Characterization of *Pseudoterranova ceticola* (Nematoda: Anisakidae) larvae from meso/bathypelagic fishes off Macaronesia (NW Africa waters)

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The genus *Pseudoterranova* includes parasite species of cetaceans and pinnipeds. The third stage larva (L3) of seal-infecting species occur in second intermediate or paratenic fish hosts mainly in neritic waters. This study firstly describes a *Pseudoterranova* L3 from meso/bathypelagic fishes off Macaronesia. L3s were morphologically and genetically studied by light microscopy and sequencing of the mtDNA *cox2* and entire ITS rDNA genes. Bayesian inferences were performed with sequences from the larvae and selected sequences from GenBank. The nematode L3s were molecularly identified as *Pseudoterranova ceticola*, a parasite of kogiid whales. Such larvae were collected from *Bolinichthys indicus*, *Chauliodus danae*, *Eupharynx pelecanoides*, *Diaphus rafinesquii*, *D. mollis*, *Diretmus argenteus* and *Maulisia argipalla*. They mainly occurred in the viscera of these fishes. *Pseudoterranova ceticola* L3 were small (< 12 mm) and whitish, and a prominent characteristic is a circumoral ridge extending from the ventral boring tooth which differentiate them from *Pseudoterranova* spp. maturing in pinnipeds and *Terranova* sensu lato larvae that mature in poikilotherms. The shape of the tail: conical, long, pointed, ventrally curved and lacking mucron also distinguish these larvae from those of the pinniped-infecting *Pseudoterranova* spp. Phylogenetic analyses based on mtDNA *cox2* and ITS rDNA sequences suggest that *P. ceticola* is closely related to *Skrjabinisakis* spp., and not with *Pseudoterranova* spp. parasitizing pinnipeds. The related species *Skrjabinisakis paggiae*, *S. brevispiculata* and *S. physeteris* (until recently belonging to genus *Anisakis*), are as *P. ceticola* also parasites of physeteroïd cetaceans. The morphology and morphological variation of the larvae of the cetacean parasite *P. ceticola* is thoroughly described for the first time. These L3 can readily be morphologically distinguished from those of the pinniped-infecting *Pseudoterranova* spp. The parasite likely completes its life cycle in the mesopelagic and bathypelagic realm, with meso/bathypelagic fish as 2nd intermediate or paratenic hosts and kogiids as final host. Thus, *Pseudoterranova* from cetaceans appear to be morphologically, genetically, and ecologically differentiated to those from pinnipeds, suggesting that they are not congeneric.

The taxonomy of ascaridoid nematodes remains confusing and unresolved. The issue is of particular importance since species from the genera *Anisakis*, *Pseudoterranova* and *Contracaecum* are recognized as causative agents of fish-borne zoonotic diseases of worldwide concern, i.e. anisakidoses1,2. Generally, these anisakids use crustaceans as first intermediate hosts, fish and squid as second intermediate or paratenic hosts, and marine mammals (i.e. cetaceans for *Anisakis* spp. and pinnipeds for *Pseudoterranova* spp. and *Contracaecum* spp.) as final hosts of their life cycle [reviewed by 3]. In addition, anisakid species belonging to the genus *Terranova* (which is now considered taxon inquirendum, see4 and further comments at the discussion section) englobed species parasites of elasmobranchs, teleosts, crocodilians, colubrid snakes and marine mammals4.
Identification of Terranova-like third-stage larvae (L3) present in fish intermediate or paratenic hosts is difficult. Larvae belonging to Pseudoterranova, Pulchrascaris and Terranova sensu lato are too morphologically similar to identify them even to genus⁴. The common morphological features are the presence of the excretory pore opening ventrally at the anterior end, presence of ventriculus without an appendix and having an intestinal caecum⁵–⁸. Molecular identification is therefore needed. Robust identification of anisakid parasites is crucial for understanding their distribution and epidemiology.

In the present study, a new Terranova-like larval type, collected from mesopelagic/bathypelagic fish species of Macaronesia, North West (NW) African waters, is morphologically described, and molecularly recognized as a potentially zoonotic member of the genus Pseudoterranova.

**Methods**

**Fish collection.** During May 2019, mesopelagic and bathypelagic fishes including the following host species: Bolinichthys indicus, Chauliodus danae, Eupharynx pelecanoides, Diaphus rafinesquii, Diaphus mollis, Diretmus argenteus and Maulisia argipalla were caught in waters off NW Africa from Cape Verde to North East (NE) of Madeira during a research cruise on board of the Norwegian vessel “RV Kronprins Haakon” (Table 1, Fig. 1). Hauls were conducted with 2 different gears: a macroplankton trawl (theoretical mouth opening 6 × 6 and 8 mm stretched mesh size) and a Multipelt trawl (mouth opening height of 35 m and 20 mm mesh in the cod-end).

Fishes were frozen on board at −20 °C for later parasite inspection on land. Fish samples were collected within the MEESO project (EU H2020 research and innovation programme, Grant Agreement No 817669) and procedures were carried out in accordance with the relevant EU legislation including EU Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Norwegian research vessels have authorization to collect fishes for research purposes; in addition, permission for the collection of the present fishes was obtained from coastal countries.

**Parasite collection.** Fishes were thawed at room temperature, opened and the visceral organs and emptied body cavity were placed in a petri dish with physiological saline solution and examined under stereomicroscope for ascaridoid nematodes. The parasites were collected, and the internal organs and carcass were then placed into plastic bags and refrozen. These were later examined using the UV-press method⁹, to detect any larvae not recovered during dissection, specially from the musculature.

**Morphological study.** The nematode larvae were examined in temporary mounts in physiological saline solution, and photographed. Various morphotypes were recognized, but only findings concerning Terranova-like larvae⁶,¹⁰ (N = 35) will be presented here. In addition, infection levels such as parasite prevalence and abundance will be published elsewhere.

**Morphometric measurements.** Series of digital photographs were obtained from 33 larvae. Measurements were taken from the digital images, except larval body lengths that mostly were measured at a mm scale.

Measurements from images were obtained using the software Image J (https://imagej.nih.gov/ij/). The oesophagus, ventricle and tail lengths were taken along the midline. The caecum length was measured from the aperture into the ventricle to the caecum end (Fig. 2).

**Molecular analyses.** DNA was extracted from a randomly selected subsample of 19 nematode larvae using the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions with the modification that sample lysis was enhanced by mechanical disruption with ceramic bead-beating system (Precellys ceramic kit 2.8 MM, VWR and Precellys 24 Tissue Homogenizer, Bertin Technologies). DNA was eluted with 30 µl AE buffer.

Polymerase chain reaction (PCR) were done with primers that amplify the entire internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (ITS1-5.8SrRNA -ITS2). The NC5F (5’–GTAGGTGAACCTGCG
**Figure 1.** Trawl stations from which infected fish hosts were obtained. Positions: station (st.) 4601: 17.969 N, 23.956 W; st. 4604: 26.899 N, 19.232 W; st. 4606: 29.767 N, 16.087 W; st. 4610: 33.695 N, 13.232 W. Figure 1 was created using R version 4.95 (2021–03-31) [https://www.r-project.org/](https://www.r-project.org/) implemented in RStudio 1.4.1106 [https://www.rstudio.com/](https://www.rstudio.com/) using.

**Figure 2.** Measurements taken from the images of *Terranova*-like larvae. The oesophagus length is taken along the midline from the start of the oesophagus (i.e. slightly sub-terminally in the roundworm) to the ventricle. The caecum length was measured from the aperture into the ventricle to the caecum end. The tail length represents the distance along the midline, from the level of the anus/cloaca to the posterior end. Figure 2 was created in Adobe Photoshop 23.5.0 [https://www.adobe.com/photoshop].
GAA GGA TCA TT-3’) and NC2R (5’ TTA GTT TCT TTT CCT CCG CT -3’) primers were used. PCR conditions followed Zhu et al.11, but annealing temperature was 54 °C instead of 55 °C.

The mitochondrial cytochrome c oxidase subunit II (cox2) gene was amplified using the primers 211F (5′-TTT TCT AGT TAT ATA GAT TGR TTT Y AT-3′) and 210R (5′-CAC CAA CTC TTA AAA TTA TC-3′)12 according to Mattiucci et al.13 with the following modifications. The cycling conditions were an initial denaturation at 94 °C for 5 min, followed by 35 cycles of: denaturation at 94 °C for 30 s, annealing at 46 °C for 1 min, extension at 72 °C for 90 s; followed by final step of final extension at 72 °C for 10 min, and hold at 4 °C.

PCR products were sent for purification and sequencing (using the primers NC5F and 210R) to Eurofins (Cologne, Germany). The National Center for Biotechnology Information (NCBI) sequence database (henceforth ‘GenBank’) was searched for similar sequences using BLAST (Basic Local Alignment Search Tool) (USA)14.

| Nematode species | GenBank accession number | Isolate | Stage | Host species | Location | References |
|------------------|--------------------------|---------|-------|--------------|----------|------------|
| *Skrjabiniasis physteteris* | JQ912693 | G | Adult | *Physeter macrocephalus* | Mediterranean Sea | Mattiucci et al.13 |
| *Skrjabiniasis breviplicata* | JQ912694 | H | Adult | *Kogia breviceps* | NW Atlantic Ocean | Mattiucci et al.13 |
| *Skrjabiniasis poggiae* | JQ912695 | I | Adult | *K. breviceps* | NW Atlantic Ocean | Mattiucci et al.13 |
| *Anisakis* sp. B | MK325217 | 21 | Adult | *K. breviceps* | Australia | Shamsi et al.63 |
| *Anisakis* sp. | JN005761 | MAD17OH3 | L3 | *Pagellus bogaraveo* | Madeira | Hermida et al.60 |
| *Anisakis* sp. | KCS82170 | 10 | Pre-adult | *Kogia sima* | Philippine | Quiazon et al.91 |
| *Anisakis* sp. | None | Seq_ASp.HC_2005 | L3 | *Epinephelus areolatus* | Indonesia | Kleinertz et al.94 |
| *Pseudoterranova decipiens* (s. s.) | AY825253 | N241 | Adult | *Phoca vitulina* | California | Nadler et al.92 |
| *Pseudoterranova cattani* | KF781284 | CL3 | L3 | *Homo sapiens* | Chile | Weitzel et al.83 |
| *Pseudoterranova azauresi* | AB576757 | Pst-2 | L3 | *Gadus macrocephalus* | Japan | Arizono et al.84 |
| *Pseudoterranova decipiens* s. e. | KF017610 | PDF2 | L3 | *Notothenia coriiceps* | Antarctica | Timi et al.96 |
| *Pseudoterranova ceticola* | ON128286 | SU6V1cet | L3 | *Saarsida undosquamis* | Tanzania | Cipriani et al. in prep |
| *Pseudoterranova krabbei* | OP355454 | GMLOB31PF2 | L3 | *Gadus morhua* | Lofoten, Norway | This study |
| *Pseudoterranova bulbosa* | OP355455 | GMHS76PV1 | L3 | *Gadus morhua* | Finnmark, Norway | This study |
| *Pseudoterranova ceticola* | OP352234 | DiMo53T | L3 | *Diaphus mollis* | Station 4606 | This study |
| *Pseudoterranova ceticola* | OP352235 | DiMo41T | L3 | *Diaphus mollis* | Station 4606 | This study |
| *Pseudoterranova ceticola* | OP352236 | DiRa23T | L3 | *Diaphus rafinesquii* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352237 | DiArg15-13 T | L3 | *Diretmus argentus* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352238 | DiRa37T | L3 | *Diaphus rafinesquii* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352239 | DiRa34-1 T | L3 | *Diaphus rafinesquii* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352240 | DiRa29 T | L3 | *Diaphus rafinesquii* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352241 | DiRa35-2 T | L3 | *Diaphus rafinesquii* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352242 | DiRa49T | L3 | *Diaphus rafinesquii* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352243 | DiRa38T | L3 | *Diaphus rafinesquii* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352244 | ChaDa53T | L3 | *Chauliodus danae* | Station 4604 | This study |
| *Pseudoterranova ceticola* | OP352245 | EuPele 13 T | L3 | *Eurypharynx plecanoides* | Station 4610 | This study |
| *Pseudoterranova ceticola* | OP352246 | DiArg14-14 T | L3 | *Diretmus argentus* | Station 4604 | This study |

Table 2. Samples used for analysis of the entire ITS rDNA. *Species formerly belonging to the genus Anisakis (see20,21).*

GAAGGATCATT-3’) and NC2R (5’-TTAGTTTTTCTTTTCTCCGGCT-3’) primers were used. PCR conditions followed Zhu et al.11, but annealing temperature was 54 °C instead of 55 °C.

The mitochondrial cytochrome c oxidase subunit II (cox2) gene was amplified using the primers 211F (5’-TTTCTTAGTTATATAGTTGRITTYAT-3’) and 210R (5’-CACCAACTCTTTAAAATTAC-T-3’)12 according to Mattiucci et al.13 with the following modifications. The cycling conditions were an initial denaturation at 94 °C for 5 min, followed by 35 cycles of: denaturation at 94 °C for 30 s, annealing at 46 °C for 1 min, extension at 72 °C for 90 s; followed by final step of final extension at 72 °C for 10 min, and hold at 4 °C.

PCR products were sent for purification and sequencing (using the primers NC5F and 210R) to Eurofins (Cologne, Germany). The National Center for Biotechnology Information (NCBI) sequence database (henceforth ‘GenBank’) was searched for similar sequences using BLAST (Basic Local Alignment Search Tool) (USA)14.
| Nematode species | GenBank accession number | Isolate | Stage | Host species | Location | References |
|------------------|-------------------------|---------|-------|-------------|----------|------------|
| Anisakis berlandii | DQ116429                |        |      | Consensus sequence |         | Valentini et al.53 |
| Anisakis pegreffii | DQ116428                |        |      | Consensus sequence |         | Valentini et al.53 |
| Anisakis simplex s.s | KC810002               | ASS1   | Adult | Balaenoptera acutorostrata | Norway | Mattucci et al.53 |
| Anisakis typica | DQ116427                |        |      | Consensus sequence |         | Valentini et al.53 |
| Anisakis ziphiidarum | DQ116430               |        |      | Consensus sequence |         | Valentini et al.53 |
| Anisakis nascetii | DQ116431                |        |      | Consensus sequence |         | Valentini et al.53 |
| Skrjabinisakis physeteris* | DQ116432         |        | Adult | Consensus sequence |         | Valentini et al.53 |
| Skrjabinisakis brevispiculata* | DQ116433   |        | Adult | Consensus sequence |         | Valentini et al.53 |
| Skrjabinisakis paggiae* | KF214801             |        | Adult | Consensus sequence |         | Valentini et al.53 |
| Anisakis typica | KF701409                |        | Ani1 | Tursiops aduncus | Northern Red Sea | Kleinen et al.85 |
| Skrjabinisakis sp. 2* | MW074868               | TMCRP20 | L3 | Trachurus murphyi | Peru | Aco Albuquerque et al.86 |
| Skrjabinisakis cf. paggiae* | KF693770            |        | AV60.8 | Kogia simia | Brazil | Di Azevedo et al.87 |
| Anisakis sp. n. 1 KMAQ-2013 isolate 2 | KF214801 | 2 | Adult | Mesoplodon hotaula | Philippine | Quaison et al.88 |
| Pseudoterranova ceticola | DQ116435            |        |      | Kogia breviceps | Caribbean Sea | Valentini et al.53 |
| Pseudoterranova ceticola | ON155434              | SU6V1  | L3 | Sardina undosquamosis | Tanzania | Cipriani et al.89 |
| Pseudoterranova ceticola | LC712859              | R2     | L3 | Katsuwonus pelamis | Japan: Mic, off Kumano | Takano & Sata90 |
| Pseudoterranova decipiens s.s | MT347695           | Pd03   | L3 | Gadus morhua | Lofoten (Norway) | Bao et al.91 |
| Pseudoterranova cattani | KUS58721             |        |      | Otaria byronia | Chile | Liu et al.92 |
| Pseudoterranova bulbosa | KU558720              |        |      | Erinipathus barbatus | Newfoundland | Liu et al.93 |
| Pseudoterranova azasai | MT912398              | ZC17_335 | L3 | Zalophus californianus | California | Harbar et al.94 |
| Pseudoterranova krabbei | KUS58724             |        |      | Halichoerus grypus | Norway | Liu et al.95 |
| Pseudoterranova ceticola | OP380493              | Maar1T  | L3 | Maulisia argippila | Station 4604 | This study |
| Pseudoterranova ceticola | OP380494              | DiMo53T | L3 | Diaphus mullis | Station 4606 | This study |
| Pseudoterranova ceticola | OP380495              | DiMo41T | L3 | Diaphus mullis | Station 4606 | This study |
| Pseudoterranova ceticola | OP380496              | DiRa23T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380497              | DiArg15-13T | L3 | Diretmus argenticeps | Station 4604 | This study |
| Pseudoterranova ceticola | OP380498              | DiRa37T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380499              | DiRa34-1T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380500              | DiRa29T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380501              | DiRa35-2T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380502              | DiRa49T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380503              | DiRa34-2T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380504              | DiRa36-1T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380505              | DiRa38T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380506              | ChaDa53T | L3 | Chausiodus danae | Station 4606 | This study |
| Pseudoterranova ceticola | OP380509              | DiRa22T | L3 | Diaphus mullis | Station 4604 | This study |
| Pseudoterranova ceticola | OP380508              | DiArg7-1T | L3 | Diretmus argenticeps | Station 4606 | This study |
| Pseudoterranova ceticola | OP380510              | DiRa37-3T | L3 | Diaphus Rafinesquii | Station 4604 | This study |
| Pseudoterranova ceticola | OP380507              | DiArg14-14T | L3 | Diretmus argenticeps | Station 4604 | This study |
| Ascaris lumbricoides | AF179907              |        |      | Homo sapiens | Louisiana | Nadler and Hudspeth96 |
| Toxocara canis | AF179923              |        |      | Canis familiaris | Illinois | Nadler and Hudspeth97 |

Table 3. Samples used for analysis of the cox2 gene. *Species formerly belonging to the genus Anisakis (see20,21).

Phylogenetic analyses. Sequences generated in this study were aligned with selected sequences obtained from GenBank, using CLUSTAL W in MEGA X (Table 2 and 3)21. High similarity scores in the BLAST as well as larvae morphological similarity were used as the criteria to select the sequences. The default setting parameters of ClustalW were used, and the alignments were manually edited and trimmed in MEGA X. Toxocara canis and Ascaris lumbricoides were set as outgroup for the cox2 phylogenetic analysis. Due to indel-induced alignment problems in ITS16, only the closely related Anisakis spp., and Pseudoterranova spp. could be aligned with confidence in homology. For the same reason, no outgroup was included. The entire ITS sequences of Pseudoterranova krabbei and Pseudoterranova bulbosa identified from two cod (Gadus morhua) caught in northern Norwegian waters were sequenced and used in the analysis (Table 2).
next best model available, i.e. HKY + G for the BI. The optimum model was HKY + G + I for cox2 dataset based on BIC criteria. The BEAST file was previously generated in BEAUti with the following characteristics: sites: entering the best substitution model and otherwise default settings; clock type: strict clock; tree prior: Speciation: Yule process; MCMC: length of chain = 10,000,000, echo state to screen every = 1000, log parameters every = 1000. Effective sample size of parameters (i.e., > 200) was checked in Tracer v1.7.2.18. The created tree was drawn in TreeAnnotator v1.10.4 and the burnin as the number of states was specified at 100,000. Figtree v1.4.4 was used to visualize the phylogenetic trees.

Results
A total of 35 Terranova-type larvae were recovered. All these were morphologically similar. They were found in 7 fish species (Table 1). In-situ, the larvae were coiled like a coil or watch spring. Most (94%) were found in the visceras, but two larvae were found in the muscle of D. rafinesquii. The larvae had a light neon-bluish colour when exposed to UV-light, after freezing.

Figure 3. Digital photographs of P. ceticola L3 from Macaronesian deepwater fishes. (a) Entire P. ceticola larva from Diaphus mollis. (b) Anterior end showing the ventral boring tooth connected to the cuticular ridge, arrow indicates the excretory pore. (c) out-of-focus view of the anterior end, showing bulbs (arrows). (d) Anterior end showing nerve ring (NR). (e–f) ventricle region, showing ventricle (V) and caecum (C), intestine (Int), and oesophagus (Oe). Arrow in (f) indicates aperture intestine-ventricle. (g) Tail region, arrow indicating anus. (h) Part of tail, showing transverse cuticular striation. Specimens were collected from D. mollis (a,b,d,f,g) and D. rafinesquii (c,e,h). Scale bars: (a) 500 μm, (b, c) to same scale 50 μm, (d) 100 μm, (e, f) to same scale 200 μm, (g) 100 μm, (h) 50 μm.
Morphology. Larvae were small and pale, with a thick-set appearance (Fig. 3, Table 4). The body was widest at the middle and posteriorly. Body length: max width ratio was 22—36:1 (mean 28 ± 4, N = 27). The cuticle was smooth, but with inconspicuous transverse striae which were most evident in tail. At anterior end, lip anlagen were visible through the cuticle, and associated with surface bulbs (Fig. 3c). Prominent conical boring tooth at the anterior extremity between ventro-lateral lip anlagen, projecting anteroventral at an angle of about 130° (115°–145°) to main axis. The boring tooth base gives rise to a circumoral cuticular ridge (Fig. 3b). The dorso-ventral extent of this ridge from the boring tooth tip was 40.8 ± 3.2 µm (mean ± SD) (range = 36–49 µm; N = 25). The excretory pore was ventrally located, near the base of the boring tooth. The oesophagus was clavate, widest posterior. The nerve ring was positioned within the anterior 25 (20–27) % (mean (range); N = 27). The caecum was normally shorter than the ventricle, averaging 74% of its length (range = 50–119, SD = 18). The tail was elongate conical, curved ventrally, pointed and without a distinct mucron (Fig. 3–G).

Molecular identification. The ITS sequences (801—842 bp) obtained from 13 Terranova type larvae were 100% identical. However, ambiguous positions (i.e. double signals) were seen in the sequences from five of these worms. The cox2 sequences (570–580 bp) obtained from 18 Terranova type larvae showed 97.1—99.4% identity. With a single exception (in DIRa38), all substitutions were silent. The cox2 sequences were 96.9% to 97.9% similar to a Pseudoterranova ceticola cox2 sequence from a Caribbean Sea K. breviceps (GenBank accession number DQ116435). Blast searches with the ITS sequence revealed 99.6—100% identity to sequences from adult worms (found in kogid whales) or larvae (from marine fish and agnathans) identified as Anisakis sp. (see Supplementary file: Table S1, and further comments at discussion section). In addition, the ITS sequence of a P. ceticola larva from the Tanzanian fish Saurida undosquamis (ON128286)19 was 100% identical. Sequences of the presently identified P. ceticola L3 were deposited in GenBank with the accession numbers (ITS: OP352234–OP352246) and (cox2: OP380493–OP380510) (see also Supplementary file: Table S2).

Phylogenetic analyses. Phylogenetic analyses were performed on ITS rDNA and mt DNA cox2 datasets. In the cox2 BI tree, adult P. ceticola from K. breviceps (DQ116435), larva from the fish S. undosquamis (ON155434), larva from the fish Katsuwonus pelamis (LC712860) and the sequences of the present Terranova-like larvae group together in a well-supported clade, representing a sister group to a clade with Skrjabinisakis physteris, S. brevispiculata and S. paggiae (until recently belonging to the genus Anisakis) (see20,21) and related sequences (Fig. 4 and see also Figure S1 at Supplementary files which details the intraspecific variations and relationships of P. ceticola). The major clade (Clade A) with these two subclades is a well-supported sister group to a clade containing the A. simplex complex (A. simplex (s.s.)), A. pegreffii, A. berlandi), Anisakis typica, Anisakis nascettii, Anisakis ziphidarium and Pseudoterranova spp. from pinnipeds (P. azarasi, P. bulbosa, P. cattani, P. decipiens (s.s.) and P. krabbei) (Clade B).

The unrooted tree obtained based on the ITS region sequences also supported Clade A and its two sub-clades (Fig. 5). Again, the pinniped Pseudoterranova spp. grouped separately. Also, the sequences of the present Terranova-like larvae grouped with larval and adult genotypes of worms identified as belonging to genus Anisakis, from fish and kogid whales. These included worms from an Australian K. breviceps and a Philippine K. sima, a larva from the teleost fish Pagellus bogaraveo from Madeira and from the fish Epinephelus areolatus from Indonesia.

Discussion
Genus Terranova was erected for a New Zealand shark parasite, T. antarctica22,23. Later additions of Terranova spp. represent further parasites from elasmobranchs, but also parasites from teleosts, crocodilians, colubrid snakes and marine mammals (reviewed by4). Attempts have been made to split the genus, and now the best-known species from poikilotherms (i.e. elasmobranchs, teleosts and reptiles) are allocated to genera Euterranova, Neoterranova or Pulchrascaris4. Several lesser known species are retained in Terranova sensu lato (species inquirenda, see4). Genus Phocanema was proposed for Porrocaecum decipiens24, a Terranova-like pinniped parasite that subsequently was shown to represent several cryptic species (see25). A new genus, Pseudoterranova26, was proposed for Terranova kogiae from an Australian kogid whale Kogia breviceps27, based on an erroneous interpretation

| Measurement         | N  | Mean | SD  | Min–Max |
|---------------------|----|------|-----|---------|
| Total length (mm)   | 27 | 9.1  | 1.1 | 7.0—11.7|
| Maximum width       | 31 | 319  | 42  | 217—385 |
| Oesophagus length   | 28 | 978  | 126 | 803—1472|
| Ventricle length    | 28 | 485  | 79  | 385—703 |
| Ventricle width     | 26 | 154  | 25  | 109—199 |
| Caecum length       | 27 | 360  | 98  | 197—637 |
| Tail length         | 30 | 200  | 20  | 155—237 |

Table 4. Measurements of P. ceticola L3 from seven Macaronesian meso- and bathypelagic fish species. N = number of measurements, SD = Standard deviation. Measurements in µm unless specified.
Figure 4. Phylogenetic tree from Bayesian inference based on cox2 sequences. A: clade A; B: clade B. Figure 4 was created in Figtree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Figure 5. Unrooted tree from Bayesian inference based on ITS sequences. Figure 5 was created in Figtree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).
found to survive in and penetrate the stomach wall of rats, demonstrating a zoonotic potential. This suggests
respectively, whereas the mean ± SD (range) for
were reported as 44 ± 7 and 48 (42–54), 36  ± 3 and 35 (31–43), 47  ± 5 and 48 ± 7, 40 ± 9 (26–62) and 49 ± 3 mm
around the Hawaii islands (see Bloodworth and Odell). Pseudoterranova
appear to show a caecum equal or longer than the ventricle, even in juvenile specimens of
P . ceticola
reported
in Australia. *Reported as third larval stage.

| Designation          |  P . ceticola (this study) | Terranova type HA | P . ceticola | P . cf. ceticola* | Pseudoterranova sp. |
|---------------------|---------------------------|-------------------|-------------|------------------|---------------------|
| Host                | See Table 1               | Aprion virencus   | K. pelamis  | K. breviceps     | K. breviceps and     |
|                     |                           |                   |             |                  | Grampus griseus     |
| Place               | Macaronesia               | Hawaii            | Japan       | Yucatan          | Japan               |
| Body L              | 7.0–11.7 (9.1)            | 7.0–11.0          | 5.9         | 10.1–13.6        | 6.45–9.00           |
| Oesophagus L        | 0.80–1.47 (0.98)          | 0.70–1.50         | 1.30–1.40   | 0.89–1.02        | 0.87–1.45 (1.12)    |
| Ventricle L         | 0.39–0.70 (0.49)          | 0.42–0.57         | 0.38        | 0.40–0.50        | 0.40–0.58           |
| Caecum L            | 0.20–0.64 (0.36)          | 0.30–0.52         | 0.49        | 0.50–0.60        | 0.36–0.53           |
| Tail L              | 0.16–0.24 (0.20)          | 0.11–0.28         | 0.16        | 0.10–0.20        | 0.11–0.15           |
| Reference           | Present study             |                   |             |                  | 0.09–0.20 (0.14)    |

Table 5. Comparison of P. ceticola L3 with other similar larvae (range and mean in mm) and immature worms from kogid whales. *Reported as third larval stage.

of the excretory pore position. Gibson corrected this error, but retained and redefined Pseudoterranova on the basis of the anterior extent of the glandular left filament of the excretory system. This genus now contains all the Terranova-like species from homeotherms, P. kogiae and P. ceticola from whales, and several species from pinnipeds, with Phocanema as a synonym.

The presently described nematode larvae were all morphologically similar, thus resembling a single morphotype. They show morphological characters shared by genera Terranova sensu lato, Pseudoterranova and Pulchrascaris, such as an excretory pore at the base of the ventral lip and the presence of an intestinal caecum. Recently, Terranova sp. type 1 and 2 larvae sensu Cannon from teleost paratenic hosts have been molecularly found to include Pulchrascaris australis and Terranova pectinolabiata (now Euterranova pectinolabiata) from Australian shark definitive hosts, respectively. Gonzalez-Solis et al. used morphology alone to identify Terranova type 1 larvae from Hawaiian fishes as Pulchrascaris sp. In general, larvae of Pulchrascaris spp. and Terranova spp. that are present in teleost paratenic hosts and mature in elasmobranchs, show very long and slender ventricles, alongside an even longer caecum, and they have conical pointed tails without mucron. Those larvae described in the present study however have shorter more oval ventricles, accompanied by caeca that rarely exceed the ventricle in length. These characteristics are shared with larvae of Pseudoterranova spp. from seals and sea lions. However, the tails of the Pseudoterranova spp. larvae of pinnipeding infecting species differ from those of P. ceticola in being generally short and rounded with a mucron. In addition, the boring tooth inclines ventrally and is prominent, different to those present in Pseudoterranova spp. from pinnipeds which are straighter and comparatively smaller. The larvae presented in this study also appear to be considerably smaller in size, reaching only up to 12 mm in body length, compared to P. decipiens s.l. (10–60 mm), P. cattani (17–43 mm) or P. decipiens sp. E (20–38 mm). A most prominent character that distinguish the present larvae from pinnipeding Pseudoterranova spp. is the circumceral cuticular ridge connected with the boring tooth. This character also distinguishes them from most Terranova sensu lato larvae, that may represent elasmobranch parasites. However, Deardorff et al. found a Terranova larval type in Hawaiian teleosts, designated Terranova sp. type HA. Those larvae fit the larvae presented in here in most aspects including caecum:ventricle length ratio, tail shape and the presence of an anterior circumoral ridge (Table 5). The Terranova sp. type HA larvae were found to survive in and penetrate the stomach wall of rats, demonstrating a zoonotic potential. This suggests that they mature in a homeotherm, i.e., belong in genus Pseudoterranova. Similarly, Kuramochi et al. described Pseudoterranova cf. ceticola larvae recovered from stranded Japanese K. breviceps and the Risso’s dolphin (Grampus griseus) (see Table 5), and those larvae fit in most aspects the present larvae and the Terranova sp. type HA of Deardorff et al., thus suggesting to be conspecific. Most recently, a single P. ceticola larva from the scombrid fish K. pelamis in Japan was briefly described (see Table 5). The larva, even though smaller, resembles in most aspects to our larva, however, authors did not refer to the circumoral cuticular ridge which is apparent in their Fig. 1A as the most prominent character of P. ceticola L3.

The present larvae are molecularly identified as Pseudoterranova ceticola, a parasite of kogid whales. That finding suggests that the Terranova sp. type HA larvae are also likely P. ceticola larvae. Kogia spp. are common around the Hawaii islands (see Bloodworth and Odell). Pseudoterranova ceticola was originally described from Kogia sima stranded in Mississippi, USA. Another species, P. kogiae was described from K. breviceps in Australia. Pseudoterranova kogiae differs from P. ceticola by having more pairs of caudal papillae present in the males and by having three transverse rows of plectanes which are absent in P. ceticola. These species appear to show a caecum equal or longer than the ventricle, even in juvenile specimens of P. ceticola reported from the final hosts (Table 5). Therefore, the relationship between caecum length and ventricle length in larvae from intermediate or transport hosts may not reflect the adult morphology, contrary to the arguments of Gonzalez Solis et al. In addition, these species from kogid whales are small compared to adult males maturing in pinnipeds. Pseudoterranova ceticola and P. kogiae ranged between 12 and 26 mm and 20–30 mm long, respectively, whereas the mean ± SD (range) for P. decipiens s.s., P. krabbei, P. bulbosa, P. cattani and P. azarasi were reported as 44 ± 7 and 48 (42–54), 36 ± 3 and 35 (31–43), 47 ± 5 and 48 ± 7, 40 ± 9 (26–62) and 49 ± 3 mm long, respectively.

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The present identification relies on the high identity of the cox2 sequences with the reference sequence of *P. ceticola* collected from a Caribbean Sea *K. breviceps* 53. Similarly, several *P. ceticola* collected from *K. breviceps* and *K. sima* stranded in Puerto Rico and Florida (USA) were morphologically and/or molecularly identified by sequencing analysis of the mtDNA cox2 54,55, so the association of this genotype with *P. ceticola* should not be in doubt. However, the herein presented ITS1-5.8S-ITS2 sequences from the same *P. ceticola* larvae specimens showed 100% identity to sequences from some worms identified as *Anisakis* sp. 56–60 (see Table S1 at supplementary file). It is probable that this occurred due to the lack/impossibility of a morphological examination (probably hampered by a poor condition of worms, hence it might be overlooked (per. obs., see also 61). The diagnostic RFLP pattern of *P. ceticola* with the HhaI restriction enzyme produce 4 bands, of about 80, 180, 200 and 400 bp length, and a single undigested band of about 1000 bp with Hinfl 75,62. However, this pattern has also been obtained with worms that may have been erroneously identified as *Anisakis* sp. 57–59,63–65, leading to confusion. A list of the anisakids likely representing *P. ceticola* but identified as *Anisakis* sp. is provided in the Supplementary file Table S3.

Clearly, one reason for this confusion is the lack of a morphological account of *P. ceticola* larvae, which we provide here. However, trustable sequence information from *P. kogiae* is not available. Either *P. kogiae* and *P. ceticola* are two valid species, or they are synonymous. Gibson examined the types of *P. kogiae* and also had at hand specimens of *P. ceticola* from South African *K. breviceps*, and seems to consider them valid species 66. Shamsi et al. 52 provided an ITS region sequence (MK325217) of an “Anisakis” sp. from an Australian *K. breviceps*, interestingly being the same type host and geographical area where *P. kogiae* was described 72. The sequence was considerably different to our *P. ceticola* sequences by having a unique 3 bp insert plus 2 substitutions (see also Fig. S). Clearly, more research is necessary in order to clarify this relationship between these *Pseudoterranova* spp.

Phylogenetic analysis obtained from the mtDNA cox2 sequences, suggests that the clade with *P. ceticola* sequences is closely related to a clade formed by *S. p aggiae*, *S. cf. p aggiae*, *S. brevispiculata*, *S. physeteris* and *Skrjabinisakis* sp. 2 (species which until most recently belonged to the genus *Anisakis* 56,21). The BI analysis of the ITS region also supports that *P. ceticola* is closely related to the former *S. physeteris* complex clade. These results are similar to those by Cavallero et al. 28 and most recently to those by Takano & Sata 31. The monophyly of the clade formed by *P. ceticola* and *S. p aggiae*, *S. brevispiculata* and *S. physeteris* was suggested by Cavallero et al. based on phylogenetic analyses of the cox2 and ITS regions, as found here. However, Takano & Sata found the species *Neoterranova caballeroi* from reptiles as the most related species to *P. ceticola* which raises concerns 31. We have performed an additional BI analysis including the cox2 sequence of *N. caballeroi* (AF179921), and *N. caballeroi* was placed as an offshoot of *Anisakidae* species (i.e. *Anisakis, Skrjabinisakis* and *Pseudoterranova*), which we believe is more congruent with the ecology and morphology of this parasite (see figure S2 at Supplementary files).

In this study, *P. ceticola* was collected from fishes which distributions span the meso- and bathypelagic zones, caught off Cape Verde, Canary Islands and Madeira. *Pseudoterranova ceticola* larva in the deep-water shark *Centrophorus squamosus* taken off Madeira was also reported by Costa et al. 52, who identified it by ITS-RFLP but provided no sequence information. Recently, Cipriani et al. identified a single *P. ceticola* larva from the reef-associated fish *S. undosquamis* caught between 100–600 m depth off the coast of Tanzania, by sequencing analysis of the ITS rDNA and mtDNA cox2 sequences 9. Most recently, a single *P. ceticola* larva from the scombroid fish *K. pelamis* was also identified in Japan 31. It appears possible that the former fish species, *i.e.* *C. squamosus, S. undosquamis* or *K. pelamis*, could have acquired *P. ceticola* though predation upon parasitized meso/bathypelagic fish.

It appears then that *P. ceticola* may have different host specificity depending on life stages, being a host specialist in the final host (i.e. kogiid whales), and generalist in the second intermediate or paratenic host (i.e. fishes). Adult *P. ceticola* has only been found in kogiid whales (i.e. *K. sima* and *K. breviceps*) suggesting stenoxeny at the final host level. *Pseudoterranova ceticola* was reported in *K. sima* from the Gulf of Mexico, Japan, Caribbean and SE Atlantic coasts of USA, and from *K. breviceps* in the same geographical region (presumably only as larvae in Japan), Atlantic Canada, NW Spain and South Africa 28,42,44,47,53–55,66–68. In addition, *Pseudoterranova* sp. (as *Terranova* sp.) has been reported in *K. breviceps* from Brazil, Pacific Gulf of California (Mexico) and France, and in *K. breviceps* and *K. sima* from the Caribbean region 45,66–70. *Pseudoterranova* cf. *ceticola* L3 has been reported from two Japanese *G. griseus* and *Pseudoterranova* sp. has been reported from Caribbean pygmy killer whale (*Feresa attenuata*) 72,68.

Thus, *P. ceticola* has so far been found in temperate waters of western and eastern Atlantic and Pacific Oceans (see above and Table S3), a distribution apparently overlapping that of its final kogiid hosts 1. In addition, kogiids have been reported stranded or observed in Macaronesia areas 46,71–73. Contrarily, *Pseudoterranova* spp. from pinnipeds are mainly distributed in Boreal and Austral waters where their final hosts thrive 74. Similarly, several *P. ceticola* have been reported stranded or observed in Macaronesia areas 46,71–73. Contrarily, *Pseudoterranova* spp. from pinnipeds are mainly distributed in Boreal and Austral waters where their final hosts thrive 74.

Our results suggest that the life cycle of *P. ceticola* occur in the mesopelagic and bathypelagic realm. In addition, the parasite also appears to occur in benthopelagic, demersal and even reef-associated fish hosts (see Table S3). Contrarily, primarily neritic, benthic and demersal fishes appears to be involved in transmitting *Pseudoterranova* spp. to pinnipeds 36,37,46–77. Indeed, it has been observed that *P. secicola* sp. larvae hatched from eggs adhered to the substrate by their tails 76,78, being eaten by benthic meiofauna (mainly copepods) first intermediate hosts leading to transmission up a benthic food-web 76,79.

There are some studies which have indicated that meso- and bathypelagic fish are prey for *K. breviceps* and *K. sima* 46,72,80. West et al. 80 analysed the stomach content of stranded *K. breviceps* of the Hawaiian archipelago and identified among others *D. argenteus, Diaphus* sp. and *E. plecanoides*, which are species that were found infected by *P. ceticola* in the present study. The parasite might also be transmitted through the food web from mesopelagic fish to squids (and other fishes) and then to the whales, since mid and deep-water cephalopods are also known as a very important part of the diet of these kogiids. 71,72,80.
Conclusions

_Pseudoterranova ceticola_ third-stage larva (L3) was herein fully described for the first time. The parasite was recovered from meso- and bathypelagic fishes from off Macaronesia archipelagos (NW Africa). L3 were small, pale, with a thick-set appearance and bluish when exposed to UV-light after thawing. Ventricles morphology, presence of a caecum, tail shape and the presence of a circumoral cuticular ridge extending dorsally from boring tooth are morphological characteristics that aid identification. _Pseudoterranova ceticola_, which has kogiid (Physeteroidea) whales as final hosts, is related to _Skjærbønnsakis_ spp. (whose species formerly belonged to the genus _Anisakis_) maturing in physeteroide whales, rather than to _Pseudoterranova_ spp. from pinnipeds. This is evidence that genus _Pseudoterranova_ may have to be split.

Data availability

Data supporting the conclusions of this article are included within the article and its supplementary files. The DNA sequences of the _P. ceticola_ specimens identified were deposited in the public sequence repository GenBank (NCBI National Center for Biotechnology Information—https://www.ncbi.nlm.nih.gov/genbank), and their accession numbers can be found in the present manuscript (Table 2 and 3) and/or supplementary files (Table S2).

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supervising the molecular analyses. E.G.S. participated in sample collection and identification of the fish species. M.B. and E.K. analysed and interpreted the data and wrote the original manuscript. L.G. and J.E.S. helped K.M.O., who carried out the parasite sampling, morphological and molecular identification, and took parasite images. M.B. and E.K. were involved in the conception and design of the study. M.B. and E.K. supervised the cruise. We acknowledge the projects HARMES (Research Council of Norway project number 280546) and MEESO (EU H2020 research and innovation programme, Grant Agreement No 817669) for supporting the sampling collection.

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Author contributions
M.B., A.L., P.C. and E.K. were involved in the conception and design of the study. M.B. and E.K. supervised K.M.O., who carried out the parasite sampling, morphological and molecular identification, and took parasite images. M.B. and E.K. analysed and interpreted the data and wrote the original manuscript. L.G. and J.E.S. helped supervising the molecular analyses. E.G.S. participated in sample collection and identification of the fish species. All authors reviewed and contributed to the manuscript and approved its final version.

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Competing interests
The authors declare no competing interests.

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