International Journal for Parasitology: Parasites and Wildlife 13 (2020) 160–170

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Ventral dermatitis in rowi (*Apteryx rowi*) caused by cutaneous capillariasis

Adrienne F. French [a,*], Fernanda Castillo-Alcala [a], Kristene R. Gedye [a], Matthew A. Knox [a], Wendi D. Roe [a], Brett D. Gartrell [a]

[a] School of Veterinary Science, Massey University, Private Bag 11- 222, Palmerston North, New Zealand

ARTICLE INFO

Keywords:
Kiwi
Apteryx
Cutaneous nematodiasis
Capillaria
Eucoleus

ABSTRACT

In 2013 there was an outbreak of crusting ventral dermatitis among a group of juvenile rowi (*Apteryx rowi*), a species of the endangered New Zealand kiwi, that were being raised on an off-shore island sanctuary. Biopsies taken at the time found nematodes migrating within the epidermis of affected skin but the specific identity and origin of the organisms was not established, and sporadic cases of similar skin disease continue to occur on the island. On examination of additional sections from the original skin biopsies, adult nematodes and eggs were identified, the histomorphology of which was consistent with *Capillaria sensu lato*. PCR was performed on DNA extracted from archived formalin-fixed, paraffin-embedded tissue blocks of skin from eight affected rowi, using primers targeting the 18S region of nuclear ribosomal DNA and the COI gene of mitochondrial DNA of capillarid nematodes. The 18S sequences from all rowi samples were identical and matched sequences from members of the genus *Eucoleus*. In contrast, two distinct capillarid COI sequences were obtained, in one case both from the same rowi skin biopsy. While there were no close matches, both COI sequences also aligned nearest to sequences identified as *Eucoleus* spp. It is considered unlikely that two different nematode species are involved in the rowi skin lesions and the possible amplification of a COI pseudogene or "numt" is discussed. A species-level identification of the capillarid nematodes causing skin disease in rowi was not obtained, however based on histological evaluation the infections include reproductively-active adult nematodes. This finding indicates the possibility of perpetuation of the skin disease in the absence of the original source, as well as raising potential for the transfer of infection from the island when the juvenile rowi are translocated to their new habitats.

1. Introduction

The kiwi family (Apterygidae) comprises five recognised species that occupy different geographic ranges within New Zealand. Of these, rowi (*Apteryx rowi*, also known as Okarito brown kiwi) were the most recently confirmed as a distinct species through phylogenetic testing (Burbidge et al., 2003; Tennyson et al., 2003). In 2018, rowi were estimated to number around 600 and were classified by the New Zealand Department of Conservation (DOC) as "threatened: nationally vulnerable", up-graded from their initial classification of "nationally critical" as a direct result of successful conservation management programmes (Robertson et al., 2016; Germano et al., 2018). The IUCN Red List (BirdLife International, 2017) also classifies rowi as "vulnerable" with an increasing population trend; at the present time however, their continued recovery remains conservation dependent.

Prior to the arrival of humans and predatory mammals to New Zealand, the distribution of rowi is believed to have included the northwest coast of the South Island and the southern tip of the North Island (Germano et al., 2018). At its lowest numbers, the extant population was restricted to a small area of lowland forest at Okarito on the west coast of the South Island, but now includes some newly established populations in the Omoeroa ranges and on two predator-free offshore islands, following translocations of birds as their numbers have slowly increased. Rowi have more than doubled in population size since the Operation Nest Egg (ONE) programme, managed by DOC, was initiated in 1994 (Robertson et al., 2016). In brief, eggs are located and removed from their burrows in the wild, artificially incubated and hatched, and the chicks raised in predator-free sanctuaries known as crèches. Once they reach a size where they are deemed to have a greater chance of survival against predators, they are released back into the wild (Colbourne et al., 2005).

In the case of rowi, ONE chicks are raised on a single off-shore crèche

* Corresponding author.
E-mail addresses: A.French@massey.ac.nz (A.F. French), F.Castillo-Alcala@massey.ac.nz (F. Castillo-Alcala), K.Gedye@massey.ac.nz (K.R. Gedye), M.Knox@massey.ac.nz (M.A. Knox), W.D.Roe@massey.ac.nz (W.D. Roe), B.Gartrell@massey.ac.nz (B.D. Gartrell).

https://doi.org/10.1016/j.ijppaw.2020.10.003
Received 9 August 2020; Received in revised form 11 October 2020; Accepted 12 October 2020
Available online 14 October 2020

island where they are intermittently monitored by DOC rangers. In 2013, 15 out of 30 juvenile rowi on the crèche island developed a crusting dermatitis affecting the ventral abdominal skin and vent margin (Gartrell et al., 2015). Histology of skin biopsies taken from several of the affected birds showed nematodes present within the epidermis, suggesting the condition cutaneous *larva migrans*. Molecular sequencing of nematode DNA extracted from the biopsies aligned among *Trichostrongylus* spp; however, the authors concluded that this result should be viewed with caution as concurrent morphological evaluation was limited and members of this nematode genus have not previously been associated with cutaneous migratory lesions (Gartrell et al., 2015).

The full investigation, management, and outcome of the 2013 outbreak of cutaneous nematodiasis in juvenile rowi have been described elsewhere (Gartrell et al., 2015). Sporadic cases of similar skin lesions have been diagnosed on the crèche island in the years since. The purpose of this study was to investigate further, through histomorphological and molecular evaluation, the identity of the nematodes causing skin disease in juvenile rowi.

2. Materials and methods

2.1. Case selection

A retrospective search of the School of Veterinary Science (SoVS) pathology database (Massey University, Palmerston North, New Zealand) was performed to identify cases of ventral dermatitis occurring in rowi in which nematodes were identified histologically in the lesional skin. Skin biopsies from the cohort of affected rowi taken during the 2013 investigation in which larval organisms were not identified in the original histological sections were also included in the initial selection. To obtain comparative sequences from capillarid nematodes previously identified histologically in New Zealand, a retrospective search was also performed to identify cases of oral or oesophageal/crop infection in kahu (Australasian harrier hawk, *Circus approximans*), as described by Alley et al. (2004).

Where sufficient tissue was available from the rowi samples, the majority of which were small skin biopsies, multiple "sandwich" sections were cut from the archived formalin-fixed, paraffin-embedded (FFPE) tissue blocks, comprised of a 4 µm section mounted on a slide and stained with Haematoxylin and Eosin (H&E), followed by a 10 µm tissue scroll for molecular analysis, a further 4 µm section mounted and stained with H&E, a further 10 µm tissue scroll, and a final 4 µm section mounted and stained with H&E. The original diagnostic slides (where available) and the three H&E-stained sandwich sections were examined for the presence of nematodes for the purposes of describing histomorphology of the organisms and the associated histopathology, as well as to assess for the probability of nematode DNA being present in the tissue scrolls taken for molecular analysis. For the larger necropsy samples from kahu, a single 4 µm H&E stained section to confirm histological presence of organisms followed by a 10 µm tissue scroll for molecular analysis were taken from the archived FFPE blocks.

For use as a positive control, DNA from an unidentified capillarid nematode retrieved from the proventriculus of a red-billed gull (*Chroicocephalus novaehollandiae scopulinus*) originating from Otago, New Zealand, was supplied by Jerusha Bennett and Dr. Bronwen Presswell from the Department of Zoology, University of Otago, New Zealand. For use as an alternative positive control in the internal transcribed spacer (ITS)-2 PCR, a *Trichostrongylus axei* nematode was sourced from the intestine of a sheep (*Ovis aries*) at routine necropsy, identified morphologically by the Massey University parasitology laboratory (Palmerston North, New Zealand), and preserved in 70% ethanol prior to DNA extraction.

2.2. DNA extraction

DNA extraction was performed on rowi and kahu FFPE tissue scrolls using a commercial kit (NucleoSpin DNA FFPE XS, Macherey-Nagel, Germany) per the manufacturer's instructions, with the exception of the lysis step which was carried out in a 56 °C water bath overnight. For extraction of DNA from the *T. axei* positive control, a mix of 100 µl DirectPCR Lysis Reagent (Tail) (Viagen Biotech Inc., USA) and 2.5 µl Proteinase K solution (20 mg/ml, Ambion, CA, USA) was prepared and 10 µl of this solution added to a single nematode in a PCR tube. This was incubated in an Applied Biosystems GeneAmp PCR system 2400 thermocycler (Thermofisher, USA) for 16 h at 55 °C followed by 1 h at 90 °C.

2.3. Molecular analysis

Primers used for molecular analysis are presented in Table 1 and include (sourced from literature) a primer set designed for the gender identification of kiwi tissue (Huynen et al., 2003); a primer set targeting the ITS-2 region of nuclear ribosomal DNA (rDNA) of nematodes, as used in the previous investigation (Gasser et al., 1993; Gartrell et al., 2015); and a primer set targeting the 18S region of rDNA of capillarid nematodes (Fischer et al., 2018).

Several literature-sourced primer sets targeting the cytochrome oxidase *c* subunit I (COI) gene of mitochondrial DNA (mtDNA), which had been used successfully on trichurid and/or capillarid nematodes of mammals (Zhu et al., 2000; Di Cesare et al., 2012; Guardone et al., 2013), were also trialled with no result, or with production of sequences aligning to avian, insect, or bacterial DNA. Only a single forward sequence that aligned most closely to a capillarid nematode sequence (*Eucoleus boehmi*, KX027312) was obtained using the primer set JB3-JB4.5 (Zhu et al., 2000) on one case of crop capillariasis from a kahu (#11). As no COI sequences originating from avian *Eucoleus* species were available in the National Center for Biotechnology Information database (GenBank Internet, 1982) at the time of investigation, this sequence was used in the design of a degenerate COI primer set (Eu COI F1B–Eu COI R1) for the purpose of this study. The primers were created manually using Geneious v. 10.2.3 (Kearse et al., 2012) following alignment of COI sequences from the mammalian capillarids *Eucoleus aerophilus* and *E. boehmi* available in GenBank (KC341988 to KC341992, KR186213 to KR86215, and KX027311 to KX027314), along with the single forward sequence obtained from the kahu.

For all primer sets, each PCR contained 1X HOT FIREPol Blend Master Mix (10 mM $MgCl_2$, Solis Biodyne, Estonia), 300 nM each of forward and reverse primers (IDT, IA, USA), and 1 µl of template DNA, made to a total of 20 µl with nuclease free water. A negative (blank) control containing 1 µl of nuclease free water in place of template DNA was run simultaneously. PCR was repeated on all rowi samples with the capillarid 18S primers, and on all positive rowi and kahu samples with the capillarid COI primers, in order to confirm sequences.

PCR was performed on a Labcycler (SensoQuest, Germany) using a touchdown PCR protocol. For the kiwi gender, ITS-2, and 18S primer sets, the following conditions were used: initial activation of 15 min at 95 °C, 12 cycles of 95 °C for 30 s, annealing for 30 s (starting at 60 °C, reducing by 0.5 °C per cycle), and 72 °C for 30 s, followed by a further 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and final elongation at 72 °C for 7 min. For the COI primer set a similar touchdown PCR protocol was used but with lower annealing temperatures, starting at 51 °C and reducing by 0.5 °C per cycle for 12 cycles, then 35 cycles at the final annealing temperature of 45 °C.

The PCR product was separated by electrophoresis on a 1% w/v agarose gel (Bioline, UK) using RedSafe (iNtRON Biotechnology, South Korea) to stain the DNA, and visualized with a gel image system (MultiDoc-It Imaging System, UVP, CA, USA). Size of PCR products was estimated in comparison to a HyperLadder™ 100 bp molecular ladder (Bioline, UK).

2.4. Sequencing and BLAST analysis

Amplicons of the appropriate size were cut from the gel, eluted

Table 1
Primer sets used in this study, including one designed for the gender identification of kiwi, and others targeting the ITS-2 region or 18S gene of nuclear ribosomal DNA and the COI gene of mitochondrial DNA of nematodes.

Primer name	Primer sequence (5′-3′)	Target	Approximate amplicon size (bp)	Reference
W5 W7	AATCACCCTTTAAACAAGCTGTTAAAGCAA CCTTTCTCAAATCTCTCTTTTGTTCTAGACAC	Uncertain – Kiwi W-linked and Z-linked or autosomal	350 (males and females) ±200 (females only)	Huynen et al. (2003)
NC1 NC2	ACGTCTGGTTCAGGGTTGTT TTAGTTTCTTTTCCTCCGCT	Nematode ITS-2 region	330	Gasser et al. (1993)
Kt875351.1 Capillaria 18S1R	CCCTAGTTGCGACTTTAAACGA TCCACCAACTAAGAACGGCC	Capillarid 18S gene	290	Fischer et al. (2018)
JB3 JB4.5	TTTTTTGGGCATCCTGAGGTTTAT TAAAGAAAGAACATAATGAAAATG	Capillarid COI gene	450	Zhu et al. (2000)
Eu COI F1B Eu COI R1	GGTCCWYTAGGWATAATYTATGC ARATCTAAAGATGCATTRGAAAG	Capillarid COI gene	290	Designed for this study

overnight in elution buffer (10 mM Tris-HCl, pH 8.0), and the eluate submitted to the Massey Genome Service (Massey University, Palmerston North, New Zealand) for bi-directional Sanger sequencing. The resultant forward and reverse sequences were aligned using Geneious v. 10.2.3; where identical sequences were obtained from repeat PCR on individual template DNA, these were all aligned together. The alignments were manually trimmed and the resultant sequences subjected to BLAST (Basic Local Alignment Search Tool) analysis. Only those sequences from capillarids identified to species level are considered in the BLAST results discussion.

Nucleotide sequences obtained in this study have been deposited in GenBank under the accession numbers MT678491 to MT678507 (18S) and MT782133 to MT782145 (COI).

2.5. Phylogenetic analysis

To further assess the relationship between the rowi, kahu, red-billed gull, and published capillarid sequences, phylogenetic trees were created. To minimise the inclusion of potentially misidentified sequences in the GenBank database, as well as to exclude sequences that lacked significant cover of the study sequences, the top 100 discontiguous megaBLAST results for the 18S and COI sequences obtained in this study were filtered to include only sequences identified as from the nematode families Capillariidae or Trichuridae, giving 99 sequences included in the 18S analysis, and 47 sequences in the COI analysis. As only a small cluster of four unidentified *Trichuris* sp. sequences were incorporated into the COI tree using this methodology, four further COI sequences originating from identified *Trichuris* species were selected from GenBank and included in the COI analysis, in order to cover a similar taxonomic scope as the 18S analysis. A sequence originating from *Enoplus* species was used in each case as an outgroup. Sequences were aligned using MAFFT (Katoh and Standley, 2013) employing the E-INS-i algorithm in Geneious v. 10.2.6 (Kearse et al., 2012) resulting in alignments of 293 and 261 nucleotides for 18S and COI respectively. For both alignments, maximum likelihood analysis was performed using PhyML version 3.0 (Guindon et al., 2010), available on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/). Phylogenetic trees were inferred employing Subtree Pruning and Regrafting (SPR) branch-swapping and nucleotide substitution models determined by Smart Model Selection (SMS) (Lefort et al., 2017). Branch support was assessed using an approximate likelihood ratio test (aLRT) with the Shimodaira–Hasegawa-like procedure. The 18S tree was produced using the Kimura 80 model with invariable sites and gamma distribution (K80 + G [0.87] + I [0.5]). The COI tree was produced using a general time-reversible model with invariable sites and gamma distribution (GTR + G [0.208] + I [0.159]). Tree visualization was performed with Interactive Tree Of Life (https://itol.embl.de) (Letunic and Bork, 2019).

3. Results

3.1. Cases

Ten (nine biopsy and one necropsy) cases of ventral dermatitis in rowi were identified in the database, diagnosed between 2013 and 2018. Of these, eight had confirmed nematode organisms identified histologically in the original diagnostic sections, while two that were lacking histologically identifiable nematodes were initially included as they were biopsies taken from the same cohort confirmed to be affected by cutaneous nematodiasis in the 2013 investigation. One of these two cases was subsequently excluded as there was insufficient tissue remaining in the FFPE block for molecular analysis. Of the nine remaining cases, seven had confirmed nematode organisms identified in histological sections both before and after the two tissue scrolls taken for molecular analysis, including the one remaining biopsy case that had been negative for organisms in the original diagnostic section. Conversely, one biopsy that had confirmed organisms in the original diagnostic section was negative in all sandwich sections and was subsequently excluded from molecular analysis. The final biopsy (rowi #8), which did contain nematode organisms in the original diagnostic section, had insufficient tissue for sandwich histology sections but remaining tissue was sufficient for DNA extraction and molecular analysis (Table 2).

Additional to the skin disease, one necropsy case from the affected 2013 rowi cohort also had a single larval nematode identified histologically in the liver; unfortunately, the organism was not present in histological sandwich sections from around the scroll taken for DNA extraction, and so molecular analysis was not performed on this tissue.

Thirteen cases of oral or crop capillariasis were identified in kahu, diagnosed between 2002 and 2010, originating from around the central North Island. In one case the archived H&E slides and FFPE blocks could not be located. Eleven of the remaining cases (eight from oral cavity and three from crop) had confirmed presence of capillarid organisms present histologically in H&E-stained sections adjacent to the tissue scroll taken for DNA extraction and were included in the nematode molecular evaluations (Table 2).

3.2. Histology

In areas of affected skin, the epidermis was mildly to moderately hyperplastic, typically characterised by papillary hyperplasia of the basal epidermis, acanthosis, and compact to laminar orthokeratotic hyperkeratosis. In some sections there was mild to moderate spongiosis, multifocal small intra-epidermal pustules, and/or superficial serocellular crusting. There were mild to moderate superficial dermal perivascular to interstitial infiltrates of lymphocytes and granulocytes (presumably eosinophils), and frequent exocytosis of lymphocytes and granulocytes into the overlying epidermis. Multifocal nodular lymphoid

Table 2
Case information and PCR results for all samples tested in this study. Case information includes: sample type (biopsy and necropsy samples both in the form of archived formalin-fixed, paraffin-embedded tissue blocks); year the sample was taken; anatomic origin of the sample within the host; and geographic origin of the host within New Zealand. For PCR results: F = PCR positive female; - = negative result; + = positive result (produced amplicon of appropriate size, confirmed by sequencing); A or B = rowi or kahu capillarid COI sequences A or B as produced on duplicate PCR runs.

Case information					PCR results (approximate size of target (bp))			
Source ID	Sample type	Year	Sample origin	Geographic origin	Kiwi gender (350 (M&F)± 200 (F))	Nematode ITS-2 (330)	Capillarid 18S (290)	Capillarid COI (290)
Rowi #1	Biopsy	2013	Skin	Crèche island	F	–	+	+A/A
Rowi #2	Biopsy	2013	Skin	Crèche island	F (200 only)	–	+	–
Rowi #3	Biopsy	2013	Skin	Crèche island	F	–	+	+A/B
Rowi #4	Biopsy	2013	Skin	Crèche island	–	–	+	+B/-
Rowi #5	Biopsy	2013	Skin	Crèche island	F	–	+	+A/A
Rowi #6	Necropsy	2013	Skin	Crèche island	F	–	+	+A/A
Rowi #7	Biopsy	2015	Skin	Crèche island	F	–	+	+A/A
Rowi #8	Biopsy	2018	Skin	Crèche island	F	–	+	+B/B
Kahu #1	Necropsy	2010	Oral	Hawke's Bay			–	–
Kahu #2	Necropsy	2007	Crop	Manawatu			+	+A/A
Kahu #3	Necropsy	2005	Oral	Manawatu			+	–
Kahu #4	Necropsy	2003	Oral	Wellington			–	–
Kahu #5	Necropsy	2003	Oral	Manawatu			+	+A/A
Kahu #6	Necropsy	2003	Oral	Whanganui			+	+A/A
Kahu #7	Necropsy	2002	Oral	Manawatu			+	+A/-
Kahu #8	Necropsy	2002	Oral	Whanganui			+	+A/A
Kahu #9	Necropsy	2002	Crop	Manawatu			+	+B/B
Kahu #10	Necropsy	2002	Oral	Manawatu			–	–
Kahu #11	Necropsy	2002	Crop	Manawatu			+	+B/B
Red-billed gull	Nematode DNA	2019	Proventriculus	Otago		–	+	+

aggregates were present in the mid to deep dermis (Fig. 1A). Two biopsies included junction of skin and cloacal mucosa, with some focal erosion of the mucosal epithelium and mild to moderate infiltration of the submucosa by lymphocytes and granulocytes.

Nematode sections were predominantly located within the epidermis, less commonly within the superficial keratin or appearing free over the skin (or, in one case, the cloacal) surface. In the single post-treatment (necropsy) case included in the study, one nematode was present at or just underlying the dermo-epidermal junction, but dermal localisation was otherwise not a feature. Both male and female adult nematodes were identifiable, as well as some suspected larval forms. Occasional free eggs were identified within the epidermis or superficial keratin. Cross-sectional or near cross-sectional diameters of adult nematodes ranged from ~50 to 75 μm through levels with identifiable sex organs, with no dramatic difference in size of males versus females noted. Musculature was coelomyarian and bilateral bacillary bands were apparent (Fig. 1B). Sections through the oesophageal region contained large cells with granular basophilic cytoplasm consistent with stichocytes (Fig. 1C). Eggs measured variably ~62–72 μm long by ~25–32 μm wide and were non-embryonated with a thick shell and bipolar plugs (Fig. 1D).

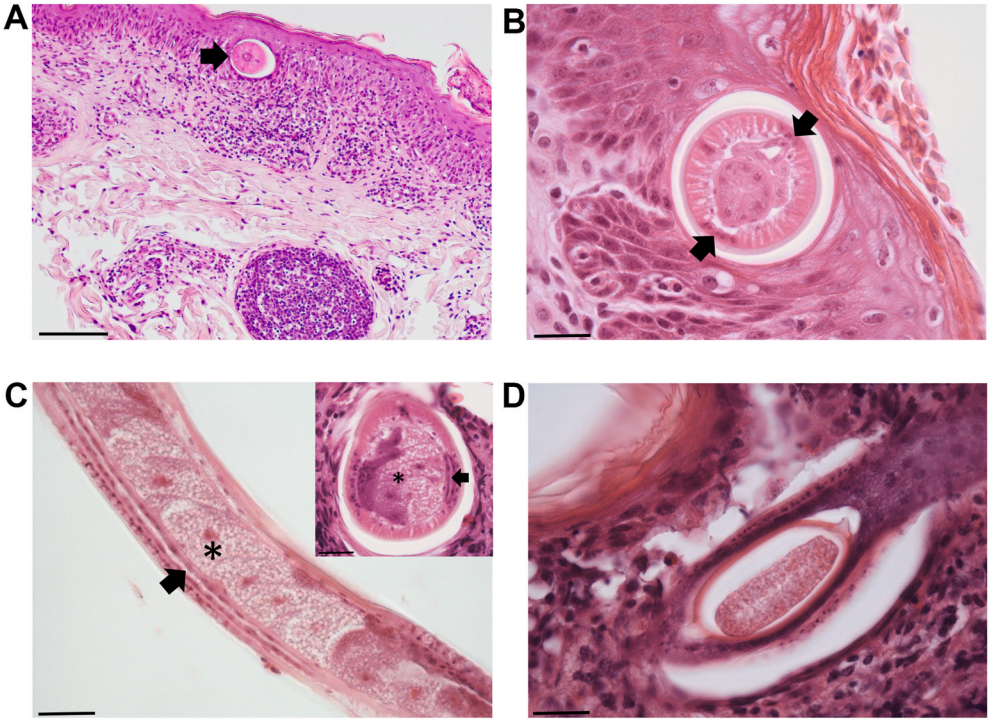

Fig. 1. Histological images of capillarid nematodes in rowi skin biopsies. 1A: Cross-section of a nematode within the epidermis (arrow), associated with epidermal hyperplasia and dermal inflammatory infiltrates (H&E, bar = 100 μm). 1B: Cross-section of a capillarid nematode within the epidermis, demonstrating low, bilateral bacillary bands (arrows) (H&E, bar = 20 μm). 1C: Longitudinal (main image) and cross-section (inset) of nematodes through the level of the oesophagus (arrow) and stichocytes (asterisk) (H&E, bar = 20 μm). 1D. Near-longitudinal section of a non-embryonated egg within a nematode present in the epidermis, demonstrating the typical barrel shape, thick shell, and bipolar plugs (H&E, bar = 20 μm).

3.3. Molecular analysis

3.3.1. Kiwi gender-specific primers

Seven out of eight extracts from the rowi samples produced appropriate amplicons using the kiwi gender-specific primers (W5–W7); all positives were consistent with females, although one (rowi #2) yielded only the 200 bp amplicon and not the 350 bp amplicon, suggesting overall lower quality DNA. The one extract that was negative (rowi #4) was reportedly a male, and so only a 350 bp amplicon would have been anticipated (Table 2).

3.3.2. Nematode ITS-2 primers

None of the eight rowi skin samples or the red-billed gull capillarid control DNA amplified with the ITS-2 primers (NC1-NC2). The *T. axei* positive control produced an amplicon of ~330 bp which on sequencing and BLAST analysis showed 100% homology with sequences from *T. axei* in GenBank (e.g. KC998725).

3.3.3. Capillarid 18S primers

All rowi extracts produced an amplicon of appropriate size (~290 bp) using the capillarid 18S primers (Kt875351.1-Capillaria 18S1R), including the sample that was negative with the kiwi gender-specific primers. Trimmed sequence lengths were 270–279 bases and on alignment had identical nucleotide sequences apart from a single degenerate base within the sequence from rowi #5. On BLAST analysis, the consensus of all eight sequences aligned 100% with GenBank sequences from a range of *Eucoleus* species identified as *E. aerophilus* (MF599385), *E. garfiai* (LC484432), *E. perforans* (LC424998), *E. boehmi* (JX456628), and *E. dispar* (EU004821), and additionally showed only a single base difference to a sixth *Eucoleus* sequence identified as *E. contortus* (LC424996) (Table 3).

Eight out of 11 kahu extracts produced an amplicon of the appropriate size using the capillarid 18S primers, with final sequence lengths of 239–287 bases. On alignment, seven of the eight had identical nucleotide sequences, with one sequence (kahu #3) containing a single base difference (repeat PCR to confirm was not performed). Alignment of the rowi capillarid 18S consensus and the consensus of all eight kahu capillarid 18S sequences was identical, and BLAST results were therefore very similar (Table 3).

The control capillarid DNA extraction derived from a red-billed gull also produced an amplicon of the appropriate size using the capillarid 18S primers, with a final sequence length of 287 bases that showed a single base different to the rowi and kahu capillarid 18S consensus sequences, confirmed on repeat sequencing; this was at a different position to the base substitution within the sequence from kahu #3. On BLAST analysis, the seagull capillarid 18S sequence aligned with 100% pairwise identity to the sequence from *E. contortus* (LC424996) (Table 3).

3.3.4. Capillarid COI primers

Seven out of eight rowi extracts produced amplicons of an appropriate size (~290 bp) using the capillarid COI primers (Eu COI F1B–Eu COI R1); the one negative case (rowi #2) had only produced the 200 bp amplicon with the kiwi gender-specific primers suggesting overall poorer quality DNA, although an amplicon of ~290 bp was obtained with the capillarid 18S primers (Table 2). Sequencing identified two distinct COI sequences with ~11% difference in base composition; one (rowi capillarid COI sequence A) from five rowi, and one (rowi capillarid COI sequence B) from two. On repeat PCR to confirm results, one sample (rowi #3) which had amplified sequence A the first time subsequently amplified sequence B from the same template DNA, and one other (rowi #4, which had also failed to amplify with the kiwi gender-specific primers) failed to amplify the second time. The trimmed COI sequences ranged from 167 to 271 bases in length. On alignment, all A sequences were identical, as were all B sequences. On BLAST analysis, both A and B consensus sequences aligned most closely with sequences from GenBank identified as *E. aerophilus* (sequence A to KC341990 and sequence B to KC341989) with 100% cover but only ~86% pairwise identity (Table 3). Rowi capillarid COI sequence B showed a consistent five base gap when aligned with all other capillarid COI sequences obtained from this study and with capillarid COI sequences available in GenBank; possibly as a result of this, the three sequences designated as rowi capillarid COI sequence B were not accepted on submission to GenBank (presented instead in Supplemental Fig. 1).

Seven out of 11 kahu samples produced amplicons of the appropriate size using the capillarid COI primers. Two distinct sequences with ~15% difference in base composition were produced, one from five birds (kahu capillarid COI sequence A), the other from two (kahu capillarid COI sequence B), which in six out of the seven birds was confirmed on repeat PCR (kahu #7 failed to amplify the second time) (Table 2). The two B sequences were identical. Of the five A sequences, one (kahu #5) included 4 individual degenerate bases, while another (kahu #7, which did not amplify on repeat PCR) showed a single base difference from the remaining three. Similar to the rowi sequences, BLAST analysis found no close matches; the kahu COI consensus sequence A aligned nearest to a GenBank sequence identified as *Capillaria aerophila* (JQ905059, syn.

Table 3
Top BLAST results for consensus sequences obtained in this study from capillarids of rowi, kahu, and red-billed gull origin.

Sequence	BLAST program	Sequence length (bp)	Aligned to organism (GenBank #)	Cover	Pairwise identity	Bit-score	e-value
Rowi capillarid 18S sequence	Megablast	279	*Eucoleus aerophilus* (MF599385) *Eucoleus garfiai* (LC484432) *Eucoleus perforans* (LC424998) *Eucoleus boehmi* (JX456628) *Eucoleus dispar* (EU004821)	100%	100%	516.336	2.50^{e-142}
Kahu capillarid 18S sequence	Megablast	287	*Eucoleus aerophilus* (MF599385) *Eucoleus garfiai* (LC484432) *Eucoleus perforans* (LC424998) *Eucoleus boehmi* (JX456628) *Eucoleus dispar* (EU004821)	100%	100%	531.109	8.68^{e-147}
Red-billed gull capillarid 18S sequence	Megablast	287	*Eucoleus contortus* (LC424996)	100%	100%	531.109	8.68^{e-147}
Rowi capillarid COI sequence A	Megablast	260	*Eucoleus aerophilus* (KC341990)	100%	86.2%	281.811	8.70^{e-72}
Rowi capillarid COI sequence B	Megablast	255	*Eucoleus aerophilus* (KC341989)	100%	85.8%	270.731	1.84^{e-68}
Kahu capillarid COI sequence A	Megablast	260	*Capillaria aerophila* (JQ905059)	87.3%	89.4%	287.351	1.85^{e-73}
Kahu capillarid COI sequence B	Discontiguous megablast	260	*Eucoleus boehmi* (KC341992)	100%	83.1%	271.792	8.90^{e-69}
Red-billed gull capillarid COI sequence	Discontiguous megablast	260	*Eucoleus boehmi* (KR186213)	95%	81.0%	236.626	6.35^{e-58}

E. aerophilus) and the kahu COI consensus sequence B aligned nearest to a sequence identified as *E. boehmi* (KC341992), with only ~83–89% pairwise identity (Table 3).

The control capillarid of red-billed gull origin also produced an amplicon of appropriate size. On BLAST analysis, closest alignment was to a sequence from *E. boehmi* (KR186213) with only ~81% pairwise identity (Table 3).

3.3.5. Phylogenetic analysis

Maximum likelihood trees comparing the sequences generated in this study with near relatives from GenBank confirmed their grouping within the capillarid group. The 18S analysis (Fig. 2; Supplemental Fig. 2) placed the study sequences within a clade containing several identical sequences identified as members of the *Eucoleus* genus. Other members identified as *Capillaria* genus also grouped together with the study sequences, which were distinct from additional capillarid genera (*Aonchotheca, Baruscapillaria, Calodium, Pearsonema, Pseudocapillaria*). Members of the *Trichuris* genus grouped separately from the capillarid sequences. Results strongly suggest that the study sequences belong in *Eucoleus/Capillaria*.

Unlike the 18S sequences generated in this study, which showed >99% similarity, the COI sequences were all relatively distinct from one another (79–90% similar). As expected from this, and a lack of close matches on BLAST analyses, each of the COI sequences were distinct from others and grouped within a diverse clade including several members of the capillarid group (Fig. 3). These results support 18S findings by placing the study sequences within the capillarid group, separate from *Trichuris*. A species-level identification based on COI data could not be reached due to the lack of close matches in GenBank; however, results show sequences representing novel genotypes with levels of divergence consistent with other, distinct species in the capillarid group.

4. Discussion

Cutaneous *larva migrans*, historically known as “creeping eruption”, can be broadly defined as the migration of a larval parasite in the skin of an abnormal host in which the parasite is unable to mature and complete its life cycle (Beaver, 1956; Caumes and Danis, 2004). Various nematode species originating from animal definitive hosts have been associated

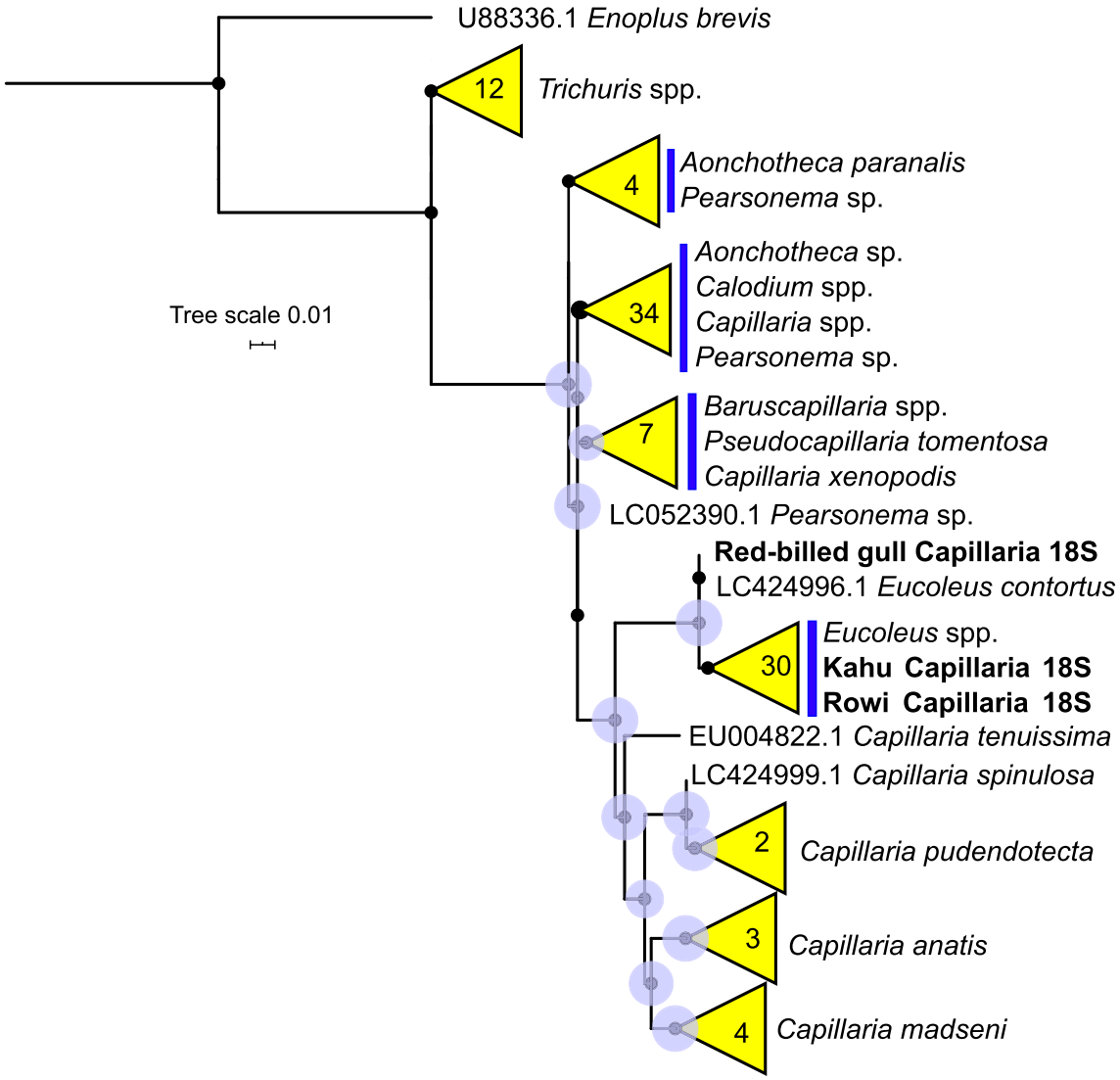

Fig. 2. Maximum likelihood 18S phylogenetic tree of capillarid taxa recovered from rowi, kahu, and red-billed gull with GenBank representatives. Yellow triangles are collapsed nodes containing multiple closely related sequences (the uncollapsed 18S tree including GenBank accession numbers is presented in supplemental figure 2). Numbers within triangles represent the number of sequences. Blue vertical lines are used to partition multiple taxa present within collapsed nodes. Coloured nodes indicate branch support determined with an approximate likelihood ratio test (aLRT) at >80%. An Enoplus brevis 18S sequence was used as outgroup taxon.

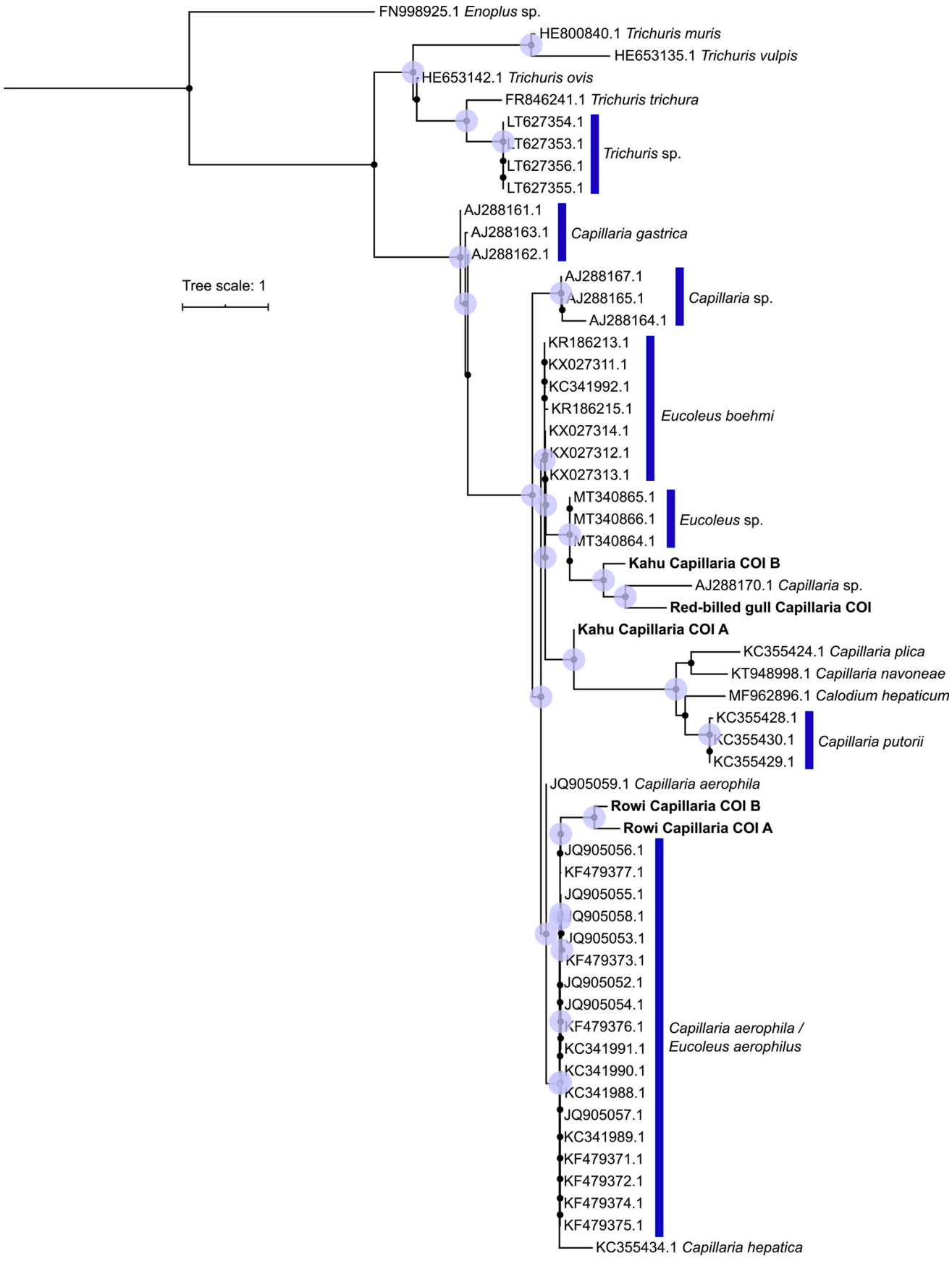

Fig. 3. Maximum likelihood cytochrome oxidase c subunit I (COI) phylogenetic tree of capillarid taxa recovered from rowi, kahu, and red-billed gull with GenBank representatives. Blue vertical lines are used to show taxonomic information for multiple sequences. Coloured nodes indicate branch support determined with an approximate likelihood ratio test (aLRT) at >80%. An Enoplus sp. COI sequence was used as outgroup taxon.

with cutaneous lesions in humans, including hookworms (e.g. *Ancylostoma* spp.) and threadworms (*Strongyloides* spp.), where the infective larvae, free-living in soil, penetrate and migrate within the skin. Cutaneous or subcutaneous lesions may also develop following oral infection and internal migration by other agents, for example cutaneous gnathostomiasis which is most commonly associated with the ingestion of infective *Gnathostoma* spp. larvae in raw or undercooked seafood. Larvae of the free-living nematode *Pelodera strongyloides* have also been reported to cause skin disease in humans (and, more commonly, dogs), again via percutaneous infection.

The skin disease in juvenile rowi cannot strictly be termed cutaneous *larva migrans* as the organisms identified histologically commonly included reproductively-active adults. In histological section, the nematodes lacked lateral cords, instead demonstrating hypodermal bacillary bands characteristic of the nematode class Adenophorea (aka Aphasmidia), as well as a stichosome associated with the oesophagus, a feature of the aphasmid superfamily Trichinelloidea that contains several important parasites of animals including the capillarid group (Gardiner and Poynton, 1999; Deplazes et al., 2016). Both histomorphology and molecular analysis in this case conclude that the nematode causing ventral dermatitis in juvenile rowi is a species of *Capillaria sensu lato* which, based on available sequences in GenBank, aligns among members identified as of the genus *Eucoleus*. The diagnosis of "cutaneous capillariasis" may therefore be more accurately applied to this disease.

The capillarids have a complicated and contentious taxonomy that has undergone numerous incarnations and revisions (Skrjabin et al., 1970; Moravec, 1982; Anderson and Bain, 2009). They have been classified as a subfamily, Capillariinae (e.g. Anderson and Bain, 2009), within the Trichuridae family or a family, Capillariidae, in their own right (e.g. Moravec, 1982), and have at different times been consolidated into the single genus *Capillaria* or differentiated into up to 27 proposed genera (Gibbons, 2010), all with variable acceptance by contemporary taxonomists and authors. Over 300 species have been described, parasitizing all classes of vertebrates. The lack of a universally accepted taxonomic scheme is a result, at least in part, of disagreements regarding the morphological delineations between proposed genera (Moravec, 1982; Anderson and Bain, 2009). For the purposes of this study, the term "capillarid" is used to describe species of *Capillaria sensu lato*.

Within their hosts, adult capillarids most commonly burrow within the lining of the gastrointestinal or respiratory tracts, but may also be found in liver, urinary bladder, and cutaneous sites (Yabsley, 2008; Anderson and Bain, 2009). Following the classification system proposed by Moravec (1982) up to 10 genera including *Eucoleus* have to date been identified in avian species (Yabsley, 2008; Tamaru et al., 2015), infecting sites within the gastrointestinal tract. To the authors' knowledge, cutaneous infections have not previously been reported in any host by species attributed to the *Eucoleus* genus. However, there are other capillarid species which do cause skin lesions in their hosts e.g. *Pseudocapillaroides xenopi* (syn. *Capillaria xenopodis*) in South African clawed frogs (*Xenopus laevis*) (Iglauer et al., 1997), and *Paratrichosoma recurvum* in crocodiles (*Crocodilus moreletii*) (Charruau et al., 2017).

The previous PCR and sequencing results implicating a nematode aligning among *Trichostrongylus* spp. (Gartrell et al., 2015) were unable to be replicated. The reason for this is uncertain but raises the possibility of sample contamination in the original study. Molecular characterisation of the organisms present in rowi skin lesions in the current study found that sequences from the 18S gene of rDNA were 99.6–100% identical to sequences obtained from lesions of oral and crop capillariasis in kahu and from a capillarid nematode originating from the proventriculus of a red-billed gull, as well as from a variety of organisms identified as *Eucoleus* spp. present in GenBank including some which parasitise the upper gastrointestinal tract of birds (*E. perforans*, *E. dispar*, and *E. contortus*) and some associated with mammalian hosts (*E. aerophilus* and *E. boehmi* from the respiratory tract, and *E. garfiai* in the tongue). These results support previous findings that the 18S region shows little genetic variability between closely related capillarid species (Guardone et al., 2013). This is, however, likely to have been exacerbated by the relatively short sequences obtainable from FFPE tissue samples in this study, as other studies which evaluated longer 18S sequences (~1800 bp) of capillarids did find sufficient variation to allow assessment of interspecific relationships within the group (Tamaru et al., 2015; Sakaguchi et al., 2020).

The COI gene of mtDNA is generally understood to show greater interspecies variation and has been applied as a target for genetic studies including DNA barcoding across a wide range of taxa; however, it is not without its own potential pitfalls (Derycke et al., 2010; Deagle et al., 2014). The capillarid COI PCR performed on rowi skin lesions produced two sequence types with greater than 10% divergence. The consistency of the two sequences between each bird and in duplicate PCR runs makes polymerase or sequencing errors unlikely. In no case did there appear to be co-amplification of the two sequences within the same reaction; however, in one (rowi #3) the same template DNA produced one of each sequence on duplicate PCR runs. The degree of divergence between the two sequences raises the possibility of two separate nematode species (Blouin, 2002), causing similar lesions within the same population and even, in the case of rowi #3, present in the same bird. While this is potentially feasible, it is considered biologically unlikely due to the small host population size and restricted geographical area in which the disease has been identified.

Among the concerns raised against the use of a single mitochondrial gene locus such as COI for barcoding purposes is the potential for overestimation of species diversity: for example, due to heteroplasmy; gene duplication within the mitochondrial genome; or the integration of mtDNA into the nuclear genome (Song et al., 2008; Derycke et al., 2010). The presence of non-functional copies of mtDNA within the nucleus (aka nuclear mitochondrial pseudogenes, or "numts") especially appears to be widespread among eukaryotes (Song et al., 2014). Following integration into the nucleus, the pseudogenes and their mitochondrial counterparts evolve independently and, apart from confounding barcoding studies, they have been found useful in phylogenetic analysis (Hay et al., 2004). Primer design may unwittingly favour the amplification of numts, where universal primers targeting relatively conserved regions of mitochondrial DNA are considered more likely to coamplify numts, but use of taxon-specific primers does not eliminate their presence entirely (Song et al., 2008; Moulton et al., 2010). Examination of the sequence composition can reportedly be used to identify the presence of pseudogenes, specifically the presence of indels, point mutations, and stop codons, but these are not always apparent even in relatively long COI sequences (Moulton et al., 2010).

COI pseudogenes do not appear to have been previously reported in nematodes, but other pseudogenes and gene duplications have been described within the mitochondrial genome of *Caenorhabditis* spp. (Raboin et al., 2010) and at least one parasitic nematode (Tang and Hyman, 2007). Presence of in-frame stop codons could not be confirmed in the relatively short sequences obtained from FFPE tissue in either rowi capillarid COI sequence A or B through translation using the invertebrate mitochondrial code by Geneious (v. 10.2.3). However, the rowi capillarid COI sequence B does include a five base gap evident when the sequence is aligned with other capillarid COI sequences, which could represent a microindel; a deletion such as this would lead to a reading frame shift. It is therefore tempting to speculate that rowi capillarid COI sequence B may reflect a numt or similar mitochondrial gene duplication/mutation, but techniques that may be of use to further investigate this hypothesis, for example next generation sequencing or isolation of mitochondrial DNA, are limited by the quality of DNA extracted from the available FFPE tissue samples as well as cost.

Two distinct COI sequences with ~15% divergence were also obtained from the kahu samples. In this instance the presence of two different species is more plausible. On repetition of the PCR, the two sequences were never identified in a single bird and there was no indication of co-amplification. One sequence was found in two out of the

three tissue sections originating from the crop, while the other was identified in all of the oral samples and the remaining crop sample. In contrast to the two rowi capillarid COI sequences, on phylogenetic analysis the two kahu capillarid COI sequences did not cluster together, with the kahu capillarid COI B sequence aligning nearer to the sequence obtained from the red-billed gull capillarid. The relatively short COI sequences obtainable from FFPE tissue and lack of capillarid COI sequences of avian origin present in GenBank for comparison does limit interpretation of this observation.

In fact, nematode COI sequences in GenBank remain relatively sparse in general (Poon et al., 2017); at the time of investigation, COI sequences from *Eucoleus* spp. were few in number and all originated from mammalian hosts. This paucity of data may be a result, at least in part, of the difficulty in design of universal nematode COI primers given that the mtDNA of some nematode groups has been shown to have a significantly higher mutation rate than that of many other animal taxa (Blouin et al., 1995; Anderson et al., 1998; Ahmed et al., 2019). Although the capillarid COI sequences obtained from the rowi, kahu, and red-billed gull samples in this study do suggest the recognition of up to four (or potentially five) different capillarid species, no species-level identification was possible for any of these sequences through BLAST analysis. Additionally, while the 18S sequences obtained in this study clearly grouped with members identified as from genus *Eucoleus*, it is possible that this may also be an artefact of limited sequence material available for comparison, and as increased genetic data becomes available resolution of where these parasites belong among the *Capillaria sensu lato* may be possible.

As the specific identity of the nematode causing skin lesions in rowi could not be established, its origin remains uncertain. The juvenile rowi are raised on an island sanctuary where introduced pests were eradicated in the 1990s and so the possibility of exposure to a nematode of mammalian origin is limited, although marine mammals are known to frequent the island. An avian parasite may be more likely as the borders of the island sanctuary remain porous to many flighted species. While *Eucoleus* species in birds typically parasitise the upper gastrointestinal tract, the atypical site in rowi, migrating within the epidermis around the vent, could be related to some biological peculiarities of kiwi; for example, kiwi lack a crop and have a lower resting body temperature and metabolic rate than most birds of a similar size (Sales, 2005). One report of infection of the lung and airsacs of a Peregrine falcon (*Falco peregrinus*) with nematodes identified morphologically as possible *E. aerophilus* suggests that infection of aberrant hosts, including infection of birds by a capillarid of primarily mammalian origin, may occur (Larrat et al., 2012).

The possibility of a novel capillarid species must also be considered. It is worth noting that the gastrointestinal parasites of kiwi themselves have been relatively poorly described. The presence of one or two novel species of *Capillaria* infecting kiwi has been previously postulated; however, the only published information, in the form of two conference abstracts (Clark, 1983b, a), is limited with no detail as to anatomical or geographical origin of the capillarids. No reports of the histological diagnosis of oral or oesophageal infection by capillarids in rowi or any other kiwi species were found in the SoVS pathology database, and skin lesions as seen in the crèche population of juvenile rowi have not been reported in rowi from other sites, nor in any other kiwi species. However, capillarid-type eggs are not uncommonly identified during routine faecal examinations from kiwi around the country, and are of uncertain origin and pathological significance. During the 2013 investigation of skin disease in rowi, seven of the 15 affected birds had capillarid-type eggs identified on faecal floatation (Gartrell et al., 2015) although their relationship to the cutaneous lesions remains unknown. The possibility that the juvenile rowi could have carried endemic parasites to the crèche island is low, however, given that they were previously raised from eggs in a controlled environment, and the aberrant migration of a parasite originating from another host is a more likely scenario.

Whatever the original source, histological confirmation of reproductively-active female nematodes and eggs within the epidermis of the cutaneous lesions raises a possibility that the infection may be, or have potential to become, self-sustaining within the island population, as well as the potential for spread of the nematode if infected birds are transferred off the island crèche. For other capillarids inhabiting skin, eggs are laid within the epidermis and pass into the environment as the superficial layers are sloughed as part of natural (or accelerated) turnover of cells (Charruau et al., 2017). At the time of the 2013 outbreak, the rowi chicks were provided with artificial burrows which had not been moved or spelled for some years prior to the first observation of skin disease (Gartrell et al., 2015). In contrast to most other kiwi populations rowi tend to share burrows in small groups, and so contamination of the burrow soil and environs may lead to increased potential for transmission of infection between birds. Following the outbreak, it was recommended that the artificial burrows should be moved every six months and, anecdotally, the incidence of the skin disease has subsequently decreased.

The life cycles of different *Eucoleus* species are variably reported as direct (through oral ingestion of infective eggs) and/or indirect (requiring or utilizing an earthworm intermediate host) (Anderson, 2000; Yabsley, 2008). The potential for percutaneous infection by infective capillarid larvae has not been established, even in those species known to parasite skin (Charruau et al., 2017). Oral infection with cutaneous invasion around the vent following migration from the intestinal tract seems the more likely route of infection in rowi, particularly as the necropsy finding of a larval nematode in the liver of one of the 2013 rowi cohort raises the possibility that some aberrant internal migration may also occur. Unfortunately, it was not possible to confirm through molecular analysis that this was the same organism as those in the skin, and the larval section included no distinguishing histomorphological features. However, while visceral *larva migrans* caused by *Toxocara cati* has been reported in North Island brown kiwi (French et al., 2020) this is not a valid differential in the juvenile rowi given that the crèche island has been free of the feline definitive host (*Felis catus*) for over 20 years and, similarly, the previous elimination of rodents and predatory mammals from the island reduce the possibility of hepatic capillariasis due to *Calodium hepaticum*.

Ideally, further investigation into the specific identification of the capillarids causing skin disease in juvenile rowi would benefit from the isolation of whole nematodes from lesional skin, for expert morphological examination as well as more extensive molecular analysis. However, as changes to the management of burrows on the crèche island appear to have reduced incidence of the disease, which is now well-recognised, future biopsy or necropsy specimens are unlikely to be forthcoming. Collection and comparative molecular analysis of capillarid nematodes originating from other species present on the crèche island may be of some benefit in identifying a potential original source of the infection. Additionally, molecular analysis of faecal samples containing capillarid eggs, both from the juvenile rowi and from other origins around New Zealand, may aid in expanding the currently sparse understanding of capillarid nematode infections in kiwi.

5. Conclusion

Histomorphological and molecular studies indicate that the nematode causing skin disease in rowi is a species of *Capillaria sensu lato* and, with the caveat of limited GenBank sequences available for comparison, suggest a potential member of the *Eucoleus* genus. While two distinct capillarid COI sequences were obtained from the rowi samples, it is considered more likely to be a single species causing the lesions with possible concurrent amplification of a COI pseudogene. A species-level identification could not be reached, but comparison of molecular results suggests a different species to capillarid organisms derived from several kahu (Australasian harrier hawks) and one red-billed gull originating from elsewhere in New Zealand. The cutaneous nematode infections in rowi appear patent, raising the potential for perpetuation of

infection in the absence of the original source, and of translocation of the parasite if infected rowi are removed from the island.

Funding

Funding for this research was provided in the form of a Doctorate Scholarship in Wildlife Health from the George Mason Charitable Trust. The sponsor had no role in the study design; data collection, analysis, or interpretation; or in writing up or presenting this article for publication.

Declaration of competing interest

None.

Acknowledgements

Publication costs were supported by the School of Veterinary Science (Massey University, Palmerston North, New Zealand). Grateful thanks also to Jerusha Bennett and Dr. Bronwen Presswell from the Department of Zoology, University of Otago, New Zealand; Dr. Donald Martin, North America Head of Parasitology, IDEXX Laboratories, Ontario, Canada; Barbara Adlington from the Massey University parasitology laboratory, Palmerston North, New Zealand; Evelyn Lupton from the Massey University histology laboratory, Palmerston North, New Zealand; Stuart Hunter from the Wildbase pathology service, Palmerston North, New Zealand; and to Dr George Mason.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.10.003.

References

Ahmed, M., Back, M.A., Prior, T., Karssen, G., Lawson, R., Adams, I., Sapp, M., 2019. Metabarcoding of soil nematodes: the importance of taxonomic coverage and availability of reference sequences in choosing suitable marker(s). Metabarcoding and Metagenomics 3, 77–99.

Alley, M.R., Coomer, A.R., Gartrell, B.D., 2004. Mycobacterial stomatitis and associated capillariasis in an Australasian harrier, *Circus approximans*. Kokako 11, 3–5.

Anderson, R.C., 2000. Nematode Parasites of Vertebrates: Their Development and Transmission, 2nd Edition. CAB International, Wallingford, Oxon UK.

Anderson, R.C., Bain, O., 2009. Enoplida. In: Anderson, R.C., Chabaud, A.G., Willmott, S. (Eds.), Keys to the Nematode Parasites of Vertebrates: Archival Volume. CAB International, Wallingford, Oxon UK.

Anderson, T.J.C., Blouin, M.S., Beech, R.N., 1998. Population biology of parasitic nematodes: applications of genetic markers. Adv. Parasitol. 41, 219–283.

Beaver, P.C., 1956. Larva migrans. Exp. Parasitol. 5, 587–621.

BirdLife International, 2017. *Apteryx Rowi*. The IUCN Red List of Threatened Species. Version 2020-2.. Available from: https://www.iucnredlist.org. Accessed 30 July 2020.

Blouin, M.S., 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Int. J. Parasitol. 32, 527–531.

Blouin, M.S., Yowell, C.A., Courtney, C.H., Dame, J.B., 1995. Host movement and the genetic structure of populations of parasitic nematodes. Genetics 141, 1007–1014.

Burbidge, M.L., Colbourne, R.M., Robertson, H.A., Baker, A.J., 2003. Molecular and other biological evidence supports the recognition of at least three species of brown kiwi. Conserv. Genet. 4, 167–177.

Caumes, E., Danis, M., 2004. From creeping eruption to hookworm-related cutaneous larva migrans. Lancet Infect. Dis. 4, 659–660.

Charruau, P., Pérez-Flores, J.S., Labarre, D., 2017. Skin parasitism by *Paratrichosoma recurvum* in wild American crocodiles and its relation to environmental and biological factors. Dis. Aquat. Organ. 122, 205–211.

Clark, W.C., 1983a. Nematodes of kiwis. In: Proceedings of the New Zealand Society for Parasitology, vol. 10, p. 129, 10th Annual Conference (1982) Dunedin, New Zealand. N. Z. J. Zool.

Clark, W.C., 1983b. Parasites of kiwi. In: Fowler, M.E. (Ed.), Wildlife Diseases of the Pacific Basin and Other Countries. Fruitridge Printing, Sacramento, California, pp. 93–95.

Colbourne, R., Bassett, S., Billing, T., McCormick, H., McLennan, J., Nelson, A., Robertson, H., 2005. The Development of Operation Nest Egg as a Tool in the Conservation Management of Kiwi (Wellington, New Zealand. Department of Conservation).

Deagle, B.E., Jarman, S.N., Coissac, E., Pompanon, F., Taberlet, P., 2014. DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biol. Lett. 10, 20140562.

Deplazes, P., Eckert, J., Mathis, A., Zahner, H., 2016. Parasitology in Veterinary Medicine. Wageningen Academic Publishers, The Netherlands.

Derycke, S., Vanaverbeke, J., Rigaux, A., Backeljau, T., Moens, T., 2010. Exploring the use of cytochrome oxidase c subunit 1 (COI) for DNA barcoding of free-living marine nematodes. PLoS One 5, e13716.

Di Cesare, A., Castagna, G., Otranto, D., Meloni, S., Milillo, P., Latrofa, M.S., Paoletti, B., Bartolini, R., Traversa, D., 2012. Molecular detection of *Capillaria aerophila*, an agent of canine and feline pulmonary capillariosis. J. Clin. Microbiol. 50, 1958–1963.

Fischer, K., Gankpala, A., Gankpala, L., Bolay, F., Curtis, K.C., Weil, G.J., Fischer, P.U., 2018. *Capillaria* Ova and diagnosis of *Trichuris trichiura* infection in humans by kato-katz smear. Liberia. Emerg. Infect. Dis. 24, 1551–1554.

French, A.F., Castillo-Alcala, F., Gedye, K.R., Roe, W.D., Gartrell, B.D., 2020. Nematode *larva migrans* caused by *Toxocara cati* in the North Island brown kiwi (*Apteryx mantelli*). Int. J. Parasitol. Parasites Wildl. 11, 221–228.

Gardiner, C.H., Poynton, S.L., 1999. An Atlas of Metazoan Parasites in Animal Tissues. Armed Forces Institute of Pathology, Washington, DC.

Gartrell, B.D., Argilla, L., Finlayson, S., Gedye, K., Gonzalez Argandona, A.K., Graham, I., Howe, L., Hunter, S., Lenting, B., Makan, T., 2015. Ventral dermatitis in rowi (*Apteryx rowi*) due to cutaneous larval migrans. Int. J. Parasitol. Parasites Wildl. 4, 1–10.

Gasser, R.B., Chilton, N.B., Hoste, H., Beveridge, I., 1993. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. Nucleic Acids Res. 21, 2525–2526.

GenBank [Internet], 1982. National Library of Medicine (US), National Center for Biotechnology Information, Bethesda (MD). Available from: https://www.ncbi.nlm.nih.gov. Accessed 2019-Feb to Apr.

Germano, J., Barlow, S., Castro, I., Colbourne, R., Cox, M., Gillies, C., Hackwell, K., Harawira, J., Impey, M., Reuben, A., 2018. Kiwi Recovery Plan 2018-2028. Department of Conservation), Wellington, New Zealand.

Gibbons, L.M., 2010. Keys to the Nematode Parasites of Vertebrates: Supplementary volume, 10. Cabi, Oxon, Wallingford, UK.

Guardone, L., Deplazes, P., Macchioni, F., Magi, M., Mathis, A., 2013. Ribosomal and mitochondrial DNA analysis of Trichuridae nematodes of carnivores and small mammals. Vet. Parasitol. 197, 364–369.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Hay, J.M., Sarre, S.D., Daugherty, C.H., 2004. Nuclear mitochondrial pseudogenes as molecular outgroups for phylogenetically isolated taxa: a case study in *Sphenodon*. Heredity 93, 468–475.

Huynen, L., Lambert, D.M., McLennan, J.A., Rickard, C., Robertson, H.A., 2003. A DNA test for sex assignment in kiwi (*Apteryx* spp.). Notornis 50, 231–233.

Iglauer, F., Willmann, F., Hilken, G., Huisinga, E., Dimigen, J., 1997. Anthelmintic treatment to eradicate cutaneous capillariasis in a colony of South African clawed frogs (*Xenopus laevis*). Comp. Med. 47, 477–482.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.

Larrat, S., Locke, S., Dallaire, A.D., Fitzgerald, G., Marcogliese, D.J., Lair, S., 2012. Fatal aerosacculitis and pneumonia associated with *Eucoleus* sp. (Nematoda: Capillaridae) in the lungs of a peregrine falcon (*Falco peregrinus*). J. Wildl. Dis. 48, 832–834.

Lefort, V., Longueville, J.-E., Gascuel, O., 2017. SMS: smart model selection in PhyML. Mol. Biol. Evol. 34, 2422–2424.

Letunic, I., Bork, P., 2019. Interactive Tree of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res 47, W256–W259.

Moravec, F., 1982. Proposal of a new systematic arrangement of nematodes of the family Capillariidae. Folia Parasitol. 29, 119–132.

Moulton, M.J., Song, H., Whiting, M.F., 2010. Assessing the effects of primer specificity on eliminating numt coamplification in DNA barcoding: a case study from Orthoptera (Arthropoda: Insecta). Mol. Ecol. Resour. 10, 615–627.

Poon, R.W.S., Tam, E.W.T., Lau, S.K.P., Cheng, V.C.C., Yuen, K.-Y., Schuster, R.K., Woo, P.C.Y., 2017. Molecular identification of cestodes and nematodes by cox 1 gene real-time PCR and sequencing. Diagn. Microbiol. Infect. Dis. 89, 185–190.

Raboin, M.J., Timko, A.F., Howe, D.K., Félix, M.-A., Denver, D.R., 2010. Evolution of *Caenorhabditis* mitochondrial genome pseudogenes and *Caenorhabditis briggsae* natural isolates. Mol. Biol. Evol. 27, 1087–1096.

Robertson, H.A., Baird, K., Dowding, J.E., Elliott, G.P., Hitchmough, R.A., Miskelly, C.M., McArthur, N., O'Donnell, C.F.J., Sagar, P.M., Scofield, R.P., Taylor, G.A., 2016. Conservation Status of New Zealand Birds. Department of Conservation), Wellington, New Zealand.

Sakaguchi, S., Yunus, M., Sugi, S., Sato, H., 2020. Integrated taxonomic approaches to seven species of capillariid nematodes (Nematoda: Trichocephalida: Trichinelloidea) in poultry from Japan and Indonesia, with special reference to their 18S rDNA phylogenetic relationships. Parasitol. Res. 119, 1–16.

Sales, J., 2005. The endangered kiwi: a review. Folia Zool 54, 1.

Skrjabin, K.I., Shikhobalova, N.P., Orlov, I.V., 1970. Trichocephalidae and Capillariidae of Animals and Man and the Diseases Caused by Them. Israel Program for Scientific Translations, Jerusalem.

Song, H., Buhay, J.E., Whiting, M.F., Crandall, K.A., 2008. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proc. Natl. Acad. Sci. Unit. States Am. 105, 13486–13491.

Song, H., Moulton, M.J., Whiting, M.F., 2014. Rampant nuclear insertion of mtDNA across diverse lineages within Orthoptera (Insecta). PLoS One 9, e110508.
Tamaru, M., Yamaki, S., Jimenez, L.A., Sato, H., 2015. Morphological and molecular genetic characterization of three *Capillaria* spp.(*Capillaria anatis, Capillaria pudendotecta*, and *Capillaria madseni*) and *Baruscapillaria obsignata* (Nematoda: Trichuridae: Capillariinae) in avians. Parasitol. Res. 114, 4011–4022.
Tang, S., Hyman, B.C., 2007. Mitochondrial genome haplotype hypervariation within the isopod parasitic nematode *Thaumamermis cosgrovei*. Genetics 176, 1139–1150.
Tennyson, A.J.D., Palma, R.L., Robertson, H.A., Worthy, T.H., Gill, B.J., 2003. A new species of kiwi (Aves, Apterygiformes) from Okarito, New Zealand. Rec. Auckl. Mus. 55–64.
Yabsley, M.J., 2008. Capillarid nematodes. In: Atkinson, C.T., Thomas, N.J., Hunter, D.B. (Eds.), Parasitic Diseases of Wild Birds. Wiley-Blackwell, Ames, Iowa, pp. 463–497.
Zhu, X., Spratt, D.M., Beveridge, I., Haycock, P., Gasser, R.B., 2000. Mitochondrial DNA polymorphism within and among species of *Capillaria sensu lato* from Australian marsupials and rodents. Int. J. Parasitol. 30, 933–938.