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Genetic structure in a progenetic trematode: signs of cryptic species with contrasting reproductive strategies ☆

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ABSTRACT

Complexes of cryptic species are rapidly being discovered in many parasite taxa, including trematodes. However, after they are found, cryptic species are rarely distinguished from each other with respect to key ecological or life history traits. In this study, we applied an integrative taxonomic approach to the discovery of cryptic species within *Stegodexamene anguillae*, a facultatively progenetic trematode common throughout New Zealand. The presence of cryptic species was determined by the genetic divergence found in the mitochondrial cytochrome *c* oxidase I gene, the 16S rRNA gene and the nuclear 28S gene, warranting recognition of two distinct species and indicating a possible third species. Speciation was not associated with geographic distribution or microhabitat within the second intermediate host; however frequency of the progenetic reproductive strategy (and the truncated life cycle associated with it) was significantly greater in one of the lineages. Therefore, two lines of evidence, molecular and ecological, support the distinction between these two species and suggest scenarios for their divergence.

1. Introduction

Modern molecular genetic methods have enabled biologists to distinguish between morphologically similar species that are genetically distinct from each other (Avise, 2004). Recently, these techniques have elucidated complexes of cryptic species within trematodes, parasitic flatworms with complex life cycles and multiple hosts (e.g. Donald et al., 2004; Miura et al., 2005; Leung et al., 2009; Blasco-Costa et al., 2010; Detwiler et al., 2010), at a higher rate than within any other helminth taxon (Poulin, 2011). Contrary to popular hypotheses about parasite speciation occurring by specialisation for different hosts (Blair et al., 1997; Poulin and Keeney, 2008; Malenke et al., 2009), cryptic trematode species can be found within the same host species at a particular life stage (Donald et al., 2004; Detwiler et al., 2010). Beyond the genetic discrimination of cryptic species, too often little effort is made to elucidate how they differ from each other with respect to ecological and life history properties. This would shed some light on the context in which they speciated and highlights the need for molecular studies to be performed alongside ecological research.

Stegodexamene anguillae (Lepocreadiidae) is a common freshwater parasite widespread throughout New Zealand (NZ), with a three-host life cycle, typical of many trematodes. Eggs hatch in the water and larvae infect snails, *Potamopyrgus antipodarum*, as the first intermediate host. After asexual reproduction within the snail, numerous cercariae leave this host and search for the second intermediate host, small freshwater fish, mostly *Gobiomorphus* spp. and *Galaxias* spp. (Macfarlane, 1951, 1952). After penetrating the fish host, cercariae encyst and develop into metacercariae, the infective stage to the definitive hosts, *Anguilla dieffenbachii* (NZ longfin eel) or *Anguilla australis* (short-finned eel). Metacercariae can encyst within a range of tissues (muscles, gonads, body cavity, etc.) within the fish intermediate host, and transmission to definitive hosts occurs when eels feed on a fish infected with metacercariae (Macfarlane, 1951, 1952). Development into the adult stage and sexual reproduction occur in the digestive tract of the eels and trematode eggs are shed into the water with the host's faeces. Infection of a new first intermediate snail host completes the life cycle. Alternatively, *S. anguillae* is capable of progenesis, in which early maturation and reproduction by selfing occurs within the second intermediate host. Thus, progenetic *S. anguillae* have an abbreviated life cycle in which they skip the definitive eel host.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers KF516994 (ITS2), KF484005–KF484006 (28S), KF483999–KF484004 (16S) and KF589317–KF589332 (COI).

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However, the wide geographic distribution of this parasite, the occurrence of intraspecific variation in life cycle strategies (three-host life cycle versus progenetic two-host cycle) and/or the use of different encystment sites within the fish intermediate host, may all conceal the existence of cryptic species. Indeed, each of these factors is associated with either the extent of gene flow among populations or specialisation of the parasite for particular host resources.

Stegodexamene anguillae is an autogenic species (sensu Esch et al., 1988), in which the life cycle is completed among hosts that are all confined to an aquatic system. Autogenic species often have low gene flow due to their aquatic hosts having low dispersal compared with similar parasites that use highly mobile definitive hosts such as birds (Criscione and Blouin, 2004; Blasco-Costa and Poulin, 2013). Further, progenetic individuals reproducing by selfing should cause reductions in genetic diversity (Jarne, 1995; Charlesworth, 2003). Here, the population genetic structure and existence of a complex of species in *S. anguillae* in multiple localities is examined using four markers, the mitochondrial cytochrome *c* oxidase I (COI) and 16S rRNA genes, and the nuclear ribosomal second internal spacer (ITS2) and 28S rRNA gene. This selection of genes is common for trematodes due to their power to resolve species boundaries (Nolan and Cribb, 2005; Vilas et al., 2005). Using an integrative taxonomic approach (see Dayrat, 2005; integration by congruence in Padial et al., 2010), this study tests the hypothesis that *S. anguillae* is a complex of multiple cryptic species and that divergence is structured according to reproductive strategy (progenetic or non-progenetic), geography (lake/river of origin) or microhabitat within the second intermediate host (site of metacercarial encystment).

2. Materials and methods

2.1. Collections

Individual metacercariae from the second intermediate host, common bully (*Gobiomorphus cotidianus*), were obtained from five localities on the South Island, NZ: Tomahawk Lagoon, Lake Waihola, Lake Waipori, Lake Hawea, Lake Ohau (Table 1, Fig. 1). Bullies from Lake Waihola were collected between December 2007 and February 2010; those from the other localities were collected in January and February 2010. Cercariae were obtained from *P. antipodarum* snails collected from Chatto Creek (Table 1, Fig. 1) from February to April 2008. Finally, adult worms were obtained from short-finned eels (*A. australis*) purchased from Aquahaven (Christchurch, South Island, NZ) and the New Zealand Eel Processing Company (Te Kuiti, North Island, NZ) in November and December 2007. The eels were sourced, respectively, from Lake Ellesmere, South Island and from Hydro Lakes on Waikato River, North Island (Table 1, Fig. 1). Cercariae were shed from snails. Metacercariae were dissected from fish host tissue and removed from cysts. Adult worms were collected from intestinal contents of eel hosts. All specimens were rinsed in 0.22-μm-filtered fresh water. A single specimen (cercaria, metacercaria or adult) from each host was used for DNA.

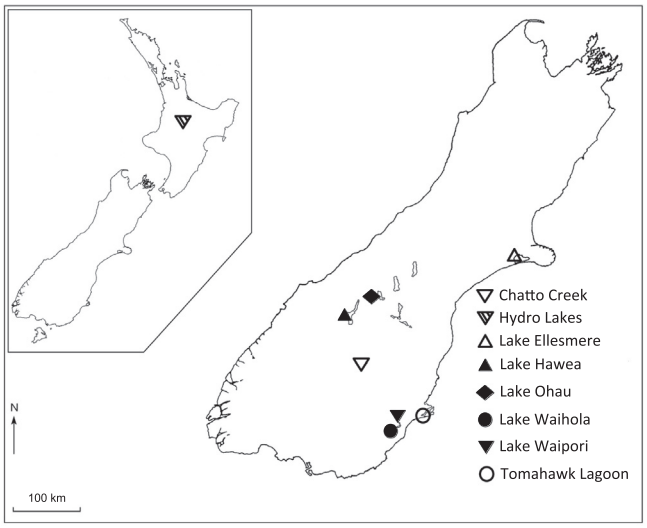

Fig. 1. Map of New Zealand showing eight localities on the South Island (enlarged) and one locality on the North Island (inset) where collections occurred.

2.2. DNA extraction, amplification and sequencing

Specimens that were not immediately used for DNA extraction were fixed in 95% ethanol and stored at −20 °C. DNA was extracted from individual cercariae, metacercariae or adults using 400 μl of 5% chelex containing 0.1 mg/mL of proteinase K, incubated overnight at 60 °C and boiled at 100 °C for 8 min. PCR amplifications were performed with 25 μl reactions containing 2.5 μl of extraction supernatant, 1× PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl at pH 8.8), 2 mM $MgCl_2$, 200 μM of each dNTP, 0.5 mM each primer and 0.7 unit of BIOTAQ DNA polymerase (Bioline, Australia). Forward and reverse COI primers used for amplification were SanCOI: 5′-GTGTGACTATGAGTAATAAAGATTC-3′ (newly designed using two specimens of *S. anguillae*) and COI-R trema: 5′-CAACAAATCATGATGCAAAAGG-3′ (Miura et al., 2005), respectively. For amplification of the mitochondrial (mt) COI gene, the following thermocycling profile was used: denaturation of DNA (95 °C for 3 min), 40 cycles of amplification (94 °C for 40 s, 54 °C for 30 s, 72 °C for 45 s), and a 4 min extension held at 72 °C. For amplification of the mitochondrial 16S rRNA region, forward and reverse primers were Platy.16Sar: 5′-ATCTGTTT(A/C)T(C/T)AAAAACAT-3′ and Platy.16Sbr: 5′-CCAATCTTAACTCAACTCATAT-3′, respectively (Donald et al., 2004). A similar thermocycling profile was used: denaturation of DNA (95 °C for 3 min), 40 cycles of amplification (94 °C for 40 s, 46 °C for 30 s, 72 °C for 1 min), and a 6 min

Table 1
New Zealand localities sampled, river catchment of locality, life stage and number (*n*) of *Stegodexamene anguillae* used for sequencing and host species collected.

Locality	Catchment	Latitude	Longitude	Life cycle stage (*n*)	Host
Chatto Creek	Clutha	45° 03′ S	169° 31′ E	Cercariae (8)	*Potamopyrgus antipodarum*
Lake Hawea	Clutha	44° 26′ S	169° 12′ E	Metacercariae (13)	*Gobiomorphus cotidianus*
Lake Ohau	Waitaki	44° 10′ S	169° 49′ E	Metacercariae (15)	*Gobiomorphus cotidianus*
Lake Waihola	Taieri	46° 00′ S	170° 06′ E	Metacercariae (49)	*Gobiomorphus cotidianus*
Lake Waipori	Taieri	45° 58′ S	170° 07′ E	Metacercariae (16)	*Gobiomorphus cotidianus*
Tomahawk Lagoon	NA (lagoon)	45° 90′ S	170° 54′ E	Metacercariae (6)	*Gobiomorphus cotidianus*
Lake Ellesmere	NA (lagoon)	43° 45′ S	172° 25′ E	Adult (2)	*Anguilla australis*
Hydro lakes, Waikato	Waikato	38° 65′ S	176° 09′ E	Adult (3)	*Anguilla australis*

NA, not applicable.

extension held at 72 °C. PCR products were purified using either PureLink PCR Purification Kits (Invitrogen, USA) or a PCR Product Pre-Sequencing Kit (USB Corporation, USA). PCR primers were used for sequencing. PCR amplicons were cycle-sequenced from both strands using an ABI BigDye™ Terminator v3.1 Ready Sequencing Kit, alcohol-precipitated and run on an ABI 3730xl automated sequencer. Contiguous sequences were assembled and edited using Sequencher™ (GeneCodes Corp. 4.10.1).

Upon finding two divergent COI clades, the ITS2 gene of nine metacercariae and two adult specimens (five from one putative clade and six from the other) and the 28S gene of six metacercariae (three from each putative clade) were sequenced to test for the presence of cryptic species. Forward and reverse PCR primers for ITS2 were LCI: 5′-CGAGTATCGATGAAGAACGCAGC-3′ and HC2: 5′-ATATGCTTAAGTTCAGCGGG-3′, respectively (Navajas et al., 1994). The PCR profile used for amplification of ITS2 was initial DNA denaturation (94 °C for 2 min), 40 cycles of amplification (94 °C for 30 s, 52 °C for 1 min, 71 °C for 1 min), and an extension held at 71 °C for 10 min. Forward and reverse PCR primers for 28S were U178: 5′-GCACCCGCTGAAYTTAAG-3′ and L1642: 5′-CCAGCGCCATCCATT TTCA-3′, respectively (Lockyer et al., 2003). The PCR profile used for amplification of 28S was initial DNA denaturation (94 °C for 2 min), 35 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min 10 s), and an extension held at 72 °C for 4 min. Purification and sequencing procedures were as above, except an additional internal primer was used in sequencing 28S, LSU1200R: 5′-GCATAGTTCACCATCTTTCGG-3′ (Littlewood et al., 2000).

2.3. Data analysis

Newly obtained sequences for COI, 16S, ITS2 and 28S were aligned in four independent data sets using Muscle implemented in MEGA 4.0 with default parameter values. The COI and 16S data sets were analysed individually and consisted of 633 and 254 bp, respectively. A combined analysis of both regions was not run since most of the specimens were sequenced for either 16S or COI, but only 20 specimens were sequenced for both genes, in order to maximise the number of different individuals sequenced. Distance matrices (absolute differences) were calculated with PAUP*. Phylogenetic trees for the COI and 16S data sets (one representative sequence per unique haplotype) were built under Bayesian Inference (BI) and Maximum Likelihood (ML) criteria. Prior to analysis, the nucleotide substitution models were estimated independently for each dataset using jModelTest 2.1.3 (Guindon and Gascuel, 2003; Darriba et al., 2012). The best fitting model for both data sets was the Hasegawa–Kishino–Yano (HKY) model, which incorporates different rates for transitions and transversions; for the COI data set it also included invariant sites (HKY + I). ML analyses were conducted using the program RAxML 7.3 (Stamatakis, 2014). All model parameters and bootstrap nodal support values (1,000 repetitions) were estimated. BI trees were constructed using MrBayes 3.2 (Ronquist et al., 2012), running two independent Markov chain Monte Carlo (MCMC) runs of four chains for 10^7 generations and sampling tree topologies every 10^3 generation. Burn-in periods were set to 10^6 generations according to the S.D. of split frequency values (<0.01). All MrBayes and RAxML computations were performed on the freely available computational resource Bioportal at the University of Oslo, Norway (http://www.bioportal.uio.no). A consensus topology and nodal support estimated as posterior probability values (Huelsenbeck et al., 2001) were calculated from the remaining trees.

In order to examine relationships between COI haplotype frequencies and either the parasite's reproductive strategy (progenetic or non-progenetic), the geographic site of origin, and encystment site within the second intermediate host, an unrooted statistical parsimony network with a connection limit of 95% was constructed using TCS 1.21 (Clement et al., 2000). This analysis was carried out on a subset of 52 individual metacercariae from the common bully host for which detailed information on development (progenetic or non-progenetic), locality of origin (Lakes Hawea, Ohau, Waihola and Waipori), and tissue of encystment (head, muscles, gonads or body cavity) was collected. Haplotype networks were not constructed for the 16S subset due to limited haplotype diversity. Differences in development, locality and encystment site between clades were assessed by Chi-square analyses with a statistical significance of $P < 0.05$.

2.4. Bayesian species delimitation

Using multi-locus data we performed Bayesian species delimitation using the distinct number of COI lineages (see below) as the maximum putative number of species, and the COI gene tree as a guiding species tree. Bayesian species delimitation was conducted using Bayesian Phylogenetics and Phylogeography (BPP v.2.0; Yang and Rannala, 2010) with the full dataset: two nuclear ribosomal loci and two mitochondrial loci. Appropriate heredity scalars were specified for each gene and among-locus variation ($\alpha = 2$) was applied. The model assumes no admixture following speciation. The reversible jump MCMC (rjMCMC) analyses were run for $5x10^5$ generations (sampling every two generations) with a burn-in period of 10^4 that produced consistent results across separate analyses initiated with different starting seeds. Adequate rjMCMC mixing involves specifying a reversible jump algorithm to achieve dimension matching between species delimitation models with different numbers of parameters. We used both algorithms 0 and 1 with the fine-tuning parameter $\varepsilon = 5$ for algorithm 0 and $\alpha = 2$, $m = 0.5$ for algorithm 1. Since both algorithms yielded congruent results we present only those for algorithm 1. Each species delimitation model was assigned equal prior probability.

The prior distributions on the ancestral population size (θ) and root age (τ_0) can affect the posterior probabilities for models, with large values for θ and small values for τ_0 favouring more conservative models containing fewer species (Yang and Rannala, 2010). We evaluated the influence of these priors by considering three different scenarios as in Leaché and Fujita (2010). A gamma $G(\alpha, \beta)$ distribution is assigned to both θ and τ_0. For the first scenario we assumed relatively large ancestral population sizes and deep divergences: priors $\theta \sim G(1,10)$ and $\tau_0 \sim G(1,10)$. The second scenario assumed relatively small ancestral population sizes and shallow divergences among species: priors $\theta \sim G(2,2000)$ and $\tau_0 \sim G(2,2000)$. The third scenario represented a mixture of the above priors that assume large ancestral populations sizes $\theta \sim G(1,10)$ and relatively shallow divergences among species $\tau_0 \sim G(2,2000)$, which is a conservative combination of priors that should favour models containing fewer species (Yang and Rannala, 2010), and may be the most appropriate for *S. anguillae*. Each analysis was run at least twice to confirm consistency between runs. Following a conservative approach only speciation events simultaneously supported by probabilities superior or equal to 0.99 for all three combinations of priors were considered for species delimitation.

3. Results

3.1. Molecular characterisation

In total, the mitochondrial COI gene was sequenced for 60 *S. anguillae*, consisting of 58 metacercariae (all from common bully) and two adults from the South Island. For the 16S rDNA region, 72 *S. anguillae* isolates were sequenced, comprising eight cercariae,

59 metacercariae, two adults from the South Island and three adults from the North Island.

There were 71 variable sites found over the 633 bp fragment of COI (11.2%), of which 16 resulted in non-synonymous amino acid mutations. For 16S, seven variable sites were found across 254 sites (2.8%). A total of 16 and six unique haplotypes for COI (GenBank accession Nos. KF589317–KF589332) and 16S (KF483999–KF484004), respectively, were recovered. ML and BI phylogenetic analyses yielded trees with similar topologies for both COI and 16S data sets. Specifically, analysis of COI sequences (Fig. 2A) revealed a strongly supported genetic split within *S. anguillae* into two main clades, corresponding to a similar split seen in the analysis of 16S sequences (Fig. 2B). Isolates sequenced for both genes consistently clustered within either Clade I or II (Supplementary Table S1). COI depicted a total of three lineages (two closely related within Clade I) while 16S depicted two lineages and two somewhat distant single haplotypes. Lineages within Clade I had higher bootstrap support values than posterior probabilities (Fig. 2). Hereafter, the COI lineages will be referred to as *S. anguillae* lineage 1, *S. anguillae* lineage 2 and *S. anguillae* lineage 3, which consisted of 20, 32 and eight isolates, respectively. Isolates clustering within Clade II in the COI tree had a single identical 16S haplotype (h6 in Fig. 2B). However, three isolates belonging to COI lineage 1 and one isolate within COI lineage 3 (Clade I) shared an identical 16S haplotype (h3). Two adult isolates from the South Island and one isolate from the North Island clustered within *S. anguillae* Clade II, whereas the other two adult isolates from the North Island fell into Clade I. Cercarial isolates were only sequenced for the 16S rRNA gene. They shared the same 16S haplotype (h1) within Clade I, with the exception of one isolate (h2). 16S haplotypes h2 and h5 were found in one isolate each out of 72 sequenced isolates for this gene. Haplotype divergence between the two COI clades ranged between 7.1% and 11.2%. Between *S. anguillae* lineage 1 and lineage 3, genetic divergence ranged between 2.4% and 3.0% (COI). Within each lineage, genetic divergence at the COI gene varied between 0.2% and 0.8%. For the 16S gene, divergence between the two main clades ranged between 1.2% and 2.4%, within Clade I a 0.5% divergence was estimated. Although not a single base pair difference was found over the 385 bp fragment of ITS2 ($n = 11$, KF516994), there were five variable sites in the 1314 bp fragment (0.4%) of 28S rRNA gene between isolates of *S. anguillae* from both clades ($n = 6$, KF484005–KH484006). *Stegodexamene anguillae* isolates from COI lineages 1 and 3 had identical 28S genotypes.

3.2. Network analysis, differentiation in reproductive strategies and geographical distribution

Network analysis of COI included 13 distinct haplotypes out of 52 sequences (some isolates were excluded due to missing ecological information), split into the three groups (corresponding to *S. anguillae* lineage 1, lineage 2 and lineage 3) that could not be joined parsimoniously at the 95% connection limit (Fig. 3). All three isolated networks consisted of non-progenetic and progenetic individuals, although *S. anguillae* lineage 2 had a larger proportion of progenetic individuals (76.7%) than *S. anguillae* lineage 1 (10.5%) and *S. anguillae* lineage 3 (20.0%; $\chi^2 = 22.1$, df = 2, $P < 0.001$; Fig. 3A). There was no statistical difference in the number of isolates of each lineage among lakes ($\chi^2 = 7.2$, df = 6, $P = 0.3$;

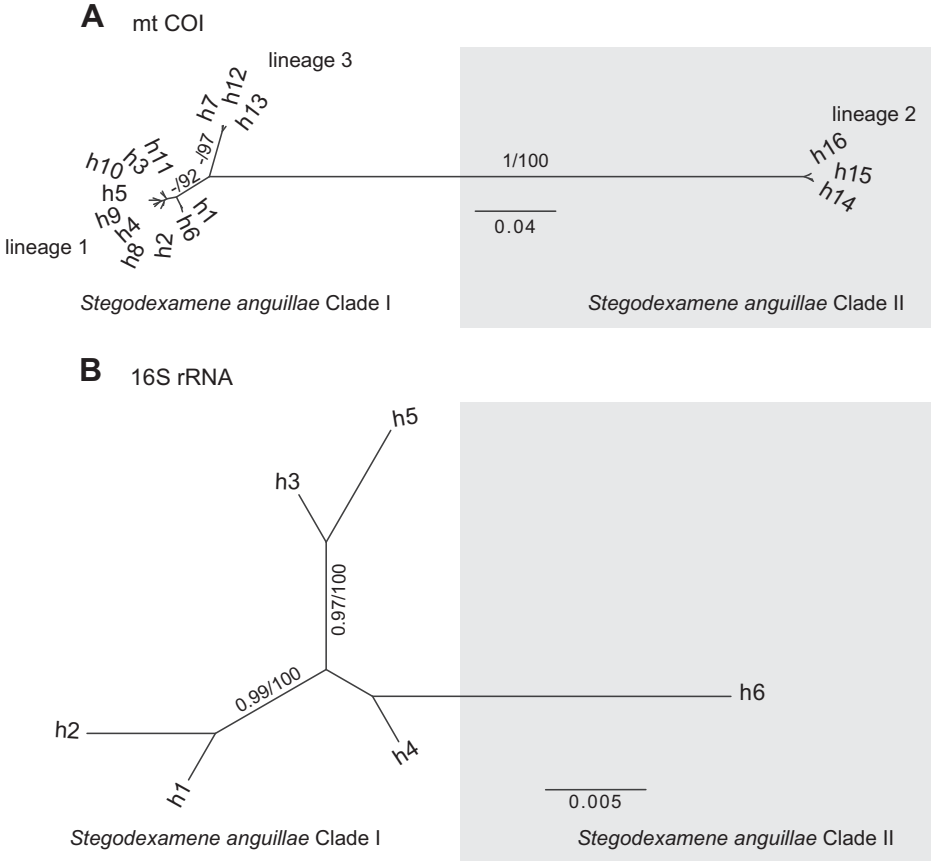

Fig. 2. Unrooted phylogenetic trees of unique haplotypes (h) detected for *Stegodexamene anguillae* isolates from all life stages and localities inferred from (A) mitochondrial (mt) cytochrome *c* oxidase subunit I gene sequences and (B) mt 16S rDNA sequences. The numbers associated with each node are the Bayesian posterior probabilities followed by bootstrap support values (posterior probabilities < 0.95 and bootstrap values < 0.60 not shown). The shading indicates distinction between putative species.

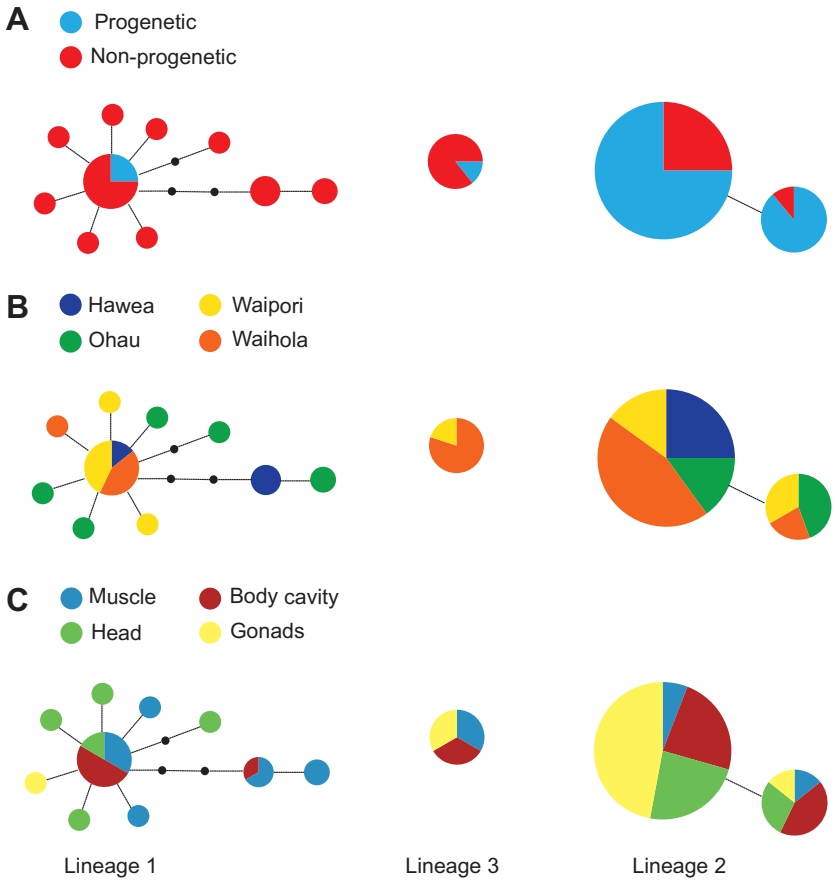

Fig. 3. Haplotype network diagram of *Stegodexamene anguillae* metacercariae based on mitochondrial DNA (cytochrome *c* oxidase subunit I), with circle area representing haplotype frequency and showing (A) reproductive strategy, (B) New Zealand lake of origin and (C) tissue of encystment. Small black dots represent inferred intermediate haplotypes not observed in the data.

Fig. 3A), although lineage 3 was only found at two out of the four lakes, perhaps due to its lower overall frequency in our samples. Finally, individual metacercarial isolates belonging to lineages 1 and 2 were found in all tissues sampled from the common bully, and isolates of lineage 3 were found in three of the four tissue types (Fig. 3C), with no statistical difference between lineages in the frequency with which metacercariae encyst in each tissue (muscle, head, body cavity or gonads; $\chi^2 = 11.0$, df = 6, $P = 0.087$). However, 37.5% of those in *S. anguillae* lineage 2 encysted in the gonads whereas only 5.6% from lineage 1 encysted in the gonads (Fig. 3C). Overall, the most common haplotype of each lineage included progenetic individuals as well as non-progenetic ones (Fig. 3A), was found in at least two of four lakes (Fig. 3B), and encysted in at least three of four types of second intermediate host tissue (Fig. 3C).

3.3. Bayesian species delimitation

Using a guide tree with three species, the Bayesian species delimitation results (Fig. 4) supported, consistently and strongly, a two species hypothesis, corresponding to *S. anguillae* Clade I and Clade II, with a speciation probability of 1.0. Different prior distributions for θ and τ_0 affected the probability of the speciation event within Clade I (generating *S. anguillae* lineages 1 and 3). Support for this speciation event (speciation probability $\geqslant 0.99$) was found only under one scenario (Fig. 4, model 2: small population size and shallow divergence) for *S. anguillae*. The influence of the priors on this event does not allow us to confidently propose three cryptic *S. anguillae* spp.

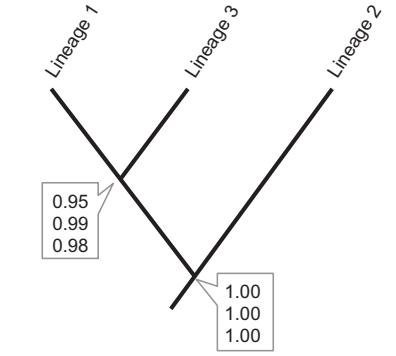

Fig. 4. Bayesian species delimitation tree of *Stegodexamene anguillae* assuming three species. The speciation probabilities are reported for each node under each combination of priors for ancestral population size (θ) and root age (τ_0).

4. Discussion

Our data and analysis confidently suggest the presence of two cryptic species corresponding to the aforementioned *S. anguillae* Clade I and *S. anguillae* Clade II. Typically, pairwise divergence at the COI gene between trematode congeners ranges from 3.9% to 25.0% (Vilas et al., 2005; Moszczynska et al., 2009); this study found levels of genetic divergence ranging from 7.1% to 11.2% at

the COI gene between the two clades. The additional molecular markers employed showed a range of variation levels. Genetic divergence levels in the 16S gene (from 1.2% to 2.4% between clades) were two to 14 times lower than in other trematode taxa (*Curtuteria* spp.: 13.1–13.7% and *Acanthoparyphium* spp.: 5.8–11.1% (Leung et al., 2009); Opecoelids: 10.8–17.5% (Donald et al., 2004) and more similar to those detected within clades (intra-specific) (0–2.9% in Donald et al., 2004)). Further, sequence divergence at the 28S gene of 0.4% supports the two cryptic species hypothesis corresponding to the two *S. anguillae* clades since typically low 28S divergence is found between closely related and cryptic trematode species (e.g. 0.4% in Miller and Cribb, 2007; 0.8% in Blasco-Costa et al., 2010). In contrast, inter-species divergence at the ITS2 gene is typically greater than 1% (Vilas et al., 2005), but our study found no divergence at ITS2. Identical sequences at ITS2 are usually an indication of a single species in trematodes, although identical ITS2 sequences have been found in two morphologically distinct species, *Paragonimus miyazakii* and *Pagumogonimus skrjabini* (Blair et al., 1997, 1999; Nolan and Cribb, 2005). These two paragonimid species also had substantial differences at the COI gene. Thus, although differences at ITS2 are typical between species, they are not necessary if a biological, morphological or ecological basis for distinguishing between two putative species exists (Nolan and Cribb, 2005).

We cannot completely rule out the possibility of a third species within Clade I, supported by genetic divergence in COI, network analysis and Bayesian species delimitation based on one of the population scenarios tested. Genetic divergence at COI between the closest *S. anguillae* lineages 1 and 3 (from 2.4% to 3.0%) exceeds previously reported intra-specific variation (Vilas et al., 2005). However, the lack of divergence on typically more conserved genetic markers (16S, 28S and ITS2), as well as a three-species hypothesis supported exclusively under the model assuming small population size and shallow divergence time among species, suggest that this may represent a special situation of incomplete speciation with secondary contact between the lineages (distribution of the three lineages is sympatric; see Fig. 3 and discussion below). Addressing this issue would require a more detailed population genetics study, preferably targeting adult instead of metacercarial stages in order to evaluate their opportunities to cross-fertilise and the degree of phenotypic differentiation, if any.

Here, the genetic divergence between species was associated with a propensity for one or the other reproductive strategy. Despite non-progenetic and progenetic individuals being found in both clades, *S. anguillae* Clade II consisted of a significantly greater proportion of progenetic individuals. *Stegodexamene anguillae* Clade II has relatively low genetic diversity with only three COI haplotypes found in this study. Selfing species tend to have lower genetic diversity than those in which reproduction occurs by cross-fertilisation with other individuals (Jarne, 1995; Charlesworth, 2003). Thus, *S. anguillae* Clade II, which consists mostly of progenetic individuals, may have low diversity due to high levels of self-fertilisation if progenesis is maintained across generations as the principal mode of reproduction. However, in another progenetic trematode, *Coitocaecum parvum*, the reproductive strategy is not heritable and may differ between parents and offspring (Lagrue and Poulin, 2009).

The divergence found in this study lacks geographic structure, with metacercariae from both clades co-occurring in all four lakes from three different river catchments. Further, the two dominant haplotypes (one in lineage 1 and one in lineage 3) of *S. anguillae* Clade I were found in three of the four lakes and the two haplotypes of *S. anguillae* Clade II were found in all four lakes, suggesting gene flow has occurred among these populations for both species. *Stegodexamene anguillae* Clade I and *S. anguillae* Clade II are autogenic parasites, completing their entire life cycle in aquatic hosts, which may limit gene flow between populations (Criscione and Blouin, 2004; Blasco-Costa and Poulin, 2013). Indeed, *P. antipodarum* snails have low gene flow between lakes (Dybdahl and Lively, 1996), indicating that the snail hosts have limited dispersal between bodies of water. Second intermediate fish hosts may move within a catchment but not between (McDowall, 1990). Eels migrate only twice in their life span, once into freshwater as parasite-free juveniles and the second time as adults migrating to spawning areas in the subtropical Pacific Ocean (McDowall, 1990). Migrating adults could be contributing to gene flow within a catchment, and in fact *S. anguillae* lacked population substructure along a 70 km stretch of river attributed to the home range movement and migration of the eel host (Blasco-Costa et al., 2012). Even though gene flow is not expected between catchments, except during rare flooding events, data from metacercariae collected from the three different catchments showed that related haplotypes were just as likely to occur across distant lakes as within a lake.

Many studies have found that movement of domesticated hosts by humans across large distances can result in mixed parasite populations (Blouin et al., 1992; Criscione and Blouin, 2004; Miura et al., 2006). If populations are being mixed by humans transferring eels among watersheds, then one would expect to see large discontinuities in haplotype phenograms, rather than a ladder-like topology indicating continuous variation in genetic distance between individuals (Blouin et al., 1995). Thus, divergence could have occurred during periods of allopatry (Bush, 1994). Historically, there may have been a geographic separation between these two species that has been muddled by hydroelectric dams and translocations of eels over the past 80 years (Beentjes and Jellyman, 2003).

Alternatively, speciation may occur by specialisation for different host species (Poulin and Keeney, 2008; Malenke et al., 2009). Both species are found in short-finned eels, however until specimens from longfin eels are tested there remains the possibility of only one species specialising in infecting longfin eels (Jousson et al., 2000). This is highly unlikely as both eel species feed on the same prey species (McDowall, 1990). Since metacercariae from both species can be found in the same second intermediate fish host, eels should become infected with individuals from both species by feeding on one fish. At the second intermediate host level, metacercariae from both species are found in common bullies. This would not be the first time cryptic trematode species have been found to be using the same hosts (Donald et al., 2004; Detwiler et al., 2010). Trematodes tend to be highly specific to their first intermediate host (Galaktionov and Dobrovolskij, 2003). Since all cercariae clustered within *S. anguillae* Clade I it is possible that *S. anguillae* Clade II uses a different snail host. Several *Potamopyrgus* spp. have been recently described in NZ, including three cryptic species closely related to *P. antipodarum* (Haase, 2008), the only known snail host of *S. anguillae*. However, if the use of a different snail host does not lead to a different definitive host species or to the same definitive host at a different point in time or location within the host in which the trematode would outcross, it may be irrelevant for the trematode speciation per se.

When two closely related but distinct species co-occur in the same host species at each life stage, the two species often specialise for different locations within the host to reduce competition for space or other resources (Holmes, 1961; Dvorak et al., 2002). Even though competitive effects have been found in trematode metacercariae (Fredensborg and Poulin, 2005; Saldanha et al., 2009), specialisation did not seem to occur in our study since metacercariae of both species can be found within the same tissues in their second intermediate host. However, slight differences in the preferred encystment site may exist. Members of the more progenetic *S. anguillae* Clade II encyst in all tissues but appear to be more likely to occur in the gonads, whereas only a small proportion

of individuals of *S. anguillae* Clade I encyst in the gonads, with the majority spread out in all other tissues. A larger sample size will be needed to confirm this apparent pattern. An alternative possibility is that the two species found in this study may specialise for different microhabitats within an eel's intestine, a phenomenon common among closely related helminth species living in the gastrointestinal tract of the same definitive host (Bush and Holmes, 1986; Stock and Holmes, 1988). This leads to the question of whether or not progenetic individuals that find themselves inside an eel will reproduce by cross-fertilisation or self-fertilisation. Additionally, if outcrossing is occurring, colonisation of different microhabitats within the eel host may have facilitated speciation.

Using an integrative taxonomic approach (Dayrat, 2005; Padial et al., 2010) applied to species discovery we have been able to identify distinct species, *S. anguillae* Clade I and *S. anguillae* Clade II, based on two lines of evidence, molecular and ecological. These two species showed genetic differentiation and propensity for different reproductive strategies. A preference for distinct microhabitats within the second intermediate host may also exist between the two species. Further, both species occurred sympatrically in the same geographic areas and the same second intermediate host species. Formal description of these species would require the examination of adult specimens of both species, preferably from the definitive host. This study was limited with respect to host species sampled. Poulin (2011) showed that the number of species in a cryptic complex was best predicted by the number of specimens sequenced and weakly correlated with the number of host species examined. Based on Bayesian species delimitation, we cannot reject the possibility of one additional species in the *S. anguillae* species complex; therefore the chance of uncovering even more cryptic species remains. To be conclusive, future studies should have more breadth by including more specimens from other second intermediate host species and longfin eels (Nolan and Cribb, 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.06.006.

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