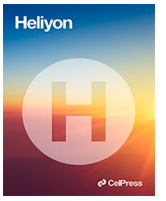
Heliyon 10 (2024) e28081

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Identification of termites from Gabon using MALDI-TOF MS

Bouthaina Hasnaoui [a,b], Adama Zan Diarra [a,b], Patrice Makouloutou-Nzassi [d,g], Jean-Michel Bérenger [a,b], Afaf Hamame [b,c], Barthelemy Ngoubangoye [e], Mapenda Gaye [b,c], Bernard Davoust [b,c], Oleg Mediannikov [b,c], Jean Bernard Lekana-Douki [f,h], Philippe Parola [a,b,*]

[a] *Aix-Marseille Univ, IRD, SSA, AP-HM, VITROME, Marseille, France*
[b] *IHU Méditerranée Infection, Marseille, France*
[c] *Aix-Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France*
[d] *Unité de Recherches en Ecologie de La Santé (URES), Centre Interdisciplinaire de Recherches Médicales de Franceville, B.P. 769, Franceville, Gabon*
[e] *Centre de Primatologie, Centre Interdisciplinaire de Recherches Médicales de Franceville, B.P. 769, Franceville, Gabon*
[f] *Unité D'Evolution, Epidémiologie et Résistances Parasitaires, Centre Interdisciplinaire de Recherches Médicales de Franceville, B.P. 769, Franceville, Gabon*
[g] *Institut de Recherches en Ecologie Tropicale (IRET-CENAREST), B.P. 13354, Libreville, Gabon*
[h] *Département de Parasitologie- Mycologie, Université des Sciences de La Santé, B.P. 4009, Libreville, Gabon*

ARTICLE INFO

Keywords:
MALDI-TOF MS
Termites
Isoptera
Gabon
Pseudacanthotermes militaris
Macrotermes bellicosus
Noditermes indoensis

ABSTRACT

Termites are one of the most common pests that damage wood and other cellulosic materials. Although Africa has more varieties of termite species than any other continent, few entomological studies have been conducted in Gabon. Identifying termites poses significant difficulties for entomologists. The aim of this study was to evaluate the reliability and confirm the significance of MALDI-TOF MS in identifying fresh termites collected in equatorial Africa.

A total of 108 termites were collected from 13 termite nests during a field mission in 2021 in Lekedi and Bongoville, Gabon. Termites were morphologically identified and subjected to MALDI-TOF MS, then molecular analyses using the *COI and 12S rRNA* genes.

Four termite species were morphologically identified in this study: *Pseudacanthotermes militaris*, *Macrotermes muelleri*, *Macrotermes nobilis*, and *Noditermes indoensis*. However, when using molecular biology, only three species were identified, namely *Macrotermes bellicosus*, *P. militaris*, and *N. indoensis,* because the specimens initially identified as *M. muelleri* and *M. nobilis* were found to be *M. bellicosus*.

The MALDI-TOF MS spectral profiles of the termites were all of good quality, with intra-species reproducibility and inter-species specificity. The spectra of 98 termites were blind tested against our upgraded database, which included the spectra of ten termite specimens. All tested spectra were correctly matched to their respective species, with log score values (LSVs) ranging from 1.649 to 2.592. The mean LSV was 2.215 ± 0.203, and the median was 2.241. However, 95.91% (94/98) of our spectra had LSVs above 1.8. This study demonstrates how a proteomic approach

* Corresponding author. VITROME, Institut Hospitalo-Universitaire Méditerranée Infection, 19–21 Boulevard Jean Moulin, 13005, Marseille, France.

E-mail addresses: bouthainahasnaoui17@gmail.com (B. Hasnaoui), adamazandiarra@gmail.com (A.Z. Diarra), patmak741@gmail.com (P. Makouloutou-Nzassi), jmberenger62@gmail.com (J.-M. Bérenger), afafhamame@gmail.com (A. Hamame), genistha@hotmail.com (B. Ngoubangoye), gaye.mapenda@gmail.com (M. Gaye), bernard.davoust@gmail.com (B. Davoust), olegusss1@gmail.com (O. Mediannikov), lekana_jb@yahoo.fr (J.B. Lekana-Douki), philippe.parola@univ-amu.fr (P. Parola).

https://doi.org/10.1016/j.heliyon.2024.e28081
Received 5 April 2023; Received in revised form 11 March 2024; Accepted 12 March 2024
Available online 14 March 2024

can overcome termites' molecular and morphological identification limitations and serve as a useful taxonomic tool.

1. Introduction

Termites are related to cockroaches, classified in the infra-order Isoptera within the order Blattodea [1]. They are eusocial insects and live in colonies comprising between a few hundred and several million individuals, composed of sterile castes (large and small workers and soldiers) and reproductive castes [2]. Termites are classified into 12 families and 330 genera [3], compromising 3015 species [4].

Higher termites, also known as Macrotermitinae, are a fascinating group behind the construction of complex mounds (fungus farming) [3]. Omnipresent in the African-tropical area [5,6], there are about 1000 species of termites in Africa [7]. In recent years, several studies have focussed on the impact of termites from a range of perspectives, including ethology, biology, cost losses regarding different crops, including sugar cane and fruit trees, diversity, their impact on the ecosystem, and the identification of harmful termites [8].

In addition to their ecological importance in soil turnover and nutrient cycling [9,10], the *Macrotermes* genus has caught the attention of scientists and engineers due to their potential to overcome the aging process [11], and as bioindicators for minerals such as gold [12,13]. The *Macrotermes* genus is mainly present in Africa, with 14 species in the savanna and six in forested areas [14].

A small number of studies have been carried out on termites in Gabon. Most of those studies have focused on the cultural diversity of the chimpanzee [15–18], with some microbiological studies on the distribution of Wolbachia [19] and the influence of termites on soil characteristics [20].

The systematic identification of termites is a complex process. The antiquity of its order and its long evolution has had little influence on its morphology, hence the morphological similarity of species within the same genus [21]. Currently, termite identification is mainly based on the characteristics of the soldiers, although this only enables identification at the genera level. The morphological identification of termite species remains challenging [22]. Molecular biology could be a reliable method for overcoming morphological limitations, especially by combining different nuclear and mitochondrial markers [23]. However, this method remains expensive and time-consuming in practice.

In recent years, MALDI-TOF MS, a proteomic tool, has been used in entomology to overcome the limitations of classical morphological identification, including the difficulty of separating closely related or damaged species and certain developmental stages due to the lack of appropriate identification keys [24]. Similarly, MALDI-TOF MS also overcomes the limitations of the molecular method, with reduced handling time and at a lower cost [25].

Several studies on identifying various arthropod species have proven the advantages of using MALDI-TOF MS in entomology [25]. In one preliminary study conducted in 2022, we used MALDI-TOF specimens belonging to 22 termite species preserved in alcohol from

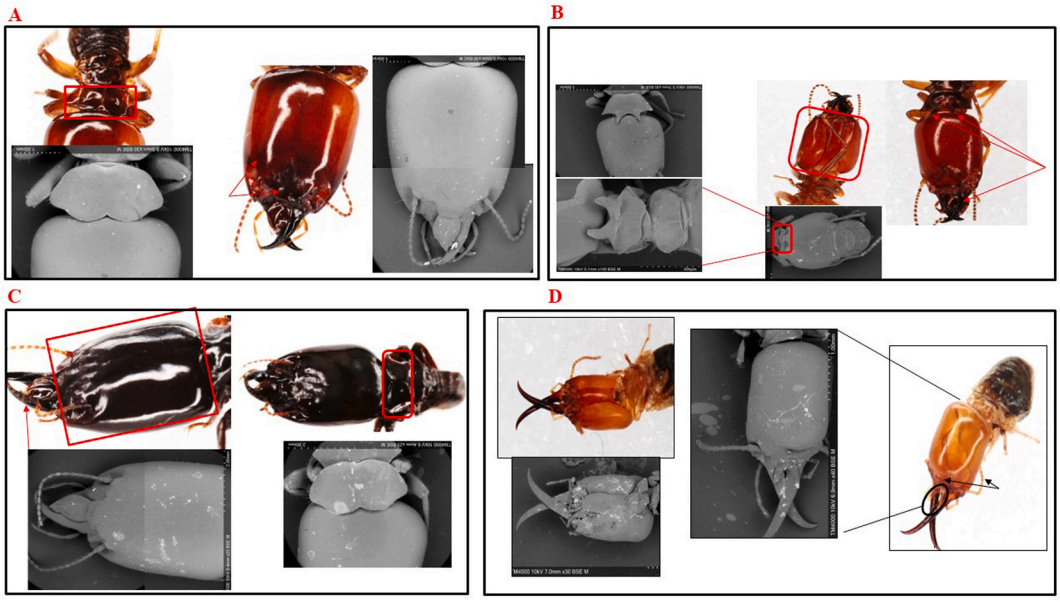


Fig. 1. Digital microscope (DM) and scanning electron microscope images (SEM) of termites from both the Bongoville and Lekedi sites. A dorsal view and pronotum view of **A** *Macrotermes bellicosus (initially labled as M. muelleri),* **B** *Pseudacanthotermes militaris*, **C** *Macrotermes bellicosus (initially labled as M. nobilis),* **D** *Noditermes indoensis,* with a ventral view of *Noditermes indoensis*.

West African countries, France and Switzerland [26]. In this study, we aimed to test the effectiveness of MALDI-TOF MS in identifying fresh termites collected in equatorial Africa (Gabon).

2. Results

2.1. Collection and morphological identification of termites

A total of 108 termite specimens were collected at the two sites (Lekedi and two at Bongoville). Termites were only found in four of the thirteen termite nests, two in each site.

Based on their morphological characteristics, four species were assumed to be identified: *P. militaris*, *N. indoensis, M. nobilis*, and *M. muelleri.* The termite species from Lekedi were identified as *P. militaris* and *N. indoensis*, whereas those from Bongoville were identified as *P. militaris* and *M. muelleri*. Two species were found in the same termite nest, namely *M. muelleri (n = 69)* and *M. nobilis* (n = 7). The morphological traits were photographed using a Canon camera, and the instrument used a scanning electron microscope, represented in Fig. 1(A-D).

2.2. Molecular identification of termites

Ten termite specimens, four of which were morphologically identified as *M. muelleri*, two as *M. nobilis*, three as *P. mulitaris* and one as *N. indoensis*, were subjected to molecular identification using the *COI* and*12S rRNA* genes sequencing. According to the NCBI BLAST results, the sequences obtained from termites which had been morphologically identified as *P. militaris,* were respectively 99.51% and 99.33% identical to sequences of *Pseudacanthotermes spiniger* (KY224401 and EU253858) for the COI and 12S rRNA genes respectively. These were linked to an article in which the formal morphological criteria were not specified [27]. The next BLAST hit revealed 99.34% and 99.26% identity to sequences of *P. militaris* (AY127731 and DQ441805, respectively) for the *COI* and *12S rRNA* genes, and the morphological characteristics were reviewed [28].

The sequences of termites morphologically identified as *N. indoensis* were 95.54% identical to the *Niditermes orthognathus* sequence for *COI* (MN646720) and 99.01% identical to the *Cubitermes ugandensis* sequence for *12S rRNA* (KP091689). However, there are no *N. indoensis* sequences in GenBank. The sequence from *M. muelleri* and *M. nobilis* were 99.01% and 98.99% identical to the sequence of *M. bellicosus* sequence for *COI* (AY127711) and *12S rRNA* (MW078940) (Table 1). Comparison of the *M. nobilis* COI sequences in our study with the one in GenBank (AY127705) showed an identity of 88.66%. Similarly, for the *M. muelleri* COI sequences in our study and that in GenBank (AY127703), showed an identity of 88.88%.

The phylogenetic position of the termites in our study and the other species in GenBank using *COI* and *12S rRNA* sequences are shown in Fig. 2A and B.

Considering the presence of clear discriminating morphological criteria and the absence or quality of sequences available in GenBank, we considered the specimens used in this work to be *P. militaris* (supported by both morphological and molecular biological identification), *N. indoensis* (morphological characteristics only), and *M. bellicosus*, for which molecular biology corrected our morphological identification, which was unsurprising, as species of the genus *Macrotermes* are morphologically close.

Table 1
Present the results of morphological and molecular identification of different termite species identified in this study. The morphological identification initially labelled, the number of each termite species, showing the first and the second hit of identification using both *12S rRNA* and *COI* accession number.

Morphological identification	Nb tested	Closest GenBank match 1st and 2nd hit (Cover& identity) % *COI*	Closest GenBank match 1st and 2nd hit (% identity) *12S rRNA*
Macrotermes nobilis	2	*Macrotermes bellicosus* AY127711 (91%/99.01%) *Macrotermes bellicosus* AY127702 (92%/98.85%)	*Macrotermes bellicosus* MW078941 (97%/98.99%) *Macrotermes bellicosus* MW078940 (97%/98.99%)
Macrotermes muelleri	4	*Macrotermes bellicosus* AY127711 (90%/99.01%) *Macrotermes bellicosus* AY127702 (92%/98.85%)	*Macrotermes bellicosus* MW078941 (96%/98.99%) *Macrotermes bellicosus* MW078941 (97%/98.99%)
Pseudacanthotermes militaris	3	*Pseudacanthotermes spiniger* EU253858 (100%/99.85%) *Pseudacanthotermes spiniger* KY224401 (100%/98.93%)	*Pseudacanthotermes spiniger* KY224401 (100%/99.51%) *Pseudacanthotermes militaris* DQ441805 (100%/99.26%)
Noditermes indoensis[a]	1	Niditermes orthognatus MN646720 (100%/95.54%) Cubitermes nr.fluvus KY224600 (100%/94.46%)	Cubitermes ugandensis KP091689 (100%/99.01%) Cubitermes nr.fluvus KY224600 (100%/94.46%)

[a] Sequence does not exist in GenBank.

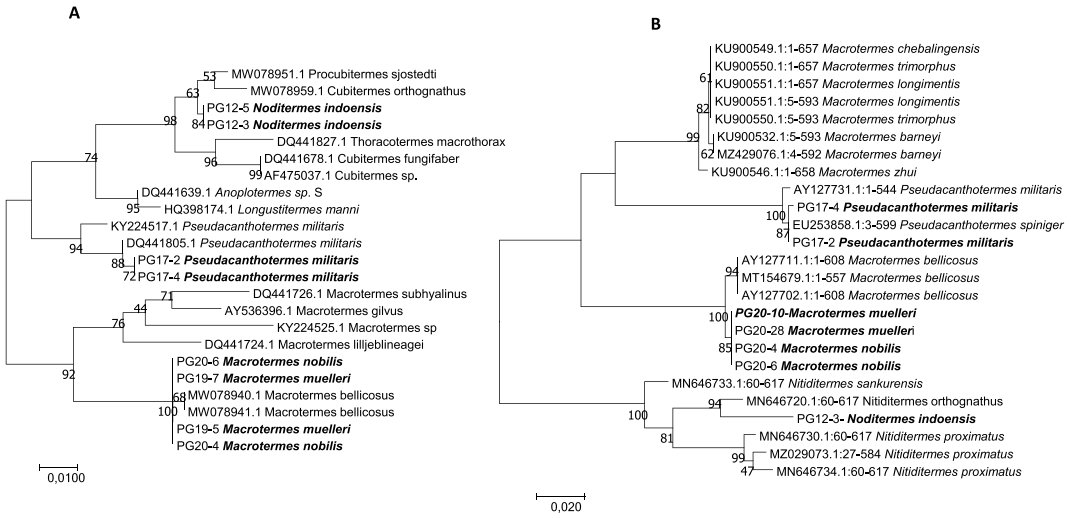


Fig. 2. Phylogenetic tree based on 380 bp and 668bp bp sequences of the *12S rRNA* (A) and *COI* (B) genes. Phylogenetic tree highlighting the position of the termites studied (in bold) in relation to other termite sequences available via GenBank. The 420 bp and 710 bp partial sequences of the *12S rRNA* and *COI* genes were aligned using CLUSTALW, and phylogenetic inferences were obtained from maximum likelihood phylogenetic analysis with 2-parameter Kimura distance (MEGA7). GenBank accession numbers are given at the beginning. Node numbers are bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree.

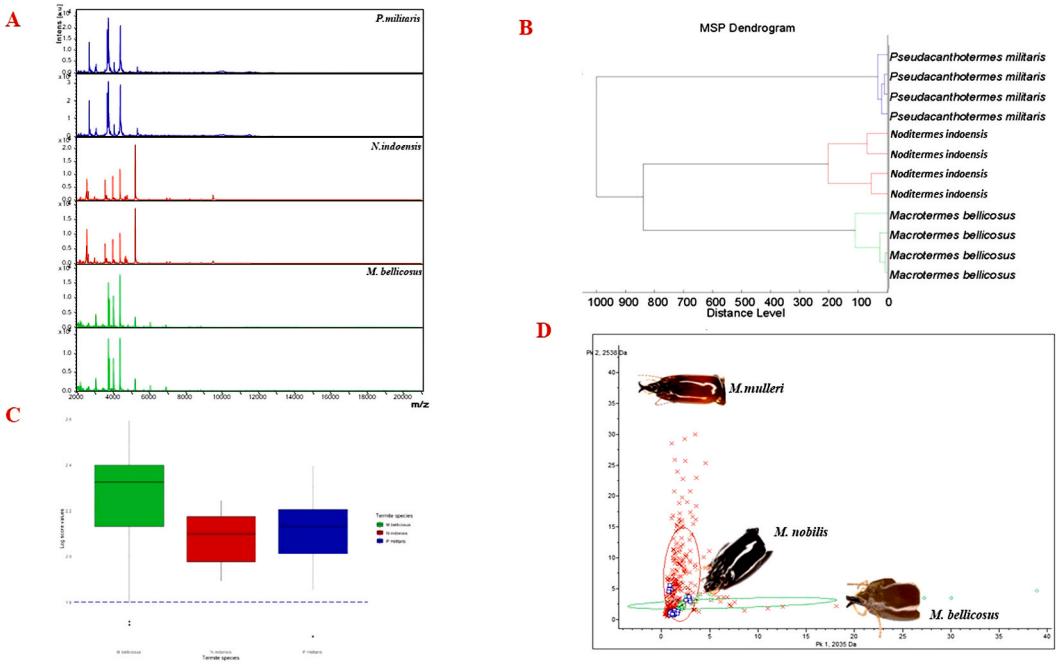


Fig. 3. MALDI-TOF MS spectra obtained from *M. bellicosus, P. militaris, N. indoensis*: **A.** Spectral alignment of MS spectra obtained from termites using flexAnalysis software v.3.3. **B**. Dendrogram using three to four representative MS spectra from three distinct species treated with MALDI Biotyper v 3.0 software and the distance units correspond to the relative similarity of MS spectra. **C**. Box plot showing the distribution of LSVs obtained by blind testing different termite species. The blue line represents the limit of the correct identification threshold of 1.8. **D.** The PCA analysis (Fig. 3. D) using spectra of *M.bellicosus* species collected from Senegal conserved on alcohol and the spectra of fresh termites species collected from Gabon morphologically ladled as *M.nobilis* and *M.mulleri,* created using ClinProTools 2.2 software. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. MALDI-TOF analyses, database upgrade, and blind test

The legs of 108 termite specimens were subjected to MALDI-TOF MS analysis. Visualization of all MS spectra using flexAnalysis v.3.3 software showed that they were all of good quality (peak intensity >3000 au, no background noise, and correct baseline subtraction) (Fig. 3A). The dendrogram generated from the MS spectra of some specimens of the three species shows intra-species reproducibility and inter-species specificity, with specimens of the same species grouped together on the same branch (Fig. 3B).

The first blind test compared the spectra of termite legs collected in Gabon with our pre-existing MALDI-TOF MS database that contained 22 termite species [26]. The results showed that the termite species morphologically identified as *M. muelleri* and *M. nobilis* were then identified as *M. bellicosus*, with log-score values (LSVs) ranging from 1.409 to 2.335, a mean of 1.866 ± 0.210, and a median of 1.886 and 75% (57/76) had an LSV>1.8 (Fig. 3C). The principal component analysis (PCA) (Fig. 3 D) using spectra of the *M. bellicosus* species collected from Senegal, conserved on alcohol, and the spectra of fresh termites species collected from Gabon and morphologically identified as *M. nobilis* and *M. mulleri,* created using ClinProTools 2.2 software, showed no differences between the spectra of the three species of the *Macrotermes* genera. However, termite specimens identified as *P. militaris* and *N. indoensis* were identified as different termite species, with LSVs<1.8, ranging from 0.981 to 1.486, a mean of 1.191 ± 0.132, and a median of 1.171.

After the addition of spectra of three *P. militaris*, six *M. bellicosus*, and one *N. indoensis* to our MALDI-TOF MS database, the remaining 98 spectra were subjected to a second blind test. The specimens de *M. muelleri* and *M. nobilis* were all identified as *M. bellicosus*, with LSVs ranging from 1.701 to 2.592, a mean of 2.258 ± 0.164, and a median of 2.323. The specimens of *P. militaris* were identified as *P. militaris,* with LSVs ranging from 1.649 to 2.395, a mean of 2.089 ± 0.182, and a median of 2.118. The *N. indoensis* were correctly identified as *N. indoensis* and had LSVs ranging from 1.891 to 2.243, a mean of 2.076 ± 0.143, and a median of 2.098 **(Table 2).**

3. Discussion

The current study focuses on the identification of fresh termites collected in the field from Lekedi and Bongoville (Gabon) by MALDI-TOF MS. Several entomological studies have provided exact morphological identification keys to a large number of termite species in the world [3,21,29,30]. These keys can be complicated to use and are also difficult to interpret for non-entomologists [22, 31]. This approach allows a significantly faster species-level identification than morphology and is less expensive than molecular methods once the device has been purchased. The tool can be utilized across multiple platforms, including entomology, mycology, microbiology, and malacology [25,32]. Several factors should be considered to ensure the quality of the spectra produced, such as the choice of the compartment of the arthropod. This compartment may be the head for bed bugs [33], the legs for ticks and mosquitos [34, 35], thoraxes with wings, legs for sand flies [36], and the cephalothorax for lice [37]. Various arthropod preservation techniques, including freezing, silica gel, and alcohol, also impact the MS profiles. When the reference database MS spectrum does not comprise all preservation methods, identification using MALDI-TOF MS is challenging [33,38,39]. For termites, we previously determined that legs appear suitable [26] when working on alcohol-preserved specimens. In the present study, we demonstrated the reliability of MALDI-TOF MS in identifying fresh Gabonese termites. To our knowledge, no study has specifically investigated Gabonese termites. Most studies have focused on termites as food for chimpanzees and gorillas [16–18].

In our study, the termites were identified to the species level based on morphological characteristics and the known distribution of termites in Gabon [3,30,40]. Interestingly in this study, morphological identification led us to speculate on the presence of two species in the same nest. *M. muelleri* and *M. nobilis* appear in fewer numbers. One hypothesis could be that a royal couple may settle in a pre-existing termite mound or that a colony may migrate there after having grown, a phenomenon that has only been reported in African termites [21,41]. The low number of inhabited termite mounds (4/12) in our study could be related to the complex interior structure of the nest, which allows termites to escape when they perceive potential danger [42]. However, if having two species in the same nest would have been possible, using molecular tools allowed us to conclude that there was only one species, that is, M. bellicosus. The genetic profiles of the two Macrotermes species were identical, suggesting that the morphological differences observed were probably the result of a misidentification. This discrepancy between morphological and molecular identification highlights the challenges associated with morphological identification based solely on external features.

To address this issue, we used mitochondrial genome sequences of the COI and 12S rRNA genes on termite molecular identification based on previous studies that demonstrated their efficiency for termite species identification and phylogenetic analysis to address this issue [43,44]. Based on research, the *12S rRNA* gene has proven to be a useful molecular marker for improved termite species identification and species diversity through the phylogenetic tree [45].

The identity percentage of *COI* sequences for *Pseudacanthotermes militaris* (morphologically identified) showed no significant variation compared to the sequences in GenBank for *P. spiniger* (99.51%) and *P. militaris* (99.34%). The sequence of *P. spiniger* deposited

Table 2
Present the result of termite's identification through MALDI-TOF MS include morphological identification after molecular confirmation, the number of termites tested, the number added to the database, MALDI-TOF MS identification and the Log Score Value (LSV).

Morphological identification	Number tested	Number added to the database	MALDI-TOF MS identification %	Score range LSV
Pseudacanthotermes militaris	23	3	100% (23/23) *P. militaris*	1.649–2.395
Noditermes indoensis	5	1	100% (5/5) *N. indoensis*	1.891–2.243
Macrotermes belicosus	70	6	100% (70/70) *M. bellicosus*	1.811–2.592

in GenBank had not been studied morphologically in detail. However, to identify this species, we used the morphological identification keys reported previously [46], namely a long and utterly rectangular head, the presence of two long spines at the anterior edge of the pronotum, a mandible curved at the extremity, 18 articles of antennae, and morphometry (e.g., the width of the head does not exceed 2.7 mm) [21,47]. It is important to indicate that both species are widespread in African forests and savannas; they may breed with one another, and generate hybrid colonies [48]. The phylogenetic tree showed that the sequences we morphologically identified as *P. militaris* were close to the *P. militaris* reference sequence about both genes.

The identity percentage of our sequences morphologically identified as *N. indoensis* showed an identification mismatch with the *COI* and *12S rRNA* genes (*Niditermes orthognatus* and *Cubitermes ugandensis*) and did not correspond to our morphology. However, the lack of reference sequences in GenBank may explain these results. The phylogenetic tree shows that both sequences are distant from our sequence (Fig. 2).

In this study, we used MALDI TOF to corroborate the results obtained by molecular biology, despite the limited number of spectral profiles of termite species (22 species) in the database. It includes spectra of Macrotermes bellicosus species obtained from termites collected in Senegal and preserved in alcohol. The results of the initial blind test for morphological species identified as M. muelleri and M. nobilis were identified as Macrotermes bellicosus with good log score values. MALDI-TOF analysis confirmed our molecular biology results, providing further evidence that the two termite species, according to their morphological classification, are indeed the same species.

The MALDI-TOF spectral analysis showed that 100% of the spectra were of excellent quality, with intra-species reproducibility and inter-species specificity. This result was consistent with those obtained from termites collected in West Africa [26]. Previous studies have shown that MS profiles within the same species may change depending on the storage method, the influence of the environment, and spatial and temporal aspects [33,38]. In this study, despite the differences between the origins and preservation techniques of the termites tested (Gabon, fresh) from those in the database (Senegal, alcohol), MALDI-TOF could still correctly identify *M. bellicosus* species. This study revealed that MALDI-TOF MS profiles of fresh *M. bellicosus* were not significantly different from those of *M. bellicosus* stored in alcohol. *P. militaris* and *N. indoensis* were not in our MALDI-TOF MS database. After enriching our database with reference spectra from these three termite species, we could accurately identify these specimens with an LSV>1.8 in 96% of cases. MALDI-TOF MS could identify specimens that molecular biology was unable to identify, making it trustworthy and accurate for identifying termites.

Although the MALDI-TOF MS tool is accurate for arthropod identification, successful identification depends on the reliability of the spectral profile database. At present, our database is still in the process of being built up, so it needs to contain more spectra representative of all known species. This absence is a limiting factor for identifying species whose specimens are outside the database, hence the need for continuous updating of the reference spectra database with new specimens, to improve the quality of protein spectra.

One crucial aspect is the need to construct a database with the most significant number of spectra. Moreover, it is essential to include spectra in the database only if the related specimen has been identified at the species level through morphological and molecular methods. By following this approach, we can ensure that the database comprises the largest possible number of spectra,

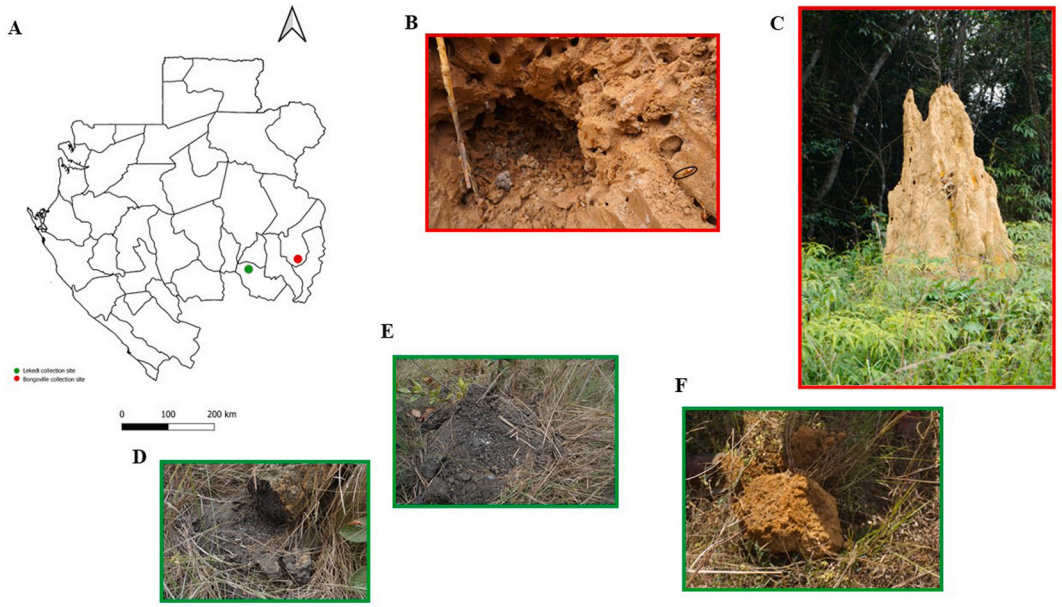


Fig. 4. **A** Map of Gabon showing both termite collection sites and some termite nests. The Bongoville site is marked with a red dot. **B** termite nest (cathedral), **C** presence of termite soldier. The Lekedi site is indicated with a green dot. **D, E** termite nest, **F** presence of termite alate. The map used in this study is not subject to copyright. Map of Gabon: QGIS.org, 2022. QGIS Geographic Information System. QGIS Association. http://www.qgis.org. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

enhancing its effectiveness and accuracy for arthropod identification.

4. Conclusion

In conclusion, our study on termite identification using MALDI-TOF MS presents a significant advancement in the field of entomology. By utilizing fresh termite specimens from Gabon and incorporating them into our extensive database, we have successfully demonstrated the reproducibility of our findings, particularly when reference spectra are available in the database. The updates made to our existing database, which now includes spectra of new species, have enhanced the credibility and accuracy of our results.

5. Materials and methods

5.1. Collection of termites

Termites were collected on August 30, 2021 at two sites in Gabon. The first site was the private nature park in Lekedi (−1.79674′ N; 13.02052′ E), and the second site was in Bongoville (−1.607275′ N; 13.939115′ E) (Fig. 4-A). Collections were made in 11 nests (labelled T1 to T11) in the park and two termite nests (T12 and T13) in Bongoville. Soil samples from each nest containing termites were collected using a shovel and forceps. The shapes of termite nests at the various collection sites were photographed, with termites represented in Fig. 4B–C from the Bongoville site, and in Fig. 4D–E from the Lekedi site. The soil samples and termites were collected in labelled plastic boxes, where they were stored at room temperature during transport to the laboratory at the University Hospital Institute (UHI) in Marseille. Prior authorization for the import of termite samples from Gabon into France was granted by the Departmental Directorate of Population Protection des Bouches-du-Rhône on August 09, 2021, under number ER-16-2021. The Gabon Ministry of the Interior authorized the shipment of this biological material out of Gabon on September 01, 2021, under number 33/MI/FPN/DGDI/AP/H.O/FCV.

In the laboratory, termites were sorted, rinsed once with 10% bleach and twice with distilled water, and dried with sterile filter paper. Each termite was placed in a 2 ml tube and stored at −20 °C before analysis.

5.2. Morphological identification

All termites collected were examined individually under a binocular loupe and ZEISS Axio Zoom V16. A few samples were selected and photographed using the Canon MP-E65mm lens (France) and a TM 4000 plus HITACHI tabletop electron microscope. The morphological characteristics of each specimen were noted and compared to different identification keys [3,21,40,47].

5.3. DNA extraction and molecular identification of termites

To confirm the morphological identification of the termites, we randomly selected ten termite specimens, consisting of four morphologically identified as *M. muelleri*, two identified as *M. nobilis*, three identified as *P. militaris*, and one identified as *N. indoensis.* Molecular identification of termite species was performed using *COI* and *12S* rRNA genes amplifying sequences of 420 and 710 bp respectively [26].

As previously described, DNA from the termites was extracted from the third part of the abdomen using an EZ1 DNA tissue kit (Qiagen) [26]. This was either used immediately or was stored at −20 °C until use. The amplified products were visualized on 1.5% agarose gel stained with SYBR Safe and then purified using a Macherey Nagel (NucleoFast 96 PCR, Düren, Germany) plate. Sequencing was performed using the BigDye Terminator v1.1, v3.1 5× Sequencing Buffer (Applied Biosystems, Warrington, United Kingdom) and run on an automated sequencer. Sequence chromatograms were assembled and edited using Chromas Pro1.77 (Technelyium Pty. Ltd, Tewantin, Australia).

The termite sequences obtained from both genes were subjected to bioinformatic comparison by comparing them to sequences in the National Center for Biotechnology Information (NCBI) database [49].

All sequences were then aligned using (MEGA7) and a phylogenetic tree was constructed and edited using the maximum likelihood method with model selection figured out by MEGA7 [50].

5.4. Sample homogenization and loading on MALDI-TOF MS target plate for analysis

Three legs from each termite specimen were dissected and individually placed in a 1.5 ml tube for MALDI-TOF MS analysis. These legs from each termite were homogenized using Tissue Lyser (QIAGEN), with a pinch of glass beads and 20 μL of a mixture of 70% formic acid and 50% acetonitrile (Fluka, Buchs, Switzerland) [26]. After homogenization and centrifugation of the sample, 1 μL of the supernatant of each sample was deposited in quadruplicate onto a MALDI-TOF MS steel plate (Bruker Daltonics, Wissembourg, France) and dried at room temperature. Each spot was covered with 1 μL of matrix solution composed of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, Lyon, France), 50% acetonitrile (v/v), 2. 5% trifluoroacetic acid (v/v) (Aldrich, Dorset, United Kingdom), and high-performance liquid chromatography (HPLC) grade water [51]. The target plate was air-dried once more at room temperature before being introduced into the Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The quality of the matrix, sample loading, and performance of the MALDI-TOF MS device were controlled using the legs of an Aedes albopictus reared in our laboratory as a positive control.

5.5. MALDI-TOF MS parameters and MS spectra analysis

Protein mass profiles were obtained using a MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics, Hamburg, Germany). Positive ion detection was performed in linear mode at a laser frequency of 50 Hz in the mass range of 2–20 kDa. The setup parameters of the MALDI-TOF MS instrument were identical to those used previously [52].

The quality of MS spectra for all specimens was inspected using ClinProTools 2.2 and flexAnalysis v.3.3 software (Bruker Daltonics) to assess the performance of MALDI-TOF MS. For further analyses, spectra of excellent quality were selected, based on their reproducibility compared to one another, the absence of background noise, and the global intensity (>3000 ua). Dendrograms were created using MALDI Biotyper v.3.0. Software, as previously described. [53].

5.6. Database updating and blind testing

MALDI-TOF MS spectra obtained from the termites' legs were tested against our homemade arthropod database containing reference spectra for 22 species of European and West African termites stored in alcohol [26]. Then, having updated this database with the spectra obtained from one to six specimens from each of the three species from Gabon, a blind test was carried out again, using the remaining spectra. The identification level was calculated using the LSVs assigned by the MALDI Biotyper v.3.0 software (Bruker Daltonics). This value, ranging from 0 to 3, assesses the similarity between a tested spectrum and the reference spectra by comparing the position of the peaks and their intensity [54].

Funding

This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the "Investissements d'avenir" programme, Reference ANR-10-IAHU-03, the Région Provence Alpes Côte d'Azur and European ERDF PRIMI funding. CIRMF is funded by Gabonese Government. It is member of CANTAM, funded by EDCTP.

BH received a grant in the form of a PhD scholarship from IHU Méditerranée Infection. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

Bouthaina Hasnaoui: Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Conceptualization. **Adama Zan Diarra:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Patrice Makouloutou-Nzassi:** Methodology, Conceptualization. **Jean-Michel Bérenger:** Writing – review & editing, Visualization, Data curation, Conceptualization. **Afaf Hamame:** Writing – review & editing, Methodology, Conceptualization. **Barthelemy Ngoubangoye:** Visualization, Funding acquisition, Data curation, Conceptualization. **Mapenda Gaye:** Writing – review & editing, Data curation, Conceptualization. **Bernard Davoust:** Writing – review & editing, Data curation, Conceptualization. **Oleg Mediannikov:** Writing – review & editing, Data curation, Conceptualization. **Jean Bernard Lekana-Douki:** Writing – review & editing, Funding acquisition, Conceptualization. **Philippe Parola:** Writing – review & editing, Visualization, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Hasnaoui reports financial support was provided by Mediterranean University Hospital Institute for Infectious Diseases.

Data availability

The data presented in this study are openly available within the link https://doi.org/10.35081/zn3f-8f26. Moreover, sequences have been deposited in GenBank under accession numbers OQ605364" title="https://doi.org/10.35081/zn3f-8f26andOQ605364">OQ605364-OQ605378 and OQ703619-OQ703627.

Acknowledgments

The authors are grateful for the termite samples from the field crew and the valuable assistance and support of our laboratory colleagues, particularly Jacques Sevestre, during this investigation.

References

[1] G. Devi, « Termite » (sept. 2020), https://doi.org/10.24941/ijcr.37571.12.2019.
[2] E.L. Vargo, Diversity of termite breeding systems, Insects 10 (2) (2019) 52, https://doi.org/10.3390/insects10020052, févr.
[3] K. Krishna, D. A. Grimaldi, V. Krishna, et M. S. Engel, « Treatise on the Isoptera of the world. (Bulletin of the American Museum of Natural History, no. 377) », *Isoptera.*, avr. 2013, Consulté le: 8 mai 2020. [En ligne]. Disponible sur: http://digitallibrary.amnh.org/handle/2246/6430.

[4] B. Penel, et al., Macrotermes termite mounds influence the spatial pattern of tree species in two African rainforest sites, in northern Congo. But were they really forests in the past? J. Trop. Ecol. (2022) 1–8, https://doi.org/10.1017/S0266467422000165, mai.

[5] J. Tuma, P. Eggleton, , et al.T.M. Fayle, Ant-termite interactions: an important but under-explored ecological linkage, Biol. Rev. 95 (3) (2020) 555–572, https://doi.org/10.1111/brv.12577.

[6] D.K. Aanen, P. Eggleton, Fungus-Growing termites originated in African rain forest, Curr. Biol. 15 (9) (2005) 851–855, https://doi.org/10.1016/j.cub.2005.03.043, mai.

[7] A. Brune, C. Dietrich, The gut microbiota of termites: digesting the diversity in the light of ecology and evolution, Annu. Rev. Microbiol. 69 (1) (2015) 145–166, https://doi.org/10.1146/annurev-micro-092412-155715.

[8] H. Sane, T. Samb, A.B. Ndiaye, C. Tidiane Ba, Etude de La diversite des termites (Isoptera) dans quelques localites de La region de kolda (haute casamance, Senegal), ESJ 12 (33) (2016), https://doi.org/10.19044/esj.2016.v12n33p263. Art. n° 33, nov.

[9] C. C, M. Baker, et al., Spatial patterning of soil microbial communities created by fungus-farming termites, Mol. Ecol. 29 (22) (nov. 2020) 4487–4501, https://doi.org/10.1111/mec.15585.

[10] Y. Zhou, A.C. Staver, A.B. Davies, Species-level termite methane production rates, Ecology 104 (2) (2023) e3905, https://doi.org/10.1002/ecy.3905, févr.

[11] S. Séité, et al., Lifespan prolonging mechanisms and insulin upregulation without fat accumulation in long-lived reproductives of a higher termite, Commun. Biol. 5 (2022) 44, https://doi.org/10.1038/s42003-021-02974-6, janv.

[12] L.C.S. Reddy, Termite mound as an effective geochemical tool in mineral exploration: a study from chromite mining area, Karnataka, India, Research Journal of Chemical Sciences ISSN 2231 (2014) 606X.

[13] W. de F. Alves, A.S. Mota, R. de Lima, R. Bellezoni, , et al.A. Vasconcellos, Termites as bioindicators of habitat quality in the caatinga, Brazil: is there agreement between structural habitat variables and the sampled assemblages? Neotrop. Entomol. 40 (2011) 39–46, https://doi.org/10.1590/S1519-566X2011000100006, févr.

[14] D.K. Aanen, P. Eggleton, C. Rouland-Lefèvre, T. Guldberg-Frøslev, S. Rosendahl, , et al.J.J. Boomsma, The evolution of fungus-growing termites and their mutualistic fungal symbionts, Proc. Natl. Acad. Sci. U. S. A. 99 (23) (2002), https://doi.org/10.1073/pnas.222313099. Art. n° 23, nov.

[15] C. Boesch, et al., Chimpanzee ethnography reveals unexpected cultural diversity, Nat. Human Behav. 4 (9) (2020), https://doi.org/10.1038/s41562-020-0890-1. Art. n° 9, sept.

[16] E. E, G. Wilfried, J. Yamagiwa, Use of tool sets by chimpanzees for multiple purposes in Moukalaba-Doudou National Park, Gabon, Primates 55 (4) (oct. 2014) 467–472, https://doi.org/10.1007/s10329-014-0431-5.

[17] W.C. McGrew, , et al.M.E. Rogers, Brief report: chimpanzees, tools, and termites: new record from Gabon, Am. J. Primatol. 5 (2) (1983) 171–174, https://doi.org/10.1002/ajp.1350050207.

[18] C. E, G. Tutin, M. Fernandez, Insect-eating by sympatric lowland gorillas (Gorilla g. gorilla) and chimpanzees (Pan t. troglodytes) in the lopé reserve, Gabon, Am. J. Primatol. 28 (1) (1992) 29–40, https://doi.org/10.1002/ajp.1350280103.

[19] V. Roy, M. Girondot, , et al.M. Harry, The distribution of Wolbachia in Cubitermes (Termitidae, Termitinae) castes and colonies: a modelling approach, PLoS One 10 (2) (2015) e0116070, https://doi.org/10.1371/journal.pone.0116070.

[20] C. Roose-Amsaleg, Y. Brygoo, , et al.M. Harry, Ascomycete diversity in soil-feeding termite nests and soils from a tropical rainforest, Environ. Microbiol. 6 (5) (2004) 462–469, https://doi.org/10.1111/j.1462-2920.2004.00579.x, mai.

[21] G. Bouillon, A. Mathot, Quel est ce termite africain? Edition de l'Université Léopoldville: Léopoldville, Congo, Edition de l'Université Léopoldville: Léopoldville, Congo, 1965, 1965. 1965.

[22] J. Korb, et al., Termite taxonomy, challenges and prospects: West Africa, A case example, Insects 10 (1) (2019), https://doi.org/10.3390/insects10010032. Art. n° 1, janv.

[23] V. Roy, C. Demanche, A. Livet, , et al.M. Harry, Genetic differentiation in the soil-feeding termite Cubitermes sp. affinis subarquatus: occurrence of cryptic species revealed by nuclear and mitochondrial markers, BMC Evol. Biol. 6 (nov. 2006) 102, https://doi.org/10.1186/1471-2148-6-102.

[24] A.S. Vidyashree, C.M. Kalleshwaraswamy, H.M. Mahadeva swamy, R. Asokan, , et al.S.K. Adarsha, Morphological, molecular identification and phylogenetic analysis of termites from western Ghats of Karnataka, India, J. Asia Pac. Entomol. 21 (1) (2018) 140–149, https://doi.org/10.1016/j.aspen.2017.11.006, mars.

[25] J. Sevestre, A.Z. Diarra, M. Laroche, L. Almeras, , et al.P. Parola, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: an emerging tool for studying the vectors of human infectious diseases, Future Microbiol. 16 (2021) 323–340, https://doi.org/10.2217/fmb-2020-0145, mars.

[26] B. Hasnaoui, et al., Use of the proteomic tool MALDI-TOF MS in termite identification, Sci. Rep. 12 (2022) 718, https://doi.org/10.1038/s41598-021-04574-0, janv.

[27] T. Bourguignon, et al., Mitochondrial phylogenomics resolves the global spread of higher termites, ecosystem engineers of the tropics, Mol. Biol. Evol. 34 (3) (mars 2017) 589–597, https://doi.org/10.1093/molbev/msw253.

[28] D.J.G. Inward, A.P. Vogler, , et al.P. Eggleton, A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology, Mol. Phylogenet. Evol. 44 (3) (2007), https://doi.org/10.1016/j.ympev.2007.05.014. Art. n° 3, sept.

[29] É. Bugnion, La différenciation des castes chez les Termites [Nevr.], Bull. Soc. Entomol. Fr. 18 (8) (1913). Art. n° 8.

[30] T. Clay, Bulletin of the British Museum (Natural History) Botany, vol. 24, Natural History) Entomology, London, 1969.

[31] S. Hellemans, J. Deligne, Y. Roisin, , et al.G. Josens, Phylogeny and revision of the 'Cubitermes complex' termites (termitidae: cubitermitinae), Syst. Entomol. 46 (2021) 224–238, https://doi.org/10.1111/syen.12458, janv.

[32] I. Sivanesan, J. Gopal, R. Surya Vinay, E.H. Luke, J.-W. Oh, , et al.M. Muthu, Consolidating the potency of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) in viral diagnosis: extrapolating its applicability for COVID diagnosis? Trends Anal. Chem. 150 (2022) 116569 https://doi.org/10.1016/j.trac.2022.116569 mai.

[33] L. Benkacimi, G. Gazelle, B. El Hamzaoui, J.-M. Bérenger, P. Parola, , et al.M. Laroche, MALDI-TOF MS identification of Cimex lectularius and Cimex hemipterus bedbugs, Infect. Genet. Evol. 85 (nov. 2020) 104536, https://doi.org/10.1016/j.meegid.2020.104536.

[34] L.N. Huynh, et al., MALDI-TOF mass spectrometry identification of mosquitoes collected in Vietnam, Parasites Vectors 15 (2022) 39, https://doi.org/10.1186/s13071-022-05149-2, janv.

[35] A.Z. Diarra, et al., Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali, PLoS Neglected Trop. Dis. 11 (7) (2017), https://doi.org/10.1371/journal.pntd.0005762. Art. n° 7, juill.

[36] A. Mathis, et al., Identification of phlebotomine sand flies using one MALDI-TOF MS reference database and two mass spectrometer systems, Parasites Vectors 8 (1) (2015) 266, https://doi.org/10.1186/s13071-015-0878-2, déc.

[37] B. Ouarti, et al., Development of MALDI-TOF mass spectrometry for the identification of lice isolated from farm animals, Parasite 27 (2020) 28, https://doi.org/10.1051/parasite/2020026.

[38] E.H.I. Ndiaye, et al., Morphological, molecular and MALDI-TOF MS identification of bedbugs and associated Wolbachia species in rural Senegal, J. Med. Entomol. 59 (3) (2022) 1019–1032, https://doi.org/10.1093/jme/tjac019, mai.

[39] S. Ngoy, et al., Using MALDI-TOF mass spectrometry to identify ticks collected on domestic and wild animals from the Democratic Republic of the Congo, Exp. Appl. Acarol. 84 (3) (2021) 637–657, https://doi.org/10.1007/s10493-021-00629-z, juill.

[40] Y. Sjöstedt, Termiten Afrikas, P.A. Norstedt & söner, 1900.

[41] A.F. Iroko, L'homme et les termitières en Afrique, KARTHALA Editions, 1996.

[42] M. Lepage, P. J, E.C. Darlington, Population dynamics of termites, in: T. Abe, D.E. Bignell, , et al.M. Higashi (Eds.), Termites: Evolution, Sociality, Symbioses, Ecology, Springer Netherlands, Dordrecht, 2000, pp. 333–361, https://doi.org/10.1007/978-94-017-3223-9_16.

[43] R. Alajmi, A. Mashaly, N. Al-Otaibi, A. Mahmoud, , et al.T. Ayaad, Efficiency of three mitochondrial genes in molecular identification and phylogenetic analysis of termites, Research journal of biotechnology 14 (sept. 2019) 30–40.

[44] N. Al-Otaibi, A. Mashaly, R. Alajmi, A. Ahmed, , et al.T. Ayaad, Genetic diversity of termites from Ta'if city, Saudi arabia, J. Environ. Biol. 40 (2019) 29–35, https://doi.org/10.22438/jeb/40/1/MRN-843, janv.

[45] R. Alajmi, R. Abdel-Gaber, , et al.N. AlOtaibi, Characterization of the 12S rRNA gene sequences of the harvester termite anacanthotermes ochraceus (Blattodea: hodotermitidae) and its role as A bioindicator of heavy metal accumulation risks in Saudi arabia, Insects 10 (2) (2019), https://doi.org/10.3390/insects10020051. Art. n° 2, févr.
[46] T. Bourguignon, et al., Delineating species boundaries using an iterative taxonomic approach: the case of soldierless termites (Isoptera, Termitidae, Apicotermitinae), Mol. Phylogenet. Evol. 69 (3) (2013), https://doi.org/10.1016/j.ympev.2013.07.007. Art. n° 3, déc.
[47] Y. Sjöstedt, Monographie der Termiten Afrikas, P.A. Norstedt & söner, 1900.
[48] S. Uehara, Seasonal changes in the techniques employed by wild chimpanzees in the mahale mountains, Tanzania, to feed on termites (Pseudacanthotermes spiniger), IJFP 37 (1–2) (1982) 44–76, https://doi.org/10.1159/000156020, févr.
[49] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, , et al.D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (oct. 1990) 403–410, https://doi.org/10.1016/S0022-2836(05)80360-2.
[50] R.H. Thomas, Molecular evolution and phylogenetics, Heredity 86 (3) (2001), https://doi.org/10.1046/j.1365-2540.2001.0923a.x. Art. n° 3, mars.
[51] A. Yssouf, L. Almeras, D. Raoult, , et al.P. Parola, Emerging tools for identification of arthropod vectors, Future Microbiol. 11 (4) (2016), https://doi.org/10.2217/fmb.16.5. Art. n° 4.
[52] I. Lafri, L. Almeras, I. Bitam, A. Caputo, A. Yssouf, C.-L. Forestier, et al., Identification of Algerian field-caught phlebotomine sand fly vectors by MALDI-TOF MS 10 janvier, PLoS Negl. Trop. Dis. 10 (1) (2016), https://doi.org/10.1371/journal.pntd.0004351.
[53] P.H. Boyer, N. Boulanger, A. Nebbak, E. Collin, B. Jaulhac, , et al.L. Almeras, Assessment of MALDI-TOF MS biotyping for Borrelia burgdorferi sl detection in Ixodes ricinus, PLoS One 12 (9) (2017), https://doi.org/10.1371/journal.pone.0185430. Art. n° 9.
[54] A. Nebbak, et al., Field application of MALDI-TOF MS on mosquito larvae identification, Parasitology 145 (5) (2018), https://doi.org/10.1017/S0031182017001354. Art. n° 5.