Genetica
DOI 10.1007/s10709-015-9839-0

Population structure and effective/census population size ratio in threatened three-spined stickleback populations from an isolated river basin in northwest Spain

A. Pérez-Figueroa[1] · C. Fernández[2] · R. Amaro[2] · M. Hermida[2] · E. San Miguel[2]

Received: 4 December 2014 / Accepted: 19 April 2015

Abstract Variability at 20 microsatellite loci was examined to assess the population genetic structure, gene flow, and effective population size (N_e) in three populations of three-spined stickleback (*Gasterosteus aculeatus*) from the upper basin of the Miño River in Galicia, NW Spain, where this species is threatened. The three populations showed similar levels of genetic diversity. There is a significant genetic differentiation between the three populations, but also significant gene flow. N_e estimates based on linkage disequilibrium yielded values of 355 for the Miño River population and 241 and 311 for the Rato and Guisande Rivers, respectively, although we expect that these are overestimates. N_e estimates based on temporal methods, considering gene flow or not, for the tributaries yielded values of 30–56 and 47–56 for the Rato and Guisande Rivers, respectively. Estimated census size (N_c) for the Rato River was 880 individuals. This yielded a N_e/N_c estimate of 3–6 % for temporal estimation of Ne, which is within the empirical range observed in freshwater fishes. We suggest that the three populations analyzed have a sufficient level of genetic diversity with some genetic structure. Additionally, the absence of physical barriers suggests that conservation efforts and monitoring should focus in the whole basin as a unit.

Keywords *Gasterosteus aculeatus* · Microsatellites · Population structure · Effective population size · N_e/N_c

✉ E. San Miguel
eduardo.sanmiguel@usc.es

[1] Departamento de Bioquímica, Genética e Inmunología, Facultad de Biología, Universidad de Vigo, 36310 Vigo, Spain

[2] Departamento de Genética, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain

Introduction

Three-spined stickleback (*Gasterosteus aculeatus*) represents a model organism in evolutionary biology (Bell and Foster 1994; Mäkinen et al. 2006; Mäkinen and Merilä 2008). It is widely distributed in the Northern Hemisphere, with the Iberian Peninsula as the southern limit in the East Atlantic Ocean (FishBase 2013), and it is found in a variety of different habitats (marine, streams, rivers, lakes) which led to a great level of phenotypic variation directly linked to adaptation to the different habitats (Bell and Foster 1994). This phenotypic variation has a genetic basis widely verified in many populations (see, for example, Colosimo et al. 2005; Cano et al. 2006; Leinonen et al. 2006; Defaveri and Merilä 2013). The formation of these stickleback morphs is recent (15,000–10,000 years), and in several cases, it happened in parallel in different independent regions through parallel evolution (Bell and Foster 1994; Rundle et al. 2000).

The global conservation status of three-spined stickleback is good, mostly because of its widespread distribution and abundance. However, this species has been subject of conservation studies and concerns both in a reduced scale (Cano et al. 2008; Von Hippel 2008) or considering the protection of its adaptive radiation as a whole (Foster et al. 2003). At the southern limit of their distribution range, three-spined sticklebacks have disappeared from much of their historic range (Doadrio 2002; Clavero et al. 2009; Araguas et al. 2012). In the Iberian Peninsula, this species is widely distributed in Galicia freshwaters (an autonomous region in northwest Spain) and limited to very fragmented populations in other regions (Doadrio 2002). Although its global threat status is least concern in the list of IUCN (2013), Galician local government (Xunta de Galicia) has included this species, as a preventive measure, in its catalogue on threatened species [Decree Act 88/2007,

Published online: 28 April 2015

Xunta de Galicia (2007)], and it is listed as vulnerable in Spain (Doadrio 2002). Despite its threatened status in Galicia, our ignorance on the effective population sizes (N_e), census sizes (N_c), and genetic variation of the Galician stickleback populations is absolute.

N_e is defined as the size of an ideal population that has the same rate of change of allele frequencies or heterozygosity as the observed population. N_e is widely regarded as one of the most important parameters in both evolutionary (Charlesworth 2009) and conservation biology (Frankham 2005; Nunney and Elam 1994) as it is closely related to loss of genetic variation, increases in inbreeding, accumulation of mutations, and effectiveness of selection. Both N_e and N_c are parameters strongly correlated with population viability (Allendorf and Luikart 2007; Allendorf and Ryman 2002). Understanding the relation between N_e and N_c is important from a conservation point of view as it could help to understand the ecological factors that reduce N_e below N_c (Kalinowski and Waples 2002). Estimation of both parameters with adequate precision is difficult given that most methods rely on several assumptions frequently violated in natural populations (Luikart et al. 2010), especially for fish species. In fact there are less than a dozen studies that estimated N_e/N_c in freshwater or anadromous fish species, and they are mainly focused on salmonids (reviewed in Palstra and Ruzzante 2008) and even those estimates could be biased (Palstra and Fraser 2012). Reductions in N_e can occur even when N_c remains large, and the only way to detect that is empirical observation of genetic variation over time (Luikart et al. 2010). Monitoring strategies using genetic-based methods have been suggested as powerful tools for early detection of population declines (Antao et al. 2011).

The upper basin of the Miño River (Galicia, NW Spain) is isolated from the rest of the basin by the Belesar dam (Fig. 1), impeding movement of fishes and configuring a drainage area of 4344 Km^2 (15 % of whole area of Galicia). Such isolation could enhance risks to population if there is any further fragmentation within the basin. Galicia is an autonomous region within Spain, and its government has large conservation responsibility. Hence, genetic monitoring of stickleback populations in the upper basin of river Miño could provide important information toward populations conservation or restoration plans for stickleback or similar species in this and other isolated basins.

In this study, we used microsatellite markers to assess gene diversity, genetic structure, effective population size, and migration rates in three Galician three-spined stickleback populations. We were interested in evaluating the magnitude of fragmentation in order to understand the risks of loss of genetic diversity or population extinction at the long-term in these threatened Galician populations. We also estimated the census size of one of these populations in order to estimate the N_e/N_c for that population.

Materials and methods

Study area and sampling collection

Samples of *G. aculeatus* were obtained from three different places (Fig. 1): Miño River (MINO; 29T616530E, 4761933N), the main river in Galicia, upstream from the Belesar dam but downstream from the other two samples, taken from two tributaries of the Miño River: Guisande River (GUI; 29T617656E, 4789150N; 38.8 km from MINO) and Rato River (RATO; 29T619547E, 4761482N; 2.8 km from MINO, 41.6 km from GUI). In the last two sites, repetitive sampling was made to obtain three and four consecutive generations, as generations are easily differentiated (Fernández et al. 2000). Fish breed the next year following their birth and die shortly after. Analyzing otoliths (unpublished data 2005), we found that more than 97 % of reproductive adults (collected in June or July) are 1+, born in the previous year, with more than 46 mm of standard length, while fishes with standard lengths between 21 and 25 mm, born in those same months and their age were 0+, born in the current year. Most of fishes collected for our analyses in early June or July were reproductive adults, born the preceding year. In September, all fishes collected measured more than 45 mm, (born in the previous spring), and therefore, they belonged to a new generation. Annual cycle is usual in southern populations (Clavero et al. 2009; Crivelli and Britton 1987) and also (but not so strictly) in some European northern populations (Wootton et al. 2005). Each individual was released after clipping a piece of caudal fin with sterile scissors. Tissue was stored in 95 % ethanol until analysis. Detailed dates and sample sizes are shown in Table 1.

DNA extraction and microsatellite analysis

Total DNA was extracted from fin tissue using DNeasy® Blood and Tissue Kit (QIAGEN). Twenty microsatellite loci from *G. aculeatus* were amplified using the primers reported by: *2142PBBE* (Heckel et al. 2002), *1125PBBE, 7033PBBE* (Largiader et al. 1999), *Stn3, Stn9, Stn12, Stn19, Stn21, Stn26, Stn38, Stn57, Stn94, Stn110, Stn131, Stn132, Stn135, Stn152, Stn163, Stn174*, and *Stn208* (Peichel et al. 2001).

Microsatellites were amplified through singleplex PCR in 15-μL final reaction volume mixes consisting of 1 × PCR Gold Buffer (Applied Biosystems, ABI), 1.5 mM $MgCl_2$, 0.1 mm of each dNTP (GE Healthcare), 0.13 μm

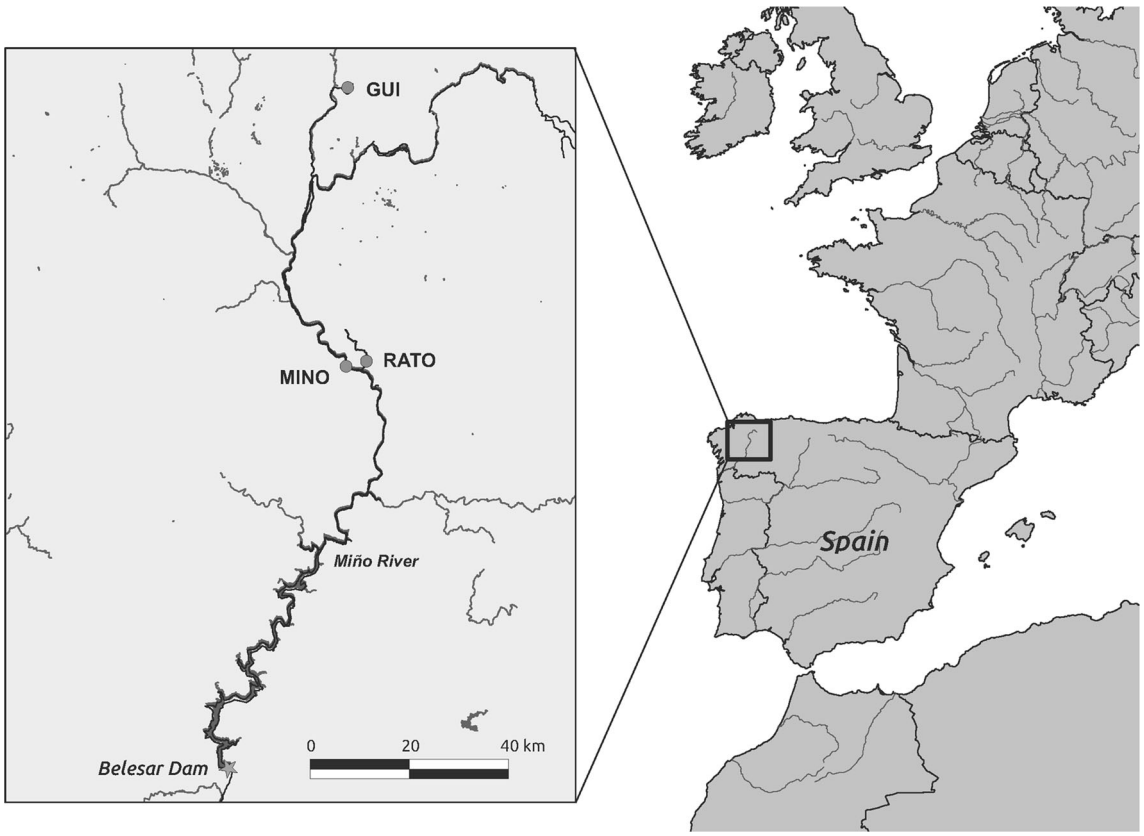

Fig. 1 Map of Miño River basin and its situation in Spain. *Circles* show the sampling points. The *star* represents the situation of Belesar dam that isolates upstream basin

Table 1 Geographical, temporal, and genetic characterization of samples

	Date sampled	*n*	*A*	*Ar*	*Ap*	*Ho*	*He*
Miño River							
MINO	June 2007	30	9.6 (1.1)	8.9 (1.0)	33	0.65 (0.06)	0.68 (0.06)
Guisande River							
GUI-0	July 2006	30	7.0 (1.1)	6.4 (1.0)	10	0.51 (0.07)	0.50 (0.06)
GUI-1	September 2006	30	6.5 (1.0)	6.1 (0.9)	6	0.49 (0.07)	0.49 (0.06)
GUI-2	July 2007	30	6.5 (0.9)	6.0 (0.8)	2	0.51 (0.06)	0.52 (0.06)
Rato River							
RATO-0	June 2006	30	5.3 (0.6)	5.1 (0.6)	2	0.61 (0.05)	0.60 (0.05)
RATO-1	September 2006	30	5.0 (0.6)	4.9 (0.6)	1	0.64 (0.05)	0.60 (0.04)
RATO-2	June 2007	30	5.0 (0.6)	4.9 (0.5)	1	0.60 (0.05)	0.61 (0.04)
RATO-3	June 2008	24	4.9 (0.6)	4.9 (0.6)	3	0.58 (0.05)	0.60 (0.04)

Sampling dates and sizes (*n*) along with the results of mean number of alleles per locus (*A*), allelic richness (*Ar*), total number of private alleles (*Ap*), unbiased (*He*), and observed (*Ho*) heterozygosities. Standard errors in brackets

of each primer, 5 U AmpliTaq® Gold DNA polymerase (ABI), and 40 ng template DNA. All loci were amplified using the following profile: denaturation (95 °C, 10′), 35 cycles of denaturation (94 °C, 45″), annealing (60 °C, 50″), and extension (72 °C, 50″); final extension (72 °C, 10′). Exceptions in the annealing temperatures were 58 °C for loci *7033PBBE, Stn12*, and *Stn21*; 56 °C for loci *Stn3, Stn132, Stn135, Stn163*, and *Stn174*; and 55 °C for locus *Stn38*. PCR products were separated in an ABI Prism 3730xl (ABI).

Genetic variation and population structure

Population characterization of allelic (number of alleles, allelic richness and number of private alleles) and gene (expected and observed heterozygosity) diversity for each sample was performed with the function POPGENE from the package PopGenkit (Rioux Paquette 2012) for the R statistical environment (R Development Core Team 2011). Allelic richness was calculated by jackknife resampling (1000 replicates), and the sample size for each locus is determined as the smallest number of individuals sampled among all populations for that specific locus (Rioux Paquette 2012). Pairwise comparisons for allelic and gene diversity were assessed by paired *t* test using R and applying BH multiple test correction. To assess the structure of these populations, we calculated a statistic of genetic differentiation, ρ_{ST} (Michalakis and Excoffier 1996), and Nei's genetic distance using SPAGeDi 1.4 (Hardy and Vekemans 2002). These statistics were calculated for every pair of samples, and then a permutation test of individuals in samples (1000 permutations) was used to assess significant contributions to the overall differentiation. Correlation between genetic and geographic distances was tested using a Mantel test implemented in vegan package (Oksanen et al. 2013) for R. Furthermore, we performed a Bayesian analysis of population structure using STRUCTURE 2.3.4 (Pritchard et al. 2000), testing all the samples for $K = 1$–8 clusters with an admixture model of correlated allele frequencies (10^5 burnin steps and 2×10^5 run steps, enough for the convergence of summary statistics). Hundred runs were evaluated using all samples or only those contemporary (June/July 2007: MINO, GUI-2 and RATO-2). The optimum number of clusters was assessed using the ΔK method (Evanno et al. 2005).

Effective population size (N_e) and census size (N_c)

We estimated local N_e using two different sets of estimators depending on the use of single or temporal samples. The first estimate was obtained using the single-sample linkage disequilibrium method implemented in the software LDNe (Waples and Do 2008), assuming a random mating system. Point estimates and 95 % confidence intervals (jackknife) are obtained using only those alleles with a frequency at least of 0.033 (two copies or more in the sample) to provide an acceptable balance between precision and bias (Waples and Do 2010). A second single-sample estimate used the sibship assignments (SA) method (Wang and Santure 2009) implemented in COLONY v. 2.0.1 (Jones and Wang 2010) assuming random mating. This method obtains N_e estimates from the frequencies of a pair of offspring taken at random from the population being sibs sharing the same one or two parents. We discarded the popular ONeSAMP (Tallmon et al. 2008), based on approximate Bayesian computation, because we found sensitivity of our data to the priors.

Temporal N_e was estimated using the modification of the standard temporal method proposed by Jorde and Ryman (2007) as implemented in NEESTIMATOR 2.01 (Do et al. 2014). We performed the analysis for both temporally sampled populations, GUI and RATO, with all the possible combinations of temporal samples within location. Point estimates and 95 % confidence intervals (parametric) are obtained using only those alleles with a frequency at least of 0.033. A second kind of temporal approach was the pseudo-ML (maximum likelihood) method implemented in NME (Wang 2001; Wang and Whitlock 2003) that allows joint estimation of N_e and migration rate (m) for continent-island metapopulation model. In this case, we allowed a prior N_e of 5000. We considered migration (and estimated) from MINO population without assuming drift–migration equilibrium.

In order to obtain an estimate of the N_e/N_c for some of the samples, we obtain maximum likelihood estimates of population census (N_c) for generation 3 of river Rato. We follow the procedures described by Moran (1951) and Zippin (1956). We used the results of a series of trappings in which the adult animals collected were removed from the river Rato (June 2008, generation 3). At the end of the experiment, all trapped fishes were returned to the river. Four consecutive samplings were practiced during 3 h in a small stretch (110 m long, 4 m width) by hand nets and electrofishing: 195, 168, 117, and 88 fishes were collected, respectively. From these data, we estimated of population size and its standard error by the regression method suggested by Zippin (1956).

Results

Genetic variability and population structure

Basic genetic diversity parameters of *G. aculeatus* populations, based on 20 microsatellite *loci* employed, appeared in appendix 1. The number of alleles detected at each microsatellite locus ranged from three (*2142PBBE, Stn135, Stn26, Stn38*) to thirty-four (*Stn208*) averaging 12.6 alleles per locus. Table 1 shows the genetic characterization of the samples including estimates of allelic and gene diversity.

Allelic diversity was higher in the main river than in the tributaries. MINO showed an allelic richness of 8.9 alleles and 33 private alleles across the 20 markers. GUI showed an averaged allelic richness of 6.2 alleles with six private alleles, and RATO an averaged allelic richness of 5.0 alleles with 1.8 private alleles. Gene diversity, measured by

the unbiased heterozygosity, was also higher in MINO (0.68) than in the tributaries. However, in contrast to allelic diversity, RATO showed more gene diversity (0.61) than GUI (0.50). There were no evidence departures from Hardy–Weinberg equilibrium as measured by F_{IS}. Both allelic and gene diversity were stable across the temporal samples in GUI and RATO. Pairwise comparisons using paired t test yield only significant ($P < 0.05$) between MINO and all the remainder samples for both allelic richness and heterozygosity.

Table 2 shows the pairwise microsatellite differentiation (ρ_{ST}) as well as the pairwise genetic distance (Nei's D), confirming GUI as the most differentiated population. Mantel test on geographical distances was not statistically significant ($P = 0.34$ for ρ_{ST} and $P = 0.16$ for Nei's D). Admixture analyses of the all the samples or only the contemporary samples using STRUCTURE gave the highest likelihood to a model of $K = 3$ homogeneous groups, (Fig. 2a). When consider the ΔK method (Evanno et al. 2005; Fig. 2b) and all the samples, however, the inferred number of clusters is $K = 2$. This seems to be an artifact given the homogeneity of the temporal samples of GUI and RATO yielding very unbalanced sample sizes of the clusters and then violating model assumptions of the ΔK method (Kalinowski 2011). In that case, it yields a scenario where MINO population is a full mix from subsidiaries populations (Fig. 2c). With $K = 3$, there is a scenario where the three rivers are very homogeneous, with MINO showing a very low degree of admixture.

Effective population size and census size

All the estimates of effective population size by the different methods used are given in Table 3. For the harmonic mean of the temporal samples (excluding infinites), $N_e = 311$ for GUI and $N_e = 241$ for RATO. When migration from MINO population is considered using the MNE method, then the N_e estimated for both populations were the smallest of all estimators. This estimator yielded for GUI a migration rate of $m = 0.08$ (confidence interval at 0.05 level: 0.04–0.12) and $N_e = 56$ (38–94). For RATO, $m = 0.10$ (0.05–0.17) and $N_e = 30$ (22–44). So, similar migration rate was estimated for both populations.

Estimates of effective population size by single-sample method based on linkage disequilibrium (Waples and Do 2008) for all the samples are also given in Table 3. For MINO, where single-sample estimations are the only estimates we obtained, $N_e = 355$. Estimates for GUI and RATO were slightly lower than MINO, but much higher than temporal estimates. The SA method obtained similar estimates (around 300–400) for the three populations.

An estimate of $N_c = 880$ was obtained for RATO population in the generation three. The 95 % confidence limits for N_c are approximately 836–924. The estimates of N_e/N_c obtained by dividing the different estimates of N_e in RATO by N_c are given in the last row of Table 3. N_e/N_c estimates ranged between 0.034 and 0.326.

Discussion

Genetic diversity and population structure

Microsatellite variation showed that genetic diversity (both allelic richness and heterozygosity) was higher in the main river than the tributaries as expected because of stream sizes. For RATO and GUI, all the temporal samples showed stability in the genetic diversity across successive generations. MINO diversity was slightly higher than diversity estimated for another Galician population and other Atlantic and Mediterranean populations (Araguas et al. 2012; Mäkinen et al. 2006) The values we obtained were similar to those reported by (Raeymaekers et al. 2008) averaging 20 sampling locations, separated by several physical barriers, of the Scheldt River in Belgium. In that study, authors concluded that anthropogenic barriers had a severe impact on genetic diversity and population structure increasing the risk of stochastic population extinction, lower survival, and population sizes. In our study, however,

Table 2 Average pairwise statistics of differentiation (ρ_{ST}, below diagonal) and genetic distance (Nei's D, above diagonal) between populations

	GUI-0	GUI-1	GUI-2	MINO	RATO-0	RATO-1	RATO-2	RATO-3
GUI-0		−0.001	0.012	**0.209**	**0.342**	**0.346**	**0.404**	**0.394**
GUI-1	−0.003		0.005	**0.194**	**0.341**	**0.341**	**0.392**	**0.374**
GUI-2	0.021	0.009		***0.203***	**0.352**	**0.350**	***0.380***	**0.366**
MINO	**0.212**	**0.202**	***0.196***		**0.118**	**0.114**	***0.121***	**0.125**
RATO-0	**0.317**	**0.315**	**0.309**	**0.106**		0.002	**0.026**	0.022
RATO-1	**0.325**	**0.321**	**0.315**	**0.106**	0.004		**0.023**	**0.024**
RATO-2	**0.338**	**0.331**	***0.314***	***0.103***	**0.030**	**0.027**		0.003
RATO-3	**0.341**	**0.331**	**0.315**	**0.109**	0.026	**0.030**	0.002	

Indicated with italic are the values comparing the contemporary samples in the three populations. In bold, values that are highly significant $P < 0.001$ in the permutation test

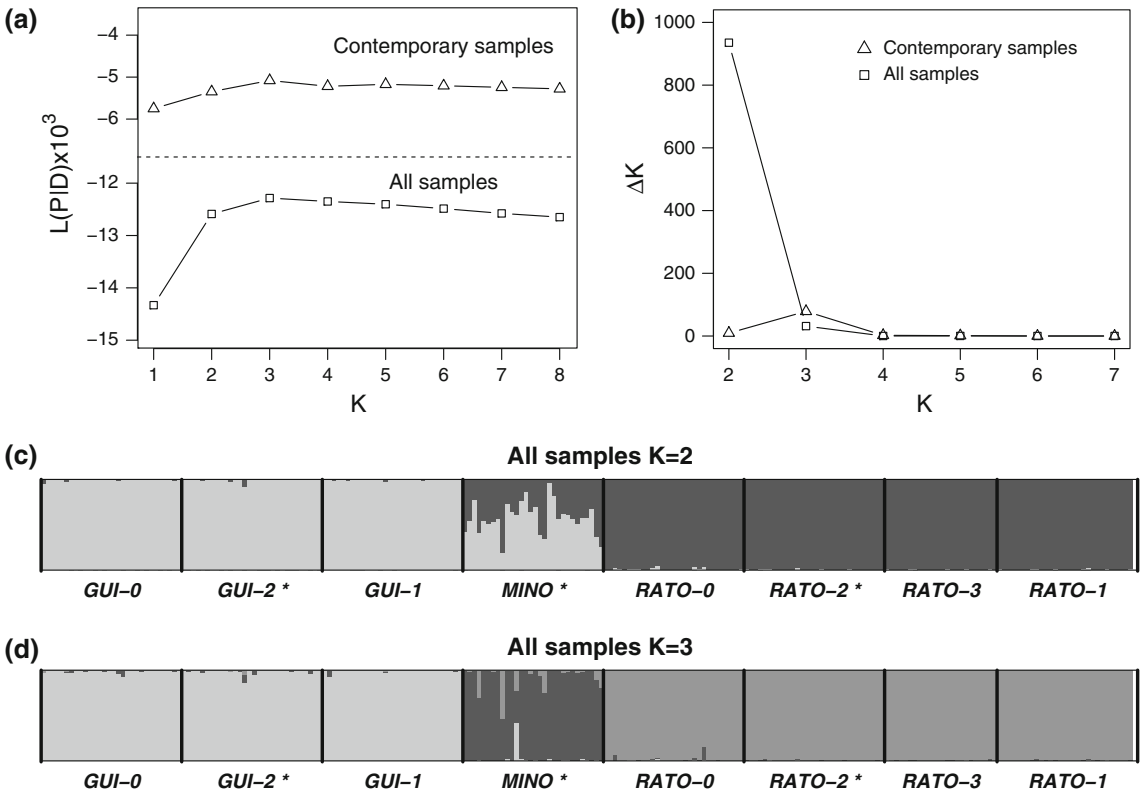

Fig. 2 Admixture analysis by STRUCTURE using all the temporal samples or only those contemporary samples (GUI-2, MINO, and RATO-2). **a** Mean log probability of the data under different number of *K* clusters. **b** Evanno et al. (2005) ΔK statistic. **c–d** *Bar plots* with assignment probabilities of all analyzed samples to the inferred clusters (**c**: $K = 2$; **d**: $K = 3$). Each individual is represented as a *vertical bar* partitioned into segments according to the proportion of markers belonging to each identified clusters, represented by different grades of *gray*. Contemporary samples are marked with an *asterisk*

Table 3 Effective population size estimated by temporal (JR07: Jorde and Ryman 2007; MLN: Wang and Whitlock 2003) and one sample methods (LDNe: Waples and Do 2008; SA: Wang and Santure 2009) in the studied rivers

	Temporal method		Single-sample methods	
	JR07	MLN	LDNe	SA
MINO	–	–	355 (150–∞)	348 (171–8206)
GUI	47[a] (34–62)	56 (38–94)	311[b] (106–∞)	373 (181–∞)
RATO	56[a] (41–74)	30 (22–44)	241[b] (98–∞)	287 (142–∞)
N_e/N_c (RATO-3)	0.064	0.034	0.273	0.326

Point estimates of N_e/N_c are given, in the last row, for generation 3 in RATO using the different estimates of N_e

[a] Temporal estimate considering the whole sampled range

[b] Harmonic mean of the different generations sampled

there are no such barriers between sampling locations which are connected by gene flow.

Our analyses detected some genetic differentiation (up to 34 %) between GUI and the other two localities, located more than 40 km away from GUI. This was expected as is common that freshwater fishes tend to lead to isolation-by-distance models of divergence because of the hydrographical pattern (Araguas et al. 2012). However, in our data, we did not find significant correlation between geographical distance and genetic differentiation measures, likely because lack of statistical power due to the low number of localities. The values we obtained for population differentiation and genetic distances were lower than those obtained by Hendry et al. (2002) in the Misty Lake system (Vancouver Island, British Columbia). Their sampling scheme was similar to that in our study, with temporal samples, sampling a population in a larger mass of water (lake in their case, the main river in ours) that the other

sampled populations (rivers), and without physical barriers between sampled populations. They found a negative association between morphological divergence and gene flow in the Misty Lake system, concluding that while natural selection promotes adaptive divergence of lake and stream sticklebacks, gene flow constrains this divergence. In our case, there is no evidence of adaptive differences in these populations, and the connectivity between rivers seems to be enough to keep genetic structure at moderate level.

Effective population size

We used several different estimators of effective population size, all with different assumptions, strengths and weaknesses. LDNe and SA are estimators of N_eI (inbreeding N_e; long term or historical N_e), being concerned with the loss of heterozygosity so they are mainly influenced by the number of breeders, while the temporal methods are estimators of N_{eV} (variance N_e, contemporary N_e), concerned with change in allele frequencies with time and therefore mostly influenced by the number of offspring (Luikart et al. 2010; Waples 2002). LDNe is biased when the sample size is not close to the effective population size (Waples and Do 2008). The high values obtained by LDNe could correspond to a metapopulations scale if migration is large enough (Waples and England 2011). Furthermore, all estimates of N_e provided should be taken with caution as our system cannot remove all possible sources of bias for both estimators, specially the sample size, the number of generations sampled, and the gene flow between localities.

It has been argued that retention of long-term evolutionary potential requires N_e over 500 and avoiding of a high increase in inbreeding rate requires N_e over 50 (Frankham et al. 2010), although a recent review of these values suggest to double them (Frankham et al. 2014). In the populations we studied, the estimates of effective population sizes were clearly below the long-term threshold, but MINO was above the threshold required for short-term conservation. However, the very wide confidence intervals, up to infinity, request some caution about conclusions. Temporal estimates in GUI and RATO showed N_e close to the recommended threshold for avoiding excessive inbreeding in the short term.

By definition, the ratio between effective population size and census size (N_e/N_c) is expected to be equal to one under Wright–Fisher ideal population conditions (panmixia, equal sex ratio, stable population size; Caballero 1994) although N_e is generally much lower than N_c in natural populations due to violations of those conditions and estimates of N_e/N_c averaged to 0.11 for studies with such violations included (Frankham et al. 2010). Palstra and Fraser (2012) reviewed a large number of genetic N_e estimates (508) and identified those that correctly linked N_e with N_c (31), obtaining a median estimate of 0.231. Despite of the importance of this ratio, only 28 % of the studies with N_e estimates have explicitly attempted to link N_e to N_c (Palstra and Fraser 2012). In this study, estimates of N_c were only possible in the Rato River which was the smallest population of those sampled. The N_e/N_c ratio estimated was very small (3–6 % for temporal estimation of N_e), but it is within the range usually observed in freshwater fish populations (see Table S1 in Palstra and Fraser 2012). Up to our best knowledge, this is the first published estimate of N_e/N_c ratio in stickleback. It has been argued that for a correct calculation of N_e/N_c, one needs explicitly assume that population size has been constant during the time period when the data were collected (Palstra and Fraser 2012). This is the assumption we made from our results. Unfortunately, we cannot discern, from our data, how the other two possible factors (mating system or sex ratio) might influence this reduction in the N_e/N_c ratio. However, there are samples from these same populations that suggest a sex ratio bias with a proportion close to 1.6 females per male (unpublished data) and this could explain the low N_e/N_c ratio obtained.

Implications for conservation

Identification of appropriate population units for protection and management is essential for the conservation of biological diversity (Allendorf and Luikart 2007). The knowledge of the geographical scale at which populations are genetically differentiated and the level of gene flow between them is key for any management plan for endangered species in order to define the appropriate units for short-term conservation (Giller 2005; Salgueiro et al. 2003). Given the results we obtained, we suggest that the three populations analyzed in the upper basin of the Miño River have an appropriate level of genetic diversity. Although there is some genetic structure, the absence of physical barriers between populations suggest that conservation efforts and monitoring do not need to focus in any of the populations but in the whole upper basin as a unit. It is important to note that such upper basin of Miño River is defined here as the entire watershed upstream to Belesar Dam, located about 60 km downstream of MINO (Fig. 1). Belesar Dam, lacking fish ladder, is a physical barrier that isolates all the upper basin from the rest of the Miño-Sil basin.

We think that moderately fragmentation and low N_e found in the upper basin of Miño River is not a drawback from the evolutionary point of view, despite of being isolated by a dam, and then it can hardly be rescued by recolonization. Extending the methods of this study using populations from other rivers and basins would be necessary to have a whole picture of the genetic health of three-

spined stickleback in Galicia. Furthermore, the results presented here could be taken as reference for future monitoring of these populations to evaluate possible changes that threaten the basin, specially monitoring of N_e/N_c ratio could help to understand its ecological determinants and take management decisions.

A relevant aspect of the interplay between genetics and conservation is to ascertain whether genetic consequences of fragmentation contribute to extinction. Given the results we found, we can conclude that there is stability enough in the genetic diversity of three-spined stickleback in upper basin of the Miño River to be optimistic about the fate of this species in Galicia, despite of its current local conservation status, at least as long as environmental conditions would remain also stable. This is important beyond the local interest of these populations as this isolated basin, in the southern limit of the distribution of sticklebacks, could be an ideal scenario to evaluate the response of the species to global climate changes.

Acknowledgments We thank J. Galindo and four anonymous reviewers for helpful discussions on a previous version of the manuscript. We also thank to all the forest guards, especially to Mr. Latas, that accompanied us in the sampling trips. This work was funded by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA; RZ2012-00011-C02-01), Xunta de Galicia (GPC2013-011 and INCITE08ENA261066ES) and Fondos Feder: "Unha maneira de facer Europa."

References

Allendorf FW, Luikart G (2007) Conservation and the genetics of populations. Blackwell Publishing, Malden

Allendorf FW, Ryman N (2002) The role of genetics in population viability analysis. In: Beissinger SR, McCullough DR (eds) Population viability analysis. University of Chicago Press, Chicago, pp 50–85

Antao T, Pérez-Figueroa A, Luikart G (2011) Early detection of population declines: high power of genetic monitoring using effective population size estimators. Evol Appl 4:144–154

Araguas RM, Vidal O, Pla C, Sanz N (2012) High genetic diversity of the endangered Iberian three-spined stickleback (*Gasterosteus aculeatus*) at the Mediterranean edge of its range. Freshw Biol 57:143–154

Bell MA, Foster SA (1994) The evolutionary biology of the threespine stickleback. Oxford University Press, Oxford

Caballero A (1994) Developments in the prediction of effective population size. Heredity 73:657–879

Cano JM, Matsuba C, Mäkinen H, Merilä J (2006) The utility of QTL-linked markers to detect selective sweeps in natural populations—a case study of the EDA gene and a linked marker in threespine stickleback. Mol Ecol 15:4613–4621

Cano JM, Mäkinen HS, Leinonen T et al (2008) Extreme neutral genetic and morphological divergence supports classification of Adriatic three-spined stickleback (*Gasterosteus aculeatus*) populations as distinct conservation units. Biol Conserv 141:1055–1066

Charlesworth B (2009) Fundamental concepts in genetics: effective population size and patterns of molecular evolution and variation. Nat Rev Genet 10:195–205

Clavero M, Pou-Rovira Q, Zamora L (2009) Biology and habitat use of three-spined stickleback (*Gasterosteus aculeatus*) in intermittent Mediterranean streams. Ecol Freshw Fish 18:550–559

Colosimo PF, Hosemann KE, Balabhadra S et al (2005) Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. Science 307:1928–1933

Crivelli AJ, Britton RH (1987) Life history adaptations of *Gasterosteus aculeatus* in a Mediterranean wetland. Env Biol Fish 18: 109–125

Defaveri J, Merilä J (2013) Evidence for adaptive phenotypic differentiation in Baltic Sea sticklebacks. J Evol Biol 26:1700–1715

Do C, Waples RS, Peel D et al (2014) NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. Mol Ecol Resour 14:209–214

Doadrio I (2002) Atlas y libro rojo de los peces continentales de España. Dirección General de Conservación de la Naturaleza: Museo Nacional de Ciencias Naturales, Madrid

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14(8):2611–2620

Fernández C, Hermida M, Amaro R, San Miguel E (2000) Lateral plate variation in Galician stickleback populations in the rivers Miño and Limia, NW Spain. Behaviour 137:965–979

FishBase (2013) Reviewed native distribution map for *Gasterosteus aculeatus aculeatus* (Three-spined stickleback). http://www.aquamaps.org, version of Aug. 2013. Accessed 4 Dec 2013

Foster SA, Baker JA, Bell MA (2003) The case for conserving threespine stickleback populations. Fisheries 28:10–18

Frankham R (2005) Genetics and extinction. Biol Conserv 126:131–140

Frankham R, Ballou JD, Briscoe DA (2010) Introduction to conservation genetics, 2nd edn. Cambridge University Press, Cambridge

Frankham R, Bradshaw CJA, Brook BW (2014) Genetics in conservation management: revised recommendations for the 50/500 rules, red list criteria and population viability analyses. Biol Conserv 170:56–63

Giller PS (2005) River restoration: seeking ecological standards. Editor's introduction. J Appl Ecol 42:201–207

Hardy OJ, Vekemans X (2002) SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Mol Ecol Notes 2:618–620

Heckel G, Zbinden M, Mazzi D, Kohler A, Reckeweg G, Bakker TCM, Largiadèr CR (2002) Microsatellite markers for the threespined stickleback (*Gasterosteus aculeatus* L.) and their applicability in a freshwater and an anadromous population. Conserv Genet 3:77–79

Hendry AP, Taylor EB, McPhail JD (2002) Adaptive divergence and the balance between selection and gene flow: lake and stream stickleback in the Misty system. Evolution 56:1199–1216

IUCN (2013) IUCN Red List of Threatened Species. Version 2013.1. http://www.iucnredlist.org. Accessed 04 Dec 2013

Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. Mol Ecol Resour 10:551–555

Jorde P, Ryman N (2007) Unbiased estimator for genetic drift and effective population size. Genetics 177:927–935

Kalinowski ST (2011) The computer program STRUCTURE does not reliably identify the main genetic clusters within species: simulations and implications for human population structure. Heredity 106:625–632

Kalinowski ST, Waples RS (2002) Relationship of effective to census size in fluctuating populations. Conserv Biol 16:129–136

Largiader CR, Fries V, Kobler B, Bakker TCM (1999) Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.). Mol Ecol 8:342–344

Leinonen T, Cano JM, Mäkinen H, Merilä J (2006) Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of threespine sticklebacks. J Evol Biol 19:1803–1812

Luikart G, Ryman N, Tallmon D, Schwartz M, Allendorf FW (2010) Estimation of census and effective population sizes: the increasing usefulness of DNA-based approaches. Conserv Genet 11:355–373

Mäkinen HS, Merilä J (2008) Mitochondrial DNA phylogeography of the three-spined stickleback (*Gasterosteus aculeatus*) in Europe-evidence for multiple glacial refugia. Mol Phylogenet Evol 46: 167–182

Mäkinen HS, Cano JM, Merilä J (2006) Genetic relationships among marine and freshwater populations of the European three-spined stickleback (*Gasterosteus aculeatus*) revealed by microsatellites. Mol Ecol 15:1519–1534

Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. Genetics 142:1061–1064

Moran PAP (1951) A mathematical theory of animal trapping. Biometrika 38:307–311

Nunney L, Elam DR (1994) Estimating the effective population size of conserved populations. Conserv Biol 8:175–184

Oksanen J, Guillaume Blanchet F, Kindt R, et al (2013) vegan: community ecology package. R package version 2.0–9. http://CRAN.R-project.org/package=vegan

Palstra FP, Fraser DJ (2012) Effective/census population size ratio estimation: a compendium and appraisal. Ecol Evol 2:2357–2365

Palstra FP, Ruzzante DE (2008) Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? Mol Ecol 17:3428–3447

Peichel CL, Nereng KS, Ohgi KA, Cole BL, Colosimo PF, Buerkle CA et al (2001) The genetic architecture of divergence between threespine stickleback species. Nature 414:901–905

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

R Development Core Team (2011) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Raeymaekers JAM, Maes GE, Geldof S, Hontis I, Nackaerts K, Volckaert FAM (2008) Modeling genetic connectivity in sticklebacks as a guideline for river restoration. Evol Appl 1:475–488

Rioux Paquette S (2012) PopGenKit: useful functions for (batch) file conversion and data resampling in microsatellite datasets. R package version 1.0. http://CRAN.R-project.org/package=PopGenKit

Rundle HD, Nagel L, Boughman JW, Schluter D (2000) Natural selection and parallel speciation in sympatric sticklebacks. Science 287:306–308

Salgueiro P, Carvalho G, Collares-Pereira MJ, Coelho MM (2003) Microsatellite analysis of genetic population structure of the endangered cyprinid *Anaecypris hispanica* in Portugal: implications for conservation. Biol Conserv 109:47–56

Tallmon DA, Koyuk A, Luikart G, Beaumont MA (2008) ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. Mol Ecol 8:299–301

Von Hippel F (2008) Conservation of threespine and ninespine stickleback radiations in the Cook Inlet Basin, Alaska. Behaviour 145:693–724

Wang J (2001) A pseudo-likelihood method for estimating effective population size from temporally spaced samples. Genet Res 78:243–257

Wang J, Santure A (2009) Parentage and sibship inference from multilocus genotype data under polygamy. Genetics 181:1579–1594

Wang J, Whitlock MC (2003) Estimating effective population size and migration rates from genetic samples over space and time. Genetics 163:429–446

Waples RS (2002) Effective size of fluctuating salmon populations. Genetics 161:783–791

Waples RS, Do C (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. Mol Ecol Resour 8:753–756

Waples RS, Do C (2010) Linkage disequilibrium estimates of contemporary Ne using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. Evol Appl 3:244–262

Waples RS, England PR (2011) Estimating contemporary effective population size based on linkage disequilibrium in the face of migration. Genetics 189:633–644

Wootton RJ, Adams CE, Attrill MJ (2005) Empirical modeling of the population dynamics of a small population of the threespine stickleback, *Gasterosteus aculeatus*. Env Biol Fish 74:151–161

Xunta de Galicia (2007) DECRETO 88/2007, do 19 de abril, polo que se regula o Catálogo galego de especies ameazadas. Diario Oficial de Galicia 89:7409

Zippin C (1956) An evaluation of the removal method of estimating animal populations. Biometrics 12:163–189