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RESEARCH ARTICLE

Distinctive insular forms of threespine stickleback (*Gasterosteus aculeatus*) from western Mediterranean islands

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Abstract Neutral and adaptive variation among populations within a species is a major component of biological diversity and may be pronounced among insular populations due to geographical isolation and island specific evolutionary forces at work. Detecting and preserving potential evolutionary significant units below the species rank has become a crucial task for conservation biology. Combining genetic, phenotypic and ecological data, we investigated evolutionary patterns among the enigmatic threespine stickleback populations from western Mediterranean islands, all of which are threatened by habitat deterioration and climate change. We find indications that these populations derive from different genetic lineages, being genetically highly distinct from the stickleback of mainland Europe and the northern Atlantic as well as from each other. Mediterranean island stickleback populations are also phenotypically distinct from mainland populations but interestingly stickleback from Iceland have converged on a similar phenotype. This distinctive island stickleback phenotype seems to be driven by distinct selective regimes on islands versus continents. Overall, our results reveal the status of western Mediterranean island stickleback as evolutionarily distinct units, important for conservation of biodiversity.

Keywords Island rule · *Gasterosteus aculeatus* · Glacial refugium · Mediterranean biota

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Introduction

Species and even entire ecosystems are currently threatened by an array of anthropogenic changes to the environment, including global warming (Pereira et al. 2010), eutrophication (Seehausen et al. 1997; Taylor et al. 2006; Vonlanthen et al. 2012) or the introduction of nonnative species (Elton 1958; Lockwood et al. 2007). As a result, many endemic species and distinct populations with small geographic ranges have either become extinct, experienced severe genetic bottlenecks that threaten their ability to adapt to changing environments (Lande 1998), or become genetically homogenized with introduced populations of related species (Olden et al. 2004). In particular, insular ecosystems, which in many cases harbor the phenotypically and genetically most distinctive populations within otherwise widely distributed species (Foster 1964; Case 1978; Whittaker and Fernández-Palacios 2007), are vulnerable to climatic and human-induced perturbations (Ricketts et al. 2005; Courchamp et al. 2014). Freshwater organisms on Mediterranean islands belong to the most threatened species, but assessment of genetic and phenotypic distinctiveness is in most cases still lacking (Kottelat and Freyhof 2007; Cuttelod et al. 2008; Geiger et al. 2014).

Islands may generally harbor genetically and phenotypically distinct populations more often than mainland

sites, following the "island rule" (Foster 1964; Case 1978; Lomolino 1985). The latter suggests that insular populations of small vertebrates evolve an increased body size, whereas large vertebrates evolve dwarf populations on islands, potentially due to partial release from competitors and predators, often leading to further phenotypic adaptation (Case 1978; Lomolino et al. 2012). Albeit the island rule has been validated in many vertebrate taxa, examples from freshwater fish are relatively rare (Lomolino 2005, but see MacColl et al. 2013).

The threespine stickleback (*Gasterosteus aculeatus* species complex) is an ancestrally marine species with a circumpolar distribution in the Northern Hemisphere. Throughout its range, it has repeatedly colonized freshwater systems, and most extant freshwater populations are young, having been formed after the last glacial maximum about 12,000 years ago (McKinnon and Rundle 2002). In Europe, freshwater stickleback exist from Iceland down to southern Italy and Greece, having been relatively widespread in freshwaters along the Mediterranean coast (e.g., Crivelli and Britton 1987; Lobón-Cerviá et al. 1988; Mäkinen et al. 2006; Mäkinen and Merilä 2008; Araguas et al. 2012) and reaching as far south as Syria (Krupp and Coad 1985). The repeated evolution of freshwater populations from marine ancestors was often facilitated by the maintenance of freshwater-adapted alleles at low frequencies in the ancestral marine gene pool due to some ongoing gene flow from freshwater adapted populations to the ancestral marine populations (Schluter and Conte 2009; Jones et al. 2012). Consequently marine populations harbor a large standing genetic variation, upon which selection can rapidly act during the colonization of new freshwater bodies. However, marine stickleback do not occur farther south in the Atlantic ocean than the Bay of Biscay and are absent from the Mediterranean Sea (Münzing 1963; Mäkinen et al. 2006). Freshwater stickleback populations from southern Europe are thus geographically strongly isolated from marine stickleback and cannot have exchanged genes for a significant length of time. Despite their patchy but geographically wide distribution in the Mediterranean bioregion, only three Mediterranean islands are known to harbor freshwater stickleback, all in the western Mediterranean: Mallorca (Riera 1980; Lobón-Cerviá et al. 1988), Corsica (Gauthier and Rose 1974) and Sardinia (Bertin 1925; Münzing 1963; Bianco 1980; Kottelat and Freyhof 2007; Orru et al. 2010).

The absence of a contemporary marine stickleback population has rendered the current biogeographic distribution of Mediterranean freshwater stickleback somewhat of an enigma (Bertin 1925; Münzing 1963). Some have suggested that the existing populations may represent glacial relicts, where marine fish from the Atlantic Ocean had occurred in the Mediterranean Sea and colonized Mediterranean freshwater sites from there during the last glacial maximum when water temperatures and salinities were lower (Münzing 1963). Genetic analyses of mainland populations imply that Mediterranean freshwater stickleback may indeed be remnants of several glacial refugia (Mäkinen et al. 2006). The absence of potential gene flow through the Mediterranean Sea among these freshwater mainland populations has moreover led to high genetic differentiation amongst them, even between geographically close populations (Cano et al. 2008; Mäkinen and Merilä 2008; Araguas et al. 2012; DeFaveri et al. 2012).

Albeit stickleback are listed as a species of least concern for conservation by the IUCN, they are locally threatened, especially in the Mediterranean region (Araguas et al. 2012, NatureServe 2015). Recent conservation-related assessments of mainland Mediterranean stickleback suggested that many of the contemporary populations represent distinct evolutionary significant units, i.e., forming unique adaptive groups due to their genetic and ecological distinctiveness (Ryder 1986; Moritz 1994). They differ from other European populations genetically and phenotypically (Mäkinen et al. 2006; Cano et al. 2008; Mäkinen and Merilä 2008; DeFaveri et al. 2012) as well as on a few occasions in their life history (Crivelli and Britton 1987; Clavero et al. 2009). Many of the populations have declined or even become extinct since the beginning of the 20th century. The remaining populations are currently threatened by an array of factors, including habitat fragmentation, the release of non-native fish species or anthropogenic changes of the flow regime due to the withdrawal of water for agricultural irrigation (Clavero et al. 2009; Orru et al. 2010; Araguas et al. 2012). Moreover, the Mediterranean climate itself with its alternate dry and wet periods and irregular flash floods may negatively affect the remaining populations (Gasith and Resh 1999), with climate change likely to result in extended dry periods with often very low residual water flow (Palmer and Räisänen 2002).

Much of the current knowledge on the genetic and phenotypic distinctiveness of Mediterranean stickleback is based on mainland populations, whereas island populations are understudied. Using microsatellite markers, we first infer the genetic relationships between populations from the three Mediterranean islands where stickleback were known to occur in relation to other mainly mainland populations from across Europe. Based on mitochondrial DNA, we further assess if the observed population genetic structure may be a result of different colonization histories and/or glacial refugias. Subsequently, we test to which degree these Mediterranean island populations may differ in their ecology and phenotype from their mainland counterparts and moreover compare them to an island population in northern Europe (Iceland).

Materials and methods

Stickleback were collected from all Mediterranean islands where their presence had previously been indicated, i.e., Corsica (Gauthier and Rose 1974), Mallorca (Lobón-Cerviá et al. 1988) and Sardinia (Bianco 1980). To assess the degree of genetic and phenotypic distinctiveness of these Mediterranean island stickleback in relation to other European populations, ten populations from across Europe were included (Fig. 1; Table 1). Individuals were collected using minnow traps and hand nets. In all cases, fish were sacrificed with an overdose of clove oil and preserved in 70 % ethanol. A fin clip was additionally taken for genetic analysis and preserved in absolute ethanol. Our studied populations originate from different freshwater habitats (i.e., lakes and streams) in order to cover much of the ecological variation among European freshwater stickleback to which the Mediterranean populations can be compared. In addition, one marine population from Iceland, representing a putative ancestral state of all European freshwater stickleback was included (Orti et al. 1994; Mäkinen et al. 2006). Populations were further assigned to belong either to northern or southern Europe, depending on whether the respective catchment was in reach of the contemporary population of Atlantic marine stickleback (populations A–F in Fig. 1) or not (populations G–M).

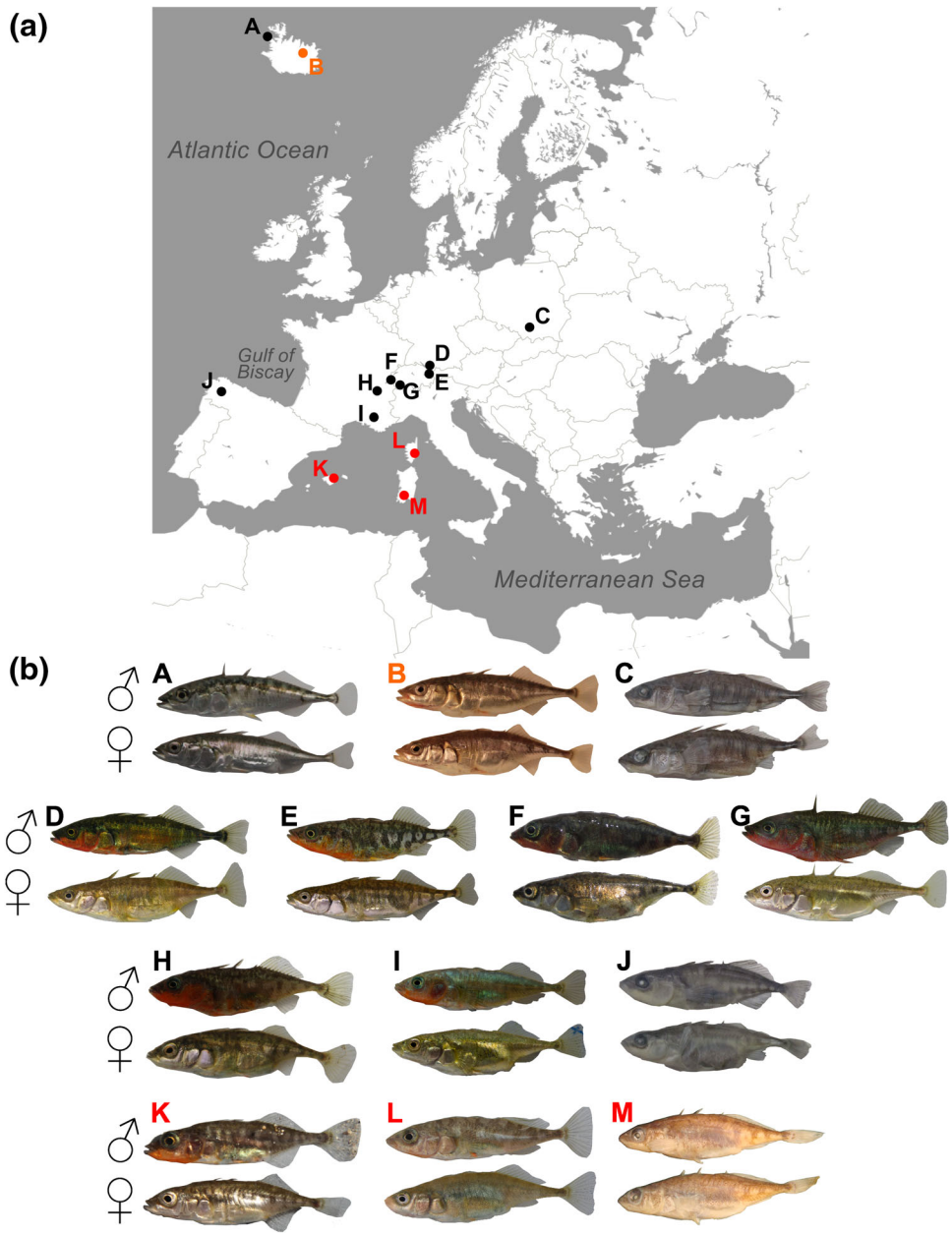

Fig. 1 Overview of the studied populations: **a** Map of Europe (© Wikimedia) with the populations included in this study highlighted. Populations from islands are highlighted in *color* (*orange* Iceland, *red* Mediterranean). **b** Representative examples of each sex for the different stickleback populations: *A* Iceland Marine, *B* Iceland Lake, *C* Poland, *D* Constance Lake, *E* Constance Stream, *F* Geneva Lake, *G* Geneva Stream, *H* France Stream 2, *I* France Stream 1, *J* Galicia, *K* Mallorca, *L* Corsica, *M* Sardinia. Pictures for populations *C*, *J* and *M* were taken from preserved specimens. Note that individuals are not scaled by size. See Table 1 for details. (Color figure online)

Table 1 Summary of all populations used in this study with their coordinates and country of origin, their relative geographical origin within Europe and their connectivity as well as the habitat they were sampled in and the sample size for morphology, stomach content analysis and microsatellites respectively (see main text for details)

Population	Country	Geography	Connectivity	Habitat	Latitude (N)	Longitude		$N_{Morphology}$	$N_{Stomachs}$	$N_{\mu sats}$
Mallorca	Spain	South	Island	Stream	39°47′18.5″	3°04′39.1″	E	26	26	29
Galicia	Spain	South	Mainland	Stream	42°36′13.2″	7°43′16.1″	W	9	9	30
Corsica	France	South	Island	Stream	41°59′46.1″	9°22′41.5″	E	32	32	29
Sardinia	Italy	South	Island	Stream	39°26′06.2″	8°45′13.0″	E	–	–	21
Sardinia[a]	Italy	South	Island	Stream	39°16′9.32″	8°48′40.6″	E	14	–	–
France Stream 1	France	South	Mainland	Stream	43°54′33.6″	5°05′08.7″	E	20	20	21
France Stream 2	France	South	Mainland	Stream	45°58′03.6″	5°17′39.6″	E	32	–	26
Geneva Lake	Switzerland	South	Mainland	Lake	46°31′02.0″	6°34′40.7″	E	40	37	29
Geneva Stream	Switzerland	South	Mainland	Stream	46°12′47.7″	7°18′25.5″	E	51	33	30
Constance Lake	Switzerland	North	Mainland	Lake	47°29′08.4″	9°32′38.4″	E	30	25	27
Constance Stream	Switzerland	North	Mainland	Stream	47°19′36.8″	9°34′04.5″	E	30	32	23
Poland	Poland	North	Mainland	Stream	50°03′13.1″	19°14′46.4″	E	20	–	22
Iceland Lake	Iceland	North	Island	Lake	65°36′52.1″	17°03′30.1″	W	30	–	31
Iceland Marine	Iceland	North	Island	Marine	65°02′39.5″	22°27′28.6″	W	45	–	41

[a] Samples from the Natural History Museum Vienna (NHW-83118)

Because only subadult individuals were caught in Sardinia, which differ phenotypically from adults (Bell 1981), the respective phenotypic data was collected from adult museum specimens that were collected from a different site in the same catchment ~20 km away (natural history museum in Vienna ID: NHW-83118). Due to formaldehyde preservation, none of the museum specimens were available for DNA extraction.

Morphology

Sixteen linear morphological traits that are known to be linked or involved in ecological diversification of stickleback (e.g., Kristjánsson et al. 2002; Mori and Takamura 2004; Berner et al. 2008) were measured to the nearest 0.01 mm using a digital caliper. These traits were either related to anti-predator defense (length of the first and second dorsal spine, the pelvic spine and the pelvic girdle), feeding ecology (head length, upper jaw length, snout length, eye diameter) or to general body shape and swimming performance (standard length, width of the pelvic girdle, body depth measured after the first and second dorsal spine, caudal peduncle length, basal length of the anal and dorsal fin, total length of the pelvic fin). Because all linear traits were significantly correlated with standard length (results not shown), a size correction was applied by using the residuals of a regression of each trait against standard length, pooling all individuals and populations. In addition to the linear morphological measurement, the number of lateral plates was counted for each individual on its left flank.

To estimate the overall phenotypic differentiation among individuals, a principal component analysis (PCA) was conducted combining all size-corrected linear measurements. A second PCA was performed using only size-corrected defense-related linear measurements, as these traits commonly differ between stickleback from northern and southern Europe (e.g., Gross 1978; Lobón-Cerviá et al. 1988; Cano et al. 2008). To subsequently test if *geography* (northern or southern Europe) or *insularity* (island or mainland) may account for the observed phenotypic variation among freshwater populations along the two leading PC axes of either analysis, a linear mixed model was employed using the population identity as a random factor. The best fitting model was determined using a backward elimination procedure starting with a model including both factors. Given the low levels of statistical replication for each factor (Table S1), no interaction was tested. Similarly, both the number of lateral plates and standard length were tested for potential effects of *geography* or *insularity* among freshwater populations. All statistical analyses were performed in R 2.15.1 (R Core Team 2012).

Feeding ecology

To study potential trophic specialization among Mediterranean stickleback, the stomachs of 214 adult individuals from eight populations (Corsica, Mallorca, Galicia, France Stream 1, Geneva Lake and Stream, Constance Lake and Stream) were extracted and all food items were counted using a dissection microscope. Due to access restrictions, stomachs could not be extracted for the Sardinian museum

specimens. Food items were assigned to the following taxonomic classes: *Amphipoda, Isopoda, Cladocera, Copepoda, Ostracoda, Decapoda, Diptera* imagos, *Chironomidae, Ephemeroptera, Trichoptera, Odonata, Acaria*, *Lumbricidae, Gastropoda,* parasites and stickleback fry and eggs. To test for feeding-related divergence along the limnetic-benthic feeding axis, the percentage of planktonic prey (%PPP) was then calculated as the fraction of *Cladocera* and *Copepoda* to the total number of all food items present for each individual following Lucek et al. (2012). Similarly the percentage of each prey category per population was calculated to allow a qualitative comparison of feeding habits across populations.

Genetic analysis

DNA for all individuals was extracted using a 10 % Chelex solution, following the manufacturer's protocol (Biorad, California, USA). Seven species-specific microsatellite markers (Gaest66, Stn26, Stn30, Stn96, Stn130, Stn173 and Stn196) were amplified in one multiplex kit. Detailed information on the multiplexing setup and the PCR protocol can be found in Lucek et al. (2014b). Alleles were visualized on an ABI 3130XL and scored with GeneMapper 4.0 (Applied Biosystems, Zug, Switzerland).

Deviation from Hardy–Weinberg equilibrium was calculated using GenoDive 2.0 using 10,000 bootstrap replicates (Meirmans and Van Tienderen 2004). To estimate the genetic variation within a population, a number of genetic indices were calculated in GenoDive 2.0: the average number of observed alleles (A_N), the effective number of alleles, i.e., the number of alleles weighted for their frequency (A_R), the observed heterozygosity (H_O) and the expected heterozygosity within a population (H_S). The inbreeding coefficient (F_{IS}) was estimated for each population. All genetic indices were subsequently compared between populations from northern and from southern Europe (Table S1) as well as between insular populations from the south, south European mainland populations and populations from northern Europe using Wilcoxon tests. In addition, pairwise genetic differentiation (F_{ST}) among all populations was estimated in GenoDive using 10,000 bootstrap replicates to assess significance. Pairwise F_{ST} were also compared between populations from northern and southern Europe as well as between the marine population and the freshwater populations using an ANOVA with a Tukey HSD post hoc test. Because F_{ST} estimates can be biased towards lower values in cases where the level of heterozygosity within populations is high (Meirmans and Hedrick 2011), the analysis was repeated using F'_{ST} (Hedrick 2005). F'_{ST} was calculated in R with the package diveRsity (Keenan et al. 2013), calculating its 95 % confidence interval using 10,000 bootstrap replicates. The genetic structuring was estimated using an admixture model implemented in Structure 2.3.3 (Falush et al. 2007) with 30,000 burnin steps followed by 300,000 MCMC steps. The simulation was performed assuming 1–13 genetic clusters (K) with 10 replicates for each assumed K. The optimal number of clusters was determined based on the estimated log likelihood of each run and its variation among runs for the same K, following Evanno et al. (2005). To further infer the genetic relationship among populations, a neighbour joining tree was established based on Cavalli-Sforza distances among populations based on microsatellite allele frequencies. Statistical support for each node of the inferred tree was obtained using a bootstrap procedure with 1000 replicates in Phylip 3.69 (Felsenstein 2012). Lastly, a PCA was conducted on population-based allele frequencies in GenoDive.

To assess the phylogenetic relationships between the west Mediterranean island populations and other European stickleback, and to estimate the divergence time among different lineages, a fragment of the mitochondrial control region spanning over 426 consecutive base pairs was amplified with primers and protocols described in Mäkinen and Merilä (2008) using one individual from Mallorca and Sardinia and including the previously identified haplotype from Corsica (Lucek et al. 2010). The phylogenetic analyses were performed in Beast 2.2.1 (Mäkinen and Merilä 2008; Bouckaert et al. 2014), adding all available stickleback sequences from GenBank, including samples from Europe, Atlantic North America (Mäkinen and Merilä 2008; Lucek et al. 2010) and Pacific North America (California; Richmond et al. 2014). The best model of molecular evolution was determined in jModeltest 2.1.7 (Darriba et al. 2012) using a Bayesian information criterion (BIC). An uncorrelated lognormal relaxed clock model was selected with a constant size coalescent tree prior. Substitution rate was set to 0.028 substitutions/site/million years following (Aldenhoven et al. 2010). The analysis was run over 50 million generations, sampling every 5000 generations. Tracer 1.6.0 (Rambaut et al. 2014) was then used to examine convergence and mixing based on effective sample sizes (ESS). Lastly, TreeAnnotator 2.2.1 was used to generate a maximum clade credibility tree using 50 % of trees as burnin. In addition, a maximum likelihood tree was estimated in Mega 5 (Tamura et al. 2011) with 1000 bootstrap replicates.

Results

Feeding ecology

Out of the 214 extracted stomachs, 13 were empty (6.1 %) and were subsequently excluded from all analyses. The

percentage of planktonic prey (%PPP) was highest in the two lake populations from Lake Geneva and Lake Constance, feeding predominantly on copepods in comparison to all stream populations ($F_{1,199} = 52.8$, $p < 0.001$; Fig. 2). Stream-dwelling individuals in contrast fed predominantly on chironomid larvae. In both populations from Galicia and the France Stream 1, several individuals had fish eggs or even stickleback fry in their stomachs.

Genetics

Deviation from Hardy–Weinberg equilibrium occurred in three out of 91 estimates following a sequential Bonferroni correction (STN26: Iceland Lake; STN96: Sardinia and Corsica). Genetic diversity was overall significantly reduced in land-locked south European populations, showing a lower number of alleles (A_N: $W = 6$, $p = 0.045$; A_R: $W = 0$, $p = 0.002$; Table 2) and decreased heterozygosity (H_O: $W = 1$, $p = 0.007$; H_S: $W = 0$, $p = 0.004$). But there was no overall difference in the degree of inbreeding between populations from northern and southern Europe (F_{IS}: $W = 21$, $p = 0.943$). Comparing populations from northern Europe with insular Mediterranean populations, the latter showed a significantly lower effective number of alleles (A_R: $W = 0$, $p = 0.036$) but not observed alleles (A_N: $W = 4$, $p = 0.365$) and decreased heterozygosity (H_O: $W = 0$, $p = 0.036$; H_S: $W = 0$, $p = 0.036$), where inbreeding coefficients did not differ (F_{IS}: $W = 10$, $p = 0.571$). None of the genetic indices differed between insular Mediterranean populations and the populations from the south European mainland (all $p > 0.100$).

Table 2 Measures of genetic diversity: A_N—average number of observed alleles within a population, A_R—effective number of alleles (number of alleles weighted for their frequencies), H_O—observed heterozygosity, H_S—within population heterozygosity, G_{IS} inbreeding coefficient

Population	A_N	A_R	H_O	H_S	G_{IS}
Mallorca	2.857	1.609	0.291	0.315	0.078
Galicia	2.714	1.669	0.261	0.282	0.076
Corsica	3.286	1.457	0.207	0.286	0.277
Sardinia	3.000	1.863	0.279	0.308	0.094
France Stream 1	2.571	1.557	0.291	0.286	−0.017
France Stream 2	2.571	1.504	0.211	0.270	0.219
Geneva Lake	2.857	1.683	0.324	0.327	0.011
Geneva Stream	3.000	1.851	0.290	0.314	0.075
Constance Lake	3.571	2.356	0.506	0.502	−0.009
Constance Stream	3.000	2.131	0.461	0.497	0.073
Poland	2.857	1.999	0.414	0.465	0.110
Iceland Lake	4.571	2.170	0.307	0.445	0.310
Iceland Marine	8.571	4.350	0.690	0.713	0.033

Pairwise F_{ST}s (Table S1) and F'_{ST}s (Table S2) were highly correlated ($R^2 = 0.884$; $F_{1,76} = 588.6$, $p < 0.001$), where F'_{ST} values were on average significantly higher than F_{ST} (paired t test: $t_{1,77} = 24.8$, $p < 0.001$). The pairwise comparisons differed overall significantly among the

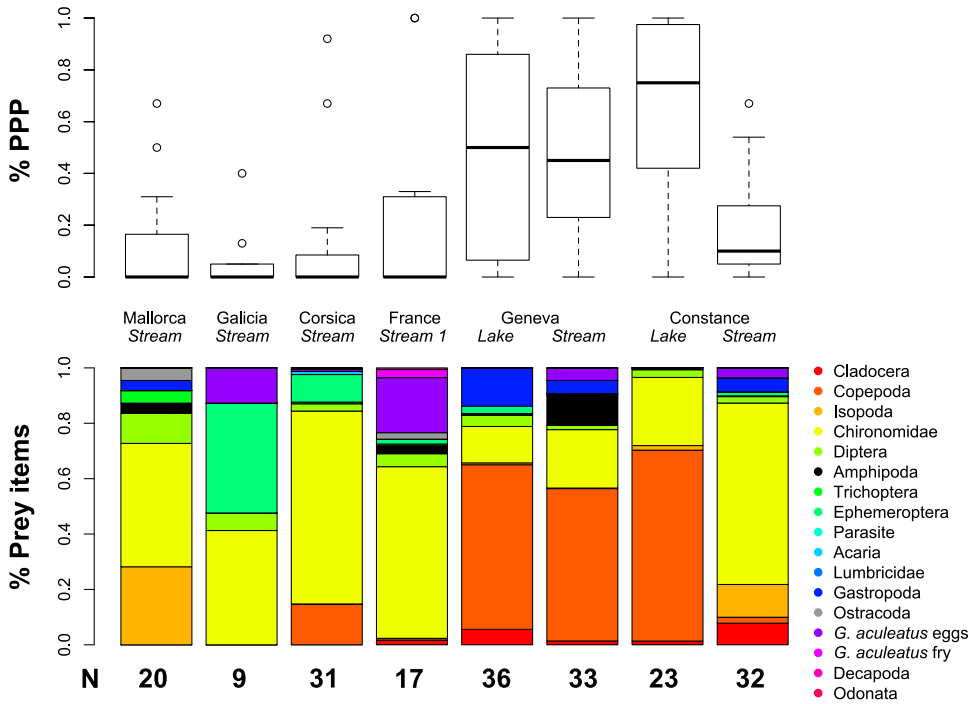

Fig. 2 Stomach content analysis for eight populations. *Top* percentage of planktonic prey found within a population sample (see main text for details). *Bottom* percentage of each prey category found in each population. N indicates the number of individuals that had at least one prey item in their stomachs

geographic groups for both F_{ST} ($F_{4,73} = 18.1$, $p < 0.001$; Fig. 3a) and F'_{ST} ($F_{4,73} = 5.6$, $p < 0.001$; Fig. 3b). Comparisons involving the marine population yielded significantly lower F_{ST} (average F_{ST} marine vs. north European populations = 0.178 ± 0.016 SE; average F_{ST} marine vs. south European populations = 0.309 ± 0.021 SE) than allopatric comparisons between freshwater populations from north and south Europe (average $F_{ST} = 0.483 \pm 0.013$ SE, post hoc $p < 0.001$). They were also lower than comparisons among south European populations (average $F_{ST} = 0.579 \pm 0.032$ SE, post hoc $p < 0.001$) but was not statistically different from comparisons among freshwater populations from northern Europe only (average $F_{ST} = 0.296 \pm 0.060$ SE, post hoc $p = 0.960$). The pairwise genetic differentiation among south European populations was furthermore significantly higher than all other comparisons (all $p < 0.018$), but did not differ between insular populations and populations from the south European mainland ($t_{1,11} = 1.87$, $p = 0.092$). The increased level of heterozygosity in the marine population (Table 2) may bias the F_{ST} estimation towards lower values (Hedrick 2005; Meirmans and Hedrick 2011). Using F'_{ST} to correct this bias (Hedrick 2005), the level of genetic differentiation between the marine population and populations from northern Europe (average $F'_{ST} = 0.406 \pm 0.038$ SE) was still significantly lower than that among populations from southern Europe (average $F'_{ST} = 0.783 \pm 0.047$ SE, post hoc $p = 0.005$) and between populations from northern and southern Europe (average $F'_{ST} = 0.726 \pm 0.026$ SE, post hoc $p = 0.023$). Also the differentiation among south European populations was higher than that among north European populations (average $F'_{ST} = 0.505 \pm 0.100$ SE, post hoc $p = 0.018$). Lastly, F'_{ST} did also not differ between insular populations and populations from the south European mainland ($t_{1,11} = 1.99$, $p = 0.074$).

Ten genetic clusters (K = 10) was the best supported K in STRUCTURE based on the observed variation of the estimated log likelihood values within a run and the increase in the log likelihood for each K (Fig. 4a; Figure S1). Consistent with previous findings (Lucek et al. 2014a), both the Geneva lake and stream populations as well as the France Stream 2 population were all assigned to a single genetic cluster with evidence for some introgression from the Constance cluster, comprising both the Constance lake and stream site. All other populations formed their own distinct genetic cluster except for the Iceland lake population showing some evidence for introgression from the marine population.

The two leading PC axes that were constructed using population-based microsatellite allele frequencies accounted for 30.4 and 15.5 % of the total variance among populations respectively (Fig. 4b). The first PC axis separated the Mediterranean island populations from all other populations, where the populations from Geneva and France Stream 2 are genetically most distinct from the latter. The second PC axis separated the France Stream 1 population from all other populations, whereas populations from Iceland, Constance, Poland and Galicia were intermediate to the other populations along the two PC axes. Interestingly the France Stream 1 population clusters closely with the population from Mallorca in the population based neighbor-joining tree (Fig. 4c),

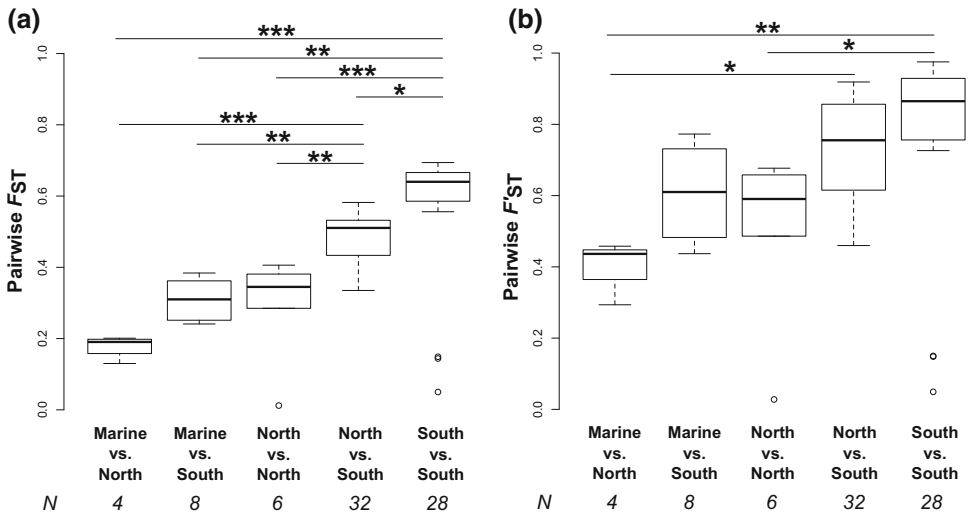

Fig. 3 Boxplots summarizing of the pairwise genetic differentiation among different geographic groups based on **a** F_{ST} and **b** F'_{ST}. Genetic differentiation was calculated between the marine and freshwater populations from either northern or southern Europe, between freshwater populations from northern Europe, between freshwater populations from northern and southern Europe and between south European populations respectively (see Tables S1, S2 for the actual F_{ST} and F'_{ST} values respectively). *Asterisks* indicate the level of statistical significance of comparisons between geographic groups, indicated by *horizontal bars*, based on post hoc ANOVA comparisons (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The number of pairwise comparisons are indicated for each geographic group (N)

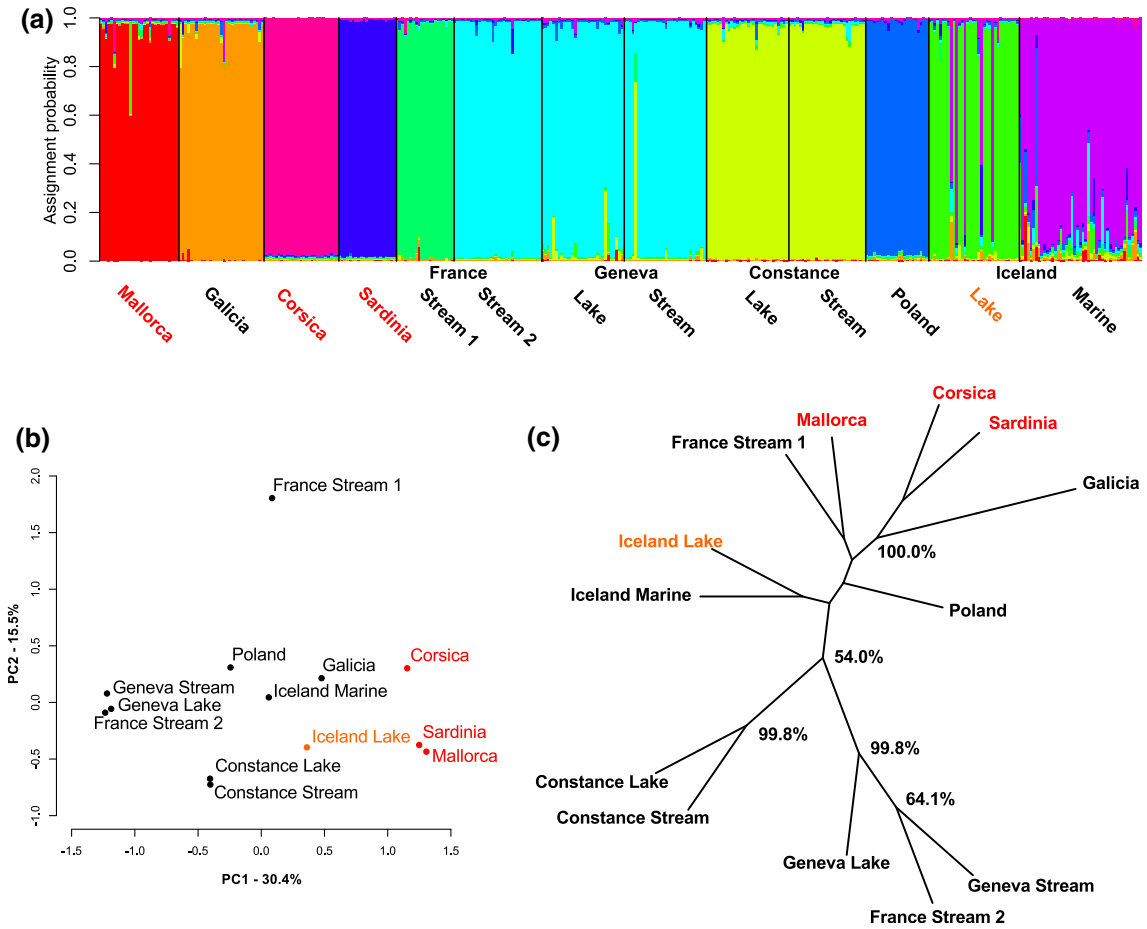

Fig. 4 Genetic relationship between populations based on seven microsatellites: **a** Individual genetic assignment using STRUCTURE. Shown is the best run, where ten genetic clusters were inferred (K = 10), which showed the highest posterior likelihood. **b** Principal component analysis, where the two leading axes capture 45.9 % of the genetic variation among populations. **c** Phylogram showing the genetic relationship among populations based on Cavalli-Sforza distances. *Numbers* indicate statistical support based on 1000 bootstrap replicates (only values >50 % are shown). Populations from islands are highlighted in *color* (*orange* Iceland, *red* Mediterranean). (Color figure online)

but is otherwise consistent with the PC analysis, where Corsica, Sardinia and Galicia form a distinct clade.

JMODELTEST selected the HKY+G+I model of molecular evolution as the best fitting model for the partial mitochondrial control region. All ESS values for the BEAST analysis were >200, suggesting an adequate sampling of the posterior. The BEAST analysis shows a deep divergence between a clade that comprises all haplotypes from the North American Pacific, North American Atlantic, the Black Sea, and the Mediterranean Lake Skadar and a clade that includes all other haplotypes from across Europe plus one North American Atlantic haplotype (Fig. 5). However, whereas this deeper split is not statistically supported, several clades within these groups are well supported (see below). Mediterranean stickleback populations occur widely scattered across both clades with several Mediterranean specific clusters, including ancient splits as well as recent ones. Two ancient populations from the Balkans (Ska, Ner) are strongly supported monophyletic groups with deep divergence from other populations, confirming previous studies (DeFaveri et al. 2012). In particular, the haplotypes of Lake Skadar fall into a relatively old clade (divergence from other stickleback 0.0419-0.3886 mya; diversification within the Skadar clade started 0.0069–0.1212 mya as suggested by the 95 % highest posterior density for this node height). Its closest relatives are haplotypes from North America, which is consistent with an ancient colonization of Europe that may be independent of that of the main clade (Bell and Foster 1994).

The haplotypes found on the three Mediterranean islands are spread across the European main clade: The haplotype from Mallorca (estimated age: 0.0070–0.1160 mya) forms a clade together with haplotypes from the Font Dame springs in southern France and the River Sorgue in the southern Rhone valley albeit with only weak support (BPP = 0.65). This clade has an estimated age of 0.0426–0.2360 mya. The

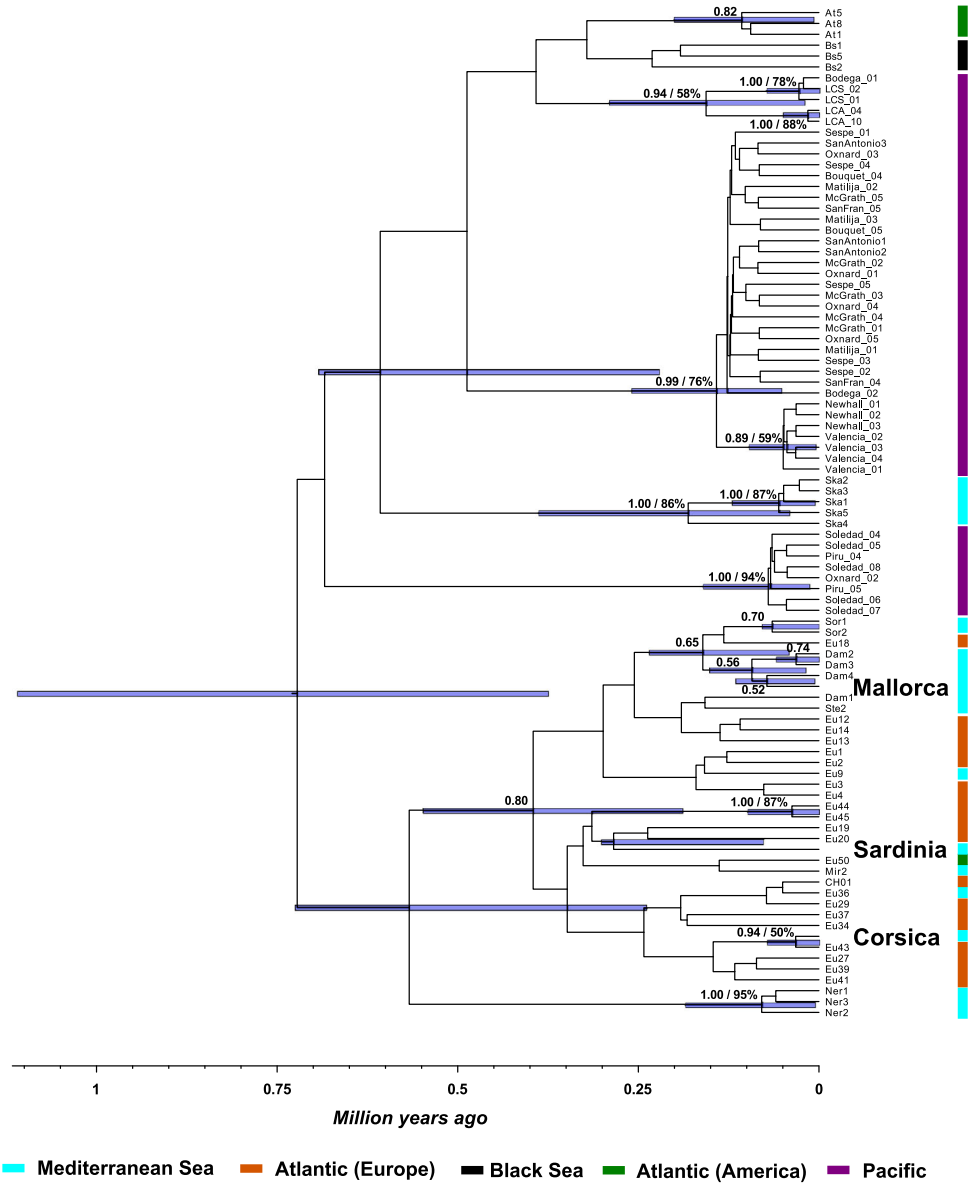

Fig. 5 Bayesian maximum clade credibility tree based on a 426 base pair fragment of the mitochondrial control region showing the genealogical relationships among threespine stickleback. Node values represent Bayesian posterior probabilities ≥0.50. Additional percentages reflect bootstrap support from an additional maximum likelihood tree for the same node, based on 1000 bootstrap replicates (only values ≥50 % are shown). *Error bars* depict 95 % highest posterior density for node heights (see main text for details)

Corsican haplotype with a divergence time of 0.0001–0.0724 mya, clustered closely with the EU43 haplotype, which has formerly been reported from the Loire drainage in Central France that drains into the Atlantic (Mäkinen and Merilä 2008). The Sardinian haplotype clustered closest to the EU19 and EU20 haplotypes, which derive from the Baltic Sea and the Northern Atlantic. However, the age of the Sardinian haplotype could not be reliable dated.

Phenotypic differentiation

The PC analysis comprising all size-corrected linear measurements accounted for 39.2 and 17.2 % of the overall phenotypic variation on the first and second PC axis respectively (Fig. 6). Defense-related traits accounted for most of the variation observed along the first PC axis, whereas the second PC axis was mainly driven by differences in fin sizes and body depth (Table S3). The best statistical model explaining the variation in PC scores along PC1 for freshwater populations using all linear traits combined included only the factor *island*, where island populations differed significantly from mainland populations ($F_{1,10} = 19.65$, $p = 0.001$). The difference between island and mainland population was also the best fitting model to explain variation on the second axis albeit being not significant ($F_{1,10} = 4.57$, $p = 0.058$). Using only defense-related linear morphology, the two leading PC

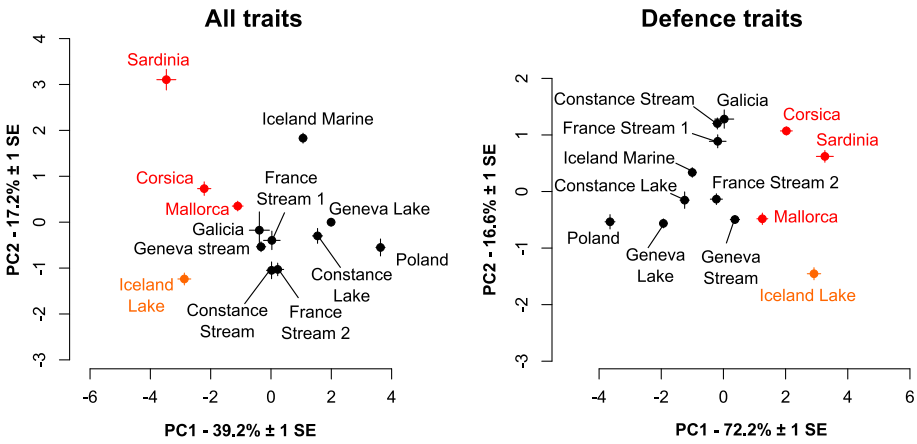

Fig. 6 Phenotypic variation across populations: Principal component analyses using either all size-corrected linear morphological traits (*left*) or only size-corrected defense-related traits (*right*, see main text for details). Shown are the mean PC scores for each population ± 1 SE. Populations from islands are highlighted in *color* (*orange* Iceland, *red* Mediterranean). (Color figure online)

axes accounted for 72.2 % and 16.6 % of the total phenotypic variation respectively, where both axes were mainly driven by variation in pelvic girdle and spine length (Table S3). The best fitting statistical model to explain the variation among freshwater populations on the first PC axis also included only a significant effect of *island* ($F_{1,10} = 22.17$, $p < 0.001$). Similarly to the analysis comprising all traits, all the island populations are separated from all the mainland populations and from the marine population, where mainland south European populations seem to resemble the insular populations most (Fig. 6). None of the tested factors was significantly associated with the variation observed along the second PC axis for defense-related traits (all $p > 0.1$, results not shown).

The best statistical model for body size (standard length) retained only *geography* as a factor, where south European individuals were significantly smaller than individuals from the north ($F_{1,10} = 11.23$, $p = 0.007$; Fig. 7). For the number of lateral plates, the best fitting model showed significant effects of both *geography* and *island*, where individuals from the south had significantly fewer plates ($F_{1,9} = 44.31$, $p < 0.001$), which was equally true for individuals originating from islands ($F_{1,9} = 15.08$, $p = 0.004$).

Discussion

Many insular ecosystems are currently threatened by climatic changes and other human-induced perturbations (Ricketts et al. 2005; Courchamp et al. 2014). Detecting and preserving insular endemics thus is pressing as, in many cases, islands harbor populations of otherwise widely distributed species that are phenotypically and genetically most distinct (Foster 1964; Case 1978; Lomolino 2005). Combining genetic, phenotypic and ecological data we aimed to determine the status of the little known and biogeographically exceptional populations of the threespine stickleback on Mediterranean islands. We find that these populations are both phenotypically and genetically distinct from mainland populations underlining their status as evolutionary significant units for conservation (Ryder 1986; Moritz 1994). Convergence on a similar phenotype was furthermore observed between these populations and an insular population from Iceland, northern Europe, suggesting that similar evolutionary forces may act during phenotypic adaptation to insular ecosystems.

Genetic relationship and divergence of Mediterranean populations

For many organisms, the Mediterranean region has acted as a glacial refugium from which subsequent postglacial expansions have taken place (Hewitt 2000). The contemporary distribution of freshwater-dwelling threespine stickleback in the Mediterranean region is likewise thought to originate from a distinct glacial refugium, but expansion to northern Europe is thought to not have occurred from this refugium. In contrast, freshwater stickleback populations from northern Europe are believed to derive from recolonization events from Atlantic marine populations (Mäkinen et al. 2006). Analyses of mitochondrial haplotypes further suggested that some Mediterranean populations may have survived the last Pleistocene glaciation (Mäkinen and Merilä 2008; DeFaveri et al. 2012). Whereas earlier phylogeographic work focused on populations from the Eastern Mediterranean, we focused on island populations in the western Mediterranean. Our phylogeographic estimate based on mitochondrial control region sequences suggests that each western Mediterranean island population represents a distinct genetic lineage (Fig. 5). Interestingly, the haplotype found on Mallorca clusters closely to haplotypes originating from a population in southern France, and together may be remnants of one glacial freshwater refugium as indicated by our Bayesian estimate of

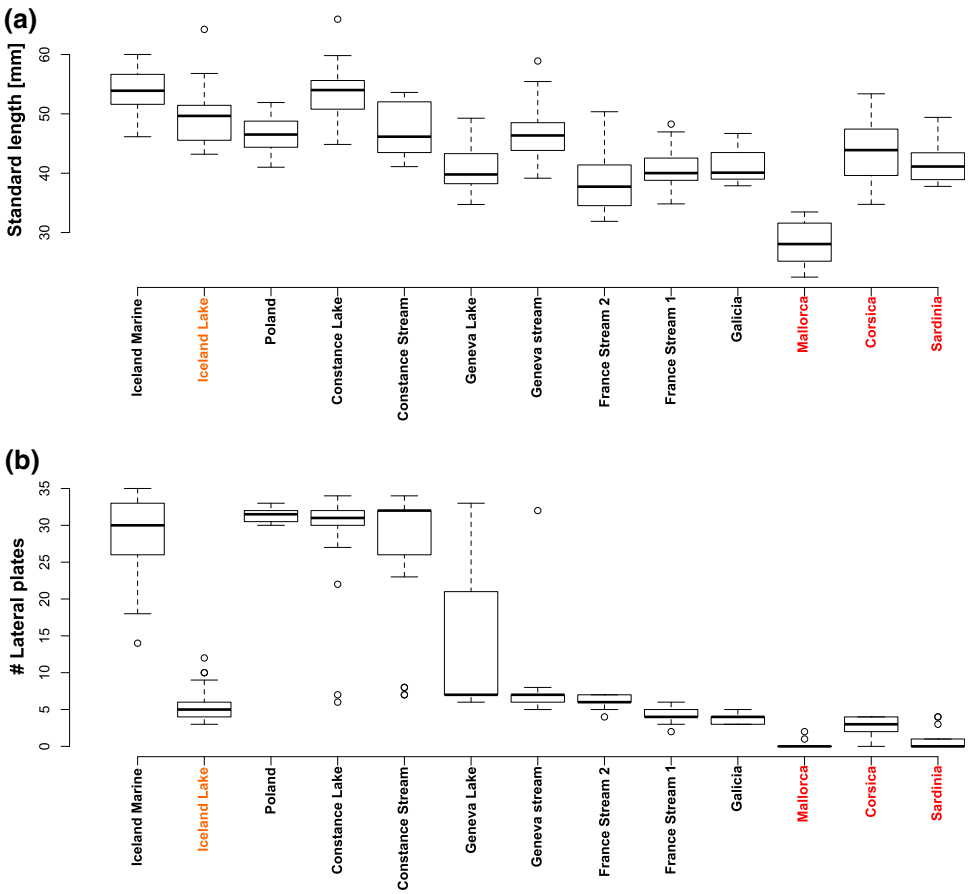

Fig. 7 Phenotypic differentiation among the sampled populations for **a** size (standard length) and **b** the number of lateral plates. Populations from islands are highlighted in *color* (*orange* Iceland, *red* Mediterranean). (Color figure online)

divergence. The haplotypes of Corsica and Sardinia, not known from anywhere else, are most closely related to haplotypes found in central or northern Europe. They may either originate from a more recent colonization event of the Mediterranean region, or may represent lineages that expanded northward again after the glacial maximum.

Threespined stickleback likely originated in the Pacific Ocean, with the most divergent lineage being the Japanese Pacific clade (Watanabe et al. 2003; Jones et al. 2012; Rezansoff et al. 2015). We could not include this lineage in our tree because sequences of the mitochondrial control region are not present in GenBank. However, studies based on other mitochondrial genes have shown that this lineage diverged from the clade that we studied about 2.5 mya (Rezansoff et al. 2015). Our tree is consistent with the hypothesis that stickleback of Europe derive from several distinct colonization events (Bell and Foster 1994). Remnants of one relatively ancient colonization event seem to persist in the Black Sea and in Lake Skadar, being known otherwise from both sides of North America (Fig. 5). The other colonization event, similarly ancient, would have formed the Neretva River clade from the Balkans and to the main European clade of freshwater stickleback. The deep divergence between these two, and the lack of statistical support for nodes deeper in the tree make it plausible that Europe was colonized more than twice. Indeed, stickleback from the Black Sea, Lake Skadar, Neretva and the clade comprising all other European haplotypes may derive from four colonization events. Clearly much more sequencing effort is needed to resolve the phylogeography of the genus *Gasterosteus* in Europe and beyond.

The potential for a Mediterranean-based refugium is further indicated by the levels of pairwise genetic differentiation found with nuclear markers, where the levels of genetic differentiation involving populations from southern Europe tend to be higher than comparisons involving north European or the marine population (Fig. 3; Tables S1, S2). Also, the levels of pairwise genetic differentiation between populations from southern Europe and the marine population tend to be higher than the levels of genetic differentiation between the marine population and populations from northern Europe. This may reflect the absence of contemporary gene flow from the Atlantic marine population into the Mediterranean region or could indicate that the overall split between the Atlantic marine and the freshwater populations from southern Europe predates the one between the marine and northern European freshwater populations. In contrast, the pattern found in northern

Europe is consistent with a recent postglacial recolonization of freshwater bodies from the Atlantic, with a significantly lower genetic differentiation between marine and freshwater (Jones et al. 2012). Lastly, almost all populations from southern Europe cluster closely together in the distance-based tree based on microsatellites (Fig. 4). A second genetic southern European cluster is formed by the three populations in the upper Rhone drainage (i.e., France Stream 2, Geneva Lake and Stream). This is in agreement with another study showing that the latter populations originate from a very recent (less than 140 years) expansion event following the translocation of stickleback within this region (Lucek et al. 2014a).

Despite clustering together in the distance-based tree, almost all populations from southern Europe represent distinct genetic clusters in the individual based assignment (Fig. 4). Moreover, the same populations show the highest degree of pairwise genetic differentiation, which in most cases even surpasses the level of genetic differentiation between populations from southern and northern Europe (Fig. 3; Tables S1, S2). This is in line with other studies on Mediterranean stickleback, where genetic differentiation was found to be high even between geographically proximate populations (Cano et al. 2008; Mäkinen and Merilä 2008; Araguas et al. 2012; DeFaveri et al. 2012). This pattern, combined with the significantly decreased genetic variation within south European populations is consistent with a reduction of gene flow due to the absence of a connecting marine population. The reduced genetic variation may furthermore be explained by a decrease in their population size due to habitat loss, fragmentation or the introduction of non-native species that compete with stickleback (Clavero et al. 2009; Orru et al. 2010; Araguas et al. 2012).

Evolution of phenotypically distinct island populations

The island rule predicts the evolution of phenotypically distinct insular populations (Foster 1964; Case 1978; Lomolino 1985), where on the one hand insular populations of small vertebrate species evolve an increased body size as a consequence of partial release from competitors and predators. On the other hand, large vertebrate species may evolve dwarfism due to resource limitations. These changes may in both cases trigger subsequent phenotypic adaptations (Foster 1964; Case 1978; Lomolino 1985; Lomolino et al. 2012). In stickleback, differences in anti-predator related phenotypes have been commonly attributed to distinct selection pressures imposed by differences in the strength and composition of the predation regimes (Reimchen 1994). Specifically, stickleback may evolve long spines and an increased number of lateral plates in environments with strong predation pressure dominated by piscivorous fishes and birds, such as in the marine environment as opposed to freshwater populations (Reimchen 1994). The evolution of low armour plate phenotypes among freshwater stickleback can occur rapidly through selection on standing genetic variation from the marine population (Colosimo et al. 2005). Indeed, the best example so far for insular phenotypes in stickleback derives from Scotland, where stickleback reduced their body size and defense-related traits, following the island rule (MacColl et al. 2013). The evolution these insular phenotypes was potentially mediated by a distinct water chemistry (i.e., a low pH and few dissolved alkaline metals (Spence et al. 2013)) and the presence of small predators (MacColl et al. 2013).

The phenotypic differentiation observed in our study is consistent with the evolution of distinct insular phenotypes, where populations from islands, including Iceland, differ phenotypically from populations on the European continent. This pattern is largely driven by a reduction in defense-related traits in insular populations (Figs. 6, 7; Table S3). Albeit the number of lateral plates differs overall between northern and southern Europe, where populations from the latter region have a much lower plate number (Fig. 7b), Mediterranean island populations show nevertheless more extreme phenotypes, in comparison to populations from the southern European mainland (Fig. 7). Similarly reduced lateral plate numbers, below the common freshwater phenotype with 5-7 lateral plates, are rare across Europe, where they occur mainly in the south (Gross 1977, 1978) or among insular populations in Scotland (Spence et al. 2013). Thus, the reduction in anti-predator related traits among our studied insular populations and insular populations from Scotland (Spence et al. 2013), may reflect common features in terms of the reduced predator and competitor communities on islands (Case 1978; Lomolino et al. 2012).

Body size itself differed overall mainly between freshwater populations from northern and southern Europe, where populations from the latter were significantly smaller (Fig. 7a). The evolution of distinct body sizes among freshwater stickleback has been associated previously with differences in water chemistry (McGuigan et al. 2011) and foraging strategies (Nagel and Schluter 1998). Whereas we cannot exclude differences in water chemistry, we found no support for feeding-related ecological differentiation among populations from southern Europe (Fig. 2). Remarkably, adult individuals from Mallorca showed a more reduced body size, resembling subadult phenotypes from northern Europe (i.e., Frommen et al. 2011; Lucek et al. 2012). Similar dwarfism has otherwise only rarely been recorded in stickleback. The best examples are the insular populations from Scotland that have reduced their body size potentially as a response to water chemistry and the presence of small

predators (MacColl et al. 2013). The Mallorcan population is moreover exceptional as we observed breeding individuals in November (Lucek *personal observation*), suggesting either an extended (from spring to November) or shifted breeding period. Analogous life-history adaptations in stickleback where stickleback shift their breeding effort to periods with decreased water temperatures have only been recorded in populations from Japan (Mori 1987) and the southern Rhone valley in France (Crivelli and Britton 1987). The latter is in the range of the other French populations that form a distinct cluster with the Mallorcan haplotype in the phylogenetic analysis (Fig. 5).

Conclusions

Taken together, our results suggest that endemic insular forms exist in threespine stickleback, where the populations from the Mediterranean islands are both phenotypically and genetically highly distinct from all mainland populations including those from southern Europe, and from each other. The genetic evidence suggests that they likely derive from glacial refugia in the Mediterranean region. Thus given their genetic distinctiveness (Figs. 3, 4) and phenotypic differentiation (Figs. 6, 7), Mediterranean island populations should be considered as evolutionary significant units (Ryder 1986; Moritz 1994), each endemic to one island. The status as evolutionary significant unit is particularly highlighted in the Mallorcan population, which shows specific life-history adaptations that have otherwise only been reported in a single population in Southern France in the Mediterranean region (Crivelli and Britton 1987). However, no such data was available for Sardinian and Corsican populations.

The low genetic variation observed within these populations, combined with their restricted range, and ecological requirements (such as year round cool water which is rare in the Mediterranean region), renders them especially vulnerable to habitat loss, fragmentation and alteration (including deforestation) (Araguas et al. 2012) or non-native fish species that outnumber native stickleback [(Orru et al. 2010); *Gambusia holbrooki* in Mallorca (Lucek *personal observation*)]. For instance the Corsican population is historically only known from two neighboring rivers in a small part of the island's coastal lowlands (Gauthier and Rose 1974). Despite intensive search, we found it in only one of them. This river is exceptional among Corsican coastal lowland rivers in having relatively cold water coming from the nearby mountains, and is well shaded by a thin strip of riparian forest. Further protective measures are thus needed that allow the monitoring of these populations, the safe-guarding of their habitats, and aim to decrease the threat made by non-native species.

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