Worms from hell: *Nematoda* from the terrestrial deep subsurface of South Africa

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Since its discovery over two decades ago, the deep subsurface biosphere has been considered to be the realm of single cell organisms, extending >3 km into the Earth's crust and comprising a significant fraction of the global biosphere[1,2,3,4]. The constraints of temperature, energy, O_2 and space seemed to preclude the possibility of more complex multi-cellular organisms from surviving at these depths. Here we report species of the phylum *Nematoda* that have been detected in or recovered from 0.9-3.6 km deep fracture water encountered in the deep mines of South Africa, but have not been detected in the mining water. These subsurface nematodes, including a new species *Halicephalobus mephisto*, tolerate high temperature, reproduce asexually and preferentially feed upon subsurface bacteria. ^{14}C data indicate that the fracture water in which the nematodes reside is 3-12 kyr old paleometeoric water. Our data suggest that nematodes should be found in other deep hypoxic settings where temperature permits and that they may control the microbial population density by grazing upon fracture surface biofilm patches. Our results expand the known *Metazoan* biosphere and demonstrate that deep ecosystems are more complex than previously accepted. The discovery of multi-cellular life in the deep subsurface of the Earth also has important implications for the search for subsurface life on other worlds in our solar system.

Halicephalobus mephisto sp. nov.; Phylum Nematoda Potts, 1932; Cephalobina, Panagrolaimidae. One holotype and nine paratypes deposited in the collection at the Museum voor Dierkunde (collection number UGMD 104182), Ghent University, Belgium. Six paratypes deposited at the University of the Free State, Bloemfontein, Republic of South Africa (collection number UFS GB0035). Type population collected from Beatrix Au mine at 1.3 km depth, shaft 3, level 26, corridor 28 approximately 1 km north of shaft 3 (28°14' 24.06"S 26°47'45.25"E). Nematodes were collected from fracture

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water expelling from a high-pressure valve. Etymology - Mephisto ("he who loves not the light") refers to the devil, Lord of the underworld, in medieval mythology from the Faust legend since the new species is found at -1.3 km into the Earth's crust. Differential diagnosis - Although *Halicephalobus* is a morphologically minimalistic genus, *H. mephisto* is a new typological morphospecies as it can be easily differentiated from all other species of *Halicephalobus* by the presence of a long tail (110-130 µm; c': 9-10) with filiform terminus and the absence of reflexed ovary tip (Fig. 1). Phylogenetically, *H. mephisto* n. sp. has a maximally supported sister relationship with *H. gingivalis-Halicephalobus spp.*, differing by 10% from *H. gingivalis* and by 8% from other *Halicephalobus* species. In comparison, the most closely related different genus, *Procephalobus*, differs by 17% from *H. mephisto* n sp. Alternative alignment methods did not have a single effect on the tree topology outcome and resulted in a similar or increased branch support for the *H. gingivalis-Halicephalobus* spp. clade. The monophyly of *Halicephalobus* was always maximally supported, independently of the alignment method. Furthermore, 13 and 6 autapomorphic characters were present in the SSU rRNA sequences for the new species and its sister clade, respectively. Although based on limited available homologous sequences, multiple autapomorphic characters from two loci (18S and D2D3) and multiple autapomorphic characters in both sister lineages (18S) indicate lineage exclusivity for *H. mephisto* n. sp. with respect to other *Halicephalobus* species (Fig. 2). Hence, the species status of *H. mephisto* fulfills the requirements of an amalgamation of evolutionary and phylogenetic species concepts. Description - Body straight to slightly ventrally arcuate after fixation, 0.52 to 0.56 mm long with annulations. The tail is relatively long and the tail tip filiform, terminus straight to variably curved. The reproductive system was monodelphic, prodelphic, on right side of the intestine with posterior reflexed ovary extending 99 – 135 µm posterior vulva. Ovary tip is not reflexed back anteriorly. Temperature tolerant and parthenogenetic (See Supplementary Discussion for more detailed description).

Although eukaryotes, bacteria and archaea cohabitate in almost all surface environments on the Earth, very few searches for eukaryotes in the subsurface have been published. Sinclair and Ghiorse[5] discovered 0.1 - 10 eukaryotes g^{-1} comprised of algae, fungi, amoebae and flagellates at 200 m depth in South Carolina. Ekendahl et al.[6] found 0.01 - 1 fungal cells mL^{-1}, ~3 µm in size, in 200 - 450 m deep fractures in Sweden. In this study of the South African subsurface, we expanded the search for subsurface life to nematodes for the following reasons: 1) nematodes are one of the most successful metazoan phyla with respect to their abundances, distribution and physiological tolerance[7,8]; 2) nematodes can enter a state of anabiosis for extended periods; 3) nematodes continue to metabolize aerobically in hypoxic environments where the pO_2 is only 0.4 kPa[9].

The steps taken to determine whether the nematodes recovered were indigenous and not recent surface or mining contaminants were: 1) adaptation of filtration procedures that had been successfully used to collect planktonic microorganisms from thousands of liters of borehole water[10] (Supplementary Methods); 2) soil samples around the boreholes and the mining water were tested for nematodes; 3) the chemical composition and the microbial community structure of the fracture water were determined; 4) the ^{3}H and ^{14}C

concentrations were measured. Twenty-two water samples were collected from 6 boreholes ranging in depth from 0.5 to 3.6 km located in 5 different South African mines (Table 1) Between 475 to 9,792 L of water were filtered for each sample. Eighteen soil samples were collected, 6 of which were from Beatrix Au mine. Seven mining water samples were collected, 3 of which were from Beatrix Au mine and for which 2 to 31,582 L of water were filtered.

Beatrix Au Mine borehole water yielded a new species, *Halicephalobus mephisto* sp. n. Borehole water from Driefontein Au Mine yielded 2 nematode species, *Plectus aquatilis* and a monhysterid specimen that survived but did not reproduce. Borehole water from the shallowest site, Star Diamonds Mine and from Northam Pt Mine did not yield any nematodes, but a fourth nematode, a monhysterid species, was detected in DNA extracted from borehole water from the deepest site at Tau Tona Au Mine. All three living nematode species preferentially fed on the borehole water bacteria versus *Escherichia coli* (Supplementary Methods), and the two that were able to reproduce did so by parthenogenesis (Table 2). None of these species were found in more than one borehole. Although these three boreholes were sampled on multiple dates, nematodes were only recovered from the first sample (Table 1). No other metazoans were detected. Only two of the soil samples yielded nematodes, but only when the soil was wet, and all were taxonomically distinct from the three borehole nematodes (Table 1). None of the soil samples from Beatrix Au Mine yielded nematodes. Nematodes were also not detected in any mining water samples.

The Beatrix and Driefontein Au mine borehole valves were closed for at least several months to a year prior to sampling and the high water pressure upon opening the valve and flushing the filtration apparatus precluded contamination from air. The possibility of nematodes being contaminants from borehole drilling is unlikely because of the mining water used for drilling is treated with Na hypochlorite and H_2O_2 to the point that its DNA is highly degraded and because the high fracture water pressure encountered during drilling flushes out drilling water (Supplementary Discussion). Nonetheless we tested for this possibility at Beatrix Au Mine, where a 6,480 L borehole water sample yielded *H. mephisto*, by filtering 31,582 L of the mining water used for drilling from a valve close to the borehole and no nematodes were detected. We cannot preclude the possibility that nematodes were present in the mining water at the time the borehole was drilled a year prior to sampling, but this seems unlikely given the disinfection procedures are standard operating protocols. Because the nematodes were parthenogenetic, crossing with surface species was impossible, and nematode morphology and genetics cannot be used as indicators of long-term isolation[11,12]. Further evaluation of their indigeneity, therefore, relied upon environmental data.

The water sampled was hypoxic with dissolved O_2 concentrations ranging from 13 to 72 μM (Supplementary Tables). The Beatrix Au Mine borehole water yielded sulfate concentrations that were similar to that of the 3-5 myr old fracture water from 1.5 km depth[14] and was 100 times less than that of the mining water[13]. The geochemistry of the Driefontein Au Mine borehole water was consistent with groundwater from the karstic, sulfidic, Transvaal dolomite aquifer[13]. The Tau Tona Au Mine borehole water was

consistent with other highly saline fracture water from >3 km depth and distinct from the mining water and acid mine drainage water in this mining district[13,15]. The 16S rRNA gene clone libraries were comprised of sulfate reducing *Firmicutes* and δ *Proteobacteria*, heterotrophic *Proteobacteria* (fermenters and/or methanotrophs), *Nitrospira* and chemolithotrophic *Proteobacteria* that have been previously detected in the fracture water of these mines[15] (Supplementary Tables and Discussion). With the exception of the Driefontein borehole water, the ^{3}H concentrations were within 2 S.D. of the detection limit (Table 1). Compared to the 10-100 TR of regional precipitation for the late 80's[16], these values indicate that <1% of the borehole water is comprised of post-1980's surface water and no more than ~3% of the Driefontein borehole water could be modern. The $\Delta^{14}C$ values for the dissolved inorganic carbon ranged from -932.8 to -619.6 and using these values along with regional recharge values and corrections for dead carbon (Supplementary Tables and Discussion), the estimated ^{14}C ages ranged from ~2.9 to 12.1 kyr (Table 1).

The geochemical, isotopic and molecular data indicate that the nematode-bearing water represents paleometeoric, hypoxic water that contains a microbial assemblage comprised of both aerobic and anaerobic bacteria. The cultured nematodes preferentially fed upon these bacteria as opposed to *E. coli*. Nematodes were absent from the mining water, and when found twice in soils they were taxonomically distinct from those found in the paleometeoric water. No other metazoans were found in the paleometeoric water. The nematodes, therefore, do not appear to be contaminants from mining contamination or from modern water incursion, but appear to be indigenous to the paleometeoric water.

Can the subsurface microbial population density sustain nematodes for thousands of years? The ratio of planktonic microbial cells to nematodes was 10^{8} to 10^{10} (Table 1) greatly exceeding the 10 to 100 microbial cells to protists ratios reported for other terrestrial subsurface environments[6]. More likely the nematodes are grazing on the ~$5x10^{4}$ cells cm^{-2} patches of bacteria attached to the fracture surfaces[17]. This density corresponds to a sessile microbial concentration 100 times that of the planktonic cells making the ratio of microbial cells to nematodes ~10^{10} to 10^{12}. Given the $2.6x10^{-8}$ g dry weight for *H. mephisto* (Supplementary Discussion), measured bactivory rates of 6.6 to 15.2 $x10^{5}$ bacterial cells (mm of nematode)$^{-1}$ day^{-1} and respiration/total C consumption ratios of 0.16 to 0.72[18] for nematodes, ~10^{4} bacterial cells could readily sustain *H. mephisto* for one day and 10^{11} for ~30 kyr. The association of nematodes with biofilms may explain why they were detected only when the boreholes were first opened and not in subsequent samples. The initial release of high-pressure water would have dislodged biofilms and nematodes from the fracture surfaces and some time would be required after the borehole was sealed again for the biofilm community to reform. This lack of reproducibility in eukaryote presence was also observed for subsurface fungi[6].

Is O_2 limiting? Water samples yielded 13 to 72 μM O_2 concentrations (equivalent to 1.3 - 6.8 kPa of O_2 in Table 1), values that can sustain the maximum metabolic rate of $3x10^{-4}$ mols of O_2 (gm of nematode)$^{-1}$ hr^{-1} for *Caenorhabditis elegans*[19,20]. If the paleometeoric water had an O_2 concentration of ~350 μM upon recharge, then the ^{14}C ages constrain the O_2 consumption rate to be ~3-$8x10^{-8}$ M yr^{-1}, values which overlap those reported for the

Middendorf aquifer[21]. Given *H. mephisto*'s mass, its maximum metabolic rate would be ~$7x10^{-8}$ mols of O_2 nematode^{-1} yr^{-1}. The observed O_2 consumption rate could support ~1 nematode L^{-1} at its maximum metabolic rate compared to the 1 nematode per 10^4 liters detected. Even at 4 μM O_2 nematodes metabolize aerobically[9], but at much slower rates[19].

Temperature imposes a limit to the depth that nematodes could live. The temperature of Beatrix BH2 is higher than most terrestrial nematodes can tolerate, but *H. mephisto* exhibited a high temperature tolerance (Table 2) like that of the opportunistic pathogen *H. gingivalis*. The detection of the monhysterid species in the 48°C Tau Tona borehole is significantly higher than a reported occurrence of this order in a 43°C hot spring[22], but comparable to the 51 to 61°C, hot spring nematode occurrences[23,24,25].

Our data suggest that the interactions between meiofaunal communities and chemolithotrophic biofilms found in hypoxic, sulfide, cave systems, e.g. Movile Cave in Romania[26], extend to greater depths and smaller confines and could control the size and turnover rate of subsurface microbial communities. Given their abundance on the sea floor and around hydrothermal vents nematodes should also be found beneath the seafloor in the sediments or in mid-ocean ridge basalt[27]. Other meiofauna, such as the Loricefera inhabiting the deep anoxic hypersaline L'Atalante basin[28] could also be present. The ability of multi-cellular organisms to survive in the subsurface should be considered in the evolution of Eukaryotes and the search for life on Mars.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions [§]AGM, DL and WB all contributed equally to this study. GB, AGM, DL, AB and ME collected the filtered samples, the control samples and performed field analyses. GB carried out the enrichments. AGM performed microbial DNA extraction and 16S rRNA amplification, sequencing and tree construction. WB contributed the nematode identification, their morphological description and their molecular analyses. TCO modeled the geochemical, ^{3}H and ^{14}C data. GB wrote the paper with input from WB, AGM, TCO and EvH. We are grateful to E. Botes and K. Albertyn of the Univ. of Free State for their contributions to the data analysis.

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Table 1. Geochemical, isotopic and nematode results

	Star Diamonds	Driefontein	Beatrix BH1	Beatrix BH2	Northam	Tau Tona
Depth (km)	0.5	0.9	1.3	1.3	1.7	3.6
T°C	32	24	30	37	48	48
pH	8.3	7.5	7.7	7.9	8.3	7.7
pO_2(kPa)[1]	4.6	2.5	3.1	1.3-6.8	2.4	2.9
microbial counts (cells L^{-1})	NA[2]	$<10^5$	NA	$3x10^6$	$2x10^5$	$3.4x10^6$
samples with nematodes/total samples	0/2	1/3[3]	0/6	1/6	0/1	1/4
Nematodes/L	NA	$\sim 7x10^{-5}$	NA	$\sim 3x10^{-5}$	$<10^{-4}$	$>\sim 5x10^{-4}$
^3H (TR[4])	NA	0.270±0.026	NA	0.014±0.024	0.062±0.045	0.034±0.021
$\delta^{13}C$ ($^o/_{oo}$ VPDB[5])	NA	-8	NA	-32	-17.4	-17.7
$\Delta^{14}C$	NA	-932.8±1.0	NA	-704.1±2.2	-645.9±2.4	-619.6±2.9
^{14}C age (yr)	NA	10,104-12,084	NA	4,413-6,247	5,798	2,919-5,165
H. mephisto	-	-	-	+	-	-
P. aquatilis	-	+	-	-	-	-
monhysterid sp1	-	+	-	-	-	-
monhysterid sp2	-	-	-	-	-	+[6]
			Control Samples			
Soil[7]	dorylaimids, mononchids and Annelids	*Diploscapter coronatus* and *Rhabditis regenfussi*	-	-	-	-
Mining water[8]	-	-	-	-	-	-

[1] pO_2 (kPa) = 101.325 x $[O_2]$ x $1.8x10^4/K_H$, where K_H is Henry's solubility constant (moles of O_2/moles of H_2O-atm) = EXP{[-286.942+15450.6/T°K+36.5593Ln(T°K)+ 0.0187662T°K] /1.987} and $[O_2]$ is the dissolved O_2 concentration in µM.

[2] NA = not analyzed.

[3] One sample yielded two nematodes of two separate species. In the case of Beatrix, only one nematode was found.

[4] TR = Tritium units = 1 ^3H per 10^{18} H atoms.

[5] VPDB = Vienna Pee Dee *Belemnitella americana*.

[6] Samples did not yield a cultivable nematode, but the DNA from the fracture yielded an 18S rRNA gene sequence belonging to *Monhysteridae*.

[7] Approximately 900 grams of soil were plated and in the cases where the soil was dry, an additional 900 grams was wetted and plated. The total number of nematodes was 17 for Star Diamonds and 18 for Driefontein.

[8] Two to six liters of mining water were filtered for metazoans with the exception of Beatrix Au Mine where 31,582 liters of mining water was filtered (Supplementary Tables and Discussion).

Table 2. Nematode characteristics

Species	Maximum growth temperature (°C)	reproduction	feeding type
H. mephisto	41	parthenogenetic	bacteriophagous
P. aquatilis	31	parthenogenetic	bacteriophagous
monhysterid sp1	unknown	sexual?	bacteriophagous
monhysterid sp2	unknown	unknown	bacteriophagous[1]

[1] *Monhysteridae* are classified as bacterial feeding and/or substrate ingestion based upon their buccal morphology[29]. Substrate ingestion is theoretically identical to bacterial feeding, predation and unicellular eukaryote feeding in many groups, because more than a pure food source is ingested.

Figure 1. General morphology of *Halicephalobus mephisto* n. sp. LM drawings of female holotype and SEM photograph of head. A: Entire body; B: Neck region; C: anterior region; D: SEM *en face* view; E: reproductive system; F: tail.

Figure 2. Bayesian interference 50% majority rule consensus phylogenies based on SSU rDNA data. *Halicephalobus mephisto* with GenBank sequences of closely related taxa. Branch support is indicated with Posterior Probability values. Scale bar indicates expected substitutions per site.