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# Population genomics of a culturally important amphibian on a remote archipelago reveals a distinct and depauperate population

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# Abstract

Amphibian populations are at risk globally and understanding their evolutionary history is critical to their future conservation. The Western Toad (*Anaxyrus boreas*) is widely distributed across western North America, but is the only native amphibian on Haida Gwaii, a large and remote archipelago off the northwest coast of British Columbia (BC), Canada. Hlk'yáan K̲'ust'áan/Hlk'yan k̲'uust'an (Western Toad) is a culturally important species for the Haida Nation, with recent concerns of possible extirpations of breeding sites. To examine genetic diversity and differentiation within populations on Haida Gwaii as compared to elsewhere in coastal BC, we sampled 1,370 individuals from 47 breeding sites across Haida Gwaii, Vancouver Island to the south, the southwest Lower Mainland, and the Northwest BC mainland. Genotyping-by-sequencing generated ~ 1,368 single nucleotide polymorphisms (SNPs) for the full dataset after quality control filtering. Haida Gwaii toads are strongly differentiated from other coastal BC populations (pairwise $F_{ST}$ = 0.363). Genetic connectivity between breeding sites is higher within Haida Gwaii ($F_{ST}$ = 0.080) than within other regions ($F_{ST}$ = 0.105). Genetic diversity is also lower in Haida Gwaii (nucleotide diversity π = 0.0004) than elsewhere (0.0007). The site frequency spectrum suggests a founder event or bottleneck may have caused this reduced diversity in Haida Gwaii. Our results suggest that Haida Gwaii toads should be treated as a distinct population for management, targeting protection of breeding habitats and connectivity between breeding sites to promote persistent populations and prevent declines of this culturally and ecologically important species.


# 1. Introduction

Amphibians are severely endangered globally, with over 30% of species facing extinction (Wake and Vredenburg 2008) due to habitat loss and fragmentation, climate change, disease, non-native species, and UV-B radiation (Beebee and Griffiths 2005; Hof et al. 2011). Many of these drivers act together to reduce population size and genetic diversity (Frankham 1996), thereby threatening the persistence of populations by increasing inbreeding and compromising fecundity, survival, competitive ability, and adaptive capacity (Keller and Waller 2002; Allentoft and O'Brien 2010; Chen et al. 2012). In particular, habitat fragmentation and population isolation can decrease genetic diversity through reduced gene flow and a smaller effective population size (Johansson et al. 2007). To slow or reverse amphibian declines through management interventions, it is important to understand population genetic structure and diversity across the landscape.

The Western Toad (*Anaxyrus boreas*) is widely distributed in western North America – from southern Alaska south to the Baja Peninsula in Mexico, and as far east as Colorado. Though globally assessed as "Least Concern", population trends across its range are declining (IUCN SSC Amphibian Specialist Group 2021). Local declines have occurred throughout the species' range, with extirpations of entire populations on Vancouver Island documented on the Pacific coast of British Columbia, Canada (Davis and Gregory 2003), and in Colorado (Carey 1993) and New Mexico (Jackson 2004) in the United States. This species is of conservation concern in Canada, listed as "Special Concern" under the Canadian Species at Risk Act (SARA, Environment and Climate Change Canada 2020). There are two Designatable

Units under SARA within Canada based on behavioural and morphological observations – denoted as calling and non-calling populations. The majority of the non-calling population in Canada occurs within the province of British Columbia, where the Western Toad is ranked as S4 - 'apparently secure' - because the species is wide-ranging across the province, though local declines have been noted, as have threats of habitat loss and fragmentation (BC Conservation Data Centre 2022).

Western Toads are highly philopatric (loyal) to their breeding sites (COSEWIC 2012), elevating the importance of habitat protection for the species' conservation. Sexually mature toads come together from across a large area to a single breeding site, breeding *en masse* for a few weeks every spring (COSEWIC 2012). Adult toads have broad environmental tolerances, relatively high dispersal ability, and may quickly colonize new habitats under suitable conditions in connected landscapes (Holmes 2015). However, mountain ranges and saltwater inlets such as those found along the North American Pacific coast can act as barriers to toad movements (Moore et al. 2011). At the northern extent of the species' range, breeding sites are often separated by distances up to 30km (Slough 2004). Breeding behaviour in landscapes constrained by topography, geography, and anthropogenic disturbance can make for highly structured populations that are vulnerable to threats to core breeding habitats.

Goebel et al. (2009) used mitochondrial DNA to identify three main clades of Western Toads across the species' range – northwestern, southwestern and eastern – though there were only four samples north of the 49th parallel, in British Columbia and Alaska, included. Of the handful of studies that have investigated the population genetics of Western Toads, few have investigated more than a single region (Moore et al. 2011) or have used high-resolution genomic methods. One recent study using mitochondrial DNA markers found no genetic structure in northern Idaho and northwest Washington toad populations (Lucid et al. 2021), similar to the findings of (Murrell 2013) using microsatellite loci to genotype toads in California. Both Moore et al. (2011) and Holmes (2015) found moderate structuring in southeast Alaska populations at the northern edge of the species range using microsatellites. By contrast, in a much smaller study area, Myers (2020) found genetic structuring in Western Toad populations in Snoqualmie Pass, Washington State using single nucleotide polymorphisms (SNPs).

The Western Toad is the only native amphibian on Haida Gwaii, a large and remote archipelago off the northwest coast of British Columbia (BC), Canada (Fig. 1). Haida Gwaii has been separated from mainland North America since a connecting continental land bridge was submerged approximately 10,000 years ago following the last glacial maximum (LGM) (McLaren et al. 2020). Haida Gwaii's history of geographic isolation combined with its relatively mild climate for its latitude (52–54ºN) has led to the evolution of a plethora of endemic taxa (Reimchen et al. 2005). It is considered a biodiversity hotspot, harbouring unique species and subspecies of mammals, fish, birds, plants, lichens, and mosses, though also characterized by depauperate insular species assemblages (Osgood 1901; Byun et al. 1997; Reimchen et al. 2005; Withrow et al. 2014; Colella et al. 2018; Geraldes et al. 2019).

The Western Toad, called Hlk'yáan K̲'ust'áan in the X̲aad Kíl dialect of the Haida language (Haida Language Council 2022), and Hlk'yan k̲'uust'an, in X̲aayda Kil (Skidegate Haida Immersion Program

2015), is a culturally important species to the Haida Nation, present in many oral histories and depicted in ancient and modern art from totem poles to jewellery. There is concern that local toad populations may have been extirpated in the last few decades, possibly due to the introduction of terrestrial vertebrate predators (e.g., raccoons) and competitors (e.g., frogs) in landscapes under stress from forest harvest disturbance and climate change (Ovaska et al. 2002; Gaston et al. 2008; Gamlen-Greene 2022). A recent experiment found that Haida Gwaii toads had weaker competitive abilities in the presence of introduced Northern Red-legged Frogs (*Rana aurora*) as compared to other toads from within the sympatric range of these two species on the southwest coastal BC mainland (Gamlen-Greene 2022).

Our goal in this study was to provide information for effective conservation management of the Western Toad and its habitats by assessing genetic differences of populations on Haida Gwaii compared to insular and mainland populations found elsewhere in coastal BC, and by investigating population genetic structure within Haida Gwaii. We addressed three specific questions:

1. *How genetically divergent are the Western Toads on Haida Gwaii from other coastal BC populations?*
2. *Are levels of genetic diversity in Haida Gwaii Western Toads comparable to those in other insular and mainland coastal BC populations?*
3. *What is the genetic diversity and divergence among breeding sites across the island archipelago within Haida Gwaii?*

We anticipated toad populations on Haida Gwaii would be significantly differentiated from and less genetically diverse than other coastal populations, given the remote geographic and historic isolation of the archipelago.

To address our questions, we sampled toad breeding sites from Haida Gwaii to compare to other insular and mainland regions in coastal BC, and generated a set of genome-wide SNPs using Restriction site Associated DNA Sequencing (RADseq). These data were analyzed to determine patterns of diversity within and divergence among populations of Haida Gwaii and other coastal populations. As part of the research, we involved the community throughout the duration of the project as we believe this to be an important and integral part of the research methods. This meaningful engagement improved both the science and real-world outcomes.

## 2. Materials and Methods

# 2.1. Field sampling

Western Toad larvae (i.e., tadpoles) were collected from 47 breeding sites across coastal British Columbia (Fig. 1), in Haida Gwaii (13) and three “other coastal BC” regions: Vancouver Island including Lasqueti Island (14), the Lower Mainland (17), and Northwest BC (3); Fig. 1. To reflect differences in land use and landscape condition, Haida Gwaii was further divided into two sub-regions: 1) the co-managed

protected area of Gwaii Haanas National Park Reserve, National Marine Conservation Area Reserve, and Haida Heritage Site (hereafter referred to as “Gwaii Haanas”) comprised of south Moresby Island and all the small islands south of it; and 2) north Moresby Island and Graham Island (largest in the archipelago) (referred to here as “Northern Haida Gwaii”) with settlements, roads, and commercially managed forests (Fig. 1).

Before field sampling for this study, only five toad breeding sites were recorded on Haida Gwaii, all within Gwaii Haanas. Given the low apparent density of toads, systematic grid surveys on Graham and north Moresby Islands did not locate additional sites. Eight more sites (13 total) were later identified through intensive searches by research teams and local volunteers over three years (2017–2019; Fig. 2a). Local observations of adult toads were solicited via community outreach, with reported locations used to define 3 km search radii on satellite imagery; all waterbodies within these areas were surveyed during the tadpole season (May–early July), under favourable conditions to maximise detection. From 2017–2022, extensive community engagement—including schools, field technicians, volunteers, and landowners—supported site discovery, monitoring, and protection of sites where possible (Fig. 2b).

We compiled inventories of known breeding sites across the three other coastal BC study regions targeted, with more confirmed sites in the populated southern regions of Vancouver Island (37) and the Lower Mainland (40) than in the less populated coastal Northwest (18), where reports were often anecdotal or historic. From May–July (2018–2022), we visited > 50% of sites in each region at least once. Detection probability depended on inventory reliability, site access (especially in the Northwest), aquatic feature size, and temporal variability in tadpole distribution. Breeding was detected at 14 of 20 sites on Vancouver Island (70%), 17 of 22 in the Lower Mainland (77%), and 3 of 16 visited sites in Northwest BC (18.8%), all near Terrace.

At each breeding site, 18–30 larvae or newly emerged toadlets were collected (1,370 total across four regions), distributed within sites to reduce sampling of siblings. Individuals were euthanized in buffered tricaine methanesulfonate, rinsed, preserved in 100% ethanol, kept cool in the field, and stored at − 20°C in the lab. Sampling was conducted under relevant wildlife, park, animal care, Indigenous, and landowner permissions (see Acknowledgements).

# 2.2. DNA extraction and sequencing

DNA was extracted in the Hamelin Genomics and Forest Health Lab at UBC from 10–20 mg of tissue using a MagMAX™ DNA Multi-Sample Ultra 2.0 Kit and KingFisher Duo Prime (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA quantity and quality was tested using the NanoDrop 1000 spectrophotometer and the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Whole genomic DNA fragment size was assessed via 0.7% agarose gel electrophoresis.

Genotyping-by-sequencing (GBS) (Elshire et al. 2011) was used to generate a dataset of genome-wide SNPs (single nucleotide polymorphisms). Samples were sent to the Plateforme d’Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (PAG-IBIS, Université Laval, Quebec, Canada) for

GBS library preparation. The PstI/MspI double-digest procedure first described in Poland et al. (2012) was used with the following modifications: the adapters were modified to include plate indexes as described in Colston-Nepali et al. (2019) and a BluePippin (Sage Biosciences, Beverly, MA, USA) size selection step prior to the final PCR was added as described in Abed et al. (2019) with elution between 56 and 65 minutes. Nine plate-indexed pools of GBS libraries, each containing up to 96 in-line barcoded samples, were prepared. Breeding sites were split across several plates to reduce bias. Each library was quantified using a fluorometric method and the profile was checked on a Bioanalyzer high sensitivity DNA chip (Agilent, Santa Clara, CA, USA). The libraries were sent for sequencing at the Génome Québec Innovation Centre (McGill University) on three Illumina NovaSeq 6000 S4 lanes with 2 x 100 cycles (paired-end mode). The libraries were normalized and pooled and then denatured in 0.05N NaOH and neutralized using HT1 buffer. The pool was loaded at 225pM on an Illumina NovaSeq 6000 S4 PE100 lane using Xp protocol as per the manufacturer's recommendations. The run was performed for 2 x 100 cycles (paired-end mode). A phiX library was used as a control and mixed with libraries at 10% level. Base calling was performed with RTA v3. The program *bcl2fastq2* v2.20 was then used to demultiplex samples by plate barcode, trim the Illumina adapters, and generate raw fastq reads (Illumina 2019).

# 2.3. Cleaning, filtering, and calling SNP genotypes

Sequencing of all 1,370 individuals resulted in over 2 billion reads. We used *fastqc* (Andrews 2017) to check for overall sequence quality and found that the MspI restriction cut site on the reverse reads had poor per base sequence quality. *Trimmomatic* (v 0.39 (Bolger et al. 2014) was used to trim the MspI cut site from the reserve reads which resulted in 98 bp fragments for the reverse read (the forward reads were 93 bp long). Samples were demultiplexed into individuals using the *STACKS* (v 2.53; Rochette and Catchen 2017; Rochette et al. 2019) *process_radtags* module (settings: --inline_null, --renz_1 pstI, --quality, --rescue, --barcode_dist_1, s = 20, w = 0.25) to remove the barcode and discard reads that had either a PHRED quality < 20 in a sliding window of 25 bases, or those with more than one mismatch in the barcode or cut site (Rochette et al. 2019). This resulted in a small fraction of reads being discarded from each plate (3.1% to 3.6%). We aligned reads to an *Anaxyrus boreas* reference genome (Trumbo et al. 2023). Ninety three percent of reads mapped with mapping quality over 30. The *STACKS* reference pipeline generated 2,870,202 loci ("stacks") for the full dataset with a mean depth of 4.2 reads per locus, and 1,735,683 SNPs for the Haida Gwaii dataset with a mean depth of 3.4 reads per locus. As per the recommendation of Rochette et al. (2019), we did not remove PCR duplicates because we used a GBS protocol that produces identical length inserts, and removing PCR replicates only works for variable length inserts.

All filtering steps and numbers of remaining SNPs and individuals are in Table 1. The *STACKS populations* module was used to filter reads and generate an ordered vcf file. Loci with a maximum observed heterozygosity ($H_o$) > 0.6 across all populations were removed as these were likely the result of paralogs rather than allelic variation (Geraldes et al. 2019). Loci with a minor allele frequency (MAF) ≥ 0.01 were retained (Gallego-García et al. 2019). Only loci that were genotyped in ≥ 60% of individuals within each population and in ≥ 60% of all individuals were retained. To calculate nucleotide diversity (π),

the “write single SNP” filter (and all additional filters below) was not applied to ensure the dataset had both variant and invariant loci. The dataset used to calculate π had 1,370 individuals and 165,731 sites (both invariant and variant) for the full dataset and 385 individuals and 75,256 sites for Haida Gwaii. For all other analyses besides nucleotide diversity, SNPs were filtered to only include the first SNP per read because SNPs in the same stack/locus are linked (Morin et al. 2009).

Next, using *vcftools* (Danecek et al. 2011), we removed SNPs that: (1) had insertion/deletion (indel) polymorphisms; (2) were not biallelic; (3) had a genotype and site quality less than 20; or (4) had less than 5x coverage (minDP 5).

SNPs and individuals then were cyclically filtered for missingness again in *vcftools* as recommended by O’Leary et al. (2018) for datasets with high missingness. To avoid losing all SNPs: (1) SNPs with more than 40% missing data were removed; (2) then individuals with more than 40% missing data were removed; (3) then SNPs with more than 20% missing data were removed; and (4) finally then individuals with more than 30% missing data were removed. The differentiation trends were consistent across missingness thresholds, so we used the dataset with the most SNPs and individuals.

We filtered for MAF again in *vcftools* and removed SNPs with MAF ≤ 0.03 across the whole dataset. Following this filtering, 46 of the 47 breeding sites had between two (one site in the Lower Mainland) and 26 samples remaining except for the site on Lasqueti Island, which had no samples left.

SNPs were then filtered for maximum heterozygosity within each region – SNPs with a maximum heterozygosity > 0.6 were removed using *filter* from the *R* package *dplyr* (Hadley Wickham, Romain François 2021).

Although numerous studies advocate for removing siblings (O’Connell et al. 2019), if not done correctly, removing siblings can do more harm than good (Waples and Anderson 2017). As a result, we did not filter for potential siblings, and followed the protocol in Forester et al. (2022), because we sampled widely at each site to avoid sibling groups.

SNPs that did not meet Hardy-Weinberg expectations (HWE) ($p < 0.01$) were removed using the *seppop* and *hw.test* functions from the *R* packages *adegenet* and *pegas* (Jombart 2008; Paradis 2010). HWE was tested within each breeding site and SNPs were removed that were out of HWE in at least two thirds of breeding sites.

SNPs that had outlier estimates of inbreeding (average $F_{IS}$) were removed per breeding site (Shafer et al. 2017), using the *basic.stats* function from the *hierfstat R* package (Goudet 2005). The distribution of $F_{IS}$ values before and after filtering can be seen in Fig. S1. The final filtered dataset included 766 individuals and 1,368 SNPs for the full dataset (46 sites), and 225 individuals and 603 SNPs for the Haida Gwaii dataset (13 sites).

# 2.4. Analyses

# 2.4.1. Genetic differentiation

To assess genetic differentiation across the sampled range of the Western Toad, we ran a PCA using the *glPca* function from the *adegenet R* package (Jombart 2008) for 1) the full dataset, and 2) just within Haida Gwaii. Our *a priori* number of populations for the full dataset was four: Haida Gwaii, Vancouver Island, Lower Mainland, and Northwest BC. The *a priori* number of populations within Haida Gwaii was two as discussed above: the southern locations within Gwaii Haanas, and northern Haida Gwaii locations on Graham Island plus the northernmost site on Moresby Island.

We ran a *STRUCTURE* analysis, a Bayesian clustering method, to investigate individual population assignment (Pritchard et al. 2000), for 1) the full dataset, and 2) just within Haida Gwaii. We converted the filtered genind file to the *STRUCTURE* format using the *genind2structure* conversion in R (Clark 2017), then ran *K* values 1 to 13 for the full dataset, and also *K* values 1 to 13 for Haida Gwaii (maximum sites), with five replicates for each *K* value. The upper *K* limit of 13 was a compromise between the maximum possible total number of sites included in analysis (46) and computational time. Each replicate had a burn-in period of 75,000 generations, followed by 250,000 iterations. Structure Harvester was used to determine the optimal *K* value with the Evanno method (Evanno et al. 2005; Earl 2012). Note that the Evanno cannot select K = 1. We used *CLUMPAK* to average across independent replicates and create membership plots of the optimal *K* value and a number of other *K* values (Kopelman et al. 2015).

A DAPC with k-means clustering and model selection was also conducted to test for the number of populations for both datasets (Jombart et al. 2010). We used the *dapc*, *find.clusters* and *scatter* functions in the *adegenet R* package (Jombart 2008). To choose the number of principal components for DAPC, we used the *optim.a.score* function. From this, the Bayesian Information Criterion (*BIC*) was plotted against the number of clusters to determine the optimal number of clusters.

$F_{ST}$ among regions and within regions was calculated with the Weir and Cockerham (1984) method, using the *hierfstat R* package (Goudet 2005) for both datasets. The *pairwise.WCfst* function was used for the among cluster pairwise weighted $F_{ST}$, and the *wc* function was used to calculate within cluster $F_{ST}$. The statistical significance of the pairwise $F_{ST}$ estimates was calculated with the *stamppFst* function from the *StAMPP R* package (Pembleton et al. 2013). The distribution of (non-weighted) pairwise $F_{ST}$ estimates per locus between Haida Gwaii and elsewhere in coastal BC was plotted using the *hierfstat R* package.

To test whether isolation-by-distance (IBD) was a possible explanation for genetic divergence between breeding sites within Haida Gwaii, a Mantel test was conducted using the *mantel.randtest* function from the *R adegenet* package (Jombart 2008). We took the natural log of the distance (in kilometers) between geographic coordinates for the 13 breeding sites on Haida Gwaii as calculated by the *earth.dist* function in the *R fossil* package (Vavrek and Vavrek 2020), and calculated genetic differentiation between the same sites as ($F_{ST}$ / 1- $F_{ST}$) (Rousset 1997; Diniz-Filho et al. 2013). The *p* value of the Mantel test

provides an indication of the significance of a potential isolation-by-distance signal (i.e., genetic differentiation and geographic distance are correlated).

## 2.4.2. Genetic diversity

We assessed whether levels of genetic diversity and inbreeding differed between Western toads on Haida Gwaii and those elsewhere in coastal BC, and how genetic diversity varied spatially within Haida Gwaii. Nucleotide diversity (π), the number of variant sites out of the total number of sites, was calculated in the *STACKS populations* module across all assembled reads (Hohenlohe et al. 2010). Expected heterozygosity was calculated using allele frequencies generated with the *summary* function in the *adegenet R* package (Jombart 2008). The inbreeding coefficient ($F_{IS}$) was calculated using the *basic.stats* function in the *hierfstat R* package (Goudet 2005). We tested for significant differences for all the above analyses in section 2.4.2 by calculating 95% confidence intervals.

For the full dataset (46 breeding sites; 766 individuals; 1368 SNPs), a site frequency spectrum (SFS) was generated for each of the four regions to assess whether there may have been a population bottleneck or founder effect on Haida Gwaii. The SFS was generated using the *maf* function in the *vcfR* package (Knaus and Grünwald 2017), after splitting the VCF file into four regions.

# 3. Results

## 3.1. Genetic differentiation between Haida Gwaii and other coastal BC populations

Western Toads on Haida Gwaii were strongly differentiated from other coastal BC populations. This result was consistent across the PCA, *STRUCTURE*, and $F_{ST}$ analyses. In the PCA (Fig. 3a), the first principal component (PC1) explained 28.2% of the variation and clearly separated Haida Gwaii from the two other regions. PC2 explained just 3.9% of the variation and did not separate Haida Gwaii from the other regions, but somewhat separated Vancouver Island populations from the Lower Mainland. PCs 3 and 4 explained substantially less variation (2.5% combined) (Supp. mat.; Fig. S2).

The Evanno method used in *STRUCTURE*, and *BIC* test on the DAPC analysis suggested the most probable number of genetic clusters to be $K = 2$ and K = 5, respectively (Supp. mat.; Fig. S3; Table S2). However, the *STRUCTURE* population assignment plots showed support for both $K = 2$ and $K = 3$. For the *STRUCTURE* plot of $K = 2$, all toads from Haida Gwaii showed 100% membership to one cluster, while all non-Haida Gwaii sites had membership to the second cluster (Fig. 3b). All toads in Northwest BC, and some in the Lower Mainland and Vancouver Island had small to moderate amounts of membership to the cluster predominantly composed of individuals from Haida Gwaii (Fig. 3b). For the $K = 3$ *STRUCTURE* plot, all toads on Haida Gwaii showed 100% membership to one cluster, while Vancouver Island toads showed membership to a distinct cluster, with some Lower Mainland toads sharing membership to this cluster. Northwest BC toads showed membership to all three clusters. The pairwise $F_{ST}$ estimate

between Haida Gwaii and the rest of coastal BC was very high (0.363; $p < 0.05$), indicating strong genetic divergence between Haida Gwaii and all other coastal BC populations. When considering the four geographic regions, pairwise $F_{ST}$ estimates were highest between sites on Haida Gwaii and Northwest BC (0.640), followed by Haida Gwaii vs Vancouver Island (0.480), and Haida Gwaii vs the Lower Mainland (0.439). Pairwise $F_{ST}$ estimates among Northwest BC, Vancouver Island, and the Lower Mainland were much lower (≤0.112, $p < 0.05$; Table S1 shows all pairwise comparisons).

For the K = 2 clusters, $F_{ST}$ estimates among breeding sites were lowest for Haida Gwaii (0.080), with the cluster containing the rest of the BC sites having an $F_{ST}$ of 0.105. When comparing $F_{ST}$ estimates among breeding sites within regions, Haida Gwaii (0.080) sites had the highest pairwise $F_{ST}$ estimates, followed by the Lower Mainland (0.074), Vancouver Island (0.058) and Northwest BC (0.011). The distribution of pairwise $F_{ST}$ estimates per locus between Haida Gwaii and elsewhere in coastal BC was left skewed but also contained some high outliers (Supp. mat.; Fig. S4).

## 3.2. Genetic diversity

Genetic diversity was considerably lower on Haida Gwaii than in the other coastal BC study regions for nucleotide diversity and expected heterozygosity, but not the inbreeding coefficient ($F_{IS}$). Nucleotide diversity (π) was significantly lower on Haida Gwaii ($\pi_{mean}$ ± st. dev = 0.00003 ± 0.000007) than elsewhere in coastal BC (0.00026 ± 0.00008), but there were no significant differences between the three regions (overlapping CIs; Fig. 4a). Similarly, expected heterozygosity ($H_e$) across all SNPs passing filters was lowest on Haida Gwaii ($H_e$ mean ± SE = 0.008 ± 0.001; Fig. 4b). Among the other regions, Northwest BC (0.010 ± 0.004), with only three breeding sites sampled within relatively close geographic proximity, had significantly lower $H_e$ than the Lower Mainland and Vancouver Island regions (0.140 ± 0.002 and 0.138 ± 0.003 respectively). The inbreeding coefficient ($F_{IS}$) was near zero in the Lower Mainland and northwest BC and differed significantly from the negative values of Haida Gwaii and Vancouver Island (Fig. 4c; Table S4).

The distribution of allele frequencies (SFS) across all loci shows the Haida Gwaii population had more monomorphic loci and rare alleles than Northwest BC, Lower Mainland and Vancouver Island regions (Fig. 5). These SFS patterns are consistent with a population bottleneck or founder effect on Haida Gwaii (consistent with an isolated island). Vancouver Island also has a skewed distribution reflective of an island population, though it is less geographically isolated than Haida Gwaii, and much larger. Both Haida Gwaii and Northwest BC had a high number of fixed SNPs.

## 3.3. Population structure and genetic diversity within Haida Gwaii

The Mantel test revealed no significant correlation between genetic and geographic distance within Haida Gwaii (R = 0.16, $p = 0.052$; Fig. 6c). The PCA of samples from within Haida Gwaii showed three clusters: Gwaii Haanas, Northern Haida Gwaii, and Gudal Lake – a single site on the southwest coast of

Graham Island, and the only known breeding site on the west coast of Haida Gwaii (Fig. 2). PC1 explained 9.6% of the variation and separated Gwaii Haanas samples from Northern Haida Gwaii individuals (Fig. 6a); PC2 explained 4.9% of the variation and separated Gudal Lake from the rest of Haida Gwaii. PCs 3 and 4 explained a total of 5.9% of the genetic variation (Supp. mat.; Fig. S5). The one other breeding site on Moresby Island that was outside of Gwaii Haanas (Moresby Adventure Camp) clustered with the sites on Graham Island, rather than those on Moresby Island and hence was part of the “Northern Haida Gwaii” group.

The Evanno method used in *STRUCTURE*, and *BIC* test on the DAPC analysis of Haida Gwaii sites suggested the most probable number of genetic clusters to be $K = 2$ and K = 3–7, respectively (Supp. mat.; Fig. S6; Table S3). However, the *STRUCTURE* population assignment plots showed support for both $K = 2$ and $K = 3$. For $K = 2$, all Gwaii Haanas samples had > 80% membership to the Gwaii Haanas cluster, and most Northern Haida Gwaii samples had > 80% membership to the Northern Haida Gwaii cluster (Fig. 6b). For $K = 3$, all the individuals from Gudal Lake had > 80% membership in a third cluster (Fig. 6b). Pairwise $F_{ST}$ for the two clusters identified by *STRUCTURE* indicated moderate differentiation between Gwaii Haanas and northern Haida Gwaii (which includes Gudal Lake) ($F_{ST} = 0.076$; $p < 0.05$). When considering $K = 3$ clusters, pairwise $F_{ST}$ estimates were highest between sites from Gwaii Haanas and Gudal Lake (0.253), followed by Gudal Lake versus northeastern Haida Gwaii (0.185), and then Gwaii Haanas versus northeastern Haida Gwaii (0.072) ($p < 0.05$). In line with the PCA and structure results, differentiation between Gwaii Haanas and Northern Haida Gwaii was stronger than between all Moresby Island sites combined versus Graham Island (0.067; $p < 0.05$).

Genetic differentiation ($F_{ST}$) among individual breeding sites on Haida Gwaii ranged from 0.007 to 0.263 ($p < 0.05$; Supp. mat.; Table S5). $F_{ST}$ estimates among breeding sites were higher within Northern Haida Gwaii (0.142), indicating comparatively lower population connectivity than among breeding sites within Gwaii Haanas (0.059). This result remained consistent whether including or excluding Gudal Lake from the Northern Haida Gwaii cluster ($F_{ST}$ within the Northern Haida Gwaii cluster without Gudal Lake was 0.111).

# 4. Discussion

Most landscape genetics studies examine populations within a single region, but analysis of multiple regions allows for comparisons between areas that differ in structure or environment (Moore et al. 2011). Motivated by apparent low density, recent concern for possible extirpations in Haida Gwaii, threats from invasive species, landscape disturbance in an isolated insular environment, and the apparent lower competitive abilities of Haida Gwaii Western Toads when compared to their coastal British Columbia counterparts (Gamlen-Greene 2022), we performed genomic analyses of 47 Western Toad breeding sites from four regions in coastal BC to compare genetic structures and inform the conservation of toads in Haida Gwaii. We found that the Haida Gwaii population was much less genetically diverse than and highly differentiated from other populations in coastal BC. Interestingly,

populations within Haida Gwaii were not segregated by the two main islands (Graham and Moresby). Instead, the dominant population structure corresponded to the southern islands of Gwaii Haanas including south Moresby Island, Gudal Lake on the southwest coast of Graham Island, and the rest of the Northern Haida Gwaii breeding sites across north Moresby and Graham Island.

# 4.1. Genetic diversity and differentiation between Haida Gwaii and other coastal BC populations

Population clustering and $F_{ST}$ analyses showed that the Haida Gwaii population is genetically distinct from other coastal BC populations. In comparison, Vancouver Island and Lower Mainland populations are much less differentiated from one another. The pairwise $F_{ST}$ estimate (0.363) between Haida Gwaii and all other sites in coastal BC is relatively high, and is similar to levels of differentiation often seen between subspecies using SNP data (Fredrickson et al. 2015; Colella et al. 2018; Winker 2021). The $F_{ST}$ is higher than that found between Northern Goshawk (*Accipiter gentilis*) populations, the only other study that calculated pairwise $F_{ST}$ between populations of a species on and off Haida Gwaii using SNP data. For the Northern Goshawk, the weighted pairwise $F_{ST}$ between Haida Gwaii and Vancouver Island was 0.079, and weighted pairwise $F_{ST}$ between Haida Gwaii and other coastal BC populations was 0.093 (Geraldes et al. 2019).

Our results suggest that populations on Haida Gwaii have historically been smaller than and less genetically connected to those on the mainland compared to Vancouver Island, resulting in higher levels of genetic drift, stronger population differentiation, and lower genetic diversity. Both Haida Gwaii's smaller land area and large distance from the mainland may have contributed to greater genetic drift on the archipelago. Toad populations on Haida Gwaii may have lower genetic diversity because a small number of toads founded the original Haida Gwaii population, or because of a population bottleneck that occurred at some point, for example, if toads were present on the island in unglaciated refugia during the Last Glacial Maximum.

The Haida Gwaii population is strikingly less genetically diverse than toad populations elsewhere in coastal BC – consistent both with expectations for small island populations (Frankham 1997, 1998) and nucleotide diversity levels seen in amphibian populations in a historically glaciated landscape (Chiocchio et al. 2021). Although Vancouver Island is also insular, it is three times larger than Haida Gwaii, and though both Haida Gwaii and Vancouver Island are ~ 50km from the mainland at their closest point, most of Haida Gwaii is much further from the mainland (up to 144km) with nothing between the archipelago and the mainland but the open and shallow Hecate Strait (Fig. 1). Vancouver Island is on average closer to the mainland, and the Johnstone Strait separating the north half of Vancouver Island from the mainland is filled with many small islands, some occupied by Western Toads, potentially acting as stepping stones to and from the mainland. This may have allowed for multiple founding events and reduced the impact of genetic drift on Vancouver Island.

Haida Gwaii also had a higher frequency of rare alleles and fixed loci compared to other coastal BC populations. Together, low genetic diversity, high genetic differentiation and more rare alleles suggest that the Haida Gwaii population has been isolated for a long time, and that toads on Haida Gwaii may have experienced a population bottleneck or founder effect, resulting in a small effective population size, strong genetic drift, and ultimately a loss of genetic variation. Genetic diversity (nucleotide diversity, expected heterozygosity) is similarly low across all Haida Gwaii breeding sites, which may indicate the founder effect or bottleneck occurred long ago (Nei et al. 1975; Allendorf et al. 2022).

Several studies have tested for founder or bottleneck effects on different species on Haida Gwaii. In one such study, Reimchen et al. (2013) detected the signature of a founder effect in three-spined stickleback (*Gasterosteus aculeatus*), and in another study, two vascular plants (*Senecio newcombei* and *Saxifraga taylori*) showed levels of diversity consistent with bottleneck or founder effect (Goertzen 1996). Conversely, evidence of these events was not detected in several birds (Pruett et al. 2013), or kelp (Brooks 2020), likely due to the high dispersal ability of these species.

It is unknown when toads arrived on Haida Gwaii, but they likely colonized the archipelago prior to the submersion of the Hecate Strait land bridge ~ 10,000 YBP given that they are unlikely to disperse through ~ 100km of seawater, and are now highly genetically differentiated from the mainland. The Hecate land bridge in the Holocene existed after the LGM between ~ 11,700 and 10,000 years ago (Hetherington et al. 2004; McLaren et al. 2020), and may have acted as a migration corridor across the Hecate Strait for toads, as it likely did for a number of other species (Reimchen et al. 2005). The land bridge is thought to have been a mosaic of forest, open freshwater, and marine marsh habitat (Fedje and Josenhans 2000; Lacourse et al. 2003), ideal for toad dispersal. Alternatively, toads may have arrived on Haida Gwaii before the LGM and persisted. There is evidence that parts of Haida Gwaii were likely unglaciated refugia during the LGM (Clague et al. 1989; Shafer et al. 2010; Pruett et al. 2013; Barrie et al. 2014). It is unknown whether there were coastal toad populations at Haida Gwaii latitudes prior to the LGM, but Western Toad remains from 18,000 to 16,000 years ago have been recorded in karst caves on the northern tip of Vancouver Island (Harington 2011). Future research could investigate divergence time, to better test whether Haida Gwaii toads diverged from other coastal BC toad populations before or after the last glaciation. Haida Gwaii has been posited to be a source of post-glacial recolonization of the continental Pacific Northwest via the Hecate land bridge for some species, such as the black bear (*Ursus americanus*) (Reimchen et al. 2005).

- The inbreeding coefficient was near zero in Lower Mainland and Northwest BC populations, but both Vancouver Island and Haida Gwaii had slightly negative $F_{IS}$ estimates, indicating less inbreeding. The sample size in the Northwest region was only three breeding sites within ~ 21 km of one another, while other regions had a much wider spatial sampling distribution (Fig. 1), potentially explaining the result. The Lower Mainland region is complex in terms of topography and human land use, with samples sites distributed across fjords, coastal mountains and high-density human settlements, potentially isolating sub-regions and influencing the result (Fig. 1). Alternatively, results

might indicate different breeding behaviour in the two insular populations. Further research is needed to evaluate this result.

## 4.2. Population structure within Haida Gwaii

Toad breeding sites within Haida Gwaii were grouped into two or three populations based on PCA, *STRUCTURE* and DAPC results. Since isolation-by-distance is absent, the genetic structure could reflect saltwater and topographic barriers, glaciation history, genetic drift, or a combination of these factors. The influence of saltwater and topographic barriers varies widely within southeast Alaska. Miller et al. (2011) reported a significant influence on Western Toad genetic structure, while Holmes (2015) did not find that such landscape barriers isolated populations. Both landscape variability (Moore et al. 2011) and seasonal variability in breeding (Holmes 2015) influenced the study and interpretation of barriers in these Alaskan landscapes, and similar caution should be taken when interpreting the Haida Gwaii results. Northern Haida Gwaii breeding sites are more strongly structured than Gwaii Haanas breeding sites (higher within-region $F_{ST}$), even when controlling for geographic distance. Higher differentiation among northern Haida Gwaii breeding sites may have resulted from smaller populations or less gene flow. While Gwaii Haanas is a protected area, Northern Haida Gwaii (Graham Island and north Moresby Island) is subject to the pressures of commercial forestry and human settlement, with the associated fragmentation of landscapes due access development and logging potentially affecting movement behaviour and connectivity between breeding sites.

The strong differentiation of the Gudal Lake population on the west coast of Graham Island, despite proximity to several other breeding sites, may be evidence for it being descended from a different glacial refugium. There was likely little to no ice cover on the west coast of Haida Gwaii during the last glaciation (Barrie et al. 2014). Alternatively, the high differentiation could be due to topographic barriers preventing gene flow. The Gudal Lake site is isolated from the other sampled breeding sites by mountains over 1000m high, and rugged areas are known to reduce amphibian dispersal (Funk et al. 1999). Locating more west coast sites for genotyping would help assess this.

## 4.3. Conservation implications

Our results demonstrate the genetic uniqueness and vulnerability of the Haida Gwaii Western Toad population within coastal BC, as well as the importance of maintaining connected breeding habitats within landscapes. Based on our findings, we recommend the Haida Gwaii Western Toad be considered as a distinct population segment in the conservation management of the species in BC. In partnership with the Haida Nation, COSEWIC (Committee on the Status of Endangered Wildlife in Canada) might consider establishing Haida Gwaii as a Designatable Unit for Western Toads under Canada's Species At Risk Act. Designatable Units (DU), comparable to Evolutionary Significant Units (ESU) under the United States Endangered Species Act (Moritz 1994), represent discrete and evolutionarily significant units where there is little transmission of heritable (cultural or genetic) information from other units, and there is either evolutionary history or heritable adaptive traits not found elsewhere in the Canadian range of the species (COSEWIC 2023). The Haida Gwaii population appears to meet these conditions. These animals

are isolated geographically with no interaction with other populations in coastal BC, and of cultural significance to the Haida Nation, the entirety of which reside within the remote Haida Gwaii archipelago. Recent research (Gamlen-Greene 2022) suggests the Haida Gwaii population may be behaviourally distinct, making them vulnerable and less adaptable to novel threats. This current research finds the Haida Gwaii toad population to be genetically distinct from the rest of coastal BC with very low diversity suggesting a unique evolutionary history where populations have been isolated for a long time and may have experienced a population bottleneck or founder effect long ago.

Of the endemic taxa on Haida Gwaii, most are differentiated from their mainland relatives at the level of subspecies, while some are even classified as different species (Reimchen et al. 2005). Given the relatively high level of observed differentiation and physical isolation, Haida Gwaii Western Toads may meet the criteria for describing a subspecies (Winker 2021). Anecdotal observations suggest that they are morphologically similar to their coastal BC relatives, though this has not been quantified. Additional genetic data, including mitochondrial DNA sequencing, and whole genome sequencing of a small number of mainland and island individuals would be informative to help resolve whether this distinction is necessary.

Designating Haida Gwaii as a DU for Western Toads would more accurately reflect the unique evolutionary history of this insular lineage and potentially provide land managers with more tools to protect and conserve this species on Haida Gwaii. Designation would allow the conservation status of Haida Gwaii toad populations to be tracked independently of the other non-calling Western Toad populations in BC over time, encouraging additional resources for research and conservation.

Low genetic diversity of the Haida Gwaii population may be cause for concern. Reductions in genetic diversity can reduce fitness and the potential of populations to adapt to future environmental changes (Frankham 2005; Bouzat 2010; DeWoody et al. 2021). Low diversity may result in the Haida Gwaii population being more vulnerable to diseases (such as chytrid fungus; *Batrachochytrium dendrobatidis*), having a reduced ability to adapt to climate change, or lacking adaptations to avoid invasive predators. Our results suggest that translocations of toads from any of the other coastal BC populations to Haida Gwaii for genetic rescue or restoration would not be advisable without further evaluation because this could potentially lead to outbreeding depression (Frankham et al. 2011). The Haida Gwaii population may have developed local adaptations to the environment or coadapted gene complexes that could be disrupted if they interbred with other coastal BC populations.

As noted in this study, known occupied breeding sites in coastal BC are widely dispersed within landscapes and regions and the effort and constraints to identify occupied breeding sites limits their known abundance and distribution. Given that Haida Gwaii consists of many islands, habitat connectivity is expected to be lower, making toad populations more vulnerable to habitat loss or degradation from forest harvest, urban and rural development, or climate-related drought or fire. If one population within Haida Gwaii declines, it is unlikely there will be enough movement or dispersal to replenish population size and genetic diversity in a short timeframe. Given the relatively high differentiation among breeding

sites within Haida Gwaii, particularly in Northern Haida Gwaii, outside of Gwaii Haanas, maintaining the potential for movement of individuals and genes between better-connected sites is especially important. This study was limited to a small number of known sites located through years of effort, but much of the archipelago, including the west coast, remains unsurveyed. Improving the ability to survey and expand the number of known breeding populations within Haida Gwaii will better inform our understanding of population distribution and isolation within topographically and geographically challenged coastal landscapes. Further research into the drivers of apparent population declines on Haida Gwaii will facilitate refinement of land use management strategies and practices to protect this unique native amphibian population.

To aid conservation of Western Toads on Haida Gwaii, results strongly suggest protecting all known aquatic breeding sites from disturbance or degradation, protecting riparian habitats immediately around breeding sites to maintain access to and from aquatic habitats for the seasonal movements of breeding adults and dispersing juveniles, and developing strategies and best practices in managed forests to maintain habitat connectivity between known breeding sites. A similar conservation approach for Western Toad breeding habitats elsewhere across coastal BC may prevent potential loss of habitats, population declines, and further genetic isolation of broadly spaced core breeding habitats.

This study of such a low-density, cryptic amphibian within Haida Gwaii would not have been possible without the support of the Haida Gwaii community. We stress the importance of engaging local communities in conservation research and actions. The cultural significance the Western Toad has for the Haida emphasizes the need for sensitivity and engagement when making conservation decisions. Becoming involved in the local and Indigenous communities improved this research and will be critical to the effectiveness of conservation of the species moving forward. Our outreach in this research through classroom visits to local schools and contacting property owners has already improved monitoring and conservation of toads on Haida Gwaii.

## Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

# Author Contribution

RGG and MT conceived the idea; RGG, MT and JG designed the sampling; RGG led the discovery of toad breeding sites outside of Gwaii Haanas National Park Reserve, National Marine Conservation Area Reserve, and Haida Heritage Site; RGG led and funded the community engagement on Haida Gwaii; RGG conducted the field work on Haida Gwaii and around Squamish and MT conducted and led the field work elsewhere; MT provided critical technical amphibian and survey expertise and logistical assistance; JG suggested GbS; RGG conducted the bioinformatics and analyses with help from JG, HT and SNA; RGG funded the field work on Haida Gwaii and Squamish; MT funded the other field work, lab work and sequencing; RGG led the writing of the manuscript with assistance from JG, HT, SNA and MT. All authors contributed critically to the drafts and gave final approval for publication.

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# Data Availability

*R* code and scripts for the bioinformatics pipeline is available here: [https://github.com/RoseannaGG/Toad_HaidaGwaii_GBS](https:/github.com/RoseannaGG/Toad_HaidaGwaii_GBS)Data from Haida Gwaii are available on request from the BC Ministry of Forests, subject to review by the Council of the Haida Nation. Genomics for locations other than Haida Gwaii will be available on the UBC Dataverse Collection repository one year after publication (https://borealisdata.ca/dataverse/ubc).

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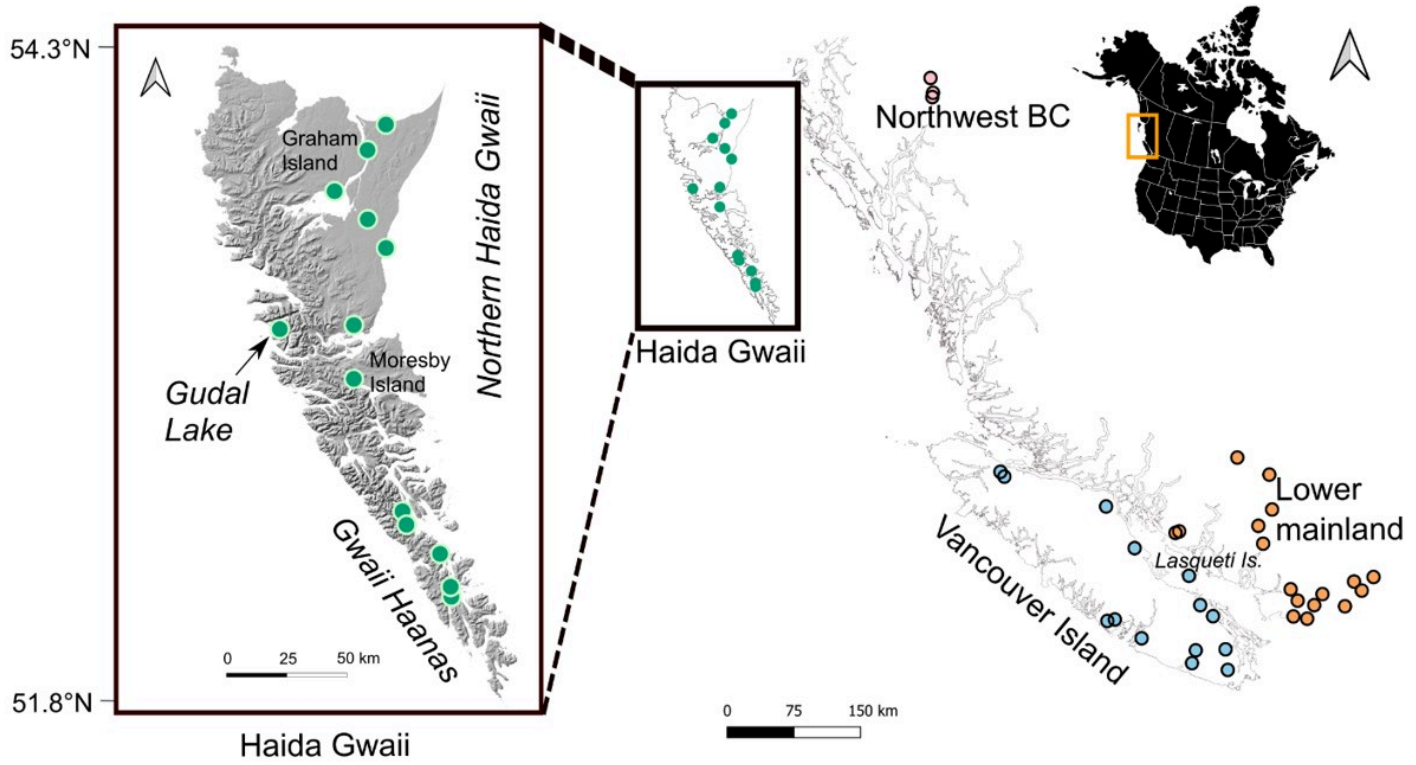
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# Figures




## Figure 1

Sampling locations of the 47 toad breeding sites used in this study.

Thirteen on Haida Gwaii (green; five of which are in Gwaii Haanas), 17 on the Lower Mainland (orange), three in Northwest BC (pink), and 14 on Vancouver Island and Lasqueti Island (blue).

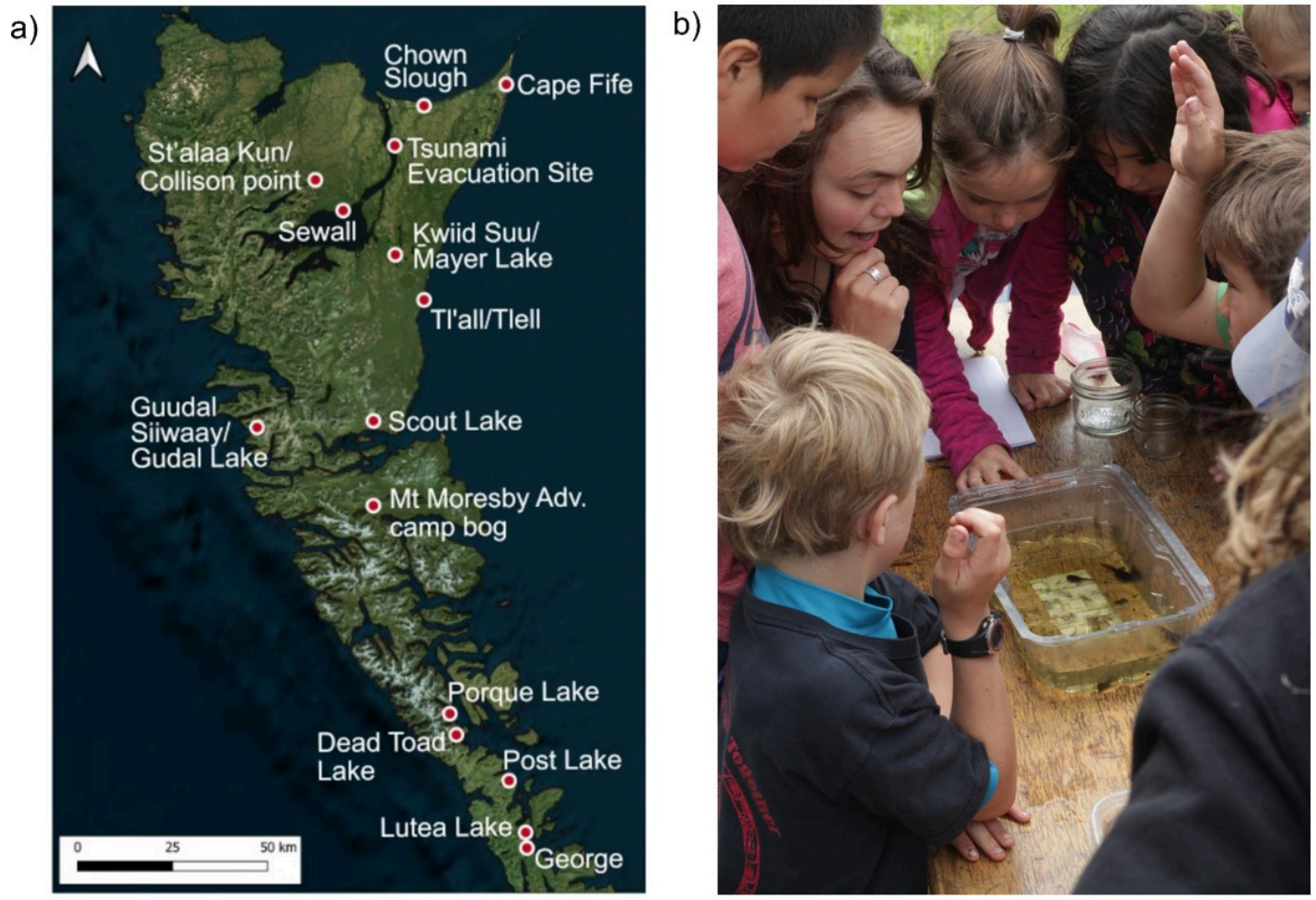
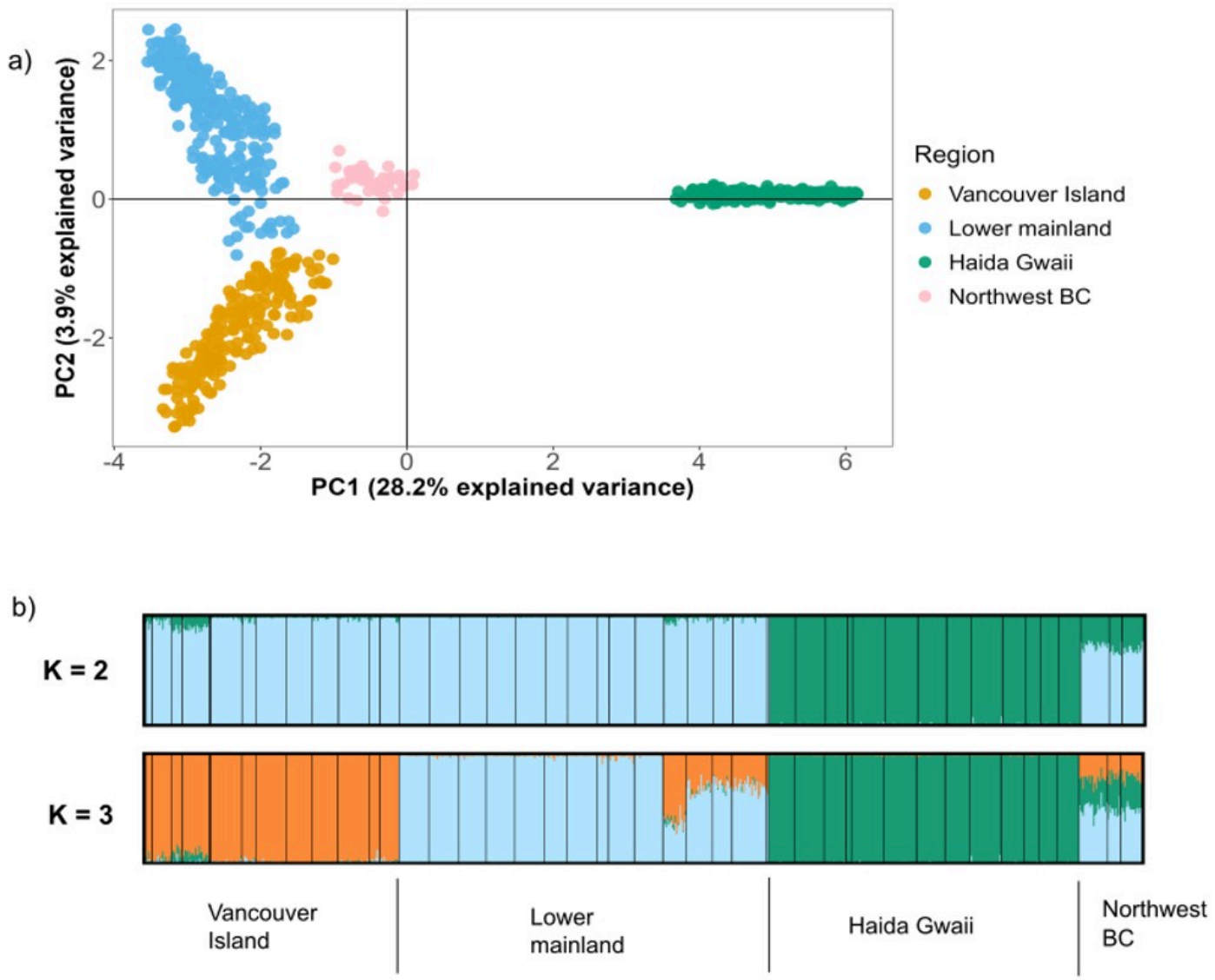



Figure 2

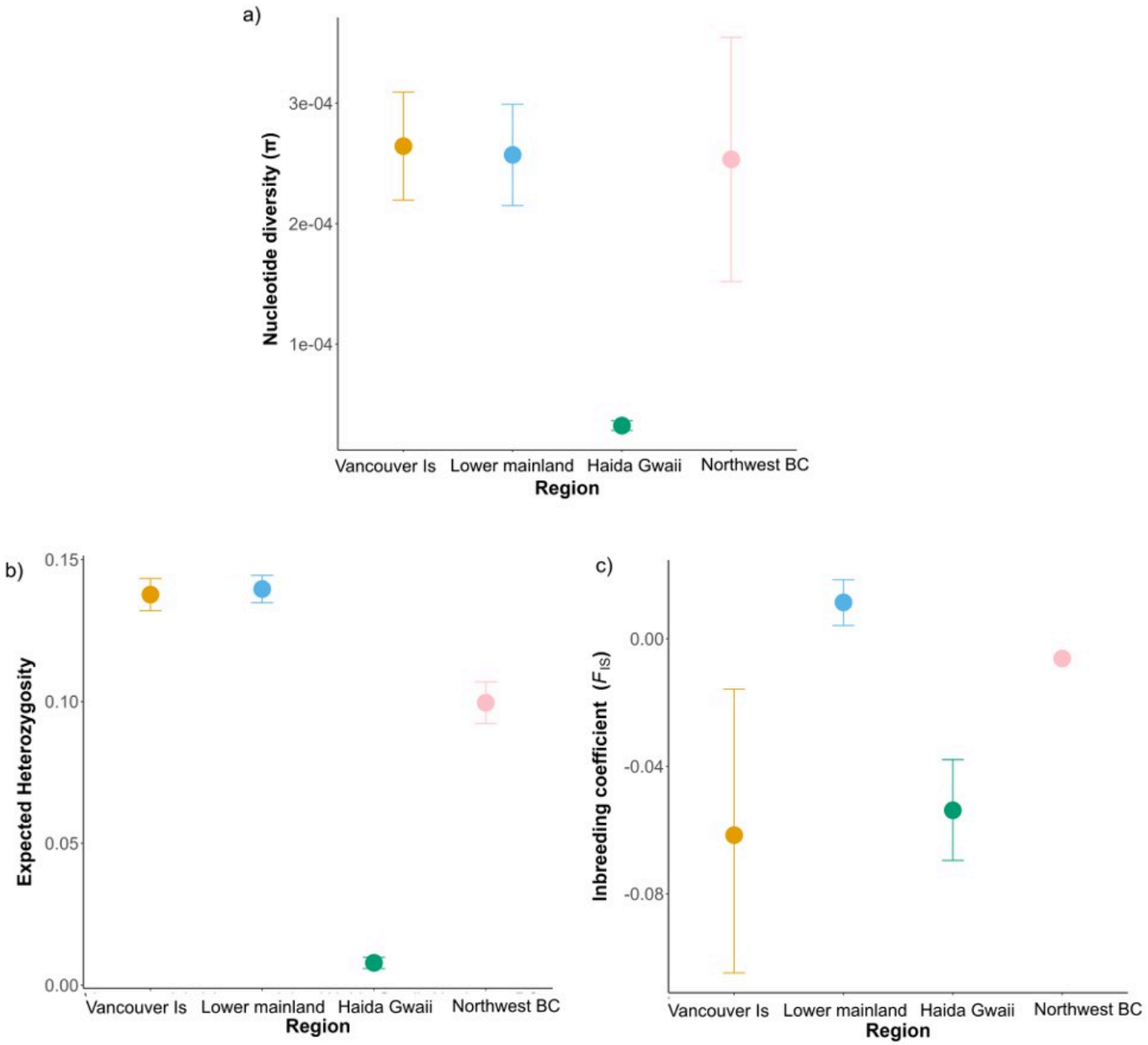
a) Toad breeding sites on Haida Gwaii identified in 2017-2019 surveys. b) Science engagement with Tahayghen Elementary School. Photo credit Andrew Hudson, Haida Gwaii Observer, 2017.




## Figure 3

Population structure of Haida Gwaii, Vancouver Island, Lower Mainland and Northwest BC toads.

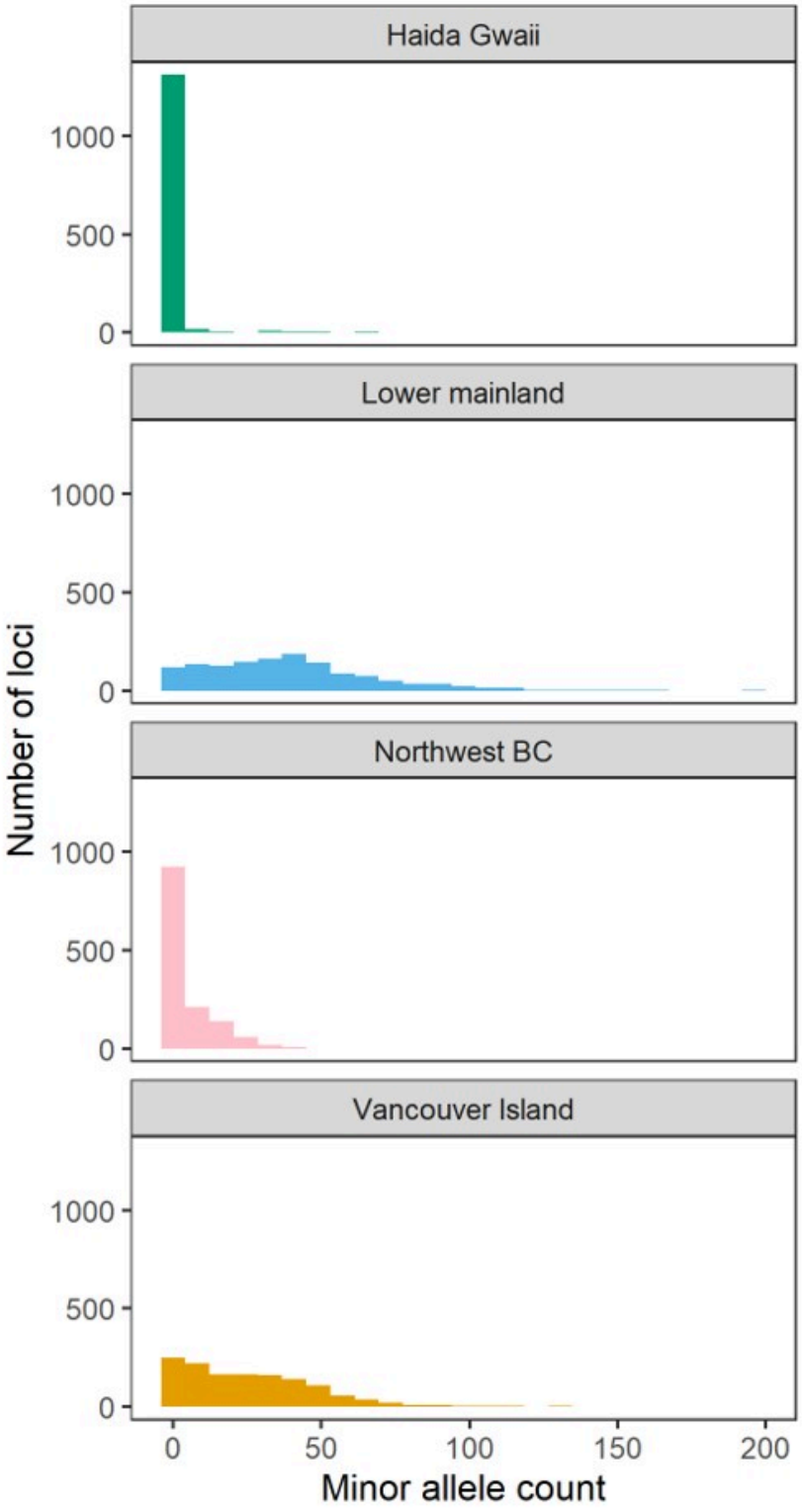
a) Plot of first two dimensions of a PCA where each point represents an individual and points are colour coded by study region and b) membership plot from the STRUCTURE analysis with K = 2 & 3, where each colored vertical bar represents an individual, colored according to cluster assignment, and black vertical lines separate breeding sites.




## Figure 4

Genetic diversity of the four regions (Vancouver Island – yellow, Lower Mainland – blue, Haida Gwaii – green, Northwest BC – pink) as described by:

a) Nucleotide diversity (π), b) expected heterozygosity ($H_e$), and c) inbreeding coefficient ($F_{IS}$) – all with mean (point) and 95% confidence intervals (whiskers).




## Figure 5

Site frequency spectrum (distribution of allele frequencies) for the Western Toad, for the four study regions in coastal BC.

Bars represent the number of loci with a given minor allele count.

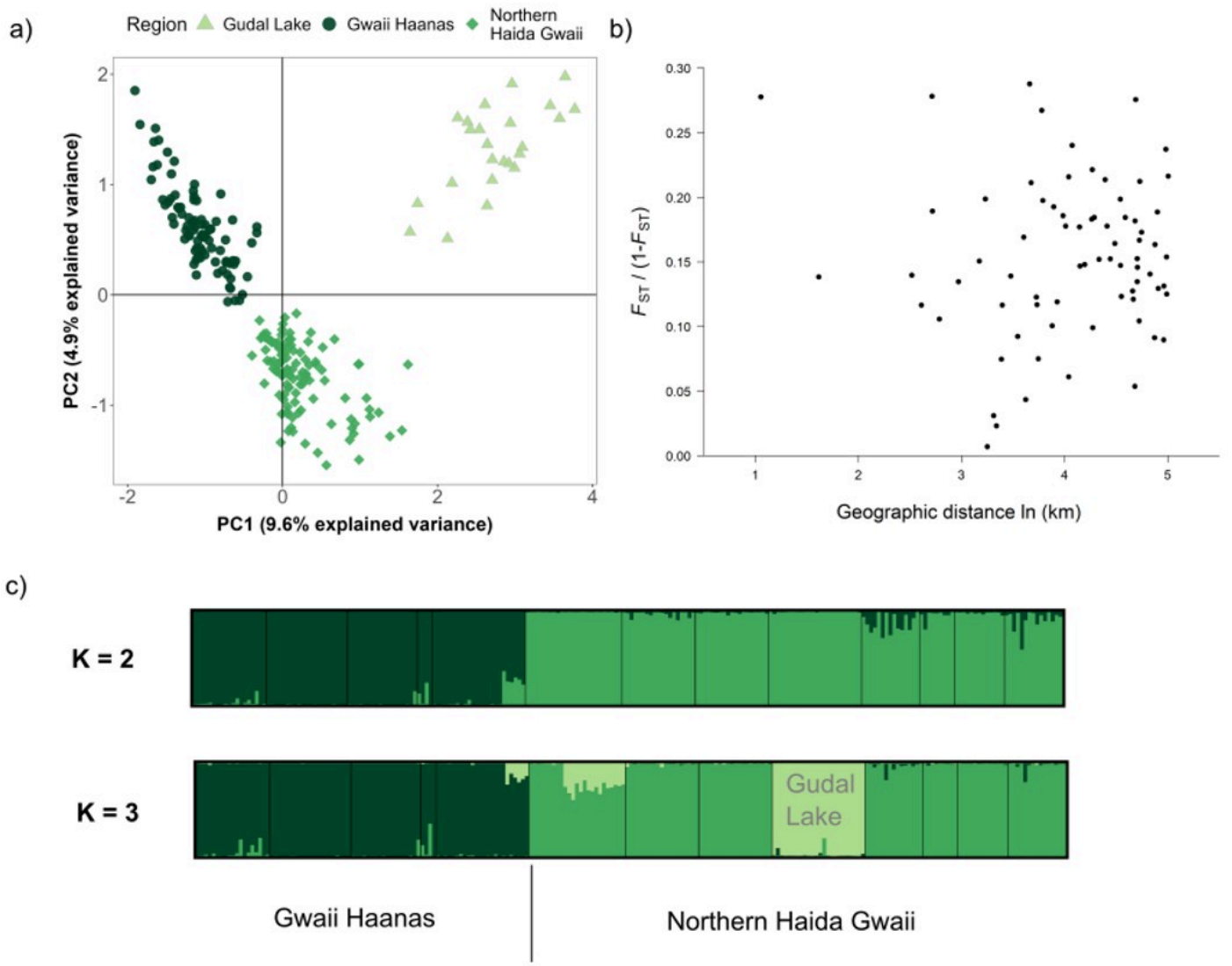



**Figure 6**

Population structure within Haida Gwaii displayed via a plot of the first two dimensions of a PCA (a) with each point representing an individual sample that is colour-coded based on cluster assignment from a *K*=3 *STRUCTURE* analysis. A plot showing the relationship between geographic and genetic distance (b) with genetic distance measured using $F_{ST}$ (y-axis) versus the geographic distance between breeding sites (points). Results from a STRUCTURE analysis of populations only from Haida Gwaii (c) using *K*=2 (top) and 3 (bottom) where each bar represents an individual colour-coded for cluster assignment and black vertical lines separate breeding sites. Note Gudal Lake labeled in the *K*=3 plot as a single breeding site clustering out separately.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GamlenGreeneetalSupMat080226.docx