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RESEARCH ARTICLE

Molecular characterization of *Amitermes rhizophagus* Belyaeva, 1974 (Blattodea: Isoptera), newly recorded from Uzbekistan

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Abstract

This study presents the first molecular confirmation of *Amitermes rhizophagus* Belyaeva, 1974 (Blattodea: Termitidae) from Uzbekistan. Specimens collected in the Kashkadarya Region were identified through combined morphological and molecular analyses. Ribosomal DNA (ITS) and mitochondrial DNA (COI) fragments were amplified, sequenced, and compared with reference sequences from GenBank. Phylogenetic analyses using the Maximum Likelihood method placed *A. rhizophagus* within a well-supported *Amitermes* clade (bootstrap = 98-100%), closely related to *A. deplanatus*, *A. conformis*, and *A. foreli*. Genetic distances were low within *Amitermes* (0.017-0.034) but high between genera (0.13-0.19), confirming clear evolutionary separation from *Drepanotermes* and *Microcerotermes*. These findings validate the taxonomic identity of *A. rhizophagus* and provide the first molecular evidence for its occurrence in Uzbekistan, enriching knowledge of Central Asian termite diversity and phylogeny.

Keywords
Amitermes rhizophagus, Termitidae, ITS, COI, phylogeny, Central Asia

Introduction

Termites are insects belonging to the order Isoptera, with around 3000 species identified worldwide. Of these, roughly 300 are known to cause considerable damage to buildings and wooden materials (Edwards and Mill 1986; Abe et al. 2000; Krishna et al. 2013). They are among the most abundant and diverse terrestrial insects, making up an estimated 10-20% of the total animal biomass in tropical regions (Eggleton et al. 1996; Ellwood and Foster 2004). Termites are capable of digesting plant material, especially lignocellulose, the most common organic compound on Earth, as well as organic matter dissolved in soil (Norkrans 1963; Dixon et al. 1994). By decomposing dead wood and plant material, they contribute significantly to nutrient cycling and soil health, sometimes consuming up to 90% of available organic matter in their habitats (Bignell 2006; Jouquet et al. 2016).

Although shrubification driven by alder, willow, and dwarf birch expansion (TapFossil records suggest that termites first appeared approximately 140 million years ago (Engel et al. 2007). However, later molecular studies involving 66 termite species estimated their evolutionary origin to be around 54 million years ago (Bourguignon et al. 2015). In addition, Thomas (2016) explored the evolutionary history and geographical distribution of termites by analyzing complete mitochondrial genomes from 415 species.

For identifying species and studying their evolutionary relationships, researchers often analyze a short segment of mitochondrial DNA, particularly the cytochrome c oxidase subunit I (COI) gene (Hebert et al. 2003a, 2003b). DNA barcoding, a widely used technique among taxonomists, links biological samples to a specific DNA sequence, most commonly the COI gene (Ebach 2011). This fragment is highly effective for distinguishing closely related species and has greatly improved taxonomy-based biodiversity assessments (Hebert et al. 2004). The COI gene has been successfully applied across numerous insect orders, including Coleoptera (Raupach et al. 2010), Diptera (Scheffer et al. 2006), Ephemeroptera (Ball et al. 2005), Hemiptera (Lee et al. 2011), Hymenoptera (Smith et al. 2009), and Lepidoptera (Hajibabaei et al. 2006).

This study aims to characterize *Amitermes rhizophagus* Belyaeva, 1974, a species belonging to the genus *Amitermes*, distributed in the Kashkadarya regions of Uzbekistan, using ribosomal DNA (ITS) and mitochondrial DNA (COI) gene markers.

Materials and methods

Morphological Basis

Before the molecular analysis, the morphology of *Amitermes rhizophagus* Belyaeva, 1974 was comprehensively examined and described by Rustamov et al. (2024), who confirmed the species' taxonomic identity based on diagnostic features of the soldier, nymph, and worker castes, and documented its occurrence in southern Uzbekistan. Their research provided essential baseline information on external morphology, caste structure, and habitat preferences, establishing A. rhizophagus as a newly recorded species for the fauna of Uzbekistan.

Building upon this morphological foundation, the present study focuses on the molecular characterization and phylogenetic placement of *A. rhizophagus* using ribosomal DNA (ITS) and mitochondrial DNA (COI) markers (White et al. 1990; Simon et al. 1994)

DNA Extraction, Amplification, and Sequencing

Specimens were collected from the antennae and leg tissues of soldiers and workers of *Amitermes rhizophagus* from the Kashkadarya Region, Uzbekistan. Genomic DNA was extracted using the GeneJet Genomic DNA Purification Kit following standard protocols (Vogelstein and Gillespie 1979; Marko et al. 1982; Boom et al. 1990).

To amplify the ribosomal DNA ITS region, PCR reactions were prepared with reagents from the Silex kit, containing sterile water, 10× PCR buffer, dNTP solution, Taq polymerase, and primers AB28 forward (ATA TGC TTA AGT TCA GCG GGT) and TW81 reverse (GTT TCC GTA GGT GAA CCT GC) (Curran 1994).

For amplification of the mitochondrial COI region, primers UEA-3 forward (TAT AGC ATT CCC ACG AAT AAA TAA) and UEA-10 reverse (TCC AAT GCA CTA ATC TGC CAT ATTA) were used (Zhang and Hewitt 1997).

PCR cycling conditions were as follows: initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute; with a final extension at 72°C for 10 minutes.

Sequencing of PCR products was performed using the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit, and products were analyzed on an ABI PRISM 3100-Avant automated sequencer at the Center for Collective Use "Genom" (GenTech, Moscow).

Sequence Alignment and Phylogenetic Analysis

The obtained nucleotide sequences were analyzed using BioEdit, Clustal W, DNASTAR™, PAUP*4, and MEGA 11 software (Thompson et al. 1994; Hall 1999;

Burland 2000; Tamura et al. 2021). Reference sequences representing other *Amitermes* species and related taxa were retrieved from the NCBI GenBank database (NCBI 1988). All sequences were checked and manually curated using Geneious Prime, and consensus sequences were generated in MEGA 11 (Kearse et al. 2012; Tamura et al. 2021).

Multiple sequence alignments for ITS and COI regions were performed using MAFFT v.7 and Clustal Omega 1.2.2 with standard settings, followed by manual refinement (Katoh and Standley 2013; Sievers and Higgins 2014). Phylogenetic relationships were inferred using the Maximum Likelihood (ML) method implemented in IQ-TREE v1.6.12 with 1,000 ultrafast bootstrap replicates, executed through the CIPRES Science Gateway v3.3 (Miller et al. 2010; Nguyen et al. 2015).

To root the trees, *Microcerotermes turmenicus* was used as an outgroup for their respective genera. Phylogenetic trees were visualized and annotated using iTOL v6.6.

Molecular Data Overview

Analysis of the mitochondrial COI region yielded a 658 bp sequence for *Amitermes rhizophagus*. Comparative alignment with GenBank sequences revealed high similarity to other *Amitermes* lineages, supporting its molecular identity and confirming its occurrence in Uzbekistan. The integration of morphological and molecular evidence provides robust confirmation of *A. rhizophagus* as part of Uzbekistan's termite fauna and contributes valuable data for understanding its evolutionary relationships and biogeography.

Phylogenetic reconstruction based on the internal transcribed spacer (ITS) gene revealed three well-supported clades corresponding to the genera *Microcerotermes*, *Amitermes*, and *Drepanotermes*. *Microcerotermes turkmenicus* (PQ058150) was used as the outgroup to root the tree. The *Amitermes* species formed a distinct, highly supported cluster (bootstrap values 88-100%), confirming their close evolutionary relationships. Within this clade, both *A. rhizophagus* specimens from Uzbekistan grouped together with strong support (bootstrap = 100%), clustering near *A. deplanatus* and *A. conformis*. The genetic distance data indicated that *A. rhizophagus* differs from *A. deplanatus* and *A. conformis* by an average of 0.021-0.034 substitutions per site, confirming their close affinity within the same lineage.

In contrast, *Drepanotermes* species (including *D. tamminensis*, *D. peringer*, *D. columellaris*, and *D. gayi*) formed a separate and monophyletic group (bootstrap = 94-100%), clearly divergent from the *Amitermes* cluster. Pairwise genetic distances among *Drepanotermes* species ranged from 0.012 to 0.045, whereas intergeneric divergence between *Amitermes* and *Drepanotermes* averaged 0.136, indicating strong genetic differentiation. These findings demonstrate that the newly sequenced *A. rhizophagus* from Uzbekistan is phylogenetically consistent with other *Amitermes* species and forms a coherent, well-supported group distinct from *Drepanotermes*.

The mitochondrial cytochrome oxidase subunit I (COI) gene-based phylogeny confirmed the taxonomic placement of *Amitermes rhizophagus* within the *Amitermes* clade. *Microcerotermes arboreus* (OM415341) was used as the outgroup. The analysis recovered a single, well-supported *Amitermes* lineage (bootstrap = 87-100%), subdivided into several regional subclades. The two *A. rhizophagus* samples from Uzbekistan clustered together (bootstrap = 98%) and showed close affinity to *A. foreli* and *A. kriptodon*, indicating low intraspecific divergence (genetic distance ≈ 0.017) and moderate interspecific divergence (0.048-0.069).

The *Amitermes* group also displayed several internal lineages corresponding to *A. amifer*, *A. beaumonti*, *A. californicus*, and *A. meridionalis*, each supported by high bootstrap values (>90%). Average genetic distances within *Amitermes* species ranged from 0.009 to 0.072, suggesting moderate differentiation but overall genetic cohesion within the genus. The large divergence between Microcerotermes and *Amitermes* (0.152-0.185) highlights their long evolutionary separation.

Together, the ITS and COI phylogenies, supported by genetic distance analysis, consistently indicate that *A. rhizophagus* belongs firmly within the *Amitermes* clade, forming a closely related lineage to *A. deplanatus* and *A. foreli*. Both markers confirmed clear separation from *Drepanotermes* and *Microcerotermes*, underscoring the genetic distinctness and evolutionary stability of *Amitermes rhizophagus* within the genus.

Results

Phylogenetic analysis identified three termite genera: *Microcerotermes*, *Amitermes*, and *Drepanotermes*. In both ITS and COI gene trees, *Microcerotermes turkmenicus* was selected as the outgroup and used as the root to determine evolutionary relationships among *Amitermes* and *Drepanotermes* species.

According to the ITS gene analysis (Fig. 1), species belonging to the genus *Amitermes* clustered together and formed a distinct, well-supported monophyletic clade (bootstrap > 88%). The two *A. rhizophagus* sequences from Uzbekistan grouped together with 100% bootstrap support, confirming their identical or nearly identical genetic composition. This *A. rhizophagus* cluster was closely related to *A. deplanatus* and *A. conformis*, with moderate genetic distances ranging from 0.021 to 0.034, indicating close affinity within the genus. In contrast, *Drepanotermes* species such as *D. tamminensis*, *D. peringier*, *D. columellaris*, and *D. gayi* formed a separate lineage (bootstrap > 94%) with intrageneric genetic distances ranging from 0.012 to 0.045. The average intergeneric divergence between *Amitermes* and *Drepanotermes* was approximately 0.136, demonstrating substantial evolutionary differentiation between these genera. All major nodes in the ITS tree were strongly supported by bootstrap values above 87%, confirming the robustness of the inferred relationships.

The COI gene-based phylogenetic tree (Fig. 2) similarly revealed strong clustering of *Amitermes* species (bootstrap > 87%). The two *A. rhizophagus* sequences from

Uzbekistan again formed a highly supported clade (bootstrap = 98%), closely allied with *A. foreli* and *A. kriptodon*. Genetic distance values between *A. rhizophagus* and *A. foreli* were relatively small (≈ 0.017-0.032), while interspecific divergence within the *Amitermes* group ranged from 0.009 to 0.072. In contrast, the separation between *Microcerotermes* and *Amitermes* was pronounced (≈ 0.152-0.185), confirming their distant phylogenetic relationship. Species such as *A. beaumonti*, *A. californicus*, and *A. meridionalis* each formed distinct sublineages within *Amitermes*, all supported by bootstrap values above 90%.

Overall, both ITS and COI analyses revealed congruent topologies that consistently placed *Amitermes rhizophagus* within a well-supported *Amitermes* clade. Genetic distance estimates and high bootstrap support jointly confirm that *A. rhizophagus* represents a distinct but closely related lineage within the genus, clearly separated from *Drepanotermes* and *Microcerotermes*. These findings provide molecular evidence that the *A. rhizophagus* population from Uzbekistan belongs to the genus *Amitermes* and shares a recent common ancestor with *A. deplanatus* and *A. foreli*.

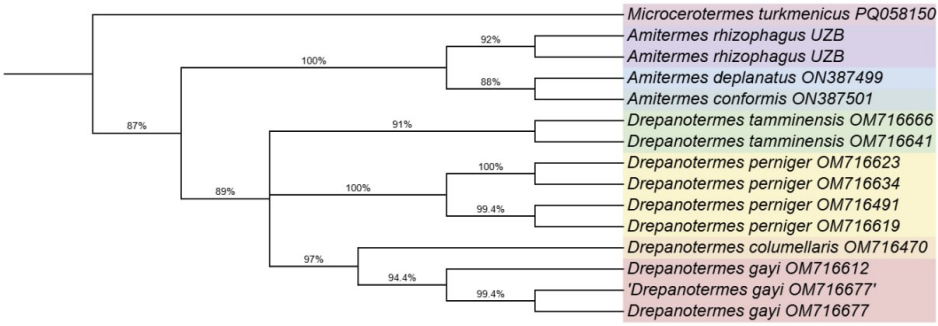

Figure 1. Phylogenetic analysis of *Amitermes rhizophagus* and closely related species based on the ITS gene.

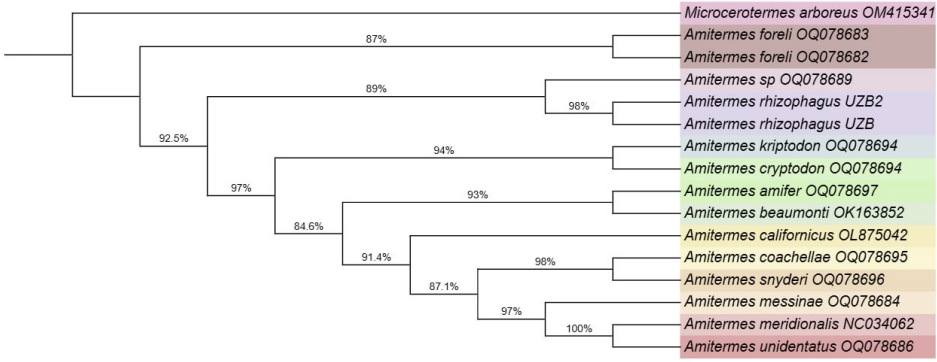

Figure 2. Phylogenetic analysis of *Amitermes rhizophagus* and closely related species based on the COI gene.

Discussion

This study provides the first molecular confirmation of *Amitermes rhizophagus* in Uzbekistan, combining morphological and genetic data to verify its taxonomic identity. Both ITS and COI gene analyses consistently placed the species within a well-supported *Amitermes* clade, closely related to *A. deplanatus* and *A. foreli*. High bootstrap support and low intraspecific divergence (≤ 0.03) confirm its genetic coherence and recent evolutionary origin within the genus.

The moderate genetic distances among *Amitermes* species (0.017-0.034) and large divergence from *Drepanotermes* and *Microcerotermes* (0.13-0.19) demonstrate clear intergeneric boundaries. These values correspond with previous studies showing long-term divergence among Termitidae lineages (Bourguignon et al. 2015; Thomas 2016). The molecular data also align with morphological differences previously documented for *A. rhizophagus*, reinforcing its diagnostic validity (Rustamov et al. 2024).

The detection of *A. rhizophagus* in the Kashkadarya Region extends the known distribution of the genus into Central Asia, indicating that arid landscapes of Uzbekistan support greater termite diversity than previously recognized. This may reflect historical dispersal from adjacent arid regions, supported by similar ecological conditions.

Overall, this research highlights the power of molecular approaches in termite taxonomy. The congruence of nuclear and mitochondrial evidence confirms the distinct status of *A. rhizophagus* and provides a foundation for future studies on the evolutionary history, ecology, and biogeography of Central Asian termites.

Conclusions

The results of the phylogenetic tree and genetic distance analyses complement each other and clearly define the evolutionary relationships within the genus *Amitermes* and its related taxa. Both ITS and COI gene-based phylogenies consistently supported the monophyly of *Amitermes*, with high bootstrap values (≥ 87%) across major nodes. The *Amitermes rhizophagus* specimens collected from Uzbekistan formed a distinct but closely related lineage within the *Amitermes* clade, showing low genetic distances from *A. deplanatus* and *A. foreli* (≤ 0.03), which confirms their close evolutionary affinity.

In contrast, *Drepanotermes* species were clearly separated from *Amitermes*, exhibiting higher intergeneric divergence (≈ 0.13-0.18), while *Microcerotermes* showed even greater genetic distance (≈ 0.15-0.19), indicating long-term evolutionary divergence between these genera. The congruent topologies obtained from both nuclear (ITS) and mitochondrial (COI) markers highlight the stability and reliability of the molecular data used in this study.

Overall, these findings demonstrate the effectiveness of molecular phylogenetic approaches in resolving termite taxonomy and provide robust evidence for the phylogenetic placement of *Amitermes rhizophagus* within the genus *Amitermes*. This study represents the first molecular confirmation of *A. rhizophagus* from Uzbekistan and contributes valuable data toward understanding the evolutionary history and biogeographic relationships of Central Asian termites.

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