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Research Article

Distribution, Morphometrics and DNA barcoding of *Archotermopsis wroughtoni* Desneux (Termopsidae: Blattodea) in District Mansehra, Pakistan

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Abstract: *Archotermopsis wroughtoni* is a primitive termite species with distinct biological and behavioral features. Despite its presence in temperate regions worldwide, including the Oriental region, there is a lack of data on the current distribution of this species in the Mansehra district of Pakistan. Samples were collected from forested areas, including the Kaghan, Naran, Mahandri, and Shogran valleys. The distribution of *A. wroughtoni* was determined by observing decayed and fallen wooden logs, and host plants were identified to assess the species' host preference. Morphometric identification was performed following relevant literature, and the barcoding technique of mtDNA *COII* was used to authenticate the species. Phylogenetic analyses were conducted using the neighbor-joining and maximum parsimony methods. The study revealed the presence of *A. wroughtoni* in the forests of northern Mansehra, where it preferred tree species such as *Cedrus deodara* and *Pinus excelsa* for nest construction and foraging. The findings of this research will contribute to future studies on the biology and ecology of *A. wroughtoni* and aid in developing conservation strategies for this species and other social insects.

Keywords: Termites, Termopsidea, *Archotermopsis wroughtoni*, distribution, morphometric, DNA barcoding, Mansehra

1. INTRODUCTION

Termopsidea family is distinguished as a small and primitive group comprised of three surviving genera: *Archotermopsis*, *Hodotermopsis*, and *Zootermopsis* [1]. These termites are geographically restricted to the Oriental and Nearctic regions [2]. Oriental region covers Asia's tropical territories, including India, Pakistan, Sri Lanka, Indonesia, and the Philippines. In contrast, the Neartic region encompasses North America, extending southward to the middle part of Mexico [3]. Among the three genera, *Archotermopsis* is the sole genus found dwelling in the foothills of the Himalayas and Vietnam [1]. On the other hand, *Hodotermopsis* and *Zootermopsis* inhabit Vietnam, South China, Japan, and the western region of the United States [1, 2].

Archotermopsis wroughtoni, commonly called the Himalayan termite, is a primitive and extant species that fall under the subfamily Termopsinae within the Termopsidae family. It inhabits the northern regions of Pakistan, the northwestern Himalayas in India, and the eastern parts of Afghanistan [2]. Termites can be classified into two distinct groups, namely lower termites and higher termite species, based on the specific nature of their symbiotic partners residing within their gastrointestinal tracts [4]. Lower termites exhibit a symbiotic relationship with prokaryotes and flagellated protozoans inhabiting their intestinal tracts [5]. In contrast, higher termites do not possess flagellated protozoa but rather harbor symbiotic prokaryotes within their intestinal tracts [4]. *A. wroughtoni* is categorized as a member of the lower termite group [6].

Archotermopsis wroughtoni serves as a keystone species in the ecosystem, playing a vital role in maintaining the balance of the forest. Through its

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breakdown of dead wood and subsequent return of nutrients to the soil, this species helps to sustain the forest's vitality [7-8]. Furthermore, *A. wroughtoni* is a crucial food source for other organisms, including birds and insects. The termite's role in the carbon cycle is also noteworthy, as it facilitates the decomposition and recycling of carbon-rich plant material. Additionally, its activity improves soil structure, thus contributing to the growth of new vegetation [8].

A. wroughtoni termite species in Pakistan is experiencing a decline and is presently considered endangered [9]. This species breeds in decaying logs commonly found in pine tree forests [2]. However, due to the escalating human population in these areas, these logs are rapidly being harvested for fuel, which results in *A. wroughtoni* losing suitable breeding sites. This human activity significantly threatens the species' survival in the wild [2, 9].

The existing body of literature regarding the historical distribution of *A. wroughtoni* documents its occurrence in Pakistan. Specifically, this species has been recorded in various northern regions of Pakistan, including Kumrat, Kalam, Roringar, Murree, Hazara, Kaghan, Naran, and Shogran [7, 10-12]. However, there is limited knowledge regarding its recent distribution, host preferences, and molecular characterization. Moreover, the phylogenetic relationships among different populations of *A. wroughtoni* still need to be adequately understood.

Therefore, this research aimed to conduct DNA barcoding and phylogenetic analysis, in addition to morphometric characterization and distribution, of *A. wroughtoni* in the Mansehra district of Pakistan. To achieve this, partial sequences of mitochondrial *COII* genes were analyzed to explore the genetic diversity and phylogenetic relationships among the regional populations of *A. wroughtoni*.

2. MATERIALS AND METHODS

2.1 Sampling

This study was conducted within the geographical boundaries of the Mansehra district in Pakistan, which can be located by coordinates situated in the northern latitudes between 34° 14' and 35° 11' and the eastern longitudes between 72° 49' and 74° 08' [13].

A survey of the district Mansehra was conducted between March and November 2020 and 2022. The belt transect method was utilized to collect samples from fallen logs [14]. Visible galleries were identified, and on-site samples were collected and preserved in vials containing 80 % ethanol for morphometric analysis and 99 % ethanol for DNA extraction [15]. Additionally, the coordinates of the sampling sites were recorded using a handheld GPS device (Garmin, GPSMAP 64sx), and the forage substrate for all locations was documented.

2.2 Morphometric Identification

We conducted morphometric identification of soldiers using available literature on keys, illustrations, pictures, characters, and indices. Measurements were taken using a binocular microscope equipped with built-in magnification, and statistical measures were calculated, including means [16]. A digital camera-equipped stereo zoom trinocular microscope (Olympus, SZX7) was employed to capture photographs. We documented and noted down ten distinct features/metrics, comprising the distance between the head and the lateral base of mandibles, the widest point of the head, the longest measurement of the labrum, the broadest point of the labrum, the length of the left mandible, the shortest median length of the postmentum, the middle point width of the postmentum, the median length of the pronotum, the median width of the pronotum, and the count of segments in the antennae [17].

2.3 Distribution and Mapping

The sampling sites' coordinates were recorded using a GPS device and projected onto a map utilizing ArcGIS 10.7 [18].

2.4 DNA Extraction and Amplification

In order to extract DNA, a single soldier termite specimen was selected as a representative and identified from available specimens. The specimen was washed with distilled water and air-dried, after which its legs were placed in a 2.5 mL eppendorf tube [19]. Liquid nitrogen was added to the tube to

freeze the specimen, which was then crushed with a pestle. The CTAB method was employed to extract DNA from the crushed specimen [20]. To assess the amount and purity of the genomic DNA extracted, an agarose gel containing 1 % concentration was utilized, which was subsequently stained with ethidium bromide. The samples were preserved at -20 °C for future experimentation after this analysis. For the PCR amplification, a 2 µL volume of the upper phase of the extracted DNA was combined with 23 µL of a master mix. The target fragment was a 684 bp segment of the COXII gene, amplified using forward and reverse primers 5′-TCTAATATGGCAGATTAGTGC-3′ and 5′-GAGACCAGTACTTGCTTTCAGTCATC-3′ [21, 22].

The PCR master mix (Thermo Fisher Scientific, Waltham, MA) included several compounds at specific concentrations: 2.5 µL of PCR buffer (10X), 1 unit of Taq polymerase (3U/µL), 2.5 µL of Bovine Serum Albumin (BSA) (100 µg/mL), 1.5-2.0 µL of Magnesium Chloride (25 mM), 0.5 µL of dNTPs Mix (10 mM), 1.25 µL of Primer (F) 10 pM, 1.25 µL of Primer (R) 10 pM, and 1 µL of Genomic DNA Template (20-50 ng). PCR-grade water was added to adjust the final volume of the mixture to 24 µL [23].

The PCR reaction was subjected to thermal cycling, utilizing the following conditions: an initial denaturation step was conducted at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 45 seconds. A final extension step was performed at 72 °C for 10 minutes. The amplified PCR products were subsequently analyzed through 1 % agarose gel electrophoresis [24].

2.5 DNA Sequencing and Phylogenetic Analysis

To sequence the PCR products, 20 µL of each sample was transferred to an eppendorf tube, which was subsequently dispatched to Celemics (Celemics, Korea) for Sanger sequencing. The obtained *COII* sequences were trimmed to an approximate length of 684 bp with the elimination of specific starting and ending fragments to ensure uniform sequence length and minimize interference [21, 25]. In order to assess the nucleotide sequence similarity, only the 30 most pertinent GenBank sequences (selected based on % query coverage, E-value, % identity, and relevant taxon matching using BLASTn) were scrutinized [26].

To maintain the precision of species identification for sequences forwarded to GenBank through BLASTn, a process of aligning the matching sequences of the target sequence with the reference sequence was conducted. The top ten sequences most closely related to *Archotermopsis* species were chosen to construct neighbour-joining and maximum likelihood trees and alignments of sequences using ClustalW in MEGA 11 [27-30]. The outcome of this process was a total of 12 *COII* sequences with accession numbers OQ753771, OQ753772, EU253892.1 (*Archotermopsis wroughtoni*), MF477197.1 (*Zootermopsis* laticeps), DQ442267.1 (*Zootermopsis* angusticollis), GQ922444.1:1-708 (*Zootermopsis* nevadensis), MZ058037.1 (*Cryptocercus matilei*), OM991373.1 (*Postelectrotermes* sp.), OM991347.1 (*Glyptotermes* sp. 11), OM991330.1 (*Comatermes perfectus*), KY224587.1 (*Mirocapritermes* sp.), and KY224622.1 (*Postsubulitermes parviconstrictus*) retrieved from GenBank. Furthermore, two newly generated sequences were deposited into the GenBank database and identified as OQ753771 and OQ753772.

3. RESULTS

3.1 Recorded Species of *Archotermopsis wroughtoni*

The head exhibits a posterior margin that is bilobed, while the cerci consist of 6 to 7 segments, and the antenna displays 22 to 27 articles (*Archotermopsis wroughtoni* Desneux) (Figure 1). The specimens gathered were identified for their taxonomic classification following the guidelines outlined by Roonwal *et al.* [2] and Imms and Hickson [16] and were found to conform to the published descriptions. The measurement of the soldier caste was conducted, considering several characteristics, and then compared to the previously reported range (Table 1).

Based on the statistical analysis, it can be inferred that all means fall within the established ranges, thus suggesting that the samples are a

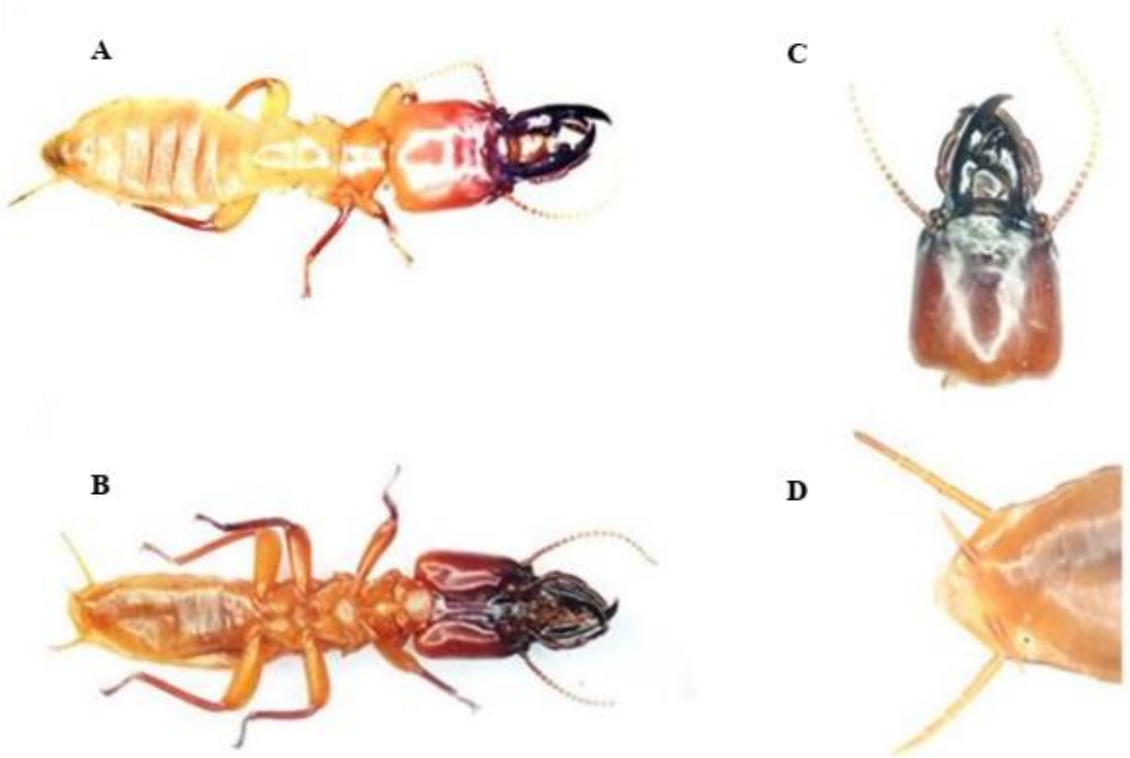

Fig. 1. Different morphometric indices of the Soldier caste of *Archotermopsis wroughtoni* **A**. *A. wroughtoni* soldier dorsal view, **B**. Ventral view of the soldier **C**. Head dorsal view, **D**. Cerci

probable reflection of the known population or that the pre-established range encompasses the population means.

3.2 Habitat Description and Wood Preference

Archotermopsis wroughtoni has been observed to inhabit high-altitude coniferous forests between 900 and 3000 meters. This particular species has demonstrated the ability to flourish by consuming decayed wood particularly that of *Cedrus deodara*, *Pinus roxburghii*, and *Pinus wallichiana* without causing harm to living trees. Its colonies, which their extended, vertical galleries characterize, are generally modest, typically consisting of 30-40 individuals.

Despite its wood consumption, no visible external signs of damage are evident. Furthermore, it has been observed that this species reproduces

Table 1. Comparison of index measurements (in mm) in the *Archotermopsis wroughtoni* soldier caste

S. No.	Indices	Measurements in (mm) of *A. wroughtoni*
1	Head length up to the lateral base of the mandibles	3.5*, 2.80-5.20**
2	Maximum width of the head	3.3*, 2.55-4.55**
3	Maximum labrum length	0.625*, 0.33-0.80**
4	Maximum labrum width	0.825*, 0.63-1.10**
5	Left mandible length	3.5*, 2.10-4.80**
6	Median length of postmentum	3.475*, 2.23-4.43**
7	Maximum postmentum width	2.315*, 1.53-3.03**
8	Maximum pronotum length	1.465*, 0.95-1.95**
9	Maximum pronotum width	2.315*, 1.53-3.03**
10	Number of segments in the antenna	23*, 22-27**

*Designates measurements recorded in the current study, ** denotes reference range [2]

within the wood, and during the monsoon season, particularly from June to August, swarming behaviour has been documented.

3.3 Local Distribution and Mapping

The distribution of *Archotermopsis wroughtoni* species was found to be restricted to specific localities within the Mansehra district, namely Kaghan Valley, Naran, Kiwai, Hangrai, Mahandri, Shogran, Pae, Paras, Garlat, and Ghannol. The species was not observed at lower elevations in the district. The coordinates of the collected and analyzed specimens were systematically plotted on a geographical map. The black dots on the map indicate the locations where the *Archotermopsis* species were encountered during the sampling process (Figure 2).

3.4 Remarks

The present study pertains to observations on *A. wroughtoni*, a termite species whose unique soldier caste morphology allows for straightforward identification. Notably, the species exhibits an uneven distribution within the Mansehra district and is confined to the upper mountainous regions characterized by the prevalence of pine forests. The species primarily occupies decaying logs as its preferred habitat, posing challenges to its detection owing to the propensity of local inhabitants to collect windfall trees.

3.5 DNA Barcoding

3.5.1. Sequences Alignment and Similarity Validation

The sequences of *Archotermopsis wroughtoni* corresponded to 97.49 % similarity with EU253892.1, as indicated by the results of the BLASTn search.

3.5.2. Neighbor-Joining Method Tree

The study employed the neighbour-joining [26]

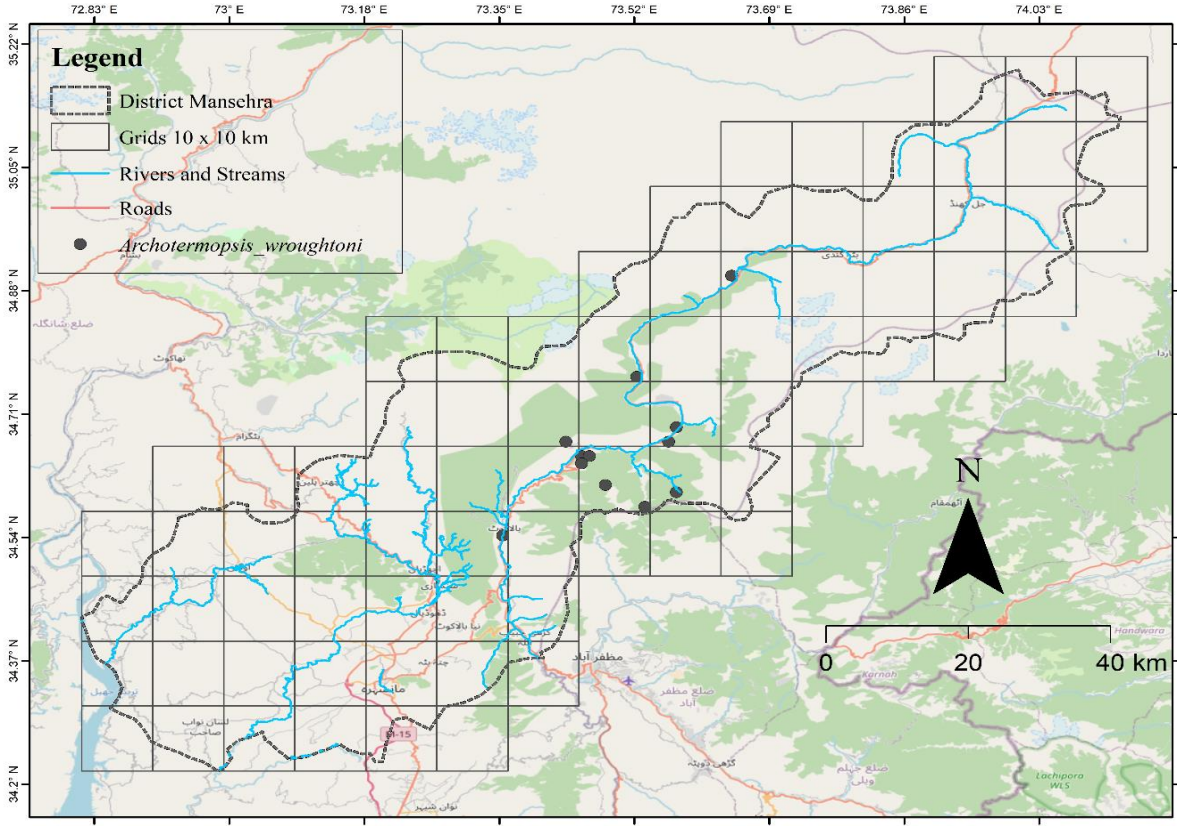

Fig. 2. Distribution of *Archotermopsis wroughtoni* in district Mansehra, Pakistan. The map was created using ArcGIS 10.7.

method to derive the evolutionary relationships, and the resulting optimal tree is presented herein (Figure 3). The bootstrap test (1000 replicates) illustrates the proportion of trees in which the associated taxa clustered with the branches.

The tree is drawn to scale, with the branch lengths representing the same units used for calculating the evolutionary distances, which were determined using the Maximum Composite Likelihood method and expressed as base substitutions per site [24, 25]. The analysis involved 12 nucleotide sequences, with all ambiguous positions removed for each sequence pair using the pairwise deletion option. The final dataset consisted of 736 positions. The evolutionary analyses were performed using MEGA11 [23]. The phylogenetic tree of the *Archotermopsis* sequence successfully matched the top sequence obtained from the BLASTn searches and isolated the *Archotermopsis* clades from other termite species (Figure 3).

3.5.3. Maximum Parsimony Method Tree

The Maximum Parsimony method was used to infer the evolutionary history, and Tree #1 out of the two most parsimonious trees (with a length of 0) is presented. The consistency index (0.644979) and the retention index (0.639885) indicate the degree of homoplasy in the dataset.

In contrast, the composite index (0.412712) measures the overall fit of the data to the phylogenetic tree. The tree branches are annotated with the percentage of replicate trees in which the associated taxa clustered together, as determined by the bootstrap test (1000 replicates) [24].

The MP tree was generated using the Subtree-Pruning-Regrafting (SPR) algorithm [26], with search level 1, and 10 initial tree additions were performed. The final dataset consisted of 12 nucleotide sequences with 736 positions. The phylogenetic tree, which includes the top 10 sequences from BLASTn searches, effectively distinguished the clades of *Archotermopsis wroughtoni* from other species, as illustrated in Figure 4. Evolutionary analyses were performed using MEGA11 [23].

4. DISCUSSION

The present investigation aimed to assess the distribution of *Archotermopsis wroughtoni* and

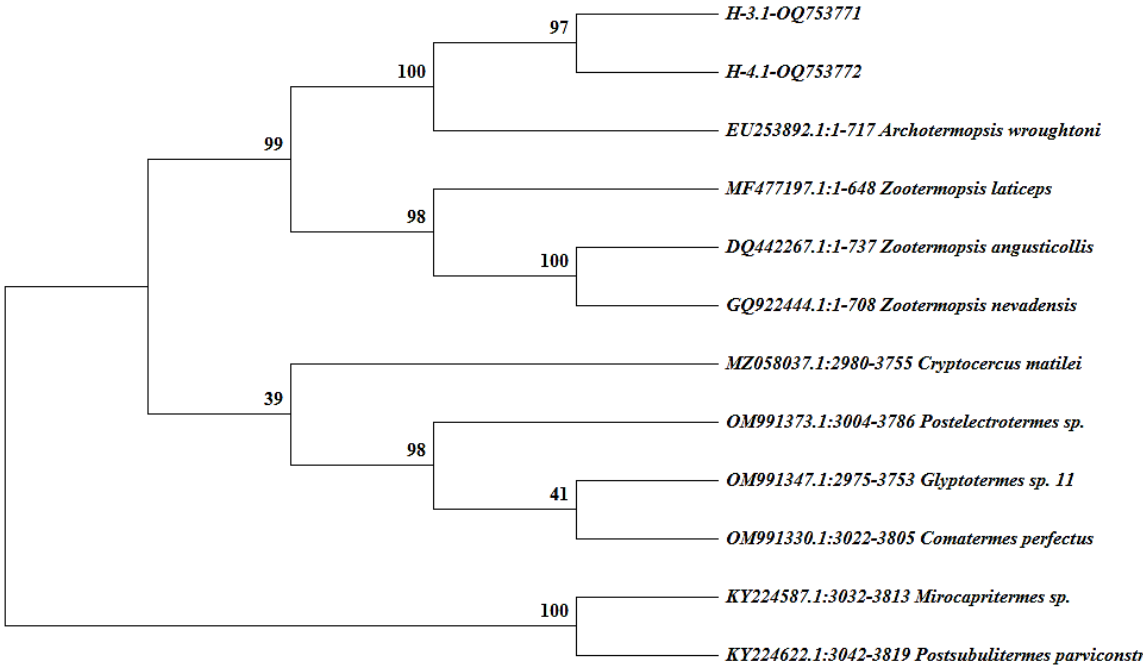

Fig. 3. Phylogenetic analysis of Archotermopsis species gathered from different regions of the Mansehra district using the Unrooted Neighbor-Joining method. The type specimens submitted by the sequence uploader are represented by sequences H-3.1-OQ753771 and H-4.1-OQ753772.

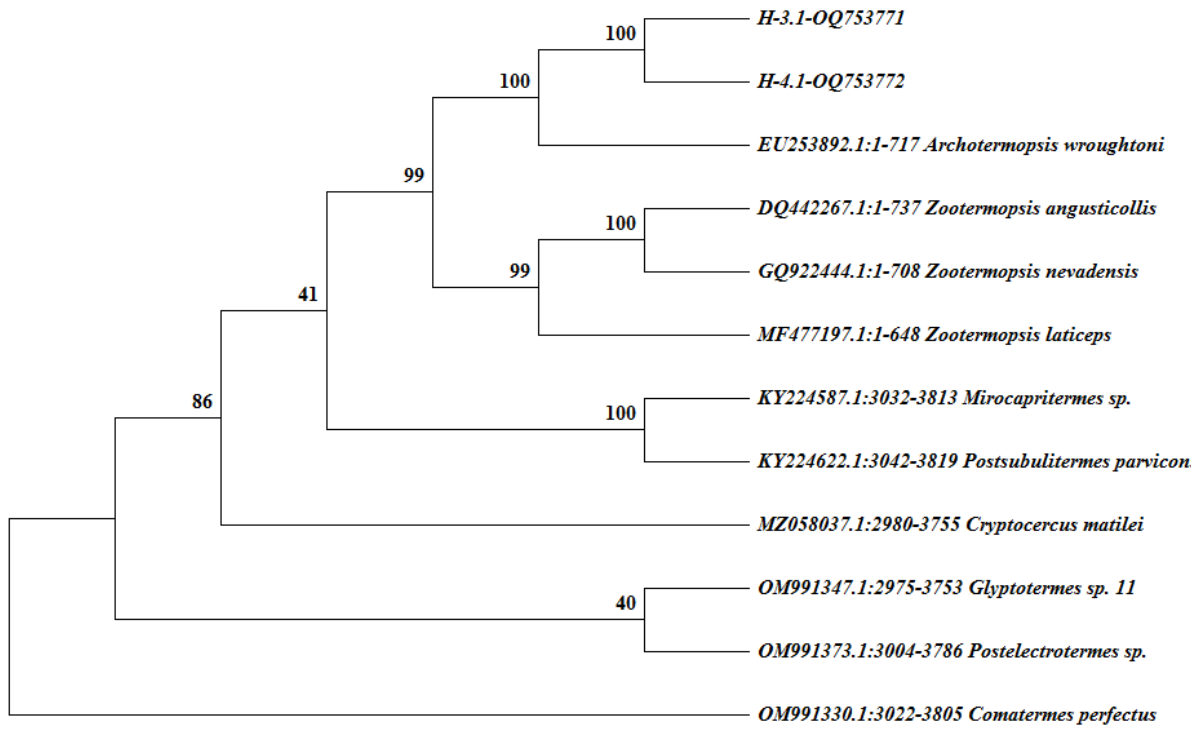

Fig. 4. Unrooted MP phylogeny of Archotermopsis species collected from the various areas of district Mansehra, Pakistan. The type specimens submitted by the sequence uploader are represented by sequences H-3.1-OQ753771 and H-4.1-OQ753772.

perform DNA barcoding and morphometric characterization in the Mansehra district. This species was found to be sparsely distributed across the district [2]. The lesser Himalayan region, ranging from 750–1700 meters in elevation, was found to be the primary distribution area for *Cedrus deodara*, *Pinus roxburghii*, and *Pinus wallichiana* [31], and it was observed that *A. wroughtoni* inhabits the decaying and fallen logs of these tree species. The range of *A. wroughtoni* was restricted to specific locations such as Kaghan Valley, Naran, Kiwai, Hangrai, Mahandri, Shogran, Pae, Paras, Garlat, and Ghannol within the district, with no presence observed at lower elevations or other areas.

The current research is consistent with previous investigations on the geographic range of *Archotermopsis wroughtoni* in the Himalayan region. This termite species has been well-documented in various regions of Pakistan, including Kumrat, Kalam, Hazara, Kaghan, Naran, and Shogran [2]. Ahmad [32] was the first to report the presence of it in West Pakistan, and later it was discovered in the Murree Hills of Punjab, Pakistan [12]. Chaudhry and Akhtar reported the species in their technical reports on the termites of Pakistan [11] and the zoogeography of the termites of Pakistan [10], respectively. Akhtar's most recent report on the species' distribution in Pakistan, published in 2000, recorded its presence in Roringar, Swat, consistent with the current study's results [9]. *A. wroughtoni* was the least commonly found termite species in the present study, which aligns with Akhtar's findings [9]. He classified the species as endangered due to human activities leading to the removal of felled logs used as breeding sites for *A. wroughtoni*.

Archotermopsis wroughtoni is found in the Himalayan regions of Pakistan, India, and Afghanistan. It has been observed in Himachal Pradesh, Jammu and Kashmir, and various districts of Uttar Pradesh in India, as well as in Nangarhar Province's Barikot in Afghanistan [1, 2].

The study included an analysis of *Archotermopsis* using DNA barcoding and phylogenetic methods, focusing on a specific segment of the mitochondrial *COII* DNA sequence. The resulting relationships between taxa were well-supported, as indicated by the bootstrap analysis. Interestingly, the relationships inferred through

both parsimony and distance analyses were highly similar, with a maximum support of 100 in MP and 97 in neighbour-joining. The phylogenies obtained from molecular data and morphological characters exhibited no significant differences in species-level relationships.

DNA barcoding, which utilizes short, standardized DNA sequences from genes like *COI*, has revolutionized the identification and classification of biological specimens [33-34]. Compared to traditional morphological identification methods, DNA barcoding offers faster, more accurate, and more objective species identification [35-40]. Its applications span conservation biology, wildlife forensics, and food safety, contributing to the understanding and management of invasive and endangered species while enriching our knowledge of biodiversity [41].

DNA barcoding employs genetic markers such as *COI* to identify and differentiate species [39]. However, for several reasons, *COII* (cytochrome c oxidase subunit II) has emerged as the most effective marker for DNA barcoding. *COII* exhibits higher variability and evolutionary rates than other markers, making it a suitable candidate for differentiating closely related species and improving the resolution of DNA barcoding analysis. Additionally, *COII* is a universally conserved region of the mitochondrial genome. It can be applied to diverse taxa and easily amplified and sequenced using standard laboratory methods [40].

Moreover, the accuracy and effectiveness of *COII* as a DNA barcoding marker have been demonstrated by large-scale projects such as the Barcode of Life Initiative (BOLD). *COII* is thus considered the best option for DNA barcoding due to its high variability, universality, and accuracy [41]. In this study, the amplified *COII* region of *Archotermopsis wroughtoni* was subjected to sequence cleaning and BLAST analysis to retrieve similar sequences. Subsequently, phylogenetic trees were constructed using the neighbour-joining and maximum parsimony methods.

The neighbor-joining algorithm is commonly used to construct phylogenetic trees based on genetic distances, connecting taxa iteratively [39-40]. This distance-based method is efficient for analyzing large datasets. However, its accuracy relies on suitable distance metrics and either homoplasy or conflicting signals. To mitigate uncertainties, we also employed the Maximum Parsimony method for phylogenetic inferences [40]. Maximum parsimony seeks to identify the tree with the fewest evolutionary changes needed to explain the observed data using a heuristic approach.

In our investigation, we observed that the clade most closely related to our sequences following *Archotermopsis*, as indicated by both the neighbour joining and maximum parsimony trees, was that of *Zootermopsis*. Notably, the family Archotermopsidae was determined to be monophyletic, despite previous research [36] highlighting a close relationship between two of the three genera within this family, namely *Archotermopsis* and *Zootermopsis* [1]. Living *Zootermopsis* occurred in western North America and was introduced in Japan [42].

As previously documented, the base composition of insect mitochondrial DNA exhibits a marked preference for adenine and thymine [43]. The present study examined the *Archotermopsis* species of termites and found a similar bias, with an average of 66.20 % adenine and thymine and 33.79 % guanine and cytosine.

5. CONCLUSION

The present study has significantly contributed to our understanding of the distribution, morphology, and DNA barcoding of *Archotermopsis wroughtoni* in the Mansehra district of Pakistan. We have confirmed the existence of this primitive termite species in the region and gained insight into its preferred host plants for nesting and foraging. Our findings provide a foundation for future research on the biology and ecology of *A. wroughtoni*.

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7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

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