



## Genetic structure and host–parasite co-divergence: evidence for trait-specific local adaptation

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Host–parasite co-evolution can lead to genetic differentiation among isolated host–parasite populations and local adaptation between parasites and their hosts. However, tests of local adaptation rarely consider multiple fitness-related traits although focus on a single component of fitness can be misleading. Here, we concomitantly examined genetic structure and co-divergence patterns of the trematode *Coitocaecum parvum* and its crustacean host *Paracalliope fluviatilis* among isolated populations using the mitochondrial cytochrome oxidase I gene (COI). We then performed experimental cross-infections between two genetically divergent host–parasite populations. Both hosts and parasites displayed genetic differentiation among populations, although genetic structure was less pronounced in the parasite. Data also supported a co-divergence scenario between *C. parvum* and *P. fluviatilis* potentially related to local co-adaptation. Results from cross-infections indicated that some parasite lineages seemed to be locally adapted to their sympatric (home) hosts in which they achieved higher infection and survival rates than in allopatric (away) amphipods. However, local, intrinsic host and parasite characteristics (host behavioural or immunological resistance to infections, parasite infectivity or growth rate) also influenced patterns of host–parasite interactions. For example, overall host vulnerability to *C. parvum* varied between populations, regardless of parasite origin (local vs. foreign), potentially swamping apparent local co-adaptation effects. Furthermore, local adaptation effects seemed trait specific; different components of parasite fitness (infection and survival rates, growth) responded differently to cross-infections. Overall, data show that genetic differentiation is not inevitably coupled with local adaptation, and that the latter must be interpreted with caution in a multi-trait context. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, 118, 344–358.

**KEYWORDS:** *Coitocaecum parvum* – cross-infections – isolated host–parasite populations – *Paracalliope fluviatilis* – sympatric versus allopatric hosts.

### INTRODUCTION

Host–parasite interactions proceed in a co-evolutionary context; both species must continually adapt to each other as well as to other environmental factors (May & Anderson, 1983; van Baalen, 1998; Dieckmann *et al.*, 2002; Vale & Little, 2009). In heterogeneous landscapes resulting in geographically isolated host–parasite populations, differences in local environmental factors, genetic drift and/or host–parasite co-evolution can lead to genetic differentiation among populations. In some cases, host–parasite co-evolution can also lead to the local adaptation of parasites to their sympatric hosts (Gandon & Michalakis,

2002; Morgan, Gandon & Buckling, 2005). As a result, parasites are often predicted to have higher mean performance in their sympatric (i.e. home) hosts than in allopatric (i.e. away; see Kawecki & Ebert, 2004; Greischar & Koskella, 2007 for ‘home vs. away’ terminology) individuals of the same host species (Lively, 1996; Gandon & Van Zandt, 1998). Parasites are generally considered to stay ahead of host defenses during co-evolution and are therefore expected to be more locally adapted to hosts, than are hosts to parasites (Kaltz & Shykoff, 1998; Morgan *et al.*, 2005). Many studies using reciprocal cross-infections between local and foreign hosts and parasites support this prediction, showing that parasites perform better in their home hosts. However, several counter-examples exist (Lively, 1989;

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Ballabeni & Ward, 1993; Kaltz & Shykoff, 1998; Lively & Dybdahl, 2000; Gandon & Michalakis, 2002; Lively *et al.*, 2004). Hosts are also continuously mounting counter-adaptations specifically targeting local parasites while developing adaptations to other local environmental factors. Overall, local environmental factors and host and parasite genotypes may be highly variable among populations. Parasite and host performances in cross-infections between local and foreign hosts and parasites may thus result from complex and unpredictable interaction effects between individual hosts and parasites, and depend strongly on particular combinations of host and parasite populations/lineages (Vale & Little, 2009). As shown by Ballabeni & Ward (1993), conspecific hosts and parasites from different populations can differ widely in their infectivity/vulnerability to local and foreign hosts/parasites in unpredictable and contrasting ways.

Generally, the degree of host-parasite local adaptation should increase over time within each population as a result of coevolution. If isolated populations achieve co-adaptation through different mechanisms (behaviour, immunity, different genes involved in adaptations, etc.), this may result in high levels of genetic co-divergence and genetic structure among these populations. However, host-parasite local adaptation and co-divergence patterns can also be modulated by gene flow and migration rates among host and parasite populations (Gandon, 2002; Lajeunesse & Forbes, 2002; Criscione, Poulin & Blouin, 2005; Morgan *et al.*, 2005). Dispersal among populations tends to maintain genetic connectivity across host and parasite geographical ranges and genetically homogenize populations. Genetic structure and co-divergence of host-parasite populations may thus be higher in parasites infecting hosts with limited dispersal abilities (Blasco-Costa & Poulin, 2013). However, whether genetic structures in hosts and parasites are linked (i.e. host-parasite co-divergence) and affect local adaptation patterns is often unclear.

Only one parasite trait is usually considered in cross-infection studies of local adaptation, most often infection rate (i.e. the proportion of individual parasites able to infect the host), even though multiple traits influence overall parasite fitness (Combes, 1997; Vale & Little, 2009). Different traits may respond differently to local host-parasite adaptation, and trait-specific responses may affect overall effectiveness of local adaptation in terms of parasite fitness (Laine, 2008; Vale & Little, 2009). For instance, lower infection rates in away compared to home hosts may be compensated by higher within-host survival or growth, thus modulating differences in overall parasite fitness among hosts (Laine, 2008). Alternatively, parasites achieving similar infection

rates in home and away hosts may experience lower survival and reach smaller sizes in away hosts; parasites may perform better against home host immune defenses thus achieving higher survival and growth in local hosts (Laine, 2007). Depending on the host-parasite system, trait-specific responses of parasites to local adaptation may or may not modulate the overall impact of host-parasite co-divergence on parasite fitness, but they should nonetheless be accounted for before drawing conclusions.

The trematode *Coitocaecum parvum* (Opecoelidae) is a parasite of small freshwater fish in New Zealand (MacFarlane, 1939; Holton, 1984), mainly the common bully (*Gobiomorphus cotidianus*). Eggs are released in fish feces and hatch into free-swimming larvae. These larvae penetrate the mud snail *Potamopyrgus antipodarum* in which they multiply and develop into sporocysts. Sporocysts asexually produce free-living larvae (cercariae) that leave the snail and then infect, through direct penetration of the cuticle, the amphipod *Paracalliope fluviatilis* in which they encyst as metacercariae in the body cavity. The life cycle is completed when infected amphipods are eaten by the fish definitive host. Both the parasite and its hosts are common and widespread throughout New Zealand, but *C. parvum* free-living larvae and all its hosts have very low dispersal abilities and are constrained to freshwater. Wide geographic distributions, coupled with low dispersal, can be associated with strong genetic structure among geographically isolated host and parasite populations (Criscione & Blouin, 2004; Louhi *et al.*, 2010; Herrmann *et al.*, 2014). For example, spatial genetic structure and isolation by distance were detected in *C. parvum*, even on a very small spatial scale (Blasco-Costa, Waters & Poulin, 2012). Similarly, although on a wider scale, *C. parvum*'s crustacean host, the amphipod *P. fluviatilis*, showed considerable genetic divergences among geographically isolated populations (Hogg *et al.*, 2006; Sutherland, Hogg & Waas, 2010). Therefore, both *C. parvum* and its amphipod host exhibit genetic divergence among populations. However, genetic structures of *C. parvum* and *P. fluviatilis* have never been documented concomitantly, and whether hosts and parasites display genetic co-divergence among geographically isolated populations remains unknown.

In co-diverging host-parasite populations, parasites are often assumed to be better adapted to interact with their home hosts (Westram *et al.*, 2011). Even if parasites are able to infect other, distant host genetic lineages, there may be differences in infection success, survival or growth in away hosts compared to that achieved in home hosts. Trematode parasite larvae can fail to infect a host, and even if they succeed they can be killed, melanized and

encapsulated by the immune system of their *Paracalliope* sp. amphipod hosts (Bryan-Walker, Leung & Poulin, 2007). Surviving metacercariae also attain widely different sizes in amphipod hosts (Lagrue & Poulin, 2009a). Since *C. parvum* size influences fecundity, larger metacercariae should achieve higher fitness (Lagrue & Poulin, 2007, 2008). Local host–parasite adaptation effects on *C. parvum* larvae infection success, survival and size remains unknown but it is likely that a combination of traits influences overall *C. parvum* fitness in its amphipod host, and thus the extent of local adaptation.

Here, the population genetic structures of the trematode parasite *C. parvum* and its amphipod host *P. fluviatilis* were concomitantly examined using the mitochondrial cytochrome *c* oxidase subunit I (COI) to document potential host–parasite genetic co-divergence patterns among geographically isolated populations. Experiments using reciprocal cross-infections among genetically divergent, geographically isolated populations were then used to test for local adaptation between hosts and parasites. Multiple components of parasite performance and fitness were measured (infection success, host-induced parasite mortality and parasite growth) to test for potential trait-specific responses to local host–parasite co-adaptation.

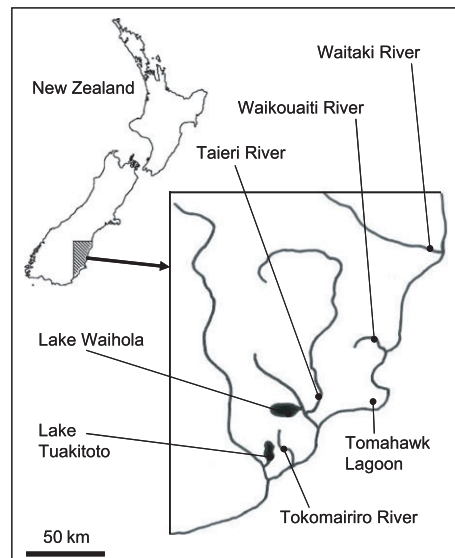
## MATERIAL AND METHODS

### SAMPLING

*Paracalliope fluviatilis* amphipods were collected in February and March 2014 (Austral summer) from seven locations in the South Island of New Zealand; Lake Waiholo (46°01'14.1"S; 170°05'05.8"E), Tomahawk Lagoon (45°54'06.0"S; 170°33'02.2"E), Lake Tuakitoto (46°13'42.5"S; 169°49'29.2"E), Taieri River (45°54'39.3"S; 170°15'42.6"E), Tokomairiro River (46°09'17.8"S; 169°58'35.4"E), Waitaki River (44°55'51.9"S; 171°05'55.5"E) and Waikouaiti River (45°36'29.3"S; 170°37'22.4"E; Fig. 1). Amphipods were sampled using kick nets (500 µm mesh size) and brought back alive to the laboratory. Amphipods were killed in 70% ethanol, rinsed and dissected in water under a stereomicroscope to determine their infection status and retrieve metacercariae of the trematode parasite *Coitocaecum parvum*. Legs of infected amphipods and metacercariae of *C. parvum* were preserved in 100% ethanol for genetic analysis.

### DNA EXTRACTION, COI AMPLIFICATION AND MOLECULAR ANALYSIS

DNA was extracted from amphipod legs and whole trematode metacercariae. About 30–40 DNA isolates of both hosts and parasites were obtained from each



**Figure 1.** Geographical distribution of the seven localities (sample sites) where the amphipod host *Paracalliope fluviatilis* and its trematode parasite *Coitocaecum parvum* were concomitantly sampled.

of the seven sampling sites. Legs from individual amphipods or individual metacercariae were placed individually into 1.5 mL Eppendorf tubes and DNA was extracted in 200 µL of 5% Chelex suspension containing 0.1 mg/mL of proteinase K by incubation at 56 °C overnight. Samples were then incubated at 90 °C for 8 min and centrifuged at 14 000 rpm for 10 min.

A fragment of the protein coding mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified to evaluate the genetic structure of the populations of both hosts and parasites. We used the primers JB3 (forward, 5'-TTTTTTGGGCATCCTGAGGTTTAT-3'; Bowles *et al.*, 1993) and PlagCOIdR (reverse, 5'-TCGGGGTCTTTCCGTCT-3'; Blasco-Costa *et al.*, 2012) for *C. parvum* and primers LCO1490 (forward, 5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (reverse, 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer *et al.*, 1994) for *P. fluviatilis* amphipods. Polymerase chain reaction (PCR) amplifications were performed in 20-µL reactions containing 10.9 µL of MilliQ water, 3 µL of DNA extraction supernatant, 4 µL of PCR buffer (Bioline 5× My *Taq* Red Reaction Buffer), 1 µL of each primer at a concentration of 10 pmol µL<sup>-1</sup> and 0.1 µL of Bioline My *Taq* DNA polymerase. The following thermocycling profile was used for *Coitocaecum parvum*: initial denaturation of DNA (95 °C for 3 min); 45 cycles of amplification (94 °C for 40 s, 49 °C for 30 s and 72 °C for 60 s); and 4 min extension hold at 72 °C. The thermocycling profile used

for *Paracalliope fluviatilis* consisted of four steps: initial denaturation of DNA (95 °C for 3 min); five cycles of amplification (94 °C for 60 s, 45 °C for 90 s and 72 °C for 60 s) followed by 35 cycles of amplification (94 °C for 60 s, 51 °C for 90 s and 72 °C for 60 s); and 5 min extension hold at 72 °C. Additionally, different sets of primers and annealing temperatures were used for amphipods and parasites of a few populations. For Tomahawk Lagoon, jgLCO1490 (forward 5'-TITCIACIAAYCAYAAARGAYATTGG-3') and jgHCO2198 (reverse, 5'-TAIACYTCIGGRTGICCRARAAAYCA-3'; Geller *et al.*, 2013) were used at 52.7 °C annealing temperature, and JB3 and Co1R-trema (reverse 5'-CAACAAATCATGATGCAAAAGG-3'; Miura *et al.*, 2005) at 49.5 °C for *P. fluviatilis* and *C. parvum* respectively. For Lake Tuakitoto, JB3 and Cpa-CO1R2 (reverse, 5'-AACYACTACACAACC-CAACG-3') were used at 52.7 °C annealing temperature. Cpa-CO1R2 was designed specifically for this study as existing primers did not work or yielded insufficient concentrations of PCR products. PCR amplicons were purified using exonuclease I and shrimp alkaline phosphatase (Werle *et al.*, 1994), and then cycle-sequenced from both strands by the company Macrogen Inc. (South Korea).

Contiguous sequences were assembled and edited using Sequencher software (GeneCodes Corp. 5). Consensus COI sequences were aligned into two independent datasets, one for amphipod hosts (651bp long) and one for the parasite (726bp long), using MUSCLE implemented in MEGA 6.0 software (Tamura *et al.*, 2011). Phylogenetic trees were built under Bayesian inference (BI) and maximum-likelihood (ML) criteria. Prior to analysis, nucleotide substitution models were estimated for each dataset using jModelTest 2.1.3 software (Guindon & Gascuel, 2003; Durrin *et al.*, 2012). The best fitting model for *C. parvum* was the Hasegawa-Kishino-Yano (HKY) model. For *P. fluviatilis* hosts, the general time reversible model with gamma-distributed across-site rate variation (GTR+ $\Gamma$ ) was the best fit. Maximum-likelihood analyses were conducted using the program RAxML 7.3 (Stamatakis, 2014). All model parameters and bootstrap nodal support values (1000 repetitions) were estimated. Bayesian inference 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<sup>7</sup> generations and sampling tree topologies every 10<sup>3</sup> generation. Burn-in periods were set to 10<sup>6</sup> generations according to the standard deviation of split frequency values (< 0.01). All MrBayes and RAxML computations were performed on CIPRES (www.phylo.org/). In order to examine potential relationships between COI haplotype frequencies of hosts and parasites and their geographical origin (i.e.

sample site), unrooted statistical parsimony networks with a connection limit of 95% were built using TCS 1.21 (Clement, Posada & Crandall, 2000). Genetic population structure of *C. parvum* and *P. fluviatilis* COI haplotypes among all localities and between pairs of sample sites was examined using analysis of molecular variance (AMOVA) with Arlequin 3.5 (Excoffier, Laval & Schneider, 2005), applying Tamura and Nei's (1993) model of sequence evolution and gamma-distributed substitution rate variation with shape parameter  $\alpha = 0.2$ . The relationship between genetic ( $\Phi_{ST}1 - \Phi_{ST}$ ; Rousset, 1997) and geographic (log-transformed) pair-wise distances among all populations was examined for each species using Mantel tests (Mantel, 1967) with 1000 randomizations as implemented in the Isolation by Distance Web Service version 3.23 (Jensen, Bohonak & Kelley, 2005). Geographic distances between sample sites were calculated as straight line distances. We tested the congruence between the phylogenies of hosts and parasites using their respective phylogeny topologies derived from the haplotype sequences using two co-phylogenetic approaches, PACo and ParaFit (Legendre, Desdevises & Bazin, 2002; Balbuena, Miguez-Lozano & Blasco-Costa, 2013). Significant congruence between the topologies would suggest a coevolutionary or local adaptation scenario between host and parasite haplotypes. As both methods require two distance matrices, each representing the phylogenetic distances among host and parasite taxa, squared patristic distance matrixes were estimated from the Bayesian maximum clade credibility trees of both hosts and parasites. In addition to overall congruence, the contribution of each individual host-parasite link to the overall fit was tested with ParaFitLink2 (Legendre *et al.*, 2002) and evaluated based on estimates of squared residuals and 95% confidence intervals of each link using a jack-knifed procedure (Balbuena *et al.*, 2013).

#### EXPERIMENTAL INFECTIONS

We used experimental infections to test for potential effects of host-parasite coevolution and local adaptation on parasite infection success, host immunological response and parasite growth within the host. Amphipod hosts were exposed to parasites from their own or a different locality (i.e. sample site); i.e. either local, co-occurring or foreign, never naturally encountered *C. parvum* lineages (; see Kawecky & Ebert, 2004; Greischar & Koskella, 2007 for 'local vs. foreign' terminology). We used *P. fluviatilis* amphipods and *C. parvum* parasites from Lake Waiholo (coded Lw) and the Waitaki River (Wr) for logistical and genetic reasons. Amphipods and the mud snail *Potamopyrgus antipodarum*, *C. parvum*'s first

intermediate host, are abundant in these two localities and can be obtained in large numbers. *Paracalliope fluviatilis* amphipods and *C. parvum* are genetically different between Lake Waihola and the Waitaki River but genetically homogeneous within each locality; i.e. all within locality haplotypes are clustered within the same lineage and grouped in the same network (Table 1; Fig. 2).

Large numbers of the snail *P. antipodarum* were collected at both localities and brought back alive to the laboratory. Snails were screened visually and infected individuals were selected from the shape of their shell; *C. parvum*-infected snails display a characteristic shell shape due to parasite-induced alterations (Lagrue *et al.*, 2007a). Around 500 naturally infected snails from each locality, Lake Waihola and the Waitaki River, were then kept in separate tanks filled with continuously aerated water and

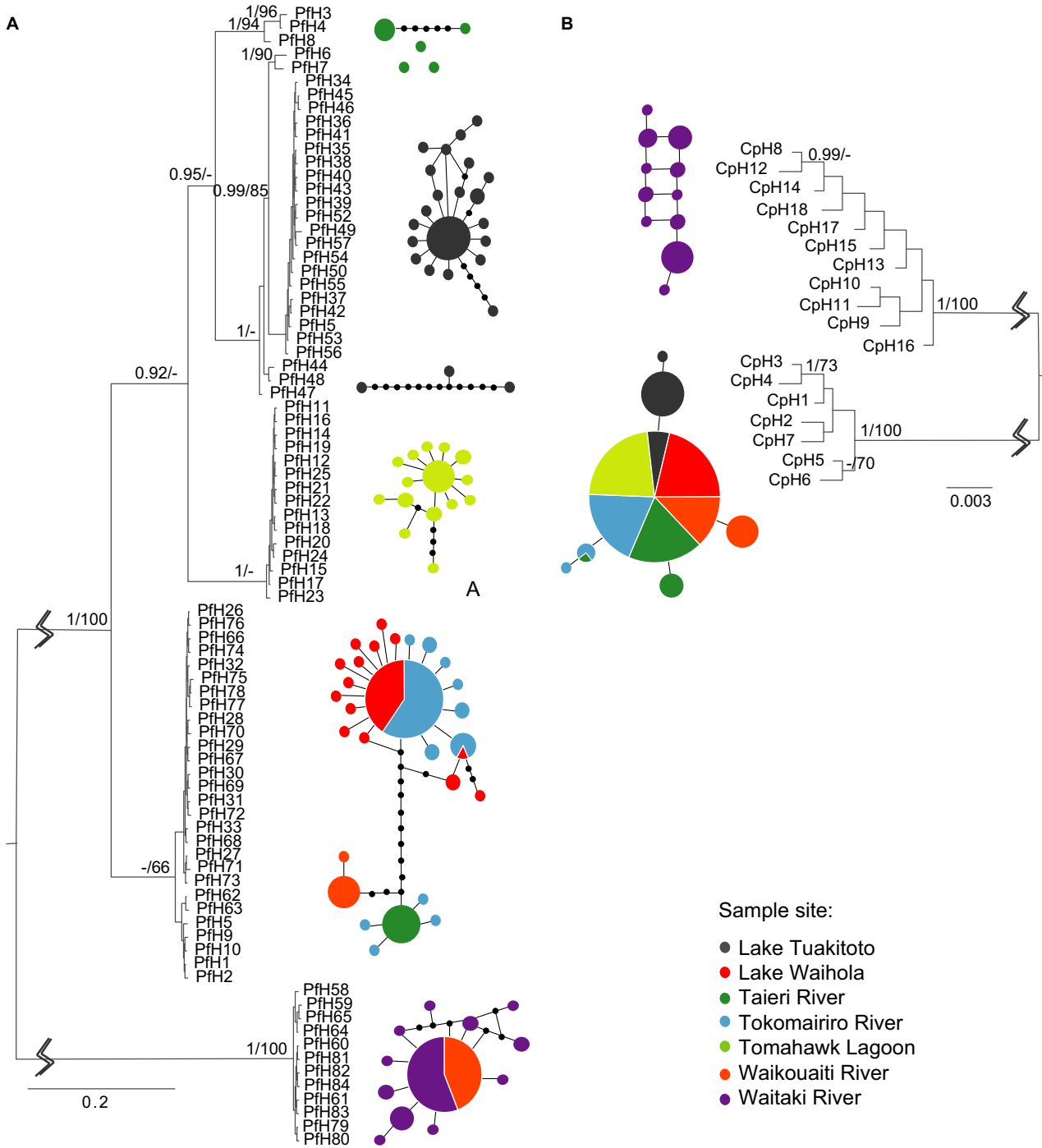
macrophytes (*Elodea canadensis*) for food, until required for experimental infections. Amphipods were collected at each sample site, separated from *P. antipodarum* snails to avoid further infection, and kept in stock tanks in the laboratory under similar conditions to snails for at least a week, allowing pre-existing *C. parvum* metacercariae to increase in size, but no more than 2 weeks to standardize delay between capture and infection. Uninfected amphipods were obtained by inspecting individuals under a dissecting microscope and discarding those that showed signs of infection. This method allows the selection of only uninfected individuals with an accuracy of about 95% (Lefebvre & Poulin, 2005). Because host condition (age, size, sex) can influence the growth of larval helminths in their intermediate hosts (Parker *et al.*, 2003), we used only male *P. fluviatilis* amphipods of similar size.

Four different treatments were used during this experiment. Two control treatments, in which uninfected *P. fluviatilis* amphipod hosts (coded H) were exposed to *C. parvum* cercariae (coded P for parasite) of their own locality (i.e. local parasite lineage), coded Lw-H × Lw-P and Wr-H × Wr-P (Lw = Lake Waihola and Wr = Waitaki River), were used to determine local host–parasite infection patterns (parasite infection success, survival and growth, and host immune response). Two cross-infection treatments were also used, in which amphipods were exposed to *C. parvum* cercariae from the other locality (Lw-H × Wr-P and Wr-H × Lw-P). Results of cross-infection treatments were compared to those of the controls to assess potential effects of host–parasite local adaptations on infection parameters.

*Coitocaecum parvum* cercariae were obtained from naturally infected snails under controlled conditions to standardize time between the release of cercariae from the snail and exposure to the amphipod host (Lagrue, Poulin & Keeney, 2009). That delay was also kept to a minimum (< 20 min) as *C. parvum* cercariae have a limited life span (~ 5 h; Lagrue & Poulin, 2007, 2009a). Infected snails were transferred from the stock tank to Petri dishes (around 50 haphazardly-chosen snails per dish) filled with water and then incubated at 25 °C for 20 min under constant light, conditions that induce cercarial release (Lagrue & Poulin, 2007, 2009a). The Petri dishes were then screened under a microscope and the cercariae found were transferred to 500 µL Eppendorf tubes using a 20 µL micropipette. Two cercariae were placed in each tube with 4 µL of water and an amphipod was then added. Amphipods were left in the tube along with the two cercariae for 5 h, time after which unsuccessful cercariae stop moving and die. Amphipod survival, at this stage, approaches 100% (Lagrue & Poulin, 2007). Amphipods were then

**Table 1.** Population pair-wise  $\Phi_{ST}$  values for parasite and host (significance level at  $P < 0.05$ )

Sites compared	<i>Coitocaecum parvum</i>		<i>Paracalliope fluviatilis</i>	
	$\Phi_{ST}$	P-value	$\Phi_{ST}$	P-value
Taieri vs. Tokomairiro	0.089	0.007	0.321	< 0.001
Taieri vs. Tomahawk	0.127	0.025	0.802	< 0.001
Taieri vs. Tuakitoto	0.586	< 0.001	0.774	< 0.001
Taieri vs. Waihola	0.122	0.028	0.288	< 0.001
Taieri vs. Waikouaiti	0.271	< 0.001	0.646	< 0.001
Taieri vs. Waitaki	0.982	< 0.001	0.986	< 0.001
Tokomairiro vs. Tomahawk	0.079	0.054	0.998	< 0.001
Tokomairiro vs. Tuakitoto	0.600	< 0.001	0.994	< 0.001
Tokomairiro vs. Waihola	0.075	0.113	0.007	0.175
Tokomairiro vs. Waikouaiti	0.268	< 0.001	0.661	< 0.001
Tokomairiro vs. Waitaki	0.982	< 0.001	1.000	< 0.001
Tomahawk vs. Tuakitoto	0.710	< 0.001	0.987	< 0.001
Tomahawk vs. Waihola	0.000	1.000	0.998	< 0.001
Tomahawk vs. Waikouaiti	0.358	< 0.001	0.753	< 0.001
Tomahawk vs. Waitaki	0.984	< 0.001	1.000	< 0.001
Tuakitoto vs. Waihola	0.703	< 0.001	0.993	< 0.001
Tuakitoto vs. Waikouaiti	0.588	< 0.001	0.726	< 0.001
Tuakitoto vs. Waitaki	0.979	< 0.001	1.000	< 0.001
Waihola vs. Waikouaiti	0.349	< 0.001	0.635	< 0.001
Waihola vs. Waitaki	0.984	< 0.001	1.000	< 0.001
Waikouaiti vs. Waitaki	0.980	< 0.001	0.293	< 0.001



**Figure 2.** Phylogenetic relationships based on COI unique haplotypes and statistical parsimony haplotype networks in (A) the amphipod host *Paracalliope fluviatilis* and (B) its trematode parasite *Coitocaecum parvum*. Bayesian inference tree topologies with posterior probabilities at nodes followed by maximum likelihood bootstrap support values. Network pie chart size is scaled to haplotype frequencies and the proportion of each haplotype recovered from each locality (i.e. sample site) is represented by different shading. Small black circles represent inferred intermediate haplotypes not observed in the data. All connections between haplotypes represent one mutation. Abbreviations: Cp, *Coitocaecum parvum*; Pf, *Paracalliope fluviatilis* and H, haplotype followed by haplotype number.

transferred to plastic containers in groups of about 25 individuals separated by treatment. Plastic containers were filled with 400 mL of continuously aerated water and strands of macrophytes (*Eloдея canadensis*) were added for food. In total, 621 amphipods served as control (303 in Lw-H  $\times$  Lw-P and 318 in Wr-H  $\times$  Wr-P, respectively) and 589 were used in cross-infection treatments (324 in Lw-H  $\times$  Wr-P and 265 in Wr-H  $\times$  Lw-P, respectively).

After 6 days, all surviving amphipods (265, 235, 285 and 204 in Lw-H  $\times$  Lw-P, Wr-H  $\times$  Wr-P, Lw-H  $\times$  Wr-P and Wr-H  $\times$  Lw-P, respectively) were killed in 70% ethanol, rinsed in water and dissected immediately afterwards. This method kills the amphipod but not its internal parasites; therefore it has no effect on *C. parvum* measurements (Lagrue & Poulin, 2008). Amphipods were measured (body length) and dissected under the microscope. Any *C. parvum* individual they contained was recorded as dead (immobile and partially or totally melanized by the host) or alive (active once removed from its translucent metacercarial cyst). Metacercariae were measured (length and width) and the body surface, used as a surrogate for body size, of each parasite was calculated using the formula for an ellipsoid,  $(\pi LW)/4$ , where  $L$  and  $W$  are the length and width of metacercariae. As amphipods were experimentally exposed to two cercariae, some individuals contained two individuals of *C. parvum*, whereas others had only one or were uninfected. Metacercariae were thus divided into two different classes under 'infection status': single infections (one individual per amphipod) and double infections (two individuals per amphipod).

Potential differences in amphipod size (body length) among the four cross-infection treatments (Lw-H  $\times$  Lw-P, Lw-H  $\times$  Wr-P, Wr-H  $\times$  Wr-P and Wr-H  $\times$  Lw-P, respectively) were tested using a General Linear Model (GLM) with the treatment as the main factor and the groups in which the amphipod were maintained after experimental infections as a nested factor. Amphipod survival (i.e. proportion of experimentally infected amphipod host alive after 6 days), infection rate (i.e. proportion of *C. parvum* cercariae successfully penetrating amphipod hosts), parasite survival (i.e. proportion of successful cercariae alive and encysted as metacercariae) and overall infection success (i.e. proportion of *C. parvum* cercariae reaching the metacercarial stage and alive at dissection) were compared among treatments in a pair-wise manner using Fisher's exact tests. Effects of amphipod and parasite locality of origin (i.e. treatment), and infection status (single or double infection) on parasite body size were tested using a GLM with the size of metacercariae used as the dependent variable and the groups in which the amphipod were

maintained after experimental infections as a nested factor. Amphipod length and parasite body surface area were log-transformed before analyses to normalize the data.

## RESULTS

### MOLECULAR ANALYSIS

Overall, 222 COI sequences of the trematode *C. parvum* and 235 of the amphipod *P. fluviatilis* were obtained. In total, 84 unique haplotypes of *P. fluviatilis* were detected (Fig. 2A; GenBank accession numbers KR336865-KR336948). These unique haplotypes were distributed amongst six disconnected haplotype networks and three unlinked haplotypes. Only three haplotypes were present at more than one locality (i.e. sample site). The two most common were restricted to two localities each; haplotype 26 (Pfh26) was found in the Tokomairiro River and Lake Waihola while Pfh60 was present in the Waikouaiti and Waitaki rivers. Haplotypes of amphipods from Tomahawk Lagoon formed a single network. Amphipods from Lake Tuakitoto could be separated into two disconnected, although closely related networks (Fig. 2A). Two networks were composed of closely related haplotypes from several localities. One network contained all haplotypes present in the Waitaki River together with most haplotypes from the Waikouaiti River. Another comprised all haplotypes recovered from Lake Waihola and the Tokomairiro River, together with some from the Waikouaiti and Taieri rivers. Haplotypes from the Taieri River clustered in two distinct networks and three single haplotypes were disconnected (Fig. 2A).

In *C. parvum*, 18 unique haplotypes were detected across all COI sequences (Fig. 2B; GenBank accession numbers KR336847-KR336864). *Coitocaecum parvum* haplotype 1 (CpH1) was the dominant haplotype (141 individuals) and was also widely distributed (present at six of the seven sampled localities; Fig. 2B). Haplotype network analysis of *C. parvum* detected two disconnected networks (Fig. 2B). One network contained seven haplotypes, representing sequenced individuals from all localities except the Waitaki River. The other network included 11 haplotypes only found in the Waitaki River. The two disconnected *C. parvum* haplotype networks corresponded to the two distantly related clades depicted by both BI and ML trees (Fig. 2B).

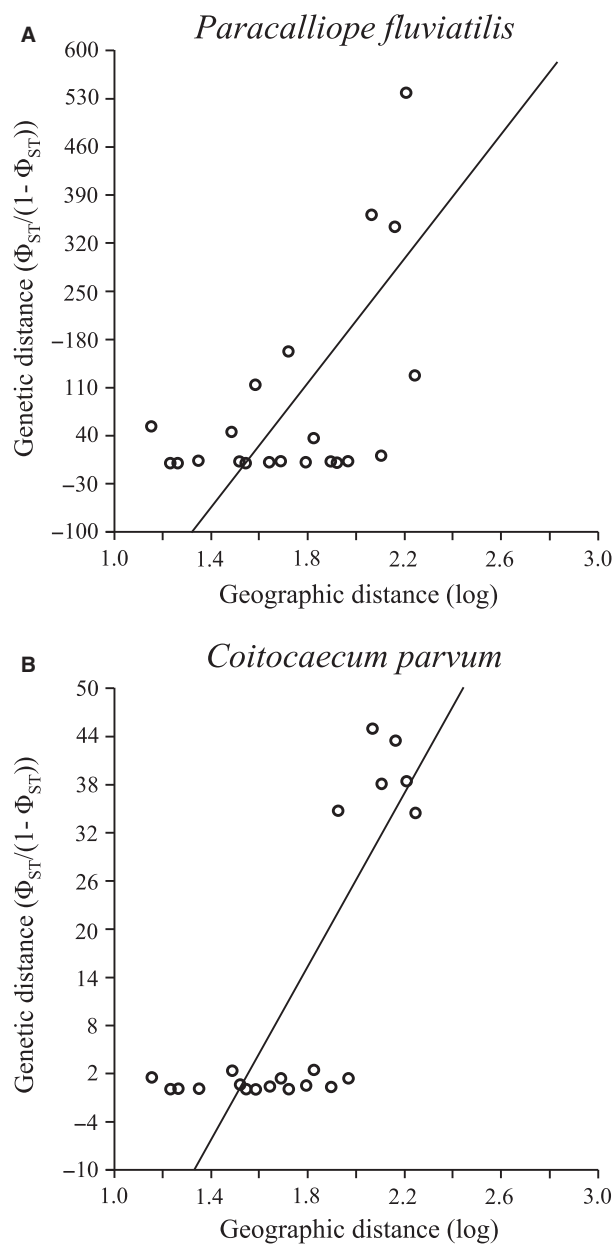
Overall, significant genetic differentiations were detected among populations (i.e. sample sites) of both amphipod hosts and parasites (*P. fluviatilis*  $\Phi_{ST} = 0.852$ ,  $P < 0.001$ ; *C. parvum*  $\Phi_{ST} = 0.971$ ,  $P < 0.001$ ). Pair-wise comparisons indicated a lack of genetic differentiation only among *C. parvum*

populations of the Tokomairiro River, Tomahawk Lagoon and Lake Waihola, and between *P. fluviatilis* populations of the Tokomairiro River and Lake Waihola; all other pair-wise comparisons indicated significant genetic differentiation between host and parasite population pairs (see Table 1 for details). A significant correlation between genetic and geographic distances was also detected with the Mantel test for both *P. fluviatilis* and *C. parvum* ( $r = 0.544$ ,  $P = 0.014$  and  $r = 0.758$ ,  $P = 0.034$ , respectively; Fig. 3). The existence of co-divergence signals among host-parasite haplotype combinations was investigated for 17 parasite and 59 host haplotypes (all combinations for which we obtained sequence data for both parasites and their infected hosts), with a total of 75 host-parasite links. Both co-phylogenetic analyses indicated that the parasite phylogram was congruent with the host phylogram (PACo:  $m^2_{XY} = 10.471$ ,  $P < 0.001$ ,  $10^4$  permutations; ParaFit: global fit statistic = 5.198,  $P < 0.001$ ,  $10^4$  permutations), which supports an overall co-divergence scenario among the host-parasite haplotype combinations found in our sample sites.

Three clusters of host-parasite haplotypes were detected suggesting three co-diverging groups, while two parasite haplotypes were also linked to more distantly related host haplotypes (Fig. 4A). The associations of the most common *C. parvum* haplotype (CpH1) with amphipod haplotypes from Tomahawk Lagoon and Lake Tuakitoto, and some amphipod haplotypes from the Taieri River (Pfh6) and Waikouaiti River (Pfh60) showed high squared residual values suggesting host switching events among *P. fluviatilis* host haplotypes rather than host-parasite co-divergence (Fig. 4B). Associations of Pfh60 and 79 with *C. parvum* haplotypes CpH7, 8 and 12 showed similarly high residuals (Fig. 4B), whereas the remaining associations seemed to represent co-divergence among host-parasite combinations, which at the geographic scale of our study is most likely related to co-adaptation between sympatric host and parasite genetic lineages. The ParaFitLink2 function considered all but the association *C. parvum* haplotypes CpH1 and 7 with *P. fluviatilis* haplotype 60 as putative co-divergence events (Fig. 4B).

#### EXPERIMENTAL INFECTIONS

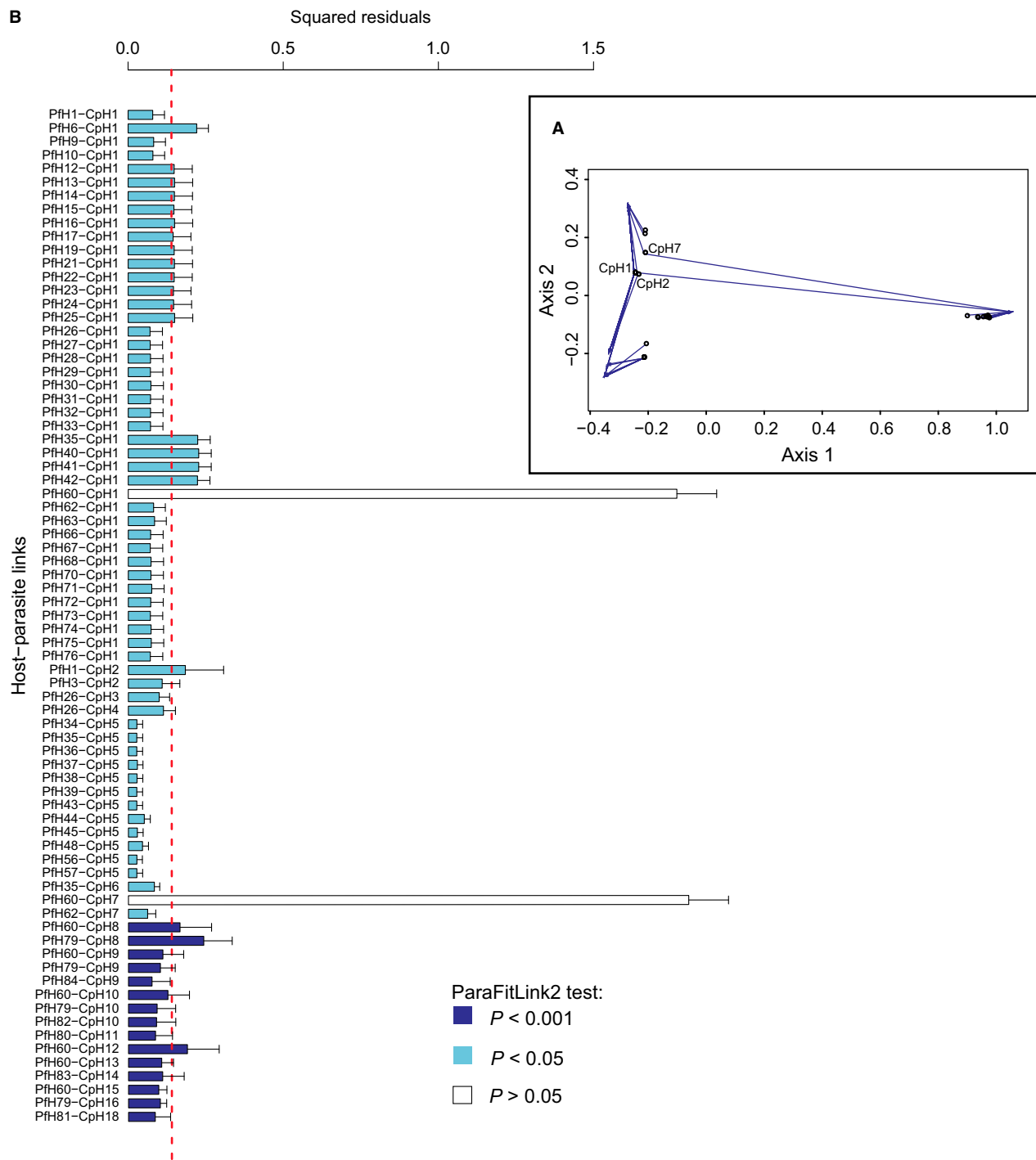
Survival of amphipod hosts was similar between control and cross-infection treatments in both Waihola (87.5% and 88% in Lw-H  $\times$  Lw-P and Lw-H  $\times$  Wr-P, respectively; Fisher's exact test,  $\chi^2 = 0.04$ ,  $P = 0.848$ ) and Waitaki amphipods (73.9% and 77% in Wr-H  $\times$  Wr-P and Wr-H  $\times$  Lw-P, respectively; Fisher's exact test,  $\chi^2 = 0.74$ ,  $P = 0.390$ ). Survival of Waihola amphipods was significantly higher than that of



**Figure 3.** Relationship between pair-wise genetic distances ( $\Phi_{ST}/(1-\Phi_{ST})$ ; Rousset, 1997) and geographical distances (log-transformed) between sites, for (A) *Paracalliope fluviatilis* and (B) *Coitocaecum parvum*.

Waitaki amphipods regardless of the origin of *C. parvum* cercariae these were exposed to (Fisher's exact tests,  $\chi^2 = 10.78$  and 20.62,  $P = 0.001$  and  $P < 0.0001$  for amphipods in Lw-H and Wr-H treatments, respectively). There was no difference in the size of surviving amphipods among treatments (mean body length in mm  $\pm$  SE =  $3.39 \pm 0.01$ ,  $3.40 \pm 0.01$ ,  $3.41 \pm 0.01$  and  $3.40 \pm 0.01$  in Lw-H  $\times$  Lw-P, Lw-H  $\times$  Wr-P, Wr-H  $\times$  Wr-P and Wr-H  $\times$  Lw-P,

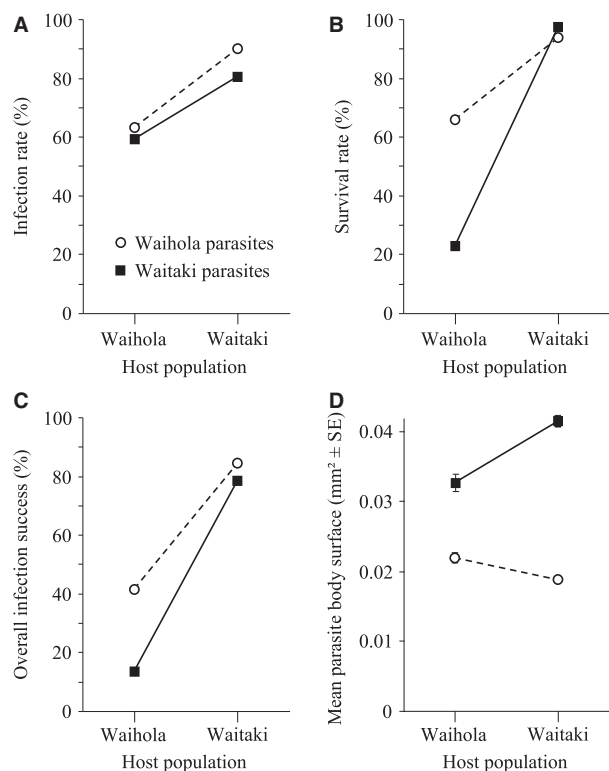




**Figure 4.** (A) Procrustean superimposition plot based on principal correspondence coordinates of squared patristic distances. Parasite configuration (represented by dots), rotated and scaled to fit the ordination of the hosts (arrow tips). (B) Barplot of the contribution of individual host–parasite links to procrustean fit. Jack-knifed squared residuals (bars), upper 95% confidence intervals (error bars) and expected squared residual value under perfect co-speciation scenario (dashed line). Significance level of the ParaFitLink2 analysis for each link is indicated by the bar colour. Abbreviations: Cp, *Coitocaeum parvum*; Pf, *Paracalliope fluviatilis* and H, haplotype followed by haplotype number.

respectively;  $F_{3,933} = 0.2$ ,  $P = 0.867$ ). There was also no significant effect of the group in which amphipod hosts were maintained on host size ( $F_{49,933} = 0.2$ ,  $P = 0.089$ ). Host size was thus unlikely to account for potential differences in infection levels or parasite size detected among treatments or even among groups.

Infection rate (proportion of *C. parvum* cercariae successfully penetrating amphipod hosts) was highly variable among treatments (Fig. 5A). *Coitocaecum parvum* infection rate was significantly lower in amphipod hosts from Waihola (Lw-H) with around 40% of cercariae failing to penetrate the amphipod host, regardless of the parasites origin (Lw-P or Wr-P, Table 2). While the proportion of *C. parvum* cercariae from Lake Waihola and the Waitaki River successfully penetrating Waihola amphipod hosts was similar, the infection rate of Waihola parasites



**Figure 5.** Results of cross-infections among amphipod hosts (*Paracalliope fluviatilis*) and *Coitocaecum parvum* parasites from Lake Waihola and the Waitaki River for (A) infection rates (proportion of *C. parvum* cercariae successfully penetrating amphipod hosts), (B) survival rates (proportion of successful cercariae alive and encysted as metacercariae), (C) overall infection success (proportion of *C. parvum* cercariae reaching the metacercarial stage and alive at dissection) and (D) body surface (mean  $\pm$  SE in mm<sup>2</sup>) in the four possible host  $\times$  parasite combinations (i.e. treatments).

in Waitaki amphipods (Wr-H) was slightly, but significantly, higher than that of Waitaki parasites in their home Wr-H hosts (Table 2). Even when *C. parvum* cercariae successfully penetrated the amphipod host, parasites could still be killed and melanized by the amphipod host's immune system before forming the cyst protecting *C. parvum* metacercariae. Survival rate (proportion of successful cercariae alive and encysted as metacercariae) was also highly variable among treatments (Fig. 5B). *Coitocaecum parvum* survival rate was significantly lower in hosts from Waihola (Lw-H) compared to survival in Waitaki amphipods (Wr-H; Table 2). In Waihola amphipods, the survival rate of local parasites (Lw-P) was significantly higher, around three times, than that of foreign parasites (Wr-P; Table 2). The same, albeit weaker, trend was observed in Waitaki amphipods in which local parasites had a higher survival rate than foreign Waihola *C. parvum*. Overall infection success (proportion of *C. parvum* cercariae reaching the metacercarial stage and alive at dissection) was consequently extremely variable among treatments (Fig. 5C). The proportion of *C. parvum* cercariae reaching the metacercarial stage was significantly lower in Waihola amphipods compared to amphipods from the Waitaki River (Table 2). In hosts from Waihola, only about one in ten Wr-P cercariae were recovered as live metacercariae during dissection. This is an infection success significantly lower than that of Waihola parasites in their home amphipod hosts (Lw-H) in which about four in ten cercariae reach the metacercarial stage (Table 2). Overall infection success in Waitaki amphipods was high for both local (Wr-P) and foreign (Lw-P) parasites but, in contrast to the trend observed in Waihola amphipods, foreign *C. parvum* (Lw-P) infection success was slightly, but significantly, higher than that of local parasites (Wr-P) in Waitaki amphipods (Wr-H; Table 2).

Parasite size was not influenced by infection status and *C. parvum* metacercariae found in single and double infections achieved similar growth (mean body surface in mm<sup>2</sup>  $\pm$  SE = 0.0284  $\pm$  0.0008 and 0.0292  $\pm$  0.0005, respectively;  $F_{1,954} = 0.004$ ,  $P = 0.952$ ). There was also no significant effect of the group in which amphipod hosts were maintained on parasite body size ( $F_{48,954} = 1.332$ ,  $P = 0.068$ ). Size of *C. parvum* metacercariae varied significantly among the four treatments (ANOVA,  $F_{3,954} = 489.1$ ,  $P < 0.0001$ ). Body size of parasites in each treatment was significantly different from that of *C. parvum* metacercariae in all other treatments (Fisher's LSD, d.f. = 954, all  $P < 0.0001$ ; Fig. 5D). *Coitocaecum parvum* metacercariae from the Waitaki River were significantly larger than parasites from Lake Waihola, regardless of the treatment. Parasites also reached

**Table 2.** Results of Fisher's exact tests for pair-wise comparisons of the proportion of *Coitocaecum parvum* cercariae successfully penetrating the amphipod host (infection rate), the proportion of successful cercariae alive and encysted as metacercariae 6 days post-infection (survival) and the proportion of *C. parvum* cercariae encysted as live metacercariae after 6 days (overall infection success) among the four experimental infection treatments (significance level at  $P < 0.05$ )

Fisher's exact tests Treatments compared	Infection rate		Survival rate		Overall infection success	
	$\chi^2$	<i>P</i> -value	$\chi^2$	<i>P</i> -value	$\chi^2$	<i>P</i> -value
Lw-H × Lw-P vs. Lw-H × Wr-P	1.75	0.1860	125.8	< 0.0001	109.3	< 0.0001
Lw-H × Lw-P vs. Wr-H × Wr-P	36.81	< 0.0001	125.1	< 0.0001	140.9	< 0.0001
Lw-H × Lw-P vs. Wr-H × Lw-P	90.43	< 0.0001	86.66	< 0.0001	178.4	< 0.0001
Lw-H × Wr-P vs. Wr-H × Wr-P	54.41	< 0.0001	424.4	< 0.0001	444.4	< 0.0001
Lw-H × Wr-P vs. Wr-H × Lw-P	114.1	< 0.0001	368	< 0.0001	489.4	< 0.0001
Wr-H × Wr-P vs. Wr-H × Lw-P	16.32	0.0001	6.8	0.0091	5.26	0.0218

Lw, Lake Waiholā; Wr, Waitaki River; H, host (*Paracalliope fluviatilis*); P, parasite (*Coitocaecum parvum*)

larger sizes in their home amphipod hosts (controls) than in hosts from the other locality (cross-infections); similar patterns were observed in both Waiholā and Waitaki *C. parvum* metacercariae. Finally, there was no interaction between treatment and infection status ( $F_{3,954} = 0.329$ ,  $P = 0.804$ ).

## DISCUSSION

There was significant genetic differentiation among populations of both the amphipod host and its trematode parasite *C. parvum*. Most of the variability observed was found among geographically isolated populations. Such geographic structure is consistent with the low dispersal abilities of the parasite and its different hosts (Jarne & Théron, 2001; Prugnolle *et al.*, 2005a). *Coitocaecum parvum* hosts are all small and exclusively freshwater organisms, which should limit gene flow among geographically isolated host and parasite populations (Criscione & Blouin, 2004; Blasco-Costa & Poulin, 2013). Indeed, both snail and amphipod hosts have low gene flow among isolated freshwater systems (Dybdhal & Lively, 1996; Hogg *et al.*, 2006; Sutherland *et al.*, 2010). Although intrinsically more mobile, the fish definitive hosts have small home ranges and sedentary habits, even within rivers or lakes, further limiting gene flow and genetic connectivity among parasite populations (McDowall, 1990; Prugnolle *et al.*, 2005a; Blasco-Costa *et al.*, 2012). However, haplotype diversity and divergence among populations was lower in *C. parvum* than in its amphipod host (Fig. 2). Although only one host species is used by *C. parvum* at the metacercarial stage, multiple widely different hosts are necessary to complete the parasite's life cycle. Coevolution and local adaptation in parasites

with complex life cycles may differ from those in parasites with direct cycles (i.e. requiring a single host per generation; Prugnolle *et al.*, 2005a). An individual *C. parvum* reaching adulthood in the fish definitive host will have successfully infected another two host species along the way, and will have thus been facing multiple host-parasite infection filters. Potential trait combinations allowing parasites to successfully infect, survive and develop in, and be transmitted to three different host species are likely limited, potentially reducing genetic diversity within and divergence among parasite populations (Prugnolle *et al.*, 2005a). Furthermore, trematode parasites typically alternate sexual and asexual reproduction during their life cycle and, as hermaphrodites, can use selfing for reproduction, potentially reducing genetic diversity (Prugnolle *et al.*, 2005b). Additionally, *C. parvum* can use progenesis and selfing while still inside the amphipod host, thus eliminating the need for the definitive host and any opportunity of sexual reproduction, further increasing inbreeding and reducing genetic diversity (Lagrue *et al.*, 2007b, 2009; Lagrue & Poulin, 2009b).

Although haplotype diversity and genetic structure among populations were less pronounced in *C. parvum* than *P. fluviatilis* amphipods, we found a significant co-divergence pattern between the parasite and its host. Sympatric host-parasite lineage combinations seem to be genetically diverging concomitantly from other isolated host and parasite populations. This pattern may indicate the existence of local co-adaptation between host and parasite genetic lineages (Gandon *et al.*, 2008); although genetic drift and adaptation to local environmental factors could also influence genetic structure among populations. Nevertheless, genetic differentiation among populations may be linked to coevolution between hosts and

parasites among these isolated populations and result in local adaptation of parasites (Thrall, Burdon & Bever, 2002; Morgan *et al.*, 2005). Local adaptation occurs when parasite fitness is higher in its home than in away hosts (Gandon & Michalakis, 2002). However, while there was clear genetic differentiation in both hosts and parasites between the two populations used in cross-infections, results show highly contrasting patterns among host-parasite combinations. *Coitocaeum parvum* infection and survival rates, and consequently overall infection success were significantly higher in amphipods from the Waitaki River than from Lake Waihola, regardless of parasite origin. As a result, while Waitaki parasites do seem to achieve higher infection levels in their home hosts than in away hosts, the trend is reversed in Waihola parasites in which infection success is higher in away Waitaki amphipod hosts. Waitaki amphipod may generally be more vulnerable to *C. parvum* infection than Waihola amphipods, local adaptation having little effect on infection patterns. Higher infection rates (proportion of *C. parvum* larvae successfully penetrating the host) in Waitaki compared to Waihola amphipods are likely due to behavioural differences. *Paracalliope fluviatilis* amphipods can be observed actively defending themselves against *C. parvum* larvae through self-grooming and predation on the parasite. Waihola amphipods may thus be more efficient groomers than Waitaki individuals. Indeed, during experimental infections, when introduced in the tubes containing *C. parvum* cercariae for experimental infections (see material and methods section for details), many Waihola amphipods became very agitated and started grooming when first contacted by a cercaria. In sharp contrast, Waitaki amphipods remained comparatively subdued and almost never groomed upon contact with *C. parvum* cercariae. Waihola *C. parvum* may concomitantly be more efficient at escaping predation by the host than Waitaki parasites, thus achieving higher infection rates in both amphipod host populations. However, further experiments need to be designed to accurately and quantitatively measure these behavioural differences and to test these hypotheses. Once *C. parvum* larvae have successfully penetrated amphipods, the parasite can still be killed and melanized by the host immune system. Parasite survival rates were much higher in Waitaki than in Waihola amphipods, regardless of parasite origin. Overall, amphipods from Waihola seem to have a more efficient immune system against *C. parvum* larvae. Although *C. parvum* prevalence (proportion of infected individuals in the host population) was similar in snail hosts from Lake Waihola and the Waitaki River (1–2%), snail host density was much higher in Lake Waihola

(~100-fold). Densities of *C. parvum*-infected snails and that of the parasite larvae are thus augmented by the same factor in Lake Waihola. In contrast, amphipod host density was higher in the Waitaki River (~10-fold; C. Lagrue, Pers. Obs.). More amphipod hosts are exposed to much fewer *C. parvum* parasite larvae in the Waitaki River than in Lake Waihola. As a result, natural *C. parvum* prevalence in *P. fluviatilis* amphipod hosts was very low (2–3%) in the Waitaki River compared to that in Lake Waihola (10–12%). Since parasite survival is also much higher in Waitaki amphipods, it is likely that the difference in infection exposure between the two populations is even higher. Selection pressure on Waihola amphipods to develop defense mechanisms, behavioural or immunological, against *C. parvum* infection is thus likely much higher than for Waitaki amphipods. This difference in selection pressure between Waihola and Waitaki amphipods may explain the variation observed here in vulnerability to *C. parvum* infection between hosts from the two populations.

However, within amphipod lineages, local parasites had higher survival than foreign *C. parvum* individuals (Fig. 5B). Local parasites were thus more efficient at escaping the immune system of their home hosts than foreign parasites; some degree of local adaptation therefore seems to influence host-parasite interactions. This trend is confirmed when considering *C. parvum* larval growth in cross-infections. Although *C. parvum* from the Waitaki River were larger than Waihola individuals, regardless of host-parasite combinations, both parasite lineages achieve larger sizes in their local hosts than in away amphipods, a pattern more commonly associated with local host-parasite coevolution (Ballabeni & Ward, 1993). Although parasites are expected to become adapted to their home hosts, the outcome of host-parasite coevolution may be trait specific and its overall effects on parasite fitness difficult to predict as shown by our results and a few other studies (Kaltz & Shykoff, 1998; Van Zandt & Mopper, 1998; Lajeunesse & Forbes, 2002). Other aspects of parasite and host natural history and evolution may also be important determinants of the occurrence or strength of local adaptations.

Parasites that interact with multiple host species may be less locally adapted as they enter into diffuse coevolutionary interactions (Lajeunesse & Forbes, 2002). This is true for parasites with broad host ranges at a given life stage (e.g. adult parasites infecting multiple host species) but also for parasites with complex life cycles (Westram *et al.*, 2011). As a trematode parasite, *C. parvum* needs up to three host species to complete its life cycle, potentially influencing local adaptation patterns between

*C. parvum* and *P. fluviatilis* hosts. Furthermore, although *C. parvum* snail and amphipod hosts show high genetic structure among populations, the fish definitive host may display less pronounced structure and be genetically more homogeneous than invertebrate hosts. As a result, adult *C. parvum* in fish hosts could face similar selective pressures across populations, potentially reducing local host–parasite co-adaptation at the amphipod host level.

The degree of local adaptation may also be influenced by the spatial scale over which comparisons are made (Thrall *et al.*, 2002). Our study area is relatively small and cross-infections among populations over a larger geographic area may yield different patterns. Genetic differentiations among populations of the amphipod *P. fluviatilis* showed a clear correlation between genetic and geographic distances (also in Sutherland *et al.*, 2010). The correlation was even stronger for *C. parvum* and increasing genetic divergence among populations may induce higher levels of apparent host–parasite local adaptation among highly divergent populations at larger distances. This could ultimately drive cryptic speciation in parasites and hosts as already suggested for *P. fluviatilis* amphipods (Hogg *et al.*, 2006; Sutherland *et al.*, 2010). Further studies would be required to confirm this trend in *C. parvum*, however.

Overall, assessing pathogen performance in home vs. away host populations can provide a misleading test of local adaptation if too few traits, or traits not actually influencing local adaptation, affecting parasite fitness are taken into account; which and how many traits should be considered will most likely vary among host–parasite systems. Furthermore, local adaptation effects on parasite fitness may be swamped or confounded by local, intrinsic host or parasite characteristics. For instance, if host populations differ in overall resistance or parasites differ in overall infectivity or growth rate (Thrall *et al.*, 2002). Here, Waitaki amphipods seemed less capable of behaviourally resisting infection and immunologically less efficient at killing *C. parvum* larvae, regardless of parasite origin, and may be generally less resistant to *C. parvum*; higher mortality in Waitaki amphipods during our experiments could thus be a direct consequence of the higher vulnerability of Waitaki amphipods to *C. parvum*, although alternative explanations exist. Conversely, Waiholā parasites were more efficient at evading immune defenses and surviving in Waiholā amphipod hosts than Waitaki parasites, providing some evidence for adaptation by these parasites to their home host population. The combination of the overall higher susceptibility of Waitaki amphipods to *C. parvum* infection with local adaptation of Waiholā parasites

to their home hosts renders results of cross-infections between these two populations difficult to interpret. Overall, our results show clearly that genetic differentiation among populations observed in the field is not inevitably coupled with clear local adaptation patterns. Generally, trait-specific local adaptation and population specific host–parasite coevolution are likely to influence apparent patterns of local adaptation among host–parasite populations; such patterns should thus be interpreted with caution. Admittedly, our conclusions are based on the use of only two populations for the test of local adaptation, which limits any inference that can be made (Kawecki & Ebert, 2004; Blanquart *et al.*, 2013). Nevertheless, the trait-specific nature of our findings still reveals the complexity of host–parasite co-adaptation.

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