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**Caenorhabditis monodelphis** sp. n.: defining the stem morphology and genomics of the genus Caenorhabditis

Dieter Slos<sup>1</sup>*, Walter Sudhaus<sup>2</sup>, Lewis Stevens<sup>3</sup>*, Wim Bert<sup>1</sup> and Mark Blaxter<sup>3</sup>

**Abstract**

**Background:** The genus *Caenorhabditis* has been central to our understanding of metazoan biology. The best-known species, *Caenorhabditis elegans*, is but one member of a genus with around 50 known species, and knowledge of these species will place the singular example of *C. elegans* in a rich phylogenetic context. How did the model come to be as it is today, and what are the dynamics of change in the genus?

**Results:** As part of this effort to “put *C. elegans* in its place”, we here describe the morphology and genome of *Caenorhabditis monodelphis* sp. n., previously known as *Caenorhabditis* sp. 1. Like many other *Caenorhabditis*, *C. monodelphis* sp. n. has a phoretic association with a transport host, in this case with the fungivorous beetle *Cis castaneus*. Using genomic data, we place *C. monodelphis* sp. n. as sister to all other *Caenorhabditis* for which genome data are available. Using this genome phylogeny, we reconstruct the stem species morphological pattern of *Caenorhabditis*.

**Conclusions:** With the morphological and genomic description of *C. monodelphis* sp. n., another key species for evolutionary and developmental studies within *Caenorhabditis* becomes available. The most important characters are its early diverging position, unique morphology for the genus and its similarities with the hypothetical ancestor of *Caenorhabditis*.

**Keywords:** Taxonomy, Systematics, Evolution, Genome, Phylogeny, Description

**Background**

The nematode genus *Caenorhabditis* includes the well-known model organism *C. elegans*, which has provided key insights into molecular and developmental biology [1]. Over the past ten years, numerous new *Caenorhabditis* species have been discovered and described [2, 3]. These putative new taxa are generally indistinguishable morphologically, and thus the most recent descriptions of new species within *Caenorhabditis* have been based on DNA sequences and mating tests only [2]. This streamlined species-description methodology has been driven by the need to have names to attach to real biological entities, and the fact that traditional taxonomy has been unable to keep up with species discovery. The method is relatively simple to implement, and delivers taxa that have a biological reality [2]. However, as the number of species discovered in *Caenorhabditis* grows, traditional, morphological descriptions are still valuable for the understanding of patterns of trait evolution and inference of ecological functions [4, 5]. Although morphology cannot be used to definitively delineate species, it should not be abandoned all together.

M-A Félix, C Braendle and AD Cutter [2] provided new species name designations for 15 biological species, considerably increasing the number of named *Caenorhabditis* species in laboratory culture. However, several key *Caenorhabditis* species remain undescribed. A well-known but undescribed species of *Caenorhabditis*, informally referred to as *Caenorhabditis* sp. 1, has been analysed in several evolutionary and developmental studies [3, 6–8]. *C. sp. 1* was previously found only once inside a fruiting body of the fungus *Ganoderma applanatum* (Pers.) Pat.
(Polyporaceae), growing on the stump of tree in Berlin, Germany. Galleries inside the fungus were frequently visited by beetles of the species *Cis castaneus* (Ciidae), a beetle with a host preference for *Ganoderma* [9]. Associations between nematodes and insects, where the nematode uses the insect as a transport carrier (phoretism), have already been described for several *Caenorhabditis* species, including *Caenorhabditis angaria*, *C. remanei*, and *C. bovis*, and similar phoretic associations could be expected for many or possibly all other *Caenorhabditis* species [10].

Here we use both morphological and molecular analyses to characterise and describe *C. sp. 1* as a new species, *Caenorhabditis monodelphis* sp. n., and explore its relationship with the beetle *Cis castaneus*. Molecular phylogenetic analysis based on whole genome sequencing of an inbred derivative of the type strain affirms the placement of *C. monodelphis* sp. n. as sister to other analysed *Caenorhabditis*, and we analyse the evolution of phenotypic traits to infer those present in the hypothetical ancestor of *Caenorhabditis*.

**Methods**

**Isolation and culture**

*Caenorhabditis monodelphis* sp. n. (strain SB341) was originally isolated from fruiting bodies of *Ganoderma applanatum* (Pers.) Pat. 1887 collected in Berlin-Grunewald, Germany (April, 2001) and later from four locations in Belgium (strain DSC001 collected from 51°06′24″N, 3°18′13″E, March 2014, strain DSC002 collected from 50°52′7″N, 4°06′54″, February 2014, and an uncultured population 51°02′41″N, 3°27′17″ June 2014) and from one location in the Botanical Garden in Oslo, Norway (strain JU2884; 59°55′04″N 10°46′01″E, 22 July 2015). These collections were from the same mushroom species. We also found *C. monodelphis* sp. n. in the fruiting body of *Fomes fomentarius* (L.) Fr. 1849 (50°43′02″N, 4°05′06″E, February 2015). Strain SB341 was chosen as type.

Nematodes were extracted from the fruiting bodies of *G. applanatum* using the modified Baermann method [11]. Dauer larvae were isolated from the beetle *Cis castaneus* (Herbst, 1793) that had been extracted from the same mushroom from multiple locations (except the type population and from 51°02′41″N, 3°27′17″). Adults and dauer larvae were picked out and cultured on nutrient agar plates seeded with *E. coli* OP50 at 15 °C.

**Morphological characterisation**

Cultures of nematodes from two populations (strain SB341 and DSC001) were used for the description. Measurements and drawings were made with an Olympus BX51 equipped with differential interference contrast (DIC). Light microscopic images were taken with a Nikon DS-FI2 camera. For Scanning Electron Microscopy (SEM), two fixation methods were used. For the first fixation method, live animals were fixed in a microwave in Trump's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in a 0.1 M Sorensen buffer) for a few seconds. Specimens were subsequently washed three times in double-distilled water. For the second method, specimens were put in a refrigerator at 4 °C for 1 h, then Trump's fixative was added and specimens were left overnight at 4 °C. The specimens were then washed with a 0.2 M phosphate buffer followed by 1 h post-fixation in a 1% OsO4 solution at room temperature and subsequently washed 4 times in double-distilled water. For both methods, the specimens were dehydrated by passing them through a graded ethanol concentration series of 30, 50, 75, 95% (20 min each) and 3x 100% (10 min each). The specimens were critical point-dried with liquid CO2, mounted on stubs with carbon discs and coated with gold (25 nm) before observation with a JSM-840 EM (JEOL, Tokyo, Japan) at 15 kV. Sperm cells were observed in the female post-uterine sac with Transmission Electron Microscopy (TEM), processing samples as described [12], except for ultramicrotomy with a Leica EM UC7 and 1 h 1% osmium post-fixation (Slos et al. unpublished).

**Molecular characterisation**

For DNA barcoding analyses, temporary slides of individual nematodes were made in tap water and digital light microscope pictures were taken as a morphological voucher. The nematode was then transferred to a PCR tube with a solution containing 10 µl 0.05 M NaOH and 1 µl Tween20, heated for 15 min at 95 °C, and 40 µl of double-distilled water was added. PCR was carried out targeting either the 28S (large subunit) ribosomal RNA gene (nLSU) or the ribosomal internal transcribed spacer 2 (ITS2) locus, and PCR products were cleaned and sequenced directly. Forward and reverse primers for the nLSU were D2Ab (ACAAGTACCGTGAGGGAAAGTTG) and D3b (TCGGAAGGAACCAGCTACTA). For ITS2 we used VRAIN2F (CTTTGTACACACCGCCCGTCGCT) and VRAIN2R (TTCACACTCGCCGTACTAAGG GAATC). The sequences obtained were 100% identical to published sequences for *Caenorhabditis* sp. 1 [3].

**Genome sequencing**

Genomic DNA was extracted from an inbred strain, JU1667, of *C. monodelphis* sp. n. (derived from strain SB341), maintained on *E. coli* OP50, using the proteinase K-spin column protocol (detailed in Additional file 1). Total RNA from the same culture was also extracted (methods detailed in Additional file 1). Two paired-end genomic libraries (insert sizes of 300 bp and 600 bp, respectively) and a single paired-end RNA-seq library (insert size 180 bp) were constructed using TruSeq reagents and sequenced on the Illumina HiSeq 2000 by Edinburgh Genomics. We obtained 124.3 million genomic read pairs (100 base, paired end) and 46.2 million pairs of RNA-Seq reads (also 100 base, paired end).
De novo genome assembly and gene prediction
Details of software versions and parameters are available (see Additional file 2). We performed initial quality control of our genomic sequence data using FastQC [13] and used Skewer [14] to remove low quality (Phred score < 30) and adapter sequence. Using bloattools [15], we generated taxon-annotated GC-coverage (TAGC) plots to identify and remove bacterial contamination. Sequence data were assembled with CLC assembler (CLCBio, Copenhagen, Denmark) and reads mapped back to this assembly using CLC mapper. Each assembly contig was compared to the NCBI Nucleotide (nt) database using megablast from the NCBI BLAST+ suite [16]. Genomic read pairs were aligned to genome references from five E. coli (strains: BL21 (DE3), ETEC H10407, K12 substr. DH10B, K-12 substr. MC4100 and B str. REL606) using Bowtie [17], and aligned pairs discarded. We identified laboratory-induced contamination with Caenorhabditis elegans in the 600 bp insert library data. To remove this, we aligned read pairs of the uncontaminated 300 bp-insert library to the C. elegans N2 reference genome. Regions of similarity between the genomes of Caenorhabditis monodelphis sp. n. and C. elegans (i.e. those regions of C. elegans with aligned C. monodelphis sp. n. reads) were masked with Ns using BEDtools [18]. Read pairs of the 600 bp-insert library were subsequently aligned to this masked C. elegans reference and any aligned read pairs discarded.

Cleaned sequence data were assembled with ABySS [19] (k = 83) and contigs were scaffolded with transcript evidence using SCUBAT [20]. RepeatModeler [21] was used to identify repetitive regions which were then masked using RepeatMasker [22]. RNA-Seq read pairs were aligned to the assembly using STAR [23], and the resulting BAM file was used to guide the prediction of protein-coding genes by BRAKER [24].

Gene structure comparisons
Genome sequences and annotation GFFs were downloaded from WormBase [25] and imported into a custom Ensembl database (version 84) [26]. Using the Ensembl Perl API, the canonical transcript from each protein-coding gene was identified and exon and intron statistics were calculated. To compare the gene structures of C. monodelphis sp. n. with that of C. elegans, we identified all orthologous clusters (details below) in which C. monodelphis sp. n. and C. elegans proteins were present as single-copy. Exon and intron statistics were calculated for each gene pair, as described previously. Plots were generated using the ggplot2 package [27] and GenePainter [28].

Phylogenetic analyses
Pairwise comparisons of protein sequences derived from genomic data for 23 species of Caenorhabditis and two outgroup species, Oscheius tipulae and Heterorhabditis bacteriophora, (see Additional file 3 for details) were performed using NCBI BLAST+ [16] and clustered into orthologous groups using OrthoFinder [29]. The sequences of 303 one-to-one orthologues (allowing for up to two species to have missing data) were extracted and aligned using ClustalOmega [30]. Poorly aligned regions were removed from the alignments using trimAL [31] and trimmed alignments concatenated using FASConCAT [32] to yield a supermatrix. We performed maximum-likelihood (ML) analysis using RAxML [33] (PROTGTR + Γ substitution model) with 1,000 bootstrap replicates. Bayesian analysis was performed using PhyloBayes [34] (CAT-GTR), with two independent Markov chains, and convergence was assessed using Tracer [35].

Nomenclatural acts
This published work and the nomenclatural acts it contains have been registered in Zoobank: http://zoobank.org/urn:lsid:zoobank.org:pub:0E6F137B-9975-4A8E-91F2-D588A572076E. The LSID for this publication is: urn:lsid:zoobank.org:pub:0E6F137B-9975-4A8E-91F2-D588A572076E.

Results
Here we provide a formal description of SB341 as the type strain of C. monodelphis sp. n.

Caenorhabditis monodelphis sp. n. Slos & Sudhaus

= Caenorhabditis sp. SB341 [7]
= Caenorhabditis sp. SB341 and Caenorhabditis sp. n. SB341 [36]
= Caenorhabditis sp. n. 1 (SB341) and (lapse)
Caenorhabditis sp. n. 4 (SB341) [10]
= Caenorhabditis sp. 1 SB341 [6, 8, 37]
= Caenorhabditis sp. 4 SB341 [38]

(Figs. 1, 2, 3 and 4; Table 1)

Adult
Small species (female 0.72 - 1.04 mm, male 0.65 – 0.77 mm); cuticle thin, ca. 1 μm wide and finely annulated, 0.8 μm wide at midbody. Lateral field inconspicuous, about 9% of body width, consisting one ridge that can be traced anteriorly to the level of the median bulb and posteriorly at level of rectum in females and about 1½ spicules length anterior of the cloacal aperture in males. Six lips slightly protruding, each with one apical papilliform labial sensillum and a second circle of four sublateral cephalic sensilla in both sexes; amphids opening on the lateral lips, hardly discernible. Buccal tube long and slender, more than twice the width in lip region, pharyngeal sleeve envelopes nearly half of the stoma, the anterior as well as the posterior end of the tube appear slightly thickened, cheilostom inconspicuous, arcade cells forming the
gymnostom sometimes visible; glottoid apparatus completely absent. Pharynx with a prominent median bulb, diameter more than 90% of diameter of terminal bulb; terminal bulb pyriform, with double chambered haustrulum, the anterior chamber smallish; cardia conspicuous, opens funnel-like in intestine. Nerve ring encircles isthmus in its anterior part in living specimens, more to the middle of the isthmus in heat relaxed or preserved specimens; deirids usually conspicuous in the lateral field at level of beginning of terminal bulb, sometimes not visible in heat relaxed animals; pore of excretory-secretory system hard to discern posterior of deirid level. Two gland cells ventral and slightly posterior of terminal bulb conspicuous in live specimens. Lateral canals visible in live specimens extending anteriorly to two stoma length from the anterior end and ending at rectum level in the female. Postdeirids usually very conspicuous dorsally of the lateral field at about 75% of body length in both
sexes and about half the length between vulva and beginning of rectum (or at level of posterior end of uterus remnant) in females, sometimes not visible in heat relaxed specimens.

Female
Maximum body diameter clearly anterior of the vulva, vulva position 65% body length, a transverse slit, bordered in both ends by cuticular longitudinal flaps, vulva lips moderately protruding, four diagonal vulval muscles conspicuous; one pseudocoelomocyte exists anterior of gonad flexure ventrally. Genital tracts asymmetrical; posterior branch rudimentary, sac like, on the left hand side of intestine, without flexure, almost as long as body diameter at the level of the vulva, containing spermatozoa (Fig. 2); anterior branch right of intestine, reflexed dorsally close to the pharynx, flexure more than half the length of the gonad (measured from vulva to flexure); at the flexure oocytes in several rows, downstream in one row, oocytes predominantly growing in the last position, where granules are stored inside; sphincter between oviduct and uterus, only a few sperm cells in oviduct, most of them in uterus and blind sac; oviparous, one egg at a time in uterus (rarely two), segmentation starts in the uterus. Rectum a little S-shaped, rectal gland cells very small, posterior anal lip slightly protuberant. Tail short, panagrolaimid, dorsally convex, with offset tip tapering, smooth to somewhat telescope-like by cuticle forming a sleeve-like structure; tail tip with tiny hooks, mostly one dorsal, but also subventral (compare with Poikilocolaimus); opening of phasmids located at 60–65% of tail length, shortly anterior of tip, phasmid glands not reaching anus level.

Male
Testis right of intestine, ventrally reflexed in a certain distance posterior of pharynx; flexure relatively short. One pseudocoelomocyte between pharynx and flexure ventrally. Bursa well developed, peloderan, anteriorly open, with smooth margin and sometimes terminally indented, posterior part of velum transversely striated.

Nine pairs of genital papillae (GP) present, two of them anterior of the cloaca, genital papilla 1 (GP1) and GP2 spaced, GP3 to GP6 and GP7 to GP9 clustered, GP5 and GP7 point to the dorsal side of the velum, GP6 slightly bottle shaped, GP8 and GP9 fused at base, GP2 and GP8 not reaching the margin of velum. Phasmids forming small tubercles to the ventral side posterior of the last GP; formula of GPs: v1,v2/(v3,v4,ad,v5) (pd,v6,v7)ph. Precloacal sensillum small, precloacal lip simple (according to type A of W Sudhaus and K Kiontke [39]), postcloacal sensilla long filamentous. Spicules short and stout, tawny, separate, slightly curved, with prominent head; shaft with a transverse seam, with a prominent longitudinal ridge, a dorsal lamella, and an oval “window”, the tip notched. Gubernaculum dorsally projecting, flexible, in the distal part following the contour of the spicules, spoon shaped in ventral view.

Dauer larva
Unsheathed, mouth closed; stoma long, slender. Pharyngeal sleeve covering about half of the stoma; pharynx with well-developed median and terminal bulbs; corpus length ca. 52% of pharynx length. Nerve ring somewhat in the middle between the middle and terminal bulb. Genital primordium at about 60% of body length, elongated oval in shape. Tail conical. Amphids, lateral lines, position excretory pore, deirids and phasmids not observed.

Aberration
In one female a second set of “sensilla” were observed a short distance posterior to postdeirids, possibly a duplication of the postdeirids.

Type carrier and locality
Holotype and paratypes of Caenorhabditis monodelphis sp. n. were isolated from the tunnels of Cis castaneus (Herbst, 1793) (Ciidae, Coleoptera) in the bracket fungus Ganoderma applanatum (Polyporales) on a stump of the common beech (Fagus sylvatica) a few centimetres above the ground in Berlin-Grunewald in April 2001. The same sample included individuals of Diploscapter sp., Plectus sp., Oscheius dolichura and one individual dorylaimid and mononchid.

Type material
Holotype male (collection number WT 3684) and five female and four male paratypes (WT 3685, WT 3686) are deposited in the National Plant Protection Organization Wageningen, The Netherlands. In addition, four female and four male paratypes, are deposited in the collection of Museum Voor Dierkunde at Ghent University, Ghent, Belgium, five female and three male paratypes in Museum.
Diagnosis and relationship

*Caenorhabditis monodelphis* sp. n. can be recognised as a *Caenorhabditis* based on the thickened GP6 and the clearly visible postdeirids. *Caenorhabditis monodelphis* sp. n. is distinguished from all other described *Caenorhabditis*
species by the presence of a monodelphic genital tract in the female with a blind sac posterior the vulva, a panagrolaimid female tail shape, adults with only one ridge on the lateral field, a very long and slender stoma without visible glottoid apparatus and male with short, stout spicule with bifurcate tip.

Ecology and biology

Caenorhabditis monodelphis sp. n. is a gonochoristic species with both males and females. Females are oviparous and carry only one egg (rarely two eggs). Development from egg to adult took about 5–6 days in juice prepared from brown algae at room temperature. Development
from dauer larva to adults was completed in less than 3 days at 20 °C on NA seeded with OP50. The lifespan of adults is at minimum 14 days for males and 17 days for females. One pair of adults produced 167 offspring in 8 days and the daily production of fertile eggs was 6–31 (mean 18; \( n = 14 \)). After the reproductive phase, females lived 9–14 days (\( n = 3 \)) with males present.

**Caenorhabditis monodelphis** sp. n. has until now only been found in *Ganoderma* and *Fomes* in Germany and Belgium in relation with the c iid beetle *Cis castaneus*. The *Ganoderma* carrying *C. monodelphis* sp. n. from Oslo was not investigated for the presence of *C. castaneus*. In fungal fruiting bodies lacking the beetle *C. monodelphis* sp. n. was not found. Dauers of *C. monodelphis* sp. n. were found under the elytra of the beetle, but were not found internally when the beetle was further dissected. These findings indicate a phoretic association with the beetle. As only dauer larvae were isolated from beetles, while adults and larvae were present in the fruiting bodies, we infer that *C. monodelphis* sp. n. exit from dauer within the mushroom, develop to adulthood and start to reproduce. The food source of the species in natural conditions is not known, but they survive and reproduce easily on *E. coli* OP50 in culture.

### Genome sequence of an inbred strain of Caenorhabditis monodelphis sp. n.

We sequenced the genome of an inbred strain (JU1677) of *C. monodelphis* sp. n. using Illumina sequencing technology to ~110x coverage. The genome was assembled into 6,864 scaffolds, spanning 115.1 Mb with a scaffold N50 of 49.4 kb (Table 2). CEGMA (Core Eukaryotic Gene Mapping Approach) [40] scores suggested the assembly is of high completeness. We predicted 17,180 protein coding gene models using RNA-Seq evidence. These statistics, and the overall gene content and structure of the assembly were largely in keeping with those determined for other *Caenorhabditis* species. The genome was larger than that of *C. elegans* and *C. briggsae*, which are hermaphroditic species, but smaller than that of *C. remanei*, a gonochoristic species.

We carried out preliminary comparisons of the structure and content of the *C. monodelphis* sp. n. genome with those of other sequenced *Caenorhabditis* species. The number of genes identified was lower than estimates for most other *Caenorhabditis* species. To compare the gene structures of *C. monodelphis* sp. n. to that of *C. elegans*, we identified 6,174 orthologous gene pairs and calculated gene structure statistics (Table 3, Fig. 5.). To minimize bias from erroneous gene predictions (such as merged or split genes), orthologous gene pairs which differed in CDS length by 20% were considered outliers. *C. monodelphis* sp. n. genes were typically longer than their orthologues in *C. elegans* length by 20% were considered outliers. *C. monodelphis* sp. n. genes were typically longer than their orthologues in

### Table 1 Measurements (in μm) of heat relaxed specimens of Caenorhabditis monodelphis sp. n.

| Character                          | Female | Male | Dauer |
|-----------------------------------|--------|------|-------|
| N                                 | 11     | 10   | 10    |
| L                                 | 870 ± 105 | 694 ± 36 | 456 ± 24 |
| A                                 | 171 ± 0.8 | 22 ± 1.6 | 23 ± 1.2 |
| B                                 | 4.9 ± 0.5 | 4.1 ± 0.3 | 3.6 ± 0.1 |
| C                                 | 20.5 ± 2.6 | 22 ± 2.3  | 98 ± 0.7 |
| c’                                | 1.99 ± 0.17 | 1.8 ± 0.2 | 3.9 ± 0.30 |
| V                                 | 65 ± 1.8 | -    | -     |
| Body width                        | 51 ± 6.9 | 32 ± 3 | 20 ± 0.6 |
| Stoma length                      | 27 ± 2.3 | 27 ± 2 | 21 ± 1.1 |
| Stoma diameter                    | 1.9 ± 0.6 | 1.2 ± 0.2 | 0.6 ± 0.1 |
| Cheilostom                        | 2.5 ± 0.2 | 2.4 ± 0.2 | - |
| Gymnostom                         | 10 ± 0.7 | 9.7 ± 0.9 | - |
| Stegostom                         | 15 ± 1.5 | 15 ± 1.5 | - |
| Pharyngeal sleeve                 | 12.4 ± 1.6 | 13 ± 1.2 | - |
| Pharynx length                    | 150 ± 6.7 | 141 ± 9.2 | 107 ± 3.3 |
| Procorpus length                  | 55 ± 3.1 | 52 ± 3.6 | - |
| Metacorpus length                 | 26 ± 2.1 | 22.8 ± 1.1 | - |
| Isthmus length                    | 39 ± 3.2 | 40 ± 4.9 | - |
| Nerve ring to terminal bulb       | 11 ± 4.9 | 19 ± 3.3 | - |
| Terminal bulb length              | 30 ± 1.8 | 27 ± 1.7 | - |
| Diameter of median bulb           | 22 ± 2.5 | 17 ± 1.3 | 9 ± 0.5 |
| Diameter of terminal bulb         | 25 ± 2  | 19 ± 1 | 11 ± 0.4 |
| Anterior end to deirid            | 150 ± 8 | 150 ± 8.3 | - |
| Postdeirid to anus                | 170 ± 29.8 | 141 ± 14 | - |
| Length intestine                  | 651 ± 100 | 494 ± 32 | - |
| Rectum length                     | 25 ± 2.6 | 24 ± 1.9 | - |
| Anal body width                   | 22 ± 2.1 | 17 ± 1.1 | 12 ± 0.6 |
| Tail length                       | 43 ± 4.3 | 32 ± 3.2 | 46 ± 2.4 |
| Anus to phasmid distance          | 26 ± 2.2 | - | - |
| Gonad length \(^a\)              | 303 ± 68 | 342 ± 44 | - |
| Gonad flexure length              | 226 ± 67 | 46 ± 6.8 | - |
| Postuterine sac                   | 45 ± 6.8 | - | - |
| Sperm diameter                    | - | 9.8 ± 1.3 | - |
| Egg length \(^b\)                 | 53 ± 3.1 | - | - |
| Egg diameter \(^b\)               | 29 ± 2.9 | - | - |
| Spicule length                    | - | 25 ± 1 | - |
| Gubernaculum length               | - | 15 ± 0.9 | - |

\(^a\)from anus to flexure in the female; from cloaca to flexure in the male

\(^b\)\( n = 7 \)
Table 2 Genome assembly statistics for C. monodelphis sp. n. and other Caenorhabditis species

| Species         | C. monodelphis | C. brenneri | C. briggsae | C. elegans | C. japonica | C. remanei | C. sinica | C. tropicalis |
|-----------------|----------------|-------------|-------------|------------|-------------|------------|-----------|---------------|
| Version         | 1.0            | WS254       | WS254       | WS254      | WS254       | WS254      | WS254     | WS254         |
| Mating type     | gonochoristic  | gonochoristic| hermaphroditic| gonochoristic| gonochoristic| gonochoristic| gonochoristic| hermaphroditic|
| Strain          | JU1667         | PB2801      | AF16        | N2         | DF5081      | PB4641     | JU800      | JU1373        |
| Span (Mb)       | 115.12         | 190.37      | 108.38      | 100.29     | 166.25      | 118.55     | 130.76     | 79.32         |

Table 3 Gene structure comparison of orthologous gene pairs from C. monodelphis sp. n. and C. elegans

|                     | C. monodelphis sp. n. | C. elegans |
|---------------------|------------------------|------------|
| Gene length (bp)    | 3359                   | 2854       |
| Coding exon length (bp) | 109                  | 144        |
| Coding exon count (nt) | 10                    | 6          |
| CDS span (bp)       | 1167                   | 1182       |
| Intron length (bp)  | 69                     | 76         |
| Total intron span per gene (bp) | 1918                 | 1187       |

All values are medians

*C. monodelphis* genes typically have a longer total span of introns than *C. elegans* transcripts (Table 3, Fig. 5).

**C. monodelphis sp. n. is sister to other known Caenorhabditis**

We clustered a total of 634,564 protein sequences from *C. monodelphis* sp. n., twenty-two other *Caenorhabditis* species, and two rhabditomorph outgroup species (*Oscheius tipulae*; data courtesy of M. A. Félix, and *Heterorhabditis bacteriophora*) to define putative orthologues. We identified 34,425 putatively orthologous groups containing at least two members, 303 of which were either single copy or absent across all 25 species. These single copy orthologues were aligned, and the alignments concatenated and used to perform maximum-likelihood and Bayesian inference analysis using RAxML and PhyloBayes, respectively. Both analyses methods resulted in an identical topology, with the placement of *C. monodelphis* sp. n. arising basally to all other *Caenorhabditis* species (Fig. 6). All branches had maximal support except for three nodes within the *Elegans* super-group. Our analysis included data from several new and currently undescribed putative species of *Caenorhabditis*, including *C. sp. 21* which is the sister taxon to the genus. *C. sp. 31* which is sister to other *Caenorhabditis* species and two rhabditomorph outgroup species (*Oscheius* super-group: mouth opening triangular (Fig. 4b), spicule having a complicated tip (notched or dentated) and a longish thin walled “window” in the blade (Figs. 1i, 4l), postcloacal sensilla being filiform (Fig. 4k), and the female tail shortened to less than three times anal body width. Other similarities between both these species are plesiomorphic.

**Stempecies pattern reconstruction**

Our phylogenetic analyses were based on species with whole genome data available, and thus did not include the full known diversity of the genus. The stemspecies pattern was reconstructed based on ingroup and outgroup comparison. Previous molecular phylogenetic analyses of *Caenorhabditis* species using a small number of marker genes [10] placed *C. monodelphis* sp. n. and *C. sonorae* [41] as sister species, again arising at the base of the genus.

The following morphological synapomorphies can be hypothesised to support a *C. monodelphis* sp. n. – *C. sonorae* clade: mouth opening triangular (Fig. 4b), spicule having a complicated tip (notched or dentated) and a longish thin walled “window” in the blade (Figs. 1i, 4l), postcloacal sensilla being filiform (Fig. 4k), and the female tail shortened to less than three times anal body width. Other similarities between both these species are plesiomorphic.

*Caenorhabditis* and its sister group constitute the monophylum Anarhabditis within the Rhabditina. For convenience, we will call the sister clade of *Caenorhabditis* Protoscatper (Fig. 7): it comprises “Protorhabditis”, *Prodontorhabditis*, *Diploscatper* and *Sclerorhabditis* [42]. To reconstruct the characters of the stemspecies of *Caenorhabditis* it is necessary to consider the morphologies of all these taxa, and not only the taxa for which we have molecular data. “Protorhabditis” is paraphyletic. The *Oxyuroides* group is sister taxon of *Prodontorhabditis* [43, 44], and the *Xylocola* group may be sister taxon of *Diploscatper/Sclerorhabditis*. However, the two species *Protorhabditis elaphri* (Hirschmann in Osche, 1952) and *P. tristis* [45] appear to represent basal branches in Protoscatper (compare [43]). These last two species, despite...
the paucity of information available for them, are crucial for comparisons that will illuminate the stemspecies patterns of Anarhabditis, Protoscoptera and Caenorhabditis.

By ingroup comparison we reconstruct the following characters of the stemspecies of Anarhabditis without differentiating them into apomorphies or plesiomorphies (on apomorphies see the legend of Fig. 7):

- adults of small size (less than 1 mm);
- lips not offset from anterior end;
- four cephalic sensilla present in male and female;
- stoma with pharyngeal sleeve (stegostom length nearly that of gymnostom);
- median bulb of pharynx strongly developed, corpus intima with transverse ridging, terminal bulb with double haustrulum;
- gonochoristic;
- female tail elongate conoid;
- gonads amphidelphic, the anterior branch right and the posterior left of intestine;
- vulva at midbody, a transverse slit;
- oviparous, usually only one egg at a time in the uteri;
- male gonad on the right side, reflexed to the ventral;
bursa peloderan and anteriorly open, oval-shaped in ventral view, with smooth margin, terminally not notched; – 9 pairs of even genital papillae, two precloacal largely spaced, GP3–6 evenly spaced, the last three GPs forming a tight cluster; GP1, GP5 and GP7 terminate on the dorsal surface of the bursa velum; – phasmids open behind GP9, inconspicuous; – bursa formula thus v1,v2/v3,v4,ad,v5 (pd,v6,v7)ph; – male tail tip present; – 1 + 2 circumcloacal sensilla inconspicuous, precloacal lip simple; – spicules separate, stout, head not rounded, behind the shaft a slight ventral projection, dorsal part of blade weakly cuticularised (velum), its tip possibly not even (argued below); – gubernaculum simple spatulate; – dauerlarvae with double cuticle (ensheathed), not waving.

Discussion
Taxonomy of Caenorhabditis monodelphis sp. n.
Caenorhabditis monodelphis sp. n. is a new species of Caenorhabditis supported by its phylogenetic position as inferred from 303 molecular markers, two precloacal largely spaced, GP3–6 evenly spaced, the last three GPs forming a tight cluster; GP1, GP5 and GP7 terminate on the dorsal surface of the bursa velum; – phasmids open behind GP9, inconspicuous; – bursa formula thus v1,v2/v3,v4,ad,v5 (pd,v6,v7)ph; – male tail tip present; – 1 + 2 circumcloacal sensilla inconspicuous, precloacal lip simple; – spicules separate, stout, head not rounded, behind the shaft a slight ventral projection, dorsal part of blade weakly cuticularised (velum), its tip possibly not even (argued below); – gubernaculum simple spatulate; – dauerlarvae with double cuticle (ensheathed), not waving.

Previously, Caenorhabditis has been characterised as having the following apomorphic characteristics: the presence of a dorsal velum on the spicule, a lateral field with three ridges, an unsheathed dauer juvenile and a slightly thickened GP6 [42]. With the discovery and description of C. monodelphis sp. n. the number of lateral ridges is no longer an apomorphic character of Caenorhabditis, since C. monodelphis sp. n. only has one lateral ridge.

Association with fungivorous beetles
Species of Caenorhabditis are known to occur in soil, compost, cadavers of insects, some plant material and the intestine of birds [10], and can most easily be isolated from rotting fruits, flowers and stems [3]. Caenorhabditis elegans has also been found infesting cultures of the mushroom Agaricus bisporus [47]. Wild mushrooms are an under-explored habitat for this genus, but our limited geographical sampling indicates that they could be an important habitat. Caenorhabditis monodelphis sp. n. was present in galleries made by Cis castaneus inside Ganoderma applanatum in Belgium, Norway, Germany and in an old fruiting body of Fomes fomentarius in Belgium. Although the true distribution of C. monodelphis sp. n. is not yet known, it is expected that this species will be found throughout Europe where Ganoderma (or in lesser extent Fomes) co-occurs with the mycophagous beetle Cis castaneus.

That Caenorhabditis species have phoretic relationships with insects and other invertebrates is well known [10]. For C. monodelphis sp. n., all records are from

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Fig. 6 Phylogenetic relationships of Caenorhabditis species and two outgroup species. Maximum-likelihood based tree from RAxML. Bootstrap support values (1000 replicates) were 100 all branches, unless noted otherwise as numbers on branches. Bayesian Posterior Probabilities were 1 for all branches and are not shown. Coloured boxes indicate supergroups, as defined in [3]. Strain names from which protein sequence were derived are noted.
mushroom fruiting bodies that are also inhabited by different insect groups, and dauer larvae were found under the elytra of *Cis castaneus*. Based on this evidence, we propose that *C. monodelphis* sp. n. propagates in galleries generated by *Cisidae* and the dauer larvae are transported by these beetles to uninested mushrooms. Records of *C. monodelphis* sp. n. in both *Ganoderma* and *Fomes*, respectively the preferred [9] and the known [48] host indicate a beetle-specific rather than a mushroom-specific relationship. The only other known *Caenorhabditis* species which appears to be phoretically associated with fungivorous organisms, most likely insects, is *C. auriculariae* Tsuda & Futai, [49] of the *Elegans* super-group. This species was found only once in the fruit bodies of *Auricularia polytricha* (Agaricomycetes) in Japan, but the vector needed to infest the mushroom is unknown [49]. *C. elegans* was also found to infest cultures of the champignon mushroom *Agaricus bisporus* [47], but most likely originated from mushroom compost where it can be frequently found. Several samples of different mushrooms on wood in Europe, USA and Japan did not yield other *Caenorhabditis* spp. However, given that many more insect species are known to feed and reproduce on mushrooms [50] and Rhabditida are known to use insects as a phoretic transport carrier [51], it is possible that mushroom species are habitats for many other rhabditid species, including new species of *Caenorhabditis*.

**Genome sequence and gene structures of *C. monodelphis* sp. n.**

Using next generation sequencing technologies and advanced bioinformatics toolkits, we have generated a good first-draft genome sequence for an inbred line derived from the type strain of *C. monodelphis* sp. n.. Although assembly metrics and CEGMA scores indicate the assembly is relatively contiguous and complete, it is likely that a proportion of *C. monodelphis* sp. n. genes are assembled only partially. This may have affected gene prediction, with the number of predicted gene models (17,180) being lower than estimates from most other *Caenorhabditis* species with available sequence data [25]. Comparisons of orthologous gene pairs revealed a significant divergence in gene structure between *C. monodelphis* sp. n. and *C. elegans*. *C. monodelphis* sp. n. genes are typically longer, contain more coding exons and a longer span of introns than *C. elegans* genes (Table 3). This increase in gene length may, in part, account for the difference in genome span between *C. monodelphis* sp. n. and *C. elegans*. The clear trend towards more coding-exons in *C. monodelphis* sp. n. relative to *C. elegans* (Fig. 5) could be explained by extensive intron loss or gain in either species. Previous studies using a small number of genes have shown that intron losses have been far more common in *Caenorhabditis* evolution than intron gains [7, 52, 53]. Thus, it is possible that the gene structures seen in *C. monodelphis* sp. n. reflect an intron-rich ancestral state, and intron loss has predominated during the evolution of *C. elegans*. In *Pristionchus pacificus*, which is distantly related to *Caenorhabditis*, genes typically have roughly twice as many introns as their orthologues in *C. elegans* [54]. Further analysis using genomes from more closely related outgroup species and other *Caenorhabditis* species is necessary before we can infer the dynamics of intron evolution in the genus.

**Phylogenetic analyses**

Phylogenetic analysis of 303 clusters of putatively orthologous protein sequences derived from whole genome sequence data of 23 species of *Caenorhabditis* and two outgroup species resulted in a well resolved phylogenetic diagram and confirmation of *C. monodelphis* sp. n. as basal to all other analysed *Caenorhabditis* species (Fig. 6). The topology is largely congruent with previously published analyses performed using a smaller number of molecular loci [1]. However, in contrast to the analyses of Kiontke et. al. [1] and Felix et. al. [2] which show *C. brenneri* and *C. doughertyi* as sister species, our phylogenetic
hypothesis places *C. doughertyi* as more closely related to *C. wallacei* and *C. tropicalis*. This node, however, has low bootstrap support. Genome sequencing projects for several *Caenorhabditis* species, including those from the currently under-sampled *Drosophilae* super-group, are currently underway. These data will be essential to resolving the phylogenetic relationships of this important genus where morphology can be misinformative and/or misleading.

**Reconstruction of stemspecies pattern**

Details of our inference of ASR depends on the placement of *P. elaphri* in “*Protorhabditis* versus as sister taxon of Anarhabditis (because of its distinct pharynx morphology)" (Fig. 7). Molecular data resolving this issue are urgently required. *Caenorhabditis monodelphis* sp. n. and *P. elaphri* share a conspicuously long and narrow stoma due to an extended stegostom (long pharyngeal sleeve) without a glottoid apparatus (bulging of the three sectors of metasemistostom) and one ridge in the lateral field. Based on current evidence, we interpret these peculiar similarities as homologous and thus as further characters of the Anarhabditis stemspecies as well as the *Caenorhabditis* stemspecies. The narrowing of the buccal cavity could have restricted the formation of sectoral swellings of the metasemistostom, so that the typical glottoid apparatus disappeared. This happened in parallel in the rhabditid *Matthesonema eremitum* [55]. The hypothesis of a reduction of the glottoid apparatus and its denticles in the stemspecies of Anarhabditis is in conflict with the structure of the metasemistostom in most species of *Caenorhabditis*, where it looks like a transformation of a glottoid apparatus [39], and in *C. sonorae* is credibly described as a glottoid apparatus [41]. To solve this conflict we must assume a partial reversion both in *C. sonorae* and in the sister-lineage of *C. sonorae/C. monodelphis* sp. n. However, instead of proposing two independent reversions, the possibility of an independent reduction of the glottoid apparatus in Protoscoptera and *C. monodelphis* sp. n. remains an equally parsimonious alternative. A reinvestigation of *P. elaphri* could resolve this question.

In the stemspecies pattern of Anarhabditis the morphology of the tip of the spicules remains unclear. In the description of *P. elaphri* some drawings show the tip to be nearly pointed [45], but in other drawings (Figure twelve m of [46]) it is terminally notched. In *P. tristis*, I. Andrásy [56] depicted a small terminal hook. These characters were not mentioned in the text in either species’ description. Nevertheless, the dentation of the spicule tips in the first branching *Caenorhabditis sonorae/C. monodelphis* sp. n. is different and distinct enough to judge this character as synapomorphic for these sister species (Fig. 7). Starting from the characters of the last common stemspecies of both these species, in *C. sonorae* the lateral ridge must have been reduced, so that its lateral field is smooth, and the male tail tip was retracted, so that the tail ends ob- tusey between the last GPs. In *C. monodelphis* sp. n. both these characters remain plesiomorphic, but the female posterior gonad branch is in the process of reduction. The ecological requirements of *C. sonorae* (inhabitant of cactus rot) and *C. monodelphis* sp. n. (living in the tunnels of Ciidae beetles in bracket fungi) are so different, that no statement on the ecology of their last common stemspecies is possible. However, as *P. elaphri* and *C. monodelphis* sp. n. exhibit a phoretic relationship with beetles and their dauer larvae seek a place under the elytra, we cautiously suggest that this behaviour could be found in the stemspecies of Anarhabditis and that of *Caenorhabditis*, respectively.

Transformations from the stemspecies pattern of Anarhabditis to *Caenorhabditis* can be traced in the cladogram (Fig. 7). With respect to the hypothesis of the stemspecies pattern of *Caenorhabditis* formulated by W Sudhaus and K Kiontke [39] only the character of the lateral field must be revised: a single ridge in the lateral field of adults must be assumed in the stemspecies pattern of Anarhabditis and of *Caenorhabditis*, respectively. Therefore, the evolution of three lateral cuticular ridges must have occurred first within *Caenorhabditis* (Fig. 7).

**Degenerative evolution towards monodelphy**

Uniquely for *Caenorhabditis* species, in *C. monodelphis* sp. n. the posterior female gonad branch has been reduced to a blind sac without gamete forming function. This vestigial branch serves mainly in storing sperm. In contrast to most mono-prodelphic rhabditids, the vulva is not shifted posteriorly in *C. monodelphis* sp. n.. A relict posterior gonad together with a nearly median vulva also occurs in *Oscheius guentheri* (Sudhaus & Hooper, 1994) [57] and an undescribed *Diplogastrellus* species from India (Sudhaus, unpublished data). Remarkable, in all these cases the anterior branch does not extend into the body posterior to the vulva, in contrast to monodelphic cephalobids, panagrolaimids and the rhabditid *Rhabpanus*. In *Rhabpanus ooniculus* Massey, [58] and *R. uniquis* Tahseen, Sultana, Khan & Hussain, [59] the prodelphic reflexed gonad reaches almost to the rectum while the vulva is located at 65–69% of body length and a short post-uterine sac filled with sperm is present [58, 59]. In contrast to species of *Acrobelloides, Cephalobus, Mesorhabditis and Panagrolaimus*, the posterior branch of the gonad of *O. guentheri* is not reduced by apoptosis of the distal tip cell [60], and the vestigial branch is very variable within this species [57]. These patterns argue for a relatively recent reduction in *O. guentheri*. Based on the similarities (in the female gonad and nearly median vulva) between *C. monodelphis* sp. n. and *O. guentheri*, the gonadal system of female *C. monodelphis* sp. n. may also represent a relatively recent evolutionary shift.
Endnote

1Named after the monodelphic reproductive system in the female.

Additional files

Additional file 1: Nucleic acid isolation from Caenorhabditis monodelphis sp. n. strain JU1677. (DOCX 13 kb)

Additional file 2: Details of software, versions and parameters used in analysis. (TSV 5 kb)

Additional file 3: Accessions and links to genome-derived protein sequence data used in phylogenetic analysis. (TSV 4 kb)

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