Origin of a new *Reticulitermes* termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data

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Abstract

The Holoarctic termite genus *Reticulitermes* is widely distributed in Europe. A new *Reticulitermes* species, *R. sp. nov.*, was recently found in France and Italy. Its phylogenetic position was investigated using a 743-bp fragment of mitochondrial 16S rRNA-ND1 genes and 382-bp of the nuclear ITS2 region. Phylogenies for these sequences were estimated by neighbor-joining, maximum-parsimony and maximum-likelihood analysis. The results strongly supported a relationship between *R. sp. nov.* and the termite species from the eastern Mediterranean area including *Reticulitermes balkanensis* from the Balkans, *Reticulitermes lucifugus* from Turkey and *Reticulitermes clypeatus* from Israel. The hypothesis of a relationship between *R. sp. nov.* and the Japanese *Reticulitermes speratus* was rejected by parametric bootstrap. The current distribution of *R. sp. nov.* could be linked to postglacial colonization routes between Balkan refuge and northern regions.

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1. Introduction

The nearly 2000 species of Isoptera in the world (Kambhampati and Eggleton, 2000) play an important natural role in the breakdown of cellulose in natural environments. However they are highly destructive pests causing extensive damage in urban settings. The cost of treatment against termites in Europe is expected to top 1 billion euros by 2005 (UNEP and FAO, 2000).

*Reticulitermes* is a Holarctic genus whose distribution in the Palearctic region is the result of historical climatic events, i.e., glaciation during the Quaternary period (Clément, 1978), as well as current factors, i.e., environmental conditions and human activity. They are subterranean termites, whereby their colonies usually consist of galleries constructed below ground level. Although *Reticulitermes* can feed on living tissue, their chief food source is dead wood and other cellulose-containing items that workers collect and carry back to the colony. This reclusive lifestyle has hindered studies of colonial social structure. New colonies are founded either by a pair of swarming winged reproductives and/or by secondary reproductives cut off from the main colony.

Rossi (1792) described the first *Reticulitermes* termites in the western Palearctic region from samples collected in Italy. Subsequently other species were identified based on morphology, behavior, cuticular hydrocarbons, enzymatic polymorphism, and mitochondrial DNA (mtDNA) (Clément, 1978; Clément et al., 2001), including *Reticulitermes grassei* and *Reticulitermes banyulensis*.
in France and Spain, *Reticulitermes balkanensis* in the Balkans, *Reticulitermes lucifugus* in Italy and France, and the *Reticulitermes lucifugus corsicus* subspecies on the islands of Corsica (France) and Sardinia (Italy). Recent studies have provided evidence of a transyrianren distribution of the Corsican subspecies due either to paleo-geographical events or human intervention (Marini and Mantovani, 2002; Uva et al., unpublished data). According to some authors, *Reticulitermes santonensis* found in France and some urban areas of Europe is synonymous with North American *Reticulitermes flavipes* (Bagnères et al., 1990; Clément et al., 2001; Jenkins et al., 2001; Marini and Mantovani, 2002; Vieau, 2001; Dronnet et al., unpublished data).

The taxonomy of termites in the eastern Mediterranean area remains obscure. In his review Harris (1970) stated that all *Reticulitermes* found in this area were *R. lucifugus* as classically defined by Rossi (1792) except for those found in Israel which were *Reticulitermes clypeatus* Lash (1952). Thus the name *R. lucifugus* is currently applied to two distinct species from Italy and Turkey. To avoid any confusion due to this homonymy, we will use the name *R. lucifugus* for populations in Italy where the species was first described (Rossi, 1792) and the name *R. l.-Turkey* for population in Turkey. A recent study should clarify the exact classification of *Reticulitermes* termites in these regions (Austin et al., 2002).

In a recent paper on termite species description rates, Eggleton (1999) reported that the cumulative number of species description in the western Palearctic region had leveled off considerably since 1949. This finding suggests that most of the termite species have been identified in this area where terrestrial fauna has been exhaustively studied. On the assumption that the classification of termites was definitive, samples have been identified based on geographic origin alone. Some *Reticulitermes* samples showing phenotypical differences from cogenic European species have been classified as *R. lucifugus* simply because they were collected in Italy (Springhetti, 1965; Marini and Ferrari, 1998). However since further study has revealed significant differences with other *Reticulitermes* species with regard to morphology, behavior and cuticular hydrocarbons, these samples have been reclassified as a separate entity designated as *R. sp. nov.* (Bagnères, pers. commun.; Clément et al., 2001). Afterwards, we identified termites displaying the *R. sp. nov.* phenotype in urban areas located in northern and southern Italy, in southeastern France and in one natural site located on the French Riviera (Uva et al., 2002).

The exact relationship of *R. sp. nov.* with other *Reticulitermes* species is uncertain. Based on mtDNA sequences, Marini and Mantovani (2002) proposed kinship with the Japanese *R. speratus*. However Clément et al. (2001) found similarities between *R. sp. nov.* and *R. balkanensis, R. santonensis* (cuticular hydrocarbons) and *R. clypeatus* (morphology).

Previously mtDNA was used to study phylogenetic relationships within the *Reticulitermes* genus (Jenkins et al., 2001; Marini and Mantovani, 2002) and to reveal matrilarchal genetic structure of *Reticulitermes* colonies in North America (Jenkins et al., 1998). Moreover, a recent study for the *Reticulitermes* genus that included the internal transcribed spacer sequence of a nuclear ribosomal RNA (Jenkins et al., 2001) provided an indication of the utility of this nuclear marker for closely related species, as previously pointed out by other authors (Schlötterer et al., 1994; Vogler and DeSalle, 1994). In this study we attempted to use both mtDNA and ribosomal ITS2 to clarify the ambiguous morphological and chemical findings concerning the phylogenetic relationship between *R. sp. nov.* and western Palearctic *Reticulitermes* termites, to assess its taxonomic status and to provide information in the general pattern of *R. sp. nov.* evolution. We also tested several hypotheses of monophyly for the termites in the eastern Mediterranean area.

2. Materials and methods

2.1. Samples

Various *Reticulitermes* samples from different Mediterranean countries (Fig. 1) were studied. DNA extraction was performed either immediately after collection in the field or after storage in alcohol at −20°C. In addition to Mediterranean *Reticulitermes*, *R. speratus* from Japan (Ibarakky) and *R. flavipes* from North America (Raleigh, NC) were included in the study. Full information concerning the samples is given in Table 1. To allow for intraspecific variations, two or three specimens were included for each species. A total of 42 specimens belonging to 20 taxa were analyzed. Sequences of *R. lucifugus* were drawn from GenBank for comparison (Uva et al., unpublished data).

Fig. 1. Map of *Reticulitermes* collection sites in Europe. See Table 1 for reference numbers.
2.2. DNA extraction, amplification, and purification

Total DNA was extracted from a single termite head using a modified version of the method described by Kocher et al. (1989).

PCR amplification was performed with a Biometra 96 T1 thermal cycler in a 50 μl reaction containing 37.75 μl of distilled H2O, 5 μl of a 10× Taq DNA polymerase buffer (500 mM KCl, 100 mM Tris–HCl, and 15 mM MgCl2, pH 9.0), 1 μl of dNTPs (10 mM each dNTP), 2.5 μl of a 10× solution of each primer, 1.25 U of Taq polymerase (Qbiogene), and 1 μl of DNA template. The primers used are shown in Table 2. The amplification profiles were: ND1—2 min denaturation at 92°C followed by 40 cycles of 15 s at 92°C, 45 s at 50°C, 2 min at 62°C, and a 7 min final extension at 62°C; ITS2—2 min denaturation at 92°C followed by 30 cycles of 30 s at 92°C, 45 s at 50°C, 45 s at 72°C, and a 7 min final extension at 72°C. Both strands of each sample were sequenced. Nucleotide sequences were entered in the GenBank database (see Table 1 for the accession numbers).

2.3. Phylogenetic analysis

Consensus sequences were aligned using the Clustal W algorithm (Thompson et al., 1994) from the BioEdit 4.8.10 sequence editor (Hall, 1999), and corrected manually. Sequence data were analyzed using the PAUP 4.0b10 package (Swofford, 2001).

To polarize the phylogenetic trees, i.e., distinguish plesiomorphic from apomorphic states, we used one subterranean species of the Coptotermitinae subfamily (Rhinotermitidae), Coptotermes formosanus Shiraki, from which the Reticulitermes genus reportedly evolved (Krishna, 1970). Trees were drawn using TreeView (Page, 1996).

The DNA sequences were analyzed by neighbor-joining (NJ) method (Saitou and Nei, 1987). Genetic distances were corrected according to the transition/transversion rate (Kimura’s two-parameter method, Kimura, 1980). Bootstrap confidence values were calculated from 2000 replications.

Maximum parsimony (MP) analyses were performed using a heuristic search with 100 random-addition
replications and Tree-Bisection-Reconnection (TBR) as the branch-swapping algorithm under the assumption of equal weight for all changes. Starting trees for branch swapping were obtained by stepwise addition. Branches were collapsed if maximum branch length was zero. Gaps were treated as missing data. Clade stability was estimated by nonparametric bootstrapping (Felsenstein, 1985, with 2000 replications as suggested by Hedges (1992) for reliable estimation of bootstrap support. Branches having bootstrap node support less than 50% were collapsed.

Prior to maximum likelihood (ML) analyses, the MODELTEST program (Posada and Crandall, 1998) was used to choose the DNA substitution model best fitting our data. Using a series of likelihood ratio tests (Huelsenbeck and Crandall, 1997), the program implements a model test routine between the simpler (null hypothesis) and the more parameter-rich model (alternative hypothesis). If the alternative model significantly improved the likelihood score, the parameters were added to the model. After selection, addition of the gamma correction for different rates over sites (Yang, 1993, 1996), and estimation of proportion of invariable sites were tested to improve the model. Heuristic tree searches were performed with TBR branch swapping with starting trees obtained by stepwise addition. Branches of zero length were collapsed. Nonparametric bootstrap analysis with 100 replicates was performed.

We used two different data sets for phylogenetic analyses: the nuclear ITS2 region and the mitochondrial ND1-16S region. Before being combined, ND1 and 16S data partitions were checked using the partition homogeneity test (Farris et al., 1995) implemented in PAUP (1000 replicates). The tRNA sequences were not included in the alignment.

Some additional analyses were performed for the ND1-16S data set by defining constraints to compare alternative phylogenetic hypotheses. Constraints were enforced in PAUP searches for minimum length trees under the hypothesis of Reticulitermes speratus basal to R. sp. nov. (accidental introduction from R. speratus, as proposed by Marini and Mantovani, 2002) and to the R. sp. nov., R. clypeatus, R. l.-Turkey, and R. balkanensis group (origin from a common ancestor in the eastern Palearctic area).

Optimal (H1) and constrained (H0) ML trees were compared by parametric bootstrapping that produced a null distribution of likelihood ratios between the two hypotheses obtained based on replicate data sets. This was accomplished using the following procedure (Hillis et al., 1996). First ML searches (constrained and unconstrained tree) were conducted in PAUP using the model previously chosen by MODELTEST. After the difference in log likelihood score between trees (observed δ) were recorded, 100 replicates were generated with SeqGen 1.2.5 (Rambaut and Grassly, 1997) based on the constrained ML tree and with the previous model of evolution. Finally the replicate data sets were analyzed with PAUP (constrained and unconstrained) and differences plotted in a histogram. PAUP instruction blocks are available upon request.

For the ND1-16S data partition, genetic pairwise distances were evaluated using the distance matrix option in PAUP according to the method described by Tamura and Nei (1993).

The molecular clock behavior of the ND1 gene in this data set was tested by comparing likelihood scores obtained for trees with and without enforcement of the molecular clock hypothesis. The molecular clock hypothesis was retained if likelihood scores were not significantly different and topologies were equivalent (Huelsenbeck and Rannala, 1997).

3. Results

3.1. Phylogenetic analysis

3.1.1. 16S rRNA-ND1 partial sequences

PCR amplification yielded a fragment of 743 bp (12.147–12.892 in Drosophila yakuba, Clary and Wolstenholme, 1985) containing a part of the ND1 gene, tRNA Leu, and a part of the 16S rRNA gene. The tRNA sequences and a highly variable block corresponding to an extension in the outgroup species were excluded from the alignments. The partition homogeneity test (ND1 and 16S) showed a p value of 0.82. Based on this finding, the null hypothesis cannot be rejected (homogeneous partitions) and data were combined for phylogenetic analysis. Moreover, no intraspecific differences in sequences were observed. In the combined data set (667 bp), among the 19 ingroup sequences obtained, there were 134 (20.1%) variable sites and 17 haplotypes were detected (Table 3).

The MP heuristic search produced four trees of equal length (276 steps, CI = 0.701, and RI = 0.722). The strict consensus tree (Fig. 2a) showed some collapsed nodes, but terminal branching patterns were well supported (bootstrap values > 70). Four groups were clearly distinguishable. Group 1 included Reticulitermes sp. nov. from Italy and France clustering with R. balkanensis (Balkans), R. l.-Turkey, R. clypeatus (Israel) and R. speratus (Japan). Group 2 was made up of R. banyulensis from France and R. grasseri from Spain and France. Group 3 comprised R. lucifugus from Italy and the Corsican sub-species R. l. corsicus. Group 4 contained R. santonensis from France clustering with the North-American R. flavipes. Observed polytomy derived from variations in group 3 branch topology which was basal to the group 1 in some trees and clustered with group 2 in others.

MODELTEST selected the Tamura-Nei model (1993) with among-site rate heterogeneity (TrN + G) as
the best fit for our data set. Rate matrix parameters were estimated on a NJ tree (PAUP rate matrix: abaaea; $a = 1$, $b = 17.43$, and $e = 11.89$). Base frequencies were $A = 0.19$, $C = 0.11$, $G = 0.21$, and $T = 0.49$. The shape of gamma distribution was 0.1922 with four rate categories. Results of ML analysis are shown in Fig. 2b.
Terminal branching was consistent with the MP tree except that the position of *R. speratus* was subtracted from group 1. All terminal branches were well supported (bootstrap values \(P > 70\)). Deep branching topology differed between MP and ML trees: the group 4 (*R. santonensis* and *R. flavipes*) was basal to the other *Reticulitermes* species in the MP tree, and showed a not resolved position in the ML tree (bootstrap values < 50).

The bootstrap consensus NJ tree was similar to the tree obtained by ML analysis. The differences were a strongly supported node (80) for the monophyletic clade including groups (2) and (3), and the position of *R. speratus* that was basal to the group (1*) although the node showed a low bootstrap support (63) (data not shown).

In all analyses *R. sp. nov.* samples from Italy and France clustered together, showing an affinity with *Reticulitermes* termites collected in the eastern Mediterranean area. *R. speratus* from Japan clustered basal to this group only in the MP and NJ analyses.

Two hypotheses were tested using ML trees. The first hypothesis (CONSTRAINT 2) implied that *R. sp. nov.* and *R. speratus* were monophyletic based on the assumption that *R. sp. nov.* was accidentally introduced from Japanese populations (Marini and Mantovani, 2002). The second hypothesis (CONSTRAINT 1) was that *R. sp. nov.*, *R. clypeatus*, *R. l.-Turkey*, and *R. balkanensis* (CONSTRAINT 1) formed a monophyletic group could not be rejected but the hypothesis that *R. sp. nov.* and *R. speratus* were monophyletic (CONSTRAINT 2) was rejected (\(p < 0.01\)) (Fig. 3). Since these findings indicate that the monophyletic *R. sp. nov.* *R. speratus* group did not result from stochastic variations in ML analysis, the hypothesis that *R. sp. nov.* populations originated from *R. speratus* cannot be accepted.

Pairwise Tamura–Nei distances showed a more restricted range of values for termites in the western European area (except *R. santonensis*) than termites in eastern area (*R. balkanensis*, *R. clypeatus*, *R. l.-Turkey*). Within the western area distances ranged from 0.0015 (Rg-For vs Rg-Ad) to 0.0046 (Rl-Cdm vs Rl-Via) at the intraspecific level and from 0.0322 (*R. banyulensis* vs *R. grassei*) to 0.1010 (*R. sp. nov.* vs *R. lucifugus*) at the interspecific level. In the eastern area distances ranged from 0.0276 (Rl-Kon vs Rb-Dio) to 0.0481 (Rl-Ank vs Rcly). *R. balkanensis* showed the lowest divergence with *R. sp. nov.* (0.0635).

### 3.1.2. ITS2 complete sequence

We obtained the complete sequences of the ribosomal internal transcribed spacer (ITS2, 382 bp). The 10 haplotypes scored (Table 3) showed only 14 variable sites (3.7%), demonstrating little sequence variability compared to the partial 16S-ND1 region. Interestingly, we observed a same haplotype for *Reticulitermes banyulensis* (Rb-Bez, Rb-Vid), for *R. grassei* (Rg-For, Rg-Ad), for *R. santonensis* and *R. flavipes* (Rs-Ole, Rs-Tc, Rs-Vid, Rs-Ad, Rs-Bez, Rs-Ad, Rs-Bez, Rs-Dio, Rs-Dio, Rs-Dio).
Rf), for R. lucifugus (Rl-Cdm, Rl-Via), for R. balkanensis from Greece and R. clypeatus from Israel (Rbk-Dio, Rcly), and for the four R. sp. nov. samples (Rsp-Bag, Rsp-Dom, Rsp-Gom, Rsp-Sa).

Construction of phylogenetic trees using NJ method did not fully resolve the relationships between the ten haplotypes. The bootstrap analysis strongly supported the branch including the haplotypes 7, 9, and 10 corresponding to the samples from the Eastern Mediterranean area (data not shown). ML analysis produced similar results (data not shown), while MP analysis resulted in 120 most-parsimonious trees. In the last case the strict consensus tree derived from these trees did not show any clear relationships between clades (data not shown).

3.2. Molecular clock

The molecular clock hypothesis for the ND1 gene (535 bp) could not be rejected. The likelihood of the ND1 tree was \(-\ln L = 1877.3070\) with molecular clock enforced versus \(-\ln L = 1868.6420\) without the molecular clock enforced. This difference was not significant (p value = 0.30; df = 15). This finding suggested that lineages in our data set evolved at the same rate. Thus the ND1 clock could be used to estimate divergence time between lineages, along with others genes showing a molecular-clock behavior. In fact, several protein clocks should be used to estimate divergence time accurately (Ayala, 1997).

4. Discussion

4.1. Phylogeny and distribution of Reticulitermes termites

All terminal clades in this study were well supported, according to previous studies demonstrating important phenotypic differences in cuticular hydrocarbon profiles (Bagnères et al., 1988, 1991) and soldier-gland secretion composition (Bagnères et al., 1990; Parton et al., 1981; Quintana et al., 2003) between the different Reticulitermes species.

Reticulitermes banyulensis and R. grassei which are found in France and Spain and live sympatrically in eastern Spain appeared to be closely related (Clément et al., 2001). This finding is in agreement with the hypothesis that the two species evolved from a common ancestor that survived the glacial period in a refugium in southern Spain. The area where the R. banyulensis sample was collected in southern France, Rb-Vid, currently represents the easternmost limit of the species distribution range. In that collection site R. banyulensis lives sympatrically with R. lucifugus.

The Italian species R. lucifugus and Corsican subspecies R. l. corsicus were placed in a well supported clade distinct from that of the R. sp. nov. samples collected in Italy. Thus, R. sp. nov. can not be classified as R. lucifugus.

Reticulitermes santonensis from France and R. flavipes from USA clustered together in phylogenetic trees obtained with 16S-ND1 sequences. However, they formed a clade basal to the other Reticulitermes species in the MP tree and were included in the polytomy in the ML and NJ trees. Interestingly, R. santonensis and R. flavipes had the same consensus sequences for the ITS2 region, as observed by Jenkins et al., 2001. The limited distribution range of R. santonensis in Europe (Vieau, 2001) and the wide distribution range of R. flavipes in North-America are in agreement with the hypothesis that the species could be native to North America and was accidentally introduced into Europe (Bagnères et al., 1990).

Turkish R. lucifugus (R.l.-Kon, R.l.-Ank), R. balkanensis (Greece) and R. clypeatus (Israel) formed a single clade well supported. Moreover, R. balkanensis
and *R. clypeatus* shared a same ITS2 haplotype. Similar results were obtained by Austin et al. (2002) with the mitochondrial DNA COII region. These results could be explained by a common origin of the termite populations during glacial movements (see below for a more detailed explanation). The Authors suggest that the taxonomy in this area should be considered carefully: further studies could detect some homonymies.

### 4.2. Origin of *R. sp. nov.*

The four samples of *R. sp. nov.* studied formed a single clade when 16S-ND1 sequences were analyzed. Moreover they had the same sequence for the ITS2 region. This suggests that the *R. sp. nov.* samples belong to the same taxonomic group. Prior to the recent discovery of a natural site in southern France (Sophia Antipolis), all samples of *R. sp. nov.* had been collected in urban settings. Such a distribution suggested that *R. sp. nov.* might be a non-western European species imported by man. However, results obtained in this study by non-parametric bootstrap strongly support a relationship between *R. sp. nov.* and eastern Mediterranean termites including *R. clypeatus* from Israel, *R. lucifugus* from Turkey and *R. balkanensis* from the Balkans. The species are characterized morphologically by the presence of a similar post-clypeus (Clément et al., 2001). The hypothesis of a relationship with *R. speratus* was rejected by parametric bootstrap.

The Quaternary cold periods are thought to have influenced distribution ranges of both animal and plant life in the Mediterranean area. Several authors have attributed the dispersion patterns shared by many taxa to these periods (De Jong, 1998; Taberlet et al., 1998). Successive contraction and expansion of distribution ranges during these periods may have resulted in a loss of genetic diversity in northern species (bottlenecks). Similarity between species in different geographical locations could be linked to postglacial colonization routes between Balkan refugia and northern regions. The Alps could act as a barrier preventing the northward progression. *R. sp. nov.* may have reached southern France by crossing Italy and passing along the Mediterranean coast. Notwithstanding the fact that most *R. sp. nov.* samples have been collected in urban sites, the geographical location of collection sites is in agreement with the hypothesis of postglacial colonization. These termites may have been introduced into urban zones from neighboring regions. The small number of *R. sp. nov.* sites discovered up to now (*n* = 15; Mira, Salsomaggiore, Bagnacavallo, S. Agata sul Santerno, Galatina, Ravenna in Italy and Domène-38, Grenoble-38, Château Gombert-13, La Ciotat-13, St-Cyr-Les-Lecques-83, St. Laurent du Var-06, Sophia Antipolis-06, St. Paul de Vence-06, Roquebrune Cap Martin-06, in France) could be linked either to the recent availability of new technology for species identification or to successive contraction and expansion of distribution range during Quaternary cold periods. In the latter case, remnants of present-day sites would be left over from wider distribution of the past.

This work demonstrated the value of the use of both nuclear and mitochondrial markers to assess phylogenetic patterns and taxonomic status. Both markers agreed in defining the specific status for the *R. sp. nov.* populations, and provided evidence to identify the area from which *R. sp. nov.* is likely to have originated from. The genetic distance between *R. sp. nov.* and species in eastern Europe are of the same magnitude as interspecific distances observed in western Europe. Several features may have contributed to the development of reproductive isolation mechanisms in *R. sp. nov.* (Clément et al., 2001) and to its specific differentiation status. Description of the new species will be published elsewhere (Bagnères and Uva, unpublished data). Samples will be sent to the Museum d’Histoire Naturelle in Paris, France, as voucher specimens.

Future studies will demand that *Reticulitermes* termites from other countries in the eastern-Mediterranean area be examined. However, investigators in that part of Europe still use the old Rossi classification system (1792) which includes only one *R. lucifugus* species. A clear classification of the genus will be needed to study the mechanisms underlying speciation in the western Palearctic region.

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