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*Article*

# Molecular Species Identification and Genotyping of Free-Living Amoebae in Soil of Recreational Mountain Areas in the Babiogórski National Park and Surroundings, Southern Poland

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**Abstract**

Free-living amoebae (FLA) are widely present in the environment and may be pathogenic for animals and humans. Studies on the prevalence of FLA in European soils are few. This study aimed to molecularly identify the species and genotype of FLA occurring in soil from Southern Poland. Forty soil samples were collected in June 2024 in the Babiogórski National Park. Amoebae cultures and a thermal-tolerance test were conducted. Five PCR protocols were applied for amplification of FLA SSU rRNA fragments. The following FLA, including potentially pathogenic ones, were detected: *Acanthamoeba* T4 and T13 genotypes in 79.1% of positive samples, *Naegleria gruberi* and *Naegleria galeacystis* in 25%, *Vermamoeba vermiformis* in 12.5%, and *Paravahlkampfia* sp. and *Ptolemeba bulliensis* in 8.3%. Species and genotype identification were determined by sequence comparison and phylogenetic analysis. This study reports the first time isolation of *N. galeacystis* from soil and *N. gruberi* and *V. vermiformis* from soil collected in Europe. The used primer sets have different usefulness for *Naegleria* species identification and their phylogenetic analysis. The primers applied in this study may not reveal the full diversity of amoebae in soil; therefore, it is necessary to design new primers for this purpose.

**Keywords** free-living amoebae; soil; SSU rRNA; genotyping

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## 1. Introduction

Free-living amoebae (FLA) are a polyphyletic group of protists that belong to three supergroups within the kingdom Protozoa: Amoebozoa, e.g., *Acanthamoeba* spp., *Vermamoeba vermiformis* (formerly *Hartmannella vermiformis*), *Balamuthia* spp., and *Sappinia* spp.; Excavata, e.g., *Naegleria* spp.; and Rhizaria, e.g., *Paulinella* [1–3]. FLA can survive and reproduce in the environment, and depending on external conditions, they may occur as active, feeding trophozoites or dormant, resistant cysts. They play critical ecological roles and interact with other microorganisms, forming a predator-prey, symbiotic, or host-parasite relationship with bacteria. FLA may serve as hosts for many animal and human pathogens, contributing to their survival, spread, and transmission [1,4,5]. Some free-living amoebae are pathogenic and may cause infections of the central nervous system (*Acanthamoeba* spp., *Balamuthia mandrillaris*, *Sappinia pedata*, and *Naegleria fowlerii*) or keratitis (*Acanthamoeba* spp. and *Vermamoeba vermiformis*) [2,3,5,6,7]. A few authors suggest the possibility of FLA presence in the gut microbiome of mammals and other animals [8].

Some FLA infecting humans have been well genetically analyzed and divided into genotypes, linked to their pathogenicity. Based on 18S rRNA gene sequence analysis, 23 genotypes (T1 to T23) of *Acanthamoeba* species have been distinguished. Some genotypes cause granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis, or both. In the case of *N. fowlerii*, five of the eight

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genotypes, established based on the sequence of ITS1 and 5.8S rRNA gene, are pathogenic and cause primary amoebic meningoencephalitis (PAM). There is no genotype classification for *B. mandrillaris* due to a lack of or low variation in its 18S rRNA and mitochondrial 16S rRNA genes, respectively [2]. The pathogenicity of *V. vermiformis* is questionable, and limited molecular analyses of this species demonstrated a low diversity of its 18S rRNA gene. However, the latest research, based on multi-locus analysis, revealed a high degree of genetic diversity and the presence of multiple cryptic species within *V. vermiformis* [2,9]. *S. pedata* is linked with only one case of encephalitis. The sequences of the 18S rRNA gene and ITS1, 5.8S, and ITS2 regions of the four analyzed isolates demonstrated a size variation, but little is known about the genetic diversity of this species [2]. In the case of *Acanthamoeba*, thermotolerance and osmotolerance are also considered to evaluate the strain's pathogenicity. However, further studies are required to clarify this question [10,11].

FLA are widely present in various natural and artificial environments and have been isolated from different water sources, solid matrices, and air [5,12]. Many studies have been conducted on the prevalence and genetic diversity of FLA in various solid matrices, including soil, mud, sand, sediments, compost, and dust [12]. However, there are few European studies on this topic, especially regarding non-*Acanthamoeba* FLA. Almost all previous studies were based on amoebae cultures before their identification. Using environmental DNA (eDNA), total DNA isolated from environmental samples (e.g., soil) originating from various organisms, helps reduce the time needed for examinations. It also allows for the detection of more species from the same sample. The challenge of the eDNA approach is the presence of PCR inhibitors or the abundance of different organisms in the same sample [13,14], which can lead to false-negative or false-positive results, respectively.

This study aims to identify FLA species occurring in soil at recreational mountain areas in Southern Poland, using PCR and sequencing, their genotyping, and verification of thermal tolerance. The next aim is to compare the usefulness of eDNA and DNA isolated from amoebae cultures for PCR amplification of FLA 18S rRNA gene fragments. The purpose is also to compare the sensitivity and specificity of five PCR protocols for FLA detection. The next aim was to evaluate the usefulness of the obtained SSU rRNA gene fragments for species identification, genotyping, and phylogenetic analysis. The knowledge regarding the prevalence, genetic diversity, and phylogenetic relationship of the detected amoebae will shed light on their biogeography and ecology and help evaluate their pathogenic potential and the health threat to visitors in the analyzed area.

## 2. Results

### *2.1. Results of Amoebae Cultures, Thermal Tolerance Test, PCRs, and Sequencing*

The PCR with JDP1/JDP2 primers and eDNA isolated directly from soil yielded a negative result. Using other primers, only non-specific products were obtained with eDNA.

Among all forty soil samples cultured at 28°C, FLA were observed under a microscope in 24 (60%). After incubation of the transferred trophozoites at 37°C and 42°C, FLA were observed respectively on 24 plates (60% of all samples, 100% of all observed FLA) and 12 plates (30% of all samples, 50% of all observed FLA). All the FLA that grew at 37°C were washed off the plates, and DNA was extracted from the rinsed material. Next, PCR reactions were performed to detect FLA. The *Acanthamoeba* genus was detected using the JDP1/JDP2 primer set in 19 of 24 (79.1%) DNA samples. The *Naegleria* genus was detected using the FLA-F/FLA-R, Ami6F1/Ami9R, and AmeF977/AmeR1534 primer sets in 6/24 (25%) DNA samples. *V. vermiformis* was detected using the FLA-F/FLA-R, AmeF977/AmeR1534, and HARTfor/HARTrev primer sets in 3/24 (12.5%) DNA samples. Other amoebae (*Paravahlkampfia* sp. and *Ptolemeba bulliensis*) were detected using the FLA-F/FLA-R and AmeF977/AmeR1534 primer sets, respectively, in 2/24 (8.3%) DNA samples. Co-occurrence of two different amoebae was detected in 7/24 (29.16%) of the DNA samples. Among all *Acanthamoeba* strains, 63.2% (12/19) represent the T4 genotype, and 36.8% (7/19) the T13 genotype. The detailed results of amoebae cultures at 42°C, PCRs with individual primer pairs, and sequencing are presented in Table 1.

**Table 1.** The results of amoebae cultures at 42°C, PCRs, and sequencing for the 26 strains that were grown at 30°C and 37°C.

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| Sample number | Culture at 42°C | PCR and sequencing results with individual primer pairs | | | | |
|---|---|---|---|---|---|---|
| | | JDP1/JDP2 | FLA-F/FLA-R | Ami6F1/Ami9R | AmeF977/AmeR1534 | HARTfor/HARTrev |
| **1.** | | | | | *Naegleria* sp. | |
| 2. | + | *Acanthamoeba* T13 | | | | *V. vermiformis* |
| 4. | | *Acanthamoeba* T4 | *Paravahlkampfia* sp. | | | |
| 6. | + | *Acanthamoeba* T4 | | | | |
| 7. | | | *N. galeacystis* | | *N. galeacystis* | |
| 8. | + | | *N. gruberi* | *N. gruberi* | | |
| 10. | | *Acanthamoeba* T13 | *N. galeacystis* | | *N. galeacystis* | |
| 11. | | | *N. gruberi* | *N. gruberi* | *Naegleria* sp. | |
| 12. | | *Acanthamoeba* T4 | | | | |
| 15. | + | *Acanthamoeba* T4 | | | | |
| 19. | + | *Acanthamoeba* T4 | *V. vermiformis* | | *V. vermiformis* | *V. vermiformis* |
| 21. | + | *Acanthamoeba* T4 | | | | |
| 23. | | *Acanthamoeba* T13 | | | | |
| 24. | + | | *N. gruberi* | *N. gruberi* | *Naegleria* sp. | |
| 27. | | *Acanthamoeba* T4 | | | | |
| 28. | | *Acanthamoeba* T13 | | | *P. bulliensis* | |
| 29. | + | *Acanthamoeba* T4 | | | | |
| 31. | + | *Acanthamoeba* T4 | | | | |
| 32. | + | *Acanthamoeba* T13 | | | | *V. vermiformis* |
| 33. | + | *Acanthamoeba* T13 | | | | |
| 35. | + | *Acanthamoeba* T13 | | | | |
| 37. | | *Acanthamoeba* T4 | | | | |
| 39. | | *Acanthamoeba* T4 | | | | |
| 40. | | *Acanthamoeba* T4 | | | | |

### *2.2. Results of Sequence Comparison and Phylogenetic Analysis*

The *Acanthamoeba* genotype and the species of the remaining amoebae were identified by aligning their sequences with those in the GenBank database using the BLAST tool, as well as by analyzing the topology of the phylogenetic trees (Figures 1-4). The detailed alignment results for *Acanthamoeba*, *Naegleria,* and *Vermamoeba vermiformis* are presented in Tables 2–4. The sequence obtained from sample 4 using the FLA-F/FLA-R primer set shows 99.25% identity to the sequence DQ388521 from the GenBank database, representing *Paravahlkampfia* sp. The sequence obtained from sample 19 using the AmeF977/AmeR1534 primer set shows 98.94% identity to the sequence PP732398 from the GenBank database, representing *Ptolemeba bulliensis* isolated from water (Philippines).

**Table 2.** The results of the sequence alignment of the sequences obtained in this study and the sequences from GenBank using the BLAST tool for the *Acanthamoeba* genus (JDP1/JDP2 primer set).

| The sequences obtained in this study (sample number and primer set) | The sequences from the GenBank database – accession number, isolation source, and country | Identity |
|---|---|---|

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| | | |
|---|---|---|
| Identical sequences: 12-JDP, 15-JDP, 19-JDP, 27-JDP, 29-JDP, 31-JDP | ON408415 *Acanthamoeba* T4 isolated from soil (USA) | 100% |
| 6-JDP | KT985967 *Acanthamoeba* T4 isolated from soil (Iran)<br>KU894800 *Acanthamoeba* sp. isolated from water (Uganda)<br>MZ404332 *Acanthamoeba* sp. isolated from a patient with keratitis (Brazil) | 100% |
| 39-JDP | KC164232 *Acanthamoeba* sp. isolated from compost (Switzerland)<br>PQ431522, PQ431528 *Acanthamoeba* sp. isolated from water (France)<br>AF019060 *A. hatchetti* T4 | 100%<br>99.48% |
| Identical sequences: 4-JDP, 21-JDP, 37-JDP, 40-JDP | JQ669659 *Acanthamoeba* T4 isolated from soil (USA)<br>KT892868 *Acanthamoeba* T4 isolated from soil (Chile)<br>FJ807650 *Acanthamoeba* sp. isolated from marsh sediment (Austria)<br>LR813621 *A. castellani* isolated from coprolite (Belgium)<br>KC164234 *A. castellani* isolated from compost (Switzerland)<br>MN700280 *Acanthamoeba* T4 isolated from water (Malaysia)<br>MT378239 *Acanthamoeba* T4 isolated from water (Iran) | 100% |
| Identical sequences: 2-JDP, 10-JDP, 23-JDP, 28-JDP, 32-JDP, 33-JDP, 35-JDP | MZ686722 *Acanthamoeba* T13 isolated from soil (Malaysia) | 99.48% |

**Table 3.** The results of the sequence alignment of the sequences obtained in this study and the sequences from GenBank using the BLAST tool for the *Naegleria* genus (FLA-F/FLA-R, AmeF977/AmeR1534, and Ami6F1/Ami9R primer sets).

| **The sequences obtained in this study (sample number and primer set*)** | **The sequences from the GenBank database – accession number, isolation source, and country** | **Identity** |
|---|---|---|
| Identical sequences: 7-FLA and 10-FLA | DQ768717 *Naegleria* sp. isolated from fish (Czech Republic)<br>PP174311 *Naegleria* sp. | 100% |
| Identical sequences: 8-FLA and 24-FLA;<br>Identical sequences: 8-Ami and 24-Ami | OR769034 *N. gruberi* isolated from soil (USA)<br>MG699123, AB298288 – *N. gruberi* | 100% |
| 11-FLA/11-Ami | | 98.64%/ 99.17% |
| Identical sequences: 7-Ame and 10-Ame | AF011457 *N. galeacystis*<br>DQ768717, DQ768718 – *Naegleria* sp. isolated from fishes | 100% |
| Identical sequences:<br>1-Ame, 11-Ame, 24-Ame | OR769034 *N. gruberi* isolated from soil (USA)<br>MG699123, AB298288 – *N. gruberi*<br>AF338417, AF338419, JQ271691, JQ271692, JQ271697, JQ271705 – *N. clarki* isolated from fishes; | 100% |

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| | |
|---|---|
| | JQ271704 – *N. clarki* isolated from sediment (Czech Republic)<br>OR045415 – *N. pringsheimi* |

*FLA - FLA-F/FLA-R, Ame - AmeF977/AmeR1534, Ami - Ami6F1/Ami9R.

**Table 4.** The results of the sequence alignment of the sequences obtained in this study and the sequences from GenBank using the BLAST tool for *Vermamoeba vermiformis* (FLA-F/FLA-R, AmeF977/AmeR1534, and HARTfor/HARTrev primer sets).

| **The sequences obtained in this study (sample number and primer set*)** | **The sequences from the GenBank database – accession number, isolation source, and country** | **Identity** |
|---|---|---|
| 19-FLA | KU746978 *V. vermiformis* isolated from soil (Spain)<br>KP792383, KP792388 *V. vermiformis* isolated from water (Italy)<br>MF112024 *V. vermiformis* isolated from water (Pakistan)<br>JQ271688 *H. vermiformis* isolated from a tapeworm (Croatia)<br>HM363627 *Hartmannella* sp. isolated from a fish (Germany) | 100% |
| 19-AME | LC764480 *V. vermiformis* isolated from soil (Japan)<br>LN650671 *V. vermiformis* isolated from water (Italy)<br>JQ271687 *V. vermiformis* isolated from a fish (Czech Republic) | 100% |
| Identical sequences: 2-HART, 19-HART, 32-HART | PP732395, PP732396 *V. vermiformis* isolated from water (Philippines) | 100% |

*FLA - FLA-F/FLA-R, Ame - AmeF977/AmeR1534, HART - HARTfor/HARTrev.

The phylogenetic trees based on the sequences obtained in this study and derived from the GenBank database are presented in Figures 1-4. The twelve *Acanthamoeba* sequences (samples 4, 6, 12, 15, 19, 21, 27, 29, 31, 37, 39, and 40) obtained in this study belong to a clade consisting of T4 genotype sequences from the GenBank database. The seven sequences (samples 2, 10, 23, 28, 32, 33, and 35) belong to a clade that contains T13 genotype sequences from GenBank (Figure 1). These results are consistent with the results of sequence comparison (Table 3). The trees based on the products of the FLA-F/FLA-R and Ami6F1/Ami9R primer sets for the *Naegleria* genus (Figures 2 and 3) have a similar topology. The three sequences obtained in this study (samples 8, 11, 24) make a group with the *N. gruberi* sequences from GenBank, while the two sequences (samples 7 and 10) are grouped with the *N. galeacystis* sequence from GenBank. These results are consistent with the results of sequence comparison (Table 3). The tree based on the products of the AmeF977/AmeR1534 primer set (Figure 4) has a different topology than the other two trees for the *Naegleria* genus. The sequences obtained from the two samples (7 and 10) belong to the same clade as the *N. galeacystis* sequence from GenBank, which is consistent with the sequence comparison (Table 3) and the trees' topology presented in Figures 2 and 3. The remaining sequences, obtained from samples 8, 11, and 24, form a clade with different *Naegleria* species within this tree. Using the AmeF977/AmeR1534 primer set, it is not possible to determine the species of the *Naegleria* strains detected in samples 8, 11, and 24 based on either the tree topology or sequence comparison.

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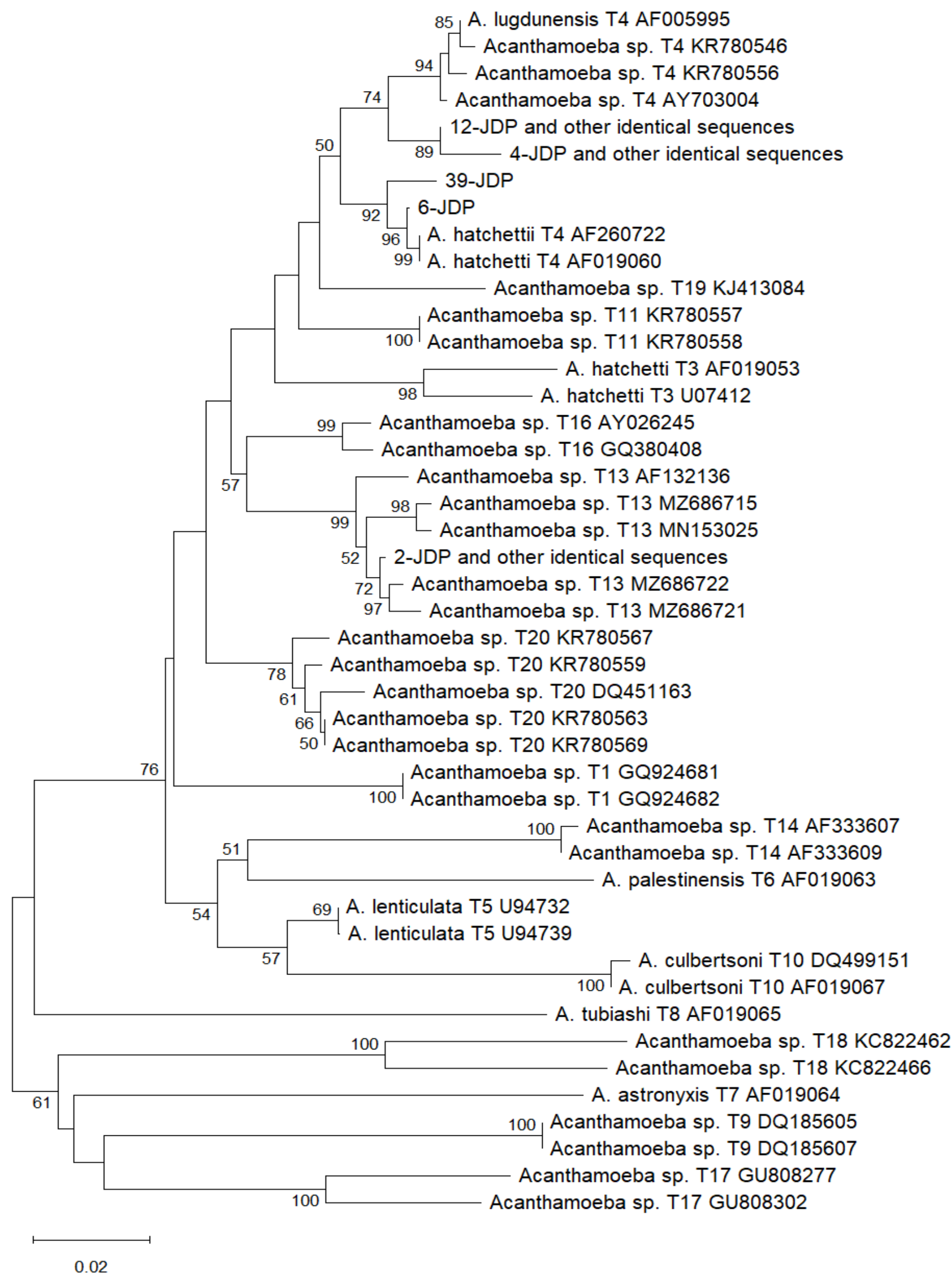


**Figure 1.** Phylogenetic tree constructed using the neighbor-joining statistical method and the Kimura 2-parameter model with 1000 bootstrap sampling, based on forty-five SSU rRNA sequences of *Acanthamoeba*. The forty sequences with accession numbers are from GenBank, and the remaining five sequences were obtained in this study using the JDP1/JDP2 primer set. Identical sequences are counted as a single sequence. Only bootstrap values ≥50 are shown.

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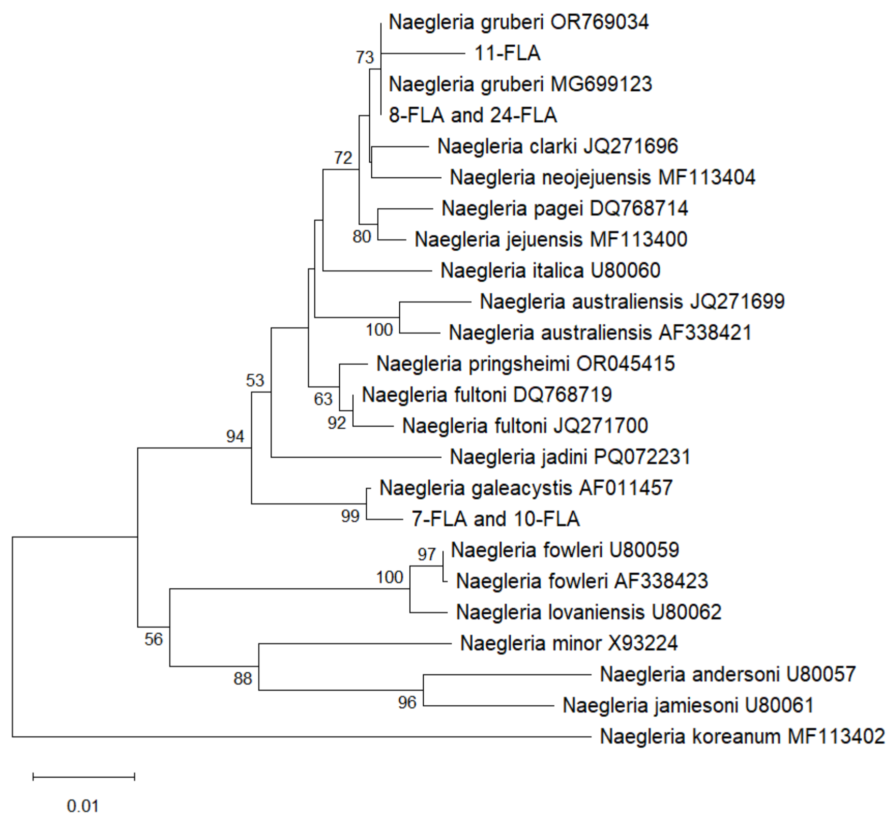


**Figure 2.** Phylogenetic tree constructed using the neighbor-joining statistical method and the Kimura 2-parameter model with 1000 bootstrap sampling, based on twenty-four SSU rRNA sequences of the *Naegleria* genus. Twenty-one sequences with accession numbers are from GenBank, and the remaining three sequences were obtained in this study using the FLA-F/FLA-R primer set. Identical sequences are counted as a single sequence. Only bootstrap values ≥50 are shown.

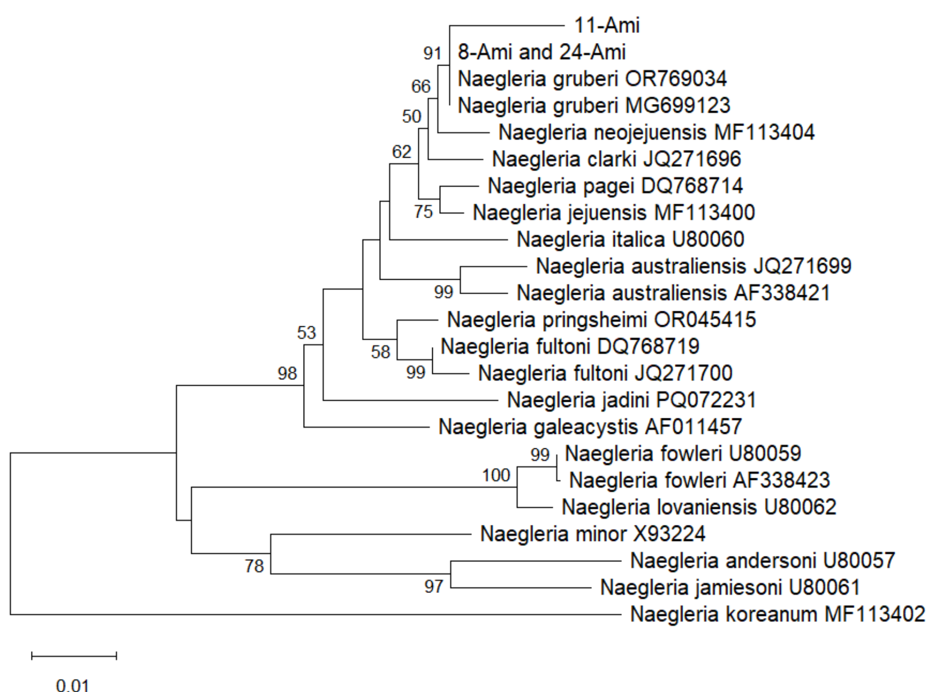


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**Figure 3.** Phylogenetic tree constructed using the neighbor-joining statistical method and the Kimura 2-parameter model with 1000 bootstrap sampling, based on twenty-three SSU rRNA sequences of the *Naegleria* genus. Twenty-one sequences with accession numbers are from GenBank, and the remaining two sequences were obtained in this study using the Ami6F1/Ami9R primer set. Identical sequences are counted as a single sequence. Only bootstrap values ≥50 are shown.

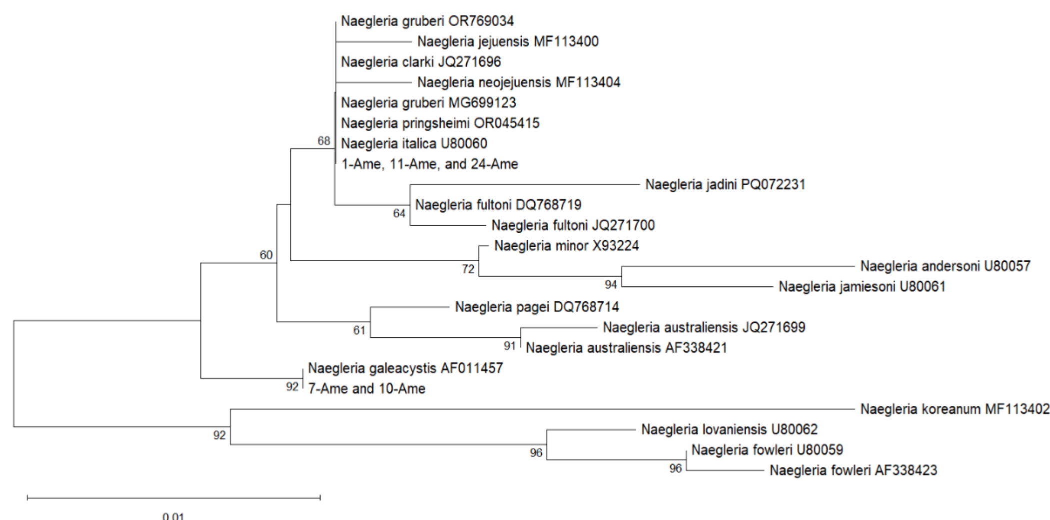


**Figure 4.** Phylogenetic tree constructed using the neighbor-joining statistical method and the Kimura 2-parameter model with 1000 bootstrap sampling, based on twenty-three SSU rRNA sequences of the *Naegleria* genus. Twenty-one sequences with accession numbers are from GenBank, and the remaining two sequences were obtained in this study using the AmeF977/AmeR1534 primer set. Identical sequences are counted as a single sequence. Only bootstrap values ≥50 are shown.

*2.3. Results of Statistical Analysis*

Statistical analysis shows significant differences in the prevalence of genera *Acanthamoeba* and *Naegleria*; *Acanthamoeba* genus and *Vermamoeba vermiformis*; and *Acanthamoeba* genus and other amoebae (*Paravahlkampfia* sp. and *Ptolemeba bulliensis*). The p-values were 0.0262, 0.314, and 0.0338, respectively. The differences between *Naegleria* genus, *V. vermiformis*, and other amoebae, as well as between T4 and T13 genotypes of *Acanthamoeba,* are not statistically significant. The analysis does not show statistically significant differences in the prevalence of different amoebae between the samples collected in the particular zones.

## 3. Discussion

Studies on FLA prevalence in the environment include cultures of amoebae, which increase their number and reduce the amount of inhibitors. However, this approach is time-consuming and allows the detection of only those amoebae that can be grown under the used conditions. In this study, an attempt was made to molecularly detect FLA in eDNA isolates obtained directly from the soil. The use of a kit intended for DNA isolation from soil enabled the effective removal of inhibitors, as PCR products were obtained from eDNA using all primer pairs except the JDP1/JDP2 pair. However, all obtained products were nonspecific and derived from non-amoebae organisms. Using DNA isolates obtained from amoebae cultures, specific products were obtained with all primer pairs. The *Acanthamoeba* genus-specific primer set, JDP1/JDP2, was the most specific of all used, but it was not sensitive enough to amplify amoebae DNA without prior culture. Thus, amoebae cultures are necessary to detect these organisms in soil samples using the primers applied in this study.

The primer pairs used in this study have been applied in other papers for FLA detection in cultures from soil samples [15–19]. However, the authors did not compare their sensitivity and specificity. In this study, FLA-F/FLA-R and AmeF977/AmeR1534 sets appeared to be the most sensitive among the primers intended for different FLA DNA amplification. They both amplified the

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amoebae DNA in seven samples. However, in one sample, *Paravahlkampfia* sp. was detected only with the FLA-F/FLA-R set, and in another two samples, *Ptolemeba bulliensis* and *Naegleria* sp. were detected only with the AmeF977/AmeR1534 set. The Ami6F1/Ami9R set was the least sensitive and allowed for the detection of *N. gruberi* in the three samples in which this species was detected using the FLA-F/FLA-R set. The sequences amplified using FLA-F/FLA-R and Ami6F1/Ami9R pairs overlap to a large extent, and their use in phylogenetic analysis resulted in the creation of phylogenetic trees with very similar topologies. Topology of these trees, combined with sequence comparison, enabled the identification of species in *Naegleria* strains isolated in this study. The disadvantage of the AmeF977/AmeR1534 set was the inability to identify the species of *Naegleria* in 3 out of 5 samples based on both sequence comparison and phylogenetic tree topology. The FLA-F/FLA-R set seems to be the best choice, but it was not sensitive enough to amplify amoebae DNA in the two samples positive with the AmeF977/AmeR1534 set. None of the primer pairs intended for different FLA allowed for the detection of *Acanthamoeba*. Reyes-Batlle et al. [20] used the FLA-F/FLA-R primer set, and they also failed to detect *Acanthamoeba* in any of the 24 soil samples from El Hierro Island (Canary Islands), despite this species being one of the most common in soil and occurring in the Canary Islands [21]. They identified only *V. vermiformis* in 20.8% of the samples. In this study, *V. vermiformis* was detected in only one sample using FLA-F/FLA-R and AmeF977/AmeR1534 pairs, in contrast to three samples with the use of the HARTfor/HARTrev set, which is intended for the detection of the former genus *Hartmannella*. The application of the primers for a wide range of FLA used in this study does not reveal the full diversity of soil amoebae. It is necessary to design new, sensitive primers enabling the detection and species identification of FLA in soil and other environmental samples. A major challenge would be to create these primers so that their sensitivity and specificity are sufficient to detect FLA in eDNA isolated directly from soil. The use of metagenomics techniques would provide complete knowledge of the amoebae diversity in soil.

Studies on the prevalence of a wide range of FLA in soil and their genotyping are few in Europe. In Poland, Hendiger-Rizo et al. [16] detected *Acanthamoeba* spp. in most of the soil samples (78.2%) from Warsaw parks and squares, followed by *Platyamoeba placida*, *Stenamoeba berchidia*, and *Allovahlkampfia* sp. Denet et al. [22] examined the diversity of culturable FLA at the genus level in French alpine soils, based on PCR and morphological analysis. They revealed that *Acanthamoeba* was the dominant genus (77%) among all detected FLA, and the remaining detected amoebae belonged to *Tetramitus* and *Stachyamoeba* genera, or were unidentified. In this study, *Acanthamoeba* sp. was also detected in the majority of positive cultures (79.1%) and occurred significantly more frequently than other identified amoebae: *Naegleria* spp., *H. vermiformis*, *P. bulliensis*, and *Paravahlkampfia* sp. The differences between this study and the others may result from distinct climate or soil properties, or, in the case of the French study, may be caused by methodological differences. Other studies on the presence of different amoebae in soil used samples from outside Europe. In samples from Turkey, Iran, Tenerife, Santiago Island of Cape Verde, and the Canary Islands, *Acanthamoeba* sp. was the most prevalent FLA [17–19,21,23,24]. *V. vermiformis* was the most prevalent FLA in Northern Iran, Bolivia, and Guadeloupe [25–27]. *Naegleria* and *Tetramitus* genera dominated in Vietnam and Burkina Faso, respectively [22]. *Acanthamoeba* sp. seems to be a dominant genus in European soils, and there are considerable differences in FLA composition between sampling areas worldwide. Additional studies on the prevalence and distribution of FLA in soil are necessary, and this research provides valuable insights into the subject. *Acanthamoeba* spp., *Naegleria* spp., and *V. vermiformis* have frequently been found in soil [9,12,15,17–20,23–25,28]. However, the Hartmannellid *genus Ptolemeba* and the Valkhampfiidae amoebae *Paravahlkampfia* sp. and *Naegleria* other than *N. fowleri* have been rarely detected in environmental samples. *Ptolemeba bulliensis* and *Ptolemeba noxubium* have been isolated from soil and water in Mississippi, respectively [29]. *P. bulliensis* has also been found in the gills of rainbow trout (*Oncorhynchus mykiss*) in Russia [30] and in water from the Philippines (PP732398, GenBank, unpublished). *Paravahlkampfia lenta* has been isolated from soil samples collected from a Scottish farm [31], and *Paravahlkampfia ustiana* from soil samples collected in Thailand [28]. *N. gruberi* has been isolated from soil samples from the UK and California [32]. This study is the first to reveal the presence of *N. galeacystis* in soil, as well as *N. gruberi* and *V. vermiformis* in soil from Europe.

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Studies on the prevalence of *Acanthamoeba* genotypes in European soils were conducted in Poland, Austria, the Netherlands, Hungary, and Sardinia. The composition of *Acanthamoeba* genotypes was different depending on the examined area. The T4 genotype was the only one detected in the soil [16] and sandboxes [33] in Poland, as well as in rhizosphere samples from Hungary [34]. This genotype dominated in soils from Austria [35] and Sardinia [36], and accounted for half of all the detected *Acanthamoeba* strains in soil from the Netherlands [36]. Additionally, the T2 genotype has been found in soil from Austria and the Netherlands [35,36], T16 in soil from the Netherlands, and T13 in soil from Sardinia [36]. In this study, the T4 and T13 genotypes were identified. The first one dominated; however, the difference was not statistically significant. The T4 genotype was the most common in the European soil samples examined so far, and the share of the remaining genotypes was different in each examined area. Additional studies are necessary to investigate whether T4 predominance is typical in European soils and whether other genotypes, not detected so far, occur. The share of FLA species and *Acanthamoeba* genotypes differed even between relatively close locations, such as central [16,33] and southern Poland (this study). The composition of FLA in soil, therefore, appears to be influenced by local conditions, such as soil properties. However, in this study, there were no significant differences in the occurrence of individual FLAs in different zones of the Babiogórski National Park.

Both *Acanthamoeba* genotypes identified in this study and *V. vermiformis* can cause keratitis, and the T4 *Acanthamoeba* genotype is an etiological agent of granulomatous amoebic encephalitis [6,37]. The *Paravahlkampfia* genus was previously considered a human pathogen [38–40]. The presence of these amoebae in the examined samples may indicate a potential health threat to individuals visiting the studied area. Tourists frequently visit the Babiogórski National Park, and maintaining hygiene, such as hand washing, is difficult in this location, which increases the risk of infection. Thermal-tolerant *Acanthamoeba* strains are considered potentially pathogenic [10,11]. All *Acanthamoeba* strains detected in this study were grown at a temperature of 28°C and 37°C. Half of the T4 strains (6/12) and 42,8% of the T13 strains (3/7) were grown at a temperature of 42°C. However, the study of Kahraman et al. [10] suggests that thermotolerance does not indicate the pathogenicity of *Acanthamoeba*.

## 4. Materials and Methods

### *4.1. Study Area and Soil Sampling*

Babia Góra National Park is situated in the Western Carpathians and is a popular destination for tourists. Babia Góra is the highest peak in the park (1,725 meters above sea level), and mixed forests cover most of the park area. The main tree species growing in the park are common beech (*Fagus sylvatica*), Norway spruce (*Picea abies*), and silver fir (*Abies alba*). Fir and beech forests, including Norway spruce and sycamore (*Acer pseudoplatanus*), dominate the lower montane zone (up to 1,150 m). Norway spruce grows in the upper montane zone (up to 1,390 m) with an admixture of rowan (*Sorbus aucuparia*). The next zone (up to 1,650 m) is dominated by dwarf mountain pine (*Pinus mugo*). However, other species, such as dwarf forms of spruce, rowan, Silesian willow (*Salix silesiaca*), rock currant (*Ribes petraeum*), and mountain juniper (*Juniperus communis* subsp. *alpina*) are also present. Only low grasslands, mosses, and lichens grow in the Alpine zone (up to 1,725 m).

Forty soil samples (approximately 5.0 mL each) were collected in June 2024 from Babia Góra National Park and its surrounding areas in southern Poland (Figure 5). Thirty-two samples (1-15, 21-28, 31-37, 39, and 40) were taken from the lower montane zone, four samples (16, 29, 30, 38) from the upper montane zone, three samples (17-19) from the zone of dwarf mountain pine, and one sample (20) from the Alpine zone. The samples were taken directly from the ground surface along marked tourist trails. During the sampling, the air temperature oscillated between 15 and 20°C. The obtained material was placed in sterile Eppendorf tubes and stored at 4°C until further processing and analysis in the laboratory.

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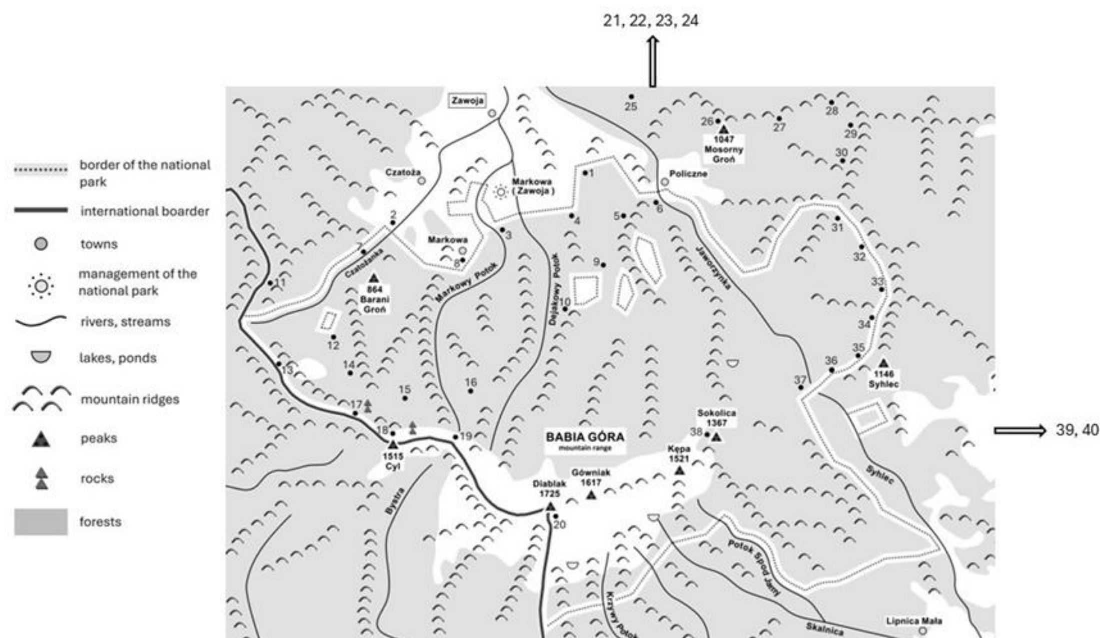


**Figure 5.** Collection sites of soil samples in the Babiogórski National Park and its surroundings.

### *4.2. Amoebae Cultures and Thermal Tolerance Test*

1g of each soil sample was seeded onto a Petri dish with NN-agar coated with heat-killed *Escherichia coli*. The agar plates were incubated at 28°C and examined daily under an optical microscope until amoebae were observed (up to 72h). Then, two sets of new Petri dishes were prepared as described above. Small pieces of agar (approximately 5 x 5 mm) with trophozoites were transferred onto new Petri dishes and placed top side down on the agar surface. One set was incubated at 37°C and the second at 42°C until amoebae were observed under the microscope (up to 72 hours). The amoebae obtained from cultures incubated at 37°C and 42°C were washed with sterile PBS buffer (1 ml per plate).

### *4.3. DNA Extraction from Soil and Cultures, and PCR Protocols*

Two hundred microliters of PBS with washed amoebae from each agar plate were used for DNA extraction with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The Soil DNA Mini Kit (Syngen, Wrocław, Poland) was used for direct DNA extraction from soil samples (0.5 g of soil per isolation). DNA extractions were performed according to the manufacturer's protocols. Five PCR protocols, as previously described by the authors, were used to amplify the SSU rRNA gene fragments and detect the presence of FLA in the examined samples (Table 5). Except for three primer pairs specific for a broad spectrum of FLA, genus-specific primers were used for *Acanthamoeba* and the former genus *Hartmannella*, as they are common in the environment [12] and have been isolated earlier from environmental samples in Poland [41]. PCR products were separated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

**Table 5.** Primer sets used in this study for FLA detection.

| The primer set | Detecting FLA | The amplifying fragment of the 18S rRNA gene and the product size | Hybridization temperature | References |
|---|---|---|---|---|
| JDP1/JDP2 | *Acanthamoeba* spp. | 897-1358 bp of *A. castellani* sequence (U07400); 462 bp | 55°C | [42] |

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| | | | | |
|---|---|---|---|---|
| HARTfor/HARTrev | Former genus *Hartmannella* | 562-1095 bp of *V. vermiformis* sequence (EU137741); 534 bp | 58°C | [43] |
| FLA-F/FLA-R | All FLA except *Balamuthia* and *Sappinia* | 631-1614 bp of *A. castellani* sequence (U07400); 984 bp | 55°C | [44] |
| Ami6F1/Ami9R | All amoebae | 641-1468 bp of *A. castellani* sequence (U07400); 828 bp | 55°C | [45] |
| AmeF977/AmeR1534 | All amoebae | 1179-1829 bp of *A. castellani* sequence (U07400); 651 bp | 60°C | [46] |

*4.4. Sequencing, Genotyping, and Phylogenetic Analysis*

Both strands of all obtained PCR products were sequenced using the amplification primers (Table 1). The sequencing was performed at Macrogen Europe (Amsterdam, the Netherlands). The obtained sequences were compared with other homologous sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information. Separate alignments were performed for sequences of different genera of FLA, using the MUSCLE algorithm (the MEGA 12 software), which allowed for comparison to each other and to other homologous sequences from GenBank. The ends of the sequences in the alignments were trimmed to form blunt ends. The multiple alignment for the *Acanthamoeba* genus covered nucleotides corresponding to positions 720 to 1,300 of the *Acanthamoeba* sp. genotype T4 sequence with GenBank accession number AY702983 (JDP1/JDP2 primer set). For the *Naegleria* genus, the multiple alignment covered nucleotides corresponding to positions 680-1343 (Ami6F1/Ami9R primer set), 756-1476 (FLA-F/FLA-R primer set), and 1277-1662 (AmeF977/AmeR1534 primer set) of the *N. gruberi* sequence with GenBank accession number OR769034. The phylogenetic trees were constructed using Mega 12 software, based on a multiple alignment and the neighbor-joining statistical method with the Kimura 2-parameter model, with 1,000 bootstrap samples [47]. Different fragments of the same sequences from GenBank were used to construct the trees based on the 18S rRNA sequence of *Naegleria* spp. (Figures 2-4). The results of the sequence comparison using BLAST and analysis of the phylogenetic trees' topology were used to determine the genotype of *Acanthamoeba* and the species of *Naegleria* strains examined in this study. The genus or species of the remaining amoebae was determined based on the sequence comparison using BLAST. Phylogenetic trees for *V. vermiformis*, *P. bulliensis*, and *Paravahlkampfia* sp. were not constructed, as all obtained *V. vermiformis* sequences represent the same species and are all identical to those in the GenBank database. Sequences of the *Ptolemeba* and *Paravahlkampfia* genera deposited in GenBank are too few to use for phylogenetic analysis. The sequences analyzed in this study have been deposited in the GenBank database under accession numbers: PV867401-PV867419 (*Acanthamoeba* spp.), PV867462-PV867474 (*Naegleria* spp.), PV867476-PV867480 (*V. vermiformis*), PV867809 (*P. bulliensis*), and PV873343 (*Paravahlkampfia* sp.).

*4.5. Statistical Analysis*

Statistical analyses were performed using a chi-squared test to investigate the differences in the prevalence of various amoebae and the prevalence of amoebae in samples collected from specific zones in the Babiogórski National Park. Statistical significance was defined as $p < 0.05$. The Statistica 13.3 software (StatSoft Inc., USA) was used for the analysis.

## 5. Conclusions

The knowledge of FLA diversity in European soils is insufficient, and more studies regarding this topic are needed. This study revealed that the *Acanthamoeba* genus (T4 and T13 genotypes)

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dominates among FLA occurring in soil from the Babiogórski National Park and its surroundings, followed by *Naegleria* spp. and *V. vermiformis*. It also reports the isolation of *Paravahlkampfia* sp., *P. bulliensis*, and *N. gruberi*, which are rarely detected in soil, as well as the first-time isolation of *N. galeacystis* from soil and *N. gruberi* and *V. vermiformis* from soil collected in Europe. Some of the detected FLAs are potentially pathogenic and may pose a health threat to humans. Among the used primer sets, genus-specific primers (JDP1/JDP2 and HARTfor/HARTrev) are more sensitive than the three pairs specific for a wide range of FLA: FLA-F/FLA-R, Ami6F1/Ami9R, and AmeF977/AmeR1534. It is necessary to design new, sensitive, and specific primers enabling the detection, species identification, and genotyping of FLA in soil.

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**Data Availability Statement:** All data generated or analyzed in this study are included in this published article. The accession numbers of the DNA sequences obtained in this study are provided in the Materials and Methods section and are available in the GenBank database.

**Conflicts of Interest:** The author declares no conflicts of interest.

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