The Journal of Experimental Biology 214, 956-962

doi:10.1242/jeb.050419

RESEARCH ARTICLE

Comparison of volatile blends and gene sequences of two isolates of *Metarhizium anisopliae* of different virulence and repellency toward the termite *Macrotermes michaelseni*

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Accepted 29 November 2010

SUMMARY

Previously, we reported an interesting relationship between virulence and repellency of different isolates of the fungus *Metarhizium anisopliae* towards the termite *Macrotermes michaelseni*: the higher the virulence of a given isolate, the greater its repellency. In the present study, we compared the volatile profiles of two isolates, one that was more virulent (and repellent) and one that was less virulent (and repellent) to the termite. The prominent components of the two blends were characterized by gas chromatography-mass spectrometry and authenticated by gas chromatography coinjections with synthentic standards. There were both qualitative and quantitative differences between the two blends. The repellencies of synthetic blends of 10 prominent constituents of the volatiles of the two isolates were compared and that of the more virulent isolate was found to be significantly more repellent. Subtractive bioassays were carried out with one of the constituents of each of the two 10-component blends missing at a time to determine its relative contribution to the overall repellency. The results indicated that the repellency of the volatiles of each isolate was primarily due to synergistic effects of a smaller number of constituents. Intraspecific differences between the two isolates were also reflected in their nucleotide sequences.

Key words: *Macrotermes michaelseni*, *Metarhizium anisopliae*, olfactory chemical signature, physiological interaction, ecological interaction.

INTRODUCTION

Termites represent useful models for studying social adaptations to infective pathogens such as bacteria, nematodes, viruses and fungi as these eusocial insects are constantly exposed to these microbes because of their nesting and foraging ecology (Rosengaus et al., 2003; Cremer et al., 2007; Rich et al., 2007). To mitigate disease risks from these microbes in their microenvironments, termites have evolved behavioural, biochemical and immunological adaptions (Rosengaus et al., 1999; Rosengaus et al., 2000; Traniello et al., 2002; Rosengaus et al., 2004). These traits account for the lack of microbial epizootics in nests of termites (Hänel and Watson, 1983; Rath, 2000; Cremer et al., 2007).

Insects often show repeated odour sampling behaviours such as antennal flicking that may be associated with their perception of important volatile signals in their niches (Brandy et al., 1990; Fäldt et al., 1999; Byers, 2008; Witzgall et al., 2010). These volatiles elicit olfactory-guided responses such as attraction or repellency (Zhang and Schlyter, 2004; Saini and Hassanali, 2007; Sullivan and Mori, 2009; Sun et al., 2010). Specific constituents of the volatile blends are perceived in a discontinuous pattern at variable concentrations in space (Vickers et al., 2001; Gikonyo et al., 2003; De Bruyne and Baker, 2008; Myrick et al., 2009). Previously, we showed that the termite *Macrotermes michaelseni* Sjölstedt (Isoptera: Macrotermitidae) detects at a distance the presence of *Metarhizium anisopliae* Solokin (Metschonikof) (Ascomycetes: Hyphomycetes) through olfaction and thus avoids direct physical contact with the fungus (Mburu et al., 2009). A strong positive correlation between virulence and repellency of different isolates of fungi towards the termite *M. michaelseni* was found (Mburu et al., 2009). The inherent potential to detect specific repellent signatures from potentially infective fungi may be a key part of the adaptive repertoire for survival of these insects (Dicke and Grostal, 2001; Myles, 2002; Mburu et al., 2009).

A key objective of the present study was to characterize and compare volatile profiles of the blends emitted by two isolates of *M. anisopliae*, one that was found to be highly virulent/repellent (ICIPE 51) and another that was relatively less virulent/repellent (ICIPE 69). We also carried out subtractive repellency bioassays with synthetic blends of the prominent constituents of the two isolates to identify those that primarily contribute to their repellency. In addition, we compared the genomic DNA of these isolates to explore possible differences in their nucleotide sequences.

MATERIALS AND METHODS

Termite trapping and maintenance

Worker castes of the termite *M. michaelseni* were trapped overnight from a mound located 1612 m above sea level at Kasarani, Nairobi, Kenya (01°13.366′S, 36°53.766′E) using a modified method described by Tamashiro et al. (Tamashiro et al., 1973). The traps were covered with dark polyethylene plastic sheets and transported to the laboratory. Termites were individually collected using a pair of soft forceps and placed in Petri dishes (9 cm diameter) lined with

wet filter papers (Whatman No. 1, 9 cm diameter). A piece of wet cotton wool was used to maintain high humidity in each Petri dish. The lids of the Petri dishes had five aeration holes (2 mm diameter) to ensure free flow of air. Two pieces of sterile cypress wood, *Cupressus lusilanica* Dallimore (~50×30×1.5 mm), and 0.5 g of fungal garden [*Termitomyces* sp. (Basidiomycetes: Agaricatidae)] from the termite mounds were provided as shelter and for food, respectively. The termites were transferred into an incubator (26±2°C, 90±5% relative humidity in the dark) where they were kept for 20 min for acclimatization before the repellency bioassays. The relative humidity in the incubator was controlled using a stable saturated solution of K_2SO_4 (Sigma-Aldrich, Gillingham, Dorset, UK).

Microbiology work

Scaled-up production of dry conidia for volatile collection

Volatile collections required substantial numbers of dry conidia. This was achieved by growing conidia on long white rice substrates following the technique described by Maniania et al. (Maniania et al., 2003). Two kilograms of the rice (Pishori) were soaked in sterilized distilled water for 10 min, rinsed three times and transferred to steel trays (33×25×13 cm). The trays were wrapped with polyethylene autoclave bags and sterilized for 1 h at 121±1°C. The substrates were left to cool at room temperature, after which they were inoculated with 3-day-old cultures of blastospores (50 ml) and thoroughly mixed for complete coverage of the rice with the inocula. The cultures were incubated in a controlled-temperature room (26±2°C, 60–70% relative humidity). After 21 days, the conidial substrates were allowed to dry overnight at room temperature (22–25°C). The conidia were harvested by sifting the substrate through a sieve (295 μm mesh size) into hazard polyethylene bags that were then sealed. Conidia were stored in a refrigerator (4°C) before use and only 1-month-old dry conidia were used for collection of volatiles.

Conidial germination tests

The germination tests were performed on all isolates used in the collection of volatiles. The viability of conidia was determined for each isolate by spread-plating 0.1 ml of conidial suspension at 3×10^6 conidia ml^{-1} on Petri dishes containing Sabouraud dextrose agar (SDA). Four sterile microscopic cover slips (22×22 mm) were placed on each plate. The plates (N=6 per replicate) were sealed with Parafilm®, incubated at 26±2°C and 90±5% relative humidity, and examined after 18 h under a phase-contrast microscope. The percentage germination of conidia was determined from 100 spore counts under cover slips at 400× magnification. Only those conidia with germ tubes twice the length of the conidial diameter were designated as having germinated. The viability of the isolates varied between 85.8 and 99.4%.

Chemical ecology work

Collection of volatiles from isolates of *M. anisopliae*

Two- to three-week-old isolates of *M. anisopliae* that had been previously identified as the most (ICIPE 51) and the least (ICIPE 69) virulent/repellent were used. Forty grams of dry conidia of each isolate were weighed on a balance (Mettler AT 261 Delta, Listers 2000, Bordentown, NJ, USA), put in volatile collection jars (ARS, Gainesville, FL, USA) and tightly sealed with lids. A 3-cm-long filter trap made of Teflon® tubes packed with 3 mg of Super-Q polymer (80–100 mesh size; ARS) as an adsorbent, held in place between two plugs of glass wool, was used. Each trap was cleaned by flushing 1 ml of dichloromethane (HPLC grade, 99.9%, Sigma-Aldrich) through the adsorbent before headspace trapping. Traps were then dried by passing purified nitrogen (BOC Gases, Nairobi, Kenya) through each at a rate of 3 ml m^{-1}. Each trap was then sealed with Teflon® thread tape on both ends to prevent contamination. For storage before use, traps were wrapped with dry aluminium foil.

To collect volatiles using an air entrainment system, a continuous flow at 3 ml m^{-1} of purified medical air (BOC Gases) was cleaned by passing through a carbon filter (ARS) and ultimately into the volatile collecting jars, each of which contained a different fungal isolate. A flowmeter (Aalborg, Orangeburg, NY, USA) regulated airflow into each jar. The adsorbent traps were each then firmly held in place by Teflon® screw caps that were connected to a Teflon® tube at the top of each volatile collecting jar. The fungus-containing jars were tightly sealed with lids to prevent contamination. Air was sucked from the closed system at a rate of 3 ml m^{-1} at one end of each of the filter traps through a Teflon® tube connected to a diaphragm vacuum pump (Wertheim, Baden-Württemberg, Germany). Trapping of the volatile blends was done for 12 h in the dark.

Elution of volatiles from Super-Q

Traps were then removed from the system and adsorbents eluted with 100 μl of dichloromethane into 2 ml vials (Sigma-Aldrich) under ice; to ensure complete elution, purified nitrogen was pushed through the filter traps at 3 ml m^{-1}. The vials with the volatile extract were then tightly sealed with lids, which were then covered with Teflon® tape. The adsorbent traps were thoroughly cleaned as above before they were re-used. In the control setups, the same protocol as described above was followed with the exclusion of the fungus in the volatile collecting jars.

Identification of volatile constituents of isolates of *M. anisopliae*

Gas chromatography

An HP 5890 A series II gas chromatograph (GC) equipped with a flame ionization detector (FID) and an HP 7673 autosampler injector were used to separate the eluted volatile collections (Agilent Technologies, Johannesburg, South Africa). The GC was interfaced to a computer monitor (Dell OptiPlex GX520, Round Rock, TX, USA) using 3365 MSD ChemStation software (G1701EA E.02.00.493, Agilent Technologies), on the screen of which the chromatograms of each analyzed blend appeared. An HP-1 methyl silicone non-polar capillary column measuring 30 m×0.25 mm×0.25 μm (length×internal diameter×film thickness) was employed for separation with sample injections done in splitless mode. The oven temperature program was set at 35°C for 3 min, followed by a temperature rise of 10°C min^{-1} up to 280°C and maintained at this final temperature for 10 min. The injector and detector temperatures were set at 280°C. Nitrogen (BOC Gases) was used as the carrier gas at a flow rate of 1.2 ml min^{-1}. The FID had a mixture of clean (medical) air and hydrogen gas (from a generator; Domnick Hunter, Gateshead, Tyne and Wear, UK) flow at 31 and 405 ml min^{-1}, respectively. A delay of 0.5 min was maintained before injection purging. Before injection of the trapped sample, an equivalent of 0.2262 μg methyl salicylate (Sigma, St Louis, MO, USA) was added (as an internal standard) to 40 μl of each extract of ICIPE 69 and double this amount to each extract of ICIPE 51. A volume of 1 μl of each of the sample was injected for all analyses.

Gas chromatography-mass spectrometry

Volatile eluates of isolates of *M. anisopliae* and of controls were analyzed using an HP 7890 A series GC (Agilent Technologies, Wilmington, DE, USA) coupled to a 5975 C series mass

spectrometer (GC-MS) fitted with an 7683 B series autosampler and a Triple Axis Detector (Agilent Technologies). The GC was equipped with a non-polar capillary column (HP-5 methyl silicone) measuring 30 m×0.25 μm×0.25 μm (length×internal diameter× film thickness) for separation of the constituents. Oven temperature programming was the same as that described for the GC analyses above, and the splitter was turned off for 0.5 min during the experiment. The carrier gas was helium at a constant flow rate of 1.2 ml min^{-1}. For electron impact (EI) of the MS, the ionization voltage was 70 eV and temperature of the ion source and the interface were 230 and 150°C, respectively. The GC was coupled to an HP monitor (L1710) to display chromatographic data, which were evaluated using 3365 MSD ChemStation software (version 2.0; Agilent Technologies). Identification of the constituent compounds was based on comparison with the mass spectral fragmentation patterns provided in the NIST/NIH/EPA mass spectral library (http://www.sisweb.com/software/ms/nist.htm) in ChemStation version 2.0. The identity of each component was confirmed by coinjections with synthetic compounds on an HP 5890 gas chromatograph described above.

Olfactometric assays of synthetic blends

Repellency indices of synthetic blends of 10 prominent constituents of volatiles (relative concentrations varying from 10^{-6} to 10^{-1} μg ml^{-1}) of the two isolates of *M. anisopliae* towards the termite and nine constituents with one of each of the 10-constituent blends subtracted at a time were evaluated using a Y-tube olfactometer in a fume hood as described previously (Mburu et al., 2009). Briefly, six serial dilutions (10^{-6}, 10^{-5}, 10^{-4} 10^{-3}, 10^{-2} and 10^{-1} μg ml^{-1}) of the two 10-component blends in relative proportions found in GC-MS profiles were prepared in acetone (97–99%, Sigma Aldrich). At the onset of each bioassay, Whatman No. 1 filter paper (2×2 cm) was loaded with 50 μl of each of the concentrations and placed in either compartment B or C of the Y-olfactometer at a time. The other compartment functioned as the control, with the Whatman filter paper containing only acetone (50 μl). Acetone was allowed to evaporate (3 min) before introduction of groups of 20 worker termites into compartment A. Nylon gauze (40-wire mesh) separated each of compartments B and C and the arms attached to them to prevent contact between the filter papers and the termites. Glass lids were loosely placed at the end of each of the compartments to prevent escape of the termites. The number of termites in the treatment and the control compartments, together with those in their respective arms (past the 2 cm score line off the intersection) were recorded at an interval of 10 min for 60 min to give six readings for each replicate dose. Protocol for handling the black cloth and cleaning of the olfactometer (after the bioassays) was as described previously (Mburu et al., 2009). After each replicate, the location of treated and control filter papers in compartments B and C were alternated as previously described (Mburu et al., 2009). Repellency assays of each concentration of the blends were replicated six times. The dose-response assays for the two 10-component blends of ICIPE 51 and ICIPE 69 gave 50% repellency indices (RD_{50}) of 0.227 and 0.427 μg μl^{-1} cm^{-2}, respectively. For subtractive assays, each of the 10 constituents was excluded one at a time at these RD_{50} values to give two sets of 10 blends of nine components each. Each of the 9-component blends was then assayed for repellency at the above serial dilutions. In addition, blends of six components of each of the two 10-component blends that were found to make significant contributions to their respective repellency were also assayed as described above.

Molecular biology and biotechnology work

DNA extraction and barcoding

Pure culture of dry conidia of ICIPE 51 and ICIPE 69 were mass-produced on rice as described earlier. Equal amounts (0.1 g) of conidia of each of the isolates were weighed in microcentrifuge tubes on a weighing balance (Mettler AT 261 Delta, Listers 2000). DNA was extracted using a slight modification of the CTAB method described by Doyle and Doyle (Doyle and Doyle, 1990) and resuspended in pre-warmed sterile deionized water. The primer pairs n-SSU-1766-5′ (ITS5) and nu-LSU-0041-3′ (ITS4) (White et al., 1990) were used to amplify the internally transcribed spacers of the genomic DNA. Primer nomenclature follows Gargas and DePriest (Gargas and DePriest, 1996). PCR amplification reactions were carried out in a total volume of 20 μl containing PCR buffer (Genscript, Piscataway, NJ, USA), 2.5 mmol l^{-1} of each dNTP (Genscript), 0.2 μl of each primer, 2.5 mmol l^{-1} of $MgCl_2$, 0.5 units *Taq* DNA polymerase (Genscript) and ~25 ng of genomic DNA. PCR amplification conditions involved initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, annealing temperature of 52°C for 40 s with an extension at 72°C for 1 min and final elongation at 72°C for 10 min. These reactions were carried out on a PTC-100 thermocycler (MJR Inc., Minneapolis, MN, USA). Negative controls without fungal DNA were run for each experiment to check for contamination of reagents.

DNA quantification, purification and sequencing

The amplification products were separated by electrophoresis in agarose gels containing 1% agarose (Fisher Scientific, Loughborough, Leicestershire, UK) run in 1× TAE buffer for 1 h at 70 V cm^{-1}. DNA was stained with ethidium bromide (3–5 μl) and visualized under UV light using a Kodak Gel imaging system (Gel logic 200, Carestream Health, New Haven, CT, USA) on a Dell monitor interfaced with the molecular imaging software. The lengths of the amplicon products were estimated by comparison with 1 kb Smart DNA ladder (Noxo, Tallinn, Estonia). The PCR products were purified using QuickClean DNA gel extraction kit (Genscript) and sequenced using ABI PRISM 3700 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Data analyses

Chemical ecology data

Repellency indices of the 10-, 9- and 6-component blends were calculated (derived) using the formula:

$$\frac{P_{nc} - P_{nt}}{P_{nc} + P_{nt}} \times 100 ,$$

where P_{nc} and P_{nt} represent the mean percentage of worker termites in control and treatment arms, respectively (Wanzala et al., 2004).

In all the tests, stochastic data for repellency were individually pooled before analyses and arcsin-transformed to normality before invoking repeated-measures ANOVA using PROC MIXED in SAS version 9.1 (SAS Institute, 2003). Means were separated using the Student–Newman–Keuls (SNK) test. The RD_{50} values of each of the blend were estimated with repeated-measures logistic regression *via* generalized estimating equations (Throne et al., 1995; Stokes et al., 2000). These analyses were carried out using the GENMOD procedure in SAS version 9.1. The level of significance was set at 5% for all analyses to identify significant differences among the values of RD_{50} for the different synthetic blends.

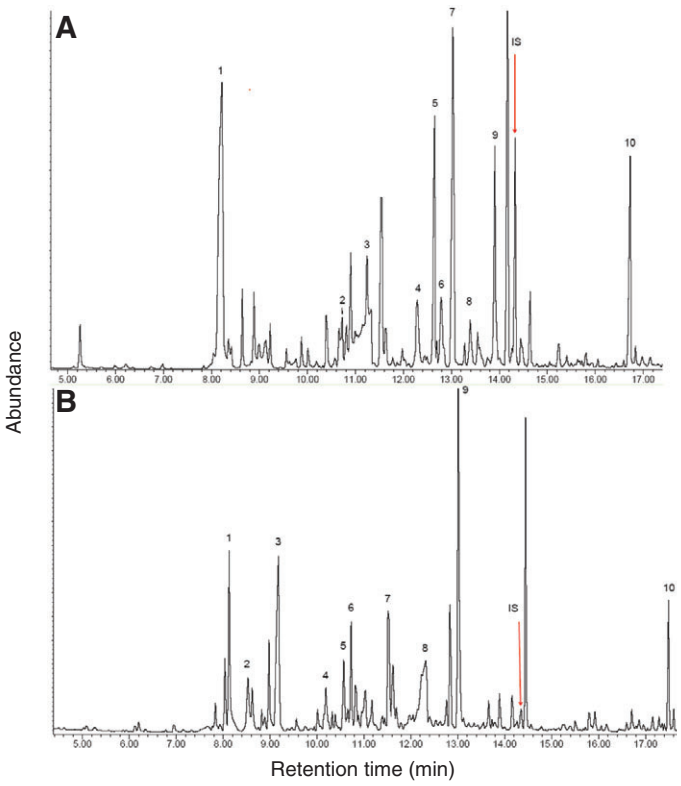

Fig. 1. Representative gas chromatograms of the more repellent (A; ICIPE 51) and less repellent (B; ICIPE 69) isolates of *Metarhizium anisopliae*. IS, internal standard.

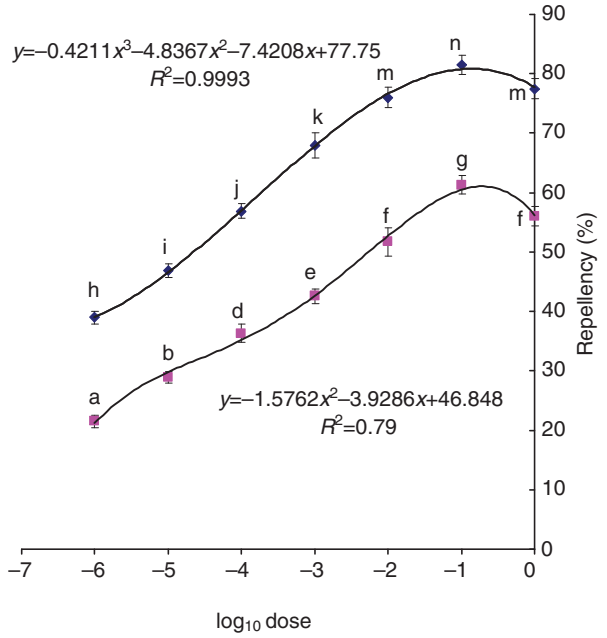

Fig. 2. Repellency dose response of the two 10-component blends of synthetic components of ICIPE 51 (blue diamonds) and ICIPE 69 (pink squares), two *M. anisopliae* isolates. Repellencies represented by the same lowercase letters are not significantly different (SNK test, $P>0.05$).

Molecular biology and biotechnology data

The sequences of the PCR products were edited and aligned using ChromasPro (version 6, Technelysium, Tewantin, QLD, Australia) and Clustal W (version 2.012; Larkin et al., 2007) software, respectively. Sequence divergences were calculated using the Kimura 2-Parameter (K2P) distance model (Kimura, 1980) and bootstrap values were based on 10,000 replicates. Maximum composite likelihood values of the pattern of nucleotide substitution (Tamura et al., 2004) between the two sets of sequences were estimated using *MEGA* version 4 (Tamura et al., 2007). The differences in the composition bias among sequences were considered in evolutionary comparisons with the first, second and third codons and noncoding positions (Tamura, 1992; Tamura and Kumar, 2002). All positions containing gaps were excluded from the data set.

RESULTS

GC and GC-MS analyses

GC profiles of the headspace odour blends from the more repellent (ICIPE 51) and less repellent (ICIPE 69) isolates of *M. anisopliae* are shown in Fig. 1. There were quantitative and qualitative differences in volatile profiles of the two isolates. Ten of the more prominent constituents of the two volatile blends identified by comparison of their mass spectra with those in the NIST/NIH/EPA library, followed by coinjections with authentic standards, are shown in Table 1. Some of the more prominent components of ICIPE 51 were present in smaller amounts in the volatile blend of ICIPE 69.

Repellency-dose responses of different blends

The repellencies of the two 10-component blends were significantly different at all doses (SNK test, $F_{5,173}=214.40$, $P<0.05$, $N=180$), as depicted in Fig. 2 and reflected in their RD_{50} values ($0.227\,\mu g\,\mu l^{-1}\,cm^{-2}$ for ICIPE 51 and $0.427\,\mu g\,\mu l^{-1}\,cm^{-2}$ for ICIPE 69). ANOVA revealed significant differences in repellency among

Table 1. Prominent components identified in the volatiles of the most (ICIPE 51) and least repellant (ICIPE 69) isolates of *Metarhizium anisopliae* and their relative amounts

ICIPE 51			ICIPE 69		
Constituent	Identity	Relative amount (μg)	Constituent	Identity	Relative amount (μg)
1	Hexanol	15.3±0.62	1	Hexanol	6.97±0.06
2	3-Octanone	3.74±0.64	2	N-Ethylacetamide	3.88±0.09
3	Acetic acid	7.5±0.41	3	Butylolactone	9.57±0.19
4	1-Octene	2.89±0.26	4	1-Ethyl-2-methylbenzene	3.76±0.22
5	2-Nonanone	5.36±0.32	5	1-Octen-3-ol	2.90±0.11
6	2-Nonanol	2.58±0.19	6	3-Octanol	4.43±0.44
7	Phenylethyl alcohol	8.29±0.29	7	2-Propyl-1-pentanol	7.64±0.23
8	3-Nonen-2-one	1.99±0.15	8	2-Pyrrolidinone	8.69±0.24
9	Borneol	4.88±0.12	9	Phenylethyl- alcohol	11.36±0.12
10	4,5-Dihydro-5-pentyl-2(3H)furanone	4.14±0.15	10	Cedrene	1.96±0.12

Table 2. RD_{50} values of repellency-dose responses of various blends [10-component (full blend), 9-component and 6-component] of *Metarhizium anisopliae* towards the termite *Macrotermes michaelseni*

ICIPE 51			ICIPE 69		
Composition	RD_{50} (ng μl^{-1} cm^{-2})	Significance	Composition	RD_{50} (ng μl^{-1} cm^{-2})	Significance
10-component [full blend (FB)]	$3.52\ (2.42–4.45)\times10^{2}$	h	10-component [full blend (FB)]	$2.32\ (2.24–2.45)\times10^{3}$	s
FB minus 4,5-dihydro-5-pentyl-2-(3H)furanone	$5.51\ (3.91–9.58)\times10^{-1}$	b	FB minus 2-pyrrolidinone	$7.78\ (6.98–8.25)\times10^{2}$	k
FB minus borneol	$5.64\ (3.16–5.84)\times10^{1}$	c	FB minus butyrolactone	$9.41\ (9.23–9.78)\times10^{2}$	m
FB minus 3-octanone	$8.26\ (7.18–9.24)\times10^{1}$	d	FB minus N-ethylacetamide	$1.19\ (9.81–1.21)\times10^{3}$	n
FB minus 3-nonen-2-one	$1.25\ (1.11–1.57)\times10^{2}$	e	FB minus cedrene	$1.31\ (1.33–1.43)\times10^{3}$	p
FB minus 2-nonanone	$2.02\ (1.82–2.07)\times10^{2}$	f	FB minus phenylethylalcohol	$1.71\ (1.58–1.73)\times10^{3}$	q
FB minus acetic acid	$2.31\ (2.16–2.32)\times10^{2}$	g	2-Propyl-1-pentanol	$1.84\ (1.77–1.96)\times10^{3}$	r
FB minus phenylethylalcohol	$3.58\ (3.83–4.49)\times10^{2}$	h	FB minus 1-ethyl-2-methylbenzene	$2.44\ (2.23–2.58)\times10^{3}$	s
FB minus 2-nonanol	$3.61\ (3.39–5.30)\times10^{2}$	h	FB minus hexanol	$2.57\ (2.51–3.27)\times10^{3}$	s
FB minus hexanol	$3.72\ (3.91–5.82)\times10^{2}$	h	FB minus 3-octanol	$3.71\ (3.17–5.05)\times10^{3}$	s
FB minus 1-octene	$4.69\ (4.25–5.96)\times10^{2}$	h	FB minus 1-octen-3-ol	$4.72\ (4.31–6.93)\times10^{3}$	s
6-component blend	$2.26\ (1.98–3.14)\times10^{-1}$	a	6-component blend	$6.58\ (6.02–6.82)\times10^{2}$	j

RD_{50} values followed by the same letter within a column are not significantly different (Proc GENMOD, $P>0.05$; SNK test, $P=0.0001$). Failure of 95% confidence limits (in parentheses) to overlap was used as the criteria for identifying significant differences among RD_{50} values.

the ten 9-component blends of ICIPE 51 at different doses (SNK test, $F_{5,840}=1945.09$, $P<0.05$, $N=900$). Likewise, the repellency indices of the ten 9-component blends of ICIPE 69 varied significantly (SNK test, $F_{5,840}=1217.79$, $P<0.05$, $N=900$). The repellency indices of the 6-component blend of ICIPE 51 were significantly higher than those of ICIPE 69 at all doses (SNK test, $F_{5,168}=181.76$, $P<0.05$, $N=180$; Fig. 3). The repellency of these two 6-component blends were significantly higher than those of the two 10-component blends at all doses (SNK test, $F_{5,336}=530.77$, $P<0.05$, $N=360$). Likewise, the repellency indices of the two 6-component blends were higher than those of the two 9-component blends at all doses (SNK test, $F_{5,1848}=3189.85$, $P<0.05$, $N=1980$). RD_{50} values of all the blends are shown in Table 2. At the highest concentration of the 6-component and 10-component blends of both isolates of *M. anisopliae*, there appeared to be a small but insignificant drop in the repellency indices (Figs 2, 3). This probably arose from some diffusion of the volatiles into the control arm near the junction of the olfactometer, which would increase with increasing concentrations of the repellent blends.

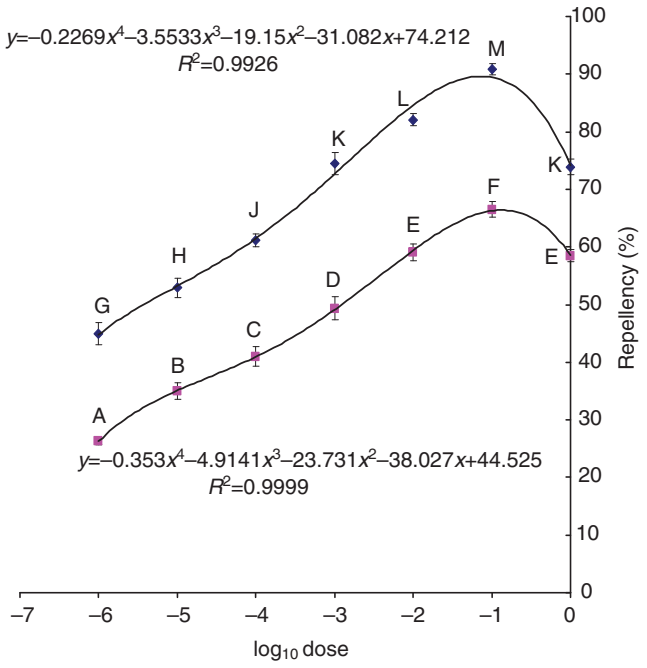

Fig. 3. Repellency dose response of the two 6-component blends of synthetic components of ICIPE 51 (blue diamonds) and ICIPE 69 (pink squares), two *M. anisopliae* isolates. Repellencies represented by the same uppercase letters are not significantly different (SNK test, $P>0.05$).

DNA barcoding

Sequence alignments showed areas of differences corresponding to 449 and 429 bases for ICIPE 51 and ICIPE 69, respectively (Fig. 4). The pairwise genetic distance between the two groups of nucleotides sequence was 0.01, indicating intraspecific divergence (Fig. 4). Instantaneous probability of substitution from one nucleotide base (row) to another (column) for each entry indicates possible point mutations (Table 3). The nucleotide frequencies were 0.254 (A), 0.239 (T/U), 0.246 (C) and 0.261 (G). The transition and transversion rate ratios were $k_1=0$ (purines) and $k_2=17.35$ (pyrimidines), respectively. The overall transition/transversion bias was $R=4.198$, where $R=[(A\times G\times k_1)+(T\times C\times k_2)]/[(A+G)\times(T+C)]$. There were a total of 426 positions in the final data set (Tamura et al., 2007).

DISCUSSION

One of the aims of the present study was to compare the composition and relative repellency (to the termite *M. michaelseni*) of the blend of prominent components of the volatile emission of a more virulent isolate of *M. anisopliae* (ICIPE 51) with that of a less virulent isolate (ICIPE 69). Our results showed both qualitative and quantitative

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution in the ICIPE 51 and ICIPE 69 isolates of *Metarhizium anisopliae*

	A	T	C	G
A	–	*2.3*	*2.36*	**0**
T	*2.43*	–	**40.88**	*2.51*
C	*2.43*	**39.91**	–	*2.51*
G	**0**	*2.3*	*2.36*	–

Entries within a row are comparable. Rates of different transitional and transversional substitutions are shown in bold and italics, respectively.
A, adenine; C, cytosine; G, guanine; T, thymine.

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ICIPE69
TCAACTATAAAAAGTTGGGGGGTTTTACGGCAGTGGACCGCGCCGCGGGCTCCTGTTGCG 60
ICIPE51
TCAACTATAAAAAGTTGGGGGGTTTTACGGCAGTGGACCGCGCCG-GGCTCCTTGTTGCG 59
******************************************** **   * *******
ICIPE69
AGTGCTTTACTACTGCGCAGAGGAGGGCCACGGCGAGACCGCCAATTAATTTAAGGGACG 120
ICIPE51
AGTGCTTTACTACTGCGCAGAGGAGGGCCACGGCGAGACCGCCAATTAATTTAAGGGACG 119
************************************************************
ICIPE69
GCTGTGCTGGAAAACCAGCCTCGCCGATCCCCAACACCAAGTCCCACAGGGGACTTGAGG 180
ICIPE51
GCTGTGCTGGAAAACCAGCCTCGCCGATCCCCAACACCAAGTCC-ACAGGGGACTTGAGG 178
****************************************** ***************
ICIPE69
GGCGTAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGACGGGCGCAATGTGCGTTC 240
ICIPE51
GGCGTAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGACGGGCGCAATGTGCGTTC 238
************************************************************
ICIPE69
AAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTT 300
ICIPE51
AAAGATTCGATGATTCACTGAATTTTGCAATTCACATTACTTATCGCATTTCGCTGCGTT 298
********************** **********************************
ICIPE69
CTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTTTTT--- 357
ICIPE51
CTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTTTTTTTA 358
******************************************************
ICIPE69
AACCACTCAGAAGATACTTATTAAAAAATTCAGAAGGTTTGGGTCCCCGGCGGGCGCGAA 417
ICIPE51
AACCACTCAGAAGATACTTATTAAAAAATTCAGAAGGTTTGGGTCCCCGGCGGGCGCGAA 418
************************************************************
ICIPE69         GTCCCGCCGAA 428
ICIPE51         GTCCCGCCGAA 429
                ***********
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Fig. 4. Sequence alignments of DNA of two *M. anisopliae* isolates, ICIPE 51 and ICIPE 69. Sections of the two sequences marked and unmarked with asterisks indicate homology and divergence, respectively, between the two.

differences in the relative amounts of the constituents of the volatile profiles of the two isolates (Fig. 1A,B; Table 1). Most of the 10 prominent components of ICIPE 51 were present in minor amounts in ICIPE 69. In Y-olfactometric assays, the blend of the 10 prominent components of ICIPE 51 was significantly ($P<0.05$) more repellent ($RD_{50}=0.227\,\mu g\,\mu l^{-1}\,cm^{-2}$) than that of ICIPE 69 ($RD_{50}=0.427\,\mu g\,\mu l^{-1}\,cm^{-2}$). These results confirm our previous finding that the more virulent isolate of *M. anisopliae* is more repellent to the termite than the less virulent isolate (Mburu et al., 2009).

The second aim of the present study was to identify the constituents of the volatiles of the two isolates that contributed to their relative repellency through subtractive bioassays. Subtraction of each of six constituents of ICIPE 51 resulted in a significant decrease in the activities of the resulting blends, with the exclusion of 4,5-dihydro-5-pentyl-2-(3H)-furanone resulting in the largest drop (Table 2). Thus, furanone contributes most to the repellency of the 10-component blends of this isolate. Coincidently, subtraction of each of six constituents of the 10-component blends of ICIPE 69 led to a significant drop in repellency, with exclusion of 2-pyrrolidinone giving the highest decrease. As expected, each of the two 6-component blends was found to be more repellent than the corresponding 10-component blend (Table 2, Figs 2, 3). Our results of blend effects suggest that synergism of some repellent constituents produces mixtures that are more active than linear summations of their individual activities (Bekele and Hassanali, 2001).

Comparison of the nucleotide sequences of the two isolates of the fungus confirms within-species genotypic variation (Hawksworth, 2001; Handelsman, 2004; Tamura et al., 2007; Humber, 2008; Dalziel et al., 2009). It would be interesting to extend this chemical and molecular ecology study to the volatile blends and genomic sequences of the other seven isolates of *M. anisophliae* that have previously shown varying levels of virulence and repellency to *M. michaelseni* (Mburu et al., 2009) (and perhaps other termite species). In addition, phylogenetic comparisons and gene expression studies of all nine isolates (and perhaps others) could shed light on possible relationships between evolutionary distance and virulence and repellency to the termite. Moreover, correlation of genetic differences in different isolates with changes in proteins will help to characterize genes associated with virulence and biosynthesis of the repellent volatile semiochemicals.

ACKNOWLEDGEMENTS

We acknowledge the ICIPE Management, Behavioural and Chemical Ecology Department, the Arthropod Pathology Unit and the Molecular Biology and Biotechnology Department for facilitating this work. The Dutch Programme for Cooperation with International Institutions-SII (grant no. B7102D-031-42389) supported a fellowship (D.M.M.) and this research. We also thank Joel Ltilitan Bargul and anonymous reviewers for technical assistance with the DNA work and positive critiques of this manuscript, respectively. D.M.M. wishes to dedicate this paper to Prof. Ahmed Hassanali, Emeritus Scientist – ICIPE, Professor of Chemistry, Kenyatta University.

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