

Comparison of Volatile Blends and Nucleotide Sequences of Two *Beauveria Bassiana* Isolates of Different Virulence and Repellency Towards the Termite *Macrotermes Michealseni*

D. M. Mburu · N. K. Maniania · A. Hassanali

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Abstract Isolates of the fungus *Beauveria bassiana* have different levels of virulence and repellency against the termite *Macrotermes michaelseni*. In the present study, we compared the volatile profiles and gene sequences of two isolates of the fungus with different levels of virulence and repellence to the termite. Gas chromatography–mass spectrometric analyses showed quantitative and qualitative differences in the composition of volatiles of the two isolates. The repellencies of synthetic blends of 10 prominent components that mimicked the volatiles of each of the two isolates were significantly different. Subtractive bioassays showed that the repellency of each isolate was due to synergistic effects of a few constituents. As previously reported for isolates of *Metarhizium anisopliae*, some differences also were found in the nucleotide sequences of the two isolates of *B. bassiana*, suggesting a genetic basis for

the observed intra-specific differences in their repellency and virulence against the termite.

Keywords *Beauveria bassiana* isolates · *Macrotermes michaelseni* · Virulence · Repellency · Olfactory blends · Nucleotide sequences

Introduction

Insects live in a complex world of chemical signals and are able to discriminate between olfactory cues in their microhabitats (Beyaert et al., 2010; Adfa et al., 2011; Behie et al., 2012; Holopainen and Blande, 2012; Tasin et al., 2012; Weerakoon and Chin, 2012). Detection of enemy-specific signals such as volatile chemicals at a distance and avoidance of an enemy is a key part of the defensive behavior of many insect orders, including isoptera (Gripenberg, et al., 2010; Najjar-Rodriguez et al., 2010; Rohlf and Churchill, 2011; Turcotte et al., 2012). Several authors have recognized that pathogenic fungal isolates can be repellent to termites, which may affect their effective deployment as mycoinsecticides (Milner, 2000; Cheragi et al., 2012; Chouvenec and Su, 2012). Previously, we reported that the termite *Macrotermes michaelseni* Sjöstedt (Isoptera: Macrotermitidae) is able to detect and avoid direct physical contact with isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycetes: Hypocreales) and *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycetes: Hypocreales) (Mburu, et al., 2009). Moreover, that study showed a strong correlation between virulence and repellency for different isolates of these fungi. In a subsequent study, we found clear qualitative and quantitative differences in the volatile blends emitted by two selected isolates of *M. anisopliae* that had different levels of virulence and repellency towards the termite (Mburu et al., 2011). Intra-specific differences between the two isolates of

D. M. Mburu · N. K. Maniania · A. Hassanali
Behavioral and Chemical Ecology Department,
International Centre of Insect Physiology and Ecology (ICIPE),
Kasarani, Nairobi County, Off-Thika Road,
Nairobi, Kenya

N. K. Maniania
e-mail: nmaniania@icipe.org

A. Hassanali
e-mail: hassanali.ahmed@ku.ac.ke

D. M. Mburu (✉)
Biological Sciences Department, Pwani University College,
Kilifi County, Coastal Resort City, Kenyan Coast,
Mombasa, Kenya
e-mail: Davmburu12@gmail.com

D. M. Mburu
e-mail: d.mburu@pwaniuniversity.ac.ke

A. Hassanali
Chemistry Department, Kenyatta University,
Kahawa, Kiambu County, Off-Thika Road,
Nairobi, Kenya

the fungus also were reflected in their nucleotide sequences (Mburu et al., 2011).

In the present study, we sought to assess whether the relationship between repellency and virulence for different isolates of *B. bassiana* towards *M. michaelseni* (Mburu et al., 2009) is reflected in the composition of their volatiles and gene sequences. Specifically, the volatile blends emitted by two isolates (ICIPE 276 and ICIPE 278) of this fungus with different levels of virulence and repellency (Mburu et al., 2009) were characterized by GC-MS, and the constituents contributing to the active blends were elucidated by subtractive bioassays. In addition, DNA sequencing was used to assess intra-specific metagenomic variations between the fungal isolates.

Methods and Materials

Termite Trapping and Maintenance Worker castes of the termite, *Macrotermes michaelseni*, trapped in the field (Tamashiro et al., 1973) were transported to the laboratory and maintained as previously described (Mburu et al., 2009, 2011). Before repellency bioassays, termites were transferred into an incubator (26 ± 2 °C and 90 ± 5 % relative humidity in the dark) where they were kept for 20 min for acclimatization (Mburu et al., 2009, 2011).

Scaled-up Production of Dry Conidia for Volatile Collection Conidia for collection of volatiles were grown on long white rice substrates following the technique described previously (Mburu et al., 2011). Conidia were harvested and

stored in a refrigerator (4 °C) for 1 month before use. Viability (81–90 %) of the conidia was confirmed prior to all bioassays. Each conidium was designated as having germinated only if its germ tube was twice the length of its diameter.

Collection of Volatiles from Isolates of *B. bassiana* Two- to three-week-old isolates of *Beauveria bassiana* that had been previously identified (Mburu et al., 2009) as the most (ICIPE 276) and least (ICIPE 278) virulent/repellent were used. Volatile blends from dry conidia (40 g) of each isolate were trapped separately using Super-Q polymer (80–100 mesh size; ARS) as previously described (Mburu et al., 2011). Volatiles were eluted with dichloromethane (100 μ l) into 2 ml vials on ice and concentrated by flushing with purified nitrogen (3 mlmin⁻¹).

Gas Chromatography–Mass Spectrometry (GC-MS) Volatiles were analyzed using an HP 7890 A series GC (Agilent Technologies) coupled to a 5975 C series MS (Agilent Technologies) as previously described (Mburu et al., 2011). Constituents of each blend were identified by comparison of their mass spectral fragmentation patterns with those provided in the NIST/NIH/EPA mass spectral library (NIST 08) and confirmed by co-elution with standards (97–99 %, Sigma-Aldrich).

Olfactometric Assays of Synthetic Blends Standard compounds representing the 10 most abundant constituents in each isolate were dissolved in acetone and mixed in the proportions that were found in the natural volatile emission from each

Table 1 Prominent components identified in the volatiles of the more repellent (ICIPE 276) and less repellent (ICIPE 278) isolates of *Beauveria bassiana* and their amounts (μ G \pm SE) in volatiles collected from 40 g of each isolate in 12 h

ICIPE 276			ICIPE 278		
Constituent ¹	Identity	Amounts (μ G \pm SE) ²			
1	(<i>R</i>)-2-Methyl-1-butanol	1.43 \pm 0.78 ^a	1	(<i>R</i>)-2-Methyl-1-butanol	1.11 \pm 0.23 ^a
2	1-Pentanol	4.60 \pm 0.97 ^c	2	1-Pentanol	2.10 \pm 0.10 ^b
3	Hexanol	18.67 \pm 0.67 ^d	3	Hexanol	5.47 \pm 1.41 ^c
4	Butyrolactone	1.47 \pm 0.76 ^a	4	Butyrolactone	0.77 \pm 0.40 ^a
5	Phenol	1.63 \pm 0.19 ^b	5	Phenol	0.67 \pm 0.21 ^a
6	Octanol	1.05 \pm 0.12 ^a	6	Octanol	0.72 \pm 0.30 ^a
7	3-Octanol	2.37 \pm 0.16 ^b	7	3-Octanol	1.87 \pm 0.20 ^b
8	4-Nonanone	1.40 \pm 0.53 ^a	8	4-Nonanone	0.72 \pm 0.14 ^a
9	2-Nonanone	2.20 \pm 0.69 ^b	9	2-Nonanone	0.81 \pm 0.30 ^a
10	Camphor*	1.11 \pm 0.42 ^a	10	Camphor	0.45 \pm 0.21 ^a
11	Borneol*	4.74 \pm 0.25 ^c	11	Borneol	2.20 \pm 0.09 ^b
12	4,5-Dihydro-5-pentyl-2-(3 H) furanone*	1.98 \pm 0.52 ^b	12	4,5-Dihydro-5-pentyl-2-(3 H) furanone	0.50 \pm 0.21 ^a

¹ The numbering of the constituents in ICIPE 276 and ICIPE 278 refer to the peaks that are numbered in Fig. 1a and b, respectively. ² SE denotes standard error of the mean

Numbers labeled with the same letters across a given row are not significantly different ($P>0.05$, SNK Test). *Chirality of these constituents was not determined

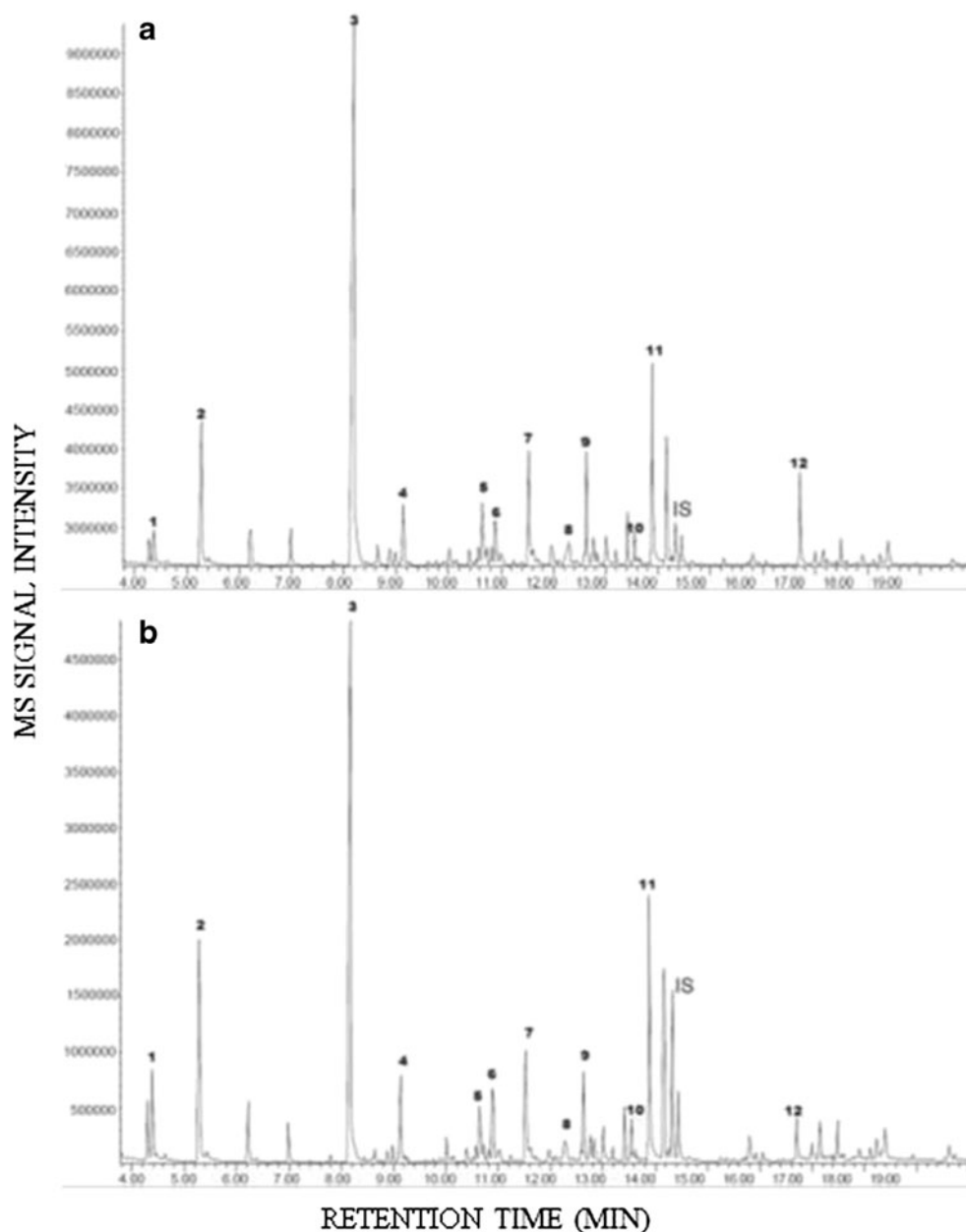
fungus isolate (Table 1). Each stock solution was diluted to five concentrations ranging between 10^{-1} to 10^{-6} $\mu\text{g}\mu\text{l}^{-1}$ total volatile components. The repellency of each dilution of each blend was evaluated in six replicates as described previously (Mburu et al., 2009, 2011). The number of termites in the treatment and the control arms of the olfactometer were recorded at intervals of 10 min for 60 min to give 6 readings for each replicate of each dose. In all assays, the location of treatment and control filter papers in the olfactometer were alternated after each replicate (Mburu et al., 2011).

Subtractive Assays with Blends of Selected Components The same methods were used to prepare mixtures of nine components, so that each component was individually omitted from

the 10-component mixture characteristic of each isolate. These mixtures were tested in six replicates at six concentrations ranging between 10^{-1} to 10^{-6} $\mu\text{g}\mu\text{l}^{-1}$. The results showed that six constituents of each isolate contributed significantly to repellency. Blends of the six respective components also were assayed at different dilutions as outlined above.

DNA Extraction and Barcoding Pure cultures of dry conidia of ICIPE 276 and ICIPE 278 were mass-produced on rice as previously described (Mburu et al., 2011) and the genomic DNA was extracted from 0.1 g samples of conidia using a slight modification of the CTAB method (Doyle and Doyle, 1990). Each extract was re-suspended in pre-warmed sterile deionized water and exposed to primer pairs n-SSU-1766-5'

Fig. 1 a and b Gas chromatograms in 40 g of both the more repellent (A; ICIPE 276) and less repellent (B; ICIPE 278) isolates of *Beauveria bassiana* (concentration of internal standard, IS, was 11.3 $\mu\text{g}\text{ml}^{-1}$ in 1A and 5.65 $\mu\text{g}\text{ml}^{-1}$ in 1B)



(ITS5, White et al., 1990) and nu-LSU-0041-3' (ITS4, White et al., 1990) to amplify the internally transcribed spacers of the genomic DNA. PCR amplifications were carried out as previously described (Mburu et al., 2011). Negative controls without fungal DNA were run for each amplification routine to check for contaminants.

DNA Quantification, Purification and Sequencing Agarose gel electrophoresis (Loughborough, Leicestershire, UK), run in 1× Tris acetate EDTA buffer for 1 h at 70 V cm⁻¹ followed by ethidium bromide staining and visualization under UV light was used to separate the DNA (Mburu et al., 2011). The lengths of the amplicon products were estimated by comparison with a DNA ladder (1 kb Smart) (Noxo, Tallinn, Estonia). Purification and sequencing of the amplified products was performed with a DNA gel extraction kit (Quick-Clean, Genscript, USA) and genetic analyzer (ABI PRISM 3700, Applied Biosystems, Foster, CA, USA), respectively.

Bioassay Data Analyses Repellency of the 10-component, 9-component and 6-component blends were calculated 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; Mburu et al., 2011).

Each set of repellency data was arcsin-transformed to normality before invoking repeated measures analysis of variance (ANOVA) using Proc Mixed in SAS version 9.2 (SAS Institute, 2008). The Student-Newman-Keuls (SNK) test was used to separate the means. Repeated-measures logistic regression via generalized estimating equations (GEE) (Throne et al., 1995; Stokes et al., 2000; Shibata and Shimizu, 2011) was used to estimate the repellency doses (RD₅₀) values required to give 50 % repellency (RD₅₀) for each of the blends. These analyses were carried out using the GENMOD procedure in SAS version 9.2 (SAS Institute, 2008). For the different synthetic blends, the level of significance was set at 5 % for all the analyses to identify significant differences among the values of RD₅₀.

PCR Data Editing and alignment of the sequences of the PCR products were performed by using ChromasPro version 6 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) and ClustalW version 2.0 (Larkin et al., 2001). Kimura (1980) 2-parameter and Tamura and Nei (1993) models were used to estimate substitution patterns and rates. Evolutionary analyses were conducted in MEGA5 version 5.05 (Tamura et al., 2004, 2011). For estimating maximum Log-likelihood values, the data were bootstrapped to 10000 replicates. Differences in composition bias

among sequences were considered in evolutionary comparisons with first, second and third codons and noncoding positions (Tamura, 1992; Tamura and Kumar, 2002). All positions containing gaps were excluded from the dataset.

Results

GC and GC-MS Analyses The most abundant constituents (representing ~80 % of the compounds in the blends) of the two volatile blends were identified by comparison of their mass spectra with those in the Wiley and NIST libraries (NIST 08) and by co-elution with authentic standards (Table 1). There were mainly quantitative differences in the volatile profiles of the two isolates with some of the more prominent components of ICIPE 276 present in smaller amounts in the volatile blend of ICIPE 278 and *vice versa* (Fig. 1a, b; Table 1). In addition, there were consistently lower concentrations of volatiles emitted by strain ICIPE 278 than ICIPE 276. The chiralities of borneol, camphor, and 4,5-dihydro-5-pentyl-2 (3 H)-furanone were not determined.

Olfactometric Assays of Synthetic Blends The percentage repellencies of the two sets of 10-component blends were different at all doses (SNK test, $F_{5,65}=40.14$, $P<0.05$, $N=72$) (Fig. 2), and this is reflected in their RD₅₀ values (Table 2). ANOVA revealed differences in repellency among the ten 9-component blends of ICIPE 276 at different doses (SNK test, $F_{9,140}=8.77$, $P<0.05$, $N=150$). Likewise, there were differences in repellency of the ten 9-component blends of ICIPE 278 (SNK test, $F_{9,140}=4.03$, $P<0.05$,

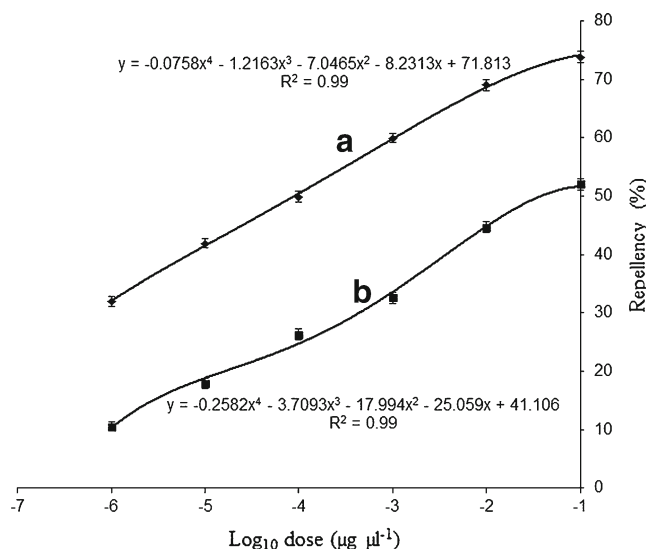


Fig. 2 Percentage dose–response of the two sets of 10-component blends of synthetic components of ICIPE 276 (a) and ICIPE 278 (b), two *Beauveria bassiana* isolates. Each point represents the average for $N=72$ with the error bars indicating the standard errors of the respective means

Table 2 RD₅₀ values of repellency dose-responses of various blends [10-component (full blend), 9-component and 6-component] of *Beauveria bassiana* volatiles towards the termite *Macrotermes michaelseni*

ICIPE 276		ICIPE 278	
Composition	RD ₅₀ , µgµl ⁻¹ (95 % confidence limits)		RD ₅₀ , µgµl ⁻¹ (95 % confidence limits)
10-components {Full blend (FB)}	4.22 (3.82-4.48) × 10 ^{2h}	10-components {Full blend (FB)}	3.82(3.67-4.08) × 10 ^{3s}
FB minus (R,S)- 4,5-Dihydro-5-pentyl-2-(3 H)furanone	6.55 (5.91-7.51) × 10 ^{-1b}	FB minus (R,S)-4,5-Dihydro-5-pentyl-2-(3 H)furanone	8.28 (8.25-8.97) × 10 ^{2k}
FB minus (R,S)- Borneol	6.63 (6.56-6.72) × 10 ^{1c}	FB minus (R,S)-Borneol	11.11 (10.43-12.24) × 10 ^{2m}
FB minus 4-Nonanone	9.21 (8.21-9.51) × 10 ^{1d}	FB minus 4-Nonanone	(12.87 × 10 ²) 12.91 × 10 ² -2.21 × 10 ³ⁿ
FB minus 2-Nonanone	2.27 (2.01-2.67) × 10 ^{2e}	FB minus 2-Nonanone	2.74 (2.45-2.79) × 10 ^{3p}
FB minus Butyrolactone	3.01 (2.91-3.54) × 10 ^{2f}	FB minus Butyrolactone	2.91 Camphor (2.85-3.01) × 10 ^{3q}
FB minus Camphor	3.68 (4.44-4.69) × 10 ^{2g}	FB minus Camphor	3.21 (3.12-3.51) × 10 ^{3r}
FB minus 1-Pentanol	4.69 Phenol (4.46-5.34) × 10 ^{2h}	FB minus 1-Pentanol	3.51 (3.42-3.67) × 10 ^{3s}
FB minus Hexanol	5.54 (5.33-5.66) × 10 ^{2h}	FB minus Hexanol	3.64 (3.51-3.72) × 10 ^{3s}
FB minus 3-Octanol	5.69 (5.55-5.82) × 10 ^{2h}	FB minus 3-Octanol	3.93 (3.67-5.23) × 10 ^{3s}
FB minus Octanol	6.24 (5.95-6.71) × 10 ²ⁱ	FB minus Octanol	5.54 (4.42-6.03) × 10 ^{3t}
6-components blend	3.45 (2.98-3.78) × 10 ^{-1a}	6-components blend	7.08 (6.98-7.23) × 10 ^{2j}

RD₅₀ values followed by the same letter within each column are not significantly different (Proc GENMOD). Failure of 95 % confidence limits (in parentheses) to overlap was used as the criteria for identifying significant differences among RD₅₀ values. 6-components blend was made up of (R,S)-4,5-dihydro-5-pentyl-2-(3 H)furanone, (R,S)-borneol, 4-nonanone, 2-nonanone, butyrolactone, and camphor

Fig. 3 Sequence alignments of genomic DNA of the two isolates of *Beauveria bassiana*, ICIPE 276 and ICIPE 278. Sections of the two sequences marked and unmarked with asterisks indicate divergence and identity, respectively, between the two isolates

ICIPE278	TCAACGTTTCAGAAAGTTGGGTGTTTTACGGCGTGCCCGCGTCGGGGTT	47
ICIPE276	TCAACGTTTCAGAAAGTTGGGTGTTTTACGGCGTGCCCGCGTCGGGGTC	60
		*
ICIPE278	CCGGTGCGAGCTGTATTACTGCGCAGAGGTGCGCGGACGGGCGCCACTCCATTTCAG	107
ICIPE276	CCGGTGCGAGCTGTATTACTGCGCAGAGGTGCGCGGACGGGCGCCACTCCATTTCAG	120
ICIPE278	GGCCGGCGGTGTGCTGCCGGTCCCAACGCCGACCTCCCCAAGGGGAGGTGAGGGTTGA	167
ICIPE276	GGCCGGCGGTGTGCTGCCGGTCCCAACGCCGACCTCCCCAAGGGGAGGTGAGGGTTGA	180
ICIPE278	AATGACGCTCGAACAGGCATGCCCGCCAGAAATGTGCGGGCGCAATGTGCGTTCAAAGA	227
ICIPE276	AATGACGCTCGAACAGGCATGCCCGCCAGAAATGTGCGGGCGCAATGTGCGTTCAAAGA	240
ICIPE278	TTCGATGATTCACTGGATTCTGCAATTCACATTACTTATCGCGTTTCGCTGCGTTCTTCA	287
ICIPE276	TTCGATGATTCACTGGATTCTGCAATTCACATTACTTATCGCGTTTCGCTGCGTTCTTCA	300
ICIPE278	TCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTGATTCAATTTGTTTGCCTTGCG	347
ICIPE276	TCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTGATTCAATTTGTTTGCCTTGCG	360
ICIPE278	GCGTATTTCAGAAAGATGCTGGAATACAAGAGTTTGAGGTCCCGGCGGGCCGCTGGTCCAG	407
ICIPE276	GCGTATTTCAGAAAGATGCTGGAATACAAGAGTTTGAGGTCCCGGCGGGCCGCTGGTCCAG	420
		*
ICIPE278	TCCGCGTCCGGGCTGGGGCGAGTCCGCCGAA	438
ICIPE276	TCCGCGTCCGGGCTGGGGCGAGTCCGCCGAA	451

$N=150$). The interspecific repellency of the 6-component blend of ICIPE 276 was higher than the 6-component blend of ICIPE 278 at all doses (SNK test, $F_{5, 65}=51.03$, $P<0.05$, $N=72$ (Table 2).

The repellencies of the two 6-component blends were higher than those of the respective two 10-component blends at all doses (SNK test, $F_{5, 135}=61.90$, $P<0.05$, $N=144$). Likewise, the repellency of each of the two 6-component blends was higher than the repellency of any of the corresponding 9-component blends at all doses (SNK test, $F_{5, 135}=68.52$, $P<0.05$, $N=144$).

DNA Sequence Analysis Sequence alignments indicated that there were two nucleotides that were different out of a total of 438 in the two amplified sequences (Fig. 3). There was instantaneous probability of substitution from one nucleotide base (row) to another (column) for each entry (Table 3). The nucleotide frequencies were 17.81 % (A), 23.52 % (T/U), 31.96 % (C), and 26.71 % (G). The transition/transversion rate ratios were $k_1=1$ (purines) and $k_2=1000$ (pyrimidines), respectively. The overall transition/transversion bias was $R=2.51$, where $[(A \times G \times k_1) + (T \times C \times k_2)] / [(A + G) \times (T + C)]$. There were a total of 438 positions in the final data set (Tamura et al., 2011).

Discussion

Our results show mainly quantitative differences in the volatile profiles of the two isolates (Fig. 1a, b; Table 1). In Y-tube olfactometric assays, the blend of the 10 most abundant components of ICIPE 276 was significantly more repellent than that of the 10 most abundant components of ICIPE 278. Most of the 10 most abundant components of ICIPE 276 were present in minor amounts in ICIPE 278. For example, 4,5-dihydro-5-pentyl-2-(3 H)furanone, which contributed most to the repellencies of both isolates, was present in larger amounts in the more repellent (and virulent) isolate (ICIPE 276). Similarly, borneol, the next most potent constituent, also was more

abundant in ICIPE 276. Two other constituents (2-nonanone and 4-nonanone) of the repellent blend of ICIPE 276 were, respectively, present at lower levels in the less repellent (and less virulent) isolate (ICIPE 278). Interestingly, 4,5-dihydro-5-pentyl-2-(3 H)furanone, which contributed most to the repellencies of the two isolates of *B. bassiana*, also was the most potent repellent component of *M. anisopliae* isolates reported previously (Mburu et al., 2011). Other common constituents of the two fungi include 2-nonanone, butyrolactone, hexanol, and 3-octanol. Thus, the two species of fungi that pose potential risks to the termite share some of the constituents that mediate their recognition and avoidance by the insect. It would be interesting to screen other pathogenic and repellent fungal species to see if they also share these constituents. Both 4,5-dihydro-5-pentyl-2-(3 H)furanone and borneol are chiral compounds. The synthetic compounds used in the bioassays were racemic, and further studies are needed to shed light on the role of chirality, if any, of the natural constituents of *B. bassiana* isolates in their repellency towards the termite.

In subtraction bioassays, six constituents of ICIPE 276 volatiles (4,5-dihydro-5-pentyl-2-(3 H)furanone, borneol, 4-nonanone, 2-nonanone, butyrolactone, and camphor) contributed to the repellency of the blend. As expected, a blend of these six constituents was more repellent than the 10-component blend from the same fungal strain (Table 2). Similarly, a 6-component blend of constituents of ICIPE 278 that contributed to its repellency was also more repellent than the corresponding 10-component blend (Table 2).

Comparison of two amplified sequences comprising of 438 bases from the two isolates of the fungus shows differences at two sites (Fig. 3), which changes their DNA sequences. These suggest some genetic basis in the virulence and repellent composition of the two isolates of the fungus in addition to any epigenetic differences between them. It would be interesting to extend the present study to other isolates of *B. bassiana* and *M. anisopliae* to elucidate the extent of within-species heterogeneity in intragenomics, metabolomics, and the relationship of these to their repellency and virulence to *M. michaelsoni* (and perhaps other termite species).

In summary, the results of the present study confirm that differential virulence of isolates of *B. bassiana* to *M. michaelsoni* is reflected in distinctive repellent volatile profiles of the fungal isolates. The termite appears to have an appropriate olfactory ability to detect specific signatures that influence its behavioral responses to risks in its habitats above and below-ground. The apparent high sensitivity of the termite to volatile emissions from these infective fungi (Mburu et al., 2009) is noteworthy and consistent with the behavioral, biochemical, and immunological adaptations observed in other termite species (Rosengaus et al., 1999; 2000; Rosengaus and Traniello, 2001; Traniello et al., 2002). The olfactory repertoire of the termite may be important in detecting volatile fingerprints that originate from potentially harmful fungi in subterranean

Table 3 Maximum composite likelihood estimate of the pattern of nucleotide substitution

ICIPE 278	ICIPE 276			
	A	T	C	G
A	-	0.05	0.05	0.06
T	0.04	-	52.92	0.06
C	0.04	46.59	-	0.06
G	0.04	0.05	0.05	-

Entries within a row are comparable. Each entry shows the probability of substitution (r) from one base (row) to another base (column) (Tamura et al., 2004). Rates of different transitional and transversal substitutions are shown in bold and italics, respectively. (A, T, C, and G represent adenine, thymine, cytosine and guanine, respectively)

habitats where emissions would be expected to diffuse in all directions and be present in relatively low concentrations. The study sheds some light on the capacity of the termite to survive in ecologies with diverse array of potentially harmful fungal species that may have varying levels of virulence (Mburu et al., 2009, 2011). This may account for failure to control termites using infective fungi during the last five decades (Chouvenc et al., 2011).

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