Intra- and interspecific genetic diversity of New Zealand hairworms (Nematomorpha)

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(*Received 7 November 2016; revised 25 January 2017; accepted 27 January 2017; first published online 9 March 2017*)

SUMMARY

Hairworms (Nematomorpha) are a little-known group of parasites, and despite having been represented in the taxonomic literature for over a century, the implementation of molecular genetics in studies of hairworm ecology and evolution lags behind that of other parasitic taxa. In this study, we characterize the genetic diversity of the New Zealand nematomorph fauna and test for genetic structure within the most widespread species found. We provide new mitochondrial and nuclear ribosomal sequence data for three previously described species from New Zealand: *Gordius paranensis, Parachordodes diblastus* and *Euchordodes nigromaculatus*. We also present genetic data on a previously reported but undescribed *Gordius* sp., as well as data from specimens of a new *Gordionus* sp., a genus new for New Zealand. Phylogenetic analyses of CO1 and nuclear rDNA regions correspond with morphological classification based on scanning electron microscopy, and demonstrate paraphyly of the genus *Gordionus* and the potential for cryptic species within *G. paranensis*. Population-level analyses of *E. nigromaculatus* showed no genetic differentiation among sampling locations across the study area, in contrast to previously observed patterns in known and likely definitive hosts. Taken together, this raises the possibility that factors such as definitive host specificity, intermediate host movement, and passive dispersal of eggs and larvae may influence host–parasite population co-structure in hairworms.

Key words: Nematomorpha, hairworms, *Euchordodes nigromaculatus*, host–parasite population structure, New Zealand.

INTRODUCTION

The remarkable diversity of parasitic taxa and life-styles makes parasitism an attractive model in which to investigate evolutionary and ecological forces (Poulin and Morand, 2004; Criscione *et al.* 2005). However, up until the past two or three decades the predominantly descriptive and systematic field of parasitology had little overlap with the more theoretical disciplines of evolutionary biology and ecology (Poulin, 2007). The decreasing cost of molecular genetic tools within biological research has led to an increase in both micro- and macroevolutionary studies of parasites. This has expanded our understanding of parasite speciation, co-evolution and biogeography, among others (Morand *et al.* 2015). However, the representation of parasitic taxa in the evolutionary literature has not been equal, with research on certain groups lagging far behind those of medical or economic importance.

Members of the phylum Nematomorpha, or hairworms, are an example of such a group, as they are considered to be one of the most understudied taxa of parasites (Hanelt *et al.* 2005). Freshwater hairworms (Gordiida) are perhaps best known for their ability to induce water-seeking behaviour in their definitive hosts (Thomas *et al.* 2002). Gordiids also have a unique life cycle, which contains two hosts and extends across aquatic and terrestrial habitats (Hanelt and Janovy, 2004). Adult hairworms emerge from their definitive host, typically an orthopteran, coleopteran, or mantid, into a stream or puddle where they can form tangled masses of mating individuals that are referred to as Gordian knots, the etymological root of the taxon. Larvae emerge from eggs laid by female worms after mating, and encyst in a wide range of aquatic invertebrate hosts, many of which are thought to constitute 'dead-ends' (Hanelt and Janovy, 2003). The transfer of the nematomorph larva to land is accomplished *via* infection of an intermediate host with an aquatic pre-adult life stage (e.g. ephemeropteran, plecopteran or culicid insects), which then metamorphoses, exits the aquatic environment, and is eaten by a definitive host, thus achieving trophic transmission of the parasite (Hanelt and Janovy, 2004).

Molecular studies involving hairworms have only become more common in the past 15 years, though in the majority of cases molecular data has only been used to investigate systematic relationships between phyla or within phyla closely related to Nematomorpha, with hairworm sequence data often solely serving as an outgroup in phylogenetic analyses (Aguinaldo *et al.* 1997; Blaxter *et al.* 1998; Sørensen *et al.* 2008). However, the implementation

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Parasitology (2017), **144**, 1026–1040.
doi:10.1017/S0031182017000233

of molecular genetics in nematomorph studies shows great potential for elucidating life cycles, discovering cryptic species and investigating biogeography (Hanelt *et al*. 2015; Harkins *et al*. 2016).

The nematomorph fauna of New Zealand is comparatively well characterized (Poinar, 1991, 2009; Schmidt-Rhaesa, 2008). One marine hairworm (Nectomatida) and four gordiid species have been reported: *Nectonema zealandica* (Poinar and Brockerhoff, 2001), *Gordius dimorphus* (Poinar, 1991), *Gordius paranensis* (Camerano, 1892), *Parchordodes diblastus* (Örley, 1881), and *E. nigromaculatus* (Poinar, 1991) (Poinar, 1991; Schmidt-Rhaesa *et al*. 2000; Poinar and Brockerhoff, 2001; see Schmidt-Rhaesa, 2008). An additional *Gordius* sp. has been found in New Zealand, but could not be formally described due to the lack of reliable diagnostic characters (Schmidt-Rhaesa, 2008). Because of deforestation and the conversion of lowlands to agriculture, nematomorphs are mostly found in montane and subalpine areas in New Zealand, and therefore occur in isolated areas separated by unsuitable habitats for their hosts. DNA sequence data has only been obtained from *G. paranensis* and *E. nigromaculatus*, which were used in a systematic study of the phylum (Bleidorn *et al*. 2002). With the exception of a sole investigation by Hanelt *et al*. (2015) into cryptic species of *Gordius robustus* in North America, no studies have used genetic data to examine allopatric speciation in hairworms.

Molecular tools can also shed light on intraspecific patterns of genetic variation. Indeed, the comparative population structure between host and parasite populations has become an increasingly popular topic within evolutionary parasitology in recent years (Criscione and Blouin, 2004; Criscione, 2008; Bruyndonckx *et al*. 2009; Keeney *et al*. 2009). The close association between host and parasite and the dependence upon host movement for parasite dispersal has led to various predicted outcomes regarding local adaptation, population structure and co-speciation (Nadler, 1995; Jarne and Theron, 2001; Gandon and Michalakis, 2002). These predictions have started to be tested, and while the results of these various studies are not always congruent, there are some general patterns beginning to emerge (Blasco-Costa and Poulin, 2013; Mazé-Guilmo *et al*. 2016). As genetic information from hairworms is largely lacking, the addition of host–parasite co-structure data from nematomorph systems should lead to a more precise understanding of evolutionary trends within parasites in general, as opposed to the current focus on a smaller subset of parasitic taxa. Given their reliance on insect hosts with limited mobility for dispersal and the fragmented nature of their habitat in New Zealand, one might expect nematomorphs to show significant genetic structure even on relatively small spatial scales.

We collected free-living adult nematomorphs from a variety of montane and subalpine locations in central Otago, South Island, New Zealand, with the aims of: (i) characterizing genetic diversity of the New Zealand nematomorph fauna, and (ii) investigating parasite population structure in the most geographically widespread species recovered. This study provides new insights into the phylogenetic relationships among hairworm taxa and the existence of potential cryptic species, as well as a discussion of comparative population structure between hairworm parasites and their orthopteran hosts.

MATERIALS AND METHODS

Nematomorph collection

Free-living, post-parasitic adult nematomorphs were collected by hand from narrow mountain streams and pools in the alpine cushionfields and subalpine tussock grasslands of five mountain ranges in Central Otago: the Rock and Pillar Range (RP), the Crown Range (CR), the Remarkables (RK), the Ida Range (ID) and the Old Man Range (OM) between February and May, 2016 (Supplementary Data S1; Fig. 1). Specimens were transported to the laboratory in containers with stream water or 70% ethanol. All specimens were placed in 70% ethanol and left overnight at 4 °C. The following day specimens were sexed and measured. For species identification by scanning electron microscopy (SEM), a ~5 mm segment of the mid-section and the posterior end were removed from all individuals and placed in a 1·5 mL Eppendorf tube containing 70% ethanol. The remaining tissue was preserved in 95% ethanol for genetic analyses. Genetic analyses and species identification through SEM were done independently from each other, i.e. their results were only compared after they were completed.

Scanning electron microscopy

Pieces for SEM were dehydrated in an increasing ethanol series, critical point dried and coated with gold in a sputter coater. Observations were made using a LEO SEM 1524, from which digital images were taken.

DNA extraction, PCR amplification and sequencing

Genomic DNA from all nematomorphs was extracted using a modified Chelex method (Walsh *et al*. 1991). For each hairworm, a ~5 mm section was removed and the cuticle was sliced longitudinally with a scalpel. The internal tissue was then scraped out and placed in 300 μL of an aqueous 5% Chelex 100 (BioRad) solution containing 0·2 mg mL^{-1} of proteinase K (Roche). Extractions were incubated at 55 °C overnight and then at 94 °C for

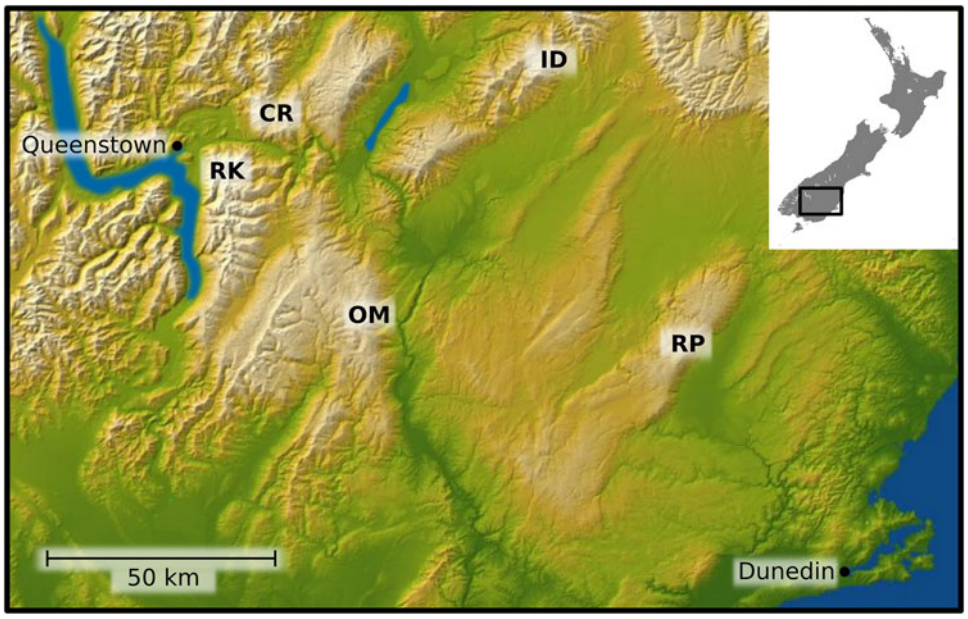

Fig. 1. Map of study area. RK, Remarkables; CR, Crown Range; ID, Ida Range; OM, Old Man Range; RP, Rock and Pillar Range.

10 m to deactivate the proteinase K. Tubes were then centrifuged at 14 000 rpm for 10 m to pellet Chelex resin and undegraded cuticle. The supernatant was then transferred to a fresh 1·5 mL Eppendorf tube or 96-well plate for storage at 4 °C.

Unless otherwise stated, all polymerase chain reactions (PCRs) were performed in a final volume of 15 μL, comprising 3 μL of MyTaq™ Red 5× reaction buffer (Bioline), 0·75 μL of 10 μM stock (75 pM) of each primer, 0·2 μL (1 U) of MyTaq™ Red DNA Polymerase, and 3 μL of extracted nematomorph genomic DNA. PCR thermal cycling profiles are provided in Supplementary Data S2. Products were purified of remaining dNTPs and oligonucleotides using the ExoSap method. Sanger sequencing by capillary electrophoresis was performed by Genetic Analysis Services, Department of Anatomy, University of Otago (Dunedin, New Zealand) or Macrogen Inc. (Seoul, Republic of Korea).

Amplification of a 709 bp partial fragment of the mitochondrial gene cytochrome c oxidase subunit 1 (CO1) was attempted for all hairworm samples. Initially, either the 'universal' primers LCO1498 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACC AAAAAATCA-3′) (Folmer *et al*. 1994), or the degenerate primers HHW-cox1-F2 (5′-TTTCWA CYAATCAYAARGAYATTGG-3′) and HHW-cox1-R2 (5′-TWACTTCAGGRTGACCAAAAA AYC-3′) (Hanelt *et al*. 2015) were used. Initial attempts with these primer sets resulted in poor amplification success and/or sequencing of contaminants for all but 18 specimens. The partial CO1 sequences from these 18 individuals were aligned in Geneious v8.1.8 (http://www.geneious.com, Kearse *et al*. 2012) and the consensus sequence was used to design the degenerate primers NZHW_ CO1_F (5′-DTTYTGRTCTGCHTTTTTRGG-3′) and NZHW_CO1_R (5′-RAAAAAWGAWGTDT TAAYATTTCG-3′), which amplify a 592 bp fragment of CO1. PCRs were performed as previously stated, but with 1·5 μL of 10 μM stock (150 pM) of each primer.

A variable length fragment of the nuclear rDNA region containing partial sequence of the 3′ end of the 18S rRNA gene, internal transcribed spacer 1, 5·8S rRNA gene, internal transcribed spacer 2, and partial sequence of the 5′ end of the 28S rRNA were amplified using the primers HHW-ITS-F2 (5′-CAC GCGCGCTACACTGAA-3′) and HHW-ITS-R2 (5′-GGCGCTGGACTCTTCCTATT-3′) (Hanelt *et al*. 2015), or the novel primers HW_Grp5_ITS_F (5′-CTGGGTATCCGCTGAACTCC-3′) and HW_ Grp5_ITS_R (5′-TAGTTTCTTTTCCTCCGC zTTATTAATATGC-3′).

Sequence processing and phylogenetics

All sequence data editing and aligning was performed using Geneious. All sequences were trimmed with the default error probability limit of 0·05, inspected by eye for incorrect base calls and ambiguities, and then edited manually. Alignments were created using the MAFFT algorithms (Katoh and Standley, 2013). For ribosomal regions, the specific MAFFT algorithm was selected using the 'Auto' function. Determination of boundaries of the various ribosomal fragments was performed by comparing regions of homology, and the sequences were annotated as such. For CO1, the 'Translation

Align' feature was used, implementing the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992). Sequences were inspected further to check for obvious incorrect base calls. Pairwise distance matrices of CO1 sequence data were created in MEGA v7 (Kumar *et al*. 2016) using the Kimura 2-parameter (K2P) measure of genetic divergence (Kimura, 1980), calculating the distance between each specimen as well as mean intra- and interspecific distances. For phylogenetic analyses, model selection and optimal partitioning schemes were determined using PartitionFinder v2.0.0 (pre-release 14) (Lanfear *et al*. 2012), which utilizes the software PhyML v3.0 (Guindon *et al*. 2010) and allows the user to restrict model testing to those implemented in MrBayes. The corrected Aikake information criterion (AICc) was used for evaluating the likelihood of the proposed models and partitioning schemes. For CO1, the optimal partitioning scheme consisted of one partition containing all codon positions. Phylogenetic tree building was performed with MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2003) and RAxML v8 (Stamatakis, 2014), and was performed remotely using the CIPRES Science Gateway (Miller *et al*. 2010). For Bayesian analyses of 18S and CO1, PartitionFinder selected the models SYM + I + Γ and GTR + I + Γ, respectively. Two runs, each consisting of four Markov chains, were allowed to run for 10 000 000 generations and were sampled every 1000. A chain heating parameter of 0·04 was used. The first 2500 trees (25%) were discarded as burn-in and posterior probabilities were obtained from a majority-rule consensus. Tracer v1.6 (Drummond and Rambaut, 2007) was used to assess convergence of the two runs and ensure high effective sample size. For maximum likelihood, the RAxML default model of GTR + Γ was used for all markers. Bootstrap support values were obtained using the rapid bootstrap method with 1000 replicates. Outgroups (two nematode species) and nematomorph reference sequences used in phylogenetic analyses were obtained from GenBank, and can be found in Supplementary Data S3. All sequences from this study are available on GenBank; accession numbers are provided in Supplementary Data S4.

Population genetics

Arlequin v3.5.2.2 (Excoffier and Lischer, 2010) was used to calculate the number of haplotypes (N_h), number of polymorphic sites (N_p), the haplotype diversity (h) and nucleotide diversity (π) for both markers for each hairworm species. For the most spatially widespread species, *E. nigromaculatus*, statistical parsimony networks of the CO1 and ITS data were created with the software PopArt (Leigh and Bryant, 2015) using the TCS method (Clement *et al*. 2000). Clustering of individual haplotypes into groups was performed with BAPS v6.0 (Corander *et al*. 2003) with the number of estimated populations set to $2 \leqslant k \geqslant 20$. For each k value, five independent runs were performed. To test for population differentiation of *E. nigromaculatus*, a single-level AMOVA (i.e. specimens from the same range treated as a distinct population with no higher-level grouping) (Excoffier *et al*. 1992) was performed in Arlequin, implementing the TrN + Γ model of nucleotide substitution with a shape parameter $\alpha = 0{\cdot}021$ for each marker, as determined by jModelTest2 (Darriba *et al*. 2012). Significance of AMOVA results was determined by 10 000 random permutations.

RESULTS

Collections and morphology

In total, 87 nematomorphs were collected (Table 1). With the exception of *G. dimorphus*, morphological identification by SEM revealed all previously reported New Zealand hairworms. *Euchordodes nigromaculatus* was the most commonly encountered species, with a total of 34 individuals from three of the five ranges, RK, RP and OM. This species has polygonal cuticular areoles and is the only New Zealand species in which males have an undivided posterior end (Fig. 2A and B). *Parachordodes diblastus* was found at three locations, the CR, RP and OM, but was most heavily represented at CR, where it comprised 12 of the 13 collected individuals. *Parachordodes diblastus* can be identified by its elevated dark superareoles (Fig. 2C and D), which are characteristic of this genus. *Gordius paranensis* was collected from the ID and RP in low numbers. This species is identified by its smooth cuticle and the presence of a semicircular pre-cloacal row of bristles, in addition to the post-cloacal crescent that is characteristic of the genus (Fig. 2E and F). Individuals of a previously reported but undescribed *Gordius* sp. were also collected (Schmidt-Rhaesa, 2008). This species displays a post-cloacal crescent but lacks the pre-cloacal bristles characteristic of *G. paranensis* (Fig. 2G and H). In addition to the previously reported New Zealand species, seven individuals of an undescribed species were also found (Fig. 2I and J). This species exhibits areoles surrounded by fine bristles and possesses a row of large bristles on the ventral side of the posterior end in males. These characters are typical of the genus *Gordionus*. A formal description of this species will be provided elsewhere. One specimen (OM 10) could not be identified by SEM or molecular methods.

Mean lengths of each hairworm species are provided in Supplementary Data S5, in which data only from intact specimens of known sex are included. The longest specimen was a female *Gordius* sp. at 565 mm.

Table 1. Summary of nematomorph collections by species, sex and location

				Rock and Pillar Range				Old Man Range				
Species	Sex	Remarkables	Crown Range	1st stream	2nd stream	Big hut	Total RP	Stream A	Stream B	Total OM	Ida Range	Overall
Euchordodes nigromaculatus		1	–	–	–	20	20	3	10	13	–	**34**
	M	1	–	–	–	14	14	2	4	6	–	21
	?	–	–	–	–	6	6	1	6	7	–	13
Parachordodes diblastus		–	11	–	–	3	3	1	3	4	–	**18**
	M	–	9	–	–	3	3	1	3	4	–	–
	?	–	2	–	–	–	–	–	–	–	–	–
Gordius paranensis		–	–	–	4	2	6	–	–	–	3	**9**
	M	–	–	–	4	1	5	–	–	–	3	8
	?	–	–	–	–	1	1	–	–	–	–	1
Gordius sp.		–	1	4	–	5	9	4	4	8	–	**18**
	M	–	1	3	–	5	8	1	4	5	–	14
	F	–	–	–	–	–	–	2	–	2	–	2
	?	–	–	1	–	–	1	1	–	1	–	2
Gordionus n. sp.		–	–	–	–	4	4	1	–	1	2	**7**
	M	–	–	–	–	4	4	–	–	–	1	5
	F	–	–	–	–	–	–	1	–	1	–	1
	?	–	–	–	–	–	–	–	–	–	1	1
unidentified		–	–	–	–	–	–	1	–	1	–	**1**
	?	–	–	–	–	–	–	1	–	1	–	1
TOTAL		**1**	**12**	**4**	**4**	**34**	**42**	**10**	**17**	**27**	**5**	**87**
	M	1	10	3	4	27	34	4	11	16	3	63
	F	–	–	–	–	–	–	3	–	–	–	3
	?	–	2	1	–	7	8	3	6	9	2	21

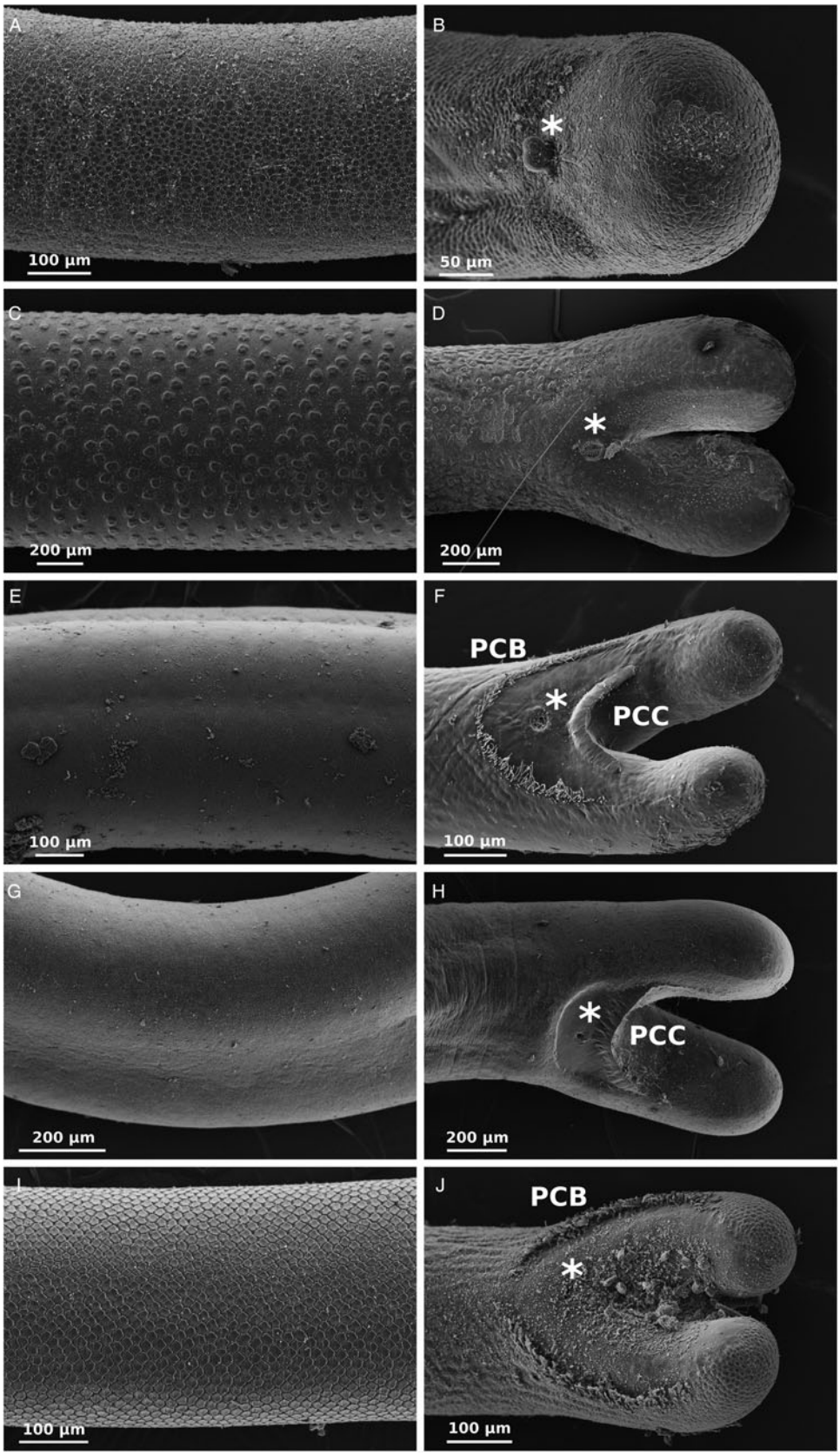

Fig. 2. SEM of nematomorph mid-body cuticular features (A, C, E, G, I) and male posterior ends (B, D, F, H, J). (A, B), *Euchordodes nigromaculatus*. (C, D), *Parachordodes diblastus*. (E, F), *Gordius paranensis*. (G, H), undescribed *Gordius* sp. (I, J), *Gordionus* n. sp. *=cloaca (in B, sperm is coming out of the cloaca), PCB, precloacal bristles; PCC, postcloacal crescent.

DNA sequences and phylogenetics

Partial CO1 sequences were successfully obtained from 71 of the 87 hairworms. The partial 18S/ITS1/5·8S/ITS2/partial 28S rDNA region was amplified and sequenced from 83 hairworms, although not all sequences were complete due to insufficient read length. Some sequences were not entirely of high quality due to long stretches of ambiguities in the ITS regions, likely driven by the bi-allelic nature of nuclear rDNA. Thus, not all of the rDNA sequences could be used for subsequent population genetic analyses. Of the 83 rDNA sequences, all but two contained high-quality sequence data for the 3′ end of the 18S rRNA gene. These 81 sequences were aligned with available nematomorph 18S reference sequences and nematode outgroups, creating a 200 bp alignment for phylogenetic analysis. The final alignment for CO1 consisted of 519 bp, with *Paragordius* spp. selected as outgroups based on the results of the 18S tree.

Bayesian and maximum-likelihood (ML) methods yielded near identical topologies for both the 18S (Fig. 3) and CO1 (Fig. 4) datasets. Therefore, the Bayesian trees are shown with posterior probabilities and bootstrap support values at branch nodes. Separate Bayesian and ML trees can be found in Supplementary Data S6–S9. Exclusion of the third codon position of CO1 did not significantly affect the topology (data not shown). The results of the phylogenetic trees are in complete agreement with the morphological identifications by SEM. With the exception of the displacement of the genus *Paragordius*, both trees support the traditional division of Gordiida into Gordiidae and Chordodidae. This is most clearly observed in the CO1 tree, which shows a well-supported bifurcation, with one branch containing only members of the genus *Gordius* and the other containing representatives from all of the remaining included genera. In the 18S tree both *Gordionus alpestris* and the putative *Gordionus* n. sp. from the present study do not form a monophyletic clade with the other *Gordionus* species. This can also be observed in the CO1 tree, although there is no available CO1 data for *G. alpestris*. Both trees support the division of Gordiidae into two groups, one containing *G. paranensis* and the other containing the unidentified *Gordius* sp. from this study. However, the *G. paranensis* 18S reference sequence is found on the branch opposite from those collected during this study. Furthermore, both trees indicate the presence of two distinct clades within the *G. paranensis* individuals from this study. Strangely, in the CO1 tree these two clades are separated by an unidentified *Gordius* sp. from New Mexico.

The rDNA region displayed substantial variation in length between the five species (Fig. 3). The longest ITS1/5·8S/ITS2 region was that of *E. nigromaculatus*, at 1880–1889 nts, whereas the shortest was that of *G. paranensis* clade 2, at 560 nts. Interestingly, there was a trend towards longer rDNA region length in species that have diverged more recently, based on the 18S tree.

CO1 sequence data for specimens OM 12 and RP 15 were excluded from phylogenetic analyses and calculations of pairwise genetic distance, as they were presumed to be nuclear pseudogene copies. The CO1 data for OM 12 contained many ambiguities, one of which led to a potential early stop codon. The CO1 data for RP 15 also contained many ambiguities and was very distant from other members of the *Gordius* sp. This coupled with the observation that the ribosomal data for this individual was highly similar to those of the other individuals of this species supports the conclusion that it is a pseudogene (data not shown). Calculations of pairwise K2P genetic distances were performed treating *G. paranensis* clades 1 and 2 as separate taxa (Table 2). Additionally, specimen ID 4 was treated as belonging to a separate clade of *Gordionus* n. sp., clade 1, due to its high degree of sequence divergence, as evidenced by its long branch length in the CO1 tree. The CO1 sequence of this specimen is unlikely to be a pseudogene due to the high quality of sequence data and additional observed differences in ribosomal sequence data and morphology. The ribosomal sequence of this specimen had numerous insertions/deletions in the ITS regions, as well as many polymorphisms in the 5·8S rRNA gene (data not shown). Mean intraspecific distance ranged from 0·01 in *G. paranensis* clade 1 to 0·044 in *G. paranensis* clade 2. Mean interspecific distance among all clades was $0{\cdot}328 \pm 0{\cdot}040$. The range of intraspecific (0·002–0·059) and interspecific (0·206–0·373) distances did not overlap, yielding a so-called 'barcoding gap' of 0·147.

Population genetics

The final dataset for analyses of population genetics was composed of 68 CO1 sequences and 66 full-length ITS1/5·8S/ITS2 sequences. Standard indices of genetic diversity for each species and sampling sites can be found in Table 3. Due to the broad range of species collected, the number of individuals of a particular species at a particular site was often low, limiting the resolution of the diversity estimates. For most species there was substantial genetic variation in both CO1 and the ITS1/5·8S/ITS2 region. In all cases nucleotide diversity (π) was higher for CO1 than for the ribosomal region. The overall number of unique haplotypes of CO1 and ITS1/5·8S/ITS2 was 54 and 45, respectively. No reliable full-length ITS1/5·8S/ITS2 sequence data were obtained from *Gordionus* n. sp. clade 2.

Due to the greater numbers in which *E. nigromaculatus* was collected, this species was selected for further analysis. Two statistical parsimony networks

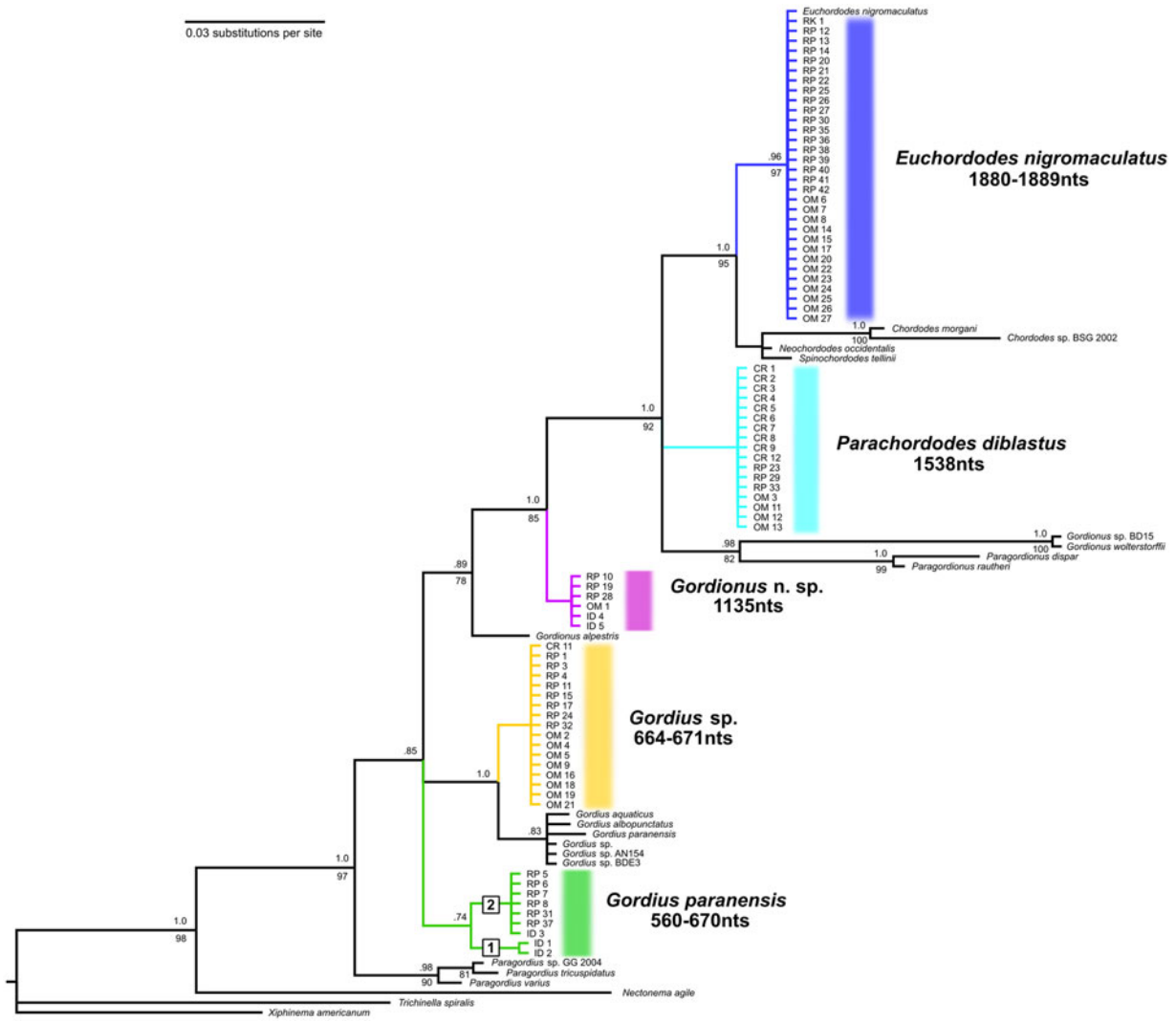

Fig. 3. Composite ML/Bayesian inference phylogenetic tree for 18S rRNA gene. Length of ITS 1, 5·8S, ITS2 region listed below species names from this study. Boxed numbers indicate two sub-clades of *Gordius paranensis* from this study.

were created to show the relationships among CO1 and ITS haplotypes (Fig. 5A and B). Twenty-six of the 34 CO1 haplotypes and 20 of the 23 ribosomal haplotypes were unique. Optimal BAPS clustering yielded three haplogroups for both the CO1 and ribosomal datasets. BAPS clustering solutions were generally inconsistent between the two markers, as the corresponding haplogroups did not tend to contain the same individuals. Proportions of haplogroups represented at each site (Fig. 5C and D) illustrate the general lack of population structure. AMOVA tests of both datasets confirmed the lack of population structure. Fixation indices Φ_{ST} for CO1 and the ribosomal region were quite low (0·036 and 0·030, respectively) and were not significant ($P = 0{\cdot}2745$ and 0·2604, respectively).

DISCUSSION

With the exception the 18S rRNA genes of *G. paranensis* and *E. nigromaculatus* (Bleidorn *et al.* 2002), no sequence data had been published for New Zealand hairworms. In this study, we present the first mitochondrial and nuclear ribosomal sequence data for *P. diblastus* and a previously reported but undescribed *Gordius* sp. (Schmidt-Rhaesa, 2008), as well as contribute mitochondrial sequences for those species already sequenced for 18S. Individuals assigned to the genus *Gordionus* were also found and molecularly characterized. This brings the total number of New Zealand gordiid species to six. We developed two novel primer sets, which may prove useful for future molecular studies of hairworms. More importantly, results from this study have implications for the systematics of the phylum Nematomorpha and the potential for cryptic species, which will be addressed below. We also compare the population structures of *E. nigromaculatus* and its potential hosts using previously published data, and provide a discussion of possible mechanisms for reduced parasite population differentiation.

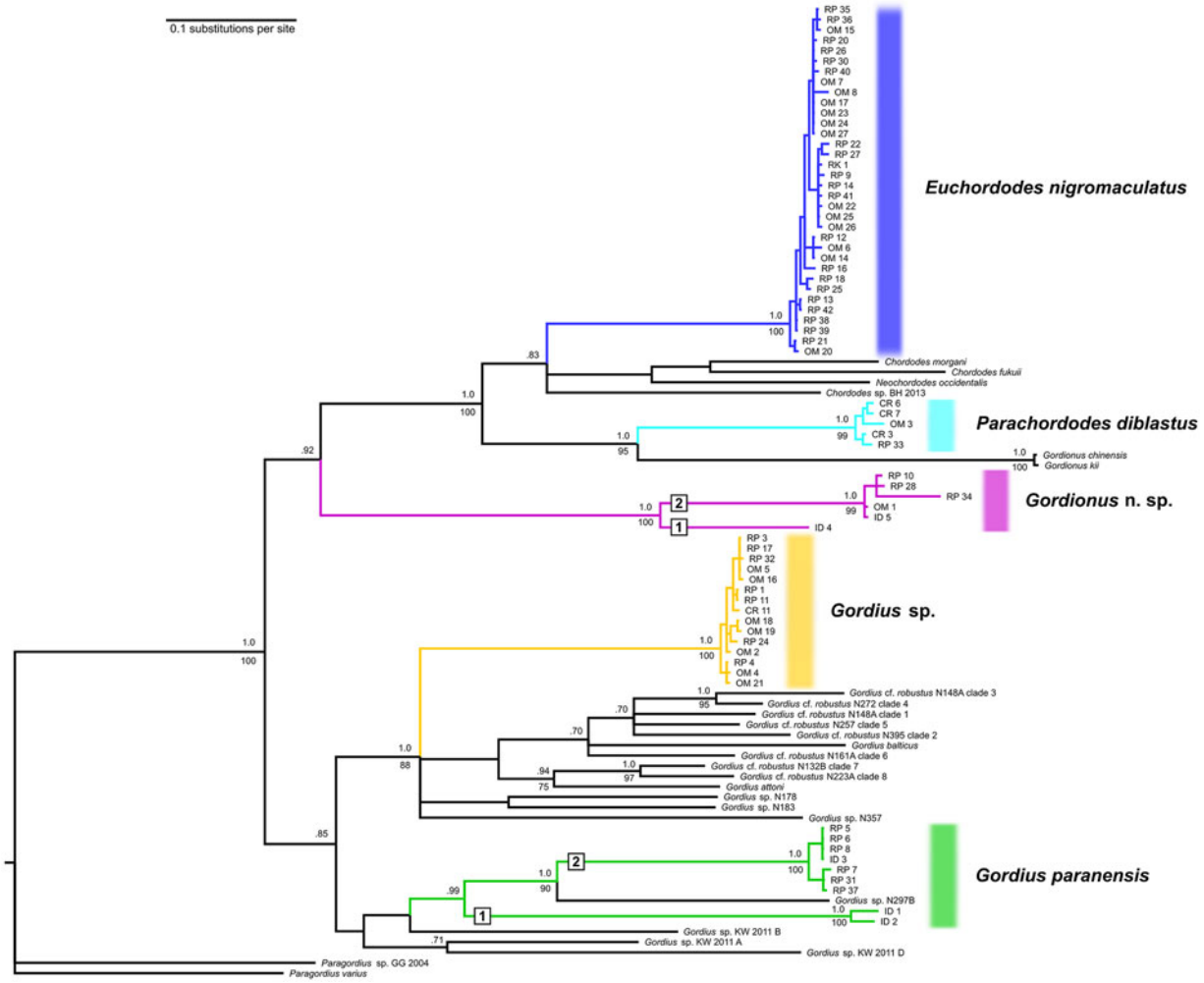

Fig. 4. Composite ML/Bayesian inference phylogenetic tree for cytochrome oxidase subunit 1 (CO1) gene. Boxed numbers indicate two sub-clades of *Gordius paranensis* and of *Gordionus* n. sp. from this study.

Nematomorph systematics and cryptic species

The results of the phylogenetic analyses are largely in agreement with previously published phylogenies within Nematomorpha. One noticeable difference, however, is the placement of the genus *Paragordius*. Our results indicate that *Paragordius* is basal to all remaining freshwater nematomorphs, whereas previous studies placed the genus within the family Chordodidae (Bleidorn *et al.* 2002; Efeykin *et al.* 2016), although this was not always well supported (Bleidorn *et al.* 2002). This discrepancy could be due in part to the relatively small size of the 18S alignment. However, this is unlikely since initial phylogenetic reconstructions using nematode outgroups for the CO1 dataset yielded the same result (data not shown). Additionally, a phylogenetic tree by Sato *et al.* (2012) placed *Paragordius* outside of Gordiidae and Chordodidae, although the tree is unrooted and therefore whether or not *Paragordius* is basal cannot be determined. This disagreement should be resolved as sequence data becomes available from additional nematomorph genera.

The presence of two well-supported clades within the *G. paranensis* specimens from this study suggests the existence of cryptic species. Hanelt *et al.* (2015) similarly found evidence of nematomorph cryptic species during an investigation of genetic lineages of *Gordius* cf. *robustus* across the contiguous USA, in which they argued the case for eight distinct species. They found a minimum among-clade CO1 K2P distance of 0·06; the distance between *G. paranensis* clades 1 and 2 of 0·306 is well above that threshold. Furthermore, the lengths of the ITS1/5·8S/ITS2 region in these two clades differ by 110 bp, much greater than the intraspecific differences observed in other species from the current study and among the eight *G.* cf. *robustus* lineages. *Gordius paranensis* has also been reported from Chile, Paraguay and Brazil, where it was originally described (Camerano, 1892, 1894; Montgomery, 1898; De Villalobos *et al.* 2005). Given the findings of the current study, it is possible that South American individuals represent cryptic species within *G. paranensis* as well, though there

Table 2. Mean CO1 K2P genetic distances of nematomorph species/clades

	Species/Clade	1	2	3	4	5	6	7
1	*Gordius paranensis* clade 1	**0·044**						
2	*Gordius paranensis* clade 2	0·306	**0·010**					
3	*Gordius* sp.	0·298	0·295	**0·010**				
4	*Gordionus* n. sp. clade 1	0·344	0·307	0·308	–			
5	*Gordionus* n. sp. clade 2	0·376	0·341	0·331	0·205	**0·030**		
6	*Parachordodes diblastus*	0·353	0·368	0·356	0·335	0·370	**0·018**	
7	*Euchordodes nigromaculatus*	0·343	0·342	0·319	0·329	0·389	0·283	**0·013**

Mean intraspecific distance on diagonal, mean interspecific below.

is no available genetic data for specimens from that region. In both the CO1 and 18S phylogenies, the genus *Gordius* was found to comprise two main clades, one containing the *G. paranensis* specimens from this study along with undescribed *Gordius* spp. from Japan (Sato *et al*. 2012), and the other containing all remaining *Gordius* spp. One obvious inconsistency in the 18S tree regarding the position of *G. paranensis* is the displacement of individuals from the current study from the sequence of a New Zealand *G. paranensis* specimen included in Bleidorn *et al*. (2002). If *G. paranensis* is a monophyletic group, it is difficult to explain how a true *G. paranensis* sequence could be included in a clade of *Gordius* spp. that lack pre-cloacal bristles. The most parsimonious explanation for this contradiction is that the specimen for that study was not properly identified, although it cannot be ruled out that pre-cloacal bristles may have evolved independently in separate lineages. This alternative scenario is supported by the separation of the two *G. paranensis* clades in the CO1 tree by an unidentified *Gordius* sp. from New Mexico (Hanelt *et al*. 2015). Although images of this specimen could not be obtained, it can be assumed that it lacked pre-cloacal bristles since the presence of this trait would have identified it as *G. paranensis* or *G. difficilis*, which also possesses pre-cloacal bristles but has cuticular areoles, instead of an undescribed species (Montgomery, 1898; Bolek and Coggins, 2002). This suggests that in addition to the potential of this trait having evolved independently more than once, it can also be subsequently lost in certain lineages. The addition of genetic data for *G. difficilis* and the newly described *G. jorriti*, which also possesses a pre-cloacal row of bristles, would shed light upon the potential multiple evolution and loss of this character (Schmidt-Rhaesa and Schwarz, 2016). The plasticity of cuticular features in nematomorphs has been illustrated previously by Hanelt *et al*. (2015). They found that two *Gordius* spp. with distinct areolar traits clustered within the *Gordius* cf. *robustus* species complex, which was classically identified by its lack of areoles. Together these findings could have implications for the interpretation of cuticular features in species delimitation for nematomorphs.

Seven specimens were identified by SEM as belonging to *Gordionus*, a genus new to New Zealand. However, monophyly of these specimens with other *Gordionus* sequences was not observed in either the CO1 or 18S trees. Schmidt-Rhaesa (2013) previously suggested that *Gordionus* may not comprise a monophyletic group due to inconsistencies within the genus and similarities to other genera regarding its supposedly characteristic areolar pattern. A revision of this genus may be necessary, but is outside the scope of this study. This issue will be addressed in a forthcoming formal species description of the specimens reported herein. From a morphological perspective, it is conceivable that slight differences in the areoles between *Gordionus* n. sp. clades 1 and 2 (data not shown) are an example of intraspecific variation, as noted elsewhere for this genus (Schmidt-Rhaesa, 2001). However, the genetic distance (0·206) is much greater than that used for species delimitation in the genus *Gordius* (see above), despite being the smallest observed inter-clade distance in the present study. Therefore, we conclude that these two clades represent distinct species. Unfortunately, only one specimen was found for *Gordionus* n. sp. clade 1, and it was not intact, preventing a comparison of the male posterior end.

Both CO1 and ribosomal molecular data support the expected placement of *P. diblastus* within the family Chordodidae but outside the subfamily Chordodinae, which includes only species without bilobed male posterior ends (Heinze, 1935; Bleidorn *et al*. 2002; Schmidt-Rhaesa, 2013). *Euchordodes nigromaculatus* was confirmed as belonging to Chordodinae using both mitochondrial and ribosomal datasets as shown by Bleidorn *et al*. (2002) and as classically defined due to its lack of tail lobes.

Lack of host–parasite co-structure

Tests of genetic differentiation of *E. nigromaculatus* using both mitochondrial and nuclear data failed to demonstrate even low levels of population structure. Because hairworms in this study were all collected in the post-parasitic adult stage, no host genetic data

Table 3. Standard population genetic indices of nematomorph species/clades of both markers

		CO1						ITS1/5·8S/ITS2					
Species	Range	bp	n	N_h	N_p	h	π	bp	n	N_h	N_p	h	π
Euchordodes nigromaculatus		526						1891					
	RK		1	1	–	–	–		1	1	–	–	–
	RP		20	18	30	0·9895 ± 0·0193	0·0125 ± 0·0069		10	10	51	1·0000 ± 0·0447	0·0088 ± 0·0048
	OM		13	9	24	0·8718 ± 0·0913	0·0114 ± 0·0065		12	10	36	0·9545 ± 0·0569	0·0061 ± 0·0033
Parachordodes diblastus		602						1538					
	CR		3	3	11	1·0000 ± 0·2722	0·0116 ± 0·0094		9	4	3	0·6944 ± 0·1470	0·0006 ± 0·0005
	RP		1	1	–	–	–		2	2	2	1·0000 ± 0·5000	0·0013 ± 0·0016
	OM		1	1	–	–	–		4	4	5	1·0000 ± 0·1768	0·0016 ± 0·0013
Gordius paranensis clade 1		631						670					
	ID		2	2	26	1·0000 ± 0·5000	0·0412 ± 0·0420		2	2	1	1·0000 ± 0·5000	0·0015 ± 0·0021
Gordius paranensis clade 2		638						560					
	RP		6	4	15	0·8000 ± 0·1721	0·0116 ± 0·0073		6	6	16	1·0000 ± 0·0962	0·0111 ± 0·0071
	ID		1	1	–	–	–		1	1	–	–	–
Gordius sp.		519						672					
	CR		1	1	–	–	–		1	1	–	–	–
	RP		7	5	10	0·9048 ± 0·1033	0·0077 ± 0·0050		8	5	14	0·8571 ± 0·1083	0·0057 ± 0·0037
	OM		7	7	14	1·0000 ± 0·0764	0·0106 ± 0·0067		8	3	8	0·4643 ± 0·2000	0·0030 ± 0·0022
Gordionus n. sp. clade 2		522											
	RP		3	3	29	1·0000 ± 0·2722	0·0370 ± 0·0285		–	–	–	–	–
	OM		1	1	–	–	–		–	–	–	–	–

bp, length of fragment in base pairs; n, number of individual sequences; N_h, number of haplotypes; N_p, number of polymorphic sites; h, haplotype diversity; π, nucleotide diversity.

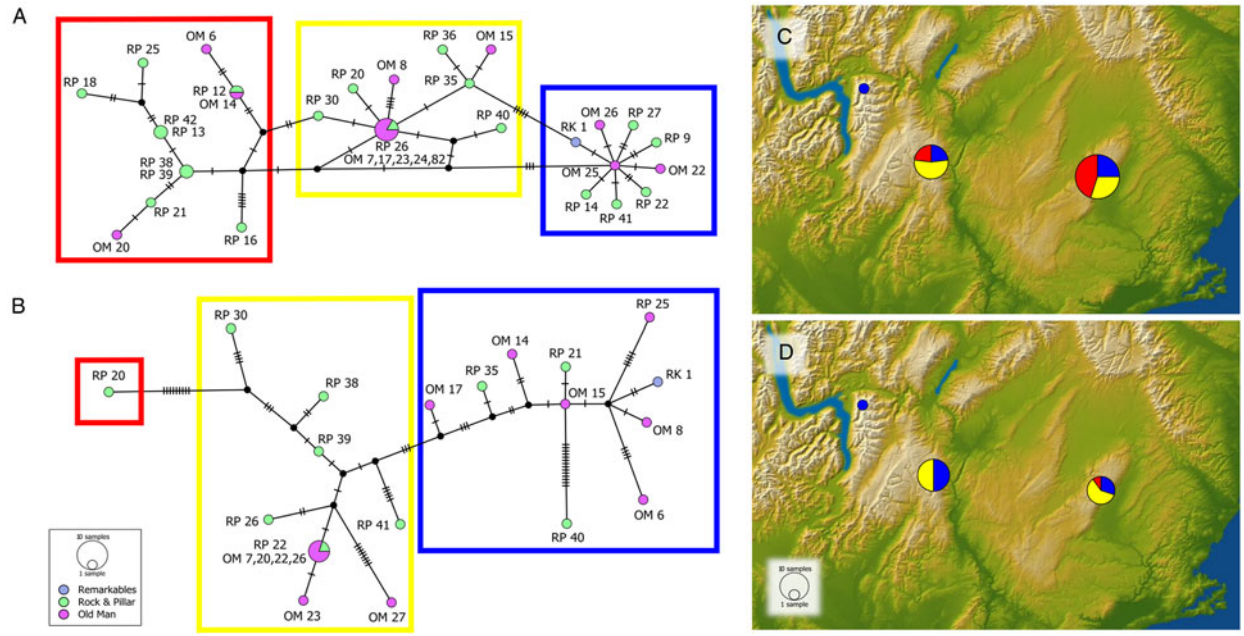

Fig. 5. Haplotypic graphics for *Euchordodes nigromaculatus*. (A, B), statistical parsimony networks of CO1 (A) and ITS1/5·8S/ITS2 (B) haplotypes. Hatch marks indicate number of mutational steps. Coloured boxes indicate haplogroups identified by BAPS. (C, D), proportion of haplogroup representation at each sampling location for CO1 (C) and ITS1/5·8S/ITS2 (D).

were obtained and therefore a direct comparison between host and parasite population structures could not be undertaken. There are, however, numerous previously published studies investigating the phylogeographical patterns of montane and subalpine insects in New Zealand (see Trewick *et al*. 2011). While there are only two confirmed definitive hosts for *E. nigromaculatus*, the wētā *Deinacrida parva* (Orthoptera: Anostostomatidae) and the grasshopper *Sigaus australis* (Poinar, 1991; Poulin, 1995), it is presumed that *E. nigromaculatus* infects a broader range of New Zealand orthopterans (Poinar, 1991; Thomas *et al*. 1999). The wētā species *Deinacrida connectens* and *Hemideina maori* are both well-studied taxa from a phylogeographical perspective (Trewick *et al*. 2000; Trewick and Wallis, 2001; King *et al*. 2003; King, 2015). Both species have been shown to display a substantial level of regional population structure, with distinct haplotype groups occurring in neighbouring ranges, even over relatively small spatial scales (~50–100 km). Genetically distinct lineages of *D. connectens* among nearby ranges in Central Otago and across the South Island were identified by both single-stranded conformation polymorphism and sequencing of CO1 (Trewick *et al*. 2000; Trewick and Wallis, 2001). Using CO1 sequence data, King (2015) similarly found genetic differentiation of *H. maori* between populations in the RP and the Black Umbrella Range, which is within the same larger mountain range as the OM, two locations from which *E. nigromaculatus* specimens were collected for the current study. Trewick (2008) also found differentiated lineages of *S. australis* populations between these two ranges. Allopatric speciation of members of the endemic cockroach genus *Celatoblatta* among South Island ranges has also been demonstrated (Trewick and Wallis, 2001; Chinn and Gemmell, 2004), with this cockroach genus being a known host taxon of New Zealand hairworms (Zervos, 1989).

A frequent assumption in studies of parasite population structure is that it should mirror that of their hosts and that, in general, parasite populations should be more differentiated (Jarne and Theron, 2001). This is based on the concept that the dispersal potential of a parasite should be dictated by its most mobile host (Prugnolle *et al*. 2005*a*), and that the low effective population sizes and short generation times typical of many parasites render them more susceptible to genetic drift (Gandon and Michalakis, 2002; Criscione and Blouin, 2005; Prugnolle *et al*. 2005*b*). Varying levels of parasite population structure as a result of differential host vagility has been demonstrated in several systems (McCoy *et al*. 2003; Criscione and Blouin, 2004; Blasco-Costa *et al*. 2012). For many parasite species with complex life cycles the most mobile host is the final host, especially in the case of parasites of vertebrates. New Zealand hairworm dispersal, however, may depend more on intermediate hosts, as flightless alpine insects such as acridid grasshoppers and wētā are known to have limited dispersal potential (White, 1978; Leisnham and Jamieson, 2002). Gordiid larvae can encyst in a wide variety of aquatic organisms, although many of these probably constitute

'dead-end' hosts (Hanelt and Janovy, 2003, 2004). Suitable intermediate hosts must be able to provide a bridge between aquatic and terrestrial habitats, and may include midges, mayflies and stoneflies (Hanelt and Janovy, 2003). While no intermediate hosts of New Zealand hairworms have been experimentally confirmed, Winterbourn (2005) found that adult stoneflies of the species *Spaniocerca zelandica* and *Cristaperla fimbria* exhibit a high prevalence of infection (69–90%) by nematomorph larvae. Winterbourn and Pohe (2016) subsequently found nematomorph larvae in two species of another New Zealand stonefly genus, *Stenoperla*. Previous studies of stonefly dispersal have demonstrated that adult stoneflies can travel hundreds or even thousands of metres along and between stream corridors (Briers *et al*. 2004; MacNeale *et al*. 2005). Correspondingly, phylogeographical analyses of two of the aforementioned New Zealand stonefly genera found little to no population differentiation/allopatric speciation over the geographical range of the current study (McCulloch *et al*. 2010). These findings together suggest that the dispersal capabilities of the potential intermediate hosts of *E. nigromaculatus* may be a factor in its observed lack of population structure.

However, intermediate host dispersal likely is not the only reason for this lack of population structure, for despite the stonefly's impressive flying ability, it is not sufficient to transport gordiid larvae directly between mountain ranges. Instead, dispersal of nematomorph lineages on this scale is likely a multi-generational process. Passive dispersal of nematomorph eggs or pre-parasitic larvae coupled with a lack of definitive host specificity may be one such avenue. Several species of hairworms deposit egg strings on debris (Hanelt and Janovy, 2002; Bolek *et al*. 2010), and these could be carried far downstream, as larval emergence can occur months after laying (Müller, 1926; Schmidt-Rhaesa, 2013). Free-living larvae could be similarly transported, as they remain infective for weeks (Hanelt *et al*. 2005). Once downstream, a larva could infect an aquatic invertebrate and subsequently be transmitted to a lowland orthopteran definitive host. While we only found *E. nigromaculatus* individuals at elevations above 1300 m, this is likely a result of bias in sampling effort, as specimens of this species occur at much lower elevations (Poinar, 1991). After emerging from this lowland definitive host, the parasite could then complete its life cycle and its offspring subsequently infect another intermediate host. As aquatic insects with flying adult stages disperse upstream as a means of combating the so-called 'drift paradox' (Müller, 1954; Hershey *et al*. 1993; Pachepsky *et al*. 2005), hairworm lineages could use this host trait to spread upstream. This process could progress in a stepping-stone manner (Kimura and Weiss, 1964) through multiple generations as seen in other parasite systems (Dybdahl and Lively, 1996; McCoy *et al*. 2005), maintaining sufficient levels of gene flow between alpine hairworm populations to prevent population structuring. Surely more studies of New Zealand nematomorphs characterizing the complete breadth of their intermediate hosts are necessary before any definitive conclusions are made regarding the influence of intermediate host movement on parasite population structure (or lack thereof).

The absence of congruence between the population structure of *E. nigromaculatus* and its potential definitive hosts stands in contrast to a once prevalent paradigm, which presumes that: (1) host mobility is the main determinant of parasite population structure; (2) population structure of parasites should mirror that of their hosts; and (3) overall levels of differentiation should be higher for parasite than for host (Price, 1980; Jarne and Theron, 2001). Recent empirical studies and meta-analyses have highlighted the importance of considering other factors in the determination of host–parasite co-structure (Bruyndonckx *et al*. 2009; Keeney *et al*. 2009; Blasco-Costa and Poulin, 2013; Couchoux *et al*. 2016; Mazé-Guilmo *et al*. 2016). Bruyndonckx *et al*. (2009) showed that while the mobility of the bat host is responsible for gene flow between populations of its tick parasite *Spinturnix bechsteini*, their population structures did not reflect one another because parasite transmission between populations did not occur during bat mating, and therefore did not result in gene flow between host populations. Their results also emphasize the influence of parasite demographic factors, such as population bottlenecks, in generating population structure. Keeney *et al*. (2009) found contrasting levels of population structure between two trematode species that both use the same marine snail as an intermediate host and shorebirds as definitive hosts. This difference was attributed in part to differences in infection intensity as well as the use of different tissues within the definitive host, which may affect transmission rates. Meta-analyses have emphasized the impact of other factors such as the per cent of free-living stages in a parasite's life cycle, parasite sexual mode and geographical range on the outcome of studies of host–parasite co-structure (Blasco-Costa and Poulin, 2013; Mazé-Guilmo *et al*. 2016). These studies and others have remodelled our understanding of the relative influence of certain aspects of parasite life-history, ecology and demography on the genetic composition of parasite populations, and provide an interesting lens through which to view the results of the current study.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017000233.

ACKNOWLEDGEMENTS

We thank Tony Stumbo and Brenah Hearne for assistance with sampling, Tania King for support with molecular work, Fátima Jorge for advice on phylogenetic analyses, Renate Walter for assistance with SEM work, and members of the University of Otago Evolutionary and Ecological Parasitology Research Group for helpful comments during manuscript preparation.

FINANCIAL SUPPORT

This research was funded by internal grants to ZJCT and RP from the Department of Zoology, University of Otago. AKY participated as an Overseas Research Associate of the Department of Biotechnology, Govt. of India at University of Hamburg.

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