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Temporal Genetic Variance and Propagule-Driven Genetic Structure Characterize Naturalized Rainbow Trout (*Oncorhynchus mykiss*) from a Patagonian Lake Impacted by Trout Farming

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Abstract  
Knowledge about the genetic underpinnings of invasions—a theme addressed by invasion genetics as a discipline—is still scarce amid well documented ecological impacts of non-native species on ecosystems of Patagonia in South America. One of the most invasive species in Patagonia’s freshwater systems and elsewhere is rainbow trout (*Oncorhynchus mykiss*). This species was introduced to Chile during the early twentieth century for stocking and promoting recreational fishing; during the late twentieth century was reintroduced for farming purposes and is now naturalized. We used population- and individual-based inference from single nucleotide polymorphisms (SNPs) to illuminate three objectives related to the establishment and naturalization of Rainbow Trout in Lake Llanquihue. This lake has been intensively used for trout farming during the last three decades. Our results emanate from samples collected from five inlet streams over two seasons, winter and spring. First, we found that significant intra-population (temporal) genetic variance was greater than inter-population (spatial) genetic variance, downplaying the importance of spatial divergence during the process of naturalization. Allele frequency differences between cohorts, consistent with variation in fish length between spring and winter collections, might explain temporal genetic differences. Second, individual-based Bayesian clustering suggested that genetic structure within Lake Llanquihue was largely driven by putative farm propagules found at one single stream during spring, but not in winter. This suggests that farm broodstock might migrate upstream to breed during spring at that particular stream. It is unclear...
whether interbreeding has occurred between “pure” naturalized and farm trout in this and other streams. Third, estimates of the annual number of breeders ($N_b$) were below 73 in half of the collections, suggestive of genetically small and recently founded populations that might experience substantial genetic drift. Our results reinforce the notion that naturalized trout originated recently from a small yet genetically diverse source and that farm propagules might have played a significant role in the invasion of Rainbow Trout within a single lake with intensive trout farming. Our results also argue for proficient mitigation measures that include management of escapes and strategies to minimize unintentional releases from farm facilities.

**Introduction**

Releases of non-native species can cause economic losses and ecological impacts to native ecosystems [1–3]. However, propagation of non-natives are likely to continue if these activities are considered beneficial or profitable [4]. Species that become invasive can be harmful to freshwater ecosystems [5,6], and the case of salmonids (i.e., salmon and trout) introduced to Patagonia in South America is especially insightful [7,8]. Revenue from recreational fishing of salmonids in both Chile and Argentina—the only two countries where salmonids have hitherto established—have been appraised in millions of dollars annually [9]. This prompted multiple publicly and privately funded initiatives to stock Patagonian rivers and lakes [10–13]. Additionally, profits from farming of salmonids in South America are even higher as Chile, in particular, has become the world’s second largest producer of farmed salmonids with revenue in the order of billions of dollars [9,14,15]. Farming is another important source of propagules mediating the establishment and naturalization of salmonids in this region as certain methods of cultivation and husbandry of farmed fish are often linked to invasions [2,16,17].

Knowledge about the genetic underpinnings of the invasion of salmonids in Patagonia has been recently accumulating [17–20] amid many studies addressing ecological effects of salmonids on native fishes, including predation and competition [21–27]. Invasion genetics, the investigation of genetic variation among non-native populations and consequences for their ecology and evolution in novel environments, is still considered an emerging discipline [28]. Investigation of genetic variation using various molecular methods can be used to: (i) evaluate founding events on introduced populations [29], (ii) gauge the consequences of population admixture on neutral genetic diversity [17] and on individual fitness [30], or (iii) trace the history of human-mediated introductions back to their donor populations [31–33]. Non-equilibrium and individual-based approaches have provided powerful insights into elucidating spatial genetic structure and hybridization among invasive populations [17,34–36], and such approaches might be more suitable for recently founded populations [35,37]. Parameters of population stochasticity, namely effective population size and the annual number of breeders ($N_b$; [38]), are also crucial to predict the intensity of genetic drift or whether populations are capable of persisting in the face of environmental change [39], but estimation of these parameters has been largely absent from invasion genetics studies (but see [17] for an exception).

Rainbow Trout (*Oncorhynchus mykiss*) is one of the most notorious invasive species around the world [40] and possibly the most conspicuous salmonid in South America based on estimates of abundance [8,22]. Successful colonization of Rainbow Trout in Patagonia was likely the result of continuous propagule pressure, high phenotypic plasticity, and low environmental resistance [41]. Two stages can be identified in the introduction and naturalization of Rainbow
Trout in the case of Chile: stocking of rivers in the early twentieth century, followed by new introductions through releases from farms (intentional or unintentional) during the late twentieth century [42].

Lake Llanquihue in the Lake District (X Region) is an exemplary case to illustrate these stages (Table 1). Following a stocking phase, Lake Llanquihue became Chile’s largest smolt producer of a domestic broodstock of Rainbow Trout [15]. Naturalized Rainbow Trout in Lake Llanquihue showed a resident but migratory (ad-fluvial) life history; mature adults used streams to breed during the austral winter and early spring, whereas juveniles used streams as nursery areas for one or two years before emigrating to the lake to continue feeding [42]. Reproductive isolation among newly established salmon populations might evolve in tens of generations [43]. We investigate whether genetic divergence has arisen among Rainbow Trout populating inlet streams of Lake Llanquihue and whether spatial divergence is temporally stable, often a trademark among native salmon and trout populations with large N_e [44,45].

In this study we used population- and individual-based inference from single nucleotide polymorphisms (SNPs) to address three objectives regarding the distribution of genetic variance within and among Rainbow Trout collections from Lake Llanquihue. First, we evaluated intra-population followed by inter-population genetic variance and their statistical significance from collections at five inlet streams off Lake Llanquihue (Fig 1). We hypothesized that intra-population (temporal) genetic variance and temporal instability might be significant among recently founded, historically small, or artificially propagated Rainbow Trout populations similar to that seen in their native range [45,46]. In addition, inter-population divergence might not follow geographic patterns, because invasive fish populations are often in disequilibrium with respect to mutation, migration, and drift [47]. Second, we inferred the putative number of gene pools of Rainbow Trout that might coexist within the lake using individual-based Bayesian inference. Trout of a putative farm origin were identified by typical traits found among farm broodstock, namely skin ulcers or abrasions, short opercula and eroded fins; they are also likely to be genetically different from naturalized trout [17]. Third, we estimated the contemporary number of effective breeders (N_e) per year among introduced populations using a linkage disequilibrium method [48]. We hypothesized that estimates of N_e might be small as other invasive Rainbow Trout populations in Patagonia were likely founded two or three generations ago [17]. These hypotheses were tested using multilocus SNP genotypes that were originally ascertained from native Rainbow Trout populations from the west coast of North America [49]. These markers are single base substitutions (mostly biallelic) that are abundant and widespread in the genome, are found in coding and non-coding regions, and can be efficiently genotyped using multiplex

Table 1. Brief history of Rainbow Trout (O. mykiss) introductions in Lake Llanquihue, Lake District (X Region) in Chile. Modified from [13] and [15].

<table>
<thead>
<tr>
<th>Period</th>
<th>Description</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1910–1916</td>
<td>“Temporary” hatchery located at the outlet of the lake (River Maullín) that maintained 50,000–100,000 eggs imported from Germany</td>
<td>For stocking of River Maullín</td>
</tr>
<tr>
<td>1969–1972</td>
<td>First farm in the south shore that bred 37,500 adults in ten net pens for the domestic market</td>
<td>“Massive” escapes reported</td>
</tr>
<tr>
<td>1975–1979</td>
<td>Second farm located near River Pescado, one inlet stream</td>
<td>Exported 40,000 kg of adults for the French market</td>
</tr>
<tr>
<td>1980–2014</td>
<td>Explosive growth of the farming industry; 15 farming companies currently authorized to maintain and breed trout</td>
<td>500,000 smolts produced annually for the domestic market</td>
</tr>
</tbody>
</table>

a One of the streams in our study.

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PCR screening [50,51]. Our goals promise to contribute to both basic and applied contexts to understand the genetic underpinnings among established populations of a successful invader as well as developing efficient management strategies for invasive species.

**Material and methods**

**Study area and sampling design of naturalized populations**

Lake Llanquihue (Lake District, X Region: Fig 1) is one of southern Chile’s largest lakes, sharing several characteristics with other Araucanian lakes: (i) a glacial origin with volcanic influences; (ii) a maximum depth greater than 100 m; and (iii) low concentration of nutrients, salts, and chlorophyll-a [52]. Lake Llanquihue harbors both native and introduced fish species, primarily salmonids [22]. We surveyed Rainbow Trout in five third-order inlet streams (Fig 1): Blanco Arenales (BAR), Yerbas Buenas (YER), Tepu (TEP), Blanco (BLA) and Pescado (PES). BAR and YER are located in the eastern shore of the lake originating on the slopes of Osorno volcano, at an altitude of approximately at 1000 m [52]. TEP, BLA and PES are located on the southern shore of the lake originating on the slopes of Calbuco volcano, at an altitude ranging from 1000 to 1600 m.
Rainbow Trout collections (N = 582; Table 2) were made using a standard two-pass back-pack electrofishing under various settings depending on water conductivity (400–700V; 40–80 Hz). Each inlet stream was sampled twice (or three times in one case: YER) over 13 months in 2012 to 2013 to gauge intra-population (temporal) genetic variance. Sampling occurred during July in austral winter (W) and during October in austral early spring (S), corresponding to the spawning period of Rainbow Trout in Lake Llanquihue [42]. Within each inlet stream, we sampled in all available habitat units (pool-run-riffle) from a 200 m reach using a uniform time effort of 1 h. Collections were labeled as stream plus the last two digits of the year and season (e.g., YER12S).

Fish were anesthetized in a bucket containing a solution of 0.015% v/v of benzocaine (BZ-20®; 20% benzocaine) before being photographed, measured, and weighed. We evaluated statistical differences in trout size (total length; cm) from different streams (and groups thereof) using non-parametric Mann-Whitney U tests in R [53]. We recorded abnormalities such as eroded fins and short opercula as these might suggest a farm broodstock origin [17]. Fin clips of 25–100 mm² in size from the adipose fin, caudal fin, or both, were non-lethally obtained and preserved in ethanol 95%. Fish were put in a recovery tank containing well oxygenated water before release.

SNP genotyping
Genomic DNA was isolated from fin clips using a Qiagen DNeasy Tissue Kit (Valencia, California, USA) following the manufacturer’s instructions. We used a panel of 96 polymorphic SNPs [54,55] which were originally ascertained from several populations spanning the native range of Rainbow Trout [49]. Multiplex PCR was carried out using Fluidigm® 96.96 dynamic array chips following established pre-amplification and multiplex PCR protocols [50,56].

SNP Selection
Prior to estimate genetic diversity within and among naturalized samples, we implemented BAYESCAN [57] to identify putative candidate SNPs that might be influenced by natural selection. We tested for deviations from Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium (LD: non-random association of alleles between loci) for all SNPs in each collection site using GENEPOP[58].

Genetic diversity within collections
We used GENALEX to estimate observed (H_o) and expected heterozygosities (H_e) within naturalized collections. Allelic richness (A_R) and inbreeding coefficients (f) were calculated in FSTAT [59]. To compare genetic diversity among collections (or groups thereof) we used permutational multivariate analysis of variance (PERMANOVA) implemented in the package vegan in R [53]. The program estimates Euclidean distances between observed and expected values following 10,000 permutations, and then tests the null hypothesis of no differences between observed and simulated distance matrices.

Intra- and inter-population genetic variance
We calculated intra-population θ (between temporal replicates within streams) as well as inter-population θ (among streams) using GENEPOP [58,60]. To test if θ were significantly greater than zero, we estimated chi-square probabilities in CHIFISH [61]. We implemented an analysis of molecular variance (AMOVA: [62]) to evaluate the significance of intra- vs. inter-population genetic variances following 10,000 permutations of multilocus genotypes using GENALEX.
Hierarchical components of genetic variance and their significance were reported using sums of squares and $F$ statistics.

Individual-based Bayesian inference of genetic structure

We used Bayesian inference to assign individual genotypes to a defined number of genetic pools ($K$) in STRUCTURE [63,64]. The program probabilistically assigns individual multilocus genotypes into a discrete number of clusters while minimizing departures from HWE and LD from admixed populations. We performed separate simulations for spring and winter sampling seasons. We ran 15 iterations per each $K$, which ranged between one and five using an admixture model and a burn-in period of 50,000, followed by 250,000 Markov Chain Monte Carlo steps after burn-in. To evaluate the most probable value for $K$, we followed the Evanno criterion [65] using STRUCTURE HARVESTER [66]. We plotted 'consensus' coefficients of individual membership ($Q$-values) in R following cluster matching and permutation in CLUMPP [67] to account for label switching artifacts and multimodality.

Estimation of the effective numbers of breeders ($N_b$)

We estimated the contemporary annual number of effective breeders ($N_b$) in naturalized collections using LDNE [48]. The method evaluates LD between unlinked loci as a proxy for genetic drift assuming selective neutrality, discrete generations, and closed populations. While the first might be met, the second might not as salmonid populations are age-structured and are likely to be connected by gene flow. When applied to age-structured populations, LD reflects a quantity closer to $N_b$ per brood year rather than the effective population size per generation [38]. The method seems robust to equilibrium gene flow, unless migration rates are higher than 5–10% [68]. LDNE was run with the following settings: (i) minor allele frequencies < 0.02 were excluded to account for the trade-off between accuracy and precision at sample sizes larger than 25 individuals per collection [38], (ii) random mating system, and (iii) 95% CIs were calculated via jackknifing of LD values among pairs of loci. Comparisons of $N_b$ between groups of naturalized trout were performed through non-parametric Mann-Whitney tests in R.

Table 2. Rainbow Trout collections and genetic statistics from inlet streams of Lake Llanquihue.

<table>
<thead>
<tr>
<th>Inlet stream</th>
<th>Date</th>
<th>Code</th>
<th>Abnormalities</th>
<th>$n$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$A_R$</th>
<th>HWE</th>
<th>$f$</th>
<th>LD $N_b$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blanco Arenales (BAR)</td>
<td>Oct 2012</td>
<td>BAR12S</td>
<td>0%</td>
<td>50</td>
<td>0.333</td>
<td>0.335</td>
<td>1.959</td>
<td>0.968</td>
<td>0.017</td>
<td>54 (43–70)</td>
</tr>
<tr>
<td></td>
<td>Jul 2013</td>
<td>BAR13W</td>
<td>0%</td>
<td>44</td>
<td>0.334</td>
<td>0.333</td>
<td>1.984</td>
<td>0.983</td>
<td>0.007</td>
<td>99 (69–163)</td>
</tr>
<tr>
<td>Yerbas Buenas (YER)</td>
<td>Oct 2012</td>
<td>YER12S</td>
<td>32%</td>
<td>31</td>
<td>0.365</td>
<td>0.369</td>
<td>1.973</td>
<td>0.955</td>
<td>0.028</td>
<td>26 (21–33)</td>
</tr>
<tr>
<td></td>
<td>Jul 2013</td>
<td>YER13W</td>
<td>0%</td>
<td>33</td>
<td>0.359</td>
<td>0.344</td>
<td>1.951</td>
<td>0.979</td>
<td>0.0273</td>
<td>35 (27–46)</td>
</tr>
<tr>
<td></td>
<td>Oct 2013</td>
<td>YER13S</td>
<td>26%</td>
<td>60</td>
<td>0.368</td>
<td>0.369</td>
<td>1.972</td>
<td>0.896</td>
<td>0.0121</td>
<td>40 (34–48)</td>
</tr>
<tr>
<td>Tepu (TEP)</td>
<td>Oct 2012</td>
<td>TEP12S</td>
<td>0%</td>
<td>51</td>
<td>0.323</td>
<td>0.316</td>
<td>1.965</td>
<td>1.000</td>
<td>0.015</td>
<td>135 (86–276)</td>
</tr>
<tr>
<td>Bianco (BLA)</td>
<td>Jul 2013</td>
<td>TEP13W</td>
<td>0%</td>
<td>95</td>
<td>0.335</td>
<td>0.322</td>
<td>1.969</td>
<td>0.999</td>
<td>0.035</td>
<td>132 (101–181)</td>
</tr>
<tr>
<td></td>
<td>Oct 2012</td>
<td>BLA12S</td>
<td>0%</td>
<td>49</td>
<td>0.326</td>
<td>0.324</td>
<td>1.977</td>
<td>0.936</td>
<td>0.003</td>
<td>69 (53–95)</td>
</tr>
<tr>
<td></td>
<td>Jul 2013</td>
<td>BLA13W</td>
<td>0%</td>
<td>88</td>
<td>0.312</td>
<td>0.315</td>
<td>1.952</td>
<td>0.967</td>
<td>0.013</td>
<td>121 (92–169)</td>
</tr>
<tr>
<td>Pescado (PES)</td>
<td>Oct 2012</td>
<td>PES12S</td>
<td>8%</td>
<td>35</td>
<td>0.332</td>
<td>0.327</td>
<td>1.971</td>
<td>0.904</td>
<td>-0.001</td>
<td>77 (54–131)</td>
</tr>
<tr>
<td></td>
<td>Jul 2013</td>
<td>PES13W</td>
<td>0%</td>
<td>46</td>
<td>0.314</td>
<td>0.308</td>
<td>1.949</td>
<td>1.000</td>
<td>0.009</td>
<td>131 (83–274)</td>
</tr>
</tbody>
</table>

$n$, sample size; $A_R$, allelic richness; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; HWE, exact probability over multiple loci (Fisher's method) to test the null hypothesis of Hardy-Weinberg equilibrium proportions; $f$, inbreeding coefficient; LD $N_b$, linkage-disequilibrium estimate for the effective number of breeders.

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Ethics statement

This study was carried out in accordance with the recommendations of the Guidelines for the Use of Fishes in Research (http://fisheries.org/guide-for-the-use-of-fishes-in-research). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidad de Concepcion. Fishing permit #1715/14 was issued by the Chilean Undersecretary of Fisheries and Aquaculture.

Results

Sampling: variation in fish size and evidence of farmed trout

Rainbow Trout from inlet streams of Lake Llanquihue varied in size from 5 to 25 cm TL with a median below 15 cm in most collections (Fig 2). We found significant differences in fish size between spring and winter collections; spring trout were larger than winter trout across collections (Mann-Whitney U test: $U = 45646, p < 0.001$). Also, trout from YER12S and YER13S were larger than trout caught at any of the remaining streams during spring (Mann-Whitney U test: $U = 10098; p < 0.001$). Further, collections PES12S, YER12S and YER13S showed trout with skin ulcers, skin abrasions, eroded fins, and short opercula, suggestive of farmed fish (Fig 3). The percentage of trout showing these characteristics varied between 8% (PES12S) and 32% (YER13S; Table 2).

SNP selection

SNPs were removed if they were monomorphic in all populations, consistently deviated from HWE proportions, showed evidence of directional selection, or exhibited significant LD (S1 Table). Three monomorphic loci were dropped from the analyses: Ocl_Okerca, Ocl_oku202 and Ocl_Oku216. We found consistent deviations from HWE proportions across populations in Omy_mcsf-268, Omy_09AAD-076, and Omy_110064–419 and removed these from the data set. A putative candidate for directional selection was found in locus Omy_107806–34 which was excluded. We found significant LD ($p < 0.001$) in more than 75% of collections (often in 100% of collections) for five groups of loci, some of which had known linkage relationships from previous studies [49,69] as presented in S1 Table. Additional linkage information was found using BLAST against contigs from whole genome sequencing of Rainbow Trout [70]. Significant matches and close proximity between SNPs suggested that LD was likely the result of physical linkage between markers (S1 Table). We thus kept the most informative of each group as suggested elsewhere [71]. We found no further evidence for deviations from either HWE proportions or LD within populations after removal of affected loci. The filtering process left a final panel of 81 SNPs for subsequent analyses.

Genetic diversity within collections

We estimated values of $H_O$ between 0.312 (BLA13W) and 0.368 (YER13S), whereas values of $H_E$ ranged between 0.315 (BLA13W) and 0.369 (YER12S, YER13S; Table 2). $A_R$ varied between 1.951 (YER13W) and 1.984 (BAR13W). Trout from YER had higher $H_E$ than trout from any of the remaining collections (PERMANOVA: $p < 0.001$).

Intra- and inter-population genetic variance

We found significant intra- and inter-population divergence measured as $\theta$ among all collections with only three exceptions—BAR12 S vs. PES12S, BLA12S vs. BAR13W, and PES12S vs. BLA13W (Table 3). Significant $\theta$ values ranged between 0.091 (TEP12S vs. YER13S) and 0.004 (PES13W vs. BLA13W) and did not follow simple geographic patterns mediated by distance.
For instance, $\theta$ values between inlet streams separated by less than 5 km (e.g., BLA12S vs. TEP12S) were larger than between inlet streams separated by more than 30 km (e.g., PES12 vs. BAR12). The highest $\theta$ values were found in inter-population pairwise comparisons involving all three YER collections. Hierarchical AMOVA suggested that both intra- and inter-population genetic variance were significant, and the latter was higher than the former if all collections were included in the analysis (Table 4). However, intra-population variance was higher than inter-population variance (which was marginally significant: $0.01 < p < 0.05$) after excluding highly distinct YER collections.

Individual-based Bayesian clustering: spring vs. winter genetic structure

The number of gene pools inferred through Bayesian clustering varied depending on sampling season (Fig 4). We found evidence for $K = 2$ during spring; asymmetric Q-values from trout sampled from from YER12S and YER13S suggested a unique ancestry in comparison to other collections. However, we found no clear evidence for genetic structure during winter ($K = 1$). Q-values among collections were relatively symmetric (0.45–0.55) when assigned to two clusters, with exception of YER13W that showed Q-values between 0.39 and 0.61.

Estimation of the effective numbers of breeders ($N_b$)

All estimates of $N_b$ were bound by finite 95% CIs, suggesting that the number of annual breeders contributing to all collections might be small (Table 2). Point estimates were below 150 and
the median $N_b$ was 73. Estimates of $N_b$ for YER12S, YER13S and YER13W were the smallest and significantly lower than any other collections (Mann-Whitney $U$ test: $U = 0, p = 0.008$).

**Discussion**

We used population- and individual-based inference of multilocus SNP genotypes to address three fundamental issues of the naturalization and invasion of Rainbow Trout in a lake historically impacted by trout stocking and farming practices. First, we found significant intra- and inter-population genetic variance, and the former (temporal) component seemed more important than the latter (spatial) component if highly distinct collections were excluded from the analyses. Spatial differentiation as revealed by estimates of inter-population $\theta$ did not follow simple geographic patterns mediated by distance. Second, individual-based Bayesian analyses further revealed that trout from one single stream, Yerbas Buenas (YER), drove genetic divergence among individuals during spring, but not in winter sampling seasons. Spring trout from YER were additionally more likely to show abnormalities and have smaller $N_b$ than trout from any other collection, suggesting they might originate from farm broodstock. Third, our estimates of $N_b$ showed that half of the collections had a breeding size of 73, consistent with
previous findings of significant temporal genetic variance. We discuss these findings in detail in the following paragraphs.

### Intra- and inter-population genetic variance

With exception of Pink Salmon where temporal divergence is prominent between odd- and even-year populations, spatial divergence at various scales as a result of homing behavior is a trademark among salmonid systems in their native range [72,73], usually surpassing the magnitude of temporal divergence in large, wild populations in pristine environments (Pacific salmon: [44,74]; Atlantic salmon, *Salmo salar*: [75,76]; brown trout, *S. trutta*: [77]). For naturalized Rainbow Trout from Lake Llanquihue, pairwise $\theta$ showed that intra-population (temporal) genetic variance was greater than inter-population (spatial) genetic variance, downplaying the importance of spatial divergence during the process of naturalization.

What is the explanation behind significant temporal genetic variance? We ruled out sources of confounding spatiotemporal effects such as family-biased sampling as no significant departures from HWE proportions or LD were evident within collections. One hypothesis is that winter and spring trout collections correspond to different cohorts and that genetic drift between cohorts was significantly higher than expected by chance. This is consistent with

### Table 3. Pairwise genetic distances ($\theta$) between collections.

<table>
<thead>
<tr>
<th>Collection</th>
<th>BAR12S</th>
<th>BAR13W</th>
<th>YER12S</th>
<th>YER13S</th>
<th>YER13W</th>
<th>TEP12S</th>
<th>TEP13W</th>
<th>BLA12S</th>
<th>BLA13W</th>
<th>PES12S</th>
<th>PES13W</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAR13W</td>
<td>0.0040*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YER12S</td>
<td>0.0530**</td>
<td>0.0527**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YER13S</td>
<td>0.0585**</td>
<td>0.0630**</td>
<td>0.0156**</td>
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</tr>
<tr>
<td>YER13W</td>
<td>0.0181**</td>
<td>0.0166**</td>
<td>0.0287**</td>
<td>0.0459**</td>
<td></td>
<td></td>
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<tr>
<td>TEP12S</td>
<td>0.0073**</td>
<td>0.0116**</td>
<td>0.075**</td>
<td>0.0911**</td>
<td>0.0330**</td>
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<tr>
<td>TEP13W</td>
<td>0.0057**</td>
<td>0.0074**</td>
<td>0.0669**</td>
<td>0.0762**</td>
<td>0.0282**</td>
<td>0.0049**</td>
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<tr>
<td>BLA12S</td>
<td>0.0066**</td>
<td>0.0032</td>
<td>0.0657**</td>
<td>0.0743**</td>
<td>0.0220**</td>
<td>0.0128**</td>
<td>0.0088**</td>
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</tr>
<tr>
<td>BLA13W</td>
<td>0.0066*</td>
<td>0.0079**</td>
<td>0.0768**</td>
<td>0.0843**</td>
<td>0.0285**</td>
<td>0.0072**</td>
<td>0.0076**</td>
<td>0.0065**</td>
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<tr>
<td>PES12S</td>
<td><strong>0.0022</strong></td>
<td></td>
<td>0.0663**</td>
<td>0.0785**</td>
<td>0.0245**</td>
<td>0.0046*</td>
<td>0.0039*</td>
<td>0.0054*</td>
<td>0.0014</td>
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<tr>
<td>PES13W</td>
<td>0.0113**</td>
<td>0.0076**</td>
<td>0.0810**</td>
<td>0.0867**</td>
<td>0.0288**</td>
<td>0.0111**</td>
<td>0.0103**</td>
<td>0.0035*</td>
<td>0.0048*</td>
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</tbody>
</table>

$p < 0.01$

*, $p < 0.001$

**, non-significant in bold.

doi:10.1371/journal.pone.0142040.t003

### Table 4. Hierarchical analysis of molecular variance (AMOVA) for spatial and temporal components.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SS</th>
<th>% Variance</th>
<th>F-statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All collections</td>
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<td></td>
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<tr>
<td>Inter-population</td>
<td>4</td>
<td>379.4</td>
<td>2%</td>
<td>0.021</td>
<td>0.001</td>
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<tr>
<td>Intra-population</td>
<td>5</td>
<td>115.2</td>
<td>1%</td>
<td>0.027</td>
<td>0.001</td>
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<td>Excluding YER collections$^a$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Inter-population</td>
<td>3</td>
<td>85.0</td>
<td>0%</td>
<td>0.002</td>
<td>0.048</td>
</tr>
<tr>
<td>Intra-population</td>
<td>4</td>
<td>85.9</td>
<td>1%</td>
<td>0.007</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$^a$YER12S, YER13S and YER13W.

doi:10.1371/journal.pone.0142040.t004
differences in size (and likely age) between spring and winter trout. Most spring trout likely comprised fish of age +1 (i.e., fish that hatched in late spring and spent one winter) and or even age +2 (e.g., YER12S and YER 13S), whereas winter trout likely comprised young-of-the-year fish [42]. Thus, differences in allele frequencies between cohorts might be an indication of a reduced number of annual breeders (small $N_b$).

Our findings are limited to collections temporally spaced 8 to 13 months and they need not reflect patterns of genetic drift at longer temporal scales. Rainbow Trout of ages 4+ to 12+ (with a mode at age 6+) breeding at the same inlet streams we examined here (PES, TEP and BLA) were described in an earlier study, suggesting an iteroparous life history [42]. Multiple breeding seasons among Rainbow Trout from Lake Llanquihue imply that genetic monitoring over longer time scales—assuming one generation occurs every five years—should be accomplished in order to appropriately assess inter-generational changes, or alternatively, estimating temporal changes from cohort-based approaches to populations with overlapping generations [78].

Lastly, significant inter-population $\theta$ which was nearly universal among all collections and lack of geographic structure should be interpreted within the context of invasive populations that might not be at migration-drift equilibrium [47]. This is a likely scenario for Rainbow Trout in Chile’s Lake District [17] and Lake Llanquihue, wherein populations might have established eight to twenty generations ago, assuming a generation time of five years [42]. Estimates of $\theta$ will fail to translate into realistic measures of gene flow if populations are in disequilibrium [37], which argues for the application of individual-based approaches to understand genetic structure and connectivity among invasive populations [35,36].

Individual-based Bayesian clustering: propagule-driven genetic structure

We identified two gene pools of Rainbow Trout coexisting in Lake Llanquihue during spring, but only one during winter sampling seasons. During spring, one of these pools comprised trout populating one single stream: Yerbas Buenas (YER), a narrow (1–2 m wetted channel...
width), fast-flowing (approximately 1 m s\(^{-1}\)), and one of the most productive inlet streams for Rainbow Trout (authors’ unpublished data). We speculate that the source of YER trout appears to be farm broodstock that migrated upstream to breed in spring, which explains why during winter they were apparently missing; it is beyond the scope of our study to determine whether YER12S and YER13S originated from one or two consecutive escaping events. Farm escapes are more likely to show abnormalities and disease that affect farmed trout [17,79]; they also had the highest heterozygosity and the smallest estimates of \(N_b\), suggesting a highly diverse and yet genetically small source. A common disease affecting Rainbow Trout in freshwater is flavobacteriosis (\(Flavobacterium psychrophilum\)) with characteristic skin ulcers and erosions as reported here (R. Avendaño, comm. pers. 2015). These results are stimulating because farm propagules of Rainbow Trout can not only be identified and distinguished from naturalized individuals; they might establish in the wild and breed with other farmed or naturalized trout. Indeed, we observed several sexually mature trout from YER during spring (authors’ field observations), suggesting a time of breeding that begins in winter but extends into early spring [42].

While identifying farm escapees can be accomplished using other methods [80], SNP genotyping might be a faster and more cost-effective method to discriminate between naturalized and farmed trout than other genetic approaches [81]. Further work should be aimed at genotyping farm broodstock from this lake and appraise their similarity with YER collections using individual assignment, to confirm our hypothesis. Such data will also help clarify if farm broodstock might have interbred with naturalized trout in YER and other streams by identifying farm-naturalized hybrids and their abundance. This is an interesting possibility and might explain why YER13W juveniles showed \(Q\)-values that departed slightly from “pure” naturalized trout found among remaining collections during winter that were symmetrically assigned to two clusters. Consuegra et al. [17] genetically characterized farmed-naturalized Rainbow Trout hybrids which were more abundant near farms, arguing that propagule-driven admixture might have facilitated establishment of Rainbow Trout in a large portion of the Lake District in southern Chile. Rainbow Trout has become the most abundant fish species at populating streams of Lake Llanquihue, displacing not only native fishes but other non-native salmonids including Brown Trout (\(Salmo trutta\)) and Coho Salmon (\(Oncorhynchus kisutch\)), which are more conspicuous in lakes with low or no propagule pressure from aquaculture farms [22,24]. Our findings argue for efficient management measures that can mitigate Rainbow Trout escapes in freshwater given that they hold a great potential to establish self-sustaining populations and spread of the invasion [16].

**Estimation of the effective numbers of breeders (\(N_b\))**

Our estimates of \(N_b\) suggest that half of naturalized trout collections have an annual breeding size of 73 individuals or less, which appears counterintuitive given their high levels of heterozygosity. This supports the notion that these populations might be experiencing substantial genetic drift and temporal genetic variance. Parallel estimates of LD \(N_b\) for Rainbow Trout populations in Patagonia were below 100 based on microsatellite DNA multilocus genotypes [17]. This is interesting because \(H_E\) among naturalized collections remained high despite small LD \(N_b\). We performed a quick comparison with native populations from North America (\(n = 10\)) [49] genotyped for matching SNPs and found that naturalized trout in Lake Llanquihue were more heterozygous than trout from ten native populations (PERMANOVA: \(p < 0.001; S1\) Fig). We hypothesize that genetic diversity has remained high due to historical, rather than recent, admixture as measures of heterozygosity are generally unaffected during bottlenecks [82,83]. High \(H_E\) is also consistent with multiple “waves” of introduction in Lake
Llanquihue likely originating from genetically distinct sources, a possible explanation of why invasive populations generally overcome founding effects and spread [84].

The paradox of genetically diverse but small-size populations might not be exclusive of Rainbow Trout outside their native range and might suggest recently founded populations [17]. Native steelhead populations (anadromous life history of Rainbow Trout) from the Skeena River in British Columbia (Canada) showed estimates of \(N_b\) below 100 in most cases, despite the fact of census population sizes (\(N\)) in the order of 1000s [46]. To our knowledge, reliable estimates of \(N\) are unavailable for inlet streams of Lake Llanquihue, and these are necessary to comprehend ecological processes affecting these populations. In addition, the ratio \(N_b/N\) is crucial to assess population stochasticity in the face of recent global environmental change [39]. This theoretical framework, conceived and often applied to native populations, can be extended to invasive populations and their management and control, for instance, to predict which populations are more likely to persist [47,85,86]. Lastly, our survey serendipitously offers a good baseline for monitoring the ecological and demographic consequences of the eruption of Calbuco volcano on aquatic communities [87], which deposited large quantities of volcanic ash on inlet streams TEP, BLA and PES.

Conclusions

Using population- and individual-based inference of SNP multilocus genotypes we provide insights on the genetic influence on the establishment and naturalization of Rainbow Trout in Lake Llanquihue, a Patagonian lake heavily affected by trout farming. First, we found that intra- population (temporal) genetic variance was greater than inter-population (spatial) genetic variance, downplaying the importance of spatial divergence during the process of naturalization. Temporal variance might be explained by allele frequency shifts between cohorts, consistent with variation in fish length between spring and winter collections. Second, individual-based Bayesian clustering suggested that genetic structure within Lake Llanquihue has been largely influenced by putative farm propagules from Yerbas Buenas (YER) stream. Yet, it is unclear whether interbreeding has occurred between “pure” naturalized and farm trout. Third, estimates of the annual number of breeders (\(N_b\)) were below 73 in half of the five collections, suggesting genetically small and recently founded populations that might experience substantial genetic drift. Our findings reinforce the notion that naturalized trout originated recently from a small yet genetically diverse source and that farm propagules might have played a significant role in the invasion of Rainbow Trout within a single lake.

Supporting Information

S1 Fig. Expected heterozygosities among Lake Llanquihue’s naturalized trout and North America’s native rainbow trout and steelhead populations genotyped for identical SNPs. (TIF)

S1 Table. List of SNP loci used in the study. Linkage information between loci is emphasized using lower case letters. (XLSX)

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organizations that supported this initiative. Carita Pascal performed all laboratory analyses, including SNP genotyping, allele scoring and database management. Fred Utter, Garrett McKinney, Zuogang Peng, and two anonymous referees provided outstanding suggestions on earlier versions of this manuscript. Ruben Avendaño helped identifying diseases among putative farmed trout escapees. We thank Morten Limborg for sharing a database of SNP genotypes from native Rainbow Trout and Steelhead Salmon populations, and Sewall Young and Nathan Campbell for sharing unpublished SNP sequence data. This research was supported by Chile’s government grants, FONDECYT 1130807 and FONDAP 15110027; and grant DIUC 212.113.082–1.0 from Universidad de Concepcion.

**Author Contributions**

Conceived and designed the experiments: DG-U LWS JES IA CEH GG. Performed the experiments: JNB DG-U MIC SSM. Analyzed the data: JNB DG-U MIC SSM. Contributed reagents/materials/analysis tools: RG GG LWS JES. Wrote the paper: JNB DG-U LWS GG IA CEH.

**References**


35. Herborg LM, Weetman D, Van Oosterhout C, Hanfling B. Genetic population structure and contemporary dispersal patterns of a recent European invader, the Chinese mitten crab, Eriocheir sinensis. Molecular Ecology. 2007; 16(2):231–42. ISSN:002433052000001. PMID: 17217341


