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Research article

Mitochondrial evolutionary rate and speciation in termites: data on European *Reticulitermes* taxa (Isoptera, Rhinotermitidae)

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Summary. The rate of mitochondrial DNA evolution and the speciation pattern in relation to glacial periods are tested in the European taxa of the eusocial genus *Reticulitermes.* The linearized tree obtained from cytochrome oxidase II sequences and a geological event calibration shows a substitution rate 100-fold higher than that usually applied for insect mitochondrial DNA. An accelerated rate of evolution has also been observed in social Vespidae (Hymenoptera); we therefore suggest the involvement of eusociality in mediating gene pool drift. The role of the last ice age in speciation pattern of *Reticulitermes* taxa is supported by molecular data, but a four refugia model better explains genetic diversity, phyletic relationships and present-day distribution of these termites.

Key words: Glacial refugia, mitochondrial DNA, molecular clock, rate of evolution, two-cluster test.

Introduction

Mitochondrial DNA analyses are widely exploited in phylogenetic reconstruction mainly because of the practical advantages that this approach offers. This genetic compartment is also often utilised as a source of historical information. Yet, little is known about the evolutionary dynamics of mitochondrial genome in organisms deviating from canonical bisexuality, i.e. females and males freely interbreeding. In eusocial animals, clusters of individuals constitute colonies in which only a single or a few females produce offspring. This leads to a consistent bias in haplotype sampling from one generation to the next. A quicker divergence of mitochondrial genomes has been proved in Hymenoptera where the mitochondrial genomic compartment shows a significantly higher evolutionary rate in social Vespidae with respect to solitary ones (Schmitz and Moritz, 1998). This has been explained as due to the presence of a unique or few reproductive females (queens) laying a large number of eggs, while thousands of infertile workers do not produce offspring. In such conditions, mutations accumulate and fix, while ancestral haplotypes are lost more rapidly than in solitary insects.

The heterometabolous order Isoptera comprises the largest and best known other group of eusocial insects. Among these, the subterranean termites of the genus *Reticulitermes* are the most abundant, naturally residing termites in Europe. Many taxa have been analysed for morphology, cuticular hydrocarbons, defensive compounds, behaviour, mitochondrial and nuclear DNA (Clément et al., 2001; Marini and Mantovani, 2002; Austin et al*.,* 2002; Uva et al*.,* 2004a,b; Luchetti et al*.,* 2004).

The distribution of *R. lucifugus* subspecific entities follows a geographic pattern. The Iberian Peninsula and southern France host *R. lucifugus grassei* on the Atlantic coasts and *R. lucifugus banyulensis* on the Mediterranean ones. *R. lucifugus lucifugus* is present on Italian mainland, *R. lucifugus corsicus* in Corsica, Sardinia and Tuscany and *R. lucifugus* subsp. nov. in Sicily (description in progress; Lozzia, 1990; Luchetti et al*.,* 2004) (Fig. 1). *R. lucifugus balkanensis* inhabits the Balkanic area*.* Recent molecular analyses suggest the existence of two genetically distinct taxa: a trans-Adriatic form, ranging from Peloponnese to north- and south-eastern Italy, and an eastern continental Greek entity (Fig. 1) (Luchetti et al*.,* 2004). While the latter may correspond to the balkanic taxon, the taxonomic position of the former is still debated (Clément et al*.,* 2001; Austin et al*.,* 2002; Uva et al*.,* 2004a; Luchetti et al*.,* 2004). Recently, a specific rank of differentiation has been proposed for the Iberian taxa and for *R. lucifugus balkanensis* (Clément et al*.,* 2001).

In the eastern Mediterranean basin, populations from Turkey were discovered as belonging to a new taxon with a high genetic divergence from *R. lucifugus* (Austin et al., 2002). The Israelian samples are classically known as *R. clypeatus* (Lash, 1952).

Finally, *R. santonensis* is present in a small area on the French Atlantic coast. This entity is genetically indistinguish-

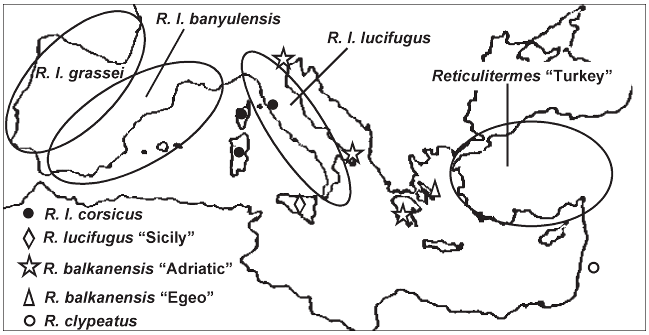

Figure 1. Map showing the present-day distribution of *Reticulitermes* taxa

Table 1. Analysed taxa and collection data (asterisks mark termites that were a kind gift of Prof. J. L. Clément)

Taxon	Sampling localities	GenBank A. N.
R. l. lucifugus	Chieti (Italy)	AF291738
	Rosarno (Italy)	AY267863
R. l. lucifugus "Sicily"	Palermo (Sicily)	AY267857
	Agrigento (Sicily)	AY267864
R. l. corsicus	*	AY267858
	Parco dell' Uccellina (Italy)	AF291727
	Flumini di Quartu (Sardinia)	AF291728
R. l. banyulensis	*	AY267859
	Béziers (France)	AF525319
R. l. grassei	Foret (France)	AF291731
	Mimizan (France)	AF291733
R. balkanensis "Adriatic"	Areopolis (Peloponnese)	AY267867
	Bagnacavallo (Italy)	AF291736
	Galatina (Italy)	AY267862
R. balkanensis "Egeo"	Schinias (Eastern Greece)	AF525318
Reticulitermes "Turkey"	Antalya (Turkey)	AF525330
R. clypeatus	Ben Shemen (Israel)	AF525320
C. formosanus	New Orleans (U.S.A.)	AF107488

able from the north-American *R. flavipes*, and its presence in Europe has been explained as the product of an anthropic introduction (Jenkins et al., 2001; Vieau, 2001; Marini and Mantovani, 2002).

Quaternary cold periods have significantly affected genome diversity in many organisms through refugia isolations and post-glacial spreading. The three Mediterranean peninsulas and the Near East are known as centres of speciation and starting points for recolonisation in different organisms (Hewitt, 2001). Biogeographic data and genetic relationships among European *Reticulitermes* termites suggest the involvement of recent climatic oscillations in their distribution and differentiation. Clément et al. (2001) have proposed two alternative hypotheses to explain the evolution of *Reticulitermes* taxa since the last ice age (Würmian era). The first model involves the three Mediterranean peninsulas as glacial refugia with the contemporary speciation of *R. balkanensis*, *R. lucifugus* and *R. l. banyulensis – R. l. grassei* group; the latter should have diverged approximately 8000 year ago by northward recolonisation. This could explain the genetic affinities between the Iberian taxa. The second model involves only the Balkanic and Iberian peninsulas as possible refugia: the Italian peninsula should have been recolonised after the end of the ice age from Spanish populations. This scenario may explain the similarities between *R. lucifugus*, *R. l. grassei* and *R. l. banyulensis*.

In the present paper, we analyse the data presented in Luchetti et al. (2004) through the "linearized tree" method (Takezaki et al., 1995) in order to verify the substitution rate of the mitochondrial compartment in these eusocial insects and to clarify their pattern of speciation. The analysis was extended to the Turkish and Israelian samples (Austin et al., 2002) to test the possibility of a fourth glacial refugium in the Near East. *R. santonensis* was excluded from this study given its allopatric origin.

Materials and methods

Cytochrome oxidase II (COII) sequences have been downloaded from Genbank for each European taxon. Accession numbers and collecting sites are given in Table 1.

For reasons of clarity, taxa of uncertain or still debated taxonomic status were hence indicated as follow:

- populations from north- and south-eastern Italy and Peloponnese = *R. balkanensis* "Adriatic"
- sample from eastern Greece = *R. balkanensis* "Egeo"
- the Turkish entity = *Reticulitermes* "Turkey".

Termites from Sicily, whose taxonomic description is still in progress, will be reported as *R. lucifugus* "Sicily".

Neighbor-Joining (NJ) on Kimura 2-parameter distances, Maximum Parsimony (MP) and Maximum Likelihood (ML) were computed with PAUP* v. 4.0b (Swofford, 2001), with the parameters described in Luchetti et al. (2004). Linearized tree and two-cluster test (Takezaki et al., 1995) were calculated through LINTREE server program, located at web site http://shangai.bio.psu.edu/lintree.html.

Two methods were applied to estimate the divergence time between analysed taxa. First, an independent molecular clock was calibrated on a geological event, i.e. the last separation between Sicily and peninsular Italy at the end of the Würmian era (12 kyr). This geological event should have isolated the Sicilian entity from *R. l. lucifugus*. Furthermore, the substitution rate commonly estimated for insects mtDNA, i.e. 2.3%/Myr (Brower, 1994), was considered.

Substitution rates for 16S rDNA (Luchetti et al., 2004 and Marini and Mantovani, 2002 datasets) and for 16S rDNA - ND1 genes (Uva et al., 2004a,b) were also calculated applying the geological calibration.

Results

The MP analysis performed on COII sequences gave two equally parsimonious trees, with length equal to 266 steps (C.I. = 0.744; not shown). The bootstrap consensus tree (268 steps; C.I. = 0.739; not shown) completely agree with the NJ one (Fig. 2): the easternmost taxa (*R. balkanensis* "Egeo", *Reticulitermes* "Turkey" and *R. clypeatus*) and the trans-Adriatic entity constitute two sister clades, well differentiated from *R. lucifugus* entities. *R. l. grassei* and *R. l. banyulensis* are basal to the cluster of Italian *R. lucifugus* sequences.

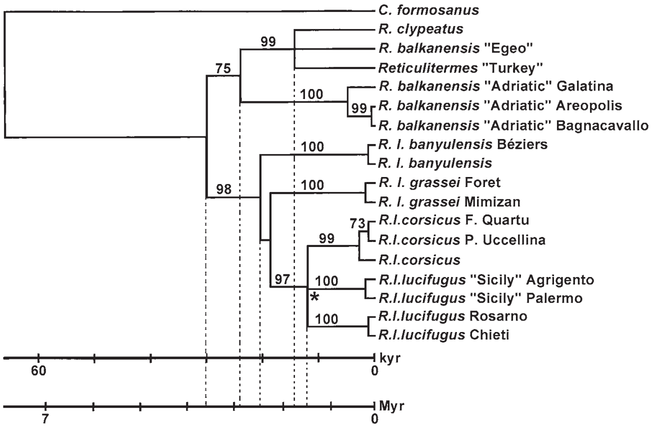

Figure 2. Schematic tree based on Neighbor-Joining and linearized tree following Takezaki et al. (1995). Bootstrap values >70% are reported above branches. The upper scale bar represents the time scale according to the geological calibration (end of the Würmian era, 12 kyr ago; asterisk marks the node to which the calibration was applied). The lower scale bar indicates the time scale obtained by applying the general molecular clock estimate (2.3%/Myr; Brower, 1994)

In this cluster, haplotypes segregate in three well differentiated groups, i.e. *R. l. lucifugus, R. l. corsicus* and *R. lucifugus* "Sicily". The ML tree (–lnL = 2199.13418; not shown) shows the same terminal branching pattern of NJ and MP trees. The only remarkable differences are the clustering of easternmost taxa in the most basal clade and the grouping of *R. l. grassei – R. l. banyulensis* in a sister clade of Italian *R. l. lucifugus*. On the whole, these results are in line with those obtained in previous studies (Marini and Mantovani, 2002; Luchetti et al., 2004).

The two-cluster test evidences a substitution rate significantly constant among clusters (available from the Authors). Taking as a reference the separation between *R. lucifugus* "Sicily" and the peninsular *R. l. lucifugus* at 12 kyr ago, the COII substitution rate is equal to 0.25–0.28%/kyr. Substitution rates of the same magnitude are also scored in the other data sets considered: ≈0.17%/kyr for the 16S rDNA gene, and ≈0.25%/kyr for the 16S rDNA-ND1 region.

Following the COII substitution rate, the main cladogenetic event between the western- and the eastern-Mediterranean lineages should date back to 31.34 +/– 3.43 kyr ago (Fig. 2).

The Iberian clades *R. l. grassei* and *R. l. banyulensis* and the Italian *R. lucifugus* have originated, almost contemporary, about 20.90 +/– 3.07 kyr ago, near the Last Glacial Maximum (LGM, 18 kyr). The trans-tyrrenian *R. l. corsicus* appears to have differentiated at the same time of *R. l. lucifugus – R. lucifugus* "Sicily" splitting.

About 24.97 +/– 3.49 kyr ago, the ancestors of *R. balkanensis* "Adriatic" should have diverged from those of the easternmost complex *R. clypeatus – Reticulitermes* "Turkey" – *R. balkanensis* "Egeo". These latter entities should have differentiated each other 14.43 +/– 2.52 kyr ago.

When the common estimate of insects mtDNA substitution rate (Brower, 1994) is applied, all cladogenetic events are significantly predated. Western- and eastern-Mediterranean clades diverged 3.74+/– 0.41 Myr ago. The differentiation of the Iberian taxa from the ancestors of the Italian ones should date back to 2.49 +/– 0.36 Myr, while the splitting of the Sicilian entity from the peninsular one is predated to 1.43 +/– 0.26 Myr ago. The easternmost taxa, whose splitting took place 1.72 +/– 0.30 Myr ago, diverged from the other eastern-Mediterranean clade, *R. balkanensis* "Adriatic", about 2.98 +/– 0.41 Myr ago.

Discussion

The most interesting datum is the extraordinary discrepancy between the substitution rate usually estimated and applied for insect mtDNA (Brower, 1994) and the one scored for the COII gene with the geological calibration: the latter is nearly 100-fold higher than the former.

It is important to understand which of these calibrations is the most tenable for both the right estimation of divergence time within Mediterranean *Reticulitermes* and the long running debate on the role of glaciations in speciation.

First of all, we should consider that the high substitution rate computed for the COII gene does not appear specific of this protein coding tract: the evolutionary rates computed for other mtDNA regions (16S and 16S-ND1) are of comparable magnitude.

A second hint derives from the comparison with nuclear data on *Reticulitermes* taxa (Uva et al., 2004a): due to its repetitive nature, ITS2 is a fast evolving region undergoing concerted evolution, which should rapidly amplify the inter-taxa divergences (Dover, 2002). Yet, the sequencing of ITS2 nuclear region does not resolve the entities identified with mtDNA markers: for example, *R. clypeatus* shows the same genotype of *R. balkanensis* "Egeo". The poor variation scored (only 14 variable sites out of the 382 sites sequenced) could be the result of gene flow between *Reticulitermes* populations, either due to recent migrations or introductions. However, this hypothesis appears consistent only for sub-specific entities, but it is unlikely for those taxa showing a specific rank of differentiation. Thus, the low variability scored could be better explained as the result of recent cladogenetic events, and this hardly fit with the divergence time computed by applying the generalised mtDNA evolutionary rate (3.7 Myr-1.5 Myr). Present analyses therefore support the conclusions drawn from Hymenoptera (Schmitz and Moritz, 1998). Obviously, it is not possible to exclude that *Reticulitermes* termites could have a dramatically accelerated molecular clock because of some undefined molecular mechanism. Further, contraction and isolation of termite populations during the glacial period could have increased the haplotype drift.

On the whole, our results support a high substitution rate, mainly due to the bias in the number of reproductive individuals per generation/population. The reproductive biology of a taxon appears, therefore, to have a leading role in determining the mtDNA evolutionary rate. This is also supported by data on organisms unrelated to insects: in the matriarchal society of macaque monkeys, characterised by few dominant females, a computer simulation showed that social and geographical population structures could significantly increase the mtDNA substitution rate (Hoelzer et al., 1998).

Concerning the pattern of *Reticulitermes* cladogenesis, our analysis confirms the role that the last cold period and the glacial refugia have played in speciation and present-day distribution of animals. Our results well fit with the climate oscillations during the last ice age and, at variance of previous hypothesis (Clément et al., 2001), point to a different timing and pattern of cladogenesis.

In particular, it appears that in the Iberian area *R. l. banyulensis* and *R. l. grassei* diverged during the LGM and not after the glacial period by the northern spreading of post-glacial recolonisation (Clément et al., 2001). It is assumable that Iberian taxa started to differentiate since their isolation at the opposite side of the Iberian Peninsula. As climate warmed, they newly become into contact producing the current distribution with sympatric zones.

For the Italian peninsula, *R. l. corsicus* and *R. lucifugus* "Sicily" have started to diverge contemporarily. During the LGM, only a strait separated the north-east of the Corsican-Sardinian plaque from the mainland (Thiede, 1978). It is impossible to suggest whether island colonisation has been anthropically mediated and how many of these events took place.

Finally, our data suggest the existence of two refugia in the eastern Mediterranean area, i.e. the southern Balkans – Greece and the Near East. When the climate warmed, the trans-Adriatic lineage recolonised Balkans and eastern Italy, while the easternmost one (comprising Israelian, Turkish and eastern Greek populations) could have further differentiated during a huge westward colonisation. Thus, the low genetic divergence observed between *R. balkanensis* "Egeo", *Reticulitermes* "Turkey" and *R. clypeatus* (Luchetti et al., 2004) could be well explained by their recent separation (14.43 +/– 2.52 kyr).

In conclusion, the present paper indicates that when tackling mitochondrial markers and divergence times in the absence of a geological calibration, the reproductive biology of the studied organism must be well defined. Otherwise, the time since isolation based on the assumption of a generalised molecular clock may be consistently biased. Further, a more clear cladogenetic picture of European *Reticulitermes* taxa is put forward.

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