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Genetic analysis of the breeding system of an invasive subterranean termite, *Reticulitermes santonensis*, in urban and natural habitats

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Abstract

***Reticulitermes santonensis* is a subterranean termite that invades urban areas in France and elsewhere where it causes damage to human-built structures. We investigated the breeding system, colony and population genetic structure, and mode of dispersal of two French populations of *R. santonensis*. Termite workers were sampled from 43 and 31 collection points, respectively, from a natural population in west–central France (in and around the island of Oléron) and an urban population (Paris). Ten to 20 workers per collection point were genotyped at nine variable microsatellite loci to determine colony identity and to infer colony breeding structure. There was a total of 26 colonies, some of which were spatially expansive, extending up to 320 linear metres. Altogether, the analysis of genotype distribution, *F*-statistics and relatedness coefficients suggested that all colonies were extended families headed by numerous neotenics (nonwinged precocious reproductives) probably descended from pairs of primary (winged) reproductives. Isolation by distance among collection points within two large colonies from both populations suggested spatially separated reproductive centres with restricted movement of workers and neotenics. There was a moderate level of genetic differentiation (F_{ST} = 0.10) between the Oléron and Paris populations, and the number of alleles was significantly higher in Oléron than in Paris, as expected if the Paris population went through bottlenecks when it was introduced from western France. We hypothesize that the diverse and flexible breeding systems found in subterranean termites pre-adapt them to invade new or marginal habitats. Considering that *R. santonensis* may be an introduced population of the North American species *R. flavipes*, a breeding system consisting primarily of extended family colonies containing many neotenic reproductives may facilitate human-mediated spread and establishment of *R. santonensis* in urban areas with harsh climates.**

Keywords: dispersal, inbreeding, Isoptera, microsatellites, Rhinotermitidae, social organization, urban invasion

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Introduction

Biological invasions are often human-facilitated and commonly create serious economic and environmental problems (Everett 2000; Pimentel *et al*. 2000; Chapman & Bourke 2001).

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Some social insect species have been highly successful in invading new regions (Moller 1996). In social insects, successful spread of invasive species largely depends on its breeding system (*sensu* Ross 1993) and mode of dispersal (Chapman & Bourke 2001; Ross 2001; Holway *et al*. 2002). The number of reproductives within a colony, the mating system, modes of colony founding and dispersal all will affect the propensity to spread in and monopolize new

habitats. In turn, arrival in a new environment may sometimes lead to an alteration in the breeding system. This might be due to new ecological conditions, such as an absence of competitors or parasites (Porter *et al*. 1997), or to genetic changes that take place during the introduction, such as a loss of nestmate recognition cues (Ross *et al*. 1996; Tsutsui *et al*. 2000, 2003; Chapman & Bourke 2001; Giraud *et al*. 2002). Therefore, detailed investigation of the breeding system of invasive social insects is fundamental to further our understanding of how social structure, dispersal, and invasion success may be linked.

In termites (Isoptera), the genera *Coptotermes* and *Reticulitermes* are prominent groups of subterranean termite pests (Rhinotermitidae); they commonly infest human-built structures where they may cause extensive damage (Gay 1969; Su & Scheffrahn 1990). A population of subterranean termites is comprised of distinct colonies. Colonies may occupy underground networks that link several foraging sites (Thorne *et al*. 1999). The breeding system and mode of dispersal of *Reticulitermes* species is complex. Colonies are generally initiated by a single pair of primary (winged) reproductives, which results in a simple-family structure (one queen, one unrelated king and their progeny). Because winged reproductives can fly relatively long distances, this mode of colony initiation can result in high levels of gene flow across large spatial scales. Later in the colony life cycle, secondary reproductives, which develop from either brachypterous nymphs or from workers (reviewed in Lainé & Wright 2003), can supplement or replace the primary reproductives. They are larvae instars, called neotenics. Neotenics do not fly, they remain and mate in the nest, resulting in inbreeding within colonies. Colonies headed by multiple neotenics can grow and expand, sometimes forming spatially diffuse networks of interconnected reproductive centres. Colony fragments can also become autonomous units that will constitute new colonies (Thorne *et al*. 1999). This 'budding' and limited dispersal would result in local isolation by distance, or 'population viscosity' (Hamilton 1964). The capacity to generate new colonies from colony fragments has also likely facilitated the spread of subterranean termites by human-mediated transportation of infested pots containing plants or timber. Thus, the diversity in breeding systems may facilitate colonization and invasion of novel environments by subterranean termites. On the one hand, once established they can produce long-range dispersers, on the other hand, they can profit from human transportation and build up populous colonies in localized areas.

The European subterranean termite *Reticulitermes santonensis* Feytaud is a severe structural pest in several parts of France, where it has been present in urban areas since at least the 18th century (de Quatrefages 1843). *R. santonensis* is a suspected introduced population(s) of the American species *Reticulitermes flavipes* (Feytaud 1924), which is supported by their qualitative similarity in cuticular hydrocarbon profiles (although they appear to differ slightly in the composition of soldier defense compounds; Bagnères *et al*. 1990) and by molecular genetic data (Clément *et al*. 2001; Jenkins *et al*. 2001; Austin *et al*. 2002; Marini & Mantovani 2002; Luchetti *et al*. 2004; Uva *et al*. 2004; Ye *et al*. 2004). Moreover, Dronnet *et al*. (2004) found that French populations of *R. santonensis* averaged fewer alleles and had lower heterozygosity than North American populations of *R. flavipes* at 11 microsatellite loci, which is consistent with a genetic bottleneck during the introduction of these termites from North America to France. The distribution of *R. santonensis* is also consistent with human introduction and spread in France because all populations are located in or close to buildings (Vieau 1993), with only a few small populations found in forests bordering the Atlantic Ocean (Vieau 2001). In addition, the populations in forest areas are close to the ports of La Rochelle and Nantes, some potential introduction sites. Invasion of new cities was probably facilitated by the development of railway transport of wood materials at the end of the 19th century, as suggested by the high incidence of infested areas near railway stations in large cities (Vieau 1993). In Paris, the species was first officially reported at the beginning of the 1950s, where termites are mostly found in buildings, but damage on trees along streets has also been observed for the last 10 years (Lohou *et al*. 1997).

The successful spread of *R. santonensis* seems associated with a high proportion of neotenic reproduction, as suggested by data on caste proportions (Vieau 1996), large spatial foraging range (Paulmier *et al*. 1997), and allozyme markers (Clément 1981). However, the breeding system of this species is still poorly known because of its cryptic lifestyle and underground foraging habits. In this study, we investigated the breeding system and mode of dispersal of *R. santonensis* using microsatellite markers. We analysed colony and population genetic structure in natural and urban regions in order to determine the prevalence of neotenic reproduction and improve our understanding of the factors facilitating the spread of subterranean termites to new urban areas.

Materials and methods

Sampling

Samples of *Reticulitermes santonensis* were collected in two contrasting habitats, a natural pine forest on or near the island of Oléron, and an urban region in the city of Paris. To obtain data both at the population and colony levels, we used a hierarchical sampling scheme, with single collection points over a large geographical scale (> 1 km) and multiple collection points within a few selected sites (*c*. 300 m).

Termites from the Oléron region were collected in April 2002 in 100-year-old pine forests, with stumps and

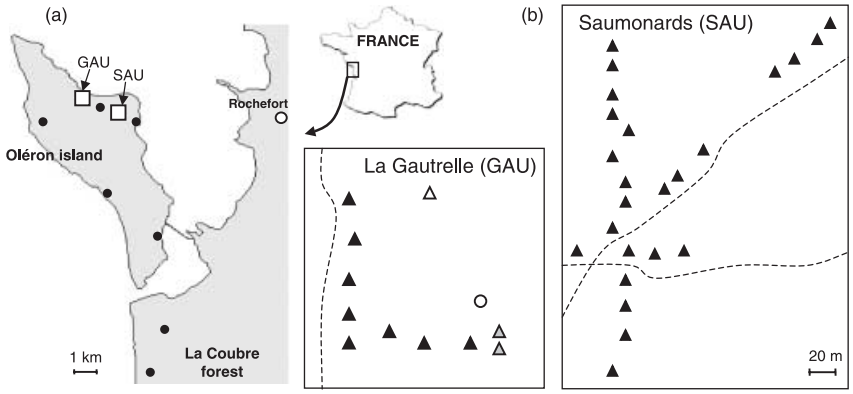

Fig. 1 Map of sample locations in the Oléron region (a), with an enlargement of the extensively sampled sites. (b) Collection points that were genetically nondifferentiated are indicated by the same symbols. Dashed lines represent forest paths.

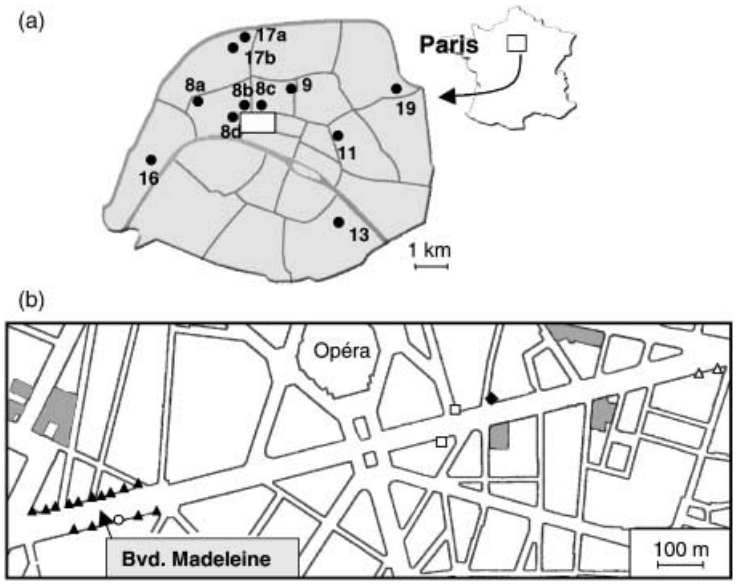

Fig. 2 Map of sample locations in Paris (a) (the numbers correspond to the different arrondissements where termites where collected), with an enlargement of the extensively sampled site. (b) Collection points along the boulevards that were genetically nondifferentiated are indicated by the same symbols. The grey areas indicate known infested buildings close to the sampling sites.

occasionally evergreen oaks. Five collection points were on the island of Oléron and two were in the continental forest of La Coubre, close to the southern tip of the island (Fig. 1a). Ten workers were sampled in each collection point (e.g. one stump). At a smaller scale, two other sites on the island of Oléron were sampled extensively (Fig. 1b), with 10 workers taken from collection points located every 20 m along two intersecting transects. Twenty-four stumps were sampled in the 'Saumonards' forest (SAU, 280 × 320 m transects), and 12 stumps were sampled in 'La Gautrelle' forest (GAU, 100 × 100 m). These two sites are separated by 2 km. In total, termites from 43 collection points were analysed in the Oléron region.

Termites were collected in September 2001 from 11 collection points within the city of Paris (Fig. 2a, b). At each collection point, 10–20 workers were sampled per building. At a smaller scale, one other site was sampled extensively (Fig. 2b), with 10 termites sampled from each of 15 trees regularly spaced approximately every 20 m lining Boulevard de la Madeleine (MAD, 8th arrondissement), and five more trees located 500 and 800 m away along the boulevard. To avoid damaging trees, termites were taken from mud tubes on the bark. In total, termites from 31 collection points were analysed in Paris.

The geographical scale of sampling was quite similar in the two regions, spreading over 14 × 11 km in Oléron (excluding samples from La Coubre) and 10 × 9.2 km in Paris. Immediately following collection, all workers from each collection point were placed in 95% ethanol and stored at 4 °C until DNA extraction.

Microsatellite genotyping

DNA was extracted from whole worker bodies using standard phenol–chloroform purification (Sambrook *et al*. 1989). In total, we determined the microsatellite genotypes of 430 termites from 43 collection points in the Oléron region and 390 termites from 31 collection points in Paris. We used nine microsatellite loci (Table 1): four loci originally isolated from *R. flavipes* (*Rf1-3*, *Rf6-1*, *Rf11-1* and *Rf15-2*; Vargo 2000) and five loci characterized from *R. santonensis* (*RS10*,

Table 1 Variability at nine microsatellite markers in Oléron region and Paris. The number of alleles and gene diversity (H_S) were calculated from the entire sample

	Oléron region (n = 430 individuals, 43 collection points, 12 colonies)		Paris (n = 390 individuals, 31 collection points, 14 colonies)	
Locus	No. of alleles	Gene diversity H_S	No. of alleles	Gene diversity H_S
Rf1-3	6	0.54	2	0.33
Rf6-1	9	0.42	6	0.49
Rf11-1	4	0.40	3	0.46
Rf15-2	4	0.37	2	0.46
RS10	5	0.35	4	0.39
RS15	4	0.40	4	0.64
RS68	3	0.18	3	0.26
RS76	3	0.20	2	0.40
RS85	4	0.19	2	0.32
Mean (± SD)	4.7 ± 1.9		3.1 ± 1.4	
Overall		0.34		0.42

RS15, *RS68*, *RS76* and *RS85*; Dronnet *et al*. 2004). Two additional markers, one from *R. flavipes* (*Rf11-2*; Vargo 2000) and one from *R. santonensis* (*RS93*; Dronnet *et al*. 2004) had to be discarded because they were difficult to score and had spurious bands. Polymerase chain reaction (PCR) amplifications were performed as described in Dronnet *et al*. (2004). PCR products were separated by electrophoresis on 6% polyacrylamide gels run on a LI-COR 4000 L sequencer. Alleles were scored using the computer program GENE PROFILER 4.03 (Scanalytics, Inc.).

Genetic data analysis

We first examined whether the collection points belonged to the same colony or not. We compared the genotypic frequencies between all pairs of collection points by means of a log-likelihood (G) based test of differentiation using the program GENEPOP on the Web (Raymond & Rousset 1995; http://wbiomed.curtin.edu.au/genepop/index.html). The overall significance was determined via Fisher's combined probability test. A Bonferroni correction was applied to account for multiple comparisons. Samples from two collection points were considered to belong to different colonies when genotypic differentiation was statistically significant, and grouped into the same colony when it was not. Admittedly, this procedure can lead to false assignments in cases in which genetic contrasts among colonies are small, such as in populations with low overall genetic diversity or high genetic diversity within colonies. However, G-tests have proven useful and are widely used to delineate colonies of social insects, which is an indispensable step for performing further analyses (Vargo 2003a, b; DeHeer & Vargo 2004).

We carried out this G-test analysis with collection points over a large spatial scale, then with multiple collection points within the extensively sampled sites, and finally among the colonies from each population to verify the genetic differences.

Once colony boundaries were defined, we investigated the breeding system of single colonies. We classified colonies as simple or extended families by comparing the observed genotypes of workers within colonies with the genotypes expected in these types of societies by using standard criteria for termites (Bulmer *et al*. 2001; Goodisman & Crozier 2002; Vargo 2003a, b; DeHeer & Vargo 2004). First, colonies could be classified as *simple families* when workers had genotypes consistent with being the direct offspring of one pair of reproductives, and when the observed frequencies of the genotypes did not differ significantly from those expected under Mendelian segregation of alleles from two parents. Significance was determined by a G-test ($P < 0.05$) combined across all loci. Second, colonies could be considered as *extended families* when the genotype distributions within colonies were not consistent with being produced by a single pair of reproductives (e.g. more than four genotypes at a locus or three or more homozygote genotypes), or genotype frequencies deviated significantly from those expected in simple families.

Genetic relatedness among workers was estimated for each colony and averaged over colonies of the same site using the computer program RELATEDNESS 5.00 (Queller & Goodnight 1989). The standard errors of the means were obtained by jackknifing over colonies. For the allelic frequencies and the average relatedness estimates, colonies were weighted equally. The Oléron and Paris regions were analysed separately because we found some genetic differentiation between these distant sites (see Results), which would tend to inflate genetic relatedness among nestmates. Because inbreeding and/or spatial genetic differentiation increase measures of genetic relatedness above what is caused by close pedigree links (Pamilo 1985, 1989), we also calculated an inbreeding-adjusted estimate of relatedness

r^* that better reflects the number of reproductives present in each colony by using Pamilo (1985) formula $r^* = r - [2F_{IT}/(1 + F_{IT})]/[1 - 2F_{IT}/(1 + F_{IT})]$.

The breeding system and genetic differentiation among colonies were further investigated with hierarchical *F*-statistics, assuming the infinite allele model and with individuals nested in colonies (Wright 1951; Weir & Cockerham 1984; Weir 1996). Again, the Oléron region and Paris were analysed separately. The hierarchical analysis was performed with the computer program FSTAT 2.9.3.2 (Goudet 1995; http://www2.unil.ch/izea/softwares/fstat.html). Our *F*-statistics followed the notation of Thorne *et al*. (1999), with the subscripts I, C and T representing the individual, colony, and total components of genetic variation, respectively. The 95% confidence intervals were obtained by bootstrapping over loci 15 000 times, and the significance of the coefficients was further tested by permuting alleles among individuals within colonies for F_{IC}, alleles among colonies for F_{IT}, and finally genotypes among colonies for F_{CT} (1000 permutations). In this special application at the colony level, the overall inbreeding coefficient (F_{IT}) reflects the deficiency of heterozygotes because of nonrandom mating within the total samples in Oléron region and Paris, respectively. F_{CT} estimates the amount of genetic differentiation (allele frequency differences) among colonies. F_{IC} provides information on the number of reproductives and relatedness among them. It is expected to be strongly negative in simple families headed by a pair of reproductives (Thorne *et al*. 1999). F_{IC} approaches zero with increasing numbers of reproductives and becomes positive in various cases: if there is assortative mating among multiple reproductives within colonies or if workers come from genetically differentiated colonies which have either fused together or which share foraging tunnels in common (Thorne *et al*. 1999). We inferred the likely breeding system of *R. santonensis* by comparing our empirical *F*-statistics and relatedness values to those of Thorne *et al*. (1999) and Bulmer *et al*. (2001) generated by computer simulations.

We investigated whether there was isolation by distance (a positive correlation between genetic differentiation and geographical distance) by calculating F_{ST} between pairs of collection points within large colonies using the computer program FSTAT 2.9.3.2, and by testing the significance of the correlation between $F_{ST}/(1 - F_{ST})$ and the logarithm of geographical distances (Slatkin 1993; Rousset 1997) with Mantel tests (Mantel 1967). We used the computer program GENEPOP, with 10 000 permutations, and the Mantel correlation coefficient r was obtained with MANTEL for Windows 1.15 (http://life.bio.sunysb.edu/morph/soft-mult.html).

We tested whether the populations from Oléron and Paris were genetically differentiated in a three-level hierarchical *F*-statistics, with individuals nested in colonies and colonies nested in region (Oléron or Paris, respectively), as implemented in the computer program GENETIC DATA ANALYSIS (Lewis & Zaykin 2000). The confidence interval of the F_{ST} between Oléron and Paris was determined by bootstrapping over loci.

Finally, we compared the number of alleles per locus between the two regions using a *t*-test, and we estimated gene diversity (Nei 1987) in the Oléron region and Paris city using FSTAT. The significance of the difference in gene diversity was estimated by permuting colonies between the two regions, as implemented in FSTAT (1000 permutations, two-tailed tests).

Results

Colony identification

The samples from the seven collection points of the Oléron region were all genetically differentiated (*G*-test of differentiation between pairs of collection point, all $P < 0.0001$), indicating that they belonged to different colonies (Fig. 1a).

The analysis at the smaller spatial scale, in the Saumonards site, revealed that workers from all of the 24 collection points belonged to a single colony that encompassed a very large area of almost 90 000 m² (Fig. 1b and 280 × 320 m, *G*-test, all $P > 0.05$). This area appeared to be a distinct entity, because just outside of this sampling area we could not find stumps containing termites. In contrast, workers from the 12 collection points in the La Gautrelle site belonged to four distinct colonies of much smaller size (Fig. 1b, *G*-test, $P > 0.05$ among two and eight collection points, respectively, while $P < 0.0001$ among all other collection points).

Hence, in the Oléron region, 12 colonies were identified because they were genetically differentiated from each other (*G*-test, all $P < 0.05$) and were included in further analyses of the breeding system and colony genetic structure.

Within Paris, there was significant differentiation among most of the 11 collection points from buildings (*G*-test: $P < 0.0001$), and these collection points were therefore considered to belong to nine different colonies. Indeed, some collection points (17th arrondissement: 17a and 17b; 8th arrondissement: 8b and 8c, see Fig. 2) in buildings were not genetically differentiated and were considered to belong to two colonies spreading over at least 190 and 175 m, respectively (*G*-test, $P = 0.53$ and 0.72). Surprisingly, neither 8b nor 8c were differentiated from another collection point (8a) located 2 km west (*G*-test, $P = 0.89$ and 0.82, Fig. 2). The distant collection point 8a was nevertheless considered to be a different colony, because it is very unlikely that it was connected to 8b and 8c by foraging tunnels in such a complex urban environment.

At a smaller scale, 14 trees along Boulevard de la Madeleine were attacked by a single large colony that spreads over 30 000 m², as revealed by nonsignificant differentiations among pairs of collection points (*G*-test, all $P > 0.05$, full triangles). Termites from a single tree in the middle of this colony were genetically differentiated from

all others (*G*-tests, $P < 0.0001$) and probably belonged to a distinct colony (Fig. 2b, open circle). Interestingly, some of the distant collection points from trees were not genetically differentiated. The 14 collection points along Boulevard de La Madeleine were not significantly differentiated from the two collection points further along the boulevard 1200 m away (Fig. 2b, open triangles). Therefore, there were five distinct colonies from trees along the boulevard. We also should notice that the collection point isolated in the middle of the large colony in La Madeleine (Fig. 2b, open circle) was not significantly differentiated from the three collection points 8a, 8b and 8c. These distant collection points were treated as separate colonies because of the presumed lack of connection to the genetically similar colonies. Hence, 14 colonies were identified in Paris and were used in further analyses.

Colony breeding system and genetic structure

Despite the presence of up to nine and six alleles per locus in the Oléron and Paris populations, respectively (Table 1), we found at most four alleles at any of the markers in all the sampled colonies. Despite the low variability of the loci, more detailed analyses revealed that worker genotypes were inconsistent with simple families comprised of pairs of primary reproductives and their offspring. In the Oléron region, nine of the 12 colonies had more than four worker genotypes at one to four loci. The three other colonies had genotypes consistent with simple families, but the genotype distributions were significantly different from the ones expected if workers were the offspring of a pair of reproductives (*G*-test across all loci, $P < 0.05$). In the city of Paris, 12 of the 14 colonies had more than four genotypes, and frequently with as many as seven to 10 genotypes at one or more loci. Another colony had workers showing two homozygote and two heterozygote genotypes while three alleles were present, and the last one had genotype frequencies deviating significantly from expected in simple families (*G*-test across all loci, $P < 0.0001$). Together, these results indicate that all sampled colonies in both Oléron and Paris had more than two reproductive individuals that most likely were the descendants of two reproductives.

The extended family structure was confirmed by the relatedness and *F*-statistic estimates. Somewhat surprisingly, the average relatedness among colonymates (r) was as high as 0.62 in colonies from the Oléron region and 0.32 in colonies from the city of Paris (Table 2). Both values differ significantly from zero. However, the high relatedness value in Oléron is largely caused by inbreeding and/or spatial genetic differentiation, which results in a high and significant F_{IT}. Indeed, the inbreeding-adjusted estimate of relatedness (r^*) was small in both regions, suggesting that many neotenics reproduce in each colony (Table 2).

The F_{CT} estimates were significantly greater than zero in the two regions, as revealed by the confidence intervals generated by bootstrapping over loci (Table 2) and by permutation tests ($P < 0.001$). A significant genetic differentiation among colonies was expected, as genetic dissimilarity of collection points was a criterion for establishing colony boundaries. However, the F_{CT} estimates were very high, indicating a strong genetic differentiation between colonies. F_{IT} estimates over all loci were high and significantly positive in both Oléron and Paris (Table 2; permutation tests, $P < 0.001$). Such a high deficit of heterozygotes overall indicates nonrandom mating and inbreeding at the population level. Interestingly, the F_{IC} estimates were close to and not significantly different from zero in Paris (Table 2; the confidence interval overlaps with zero, and $P = 0.50$ in the permutation test). A small positive F_{IC} was also found in the Oléron region, but the permutation test indicated that it was just significant ($P = 0.032$), suggesting a slight deficit of heterozygous within colonies. The low F_{IC} values suggest that there are many breeders in each colony, and that mating occurs almost at random within colonies. Interestingly, relatedness, F_{CT}, and F_{IT} were significantly lower in Paris than in Oléron, as demonstrated by the non-overlapping confidence intervals, all of which are consistent with higher numbers of neotenics in the Paris colonies.

Isolation by distance

There was significant isolation by distance among collection points within two of the three largest colonies from the small spatial scale in the two regions. Specifically, there was a significant positive correlation between geographical distance and genetic differentiation between pairs of collection points in the colonies of the Saumonards (Fig. 3A; Mantel test: $n = 24$, $r = 0.20$, $P = 0.01$) and Boulevard de la Madeleine (Fig. 3B; Mantel test: $n = 14$, $r = 0.22$, $P = 0.033$), but not in the colony of La Gautrelle (Mantel test: $n = 8$, $r = -0.051$, $P = 0.41$).

Table 2 *F*-statistics, worker relatedness estimates (r) and inbreeding-adjusted relatedness estimates (r^*) in Oléron and Paris. Confidence intervals of 95% are shown in parentheses. The sample size n refers to the number of colonies studied in each population

	F_{IC}	F_{IT}	F_{CT}	r	r^*
Oléron region ($n = 12$)	0.032 (–0.017–0.079)	0.386 (0.308–0.449)	0.366 (0.308–0.449)	0.618 (0.564–0.672)	0.138
Paris ($n = 14$)	–0.001 (–0.025–0.022)	0.168 (0.084–0.262)	0.169 (0.082–0.272)	0.322 (0.259–0.385)	0.048

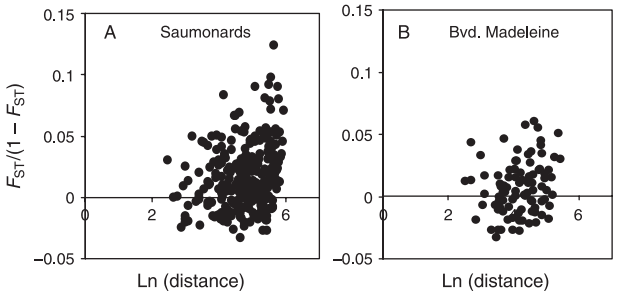

Fig. 3 Isolation-by-distance analysis within the two largest colonies. The relationship between pairwise estimates of $F_{ST}/(1 - F_{ST})$ and geographical distance is shown between pairs of collection points (stumps) within the colony of Saumonards in Oléron ($r = 0.20$, $P = 0.01$, Mantel test) and between pairs of collection points (alignment trees) within the colony of Boulevard de la Madeleine in Paris ($r = 0.22$, $P = 0.033$, Mantel test).

Genetic diversity in natural and urban regions

There was significant genetic differentiation between the Oléron region and Paris ($F_{ST} = 0.102$, 95% CIs = 0.040–0.221). The number of alleles was significantly higher in Oléron region than in Paris (Table 1, *t*-test, one-tailed, $P = 0.03$). However, the level of gene diversity H_S was not significantly different between the two populations ($H_S = 0.34$ and 0.42 for Oléron and Paris, respectively; permutation test, two-sided, $P = 0.31$).

Discussion

The genetic data indicate that all the study colonies were headed by large numbers of inbred neotenic reproductives. The genotype distribution within colonies strongly suggests that there were always more than two breeders. However, the presence of at most four alleles in each colony is compatible with the hypothesis that all breeders from a colony descend from a single pair of primary reproductives. The *F*-statistics are fully consistent with colonies being headed by multiple neotenic reproductives inbred for several generations. Taken together, the high and significant F_{IT}, F_{CT} very close to F_{IT}, and F_{IC} close to zero indicate that there are many related breeders in each colony, which results in significant genetic differentiation among colonies and inbreeding. The low inbreeding-adjusted relatedness estimates confirms that there are many breeders in each colony. Moreover, we observed many neotenic reproductives when sampling workers, up to two dozen in some single collection points, even in covered galleries on tree bark. When compared to the results of simulations corresponding to various types of breeding systems (Thorne *et al*. 1999; Bulmer *et al*. 2001), our relatedness and *F*-statistics coefficients match best with the situation in which mating occurs among multiple neotenics (from 10 to 200) who are descended from a pair of primary reproductives and then mate within colonies for at least three generations (e.g. $F_{IT} = 0.34$, $F_{CT} = 0.34$ and $F_{IC} = 0.00$ for 200 females and 100 males; Thorne *et al*. 1999).

Our finding of only neotenic-headed colonies contrasts with the variability of breeding systems in other subterranean termites. In the related North American species *Reticulitermes flavipes*, a population from central North Carolina contains some 75% of colonies consisting of simple families, about 25% contain low numbers of neotenic reproductives descended from simple families and 1–2% are mixed families (Vargo 2003a, b; DeHeer & Vargo 2004; Vargo & J. Carlson, unpublished); whereas in a Massachusetts population, most colonies contain numerous neotenics, about 33% are simple families and about 10% are mixed families (Bulmer *et al*. 2001). The frequency of simple families was also variable in French populations of *Reticulitermes grassei* (Clément 1981; DeHeer *et al*., unpublished). In *Coptotermes formosanus*, 90% of colonies infesting structures in Nagasaki, Japan, consisted of simple families (Vargo *et al*. 2003). In a previous study of the La Coubre forest population of *Reticulitermes santonensis* based on a single polymorphic allozyme marker, Clément (1981) reported a high proportion of colonies with worker genotypes consistent with simple families. The difference between these previous results and our results might be caused by a shift in colony genetic structure over time, to geographical differences, or to the higher resolution of multiple microsatellite loci over a single allozyme locus.

The prevalence of neotenic reproduction in *R. santonensis* is reflected by data on demography and seasonal changes in caste proportions. At the time of swarming, the proportion of brachypterous nymphs, which molt into neotenics, was much higher than nymphs with long wing buds, which develop into alates (Vieau 1996). Interestingly, the alate stage is also relatively rare in *R. flavipes* colonies from the extreme northern edge of its distribution in the United States, where the species is presumably introduced, which suggests that the nymphs in this region also develop into neotenics (Esenther 1969; Grace 1996). In contrast, neotenics did not exceed 0.1% of the biomass in *R. flavipes* colonies from Mississippi (US) while nymphs with long wing buds attained 13% of the biomass and showed two peaks of abundance in early fall and early summer (Howard & Haverty 1981). Similar changes in the breeding system between native and introduced populations have been found in three Australian *Coptotermes* species. Functional neotenics are infrequent in the native range, but appear to be the means by which colonies grow and spread in introduced populations of New Zealand (Lenz & Barrett 1982).

Our data indicate that in both natural and urban regions, colonies of *R. santonensis* can be spatially expansive, in accordance with a mark–release–recapture experiment which has revealed that the foraging range of a single colony exceeded 1000 m² (Paulmier *et al*. 1997). Occurrence of large

and populous colonies is consistent with the presence of extended families headed by numerous neotenics. A recent laboratory study using isolated groups of workers showed that *R. santonensis* workers could differentiate into male and female neotenics in 5 months and produce eggs 3 months later (Kutnik *et al.*, unpublished). Even if individual neotenic female lays eggs at a lower rate than primary queens, the combined reproductive capacity of large numbers of neotenics can far exceed that of a single queen (Myles & Nutting 1988; Thorne 1998; Long *et al*. 2003; Grube & Forschler 2004). With a large population size, colonies can form extensive networks of underground tunnels leading to the simultaneous exploitation of numerous food resources.

We found significant isolation by distance among the collection points within the two largest colonies, indicating that the workers within expansive colonies are not genetically homogeneous. This suggests that there were spatially separated reproductive centres, among which there was somewhat limited movement of workers and neotenics. In spatially expansive colonies, nymphs might be released from pheromonal inhibition (reviewed in Henderson 1998), permitting their differentiation into secondary reproductives and hence the development of daughter satellite nests (Myles 1999). Whether such daughter nests can completely separate from the mother colony and become autonomous colonies remains to be determined, but so far genetic data have not supported the view that budding is a common mode of reproduction in *Reticulitermes* spp. (Bulmer & Traniello 2002; Vargo 2003a; DeHeer & Vargo 2004).

The nondifferentiation of some distant colonies in the urban population and the significantly lower *F*-statistics values in Paris than in Oléron suggest that humans contribute to the dispersal and fragmentation of colonies in the city. This phenomenon has already been reported in the invasive species *Coptotermes formosanus* in which a lack of significant viscosity was found in introduced populations in New Orleans, Hawaii, and southern Japan (Husseneder *et al.*, unpublished; Vargo *et al*. 2003). Moreover, swarming is still observed in Paris (Lohou, personal observation), so a few alates of *R. santonensis* might occasionally disperse over long distances and colonize new areas. In *R. flavipes* populations in central North Carolina, long-range mating flights appear responsible for the extensive gene flow observed in this region at scales of 1–20 km (Vargo 2003a).

Finally, we found a moderate level of genetic differentiation between Paris and Oléron regions, no doubt because the populations are too far apart to permit natural gene flow. The higher number of alleles in the Oléron region than in Paris is consistent with the hypothesis that *R. santonensis* was first introduced to western France, from which it was then transported to eastern and northern cities. Studies involving more populations will be needed to test this scenario.

The results of this study, in combination with the intra- and interspecific patterns of variation in breeding systems in other subterranean termites, suggest an association between the prevalence of reproduction through neotenics and the invasion of new habitats with harsh climatic conditions. The diverse and flexible breeding systems found in subterranean termites might pre-adapt them to invade new or marginal habitats. In France, where *R. santonensis* is likely to have been introduced, colonies tend to be very large and have high numbers of neotenics, which probably facilitates the spread of colonies by human transport and contributes to the ecological success of *R. santonensis* in urban areas. A study using mitochondrial DNA and microsatellite markers is currently being conducted to establish conclusively whether *R. santonensis* is an introduced form of *R. flavipes*, and if so, whether there has been a significant change in the breeding system from the population of origin. Identifying the population of origin, whether it be North America or somewhere else, will allow comparative studies of the breeding systems to determine if the success of *R. santonensis* in France and other places is caused by the characteristics of the breeding system in the original population, or is a consequence of the introduction and subsequent colonization in these new areas. In this regard, studies of other *R. santonensis* populations, such as in Santiago and Valparaiso, Chile, or in Hamburg, Germany, as well as other introduced populations of *R. flavipes*, such as in Toronto, Canada, should help answer these questions.

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