

The rapid, mass invasion of New Zealand by North American *Daphnia* “*pulex*”

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Abstract

Nonindigenous members of the *Daphnia pulex* complex 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 precise phylogenetic origin of invasive *Daphnia*, whether each lake is invaded by a single clone or multiple clones, the lineage of the invasive clones, and whether they are obligately asexual clones. Furthermore, the source and time of arrival of the invasive genotype(s) are unclear. We address these questions by genomic sequencing of *Daphnia* populations from 13 lakes in the South Island and 1 lake in the North Island, NZ. All biallelic sites in these NZ populations have similar numbers of reads for the two parental alleles, suggesting each NZ population originates from a single asexual clone. Based on 25,643 monomorphic lineage-specific markers, the invasive *Daphnia* in the South Island were found to be *Daphnia pulicaria* Forbes, while those in the North Island are hybrids of *D. pulicaria* Forbes and *D. cf. pulex* sensu Hebert. Both the South and North Island *Daphnia* are phylogenetically clustered with North American *Daphnia*, thereby suggesting their North American origins. We found also that all South Island clones 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 clones of ~ 60 years ago, with a likely invasion route associated with the introduction of salmonids from North America.

Daphnia are small crustaceans commonly found in small lakes and ponds (Benzie 2005). Molecular data revealed that *Daphnia pulex* is a polyphyletic species complex containing at least eight genetically distinct lineages, which can be assigned to three major clades: the European *D. pulex* clade (*Daphnia pulex* Leydig, 1860), the *Daphnia tenebrosa* Sars, 1898 and European *Daphnia pulicaria* clade (*D. cf. pulicaria* sensu Alonso, 1996), and the panarctic *D. pulex* (*D. cf. pulex* sensu Hebert, 1995), *Daphnia melanica* Hebert, 1995, *Daphnia middendorffiana* Fisher, 1851, and North American *D. pulicaria* clade (*D. pulicaria* Forbes, 1893) (Colbourne et al. 1998). Two of the lineages, *D. cf. pulex* sensu Hebert and *D. pulicaria* Forbes, can still hybridize and form viable hybrids (Hebert et al. 1989; Crease et al. 2011), and their hybrids have been found to be invasive. For example, a *D. cf. pulex* sensu Hebert × *D. pulicaria* Forbes hybrid clone invaded Africa after the 1920s and has spread throughout the range of native sexual

D. “pulex” (clones that have been morphologically or genetically determined to be members of the *D. pulex* species complex but with unclear lineage affiliations), displacing the sexual population (Mergeay et al. 2006). In addition, four hybrid *Daphnia* clones invaded Japan between 680 and 3400 years ago (So et al. 2015). Recently, nonindigenous *Daphnia* species morphologically determined to be from the *D. pulex* complex have been found in several lakes in New Zealand (NZ) (Duggan et al. 2012). Despite the extreme success of *Daphnia* introductions into several continents, studies on the invasive *D. “pulex”* are scarce and somewhat ambiguous due to small sample size in certain localities, incorrect lineage identification caused by hybridization, and the lack of testing for obligate asexuality (Duggan et al. 2012; So et al. 2015).

First, most of the previous studies on invasive *D. “pulex”* were based on just a few clones from each lake or pond (Mergeay et al. 2005; Duggan et al. 2012; So et al. 2015), and when clones within a lake or pond shared a single haplotype, it was concluded that the population consisted of a single *Daphnia* clone. Because sexual and obligate asexual, indigenous, and nonindigenous *D. “pulex”* clones can coexist in the same ponds (Mergeay et al. 2006; Lehto and Haag, 2010), and the coexisting clones are often morphologically indistinguishable, by sequencing just a few clones, one or more coexisting

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genotypes could easily be missed, thus leading to incorrect conclusions.

Second, lineage identification was often based on a single marker gene in previous studies, which may be confounded by hybrids. *D. cf. pulex* sensu Hebert, *D. pulicaria* Forbes, and their hybrids are not easily distinguished morphologically, and typically are identified by allozyme electrophoresis of the lactate dehydrogenase (*Ldh*) locus, with homozygous *D. pulicaria* Forbes having two “fast” alleles, and homozygous *D. cf. pulex* sensu Hebert having two “slow” alleles (Hebert et al. 1989; Crease et al. 2011). Hybrids are generally found in disturbed ponds or deforested areas and have a heterozygous slow/fast genotype (Hebert et al. 1989). However, due to the frequent gene flow between *D. cf. pulex* sensu Hebert and *D. pulicaria* Forbes (Hebert et al. 1989; Cristescu et al. 2012), some hybrids are also homozygous for the *Ldh* locus, presumably resulting from backcrossing with either *D. cf. pulex* sensu Hebert or *D. pulicaria* Forbes (Xu et al. 2013). Therefore, homozygosity of “fast” or “slow” alleles does not guarantee a specific *D. cf. pulex* sensu Hebert or *D. pulicaria* Forbes origin of the invasive *Daphnia*. In addition to the *Ldh* locus, the mitochondrial cytochrome c oxidase subunit 1 (*CO1*) gene has also been used to distinguish invasive *D. cf. pulex* sensu Hebert and *D. pulicaria* Forbes (Mergeay et al. 2005; Duggan et al. 2012). Because mitochondrial DNA (mtDNA) is maternally inherited and hybrids could have mtDNA from either *D. cf. pulex* sensu Hebert or *D. pulicaria* Forbes (Cristescu et al. 2012; Marková et al. 2013), these hybrids may be erroneously identified as *D. cf. pulex* sensu Hebert or *D. pulicaria* Forbes, thus biasing lineage identification.

Third, a number of previous studies did not thoroughly test whether the invasive *D. “pulex”* clones are obligate parthenogens (OP). Both *D. cf. pulex* sensu Hebert and *D. pulicaria* Forbes 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 lineage have lost the ability to engage in meiosis and have become OP (Hebert and Crease 1980; Hebert et al. 1993; Paland et al. 2005). Although OP clones are thought to have much reduced or eliminated male production compared to CP clones, some OP clones can still produce functional males bearing haploid sperm (Innes et al. 2000; Tessier and Cáceres 2004). Such males from OP *D. cf. pulex* sensu Hebert can mate and form viable hybrids with females from CP *D. cf. pulex* sensu Hebert, ~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. cf. pulex* sensu Hebert share common haplotypes on chromosomes VIII and IX that arose by introgression from *D. pulicaria* Forbes (Lynch et al. 2008; Xu et al. 2015) and are transmitted through OP males without recombination (Tucker et al. 2013). Because CP and OP *D. cf. pulex* sensu Hebert clones

have different traits and evolutionary histories, false designation of the two may lead to incorrect estimation of times and routes of invasion.

To solve the above issues and understand the origin of the invasive *D. “pulex”* in NZ, we performed whole-genomic sequencing of *D. “pulex”* populations from 13 lakes across the South Island and one lake in the North Island, NZ. To determine if each lake contains more than one *D. “pulex”* genotype, we pooled 50–200 individuals from each lake for whole-genomic sequencing and then performed binomial tests on all biallelic sites to check if the numbers of reads for the two parental alleles deviated from the 1 : 1 ratio expected for a fixed heterozygote. The lineage identifications and likely sources of origin of the invasive *D. “pulex”* were determined via a survey of *D. pulicaria* Forbes-specific markers and a whole-genomic phylogenetic tree that includes *D. “pulex”* clones collected across North America, Europe, and China. A combination of experiments and genetic markers implies invasion by OP clones. With a clear lineage identification and whole-genomic molecular data, we were able to estimate the colonization time and predict the likely route of the *D. “pulex”* invasion in NZ.

Materials and methods

Nomenclatural remarks

There are extensive nomenclature issues within the *D. pulex* species complex. *D. cf. pulex* sensu Hebert, 1995 is a lineage in the complex commonly found in Panarctic regions, which is morphologically indistinguishable but genetically different from its European counterpart *D. pulex* Leydig, 1860. However, both of these two lineages continue to be referred as *D. pulex* (Hebert et al. 1989; Crease et al. 2011). Likewise, although North American *D. pulicaria* Forbes, 1893 and the European *D. cf. pulicaria* sensu Alonso, 1996 have been assigned to different clades in the species complex (Colbourne et al. 1998), they are still both referred as *D. pulicaria* (Dufresne et al. 2011). In addition, *Daphnia* clones that have been morphologically or genetically determined to be members of the *D. pulex* species complex but with unclear lineage affiliations have been referred as *D. pulex* (So et al. 2015; Geng et al. 2016). To avoid confusion in this study, we use full taxonomic description names (i.e., *D. cf. pulex* sensu Hebert, 1995) for *Daphnia* clones from known lineages. For *Daphnia* clones with undetermined or mixed lineages, we use a geographical prefix and quotation markers to describe them (i.e., South American *D. “pulex”*).

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; Supporting information Table S1). In each location, *Daphnia* were sampled by tows of a conical plankton net and preserved in DNA/RNA Shield. DNA from

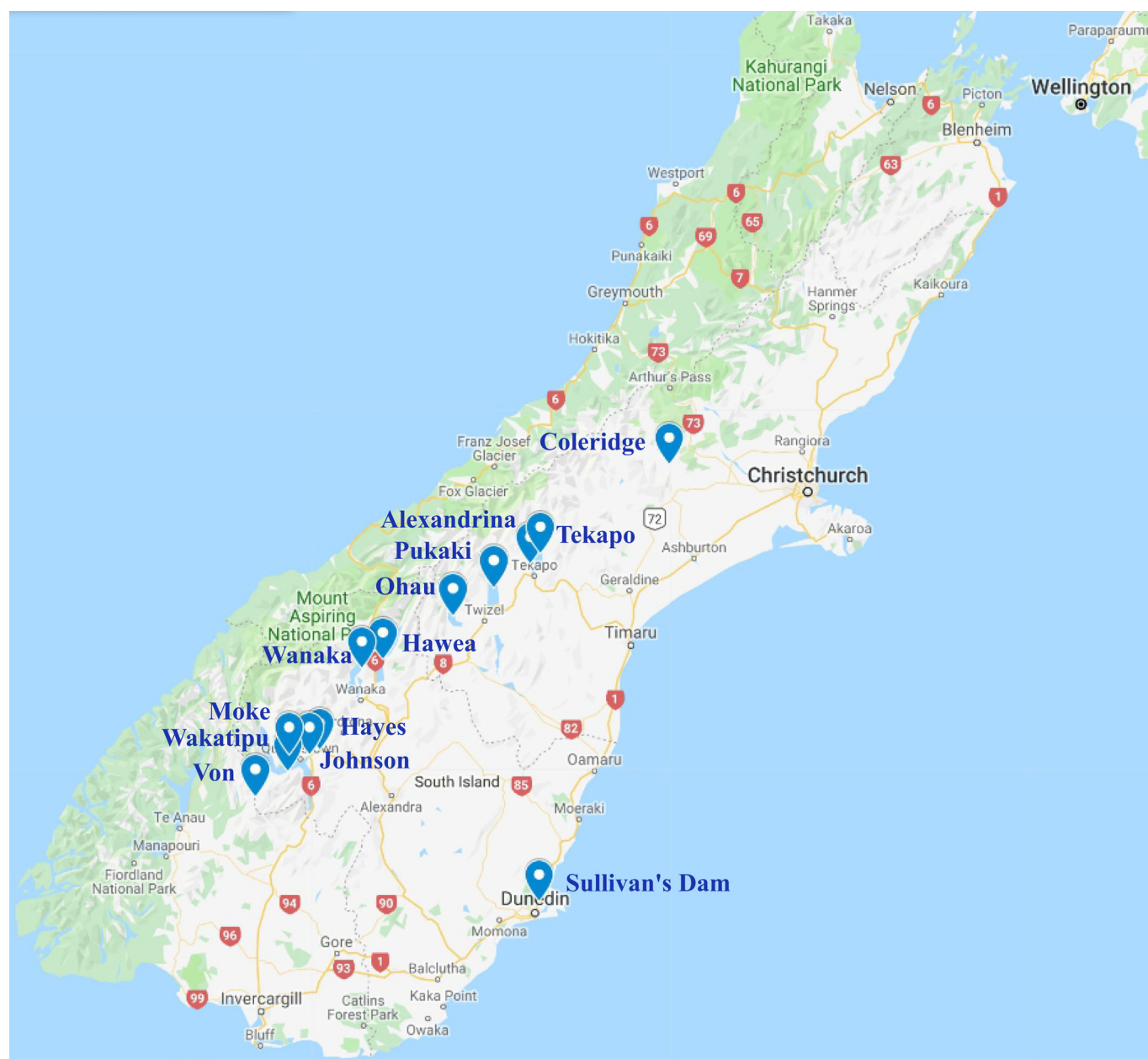


Fig 1. Location of lakes in the South Island, New Zealand, from which the *Daphnia* in this study were collected (South Island, New Zealand, Google Maps, 16 October 2019). Lake names are highlighted in blue. Lake Kapoai, North Island, not shown.

each pooled population sample (50–200 individuals) was extracted with Zymo DNA Clean and Concentrator-25 Kit and sequencing libraries were prepared using NEXTFLEX® Rapid DNA-Seq Kit 1.0. The 150-bp paired-end reads were sequenced using Highseq 2500 platform at Center for Genomics and Bioinformatics, Indiana University.

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. cf. pulex* sensu Hebert reference genome (PA42 version 4.1) using Burrows-Wheeler Alignment tool (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). Single nucleotide polymorphisms (SNPs)

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

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 biallelic 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 > three SNPs within 100 bp because additional differentiating sites will interfere with read alignment (Stevenson et al. 2013); and (3) to reduce the mapping bias toward the reference alleles (Degner et al. 2009), we generated an assembly by masking all of the biallelic sites with “N” in the PA42 4.1 reference. Only biallelic 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* Forbes-specific markers

To identify *D. pulicaria* Forbes-specific markers, we first searched for homozygous SNPs shared by all 14 *D. pulicaria* Forbes clones (Supporting Information Table S2). Then, we checked these SNPs in 30 *D. cf. pulex* sensu Hebert clones collected across North American and eliminated those that appeared in any of the *D. cf. pulex* sensu Hebert clones. For the remaining SNPs, we checked the corresponding loci in the genome in each of the *D. cf. pulex* sensu Hebert clones and only kept those that are homozygous for the same two bases across all *D. cf. pulex* sensu Hebert clones.

Origin and colonization time of the NZ populations

To estimate the origin of the South Island *D. pulicaria* Forbes clones, mitochondrial *CO1* gene sequences for *D. “pulicaria”* clones sampled across North America, South America, Europe, Asia, NZ, and the north polar region are downloaded from NCBI or generated from raw reads using Samtools (Li et al. 2009) with the command: samtools mpileup -uf ref.fasta aln.bam | bcftools call -c | vcfutils.pl vcf2fq > cns.fq. *CO1* sequences were aligned with ClustalW program from MEGA 7 and the maximum-likelihood tree based on *CO1* gene sequences was constructed using MEGA 7 with 100 Bootstrap (Kumar et al. 2016).

To estimate when the South Island genotype diverged from North American *D. pulicaria* Forbes, we extracted coding sequence from the reference genome (PA42 4.1). After mapping the reads from each clone to the coding sequences, we generated consensus sequences for each clone using Samtools (Li et al. 2009). Custom scripts were then used to count the number of synonymous/nonsynonymous changes and sites in the coding sequences.

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. 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 *Daphnia* population, *D. pulicaria* Forbes, and *D. cf. pulex* sensu Hebert 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 were used to infer a male-requiring CP. We decapsulated a total of 30 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 MF 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

Testing if each lake consists of a single clone

To understand the *Daphnia* invasion in NZ, we sampled *Daphnia* populations from 13 lakes in the South Island and one in the North Island, NZ (Fig. 1; Supporting Information

Table S1). *Daphnia* populations from each lake were determined to be *D. pulex* sensu lato based on morphological features (Benzie 2005; Ebert 2005). Each population was sequenced to an average depth of sequencing coverage of $\times 86$ (Table 1), involving more than 100 million 150-bp reads per population. On average, 1.5 million biallelic sites were identified for each population (Table 1).

If each NZ *Daphnia* population represents a single clone, assuming no mutation or gene conversion, all of its biallelic 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 biallelic sites to check for deviations from a 1 : 1 ratio. Over all 14 samples, with an average of 1.5 million significant biallelic sites per sample, only 14–667 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. cf. pulex* sensu Hebert 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 toward low-frequency alleles (Lynch et al. 2008). Therefore, our results suggest that each NZ population is a single clone. 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.

Lineage identification of *Daphnia* from NZ lakes

Because traditional lineage identifications using the mitochondrial genome and/or the nuclear-encoded *Ldh* locus may be biased due to introgression between *D. cf. pulex* sensu Hebert and *D. pulicaria* Forbes (Omilian and Lynch 2009; Cristescu et al. 2012; Marková et al. 2013), we relied on whole-genomic information to distinguish *D. cf. pulex* sensu Hebert, *D. pulicaria* Forbes, and their hybrids. To obtain whole-genomic information, we collected 30 sexual *D. cf. pulex* sensu Hebert clones from temporary ponds across North America, 6 *D. “pulex”* from Oregon, USA, 3 *D. pulex* Leydig from Europe, and 3 *D. “pulex”* from China (Supporting Information Table S2). Each clone was sequenced to an average depth of sequencing coverage of $\times 10$. By comparing whole-genomic information between these 30 *D. cf. pulex* sensu Hebert clones and 14 *D. pulicaria* Forbes (Xu et al. 2015), we identified 25,643 informative sites, in which all *D. pulicaria* Forbes are monomorphic for one nucleotide and all *D. cf. pulex* sensu Hebert are monomorphic for another nucleotide (Supporting Information File S2). These 25,643 informative sites span 11 of the 12 chromosomes in *D. cf. pulex* sensu Hebert (Supporting Information Fig. S1). We found that all South Island *Daphnia* have > 23,867 (93%) sites

homozygous for *D. pulicaria* Forbes-specific nucleotides, while only 807 (3.3%) sites are homozygous in the North Island clone. This suggests a *D. pulicaria* Forbes origin of the South Island *Daphnia* clones, and a hybrid origin of the North Island clone.

To further confirm the lineages of *Daphnia* clones in NZ, we constructed a consensus maximum-likelihood (ML) tree using whole-genomic coding sequences from 14 NZ clones, 14 *D. pulicaria* Forbes, 30 *D. cf. pulex* sensu Hebert clones, 3 *D. pulex* Leydig from Europe, and 3 *D. “pulex”* from China. Heterozygous sites in each sequence were represented using IUPAC ambiguity code. Within this tree, the South Island clones formed a monophyletic clade with all North American *D. pulicaria* Forbes clones, further supporting the view that the South Island clones are *D. pulicaria* Forbes (Fig. 2). The North Island clone is in a clade with *D. pulicaria* Forbes and *D. cf. pulex* sensu Hebert, but clusters with neither *D. pulicaria* Forbes nor *D. cf. pulex* sensu Hebert (Fig. 2), confirming its hybrid origin. Unlike the situation for nuclear data, for the ML tree based on full-length mitochondrial genomes, *D. cf. pulex* sensu Hebert and *D. pulicaria* Forbes clones interlaced outside of the NZ clones (Supporting Information Fig. S2). This discordant phylogeny between the mitochondrial and nuclear genomes may represent historical hybridization between *D. cf. pulex* sensu Hebert and *D. pulicaria* Forbes (Marková et al. 2013).

Testing a single-clone origin hypothesis for all South Island *Daphnia* clones

Because all South Island *Daphnia* clones are *D. pulicaria* Forbes, we wanted to test if they all derive from a single *D. pulicaria* Forbes clone. We compared coding sequences of the 13 South Island *Daphnia* clones and found that the average number of synonymous substitutions among clones is < 24 (over a total of 7.05 million synonymous sites), implying that all South Island *Daphnia* originated from single founder clone. In further support of the single-clone origin hypothesis, we found that all 13 South Island *Daphnia* populations have identical mitochondrial genomes. Moreover, six of the seven clones from four different South Island lakes collected > 7 years ago (Duggan et al. 2012) have 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* clones 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 clones are OP, we experimentally examined their ability to produce diapausing embryos without fertilization. CP *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. These results suggest that the *Daphnia* invasion of the South Island involved a single obligately asexual clone capable of (but not requiring) male production.

Due to the lack of available live animals in the laboratory, we were unable 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 *D. cf. pulex* sensu Hebert contain > 30,000 markers that are informative with respect to the origin of asexuality (Tucker et al. 2013; Xu et al. 2015). All of these markers are heterozygous in obligate asexual hybrids but are homozygous in CP *D. cf. pulex* sensu Hebert or *D. pulicaria* Forbes. 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 OP clone, capable of producing males.

Origin and colonization time of the NZ *Daphnia* clones

To infer the geographic origin of the *D. pulicaria* Forbes 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 NZ, 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, NZ, 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* ultimately came from North America.

To estimate when the South Island genotype diverged from North American *D. pulicaria* Forbes, 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* Forbes (T_1 , Fig. 2) can be estimated by the average pairwise distance of synonymous sites (d_s) between North American *D. pulicaria* Forbes 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

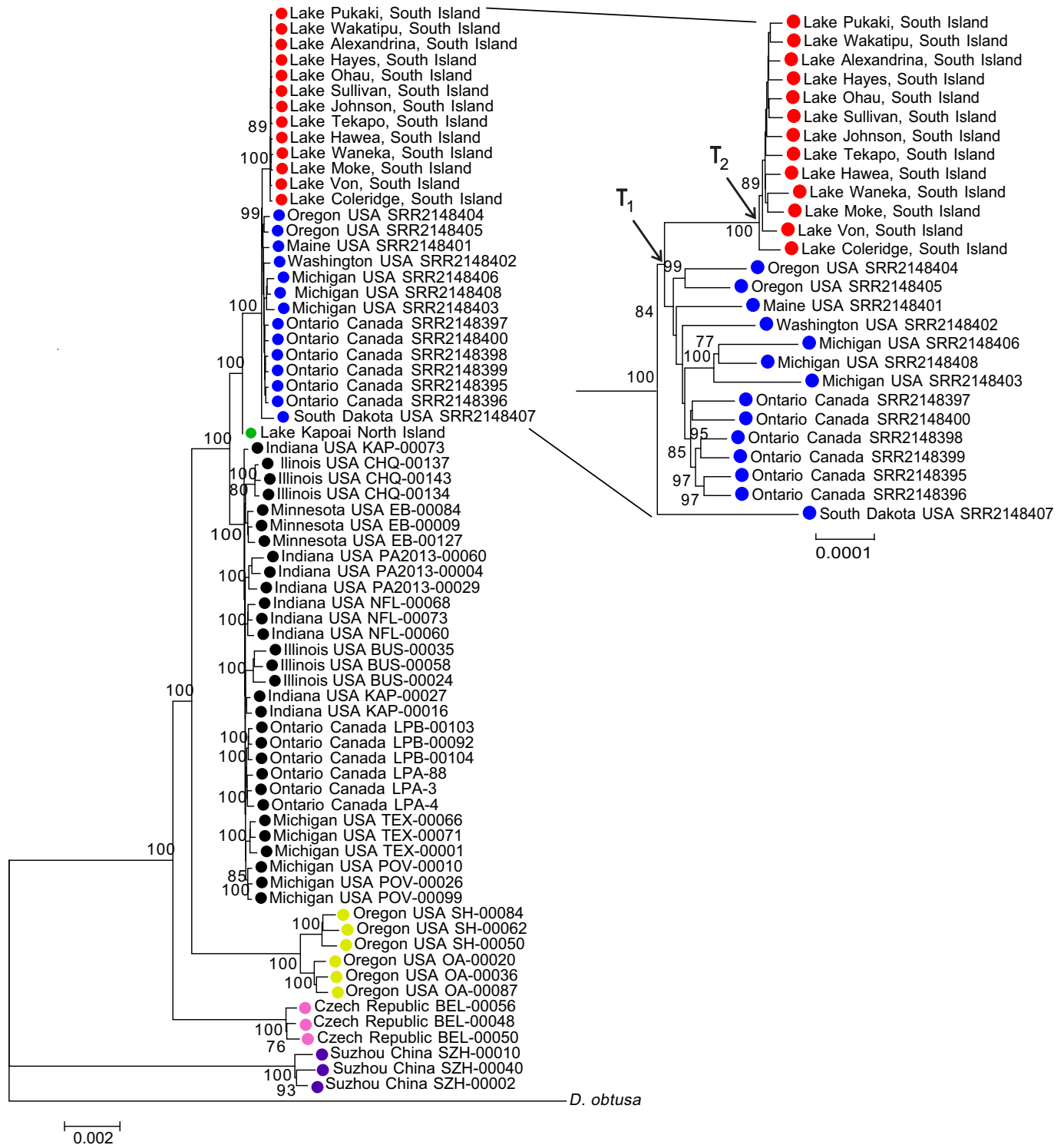


Fig 2. Maximum likelihood tree using nuclear genome sequences. South Island, New Zealand (NZ) *Daphnia* samples (red circles); North Island, NZ *Daphnia* sample (green circle); *Daphnia* cf. *pulex* sensu Hebert (black circles); Oregon *D. "pulex"* (yellow circles); *D. pulex* Leydig (pink circles), and Chinese *D. "pulex"* samples (purple circles); *Daphnia pulicaria* Forbes samples are marked with blue circles. Inset on the right shows the phylogeny of the South Island and North American *D. pulicaria* Forbes in more detail. Bootstrap values > 75/100 are shown. T_1 is the divergence time between the north American and the south Island *D. pulicaria* Forbes. T_2 is the expansion time of South Island *D. pulicaria* Forbes.

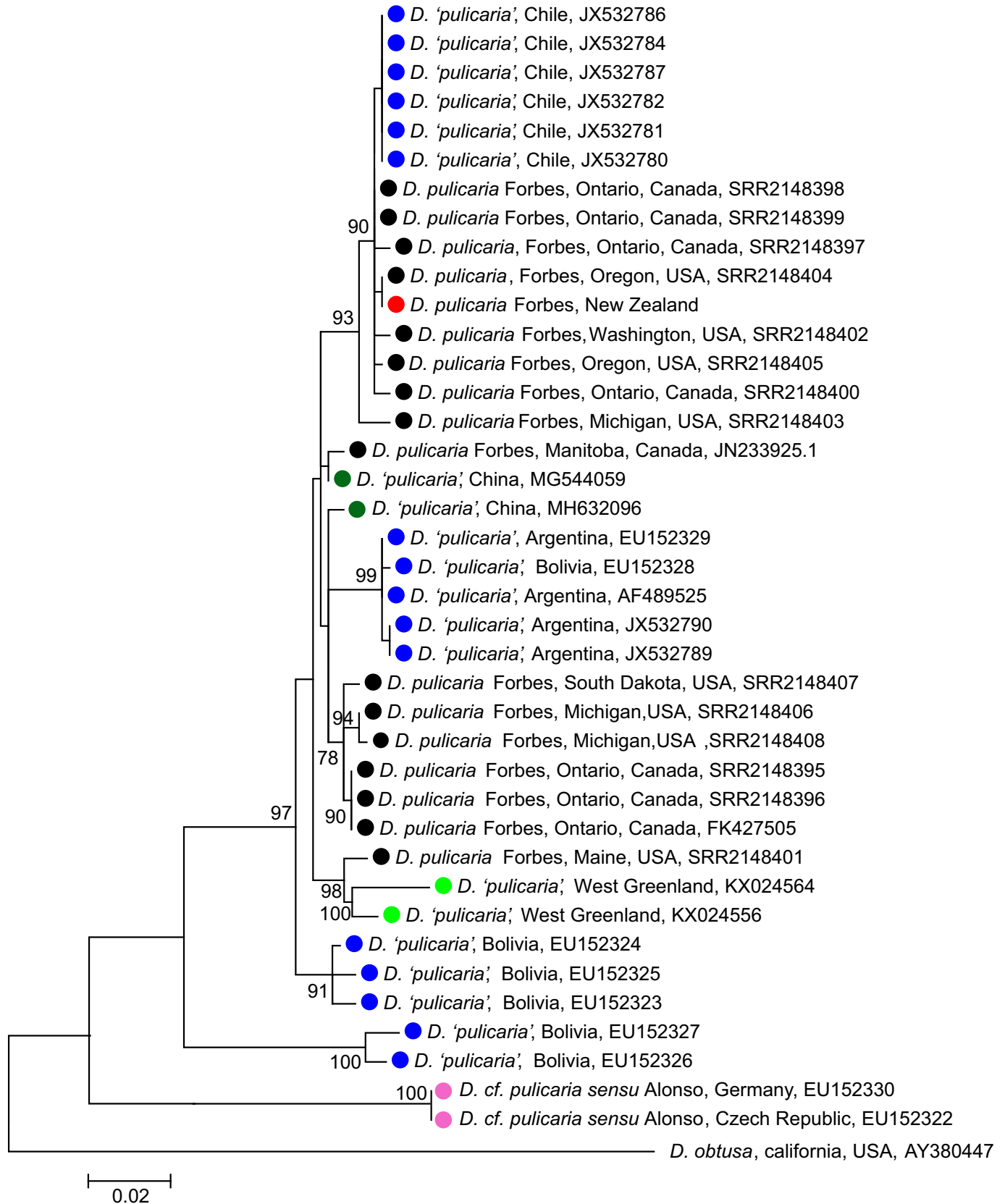


Fig 3. Maximum-likelihood tree using partial nucleotide sequences from the COI gene. Clones from different continents are color coded: South Island, New Zealand (red), Asia (dark green), South America (blue), North America (black), Europe (pink), and Greenland (light green). The codes following each sample are accession numbers from GenBank or Sequence Read Archive. We used geographical location and quotation markers to describe clones without clear lineage identification (i.e., *D. "pulicaria,"* China).

per year, we estimate that the South Island genotype diverged from other North American *D. pulicaria* Forbes ~ 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 cannot be estimated by this means.

We also estimated the expansion time of the South Island clones (T_2 , Fig. 2). Assuming these clones 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 an expansion time of $T_2 = d_s/(2\mu) = 3.4 \times 10^{-6}/(2 \times 5.85 \times 10^{-9}/\text{site/generation} \times 5 \text{ generations/year}) = \sim 58 \text{ years}$. This calculation is supported by the presence of characteristic ephippia of *D. “pulex”* 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.).

Discussion

Determining if each lake consists of a single *Daphnia* clone using pooled sequencing

Most of the previous studies on *Daphnia* invasions only sequenced a few genes from a few clones from each lake or pond (Mergeay et al. 2005; Duggan et al. 2012; So et al. 2015), possibly leading to undersampling of coexisting genotypes. In Africa, the invasive *D. cf. pullex* sensu Hebert and native *D. “pulex”* are thought to have coexisted for ~ 60 years before the native *D. “pulex”* was completely displaced (Mergeay et al. 2005). Because invasive *D. cf. pullex* sensu Hebert and native *D. “pulex”* in Africa are not easily distinguished morphologically and could only be identified by sequencing dozens of clones, which may require extensive lab work to check the genotype of each clone. Our study provided a relatively easy solution for checking for potential coexistence by pooling 50–200 individuals for whole-genomic sequencing. If all individuals in a single sample derived from a single clone, all polymorphic sites should have fixed heterozygosity and similar numbers of reads mapped to the two parental alleles.

Although the NZ lakes in our study have historically been inhabited by native NZ species of *Daphnia* (Burns et al. 2017), we found each lake sample to consist of just a single non-native *Daphnia* clone, owing to complete displacement by just two invading clones from North America (one on each island). Genome-wide sequence analyses did not reveal any evidence of adaptive divergence within the invasive

clones, but this does not rule out fixation of substantial numbers of mutations in the founder population before it spread across the landscape.

Distinguishing *D. cf. pullex* sensu Hebert and *D. pulicaria* Forbes

North American *D. cf. pullex* sensu Hebert and *D. pulicaria* Forbes 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 their hybrids to have mtDNA from either *D. cf. pullex* sensu Hebert or *D. pulicaria* Forbes, thus biasing inferences based on trees derived solely from mtDNA data (Marková et al. 2013). Although the nuclear-encoded *Ldh* locus has been used to discriminate *D. cf. pullex* sensu Hebert and *D. pulicaria* Forbes, inferring lineages solely based on the *Ldh* locus can still be misinformative due to hybridization and backcrossing (Xu et al. 2013). For example, an *Ldh* Slow-Slow *Daphnia* genotype derived from crossing between Slow-Fast hybrids and Slow-Slow *D. cf. pullex* sensu Hebert 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 25,643 *D. pulicaria* Forbes-specific nuclear markers that are monomorphic in every nonhybrid *D. pulicaria* Forbes, and unique with respect to *D. cf. pullex* sensu Hebert. These markers will be useful for diagnostic purposes in other future studies involving these two lineages, including the identification of hybrids. They may also serve as a resource for studying genes involved in potential adaptive divergence between *D. cf. pullex* sensu Hebert and *D. pulicaria* Forbes.

Invasive *D. “pulex”* in NZ

In our study, we identified independent invasions of *D. “pulex”* on the South and North Islands of NZ. Phylogenetic data from mitochondrial genomes revealed that both invasive *D. “pulex”* originated from North America. Intriguingly, all 13 lakes in the South Island were invaded by a single *D. pulicaria* Forbes clone, suggesting higher fitness of the invasive *Daphnia* clones compared to the NZ-native *Daphnia* clones. Consistent with this hypothesis, Burns (2013) found that the invasive *Daphnia* could displace the native *Daphnia* when water temperature and nutrition level are relatively high.

The invasive *Daphnia* on both islands are obligate asexuals. The origin of asexuality for the North Island *Daphnia* is likely caused by hybridization between *D. cf. pullex* sensu Hebert and *D. pulicaria* Forbes prior to the arrival in NZ (Innes and Hebert 1988), while that for the South Island *D. pulicaria* Forbes is still not clear. Obligately asexual *D. cf. pullex* sensu Hebert $\times$ *D. pulicaria* Forbes hybrids are notoriously invasive, colonizing, and spreading throughout 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). The

Daphnia invasion time in Japan overlapped with the estimated invasion time of South Island *Daphnia* clone in NZ, suggesting a potential clone transfer from Japan or origination from the same event.

Source of invasive *Daphnia* in NZ

To infer the source of *D. “pulex”* in NZ, we checked the possible routes of the *D. “pulex”* invasions. It is thought that *Daphnia thomsoni* Sars, 1894 (formerly *Daphnia carinata*, Burns et al. 2017) and *Daphnia tewaipounamu* Burns et al., 2017 are the only native *Daphnia* species in NZ, although there are several non-indigenous invasive *Daphnia*. One recent invader, *Daphnia 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 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* Forbes to the South Island.

Duggan et al. (2012) suggested introduced salmonids (trout, salmon) and associated recreational fishing equipment as potential vectors for *D. pulicaria* Forbes in the South Island. The most common salmonid in NZ, Chinook salmon (*Oncorhynchus tshawytschka*), was initially introduced from the Sacramento River, California, USA to South Island, NZ, between 1901 and 1907. Thus, the founder *D. pulicaria* clone in the South Island might be associated with one of the Chinook salmon introductions. Salmon fishing in NZ has become popular since the 1970s, attracting many international tourists. The *D. pulicaria* Forbes clone in 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 ephippia adhering to recreational equipment, people, and wildlife likely facilitated the spread of *D. pulicaria* Forbes throughout the South Island. In support of this, the invasion time estimated from the molecular data (58 years) is roughly consistent with transport during this period.

Methods and resources generated in this study

In this study, we provide methods and resources that could be of benefit in future studies of invasive *Daphnia* species: (1) we provided an easy solution to check for potential coexistence of *Daphnia* clones that are hard to distinguish morphologically; (2) we identified 25,643 *D. pulicaria* Forbes-specific nuclear markers that can be used to easily

distinguish *D. cf. pullex* sensu Hebert and *D. pulicaria* Forbes; (3) we showed that the origin and expansion time of the invasive *Daphnia* clones could be accurately estimated using whole genomic data.

Data Availability Statement

NZ population data for this study were deposited to NCBI under BioProject ID PRJNA573527. *D. cf. pullex* sensu Hebert and *D. pulicaria* Forbes 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. cf. pullex* sensu Hebert genome assembly PA42 v4.1 is available at GenBank under accession GCA_900092285.2 (Ye et al. unpubl.) and the corresponding annotation file is provided in Supporting Information File S1.

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Conflict of Interest

None declared.

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