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Life at the beach: comparative phylogeography of a sandhopper and its nematode parasite reveals extreme lack of parasite mtDNA variation

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Molecular genetics has proven to be an essential tool for studying the ecology, evolution and epidemiology of parasitic nematodes. However, research effort across nematode taxa has not been equal and biased towards nematodes parasitic in vertebrates. We characterize the evolutionary genetics of the mermithid nematode *Thaumamermis zealandica* Poinar, 2002 and its host, the sandhopper *Bellorchestia quoyana* (Milne-Edwards, 1840) (Talitridae: Amphipoda), across sandy beaches of New Zealand's South Island. We test the hypothesis that parasite population genetic structure mirrors that of its host. Sandhoppers and their parasites were sampled at 13 locations along the island's south-eastern coast. Sequencing of the mitochondrial gene cytochrome *c* oxidase subunit 1 (*CO1*) from *B. quoyana* reveals a regional pattern of population structure that suggests a northward pattern of dispersal. Surprisingly, no population structure was observed for *T. zealandica*. In fact, sequencing of three commonly used markers revealed no intraspecific parasite variation. This result suggests that mermithid mtDNA may evolve at an extraordinarily slow pace, perhaps as a result of extensive and frequent changes in gene order and mitochondrial genome length. Furthermore, a mermithid phylogeny based on sequences of the *18S* and *28S* ribosomal RNA genes suggests that a systematic revision of the family is necessary.

ADDITIONAL KEYWORDS: *Bellorchestia quoyana* – comparative population genetics – host–parasite phylogeography – Mermithidae – New Zealand – *Thaumamermis zealandica*.

INTRODUCTION

Nematodes are found in nearly every marine, aquatic and terrestrial environment, having evolved both free-living and parasitic forms, and comprising at least 100 000 species (Lambshead, 1993; Blaxter, 2003). Despite their taxonomic and ecological diversity, studies of nematodes are outnumbered by those of more charismatic animal taxa (Cobb, 2015; Baldwin, Nadler & Wall, 2000; De Ley, 2000). This is due in part to the difficulty with which nematode species are identified, owing to the dearth of easily distinguished morphological traits (Floyd *et al.*, 2002; Powers, 2004). The advent of molecular genetics, however, has been invaluable for characterizing nematode diversity and conducting studies of their ecology, evolution and epidemiology (Blaxter *et al.*, 1998; Read *et al.*, 2006; Criscione *et al.*, 2010).

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Parasitism has arisen repeatedly among nematode lineages, encompassing diverse host taxa including plants, invertebrates, and vertebrates (Blaxter *et al.*, 1998; Hu *et al.*, 2003). Due to their importance for health and agriculture, molecular studies abound for nematodes that infect humans, crops and livestock (Pritchard, 2001; Criscione *et al.*, 2007; Opperman *et al.*, 2008). Parasitic nematode taxa without obvious socio-economic implications, however, have generally been neglected, with much of the scientific literature consisting solely of species descriptions, morphological studies and host reports.

One such nematode taxon is the family Mermithidae Braun, 1883. All mermithids are obligate parasites of invertebrates, most commonly infecting insects (Poinar, 1991). Most mermithids have a direct life cycle with a free-living stage, typically emerging from hosts into an aquatic environment, mating and laying eggs; transmission to the host is accomplished via ingestion of the eggs or penetration by the hatched larvae

(Poinar, 1991). Mermithids have gained attention due to their potential as biocontrol agents of mosquitos (Platzer, 2007), their unusual mitochondrial genomes (Hyman *et al.*, 2011) and their ability to manipulate the behaviour of their hosts (Vance, 1996). However, little is known of their evolution, both at the species and population levels.

Thaumamermis zealandica Poinar, 2002 is a mermithid parasite of the sandhopper *Bellorchestia quoyana* (Milne-Edwards, 1840) (Talitridae: Amphipoda) found on the sandy beaches around Dunedin, New Zealand (Poinar, Latham & Poulin, 2002). Several studies have investigated its general ecology and potential host manipulation strategies (Poulin & Rate, 2001; Poulin & Latham, 2002a, b; Williams, Poulin & Sinclair, 2004; Rasmussen & Randhawa, 2015); however, no genetic studies have been carried out for this parasite or its host nor have surveys been conducted outside of the Dunedin area. Macro- and microevolutionary investigations of *T. zealandica* and *B. quoyana* implementing molecular genetics should yield interesting information regarding the phylogeny of the family Mermithidae and the factors influencing migration of the host, and how this might be reflected in the population genetic structure of the parasite.

Recent population genetic studies of host and parasite have sought to investigate instances of host–parasite co-structure (Dybdahl & Lively, 1996; Criscione & Blouin, 2004; McCoy, Boulinier & Tirard, 2005). Because parasites are typically dependent on their hosts for dispersal, a longstanding assumption in the field has been that the parasite's population structure should reflect that of its host (Jarne & Théron, 2001; Gandon & Michalakis, 2002). Furthermore, due to lower effective population sizes and shorter generation times, parasite populations are expected to exhibit higher levels of differentiation than those of their hosts (Jarne & Théron, 2001). Instances for which these assumptions have been upheld have proven useful for studying host phylogeography, as the increased levels of parasite population differentiation have provided increased spatiotemporal resolution in elucidating the historical, ecological and geological factors shaping host distributions (Nieberding *et al.*, 2005; Galbreath & Hoberg, 2012). While no population genetic or phylogeographical investigations have been conducted for *B. quoyana*, there are numerous studies of other supralittoral talitrids (see Pavesi & Ketmaier, 2013). These have generally provided evidence for rafting, the association with floating wrack, as a means of dispersal. Although most of these studies have focussed on talitrid species in the Mediterranean, numerous cases of kelp-mediated dispersal for invertebrate taxa in southern waters, and specifically in New Zealand, have been documented (Nikula *et al.*, 2010; Fraser, Nikula & Waters, 2011), suggesting that *B. quoyana* may also depend on this means of transport. As *T. zealandica* is a direct life-cycle parasite with only one known host, it is expected that its population structure will be similar to that of *B. quoyana* and that increased population differentiation may reveal slight patterns left uncovered by study of host genetics alone.

The aim of the current study is to investigate the population genetic structure and phylogeography of *B. quoyana* and its parasite *T. zealandica*, while testing the assumption that a parasite's population structure mirrors that of its host. Additionally, by conducting the most widespread collection of *T. zealandica* to date, we provide new information on the spatial distribution of the parasite. In obtaining the first genetic data for the host and parasite, this data set also enable us to investigate the phylogeny of the family Mermithidae. The findings of this study also have important implications for the selection of appropriate molecular markers for future population genetic studies of mermithids.

METHODS

Talitrid and mermithid collection

Bellorchestia quoyana individuals were collected from 13 sandy beaches along the south-coast of New Zealand's South Island from December 2015 to March 2016 (Supporting Information, Table S1). All specimens were collected by hand from the sediment below patches of macroalgae. Sandhoppers were placed in 2-L containers with moist sediment, transported to the laboratory and were maintained at a temperature below 20 °C while awaiting dissection. Individuals were processed within 3 days of collection. The four posteriormost pereopods (nos 6 and 7, two each) of the first 30 unparasitized individuals from each site were removed and preserved in 99% ethanol in a 1.5-mL Eppendorf tube for genetic analysis. In parasitized individuals, one or multiple mermithids were removed from the haemocoel and transferred to a separate petri dish containing sea water. Mermithids were relaxed using heated seawater and untangled with fine forceps. Individual mermithids were measured, rinsed to prevent contamination by *B. quoyana* internal tissue and ectoparasites and preserved in 95% ethanol in a 1.5-mL Eppendorf tube.

DNA extraction, PCR amplification and sequencing

Extractions of *B. quoyana* genomic DNA were performed on 20 samples each for all sites, for a total of 260 *B. quoyana* samples. DNA was extracted using a modified Chelex method (Walsh, Metzger & Higuchi, 1991). For each sandhopper, the basis, ischium and merus (segments 2–4) of one pereopod were macerated with forceps and placed in 300 µL of an aqueous 5%

Chelex 100 (Bio-Rad) solution containing 0.2 mg/mL of proteinase K (Roche). Extractions were incubated at 55 °C overnight and then at 94 °C for 10 min to deactivate the proteinase K. Tubes were then centrifuged at 14 000 rpm for 10 min 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. Extractions of *T. zealandica* were performed using the same Chelex protocol as for the talitrids, except that a ~20-mm segment of the worm was used as the source tissue. For worms smaller than 20 mm, the entire worm was used. Extractions were performed on all collected mermithids.

Unless otherwise stated, all PCRs were performed in a final volume of 15 µL, comprising 3 µL of MyTaqTM Red 5× reaction buffer (Bioline), 0.75 µL of 10 µM stock (75 pmol) of each primer, 0.2 µL (1 U) of MyTaqTM Red DNA polymerase and 3 µL of extracted sandhopper or mermithid genomic DNA. PCR thermal cycling profiles are provided in Supporting Information, Table 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) using the same primers as for amplification.

For *B. quoyana*, a 709 bp partial fragment from the 5′ end of the mitochondrial gene cytochrome *c* oxidase subunit 1 (*CO1*) was amplified using the 'universal' primers LCO1498 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). Partial *CO1* fragments were amplified from a total of 260 *B. quoyana* individuals.

For *T. zealandica*, multiple markers were amplified via PCR. For *CO1*, two partially overlapping regions were amplified. A 418 bp fragment of the I3M11 partition (Erpenbeck, Hooper & Wörheide, 2006), from the middle of the *CO1* gene, was amplified using the primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) (Bowles & McManus, 1994) and JB5 (5′-AGCACCTAAACTTAAAACATAATGAAA-3′) (Derycke *et al.*, 2005). In total, the I3M11 fragment of *CO1* was amplified from 14 individuals.

For amplification of the M1M6 partition (Folmer fragment) of *CO1*, the novel primers MM_M1M6_F (5′-GGATCTTTCTATTTTATTCTAAGATTGTGG-3′) and MM_M1M6_R (5′-ATAGTCAGATGTGATAAGATTCCG-3′) were used. Information of how these and other novel primers were designed is included in Supporting Information, File S3. In total, the M1M6 fragment of *CO1* was amplified from 83 individuals.

The amplification of a 474 bp partial fragment of the mitochondrial gene cytochrome b (*CYTB*) was carried out using the novel primers MM_CytB_F (5′-GCATTTWTAGGGTATGTKYTACCYTGR-3′) and MM_CytB_R (5′-CGWGGYATMCCATTTAADCCYAWAAAR-3′). PCRs were performed as previously stated but with 1.5 µL of 10 µM stock (150 pmol) of each primer. In total, the partial *CYTB* fragment was amplified from 37 individuals.

A 423 bp partial fragment of the mitochondrial gene NADH dehydrogenase subunit 4 (*NADH4*) was amplified using the novel primers TZ_ND4_F (5′-TCCCATATTATTTTTACTCTTAGG-3′) and TZ_ND4_R (5′-TTTTAAAATCTCTTTGGAATAAGC-3′). In total, a partial region of *NADH4* was amplified from 37 individuals.

Two partial fragments of mermithid nuclear ribosomal RNA (rRNA) genes were also amplified: the small ribosomal subunit (*18S*) and the large ribosomal subunit (*28S*). For the amplification of a ~1700 bp partial fragment of the *18S rRNA* gene, the primers 1.2F (5′-TGCTTGTCTCAAAGATTAAGC-3′) and 9R (5′-GATCCTTCCGCAGGTTCACCTAC-3′) were used (Whiting, 2002). For the amplification of a ~1100 bp fragment of the *28S rRNA* gene, the primers 28S rd1.2A (5′-CCCSSGTAATTTAAGCATATTA-3′) and 28S B (5′-TCGGAAGGAACCAGCTAC-3′) were used (Whiting, 2002). In total, partial fragments from both *18S* and *28S* were amplified from three individuals.

Sequence processing

Raw chromatogram files with base calls were uploaded into Geneious v8.1.8 (http://www.geneious.com; Kearse *et al.*, 2012). Sequences were automatically trimmed with a default error probability limit of 0.05. All sequences were manually edited for incorrect or ambiguous base calls. For all non-protein-coding markers, sequences were aligned using MAFFT (Katoh & Standley, 2013), with the specific algorithm selected by the 'Auto' function in Geneious. For protein-coding genes, sequences were aligned using the 'Translation Align' tool, implementing MAFFT and using the BLOSUM62 scoring matrix (Henikoff & Henikoff, 1992). Sequences were then inspected further for obvious incorrect base calls. For protein-coding genes, the correct reading frame was determined by toggling frame start positions and examining the alignment for stop codons. The reading frame was then confirmed by comparing to reference sequences on GenBank (www.ncbi.nlm.nih.gov). All sequences from this study are available on GenBank (accession nos KY264137–65).

Sandhopper population genetics

The final *CO1* sequence alignment for *B. quoyana* encompassed 622 bp. Calculations of pairwise genetic distance between haplotypes were performed in MEGA

v7 (Kumar, Stecher & Tamura, 2016) using the Kimura 2-parameter (K2P) measure of genetic divergence (Kimura, 1980). Haplotype networks were constructed with PopART (Leigh & Bryant, 2015) using the median joining and TCS network inference methods (Bandelt, Forster & Röhl, 1999; Clement, Posada & Crandall, 2000). Arlequin v3.5 (Excoffier & Lischer, 2010) was used to calculate the standard indices of haplotype diversity (h) and nucleotide diversity (π), perform analyses of molecular variance (AMOVAs) and spatial analyses of molecular variance (SAMOVAs) (Dupanloup, Schneider & Excoffier, 2002), as well as conduct a Mantel test (Mantel, 1967). For SAMOVA, AMOVA and Mantel tests, genetic distance was estimated as determined by a model of sequence evolution selected using the corrected Akaike information criterion (AICc) in jModelTest v2.1.6 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012), which was performed remotely using the CIPRES Science Gateway v3.3 (Miller, Pfeiffer & Schwartz, 2010). The two best models, TIM2 + G and TIM2 + I + G, are not implemented in Arlequin, so the third best model, TrN + G (gamma shape parameter $\alpha = 0.023$) was used (Tamura & Nei, 1993). The difference in AICc score between TrN + G and the two best models was low (1.5531 and 0.0515). Clustering of sampling locations into groups for hierarchical tests of population structure was performed using SAMOVA. Sequential SAMOVA tests were performed both with and without the inclusion of spatial information, with the number of groups (K) ranging from 2 to 12 (number of sites – 1). AMOVA tests (see below) were performed for all grouping schemes, and the resulting F_{ST}, F_{SC} and F_{CT} were plotted and compared to identify the most optimal number of groups, similar to the ΔK method (Evanno, Regnaut & Goudet, 2005) used for microsatellite data in STRUCTURE (Pritchard, Stephens & Donnelly, 2000), as has been done by others (Kuhn *et al.*, 2010; Domínguez-Domínguez *et al.*, 2011; Shi, Kerdelhué & Ye, 2012). Population differentiation was visualized by creating principal coordinates of analysis (PCoA) plots of Nei's uncorrected measure of nucleotide differentiation, D_{XY}, in Genalex (Nei, 1978; Peakall & Smouse, 2006). Genetic structure was investigated by three independent AMOVAs, with significance testing done by 10 000 random permutations. One treated all populations as being separate subpopulations. The second test used the population groups as determined from the SAMOVA. The third test used the SAMOVA groups except one sampling location (TT) was transferred between two groups to preserve geographical contiguity (see Results). A Mantel test was performed to investigate genetic isolation by distance. The Geographic Distance Matrix Generator (Ersts, 2000, http://biodiversityinformatics.amnh.org/open_source/gdmg) was used to calculate the linear distance in kilometres between all pairs of sampling locations from their geographical coordinates. Genetic isolation was measured as the pairwise difference in $\Phi_{ST}/(1 - \Phi_{ST})$ (Rousset, 1997). Significance testing was performed through 1000 random permutations.

Mermithid phylogenetics

Markers used in phylogenetic tree-building for the family Mermithidae were the partial *18S rRNA* gene, partial *28S rRNA* gene and partial *CO1* gene. The length of each alignment was as follows: *18S* = 755 bp, *28S* = 277 bp, *CO1* = 328 bp. These alignments are significantly shorter than the fragments amplified from *T. zealandica* due to the limited coverage of many mermithid sequences available on GenBank, as inclusion of more taxa was prioritized over alignment length and incorporation of missing data was avoided. All mermithid sequences available for each marker on GenBank that fully spanned each alignment were included, with the exception of identical duplicate sequences from the same species and source study. One additional *28S* sequence from *Amphimermis* sp. B 2007 (EF617373) was excluded. This sequence has 98.88% nucleotide sequence identity with *Octomyomermis huazhongensis*, as opposed to 79.48% identity with the conspecific *Amphimermis* sp. A 2007. These three sequences were all obtained in the same study (Wang *et al.*, 2007). A list of the mermithid and outgroup sequences from GenBank used in phylogenetic analyses is provided in Table 1.

Selection of nucleotide substitution models and optimal partitioning schemes was determined using PartitionFinder v2.0.0 (pre-release 14) (Lanfear *et al.*, 2012), which utilizes the software PhyML v3.0 (Guindon *et al.*, 2010). The AICc was used for evaluating the likelihood of the proposed models and partitioning schemes. Phylogenetic tree building was performed with MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003) and RAxML v8 (Stamatakis, 2014) and was performed remotely using CIPRES. For *CO1*, the optimal scheme partitioned the data into two partitions, one containing codon positions 1 and 2 and the other containing codon position 3. Substitution saturation of the third codon position of *CO1* was assessed by plots of transitions/transversions vs. Jukes–Cantor '69 distance (Jukes & Cantor, 1969), created in DAMBE v6.4.2 (Xia, 2013). For MrBayes, models selected by PartitionFinder for each marker were used: *18S* = GTR + I + Γ, *28S* = GTR + I + Γ, *CO1* (codon positions 1 and 2) = GTR + Γ and *CO1* (codon position 3) = HKY + Γ. For Bayesian analyses, 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

Table 1. Accession numbers and references of mermithid and outgroup sequences used for phylogenetic analyses

Species	*18S*	*28S*	*CO1*	Reference
Xiphinema americanum	AY283170	AY580056	AY382608	Neilson *et al.* (2004); DS; He *et al.* (2005)
Anatonchus tridentatus	AY284768	AY593065		Holterman *et al.* (2006); DS
Mononchus tunbridgensis	AY593954	AY593063		Holterman *et al.* (2006); DS
Mononchus truncatus	AY284762	AY593064		Holterman *et al.* (2006); DS
Longidorus helveticus	EF538759	EF538753	EF538747	Kumari *et al.* (2009)
Agamermis changshaensis	DQ628908	EF617371	EF617362	Wang *et al.* (2007)
Agamermis sp. BH-2006	DQ665653		DQ665656	DS
Agamermis xianyangensis	EF617352	EF617370	EF617361	Wang *et al.* (2007)
Allomermis solenopsii	DQ533953			Poinar *et al.* (2007
Amphimermis sp. A-2007	EF617354	EF617372	EF617363	Wang *et al.* (2007)
Amphimermis sp. B-2007	EF617355		EF617364	Wang *et al.* (2007)
Gastromermis sp. BH-2006	DQ533954			DS
Gastromermis viridis			EU876605	St-Onge *et al.* (2008)
Heleidomermis sp. BH-2006	DQ533955			DS
Hexamermis agrotis A	DQ530350	EF617369	EF617360	Wang *et al.* (2007)
Hexamermis agrotis B			EF368011	DS
Isomermis lairdi	FN400892			Crainey *et al.* (2009)
Isomermis wisconsinensis			EU876606	St-Onge *et al.* (2008)
Mesomermis camdenensis			EU876607	St-Onge *et al.* (2008)
Mesomermis flumenalis complex S1			EU876610	St-Onge *et al.* (2008)
Mesomermis flumenalis complex S2			EU876611	St-Onge *et al.* (2008
Mesomermis flumenalis complex W1			EU876608	St-Onge *et al.* (2008)
Mesomermis flumenalis complex W2			EU876609	St-Onge *et al.* (2008)
Limnomermis sp. 1 JH-2014	KJ636371			DS
Mermis nigrescens BC, CA 1	DQ518905			DS
Mermis nigrescens BC, CA 2	AF036641			Blaxter *et al.* (1998)
Mermis nigrescens New Zealand	KF886018	KF886018		Presswell *et al.* (2015)
Mermithid sp. JH-2004	AY284743			Holterman *et al.* (2006)
Mermithidae sp. 1 KCK-2013	KC243312			Kobylinski *et al.* (2012)
Mermithidae sp. 2 JH-2014	KJ636328			DS
Mermithidae sp. A-AV-2003	AY374415			Vandergast & Roderick (2003)
Mermithidae sp. B-AV-2003	AY374416			Vandergast & Roderick (2003)
Mermithidae sp. C-AV-2003	AY374417			Vandergast & Roderick (2003)
Mermithidae sp. JR-2009	FJ982324			Ross *et al.* (2010)
Mermithidae sp. MHMH-2008	FJ040480			Holterman *et al.* (2006)
Mermithidae sp. NM1	LC114020			Kubo *et al.* (2016)
Mermithidae sp. TB-2009	FJ605514			Yeates & Buckley (2009)
Nematoda sp. MQ26	JQ894731			Umbers *et al.* (2015)
Nematoda sp. MQ94	JQ894732			Umbers *et al.* (2015)
Octomyomermis huazhongensis	EF617353	EF617368	EF617359	Wang *et al.* (2007)
Ovomermis sinensis China	DQ520879			Wang *et al.* (2007)
Ovomermis sinensis Europe	KU177046			DS
Romanomermis culicivorax	DQ418791	EF417153	EF154459	DS; Sonnenberg, Nolte & Tautz (2007)
Romanomermis iyengari	JX021620		EF175764	DS
Romanomermis nielseni			EF175763	DS
Romanomermis sichuanensis	EF612769	EF617366	EF617357	Wang *et al.* (2007)
Romanomermis wuchangensis	DQ520878	EF617365	EF617356	Wang *et al.* (2007)
Strelkovimermis spiculatus	DQ665654	LN879496	LN879495	DS
Strelkovimermis spiculatus isolate Cd Punta Lara	KP270700			Belaich *et al.* (2015)
Strelkovimermis spiculatus isolate Oa Los Hornos	KP270703			Belaich *et al.* (2015)
Thaumamermis cosgrovei	DQ665655		DQ520858	DS; Tang & Hyman (2007)

DS, direct submission.

(Drummond & 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.

A fourth, concatenated alignment including sequences from all three markers was also used in phylogenetic analyses. This alignment included two dorylaimid outgroup species (*Xiphinema americanum* and *Longidorus helveticus*) and three mononchid outgroup species (*Mononchus truncatus*, *Mononchus tunbridgensis* and *Anatonchus tridendatus*). Mermithid species included were *Agamamermis chanshaensis*, *Agamermis xianyangensis*, *Amphimermis* sp. A 2007, *Hexamermis agrotis*, *Mermis nigrescens*, *O. huazhongensis*, *Ovomermis sinensis*, *Romanomermis culicivorax*, *Romanomermis sichuanensis*, *Romanomermis wuchangensis*, *Strelkovimermis spiculatus*, *Thaumamermis cosgrovei* and *T. zealandica*. Some taxa were included despite missing data at some loci. No *28S* data are available for *S. spiculatus* and *T. cosgrovei*. No *CO1* data are available for the three mononchid species or the mermithid species *M. nigrescens*. Despite the unavailability of sequence data for the aforementioned markers, these species were included to increase the number of taxa in the alignment and because missing data are not expected to have a significant impact on the results of phylogenetic analyses of multi-locus data (Wiens & Morrill, 2011; Streicher, Schulte & Wiens, 2016). These data were partitioned into *18S*, *28S*, *CO1* codon positions 1 and 2, and *CO1* codon position 3, utilizing the same models and parameters as in the individual analyses. Separate analyses were performed with the third codon position of *CO1* both included and excluded.

RESULTS

Collections

In total, 247 *T. zealandica* were isolated from 6040 talitrids. Mermithid-infected *B. quoyana* were found at 8 of the 13 sampling locations: Long Beach (LB), Aramoana (AR), Smails (SM), Tomahawk (TK), Taeri Mouth (TM), Tautuku (TT), Porpoise Bay (PB) and Oreti (OT) (Table 2). No mermithids were found north of LB. Among the sites at which infected sand hoppers were found, there was a wide range of prevalence, from 0.35% at OT to 15.56% at PB. The mean intensity (number of parasites per infected host) among all sites was 1.355 (SD = 0.694). The mean mermithid length among all sites was 67.01 mm (SD = 34.98). The longest mermithid found was 146 mm.

Bellorchestia quoyana: population genetics

From the 260 *B. quoyana CO1* sequences, 23 polymorphic sites and 20 unique haplotypes were identified, although two of these (Moeraki_06 and Oreti_08) only differed from a more common haplotype due to an ambiguous nucleotide (both T → K at position 16 in alignment). Standard indices of population genetic diversity are provided in Table 3. Three of the haplotypes (nos 1, 2 and 3) were shared among many individuals, whereas the remaining 17 were each only found in a single individual.

Table 2. Collection results, including prevalence (% parasitized), intensity (*n* mermithids per infected host) and mean mermithid length

Site	*n Bellorchestia quoyana*	*n* parasitized (%)	*n Thaumamermis zealandica*	Mean intensity ± SD	Mean *Thaumamermis zealandica* length ± SD (mm)
NB	313	0	0	N/A	N/A
SU	275	0	0	N/A	N/A
OA	298*	0	0	N/A	N/A
MO	395	0	0	N/A	N/A
WK	310	0	0	N/A	N/A
LB	967	32 (3.31)	46	1.438 ± 0.840	56.80 ± 31.35
AR	535	7 (1.31)	10	1.429 ± 0.787	73.14 ± 32.16**
SM	460	24 (5.22)	36	1.500 ± 0.885	59.28 ± 33.06
TK	133	12 (9.02)	18	1.500 ± 0.905	73.67 ± 29.75
TM	221	18 (8.14)	19	1.056 ± 0.236	50.53 ± 41.20
TT	722	10 (1.39)	14	1.400 ± 0.966	91.17 ± 32.56
PB	482	75 (15.56)	102	1.360 ± 0.629	72.72 ± 35.08
OT	568	2 (0.35)	2	1.000 ± 0.000	78.50 ± 27.58
Total	6040	180 (3.13)	247	1.355 ± 0.694	67.01 ± 34.98

*Number of *Bellorchestia quoyana* individuals at this site includes an unidentified talitrid species (see Discussion). **Length data not obtained from three *Thaumamermis zealandica* individuals at this site.

Pairwise K2P genetic distance among these haplotypes ranges from 0.2 to 1.5%. TCS and median joining methods yielded identical haplotype network topologies (Fig. 1). The most common haplotype, haplotype 1, is separated from both haplotypes 2 and 3 by single mutational steps. Haplotypes 2 and 3 are separated from each other by two mutational steps, either through haplotype 1 or an unsampled haplotype. Most unique haplotypes are separated from only one of the common haplotypes by one or two mutational steps, with just two exceptions (Fig. 1). Haplotype 1 was found at every *B. quoyana* sampling site, and at the Christchurch-area beaches New Brighton (NB) and Sumner (SU), it was the only represented haplotype (Fig. 2). Haplotype 2 was only found at sites south of the Otago Harbour entrance and was most prevalent at SM, TK and TM. Haplotype 3 was found from Oamaru (OA) to PB, but was most common at the central coast locations OA, Moeraki (MO), Waikouati (WK), LB and AR. In general, there was a pattern of high haplotype diversity in southern sampling locations, moderate diversity in central locations and no diversity in northern locations. In other words, haplotype diversity decreased with decreasing latitude. The optimal number of SAMOVA groups was determined to be three, as the largest rise in F_{CT} (differentiation among groups) and fall in F_{SC} (differentiation among populations within groups) occurred when group number (K) was increased from two to three (Supporting Information, Fig. S4). SAMOVA tests yielded identical groupings of populations when performed both with and without spatial information, largely corresponding to regional subdivision. Group 1 contains just the northern sites of NB and SU. Group 2 contains the southern sites OT, PB, TM, TK and SM. Group 3 contains the central sites of OA, MO, WK, LB and AR along with one southern site, TT. This grouping is also evident from the PCoA (Fig. 3). AMOVA tests revealed substantial levels of differentiation at the global, SAMOVA group and regional levels (Table 4). The regional hierarchy [Group 1: (Group 2 – TT): (Group 3 +

Table 3. Standard population genetic indices for the *Bellorchestia quoyana* at all collection sites

Site	n	N_h	N_p	h	π
NB	20	1	–	–	–
SU	20	1	–	–	–
OA	20	4	8	0.3635 ± 0.1309	0.001557 ± 0.001234
MO	20	5	4	0.6316 ± 0.0875	0.001320 ± 0.001101
WK	20	4	4	0.6000 ± 0.0771	0.001295 ± 0.001087
LB	20	2	1	0.5053 ± 0.0560	0.000812 ± 0.000803
AR	20	2	1	0.5053 ± 0.0560	0.000812 ± 0.000803
SM	20	4	4	0.7211 ± 0.0506	0.001794 ± 0.001363
TK	20	5	4	0.6789 ± 0.0798	0.001591 ± 0.001252
TM	20	6	5	0.6737 ± 0.0998	0.001836 ± 0.001386
TT	20	4	3	0.5368 ± 0.1042	0.001032 ± 0.000935
PB	20	4	3	0.5737 ± 0.0904	0.001092 ± 0.000970
OT	20	4	2	0.4895 ± 0.1167	0.000795 ± 0.000792

π, nucleotide diversity; h, haplotype diversity; n, number of individuals sequenced, N_h, number of haplotypes, N_p, number of polymorphic sites.

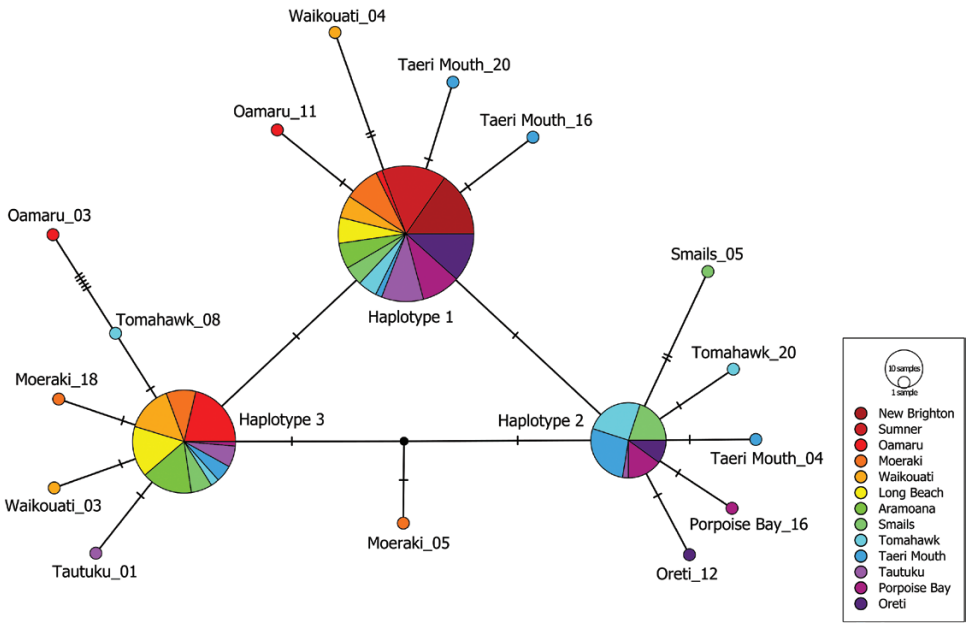

Figure 1. Statistical parsimony network of *Bellorchestia quoyana CO1* haplotypes. Hatch marks represent mutational steps. Dark circles represent inferred, unsampled haplotypes. Sampling locations are coloured according to their position along the coast, with hotter colours representing northeastern locations and cooler colours representing southwestern locations.

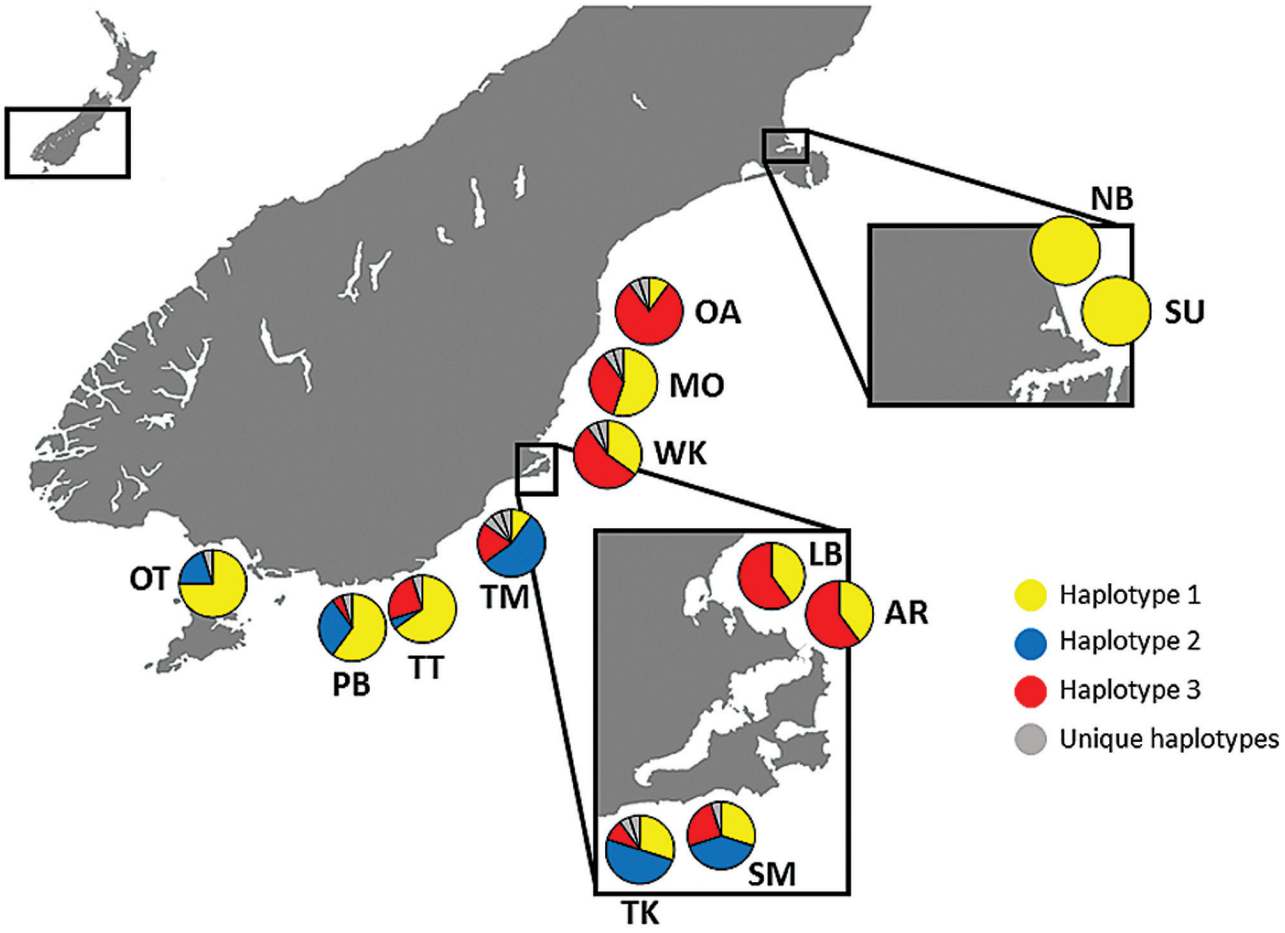

Figure 2. Map of study area indicating *Bellorchestia quoyana CO1* haplotype representation at the various collection sites. Haplotype 1 is found at all locations, while haplotype 2 is found only in southern locations. Haplotype 3 is mainly found in central locations but is also found in lower numbers further south. Note a northward trend of decreasing diversity.

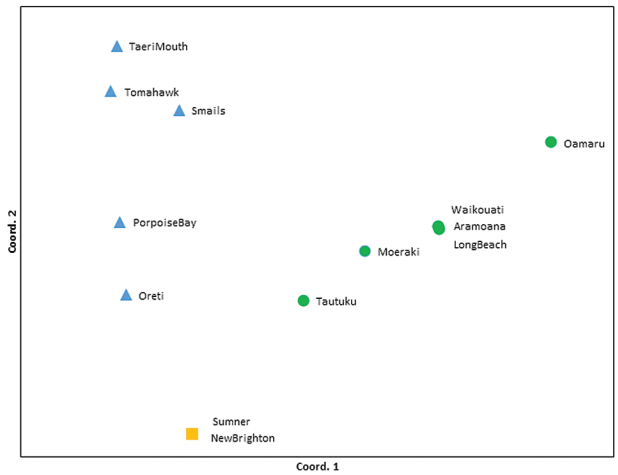

Figure 3. PCoA of Nei's uncorrected measure of nucleotide differentiation, *DXY*. Shapes and colours represent the groups identified by SAMOVA.

TT)] performed similarly to that of the SAMOVA groups. A simple Mantel test of a linear relationship linking distance between sampling location and genetic isolation [$\Phi_{ST}/(1 - \Phi_{ST})$] showed moderate levels of isolation by distance ($r^2 = 0.1496$; $p = 0.007$) (Fig. 4).

THAUMAMERMIS ZEALANDICA: SEQUENCE CHARACTERISTICS AND MERMITHID PHYLOGENY

Partial sequences of *CYTB* (alignment length = 418 bp) and *NADH4* (384 bp) were obtained from five individuals each from each of the eight locations at which mermithids were found (except for OT where only two were found), for a total of 37 sequences for each of the two markers. Partial sequence of the M1M6 partition of *CO1* (686 bp) was obtained from at least ten mermithids from each location except for OT, for a total of 83 sequences. Additionally, sequence data from the I3M11 partition of *CO1* (385 bp) were obtained from a total of 14 individuals

Table 4. Results of AMOVA tests

Analysis	Source of variation	d.f.	% var.	Fixation indices	*P*-value
Overall	Among sites	12	26.69	$\Phi_{ST} = 0.26689$	< 0.001
	Within sites	247	73.31		
SAMOVA	Among groups	2	30.70	$\Phi_{CT} = 0.30701$	< 0.001
	Among sites within groups	10	3.49	$\Phi_{SC} = 0.05038$	< 0.01
	Within sites	247	65.81	$\Phi_{ST} = 0.34192$	< 0.001
Regional	Among regions	2	28.98	$\Phi_{CT} = 0.28983$	< 0.001
	Among sites within regions	10	4.79	$\Phi_{SC} = 0.06743$	< 0.01
	Within sites	247	66.23	$\Phi_{ST} = 0.33771$	< 0.001

d.f., degrees of freedom; % var., percentage of total variation explained by each source.

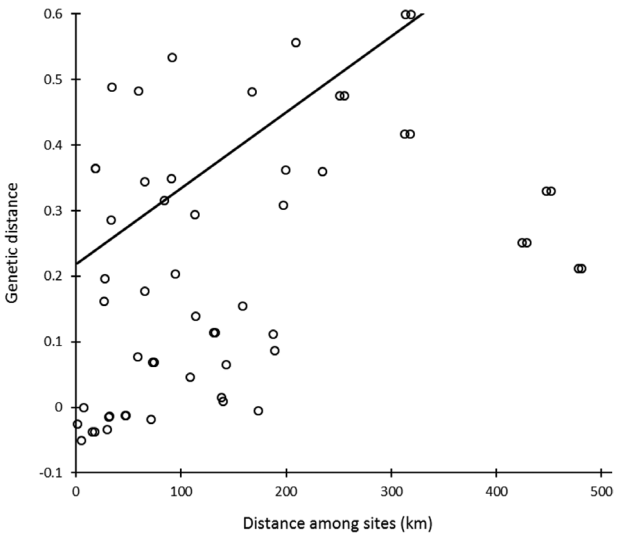

Figure 4. Plot of linear distance between sites vs. genetic distance $[\Phi_{ST}/(1 - \Phi_{ST})]$, showing moderate levels of isolation by distance ($r^2 = 0.1496$; $P = 0.007$).

among four locations (OT, PB, AR, LB). Surprisingly, for every marker, all sequences were identical to one another and not a single polymorphism was observed.

In analyses of mermithid phylogeny, Bayesian and maximum likelihood (ML) methods each yielded similar topologies for the *18S*, *28S*, *CO1* and concatenated data sets. Bayesian trees for the *18S* and concatenated data sets are displayed here with both posterior probabilities and bootstrap support values. The separate Bayesian and ML for the *18S* data set, along with the *28S* and *CO1* data sets alone (with and without third codon position), are provided in Supporting Information, Figs S5–S12. The *18S* tree (Fig. 5) shows a trifurcation at the most basal node within the family, separating it into three clades: clade 1 containing the genera *Allomermis*, *Mermis*, *Heleidomermis*, *Limnomermis*, *Isomermis*, *Gastromermis*, *Strelkovimermis*, *Amphimermis* and *Romanomermis*; clade 2 containing the genera *Octomyomermis*, *Hexamermis*, *Agamermis* and *Ovomermis*; and clade 3 containing the study species, *T. zealandica*, and its congener, *T. cosgrovei*. Plots of transitions/transversions vs. genetic distances indicated significant saturation of the third codon position of *CO1* (Supporting Information, Fig. S13). Therefore, the tree presented here is estimated from the concatenated data set from which third codon position data are excluded (Separate Bayesian and ML trees, as well as those from an alignment including third codon position, are provided in Supporting Information, Figs S14–S17). Inclusion of data from *28S* and *CO1* codon positions 1 and 2 with that from *18S* yields a similar topology (Fig. 6), except that a bifurcation at the most basal node is now observed and *T. zealandica* and *T. cosgrovei* now form a sister taxon to clade 1.

DISCUSSION

Population genetic analyses of *B. quoyana CO1* sequence data demonstrate moderate levels of population structuring that support the existence of three regional metapopulations along the south-eastern coast of New Zealand's South Island. Surprisingly, sequence from three expectedly polymorphic molecular markers commonly used in population genetic and phylogeographical studies revealed absolutely no sequence variation of mermithid individuals both within and among sampling locations. This study also provides new, multi-locus phylogenetic reconstructions of the family Mermithidae.

Range, specificity and infection levels of *T. zealandica*

Previous studies of *T. zealandica* focussed only on locations around Dunedin, with the most comprehensive collection having been conducted along roughly 60 km of coastline (Rasmussen & Randhawa, 2015). The present study collected potential hosts over at least 700

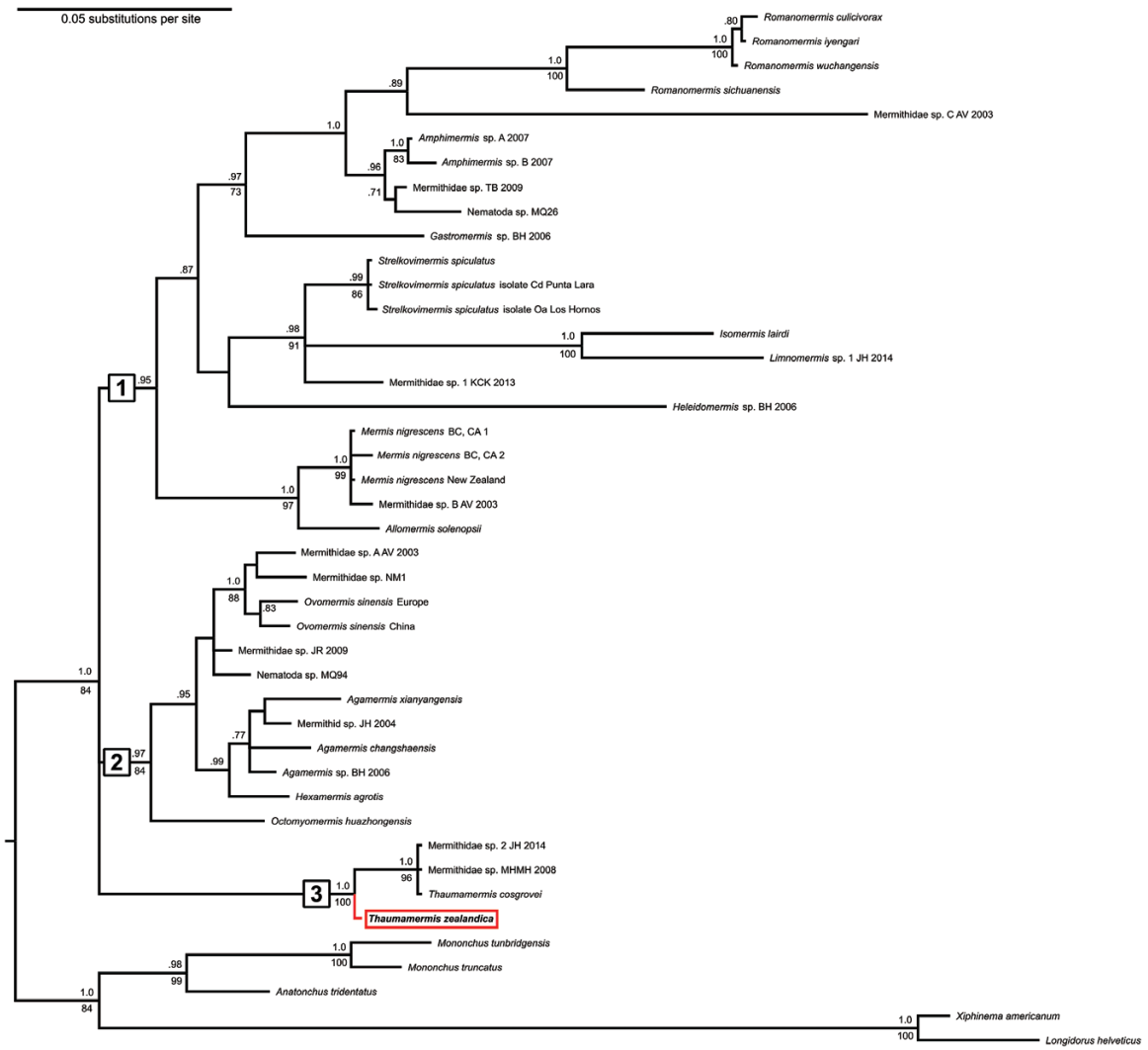

Figure 5. Combined Bayesian/ML tree of the *18S rRNA* gene. Posterior probabilities indicated above the nodes, bootstrap support values below. Only values above 0.70 or 70 are shown. Clade numbers are indicated in boxes at base of each clade.

km of coastline, from OT near Invercargill to NB in Christchurch. This revealed that *T. zealandica* is likely restricted to locations in the Dunedin area and southwards, as no mermithid was found among the 1754 talitrids collected north of LB. Additionally, no individuals were found to parasitize a genetically distinct but unidentified talitrid species collected during the course of this study. This talitrid occurred on pebbly beaches both within and outside *T. zealandica*'s range, as opposed to the sandy beaches upon which *B. quoyana* was found. *Thaumamermis zealandica* may only be able to parasitize *B. quoyana* but not the other talitrid. Alternatively, the parasite may not be a specialist of *B. quoyana* but rather there may be little opportunity for host switching, as *B. quoyana* and the other talitrid were not found in sympatry outside of OA, a beach of mixed sediment composition. Given that several mermithid species can parasitize a range of host species (Petersen *et al.*, 1969; Blackmore, 1992; Becnel & Johnson, 1998) and that *T. zealandica*'s congeneric, *T. cosgrovei*, infects multiple species of isopod (Poinar, 1981), it is likely that the latter scenario is correct. However, experimental infections would be required to determine this with certainty. Estimates of parasite prevalence from this study fall within the previously observed range. With the exception of the study by Rasmussen & Randhawa (2015), all previous investigations of *T. zealandica* were carried out at Long Beach. Prevalence at this location has varied greatly, from 0% in Rasmussen & Randhawa (2015) to 31.3% in Poulin & Latham (2002a), compared to 3.31% in the current study. This could be due to a variety of factors, both spatial and temporal. Poulin & Rate (2001) showed that parasite prevalence can vary greatly among kelp patches on an individual beach.

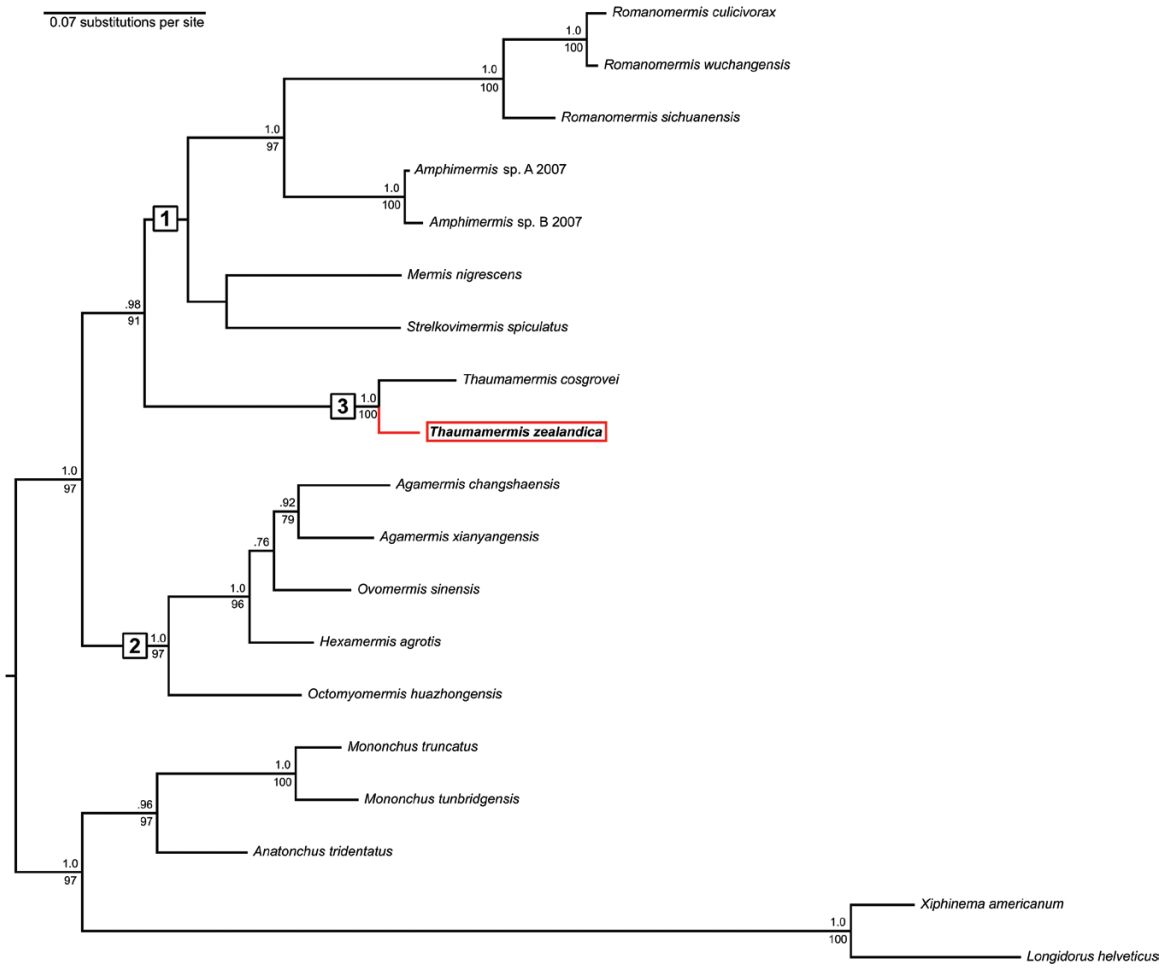

Figure 6. Combined Bayesian/ML tree of the *18S*/*28S*/*CO1* concatenated data set. Third codon position of *CO1* is excluded. Posterior probabilities indicated above the nodes, bootstrap support values below. Only values above 0.70 or 70 are shown. Clade numbers are indicated in boxes at base of each clade.

Thus, the difference in the prevalence may be explained by chance selection of high- or low-prevalence kelp patches in each study. Alternatively, seasonal or annual fluctuations in the parasite's population levels may explain this difference.

Bellorchestia quoyana: phylogeography

The population genetic structure of *B. quoyana* largely follows a pattern of regional subdivision, with three distinct groups in the north, centre and south of the collection range. Separations between these groups occur at the Otago Peninsula for the southern and central groups and at Banks Peninsula for the central and northern groups. This suggests that these two largest peninsulas on the South Island's eastern coast function as barriers to *B. quoyana* dispersal. Additionally, the Canterbury Bight, a long stretch of coast between Oamaru and Banks Peninsula comprised solely of pebbly substrate, likely contributes further to the isolation of the northern populations. A pattern of higher genetic diversity in the south, moderate genetic diversity in the centre and no diversity in the north suggests a northward direction of *B. quoyana* gene flow, indicating that consecutive population bottlenecks may have occurred at the peninsulas and/or across the Canterbury Bight. This, along with limited divergence between haplotypes, suggests that *B. quoyana* may be a recent arrival to the Christchurch area or that previous populations went locally extinct and the area has subsequently been recolonized. This recent expansion is further supported by the observation that no unique haplotypes were encountered at NB and SU, indicating that these populations have established relatively recently with little time to accumulate novel alleles. Given that the Southland Current travels from south to north, these findings are consistent with the dominant hypothesis of rafting as the dispersal mechanism

for talitrids (Wildish, 2012; Wildish & Chang, 2017). Most population genetic studies of supralittoral talitrids have been carried out in the Mediterranean and/or Atlantic and have also found evidence for current-mediated dispersal via rafting (De Matthaeis *et al.*, 2000; Henzler & Ingólfsson, 2008; Pavesi *et al.*, 2012; reviewed in Pavesi & Ketmaier, 2013). The connectivity between populations has largely been linked to the ecological characteristics of talitrids and their effects on dispersal. For example, beachfleas, which do not burrow and inhabit macroalgae or seagrass, typically show lower levels of population differentiation, indicating greater connectivity between locations (Pavesi & Ketmaier, 2013). As they directly associate with wrack, long distance dispersal via rafting may occur with relatively high frequency. Sandhoppers, in contrast, burrow into the substrate and feed on wrack rather than directly living among it. As such, dispersal occurs less frequently, as evidenced by stronger population differentiation (Pavesi & Ketmaier, 2013). The population structure of *B. quoyana* is therefore in accordance with previously observed patterns for sandhoppers from the Mediterranean.

While there are no previous phylogeographic studies of New Zealand talitrids, there have been many of other coastal benthic and littoral species (see Ross *et al.*, 2009). Many of these studies have revealed a phylogeographic break between north and south, largely attributed to the complex hydrogeographic features around the Greater Cook Strait area (Apte & Gardner, 2002; Ayers & Waters, 2005; Veale & Lavery, 2011). Most have focussed on species with a planktonic larval stage, which therefore have much greater dispersal potential than *B. quoyana*. However, there are a handful of studies that have investigated brooding littoral species or otherwise dispersal-limited organisms, often highlighting the importance of kelp rafting (Sponer & Roy, 2002; Jones, Gemmill & Pilditch, 2008). For example, Sponer & Roy (2002) found that while the brooding brittle star *Amphipholis squamata* did exhibit local population differentiation, there was evidence for connectivity between far flung populations, suggesting that stochastic dispersal via rafting is integral to its widespread present-day distribution.

Although once discounted in favour of vicariant processes (plate tectonics, glaciation etc.), the role of dispersal in explaining biogeographical patterns is increasingly being appreciated (Zink, Blackwell-Rago & Ronquist, 2000; De Queiroz, 2005). Drift/rafting has recently been shown to be an important means of dispersal for many marine taxa, specifically in southern waters (Highsmith, 1985; Donald, Kennedy & Spencer, 2005; Nikula *et al.*, 2010; Fraser *et al.*, 2011; Nikula, Spencer & Waters, 2012; see Waters, 2008). One study particularly relevant to our findings deals with the asymmetric dispersal of southern bull-kelp (*Durvillaea antarctica*) in New Zealand and its subantarctic islands (Collins *et al.*, 2010). The authors determined the source populations of stranded kelp on the shores of the Canterbury Bight using molecular markers. They showed that nearly all individuals had drifted from southern locations, some over distances of at least 500 km from the subantarctic, with the small fraction of those that displayed southward dispersal typically only having travelled tens of kilometres. This indicates that the dispersal capability and directionality of *D. antarctica*, with which *B. quoyana* is closely associated (Marsden, 1991a, b; Dufour, Probert & Savage, 2012), would be sufficient to transport the talitrid over the distances observed in the current study and contribute to the south-to-north pattern of decreasing diversity.

THAUMAMERMIS ZEALANDICA: SEQUENCE INVARIANCE

The lack of sequence variation for *T. zealandica CO1*, *NADH4* and *CYTB* indicates no population structure for the parasite and/or the unsuitability of these markers in population genetic studies of this species. This striking result is unexpected given the typically high substitution rates and widespread use of these markers in population genetic studies of many taxa, including other nematodes (Moritz, Dowling & Brown, 1987; Sunnucks, 2000; Hu *et al.*, 2003). This observed lack of sequence variation could be explained by markedly low mutation rates for these markers, the occurrence of a recent population bottleneck or a combination of the two.

Could extremely slow evolutionary rates of mtDNA somehow be characteristic of mermithids? Given the dearth of studies investigating intraspecific variation of mtDNA in mermithids, it is difficult to draw comparisons (St-Onge, LaRue & Charpentier, 2008; Belaich *et al.*, 2015). St-Onge *et al.* (2008) identified four cryptic species within *Mesomermis flumenalis*, a mermithid parasitizing blackflies in North America. Two of the four cryptic species showed no variation in a 325 bp region of *CO1*, although they were only represented by a total five specimens. The remaining two cryptic species, however, did exhibit intraspecific sequence polymorphism, albeit at low levels. Belaich *et al.* (2015) similarly found intraspecific variation of *NADH4* in the South American mosquito parasite *S. spiculatus*. It is difficult to make conclusions about rates of molecular evolution in the family Mermithidae when sampling both among and within species is so limited.

While there has been little investigation of mtDNA gene sequence evolution in mermithids, there is a larger body of work characterizing the structure of their mitochondrial genomes (Powers, Platzer & Hyman, 1986; Hyman & Azevedo, 1996; He *et al.*, 2009). Mermithids are unique in that they tend to have considerably large

mitochondrial genomes that display rapid changes in gene order both among and within species (Hyman *et al.*, 2011). A striking example is that of *T. zealandica*'s congener, *T. cosgrovei* (Tang & Hyman, 2005, 2007). The mitochondrial genome of this mermithid species is one of the largest among all metazoans (> 34 kb) and contains a 'hypervariable region' with repeated non-coding stretches and degenerate pseudogene copies, as observed in other mermithid species (Wu, 2007; He *et al.*, 2009). The sequence and length variability of this region are so pronounced that, unless recovered from the same individual host, no two *T. cosgrovei* specimens share a genomic haplotype. Interestingly, when Tang & Hyman (2007) sequenced a ~4 kb stretch of the shared mitochondrial backbone they found greater than 99.5% sequence identity among specimens, which they suggested indicates strong selection upon this region to maintain functional integrity in the face of frequent and extensive remodelling of the hypervariable region. Thus, it is possible that the rapid changes in gene order and length necessitate sequence conservation in the functional gene copies. This would support the assertion of slow mutational rates of mtDNA in mermithids and account for the observed lack of polymorphism in the current study. An interesting study would involve sequencing of the full mitochondrial genomes of many *T. zealandica* individuals. If *T. zealandica* has a hypervariable region in its mitochondrial genome, analysis of its length or gene order may provide an alternative means of elucidating its population structure and phylogeography.

A lack of haplotype diversity within a population can also indicate a recent population bottleneck, as that inferred for *B. quoyana* populations near Christchurch. If this were the case, *T. zealandica* would have to be a recent colonizer of the south-eastern coast. This scenario would necessitate the recent colonization of New Zealand by its host or a recent host switch from an exotic, unidentified host. Both scenarios are rather unlikely. For one, *B. quoyana* is only found in New Zealand. Second, as the unidentified talitrid was never found to be infected, a recent host switch is also hard to imagine. It is possible, although, that a separate and exotic unidentified talitrid sympatric with *B. quoyana* may have brought with it *T. zealandica* and transferred it to its current host. In either case, as population structuring of the host was observed over the geographical range of the parasite, the bottleneck scenario must also include slower evolutionary rates of the parasite than the host. Thus, one must conclude that rates of nucleotide substitution for mtDNA are too low for it to be of use in population-level analyses of *T. zealandica*. The application of more variable nuclear markers such as microsatellites may provide the resolution needed to investigate host–parasite co-structure in this system.

There are very few examples demonstrating invariance of molecular markers in population genetic studies of animals. Slow evolutionary rates of mtDNA in plants have been documented for some time, highlighting their unsuitability for population genetics and phylogeography (Palmer & Herbon, 1988; Schaal *et al.*, 1998). As such, many studies of plants have relied on chloroplast DNA and/or microsatellites (Schaal *et al.*, 1998; Petit *et al.*, 2005). Within animals, there is evidence of slower mtDNA evolutionary rates among basal lineages of the metazoan tree (Huang *et al.*, 2008). Substitution rates 50–100 times slower than most animals have been observed in anthozoans, such as corals and anemones (Shearer *et al.*, 2002; Hellberg, 2006). For example, in a study of the waratah anemone (*Actinia tenebrosa*), Veale & Lavery (2012) found no sequence polymorphism of mitochondrial genes or their introns among specimens collected throughout New Zealand. Slow rates of mtDNA evolution have also been observed in certain chordate lineages (Avise *et al.*, 1992; Palumbi, 1992), although not of sufficient magnitude to preclude population genetic studies at large spatial scales (Bowen *et al.*, 1994; Dutton *et al.*, 1999; Keeney *et al.*, 2005; Duncan *et al.*, 2006).

While high levels of intraspecific mtDNA sequence conservation may be a feature of plants, basal metazoans and certain vertebrate lineages, this certainly does not appear to be a feature of nematodes generally. Similar levels of intraspecific mtDNA variation have been observed in nematodes as in other animal taxa (Blouin, 1998; Blouin *et al.*, 1998; Blouin, 2002). The use of mtDNA in population genetic and phylogeographical studies of nematodes, both free living and parasitic, is widespread (Blouin *et al.*, 1995; Anderson, Blouin & Beech, 1998; Brant & Ortí, 2003; Nieberding *et al.*, 2004, 2005; Derycke *et al.*, 2008; Bik *et al.*, 2010). While the majority of these investigations focussed on nematode lineages distantly related from mermithids, studies of the order Dorylaimida, the closest taxon to Mermithidae with available data, also demonstrate expected levels of intraspecific mtDNA divergence (Kumari *et al.*, 2009; Chizhov *et al.*, 2014).

Despite the lack of mtDNA sequence data for mermithids, our results suggest that extremely low levels of intraspecific mtDNA diversity may be characteristic of the family, perhaps as a consequence of the extensive and frequent remodelling of mitochondrial genome structure. In any case, the results of our study should function as a guide for marker selection in future studies of mermithid population genetics, advising against the use of mtDNA in favour of more variable markers.

Phylogenetic relationships within the family Mermithidae

The results from all phylogenetic analyses conducted here show that *T. zealandica* and *T. cosgrovei* are indeed congeneric, demonstrating monophyly of the

genus and confirming previous morphological determinations (Poinar *et al.*, 2002). This comes as no surprise, as the autapomorphy of this genus is the extreme inequality in length between the two spicules (Poinar *et al.*, 2002).

The evolutionary relationships among mermithid taxa have not been extensively investigated. Based on morphological data alone, Gafurov (1996) published the only systematic framework within the family, establishing seven subfamilies (Supporting Information, Fig. S18). The paucity of genetic data from mermithids has made it difficult to verify congruence between molecular and morphological phylogenies. By including as many taxa as possible, using multi-locus data and applying more rigorous analytical techniques, our study provides the most comprehensive phylogenetic investigation within the family.

Our results are in accordance with Gafurov's scheme at relatively shallow nodes, for example, those separating the genera *Hexamermis* and *Agamermis*, or *Allomermis* and *Mermis* (Poinar *et al.*, 2007; Crainey, Wilson & Post, 2009). However, certain genera are occasionally placed in entirely different parts of the tree, such as *Amphimermis*, which is considered as part of the subfamily Agamermithinae but clusters more closely with *Gastromermis* and *Romanomermis* in the trees provided here and elsewhere (Wang *et al.*, 2007; Crainey *et al.*, 2009). Furthermore, relationships among subfamilies depicted by Gafurov are mostly without molecular phylogenetic support.

Many previous studies provide trees supporting two of the main clades found here (clades 1 and 2) (Wang *et al.*, 2007; Crainey *et al.*, 2009). However, few investigations have included sequences from *Thaumamermis* spp. in their phylogenetic analyses, thus preventing a thorough comparison of the position of the genus. Kubo, Ugajin & Ono (2016) demonstrate a similar topology to that of the *18S* data presented here, except that *Thaumamermis* falls among species defined here as clade 2. Wang *et al.* (2007) present a similar topology in their *18S* tree, as do Kobylinski *et al.* (2012). The phylogenetic tree from the concatenated *18S*/*28S*/*CO1* data set provided here is the first to provide support for *Thaumamermis* falling within clade 1. However, a look at the analyses of the individual markers (Supporting Information, Fig. S5–S12) reveals that this position is mostly driven by the *28S* data set, as both the *18S* and *CO1* data sets fail to provide sufficient resolution at this depth of the tree. As the length of the *28S* alignment was restricted to sequences available on GenBank, such a result may be due to differences in homology among various taxa along the length of the gene. As the number of available sequences increases, future investigators will be better equipped to reconstruct the evolutionary history of the family.

In conclusion, besides providing detailed information on the population genetics of the host and the unexpected lack of intraspecific variation in the parasite, this study provides the first genetic data for either study organism and an improved phylogeny of the family Mermithidae. Phylogeographical results from the sandhopper indicate that it likely utilizes kelp rafting as a means of dispersal and that successive population bottlenecks may have occurred due to topographical features such as peninsulas and long stretches of coarse-sediment coastline. The extensive refinement of primers for the amplification of several mitochondrial genes may assist in future studies involving molecular genetics of mermithids. By revealing the invariant nature of *T. zealandica* mtDNA, this study highlights the potential importance of mitochondrial genome rearrangement in minimizing rates of mtDNA evolution in mermithids and suggests the use of alternative markers such as microsatellites in future population genetic studies of this group.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sandhopper collection site information.
Table S2. PCR thermal cycling profiles.
File S3. Novel primer design.
Figure S4. Plot of hypothetical population number (*K*) vs. fixation indices for selection of *K* in AMOVA tests. Most drastic increase in *F*CT and decrease in *F*SC occur when raising *K* from 2 to 3.
Figure S5. Mermithid *18S rRNA* Bayesian phylogenetic tree. Posterior probabilities shown above nodes.
Figure S6. Mermithid *18S rRNA* maximum likelihood phylogenetic tree. Bootstrap support values shown above nodes.
Figure S7. Mermithid *28S rRNA* Bayesian phylogenetic tree. Posterior probabilities shown above nodes.
Figure S8. Mermithid *28S rRNA* maximum likelihood phylogenetic tree. Bootstrap support values shown above nodes.
Figure S9. Mermithid *CO1* Bayesian phylogenetic tree. Posterior probabilities shown above nodes.
Figure S10. Mermithid *CO1* maximum likelihood phylogenetic tree. Bootstrap support values shown above nodes.
Figure S11. Mermithid *CO1* Bayesian phylogenetic tree. Third codon position is excluded. Posterior probabilities shown above nodes.
Figure S12. Mermithid *CO1* maximum likelihood phylogenetic tree. Third codon position is excluded. Bootstrap support values shown above nodes.
Figure S13. Transition/transversion vs. JC69 genetic distance plot of mermithid *CO1* codon position three showing high levels of substitution saturation.

Figure S14. Mermithid *18S*/*28S*/*CO1* concatenated Bayesian phylogenetic tree. Posterior probabilities shown above nodes.
Figure S15. Mermithid *18S*/*28S*/*CO1* concatenated maximum likelihood phylogenetic tree. Bootstrap support values shown above nodes.
Figure S16. Mermithid *18S*/*28S*/*CO1* concatenated Bayesian phylogenetic tree. *CO1* third codon position excluded. Posterior probabilities shown above nodes.
Figure S17. Mermithid *18S*/*28S*/*CO1* concatenated maximum likelihood phylogenetic tree. *CO1* third codon position excluded. Bootstrap support values shown above nodes.
Figure S18. Mermithidae phylogenetic tree as proposed by Gafurov (1997). Adapted from Gafurov (1997), kindly provided by B. Hyman.