

New data on *Reticulitermes urbis* and *Reticulitermes lucifugus* in Italy: are they both native species?

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Abstract

In Italy, three species of subterranean termites in the genus *Reticulitermes* exist: the native *R. lucifugus* (Rossi), the North-American *R. flavipes* (Kollar), and *R. urbis* Bagnères, Uva et Clement. *R. urbis* is a recently described species, that is suspected to have been introduced from the Balkans to France and Italy, where until the present study it had been found only in a few urban areas. The distribution of *R. urbis* in Italy is still poorly understood. We collected 22 *Reticulitermes* samples in 17 Italian localities in order to define better the distributions of *R. urbis* and *R. lucifugus* and to determine whether *R. urbis* can be found also in natural environments. We identified termite species by sequencing a portion of the mitochondrial gene encoding for the cytochrome oxidase subunit II (COII), that we also used for the construction of haplotype networks and for phylogenetic analysis. We found *R. urbis* in three urban areas in the north, and in seven natural areas in the south. So, in Italy, *R. urbis* is more diffused than previously thought. The same pattern of distribution, restricted to urban areas in the north and including also natural environments in the south, is seen in the native *R. lucifugus*, and may be explained by the climatic requirements of these species. The lower genetic variability of *R. urbis* observed in Italy and France, compared with the Balkans, may be explained without implying an introduction by man. Moreover, many insect species exist with a range similar to that of *R. urbis*. For these reasons, we think that *R. urbis* could be native in Italy.

Key words: introduced species, native species, Italy, subterranean termites, mitochondrial DNA.

Introduction

Three species of the genus *Reticulitermes* (Isoptera Rhinotermitidae) are known to occur in Italy. *Reticulitermes lucifugus* (Rossi) is found in many localities across most of the Italian territory. The recently described species *Reticulitermes urbis* Bagnères, Uva et Clement (Bagnères *et al.*, 2003) has been found in some localities in the Adriatic side of the Po Plain and in Apulia (Marini and Mantovani, 2002; Luchetti *et al.*, 2004). The North-American species *Reticulitermes flavipes* (Kollar), whose presence in Europe is long time known (Kollar, 1837; Jucci, 1924; Feytaud, 1925; Austin *et al.*, 2005; Dronnet *et al.*, 2005), has recently been found in two localities in Lombardy (Ghesini *et al.*, 2010; 2011).

Three subspecies of *R. lucifugus* exist: *R. lucifugus lucifugus* in the Italian peninsula, *R. lucifugus corsicus* in Sardinia and on the coasts of Tuscany, and a third subspecies not yet described in Sicily and Trieste (Marini and Mantovani, 2002; Luchetti *et al.*, 2004). Outside Italy, *R. lucifugus lucifugus* is found in southern France and in Bastia (Corse), while *R. lucifugus corsicus* is found in southern France and Corse (Lefebvre *et al.*, 2008).

Outside Italy, *R. urbis* is distributed in western Greece and Peloponnese, in Croatia and in southern France (Luchetti *et al.*, 2007; Leniaud *et al.*, 2010).

In the phylogeny of European *Reticulitermes* species, two main clades are identified, the first one including west-Mediterranean species and the second one includes east-Mediterranean species (Velonà *et al.*, 2010). *R. lucifugus* belongs to the west-Mediterranean clade, together with the Franco-Iberian species *Reticulitermes grassei* Clement and *Reticulitermes banyulensis* Clement. *R. urbis* belongs to the east-Mediterranean clade, together with the Greek species *Reticulitermes bal-*

kanensis Clement, the Israeli species *Reticulitermes clypeatus* Lash and some other taxa, not yet described, distributed in eastern Greece, Turkey, Crete, and Cyprus (Velonà *et al.*, 2010; Ghesini and Marini, 2012).

While the status of *R. lucifugus* and *R. flavipes* as, respectively, a native and an introduced species in Italy, is commonly accepted, the status of *R. urbis* still presents some elements of doubt, and arguments supporting both its indigenous origin and its introduction by man have been suggested (Uva *et al.*, 2004; Luchetti *et al.*, 2007; Leniaud *et al.*, 2010).

When the existence of *R. urbis* as a distinct species was demonstrated (Clément *et al.*, 2001), only a few Italian and French populations, confined to urban areas, were known. Because Italian localities with *R. urbis* presence are situated on the Adriatic side of the peninsula, it was thought possible that the core of *R. urbis* range could be in the Balkan Peninsula. This hypothesis was supported by the finding that the defensive secretions of *R. urbis* have affinities with those of the Greek species *R. balkanensis* (Quintana *et al.*, 2003). Termite surveys carried out from Istria down to Peloponnese actually allowed to identify *R. urbis* populations in many localities, in natural environments (Luchetti *et al.*, 2005; 2007). Therefore, *R. urbis* was thought to be native to the Balkan Peninsula, and introduced by man in Italy and France (Luchetti *et al.*, 2007). Analyses on mitochondrial DNA sequences and microsatellites have confirmed that the genetic diversity of *R. urbis* is higher in the Balkan Peninsula than in Italy and France (Leniaud *et al.*, 2010). These results, together with the observation that, in Italy and France, the distribution of *R. urbis* mitochondrial DNA haplotypes is not concordant with geographic regions, have been interpreted as supportive of the hypothesis that *R. urbis* was introduced by man

(Leniaud *et al.*, 2010). The introduction of termites by man, through the displacement of infested wood, wooden objects or soil is in fact a possible occurrence, as documented by many cases (Vargo and Husseneder, 2009).

During the 20th century *R. lucifugus* has been reported in more than 200 localities in continental Italy (Sbrenna and Micciarelli Sbrenna, 2008), but the identification of these samples cannot be trusted, because, before *R. urbis* was recognized as a distinct species, all *Reticulitermes* findings in Italy were automatically identified as *R. lucifugus*. Recent analyses of samples collected in some of these localities have shown that the species involved is actually *R. urbis* (Marini and Mantovani, 2002; Luchetti *et al.*, 2004). Up to now, the presence of *R. urbis* in Italy is ascertained in eight urban areas, both in the North-East and in the South-East, in particular in Veneto, Emilia-Romagna, and Apulia (Marini and Mantovani, 2002; Luchetti *et al.*, 2004). It is possible that the presence of *R. urbis* in Italy is still underestimated.

In this study, we examine *Reticulitermes* populations from the Adriatic and Ionian sides of Italy, with the purpose of clarifying *R. urbis* and *R. lucifugus* distributions, verifying whether *R. urbis* is present in natural environments, and comparing the two species from an ecological and genetic (mitochondrial DNA) point of view.

Materials and methods

Sampling

Termite collection surveys were conducted during 2009 and 2010. Surveys were initially carried out in northern regions, in some localities where termite activity was reported by homeowners, then extended to natu-

ral environments in the same regions and in the other regions along the Adriatic and Ionian coasts. We considered natural environments as those areas that are not urbanized, and where old scrubs or woods exist, because areas with primary vegetation are scarce in Italian plains. In northern regions (Veneto, Friuli-Venezia Giulia), no termites were found in natural environments, and all the samples were collected in urban areas. In southern regions (Apulia, Basilicata, Calabria), termites were found and collected in natural environments.

Seventeen localities were sampled, and 22 samples were collected (figure 1). In most localities, a single termite colony was found. In five localities, two termite colonies, separated by a distance of at least 500 m, were sampled. The two samples from the same locality will be indicated as 1 and 2.

Genetic analysis

A preliminary analysis of the samples collected revealed that they all belonged to the genus *Reticulitermes*. The identification of Italian *Reticulitermes* species based on morphological characters is not sufficiently reliable, therefore, in order to determine our samples, we sequenced a portion of the mitochondrial gene encoding for the cytochrome oxidase subunit II (COII). This portion is a good marker for the identification of species and subspecies in the genus *Reticulitermes* and has been used in many studies on European termites (Marini and Mantovani, 2002; Luchetti *et al.*, 2007, Leniaud *et al.*, 2010).

Total DNA was extracted from termite heads with a CTAB protocol (Doyle and Doyle, 1987). Two individuals for each sample were analyzed. For each individual, a 684-bp portion of the mitochondrial gene COII, encoding for 228 amino acids, was amplified.

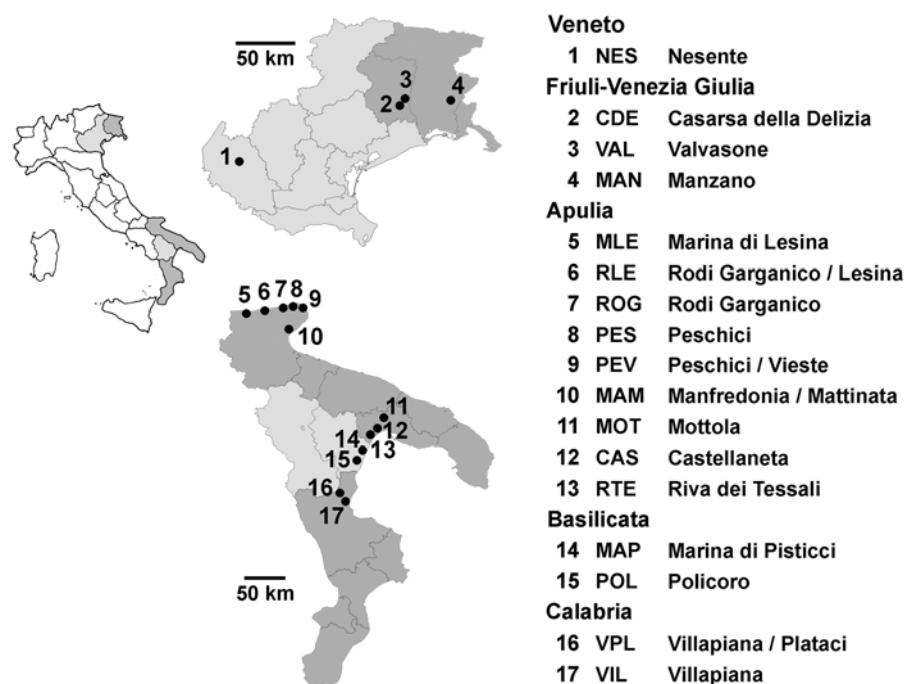


Figure 1. Sampling localities in northern and southern Italy.

Amplification was obtained with the primers TL2-J-3034 = mtD-13 (5'-AAT ATG GCA GAT TAG TGC A-3') and TK-N-3785 = mtD-20 (5'- GTT TAA GAG ACC AGT ACT TG-3').

PCR was performed in a 50 µl mixture using GoTaq® Flexi DNA Polymerase kit (Promega, USA), following the manufacturer's protocol. Reaction conditions were set as follows: initial denaturation at 94 °C for 5 minutes; 30 cycles composed by denaturation at 94 °C for 30 seconds, annealing at 48 °C for 30 seconds, extension at 72 °C for 30 seconds; final extension at 72 °C for 7 minutes. Purification and sequencing were performed by MacroGen Inc. (Seoul, South Korea). Sequencing was carried out with the same primers that were used for amplification, and both strands were sequenced.

For the haplotype network construction and phylogenetic analysis, the sequences obtained in this work were aligned with *R. urbis* and *R. lucifugus* COII sequences obtained in previous works (Austin *et al.*, 2002; Marini and Mantovani, 2002; Luchetti *et al.*, 2004; 2007; Lefebvre *et al.*, 2008; Leniaud *et al.*, 2010) and available in GenBank. For *R. lucifugus*, only sequences from the subspecies *R. lucifugus lucifugus* were considered, because our samples were collected in areas where the other subspecies are not found. A 595-bp portion was used, which was available for all the localities considered. The preliminary analysis and the alignment of DNA sequences were performed with MEGA version 5 (Tamura *et al.*, 2011). Statistical parsimony haplotype networks were obtained with TCS v. 1.21 (Clement *et al.*, 2000). A single sequence for each haplotype was used for this analysis, in order to avoid a possible bias towards the most frequent haplotype when inferring the ancestral haplotype. Median joining haplotype networks were obtained with Network 4.6.0.0, available at <http://www.fluxus-engineering.com> (Bandelt *et al.*, 1999), using as outgroups either *R. balkanensis* or *R. lucifugus* (A.N. AY954662 and AF291740) for *R. urbis* network, and either *R. grassei* or *R. urbis* (A.N. AY510576 and AF291736) for *R. lucifugus* network.

When counting the number of localities where a haplotype was found, samples from the same locality were considered as one, except in the cases where their haplotypes differed.

For *R. urbis* phylogenetic analysis, 683-bp sequences were used, one for each haplotype. COII sequences from *R. lucifugus* and *R. flavipes* (A.N. AF291740 and GU070789) were used as outgroups. For *R. lucifugus lucifugus* phylogenetic analysis, 666-bp sequences were used, one for each haplotype. COII sequences from *R. urbis*, *R. lucifugus* 'Sicily' and *R. lucifugus corsicus* (A.N. AF291736, AY267857, and AF291729) were used as outgroups. Models of nucleotide substitution were tested with ModelTest Server 1.0 (Posada and Crandall, 1998; Posada, 2006). According to Bayesian information criterion, the best substitution models resulted HKY+I (I = 0.7429) and HKY+Γ (Γ = 0.3196) for *R. urbis* and *R. lucifugus lucifugus* datasets, respectively. Maximum Likelihood and Maximum Parsimony analyses were performed with PAUP* 4.0b10 (Swoford, 2003). Bootstrap values were calculated with 200 and 2000 replicates, respectively. The bayesian tree was

obtained with MrBayes 3.1.2 (Huelsenbeck *et al.*, 2001; Ronquist and Huelsenbeck, 2003). Convergence was reached after two million generations (average standard deviation of split frequencies <0.005). Trees were sampled every 100 generations, and the first 5000 trees were discarded as burn-in, after graphic visualization. Trees were edited with the software FigTree 1.3.1, available online at <http://tree.bio.ed.ac.uk/software/figtree/>.

Figures were created using Adobe Photoshop® 6.0 software (Adobe Systems Inc., USA).

Results

Ecological notes

In northern Italy (Veneto and Friuli-Venezia Giulia), termites were only found in old buildings and in their gardens, in the oldest parts of towns. Inspections were carried out in buildings and gardens surrounding the infested areas, but no other colonies were found.

In order to ascertain if natural populations of termites existed in Veneto and Friuli-Venezia Giulia, surveys were also conducted in natural environments, in fields and woods, from the coast up to the Alpine foothills. Neither termites nor signs of former termite presence, such as tunnelling in wood, were found. Surveys conducted along the Adriatic coast (south to Veneto), in woods where conditions are suitable for termite presence such as Bosco Mesola and Mount Conero, no *Reticulitermes* termites were found. In southern Italy, searches were conducted in natural environments. Termites were found mainly in pine forests near the coasts and in Mediterranean scrubs. Sampling localities are situated mainly in Gargano and along the Ionian coast, where pinewoods and Mediterranean scrubs still exist. In the lowlands between these two areas, natural environments are virtually absent.

Genetic analysis

The COII sequences obtained in this study are deposited in GenBank under accession numbers JQ071531-JQ071533 and JQ231191-JQ231196.

Eleven samples (four in the north and seven in the south) belong to *R. urbis*, while the other 11 (one in the north and 10 in the south) belong to *R. lucifugus*, in particular to the peninsular subspecies *R. lucifugus lucifugus* (tables 1 and 2). The sample from Veneto was identified as *R. urbis*. Samples from Calabria were identified as *R. lucifugus lucifugus*. In Friuli-Venezia Giulia, Apulia, and Basilicata, both species were found.

The two samples collected in Marina di Lesina (MLE1 and MLE2) have the same sequence. In the other cases where two colonies were sampled in the same locality, their sequences are different (VAL1 and VAL2, MAP1 and MAP2), and in two cases the samples belong to different species (ROG1 and ROG2, CAS1 and CAS2).

The areas where the presence of *R. urbis* and *R. lucifugus lucifugus* has been ascertained so far, based on the data of this and of previous studies (Austin *et al.*, 2002; Marini and Mantovani, 2002; Luchetti *et al.*, 2004; 2007; Lefebvre *et al.*, 2008; Leniaud *et al.*, 2010; Ghesini *et al.*, 2011) are shown in figure 2 (for a detailed list of the localities, see tables 1 and 2).

Table 1. *R. urbis* haplotypes. The localities examined for the first time in this study and the accession numbers of the corresponding sequences are in bold.

Clade	Hapl.	Localities	GenBank A.N.	
South	A	Italy Bagnacavallo	AF291736	
	B	Greece Kato Ackaia	DQ487829	
	C	Greece Areopolis	AY267867	
	D	Italy MAM	JQ231191	
	E	Italy Sant'Agata	AF291737	
	F	Greece Kalamatas	DQ487832	
	G	Greece Kalavrita	DQ487828	
	H	Greece Neapolis	AY954668	
	I	Greece	Kiparissia, Sivota	DQ487823
		Croatia	Gradac	DQ487823
		Italy	Mira, NES, VAL2, MOT	AF291734, JQ071532
		France	Domène	AF525350
	J	Greece	Kallikomon	DQ487830
	K	Italy	Verona	AY267860
L	Greece	Pylos	DQ487831	
North	M1	Greece	Igoumenitsa	DQ866972
		Croatia	Klek, Komarna, Živogošće	DQ487822
		Italy	Ravenna, Salsomaggiore, VAL1, CDE, RTE, PES, ROG2, VPL	FJ381927, AF291735, JQ071531
		France	Antibes, Château Gombert, Carnoux, La Ciotat, Sophia Antipolis, St Cyr Les Lecques	FJ381938-42, FJ381947-51
	M2	Italy	CAS1	JQ231196
	N	Greece	Parga	DQ487824
	O	Greece	Katoki, Kastrosikia	DQ487825
	P	Italy	Galatina, Lecce	AY267862
	Q	Greece	Lefkada	DQ487826

Table 2. *R. lucifugus lucifugus* haplotypes. The localities examined for the first time in this study and the accession numbers of the corresponding sequences are in bold. “Side” refers to the side of Italy where the correspondent haplotype is found: T = Tyrrhenian; A = Adriatic and Po plain; I = Ionian. A and I sides are those where *R. lucifugus* is sympatric with *R. urbis*.

Hapl.	Localities	Side	GenBank A.N.
R	Italy Rosarno	T	AY267863
S	Italy Chieti, Canosa Sannita, MLE1, MLE2, ROG1, RLE	A	AF291738, JQ231192
T	Italy Molinella, MAP1, VIL	A/I	AY954659, JQ231193
U	Italy POL	I	JQ231195
V1	Italy	T/A/I	AF291725, EF591504, AF291739, JQ231194
	Italy		Forli, Grottaferrata, Roma, Castel Porziano, MAP2
V2	Italy	T/A/I	AF291740, EF591505-06, JQ071533 ,
	France		Marseille, Martigues, Saint Rémy, Salon de Provence, Bouc Bel-Air, Plain de Cuques EF591494, EF591497-98, EF591500-02
W	Italy	T	AF292007 EF591508-09
	France		Bastia EF590507
X	Italy Firenze, Firenze Peretola, Marina di Bibbona, Macchia Lucchese, Castiglioncello, Torre del Lago	T	AF291726 EF591510-14
Y	Italy Bologna	A	AF291723
Z	Italy Patanello	T	AF525341

For the analysis of haplotypes, we used a 595-bp portion of COII sequences, for both *R. urbis* and *R. lucifugus*. When sequences differed in loci outside the portion used for the haplotype analysis, we included them in the same haplotype, but kept them separated in Tab.1 and Tab.2 (haplotypes M1 and M2, V1 and V2).

Most of the haplotypes found in this study had been already isolated in other studies (Austin *et al.*, 2002; Marini and Mantovani, 2002; Luchetti *et al.*, 2004; 2007; Lefebvre *et al.*, 2008; Leniaud *et al.*, 2010), in other localities (tables 1 and 2). One *R. urbis* haplotype (haplotype D) and one *R. lucifugus* haplotype (haplotype U) were isolated for the first time in this study.

In Italy, seven *R. urbis* haplotypes and nine *R. lucifugus lucifugus* haplotypes have been found (tables 1 and 2). In the areas where *R. urbis* and *R. lucifugus* are sympatric, i.e. north-eastern Italy and the Adriatic and Ionian sides of southern Italy, *R. lucifugus* is represented by its subspecies *R. lucifugus lucifugus*, except in Trieste, where a colony of *R. lucifugus* “Sicily”, probably introduced by man, was found (Luchetti *et al.*, 2004). In these areas, seven *R. urbis* haplotypes (19 localities), six *R. lucifugus lucifugus* haplotypes (16 localities), and one *R. lucifugus* “Sicily” haplotype (one locality) have been identified.

Reticulitermes urbis

For *R. urbis*, the dataset for the analysis of haplotypes included 44 sequences (table 1). Fourteen sites out of 595 are variable, and 12 are parsimony-informative. The haplotype networks obtained with TCS 1.21 and with Network 4.6 are coincident (figure 3) and show the presence of two main groups, already found in previous studies (Luchetti *et al.*, 2007; Velonà *et al.*, 2010): a “southern clade”, to which all the samples from the Peloponnese belong, and a “northern clade”, including only samples collected outside the Peloponnese. In north-western Greece, Italy and France, both clades are represented.

Haplotype M, the most frequent one, isolated in many localities across *R. urbis* range except Peloponnese, is

identified by TCS 1.21 as the ancestral one. Using Network 4.6, the inferred ancestral haplotype is O, that differs from M by a silent mutation.

For *R. urbis* phylogenetic analysis, we considered sequences representative of the 17 haplotypes (table 1 and figure 3), keeping M1 and M2 separate. The topologies of the phylogenetic trees obtained with bayesian inference, maximum parsimony, and maximum likelihood methods are largely coincident, differing only in the support values of the nodes (figure 4). The splitting of the two main clades is well supported in bayesian and maximum parsimony trees, less so in maximum likelihood tree. The internal branching of the two main clades is not well supported.

Reticulitermes lucifugus lucifugus

For *R. lucifugus lucifugus*, the dataset for the analysis of haplotypes included 39 sequences (table 2). Eleven sites out of 595 are variable, and seven are parsimony-informative. The haplotype networks obtained with TCS 1.21 and with Network 4.6 are coincident (figure 5). TCS 1.21 identifies haplotype V, the most frequent and distributed in many localities across *R. lucifugus lucifugus* range, as the ancestral one. Network 4.6 identifies haplotype U as ancestral if *R. grassei* is used as out-group. It identifies haplotype U or the missing haplotype between V and W as ancestral if *R. urbis* is used as out-group. Haplotype T and its closely related haplotypes S and U are found in the Adriatic and Ionian sides of Italy. Haplotype R is included in the “Adriatic/Ionian” group in figure 5 because, despite being found on the Tyrrhenian side of Calabria, it does not belong to the “Tyrrhenian” clade in *R. lucifugus lucifugus* phylogeny (figure 6). Haplotype V is the most common one, being distributed in many localities all over Italy and in south France. It is included in the “Tyrrhenian” group in figure 5, according to its position in *R. lucifugus lucifugus* phylogeny (figure 6). Haplotypes W, X, Y, and Z, are found mainly in the Tyrrhenian side of Italy. The connection between “Adriatic/Ionian” and “Tyrrhenian” haplotypes is ambiguous, as indicated by the loop in figure 5.

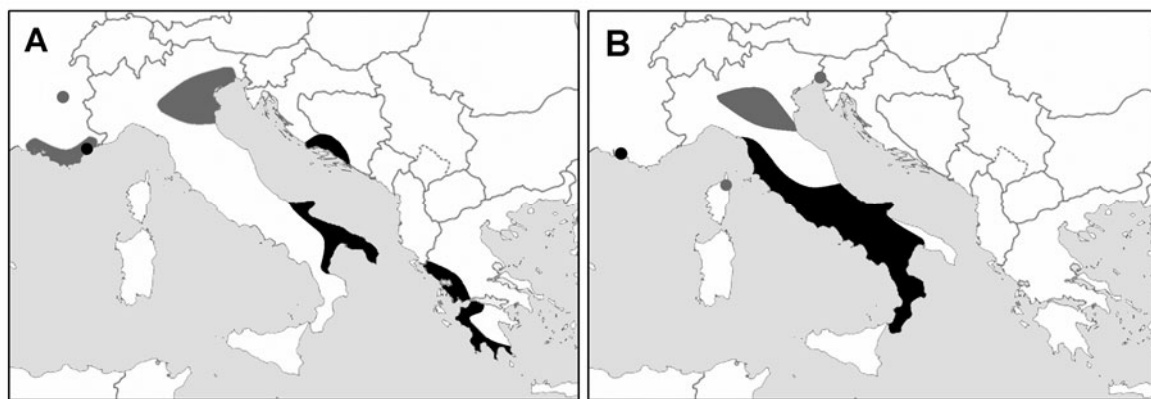


Figure 2. Ascertained ranges of *R. urbis* (A) and *R. lucifugus* (B). Grey: regions where *Reticulitermes* spp. are found only in urban areas; Black: regions where *Reticulitermes* spp. are found also in natural areas.

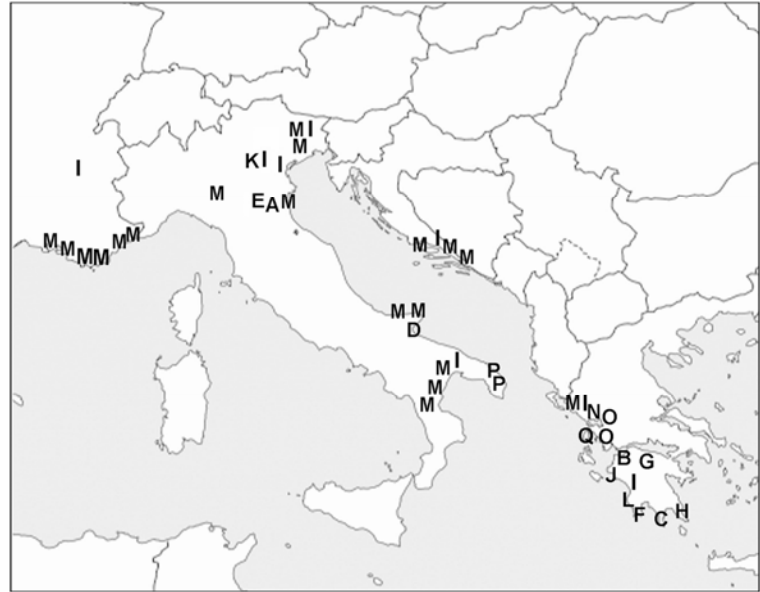
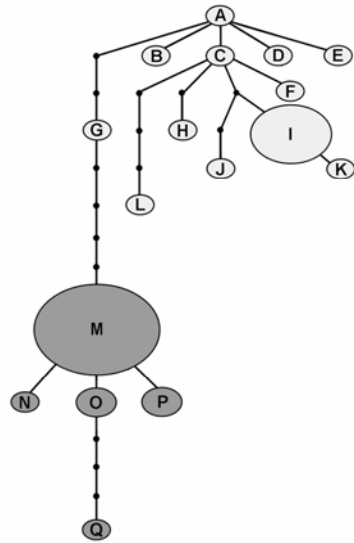


Figure 3. Haplotype parsimony network (left) and geographic distribution of *R. urbis* haplotypes (right). The size of the ovals is proportional to the number of localities where the corresponding haplotype has been identified. Letters symbolizing the haplotypes are as in table 1. Light gray: southern clade haplotypes. Dark grey: northern clade haplotypes. Black dots: missing or ideal haplotypes.

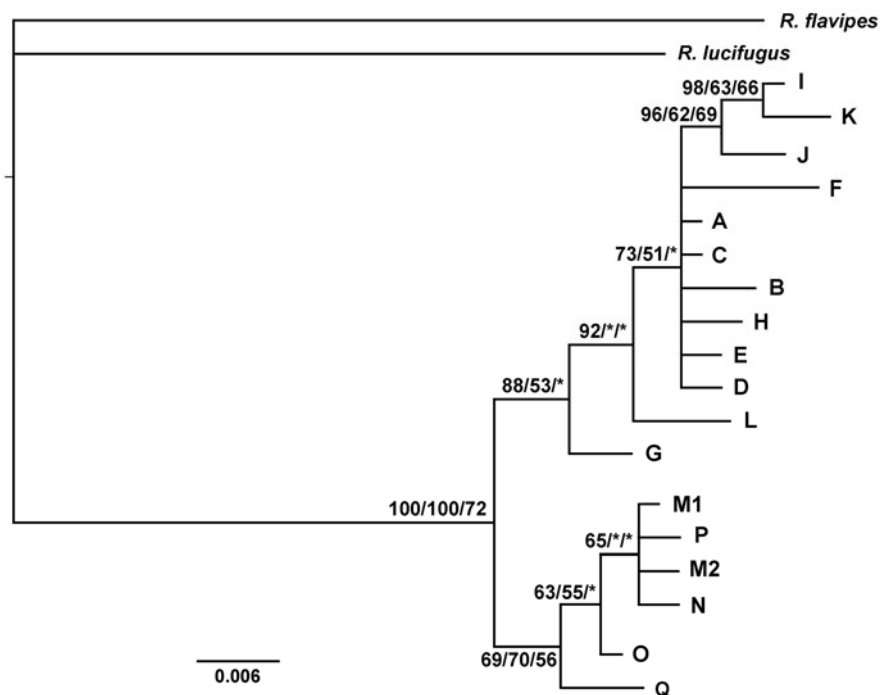


Figure 4. Bayesian inference (BI), maximum parsimony (MP), and maximum likelihood (ML) tree based on *R. urbis* COII haplotypes. Numbers at nodes are percent support values (BI/MP/ML). Values lower than 50 are not shown. Letters refer to the haplotypes listed in table 1. The bar indicates genetic distance.

For *R. lucifugus lucifugus* phylogenetic analysis, we considered sequences representative of the nine haplotypes (table 2 and figure 5), including distinct sequences for V1 and V2. The topologies of the phylogenetic trees obtained with bayesian inference, maximum parsimony, and maximum likelihood methods are largely coinci-

dent, differing only in the support values of the nodes (figure 6). The group including haplotypes R, S, and T, is not well supported, while the group including the haplotypes in the lower right of the haplotype net (from V to Z) form a better supported “Tyrrhenian” clade.

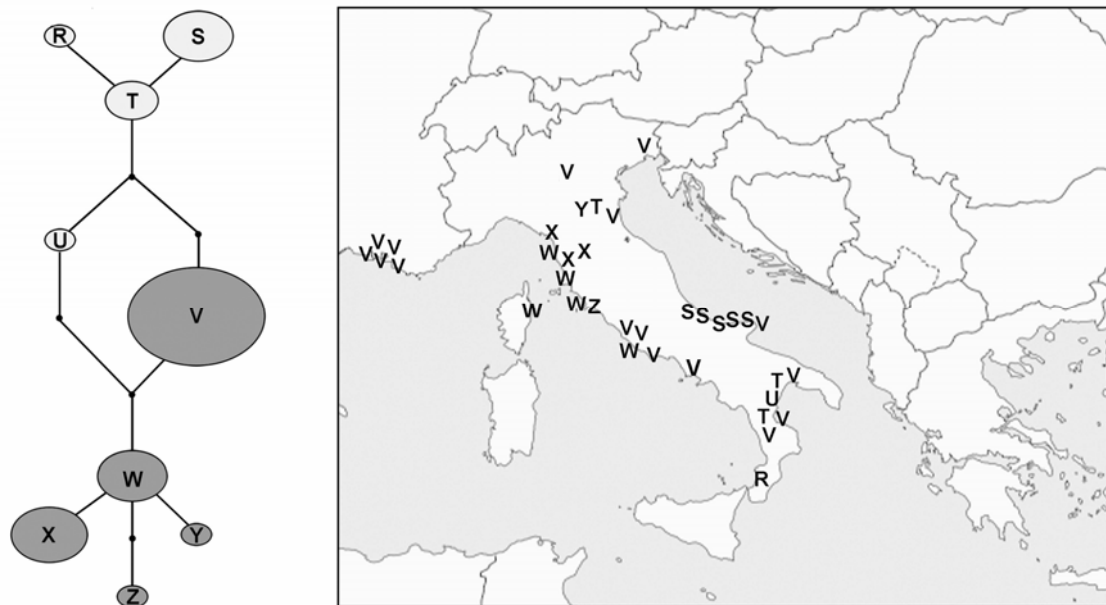


Figure 5. Haplotype parsimony network (left) and geographic distribution of *R. lucifugus lucifugus* haplotypes (right). The size of the ovals is proportional to the number of localities where the corresponding haplotype has been identified. Letters symbolizing the haplotypes are as in table 2. Light gray: “Adriatic/Ionian” haplotypes. Dark grey: “Tyrrhenian” haplotypes. Black dots: missing or ideal haplotypes.

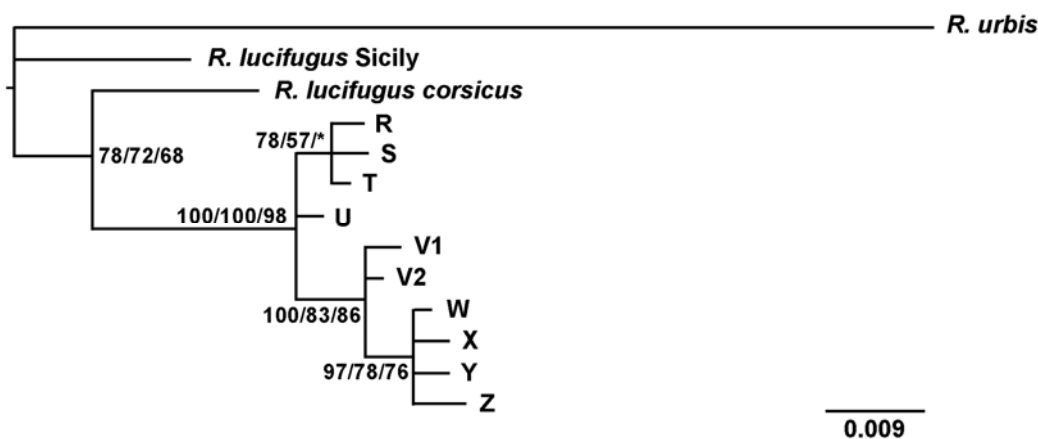


Figure 6. Bayesian inference (BI), maximum parsimony (MP), and maximum likelihood (ML) tree based on *R. lucifugus lucifugus* COII haplotypes. Numbers at nodes are percent support values (BI/MP/ML). Values lower than 50 are not shown. Letters refer to the haplotypes listed in table 2. The bar indicates genetic distance.

Discussion

R. urbis and *R. lucifugus lucifugus* distributions and genetics

In this study, we examined 22 samples from 17 Italian localities. We found *R. urbis* in ten localities. In Friuli-Venezia Giulia and Basilicata, *R. urbis* had never been reported before. We found *R. lucifugus*, in particular the peninsular subspecies *R. lucifugus lucifugus*, in nine localities.

In northern Italy, we found *R. urbis* and *R. lucifugus* only in urban areas. *Reticulitermes* termites seem to be absent from natural areas south of the Po delta, i.e. coastal woods such as the pinewoods of San Vitale, Classe (Ravenna) (Campadelli, personal communication), Bosco della Mesola wood (Ferrara), and Mount Conero (Ancona). In southern Italy (from Gargano,

about 41°57'N, southwards), where the presence of *R. urbis* and *R. lucifugus* in urban areas had already been ascertained (Luchetti *et al.*, 2004), we found both species also in natural environments.

Along the Balkan Peninsula coasts, *R. urbis* is found in natural environments from Živogošće (about 43°11'N) southwards. North of this location, along the Slovenian and Croatian coasts, *Reticulitermes* termites have not been found in natural environments, despite thorough surveys (Marini, unpublished data).

Therefore, along the Adriatic coasts, both in Italy and in the Balkans, *R. urbis* is found in natural environments in the areas with a mesomediterranean or a thermomediterranean climate (in the sense of Rivas-Martinez *et al.*, 2004). In Italy, in the same bioclimatic regions, *R. lucifugus* is also found in natural environ-

ments. Interestingly, *R. lucifugus* is found in natural areas in the south of France (Lefebvre *et al.*, 2008), where the climate is also mesomediterranean.

One *R. urbis* COII haplotype (D) and one *R. lucifugus* COII haplotype (U) were isolated for the first time in this study. On the whole, seven *R. urbis* haplotypes and nine *R. lucifugus lucifugus* haplotypes are found in Italy.

For both *R. urbis* and *R. lucifugus lucifugus*, the haplotype networks obtained with the statistical parsimony method (TCS 1.21) and with the median joining method (Network 4.6) share the same topology. This is expected when, as in the cases of our datasets, sequences are not very divergent (Mardulyn *et al.*, 2009). As to the inference of ancestral haplotypes, the two methods provide different results, possibly due to their different assumptions. For both *R. urbis* and *R. lucifugus lucifugus*, TCS 1.21 identifies as ancestral the most frequent haplotype (M and V, respectively), in accordance with coalescent theory (Posada and Crandall, 2001). In the case of *R. urbis*, Network 4.6 identifies as ancestral haplotype O, that is closely related to M. Both M and O belong to the northern clade, which is distributed all over the range of *R. urbis* except the Peloponnese. If this result were confirmed, it could indicate that *R. urbis* originated outside the Peloponnese, and that the high diversity found in the Peloponnese is the result of a more recent radiation. In the case of *R. lucifugus lucifugus*, Network 4.6 identifies haplotype U or a missing haplotype between V and W as ancestral, depending on which species is used as an outgroup (*R. grassei* or *R. urbis*, respectively). It is probable that *R. grassei* is the best outgroup, because *R. lucifugus* populations are the sister clade of the group *R. grassei* + *R. banyulensis*, while *R. urbis* is phylogenetically quite distant from *R. lucifugus* (Velonà *et al.*, 2010).

R. urbis and *R. lucifugus lucifugus* show different patterns of haplotype distribution. *R. lucifugus lucifugus* haplotypes form two groups, whose ranges correspond fairly well with the two sides of the Appennines (Tyrrhenian and Adriatic/Ionian), even though the most common haplotype is found on both sides. For *R. urbis*, both the haplotype net and the phylogenetic analysis show the presence of two major clades, as reported in previous studies (Luchetti *et al.*, 2007; Velonà *et al.*, 2010). All the haplotypes found in the Peloponnese belong to the southern clade. North of the Corinth Gulf, in Italy, and in France, haplotypes from both the northern and the southern clade can be found. In Italy, haplotypes from different clades are often found in localities near each other or even in the same locality. The presence of two clades with independent distributions could be the result of two (or more) distinct expansion events: one starting from the Peloponnese (southern clade), and the other possibly starting from the north-west region of Greece (northern clade).

Is *Reticulitermes urbis* native in Italy?

The main arguments supporting the hypothesis that *R. urbis* was introduced by man in Italy and Provence are: i) its presence only in a few urban areas, disjointed from the core range of the species; ii) its absence in natural environments; iii) its higher genetic diversity in

the Balkan peninsula, particularly in Greece (Luchetti *et al.*, 2007; Leniaud *et al.*, 2010); iv) the lack of association between mtDNA haplotypes and geographic regions (Leniaud *et al.*, 2010).

We will discuss each of these points in the light of the results of the present study.

i) *R. urbis* was initially identified in a few urban areas (Marini and Mantovani, 2002). As the analyses of new samples progress, the number of Italian localities where *R. urbis* is found increases. Until the present study, *R. urbis* was known to occur in eight towns, six in the north and two in the south (Marini and Mantovani, 2002; Luchetti *et al.*, 2004). In this study, we found *R. urbis* in ten additional localities, distributed in four regions.

A disjunct range of distribution is one of the elements that can lead to suspect that a species is not native (Webb 1985). However, the presence of *R. urbis* and *R. lucifugus* in scattered localities in northern Italy can be explained by their microclimatic requirements: In regions where winters are cold, human settlements could have provided and still provide warm refugia where termites can survive. In many cases, *Reticulitermes* infestations in northern Italy occur in old buildings, often in the oldest parts of towns, where human settlements have probably existed for centuries. In northern Italy, urban termite infestations often show a strong tendency to remain confined in the same areas. When detailed old data on termite presence in urban areas exist, such as in the cases of Salsomaggiore (Springhetti, 1965), and Mira (Jucci and Springhetti, 1957), recent inspections have revealed that termites are still confined below the same buildings where they were found 60-100 years ago (Marini, unpublished data), showing their inability to spread to surrounding natural areas where climatic conditions are now unsuitable for their survival.

It is likely that, in periods when winter temperatures were milder, termite species in northern Italy were more widespread, and that their ranges became restricted and fragmented in colder periods.

ii) We showed in this study that *R. urbis* is actually found also in natural environments, in Italian regions where winters are warmer (mesomediterranean or thermomediterranean climate). Interestingly, the native *R. lucifugus* has the same pattern of distribution: restricted to urban areas in northern Italy and including both urban and natural areas in southern Italy.

iii) As to the lower genetic diversity in Italy and Provence compared with the Balkans, it is not surprising to find that the genetic diversity of a species varies over its geographical range. Italy and Provence are outside the core of *R. urbis* range of distribution, and several cases are documented in which genetic diversity is lower at the periphery of the range (Kark *et al.*, 2008). In particular, a reduction in genetic diversity at the margin of the current range is observed in many insect species that experienced postglacial range expansions (Hill *et al.*, 2011), which is believed to have also occurred to European termites (Clément *et al.*, 2001; Luchetti *et al.*, 2004; Uva *et al.*, 2004). The hypothesis that successive contractions and expansions of *R. urbis* range could have caused a loss of genetic diversity in northern regions has been already considered in Uva *et al.*, 2004.

In the Italian areas where *R. urbis* and *R. lucifugus* are sympatric, seven COII haplotypes have been isolated in *R. urbis* and seven haplotypes have been isolated in *R. lucifugus*, on a similar number of sampling localities. Even though the comparison between the haplotype richness of two different species is not completely justifiable, the small number of *R. lucifugus* haplotypes could indicate that the number of haplotypes found for *R. urbis* is not far from what can be expected for a native *Reticulitermes* species in Italy.

iv) If the two clades of *R. urbis* are considered together, mtDNA haplotypes seem to be uncorrelated with geographical regions (Leniaud *et al.*, 2010). If the two clades, probably deriving from different expansion events, are considered separately, some level of association appears to exist. However, further data are needed to verify this point.

As an additional point, it must be noted that ranges of distribution similar to *R. urbis* range, including the Balkan Peninsula, Italy, and Provence (southern France) are not uncommonly found. Many other insect species have an eastern-Mediterranean range of distribution, including the Balkans, Italy, and often extending westwards up to the south-east of France. We cite as an example some Lepidoptera, that cannot be suspected of having been introduced by man, because they are strongly dependent upon wild plants and particular microhabitats both at the larval and at the imaginal stages: the Noctuidae *Mythimna alopecuri* (Boisduval), *Conistra veronicae* (Hubner), *Dryobotodes carbonis* Wagner, *Aporophyla canescens* (Duponchel), *Polymixis rufocincta* (Geyer), *Lithophane lapidea* (Hubner), *Ulochlaena hirta* (Hubner), *Cucullia formosa* Rogenhofer, the Arctiidae *Amata kruegeri* (Ragusa) and *Dysauxes famula* (Freyer), the Zygaenidae *Adscita mannii* (Lederer), and the Papilionidae *Papilio alexanor* Esper and *Zerynthia polyxena* (Denis et Schiffermuller) (de Freina and Witt, 1987; 2001; Ronkay and Ronkay, 1994; Descimon, 1996; Ronkay *et al.*, 2001; Hacker *et al.*, 2002; Bollino and Sala, 2004).

In the light of what is discussed above, namely: *R. urbis* is found in more Italian localities than previously thought, it is found also in natural environments in regions where the climate is suitable for *Reticulitermes* presence, many insect species exist with a similar range of distribution, variations in genetic diversity across its range may be the result of natural processes, its distribution and genetic diversity in Italy are similar to those of the native *R. lucifugus*, we believe that the distribution of *R. urbis* in Italy can be explained mostly by natural events, although the introduction by man in some localities cannot be excluded.

From the conservationist's point of view, the attribution of the status of native species to a termite species raises certain ethical issues. In natural environments, termites have an important ecological role as cellulose recyclers, but in urban areas they are considered wood pests and can cause heavy infestations and economic losses. Effective pest control strategies exist that can eliminate their colonies within a few years, as was the case for a *R. urbis* infestation in Bagnacavallo (North Italy) (Ferrari *et al.*, 2011) and Domène (France) (Leniaud *et al.*, 2009).

In northern Italy, *Reticulitermes* termites can live only in urban areas and are absent from the surrounding lands, therefore pest control has the potential to eliminate entire populations. Many of these urban termite populations will probably cease to exist within the next few years due to termite control measures, thus preventing future work studying their biology.

Acknowledgements

This study was supported by Canziani funds. We wish to thank Danilo Morano for providing the sample from Policoro.

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Received August 8, 2012. Accepted October 22, 2012.