Origin and evolution of the Italian subterranean termite *Reticulitermes lucifugus* (Blattodea, Termitoidae, Rhinotermitidae)

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Abstract

The Holarctic genus *Reticulitermes* shows seven species within the Mediterranean Basin. While phylogeny and systematics at continental level has been deeply investigated, a few studies concentrated on local ranges. To gain a clearer picture of the diversity and evolution of the Italian species *Reticulitermes lucifugus*, we analyzed the mitochondrial cytochrome oxidase II (COII) gene marker in newly collected colonies across the Peninsula. Data were gathered with all *R. lucifugus* sequences available from previous studies; COII sequences of the closely related Iberian taxa were also added to the data set. Maximum-likelihood, median-joining and statistical parsimony network elaborations on the resulting 119 colonies all agreed in indicating that: (i) the Sardo-Corsican subspecies *R. lucifugus corsicus*, strictly related to Southern Italian populations (including the Sicilian ones), is phylogenetically closer to the Iberian *Reticulitermes grassei*; and (ii) *R. lucifugus lucifugus* peninsular populations are structured into three clusters. The phylogenetic relationships and the biogeography of extant taxa suggest a scenario in which *R. lucifugus* ancestors colonized the Italian region through the Sardo-Corsican microplate during its Oligocene-Miocene anticlockwise rotation. Moreover, well after the colonization took place, northward range expansion might have produced the presently observed genetic diversity, as inferred from haplotype and nucleotide diversity estimates. On the whole, this study highlights the evolution of Italian *Reticulitermes* taxa and supports the importance of a wide taxon sampling especially when dealing with organisms easily dispersed by human activities.

Keywords: haplotype networks, Italy, phylogeny, *Reticulitermes lucifugus*, Sardo-Corsican microplate rotation

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Introduction

Termites (Blattodea, Termitoidae) are essential components of soil communities in terrestrial ecosystems of warm regions. While producing positive effects on soil structure and productivity because of their ability to degrade wood matter, they can be a serious pest for human wooden artifacts: the termite-derived annual damage, in fact, is estimated about 3–5 billion US$ in the USA and about 1 million € in the EU (Jeffery *et al.*, 2010).

Subterranean termites of the genus *Reticulitermes* Holmgren belong to the Rhinotermitidae family, the apical clade of the so-called lower termites, which includes most of the invasive termite species (Vargo & Husseneder, 2009;

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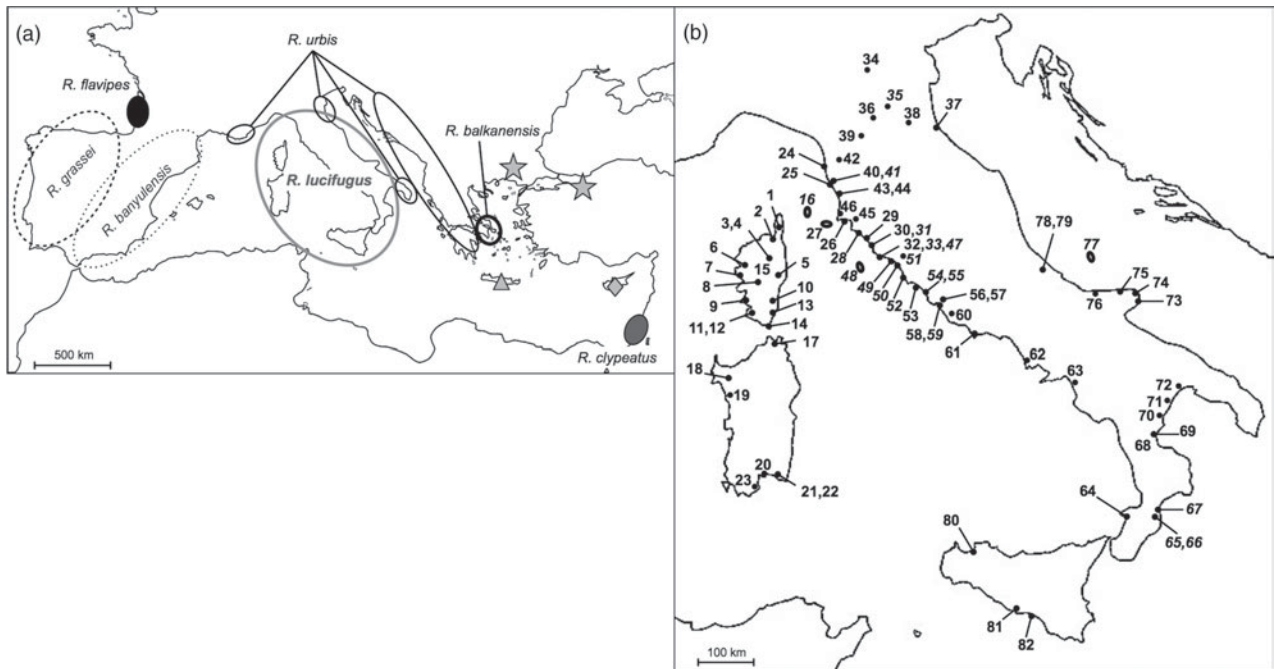E-mail: andrea.luchetti@unibo.it

Fig. 1. Geographic distribution of *Reticulitermes* taxa and sampling sites. (a) Map of European *Reticulitermes* species distribution. Main mtDNA lineages are also represented: triangle = Cretan lineage; diamond = Cypriot lineage; stars = Eastern Greece–Turkish lineage. (b) Map showing sampling localities of the considered *R. lucifugus* colonies. Numbers refer to table 1; numbers in italic indicate presently analyzed samples.

Evans, 2011). The genus is Holarctic and in Europe it is present in the Mediterranean Basin with seven species (*R. flavipes* Kollar, *R. grassei* Clément, *R. banyulensis* Clément, *R. lucifugus* Rossi, *R. urbis* Bagnères, Uva & Clément, *R. balkanensis* Clément and *R. clypeatus* Lash), plus three mitochondrial DNA (mtDNA) lineages whose taxonomic rank has not been determined yet (Lash, 1952; Clément *et al.*, 2001; Austin *et al.*, 2002, 2005; Marini & Mantovani, 2002; Bagnères *et al.*, 2003; Kutnik *et al.*, 2004; Luchetti *et al.*, 2004, 2007, 2013*a*; Uva *et al.*, 2004*a*, *b*; Lefebvre *et al.*, 2008; Leniaud *et al.*, 2010; Velonà *et al.*, 2010; Ghesini & Marini, 2012*a*, *b*; Perdereau *et al.*, 2013; fig. 1a). The high diversity observed for this termite genus within the Mediterranean region, compared with the other naturally occurring genus *Kalotermes* (Velonà *et al.*, 2011; Luchetti *et al*, 2013*b*), mirrors the complex paleogeographic–paleoclimatic history of Southern Europe that comprised significant landmasses movements, since 30–40 Myr ago, and recent climatic oscillations (Webb & Bartlein, 1992; Hewitt, 1996; Rosenbaum *et al.*, 2002; Meulenkamp & Sissing, 2003). Previous studies on the phylogeny and evolution of European *Reticulitermes* termites agreed to interpret the observed taxonomy and phyletic relationships as a consequence of the Quaternary climatic oscillations (population range contraction/expansion and physical barriers fluctuations; Clément *et al.*, 2001; Uva *et al.*, 2004*a*; Luchetti *et al.*, 2005; Lefebvre *et al.*, 2008). However, more recently, a wide phylogeographic study anticipated cladogenetic events back to the Oligocene (∼ 20–30 Myr ago; Velonà *et al.*, 2010), even if the role of climate fluctuations on the evolution of *R. grassei* and *R. urbis* populations, in particular, cannot be disregarded (Kutnik *et al.*, 2004; Luchetti *et al.*, 2007; Velonà *et al.*, 2010).

Only few studies focused specifically on the Italian range, mainly concentrating on the evolution of *R. lucifugus corsicus* or on localized samples of *R. lucifugus lucifugus* (Uva *et al.*, 2004*b*; Lefebvre *et al.*, 2008; Ghesini & Marini, 2012*b*). These studies, while pointing out a certain degree of differentiation within the peninsular *R. lucifugus*, gave only a partial view of the whole scenario.

In this study, we provide the widest analysis of the Italian *R. lucifugus* populations based on the mtDNA marker cytochrome oxidase II (COII), integrating the information of newly investigated samples together with previously obtained *R. lucifugus* sequences. The analyses are also widened to the Iberian taxa: these show, in fact, a closer relationship to the Italian ones, opposite to the high divergence with Eastern-Mediterranean clades (Uva *et al.*, 2004*a*; Luchetti *et al.*, 2007; Velonà *et al.*, 2010; Luchetti, unpublished data). On the whole, we provide the most comprehensive dataset regarding both the genetic variability and the geographic distribution of considered samples, aiming to understand the origin and evolutionary dynamics of *R. lucifugus*.

Material and methods

Twenty-seven colonies were field sampled. Collected specimens were preserved in absolute ethanol until molecular investigation (table 1, fig. 1b).

Total DNA was isolated from single termite heads with the CTAB method (Doyle & Doyle, 1987); two workers for each colony were used for PCR amplification of mitochondrial COII gene with primer TL2-J-3034 (5′-AAT ATG GCA GAT TAG TGC A-3′) and TK-N-3785 (5′-GTT TAA GAG ACC AGT ACT TG-3′; Luchetti *et al.*, 2004). PCR amplification was performed

Table 1. List of the complete *R. lucifugus* sequence dataset here considered, with all pertinent information. Numbers refer to map position in fig. 1.

Taxon		Sampling locality	Haplotype	Reference/GenBank accession numbers
R. lucifugus corsicus	1	Luri	h1	Lefebvre *et al.* (2008)
	2[1]	Saint-Florent	h1	A.N. KC576861
	3	Poggiola	h1	Lefebvre *et al.* (2008)
	4	Ponte Leccia	h1	Lefebvre *et al.* (2008)
	5	Valle d'Alesani	h1	Lefebvre *et al.* (2008)
	6	Evisa	h2	Lefebvre *et al.* (2008)
	7	Piana	h2	Lefebvre *et al.* (2008)
	8	Bocognano	h2	Lefebvre *et al.* (2008)
	9	Ajaccio 1	h1	Lefebvre *et al.* (2008)
		Ajaccio 2	h2	Lefebvre *et al.* (2008)
	10	Solenzara	h3	Lefebvre *et al.* (2008)
	11	Propriano	h2	Lefebvre *et al.* (2008)
	12	Sartene	h2	Lefebvre *et al.* (2008)
	13	Porto Vecchio	h1	Lefebvre *et al.* (2008)
	14	Bonifacio	h1	Lefebvre *et al.* (2008)
	15[2]	Corsica	h4	Luchetti *et al.* (2004)
	16	Capraia	h5	A.N. KC576862
	17	La Maddalena	h2	Lefebvre *et al.* (2008)
	18	Saccheddu	h1	Marini & Mantovani (2002)
	19	Alghero	h1	Lefebvre *et al.* (2008)
	20	Cagliari	h6	Marini & Mantovani (2002)
	21	Capitana di Quartu	h7	Luchetti *et al.* (2004)
	22	Flumini di Quartu	h6	Marini & Mantovani (2002)
	23	Pulas Is Molas	h8	Luchetti *et al.* (2004)
	24	Viareggio	h1	Lefebvre *et al.* (2008)
	25	Monti Livornesi 1	h9	A.N. KC576863
	26	Follonica	h1	Lefebvre *et al.* (2008)
	27	Elba	h1	Lefebvre *et al.* (2008)
	28	Punta Ala 1	h10	A.N. KC576885
		Punta Ala 2	h11	A.N. KC576886
	29	Castiglion della Pescaia	h1	Luchetti *et al.* (2013a)
	30	Parco dell'Uccellina	h1	Marini & Mantovani (2002)
	31	Marina di Grosseto 1	h9	Luchetti *et al.* (2013a)
		Marina di Grosseto 2	h1	A.N. KC576887
	32	Giannella	h1	Lefebvre *et al.* (2008)
	33	Feniglia	h1	A.N. KC576867
R. lucifugus lucifugus	34	Desenzano del Garda	h17	Luchetti *et al.* (2004)
	35[1]	Molinella	h12	A.N. KC576866
	36	Bologna	h13	Marini & Mantovani (2002)
	37	Cesenatico	h14	A.N. KC576869
	38	Forlì	h18	Marini & Mantovani (2002)
	39	Firenze	h16	Marini & Mantovani (2002)
	40	Migliarino	h14	Lefebvre *et al.* (2008)
	41	San Rossore	h16	A.N. KC576865
	42	Macchia Lucchese	h16	Lefebvre *et al.* (2008)
	25	Monti Livornesi 2	h16	A.N. KC576864
	43	Castiglioncello	h16	Lefebvre *et al.* (2008)
	44	Marina di Bibbona	h16	Lefebvre *et al.* (2008)
	45	Massa Marittima	h16	Luchetti *et al.* (2013a)
	46	Populonia	h14	Lefebvre *et al.* (2008)
	47	Porto Santo Stefano	h14	A.N. KC576882
	48	Isola del Giglio	h14	A.N. KC576876
	33	Feniglia	h14	Luchetti *et al.* (2013a)
	49	Montalto Marina 1	h17	A.N. KC576879
		Montalto Marina 2	h14	A.N. KC576880
	50	Riva dei Tarquini 1	h14	A.N. KC576873
		Riva dei Tarquini 2	h19	A.N. KC576874
	51	Viterbo	h14	A.N. KC576881
	52	Civitavecchia	h14	A.N. KC576872
	53	Marina di Cerveteri	h15	A.N. KC576878
	54	Maccarese	h14	A.N. KC576877
	55	Fregene	h17	A.N. KC576875
	56	Roma	h18	Marini & Mantovani (2002)
	57	Castel di Decima	h14	Marini & Mantovani (2002)
	58	Castel Porziano	h18	Luchetti *et al.* (2004)
	59	Ostia	h17	A.N. KC576883
	60	Grotta Ferrata	h18	Lefebvre *et al.* (2008)
	61	Anzio	h17	Lefebvre *et al.* (2008)
	62	Sabaudia	h17	Lefebvre *et al.* (2008)
	63	Napoli	h17	Marini & Mantovani, 2002

Table 1. (*Cont.*)

Taxon		Sampling locality	Haplotype	Reference/GenBank accession numbers
	64	Rosarno	h21	Luchetti *et al.* (2004)
	65	San Sostene	h20	A.N. KC576870
	66	Isca	h17	A.N. KC576871
	67	Marina di Davoli	h14	A.N. KC576884
	68	Spezzano Albanese	h17	Luchetti *et al.* (2004)
	69	Villapiana	h12	Ghesini & Marini (2012*b*)
	70	Policoro	h22	Ghesini & Marini (2012*b*)
	71	Marina di Pisticci 1	h12	Ghesini & Marini (2012*b*)
		Marina di Pisticci 2	h18	Ghesini & Marini (2012*b*)
	72	Castellaneta	h17	Ghesini & Marini (2012*b*)
	73	Peschici/Vieste	h17	Ghesini & Marini (2012*b*)
	74	Rodi Garganico	h23	Ghesini & Marini (2012*b*)
	75	Rodi/Lesina	h23	Ghesini & Marini (2012*b*)
	76	Marina di Lesina	h23	Ghesini & Marini (2012*b*)
	77	Tremiti Islands	h23	A.N. KC576868
	78	Chieti	h23	Marini & Mantovani (2002)
	79	Canosa Sannita	h23	Luchetti *et al.* (2004)
R. lucifugus 'Sicily'	80	Palermo	h24	Luchetti *et al.* (2004)
	81	Agrigento	h25	Luchetti *et al.* (2004)
	82	S. Stefano Quisquina	h25	Luchetti *et al.* (2004)

[1] Field-collected and kindly gifted by M. Marini.
[2] Laboratory-reared colony, kindly gifted by J.-L. Clément.

on 20 ng of template DNA in a 50 μl mixture with Go-Taq polymerase (Promega, Madison, WI, USA), following the manufacturer protocol. Thermal cycling was as follows: an initial denaturation step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, extension at 72°C for 30 s, a final elongation step at 72°C for 7 min. PCR products were purified using the Wizard SV PCR and Gel cleaning kit (Promega) and both strands were directly sequenced at Macrogen Inc. – Europe Laboratory. Newly scored haplotypes were deposited into GenBank (table 1).

To the newly obtained sequences, we added GenBank-derived ones from colonies analyzed in Marini & Mantovani (2002), Luchetti *et al.* (2004), Kutnik *et al.* (2004), Lefebvre *et al.* (2008), Ghesini & Marini (2012*b*) and Luchetti *et al.* (2013*a*) (table 1 and Suppl. Table S1), for a total data set of 90 *R. lucifugus* samples and 29 Iberian colonies. Haplotype numbering is here redefined following samples' West–Eastern geographic distribution. In order to avoid confusion with entities whose taxonomic status is still uncertain or debated, they will be referred as: the Sicilian form = *R. lucifugus* 'Sicily'; the divergent *R. grassei* clade (Kutnik *et al.*, 2004) = *R. grassei*-B.

Sequence alignment with Clustal algorithm, the search for the best substitution model (HKY + Γ) and the computation of the Maximum-likelihood tree, with 100 bootstrap replicates, were done using MEGA v. 5 (Tamura *et al.*, 2011).

Median-joining network (Bandelt *et al.*, 1999) was calculated with Network v. 4.611 (available at http://www.fluxus-engineering.com/sharenet.htm). Statistical parsimony network (Templeton *et al.*, 1992) was obtained with TCS v. 1.21 (Clement *et al.*, 2000); parsimony connection limits between potential sub-networks was set to 95%.

Haplotype diversity ($h_D \pm$ SE), nucleotide diversity ($\pi \pm$ SE) and the number of polymorphic sites (S) were obtained with DnaSP v. 5.1 (Librado & Rozas, 2009).

Results

The 27 colonies studied had 13 COII haplotypes (table 1), differing by one to 25 substitutions for a total of 34 variable positions. Seven haplotypes were already scored in previous analyses (h1, h9, h12, h14, h16, h17, h23; Mantovani & Marini, 2002; Luchetti *et al.*, 2004; Lefebvre *et al.*, 2008; Ghesini & Marini, 2012*b*; Luchetti *et al.*, 2013*a*).

Newly obtained haplotypes have been combined into a larger dataset containing all COII sequences so far produced for *R. lucifugus lucifugus, R. lucifugus* 'Sicily' and *R. lucifugus corsicus* (Marini & Mantovani, 2002; Luchetti *et al.*, 2004, 2013*a*; Lefebvre *et al.*, 2008; Ghesini & Marini, 2012*b*). COII sequences of *R. grassei* and *R. banyulensis* (Marini & Mantovani, 2002; Kutnik *et al.*, 2004) were also included, so that 119 colonies, collected from 111 localities, have been analyzed for a total of 45 haplotypes (table 1 and Suppl. Table S1). The sequence alignment showed 97 variable positions (76 parsimony informative sites) out of 677: 73 substitutions fall in the third codon position, 19 in the first and 5 at the second codon position. Haplotypes differ from each other by one to 42 substitutions.

Maximum-likelihood phylogenetic analysis (fig. 2a) recognizes two main clades: one embodying the Iberian *R. grassei* and *R. banyulensis*, and the other comprising *R. lucifugus* sequences. The first clade, as already evidenced by Kutnik *et al.* (2004), further splits in the sub-clusters given by sequences obtained from *R. grassei, R. banyulensis* and *R. grassei*-B samples.

The three known *R. lucifugus* taxa can be recognized in the second clade; the *R. lucifugus lucifugus* cluster is of particular interest as it is further structured in three, geographically partitioned lineages (fig. 2b). The first one (henceforth referred to as A lineage) is in sister relationship with the other two *R. lucifugus lucifugus* lineages and comprises colonies collected
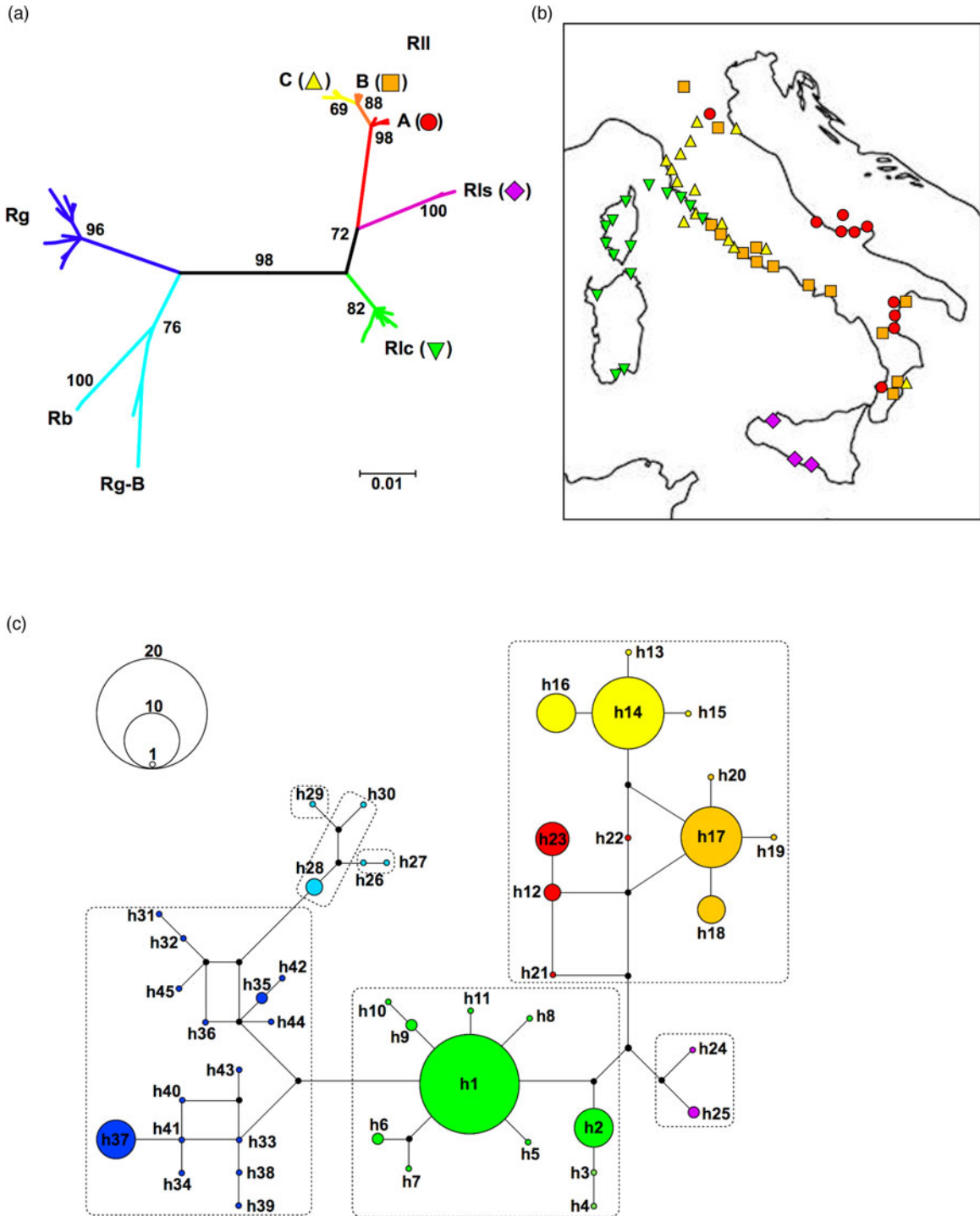
Fig. 2. Phylogenetic analysis on the COII dataset. (a) Maximum-likelihood tree ($-\ln L = 1808.53$); numbers at nodes are bootstrap values >60%. Rg = *Reticulitermes grassei*; Rg-B = *R. grassei*-B divergent lineage; Rb = *R. banyulensis*; Rlc = *R. lucifugus corsicus*; Rls = *R. lucifugus* 'Sicily'; Rll = *R. lucifugus lucifugus* with A, B and C indicating the three *R. lucifugus lucifugus* lineages; symbols as in panel (b) are also reported; (b) Map showing the distribution of *R. lucifugus* subspecies/lineages, as resulted from maximum-likelihood tree. *R. lucifugus* taxa/lineages are indicated by symbols as follows: *R. lucifugus corsicus*, reversed triangle; *R. lucifugus* 'Sicily', diamond; *R. lucifugus lucifugus* lineage A, circle; *R. lucifugus lucifugus* lineage B, square and *R. lucifugus lucifugus* lineage C, triangle; (c) Median-joining network; the magnitude of circles is proportional to the haplotype frequency; haplotypes are as in table 1. Dotted lines define sub-networks obtained with statistical parsimony 95% limit.

Table 2. Genetic diversity of the analyzed *Reticulitermes* samples. Number of sequenced colonies (N), number of haplotypes (h_N), haplotype diversity (h_D), polymorphic sites (S) and nucleotide diversity (π) are reported.

Clade	N	h_N	h_D	S	π
R. lucifugus corsicus	36	11	0.721 ± 0.071	13	0.0026 ± 0.0004
R. lucifugus lucifugus	51	12	0.857 ± 0.025	14	0.0047 ± 0.0004
A lineage	11	4	0.673 ± 0.123	4	0.0016 ± 0.0005
B lineage	18	4	0.575 ± 0.101	3	0.0010 ± 0.0002
C lineage	22	4	0.571 ± 0.081	3	0.0009 ± 0.0002
R. grassei	22	15	0.905 ± 0.057	21	0.0067 ± 0.0008

in Southern Italy, from the Tyrrhenian side (Rosarno) to the Ionian coast, up to the Tremiti Islands and on the Southern Adriatic side of the peninsula; a colony belonging to this lineage was also collected from an infested building in the Northern town of Molinella. The second lineage (the B lineage) comprises colonies collected along the Central/Southern Tyrrhenian coast and on the Ionian one; two colonies of the B lineage have been found infesting buildings in the Northern cities of Forlì and Desenzano del Garda. The third lineage (the C lineage) embodies samples collected along the Central/Northern Tyrrhenian side of the peninsula, but three: one from Bologna (infested building), one sampled in the Cesenatico pine wood (on the Adriatic side) and one collected in the very South of Italy, on the Ionian side (fig. 2b). The three lineages partially overlap in the contact zones (fig. 2b). Median-joining and statistical parsimony networks give strictly congruent results (fig. 2c). On the 95% parsimony connection limit, statistical parsimony networks analysis produces seven sub-networks, each representing a recognized species, sub-species or lineage. *R. grassei* haplotypes are directly linked to the *R. lucifugus corsicus* sub-network, which is equally separated from the sub-networks of the Sicilian taxon and of *R. lucifugus lucifugus*. The haplotypes distribution in the *R. lucifugus lucifugus* sub-network agrees with the clustering observed in the maximum-likelihood analysis. In comparison with the Iberian sub-network(s), the *R. lucifugus* complex ones are characterized by few, high-frequency haplotypes.

Intra-taxa genetic diversity (table 2) indicates *R. grassei* as the more variable ($h_D = 0.905 ± 0.057$; $\pi = 0.0067 ± 0.0008$) followed by *R. lucifugus lucifugus* ($h_D = 0.857 ± 0.025$; $\pi = 0.0047 ± 0.0004$). Among Italian peninsular lineages, the A one is the more variable ($h_D = 0.673 ± 0.123$; $\pi = 0.0016 ± 0.0005$), whereas the C lineage shows the lowest genetic diversity ($h_D = 0.571 ± 0.081$; $\pi = 0.0009 ± 0.0002$).

Discussion

Phylogeny and evolution of *Reticulitermes* species in Europe has been deeply investigated (Clément *et al.*, 2001; Austin *et al.*, 2002; Marini & Mantovani, 2002; Luchetti *et al.*, 2004, 2007; Uva *et al.*, 2004*a*, *b*; Velonà *et al.*, 2010; Ghesini & Marini, 2012*b*), as well as phylogeography and population genetics of both Iberian and Balkan clades (Kutnik *et al.*, 2004; Luchetti *et al.*, 2007; Leniaud *et al.*, 2010). On the other hand, the biosystematics of the Italian *R. lucifugus* has been only partially studied, with particular attention just paid to the trans-Tyrrhenian distribution of *R. lucifugus corsicus* (Uva *et al.*, 2004*b*; Lefebvre *et al.*, 2008).

In this paper, we provide the most comprehensive genetic analysis of *R. lucifugus* colonies ($N = 90$) based on both new and previously produced COII mitochondrial marker data, taking also into account the data on Iberian taxa (29 colonies). Despite possible limitations imposed by the use of a single mtDNA marker, this study allowed us to evidence: (i) a well-defined phylogeographic pattern explaining the origin of Italian *Reticulitermes* and (ii) the presence of three well-differentiated lineages among the Italian peninsular populations.

On the origin of *R. lucifugus* taxa, two hypotheses have been put forward so far. The first one suggested vicariance due to the contemporary separation of the Iberian, Italian and Balkan taxa in their Mediterranean peninsulas during the Quaternary climatic oscillations. The second hypothesis explained *R. lucifugus* speciation by dispersal after a post-Ice Age northward recolonization originating from the Iberian refugium and followed by the invasion of the Italian peninsula from the North (Clemént *et al.*, 2001). The latter hypothesis has been considered as the most likely, being supported by subsequent phylogenetic analyses highlighting the closer relationship between Iberian and Italian taxa and the higher divergence of Eastern-Mediterranean clades (Uva *et al.*, 2004*a*; Luchetti *et al.*, 2007; Velonà *et al.*, 2010; Luchetti, unpublished data). Following the cladogenetic event leading to the peninsular *R. lucifugus* lineage, *R. lucifugus corsicus* and the Sicilian entity would have evolved during the Pleistocene cycles of separation/connection of the mainland with the Corse, Sardinia and Sicily islands (Luchetti *et al.*, 2005; Lefebvre *et al.*, 2008). Recently, the origin of *R. lucifugus corsicus* has been predated to the separation of the Sardo-Corsican microplate from the mainland's Northern part, at the end of its anticlockwise rotation (8–5 Myr ago; Velonà *et al.*, 2010). Data presented here evidence a slightly different scenario: the maximum-likelihood tree and haplotype networks evidenced a relationship between *R. grassei* and *R. lucifugus corsicus* closer than that with the two other *R. lucifugus* taxa and, remarkably, *R. lucifugus corsicus* is further strictly related to the Sicilian entity and to the Southernmost *R. lucifugus lucifugus* A lineage. This implies that the Italian peninsula was colonized by an ancestral *R. lucifugus* lineage from the South rather than from the North and therefore we suggest a revision of the previous hypothesis on the origin and differentiation of the *R. lucifugus* complex.

The Sardo-Corsican microplate separated from the Iberian region between 30 and 25 Myr ago, as a part of the so-called Hercynian belt; it then drifted eastward and collided with the Apulian microplate ($\sim$ 18 Myr ago) (Rosenbaum *et al.*, 2002; Meulenkamp & Sissing, 2003). Successively, the Sardo-Corsican microplate separated again from the Apulian one, leaving a fragment (the Calabrian one) connected to the mainland (Rosenbaum *et al.*, 2002). This paleogeographic pattern and our data suggest the following scenario: after the separation of the Sardo-Corsican microplate, the ancestral Iberian *Reticulitermes* taxon split into two entities, one corresponding to the present-day *R. grassei* (left on the Iberian area) and the other to *R. lucifugus* (trapped within the eastward-rotating microplate). The timing of these geological events well reconciles with the timing of cladogenetic events estimated by Velonà *et al.* (2010), i.e., between 24 and 10 Myr ago. Once the microplate collided with the Apulian block, the ancestral *R. lucifugus* spread on the new land from the Southern side before the subsequent separation, accordingly

to the closer relationship between the Sardo-Corsican sub-species, the Sicilian taxon and the *R. lucifugus lucifugus* A lineage. The origin of the *R. lucifugus* complex from a small subset of the *R. grassei* ancestors appears also reflected by the difference in the genetic variability between the two taxa. Indeed, with respect to the Iberian taxon, both *R. lucifugus corsicus* and *R. lucifugus lucifugus* exhibit lower values of h_D and π: it is likely that, when Hercynian belt separated from the mainland, the populations living on that plate fragment embodied just a fraction of the whole genetic variability. Notably, even if to a limited extent, the *R. lucifugus lucifugus* A lineage appears more variable than the Northernmost B and C ones (table 2): this is consistent with the hypothesis of the Italian peninsula colonization from the South we propose here.

Cladogenetic events that followed the break off of the Sardo-Corsican microplate from Iberia have been described, until now, in only two terrestrial organisms: the plant family Araceae (Mansion *et al.*, 2008) and the ground-dweller spider genus *Parachtes* (Bidegaray-Batista & Arnedo, 2011). There-fore, our data confirm this peculiar pattern, providing a new evolutionary scenario for the *R. lucifugus* complex origin.

Maximum-likelihood analysis evidence a genetic structur-ing of peninsular Italy populations, with three divergent lineages: one distributed to the very South, another one scattered on the West side spanning from Southern to Central Italy and the last one occurring from Central- to North-Western Italian coasts. Following the cladogenesis timing calculated by Velonà *et al.* (2010), the splitting of the Sicilian-peninsular clade dates back, approximately, to the Messinian salinity crisis (∼ 5.3 Myr ago); this means that the separation of the peninsular populations took place not later than the Pliocene period. Then, from the *R. lucifugus lucifugus* ancestral populations: (i) the A lineage branched out, at present occurring under the geographic barrier given by the Southern Apennine chain (Ghesini & Marini, 2012*b*), and (ii) lineages B and C underwent northward range expansion along the Tyrrhenian coasts, eventually producing the observed South-North cline (Uva *et al.*, 2004*b*; Lefebvre *et al.*, 2008). It is to be noted, although, that the distribution areas of the three peninsular lineages are not well delimited, with overlaps in the contact zones. This may be explained by subsequent, recent dispersion.

The study of the biogeography and of phylogeographic patterns of subterranean termites can be difficult for two main reasons: first of all, they have cryptic nesting habits, making the taxon sampling difficult; second, they can be easily dispersed by human-mediated movement of wooden arti-facts/materials (see for example, Jenkins *et al.*, 2001; Perdereau *et al.*, 2013). This latter feature may mask the correct phylo-geographic signal when the taxon sampling is not enough representative. In the present analysis, we provide the first comprehensive study of the *R. lucifugus* complex, using both originally produced and literature-derived molecular data, able to highlight previously unnoticed phylogeographic patterns. The utility of the wider taxon sampling was particularly evident in the definition of the three *R. lucifugus lucifugus* lineages even if some haplotypes are scattered outside the presumed distribution area: for example, one A and two B lineages' haplotypes can be found in Northern localities, as well as one C lineage haplotype has been detected in the very South of the peninsula. The geographic isolation of these haplotypes as well as their presence in Northern localities within cities but not in the field, where natural condition would not allow the termite survival, speak in favor of secondary introduction events.

As a general consideration, this paper point out that when tackling the phylogeography of organisms closely associated with human trades, a wide taxon sampling considering both infestation and natural sites is required to obtain reliable bio-geographic and biodiversity patterns.

The supplementary materials for this article can be found at http://www.journals.cambridge.org/ber.

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