LETTER

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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 over three kilometres into the Earth's crust and comprising a significant fraction of the global biosphere[1–4]. The constraints of temperature, energy, dioxygen and space seemed to preclude the possibility of more-complex, multicellular 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-kilometre-deep fracture water 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. Carbon-14 data indicate that the fracture water in which the nematodes reside is 3,000–12,000-year-old palaeometeoric 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 on 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 multicellular life in the deep subsurface of the Earth also has important implications for the search for subsurface life on other planets in our Solar System.

Phylum Nematoda Potts, 1932
Suborder Cephalobina
Superfamily Panagrolaimidae
Halicephalobus mephisto sp. nov.

Etymology. *Mephisto* (from *Mephistopheles*, pseudo-Greek): "he who loves not the light", alluding to the Devil, Lord of the Underworld, in reference to the Faust legend in medieval mythology because the new species is found at a depth of 1.3 km in the Earth's crust.

Holotype. Museum voor Dierkunde, Ghent University, Belgium (collection number, UGMD 104182).

Paratype. Museum voor Dierkunde, Ghent University, Belgium (collection number, UGMD 104182) (nine paratypes); University of the Free State, Bloemfontein, South Africa (collection number, UFS GB0035) (six paratypes).

Locality. Type population collected from shaft 3, level 26, corridor 28 of Beatrix gold mine, South Africa, at a depth of 1.3 km, approximately 1 km north of shaft 3 (28° 14′ 24.06′′ S, 26° 47′ 45.25′′ E). Nematodes collected from fracture water expelled from a high-pressure valve.

Diagnosis. Body straight to slightly ventrally arcuate after fixation, 0.52–0.56 mm long with annulations. Tail relatively long and tail tip filiform; terminus straight to variably curved. Reproductive system monodelphic, prodelphic and on right side of intestine with posterior reflexed ovary extending 99–135 μm posterior to vulva. Ovary tip not reflexed back anteriorly. Temperature tolerant and parthenogenetic. See Supplementary Discussion and Description for more details.

Description. 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; ratio of total length to body diameter at anus, $c' = 9–10$) with a filiform terminus and no reflexed ovary tip (Fig. 1). Phylogenetically, *H. mephisto* has a maximally supported sister relationship with *Halicephalobus 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*. 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, independent of the alignment method. Furthermore, 13 and 6 autapomorphic characters were present in the small-subunit ribosomal RNA 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* with respect to other *Halicephalobus* species (Fig. 2). Hence, the species status of *H. mephisto* fulfils the requirements of an amalgamation of evolutionary and phylogenetic species concepts.

Although Eukaryota, Bacteria and Archaea cohabitate in almost all surface environments on the Earth, very few searches for eukaryotes in the subsurface have been published. In South Carolina 0.1–10 eukaryotes per gram comprising algae, fungi, amoebae and flagellates have been discovered[5] at a depth of 200 m, and in Sweden 0.01–1 fungal cells per millilitre, ~3 μm in size, have been found[6] in 200–450-m deep fractures. In this study of the South African subsurface, we expanded the search for subsurface life to nematodes because they are one of the most successful metazoan phyla with respect to their abundances, distribution and physiological tolerance[7,8]; they can enter a state of anabiosis for extended periods; and they continue to metabolize aerobically in hypoxic environments where the partial pressure of oxygen (p_{O_2}) is only 0.4 kPa (ref. 9).

We took the following steps to determine whether the nematodes recovered were indigenous and not recent surface or mining contaminants: adaptation of filtration procedures that had been successfully used to collect planktonic microorganisms from thousands of litres of borehole water[10] (Supplementary Methods); soil samples around the boreholes and the mining water were tested for nematodes; the chemical composition and the microbial community structure of the fracture water were determined; and the ^{3}H and ^{14}C concentrations were measured. Twenty-two water samples were collected from six boreholes ranging in depth from 0.5 to 3.6 km and located in five different South African mines (Table 1). Between 475 and 9,792 l of water were filtered for each sample. Eighteen soil samples were collected, six of which were from Beatrix gold mine. Seven mining water samples were collected, three of which were from Beatrix gold mine and for which 2 to 31,582 l of water were filtered.

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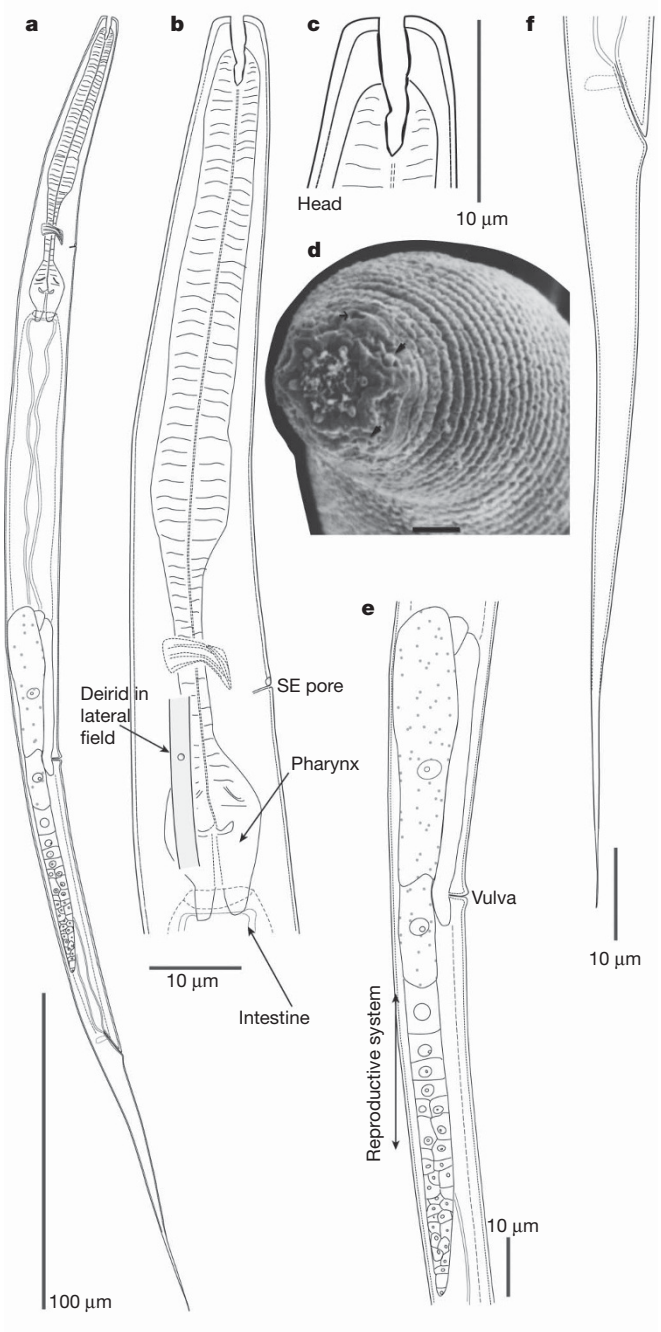

Figure 1 | General morphology of *H. mephisto*. Light microscopy drawings of female holotype and scanning electron microscopy photograph of head. **a**, Entire body; **b**, neck region; **c**, anterior region; **d**, scanning electron microscope face view (scale bar, 1 µm; black arrowheads indicate the positions of two cephalic papillae; black arrow indicates amphid opening); **e**, reproductive system; **f**, tail. SE, secretory–excretory.

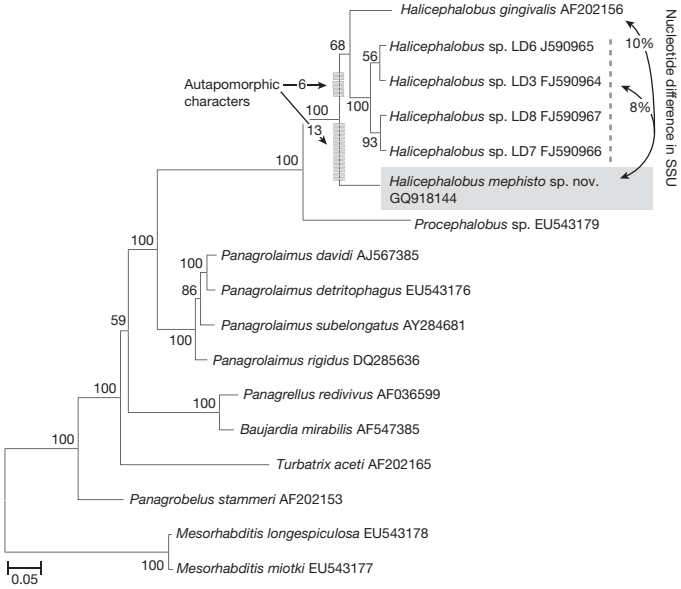

Figure 2 | Bayesian-interference 50%-majority-rule consensus phylogenies based on small-subunit rDNA data. *H. mephisto* with GenBank sequences of closely related taxa. Branch support is indicated with posterior probability values. Scale bar, expected substitutions per site. SSU, small subunit.

Borehole water from Beatrix gold mine yielded the new species, *H. mephisto*. Borehole water from Driefontein gold mine yielded two 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 Zondereinde platinum 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 gold mine. All three living nematode species fed on the borehole water bacteria in preference to *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 gold mine yielded nematodes. Nematodes also were not detected in any mining water samples.

The borehole valves at the Beatrix and Driefontein gold mines were closed for at least several months to a year before sampling and the high water pressure on opening the valves and flushing the filtration apparatus precluded contamination from air. The possibility of nematodes being contaminants from borehole drilling is unlikely because the mining water used for drilling is treated with NaClO and H_2O_2 to the point that nematode DNA is highly degraded and because the high pressure of fracture water encountered during drilling flushes out drilling water (Supplementary Discussion). Nonetheless, we tested for this possibility at Beatrix gold 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: 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 before sampling, but this seems unlikely given that 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 borehole water from Beatrix gold mine yielded sulphate concentrations that were similar to that of the 3–5-Myr-old fracture water from a depth of 1.5 km (ref. 13) and were 100 times less than that of the mining water[14]. The geochemistry of the borehole water from Driefontein gold mine was consistent with groundwater from the karstic, sulphidic Transvaal dolomite aquifer[14]. The borehole water from Tau Tona gold mine was consistent with other highly saline fracture water from depths of >3 km and was distinct from the mining water and acidic mine drainage water in this mining district[14,15]. The 16S rRNA gene clone libraries comprised sulphate-reducing Firmicutes and Deltaproteobacteria, heterotrophic Proteobacteria (fermenters and/or methanotrophs), Nitrospira and chemolithotrophic Proteobacteria that have

Table 1 | Geochemical, isotopic and nematode results

	Star Diamonds	Driefontein	Beatrix		Zondereinde	Tau Tona
			Borehole 1	Borehole 2		
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
p_{O_2} (kPa)*	4.6	2.5	3.1	1.3–6.8	2.4	2.9
Microbial counts (cells per litre)	NA	$<10^5$	NA	3×10^6	2×10^5	3.4×10^6
Samples with nematodes/total samples	0/2	1/3†	0/6	1/6	0/1	1/4
Nematodes (number per litre)	NA	$\sim7\times10^{-5}$	NA	$\sim3\times10^{-5}$	$<10^{-4}$	$>\sim5\times10^{-4}$
^{3}H (TR)	NA	0.270 ± 0.026	NA	0.014 ± 0.024	0.062 ± 0.045	0.034 ± 0.021
δ^{13}C (‰ VPDB)‡	NA	−8	NA	−32	−17.4	−17.7
Δ^{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 sp. 1	−	+	−	−	−	−
Monhysterid sp. 2	−	−	−	−	−	+‖
Control samples						
Soil¶	Dorylaimids, mononchids and annelids	*Diploscapter coronatus* and *Rhabditis regenfussi*	−	−	−	−
Mining water#	−	−	−	−	−	−

NA, not analysed; TR, tritium units (1 ^{3}H per 10^{18} H atoms).

* p_{O_2} (kPa) = $101.325\times[O_2]\times1.8\times10^4/K_H$, where K_H = exp{[−286.942 + 15,450.6/T(K) + 36.5593ln(T(K)) + 0.0187662T(K)]/1.987} is Henry's solubility constant (moles of O_2 per moles of H_2O at the partial pressure of O_2 in atmosphere) and $[O_2]$ is the dissolved O_2 concentration in micromolars.

† One sample yielded two nematodes of two separate species. In the case of Beatrix gold mine, only one nematode was found.

‡ $\delta^{13}C = (^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1$; standard used is Vienna PeeDee *Belemnitella americana* (VPDB).

§ $\Delta^{14}C = 10\times{}^{14}C_{PMC} - 1{,}000$; $^{14}C_{PMC}$ is the carbon activity as per cent modern carbon.

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

¶ Approximately 900 g of soil was plated, and in the cases where the soil was dry an additional 900 g was wetted and plated. The total number of nematodes was 17 for Star Diamonds mine and 18 for Driefontein gold mine.

Two to six litres of mining water was filtered for metazoans except at Beatrix gold mine, where 31,582 litres of mining water was filtered (Supplementary Tables and Supplementary Discussion).

been previously detected in the fracture water of these mines[15] (Supplementary Tables and Supplementary Discussion). With the exception of the Driefontein borehole water, the ^{3}H concentrations were within 2 s.d. of the detection limit (Table 1). In comparison with the concentration, 10–100 TR, in regional precipitation during the late 1980s[16], these values indicate that <1% of the borehole water comprises post-1980s surface water and that no more than ~3% of the Driefontein borehole water can be modern. The Δ^{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 Supplementary Discussion), the estimated ^{14}C ages ranged from about 2,900 to 12,100 years (Table 1).

The geochemical, isotopic and molecular data indicate that the nematode-bearing water represents palaeometeoric, hypoxic water that contains a microbial assemblage comprising both aerobic and anaerobic bacteria. The cultured nematodes preferentially fed on 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 palaeometeoric water. No other metazoans were found in the palaeometeoric water. The nematodes, therefore, do not seem to be contaminants due to mining or incursion of modern water, but seem to be indigenous to the palaeometeoric water.

The subsurface microbial population density can sustain nematodes for thousands of years. The ratio of planktonic microbial cells to nematodes was 10^8–10^{10}:1 (Table 1), greatly exceeding the 10–100:1 ratio of microbial cells to protists reported for other terrestrial subsurface environments[6]. Rather than feeding on planktonic cells, the nematodes are more likely to graze on the patches of bacteria ($\sim5\times10^4$ cells per square centimetre) 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 $\sim10^{10}$–10^{12}:1. Given the dry weight of *H. mephisto*, 2.6×10^{-8} g (Supplementary Discussion), measured bacterivory rates of 6.6×10^5 to 15.2×10^5 bacterial cells per millimetre of nematode per day and respiration/total carbon consumption ratios of 0.16 to 0.72 (ref. 18) for nematodes, $\sim10^4$ bacterial cells could readily sustain *H. mephisto* for one day and 10^{11} for about 30,000 years. 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 for the biofilm community to reform after the borehole was sealed again. This lack of reproducibility in eukaryote presence was also observed for subsurface fungi[6].

Table 2 | Nematode characteristics

Species	Maximum growth temperature (°C)	Reproduction	Feeding type
H. mephisto	41	Parthenogenetic	Bacteriophagous
P. aquatilis	31	Parthenogenetic	Bacteriophagous
Monhysterid sp. 1	Unknown	Sexual?	Bacteriophagous
Monhysterid sp. 2	Unknown	Unknown	Bacteriophagous*

* Monhysteridae are classified as 'bacterial feeding' and/or 'substrate ingestion' on the basis of 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.

Dioxygen is not a limiting factor. Water samples yielded O_2 concentrations of 13 to 72 μM (equivalent to 1.3 to 6.8 kPa of O_2 in Table 1), values that can sustain the maximum metabolic rate of 3×10^{-4} moles of O_2 per gram of nematode per hour for *Caenorhabditis elegans*[19,20]. If the palaeometeoric water had an O_2 concentration of ~350 μM on recharge, then the ^{14}C ages constrain the O_2 consumption rate to be $\sim3\times10^{-8}$ to 8×10^{-8} M yr^{-1}, values which overlap those reported for the Middendorf aquifer[21]. Given the mass of *H. mephisto*, its maximum metabolic rate would be $\sim7\times10^{-8}$ moles of O_2 per nematode per year. The observed O_2 consumption rate could support ~1 nematode per litre at its maximum metabolic rate, compared to the 1 nematode per 10^4 litres detected. Even at an O_2 concentration of 4 μM, nematodes metabolize aerobically[9], but at much slower rates[19].

Temperature imposes a limit to the depth at which nematodes could live. The temperature of Beatrix borehole 2 is higher than most terrestrial nematodes can tolerate, but *H. mephisto* showed a high temperature tolerance (Table 2) like that of the opportunistic pathogen *Halicephalobus gingivalis*. The detection of the monhysterid species in the Tau Tona borehole at 48 °C is significantly higher than a reported occurrence of this species in a hot spring at 43 °C (ref. 22), but is

comparable to the occurrences of nematodes in hot springs at 51 to 61 °C (refs 23–25).

Our data suggest that the interactions between meiofaunal communities and chemolithotrophic biofilms found in hypoxic, sulphide cave systems, for example 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 sea floor in the sediments or in mid-ocean-ridge basalt[27]. Other meiofauna, such as the Loricifera inhabiting the deep, anoxic, hypersaline L'Atalante basin[28], could also be present. The ability of multicellular 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 A.G.-M., D.L. and W.B. all contributed equally to this study. G.B., A.G.-M., D.L., A.B. and M.E. collected the filtered samples and the control samples and performed field analyses. G.B. carried out the enrichments. A.G.-M. performed microbial DNA extraction and 16S rRNA amplification, sequencing and tree construction. C.M. performed DNA analyses on filters of mining water. W.B. provided the nematode identification, their morphological description and their molecular analyses. T.C.O. modelled the geochemical, ^{3}H and ^{14}C data. G.B. wrote the paper with input from W.B., A.G.-M., T.C.O. and E.v.H.

Author Information Sequence information for *H. mephisto* has been deposited at GenBank under accession number GQ918144. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to G.B. (gborgonie@gmail.com) or T.C.O. (tullis@princeton.edu).