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Trail-Following Pheromones in Basal Termites, with Special Reference to *Mastotermes darwiniensis*

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Abstract In the framework of an evolutionary study, trail pheromones have been studied in the most basal extant termite, *Mastotermes darwiniensis* (Mastotermitidae), and two other basal termites, the Termopsidae *Porotermes adamsoni* (Porotermitinae) and *Stolotermes victoriensis* (Stolotermitinae). Although workers of *M. darwiniensis* do not walk in single file while exploring a new environment under experimental conditions and are unable to follow artificial trails in 'open field' experiments, they do secrete a trail-following pheromone from their sternal glands. This unique behavior might reflect a primitive function of communication of the sternal gland. The major component of the pheromone appears to be the same in the three basal species: the norsesquiterpene alcohol (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol. This represents a new chemical category of trail-following pheromones for termites. The quantity of pheromone was estimated as 20 pg/individual in *M. darwiniensis*, 700 pg/individual in *P. adamsoni*, and 4 pg/individual in *S. victoriensis*. The activity threshold was 1 ng/cm in *M. darwiniensis* and 10 pg/cm in *P. adamsoni*. In *M. darwiniensis*, the trail pheromone was secreted by sternal gland 4 and to a lesser degree by sternal gland 3, sternal gland 5 being almost inactive. This study highlighted phylogenetic relationships between the Mastotermitidae and two subfamilies of the Termopsidae, the Porotermitinae and the Stolotermitinae. Furthermore, it indicated a heterogeneity within the Termopsidae, with Porotermitinae and Stolotermitinae on one hand, and Termopsinae on the other. Finally, Mastotermitidae and Termopsidae, with C14 trail pheromones, are clearly separated from the Kalotermitidae, Rhinotermitidae, and Termitidae that secrete C12 or C20 trail pheromones.

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Introduction

Pheromones form the mechanistic basis of the organization of foraging in termites, which is the most significant factor affecting their social evolution as emphasized by Traniello and Leuthold (2000) in their review of behavior and ecology of foraging in termites. Termite trail-following pheromones are relatively well known for the Rhinotermitidae and Termitidae of the 'separate life type' (Abe 1987), i.e., for species in which workers forage outside the nest or move from one nesting site to another. In these termites, trail pheromones elicit recruitment and orientation (Traniello 1982; Traniello and Leuthold 2000). Their major component is either an unsaturated straight chain C12 alcohol or a diterpene hydrocarbon (Pasteels and Bordereau 1998; Wobst et al. 1999; Peppuy et al. 2001a, b; Robert et al. 2004; Sillam-Dussès et al. 2005).

In contrast, little is known about those termites that feed on wood in which they also nest ('one-piece life type'; Abe 1987) and, a priori, may have less need for a trail pheromone, but some species of the one-piece nesting termite *Prorhinotermes* may leave their nests (Roisin et al. 2006). The first trail-following pheromone of termites was nonetheless demonstrated in a 'one-piece life type' dampwood termite, *Zootermopsis nevadensis*, (Lüscher and Müller 1960; Stuart 1961), and originally identified incorrectly as hexanoic acid by Hummel and Karlson (1968) and Karlson et al. (1968). The true pheromone structure was recently identified as 4,6-dimethyldodecanal (Bordereau et al. 2006), which is chemically different from trail pheromones of more advanced termites. According to Stuart (1963, 1967, 1969), trail pheromones of the 'one piece life type' termites possibly should be considered more as alarm signals eliciting excitation and recruitment for the defense of the colony. The sternal gland secretion also may have other functions, such as a fungistatic activity (Rosengaus et al. 2004).

In the framework of an evolutionary study on the strategies of chemical communication in termite foraging, the behavioral and chemical properties of sternal gland secretions were investigated in basal termites. The sternal gland, a small ectodermal abdominal gland, is the only glandular source for trail pheromones (Noirot 1969).

We report here the results obtained on *Mastotermes darwiniensis*, which represents the most basal lineage among extant termites, although it exhibits a mixture of primitive and advanced characters, especially with the presence of a true worker caste. *Mastotermes darwiniensis*, endemic to northern Australia, is a unique living representative of a family of termites distributed worldwide in the past. Its phylogenetic relationships with the subsocial cockroach *Cryptocercus* often have been emphasized, but they remained controversial (Thorne 1990, 1991; Nalepa 1991; Grandcolas 1996; Grandcolas and Deleporte 1996; Nalepa and Bandi 2000; Nalepa and Lenz 2000; Lo et al. 2000, 2003; Eggleton 2001) until Inward et al. (2007) showed from a molecular phylogenetic study that *Cryptocercus* is indeed the sister group of termites. *Mastotermes darwiniensis* is the only extant termite whose workers possess three sternal glands located on sternites 3–5. All other termites possess only one sternal gland located on the fourth or fifth sternite. The architecture of its subterranean nest is not well known, but colonies typically feed on multiple trees over extensive areas, requiring extensive subterranean galleries (Miller 1993; Goodisman and Crozier 2002). This termite is, therefore, of an intermediate ecological life type, suggesting

that it might use a trail pheromone. However, a trail pheromone has not been demonstrated previously in this basal termite. Colonies of *M. darwiniensis* in natural woodlands are relatively moderate in size, but huge colonies composed of several million individuals can be found in disturbed habitats, such as urban areas, plantations, and the like, where they may cause severe damage (Hill 1942; French 1986).

We also extended our study to two other basal termites with uncertain phylogeny, *Porotermes adamsoni* (Termopsidae, Porotermitinae) and *Stolotermes victoriensis* (Termopsidae Stolotermitinae; Emerson 1942, 1947; Kambhampati and Eggleton 2000). There are only three species of Porotermitinae in the world; their present geographical distribution (Chile, South Africa, Australia) suggests a Gondwanian origin from the Triassic period. *Porotermes adamsoni*, a unique representative of Porotermitinae in Australia, is considered a relict species. This dampwood termite from south-eastern Australia, generally considered as a 'one-piece ecological life type' termite, although, according to Nkunika (1988), its pseudergates are able to build subterranean galleries and can move from one piece of wood to another. Its colonies are composed of a few thousand to tens of thousands of individuals (Mensa-Bonsu 1976; Lenz 1994). *Stolotermes victoriensis* shares much of its range with *P. adamsoni* in mountainous regions of south-eastern Australia. Its phylogenetic position remains controversial. Although generally considered a basal termite, the configuration of the genitalia would indicate a closer relation of Stolotermitinae with Kalotermitidae, Rhinotermitidae, and Termitidae rather than with Termopsinae and Hodotermitidae (Klass et al. 2000). *Stolotermes victoriensis* is also a 'one-piece life type' termite with pseudergates. Its colonies are composed of a few hundred small individuals.

Thus, the principal objectives of this research were threefold: (a) to investigate whether trail pheromones were present and secreted from the sternal glands in the basal termites *M. darwiniensis*, *P. adamsoni*, and *S. victoriensis*; (b) to identify, synthesize and confirm by bioassay any trail pheromones arising from such studies; and (c) to correlate these results with termite phylogeny.

Materials and Methods

Insects

Mastotermes darwiniensis termites were collected in the field in Australia (Darwin in the Northern Territory and Townsville in Queensland), or were reared in the laboratory in Canberra (ACT) and in Dijon (France). The termites used in Canberra were collected in the Northern Territory and reared in metal drums in climate-controlled rooms (30°C, 90% RH). Most termites used in Dijon came from a colony founded in Australia and taken in 1973 to the Federal Institute of Testing of Materials (BAM) in Berlin (Germany; M. Lenz) and maintained in Dijon since 1999 (28°C, 80% RH). Incipient colonies founded in Darwin in 1999 from swarming imagos, then reared in Dijon, were also used. Workers of *M. darwiniensis* are of large size (9–10 mm long). They possess three sternal glands, of about 0.006 mm^3 for the first two, and a maximum of 0.001 mm^3 for the third.

Porotermes adamsoni termites were collected from stumps or logs of *Eucalyptus* in the Brindabella Mountains near Canberra. They were used in Canberra for trail-following bioassays shortly after collection, or later in Dijon where they were maintained in a climate-controlled room (26°C, 60% RH). Pseudergates of this species also are of large size (8 mm long) and their unique sternal gland is well developed (about 0.03 mm^3).

Stolotermes victoriensis is a termite colonizing wet and decayed logs in southeastern Australia. Pseudergates are 6.5 mm long, and the volume of their sternal gland is about 0.001 mm^3. The termites were collected in the Brindabella Mountains near Canberra, and then maintained in Dijon until use.

Pheromone Extracts

The pheromone was extracted by using two procedures. (a) Whole bodies or sternal glands of workers or pseudergates were immersed in different purified solvents (pentane, hexane, dichloromethane, or methanol) for 12 h at 4°C. After removing workers or glands, extracts were stored at −20°C until used for bioassays or chemical analyses. Sternal glands were prepared by dissecting the glands from cold anesthetized individuals under a stereomicroscope with microscissors and forceps. They were extracted in 2 ml of solvent for 500 glands. (b) Cold anesthesized termites were artificially induced to expose their sternal glands under a stereomicroscope by stretching the abdominal segments with a forceps. A Supelco 65 μm-fiber for solid phase microextraction (SPME) was gently rubbed on the sternal glands. Different types of fibers were tested; the polydimethylsiloxane/divinylbenzene type gave consistently best results. Surface chemicals from 30 individual termites were accumulated on the fiber before thermal desorption. Controls were carried out by rubbing other intertergal membranes or tergites with the same type of SPME fiber. The fiber was desorbed in the injection port of a gas chromatograph for 3 min for gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses.

Fractionation of extracts of sternal glands of *M. darwiniensis* and *P. adamsoni* was carried out by preparative GC (Hewlett-Packard HP 6890 GC) connected to a Gerstel preparative fraction collector, using an HP5 capillary column (30 m×0.32 mm ID) (0.25-μm film thickness). The GC effluent was trapped in 120-μl glass U-tube traps cooled in liquid nitrogen. Fractions were collected from a total of 150 individuals, the SPME fiber being desorbed in the GC after rubbing the sternal gland of 30 individuals. The biological activity of fractions was tested in trail-following bioassays.

Cuticular Hydrocarbons

Cuticular hydrocarbons were obtained by extracting 100 workers in 5 ml hexane for 1 h at 4°C, then purifying the extract on silica gel columns (hexane elution).

Chemical Analyses

GC and GC-MS analyses were carried out with a 5973N Mass Selective Detector coupled to a 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) fitted with a split-splitless injector and a DB™-Wax column (30 m×0.32 mm ID, 0.5 μm film thickness, J&W Scientific, Folsom, CA, USA) or an Equity 5 column (30 m×0.32 mm ID, 0.25 μm film thickness, Supelco). Columns were heated from 40 to 240°C at 5°C min^{-1}. Helium was used as carrier gas at a constant velocity of 37 cm/s. The temperature of the injector was set to 250°C. The column was interfaced directly to the ion source of the mass spectrometer through a heated transfer line maintained at 250°C. Electron-ionization (EI) mass spectra were obtained at 70 eV with the instrument scanning from *m/z* 29 to 450, and the source maintained at 230°C.

Positive chemical ionization (PCI) mass spectra were generated with ammonia as reagent gas (PCI-NH_3). The instrument was scanned from *m/z* 60 to 300 in a 0.7 s cycle.

Quantification of the Pheromone

Because SPME is not a satisfactory quantitative method of extraction, the quantity of pheromone was estimated from a comparison of GC peaks of the pheromone present in sternal gland extracts and of the standard. Due to the limited biological material available in France, the measurements were made on two extracts of 500 sternal glands in *M. darwiniensis* and 100 sternal glands in *P. adamsoni* and *S. victoriensis*. Therefore, it was not possible to use statistical analyses. These qualitative estimates were then compared to the data from the trail-following bioassays.

Synthesis of (*E*) and (*Z*)-2,6,10-Trimethyl-5,9-undecadien-1-ol

Racemic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol [containing 1.5% (*Z*)-isomer] was prepared by way of the Darzens condensation of geranylacetone (Fluka, Sydney) with ethyl chloroacetate followed by hydrolysis of the intermediate ester, thermal decarboxylation, and $LiAlH_4$ reduction of the resultant aldehyde, as adapted from the method of Kulesza and Gora (1969). (*Z*)-2,6,10-Trimethyl-5,9-undecadien-1-ol [containing 2% (*E*)-isomer] was prepared similarly from nerylacetone (Fluka, Sydney).

Trail-Following Bioassays

Bioassays were carried out either in Canberra with termites freshly collected from the field or in Dijon with laboratory-reared termites. All bioassays were performed in climate-controlled rooms (30°C, 80% RH) under red light. The orientation activity elicited by extracts was assayed with two types of bioassays.

(a) Trail extracts were tested with a 'Y open field' trail-following bioassay on Whatman no.1 filter paper discs (15-cm diameter), with a 120° angle between each branch. On the Y stem (3 cm) and one of the Y branches (7 cm), an artificial trail was drawn with a microliter syringe containing 1 μl of extract per cm of trail. Another extract or solvent as a control was deposited under the same conditions on the base and the other branch of the Y. One termite was placed inside a holding chamber consisting of a small plastic vial (55 mm ID), with the 2-mm wide opening being located at the base of the Y. The distance traveled by the insect on the trail was measured. The activity threshold for an extract was defined as the minimum concentration that elicited termites to travel a mean distance of more than 3 cm, the maximum response being 10 cm. Each termite and each trail were used only once to prevent any effects from behavioral conditioning or trail reinforcement.

(b) Trail extracts were also tested with a 'T maze' bioassay. T-shaped artificial trails were drawn on a Whatman no.1 filter paper and covered with a T tunnel formed in a perspex block laid on the filter paper disc. The T base was 3 cm long, and the branches 6 cm long. The termites were thus guided by the tunnel and had the choice between the left and the right branches.

Exploratory Behavior of a New Environment

The behavior of termites while exploring a new environment was observed by using an 'open field' design: a piece of wood containing a few hundred individuals was deposited at

the center of a sheet of filter paper (40×60 cm) covering the bottom of a plastic container. Observations of termites exploring their surroundings were made under red light.

Statistical Analyses

Results of trail-following bioassays in the open field design were analyzed with a *t* test, whereas those in the T-maze were analyzed with a Kruskall-Wallis or χ^2 test. In this latter case, results were considered significantly different when choices were ≥21/9 for N=30 (significance set at 0.05).

Results

Exploratory Behavior and Trail-following Pheromone

Mastotermes darwiniensis On a clean arena, workers of *M. darwiniensis* explored their environment individually and in tandem and were never seen moving in single file as do termites known for secreting a trail pheromone (Fig. 1a). Moreover, in an open field

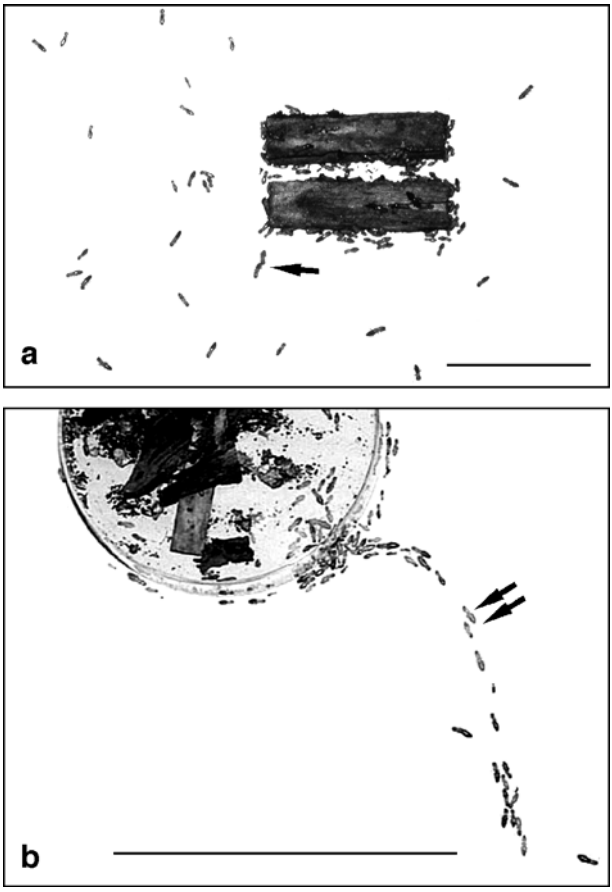

Fig. 1 **a** Exploratory behavior of termites in a new environment. Deposited at the center of a clean container, workers of *M. darwiniensis* scatter over the whole surface and explore their environment individually or in tandem (*arrow*) (scale bar=10 cm). **b** Under the same experimental conditions as for workers of *M. darwiniensis*, pseudergates of *P. adamsoni* explore their environment in single files (*double arrows*), showing the use of a trail pheromone and mass recruitment (scale bar=10 cm)

situation, workers were unable to follow artificial trails made from extracts of whole workers or sternal glands, irrespective of the solvent used for extraction. Even at a concentration of five sternal glands/cm of trail, trail-following did not reach the activity threshold of 3 cm (mean±SD: 1.9±0.5 cm, N=30). These observations raised doubts whether *M. darwiniensis* was secreting a trail-following pheromone. However, T-maze bioassays (Table 1) showed that the workers of *M. darwiniensis* were able to distinguish a trail used by nestmates from a control trail. When given a choice between a control trail and a trail previously followed by at least 50 nestmates, workers preferentially chose the previously used trail, indicating that workers actively or passively mark the substrate when moving.

Workers of *M. darwiniensis* also were able to follow an artificial trail made of hexane extracts of whole workers of at least five individuals/cm of trail in a 'guided' context, indicating a chemical origin of the 'marking' (for 30 tests, 28 choices for the trail made from worker extracts, 2 choices for the control trail made from hexane). Moreover, when given a choice between a trail made with extracts of whole workers without sternal abdominal segments and a trail made from sternal glands 3–5, workers preferentially chose the trail made with sternal gland extracts (26 choices out of 30 tests). In contrast, a trail consisting only of cuticular hydrocarbons of workers was not followed (for 30 tests, 18 choices for the trail made from cuticular hydrocarbons at a concentration of 1 worker equivalent/cm, 19 choices at a concentration of 5 worker equivalent/cm). These results suggest that *M. darwiniensis* secretes a trail-following pheromone from its sternal glands as do other species of termites.

Extracts of sternal gland 4 were about five times more active than those of sternal gland 3 (Table 2). This result was confirmed with a choice test between trails made from extracts of sternal gland 4 and sternal gland 3, at a concentration of 1 gland/cm; workers clearly preferred the trail with the sternal gland 4 extract. Trail-following was not observed for extracts of sternal gland 5, probably due to its very small size.

Porotermes adamsoni In contrast to the individual exploratory behavior of *M. darwiniensis*, pseudergates of *P. adamsoni* explored a new environment in single file with individuals following each other closely (Fig. 1b). This behavior suggested the use of trail pheromone. In contrast also to *M. darwiniensis*, pseudergates of *P. adamsoni* were able to follow artificial trails in an open field situation, secreting a very active trail pheromone from their abdominal sternal gland. The biological activity threshold of trails made from sternal gland was 10^{-2} gland/cm (mean±SD: 7±0.7 cm, N=30). Trail-following was optimal at 10^{-1} and 1 gland/cm (mean: 10 cm, n=30, and reduced at higher concentrations (5 sternal gland/cm; mean±SD: 7.5±0.5 cm, N=30). This bell-shaped dose-curve or concentration-response is characteristic of pheromones.

Table 1 Results of a T-maze trail-following bioassay. Workers of *M. darwiniensis* are able to recognize a trail previously used by at least 50 nestmates. (S: result statistically significant, NS: result statistically non significant, N=30)

Number of Passages	Number of Choices		χ^2 test
	Control	Trail	
25	14	16	NS
50	8	22	S
100	9	21	S
250	6	24	S
1,000	4	26	S

Table 2 Comparison of the trail-following activity of sternal glands 3 and 4 in *M. darwiniensis*

	Trail Concentration	1 Weq/cm	0.5 Weq/cm	0.25 Weq/cm	0.1 Weq/cm
Choices	Sternal gland 3 vs hexane	26 vs 4 (S)	22 vs 8 (S)	17 vs 13 (NS)	17 vs 13 (NS)
	Sternal gland 4 vs hexane	28 vs 2 (S)	–	–	26 vs 4 (S)
	Sternal gland 3 vs Sternal gland 4	7 vs 23 (S)	–	–	–

Extracts of sternal gland 4 (active at 0.1 Weq/cm) were at least five times more active than extracts of sternal gland 3 (active at 0.5 Weq/cm)

S, result statistically significant, *NS*, result statistically non significant, *Weq*, worker equivalent, *N*=30

Stolotermes victoriensis Pseudergates of *S. victoriensis* also followed artificial trails in the open field bioassay. Bioassays showed that the sternal gland was the only glandular source of the trail pheromone. Optimal trail-following was elicited at a concentration of 10^{-1} gland/cm of trail.

Interspecific trail-following bioassays between *M. darwiniensis*, *P. adamsoni*, and *S. victoriensis*

Interspecific trail-following bioassays showed positive responses between *M. darwiniensis*, *P. adamsoni*, and *S. victoriensis*. In a T-maze bioassay, workers of *M. darwiniensis* followed an artificial trail made from extracts of sternal glands of *P. adamsoni* pseudergates (Table 3). Moreover, when given a choice between trails prepared from extracts of *M. darwiniensis* or *P. adamsoni* sternal glands, workers of *M. darwiniensis* preferentially chose the trails of *P. adamsoni* (Table 3).

Pseudergates of *P. adamsoni* always chose the *P. adamsoni* trail when given a choice between *M. darwiniensis* and *P. adamsoni* sternal gland extract trails (Table 3). However, in 'open field' assays, they were able to follow artificial trails made of *M. darwiniensis* sternal gland but to a lesser degree than their own trails (Table 4).

In T-maze bioassays, pseudergates of *S. victoriensis* were not able to distinguish a trail of *Mastotermes* sternal gland extract from their own (Table 3), and in an open field assay, they followed a *Mastotermes* trail better than their own (Table 4).

In sum, these results suggest the presence of a trail-following pheromone component common to *M. darwiniensis*, *P. adamsoni,* and *S. victoriensis*.

Table 3 Interspecific trail-following in a T-maze bioassay

	Number of Individuals Following Each Trail				
Tested Species	Hexane	*P. adamsoni*	*M. darwiniensis*	*S. victoriensis*	χ^2
M darwiniensis	5	25	–	–	S
M. darwiniensis	–	24	6	–	S
P. adamsoni	–	30	0	–	S
S. victoriensis	–	–	17	13	NS

Workers of *M. darwiniensis* were able to follow trails of sternal gland extracts of *P. adamsoni* and that they preferentially chose trails made from extracts of *P. adamsoni* rather than their own trails

Pseudergates of *P. adamsoni* preferred their own trails to trails of *M. darwiniensis*. Pseudergates of *S. victoriensis* followed their own trails and trails of *M. darwiniensis* without preference (concentration of trails: five sternal glands/cm; *N*=30)

S, result statistically significant; *NS*, result statistically non significant

Table 4 Results of interspecific 'open-field' trail-following bioassays

Tested Species	Trail Following in cm (mean ± SD)			
	Hexane	*P. adamsoni*	*M. darwiniensis*	*S. victoriensis*
P. adamsoni	0	8.4±0.5	–	–
	0	–	5.1±0.5	–
S. victoriensis	0	–	10	–
	0	–	–	6.9±1

Pseudergates of *P. adamsoni* and *S. victoriensis* were able to follow trails of sternal glands of *M. darwiniensis* (N=30, Concentration of trails: five sternal glands/cm)

Chemical Nature of the Trail Pheromone

The existence of a compound common to the three species was confirmed by GC-MS after SPME of the surface of the sternal glands (Fig. 2). It was present in relatively high quantities in *P. adamsoni*, and in lesser amounts in *M. darwiniensis* and *S. victoriensis*, where it was detected only by selected ion monitoring to increase sensitivity. Moreover, it partly co-eluted with another compound in *M. darwiniensis*, but its presence was confirmed in two samples of 500 dissected sternal glands.

For *M. darwiniensis* and *P. adamsoni*, the accumulated SPME extracts of the sternal gland surfaces of 150 individuals were separated into three fractions by preparative GC. Fraction F1 from retention times 3 to 29.40 min comprised the most volatile compounds; fraction F2 from RT 29.40 to RT 29.60 min contained only the common compound specific to the sternal gland surface; fraction F3 from RT 29.60 to RT 48 min contained the less volatile compounds, essentially cuticular hydrocarbons from C23 to C28 for *M. darwiniensis*, and from C22 to C30 for *P. adamsoni.* Results in Table 5 show that workers of *M. darwiniensis* followed only the

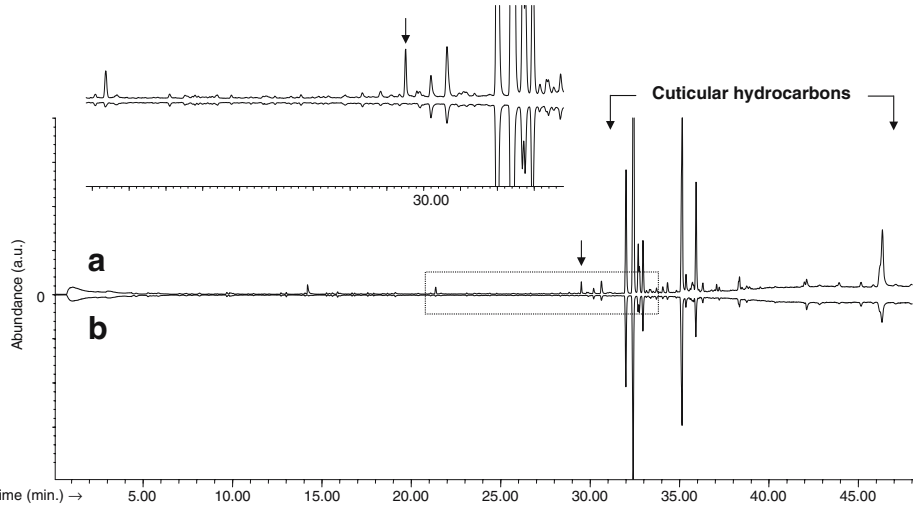

Fig. 2 Comparison of the GC profiles of SPME extracts of (*a*) the surface of sternal gland 5, and (*b*) the extra-glandular abdominal integument of 50 pseudergates of *P. adamsoni*. The majority of peaks are common to both surfaces and correspond to cuticular hydrocarbons from C22 to C30. However, the peak marked by an arrow (RT 29.5 min) is specific to the glandular surface. Detail of the window in the upper part of the figure

Table 5 Trail-following activity of preparative GC fractions of SPME extracts of the sternal gland surface from 50 individuals in *M. darwiniensis* and *P. adamsoni*

	Extracts of *M. darwiniensis* Conc: 0.5×10^{-1} ng/cm (n=30)			Extracts of *P. adamsoni*					
				Conc: 10^{-1} ng/cm (n=30)			Conc: 0.5×10^{-1} ng/cm (n=30)		
Tested species	F1/ hexane	F2/ hexane	F3/ hexane	F1/ hexane	F2/ hexane	F3/ hexane	F1/ hexane	F2/ hexane	F3/ hexane
M. darwiniensis	18/12 (NS)	21/9 (S)	15/15 (NS)	19/11 (NS)	29/1 (S)	17/13 (NS)	–	–	–
				Open field trail-following (cm; n=15)					
P. adamsoni	14/16 (NS)	21/9 (S)	18/12 (NS)	1.3±0.3	5.9±0.9	1.5±0.4	1.8±0.4	10	7.8±1.1

Fraction F1 (0 to 29.40 min); Fraction F2 (29.40 to 29.60 min)=compound specific to the sternal gland surface; Fraction F3 (29.60 to 48 min)=cuticular hydrocarbons; concentration of 10^{-1} ng/cm

Conc, concentration of the trail; *S*, result statistically significant; *NS*, result statistically non significant

trails made of fraction F2 prepared either from *M. darwiniensis* or from *P. adamsoni*. Trail-following was better with trails made from the *P. adamsoni* fraction F2.

In the same manner, pseudergates of *P. adamsoni* followed only the trails made from fraction F2 of the *M. darwiniensis* sternal gland extracts (Table 5). In open field bioassays with trails made of sternal gland extracts of *P. adamsoni*, only the trails made with fraction F2 were active in eliciting trail-following in pseudergates of *P. adamsoni* when tested at the concentration of 10^{-1} ng/cm. They were also the most active when tested at higher concentration (5×10^{-1} ng/cm), eliciting maximal trail-following, but it was noted that at this concentration, fraction F3 containing the cuticular hydrocarbons was also active (Table 5).

The common sternal gland compound of the three species had a retention index (RI) of 2161 on a polar DB-Wax column, and a RI of 1593 on a nonpolar Equity 5 column. Its EI mass spectrum (Fig. 3) suggested a terpenoid structure similar to that of 3,7,11-trimethyl-6,10-dodecadien-1-ol (Bergström et al. 1968). The molecular ion M^+ of *m/z* 210 was confirmed by positive ion chemical ionization (PCI/NH_3), which displayed an MH^+ ion of *m/z* 211. These results suggested that the compound might be the norsesquiterpene homolog of 3,7,11-trimethyl-6,10-dodecadien-1-ol, i.e., 2,6,10-trimethyl-5,9-undecadien-1-ol, which was confirmed by the synthesis of the molecule. The synthetic (*E*)-isomer of 2,6,10-trimethyl-5,9-undecadien-1-ol possessed the same EI mass spectrum and RI (RI 2163 on DB-Wax, and 1598 on Equity 5) as the natural compound. The (*Z*)-isomer was resolved clearly from the (*E*)-isomer (RI 2137 on DB-Wax, and 1572 on HP5). No trace of (*Z*)-2,6,10-trimethyl-5,9-undecadien-1-ol was detectable in extracts of *M. darwiniensis* and *P. adamsoni*.

Biological Activity of Synthetic (*E*) and (*Z*) Isomers of 2,6,10-Trimethyl-5,9-undecadien-1-ol

Mastotermes darwiniensis (*E*)-2,6,10-Trimethyl-5,9-undecadien-1-ol elicited significant trail-following at a concentration of 1 ng/cm of trail, whereas trail-following was reduced beyond a concentration of 100 ng/cm (Table 6). Surprisingly, similar results were obtained

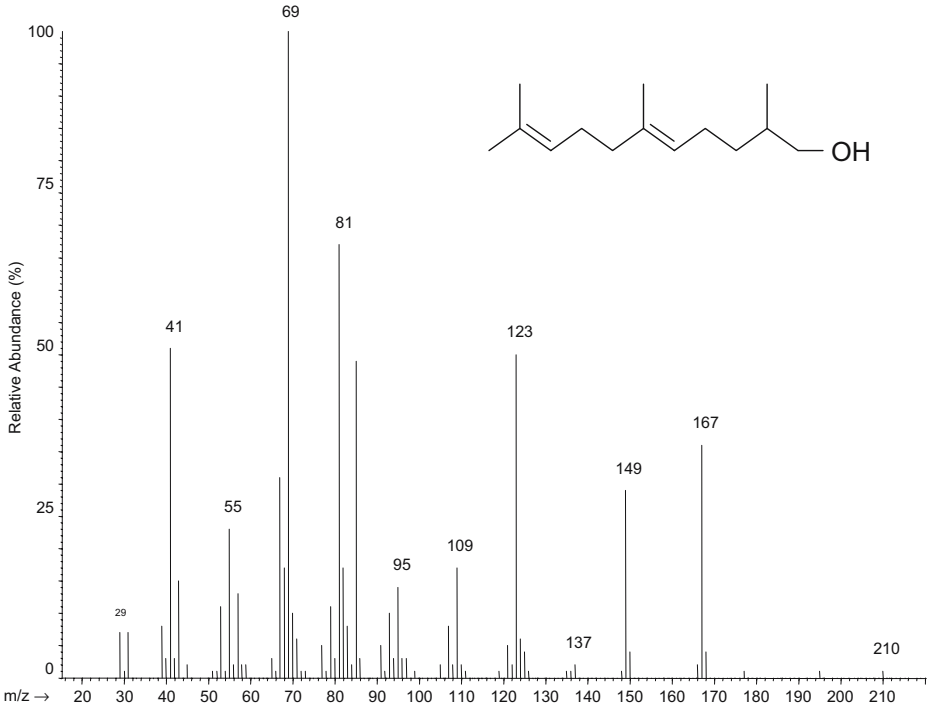

Fig. 3 EI mass spectrum of the compound specific to the sternal gland surface, identified as (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol

with the (*Z*)-isomer for the threshold activity, but higher concentrations of this isomer did not induce a reduction in trail-following. Thus, with the (*Z*)-isomer, we did not obtain the classical bell-shaped curve of responses in relation to pheromone concentration. In addition, a mixture of the two isomers was more active than the (*E*)-isomer, the activity threshold being obtained from 10^{-2} ng/cm.

Table 6 Choice of trails made with synthetic (*E*)- and (*Z*)-isomers, and a mixture of both isomers of 2,6,10-trimethyl-5,9-undecadien-1-ol for *M. darwiniensis* (T-maze trail-following bioassays)

Concentration	(*E*) Isomer Hexane	(*Z*) Isomer Hexane	(*E*)+(*Z*) Isomers Hexane
10^{-3} ng/cm	54/36 NS (n=90)	57/33 NS (n=90)	26/34 NS (n=60)
10^{-2} ng/cm	57/33 NS (n=90)	51/39 NS (n=90)	48/12 S (n=60)
10^{-1} ng/cm	57/33 NS (n=90)	51/39 NS (n=90)	46/14 S (n=60)
1 ng/cm	115/25 S (n=150)	81/9 S (n=90)	48/12 S (n=60)
10 ng/cm	69/21 S (n=90)	69/21 S (n=90)	54/6 S (n=60)
100 ng/cm	38/22 NS (n=60)	48/12 S (n=60)	48/12 S (n=60)
1,000 ng/cm	34/26 NS (n=60)	–	–

S, result statistically significant; *NS*, result statistically non significant

Porotermes adamsoni For this species, the isomers were tested in open-field bioassays (Fig. 4). With the (*E*)-isomer, we obtained an activity threshold at 10^{-2} ng/cm, optimum trail-following at 10^{-1} ng/cm, a reduction in trail-following beyond 1 ng/cm, and no response at 1 μg/cm. With the (*Z*)-isomer, the activity threshold was only reached at a concentration of 1 ng/cm and a concentration of 100 ng/cm elicited extensive trail-following. A mixture of both isomers elicited trail-following similar to that obtained with the (*E*)-isomer alone, with an activity threshold at 10^{-2} ng/cm, optimal trail-following between 10^{-1} and 1 ng/cm, and a reduction in trail-following beyond 10 ng/cm.

Stolotermes victoriensis The activity of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol could not be tested with this species, due to the death of the colony maintained in France.

Quantification of (*E*)-2,6,10-Trimethyl-5,9-undecadien-1-ol in Sternal Gland Extracts

For *M. darwiniensis*, the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was estimated to be about 20 pg per individual. This was confirmed with bioassays: artificial trails made from five sternal glands/cm were more active than trails of 10 pg of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol/cm, as active as those of 100 pg/cm and less active than those of 1 ng/cm (Table 7).

For *P. adamsoni*, the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was estimated by GC analysis to be about 700 pg/individual, 35 times more than in *M. darwiniensis*. Bioassays confirmed this result. Trails made of five sternal glands/cm were more active than trails of 1 ng of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol/cm, as active as trails of 3.5 ng/cm, and less active than those of 10 ng/cm (Table 7).

For *S. victoriensis*, the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was estimated to be 4 pg/individual, five times less than in *M. darwiniensis* and 175 times less than in *P. adamsoni*. This was confirmed by trail-following bioassays. Pseudergates of *S. victoriensis* were not able to distinguish their own trails of 5×10^{-1} sternal gland/cm from

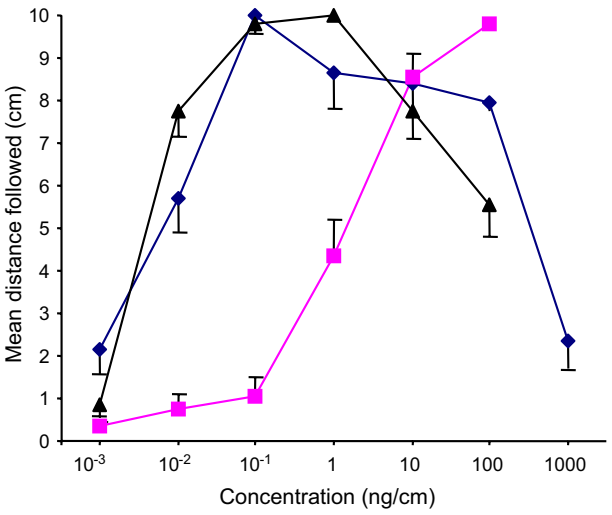

Fig. 4 Trail-following activity of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol (—◆—), (*Z*)-2,6,10-trimethyl-5,9-undecadien-1-ol (—■—), and a mixture of both isomers (—▲—) for pseudergates of *P. adamsoni* ('open-field' trail-following bioassay, *N*=30)

Table 7 Comparison of the trail-following activity of trails made from sternal gland extracts (*M. darwiniensis* or *P. adamsoni*), and synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol

Trail	Sternal Gland/cm	(*E*)-2,6,10—Trimethyl-5,9—undecadien-1-ol (ng/cm)					χ^2 test
Trail concentration	5	10^{-2}	10^{-1}	1	3.5	10	
Number of choices for *M. darwiniensis*	28	2	–	–	–	–	S
	11	–	19	–	–	–	NS
	7	–	–	23	–	–	S
Number of choices for *P. adamsoni*	24	–	–	6	–	–	S
	17	–	–	–	13	–	NS
	4	–	–	–	–	26	S

Results confirm the quantitative estimation of about 20 pg of pheromone per worker in *M. darwiniensis*, and 700 pg of pheromone per pseudergate in *P. adamsoni*

S, result statistically significant; *NS*, result statistically non significant; $N=30$

trails of 10^{-1} sternal gland of *M. darwiniensis*/cm (17 choices for the *S. victoriensis* trail, 13 choices for the *M. darwiniensis* trail, $N=30$).

Whereas the quantity of trail pheromone in individual sternal glands of *P. adamsoni* was 175 times greater than in *S. victoriensis*, it was not possible in the present study to evaluate the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol in their natural trails by direct measurement.

Discussion

The major roles of the trail-following pheromones in termites are recruitment and orientation. For example, in species of the 'one-piece life type', the pheromone alerts and recruits termites to repair a breach in the nest; in species of the 'separate life type', it also induces recruitment and orientation of foragers outside the nest (Traniello 1982; Hall and Traniello 1985; Traniello and Leuthold 2000). However, we have shown for 'separate life type' species (Pasteels and Bordereau 1998; Wobst et al. 1999; Peppuy et al. 2001a, b) and in this study for the 'one piece' species *P. adamsoni* that both ecological types were able to orientate on artificial trails in an open field experiment. Under the same experimental conditions, the situation was shown to be different for *M. darwiniensis*, which showed no tendency to follow open field trails, independent of the solvents used for extracting the pheromone or the concentration of the trail. Furthermore, whereas pseudergates of *P. adamsoni* form single files of individuals to explore a new environment, just as do workers of 'separate life type' species, workers of *M. darwiniensis* explore the same new environment individually or in tandem. Therefore, the first aim of our study was to know whether the most ancestral extant termite, *M. darwiniensis*, secreted a trail-following pheromone.

Our observations showed that workers of *M. darwiniensis* use a trail-following pheromone secreted from their sternal glands. However, this termite remains the only known species in which a trail pheromone alone does not elicit orientation. Thus, in addition to a trail pheromone, this termite requires a physical constraint, such as a tunnel or gallery, to guide its movement. The question then arose as to whether this unique situation was due to the intrinsic behavior-eliciting properties of the pheromone or to other specific termite behavior. Results obtained with *P. adamsoni* showed that the nature and the

concentration of the pheromone were not responsible for this special behavioral trait of *M. darwiniensis*. Thus, *P. adamsoni* used the same trail pheromone as *M. darwiniensis*, and it was readily able to follow artificial trails made from sternal glands of *M. darwiniensis* in open field assays, whereas the same trails were not followed by workers of *M. darwiniensis*. The unique exploratory behavior of *M. darwiniensis* workers that forage individually or in tandem but not in single file might represent a specific stage in the evolution of the recruitment strategies in termites. Indeed, according to Traniello and Leuthold (2000), mass recruitment might have evolved in social insects from a communication of tandem running type. The ancestral function of the sternal gland in termites, that is the secretion of semiochemicals that mediate the tandem running of swarming imagos at the time of the nuptial promenade, would have evolved in derived species to induce mass recruitment and orientation of foragers. The unusual formation of worker tandems as observed in *M. darwiniensis* during exploratory behavior, therefore, supports this evolution.

Our observations also highlight the uniqueness of *M. darwiniensis* regarding the glandular source of its trail pheromone. Whereas for all other termite species the trail pheromone is secreted by only one sternal gland, it is secreted by at least two sternal glands (sternal glands 3 and 4) in *M. darwiniensis*, which is the only termite known to possess three sternal glands (Noirot 1969). Extracts of sternal gland 4 were much more active than those from sternal gland 3, whereas extracts from sternal gland 5 had minimal activity. Therefore, *M. darwiniensis* might represent an intermediary taxon in a regressive evolution from lost ancestors with numerous metameric glands to the present species of termites that possess only one sternal gland located on sternite 4 or 5. The higher secretory activity of sternal gland 4 of *M. darwiniensis* is significant, given the position of the sternal gland on sternite 4 in the other basal termite families Termopsidae and Hodotermitidae.

The identification of the trail-following pheromone of the ancestral termites *M. darwiniensis, P. adamsoni,* and *S. victoriensis* was the second major aim of our study. We first showed that these three species secreted the same trail pheromone, which was identified as the norsesquiterpene alcohol (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol. This is a new structure for termite trail pheromones. Until now, only unsaturated C12 aliphatic alcohols and a C20 hydrocarbon have been identified as termite trail pheromones: (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol in numerous Rhinotermitidae and Termitidae (Matsumura et al. 1968; Tokoro et al. 1989, 1991; Laduguie et al. 1994; Pasteels and Bordereau 1998; Wobst et al. 1999), (3*Z*,6*Z*)-3,6-dodecadien-1-ol in *Ancistrotermes pakistanicus* (Termitidae Macrotermitinae; Robert et al. 2004), (*Z*)-3-dodecen-1-ol in several Termitidae (Peppuy et al. 2001a, b), and the diterpene hydrocarbon neocembrene in the Rhinotermitidae, Prorhinotermitinae (Sillam-Dussès et al. 2006), and in the Termitidae, Nasutitermitinae (Birch et al. 1972; MacDowell and Oloo 1984).

(*E*)-2,6,10-Trimethyl-5,9-undecadien-1-ol is known in the perfumery industry for its aromas of lemon and lily of the valley (Gora and Antczak 1980) but has never been identified previously in animals. However, an isomer of this compound, (*E*)-4,6,8-trimethyl-7,9-undecadien-5-ol, is secreted from sexual glands of female *Cryptocercus punctulatus* (Le Quéré et al. 1991) and *C. kyebangensis* (Grandcolas et al. 2001), subsocial cockroaches considered as sister taxa of *M. darwiniensis* (Eggleton 2001; Lo et al. 2003). Furthermore, a similar compound, 3,7,11-trimethyl-6,10-dodecadien-1-ol, is secreted by numerous species of bumblebees as a signal sex pheromone, and ants as a trail-marking pheromone (Francke and Schulz 1999; Luxova et al. 2004).

The biosynthesis of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol probably arises from mevalonate or farnesyldiphosphate (Prestwich 1981; Luxova et al. 2004; Hojo et al. 2007), whereas C12 alcohol pheromones probably arise from fatty acid biosynthesis,

especially from linoleic acid or oleic acid (Francke and Schulz 1999; Morgan 2004; Robert et al. 2004). The trail-following pheromone of *M. darwiniensis* and the Termopsidae *P. adamsoni* and *S. victoriensis* therefore represents a new chemical class of pheromones in termites.

The question arose as to whether (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was the sole compound of the trail pheromone or only its major compound. The pheromones of insects are generally composed of blends, and several observations suggested the existence of multicomponent pheromones in termites (Kaib et al. 1982; Traniello 1982; Hall and Traniello 1985; Pasteels and Bordereau 1998; Traniello and Leuthold 2000; Peppuy et al. 2001a, b). In *M. darwiniensis* and *P. adamsoni*, (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was the only specific compound detected from the surface of the sternal glands by SPME analysis. The GC fraction containing only (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was also the only fraction that elicited trail-following. These data suggested that the trail pheromone of *M darwiniensis, P. adamsoni*, and *S. victoriensis*, is comprised solely of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol. Nevertheless, trail-following bioassays performed at high concentrations showed that the GC fraction following that of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol and containing cuticular hydrocarbons also elicited trail-following, although to a lesser degree. This could be explained by the presence of an additional undetected component of the trail pheromone, but not by the presence of the (Z)-isomer of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol that is more volatile than the (*E*)-isomer. We also demonstrated that cuticular hydrocarbons from workers were inactive in eliciting trail-following in *M. darwiniensis*. More likely, it is the result of a tailing peak of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol, as has been observed previously for dodecatrienol or dodecenol after preparative GC in termite species that secrete these compounds as a trail pheromone (Peppuy et al. 2001a; Bordereau, unpublished results).

For *M. darwiniensis*, the activity threshold of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was obtained from 1 ng/cm of trail, i.e., in the high value range known for the activity of termite trail pheromones (Pasteels and Bordereau 1998; Sillam-Dussès 2004). However, this threshold might be dependent on the absolute configuration of the compound, as has been demonstrated previously in insects (Mori 1998) and especially in bumblebees for 3,7,11-trimethyl-6,10-dodecadien-1-ol (Luxova et al. 2004). The chirality of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol in *M. darwiniensis* has not yet been determined. For *P. adamsoni*, (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol elicited trail-following from 10^{-2} ng/cm of trail, a threshold typical of many other termite trail pheromones, for example with dodecenol in Macrotermitinae (Peppuy et al. 2001a; Sillam-Dussès 2004).

The third contribution of our study concerns phylogeny. Although chemical communication in termites appears to be highly conserved (Pasteels and Bordereau 1998; Sillam-Dussès 2004; Sillam-Dussès et al. 2006), our study on trail pheromones of ancestral termites highlights some novel phylogenetic data. First, female adults of the subsocial cockroach *Cryptocercus*, a sister taxon of *M. darwiniensis* (Inward et al. 2007), secrete an isomer of the trail pheromone of *M. darwiniensis* from their tergal glands. This would support the phylogenetic relationships between cockroaches and termites, although it also could be a simple phenomenon of convergence because these isomers might arise from different metabolic pathways. Second, whereas the position of the Termopsidae remains controversial from morphological and molecular phylogenetic studies (Eggleton 2001), we showed that the only extant representative of the Mastotermitidae, *M. darwiniensis*, secreted the same trail pheromone as the representatives of two subfamilies of the Termopsidae (Porotermitinae and Stolotermitinae). This supports a basal position of Termopsidae (Thompson et al. 2000) and that the Termopsidae would be socially the most

primitive termites (Thorne and Carpenter 1992; Thorne 1997; Thorne and Traniello 2003). Our results also confirmed the basal position of Stolotermitinae, in contrast to the recent morphological study of Klass et al. (2000) that suggested relationships between Stolotermitinae and Kalotermitidae, Rhinotermitidae and Termitidae. Moreover, our study indicates a heterogeneity within the Termopsidae with the Porotermitinae and the Stolotermitinae in one group and the Termopsinae in the other. Thus, the Termopsidae might not be monophyletic.

The chemical nature of trail pheromones of termites separates the basal termites Mastotermitidae, Termopsidae, and Hodotermitidae with C14 or C18 trail pheromones from the other families (Kalotermitidae, Serritermitidae, Rhinotermitidae and Termitidae) that secrete C12 or C20 trail pheromones (Sillam-Dussès 2004; Sillam-Dussès et al. 2006). This aspect deserves to be studied in other taxa.

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