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Nematofauna of the Natural Park "Devil's Town"

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Abstract: "Devil's Town" is a natural park dominated by broadleaf deciduous trees, and made up of two rare natural phenomena in the world: earthen statues, as specific forms of relief, and two springs of highly acidic water with high mineralization. Devil's Town is a "biodiversity star" with a unique ambient, flora, fauna, and microbiome. The research aimed to: investigate the concentration of chemical parameters in the soil of the natural park, identify nematodes that can survive in extreme conditions, explore feeding habits of nematodes, and infer phylogenetic relationships of nematodes based on *28S* rRNA sequences. Soil samples were collected from two sites, designated Soil under vegetation and Saxon mine soil, from which nematodes were discovered. Phylogenetic analyses were performed with *28S* rRNA gene primers, using Maximum likelihood and Bayesian inference. The presence of minerals and heavy metals, combined with high acidity created extreme environmental conditions in which specific nematode species can survive. These circumstances favored fast-moving species with teeth and spears, such as mononchids and dorylaimids, enabling them to adopt predatory feeding behavior. In contrast, *Acrobeloides*, *Prismatolaimus*, *Rhabditis* spp. etc., are saprobionts adapted to specific chemical pollutants, and they tolerate high levels of Pb, Zn, Fe, Cu, Cd and As.

Keywords: natural pollution; chemistry; nematodes; phylogeny; *28S*

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1. Introduction

"Devil's Town" (originally, Djavolja Varos) is a natural park dominated by broadleaf deciduous trees and shrub vegetation with sporadically occurring coniferous trees, located in the south of Serbia (N 43° 00′ 41″, E 21° 24′ 36″). The park is made up of two rare natural phenomena in the world: earthen statues, as specific forms of relief, and two springs of highly acidic water with high mineralization. The area was the center of intense volcanic activity several million years ago. In the Middle Ages, ores were mined there and there remains an original "Saxon mine" from the 13th century. The "Red" and "Yellow" springs and their sediments contain heavy metals in large quantities. The content of some elements (iron, potassium, copper, nickel, sulfur) is extremely high compared to ordinary drinking water, i.e., increased by 10–1000 times. Water and wind erosion have created more than 200 earthen statues of different sizes, from 2 to 15 m high, 0.5 to 3 m wide, with stone caps on them. The statues appear, grow, gradually disappear and reappear. Devil's Town is a "biodiversity star" with a unique ambient, flora, fauna and microbiome. The site was Serbia's official candidate to be elected to the "Seven Wonders of the Natural World" in 2010. In the past decades, great attention was devoted to extremophilic organisms. Such organisms survive extreme temperature conditions, high saline, acidic, and alkaline solutions or environments with elevated heavy metal levels [1]. Most contaminants are of anthropogenic origin, but some contaminants are mineral constituents that occur naturally

in soils as a result of volcanic activity, as in Devil's Town. Nematodes as the most abundant metazoans in the world (4.4×10^{20}) with a total biomass of approximately 0.3 GT [2] are ideal bioindicators of soil health. They integrate physical, chemical, and biological soil properties and processes, are accessible to a wide range of users, and are sensitive to changes [3]. Soil nematode communities under various environmental conditions have been evaluated over the past 20 years. In the study by Pavao-Zuckerman and Coleman [4], the soil nematode communities along urban vs. rural roadways were investigated in North Carolina. The number of predatory and omnivorous nematodes was lower in urban soils. The structure of soil nematode communities was studied in terrestrial sand dune systems in China. The dominant genus was *Acrobeles*, while total numbers of nematodes were positively correlated with electrical conductivity, soil moisture, total organic carbon and nitrogen contents [5]. Quist et al. [6] studied nematodes in crop fields, semi-natural grasslands, and in marine river clay and sandy soils. The widespread nematodes were bacterivorous and fungivorous taxa, predators and omnivores were common taxa, while plant parasites and entomopathogenic nematodes were rare nematode taxa. Čerevkova et al. [7] investigated soil nematode communities in managed and natural temperate forests. The highest number of nematode genera, dominated by *Rhabditis* and *Filenchus*, was in European beech forests. The lowest number of nematode genera, dominated by *Acrobeloides*, *Plectus*, and *Rhabditis* was in a Norway spruce forest. The unmanaged old-growth forest had the highest abundance of herbivorous nematodes, such as *Filenchus*, *Malenchus*, and *Paratylenchus*, and a high abundance of predators. In semi-desert ecosystem in Mexico, the most prevalent genera were *Aphelenchus* and Aphelenchoididae, *Acrobeles*, *Cephalobus*, *Eucephalobus*, *Geomonhystera*, *Plectus*, *Prismatolaimus* and *Wilsonema*, while the most prevalent herbivores were *Belonolaimus*, *Hirschmanniella*, *Hoplolaimus*, *Pratylenchus*, *Psilenchus*, *Telotylenchus*, *Trophurus*, unidentified Tylenchidae, *Tylenchorhynchus*, *Tylenchus* and *Xiphinema* [8]. In the study by Fiscus and Neher [9], the most sensitive nematodes to direct effects of tillage included *Aphelenchoides*, *Eucephalobus*, *Eudorylaimus*, *Heterocephalobus*, and *Wilsonema* compared to the tolerant genera *Achromadora*, *Anatonchus*, *Cephalobus*, *Chiloplacus*, *Clarkus*, *Epidorylaimus*, *Mylonchulus*, *Plectus*, and *Tylencholaimellus*. Yeates [10] in his study of nematodes as soil indicators, indicated that Cephalobidae were often the most abundant group in soil, the number of Rhabditidae might be increased after increasing resources and in stressed, natural environments Plectidae were the dominant group. Increased soil pH significantly increased total nematode numbers, leading to higher numbers of bacterivorous and omnivorous nematodes [11]. In the study by Sanches-Moreno et al. [12], *Mesorhabditis* and *Acrobeloides* were positively associated with ammonium ions, while *Panagrolaimus* and *Plectus* were negatively associated with some phospholipid fatty acids. *Discolaimus*, *Prionchulus*, *Mylonchulus*, and Aporcelaimidae were associated with deeper soil layers. Increased Cu content in soil resulted in tolerance to copper pollution in nematode communities [13]. Shao et al. [14] concluded that nematode c-p groups 3, 4 and 5 were negatively correlated with Pb and Zn concentrations and can be utilized as indicators of heavy metal contamination. In the study by Šalamun et al. [15], nematodes with the highest tolerance to elevated Mg content in soils were c-p 1 group nematodes and bacterivores. Certain nematode genera can predict specific type of disturbance. Cultivation decreased abundances of *Diphtherophora*, *Prismatolaimus* and *Tylenchorhynchus*. Application of synthetic chemical fertilizers reduced numbers of *Plectus*, while application of organic fertilizers resulted in increased numbers of *Cruznema*, *Mesorhabditus*, *Mesodorylaimus* and *Nygolaimus* [16]. The results of Shih et al. [17] suggested that *Auanema* spp. developed extreme arsenic resistance that enabled nematodes to survive in Mono Lake. In the study by Bonaglia et al. [18], it was presented that meiofauna played an important role in several geochemical processes as they: increase the oxygen penetration depth; decrease the overall sulfide flux; and increase the volume of oxidized, sulfide-free sediment in seasonally hypoxic environments. The effect of joint toxicity of Cu and Zn to a terrestrial nematode community was demonstrated in the study of Korthals et al. [19]. *Thonus*, *Alaimus*, and *Aporcelaimellus* were the most sensitive genera and disappeared when Cu and Zn concentrations exceeded 50 mg/kg. The

widespread success of nematodes in extreme environments indicated strong preadaptation throughout the phylum [17].

This research aimed to: investigate the concentration of chemical parameters in the soil of the natural park, identify nematodes that can survive in such extraordinary conditions, explore the feeding habits of nematodes using stomatal anatomy, and infer the phylogenetic relationships of nematodes based on the maximum likelihood (ML) and Bayesian inference (BI) of *28S* rRNA sequences.

2. Materials and Methods

2.1. Soil Collection, Nematode Extraction and Nematode Morphological Characterization

Soil samples were collected with a hand trowel as composite samples from 10 sites each, weighing about one kilogram [20]. Nematodes were found in soils covered with vegetation or rain-washed plant material. Soils were designated as Soil under vegetation (Soil No. 1) and Saxon mine soil (Soil No. 2). Nematode extraction was performed by Baermann's funnels to capture the entire population [21]. A soil sample weighing 100 g was placed on a 150 μm sieve immersed in a funnel filled with water. At the end of the funnel a rubber pad was attached to a glass flacon in which the nematodes were collected after 24 h.

Morphological characterization of nematodes was carried out using descriptions from the available literature, comparing digital photographs, drawings and morphometrical data, especially comparing nematode stomatal regions and other key morphological features.

2.2. Nematode DNA Extraction and Phylogeny

Nematode DNA was extracted with The Dneasy Plant and Tissue kit according to the manufacturer's procedure. The *28S* rRNA gene primers, D2A (5′-ACAAGTACCGTGAGGG AAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) were used in the PCR procedure described by Subbotin et al. [22]. To perform PCR assay, the reaction mixture contained 2 × PCR Master Mix, 0.4 μM each of forward and reverse primers, 2 μL of DNA template and nuclease-free water to a total volume of 50 μL. PCR cycling program for *28S* was as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s and extension at 72 °C for 2 min. A final extension was performed at 72 °C for 10 min. Phylogenetic analyses were performed with submitted nematode sequences containing accession numbers: OR625213–OR625225 and related species from the GenBank database, using Maximum likelihood (ML) and Bayesian inference (BI). The ML and BI dendrograms were generated by the computer programs PhyML 3.1 [23] and MrBayes 3.1.2 [24], respectively using the General Time Reversible Model (GTR), invariant sites, and gamma distribution (GTR + I + G). Sequence alignment was performed with the ClustalW module incorporated in Mega 4 [25]. The dendrogram obtained by Bayesian inference was generated by 10^6 generations of Markov Chain Monte Carlo, with a sampling frequency of 100 and a "burnin" function of 20%. Branch support greater than 70% was shown next to the node.

Images of nematodes were captured using an Olympus BH-2 microscope with Nomarsky contrast, a digital camera and Motic Images Plus 2.0 ML software (Motic China Group Co., Ltd., Nanjing, China) while a pictorial dendrogram was inferred using the Neighbor-joining method [26] in Mega 4 with 500 replicates.

2.3. Chemical Analyses of Soil Samples

Sample preparation for determination of specific conductivity, pH, and total nitrogen content was performed using air-dried soil samples. For moisture (dry matter) content, organic matter content, and inorganic nitrogen compounds, field-moist (undried) soil samples were used. Specific conductivity was measured according to ISO 11265 Standard [27]. The determination of electrical conductivity was made with a TetraCon 325 conductivity cell (Cond 340i Set, WTW, Weilheim, Germany) by measuring the electrical resistance of a 1:5 (soil:water, w/v) suspension. Twenty grams of air-dried soil was weighed and 100 mL

of deionized water was added into a container, covered with bottle caps and placed in a shaker. The suspension was further prepared by shaking it for 60 min at 180 r/min. After shaking, the suspension was removed from the shaker and allowed to stand for 30 min. The conductivity cell was then immersed in the supernatant, without disturbing the sediment. When it was stabilized, conductivity measurements were taken. Determination of pH is performed by measuring the potential difference between two appropriate electrodes immersed in the solution to be tested according to ISO 10390 [28] using HI2020 Edge pH-meter (HANNA Instruments Inc., Woonsocket, RI, USA) with glass electrode. Representative soil samples were mixed with deionized water in a 1:5 (soil:water, w/v) ratio. The suspension was mixed for 60 min ± 10 min, and allowed to stand for the same time. The pH was then measured.

Moisture (dry matter) content was determined by the gravimetric method according to ISO 11465 [29] using a Digitheat-TFT electric oven (J.P. Selecta, Barcelona, Spain). Soil samples were placed on a clean surface, then rocks, branches, and leaves were removed and mixed well. Before adding samples, the opened aluminum dish was dried at a temperature of 105 ± 5 °C, the closed dish was cooled in a desiccator for at least 45 min, then the weight of the closed dish was determined. Forty grams of undried soil was transfered to an aluminum dish, then the mass of the sealed dish containing the sample was measured. Opened aluminium dish with soil and lid were dried at 105 ± 5 °C, closed dish with soil sample was cooled in a desiccator for at least 45 min and then the mass of closed container with soil was measured. The drying and cooling procedure was repeated until a constant mass was obtained. Moisture (in %) was calculated as the mass difference of the dish plus field-moist soil and the dish plus oven-dried soil divided by the mass difference of the dish plus oven-dried soil and the empty dish with lid.

Organic matter content was determined by the gravimetric method according to EN 15935 standard [30] using an FT 35 electric furnace (Prederi Vittorio & Figli snc., Milano, Italy) and was calculated from the difference in mass determined before and after ignition. Before adding the sample, the ceramic crucible was placed in the furnace and heated to 550 ± 25 °C for at least 20 min, then cooled in a desiccator to room temperature and the mass of the empty crucible was measured. For incineration, about 2 g of undried soil was placed in a ceramic crucible and transferred to a cool furnace, after which the temperature was gradually increased to 250 °C over a period of 50 min, allowing pyrolysis of the soil sample. The furnace temperature was increased to 550 ± 25 °C and ignition lasted for at least 2 h, after which the ceramic crucible with ignition residue was cooled to room temperature. The mass of the crucible containing the burned residue was then measured. The drying and cooling procedure was repeated until a constant mass was obtained. Total organic carbon content was calculated by dividing the organic matter content (in %) by a factor of 1.724.

ISO 11261 [31] was applied for the volumetric determination of total nitrogen content (sum of ammonium nitrogen, nitrate nitrogen, nitrite nitrogen and organic nitrogen), using the K-424 digestion system unit (Buchi, Flawil, Switzerland) and K-350 distillation system unit (Buchi, Flawil, Switzerland). Air-dried soil (0.2 g) was placed in the digestion flask (Kjeldahl tube) and 4 mL of salicylic–sulfuric acid mixture (25 g of salicylic acid dissolved in 1 L of concentrated sulfuric acid) was added, then the flask was swirled until the contents were completely mixed and left overnight. Next, 0.5 g of sodium thiosulfate pentahydrate was added through a dry funnel with a long stem that reached down into the bulb of the digestion flask without immersion into the content, and the mixture was carefully heated in the digestion flask until foaming stopped. The flask was cooled and 1.1 g of the catalyst mixture (200 g of potassium sulfate, 6 g of copper sulfate pentahydrate and 6 g of titanium dioxide) was added and heated until the digestion mixture became clear. The digestion process continued for 2–3 h, at higher temperatures but not higher than 400 °C. Once the digestion phase was completed, the flask was cooled and 20 mL of water was added and stirred slowly. Then, the flask and its content was transferred to the distillation system unit. At the same time, in the part below the condenser of the distillation system unit, a

250 mL conical flask filled with 25 mL of boric acid solution (20 g/L) was placed so that the end of the condenser hose was immersed in the solution. Steam distillation was started while 20 mL of sodium hydroxide (10 M) was introduced into a distillation column. With distillation rates up to about 25 mL/min, the process was stopped when 100 mL of distillate was obtained. Then a few drops of indicator was added (0.1 g bromocresol blue and 0.02 g methyl red dissolved in 100 mL ethanol) to the distillate and titrated with sulfuric acid solution (0.01 M) until the purple endpoint. The same procedure was performed with the blank sample. The consumption of sulfuric acid solution in soil and the blank sample was notified.

The content of inorganic nitrogen compounds (ammonium, nitrate and nitrite) was determined by spectrophotometry according to ISO/TS 14256-1 [32] using a UV-VIS 2100 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The required reagents and solutions were prepared according to procedures described in the Standard. An undried soil sample weighing 40.0 g was homogenized by mixing thoroughly and adding 200 mL of potassium chloride solution (1 M) to a 500 mL polyethylene bottle and shaking well for 1 h at (20 ± 2) °C, enough for the extraction process to be complete. Sixty milliliters of the extract suspension was decanted into a 100 mL centrifuge tube and centrifuged on an EBA 280 (Hettich, Tuttlingen, Germany) for 10 min at 3000 g. The separated supernatant was poured into a 100 mL Erlenmeyer flask and the nitrogen fractions were measured in appropriate aliquots. The blank test was also performed by adding only potassium chloride solution (1 M) to the bottle. In the first aliquot (1.00 mL), the sum of nitrate and nitrite was determined in several steps, starting with the reduction in nitrate to nitrite (including the amount of nitrite initially present in the sample) by adding extract into a reduction column containing copperized cadmium powder. The reduction step was also performed on 1.00 mL of blank sample, nitrate standard solution, nitrite standard solution, and zero standard solution. After the reduction step, the reduced eluent was collected into a 30 mL tube and color development was performed by adding 0.20 mL of color reagent to each tube, mixing well and leaving for 90 min at room temperature. The color reagent was prepared by mixing 20 mL of sulfanilamide solution, 20 mL of N-(1-naphthyl)ethylenediamine dihydrochloride solution and 20 mL of phosphoric acid. The absorbance of the diazo compound likely to form in acidic media after the addition of the Griess-Ilosvay reagent was measured spectrophotometrically in each tube at 543 nm. In the second aliquot (1.00 mL) placed in a 30 mL tube, the nitrite content alone, was determined by adding 20 mL of water and color development was carried out by adding 0.20 mL of the same color reagent, mixed well and left for 90 min at room temperature. The same procedure was performed on each 30 mL tube containing 1.00 mL blank sample, nitrite standard solution and zero standard solution. The absorbance was measured spectrophotometrically in each tube at 543 nm. In the third aliquot (10.0 mL) placed in a 100 mL Erlenmeyer flask, the ammonium ion content was determined by adding 40 mL of color reagent no. 1, mixed well and left for 15 min. One milliliter of color reagent no. 2 was added, mixed well and left for 5 h at room temperature. Both color reagents were prepared according to the Standard. The same procedure was performed on each 30 mL tube containing 10.0 mL blank sample, ammonium standard solution and zero standard solution. Ammonium and hypochlorite ions at high pH form monochloramine, which reacts with phenol and obtains the indophenol blue complex, the absorbance of which was measured at 630 nm. Total organic nitrogen content (%) was calculated as the difference between the total nitrogen content and the inorganic nitrogen content. Heavy metal and microelement analyses were performed using an advanced MARS 6 iWave microwave digestion system (CEM Corporation, Charlotte, NC, USA) and 5110 Dual view inductively coupled plasma-optical emission spectrometer equipped with SPS-4 auto-sampler (Agilent Technologies Inc., Santa Clara, CA, USA) according to ISO 22036 [33]. Sample preparation for heavy metal and microelement analyses was performed according to US EPA method 3052 [34]. Representative samples up to 0.5 g were digested in 9 mL of concentrated nitric acid (65%) and 2 mL of hydrogen peroxide (30%) by microwave heating with microwave system. The temperature profile was specified

to trigger specific reactions starting at 16 ± 4 °C and reaching 165–170 °C for 10 min and remaining at the same temperatures for 10 min to complete reactions. After cooling for at least 30 min, the samples were filtered and analyzed. The operating conditions of ICP-OES for multi-element determination were as follows: Power: 1.3 KW; Optical spectrum range: 165–800 nm; Plasma gas flow: 12 L/min (argon); Auxiliary gas flow: 1.0 L/min; Nebulizer flow: 0.70 L/min; Pump speed: 12 rpm; Pump tubing: white-white for sample (1.02 mm id), orange-white for internal standard (0.64 mm id), blue-blue for waste (1.65 mm id); Sample uptake rate: 1.0 mL/min; Replicate read time: 10 s; Sample uptake delay time: 35 s; Fast pump: on; Rinse time: 10 s; Sampling mode: auto sampler; Background correction: fitted, Number of replicates: 3. Elements were determined at element-specific analytical wavelengths and the content in the sample was calculated using a calibration curve. Individual commercial standards (AccuStandard Inc., New Haven, CT, USA) were used to prepare standard calibration mixtures. Working (calibration) standard solutions were prepared at the following concentration range: Pb (0.0; 0.5; 1.0; 5.0; 10.0 mg/L), Cd (0.0; 0.1; 0.2; 1.0; 2.0 mg/L), Zn (0.00; 0.25; 0.50; 2.50; 5.00 mg/L), Cu (0.0; 0.1; 0.2; 1.0; 2.0; 10.0 mg/L), Ni (0.0; 0.1; 0.2; 1.0; 2.0 mg/L), Cr (0.00; 0.25; 0.50; 2.50; 5.00 mg/L), Fe (0.0; 0.5; 1.0; 5.0; 10.0 mg/L), Hg (0.0; 0.5; 1.0; 5.0; 10.0 mg/L), As (0.0; 0.5; 1.0; 5.0; 10.0 mg/L), P (0.0; 0.5; 1.0; 5.0; 10.0; 50.0 mg/L), S (0.00; 0.25; 0.50; 2.50; 5.00 mg/L) and K (0.0; 1.0; 2.0; 10.0; 20.0 mg/L).

3. Results and Discussion

3.1. Chemical Analyses of Soil Samples

The chemical analyses of the studied soil samples are presented in Table 1. The acidity of the Saxon mine soil (Soil No. 2) is extremely high, and the values of electrical conductivity, organic matter content and moisture are several times higher than the corresponding values of the Soil under vegetation (Soil No. 1). In the Saxon mine soil, low pH and higher moisture promote better salt dissolution, which is reflected in higher electrical conductivity compared to the Soil under vegetation. Additionally, in Soil No. 2, high organic matter content was observed, resulting in higher levels of total organic carbon and total organic nitrogen. The total inorganic nitrogen content of the two soil samples was similar. The phosphorus content of Soil No. 2 is nearly 3.5 times higher than that of soil No. 1 and the sulphur content of Soil No. 2 is almost twice as high as in Soil No. 1. In contrast, the potassium content of Soil No. 1 is almost twice as high as that of Soil No. 2. Regarding heavy metal content, the Soil under vegetation contains high levels of lead and copper, while the Saxon mine soil is rich in lead, zinc, iron and arsenic. The soil of the Saxon mine contains nearly 10 times more iron than the Soil under vegetation.

Table 1. Physico-chemical and chemical parameters of investigated soils.

Parameters ± SD	Soil under Vegetation (Soil No. 1)	Saxon Mine Soil (Soil No. 2)
pH	4.20 ± 0.03	3.10 ± 0.01
el. conductivity (mS/m)	7.30 ± 0.69	58.00 ± 0.15
organic matter (%)	3.70 ± 0.34	26.90 ± 1.40
moisture (%)	14.10 ± 0.20	89.70 ± 0.10
dry matter (%)	85.90 ± 0.20	10.30 ± 0.10
Pb (mg/kg)	196.00 ± 3.53	74.10 ± 1.85
Cd (mg/kg)	ND	0.60 ± 0.03
Zn (mg/kg)	25.70 ± 0.70	204.00 ± 4.69
Cu (mg/kg)	51.60 ± 1.14	10.20 ± 0.14
Ni (mg/kg)	6.60 ± 0.09	3.40 ± 0.05
Cr (mg/kg)	6.40 ± 0.09	1.30 ± 0.02
Fe (%)	2.70 ± 0.03	20.30 ± 0.49

Table 1. *Cont.*

Parameters ± SD	Soil under Vegetation (Soil No. 1)	Saxon Mine Soil (Soil No. 2)
Hg (mg/kg)	0.50 ± 1.06	0.02 ± 0.01
As (mg/kg)	20.80 ± 0.03	53.90 ± 2.37
N total (%)	2.12 ± 0.20	2.60 ± 0.18
N total organic (%)	1.60 ± 0.18	2.04 ± 0.24
N total inorganic (%)	0.50 ± 0.04	0.60 ± 0.06
C total (%)	2.10 ± 0.19	15.70 ± 0.82
P (mg/kg)	431.00 ± 13.79	1495.00 ± 42.61
K (mg/kg)	810.00 ± 23.65	488.00 ± 15.52
S (%)	2.30 ± 0.04	4.10 ± 0.07

SD = Standard Deviation, ND = Non-Detectable.

3.2. Nematode Characterization

The soils studied, although separated by less than 30 m, had different physico-chemical properties that indicate their different geological history. Even the composition of nematode species was not exactly the same and was affected by certain soil chemical properties. The list of nematode species was as follows (Figure 1a–m):

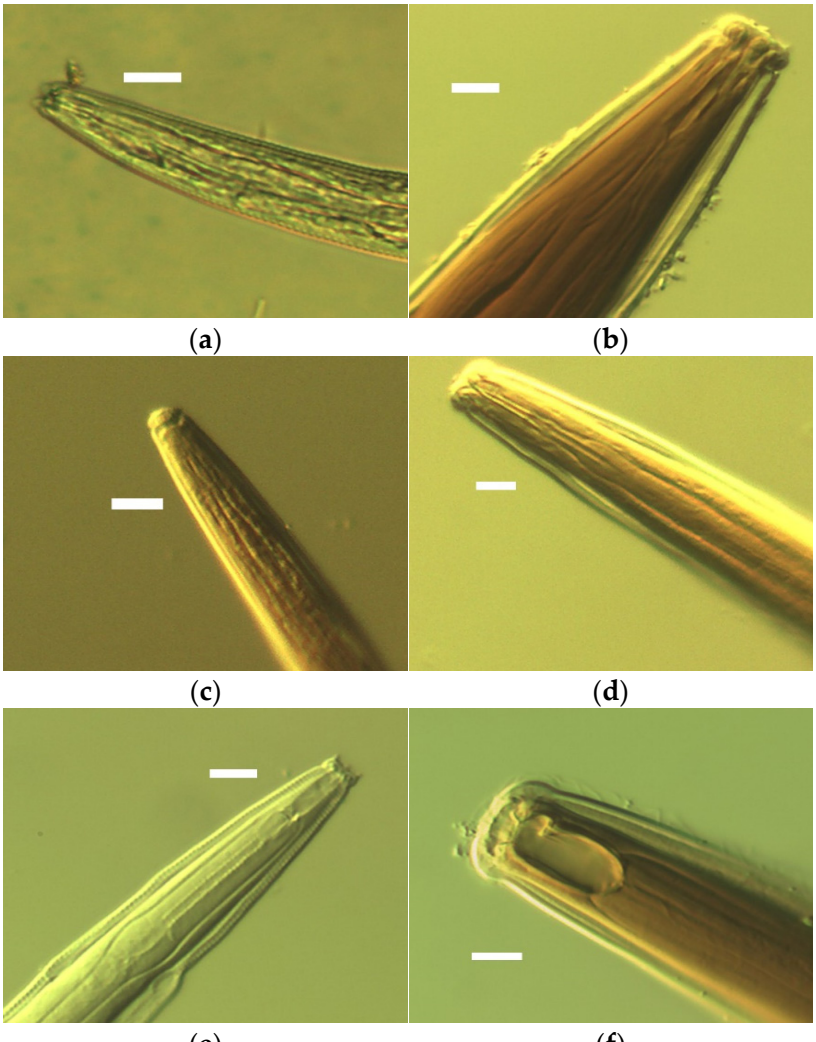

Figure 1. *Cont.*

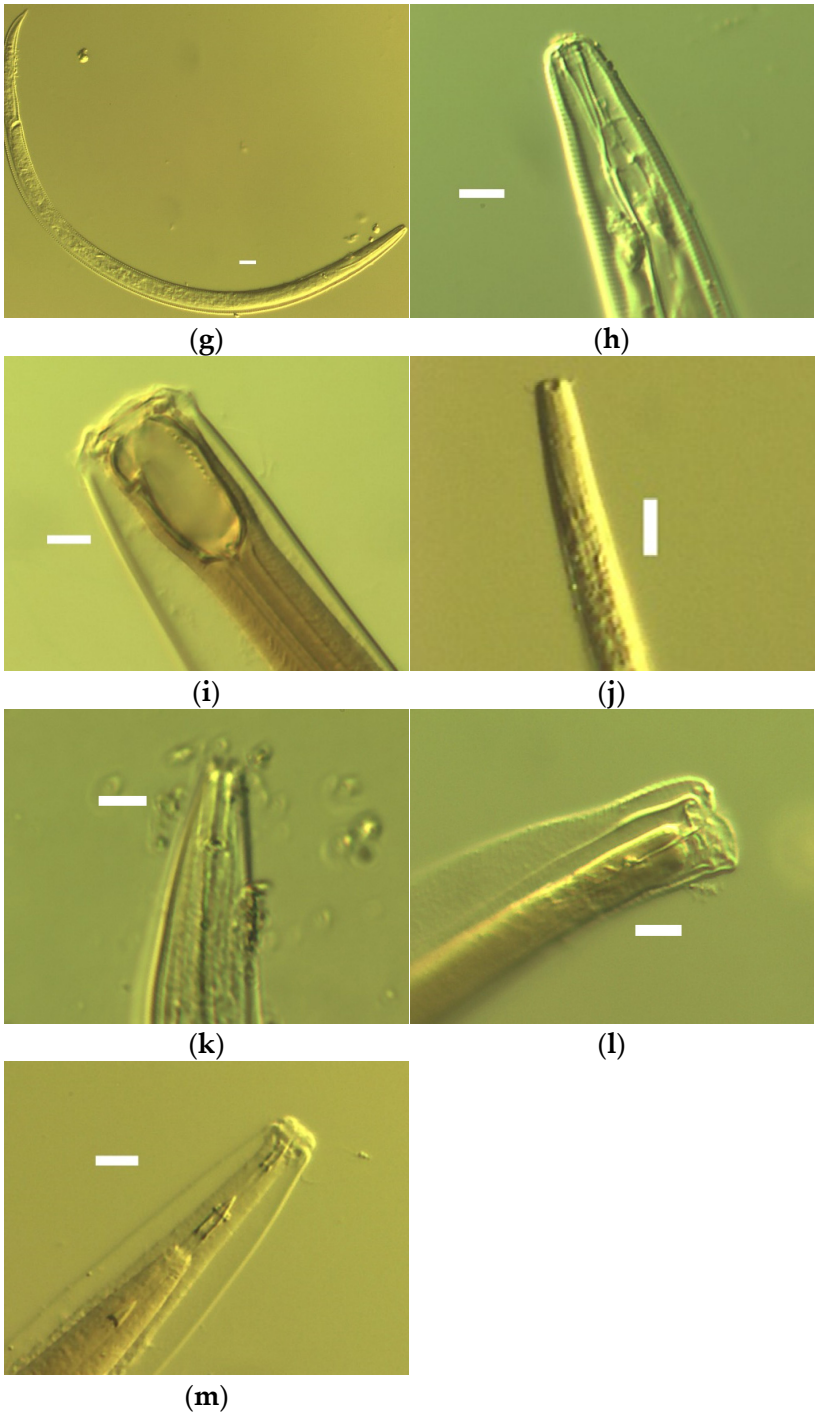

Figure 1. Nematodes from Devil's Town: (**a**) *Acrobeloides nanus* (**b**) *Aporcelaimellus obtusicaudatus* (**c**) *Calcaridorylaimus castaneae* (**d**) *Epidorylaimus consobrinus* (**e**) *Mesorhabditis simplex* (**f**) *Mononchus* sp. (**g**) *Paratylenchus* sp. (**h**) *Plectus* sp. (**i**) *Prionchulus* sp. (**j**) *Prismatolaimus dolichurus* (**k**) *Rhabditis terricola* (**l**) *Tripyla* sp. (**m**) *Tylencholaimus* sp. (bar = 10 µm).

***Acrobeloides nanus* (de Man, 1880).** Cephalobidae. In the description of Kim et al. [35], the stoma of this species is cephaloboid with a length about 1.6–2 times lip region diameter, and labial probolae are conical, not furcate, which is in accordance with Figure 1a. The nematode was observed to ingest bacteria of the *Pseudomonas* and *Clavibacter* genera [36]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 2. In the study of Bendoy et al. [38], *A. nanus* responded positively to increasing Cd concentrations. The nematode was found in the Soil under vegetation with elevated Pb, Hg, and Cu

contents. The Blast search indicated 100% homology with the same species from the NCBI sequence database.

***Aporcelaimellus obtusicaudatus* (Bastian, 1865).** Aporcelaimidae. In the description by Goodey [39], the stoma of *A. obtusicaudatus* is armed with a robust, short spear, similarly to the one shown in Figure 1b. The nematode is categorized as a predator and an omnivore [40]. An *Aporcelaimellus* population fed on algae, moss, nematodes, nematode eggs, enchytraeids and rotifers [41]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 5. *Aporcelaimellus obtusicaudatus* was found in the Soil under vegetation with elevated content of lead, mercury and copper. The Blast search revealed 100% similarity to the same species from the NCBI sequence database.

***Calcaridorylaimus castaneae* (Nedelchev et al., 2013).** Dorylaimidae. The original description of Nedelchev et al. [42] is congruent with Figure 1c. The species is characterized as an omnivore cp-4. The nematode was found in the Soil under vegetation with elevated Pb, Hg and Cu contents. The Blast search indicated 100% homology with the same species from the NCBI sequence database.

***Epidorylaimus consobrinus* (de Man, 1917)**. Quadsianematidae. According to Bongers [43], the lip area of this nematode is weakly marked by an indentation, spear 16–21 µm long, which is in accordance with Figure 1d. The nematode is categorized as a predator and an omnivore [40], and belongs to c-p 4 [37]. The nematode was found in both soil samples and can tolerate high Pb, Zn, Fe, Cu, Hg and As contents. The Blast search revealed 96% homology with the same genus from the NCBI sequence database, there were no sequences of the same species.

***Mesorhabditis simplex* (Cobb, 1893).** Rhabditidae. According to Goodey [39], the stoma is long and narrow, never prismatic. Metarhabdions each carry two inclined teeth as in Figure 1e. The nematode is classified as a bacterivore [41]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 1. In the study of Lu et al. [44], *Mesorhabditis* was tolerant to elevated mercury content. The nematode was found in both soil samples and can tolerate high Pb, Zn, Fe, Cu, Hg and As contents. The Blast search indicated 100% identity with the same species from the NCBI sequence database.

***Mononchus* sp. (Bastian, 1865).** Mononchidae. In the description of Goodey [39], this nematode has a dorsal tooth in the anterior part of stoma that is powerful and pointing forward with a non-denticulate ventral ridge ending opposite the tooth as shown in Figure 1f. The nematode is classified as a predator [40]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 4. *Mononchus* could be found most abundantly at sites with high metal contamination [45]. The nematode was found in both soil samples and can tolerate high Pb, Zn, Fe, Cu, Hg and As contents. The Blast search revealed 92% similarity to the *Mononchus* species with only three sequences existing in the NCBI sequence database.

***Paratylenchus* sp. (Micoletzky, 1922).** Tylenchulidae. According to Clavero-Camacho et al. [46], this nematode has a rounded head, long stylet, and indistinct basal bulbs as in Figure 1g. The nematode feeds ectoparasitically on plant roots. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 2. It was shown that the nematode was a good bioindicator of Cr [47]. The nematode was found in the Soil under vegetation with elevated Pb, Cu, and Hg contents. The Blast search indicated 96% homology with *P. straeleni*.

***Plectus* sp. (Bastian, 1865).** Plectidae. According to Goodey [39], the nematode has a tubular stoma lined with rod-like rhabdions as in Figure 1h, and is observed to establish colonies on bacteria [41]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 2. *Plectus murrayi* was sensitive to aqueous copper [48]. The nematode of the genus *Plectus* in our study was found in the Soil under vegetation with elevated Pb, Cu and Hg contents. The Blast search revealed 97% homology with the same genus from the NCBI sequence database.

***Prionchulus* sp. (Wu and Hoeppli, 1929).** Mononchidae. In the description of Goodey [39], the nematode has an oval-oblong stoma, with almost parallel sides. The dorsal

tooth is large, pointing forwards in the anterior half of the stoma, opposed by longitudinal ridges bearing denticles similarly to Figure 1i. It is a predatory nematode [49]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 4. The nematode was found in the Soil under vegetation with elevated Pb, Cu and Hg contents. The Blast search indicated 98% similarity to the same genus from the NCBI sequence database.

***Prismatolaimus dolichurus* (de Man, 1880).** Prismatolaimidae. In the description by Abebe and Coomans [50], the nematode has a stoma anteriorly barrel-shaped, 1.3 times longer than wide, similarly to Figure 1j, and is classified as a bacterivore [40]. According to Bongers [37], the species belongs to the c-p group 3. This nematode exhibited extreme arsenic tolerance [17]. *Prismatolaimus dolichurus* was found in the Saxon mine soil with high Pb, Zn, Fe and As contents. The Blast search indicated 100% homology with the same species from the NCBI sequence database.

***Rhabditis terricola* (Dujardin, 1845).** Rhabditidae. According to Goodey [39], the species has a tubular stoma, the three metarhabdions together form the glottoid apparatus and each bears teeth or tubercules as shown in Figure 1k. *Rhabditis* sp. is observed to establish colonies on a wide range of bacteria species [41]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 1. *Rhabditis* was found to be tolerant to Cu, Mg and Zn [15]. *Rhabditis terricola* was found in the Saxon mine soil with high Pb, Zn, Fe and As contents. The Blast search revealed 99% homology with the same species from the NCBI sequence database.

***Tripyla* sp. (Bastian, 1865).** Tripylidae. According to Goodey [39], the nematode has a stoma that is a simple tube with a tooth present in the dorsal wall as in Figure 1l. Observation of intestinal contents of various species suggests that all genera of the Tripylidae are predators and commonly consume protozoa, small nematodes, tardigrades, and rotifers [51]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 3. The nematode was found in both soil samples and can tolerate high Pb, Zn, Fe, Cu, Hg and As contents. The Blast search revealed 97% homology with the same genus from the NCBI sequence database.

***Tylencholaimus* sp. (de Man, 1876).** Tylencholaimidae. In the description by Goodey [39], bodies are robust with spear dorylaimoid and spear extensions with small basal knobs as shown in Figure 1m. The nematode is classified as a fungivore [40]. According to Bongers [37], the species belongs to the colonizer-persister (c-p) group 4. The nematode was found in both soil samples and can tolerate high Pb, Zn, Fe, Cu, Hg and As contents. The most similar species was *Tylencholaimus* from the NCBI sequence database with 87% similarity.

3.3. Phylogenetic Analyses

In spite of the fact that all but one of the nematode species found were described in the 19th and 20th centuries, their sequence records remain rare. The phylogenetic analyses based on 28S sequences of the found nematodes (OR625213–OR625225) and related species are shown in Figures 2 and 3. Both ML and BI trees are in agreement and generated several distinct clades. Beginning from the top, within the first clade there are two subclades; the first subclade includes the families Aporcelaimidae, Dorylaimidae, Quadsianematidae and Tylencholaimidae. The second one with *Mononchus* and *Prionchulus* spp. represents the family Mononchidae. The second clade contains three subclades composed of the families Tripylidae, Prismatolaimidae and Plectidae. The last clade is represented by the families Rhabditidae, Cephalobidae and Tylenchulidae. Surprisingly, the *Mesorhabditis* subclade is not a sister subclade of the *Rhabditis* subclade. This was solved by a pictorial diagram representing only the species from Devil's Town. The pictorial diagram was based on the Neighbor-joining method, and is given in Figure 4.

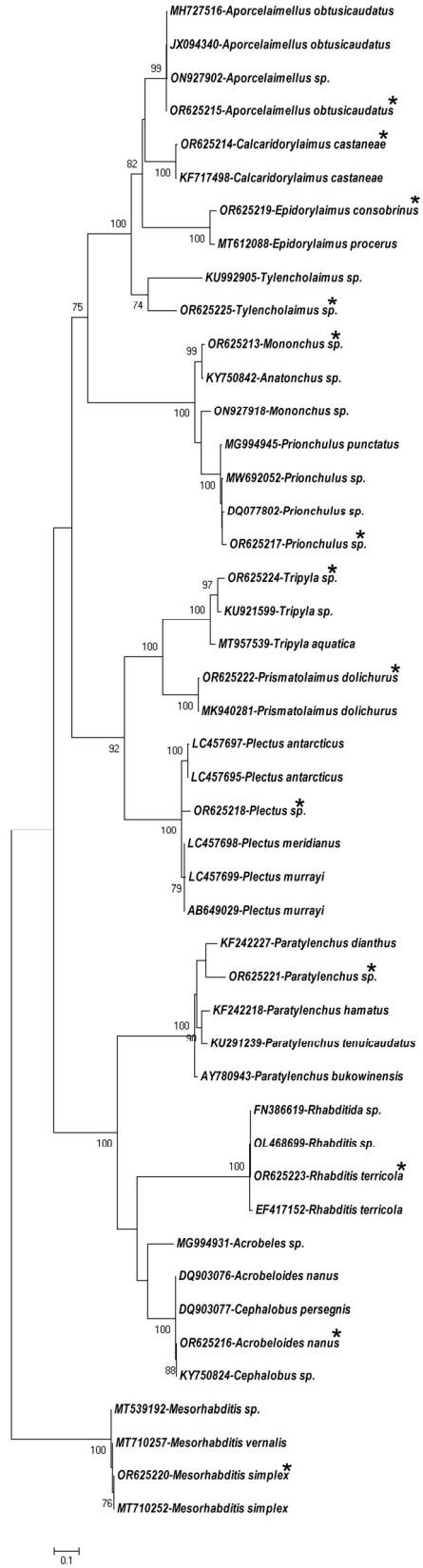

Figure 2. Maximum likelihood phylogenetic tree of nematodes based on *28S* sequence region using the GTR + I + G nucleotide evolution model. The newly obtained sequences are marked with asterisks.

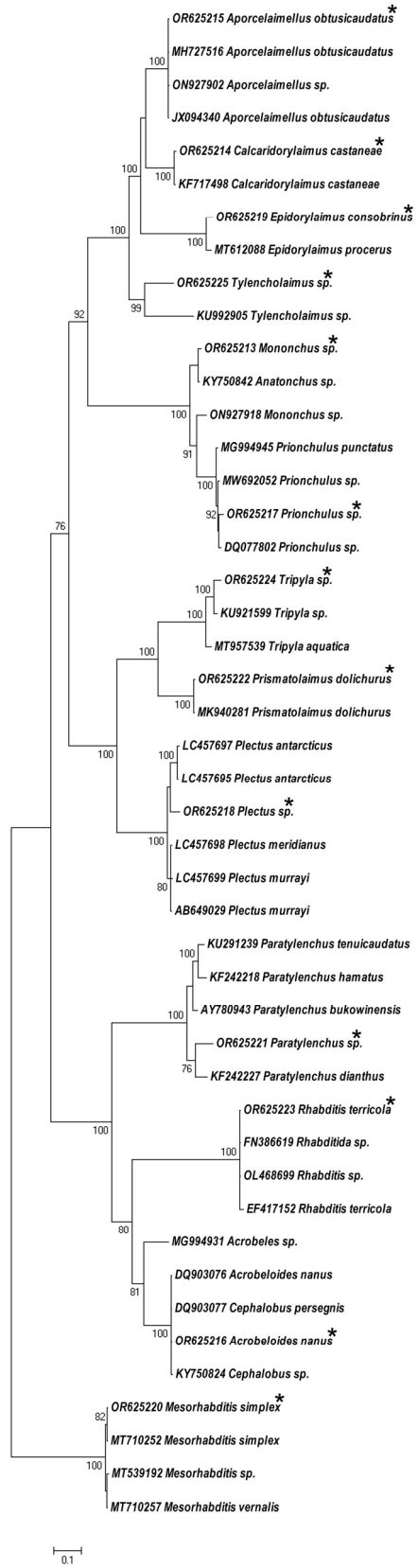

Figure 3. Bayesian phylogenetic tree of nematodes derived from consensus 50% majority rule based on *28S* sequence region using the GTR + I + G nucleotide evolution model. The newly obtained sequences are marked with asterisks.

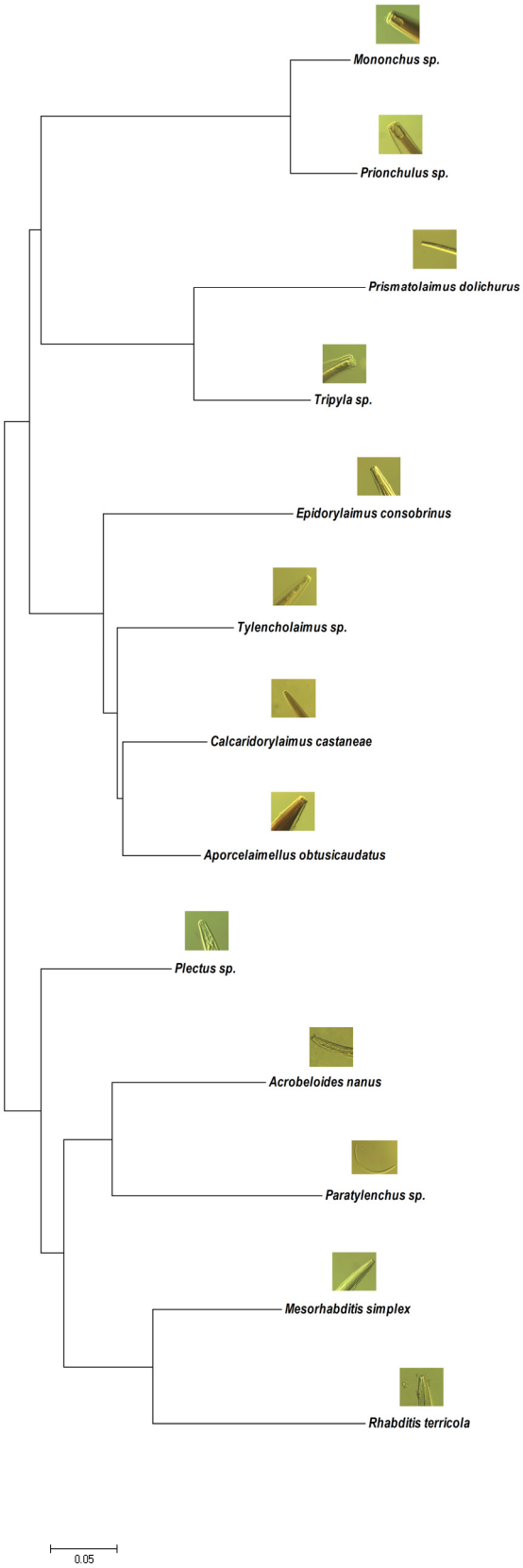

Figure 4. Pictorial phylogenetic tree of nematodes based on *28S* sequence region using the Neighbor-joining method.

The grouping of species in Figures 2 and 3 can be explained by the colonizer-persister grouping. Species within the first clade belong to c-p 4 and 5 and are the dominant

species of the locality. The percentages of mononchids were 28.2 and 25.4 in Soils No. 1 and 2, respectively. The percentages of dorylaimids were 46.2 and 59 in Soils No. 1 and 2, respectively. The second clade contains c-p 2 and 3 species, while the last clade is composed of c-p 1 and 2 species. The percentages of saprobionts were 19.2 and 13.6 in Soils No. 1 and 2, respectively. The predatory species *Tripyla* had 3.8 and 2% in Soils No. 1 and 2, respectively, while the only plant parasite *Paratylenchus* had 2.6% in Soil No. 1. The pictorial diagram (Figure 4) clustered three distinct clades representing mononchids and enoplids in the first clade, dorylaimids in the second clade, and plectids, cephalobids, rhabditids, and tylenchids in the last one. Bongers [37] defined colonizer (c-p 1) versus persister (c-p 5) nematode species. Nematodes that rapidly increase in number under favorable conditions can be considered to be colonizers. They possess a short life-cycle, high colonization ability and tolerance to disturbance, eutrophication and anoxybiosis. On the other hand, persisters have a low reproduction rate. In general, they have a long life-cycle, low colonization ability and are sensitive to disturbance. In our study, Soil No. 2 has more rigorous conditions than Soil No. 1 and consequently has fewer nematode species. *Rhabditis terricola* and *Prismatolaimus dolichurus* are two species found exclusively in Soil No. 2 and may be indicators of high acidity and high levels of lead, zinc, iron, and arsenic. They are colonizers as well. On the other hand, *Paratylenchus* sp. is the only representative of plant parasites in Soil No. 1 and indicate the presence of plants in the area. Mononchids and dorylaimids are persisters and dominant species, sensitive to disturbances. Considering this, Devil's Town presents a stable ecosystem with extremely high performances.

The exact mechanism of heavy metal resistance/tolerance in nematodes is still unknown. In this study, heavy metal-resistant nematodes are microbivores. Bacteria and fungi in nematode diets have the ability to detoxify heavy metals. Many bacteria have specific genetic mechanisms to resist toxic metals [52,53]. Fungi have evolved active defense mechanisms as biosorption, bioaccumulation, metal chelation, and efflux transport to provide resistance from heavy metal stress in natural environments [54]. Consequently, the feeding behavior and stomatal apparatus may have the key function in heavy metal detoxification and mitigate a harmful effect of metals on nematodes.

Further research will focus on nematofauna from nearby sites that are not naturally contaminated, in order to make a comparison among nematode populations and to determine dominant species in non-extreme environments.

4. Conclusions

The results of this study suggest that two proximal sites of the same locality may have different soil chemical composition and characteristics, presumably due to their different geological and exploitation history. Volcanic activity created sites with elevated heavy metal content, while wind and water erosion degraded solid mineral rocks into more porous substrates. The presence of minerals and heavy metals in combination with high acidity created harsh, i.e., extreme environmental conditions in which specific nematode species can survive. These circumstances favored fast-moving species with teeth and piercing spears in their stomatal apparatus, such as mononchids and dorylaimids, allowing them predatory feeding habits. In contrast, there are saprobionts adapted to specific chemical pollutants, such as *Acrobeloides*, *Prismatolaimus*, *Rhabditis* spp. etc., which tolerate high levels of Pb, Zn, Fe, Cu, Cd, Hg and As and serve as a food source for predators. Perhaps, the best conclusion may be borrowed from Dennis Overbye (The New York Times): "Geology is biological destiny: Whatever minerals land or are deposited in a place determine what or who can make a living there millions of years later."

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