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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

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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.

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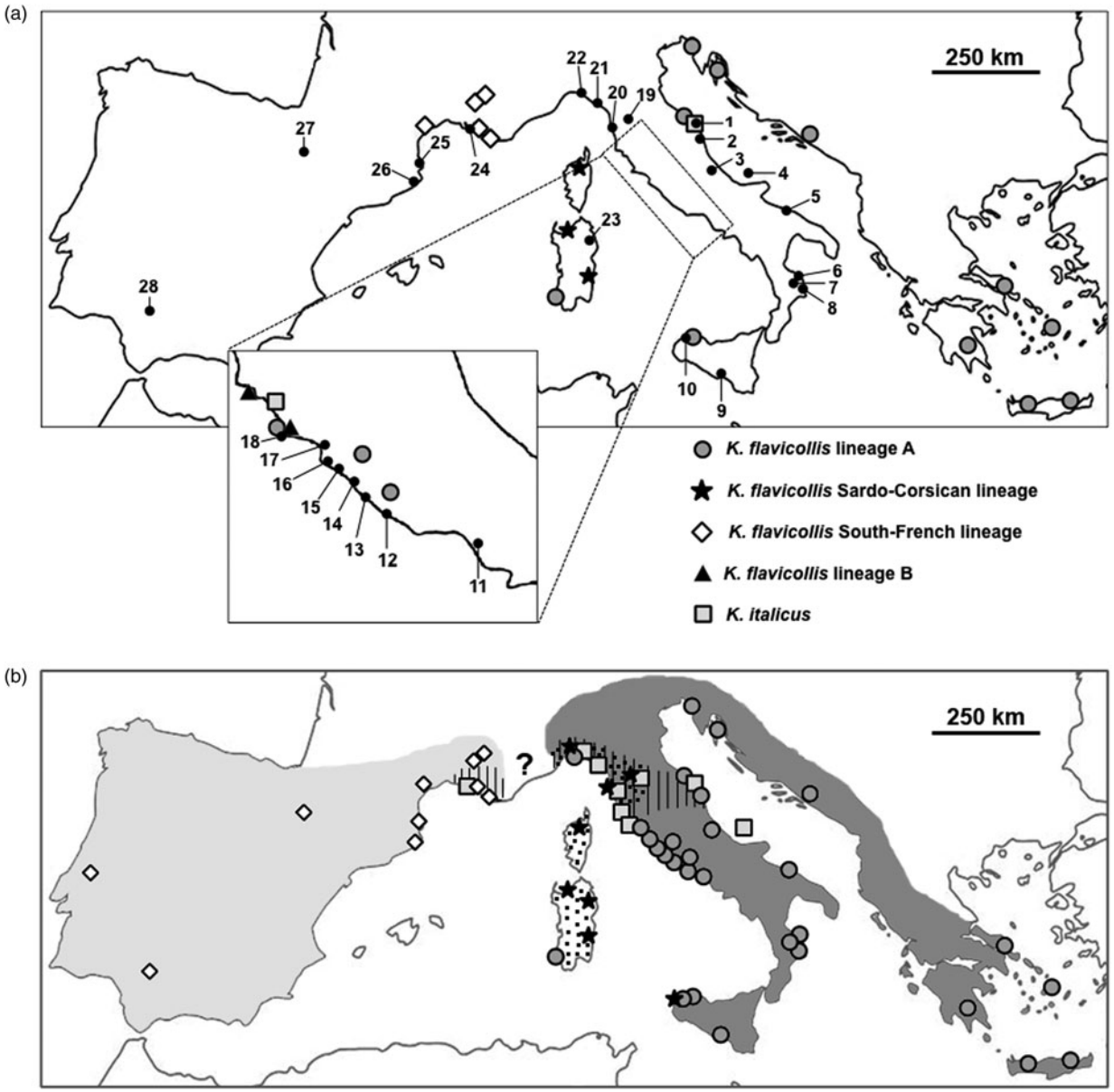

Fig. 1. (a) *Kalotermes* sampling locations and lineages distribution known so far. Numbers refer to table 1. (b) Summary of European *Kalotermes* taxa distribution as derived from the present analysis. Light gray area: *Kalotermes flavicollis* lineage IF; dark gray area: *K. flavicollis sensu strictu*; 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.

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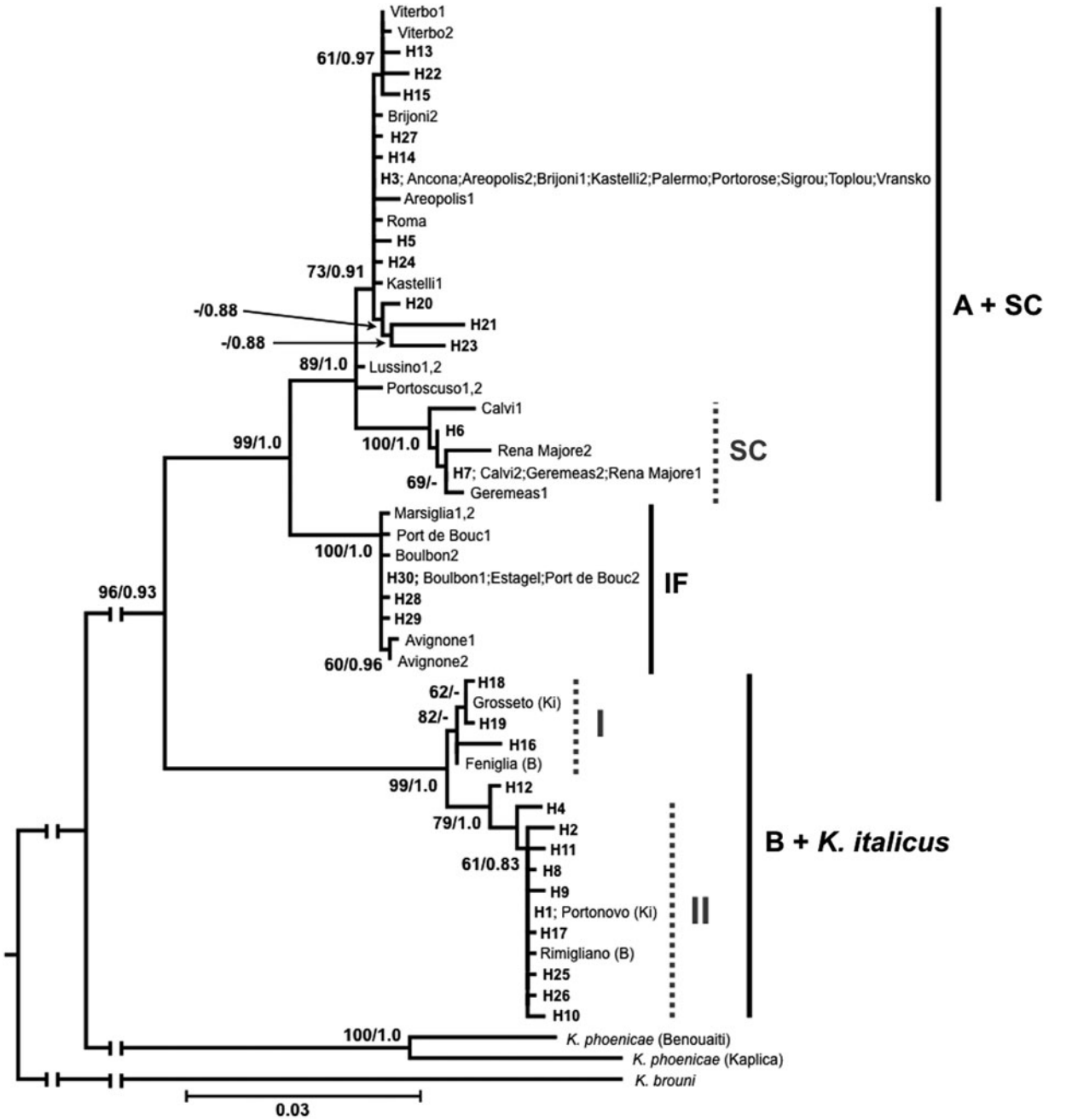

Fig. 2. Maximum Likelihood tree (−*lnL* = 2953.453) obtained from *cox1*/*trnL*/*cox2* haplotypes. Bayesian Inference analysis (−*lnL* = 2973.756) resulted in an overlapping topology. Haplotype codes as in table 1; previously identified haplotypes are reported with the name of the sampling location (consistently 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

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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

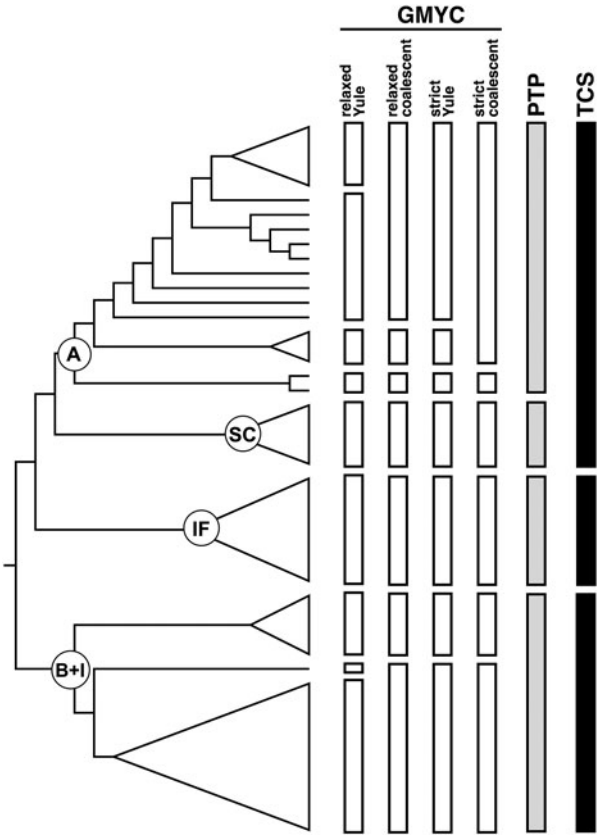

Fig. 3. Species delimitation analyses. Main clades are indicated by circles at their respective ancestral nodes. Outgroups have been omitted in the figure. Abbreviations: A, lineage A; SC, Sardo-Corsican lineage; IF, Ibero-French lineage; B + I: lineage B and *Kalotermes italicus*.

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

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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*

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(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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