	139	112	112	216	216	63	63	172	172	278	307	168	168	102	123	162	168	229	229
N_m12	115	118	139	139	105	105	216	216	63	63	172	172	307	307	168	168	123	125	168	168	229	229
N_m13	115	115	139	139	105	112	216	218	63	63	172	172	307	307	168	168	102	123	162	168	229	229
N_m14	118	118	139	139	105	112	216	218	63	63	172	172	278	307	168	168	102	125	162	168	229	229
N_m15	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	123	125	168	168	229	229
N_m16	115	115	139	139	112	112	216	216	63	63	172	172	278	307	168	168	102	102	162	168	229	229
N_m17	115	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	102	123	168	168	229	229
N_m18	115	118	139	139	105	105	216	218	63	63	172	172	278	307	168	168	123	123	168	168	229	229
N_m19	118	118	139	139	105	112	216	218	63	63	172	172	278	307	168	168	123	125	168	168	229	229
N_m20	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	123	168	168	229	229
w1	115	115	139	139	105	112	216	218	63	63	172	172	307	307	168	168	123	123	162	168	229	229
w2	118	118	139	139	112	112	216	216	63	63	172	172	307	307	168	168	102	123	162	168	229	229
w3	118	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	125	125	162	168	229	229
w4	115	118	139	139	105	112	216	218	63	63	172	172	278	307	168	168	102	123	168	168	229	229
w5	118	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	123	123	162	168	229	229
w6	115	115	139	139	105	112	216	218	63	63	172	172	307	307	168	168	123	125	162	168	229	229
w7	115	118	139	139	112	112	216	216	63	63	172	172	307	307	168	168	123	125	162	168	229	229
w8	115	118	139	139	112	112	216	216	63	63	172	172	307	307	168	168	123	125	168	168	229	229
w9	118	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	102	102	162	168	229	229
w10	118	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	102	123	168	168	229	229
w11	115	115	139	139	112	112	216	216	63	63	172	172	278	307	168	168	123	123	168	168	229	229
w12	115	118	139	139	105	105	216	218	63	63	172	172	278	307	168	168	102	102	162	168	229	229
w13	115	118	139	139	105	112	216	216	63	63	172	172	307	307	168	168	102	123	162	168	229	229
w14	115	115	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	123	162	168	229	229
w15	118	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	123	125	168	168	229	229
w16	115	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	102	162	168	229	229
w17	118	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	102	125	162	168	229	229
w18	115	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	123	123	168	168	229	229
w19	118	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	102	102	168	168	229	229
w20	115	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	123	123	168	168	229	229
w21	115	115	139	139	112	112	216	218	63	63	172	172	307	307	168	168	123	123	162	162	229	229
w22	118	118	139	139	112	112	218	218	63	63	172	172	278	307	168	168	123	123	162	168	229	229
w23	118	118	139	139	112	112	216	218	63	63	172	172	307	307	168	168	123	123	162	168	229	229
w24	118	118	139	139	105	112	218	218	63	63	172	172	278	307	168	168	102	125	162	168	229	229
w25	118	118	139	139	105	105	216	216	63	63	172	172	278	307	168	168	125	125	162	168	229	229
w26	115	118	139	139	105	112	216	216	63	63	172	172	307	307	168	168	123	125	168	168	229	229
w27	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	125	168	168	229	229
w28	118	118	139	139	105	112	216	218	63	63	172	172	307	307	168	168	125	125	162	168	229	229
w29	115	115	139	139	105	112	216	216	63	63	172	172	307	307	168	168	125	125	162	168	229	229
RG4																						
A_f1	118	118	139	139	112	112	216	218	63	63	172	172	278	307	156	168	102	121	162	168	214	214
A_m1	118	118	139	139	105	112	218	218	63	63	172	172	278	278	156	168	123	123	168	168	214	229
A_m2	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	123	162	168	214	229
A_m3	118	118	139	139	105	112	216	218	63	63	172	172	278	278	168	168	123	123	168	168	214	229
A_m4	118	118	139	139	105	112	216	216	63	63	172	172	278	278	168	168	123	123	162	168	229	229
N_f1	118	118	139	139	112	112	216	216	63	63	172	172	278	307	168	168	102	121	168	168	229	229
N_f2	118	118	139	139	112	112	216	218	63	63	172	172	278	307	156	168	121	123	168	168	214	229

Colony ID/individuals	Microsatellite loci																					
	Rg15		Rg32		Rg35		Rg39		Rg46		Rg3		Rg9		Rg23		Rg44		Rg48		Rf21-1	
RG4																						
N_f3	118	118	139	139	112	112	216	218	63	63	172	172	278	278	156	168	102	123	162	168	214	214
N_f4	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	123	168	168	214	229
N_f5	118	118	139	139	112	112	216	216	63	63	172	172	278	307	156	168	123	123	162	168	214	229
N_f6	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	121	123	168	168	229	229
N_f7	118	118	139	139	105	112	216	218	63	63	172	172	278	307	156	168	102	121	168	168	214	229
N_f8	118	118	139	139	112	112	216	216	63	63	172	172	278	307	156	168	102	123	168	168	214	229
N_f9	118	118	139	139	112	112	218	218	63	63	172	172	278	278	168	168	123	123	162	162	229	229
N_f10	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	121	168	168	214	229
N_f11	118	118	139	139	112	112	218	218	63	63	172	172	278	307	156	168	102	102	168	168	214	214
N_f12	118	118	139	139	112	112	216	216	63	63	172	172	278	307	156	168	102	123	168	168	214	229
N_f13	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	121	162	168	229	229
N_f14	118	118	139	139	105	112	216	216	63	63	172	172	278	307	156	168	121	123	162	168	214	229
N_f15	118	118	139	139	105	112	216	218	63	63	172	172	278	307	156	168	102	123	168	168	229	229
N_f16	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	121	168	168	214	214
N_f17	118	118	139	139	112	112	216	218	63	63	172	172	278	307	156	168	102	121	168	168	214	214
N_f18	118	118	139	139	112	112	216	216	63	63	172	172	278	307	156	168	121	123	162	168	214	229
N_f19	118	118	139	139	112	112	216	216	63	63	172	172	278	307	168	168	102	121	162	168	214	214
N_f20	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	121	123	162	168	229	229
N_f21	118	118	139	139	112	112	216	216	63	63	172	172	278	307	156	168	123	123	168	168	229	229
N_m1	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	121	123	162	168	214	214
w1	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	123	123	168	168	214	229
w2	118	118	139	139	112	112	216	218	63	63	172	172	278	307	156	168	102	121	168	168	229	229
w3	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	123	162	168	214	229
w4	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	123	162	168	214	229
w5	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	121	168	168	229	229
w6	118	118	139	139	112	112	216	218	63	63	172	172	278	307	156	168	121	123	162	168	214	229
w7	118	118	139	139	112	112	216	216	63	63	172	172	278	307	168	168	121	123	168	168	214	214
w8	118	118	139	139	112	112	216	216	63	63	172	172	307	307	168	168	121	121	162	168	214	229
w9	118	118	139	139	112	112	216	216	63	63	172	172	278	307	168	168	121	123	162	168	229	229
w10	118	118	139	139	105	105	216	218	63	63	172	172	278	307	168	168	102	123	168	168	214	229
w11	118	118	139	139	112	112	216	216	63	63	172	172	278	307	156	168	102	123	162	168	214	229
w12	118	118	139	139	112	112	216	216	63	63	172	172	278	278	156	156	121	123	162	168	214	229
w13	118	118	139	139	105	112	216	216	63	63	172	172	278	307	168	168	102	123	162	168	214	229
w14	118	118	139	139	105	112	216	218	63	63	172	172	278	307	156	168	123	123	162	168	214	214
w15	118	118	139	139	112	112	216	218	63	63	172	172	278	307	168	168	102	121	162	168	214	229
w16	118	118	139	139	105	112	216	218	63	63	172	172	278	307	168	168	123	123	168	168	214	229
w17	118	118	139	139	112	112	216	216	63	63	172	172	278	307	156	168	121	123	168	168	214	214
w18	118	118	139	139	105	112	216	216	63	63	172	172	278	307	156	168	123	123	168	168	214	229
RG5																						
A_f1	118	118	139	139	105	112	216	218	63	63	172	172	278	307	159	159	104	125	162	162	205	214
A_f2	118	130	139	139	112	112	216	216	63	63	172	172	278	307	159	159	104	104	162	162	205	214
A_f3	118	130	139	139	105	112	216	218	63	63	172	172	278	307	159	168	104	104	162	162	214	214
A_f4	118	130	139	139	105	112	216	216	63	63	172	172	278	307	159	159	104	104	162	162	205	214
A_f5	118	130	139	139	105	112	216	216	63	63	172	172	278	307	159	159	104	104	162	162	205	214
A_f6	118	130	139	139	105	112	216	218	63	63	172	172	278	307	159	168	104	121	162	162	214	214
A_f7	118	118	139	139	112	112	218	218	63	63	172	172	307	307	159	159	104	121	162	162	214	214
A_f8	118	118	139	139	112	112	216	218	63	63	172	172	278	307	159	159	119	121	162	162	214	214
A_f9	118	130	139	139	105	112	216	216	63	63	172	172	278	278	168	168	104	104	162	162	214	214
A_f10	130	130	139	139	112	112	216	216	63	63	172	172	307	307	159	159	104	121	162	162	205	214
A_m1	118	130	139	139	112	112	216	216	63	63	172	172	278	307	159	168	121	125	162	162	214	214
A_m2	118	118	139	139	105	112	216	216	63	63	172	172	307	307	159	159	119	121	162	162	214	214
A_m3	118	118	139	139	105	112	216	216	63	63	172	172	278	278	159	168	104	125	162	162	214	214
A_m4	118	130	139	139	105	112	218	218	63	63	172	172	307	307	159	159	104	121	162	162	214	214
A_m5	118	118	139	139	105	112	216	218	63	63	172	172	278	307	159	168	104	121	162	162	214	214
A_m6	130	130	139	139	105	112	216	218	63	63	172	172	307	307	159	159	104	121	162	162	214	214
A_m7	118	130	139	139	105	112	216	218	63	63	172	172	307	307	159	168	104	104	162	162	205	214
A_m8	118	118	139	139	112	112	216	218	63	63	172	172	307	307	159	159	121	125	162	162	214	214
N_f1	118	118	139	139	112	112	216	218	63	63	172	172	307	307	159	159	104	125	162	162	214	214

Colony ID/individuals	Microsatellite loci																					
	Rg15		Rg32		Rg35		Rg39		Rg46		Rg3		Rg9		Rg23		Rg44		Rg48		Rf21-1	
RG5																						
N_f2	118	118	139	139	105	105	216	218	63	63	172	172	307	307	159	159	104	104	162	162	205	214
N_f3	118	118	139	139	105	112	216	218	63	63	172	172	278	307	159	159	104	125	162	162	214	214
N_f4	118	118	139	139	112	112	216	216	63	63	172	172	307	307	159	159	104	121	162	162	205	214
N_f5	118	118	139	139	105	112	216	218	63	63	172	172	278	278	168	168	104	121	162	162	214	214
N_f6	130	130	139	139	105	112	216	216	63	63	172	172	278	307	159	168	104	121	162	162	214	214
N_f7	118	130	139	139	105	105	216	216	63	63	172	172	278	307	159	168	104	104	162	162	214	214
N_f8	130	130	139	139	105	105	216	216	63	63	172	172	278	278	168	168	104	104	162	162	214	214
N_f9	130	130	139	139	105	112	216	218	63	63	172	172	278	307	159	168	104	121	162	162	214	214
N_m1	118	130	139	139	105	112	218	218	63	63	172	172	278	307	159	168	104	121	162	162	214	214
N_m2	118	130	139	139	105	105	216	216	63	63	172	172	278	278	159	168	104	121	162	162	205	214
N_m3	118	130	139	139	105	112	216	218	63	63	172	172	307	307	159	159	104	121	162	162	214	214
w1	118	118	139	139	112	112	216	216	63	63	172	172	307	307	159	159	104	104	162	162	214	214
w2	118	130	139	139	105	112	216	216	63	63	172	172	307	307	159	159	104	125	162	162	214	214
w3	118	118	139	139	105	112	216	216	63	63	172	172	278	307	159	159	104	119	162	162	214	214
w4	118	130	139	139	105	112	216	216	63	63	172	172	307	307	159	159	104	104	162	162	214	214
w5	118	118	139	139	105	112	216	216	63	63	172	172	278	278	159	168	104	125	162	162	214	214
w6	118	118	139	139	112	112	216	216	63	63	172	172	278	307	159	159	121	125	162	162	214	214
w7	118	118	139	139	105	112	216	216	63	63	172	172	278	307	159	168	104	125	162	162	214	214
w8	118	118	139	139	105	112	216	216	63	63	172	172	278	307	159	159	104	119	162	162	214	214
w9	118	130	139	139	105	112	216	216	63	63	172	172	278	307	159	159	104	121	162	162	205	214
w10	118	118	139	139	105	112	216	216	63	63	172	172	307	307	159	159	121	121	162	162	205	214
w11	118	118	139	139	112	112	216	218	63	63	172	172	307	307	159	159	104	121	162	162	214	214
w12	118	130	139	139	105	112	216	218	63	63	172	172	278	307	159	168	104	104	162	162	214	214
w13	118	130	139	139	105	105	216	218	63	63	172	172	278	307	159	168	104	104	162	162	214	214
w14	118	118	139	139	112	112	216	216	63	63	172	172	278	307	159	159	104	119	162	162	205	214
w15	118	130	139	139	105	112	216	216	63	63	172	172	278	307	159	159	104	121	162	162	205	214
w16	118	130	139	139	105	105	216	218	63	63	172	172	278	278	168	168	104	104	162	162	214	214
w17	118	118	139	139	105	112	216	216	63	63	172	172	278	307	159	159	104	104	162	162	205	205
w18	118	130	139	139	105	112	216	216	63	63	172	172	307	307	159	159	104	104	162	162	205	214
w19	118	130	139	139	112	112	216	218	63	63	172	172	278	307	159	168	104	104	162	162	205	214
w20	118	130	139	139	105	112	216	218	63	63	172	172	278	307	159	159	104	121	162	162	214	214

Suppl. Table S3. Genotypes of *R. flavipes* colonies (A_f/m: alate female/male; N_f/m: neotenic female/male; w: worker)

Colony ID/individuals	Microsatellite loci															
	Rf11-1		Rf6-1		Rs1		Rf21-1		Rs43		Rs15		Rf15-2		Rf1-3	
RF1																
A_f1	225	231	173	197	273	282	223	223	220	220	262	262	208	217	200	203
A_f2	225	231	197	197	273	273	223	223	220	224	253	253	217	217	200	203
A_f3	231	231	194	197	279	279	223	223	224	224	262	262	208	208	200	203
A_f4	225	225	194	200	273	282	223	223	220	224	262	262	217	217	200	203
A_f5	225	231	197	197	273	273	223	265	220	220	253	253	208	217	200	203
A_f6	225	225	185	200	273	282	223	223	220	220	253	262	208	217	200	203
A_f7	225	231	185	185	273	273	265	265	220	220	253	253	208	217	200	203
A_f8	231	231	185	185	273	282	223	265	220	224	253	253	217	217	200	203
A_f9	225	231	194	194	273	273	223	265	220	224	253	262	208	217	200	203
A_f10	231	231	185	194	282	282	223	223	220	220	253	262	208	208	200	203
A_m1	231	231	194	194	273	282	223	223	220	224	262	262	208	217	200	203
A_m2	225	231	185	185	273	273	223	223	224	224	253	262	208	217	200	203
A_m3	231	231	185	197	279	282	223	223	220	220	262	262	208	208	200	203
A_m4	231	231	185	185	273	282	223	265	220	220	253	262	217	217	200	203
A_m5	225	231	185	194	273	279	265	265	220	220	262	262	208	217	200	203
A_m6	225	231	185	194	282	282	223	223	220	220	262	262	217	217	200	203
A_m7	231	231	185	185	273	273	223	265	220	224	253	262	208	208	200	203
A_m8	225	231	185	185	273	273	265	265	220	220	253	253	217	217	200	203
A_m9	225	231	194	197	273	273	223	265	220	224	262	262	208	217	200	203
A_m10	225	231	185	185	273	273	265	265	220	220	253	262	217	217	200	203
w1	225	231	185	185	273	282	223	223	220	224	262	262	208	208	203	206
w2	231	231	185	191	273	273	223	223	220	224	262	262	217	217	203	206
w3	225	225	185	194	273	279	223	265	220	224	253	262	208	208	200	206
w4	231	231	194	194	273	273	223	223	220	224	253	262	217	217	200	200
w5	231	231	185	185	273	279	223	223	220	220	253	253	217	217	200	203
w6	231	231	194	197	273	273	265	265	220	224	253	262	217	217	203	203
w7	225	225	185	200	273	273	223	265	220	224	253	262	208	217	200	203
w8	225	231	185	185	273	279	223	265	220	220	253	253	208	217	203	203
w9	225	231	194	197	273	273	223	223	220	220	253	262	217	217	203	203
RF2																
A_f1	231	231	182	182	270	270	238	268	220	220	256	259	211	211	206	209
A_f2	231	231	176	176	270	270	211	238	220	220	256	256	211	211	206	206
A_f3	231	231	176	182	300	300	238	238	220	220	256	259	211	211	206	206
A_f4	231	231	176	182	270	300	238	238	220	220	256	256	211	211	206	206
A_f5	231	231	182	182	270	300	211	211	220	220	259	259	211	211	200	200
A_f6	231	231	182	182	270	270	211	238	220	220	256	256	211	211	200	206
A_f7	231	231	176	182	270	300	238	238	220	220	256	259	211	211	206	206
A_f8	231	231	182	182	270	300	238	238	220	220	256	259	211	211	206	209
A_f9	223	231	182	182	300	300	238	238	220	220	256	256	211	211	206	206
A_f10	231	231	176	182	270	300	238	238	220	220	256	259	211	211	206	206
A_m1	223	223	182	182	270	300	238	238	220	220	256	259	211	211	206	206
A_m2	223	231	176	182	270	270	238	238	220	220	256	259	211	211	206	209
A_m3	231	231	176	182	270	270	211	211	220	220	256	259	211	211	206	206
A_m4	231	231	182	182	270	270	211	238	220	220	256	256	211	211	206	206
A_m5	231	231	182	182	270	300	238	238	220	220	256	259	211	211	206	209
A_m6	231	231	176	176	270	270	238	238	220	220	256	259	211	220	200	206
A_m7	231	231	176	182	300	300	238	238	220	220	256	256	211	211	206	206
A_m8	223	231	182	182	270	300	238	238	220	220	259	259	211	211	206	209
A_m9	231	231	176	182	270	270	238	238	220	220	256	259	211	211	206	206
A_m10	231	231	176	182	270	300	238	238	220	220	256	259	211	211	206	209
N_f1	223	231	176	182	270	300	238	238	220	220	259	259	211	220	206	209
N_f2	231	231	182	182	270	300	238	238	220	220	259	259	211	211	200	200
N_f3	231	231	176	182	270	270	238	238	220	220	256	256	211	211	206	206

Colony ID/individuals	Microsatellite loci															
	Rf11-1		Rf6-1		Rs1		Rf21-1		Rs43		Rs15		Rf15-2		Rf1-3	
RF2																
N_f4	231	231	176	182	300	300	238	238	220	220	256	259	211	211	206	209
N_f5	231	231	176	182	270	270	238	238	220	220	256	259	211	211	206	206
N_f6	223	231	176	182	300	300	211	238	220	220	256	259	211	211	206	206
N_f7	231	231	182	182	270	300	238	238	220	220	256	259	211	220	206	209
N_f8	231	231	182	182	300	300	238	238	220	220	259	259	211	211	200	206
N_f9	223	231	176	176	270	270	238	238	220	220	259	259	211	211	206	206
N_m1	231	231	176	176	270	300	238	238	220	220	259	259	211	211	206	206
N_m2	223	231	176	182	270	300	238	238	220	220	256	259	211	211	206	206
N_m3	223	231	182	182	270	300	211	238	220	220	256	259	211	211	206	206
N_m4	231	231	182	182	300	300	238	238	220	220	256	259	211	211	206	209
N_m5	231	231	176	182	270	300	238	238	220	220	256	259	211	211	200	200
N_m6	231	231	182	182	300	300	238	238	220	220	256	259	211	220	206	206
N_m7	231	231	176	182	270	300	238	238	220	220	259	259	211	211	206	209
N_m8	231	231	176	182	300	300	238	238	220	220	256	259	211	211	206	209
N_m9	223	223	182	182	300	300	238	238	220	220	259	259	211	211	206	209
N_m10	231	231	182	182	300	300	211	238	220	220	256	256	211	211	206	206
w1	231	231	176	182	300	300	238	238	220	220	256	256	211	211	206	206
w2	231	231	176	182	270	270	238	238	220	220	256	259	211	211	206	206
w3	223	231	182	182	270	300	211	238	220	220	256	259	211	211	206	206
w4	223	231	182	182	270	270	238	238	220	220	259	259	211	211	206	206
w5	223	231	182	182	270	300	211	238	220	220	256	259	211	211	206	209
w6	231	231	182	182	300	300	238	238	220	220	259	259	211	211	200	206
w7	223	231	182	182	270	270	238	238	220	220	256	259	211	211	206	209
w8	223	223	176	182	270	300	238	238	220	220	256	259	211	211	200	206
w9	223	223	176	182	270	270	238	238	220	220	259	259	211	211	206	206
w10	223	231	182	182	270	270	238	238	220	220	259	259	211	211	200	206
w11	223	231	182	182	270	300	238	238	220	220	256	259	211	211	206	206
w12	231	231	176	182	270	270	238	238	220	220	256	259	211	211	206	206
w13	223	231	182	182	270	270	238	238	220	220	259	259	211	211	206	206
w14	231	231	182	182	300	300	238	238	220	220	259	259	211	211	206	206
w15	223	231	182	182	270	300	238	238	220	220	259	259	211	211	206	206
w16	231	231	176	182	270	270	238	238	220	220	256	259	211	211	206	206
w17	223	231	182	182	270	270	238	238	220	220	256	259	211	211	206	206
w18	231	231	176	182	270	300	211	238	220	220	256	259	211	211	206	206
w19	223	231	182	182	270	300	238	238	220	220	259	259	211	211	200	206
RF3																
A_f1	231	231	170	182	279	282	223	232	220	224	253	262	208	208	200	203
A_f2	231	231	182	182	279	282	223	223	220	220	253	262	208	208	200	203
A_f3	227	231	182	182	279	279	223	223	224	224	253	262	208	208	203	203
A_f4	225	225	170	182	279	279	223	223	224	224	253	253	208	208	200	203
A_f5	225	225	170	191	279	279	223	223	224	224	253	262	208	208	200	203
A_f6	225	231	170	191	279	279	223	259	220	224	253	253	208	208	203	203
A_f7	231	231	170	170	279	282	259	259	220	220	259	262	208	217	203	203
A_f8	231	231	170	191	279	279	223	259	220	224	253	262	208	217	200	200
A_f9	231	231	170	182	279	279	223	259	224	224	262	262	208	217	203	203
A_f10	231	231	182	182	279	279	223	223	220	224	253	262	208	217	203	203
A_m1	225	231	170	182	279	279	223	223	220	224	253	253	208	217	203	203
A_m2	231	231	182	182	279	279	223	223	220	224	253	262	217	217	200	203
A_m3	225	231	182	182	279	279	223	223	224	224	262	262	208	208	200	203
A_m4	231	231	170	182	279	279	223	223	224	224	262	262	208	208	200	203
A_m5	225	231	182	182	279	282	223	223	220	224	262	262	208	217	200	203
A_m6	231	231	182	182	279	279	223	259	220	224	262	262	208	217	203	203
A_m7	225	231	170	182	279	282	223	223	220	224	253	253	208	208	203	203
A_m8	231	231	170	182	279	279	223	223	220	220	253	262	208	208	200	203
A_m9	225	231	182	188	279	279	223	223	220	220	253	262	208	208	200	200
A_m10	225	231	170	182	279	279	223	223	220	220	253	262	208	217	200	203
N_f1	231	231	170	191	279	279	223	259	220	224	253	262	208	217	200	203
N_f2	225	231	170	182	279	279	223	223	220	224	253	253	208	217	200	203

Colony ID/individuals	Microsatellite loci															
	Rf11-1		Rf6-1		Rs1		Rf21-1		Rs43		Rs15		Rf15-2		Rf1-3	
RF3																
N_f3	231	231	170	182	279	279	223	259	220	224	253	262	208	208	203	203
N_f4	231	231	182	182	279	282	223	223	220	224	259	262	208	208	200	203
N_f5	225	227	170	182	279	279	223	223	224	224	253	253	208	217	200	203
N_f6	225	231	182	182	279	279	223	223	224	224	253	262	208	208	200	203
N_f7	227	231	182	182	282	282	223	223	224	224	262	262	208	208	200	203
N_f8	225	231	170	182	279	279	223	223	224	224	253	262	208	208	200	203
N_f9	231	231	170	182	279	279	223	223	224	224	253	253	208	217	203	203
N_f10	231	231	170	182	279	279	223	223	220	220	262	262	208	208	200	203
N_m1	225	231	170	182	279	279	223	223	220	224	253	262	208	208	203	203
N_m2	225	225	176	182	279	279	223	223	220	224	253	253	208	208	200	203
N_m3	231	231	182	194	279	282	223	223	220	224	253	262	208	208	203	203
N_m4	231	231	182	182	279	282	223	223	220	224	253	253	208	208	203	203
N_m5	227	231	170	191	279	279	223	223	220	224	262	262	208	208	200	200
N_m6	225	231	170	182	279	282	223	223	224	224	259	262	208	208	200	203
N_m7	231	231	179	182	279	279	223	223	220	224	253	262	208	217	203	203
N_m8	231	231	182	191	279	279	223	223	224	224	262	262	208	227	203	203
N_m9	225	231	182	191	279	282	223	223	220	220	253	262	208	217	200	203
N_m10	225	231	170	182	279	279	223	223	220	220	253	262	208	217	200	200
w1	225	231	170	170	279	279	223	223	220	224	253	262	208	217	200	200
w2	225	231	182	182	279	279	223	259	220	224	253	262	217	217	200	203
w3	231	231	170	170	279	279	223	223	220	224	262	262	208	208	200	203
w4	227	231	182	182	279	279	223	223	220	224	253	253	208	217	200	203
w5	231	231	170	170	279	282	223	223	224	224	253	262	217	217	203	203
w6	231	231	170	182	282	282	223	223	220	224	262	262	208	208	200	203
w7	225	231	182	191	279	279	223	259	220	224	259	262	208	208	203	203
w8	225	231	182	182	279	279	223	223	220	224	253	262	208	217	203	203
w9	227	231	170	182	279	279	223	223	220	224	253	253	217	217	200	203
w10	225	231	170	182	279	279	223	223	220	224	253	262	208	208	203	203
w11	231	231	170	182	279	279	223	223	224	224	262	262	208	208	200	203
w12	227	231	182	182	279	279	223	259	224	224	262	262	208	208	203	203
w13	225	231	182	182	279	279	223	259	220	224	262	262	208	217	200	203
w14	231	231	170	170	279	282	223	223	220	224	253	262	217	217	200	203
w15	225	231	170	182	279	279	223	223	220	224	259	262	208	217	203	203
w16	231	231	170	191	279	279	223	259	220	224	253	259	208	208	200	203
w17	231	231	170	191	279	279	223	223	220	224	253	262	208	208	200	203
RF4																
A_f1	225	231	182	194	282	282	223	259	220	220	253	262	217	220	200	203
A_f2	225	231	194	194	282	282	259	259	220	220	253	262	208	220	203	203
A_f3	231	231	182	182	282	282	259	259	220	220	253	262	220	220	200	200
A_f4	231	231	170	182	282	282	223	259	220	220	253	262	220	220	203	203
A_f5	231	231	182	182	282	282	223	223	220	220	253	253	217	217	200	203
A_f6	231	231	170	182	282	282	223	259	220	220	253	262	208	220	200	203
A_f7	231	231	182	194	282	282	223	223	220	220	253	262	217	217	203	203
A_f8	231	231	182	194	273	282	259	259	220	220	253	259	217	220	200	203
A_f9	231	231	170	182	282	300	223	259	220	220	262	262	208	217	203	203
A_f10	231	231	182	194	273	282	223	259	220	220	253	253	217	217	200	203
A_m1	231	231	182	194	282	282	259	259	220	220	253	253	217	220	203	203
A_m2	231	231	182	194	282	282	223	259	220	220	253	262	208	217	203	203
A_m3	225	225	170	182	273	282	223	259	220	220	253	253	217	220	203	203
A_m4	225	231	194	194	282	282	223	223	220	220	253	262	208	217	203	203
A_m5	231	231	170	194	282	282	223	259	220	220	253	262	220	220	203	203
A_m6	231	231	182	194	282	282	259	259	220	220	253	253	217	220	200	203
A_m7	225	231	170	182	282	282	223	223	220	220	253	262	217	217	203	203
A_m8	231	231	182	182	282	282	223	256	220	220	253	253	217	217	203	203
A_m9	225	231	170	170	282	282	259	259	220	220	253	262	220	220	203	203
A_m10	225	231	170	182	282	282	259	259	220	220	253	253	217	217	203	203
N_f1	225	231	182	194	273	282	259	259	220	220	253	262	208	217	200	200
N_f2	231	231	170	182	282	282	259	259	220	220	259	262	208	208	200	200

Colony ID/individuals	Microsatellite loci															
	Rf11-1		Rf6-1		Rs1		Rf21-1		Rs43		Rs15		Rf15-2		Rf1-3	
RF4																
N_f3	231	231	182	182	282	282	223	259	220	220	253	262	208	217	203	203
N_f4	231	231	182	194	282	282	259	259	220	220	262	262	208	208	203	203
N_f5	231	231	194	194	282	282	223	259	220	220	253	262	208	217	203	203
N_f6	231	231	170	194	282	282	223	259	220	220	253	262	220	220	203	203
N_f7	231	231	182	194	282	282	259	259	220	220	259	262	208	217	200	203
N_f8	231	231	194	194	282	282	223	223	220	220	253	253	217	217	203	203
N_f9	231	231	182	182	282	282	259	259	220	220	253	253	208	217	203	203
N_f10	225	231	182	182	282	282	223	259	220	220	253	262	208	220	203	203
N_m1	231	231	182	194	282	282	223	259	220	220	253	253	208	220	200	203
N_m2	225	231	182	182	282	282	223	223	220	220	253	253	208	220	200	203
N_m3	231	231	182	194	282	282	223	259	220	220	253	253	217	220	200	203
N_m4	231	231	182	182	282	282	223	259	220	220	253	253	208	220	200	203
N_m5	231	231	182	194	282	282	223	259	220	220	253	253	217	220	203	203
N_m6	231	231	170	170	282	282	223	259	220	220	253	262	208	220	200	203
N_m7	231	231	182	194	273	282	259	259	220	220	253	262	208	217	200	200
N_m8	225	231	182	194	273	282	223	259	220	220	262	262	208	217	203	203
N_m9	231	231	182	182	282	282	259	259	220	220	253	253	217	217	203	203
N_m10	231	231	182	194	282	282	259	259	220	220	262	262	208	220	200	203
w1	225	231	182	194	273	282	223	223	220	220	253	262	217	217	200	200
w2	225	231	170	194	282	282	223	259	220	220	253	253	208	217	200	203
w3	231	231	182	182	282	282	259	259	220	220	253	253	217	217	203	203
w4	231	231	170	182	282	282	223	259	220	220	253	262	217	217	200	203
w5	225	231	170	194	282	282	223	259	220	220	253	253	217	217	203	203
w6	225	225	170	194	282	282	259	259	220	220	253	253	217	217	203	203
w7	225	231	182	182	282	282	223	259	220	220	253	253	217	220	203	203
w8	231	231	170	194	282	282	259	259	220	220	253	253	217	217	200	203
w9	231	231	182	194	282	282	223	259	220	220	253	262	217	220	200	203
w10	225	231	194	194	282	282	259	259	220	220	253	262	217	217	203	203
w11	225	231	170	170	282	282	223	259	220	220	253	259	217	217	203	203
w12	231	231	170	194	282	282	223	259	220	220	253	253	217	220	203	203
w13	225	231	182	182	273	282	259	259	220	220	262	262	217	217	203	203
w14	225	231	194	194	282	282	223	223	220	220	262	262	217	217	200	203
w15	225	231	194	194	282	282	223	223	220	220	253	262	217	217	203	203
w16	231	231	170	194	282	282	223	259	220	220	253	262	217	217	200	203
w17	231	231	182	194	282	282	259	259	220	220	253	253	208	220	203	203
w18	231	231	170	182	282	282	259	259	220	220	253	253	220	220	203	203

Suppl. Table S4. Genotypes of *R. lucifgus* colonies and inferred primary queens and kings (PQ: primary queen; PK: primary king; A_f/m: alate female/male; N_f/m: neotenic female/male; w: worker)

Colony ID/individuals	Microsatellite loci											
	Rf24-2		Rf21-1		Rs02		Rf5-10		Rs10		Rf1-3	
RL1												
PQ	91	94	210	216	239	239	137	140	158	158	211	211
PK	91	94	213	216	239	239	137	137	158	209	211	211
A_f1	94	94	216	216	239	239	137	137	158	158	211	211
A_f2	94	94	216	216	239	239	137	137	158	158	211	211
A_f3	94	94	210	210	239	239	137	137	158	158	211	211
A_f4	94	94	216	216	239	239	137	137	158	158	211	211
A_f5	94	94	216	216	239	239	137	137	158	158	211	211
A_f6	94	94	216	216	239	239	137	137	158	158	211	211
A_f7	94	94	216	216	239	239	137	137	158	158	211	211
A_f8	94	94	216	216	239	239	137	137	158	158	211	211
A_f9	94	94	216	216	239	239	137	137	158	158	211	211
A_f10	91	94	216	216	239	239	137	137	158	158	211	211
A_m1	94	94	216	216	239	239	137	137	158	158	211	211
A_m2	94	94	210	216	239	239	137	137	158	158	211	211
A_m3	94	94	216	216	239	239	137	140	158	158	211	211
A_m4	94	94	210	216	239	239	137	140	158	158	211	211
A_m5	94	94	216	216	239	239	137	137	158	158	211	211
A_m6	94	94	210	216	239	239	140	140	158	158	211	211
A_m7	94	94	216	216	239	239	137	137	158	158	211	211
A_m8	94	94	216	216	239	239	137	137	158	158	211	211
A_m9	94	94	216	216	239	239	137	137	158	158	211	211
A_m10	94	94	216	216	239	239	137	137	158	158	211	211
N_f1	94	94	216	216	239	239	137	137	158	158	211	211
N_f2	94	94	216	216	239	239	137	137	158	158	211	211
N_f3	94	94	210	210	239	239	137	137	158	158	211	211
N_f4	94	94	216	216	239	239	137	137	158	158	211	211
N_f5	91	91	216	216	239	239	140	140	158	158	211	211
N_f6	94	94	216	216	239	239	140	140	158	158	211	211
N_f7	94	94	216	216	239	239	140	140	158	158	211	211
N_f8	94	94	216	216	239	239	140	140	158	158	211	211
w1	94	94	213	216	239	239	137	140	158	158	211	211
w2	94	94	210	216	239	239	137	140	158	209	211	211
w3	91	94	210	216	239	239	137	140	158	158	211	211
w4	91	94	213	216	239	239	137	137	158	158	211	211
w5	91	94	216	216	239	239	137	137	158	158	211	211
w6	94	94	213	216	239	239	137	137	158	158	211	211
w7	91	94	216	216	239	239	137	137	158	158	211	211
w8	91	91	216	216	239	239	137	140	158	158	211	211
w9	91	94	216	216	239	239	137	137	158	158	211	211
w10	91	94	216	216	239	239	137	137	158	158	211	211
RL2												
PQ	91	94	210	216	239	239	137	137	158	158	211	211
PK	91	91	210	210	239	239	137	140	158	158	205	211
N_f1	94	94	210	210	239	239	137	137	158	158	211	211
N_f2	94	94	210	210	239	239	137	137	158	158	211	211
N_f3	94	94	210	210	239	239	137	137	158	158	211	211
N_f4	94	94	216	216	239	239	137	137	158	158	211	211
N_f5	94	94	210	210	239	239	137	137	158	158	211	211
N_f6	94	94	210	210	239	239	137	137	158	158	211	211
N_f7	91	91	210	210	239	239	137	137	158	158	211	211
N_f8	91	91	210	210	239	239	137	137	158	158	211	211

Colony ID/individuals	Microsatellite loci											
	Rf24-2		Rf21-1		Rs02		Rf5-10		Rs10		Rf1-3	
RL2												
N_f9	91	91	210	210	239	239	137	137	158	158	211	211
N_f10	94	94	210	210	239	239	137	137	158	158	211	211
w1	91	91	210	210	239	239	137	137	158	158	211	211
w2	91	94	210	210	239	239	137	137	158	158	211	211
w3	91	94	210	210	239	239	137	137	158	158	205	211
w4	91	94	210	210	239	239	137	137	158	158	205	211
w5	91	94	210	210	239	239	137	137	158	158	211	211
w6	91	94	210	210	239	239	137	140	158	158	211	211
w7	91	91	210	210	239	239	137	137	158	158	205	211
w8	91	91	210	210	239	239	137	137	158	158	211	211
w9	91	91	210	210	239	239	137	137	158	158	205	211
w10	91	94	210	210	239	239	137	137	158	158	205	211
RL3												
PQ	94	94	210	216	239	239	137	140	158	158	211	211
PK1	91	94	207	216	239	239	137	137	158	158	211	211
PK2	94	94	210	216	239	239	137	137	158	158	211	211
A_f1	94	94	216	216	239	239	137	137	158	158	211	211
A_f2	94	94	210	216	239	239	140	140	158	158	211	211
A_f3	94	94	210	210	239	239	137	140	158	158	211	211
A_f4	94	94	216	216	239	239	137	140	158	158	211	211
A_f5	94	94	216	216	239	239	137	140	158	158	211	211
A_f6	94	94	210	216	239	239	137	140	158	158	211	211
A_f7	94	94	210	216	239	239	137	140	158	158	211	211
A_f8	94	94	210	210	239	239	140	140	158	158	211	211
A_f9	94	94	210	216	239	239	137	137	158	158	211	211
A_f10	94	94	216	216	239	239	137	140	158	158	211	211
A_m1	94	94	216	216	239	239	137	137	158	158	211	211
A_m2	94	94	210	216	239	239	137	137	158	158	211	211
A_m3	94	94	210	216	239	239	137	140	158	158	211	211
A_m4	94	94	210	210	239	239	137	140	158	158	211	211
A_m5	94	94	210	210	239	239	137	140	158	158	211	211
A_m6	94	94	210	216	239	239	137	140	158	158	211	211
A_m7	94	94	210	216	239	239	137	140	158	158	211	211
A_m8	94	94	210	216	239	239	137	140	158	158	211	211
A_m9	94	94	210	210	239	239	137	140	158	158	211	211
A_m10	94	94	210	210	239	239	137	140	158	158	211	211
N_f1	94	94	210	216	239	239	137	140	158	158	211	211
N_f2	94	94	216	216	239	239	137	140	158	158	211	211
N_f3	94	94	216	216	239	239	140	140	158	158	211	211
N_f4	94	94	210	210	239	239	140	140	158	158	211	211
N_f5	94	94	216	216	239	239	140	140	158	158	211	211
N_f6	94	94	216	216	239	239	137	140	158	158	211	211
N_f7	94	94	216	216	239	239	140	140	158	158	211	211
N_f8	94	94	216	216	239	239	137	140	158	158	211	211
N_f9	94	94	210	210	239	239	137	140	158	158	211	211
N_f10	94	94	210	210	239	239	140	140	158	158	211	211
w1	94	94	216	216	239	239	137	140	158	158	211	211
w2	94	94	210	210	239	239	137	140	158	158	211	211
w3	94	94	210	216	239	239	137	140	158	158	211	211
w4	94	94	216	216	239	239	137	137	158	158	211	211
w5	91	94	210	216	239	239	137	140	158	158	211	211
w6	91	94	210	216	239	239	137	137	158	158	211	211
w7	91	94	210	216	239	239	137	137	158	158	211	211
w8	91	94	207	210	239	239	137	137	158	158	211	211
w9	94	94	216	216	239	239	137	140	158	158	211	211
w10	91	94	216	216	239	239	137	137	158	158	211	211

Colony ID/individuals	Microsatellite loci											
	Rf24-2		Rf21-1		Rs02		Rf5-10		Rs10		Rf1-3	
RL4												
A_f1	94	94	210	210	239	239	137	140	158	158	211	211
A_f2	94	94	210	210	239	239	137	140	158	158	211	211
A_f3	94	94	210	210	239	239	137	140	158	158	211	211
A_f4	94	94	210	210	239	239	140	140	158	158	211	211
A_f5	94	94	210	210	235	239	137	140	158	158	211	211
A_f6	94	94	210	210	239	239	140	140	158	158	211	211
A_f7	94	94	210	210	239	239	140	140	158	158	211	211
A_f8	94	94	210	210	239	239	137	140	158	158	211	211
A_f9	94	94	210	210	239	239	137	137	158	158	211	211
A_f10	94	94	210	210	239	239	137	137	158	158	211	211
A_m1	94	94	210	210	239	239	137	140	158	158	211	211
A_m2	94	94	210	210	239	239	140	140	164	164	211	211
A_m3	94	94	210	210	235	239	140	140	158	158	211	211
A_m4	94	94	210	210	235	239	137	140	158	158	211	211
A_m5	94	94	210	210	235	239	140	140	158	158	211	211
A_m6	94	94	210	210	235	239	137	137	158	158	211	211
A_m7	94	94	210	210	235	239	137	140	158	158	211	211
A_m8	94	94	210	210	235	239	140	140	158	158	211	211
A_m9	94	94	210	210	239	239	137	140	158	158	211	211
A_m10	94	94	210	210	239	239	137	140	158	158	211	211
w1	94	94	210	210	239	239	140	140	158	158	211	211
w2	94	94	210	210	239	239	140	140	158	158	211	211
w3	94	94	210	210	239	239	137	137	158	158	211	211
w4	94	94	210	210	239	239	137	137	158	158	211	211
w5	94	94	210	210	239	239	137	137	158	158	211	211
w6	94	94	210	210	239	239	137	140	158	158	211	211
w7	94	94	210	210	239	239	137	140	158	158	211	211
w8	94	94	210	210	239	239	137	140	158	158	211	211
w9	94	94	210	210	239	239	137	137	158	158	211	211
w10	94	94	210	210	239	239	137	140	158	158	211	211
RL5												
A_f1	94	94	216	216	239	239	137	137	158	158	211	211
A_f2	94	94	216	216	239	239	137	137	158	158	211	211
A_f3	94	94	216	216	239	239	140	140	158	158	211	211
A_f4	94	94	216	216	239	239	137	137	158	158	211	211
A_f5	91	91	210	210	239	239	140	140	158	158	211	211
A_f6	94	94	216	216	239	239	137	137	158	158	211	211
A_f7	94	94	210	210	239	239	137	137	158	158	211	211
A_f8	94	94	216	216	239	239	140	140	158	158	211	211
A_f9	91	91	210	210	239	239	140	140	158	158	211	211
A_f10	91	91	216	216	239	239	137	137	158	158	211	211
A_m1	94	94	210	210	239	239	137	137	158	158	211	211
A_m2	94	94	216	216	239	239	137	137	158	158	211	211
A_m3	91	94	210	216	239	239	140	140	158	158	211	211
A_m4	94	94	216	216	239	239	137	140	158	158	211	211
A_m5	91	94	210	216	239	239	137	140	158	158	211	211
A_m6	94	94	210	216	239	239	137	140	158	158	211	211
A_m7	94	94	216	216	239	239	137	140	158	158	211	211
A_m8	91	94	210	216	239	239	137	137	158	158	211	211
A_m9	94	94	216	216	239	239	137	140	158	158	211	211
A_m10	94	94	216	216	239	239	137	137	158	158	211	211
w1	94	94	216	216	239	239	137	137	158	158	211	211
w2	94	94	216	216	239	239	137	137	158	158	211	211
w3	94	94	216	216	239	239	137	140	158	158	211	211
w4	91	94	210	216	239	239	137	140	158	158	211	211
w5	94	94	216	216	239	239	137	137	158	158	211	211
w6	94	94	216	216	239	239	137	140	158	158	211	211
w7	94	94	216	216	239	239	137	137	158	158	211	211

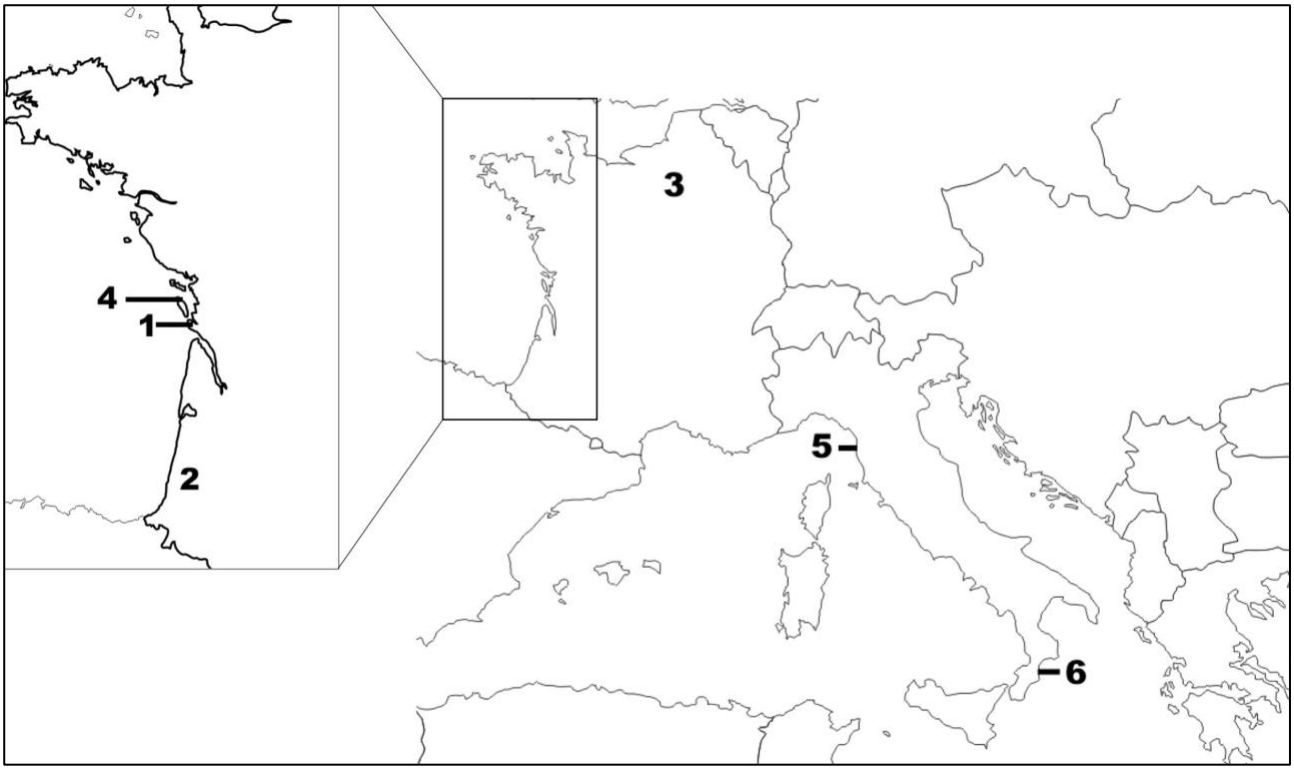
Colony ID/individuals	Microsatellite loci											
	Rf24-2		Rf21-1		Rs02		Rf5-10		Rs10		Rf1-3	
RL5												
w8	94	94	216	216	239	239	137	137	158	158	211	211
w9	94	94	216	216	239	239	137	140	158	158	211	211
w10	94	94	216	216	239	239	137	140	158	158	211	211
RL6												
A_f1	94	94	210	210	239	239	137	137	158	158	211	211
A_f2	94	94	216	216	239	239	137	137	158	158	211	211
A_f3	94	94	216	216	239	239	137	137	158	158	211	211
A_f4	94	94	210	216	239	239	137	137	158	158	211	211
A_f5	94	94	210	216	239	239	137	137	158	158	211	211
A_f6	94	94	210	210	239	239	137	137	158	158	211	211
A_f7	94	94	216	216	239	239	137	137	158	158	211	211
A_f8	94	94	210	216	239	239	137	137	158	158	211	211
A_f9	94	94	210	216	239	239	137	137	158	158	211	211
A_f10	94	94	210	210	239	239	137	137	158	158	211	211
A_m1	94	94	210	216	239	239	137	137	158	158	211	211
A_m2	94	94	210	216	239	239	137	137	158	158	211	211
A_m3	94	94	210	216	239	239	137	137	158	158	211	211
A_m4	94	94	210	216	239	239	137	137	158	158	211	211
A_m5	94	94	210	216	239	239	137	137	158	158	211	211
A_m6	94	94	210	216	239	239	137	137	158	158	211	211
A_m7	94	94	210	210	239	239	137	137	158	158	211	211
A_m8	94	94	210	216	239	239	137	137	158	158	211	211
A_m9	94	94	216	216	239	239	137	137	158	158	211	211
A_m10	94	94	210	216	239	239	137	137	158	158	211	211
w1	94	94	210	210	239	239	134	137	158	161	211	211
w2	94	94	216	216	239	239	137	137	158	161	211	211
w3	94	94	210	216	239	239	137	137	158	161	211	211
w4	94	94	210	216	239	239	134	137	158	161	211	211
w5	94	94	210	216	239	239	137	137	158	161	211	211
w6	94	94	216	216	239	239	134	137	158	161	211	211
w7	94	94	210	210	239	239	134	137	158	161	211	211
w8	94	94	216	216	239	239	137	137	158	161	211	211
w9	94	94	210	216	239	239	134	137	158	161	211	211
w10	94	94	210	210	239	239	134	137	158	161	211	211
RL7												
A_f1	94	94	210	216	239	239	137	137	158	158	211	211
A_f2	94	94	210	210	239	239	137	137	158	158	211	211
A_f3	94	94	216	216	239	239	137	137	158	158	211	211
A_f4	94	94	210	210	239	239	137	137	158	158	211	211
A_f5	94	94	210	216	239	239	137	137	158	158	211	211
A_f6	94	94	210	210	239	239	137	137	158	158	211	211
A_f7	94	94	210	216	239	239	137	137	158	158	211	211
A_f8	94	94	210	210	239	239	137	137	158	158	211	211
A_f9	94	94	216	216	239	239	137	137	158	158	211	211
A_f10	94	94	210	210	239	239	137	137	158	158	211	211
A_m1	94	94	210	216	239	239	137	137	158	158	211	211
A_m2	94	94	210	210	239	239	137	137	158	158	211	211
A_m3	94	94	210	216	239	239	137	137	158	158	211	211
A_m4	94	94	210	216	239	239	137	137	158	158	211	211
A_m5	94	94	210	216	239	239	137	137	158	158	211	211
A_m6	94	94	210	210	239	239	137	137	158	158	211	211
A_m7	94	94	210	216	239	239	137	137	158	158	211	211
A_m8	94	94	210	216	239	239	137	137	158	158	211	211
A_m9	94	94	210	216	239	239	137	137	158	158	211	211
A_m10	94	94	210	210	239	239	137	137	158	158	211	211
w1	94	94	210	216	239	239	137	137	158	158	211	211
w2	91	91	210	210	239	239	137	137	158	158	211	211

Colony ID/individuals	Microsatellite loci											
	Rf24-2		Rf21-1		Rs02		Rf5-10		Rs10		Rf1-3	
RL7												
w3	94	94	216	216	239	239	137	137	158	158	211	211
w4	94	94	210	216	239	239	137	137	158	158	211	211
w5	94	94	210	216	239	239	137	137	158	158	211	211
w6	94	94	210	216	239	239	137	137	158	158	211	211
w7	94	94	210	210	239	239	137	137	158	158	211	211
w8	91	94	210	210	239	239	137	137	158	158	211	211
w9	91	94	210	216	239	239	137	137	158	158	211	211
w10	94	94	210	210	239	239	137	137	158	158	211	211
RL8												
PQ	91	94	210	216	239	239	137	140	158	158	211	211
PK	91	94	210	216	239	239	137	140	158	158	211	211
N_f1	91	91	210	210	239	239	137	137	158	158	211	211
N_f2	91	91	210	210	239	239	137	137	158	158	211	211
N_f3	91	91	216	216	239	239	137	137	158	158	211	211
N_f4	94	94	216	216	239	239	137	137	158	158	211	211
N_f5	91	91	216	216	239	239	137	137	158	158	211	211
N_f6	94	94	210	210	239	239	137	137	158	158	211	211
N_f7	94	94	210	210	239	239	137	137	158	158	211	211
N_f8	91	91	210	210	239	239	137	137	158	158	211	211
N_f9	91	91	216	216	239	239	137	137	158	158	211	211
N_f10	91	91	210	210	239	239	137	137	158	158	211	211
N_m1	91	94	216	216	239	239	140	140	158	158	211	211
N_m2	91	91	210	216	239	239	137	137	158	158	211	211
N_m3	91	94	210	216	239	239	137	137	158	158	211	211
W1	91	91	210	216	239	239	137	140	158	158	211	211
W2	94	94	210	210	239	239	137	140	158	158	211	211
W3	91	94	210	216	239	239	137	137	158	158	211	211
W4	91	94	216	216	239	239	137	140	158	158	211	211
W5	91	91	216	216	239	239	137	137	158	158	211	211
W6	94	94	216	216	239	239	137	137	158	158	211	211
W7	91	91	210	210	239	239	137	137	158	158	211	211
W8	91	91	210	216	239	239	140	140	158	158	211	211
W9	94	94	216	216	239	239	137	137	158	158	211	211
W10	91	91	210	216	239	239	137	137	158	158	211	211
RL9												
PQ	82	106	195	219	239	239	137	137	158	158	211	211
PK	82	82	213	219	239	241	134	137	158	161	211	211
N_f1	82	82	219	219	239	239	137	137	158	158	211	211
N_f2	82	82	219	219	239	239	137	137	158	158	211	211
N_f3	82	82	219	219	239	239	137	137	158	158	211	211
N_f4	106	106	195	195	239	239	137	137	158	158	211	211
N_f5	82	82	219	219	239	239	137	137	158	158	211	211
N_f6	82	82	195	195	239	239	137	137	158	158	211	211
N_f7	82	82	195	195	239	239	137	137	158	158	211	211
N_f8	82	82	219	219	239	239	137	137	158	158	211	211
N_f9	106	106	195	195	239	239	137	137	158	158	211	211
N_f10	106	106	195	195	239	239	137	137	158	158	211	211
N_m1	82	82	219	219	239	239	134	137	158	158	211	211
N_m2	82	106	213	219	239	241	137	137	158	161	211	211
w1	82	82	195	219	239	241	134	137	158	158	211	211
w2	82	82	219	219	239	241	134	137	158	161	211	211
w3	82	106	195	219	239	241	137	137	158	161	211	211
w4	82	82	195	213	239	239	137	137	158	158	211	211
w5	82	82	213	219	239	241	134	137	158	161	211	211
w6	82	106	213	219	239	239	134	137	158	158	211	211
w7	82	106	219	219	239	239	134	137	158	161	211	211
w8	82	106	219	219	239	239	134	137	158	158	211	211

Colony ID/individuals	Microsatellite loci											
	Rf24-2		Rf21-1		Rs02		Rf5-10		Rs10		Rf1-3	
RL9												
w9	82	106	195	219	239	241	137	137	158	158	211	211
w10	82	82	213	219	239	239	134	137	158	161	211	211
RL10												
PQ	82	94	213	213	239	241	137	137	152	152	211	211
PK	82	88	213	213	239	241	137	137	152	158	211	211
N_f1	82	82	213	213	239	239	137	137	152	152	211	211
N_f2	82	82	213	213	239	239	137	137	152	152	211	211
N_f3	82	82	213	213	239	239	137	137	152	152	211	211
N_f4	82	82	213	213	239	239	137	137	152	152	211	211
N_f5	82	82	213	213	239	239	137	137	152	152	211	211
w1	82	94	213	213	239	239	137	137	152	152	211	211
w2	82	82	213	213	239	239	137	137	152	158	211	211
w3	82	82	213	213	239	239	137	137	152	158	211	211
w4	82	94	213	213	239	239	137	137	152	152	211	211
w5	82	82	213	213	239	239	137	137	152	158	211	211
w6	82	82	213	213	239	239	137	137	152	152	211	211
w7	82	88	213	213	241	241	137	137	152	158	211	211
w8	82	82	213	213	239	239	137	137	152	158	211	211
w9	82	94	213	213	239	239	137	137	152	152	211	211
RL11												
PQ	82	94	213	213	239	239	137	140	152	158	211	211
PK	82	88	213	213	239	239	137	137	158	158	211	211
N_f1	82	82	213	213	239	239	140	140	152	158	211	211
N_f2	82	82	213	213	239	239	137	137	152	152	211	211
N_f3	82	82	213	213	239	239	137	137	152	152	211	211
N_f4	82	82	213	213	239	239	137	137	152	152	211	211
N_f5	82	94	213	213	239	239	137	137	152	152	211	211
N_f6	82	94	213	213	239	239	137	137	152	158	211	211
N_f7	82	82	213	213	239	239	137	137	152	152	211	211
N_f8	82	94	213	213	239	239	137	137	152	158	211	211
N_f9	82	82	213	213	239	239	137	137	152	152	211	211
N_f10	82	94	213	213	239	239	137	137	152	158	211	211
w1	82	94	213	213	239	239	137	137	158	158	211	211
w2	82	82	213	213	239	239	137	137	158	158	211	211
w3	82	94	213	213	239	239	137	137	158	158	211	211
w4	82	88	213	213	239	239	137	137	158	158	211	211
w5	82	94	213	213	239	239	137	137	158	158	211	211
w6	82	82	213	213	239	239	137	137	158	158	211	211
w7	82	82	213	213	239	239	137	137	158	158	211	211
w8	82	94	213	213	239	239	137	137	158	158	211	211
w9	82	94	213	213	239	239	137	137	158	158	211	211
w10	82	82	213	213	239	239	137	137	158	158	211	211
RL12												
PQ	82	103	213	219	239	241	137	137	158	158	211	211
PK	103	103	213	213	239	241	137	137	158	158	211	211
N_f1	103	103	213	213	241	241	137	137	158	158	211	211
N_f2	82	103	213	213	239	241	137	137	158	158	211	211
N_f3	103	103	213	213	239	239	137	137	158	158	211	211
N_f4	103	103	213	213	241	241	137	137	158	158	211	211
N_f5	82	82	213	213	239	239	137	137	158	158	211	211
N_f6	82	82	213	213	239	239	137	137	158	158	211	211
N_f7	103	103	213	213	239	239	137	137	158	158	211	211
N_f8	103	103	213	213	241	241	137	137	158	158	211	211
N_f9	103	103	213	213	239	239	137	137	158	158	211	211
N_f10	82	82	213	219	241	241	137	137	158	158	211	211
N_m1	103	103	213	213	239	239	137	137	158	158	211	211

Colony ID/individuals	Microsatellite loci											
	Rf24-2		Rf21-1		Rs02		Rf5-10		Rs10		Rf1-3	
RL12												
N_m2	82	103	213	213	241	241	137	137	158	158	211	211
N_m3	103	103	213	213	241	241	137	137	158	158	211	211
N_m4	82	103	213	213	241	241	137	137	158	158	211	211
N_m5	103	103	213	213	239	239	137	137	158	158	211	211
N_m6	82	82	213	219	239	241	137	137	158	158	211	211
N_m7	103	103	213	213	239	241	137	137	158	158	211	211
N_m8	103	103	213	213	239	241	137	137	158	158	211	211
N_m9	103	103	213	213	239	241	137	137	158	158	211	211
N_m10	103	103	213	213	239	241	137	137	158	158	211	211
w1	103	103	213	213	239	241	137	137	158	158	211	211
w2	103	103	213	213	239	241	137	137	158	158	211	211
w3	103	103	213	213	239	239	137	137	158	158	211	211
w4	82	103	213	219	241	241	137	137	158	158	211	211
w5	82	103	213	213	239	239	137	137	158	158	211	211
w6	103	103	213	213	239	241	137	137	158	158	211	211
w7	82	103	213	213	239	241	137	137	158	158	211	211
w8	103	103	213	213	239	241	137	137	158	158	211	211
w9	82	103	213	213	241	241	137	137	158	158	211	211

Suppl. Figure S1. Map showing the *Reticulitermes* sampling sites: 1, La Tremblade; 2: Pissos; 3, Paris; 4, Saint-Georges-d'Oléron; 5: San Rossore Natural Reserve; 6: Roccelletta di Borgia



CHAPTER 7

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SEX-BIASED INVESTMENT IN ALATES OF THE SUBTERRANEAN TERMITE *RETICULITERMES LUCIFUGUS* (BLATTODEA, TERMITOIDAE, RHINOTERMITIDAE)

Sex-biased investment in alates of the subterranean termite *Reticulitermes lucifugus* (Blattodea, Termitoidae, Rhinotermitidae)

Vito Scicchitano, Barbara Mantovani and Andrea Luchetti

Abstract

Sex-biased investment is the allocation of reproductive resources toward a certain sex. One of the best examples explaining this phenomenon is social haplodiploid Hymenoptera in which kin selection, promoted by sex-biased genetic relatedness, is the force shaping the investment toward the female sex. In social diploid termites, sex allocation was thought to be unbiased because of the absence of relatedness asymmetries. However, a recent analysis described how the Asexual Queen Succession (AQS) reproductive strategy leads to a female-biased investment causing a significant deviation in the sex-ratio of swarming reproductives of two AQS termite species belonging to *Reticulitermes* genus, the Japanese *R. speratus* and the American *R. virginicus*.

To test sex-biased resource allocation in the Italian AQS species *R. lucifugus,* we quantified the sex-ratio of alates from 24 colonies collected in the San Rossore Natural Reserve. Morphometric analyses were also performed to understand whether the AQS strategy could affect sex-biased investment from a morphological standpoint. Results confirmed the presence of a female-biased allocation also in *R. lucifugus* since 18 out 24 colonies examined showed a sex-ratio significantly biased toward the female sex. On the contrary, the remaining six colonies showed a male-biased sex ratio, due to an intra-colonial seasonality in sex allocation, probably as a consequence of bivoltinism or protrandry. Finally, morphometric analyses revealed an intra-sexual size dimorphism (iSSD) among female alates

belonging to colonies with the opposite sex investment ratio. A possible explanation of such phenomenon could be linked to a side effect of protrandry and different developmental time between sexes.

Keywords: sex allocation, kin selection, AQS, iSSD, protrandry, bivoltinism, termites

Introduction

Sex allocation is the allocation of reproductive resources between sexes (Charnov, 1982). Despite Fisher (1929) predicted a greater evolutionary advantage in a balanced sex investment, several cases occur where resources are biased toward one sex (Hamilton, 1964). One of the best examples is social haplodiploid insects: in line with the Inclusive Fitness Theory (Hamilton, 1964), workers preferentially allocate resources towards female relatives to maximize the transmission rate of their own genes to the next generation (Bourke, 2015). This relatedness asymmetry is thought to be the force shaping the bias in the reproductive values in Hymenoptera (Wilson & Hölldobler, 2005), causing a significant deviation in their sex ratio. In social diploids insects, such as termites, sex allocation should be unbiased, since relatedness asymmetries are generally thought to be absent. However, a recent analysis demonstrated the presence relatedness asymmetries also in diploid termites, suggesting a new mechanism causing sex-biased investment (Kobayashi *et al.*, 2013). It has been recently discovered a peculiar reproductive strategy, called Asexual Queen Succession (AQS), occurring in several species of the Rhinotermitidae and Termitidae families (Matsuura *et al.,* 2009; Vargo *et al.*, 2012; Luchetti *et al.,* 2013a; Fougeyrollas *et al.,* 2015; Fournier *et al.,* 2016; Fougeyrollas *et al.,* 2017). In AQS termites, the primary queen, the colony founders together with the primary king, is soon replaced by parthenogenetically-produced secondary queens that start mating with the primary king. During the terminal phase of colony development, a sexually produced secondary king emerges and replaces the primary king (Kobayashi *et al.*, 2013; Matsuura *et al.,* 2017). When secondary queens and king mate, a mother-son inbreeding occurs: this generates a sex-asymmetric genetic contribution to the next offspring, leading to a female-biased sex-ratio of swarming individuals (alates; Kobayashi *et al.*, 2013). In agreement with this mathematical prediction, sex-biased investment was observed in *Reticulitermes speratus* and *R. virginicus*, two AQS species of the Rhinotermitidae family (Matsuura *et al.,* 2009; Vargo *et al.*, 2012) showing a female-biased alates sex ratio (Kobayashi *et al.*, 2013).

Moreover, the AQS strategy could affect sex-biased investment also from a morphological point of view: in *R. speratus*, the loss of heterozygosity following the parthenogenesis producing secondary queens correlated with an increased body size, because of a greater fertility (Yamamoto and Matsuura, 2012).

Also natural selection may favour sex-biased investment: sex-biased investment resulting in a sexual size dimorphism (SSD) is common among insects (Stillwell *et al.,* 2010; Tammaru *et al.,* 2010). Selection on fecundity, in fact, favours larger females that usually produce more and larger offspring (Darwin 1871, Charnov 1982, Honék 1993; Stillwell *et al.,* 2010).

In Italy, two *Reticulitermes* species are known, *R. lucifugus* and *R. urbis* (Luchetti *et al.*, 2013b; Scicchitano *et al.*, 2017): while the latter is an invasive species, only distributed in Apulia, the former can be found along the peninsula, Sardinia and Sicily. *R. lucifugus* comprises two subspecies: *R. lucifgus lucifugus*, distributed along the continental Italy, and *R. lucifugus corsicus*, that can be found in Sardinia and southern Tuscanian coasts (Luchetti *et al.*, 2013b). In this study, we tested sex-biased resources allocation in swarming individuals belonging to *R. lucifugus* colonies, collected in the San Rossore Natural Reserve where the only *R. lucifgus lucifugus* subspecies occur (Luchetti *et al.*, 2013b). We, therefore, checked sex ratio and morphometric analysis in order to investigate on the presence of a sex-biased investment trying to understand the evolutionary and ecological forces behind the onset of this phenomenon.

Materials and Methods

Sampling and sex ratio estimation

Alates of both sexes were collected from 24 *R. lucifugus* colonies in the San Rossore Natural Reserve (Figure 1), right before the flight, during the late swarming season, between the end of May and the

begin of June 2013. Collection has been performed in three days: the 21st May (N=9), the 28th May (N=10) and the 4th June (N=5) (Table 1). Individuals were stored under absolute ethanol (100%) to preserve tissues. Sex was determined from the configuration of the caudal sternites (Zimet & Stuart, 1982) and then, for each colony, alates were separated by sex to calculate the sex-ratio, as the number of females / tot. number of alates. Deviation from expected 1:1 sex ratio was checked by χ^2 test; the distribution of sex ratio over the collection period was checked with a one-way ANOVA, followed by the Tukey HSD post-hoc test with P adjusted for multiple comparisons, using R v.3.3.2 software (R Core Team, 2013).

Morphometric analyses

Among the 24 colonies, 12 *R. lucifugus* colonies were chosen for morphometric analysis, also considering sex ratio results. Samples in good conditions (not damaged) and with wings still attached were dried out in oven for 12 hours at 60°C. Using stereoscopic microscope and millimetre paper, 16-20 alates for each of the 12 selected colonies were measured for total body length, abdomen length (B) and width (C), wings length as described in Figure 2. All comparisons between sexes and between sexes based on biased sex ratio of the pertaining colony were statistically assessed by non-parametric Mann-Whitney test, using R v.3.3.2 software (R Core Team, 2013).

Results

Eighteen out 24 colonies showed a significantly higher number of female individuals with sex ratio ranging from 0.587 to 0.981; the other six colonies showed a sex ratio significantly biased toward the male sex, showing a sex ratio of 0.046-0.374 (Table 1). Sex ratio biases do not distribute evenly during

the collection ($P_{ANOVA\ F} < 0.05$), male-biased sex ratio colonies significantly concentrated during the last day of collection ($P_{Tukey\ HSD,\ adjusted} < 0.05$).

Mean total body length ranged from 4.15 ± 0.21 to 4.60 ± 0.32 mm in females and from 4.15 ± 0.38 to 4.50 ± 0.25 in males; mean abdomen length varied from 1.65 ± 0.13 to 2.10 ± 0.17 mm in females and from 1.63 ± 0.18 to 2.07 ± 0.19 mm in males (Table 2). Female and male abdomen width ranged from 0.93 ± 0.17 to 1.10 ± 0.13 mm and from 0.93 ± 0.12 to 1.03 ± 0.08 mm, respectively (Table 2). Forewing length means spanned from 7.83 ± 0.17 to 7.28 ± 0.22 in female alates and from 7.88 ± 0.19 to 6.95 ± 0.20 in males; rear wings were shorter, ranging from 7.55 ± 0.16 to 6.83 ± 0.29 in females and from 7.63 ± 0.19 to 5.74 ± 1.80 (Table 2).

Overall, females showed longer body and abdomen length ($P_{Mann\text{-}Whitney} < 0.01$), while males have wider abdomens ($P_{Mann\text{-}Whitney} < 0.01$). Moreover, females' wings (both front and rear wings) were longer than those of males ($P_{Mann\text{-}Whitney} < 0.01$). We then compared the two sexes considering the sex ration bias observed in their respective colony (Table 3). Females belonging to colonies with female-biased sex ratio always resulted having larger measure ($P_{Mann\text{-}Whitney} < 0.01$), apart from abdomen width which resulted larger in female from colonies with male biased sex ratio ($P_{Mann\text{-}Whitney} < 0.01$). Males do not show significant differences between colonies with different sex ratio regarding the body, although wings of male from female-biased sex ratio were longer ($P_{Mann\text{-}Whitney} < 0.05$).

Discussion

In the present analysis, for the first time, we analyzed the sex ratio and sex dimorphism in the subterranean termite *R. lucifugus*.

Over two weeks sampling, alates have been collected from 24 colonies and they always showed biased sex ratios. Most colonies showed a female-biased sex ratio, in agreement with previous

surveys (Jones *et al.*, 1988; Luchetti *et al.*, 2013), while six of them showed a male biased sex ratio. As modeled by Kobayashi *et al.* (2013), in AQS species such as *R. lucifugus*, female-biased sex ratio is a consequence of this reproductive system because of a female-biased genetic contribution due to mother-son inbreeding. This, however, is expected to occur during the terminal phase of colony development, when the secondary king replaces the primary one and mates with secondary queens (Kobayashi *et al.* 2013). Taking this model into account, we can explain our data as the male-biased colonies being still the early-mature phase of colony development (Matsuura, 2017). On the other hand, it is interesting that non-female biased colonies are all male-biased. Matsuura (2006) reported incidental protandry in the Japanese congeneric species *R. speratus*, i.e. the early eclosion of males, suggesting that sex ratio count may be biased if collection occur at the early phase of swarming alates eclosion. Incidental protandry may be a side effect of a sexual size dimorphism in which female alates require a longer developmental time than males to achieve a larger body size (Matsuura, 2006). This is, thus, the same observation we made in *R. lucifugus*, where females are larger than males. Alternating sex ratio biases, e.g. due to bivoltinism or protrandry, are common in insects (Werren & Charnov, 1978; Fischer & Fiedler 2001; del Castillo & Nunêz-Farfan, 2002; Zijlstra *et al.*, 2002; Mitton & Ferrenberg, 2014). The presence of seasonal variations in sex ratio is an adaptive reproductive strategy favored by natural selection that allows to maximize the number of encounters between unrelated individuals, avoiding\reducing those among nest-mates with the consequent loss of genetic variability due to inbreeding (Fagerström & Wiklund 1982; Wiklund & Solbreck 1982; Iwasa *et al*., 1983; Nylin *et al*., 1993). This phenomenon has been reported also in social insects (Evans & West-Eberhard 1970; Hunt & Amdam, 2005), like in termites (Vargo & Husseneder, 2009), where partial bivoltinism was suggested to favor eusociality (Seger, 1983; Hunt & Amdam, 2005). Accordingly, male-biased sex ratio observed in our colonies could be the result of an intra-colonial seasonality in sex allocation, probably because of protrandry (Matsuura, 2006): colonies showing a male biased sex

ratio may have been collected in an early-stage in which more mature male alates were available than females.

In addition to this, our data highlight also the presence of an intra-sexual size dimorphism (iSSD) occurring among female alates belonging to colonies with opposite sex ratio. On the contrary, this iSSD is not observed in males. Therefore, the observed female iSSD could be due to their long developmental time and it is possible that individuals from female-biased colonies were collected upon completion of their development process while those belonging to male-biased colonies had not yet finished it. In support of this hypothesis there is the absence of male ISSD: because of protrandry, such individuals did not show significant different sizes since they all had achieved the body size development. The only exception are wings, which resulted longer in males from female-biased sex ratio colonies: it can be suggested that wings, at variance of the body, continue to develop. It remains unanswered why male-biased sex ratio colonies have been mainly found later in the swarming season: although being only six out 24 colonies, it appears significant that four of them have been collected the last day of the field survey, especially considering that no further swarming individuals have been found after that date, and only one was found having a female-biased sex ratio. We can speculate about the presence of some trait that may affect the early/late development of alates in some colonies, independently from protandry. For example, at the present, we cannot completely exclude that some AQS-related trait, possibly linked to the early/mature/late colony life stage, may also determine iSSD. In this view, female-biased sex ratio colonies may grow larger because of more sex-biased allocation of resources when the genetic contribution of the founder queen become prevalent in the terminal stage of colony life, concomitantly to distortion of sex ratio in favor of females (Kobayashi *et al.*, 2013). Therefore, AQS species, such as *R. lucifugus*, appear an interesting framework where to address specific studies on sex allocation of resources; future studies may help to shed light on this peculiar reproductive strategy and on variation of sex ratio over time.

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Tables and figures

Table 1. Sex ratio observed in collected colonies ([1] colony used for morphometric analyses; * P< 0.05; ** P<0.01; *** P<0.001).

Colony	Collection date	Alates ♀	Alates ♂	Sex ratio	Pχ^2
C1[1]	28 May	33	113	0.226	***
C2	28 May	100	2	0.980	***
C3	28 May	105	74	0.587	*
C4	28 May	103	72	0.589	*
C5[1]	28 May	107	19	0.849	***
C6[1]	28 May	97	44	0.688	***
C7	21 May	40	11	0.784	***
C8[1]	21 May	102	50	0.671	***
C9[1]	21 May	120	61	0.663	***
C10	21 May	100	37	0.730	***
C11	21 May	125	9	0.933	***
C12[1]	04 June	13	103	0.112	***
C13[1]	04 June	5	104	0.046	***
C14[1]	04 June	20	110	0.154	***
C15	21 May	90	34	0.726	***
C16	04 June	89	4	0.957	***
C17	21 May	105	27	0.795	***
C18	21 May	99	42	0.702	***
C19	21 May	100	41	0.709	***
C20[1]	28 May	110	23	0.827	***
C21	28 May	101	2	0.981	***
C22[1]	28 May	46	77	0.374	***
C23[1]	28 May	126	11	0.920	***
C24[1]	04 June	39	68	0.364	**

Table 2. Average values of morphometric measures (mm ± standard deviation) per sex and per colony ([1] F: female-biased; M: male-biased; see Table 1).

Colony	Sex ratio[1]	Body length		Abdomen length		Abdomen width		Forewing length		Rear wing length	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
C1	M	4.30 ± 0.20	4.25 ± 0.17	1.73 ± 0.14	1.75 ± 0.17	1.08 ± 0.12	0.88 ± 0.13	7.53 ± 0.22	7.48 ± 0.18	7.05 ± 0.23	7.25 ± 0.17
C5	F	4.40 ± 0.21	4.25 ± 0.25	1.85 ± 0.17	1.75 ± 0.25	0.93 ± 0.17	0.79 ± 0.09	7.65 ± 0.13	7.57 ± 0.12	7.33 ± 0.17	7.18 ± 0.12
C6	F	4.38 ± 0.13	4.28 ± 0.08	1.98 ± 0.14	1.93 ± 0.12	0.98 ± 0.08	0.93 ± 0.12	7.72 ± 0.18	7.48 ± 0.18	7.43 ± 0.12	7.20 ± 0.16
C8	F	4.35 ± 0.18	4.23 ± 0.08	2.03 ± 0.18	1.78 ± 0.08	0.93 ± 0.17	0.98 ± 0.08	7.70 ± 0.20	7.55 ± 0.20	7.43 ± 0.17	7.30 ± 0.20
C9	F	4.60 ± 0.32	4.50 ± 0.25	2.10 ± 0.17	2.07 ± 0.19	0.93 ± 0.17	0.82 ± 0.12	7.58 ± 0.12	7.54 ± 0.22	7.28 ± 0.18	7.04 ± 0.22
C12	M	4.18 ± 0.21	4.28 ± 0.18	1.65 ± 0.13	1.63 ± 0.18	0.95 ± 0.16	0.85 ± 0.13	7.28 ± 0.22	7.25 ± 0.20	6.90 ± 0.24	6.73 ± 0.25
C13	M	4.25 ± 0.14	4.22 ± 0.20	2.00 ± 0.00	1.97 ± 0.20	1.00 ± 0.00	0.94 ± 0.11	7.64 ± 0.13	7.28 ± 0.20	7.14 ± 0.13	7.03 ± 0.20
C14	M	4.25 ± 0.25	4.15 ± 0.38	2.08 ± 0.14	2.03 ± 0.30	1.00 ± 0.00	0.90 ± 0.13	7.33 ± 0.29	6.95 ± 0.20	6.83 ± 0.29	5.74 ± 1.80
C20	F	4.28 ± 0.22	4.25 ± 0.33	1.85 ± 0.27	1.78 ± 0.31	0.93 ± 0.17	1.00 ± 0.23	7.75 ± 0.17	7.56 ± 0.22	7.30 ± 0.20	7.19 ± 0.12
C23	F	4.43 ± 0.12	4.38 ± 0.13	2.05 ± 0.11	2.03 ± 0.09	0.98 ± 0.08	0.97 ± 0.09	7.83 ± 0.17	7.88 ± 0.19	7.35 ± 0.21	7.63 ± 0.19
C22	M	4.15 ± 0.21	4.23 ± 0.18	1.93 ± 0.12	1.98 ± 0.18	0.93 ± 0.12	0.95 ± 0.11	7.83 ± 0.17	7.63 ± 0.13	7.55 ± 0.16	7.38 ± 0.13
C24	M	4.38 ± 0.18	4.33 ± 0.21	2.08 ± 0.24	2.05 ± 0.20	1.10 ± 0.13	1.03 ± 0.08	7.80 ± 0.20	7.75 ± 0.24	7.45 ± 0.11	7.50 ± 0.24

Table 3. Average values of morphometric measures (mm ± standard deviation) considering the colony sex ratio bias (* P< 0.05; ** P<0.01; *** P<0.001).

Measure	**Sex**	**Female bias**	**Male bias**	**$P_{Mann\text{-}Whitney}$**
Body length	♀	4.40 ± 0.22	4.26 ± 0.21	**
	♂	4.31 ± 0.21	4.24 ± 0.22	n.s.
Abdomen length	♀	1.97 ± 0.20	1.87 ± 0.22	**
	♂	1.89 ± 0.21	1.90 ± 0.25	n.s.
Abdomen width	♀	0.94 ± 0.14	1.01 ± 0.13	**
	♂	0.92 ± 0.15	0.92 ± 0.12	n.s.
Forewing length	♀	7.70 ± 0.17	7.57 ± 0.28	**
	♂	7.59 ± 0.22	7.39 ± 0.32	***
Rear wing length	♀	7.35 ± 0.18	7.18 ± 0.31	**
	♂	7.26 ± 0.24	6.94 ± 0.94	*

Figure 1. Map of sampling sites in San Rossore Natural Reserve. Underlined colony IDs indicate samples used for morphometric analyses.

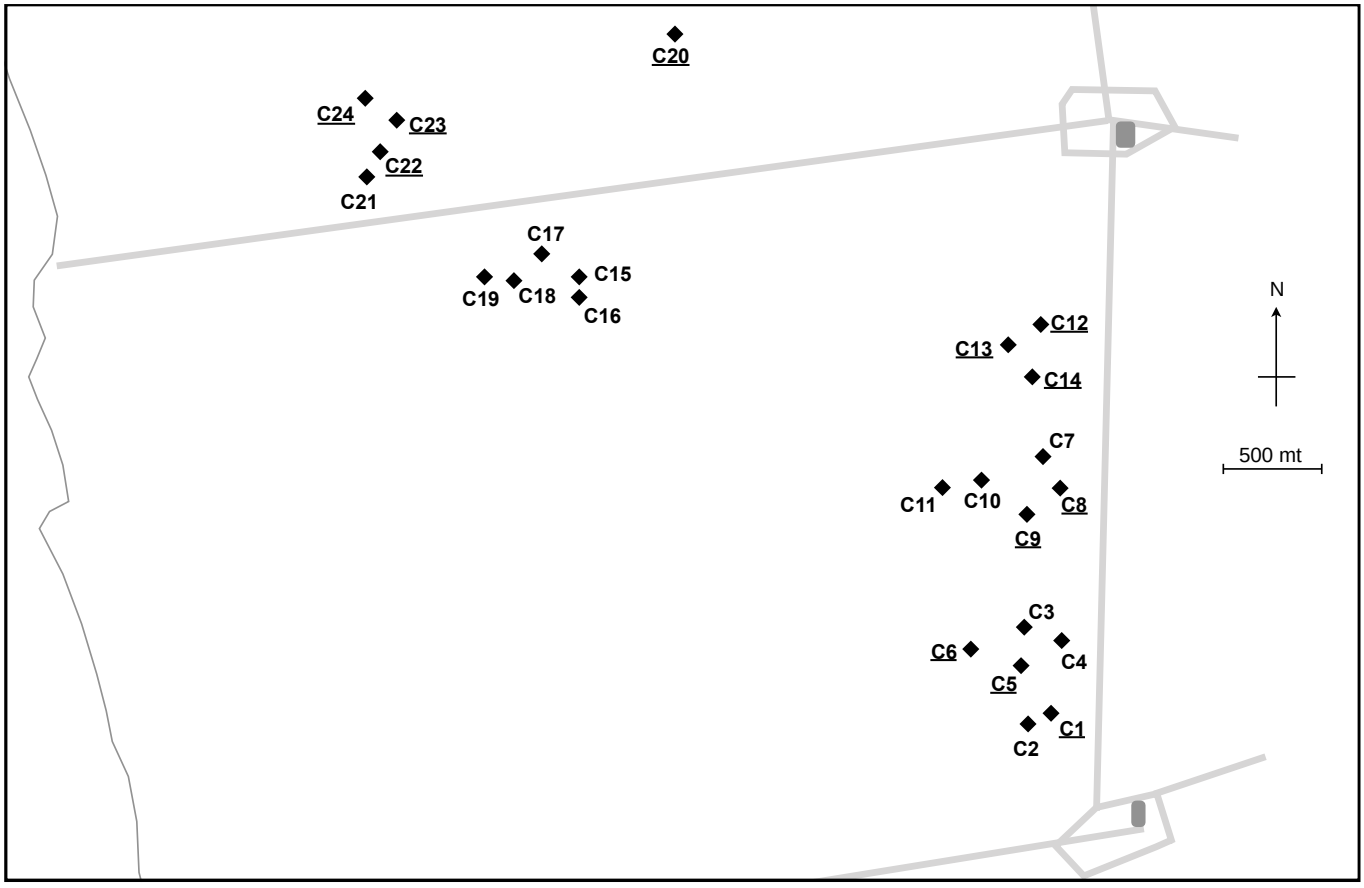
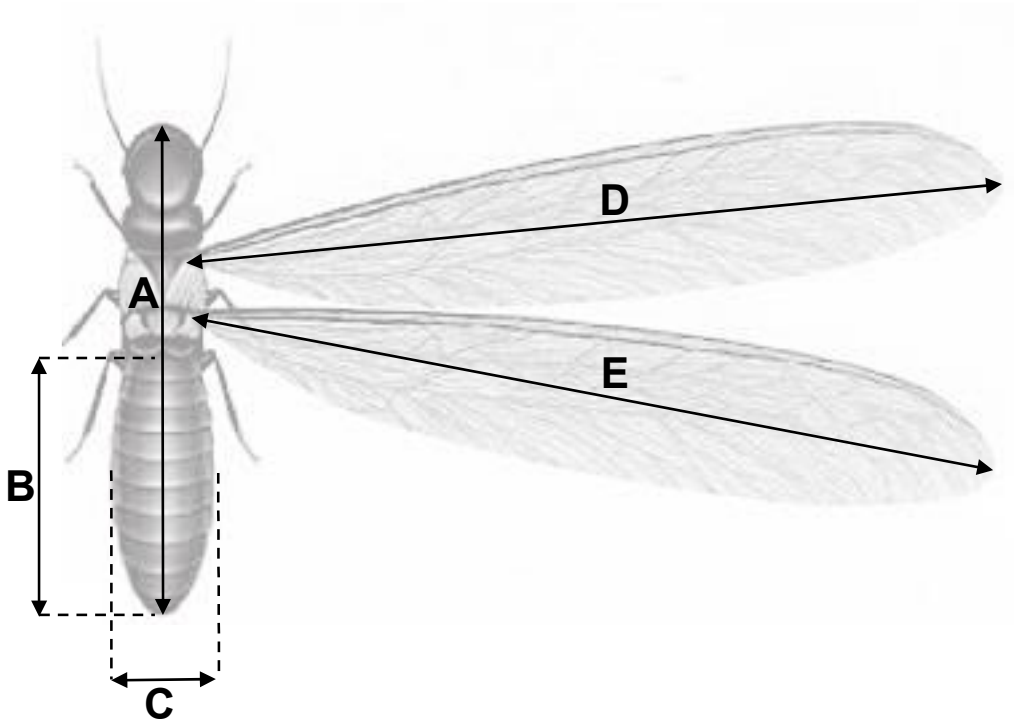


Figure 2. Schematic drawing of measures used for morphometric analyses. A: body length; B: abdomen length; C: abdomen width; E: forewing length; F: rear wing length.



CHAPTER 8

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DETECTING *WOLBACHIA* INFECTION STATUS IN EUROPEAN TERMITES OF *RETICULITERMES* AND *KALOTERMES* GENERA (INSECTA, BLATTODEA, TERMITOIDAE): RELATIONSHIP WITH THE HOST PHYLOGENY AND ITS INVOLMENT IN THE REPRODUCTIVE STRATEGIES.

This preliminary survey was carried out during my research term, in the last year of my PhD, at the the *Institut de Recherche sur la Biologie de l'Insectes* (University of Tours, France) in collaboration with Prof. Franck Dedeine.

Detecting *Wolbachia* infection status in European termites of *Reticulitermes* and *Kalotermes* genera (Insecta, Blattodea, Termitoidae): relationship with the host phylogeny and its involvement in the reproductive strategies

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Abstract

Microbial symbionts play a crucial role in the lifestyle of termites providing either benefits, through mutualism, or disadvantages, with parasitism. This is the case of the cellular endosymbiotic α-proteobacteria of the genus *Wolbachia* affecting host reproduction. To verify if this bacterium is involved in termite parthenogenesis or hybridization events, European species belonging to *Reticulitermes* and *Kalotermes* genera were analyzed for *Wolbachia* infection. In particular, *Wolbachia* occurrence was checked in *R. lucifugus* colonies to understand a possible involvement of this microbe in the Asexual Queen Succession strategy, which include the use of parthenogenesis, and in mixed *Kalotermes* colonies to verify if *Wolbachia* could be involved in the interspecific hybridization events through some kind of cytoplasmic incompatibility leading to preferential mating between divergent taxa. Preliminary data obtained point to a complex situation, and no clear link between *Wolbachia* occurrence and termite reproductive biology emerged yet.

Keywords: Accelerated Nest Inheritance, Asexual Queen Succession, *Wolbachia*, termites, Isoptera

Introduction

Microbial symbiosis plays a relevant role in the lifestyle of termites providing benefits, through mutualism, or disadvantages, as in the case of parasitism (Dedeine *et al.,* 2003). For example, termite gut microbiota is composed by bacteria, archeae and unicellular flagellated protists which enable termites to feed degrading lignin, cellulose, and hemicelluloses to fermentable carbohydrates (Berlanga *et al.*, 2011). Termites, however, harbor also parasitic endosymbiotic bacteria living within their germinal line cells. The most common of these intracellular parasites are, undoubtedly, the α-proteobacteria of the genus *Wolbachia* (Rickettsiales, Rickettsiaceae), which includes obligate intracellular bacteria that are cytoplasmically inherited in arthropods and filarial nematodes (Lo & Evans, 2007). *Wolbachia* can induce several effects on the host reproductive biology (phenotypes), such as feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (Brucker & Bordenstein, 2012). The latter is the most frequent *Wolbachia*-induced phenotype observed and it consists in the incompatibility between sperms from infected males and eggs of uninfected females or between individuals harboring different *Wolbachia* strains (Lo & Evans, 2007). This so-called cytoplasmatic incompatibility (CI) is due to the disruption of the hosts cell cycle, which results in asynchronous development of male and female pronuclei (Werren *et al.,* 2008). Therefore, CI results in a post-copulatory reproductive isolation and, for this reason, it was suggested that *Wolbachia* could be involved in speciation events (Brucker & Bordenstein, 2012).

Sixteen supergroups of *Wolbachia* are recognized (A–Q; Glowska *et al.*, 2015), and four of these infect termites: supergroups A, B and F infects the majority of termite species, including both derived (i.e., *Reticulitermes* genus) and more primitive taxa (i.e., *Kalotermes* genus), whilesupergroup H is found only in *Zootermopsis* species (Lo *et al*., 2002; Salunke *et. al*, 2010). However, even if the systematics, phylogeny and taxonomic distribution of *Wolbachia* strains have

been quite widely studied, it is poorly known if and how *Wolbachia* infection can affect the reproductive biology of termites (Matsuura *et al.,* 2004; Lo & Evans, 2007; Werren *et al.*, 2008).

European termite species belong to two genera: *Reticulitermes* and *Kalotermes*. The former genus includes six subterranean termite species: the European *R. grassei, R. banyulensis, R. lucifugus, R. urbis, R. balkanensis* and the Aegean *R. aegeus* (Austin *et al.,* 2002; Marini & Mantovani, 2002; Bagnères *et al.,* 2003; Kutnik *et al.,* 2004; Luchetti *et al.,* 2004, 2007, 2013a; Uva *et al.*, 2004; Velonà *et al.,* 2010; Ghesini & Marini, 2015a). Moreover, the North American species *R. flavipes* is distributed in Europe with some invasive populations in France and Germany (Perdereau *et al.*, 2013). Of these taxa, only *R. grassei* has been analyzed for *Wolbachia* infection and its presence was evidenced only in the soldier caste (Berlanga *et al*., 2011). On the other hand, previous analyses in the *Reticulitermes* genus revealed *Wolbachia* occurrence in the North American *R. flavipes* and in the Japanese *R. speratus*, the latter being the first AQS species identified (Matsuura *et al.,* 2004). Asexual Queen Succession (AQS) is a peculiar reproductive strategy which includes the conditional use of parthenogenesis (Matsuura *et al*., 2009, Luchetti *et al.*, 2013b). In AQS species, in fact, reigning queens produce homozygous secondary queens by parthenogenesis, while individuals of all other castes derive from mating with the king (Matsuura *et al*., 2009). AQS is now known to occur in other species such as the Italian *R. lucifugus* (Luchetti *et al.*, 2013b). In this species, no evidences about *Wolbachia* occurrence has been so far described.

As far as the *Kalotermes* genus is concerned, three dry-wood dwelling species are present in the area. The most widespread is *K. flavicollis*, showing three distinct genetic lineages (Scicchitano *et al.*, 2017): lineage A occurs from Italy to Aegean Islands; lineage SC can be found in Sardinia, Corsica and along the Tuscany coast and lineage IF in the Iberian Peninsula. The sister species *K. italicus* (Ghesini & Marini, 2013) occurs in North-central Italy and South France (Scicchitano *et al.*, 2017), its distribution overlapping with *K. flavicollis* one. Finally, *K. phoenicae* was found on Cyprus island, and

along Israeli and Lebanese coasts (Ghesini & Marini, 2015b). Within this genus, the *Wolbachia* F strain was found, so far, in one sample of the species *K. flavicollis,* at that time the only known taxon of this genus in Europe (Lo *et al.*, 2002; Casiraghi *et al.,* 2005). European *Kalotermes* colonies are known to frequently produce mixed colonies, by fusion of two or more pre-existing ones (Luchetti *et al.,* 2013c; Scicchitano *et al.*, 2017, see Chapter 5). Very interestingly, also colonies belonging to different taxa were found to fuse in the field, with instances of very high rate of colony fusion given by up to nine colonies (Luchetti *et al.*, 2013c; Schicchitano *et al.*, 2017). In accordance to the 'Accelerated Nest Inheritance' theory (Thorne *et al.*, 2003), these colony fusions should lead to the death of royal founders allowing false workers (pseudoergates) to moult into reproducers and to inherit the colony. The emergence of hybrid individuals observed after fusion events (Luchetti *et al*., 2013c) led to the hypothesis that hybridization between different genetic lineages or species could further promote the fusion of more than two colonies overcoming mechanisms of nest-mate recognition (Thorne *et al.*, 2003). Though, the underlying genetic causes allowing such hybridization events are still unknown. It is, therefore, worth investigating if *Wolbachia* may be involved in the process of interspecific hybridization and, therefore, of colony fusion in *Kalotermes*.

Here, a preliminary molecular investigation was performed on termite samples belonging to the European *Reticulitermes* and *Kalotermes* species in order to identify *Wolbachia* infection and to characterize the relevant strains. Attention has been given, in particular, to the AQS species *R. lucifugus* and to mixed colonies of the *Kalotermes* genus to verify whether *Wolbachia* presence could be related to parthenogenesis or hybridization.

Materials and Methods

For the genus *Reticulitermes*, samples of *R. banyulensis*, *R. grassei*, *R. flavipes*, *R. lucifugus* and *R. urbis* (two colonies each) were analyzed (Table 1). For the genus *Kalotermes,* one colony of *K. italicus* and one sample for each mitochondrial lineage of *K. flavicollis* (lineage A, lineage SC, lineage IF; Scicchitano *et al.*, 2017) have been surveyed (Table 1). Species identification was performed through mitochondrial characterization following Dedeine *et al.* (2016) for *Reticulitermes* samples and Scicchitano *et al.* (2017) for the *Kalotermes* ones.

DNA was extracted using Macherey-Nagel NucleoSpin® Tissue Kit. The termite head and thorax was used to confirm taxonomic species identification and colony composition through mitochondrial characterization. The abdomen, on the other hand, was utilized for microbial DNA isolation, using the same extraction kit. To test for *Wolbachia* infection, PCRs were performed following Lo & Evans (2007), amplifying the *FtsZ* gene (encoding a cell division protein; >750bp region) as marker and the HotStart Master Mix (Quiagen®) kit. The same PCR settings were used to amplify the bacterial 16S rRNA gene, chosen as a positive control to test the success of the microbial DNA isolation.

Wolbachia FtsZ sequences were produced for a subset of the positive amplicons; sequences were aligned together with others isolated from termites, retrieved from GenBank (A.N. are given in Figure 1). Portion of the *Ehrlichia ruminantium* strain Welgevonden (A.C.: NC_005295) *FtsZ* gene was used as outgroup. The best substitution model, GTR+G, was tested by jModelTest v2.1.10 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). Bayesian phylogenetic inference was performed using MrBayes v3.2.5 (Ronquist *et al.*, 2012) running four Metropolis coupled Markov chains for 1 million generations after which convergence was reached (average standard deviation of split frequencies < 0.01). Trees were sampled every 250 generations and the first 25 % were discarded as burn-in trees before constructing a 50 % majority rule consensus tree.

Results and Discussion

Reticulitermes. As resulted from the *FtsZ* PCR analyses, all individuals from the considered species did not show positive amplification for any *Wolbachia* strain. Our preliminary results, thus, indicate the absence of *Wolbachia* infection in *R. banyulensis*, *R. grassei*, *R. flavipes*, *R. lucifugus* and *R. urbis* for the castes analyzed. It is to be noted that evidences about *Wolbachia* presence have been reported for *R. grassei* soldiers (Berlanga *et al*., 2011). It is to be explained therefore why we failed to amplify the *FtsZ* gene in the single *R. grassei* individual of the soldier caste analyzed. This could obviously be due to some technical failure, but it could be argued that *Wolbachia* infection may be unequally distributed in the species, therefore calling for a population-based analysis of *Wolbachia* distribution in this taxon.

The absence of infection (besides workers and soldiers) also in female parthenogenetically produced nymphoids of the AQS species *R. lucifugus* suggests to definitively exclude a possible involvement of *Wolbachia* in the establishment of this reproductive strategy. Available results in fact shows that *Wolbachia* can be either present or absent in an AQS species (*R. speratus* and *R. lucifugus*, respectively; Matsuura *et al.*, 2004; present data). The molecular mechanisms at the basis of the switch between parthenogenesis and bisexual reproduction in AQS queen still needs to be clarified.

Kalotermes. Mitochondrial identification evidenced the presence of two colonies with different haplotype contribution, i.e. colony Km1 with *K. italicus* / *K. flavicollis* – lineage SC haplotypes and Km2 with *K. italicus* / *K. flavicollis* – lineage A haplotypes (Table 2).

Regarding the detection of *Wolbachia*, positive PCR amplifications for the *FtsZ* marker were obtained in samples belonging to *K. flavicollis* lineage SC and *K. italicus*, all analyzed specimens showing a well evident band on agarose gel (data not shown). On the contrary, no amplification product was observed from individuals belonging to *K. flavicollis* A and IF lineages. *Wolbachia*

infection was also detected in all individuals of the colony Km1, while individuals belonging to the colony Km2 did not show evidence of infection.

All the *FtsZ* sequences obtained showed 99% identity (720/723 bp) with the *FtsZ* sequence of *Wolbachia* endosymbiont from Italian *K. flavicollis* (Genbank accession number: AJ292345; Lo *et al*., 2002). All these sequences are included in the F supergroup, as evidenced by Bayesian phylogenetic analyses (Figure 1). Overall, results highlight new more detailed information about the distribution of *Wolbachia* parasite within the *Kalotermes* taxa analyzed, reporting its presence also in the SC lineage and in the newly described *K. italicus* species. Previous investigations, on the contrary, reported *Wolbachia* occurrence only in *K. flavicollis* (Lo *et al.*, 2002; Casiraghi *et al.*, 2005); this paper though did not give any information about the sample geographic origin so it is difficult to make any comparisons with our analysis. In addition, data obtained highlighted a phylogenetic signal in the history of *Wolbachia* infection. In fact, considering host phylogeny (Scicchitano *et al.*, 2017), *Wolbachia* is present in *K. italicus* and absent in the *K. flavicollis* clade, with the only exception of the Sardo-Corsican lineage. A possible explanation could be linked to i) an ancestral infection maintained in the *K. italicus* lineage and, subsequently, lost in most of the *K. flavicollis* taxa or ii) to more recent and independent infections that occurred in the *K. flavicollis* SC and *K. italicus* taxa.

As far as mixed colonies are concerned (Table 2), due to the limited sample size and the preliminary nature of these data, it is difficult to ascertain whether *Wolbachia* could be involved in hybridization and colony fusion events. It is interesting, though, that it has been found in the *K. italicus/K. flavicollis* SC colony but it was absent in the *K. italicus*/*K. flavicollis* A one, also considering that *K. italicus* resulted infected.

On the whole, present results point to the absence of *Wolbachia* from assayed *Reticulitermes* species, while revealing a patchy distribution among *Kalotermes* taxa and mixed colonies. Yet further analyses are needed at the population level, with the involvement of a higher number of

samples, to understand the apparent random occurrence of this α-proteobacterium in termites and the phenotype induced in these insects.

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Tables and figures

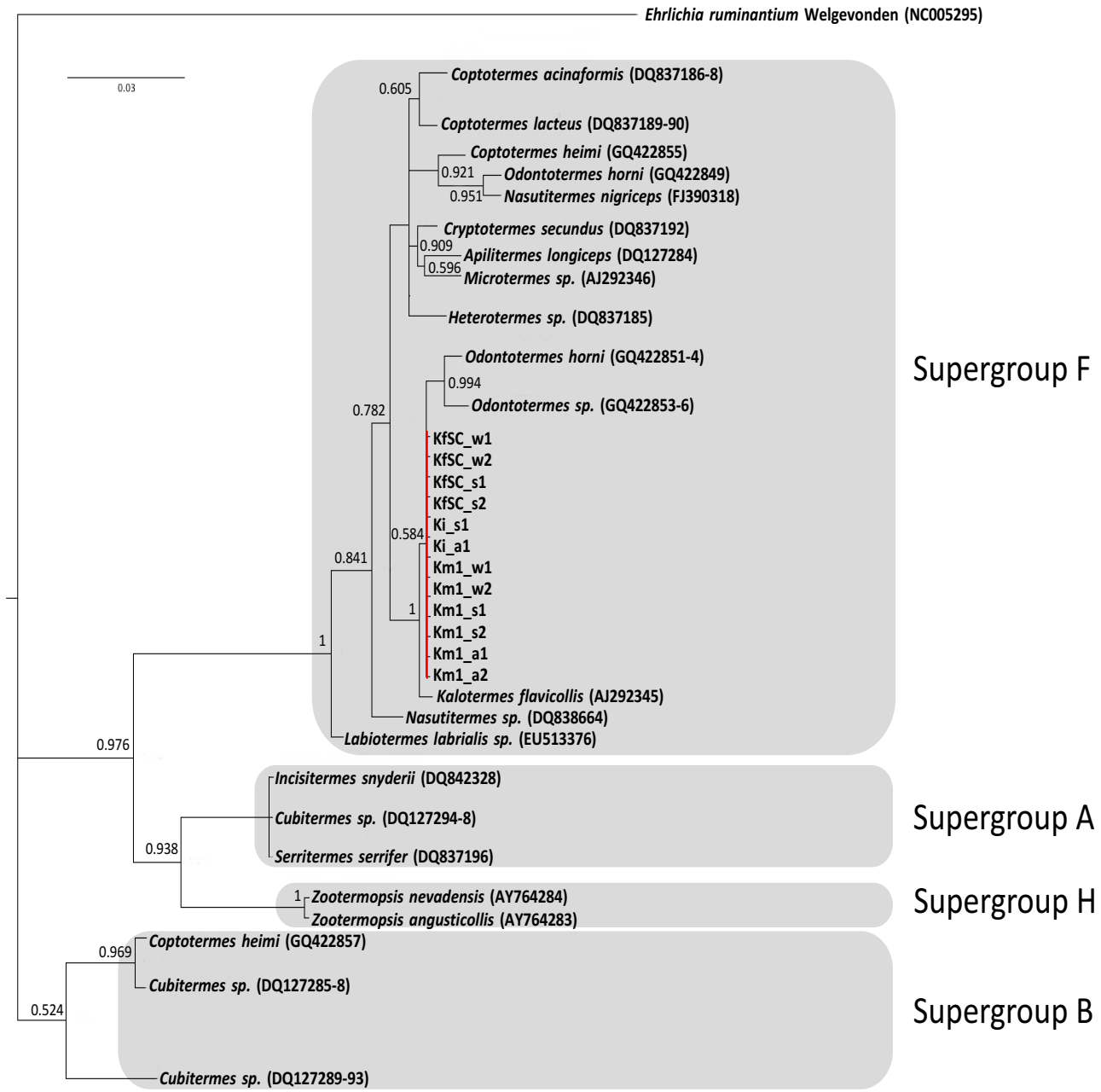
Table 1. Sample information and number of individuals analyzed. Sex and caste of each individual are reported in parenthesis (w: workers; s: soldier; nd: nymphoid; n: nymph; a: alate);

Locality	Genus	Species	Colony ID	N° individuals
France	*Reticulitermes*	*R. grassei*	RG1	3 (1w; 1nd♀; 1n♀)
			RG2	4 (1w; 1s; 1nd♀; 1n♀)
		R. banuylensis	RBN1	3 (1w; 1s; 1nd♀)
			RBN2	2 (1w; 1nd♀)
		R. flavipes	RF1	3 (1w; 1s; 1nd♀)
			RF2	4 (1w; 1s; 1nd♀; 1n♀)
		R. urbis	RU1	2 (1w; 1s)
			RU2	2 (1w; 1n♀)
Italy		*R. lucifugus*	RL1	3 (1w; 1s; 1nd♀)
			RL2	5 (1w; 1s; 3nd♀)
	Kalotermes	*K. flavicollis* - Lineage A	KfA	4 (2w; 2s)
		K. flavicollis - Lineage SC	KfSC	4 (2w; 2s)
		K. Flavicollis - Lineage IB	KfIB	4 (2w; 1s; 1a)
		K. italicus	Ki	2 (1s; 1a)
		K. italicus; K. Flavicollis - Lineage SC	Km1	6 (2w; 2s; 2a)
		K. italicus; K. flavicollis - Lineage A	Km2	3 (3w)

Table 2. Mithocondrial characterization of *Kalotermes* mixed colonies, following Scicchitano *et al.* (2017). Kalotermes COII haplotypes retrieved from GenBank show accession number in parenthesis; individuals are named as in Table 1.

		***Kalotermes* COII haplotypes**		
Colony ID	**N° individuals**	***K. flavicollis* - Lineage A (FJ750513)**	***K. flavicollis* - Lineage SC (FJ750509)**	***K. italicus* (JQ434267)**
Km1	6		w2 s1 a1 a2	w1 s2
Km2	3	w3		w1 w2

Figure 1. Bayesian tree inferred on *Wolbachia FtsZ* sequences isolated from termites. Each sequence is labeled on the basis of the relative host. Red bar indicates new sequences obtained from *Kalotermes* samples. *FtsZ* sequences retrieved from GenBank shows accession number in parenthesis; supergroups are defined as in Salunke *et al*., 2010. Number at nodes represent posterior probabilites. Genbank accession number of reference sequences are given in parentheses.



CHAPTER 9

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CONCLUSIONS

9.1 PHYLOGEOGRAPHIC CONSIDERATIONS

Through a deep genetic investigation, my survey allowed to clarify the genetic diversity of the European *Reticulitermes urbis* and its status as invasive species in Italy and France, by describing the invasion history occurred. Results obtained, in fact, support a history of multiple introductions in the Italian and French regions, in a scenario consistent with continuous exchanges between native and invasive areas, as expected along human trades routes. Similar circumstances were described also in the other invasive congeneric species, the American *R. flavipes* (Perdereau *et al.*, 2013). Phylogeographic analyses, in fact, allowed to reconstruct the invasion routes of this species in France, identifying the native source population in Louisiana (USA; Perdereau *et al.*, 2013). On the light of these findings, thus, it is evident how molecular phylogenetics and population genetics studies are excellent tools to obtain information about the source population(s), invasion routes, and the pattern of colonization, essential to develop effective prevention and management strategies against invasive species (Evans *et al*., 2013).

Furthermore, my analyses allowed to draw a more detailed picture of *Kalotermes* biogeography in Europe confirming previously identified lineages and taxa, but widening the distribution of the three *K. flavicollis* lineages and of *K. italicus*. For the latter, I provided, in addition, evidence for a synonymy with *K. flavicollis* lineage B.

On the whole, the phylogeographic pattern found in *Kalotermes* lineages, even if without time estimations, nicely mirrors that observed in Italian *Reticulitermes lucifugus* subspecies in which, paleogeographic events, during the Pleistocene, have affected the biogeographic history of this species (Luchetti *et al.*, 2013c). Comparable patterns were described in other terrestrial plant and animal organisms, including arthropods (Mansion *et al.*, 2008; Bidegaray-Batista & Arnedo, 2011). My data further provide a new evolutionary scenario for the origin of *Kalotermes* genus.

9.2 BREEDING SYSTEMS CONSIDERATIONS AND EUSOCIALITY EVOLUTION

My PhD research allowed to confirm the presence of the AQS strategy in *R. lucifugus*, and to demonstrate its absence in *R. grassei* and in the invasive populations of *R. flavipes.* These results add to the knowledge about the evolution of the AQS system providing new support to the hypothesis of multiple and independent origins of this particular reproductive strategy in the *Reticulitermes* genus (Dedeine *et al.*, 2016). Moreover, results obtained demonstrated the occurrence of king replacements in the AQS-performing *R. lucifugus* and how such phenomenon generates, from a genetic point of view, the onset of sex allocation in the swarming reproductives caste, theoretically predicted by Kobayashi *et al.* (2013). My research also provided further evidences of this phenomenon through sex ratio and morphometric data, describing, in addition the occurrence of proterandry in *R. lucifugus* dispersers as a side effect/strategy of an intra-sexual size dimorphism, previously described only in the other AQS species *R. speratus* (Matsuura, 2006). Results, thus, highlight how *R. lucifugus* appears an interesting framework where to address specific studies on AQS-related sex allocation of resources.

As far as the *Kalotermes* genus is concerned, I reported the occurrence of colony mixing between *K. italicus* and *K. flavicollis* A and SC lineages. These results suggest that this phenomenon,

previously observed only at an intraspecific level (Luchetti *et al.*, 2013a), occurs even at an interspecific level, providing interesting insights about the reproductive boundaries in these species.

Taking into account the two particular breeding systems occurring in *Reticulitermes* and *Kalotermes* (AQS strategy and intra-/inter- colony fusions, respectively), it is possible to speculate on the evolution of the eusociality in termites. Considering results explained in Chapter 6 and 7, AQS strategy seems to lead to the establishment of high levels of relatedness among individuals of the colony. On the other hand, colony fusions allow *Kalotermes* pseudoergates (false workers) to become reproductives and to inherit the colony, as predicted by the accelerated nest inheritance theory (see Chapter 1.5.1). It is possible, thus, to hypothesize that cooperation between unrelated individuals should be the basal force promoting this phenomenon. A comparison with the phylogeny of these taxa (Beccaloni & Eggleton, 2011; Cameron *et al.*, 2012) seems to indicate that primitive termite genera, such as *Kalotermes,* exhibit instances of cooperative behavior following dynamics explainable through the evolutionary game theory (Maynard-Smith & Price, 1973). On the contrary, eusociality observed in the most recently derived *Reticulitermes* genus reflects the rules described by the kin-selection theory (Maynard-Smith, 1964). However, further investigations concerning the reproductive biology of termites will better clarify these speculations.

9.3 *WOLBACHIA* INFECTION CONSIDERATIONS

The preliminary results obtained suggest the absence of *Wolbachia* infection in the European *Reticulitermes* species analyzed, including the AQS species *R. lucifugus.* Although evidences on *Wolbachia* infection were detected in another AQS species, *R. speratus* (Matsuura *et al.*, 2004), my

preliminary analyses showed that *Wolbachia* can be either present or absent in an AQS species. This appears to suggest that this microbe could not be directly involved in the establishment of this particular reproductive strategy.

The detection and taxonomic distribution of this endosymbiotic parasite in Kalotermes taxa, though, do not led to conclusive results. In fact, data point out to a patchy distribution of *Wolbachia* in *Kalotermes* species and colonies, which deserve more detailed analysis to be fully understood. However, considering the only data available in literature on *Wolbachia* infection in *Kalotermes* (Lo *et al.*, 2002; Casiraghi *et al.*, 2005), my research has provided a more detailed picture about the distribution of this parasite in *Kalotermes* species, thanks also to new phylogeographic evidences that I reported for this termite genus (Scicchitano *et al.*, 2017).

9.4 PERSPECTIVES

Results obtained during my PhD course allow to design future projects such as:

- a genetic investigation about the presence\absence of the AQS strategy in *R. urbis* also in native populations since, to date, it has been performed only in the invasive range (Luchetti *et al.*, 2013b).

- a deep genomic investigation on *R. lucifugus* in order to identify putative genetic elements involved in the onset of the AQS strategy.

- more accurate studies about the Accelerated Nest Inheritance hypothesis and the occurrence of colony mixing in the *Kalotermes* genus to provide deeper insights about the reproductive boundaries and colony mate recognition of these social insects.

- more detailed analyses, by expanding the number of termite samples and using more specific markers, to better understand the role of *Wolbachia* in termites.

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APPENDIX

Scientific production during my PhD course

In extenso papers:

Scicchitano, V., Dedeine, F., Bagnères A.-G., Luchetti, A. & Mantovani, B. Genetic diversity and invasion history of the European subterranean termite *Reticulitermes urbis* (Blattodea, Termitoidae, Rhinotermitidae). *Biological Invasions* (2017). doi:10.1007/s10530-017-1510-5

Scicchitano, V., Dedeine, F., Mantovani, B. & Luchetti, A. Molecular systematics, biogeography, and colony fusion in the European dry-wood termites *Kalotermes* spp. (Blattodea, Termitoidae, Kalotermitidae). *Bulettin of Entomological Resesearch* 1-9 (2017) doi:10.1017/S0007485317001080

Posters & Oral communications:

Scicchitano, V., Dedeine, F., Mantovani, B., Luchetti, A. Reproductive strategies and breeding systems in *Reticulitermes* subterranean termites (Isoptera, Rhinotermitidae). VII Congress of the Italian Society for Evolutionary Biology (SIBE) - Roma, 28\08 - 31\08\2015.

[Poster]

Scicchitano, V., Dedeine, F., Mantovani, B., Luchetti, A. Phylogeography of European dry-wood dwelling termites of the genus *Kalotermes* (Isoptera, Kalotermitidae). VI Congress of the International Union for the Study of Social Insects (IUSSI) - Helsinki, 08- 11\08\2016.

[Poster]

Scicchitano, V., Dedeine, F., Bagnères, A.-G., Mantovani, B., Luchetti, A. Genetic analysis reveals multiple 'introduction events' of the Balkanic *Reticulitermes urbis* (Blattodea, Termitoidae, Rhinotermitidae) in Italy and France. VI Congress of the International Union for the Study of Social Insects (IUSSI) - Helsinki, 08- 11\08\2016.

[Oral Communication]

Scicchitano, V., Mantovani, B., Luchetti, A. Global genetic analysis reveals multiple invasions of the Balkanic *Reticulitermes urbis* (Blattodea, Termitoidae, Rhinotermitidae) in Italy and France. 6° AISASP Student Meeting – Firenze, 29\02\2016.

[Oral Communication]

Scicchitano, V., Luchetti, A., Mantovani, B. Sex investment ratio and Asexual Queen Succession in Italian colonies of *Reticulitermes lucifugus* (Blattodea, Termitodae, Rhinotermitidae). LXXVI Congresso Nazionale dell'Unione Zoologica Italiana (UZI) - Viterbo, 15-18\09\2015.

[Poster]

Scicchitano, V., Luchetti, A., Mantovani, B. Phylogeography and colony structure of dry-wood dwelling termites of the genus *Kalotermes* (Isoptera, Kalotermitidae) in Southern Europe. VI Congress of the Italian Society for Evolutionary Biology (SIBE) - Bologna, 31\08 - 03\09\2015.

[Poster]

Scicchitano, V., Luchetti, A., Mantovani, B. Reproductive system and Asexual Queen Succession in *Reticulitermes lucifugus* and *R. urbis* (Blattodea, Termitodae, Rhinotermitidae). 5° AISASP Student Meeting – Bologna, 12\06\2015.

[Oral Communication]