

## RESEARCH ARTICLE

# Temporal Stability of Genetic Variability and Differentiation in the Three-Spined Stickleback (*Gasterosteus aculeatus*)

Jacquelin DeFaveri, Juha Merilä\*

Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, Helsinki, Finland

\* [juha.merila@helsinki.fi](mailto:juha.merila@helsinki.fi)
 OPEN ACCESS

**Citation:** DeFaveri J, Merilä J (2015) Temporal Stability of Genetic Variability and Differentiation in the Three-Spined Stickleback (*Gasterosteus aculeatus*). PLoS ONE 10(4): e0123891. doi:10.1371/journal.pone.0123891

**Academic Editor:** Lorenzo Zane, University of Padova, ITALY

**Received:** December 16, 2014

**Accepted:** March 2, 2015

**Published:** April 8, 2015

**Copyright:** © 2015 DeFaveri, Merilä. This is an open access article distributed under the terms of the [Creative Commons Attribution License](http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All genotype data used in this paper have been uploaded to Dryad repository: <http://doi.org/10.5061/dryad.p15h2>.

**Funding:** This research was supported by the Academy of Finland ([www.aka.fi](http://www.aka.fi); grant #:s 200940, 108601 and 118673 to JM) and LUOVA graduate school (JD). The research leading to these results has also received funding from the European Community's Seventh Framework Programme (FP/2007-2013) under grant agreement n°217246 made with BONUS (<http://www.bonusportal.org>), the joint Baltic Sea research and development programme. The funders had no role in study design, data

## Abstract

Temporal variation in allele frequencies, whether caused by deterministic or stochastic forces, can inform us about interesting demographic and evolutionary phenomena occurring in wild populations. In spite of the continued surge of interest in the genetics of three-spined stickleback (*Gasterosteus aculeatus*) populations, little attention has been paid towards the temporal stability of allele frequency distributions, and whether there are consistent differences in effective size ( $N_e$ ) of local populations. We investigated temporal stability of genetic variability and differentiation in 15 microsatellite loci within and among eight collection sites of varying habitat type, surveyed twice over a six-year time period. In addition,  $N_e$ s were estimated with the expectation that they would be lowest in isolated ponds, intermediate in larger lakes and largest in open marine sites. In spite of the marked differences in genetic variability and differentiation among the study sites, the temporal differences in allele frequencies, as well as measures of genetic diversity and differentiation, were negligible. Accordingly, the  $N_e$  estimates were temporally stable, but tended to be lower in ponds than in lake or marine habitats. Hence, we conclude that allele frequencies in putatively neutral markers in three-spined sticklebacks seem to be temporally stable – at least over periods of few generations – across a wide range of habitat types differing markedly in levels of genetic variability, effective population size and gene flow.

## Introduction

The study of evolution is ultimately about the study of changes in allele frequencies within populations over time. Allele frequencies in a given locus can change either due to deterministic (e.g. selection) or stochastic (e.g. migration, genetic drift, mutation) reasons [1]. In population genetics, as in evolutionary biology in general, allele frequency changes are more frequently and widely studied with synchronic (i.e. study of spatial genetic variation) than with allochronic approaches (i.e. study of temporal variation). With the notable exception of experimental evolution approaches undertaken in laboratory or mesocosms [2,3], the utility of allochronic approaches in population genetic studies of wild populations has traditionally been limited by

collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

access to historical samples [4,5]. Therefore, synchronic approaches have remained by far the most common way of inferring evolutionary transitions. However, inferences from patterns of spatial genetic variation at a given time point are subject to errors and biases, such as noise from intralocus sampling error [6], non-random sampling of relatives [6,7] or differentiation among sampled age classes [8]. Therefore, repeated sampling of the same localities at different time points provides an effective way of assessing if spatial genetic patterns—whether caused by neutral or selective processes—persist over time, and hence, can be reliably inferred from samples collected at one particular time point.

During the past decade, an increasing number of studies have investigated temporal changes in allele frequencies and population genetic parameters in the wild, both over long (i.e. many generations; [9,10]) and short (i.e. consecutive years [11,12]) time periods. Some have explored temporal stability of allele frequencies within high gene flow environments in order to assess the biological significance of low but statistically significant population differentiation (e.g. [11,13–16]). Others have been interested in temporal allelic shifts in small, closed populations [17], since stochastic effects such as drift are more likely to have a large effect on temporal genetic differentiation when effective population size is small [18]. However, simultaneous exploration of temporal stability of genetic parameters in populations with contrasting demographics/population structure (i.e. both with and without gene flow) is often not possible (but see [19] and [20] for interspecific comparisons) because many species do not demonstrate such contrasts in their population structure and demography, even over large areas (e.g. [16]).

In addition to informing us about population structure, temporal changes in allele frequencies in neutral loci can also allow for the estimation of effective population sizes ( $N_e$ ; reviews in: [18,21,22]).  $N_e$ -estimates in turn provide important information that can be applied in management and conservation of wild populations [22,23]: Low  $N_e$  increases the loss of genetic diversity and can thereby impede populations' capacity for adaptive change. Not surprisingly, temporal studies of genetic variability are particularly common in species of economic interest such as salmonids and marine fishes (reviews in [18,22,24,25]). However, species of more academic interest have less frequently been subject to rigorous tests of temporal stability of population genetic parameters.

The three-spined stickleback (*Gasterosteus aculeatus*) provides a case in point. This species has been subject to an increasing number of population genetic studies during the past decade (e.g. [26–33]). However, as far as we are aware, none of these studies have explicitly investigated the temporal stability of genetic variability and structuring of populations. For example, McCairns and Bernatchez [30] and Araguas et al. [34] each pooled temporal replicates sampled one year apart after finding no genetic differences between sampling years, and DeFaveri et al. [32] mentioned in passing that there was no differentiation among four Baltic Sea sites sampled six years apart. Hence, to what degree the observed patterns of structuring and variability reflect spatial, as opposed to temporal variability stemming from various factors capable of reducing effective population size [6,25], remains largely unknown. Moreover, strong reductions in genetic diversity in freshwater as compared to marine populations of three-spined sticklebacks (e.g. [31,35–37]) suggest that reductions in effective population size—and thereby pronounced generation-to-generation fluctuations in allele frequencies—are more likely to occur in freshwater as compared to marine localities. Likewise, given that the low degree of population differentiation in neutral marker loci in marine fish populations [38,39]—including sticklebacks (e.g. [31,32,40])—has been identified as making their allele frequency estimates disproportionately prone to sampling errors [6], studies in temporal stability of genetic parameters in marine stickleback populations are also warranted.

The aim of this study was to investigate temporal stability of within-population genetic diversity and among-population genetic differentiation in three-spined sticklebacks. To this end,

we recorded allele frequencies in 15 putatively neutral microsatellite loci in eight different sampling locations over a six-year period, corresponding to approximately two to three stickleback generations (cf. [41]). In order to probe whether temporal stability differs among habitat types—and therefore, between populations likely to differ in their effective sizes and the amount of gene flow between them—the sampling was conducted in two pond and two lake (i.e. closed populations) and four marine (i.e. open populations) sites. Furthermore, different single-sample and temporal methods were used to estimate effective population sizes in different sampling sites with the expectation that they would be smaller for finite freshwater habitats than for marine habitats.

## Material and Methods

### Ethics statement

The research described in this paper was conducted in strict accordance with the Finnish and Swedish legislation. Fishing rights in Finland belong to the landowner according to the Finnish Fishing Law (5§ 27.5.2011/600) and since the sampling occurred in government owned areas, the fish were collected under appropriate national fishing licenses (allowing capture and killing of the fish) possessed by persons involved in sampling. No ethical permission was required (verified from Animal Experiment Board in Finland) for the described scientific sampling according to the Finnish Animal Conservation Law (7§ 28.6.2013/498). The samples from Sweden in 2009 were collected in accordance with Swedish fishery regulation SFS 1994: 1716, Chapter 2 § 4 with permits from Länsstyrelsen Västerbotten (no. 620-4696-2009) and Länsstyrelsen Västra Götlands Län (no. 623-41555-2009). The 2003 samples were provided to us by the Swedish National Board of Fisheries under their own permits. The fish were sacrificed by an overdose of MS-222 (tricaine methanesulfonate) immediately upon their capture. Hence, suffering before anesthesia was minimal.

### Sampling

Adult sticklebacks were collected during the breeding season (May–June) in 2003 ( $n = 322$ ) and 2009 ( $n = 327$ ) from eight different localities (Table 1; Fig 1). Four of the localities are marine/brackish sites: one situated in the North Sea, and the others in the Bay of Bothnia and Gulf of Finland in the Baltic Sea. The data on 2009 samples from these four populations have earlier been used in ref [32]. The remaining four localities are freshwater sites from northern Finland, two of which are small ponds (approximately 100 m<sup>2</sup>; Table 1; Fig 1) and two are large lakes (1–12 km<sup>2</sup>; Table 1; Fig 1). The two pond populations are isolated, and hence, totally closed from migration. Although the two lake populations are connected to the Barent's Sea by rivers, they are phenotypically [42] and genotypically [43] divergent from potential source populations, and hence there is no reason to expect on-going gene flow. After collection, the fish were preserved in ethanol.

### DNA extraction and genotyping

DNA extractions were performed from pectoral fin clips using a 10% Chelex -100 resin (Bio-Rad Laboratories, Richmond CA). Fifteen microsatellite loci (STN3, STN23, STN24, STN30, STN38, STN42, STN79, STN110, STN123, STN132, STN146, STN168, STN174, STN185, STN195, [32]) were amplified in 10  $\mu$ l multiplex reactions with the following concentrations: 1  $\times$  Qiagen multiplex PCR master mix (Qiagen Inc. Valencia, CA, USA), 0.5  $\times$  Q-solution, 2 pmol of each forward (fluorescently labeled) and reverse primer and approximately 10–20 ng of template DNA. The PCR cycling profile was as follows: activation step at 95°C for 15 min,

**Table 1. Basic information and summary statistics by sampling locality for the 15 microsatellite loci.**

Locality	Coordinates	Area (km <sup>2</sup> )	Year	N	Age	H <sub>E</sub>	A	A <sub>R</sub>	A <sub>P</sub>	F <sub>IS</sub>	F <sub>ST</sub>
Baltic Sea Fiskebäckskil (FIS)	58°15'05"N, 11°27'06"E	∞	2003	35 (30)	2.7 (2–4)	0.78	10.7	9.4	0.3	-0.02	0.000
	58°14'05"N, 11°24'06"E		2009	48 (42)		0.76	11.0	9.1	0.4	0.05	
Baltic Sea Kotka (KOT)	60°27'18"N, 26°55'22"E	∞	2003	48 (45)	3 (2–4)	0.74	10.9	8.7	0.2	0.00	0.001
	60°33'55"N, 27°12'22"E		2009	43 (39)		0.75	11.1	9.0	0.3	0.02	
Baltic Sea Tvärminne (TVÄ)	59°50'20"N, 23°12'15"E	∞	2003	32 (26)	3.6 (3–5)	0.75	9.5	8.8	0.3	0.02	0.000
	59°50'20"N, 23°12'15"E		2009	29 (28)		0.74	9.3	8.5	0.2	0.01	
Baltic Sea Sikeå (SIK)	63°59'01"N, 20°53'17"E	∞	2003	32 (27)		0.75	9.8	8.6	0.1	-0.01	0.000
	64°09'34"N, 20°58'37"E		2009	35 (34)		0.74	9.7	8.6	0.2	0.02	
Lake Kevojärvi (KEV)	69°45'01"N, 27°00'55"E	1.02	2003	46 (40)	3.7 (2–6)	0.56	6.3	5.1	0.1	-0.02	0.000
	69°45'01"N, 27°00'55"E		2009	32 (29)		0.52	5.6	5.0	0.1	0.01	
Lake Pulmankijärvi (PUL)	69°58'46"N, 27°58'55"E	12.18	2003	42 (35)	2.8 (2–5)	0.63	7.0	6.0	0.1	-0.01	0.002
	69°58'46"N, 27°58'55"E		2009	44 (40)		0.62	6.5	5.5	0.0	0.05	
Pond Mieraslompolo (MIE)	69°34'10"N, 27°14'41"E	0.08	2003	41 (34)	2.5 (2–5)	0.20	2.9	2.3	0.1	-0.07	0.000
	69°34'10"N, 27°14'41"E		2009	48 (47)		0.18	2.7	2.2	0.0	0.11	
Pond Karilampi (KAR)	69°33'59"N, 27°14'35"E	0.13	2003	46 (45)	1.8 (1–3)	0.26	3.6	2.8	0.2	-0.01	0.004
	69°33'59"N, 27°14'35"E		2009	48 (44)		0.25	3.1	2.4	0.2	0.02	

N = sample size (number of complete genotypes scored), Age = average age in years (min—max) as determined in [42], H<sub>E</sub> = expected heterozygosity, A = number of alleles, A<sub>R</sub> = allelic richness, A<sub>P</sub> = number of private alleles, F<sub>IS</sub> = fixation index, F<sub>ST</sub> = pairwise genetic distance [50] between samples collected from the same locality

doi:10.1371/journal.pone.0123891.t001

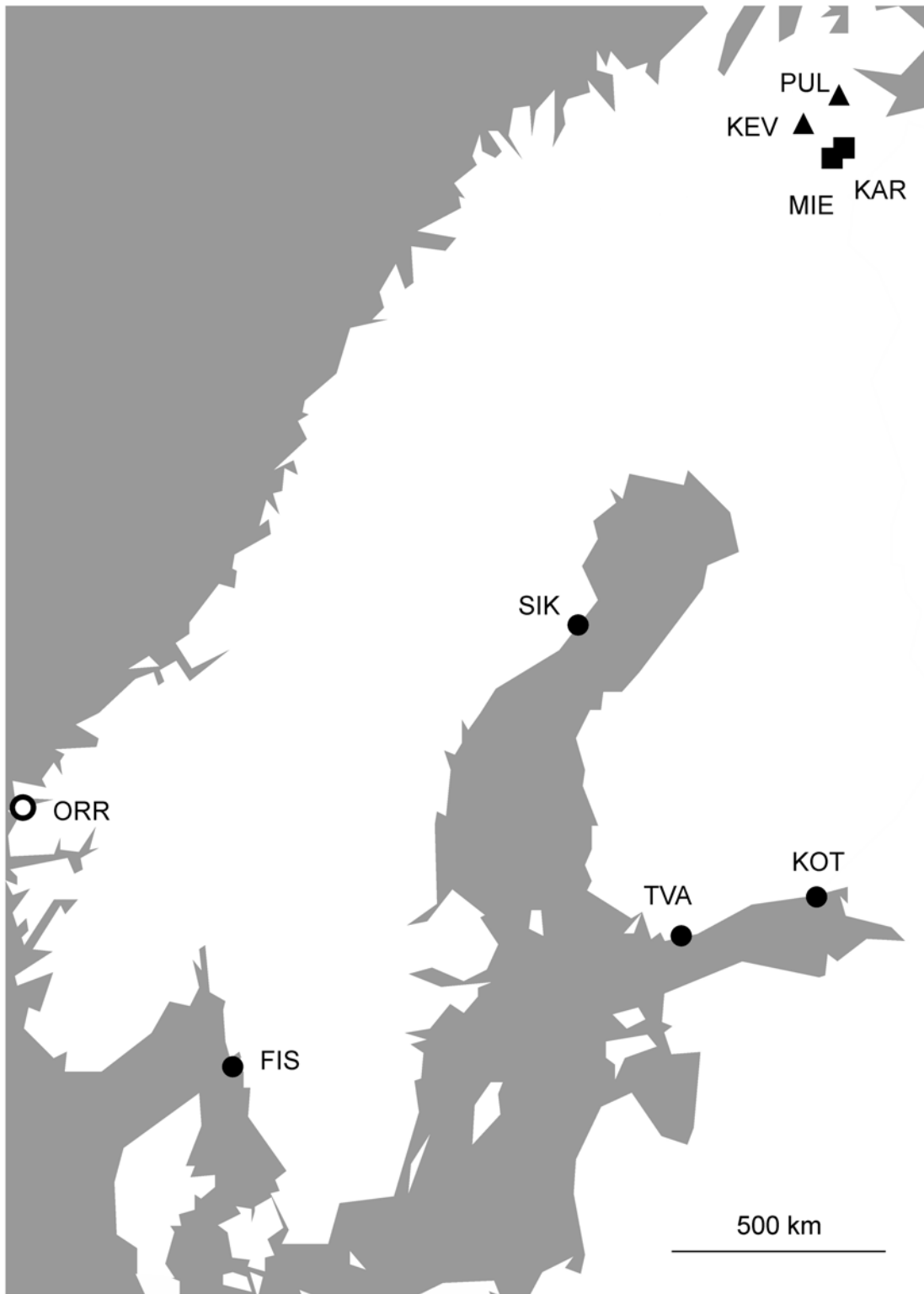
30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 90 s, and extension at 72°C for 60 s. A final extension at 60°C for 5 min was included to complete the cycle. All PCR products were resolved with a MegaBACE 1000 automated sequencer (Amersham Biosciences) following 1:500 dilution, and alleles were scored with Fragment Profiler 1.2 software (Amersham Biosciences). Automatic binning was first used to designate allele sizes, and then sizes were edited by eye (JD). All genotype data used in this paper have been uploaded to Dryad repository: <http://doi.org/10.5061/dryad.p15h2>.

### Data quality

The program MICROCHECKER [44] was used to check for the presence of null alleles. Tests for deviations from Hardy-Weinberg equilibrium, as well as the extent of linkage disequilibrium between all pairs of loci in each sample, were conducted with the program FSTAT 2.9.3.2 [45]. Selective neutrality was tested for using two outlier detection methods: the coalescent-based FDIST method as implemented in LOSITAN [46], and the Bayesian method of Foll and Gaggiotti [47] as implemented in BAYESCAN. Since divergence can accumulate between habitats as a result of ecological differences [31,37] both tests were performed in a habitat-specific fashion (i.e. among lake, pond and sea populations separately).

### Estimates of genetic variability and differentiation

The expected heterozygosity (H<sub>E</sub>), number of alleles (A), allelic richness (A<sub>R</sub>), and inbreeding coefficient (F<sub>IS</sub>) were calculated for each population at each sampling point using FSTAT 2.9.3.2 [45]. The program HP-RARE 1.0 [48] was used to calculate private allelic richness (A<sub>P</sub>). Non-parametric tests were used to test for differences in diversity estimates between sampling years. Differences in allele frequency distribution were tested for with the genic differentiation option in the online program GENEPOP 1.2 [49], using Fisher's exact probability tests. The



**Fig 1. A map showing the geographic location of the eight sampling locations.** Circles denote sea; triangles, lake; squares, pond populations. ORR indicates a source population to infer migration patterns and  $N_e$  in FIS according to the MLNE method (see text for details).

doi:10.1371/journal.pone.0123891.g001

degree of differentiation—both globally over all populations, as well as pairwise comparisons among populations—was estimated using Weir and Cockerham's theta [50] following 1000 permutations in FSTAT 2.9.3.2 [45]. POPULATIONS 1.2 [51] software was used to construct a neighbor-joining tree based on pairwise chord distance ( $D_{CE}$ ; [52]) between populations. A Bayesian clustering algorithm was used to determine population structure within freshwater and marine habitats, as performed with the program STRUCTURE 2.3.3 [53]. The eight samples from the two pond and two lake populations were run together under the admixture model for five iterations for each value of K ranging from one to eight. Each run started with 50 000 burn-in steps followed by 100 000 MCMC repetitions. All other parameters were set to the default values. The eight samples from the four marine populations from the Baltic Sea were run under similar conditions, except sampling location was used as a prior in order to assist in detecting structure when levels of divergence are low [54]. The Structure Harvester web service [55] was used to determine and visualize the most probable value of K.

### $N_e$ estimates

Given the challenges and uncertainties associated with estimation of effective population size ( $N_e$ ), we used six different methods—three temporal and three single-sample—to estimate  $N_e$  in each of the study sites. The rationale behind the use of all these methods was to affirm the robustness and reliability of the estimates by comparison: if multiple methods give similar estimates, more confidence can be placed on the conclusions (cf. [56]). We wish to further emphasize the fact that the primary goal in these comparisons was not to compare the performance of the estimators as such. Rather, the goal was to gain confidence in inferring possible  $N_e$  differences among the three different habitat types with the *a priori* expectation of  $N_{e \text{ [Sea]}} > N_{e \text{ [Lake]}} > N_{e \text{ [Pond]}}$ .

For the single-sample estimators, three methods were used to estimate inbreeding  $N_e$  from the multilocus genotype data of each sampling location at each time point.

The first was the linkage disequilibrium (LD; [57]) method, which uses the unbiased estimator of Burrow's  $\Delta$  [58] to test for non-random associations between unlinked loci. The random mating model was used for the LD option as implemented in the software  $N_E$ ESTIMATOR v2 [59], which corrects for sample size bias and accounts for missing data [60]. Allele frequencies of less than 0.05 were excluded, and confidence limits were calculated by jackknifing over loci. The second was the sibship assignment method (SA; [61]), which uses sibship frequencies estimated from randomly sampled pairs of individuals as being sibs sharing one or two parents. The program Colony2 [61] was run under the full likelihood model with high precision, polygamous breeding systems for both sexes, and no prior information on candidate parents or sibship sizes. The third was the Bayesian method as implemented with the online program ONE-SAMP [62], which uses approximate Bayesian computation (ABC) to calculate eight summary statistics—including LD—to estimate  $N_e$ . Prior  $N_e$  estimates (lower and upper) were set to two and 500 for the freshwater populations, and two and 5,000 for the Baltic Sea populations.

For the temporal estimators, three methods were used to estimate the harmonic mean of variance  $N_e$  based on the samples taken at both time points for each sampling location.

The first was the moment-based method of Waples [63], which uses F statistics to calculate the standardized variance in allele frequency between sampling events. Three different options were used to measure  $N_e$ :  $F_c$  [64],  $F_k$  [65], and the unbiased estimator  $F_s$  [66]. In the case of these F statistics, the  $N_e$  estimates were divided by the number of generations represented in the sample. The information on approximate generation length was obtained for all freshwater populations collected in 2003, as well as three Baltic Sea sites (FIS, KOT, TVA) collected in 2003, from the average age of breeding adults in the given population ([67]; see [41]). Since the average age in most populations was close to three (Table 1; [41]), this corresponded to

a maximum of two generations. In the case of KAR, the average age was two, corresponding to three generations.  $N_e$ ESTIMATOR v2 [59] was used to generate each estimate after excluding allele frequencies of less than 0.05, and confidence limits were calculated by jackknifing over loci. The second moment-based method was the pseudo-maximum likelihood method of Wang and Whitlock ([68]; MLNE), which relaxes the assumption of closed populations. As such,  $N_e$  and migration,  $m$ , were estimated jointly for the Baltic Sea populations since there is a high degree of gene flow in the Baltic Sea [28,32]. In these analyses, FIS was used as a source population for KOT, SIK and TVA, as earlier analyses of gene flow indicate that migration rate from North Sea to Baltic Sea is higher than the reverse [32]. An Atlantic population from the coast of Norway (Orrevannet; 58°44'N 05°31'E, see [28]; Fig 1) was used as a source population for FIS. Since the freshwater populations are isolated and therefore are not likely experiencing gene flow with other populations, only  $N_e$  was estimated. Two generations were assumed for all populations except KAR, in which three generations were assumed. The upper  $N_e$  priors were set to 500 for the freshwater populations and 5,000 for the Baltic Sea populations. The third method was the likelihood-based method of Berthier et al. ([69]; TM3), which uses coalescence theory and Bayesian prior information to estimate  $N_e$ . The same upper  $N_e$  priors were used as in the ABC and MLNE methods.

## Results

### Data quality

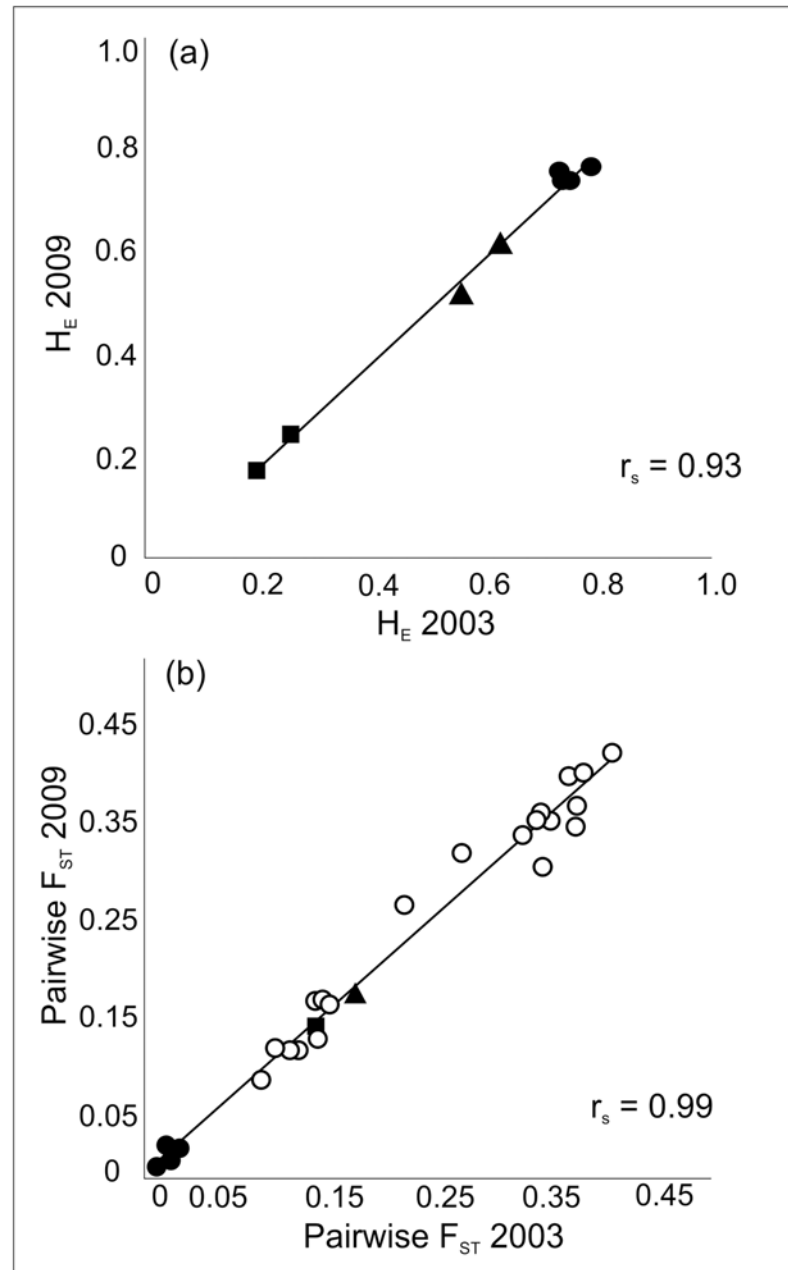
Although there was evidence of null alleles and Hardy-Weinberg deviations at some loci in some populations, there were no consistent patterns across any locus, populations or habitats. Similarly, there was no consistent pattern of linkage disequilibrium in any pair of loci across all populations. Neither of the outlier tests detected divergent loci in the pond and lake populations, however one locus (STN146) was detected as an outlier with both methods in the Baltic Sea populations. Although visual inspection of the data revealed that the elevated divergence observed in this locus arose from spatial, not temporal, shifts in allele frequencies, it was nevertheless eliminated from further analyses.

### Genetic variability

There was significant genetic heterogeneity among populations in all genetic diversity measures: marine populations were genetically more variable than the lake populations, which were more variable than the pond populations (Table 1). However, there were no temporal differences in the allele frequencies in any loci in any of the populations between the two sampling occasions (Fisher's exact probability tests,  $P > 0.05$ ). Accordingly, different estimates of genetic variability were similar (Kruskal-Wallis test,  $P > 0.05$  for  $H_E$ ,  $A$ , and  $A_R$ ) and strongly correlated ( $r_s = 0.77$ – $0.93$ ;  $n = 8$ ,  $P \leq 0.001$ ) between the two sampling occasions (e.g. Fig 2a).  $F_{IS}$  values were small and similar in all populations and across the two sampling periods (Table 1).

### Genetic differentiation

The overall  $F_{ST}$  across all populations was 0.199 (95% CI: 0.156–0.238,  $P < 0.05$ ) in 2003 and 0.209 (95% CI: 0.107–0.246,  $P < 0.05$ ) in 2009, and the pairwise estimates across populations over time were strongly positively correlated ( $r = 0.99$ ;  $n = 8$ ,  $P < 0.001$ ; Fig 2b). Within each habitat type, overall  $F_{ST}$  was similar between years (Table 2). Accordingly, the temporal replicates branched together in the neighbor-joining tree, with high bootstrap support for the freshwater populations (Fig 3). STRUCTURE also assigned temporal replicates to the same genetic cluster for each population (Fig 3). Each of the freshwater sampling locations were assigned as



**Fig 2. Correlation between (a) expected heterozygosity and (b) pairwise  $F_{ST}$  values from two different sampling occasions.** Closed circles denote pairwise  $F_{ST}$  among sea populations; triangles among lake populations; squares among pond populations; open circles between habitat comparisons.

doi:10.1371/journal.pone.0123891.g002

independent clusters ( $K = 4$ ), and the most likely  $K$  for the sea populations was three (Fig 3). Among all populations, 20.36% of the genetic variation was attributed to differences among habitats, but the variance component due to temporal changes within localities was negative and non-significant (Table 3). This was true whether all populations were analyzed together or when each habitat was analyzed separately (Table 3).



**Table 2. Comparison of estimates of population differentiation ( $F_{ST}$ ) between sampling years (2003 and 2009) in different habitat types.**

Habitat	2003	95% CI	2009	95% CI
Baltic Sea	0.004	0.002–0.007	0.009	0.002–0.015
Lake	0.174	0.115–0.235	0.174	0.123–0.222
Pond	0.141	0.023–0.272	0.148	0.038–0.270
Overall	0.199	0.156–0.238	0.209	0.107–0.246

doi:10.1371/journal.pone.0123891.t002

### $N_e$ estimates: Single-sample methods

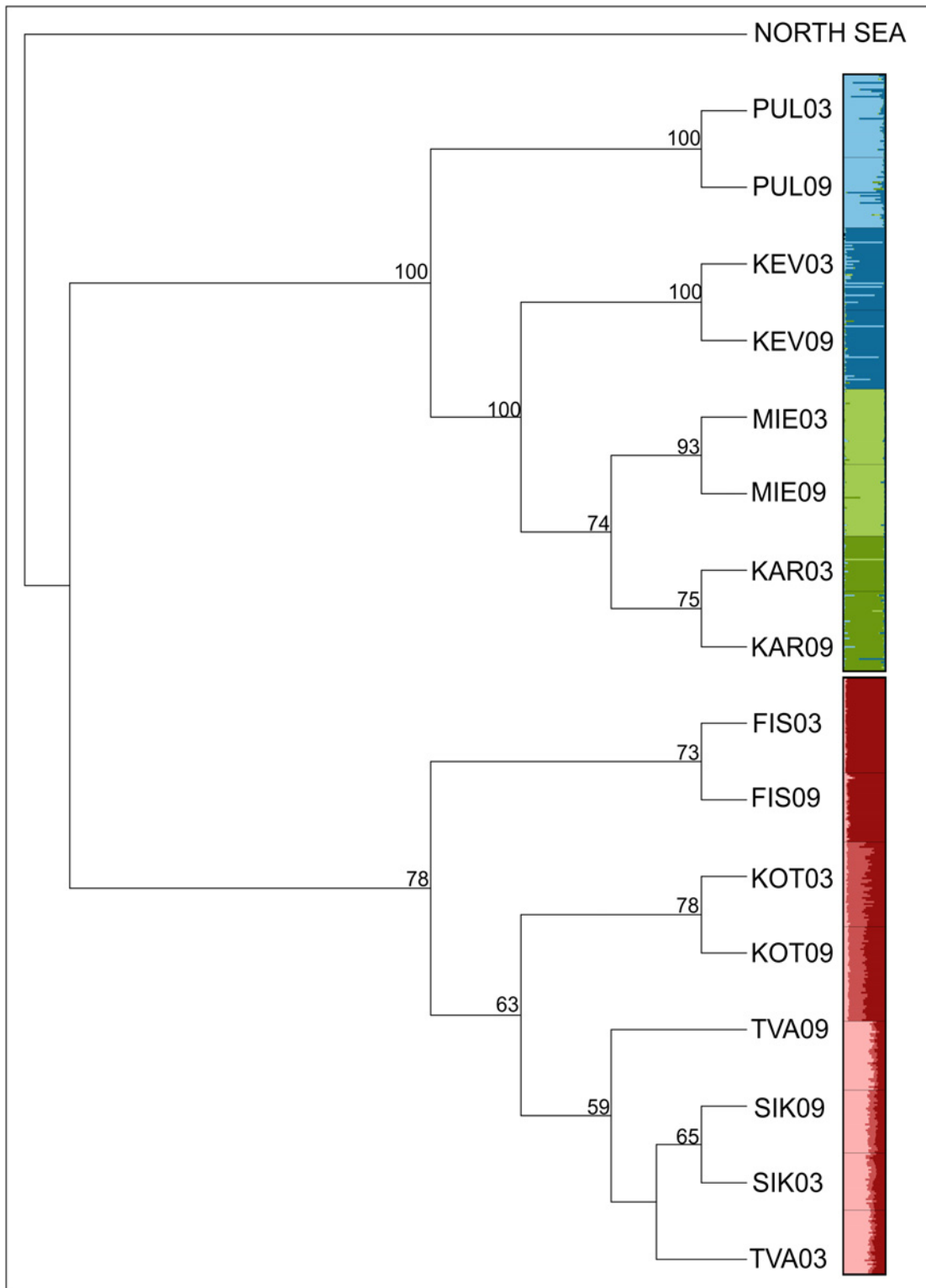
The LD method yielded infinite  $N_e$  estimates for all the sea populations (Fig 4; S1 Table). The  $N_e$  estimates were an order of magnitude higher for the lake populations (average = 238) as compared to the pond populations (average = 13; Fig 4). The SA method yielded the lowest  $N_e$  estimates across all populations, with the narrowest confidence intervals (Fig 4; S1 Table). The sea populations had similar  $N_e$  estimates (average = 53) to the lake populations (average = 43), while the pond populations had the lowest (average = 15; Fig 4; S1 Table). The ABC method also yielded  $N_e$  estimates that were similar across sea (average = 147) and lake (average = 213) populations. As with the other methods,  $N_e$  estimates in the pond populations were significantly smaller (average = 54) and more precise (narrower confidence intervals; Fig 4; S1 Table).

### $N_e$ estimates: Temporal methods

The standard moment-based method of Waples (1989) yielded similar results whether calculated according to Nei and Tajima ( $F_c$ : [64]), Pollak ( $F_k$ : [65]) or Jorde and Ryman ( $F_s$ : [66]; Fig 4; S1 Table).  $N_e$  estimates were slightly but not significantly higher in the lake (average = 271) than sea (average = 163) populations. Most estimates yielded infinite confidence intervals, except the  $F_s$  method for some of the sea populations (Fig 4; S1 Table).  $N_e$  estimates for the pond populations were an order of magnitude lower (average = 38) than for the lake and sea populations, with narrower confidence intervals (Fig 4; S1 Table). When calculated with the assumption of migration, MLNE yielded the lowest  $N_e$  estimates among the sea populations (average = 78; Fig 4; S1 Table). Assuming no migration,  $N_e$  was similar across all freshwater populations, and was slightly higher than in the sea populations (average = 135; Fig 4; S1 Table). The  $N_e$  estimates from TM3 yielded similar patterns as the single-sample SA method, where  $N_e$  was highest in the sea populations (average = 110), lowest in the pond populations (average = 16) and intermediate in the lake populations (average = 60; Fig 4; S1 Table).

## Discussion

The most salient finding of this study was that, despite the differing levels of genetic diversity, gene flow and habitat size, allele frequencies as well as genetic variability and differentiation measures within and among three-spined stickleback populations from both marine and freshwater habitats were temporally stable over the six year time period. This suggests that, in spite of the potential for sampling artifacts and low signal-to-noise ratio to affect estimated genetic parameters, these issues were of negligible concern. It is particularly noteworthy that this was true in the case of small isolated populations as well as in larger interconnected populations. Likewise, using different methods to estimate effective size of stickleback populations from different habitats, we found fairly consistent support for relatively small effective sizes (tens to hundreds) in all habitat types, although the census population sizes in this species are likely to be orders of magnitudes higher (e.g. [70–72]). In the following, we discuss these findings and



**Fig 3. Neighbor-joining tree based on  $D_{CE}$  distances [52], rooted with a population from the North Sea.** Bootstrap values above 50% are indicated at nodes. Genetic clusters as detected with STRUCTURE [53] are indicated at branch tips.

doi:10.1371/journal.pone.0123891.g003

**Table 3. Hierarchical partitioning of spatial and temporal variation in allele frequency data with AMOVA.**

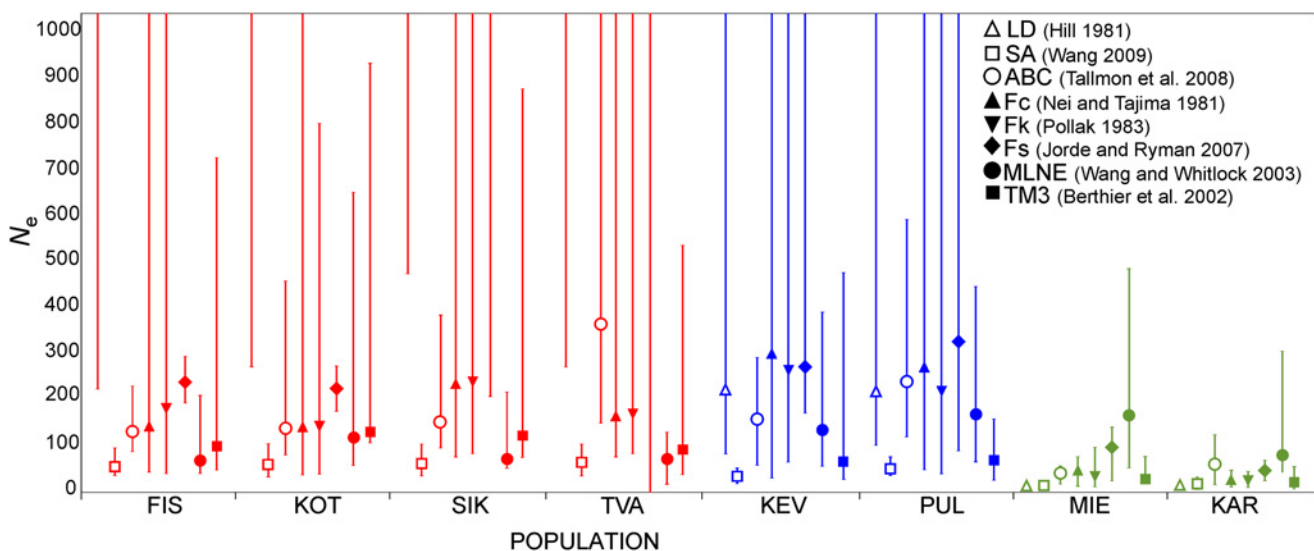
Source of variation	Degrees of freedom	Sum of Squares	Variance component	% of variation	p
Among habitat type	2	962.56	1.103	20.38	<0.001
Among years	1	5.55	-0.132	-2.64	0.986
Baltic Sea	1	5.08	-0.018	-0.33	0.912
Lake	1	6.76	-0.445	-9.39	0.328
Pond	1	1.99	-0.136	-7.58	0.322

doi:10.1371/journal.pone.0123891.t003

their implications to studies of genetic population structure, and that of the three-spined stickleback in particular.

### Temporal stability of allele frequencies

Most population genetic studies employ allochronic approaches to assess shifts in allele frequencies, with the underlying assumption of temporal genetic stability. Our results demonstrating short-term stability of genetic parameters in multiple three-spined stickleback populations across a range of habitat types validate this assumption for this species. This finding may not be surprising considering the relatively short time period—roughly two to three stickleback generations—between sampling events. The probability of detecting significant allele frequency differences when samples are collected few years apart may be low (e.g. [73]). Moreover, the temporal stability in genetic parameters in the high gene flow Baltic Sea environment was expected (see: [9,11,16,74–77] for similar examples), since genetic diversity is likely to be maintained by migration [78,79]. However, the stability in the two small and isolated pond populations is noteworthy because the stochastic component to variance in allele frequencies in small populations is expected to be large [80,81].



**Fig 4. Estimates of effective population size in each of the sea (red), lake (blue) and pond (green) populations.** Infinite confidence intervals are indicated with lines without caps. Single-sample estimates (LD, SA, ABC) represent harmonic mean of the two sampling periods. MLNE estimates for sea populations are assuming migration whereas those for the freshwater populations assume no migration.

doi:10.1371/journal.pone.0123891.g004

## Temporal stability in genetic diversity

Genetic drift is typically stronger in small than in large populations. Specifically, the rate of loss of diversity is inversely proportional to the effective population size (i.e.  $1/2N_e$ ; [80]). Therefore, loss of diversity via drift can be expected to be faster—particularly in the absence of migration—in small than in large populations. Moreover, inbreeding and the accumulation of deleterious mutations can accelerate loss of genetic variation in small populations [18,78]. In accordance with these expectations, we found that the genetic diversity in the freshwater populations was substantially lower than that in the marine populations (see also: [28,35,36] for similar evidence from this species), and yet, genetic diversity measures remained stable over the study period in all sampling sites. This temporal stability of genetic parameters in the small pond populations being similar to those of the larger lake and sea populations could come about if the difference in  $N_e$  between the pond and other populations was too small to generate noticeable allele frequency shifts over the short time span encompassed in our study (cf. [82]). For example, assuming  $N_e$  of 30 in the pond populations and 150 in the sea populations, the rate of loss in the former would “only” be five times faster than in the latter. Hence, as evidenced by their low genetic diversity, the pond populations are likely more susceptible to the negative consequences of drift than the larger lake and Baltic Sea populations. In fact, most point estimates of  $N_e$  for pond populations were lower than 50, which is far below the minimum short-term  $N_e$  required to ensure potential for long-term persistence in the face environmental changes and to avoid inbreeding problems [78,83,84].

## Temporal stability in population structure

Demonstrating temporal stability in the patterns and degree of population structure is a powerful way of confirming the reliability of observed spatial genetic patterns. For example, Tessier and Bernatchez [85] sampled salmon populations over several generations to validate their earlier finding of surprisingly high divergence within a single river system [86]. Temporal sampling is especially relevant in the case of subpopulations interconnected by gene flow, such as those of marine fishes in which low but statistically significant levels of differentiation are often observed [38]. Several studies have utilized temporal replicates—spanning few to many generations—to infer the biological significance of weak differentiation (e.g. [9,11,13]), or to clarify contrasting patterns of differentiation [74]. Moreover, when sample sizes are small in comparison to population size, as is likely the case with many marine fishes, accuracy of allele frequency estimates is reduced by small sample sizes [60,63]. Hence, our finding of stability in genetic composition among the Baltic Sea samples suggests that sample size and sampling errors did not influence the observed patterns. In addition, this result indicates that the barriers to gene flow between the North Sea and Baltic Sea have remained stable over several years. Similar findings have been reported in other fish studies comparing temporal stability genetic parameters across the Baltic—North Sea transition [14,16,87].

Although differential allele frequency changes are expected in populations subject to different levels of gene flow [88], the small, isolated pond populations showed the same degree of temporal stability in patterns and degree of population differentiation as the large, interconnected Baltic Sea populations. Albeit only one year had elapsed between sampling periods, Araguas et al. [34] also failed to find significant temporal heterogeneity in differentiation between endangered, isolated freshwater stickleback populations in the Iberian Peninsula. Similarly, Raeymaekers et al. [89] reported weak but significant temporal differentiation among fresh- and brackish water stickleback populations—connected by gene flow—collected several times during two years. McCairns and Bernatchez [30] also noted strong clustering of temporal samples collected from the high gene flow St. Lawrence estuary. With six years elapsed between

sampling events, our current study represents—to the best of our knowledge—the longest time period over which temporal stability of allele frequencies in presumably neutral marker genes in stickleback populations has been assessed. Nevertheless, our results accord with those of earlier studies in which both isolated and interconnected populations appear to be stable over short term. It is important to note that despite the apparent stability in neutral population structure of sticklebacks, several studies have identified significant seasonal and temporal changes in allele frequencies of the locus underlying lateral plate number differentiation [89–91]. Similar findings were also reported by Fraser et al. [19], who noted that although temporal replicates of guppy populations were stable in neutral loci, there were significant changes in the frequency of different MHC alleles. Hence, the stability of allele frequencies in neutral loci may differ from those subject to natural selection—a situation analogous to the differential degree of spatial genetic differentiation in neutral and selected loci (e.g. [92,93]).

Finally, we note that the results of this study in respect to the degree and patterns of genetic differentiation among Baltic and North Sea populations are largely in agreement with earlier studies conducted across these sites [28,32,36,94]. In general, as shown also by our results, the degree of neutral genetic differentiation is usually clearest between the North Sea and Baltic Sea sites, while little differentiation is usually detected among the inner Baltic Sea sites (see also [36]). However, several markers located within or close to genes of functional importance display marked differentiation even within the inner Baltic Sea [32]. The shallow genetic structuring in neutral genes within the Baltic Sea is also likely to explain why the patterns of genetic differentiation recovered in the present and earlier studies [28,94] are not always exactly concurrent. Part of these discrepancies may occur because the markers used in the present study were not exactly the same as those in the previous study [32,94], and/or that the sample size in this study were higher than in earlier studies [94]. For instance, the degree of genetic differentiation (as reflected in  $F_{ST}$ ) among the marine sites SIK and KOT, as well as between KOT and TVA, were significant in this but not in a previous study [94]. Yet, this discrepancy occurred only in two of the six possible comparisons and concurrence between the  $F_{ST}$ -estimates across the two studies is high ( $r_s = 0.84$ ,  $P = 0.036$ ).

## Effective population size

Although the assumptions associated with estimating effective population size are likely violated in many studies [18], both temporal and single-sample methods have remained popular tools in population and conservation genetics. While many studies have reported some degree of congruence among estimates obtained with different methods (e.g. [56,95, 96]), a high degree of variation surrounding estimates is more of a rule, rather than exception. This applies to some degree to our study as well. Like most other studies, we found that  $N_e$  estimates were most precise when small. This was most often seen with all methods in the pond populations, and with the single-sample sibship analysis method in all populations. However, there is likely little probability of sampling sibs in our scheme, since sibs are unlikely to be present among breeders [97], at least not in the samples from the sea. In fact, the sibship method may be biased for large  $N_e$  [61], and hence not be very appropriate for the sea populations used in our study. Therefore, although precise, the sibship results may not be the most appropriate given our sampling design. In general, the confidence intervals for  $N_e$  estimates in the sea and lake populations obtained with most other methods included infinity, which is likely to be a reflection of weak genetic signal relative to sampling noise: the precision of  $N_e$  estimates declines as  $N_e$  increases [63]. However, it is noteworthy that irrespective of the method used, the lower confidence limits were consistently (ignoring the LD-method estimates; see Fig 4) higher for sea and lake as compared to pond populations.

In general, the point estimates of  $N_e$  of the sea populations were lower than expected, as those of many marine fishes are at least an order of magnitude higher (e.g. [16,75,98]). This could derive from the fact that most methods used here assume population isolation and that shifts in allele frequencies occur strictly as a result of drift without introduction of new alleles from migrants [8,66,68]. However, gene flow may inflate diversity and variance in allele frequencies, creating a signal of instability and therefore yield low  $N_e$  estimates (e.g. [15,68]). Likewise, episodic pulses of gene flow from divergent populations—such as large influxes of freshwater or anadromous sticklebacks to a given sea area—could explain the low  $N_e$  in marine sites [99]. We tried to avoid this potential problem firstly by performing clustering analysis and subsequently estimating  $N_e$  according to the identified genetic clusters, rather than on the basis of predefined populations based on sampling location. Secondly, we used the method of Wang and Whitlock [68] which accounts for gene flow from a predefined source population. Interestingly, this method yielded slightly lower  $N_e$  estimates than the methods assuming no migration—a finding encountered in several other studies too (e.g. [10,56,95,100,101]). In addition, violation of the assumption of discrete generations can lead to downward bias in  $N_e$  estimates [8,66], but corrections for overlapping generation require considerable life-history information as well samples from consecutive cohorts (e.g. [102]) which are not available for our study.

The low  $N_e$  estimates for sea and lake populations of three-spined stickleback populations are puzzling given that census population sizes ( $N_c$ ), even in small ponds, can be in the range of thousands (e.g. [72]), and those in the Baltic Sea most likely in the millions (e.g. [70,71]). Hence, a conservative assumption of  $N_c = 10\,000$  for freshwater populations and  $N_c = 1$  million for sea populations would yield in  $N_e/N_c$  ratios far below the usual  $\approx 10\%$  observed in studies of wild animal populations [103]. Although other studies of marine species have reported similar findings [18,22], we also observed this in the lake populations. As discussed above, violation of assumptions (e.g. no migration, discrete generations) together with low signal-to-noise ratio may explain the low point estimates of  $N_e$  in the lake and sea samples: the genetic signal may simply be too weak to discern between  $N_e$  of moderate and large size (cf. [6]). It is also worth pointing out that genotyping errors and non random sampling can inflate variance in allele frequencies, and thereby lead to downwardly bias  $N_e$  estimates. However, we have no reason to assume that these sources of error would differ among habitat types, and the genotyping error rates for the used loci are likely to be low as they were selected for this study on the basis of being easily scoreable. Likewise, while non random sampling of individuals is hard to discount without extensive resampling of individuals in a given locality, the high degree of temporal stability in all genetic parameters in this study speaks against such possibility.

As pointed out by Palstra and Ruzzante [18], the possibility of low signal-to-noise ratio could be investigated by substantially increasing sample sizes and thereby also the signal-to-noise ratio, although it is noteworthy that when we combined the two temporal samples in the single-sample methods, estimates remained unchanged (data not shown). Nevertheless, regardless of the absolute magnitude of  $N_e$ -values, the sea and lake populations appear to have substantially higher (and likely underestimated)  $N_e$ s than the pond populations: for most estimators, both the lower and upper confidence intervals, as well as the point estimates, were higher for sea and lake populations than for pond populations. In spite of the uncertainties involved with  $N_e$  estimates, the temporal changes in allele frequencies in all habitat types were small over the six year study interval, and hence, from the point of view estimation of genetic population structure, negligible.

## Conclusions

In conclusion, the results of this study demonstrate that genetic diversity and population structuring among three-spined stickleback populations are temporally stable over short term: this

holds true for both small isolated freshwater populations as well as for large, interconnected marine populations. This result suggests that small scale temporal variation in sampling scheme is unlikely to bias or otherwise influence population genetic inference based on allele frequencies in neutral marker genes in this species. Although point estimates of effective population size varied depending on the estimation method, two fairly robust conclusions about them are possible. First, as expected, the effective sizes appear to be smaller in the pond than in lake and sea populations. Second, the census population sizes of sticklebacks are likely to be orders of magnitudes higher than the effective population sizes, at least in the isolated pond populations where the  $N_e$  estimates were most precise. Future studies utilizing more widely separated temporal cohort-based samples could be informative in gaining better estimates of effective size of contemporary populations (cf. [101]).

## Supporting Information

**S1 Table. Effective population size estimates for three-spined sticklebacks.**  
(XLSX)

## Acknowledgments

We thank Teija Aho, Tuomas Leinonen, Hannu Mäkinen, Jouko Pokela and Ilkka Syvänpää for help in collecting the fish samples, and Marika Karjalainen and Kirsi Kähkönen for their help in the laboratory.

## Author Contributions

Conceived and designed the experiments: JD JM. Performed the experiments: JD. Analyzed the data: JD. Contributed reagents/materials/analysis tools: JD JM. Wrote the paper: JM JD.

## References

1. Hartl DL, Clark AG. Principles of Population Genetics. Sunderland: Sinauer Associates; 1997.
2. Garland T, Rose MMR. Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments. Berkeley: University of California Press; 2009.
3. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC. Experimental evolution. Trends Ecol Evol. 2012; 27: 547–560. doi: [10.1016/j.tree.2012.06.001](https://doi.org/10.1016/j.tree.2012.06.001) PMID: [22819306](https://pubmed.ncbi.nlm.nih.gov/22819306/)
4. Wandeler P, Hoeck PE, Keller LF. Back to the future: museum specimens in population genetics. Trends Ecol Evol. 2007; 22: 634–642. PMID: [17988758](https://pubmed.ncbi.nlm.nih.gov/17988758/)
5. Nielsen EE, Hansen MM. Waking the dead: the value of population genetic analyses of historical samples. Fish Fish. 2008; 9: 450–461. doi: [10.1186/1471-2164-9-450](https://doi.org/10.1186/1471-2164-9-450) PMID: [18826589](https://pubmed.ncbi.nlm.nih.gov/18826589/)
6. Waples RS. Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. J Hered. 1998; 89: 438–450.
7. Allendorf FW, Phelps SR. Use of allelic frequencies to describe population structure. Can J Fish Aquat Sci. 1981; 38:1507–1514.
8. Jorde PE, Ryman N. Unbiased estimator for genetic drift and effective population size. Genetics. 2007; 177: 927–935. PMID: [17720927](https://pubmed.ncbi.nlm.nih.gov/17720927/)
9. Ruzzante DE, Taggart CT, Doyle RW, Cook D. Stability in the historical pattern of genetic structure of Newfoundland cod (*Gadus morhua*) despite the catastrophic decline in population size from 1964 to 1994. Conserv Genet. 2001; 2: 257–269.
10. Hoffman EA, Schueler FW, Blouin MS. Effective population sizes and temporal stability of genetic structure in *Rana pipiens*, the Northern leopard frog. Evolution 2004; 58: 2536–2545. PMID: [15612296](https://pubmed.ncbi.nlm.nih.gov/15612296/)
11. Jónsdóttir ODB, Danielsdóttir AK, Nævdal G. Genetic differentiation among Atlantic cod (*Gadus morhua* L.) in Icelandic waters: temporal stability. ICES J Mar Sci. 2001; 58: 114–122.
12. Garant D, Dodson JJ, Bernatchez L. Ecological determinants and temporal stability of the within-river population structure in Atlantic salmon (*Salmo salar* L.). Mol Ecol. 2000; 9: 615–628. PMID: [10792704](https://pubmed.ncbi.nlm.nih.gov/10792704/)

13. Nielsen EE, Nielsen PH, Meldrup D, Hansen MM. Genetic population structure of turbot (*Scophthalmus maximus* L.) supports the presence of multiple hybrid zones for marine fishes in the transition zone between the Baltic Sea and the North Sea. *Mol Ecol*. 2004; 13: 585–595. PMID: [14871363](#)
14. Hemmer-Hansen J, Nielsen EE, Frydenberg J, Loeschcke V. Adaptive divergence in a high gene flow environment: Hsc70 variation in the European flounder (*Platichthys flesus* L.). *Heredity*. 2007; 99: 592–600. PMID: [17848973](#)
15. Walter RP, Aykanat T, Kelly DW, Shrimpton JM, Heath DD. Gene flow increases temporal stability of Chinook salmon (*Oncorhynchus tshawytscha*) populations in the Upper Fraser River, British Columbia, Canada. *Can J Fish Aquat Sci*. 2009; 66: 167–176.
16. Larsson LC, Laikre L, André C, Dahlgren TG, Ryman N. Temporally stable genetic structure of heavily exploited Atlantic herring (*Clupea harengus*) in Swedish waters. *Heredity*. 2010; 104: 40–51. doi: [10.1038/hdy.2009.98](#) PMID: [19654606](#)
17. Schwartz MK, Luikart G, Waples RS. Genetic monitoring as a promising tool for conservation and management. *Trends Ecol Evol*. 2007; 22: 25–33. PMID: [16962204](#)
18. Palstra FP, Ruzzante DE. Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? *Mol Ecol*. 2008; 17: 3428–3447. PMID: [19160474](#)
19. Fraser BA, Ramnarine IW, Neff BD. Temporal variation at the MHC class IIB in wild populations of the guppy (*Poecilia reticulata*). *Evolution*. 2010; 64: 2086–2096. doi: [10.1111/j.1558-5646.2010.00965.x](#) PMID: [20148955](#)
20. Gomez-Uchida D, Palstra FP, Knight TW, Ruzzante DE. Contemporary effective population and metapopulation size ( $N_e$  and meta- $N_e$ ): comparison among three salmonids inhabiting a fragmented system and differing in gene flow and its asymmetries. *Ecol Evol*. 2013; 3: 569–580. doi: [10.1002/ece3.485](#) PMID: [23532448](#)
21. Luikart G, Ryman N, Tallmon DA, Schwartz M, Allendorf FW. Estimation of census and effective population sizes: the increasing usefulness of DNA-based approaches. *Conserv Genet*. 2010; 11: 355–373.
22. Hare MP, Nunney L, Schwartz MK, Ruzzante DE, Burford M, Waples RS, et al. Understanding and estimating effective population size for practical application in marine species management. *Conserv Biol*. 2011; 25: 438–449. doi: [10.1111/j.1523-1739.2010.01637.x](#) PMID: [21284731](#)
23. Lande R, Barrowclough GF. Effective population size, genetic variation, and their use in population management. In: Soule ME, editor. *Viable Populations for Conservation*. Cambridge: Cambridge University Press; 1987. pp. 87–123.
24. Tallmon DA, Gregovich D, Waples RS, Baker CS, Jackson J, Taylor BJ, et al. When are genetic methods useful for estimating contemporary abundance and detecting population trends? *Mol Ecol Res*. 2010; 10: 684–692. doi: [10.1111/j.1755-0998.2010.02831.x](#) PMID: [21565073](#)
25. Waples RS. Spatial-temporal stratifications in natural populations and how they affect understanding and estimation of effective population size. *Mol Ecol Res*. 2010; 10: 785–796. doi: [10.1111/j.1755-0998.2010.02876.x](#) PMID: [21565090](#)
26. Reusch TB, Wegner KM, Kalbe M. Rapid genetic divergence in postglacial populations of threespine stickleback (*Gasterosteus aculeatus*): the role of habitat type, drainage and geographical proximity. *Mol Ecol*. 2001; 10: 2435–2445. PMID: [11703651](#)
27. Raeymaekers JAM, Maes GE, Audenaert E, Volckaert FAM. Detecting Holocene divergence in the anadromous-freshwater three-spined stickleback (*Gasterosteus aculeatus*) system. *Mol Ecol*. 2005; 14: 1001–1014. PMID: [15773932](#)
28. Mäkinen HS, Cano JM, Merilä J. Genetic relationships among marine and freshwater populations of the European three-spined stickleback (*Gasterosteus aculeatus*) revealed by microsatellites. *Mol Ecol*. 2006; 15: 1519–1534. PMID: [16629808](#)
29. Cano JM, Mäkinen HS, Merilä J. Genetic evidence for male-biased dispersal in the three-spined stickleback (*Gasterosteus aculeatus*). *Mol Ecol* 2008; 17: 3234–3242. doi: [10.1111/j.1365-294X.2008.03837.x](#) PMID: [18564084](#)
30. McCairns RJS, Bernatchez L. Landscape genetic analyses reveal cryptic population structure and putative selection gradients in a large-scale estuarine environment. *Mol Ecol*. 2008; 17: 3901–3916. doi: [10.1111/j.1365-294X.2008.03884.x](#) PMID: [18662229](#)
31. Hohenlohe PA, Bassham S, Etter PD, Stiffler N, Johnson EA, Cresko WA. Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. *PLoS Genet*. 2010; 6: e1000862. doi: [10.1371/journal.pgen.1000862](#) PMID: [20195501](#)



32. DeFaveri J, Jonsson PR, Merilä J. Heterogeneous genomic differentiation in marine threespine sticklebacks: adaptation along an environmental gradient. *Evolution*. 2013; 67: 2530–2546. doi: [10.1111/evo.12097](https://doi.org/10.1111/evo.12097) PMID: [24033165](https://pubmed.ncbi.nlm.nih.gov/24033165/)
33. Drevecky CJ, Falco R, Aguirre WE. Genetic divergence of a sympatric lake-resident–anadromous three-spined stickleback *Gasterosteus aculeatus* species pair. *J Fish Biol*. 2013; 83: 111–132. doi: [10.1111/jfb.12154](https://doi.org/10.1111/jfb.12154) PMID: [23808695](https://pubmed.ncbi.nlm.nih.gov/23808695/)
34. Araguas RM, Vidal O, Pla C, Sanz N. High genetic diversity of the endangered Iberian three-spined stickleback (*Gasterosteus aculeatus*) at the Mediterranean edge of its range. *Freshw Biol*. 2012; 57: 143–154.
35. Whither RE, McPhail JD. Genetic variability in freshwater and anadromous sticklebacks (*Gasterosteus aculeatus*) of southern British Columbia. *Can J Zool*. 1984; 63: 528–533.
36. Mäkinen H, Cano JM, Merilä J. Identifying footprints of directional and balancing selection in marine and freshwater three-spined stickleback (*Gasterosteus aculeatus*) populations. *Mol Ecol*. 2008; 17: 3565–3582. doi: [10.1111/j.1365-294X.2008.03714.x](https://doi.org/10.1111/j.1365-294X.2008.03714.x) PMID: [18312551](https://pubmed.ncbi.nlm.nih.gov/18312551/)
37. DeFaveri J, Shikano T, Shimada Y, Goto A, Merilä J. Global analysis of genes involved in freshwater adaptation in threespine sticklebacks (*Gasterosteus aculeatus*). *Evolution*. 2011; 65: 1800–1807. doi: [10.1111/j.1558-5646.2011.01247.x](https://doi.org/10.1111/j.1558-5646.2011.01247.x) PMID: [21644964](https://pubmed.ncbi.nlm.nih.gov/21644964/)
38. Ward RD, Woodwark M, Skibinski DOF. A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. *J Fish Biol*. 1994; 44: 213–232.
39. DeWoody JA, Avise JC. Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *J Fish Biol*. 2000; 56: 461–473.
40. Hohenlohe PA, Bassham S, Currey M, Cresko WA. Extensive linkage disequilibrium and parallel adaptive divergence across threespine stickleback genomes. *Philos T R Soc B*. 2012; 367: 395–408. doi: [10.1098/rstb.2011.0245](https://doi.org/10.1098/rstb.2011.0245) PMID: [22201169](https://pubmed.ncbi.nlm.nih.gov/22201169/)
41. DeFaveri J, Merilä J. Variation in age and size of Fennoscandian three-spined sticklebacks (*Gasterosteus aculeatus*). *PLoS One*. 2013; 8: e80866. doi: [10.1371/journal.pone.0080866](https://doi.org/10.1371/journal.pone.0080866) PMID: [24260496](https://pubmed.ncbi.nlm.nih.gov/24260496/)
42. Leinonen T, Cano Arias JM, Mäkinen H, Merilä J. Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespined sticklebacks. *J Evol Biol*. 2016; 19: 1803–1812.
43. Shimada Y, Shikano T, Merilä J. A high incidence of selection on physiologically important genes in the three-spined stickleback, *Gasterosteus aculeatus*. *Mol Biol Evol*. 2011; 28: 181–193. doi: [10.1093/molbev/msq181](https://doi.org/10.1093/molbev/msq181) PMID: [20660084](https://pubmed.ncbi.nlm.nih.gov/20660084/)
44. van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes*. 2004; 4: 535–538.
45. Goudet J. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). 2001; Available from [www2.unil.ch/popgen/softwares/fstat.htm](http://www2.unil.ch/popgen/softwares/fstat.htm)
46. Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G. LOSITAN: a workbench to detect molecular adaptation based on a  $F_{ST}$ -outlier method. *BMC Bioinformatics*. 2008; 9: 323. doi: [10.1186/1471-2105-9-323](https://doi.org/10.1186/1471-2105-9-323) PMID: [18662398](https://pubmed.ncbi.nlm.nih.gov/18662398/)
47. Foll M, Gaggiotti OE. A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: a Bayesian perspective. *Genetics*. 2008; 180: 977–995. doi: [10.1534/genetics.108.092221](https://doi.org/10.1534/genetics.108.092221) PMID: [18780740](https://pubmed.ncbi.nlm.nih.gov/18780740/)
48. Kalinowski ST. HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol Ecol Notes*. 2005; 5: 187–189.
49. Raymond M, Rousset F. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J Hered*. 1995; 86: 248–249.
50. Weir BS, Cockerham CC. Estimating F-statistics for the analysis of population structure. *Evolution*. 1984; 38: 1358–1370.
51. Langella O. POPULATIONS 1.2. Population genetic software (individuals or populations distances, phylogenetic trees). CNRS, France; 2002.
52. Cavalli-Sforza LL, Edwards AW. Phylogenetic analysis. Models and estimation procedures. *Am J Hum Genet*. 1967; 19: 233. PMID: [6026583](https://pubmed.ncbi.nlm.nih.gov/6026583/)
53. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. *Genetics*. 2000; 55: 945–959.
54. Hubisz MJ, Falush D, Stephens M, Pritchard JK. Inferring weak population structure with the assistance of sample group information. *Mol Ecol Res*. 2009; 9: 1322–1332. doi: [10.1111/j.1755-0998.2009.02591.x](https://doi.org/10.1111/j.1755-0998.2009.02591.x) PMID: [21564903](https://pubmed.ncbi.nlm.nih.gov/21564903/)

55. Earl DA. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Res.* 2012; 4: 359–361.
56. Fraser DJ, Hansen MM, Østergaard S, Tessier N, Legault M, Bernatchez L. Comparative estimation of effective population sizes and temporal gene flow in two contrasting population systems. *Mol Ecol.* 2007; 16: 3866–3889. PMID: [17850551](#)
57. Hill WG. Estimation of effective population size from data on linkage disequilibrium. *Genet Res.* 1981; 38: 209–216.
58. Weir BS. Inferences about linkage disequilibrium. *Biometrics.* 1979; 35: 235–254. PMID: [497335](#)
59. Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR. NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size ( $N_e$ ). *Mol Ecol Res.* 2014; 14: 209–214. doi: [10.1111/1755-0998.12157](#) PMID: [23992227](#)
60. Waples RS. A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. *Conserv Genet.* 2006; 7: 167–184.
61. Wang J. A new method for estimating effective population sizes from a single sample of genotypes. *Mol Ecol.* 2009; 18: 2148–2164. doi: [10.1111/j.1365-294X.2009.04175.x](#) PMID: [19389175](#)
62. Tallmon DA, Koyuk A, Luikart G, Beaumont MA. ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. *Mol Ecol Res.* 2008; 8: 299–301. doi: [10.1111/j.1471-8286.2007.01997.x](#) PMID: [21585773](#)
63. Waples RS. Temporal variation in allele frequencies: testing the right hypothesis. *Evolution.* 1989; 43: 1236–1251.
64. Nei M, Tajima F. Genetic drift and estimation of effective population size. *Genetics.* 1981; 98: 625–640. PMID: [17249104](#)
65. Pollak E. A new method for estimating the effective population size from allele frequency changes. *Genetics.* 1983; 104: 531–548. PMID: [17246147](#)
66. Jorde PE, Ryman N. Temporal allele frequency change and estimation of effective size in populations with overlapping generations. *Genetics.* 1995; 139: 1077–1090. PMID: [7713410](#)
67. Hill WG. A note on effective population size with overlapping generations. *Genetics.* 1979; 92: 317–322. PMID: [17248921](#)
68. Wang J, Whitlock MC. Estimating effective population size and migration rates from genetic samples over space and time. *Genetics.* 2003; 163: 429–446. PMID: [12586728](#)
69. Berthier P, Beaumont MA, Cornuet J-M, Luikart G. Likelihood-based estimation of the effective population size using temporal changes in allele frequencies: a genealogical approach. *Genetics.* 2002; 160: 741–751. PMID: [11861575](#)
70. Jurvelius J, Leinikki J, Mamylovc V, Pushkin S. Stock assessment of pelagic three-spined stickleback (*Gasterosteus aculeatus*): A simultaneous up- and down-looking echo-sounding study. *Fish Res.* 1996; 27: 227–241. PMID: [8625959](#)
71. Peltonen H, Vinni M, Lappalainen A, Ponnii J. Spatial feeding patterns of herring (*Clupea harengus* L.), sprat (*Sprattus sprattus* L.), and the three-spined stickleback (*Gasterosteus aculeatus* L.) in the Gulf of Finland, Baltic Sea. *ICES J Mar Sci.* 2004; 61: 966–971.
72. Wootton RJ, Adams CE, Attrill MJ. Empirical modelling of the population dynamics of a small population of the threespine stickleback, *Gasterosteus aculeatus*. *Env Biol Fish.* 2005; 74: 151–161.
73. Palm S, Laikre L, Jorde PE, Ryman N. Effective population size and temporal genetic change in stream resident brown trout (*Salmo trutta*, L.). *Conserv Genet.* 2003; 4: 249–264.
74. Bernal-Ramírez JH, Adcock GJ, Hauser L, Carvalho GR, Smith PJ. Temporal stability of genetic population structure in the New Zealand snapper, *Pagrus auratus*, and relationship to coastal currents. *Mar Biol.* 2003; 142: 567–574.
75. Therkildsen NO, Nielsen EE, Swain DP, Pedersen JS. Large effective population size and temporal genetic stability in Atlantic cod (*Gadus morhua*) in the southern Gulf of St. Lawrence. *Can J Fish Aquat Sci.* 2010; 67: 1585–1595.
76. Cuveliers EL, Volckaert FAM, Rijnsdorp AD, Larmuseau MHD, Maes GE. Temporal genetic stability and high effective population size despite fisheries-induced life-history trait evolution in the North Sea sole. *Mol Ecol.* 2011; 20: 3555–3568. doi: [10.1111/j.1365-294X.2011.05196.x](#) PMID: [21790820](#)
77. Kennington WJ, Cadee SA, Berry O, Groth DM, Johnson MS, Melville-Smith R. Maintenance of genetic variation and panmixia in the commercially exploited western rock lobster (*Panulirus cygnus*). *Conserv Genet.* 2013; 14: 115–124. doi: [10.1186/gb-2013-14-5-115](#) PMID: [23657273](#)
78. Frankham R, Ballou JD, Briscoe DA. Introduction to Conservation Genetics. Cambridge University Press; 2002.
79. Lenormand T. Gene flow and the limits to natural selection. *Trends Ecol Evol.* 2002; 17: 183–189.

80. Crow JF, Kimura M. An Introduction to Population Genetics Theory. Harper and Row. 1970.
81. Richards C, Leberg PL. Temporal changes in allele frequencies and a population's history of severe bottlenecks. *Conserv Biol*. 1996; 10: 832–839.
82. Waples RS, Teel DJ. Conservation genetics of Pacific salmon I. Temporal changes in allele frequency. *Conserv Biol*. 1990; 4: 144–156.
83. Frankham R, Bradshaw CJ, Brook BW. Genetics in conservation management: revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biol Conserv*. 2014; 170: 56–63.
84. Traill LW, Brook BW, Frankham RR, Bradshaw CJ. Pragmatic population viability targets in a rapidly changing world. *Biol Conserv*. 2010; 143: 28–34.
85. Tessier N, Bernatchez L. Stability of population structure and genetic diversity across generations assessed by microsatellites among sympatric populations of landlocked Atlantic salmon (*Salmo salar* L.). *Mol Ecol* 1999; 8: 169–179.
86. Tessier N, Bernatchez L, Wright JM. Population structure and impact of supportive breeding inferred from mitochondrial and microsatellite DNA analyses in land-locked Atlantic salmon *Salmo salar* L. *Mol Ecol*. 1997; 6: 735–750.
87. Bekkevold D, André C, Dahlgren TG, Clausen LA, Torstensen E, Mosegaard H, et al. Environmental correlates of population differentiation in Atlantic herring. *Evolution*. 2005; 59: 2656–2668. PMID: [16526512](#)
88. Whitlock MC, McCauley DE. Indirect measures of gene flow and migrations:  $F_{ST} \neq 1/(4Nm + 1)$ . *Heredity*. 1999; 82: 117–125. PMID: [10098262](#)
89. Raeymaekers JAM, Konijnendijk N, Larmuseau MH, Hellemans B, Meester L, et al. A gene with major phenotypic effects as a target for selection vs. homogenizing gene flow. *Mol Ecol*. 2014; 23: 162–181. doi: [10.1111/mec.12582](#) PMID: [24192132](#)
90. Barrett RD, Rogers SM, Schluter D. Natural selection on a major armor gene in threespine stickleback. *Science*. 2008; 322: 255–257. doi: [10.1126/science.1159978](#) PMID: [18755942](#)
91. Kitano J, Bolnick DI, Beauchamp DA, Mazur MM, Mori S, Nakano T, et al. Reverse evolution of armor plates in the threespine stickleback. *Curr Biol*. 2008; 18: 769–774. doi: [10.1016/j.cub.2008.04.027](#) PMID: [18485710](#)
92. McKay JK, Latta RG. Adaptive population divergence: Markers, QTL and traits. *Trends Ecol Evol*. 2002; 17: 285–291.
93. Stortz JF. Using genome scans of DNA polymorphism to infer adaptive population divergence. *Mol Ecol*. 2005; 16: 671–688.
94. DeFaveri J, T. Shikano T, Ab Ghani NI, Merilä J. Contrasting population structures in two sympatric fishes in the Baltic Sea basin. *Mari Biol*. 2012; 159: 1659–1672.
95. Barker JSF. Effective population size of natural populations of *Drosophila buzzatii*, with a comparative evaluation of nine methods of estimation. *Mol Ecol*. 2011; 20: 4452–4471. doi: [10.1111/j.1365-294X.2011.05324.x](#) PMID: [21951766](#)
96. Hoehn M, Gruber B, Sarre SD, Lange R, Henle K. Can genetic estimators provide robust estimates of the effective number of breeders in small populations? *PLoS One*. 2012; 7: e48464. doi: [10.1371/journal.pone.0048464](#) PMID: [23139784](#)
97. Peuhkuri N, Seppä P. Do three-spined sticklebacks group with kin? *Ann Zool Fennici*. 1998; 35: 21–27.
98. Poulsen N, Nielsen EE, Schierup MH, Loeschcke V, Grønkjær P. Long-term stability and effective population size in North Sea and Baltic Sea cod (*Gadus morhua*). *Mol Ecol*. 2006; 15: 321–331. PMID: [16448403](#)
99. Waples RS, England PR. Estimating contemporary effective population size on the basis of linkage disequilibrium in the face of migration. *Genetics*. 2011; 1892: 633–644.
100. Consuegra S, Verspoor E, Knox D, Garcia de Leaniz C. Asymmetric gene flow and the evolutionary maintenance of genetic diversity in small, peripheral Atlantic salmon populations. *Conserv Genet*. 2005; 6: 823–842.
101. Saillant E, Gold JR. Population structure and variance effective size of red snapper (*Lutjanus campechanus*) in the north Gulf of Mexico. *Fish B-NOAA*. 2006; 104: 136–148.
102. Jorde PE. Allele frequency covariance among cohorts and its use in estimating effective size of age-structured populations. *Mol Ecol Resour*. 2012; 12: 476–480. doi: [10.1111/j.1755-0998.2011.03111.x](#) PMID: [22230371](#)
103. Frankham R. Effective population size/adult population size ratios in wildlife: a review. *Genet Res*. 1995; 66: 95–107.