The Rapid, Mass Invasion of New Zealand by North American Daphnia pulex/pulicaria

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Abstract

Non-indigenous Daphnia 'pulex' have been found in many lakes in New Zealand (NZ) in the past 20 years, suggesting a recent invasion. However, very little is known about the origin of invasive D. 'pulex', whether they are D. pulex or D. pulicaria, and whether they are obligately asexual clones or cyclical parthenogens. Furthermore, the source and time of arrival of the invasive genotype(s) are unclear. We address these questions by genomic sequencing Daphnia populations from 13 lakes on the South Island and one on the North Island, NZ. Based on ~24,000 monomorphic species-specific markers, the invasive Daphnia on the South Island were found to be D. pulicaria, while those on the North Island are D. pulex/ pulicaria hybrids. Both the South and North Island Daphnia are phylogenetically clustered with North American D. pulicaria/pulex, thereby suggesting their North American origins. We further found that the South Island Daphnia populations are fixed heterozygotes for nearly all bi-allelic sites in the nuclear genome and contain identical mitochondrial genomes, suggesting the origin and proliferation from a single founder clone, which we experimentally verified to be an obligate asexual. Estimates from molecular data imply a colonization time for the South Island populations of ~ 60 years ago, with a likely invasion route associated with the introduction of salmonids from North America. Key words: Daphnia pulex; Daphnia pulicaria; invasion; obligately asexual; hybridization

Introduction

The *Daphnia pulex* complex contains at least eight genetically distinct species, widely found throughout the northern hemisphere (Colbourne et al. 1998). Two of the species, *D. pulex* and *D. pulicaria* , can form viable hybrids, but are not easily distinguished morphologically, and typically are identified by allozyme electrophoresis of the lactate dehydrogenase locus, with homozygous *D. pulicaria* having two "fast" alleles and homozygous *D. pulex* having two "slow" alleles (Hebert et al. 1989; Crease et al. 2011). Hybrids with the heterozygous slow/fast genotype are generally found in disturbed ponds or deforested areas (Hebert et al. 1989). Due to the frequent gene flow between *D. pulex* and *D. pulicaria* (Hebert et al. 1989; Cristescu et al. 2012), some hybrids are also homozygous for the Ldh locus, presumably resulting from backcrossing with either *D. pulex* or *D. pulicaria* (Xu et al. 2013), which further complicates the species identification.

Both *D. pulex* and *D. pulicaria* can reproduce by cyclical parthenogenesis (CP), with extended periods of parthenogenesis interspersed with sexual resting-egg production (Hebert 1978; Hebert et al. 1993). However, some clones in each species have lost the ability to engage in meiosis and have become obligate parthenogens (OP) (Hebert and Crease 1980; Hebert et al. 1993; Paland et al. 2005). Interestingly, the loss of meiosis is often limited to females, and some OP *D. pulex*can still produce functional males bearing haploid sperm. Such males from OP *D. pulex* can mate with females from CP *D. pulex*and form viable hybrids, ~40% of which have the OP phenotype (Innes and Hebert 1988), indicating that partially dominant meiosis-suppressing elements are carried by OP males. Further analysis has shown that all OP *D. pulex* share common haplotypes on chromosomes VIII and IX that arose by introgression from *D. pulicaria* (Xu et al. 2015) and are transmitted through OP males without recombination (Tucker et al. 2013). These observations suggest that all OP *D.*

pulex may be descendants of a single hybridization event between *D. pulex* and *D. pulicaria.* Initially, it was thought that all F_1 hybrids between *D. pulex* and*D. pulicaria* are obligate parthenogens (Crease and Lynch 1991), but Heier and Dudycha (2009) found that F_1 hybrids between some CP *D. pulex* females and CP *D. pulicaria* males have CP phenotypes and can backcross with both parental species and themselves (Heier and Dudycha 2009). Thus, Xu et al. (2013) argued that OP hybrids originated from a unique historical hybridization and introgression event between female *D. pulex* and male *D. pulicaria* .

Although asexual clones are generally thought to suffer from long-term selective disadvantages resulting from reduced efficiency of purging deleterious mutations (Lynch et al. 1993), the OP clones with hybrid backgrounds from *D. pulex* and *D. pulicaria* are notably invasive. For example, an obligately asexual *D. pulex* ×*pulicaria* hybrid clone invaded Africa after the 1920s (Mergeay et al. 2006) and has spread throughout the range of native sexual *D. pulex* and displaced the sexual population. This asexual hybrid clone is thought to have been initially introduced from the USA via the introduction of largemouth bass in 1927-29 and completely displaced all other *D. pulex* genotypes in Africa within 60 years (Mergeay et al. 2006). In addition, an OP *D. pulex* invasion in Japan, involving four hybrid clones and thought to have happened between 680 and 3400 years ago, is unlikely due to human activity (So et al. 2015).

Recently, non-indigenous *Daphnia* species morphologically determined to be from the *D. pulex* complex have been found in several lakes on the South Island of New Zealand (NZ) (Duggan et al. 2012). Although Duggan et al. (2012) considered the invasive South Island clones to be *D. pulex,* because their mitochondrial cytochrome c oxidase subunit 1 (CO1) gene sequences formed a monophyletic group with North American *D. pulex* , their analysis only used a 658-bp fragment of CO1 nucleotide sequences from 7 South Island clones. Due to the frequent hybridization between *D. pulex* and *D. pulicaria* (Hebert et al. 1989; Heier and Dudycha 2009; Cristescu et al. 2012), the mitochondrial genomes may actually have either a *D. pulicaria* or *D. pulex* origin (Cristescu et al. 2012; Marková et al. 2013). Although members of the South Island populations are Ldh homozygotes (Duggan et al. 2012), it is unclear whether this locus is homozygous for *D. pulex* or *D. pulicaria* alleles, and again the interpretation may be clouded when hybrids exist. Moreover, due to the limited sequencing information, Duggan et al. (2012) were unable to estimate when the invasion occurred.

To obtain a better understanding of the NZ *Daphniapulex/pulicaria* invasion, we performed genome-wide analyses of*Daphnia pulex/pulicaria* populations from 13 lakes across the South Island and one lake on the North Island. The species designations and likely sources of origin were determined via a survey of *D. pulicaria* -specific markers and a whole-genomic phylogenetic tree that includes *D. pulicaria* and *D. pulex* clones collected across North America, Europe, and China.

Methods and methods

Sampling and sequencing

Daphnia populations were collected from 13 lakes in the South Island and 1 lake in the North Island, NZ in March and October 2019 (Fig. 1). In each location, *Daphnia* were sampled by tows of a conical plankton net and preserved in DNA/RNA Shield. DNA from each pooled population sample (50-200 individuals) was extracted with Zymo DNA Clean and Concentrator-25 Kit and sequenced on an Illumina HiSeq 2500 platform.

Reads mapping and SNP calling

First, we trimmed adapter sequences from the sequence reads by applying Trimmomatic (Bolger et al. 2014) to the FASTQ files. Then, we mapped the adapter-trimmed sequence reads to the *D. pulex* reference genome (PA42 version 4.1) using BWA (Li and Durbin 2009). Coverage for each site was calculated by counting the total mapped reads, and coverage for each sample is calculated as the total mapped bases divided by the mapped genomic regions. We examined the genomic distribution of the sites coverage and set minimum and maximum sample-coverage cutoffs to avoid analyzing problematic sites (Table 1; Fig. S1). SNPs were identified by Samtools (Li et al. 2009) with "samtools mpileup -uf ref.fasta sorted.bam | bcftools call -mv > raw.vcf" and "bcftools filter -s LowQual -e '%QUAL<20' raw.vcf > flt.vcf".

If each NZ population represents a single clone, all of its bi-allelic sites should have equal numbers of reads mapped to the two parental alleles. To check this, we searched for sites with reads for the two parental alleles deviating from a 1:1 ratio. To minimize mapping bias, 1) we remapped the reads using bowtie with "-q -m 1 -v 3 –best" to obtain unique mapped reads (Stevenson et al. 2013); 2) we removed regions with >3 SNPs within 100 bp because additional differentiating sites will interfere with read alignment (Stevenson et al. 2013); 3) To reduce the mapping bias toward the reference alleles (Degner et al. 2009), we generated an assembly by masking all of the bi-allelic sites with "N" in the PA42 4.1 reference. Only bi-allelic sites with reads for the two alleles deviating from a 1:1 ratio from both the original and masked assemblies are considered true deviating sites.

Characterizing D. pulicaria-specific markers

To identify *D. pulicaria* -specific markers, we first searched for homozygous SNPs shared by all 14 *D. pulicaria* clones. Then, we checked these SNPs in 42 *D. pulex* clones collected across North American, Europe, and China and eliminated those that appeared in any of the *D. pulex* clones. For the remaining SNPs, we checked the corresponding loci in the genome in each of the *D. pulex* clones and only kept those that are homozygous for the same two bases across all *D. pulex* clones.

Phylogenetic analysis

To generate clean mitochondrial and nuclear genomes for the phylogenetic analysis, we removed regions that are subjected to transposition of cytoplasmic mitochondrial DNA into the nuclear genome in the historical past (numts). We discarded read pairs for which two reads in the pair mapped to both mitochondrial genome and nuclear genome. First, trimmed reads were mapped to the mitochondrial reference genome using BWA (Li and Durbin 2009) with default parameters. We extracted paired-end reads that aligned to the mitochondrial genome from each BAM file. GSNAP (Wu and Watanabe 2005) was used to realign these reads to the mitochondrial reference genome and nuclear genome separately following the pipeline from MToolBox (Calabrese et al. 2014). Read pairs that mapped to both the mitochondrial and nuclear genomes were removed from downstream analysis.

We generated phylogenetic trees using nuclear and mitochondrial genomes, separately. For the nuclear genome, a phylogenetic tree was constructed using the consensus genomic sequences for each NZ population and*D. pulex* /*D. pulicaria*clones. Consensus sequences for each population/clone were generated using Samtools (Li et al. 2009) with command: samtools mpileup -uf ref.fa aln.bam | bcftools call -c | vcfutils.pl vcf2fq > cns.fq. We randomly selected 1% of the consensus sequences and repeated this 1000 times, constructing maximum-likelihood trees for each subset of data using iq-tree with GTR + I model and ultrafast bootstrap (Nguyen et al. 2014; Hoang et al. 2017). The consensus tree was generated using the Consense program, and branch lengths estimated by the Dnaml program in the PHYLIP package (Felsenstein 1993). A separate phylogenetic tree based on mitochondrial data was constructed with the maximum likelihood method using MEGA 7 with 100 Bootstrap (Kumar et al. 2016).

Phenotypic tests

The reproductive modes of NZ *Daphnia* clones were determined using a sexuality test. Because sexual *Daphnia* need sperm fertilization to produce diapausing embryos, the sexuality test is to check whether, in the absence of males, diapausing embryos are deposited into ephippia by females. The consistent presence of embryos in ephippia in the absence of males implies an obligately asexual clone, whereas consistent results of no ephippial embryos from at least three consecutive rounds of tests was used to infer a male-requiring cyclical parthenogen. We decapsulated a total of 32 ephippial embryos produced by clones from Lake Alexandrina in the absence of males. All 30 contained embryos and some of these hatched, consistent with the clones from this lake being obligate asexuals.

Male production was induced by adding the hormone methyl farnesoate (MF) to the medium. Adult females for each clone were isolated and placed into 50-mL tubes containing methyl farnesoate at a concentration of 400 nM (changed daily). We examined the sex of offspring using a dissection microscope. Males can be

visually distinguished from females based on the enlarged antennules and flattened ventral carapace margin. Successful production of males by [?]5 individuals was taken to be evidence for a male-producing clone.

Results

Species identification of the Daphnia from New Zealand lakes

We sampled *Daphnia* populations from 13 lakes on the South Island and one on the North Island, New Zealand (NZ) (Fig. 1; Table S1).*Daphnia* populations from each lake were determined to be *D. pulex* /*D. pulicaria sensu lato* based on morphological features (Benzie 2005; Ebert 2005), although genetic information is required to distinguish the two species. To obtain preliminary genetic information in a simple manner, we pooled all individuals (50-200) from each lake for whole-genomic sequencing. Each population was sequenced to an average depth of sequencing coverage of 86x (Table 1), involving more than 100 million 150-bp reads per population. On average, 1.5 million bi-allelic sites were identified for each population (Table 1).

To determine the species of *Daphnia* populations in NZ, we first relied on the lactate dehydrogenase locus (Ldh), a commonly invoked indicator for distinguishing *D. pulex* and *D. pulicaria*(Hebert et al. 1989; Crease et al. 2011). For this locus, non-hybrid*D. pulicaria* have two "fast" allozyme alleles and non-hybrid*D. pulex* have two "slow" allozyme alleles. The mobility difference is caused by the substitution of glutamine in *D. pulex*to glutamic acid in *D. pulicaria*at position 229 of the amino-acid sequence (Crease et al. 2011). We found that all the South Island *Daphnia* populations are monomorphic for glutamic acid at position 229, suggesting that the South Island populations are *D. pulicaria* . However, the North Island population is heterozygous for position 229, suggesting that it has a hybrid origin.

In addition to the Ldh locus, we identified 24,185 informative sites by comparing 14 *D. pulicaria* and 42 *D. pulex* genomes (Table S2), in which all *D. pulicaria* are monomorphic for one nucleotide and all *D. pulex* are monomorphic for another nucleotide (File S2). We found that all the South Island *Daphnia*populations have >23,390 (97%) sites homozygous for*D. pulicaria*- specific nucleotides, while only 807 (3.3%) sites are homozygous in the North Island population. Our result is consistent with a *D. pulicaria* origin of the South Island *Daphnia*populations, and a hybrid origin of the North Island population.

To further confirm the species of *Daphnia* in NZ, we constructed a consensus maximum-likelihood (ML) tree using whole-genomic coding sequences from 14 NZ populations, 14 *D. pulicaria,* and 42*D. pulex* clones collected across North America, Czech Republic, and China (Table S2). Within this tree, the South Island populations formed a monophyletic clade with all North American *D. pulicaria*clones, further supporting the view that the South Island populations are *D. pulicaria* (Fig. 2). The North Island population is in a clade with North American *D. pulicaria* and *D. pulex* , but clusters with neither North American *D. pulicaria* nor *D. pulex* (Fig. 2), confirming its hybrid origin. Unlike the situation for nuclear data, for the ML tree based on full-length mitochondrial genomes, *D. pulex* and *D. pulicaria* clones interlaced outside of the NZ populations (Fig. S1). This discordant phylogeny between the mitochondrial and nuclear genomes may represent historical hybridization between the two species (Markova et al. 2013).

Testing the single-clone origin hypothesis for the South Island Daphnia populations

Because both North American *Daphnia* invasions in Africa and Japan involved only one or just a few clones (Mergeay et al. 2006; So et al. 2015), we wanted to test if the NZ *Daphnia* populations are also derived from just a few clones. If each NZ *Daphnia* population represents a single clone, assuming no mutation or gene conversion, all of its bi-allelic sites should have similar numbers of reads mapped to the two parental alleles as expected for a fixed heterozygote. To test this, we performed binomial tests on all bi-allelic sites to check for deviations from a 1:1 ratio. Over all 14 samples, with an average of 1.5 million significant bi-allelic sites per sample, only 14 to 667 sites in each population have unequal numbers of reads mapped to the two parental alleles after correcting for multiple comparisons (Table 1), while a typical sexually reproducing *D. pulex* population from North America has >98% of heterozygous sites with minor-allele frequencies significantly deviating from 0.5, and a strong skew of the site-frequency spectrum towards low frequency alleles (Lynch et al. 2017). In principle, the tiny fraction of sites deviating from a 1:1 ratio might have arisen from an

inherent bias toward more effective mapping of one allele (Degner et al. 2009), although this seems very unlikely, as it would have to occur in population-specific samples. A more likely explanation is the low level of mutation and gene conversion operating in all populations, including those that are obligately asexual.

In further support of the hypothesis that all 13 South Island populations originated from the same clone, we found that all of them have identical mitochondrial genomes. Moreover, 6 of the 7 clones from four different South Island lakes collected >7 years ago (Duggan et al. 2012) share identical sequence for the CO1 gene as our South Island samples. The only exception is a clone collected from Lake Wanaka by Duggan et al. (2012) with one unique SNP, which is likely a *de novo* mutation. To understand if the South and North Island *Daphnia* have the same maternal origin, we compared their 15-kb mtDNA genome sequences, and found 54 nucleotide differences, thereby ruling out origination from a single clone.

To check if the South Island populations are obligate parthenogens, we experimentally examined their ability to produce diapausing embryos without fertilization. Cyclically parthenogenetic *Daphnia* produce haploid diapausing eggs that require fertilization by sperm, while obligately asexual *Daphnia* are capable of producing diploid diapausing eggs in the absence of males. We found that in the absence of males, the South Island *Daphnia* (from Lake Alexandrina) can generate ephippia containing embryos, indicating that the life cycle can be completed without sex. We also tested the male-producing ability for the *Daphnia* from Lake Alexandrina and found consistent male production. Our results suggest that the *Daphnia* invasion of South Island involved a single obligately asexual clone capable of (but not requiring) male production.

Due to the lack of live animals, we were not able to experimentally examine the reproductive mode and male-producing ability in the North Island *Daphnia* . Instead, we used informative markers to predict their traits. Chromosomes VIII and IX in *Daphnia pulex/pulicaria*contain >30,000 markers that are informative with respect to the origin of asexuality (Xu et al. 2015, Tucker et al. 2013). All of these markers are heterozygous in obligate asexual hybrids but are homozygous in CP *D. pulex* or *D. pulicaria* . The North Island *Daphnia* carry the heterozygous markers at >87% of these reference sites, suggesting obligate asexuality. We also examined the male-producing ability in the North Island *Daphnia* by reference to markers identified by Ye et al. (2019), finding a complete absence of markers associated with the loss of male-producing ability, thereby suggesting a capacity for male production. Thus, our results indicate that the *Daphnia* invasion of North Island also involved an obligately parthenogenetic clone, capable of producing males.

Origin and colonization time of the NZ populations

To infer the geographic origin of the *D. pulicaria* that invaded South Island, genetic data are required from throughout the natural range of this species. Although whole nuclear and mitochondrial genomes are available for samples from North America and New Zealand, the only data available for *D. pulicaria* from other continents are mitochondrial CO1 gene sequences. For the maximum-likelihood tree using mitochondrial CO1 gene sequences collected from *D. pulicaria*across North America, South America, Europe, Asia, New Zealand, and the north polar region, the South Island *Daphnia* clustered with a subset of North American clones and a few clones from South America (Fig. 3). Because South American *D. pulicaria* are thought to have been introduced from North America (Crease et al. 2012), the phylogeny suggests that the South Island *Daphnia* have a North American origin.

To estimate when the South Island genotype diverged from North American*D. pulicaria* , we calculated the nuclear genome-wide divergence between synonymous sites for these two groups. The divergence time between the South Island genotype and North American *D. pulicaria*(T_1, Fig. 2) can be estimated by the average pairwise distance of synonymous sites (d_s) between North American *D. pulicaria* and the South Island, NZ haplotypes ($d_s = 0.00023$). Letting $T_1 = d_s / 2^{*}\mu$, with $\mu = 5.85 \times 10^{-9}$being the mutation rate per site per generation (obtained from a mutation-accumulation experiment; Keith et al. 2016), and assuming ~5 generations per year, we estimate that the South Island genotype diverged from other North American *D. pulicaria* ~ 3400 years ago. Because the North Island*Daphnia* have a hybrid origin, and we do not have genomic sequences available for North American hybrids, the divergence time between the North Island genotype and North American hybrids of *D. pulex* x *D. pulicaria* cannot be estimated by this means.

We also estimated the expansion time of the South Island populations (T_2, Fig. 2). Assuming these populations descended from a single primary colonization event, then the nucleotide diversity among South Island haplotypes would have been acquired after the colonization, and the expansion time can be estimated from the average synonymous nucleotide divergence between the most distant population (Lake Coleridge) and the remaining populations. We found that the*Daphnia* population from Lake Coleridge has an average of 24 synonymous substitutions (over a total of 7.05 million synonymous sites) compared to the remaining populations, implying a expansion time of $T_2 = d_s /(2*\mu) = 3.4 \times 10^{-6}$ / (2 x 5.85 x 10^{-9} / site / generation x 5 generations / year) = ˜58 years. This calculation is supported by the presence of characteristic ephippia of*D. pulex/pulicaria* in a sediment-dated core taken in Lake Hayes that suggested appearance of this species in the lake in the late 1950s or early 1960s (Samiulah Khan, pers. comm). Moreover, we found that the North Island *Daphnia* has an identical ND5 nucleotide sequence and only a single nucleotide difference on the COI gene relative to one of the clones that invaded Japan between 680 and 3400 years ago (So et al. 2015), suggesting that these two have the same origin.

Discussion

Invasive Daphnia pulex/pulicaria in NZ

In this study, we identified two independent invasions of *Daphnia pulex/pulicaria* on the South and North Island of NZ. Phylogenetic data from mitochondrial genomes revealed that both invasive *Daphnia pulex/pulicaria* originated from North America. We examined*Daphnia pulex/pulicaria* populations from 13 lakes on the South Island, only to find an average of 24 synonymous substitutions (over a total of 7.05 million synonymous sites) among populations and all the populations share a single mitochondrial haplotype, suggesting that all South Island *Daphnia pulex/pulicaria* are decendants of a single clone. In addition, all of the South Island *Daphnia pulex/pulicaria* are homozygous at the Ldh locus and have >97% sites homozygous for *D. pulicaria* -specific nucleotides, indicating they are non-hybrid *D. pulicaria* . On the contrary, the North Island *Daphnia pulex/pulicaria* are heterozygous at the Ldh locus and have >96.6% sites heterozygous for *D. pulicaria* - and *D. pulex* -specific nucleotides, thereby suggesting a hybrid origin of the North Island population.

Both the South and North Island invasions involved obligately asexual*Daphnia* clones. The origin of asexuality for the North Island*Daphnia pulex/pulicaria* is likely caused by hybridization between*D. pulex* and *D. pulicria* prior to the arrival of NZ (Innes and Hebert 1988), while that for the South Island *D. pulicaria* is still not clear. Obligately asexual *D. pulex* x *pulicaria*hybrids are notoriously invasive, invading many African lakes within just a few decades (Mergeay et al. 2006), and invading Japan between 680 and 3400 years ago (So et al. 2015). In Africa, the invading *D. pulex* x *pulicaria* hybrids spread and replaced the native sexual*D. pulex* (Mergeay et al. 2006). This might be due to obligate asexuals avoiding the cost of producing males, although obligately asexual *Daphnia* are often capable of producing males (Lynch et al. 2008), and there are additional ecological matters to consider. For example, the subset of obligate asexuals that are successful are often generalists capable of outcompeting sexuals when environmental dispersal is limited (Baker 1965; Parker 1977; Lynch 1984).

Although the NZ lakes in our study have historically been inhabited by native NZ species of *Daphnia* (Burns et al. 2017), we could not find any native *Daphnia* species in our samples, which is likely due to them being completely displaced by just two invading clones from North America, neither of which shows significant evidence of adaptive divergence based on genome-wide sequence analyses. The invasive asexual*Daphnia* clones could be bearers of a serious pathogen for the native *Daphnia* , because complete displacement requires a fortuitous ecological advantage over presumably well-adapted, genetically variable, long-term residents of these lakes.

Invasive history of NZ Daphnia populations

To infer the source of invasive *Daphnia pulex/pulicaria* in NZ, we checked the possible routes of *Daphnia pulex/pulicaria* invasion. It is thought that *D. thomsoni* (formerly *D. carinata*,Burns et al. 2017) and *D. tewaipounamu* are the only native*Daphnia* species in NZ, although there are several invasive*Daphnia* .

One recent invader, *D. galeata,* now common in lakes throughout the North Island of NZ, may have been introduced to the South Island during translocation of farmed Chinese grass carp (*Ctenopharyngodon idella*) for the purpose of weed control in northern South Island waterways (Duggan and Pullan 2017). The presence of *D. pulex* in some shallow constructed ponds in suburban Auckland, North Island, has also been attributed to introductions of grass carp (Branford and Duggan 2017). However, Chinese grass carp have only been used for the weed control on the North Island and the northern tip of the South Island, as water temperatures in southern NZ are generally too cold for these fish. Therefore, it is highly unlikely that grass carp were a major vector for the introduction of *D. pulicaria* to the South Island.

Duggan et al. (2012) suggested introduced salmonids (trout, salmon) and associated recreational fishing equipment as potential vectors for*D. pulicaria* on the South Island. The most common salmonid in NZ, Chinook salmon (*Oncorhynchus tshawytschca*), was initially introduced from the Sacramento River, California, USA to South Island, NZ, between 1901 and 1907. Salmon fishing in NZ has become popular since the 1970s and has attracted many international tourists. The *D. pulicaria* clone on the South Island has been known to produce diapausing ephippia, which float on the water surface for long periods, adhering to any surfaces they encounter, such as fishing equipment, skin, fur, feathers, and clothing (Burns 2013). The movement of these ephippia attached to recreational equipment, people, and wildlife may facilitate the spread of *D. pulicaria* in lakes in the South Island, NZ. In support of this, the invasion time estimated from the molecular data is roughly consistent with transport during this period. As noted above, the North Island *Daphnia* has only one nucleotide difference on the COI gene compared to the JPN 1 clone that invaded Japan (So et al. 2015), which suggests the possibility of a recent transfer from Japan.

Distinguishing North American D. pulex and D. pulicaria

North American *D. pulex* and *D. pulicaria* are thought to have recently diverged from a common ancestor and adapted to distinct environments while still experiencing introgression (Omilian and Lynch 2009; Cristescu et al. 2012). Such introgression may cause the *D. pulex /pulicaria* hybrids to have mtDNA from either *D. pulex* or *D. pulicaria* , thus biasing inferences based on trees when derived from mtDNA data (Markova et al. 2013). Although the nuclear-encoded lactate dehydrogenase locus (Ldh) has been used to discriminate *D. pulex* and *D. pulicaria* , and was useful in determining the species of NZ *Daphnia* samples, inferring species solely based on the Ldh locus can still be misinformative due to hybridization and backcrossing (Xu et al. 2013). For example, an Ldh SS*Daphnia* that derived from crossing between SF *D. pulex/pulicaria* and SS *D. pulex* may be erroneously inferred as a non-hybrid clone. To eliminate the limitations of a single nuclear marker to derive inferences, we generated a set of 24,185 *D. pulicaria* -specific nuclear markers that are monomorphic in every non-hybrid *D. pulicaria,* and unique with respect to North American *D. pulex* . These markers will be useful for diagnostic purposes in other future studies involving these two species, including the identification of hybrids. They may also serve as a resource for studying genes involved in potential adaptive divergence between*D. pulex* and *D. pulicaria.*

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Author Contributions

Z.Y., C.W.B., and M.L. designed research; Z.Y., E.W., C.W.B., and M.L. performed research; Z.Y., C.Z., and M.L. analyzed data; and Z.Y., C.W.B., and M.L. wrote the paper.

Disclosure Declaration

The authors confirm that there are no known conflicts of interest associated with this publication.

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Data accession

NZ population data for this study were deposited to NCBI under BioProject ID PRJNA573527.

D. pulex/D. pulicaria genomic data can be accessed at BioProject ID PRJNA573529 and accession numbers SAMN03964756-SAMN03964769 (Xu et al. 2015), and the *D. obtusa* genomic sequences used in this study can be accessed at NCBI under accession number SAMN12816670. The*D. pulex* genome assembly PA42 v4.1 is available at GenBank under accession GCA_900092285.2 (Ye et al. unpublished) and the corresponding annotation file is provided in File S1.

Table 1 . Summary of sample information. Coverage denotes the mean depth of genomic coverage and chosen coverage range (in square brackets) of sites analyzed in each population. Mapped sites: sites covered by at least one read. Deviating sites are bi-allelic sites (minor allele frequency >0.05) with read numbers for the two parental alleles deviating significantly from 1:1 after Benjamini-Hochberg procedure (FDR=0.05). Sites with more than two alleles are not used in our analysis.

Samples	Mapped sites	Mapped sites	Coverage (X)	Coverage (X)	Coverage (X)	Bi-allelic sites	Deviating
Alexandrina	168,224,903	168,224,903	98 [40-200]	98 [40-200]	98 [40-200]	1,540,667	288
Coleridge	166,329,006	166,329,006	84 [20-150]	84 [20-150]	84 [20-150]	1,491,775	252
Hawea	165,334,107	165,334,107	51 [20-120]	51 [20-120]	51 [20-120]	1,416,048	35
Hayes	168,826,070	168,826,070	139 [50-250]	139 [50-250]	139 [50-250]	1,538,123	667
Johnson	168,204,869	168,204,869	125 [50-250]	125 [50-250]	125 [50-250]	1,523,441	572
Ohau	168,392,915	168,392,915	102 [50-200]	102 [50-200]	102 [50-200]	1,539,681	340
Pukaki	168,109,981	168,109,981	94 [50-200]	94 [50-200]	94 [50-200]	1,536,485	238
Sullivan's Dam	167,880,110	167,880,110	88 [50-200]	88 [50-200]	88 [50-200]	1,525,683	224
Moke	164,531,082	164,531,082	43 [20-100]	43 [20-100]	43 [20-100]	1,509,765	41
Tekapo	166,918,220	166,918,220	78 [30-150]	78 [30-150]	78 [30-150]	1,494,053	175
Von	166,794,759	166,794,759	82 [30-150]	82 [30-150]	82 [30-150]	1,488,926	196
Wakatipu	167,805,336	167,805,336	107 [50-200]	107 [50-200]	107 [50-200]	1,513,257	411
Wanaka	164,530,961	164,530,961	42 [20-100]	42 [20-100]	42 [20-100]	1,370,378	14
Kapoai	175,278,062	175,278,062	48[20-100]	48[20-100]	48[20-100]	2,059,905	147

Figure 1. Location of lakes on the South Island, New Zealand, from which the *Daphnia* in this study were collected (South Island, New Zealand. *Google Maps* , 16 October 2019). Lake names are highlighted in blue. Lake Kapoai, North Island, not shown.

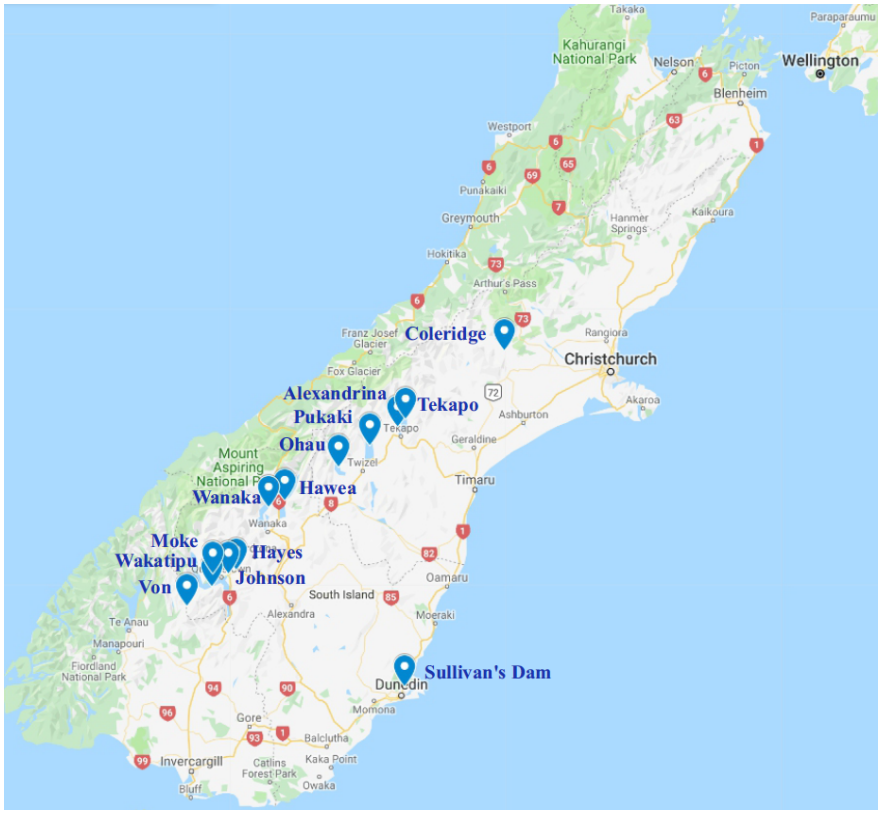

Figure 2. Maximum likelihood tree using nuclear genome sequences. South Island, NZ *Daphnia* samples (red circles); North Island, NZ *Daphnia* sample (red ring); *D. pulex* samples (black circles); *D. pulicaria* samples are marked with blue circles. *Inset* on the right shows the phylogeny of the South Island and North American *D. pulicaria* in more detail. Bootstrap >75 are shown. T_1 is the divergent time between the North American and the South Island *D. pulicaria*.T_2 is the expansion time of South Island *D. pulicaria* .

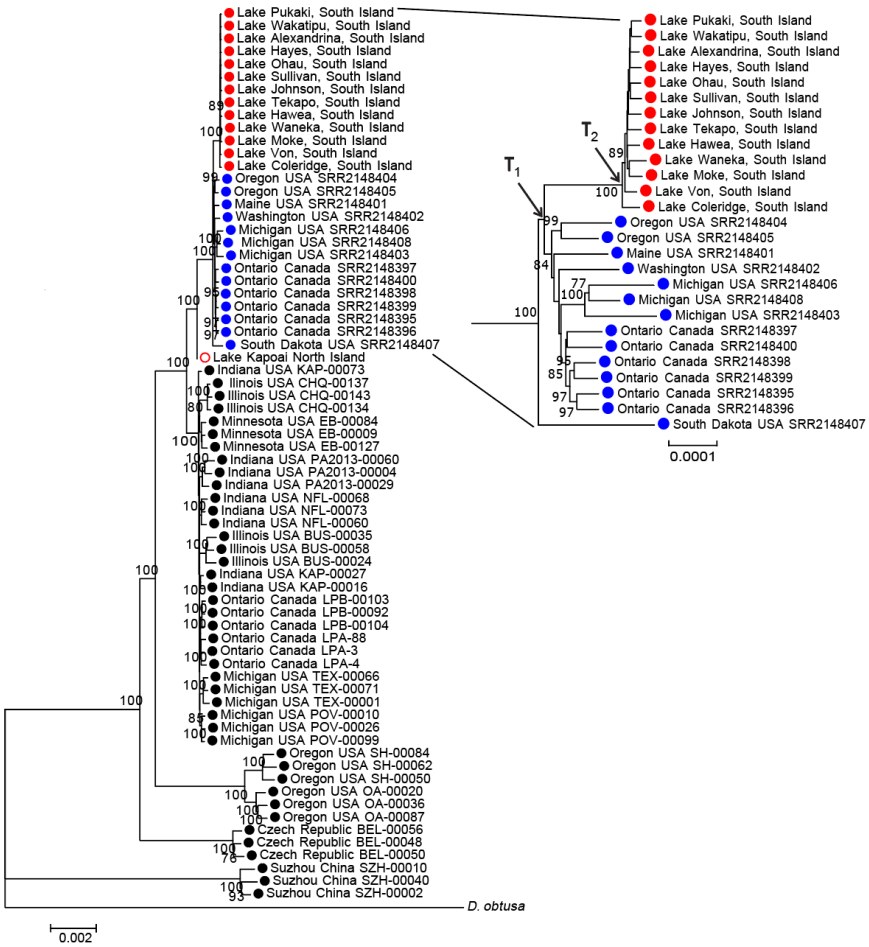

Figure 3. Maximum-likelihood tree using partial nucleotide sequences from the COI gene. Clones from different continents are color coded: South Island, New Zealand (red), Asia (dark green), South America (blue), North America (black), Europe (pink), and polar region (light green).