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Molecular and morphometrical characterization of Sicilian *Reticulitermes* termites (Blattodea, Termitoidae, Rhinotermitidae) with the description of a new subspecies

Silvia Ghesini, Andrea Luchetti, Mario Marini
Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Italy

Abstract

The subterranean termite *Reticulitermes lucifugus* (Rossi 1792) is distributed in Italy and southern France, both in natural and urban environments. It comprises three lineages: i) *R. lucifugus lucifugus* (Rossi 1792) in the mainland, ii) *R. lucifugus corsicus* Clement 1977 in Corsica, Provence, Sardinia and a portion of the Tyrrhenian coast of Italy, iii) a third lineage, not yet described, identified based on the genetic analysis of a few samples from Sicily and from an isolated population in northern Italy, where it was most probably introduced by man. In this study, we characterize genetically (COII and 16S mitochondrial genes) and morphometrically (measurements of alates and soldiers) the Sicilian lineage, based on new samples collected all over the island. Our results confirm the existence of a well supported clade comprising all the samples from Sicily, neatly separated from *R. lucifugus lucifugus* and *R. lucifugus corsicus*. No other *Reticulitermes* species or subspecies was found in Sicily, and the Sicilian lineage was not found in peninsular Italy, suggesting that the strait separating Sicily from the mainland is an effective barrier to termite dispersal. Based on these results, we describe the Sicilian lineage of *R. lucifugus* as *Reticulitermes lucifugus siculus* ssp. nov.

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Key words: molecular taxonomy, morphometry, molecular phylogenetics, Sicily, subterranean termites.

Introduction

Termites provide important ecosystem services in natural environments, both in tropical and in temperate ecosystems (Jouquet *et al.*, 2011; Myer and Forschler, 2019). In built-in areas, they are renowned as pests, as they can cause damage to wooden objects and structures. Understanding their diversity is important both from the ecological point of view and for pest control purposes.

In the last few decades, great progress has been achieved in the taxonomy and phylogeny of the two native European termite genera: *Kalotermes* (Hagen 1853) (Kalotermitidae, or dry-wood termites) and *Reticulitermes* Holmgren 1913 (Rhinotermitidae, or subterranean termites). This advance in knowledge was obtained thanks to morphological, chemical (cuticular hydrocarbons) and DNA analyses. The latter are particularly useful for taxa that show little morphological differentiation, as is often the case with termites (Korb *et al.*, 2019) and, in particular, with *Reticulitermes* species (Clément *et al.*, 2001; Vargo and Husseneder, 2009).

In Europe, the genus *Reticulitermes* was once believed to include two species: *Reticulitermes lucifugus* (Rossi 1792), widespread in most of the south of the continent, and *Reticulitermes santonensis* Feytaud 1924, found mainly on the Atlantic coast of France. The latter is now recognized as a junior synonym of the North American species *Reticulitermes flavipes* (Kollar 1837), that was most likely introduced to Europe from the USA (Austin *et al.*, 2005), while what was once considered to be a single species, *R. lucifugus*, is now recognised as a species complex, with at least eight taxa of specific or sub-specific level (Clément *et al.*, 2001; Kutnik *et al.*, 2004; Nobre *et al.*, 2006; Luchetti *et al.*, 2007; 2013; Lefebvre *et al.*, 2008; 2016; Velonà *et al.*, 2010; Ghesini and Marini, 2015).

To date, the range of *R. lucifugus* is limited to Italy and southern France, and three distinct lineages have been identified. Two of them have been formally described as subspecies: *Reticulitermes lucifugus lucifugus* (Rossi 1792), distributed over most of the Italian peninsula and southern France, and *Reticulitermes lucifugus corsicus* Clement 1977, distributed in Corsica, Provence, Sardinia and the Tyrrhenian coast of central Italy. The third lineage, naturally distributed in Sicily, has never been described and is usually referred to as *Reticulitermes lucifugus* "Sicily" (Luchetti *et al.*, 2004; 2013; Lo Pinto *et al.*, 2016).

Sicily is the largest Mediterranean island, separated from the Italian mainland by the Strait of Messina, 3.1 Km wide at its narrowest point. Most of the publications on Sicilian termites date back to times when *Kalotermes flavicollis* (F. 1793) and *R. lucifugus* were believed to be the only termite species occurring in Italy, and the existence of intra-specific divergence of *R. lucifugus* was still unrecognized (Jucci and Springhetti, 1952; Springhetti, 1963). Only a couple of recent studies have addressed taxonomy and phylogeny of Sicilian termites, and few samples have been analysed from the genetic point of view. DNA sequences of *Reticulitermes* samples are available only from three Sicilian localities - Palermo, Agrigento, and Santo Stefano Quisquina -, and were all found to belong to the Sicilian taxon (Luchetti *et al.*, 2004; 2013). Two termite samples collected from indoor structures in Trieste (NE Italy) were also found to belong to the Sicilian subspecies (Luchetti *et al.*, 2004), almost certainly as the result of the introduction of furniture from a Sicilian house.

In this study, we analyse from the genetic and morphometric point of views *Reticulitermes* samples collected in 15 Sicilian localities, in order to characterize taxonomically the *R. lucifugus* "Sicily" lineage. In the light of the data obtained, we describe this taxon as a new subspecies: *R. lucifugus siculus* ssp. nov.

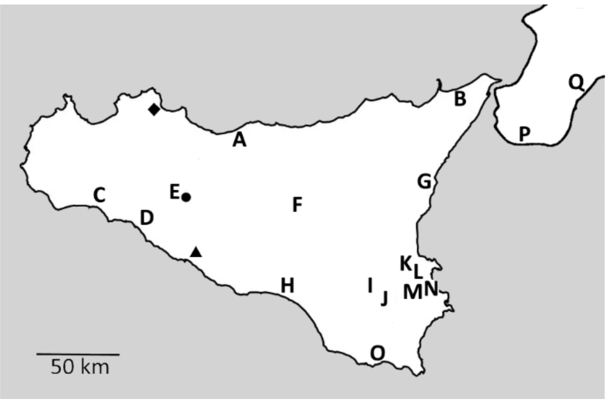

Figure 1. Collecting sites in Sicily and Calabria. Details on collecting sites are shown in table 1. Localities sampled in previous studies: ♦ Palermo, ● S. Stefano Quisquina, ▲ Agrigento.

Materials and methods

Termite sampling

Twenty-two *Reticulitermes* termite colonies were collected in 15 localities in Sicily, and three further colonies were collected in two localities in the south of Calabria (figure 1, table 1). Samples were preserved in 100% ethanol.

DNA analysis

Total DNA was extracted from termite heads following the CTAB protocol (Doyle and Doyle, 1987). Two individuals from each sample were analysed. A 685-bp fragment of the cytochrome oxidase subunit II gene (COII) was amplified with the primers 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′). A 489-bp fragment of the large mitochondrial ribosomal subunit gene (16S) was amplified with the primers LR-J-12887 (5′-CCG GTC TGA ACT CAG ATC ACG T-3′ and LR-N-13398 (5′-CGC CTG TTT AAC AAA AACAT-3′). PCR was performed using GoTaq® Flexy DNA Polymerase kit (Promega, USA), following the enclosed protocol. Purification and sequencing were performed by Macrogen Inc. (Amsterdam, The Netherlands). The sequences obtained in this study are deposited in GenBank under accession numbers OQ148384-OQ148395 and OQ162372-OQ162379.

Sequence alignment and preliminary analysis were carried out in MEGA 11 (Tamura *et al.*, 2021). Sequences of *R. lucifugus* from Sicily, *R. lucifugus lucifugus* and *R. lucifugus corsicus* obtained in previous studies (Marini and Mantovani, 2002; Luchetti *et al.*, 2004) were drawn from GenBank and added to the alignment. Sequences from *Reticulitermes urbis* Bagneres, Uva et Clement 2003 and from the North American species *R. flavipes* were used as outgroups.

The Maximum likelihood tree on concatenated COII and 16S alignments was obtained with IQ-TREE web server (Nguyen *et al.*, 2015; Trifinopoulos *et al.*, 2016; Chernomor *et al.*, 2016), implementing the model selected by ModelFinder (Kalyaanamoorthy *et al.*, 2017), and using 1000 ultra-fast bootstrap replicates for nodal support. The Bayesian inference tree was built with MrBayes 3.2.7 (Huelsenbeck *et al.*, 2001; Ronquist *et al.*, 2012). Convergence was reached after 1000000 generations (average standard deviation of split frequencies < 0.01).

Morphological analysis

Measurements were taken with a stereomicroscope equipped with an ocular micrometre. Twenty soldiers from four colonies (A, L, M and Oa), and 20 alates - 10 males and 10 females - from the only colony that contained them (M) were measured. For soldiers, the following measures were taken: head length (from the line joining mandible insertions to the posterior end of the head), maximum head width, left mandible length, pronotum length along the median line, pronotum width, tibia length in the third right leg. For alates, the following

Table 1. Sampling localities in Sicily (A-O) and Calabria (P, Q) and number of colonies sampled in each locality.

	Locality	Coordinates	Elevation (m a.s.l.)	N. colonies
A	Campofelice di Roccella	37°58'49"N 13°50'12"E	7	1
B	Villafranca Tirrena	38°14'16"N 15°26'19"E	49	1
C	Porto Palo	37°34'37"N 12°56'13"E	41	2
D	Ribera	37°28'37"N 13°15'07"E	188	1
E	Santo Stefano Quisquina	37°37'43"N 13°27'29"E	620	1
F	Enna	37°57'46"N 14°29'50"E	658	4
G	Giarre	37°42'16"N 15°10'09"E	183	1
H	Gela	37°04'53"N 14°12'50"E	10	2
I	Buccheri	37°06'36"N 14°52'23"E	830	1
J	Buscemi	37°05'19"N 14°52'43"E	754	1
K	Melilli	37°11'32"N 15°07'33"E	126	1
L	Bufalaro	37°12'55"N 15°09'16"E	33	1
M	between Sortino and Melilli	37°09'32"N 15°04'56"E	497	2
N	Priolo Gargallo	37°12'04"N 15°10'51"E	8	1
O	Pozzallo	36°43'51"N 14°51'44"E	8	1
P	Melito Porto Salvo	37°55'32"N 15°47'41"E	123	2
Q	Locri	38°12'28"N 16°14'24"E	5	1

Table 2. COII and 16S haplotypes isolated in samples from Sicily (A-Ob) and Calabria (P and Q), and corresponding GenBank accession numbers.

Colony	COII	A.N.	16S	A.N.
A	C1	OQ148384	S1	OQ162372
B	C2	OQ148385	S2	OQ162373
Ca, Cb	C3	OQ148386	S2	OQ162373
D	C4	OQ148387	S1	OQ162372
E	C5	OQ148388	S2	OQ162373
Fa, Fb, Fc, Fd	C1	OQ148384	S1	OQ162372
G	C7	OQ148390	S2	OQ162373
Ha, Hb	C6	OQ148389	S2	OQ162373
I	C6	OQ148389	S3	OQ162374
J	C8	OQ148391	S4	OQ162375
K	C9	OQ148392	S5	OQ162376
L	C6	OQ148389	S3	OQ162374
Ma, Mb	C9	OQ148392	S5	OQ162376
N	C9	OQ148392	S5	OQ162376
Oa	C10	OQ148393	S6	OQ162377
Ob	C6	OQ148389	S7	OQ162378
P	C11	OQ148394	S8	OQ162379
Q	C12	OQ148395	S8	OQ162379

measures were taken: head length (from the distal margin of the clypeus to the posterior end of the head), maximum head width (eyes included), maximum eye diameter, pronotum length along the median line, pronotum width, tibia length in the third right leg. The number of antennal segments was counted, both in soldiers and in alates. Only complete antennae were considered.

Twenty *R. lucifugus lucifugus* alates from central and southern Italy (Marina di Lesina, Pisticci Marina, Rodi Garganico) and twenty *R. lucifugus corsicus* alates from Corsica and Sardinia (Saint Florent, Dolianova) were also measured for comparison.

Statistical analyses were carried out using the software Past 4.09 (Hammer *et al*., 2001) and R-4.2.2 (R Core Team, 2022). P-values of Mann-Whitney (MW) and Kolmogorov-Smirnov (KS) tests were calculated for two tails.

Photos of alates and soldiers were obtained with a Canon EOS 750D camera mounted on a Zeiss Stemi SV11 microscope.

Results

DNA analysis

Ten COII haplotypes and seven 16S haplotypes were found (table 2). Colonies collected in the same site share the same haplotypes, except in the case of colonies Oa and Ob, that have different haplotypes for both genes.

The phylogenetic analysis of concatenated COII and 16S alignments returned fully overlapping topologies of Maximum likelihood and Bayesian inference trees (figure 2). DNA sequences from samples collected in Sicily cluster together with representative sequences from the taxon *R. lucifugus* "Sicily". Two main subclades are evident, one of which is formed by sequences from samples collected in the south-eastern part of the island (K, M, N, and O), and the other one by sequences from all over the island. The internal structure of this latter subclade is not well resolved.

Sequences from samples collected in Calabria group with sequences from the subspecies *R. lucifugus lucifugus*.

Morphology

Measurements of alates and soldiers are shown in tables 3 and 4. For most of the measured characters, the ranges of variation observed in the three *R. lucifugus* lineages are largely overlapping.

Alates of *R. lucifugus* "Sicily" differ significantly from those of *R. lucifugus lucifugus* by the width of the pronotum (MW: $P < 0.001$, KS: $P < 0.001$), the length of the tibia (MW: $P < 0.001$, KS: $P < 0.001$), and the diameter of the eye (MW: $P = 0.003$, KS: not significant). They differ from *R. lucifugus corsicus* alates by the length of the head (MW: $P = 0.040$, KS: $P = 0.008$), the width of the head (MW: $P < 0.001$, KS: $P = 0.003$), the length of the tibia, the diameter of the eye and the number of antennal segments (MW: $P < 0.001$, KS: $P < 0.001$ in all three cases).

Soldiers of *R. lucifugus* "Sicily" differ significantly from those of *R. lucifugus lucifugus* by the length of the head (MW: $P < 0.001$, KS: $P = 0.004$), the width of the head (MW: $P < 0.001$, KS: $P < 0.001$), the length of the mandible (MW: $P = 0.007$, KS = 0.038), and the length of the tibia (MW: $P < 0.001$, KS: $P < 0.001$). They differ from *R. lucifugus corsicus* soldiers by the length of the head (MW: $P < 0.001$, KS: $P < 0.001$), the width of the head (MW: $P < 0.001$, KS: $P < 0.001$), and the length of the tibia (MW: $P < 0.001$, KS: $P < 0.001$).

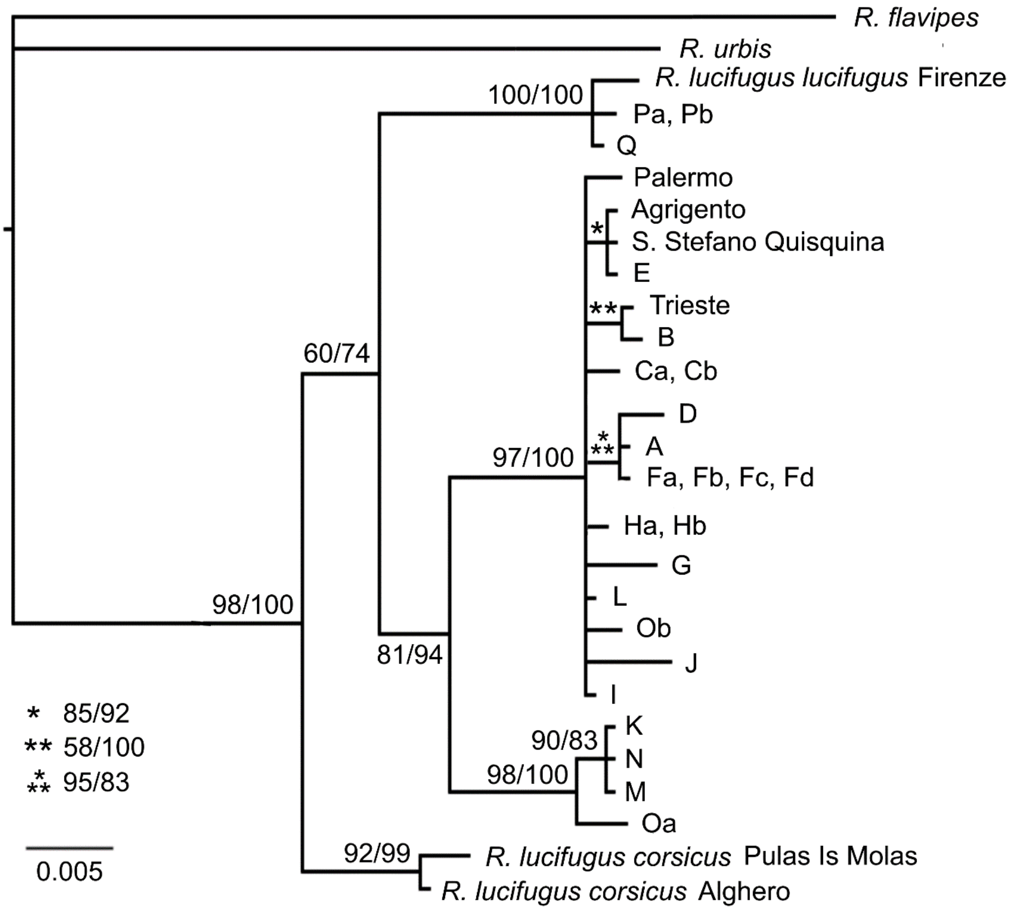

Figure 2. Maximum likelihood and Bayesian phylogenetic tree based on concatenated COII and 16S sequences. Numbers at nodes are bootstrap/posterior probability values.

Discussion

Based on the phylogenetic analysis, all the samples collected in Sicily can be ascribed to the Sicilian lineage of *R. lucifugus*, while samples collected in the south of Calabria belong to the peninsular subspecies *R. lucifugus lucifugus*. To date, no *R. lucifugus lucifugus* colony has been found in Sicily, neither in this nor in previous studies (Luchetti *et al.*, 2004; 2013). Despite the extensive samplings carried out throughout the Italian mainland, the Sicilian *R. lucifugus* subspecies has been found in a single locality (Trieste), where it was most likely introduced by man (Luchetti *et al.*, 2004). Besides *R. lucifugus lucifugus*, two other *Reticulitermes* species are found in mainland Italy: *R. urbis* and the North American *R. flavipes* (Marini and Mantovani, 2002; Luchetti *et al.*, 2004; Ghesini *et al.*, 2010; Ghesini and Marini, 2012; Luchetti *et al.*, 2013). None of these taxa was ever found in Sicily. Thus, *R. lucifugus* lineage from Sicily seems to be well segregated from the other *Reticulitermes* taxa distributed on mainland Italy, even though the presence of unsampled colonies that could change this picture cannot be ruled out. A clear separation between peninsular and Sicilian populations or species is well documented for other organisms (Schmitt *et al.*, 2021), including highly mobile insects such as butterflies (Scalercio *et al.*, 2020), despite a connection between Sicily and the mainland having existed also in recent geological times (Antonioli *et al.*, 2014). In the case of termites, this separation is even more striking, as they can easily be introduced by man to new localities through the transport of wooden materials or soil. A human-mediated introduction event has been recently documented in Sicily, involving the tropical subterranean termite *Coptotermes gestroi* (Wasmann 1896) (Ghesini *et al.*, 2011), but it did not apparently result in the successful settlement of this species.

R. lucifugus "Sicily" appears to be more closely related to *R. lucifugus lucifugus* than to *R. lucifugus corsicus*, in accordance with previous molecular analyses (Luchetti *et al.*, 2004; 2013), and as could be expected due to the geographical proximity between Sicily and the mainland.

Inside the Sicilian clade, some of the samples from the south-eastern region of the island form a distinct, well supported subclade. This area, corresponding to the Hyblaean plateau territory, is an important centre of biodiversity and speciation, and is rich in phylogenetic neo-endemisms (Brullo *et al.*, 2011; Albassatneh *et al.*, 2021).

The measurements of some characters of alates and soldiers show significant differences between *R. lucifugus* from Sicily and the other two subspecies of *R. lucifugus*. However, the ranges of variation largely overlap, making these characters scarcely useful for identification purposes. Moreover, it cannot be excluded that the extension of the analysis to a higher number of individuals from

Table 3. Measurements (mm) of alates of the three *R. lucifugus* lineages.

	R. lucifugus "Sicily"	*R. lucifugus lucifugus*	*R. lucifugus corsicus*
Head length			
range	1.05 - 1.17	1.05 - 1.21	1.05 - 1.14
mean ± s.d.	1.12 ± 0.04	1.14 ± 0.04	1.11 ± 0.02
n	20	20	20
Head width			
range	1.05 - 1.17	1.02 - 1.18	1.08 - 1.19
mean ± s.d.	1.09 ± 0.03	1.10 ± 0.03	1.12 ± 0.02
n	20	20	20
N. of antennal segments			
range	17 - 18	17 - 19	16 - 18
mean ± s.d.	17.87 ± 0.34	17.82 ± 0.48	17.35 ± 0.65
n	31	28	23
Max. eye diameter			
range	0.20 - 0.24	0.19 - 0.23	0.15 - 0.23
mean ± s.d.	0.22 ± 0.01	0.21 ± 0.01	0.20 ± 0.02
n	20	20	20
Pronotum length			
range	0.50 - 0.55	0.50 - 0.57	0.48 - 0.55
mean ± s.d.	0.53 ± 0.01	0.54 ± 0.02	0.52 ± 0.02
n	20	20	20
Pronotum width			
range	0.83 - 0.90	0.90 - 0.97	0.84 - 0.93
mean ± s.d.	0.87 ± 0.02	0.94 ± 0.02	0.88 ± 0.02
n	20	20	20
Tibia length			
range	0.93 - 1.14	1.02 - 1.21	1.08 - 1.22
mean ± s.d.	1.05 ± 0.07	1.15 ± 0.04	1.14 ± 0.04
n	20	20	20

Table 4. Measurements (mm) of soldiers of the three *Reticulitermes lucifugus* lineages.

	R. lucifugus "Sicily"	*R. lucifugus lucifugus*	*R. lucifugus corsicus*
Head length			
range	1.55 - 1.85	1.70 - 1.97	1.74 - 1.94
mean ± s.d.	1.72 ± 0.08	1.85 ± 0.08	1.86 ± 0.05
n	20	20	20
Head width			
range	1.09 - 1.18	1.14 - 1.32	1.14 - 1.29
mean ± s.d.	1.16 ± 0.02	1.22 ± 0.05	1.23 ± 0.04
n	20	20	20
Left mandible length			
range	0.95 - 1.08	0.95 - 1.21	0.93 - 1.11
mean ± s.d.	1.02 ± 0.04	1.07 ± 0.07	1.03 ± 0.05
n	20	20	20
N. of antennal segments			
range	15 - 17	16 - 17	15 - 16
mean ± s.d.	15.65 ± 0.61	16.13 ± 0.35	15.73 ± 0.47
n	18	16	11
Tibia length			
range	0.85 - 0.94	0.79 - 1.06	0.90 - 1.08
mean ± s.d.	0.89 ± 0.03	0.96 ± 0.08	1.00 ± 0.05
n	20	18	19

Figure 3. Alate: habitus, head capsule dorsal and lateral view.

different colonies could make the differences non-significant, due to the high among- and within-colony variability that is usually recorded in termites. Genetic analysis is therefore recommended for identification.

Reticulitermes lucifugus siculus **ssp. nov.**

Zoobank registration

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Type material

Holotype: female alate from a locality between Sortino and Melilli (colony M, table 1).

Paratypes (10 female alates, 10 male alates from Sortino and Melilli and 29 soldiers in total): These series are in the M. Marini collection, Department BiGeA, University of Bologna, Italy.

Morphology

Alate - (figure 3; holotype measurements in parentheses) Body dark brown, almost black. Postclypeus proximally dark brown, distally yellowish, moderately protruding. Proximal margin of postclypeus rounded. Clypeus dark brown. Labrum dark brown, with rounded tip. Tibiae proximally brown, distally light brown. Tarsi very pale. Wing membrane smoky light brown.

Maximum eye diameter: 0.20-0.24 mm (0.23 mm). Ocellus separated from the marginal line of ommatidia of the compound eye by a distance approximately equal to the ocellus diameter.

Antennae 17- or 18-segmented (18). A different number of segments in the two antennae of the same individual is quite common.

Head length: 1.05-1.17 mm (1.11 mm). Head width: 1.05-1.17 mm (1.08 mm).

Pronotum length: 0.50-1.17 mm (0.55 mm). Pronotum width: 0.83-0.90 mm (0.90 mm). Tibia length in the third right leg: 0.93-1.14 mm (1.11 mm). Arolia absent.

Soldier - (figure 4) Head ochre, postclypeus lighter. Mandibles ochre at the base, darkening toward the apex. Thorax and abdomen whitish.
Antennal segments 15 - 17. Antennal asymmetry, with different number of articles in the two antennae of the same individual, quite frequent. Third antennal segment shortest. Arolia absent. Measurements: see table 4.

Ecology

Samples were collected in diverse environments, such as Mediterranean scrub, reforestation pine woods, orchards and hedges, from 5 to 830 m a.s.l. Most colonies were found in natural environments.

Host plants: colonies were found in *Pinus* spp., - particularly *Pinus pinaster* Aiton and *Pinus nigra* Arnold -,

Figure 4. Soldier: head capsule (dorsal, ventral and lateral view).

Cupressus sempervirens L., *Eucaliptus* sp., *Prunus dulcis* (Mill.) D. A. Webb, *Olea europaea* L., *Casuarina equisetifolia* L. It is very likely that many other plants could be attacked, as is usual for other *Reticulitermes* species.

Diagnosis

Due to the high morphological similarity of *Reticulitermes* species, and to the high intra-specific variability of morphometric characters, genetic analysis is recommended for a correct identification. *Reticulitermes lucifugus siculus* sequences available in GenBank have been obtained in previous studies (A.N. AF292024, AY267857, AY267864, AY267868, AY268364, AY268361, and AY268363-AY268365) and in this study (A.N. OQ148384-OQ148395 and OQ162372-OQ162379).

Distribution

Natural range: Sicily. Human-mediated introduction: Trieste (NE Italy)

Etymology

The name refers to the distribution range of the species.

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Authors' addresses: Silvia Ghesini (corresponding author: silvia.ghesini5@unibo.it; ghe74@yahoo.it), Andrea Luchetti, Mario Marini, Department of Biological, Geological and Environmental Sciences (BiGeA), *Alma Mater Studiorum* Università di Bologna, via Selmi 3, I-40126 Bologna, Italy.

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