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Are Japanese freshwater populations of threespine stickleback derived from the Pacific Ocean lineage?

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ABSTRACT

Background: The presence of ecological opportunity can trigger adaptive radiation. Freshwater colonization of marine ancestors during the post-glacial dispersal could have triggered adaptive radiation in the threespine stickleback (*Gasterosteus aculeatus*). Japanese marine threespine stickleback can be classified into two genetically divergent groups, the Pacific Ocean group and the Japan Sea group.

Question: Are all Japanese freshwater threespine stickleback populations derived from the Pacific Ocean lineage? Or are some freshwater populations derived from the evolutionarily divergent Japan Sea lineage?

Methods: We collected stickleback from 22 different locations across Japan, including nine freshwater populations, five Pacific Ocean anadromous populations, and eight Japan Sea anadromous populations. We determined the genotypes for 11 different microsatellite markers. We created phylogenies using different measures of genetic distance based on both allele frequencies and allele lengths. We inferred population structure using Bayesian analysis.

Results: All freshwater populations analysed were genetically similar to the Pacific Ocean anadromous populations, suggesting that they are likely derived from the Pacific Ocean lineage rather than the Japan Sea lineage.

Keywords: adaptive radiation, microsatellites, phylogeography, stickleback.

INTRODUCTION

The presence of ecological opportunity can trigger adaptive radiation (Schluter, 2000; Glor, 2010; Losos, 2010). When interspecific competition or predation is reduced, phenotypic diversification occurs as a result of colonization and adaptation to vacant niches. Newly formed lakes and islands or mass extinction can provide ecological opportunities to many lineages (Schluter, 2000; Gillespie and Baldwin, 2010; Glor, 2010; Losos, 2010). However, not all lineages can exploit

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these opportunities. For example, Darwin's finches and Hawaiian honeycreepers have achieved tremendous adaptive radiations, while Galápagos mockingbirds and Hawaiian thrushes have not (Losos, 2010). Differences in dispersal rates or geographical proximity to habitats with vacant niches play an important role in shaping colonization history. Lineages that are able to colonize novel environments and diversify rapidly may therefore preclude later colonization by other competing lineages (Glor, 2010; Losos, 2010). Alternatively, colonizing taxa might lack the key traits or genetic variation that allows exploitation of vacant niches (Glor, 2010; Losos, 2010). Interactions between adaptation and colonization success mean that the phylogeography of evolutionary divergent lineages is of considerable importance (Waters, 2011). Comparisons between lineages that successfully diversified and those that did not are essential for a better understanding of the factors promoting and constraining adaptive radiations.

Temperature shifts, ice sheet movement, and sea-level change during the Quaternary period altered the range and distribution of taxa, thus influencing gene flow, genetic diversity, and divergence (Avise, 2000; Bennett, 2008). The glacial and inter-glacial cycles of the Pleistocene have driven adaptive radiations in a number of post-glacial temperate northern fish species (Schluter, 1996). Ice retreat following the Last Glacial Maximum exposed numerous freshwater habitats that were colonized by multiple ancestral marine fish species, providing novel niches and leading to rapid evolutionary diversification (Taylor, 1999; Schluter, 2000). One of the best studied is the threespine stickleback (*Gasterosteus aculeatus* L.) species complex, characterized by independent colonization of freshwater environments by marine ancestral forms throughout its circumpolar distribution, leading to the parallel evolution of extensive phenotypic diversity (Wootton, 1976; Bell and Foster, 1994; McKinnon and Rundle, 2002). Although marine ancestral sticklebacks are relatively homogeneous in phenotypic traits, freshwater sticklebacks diversified and adapted to diverse environments after colonization of a variety of newly created freshwater environments (Wootton, 1976, 1984; Bell and Foster, 1994).

Japanese marine threespine stickleback can be classified into two genetically divergent groups, the Pacific Ocean anadromous form (PA) and the Japan Sea anadromous form (JA) (Higuchi and Goto, 1996; Kitano *et al.*, 2007, 2009). Around the Japanese archipelago, the distribution of JA fish centres primarily on the Sea of Japan and Sea of Okhotsk, with populations found across the western seaboard of Honshu (Higuchi and Goto, 1996). In contrast, PA fish have a much broader distribution, occurring across the Pacific Ocean from North America to Japan (Higuchi and Goto, 1996). Both forms are sympatric in Hokkaido, the northern islands of the Japanese archipelago, sometimes co-occurring in the same drainage system (Higuchi and Goto, 1996; Kitano *et al.*, 2007). PA and JA fish probably diverged during a period of extended allopatry 0.5–2 million years ago when sea-level change isolated the Sea of Japan from the Pacific Ocean (Yamada *et al.*, 2001; Kitano *et al.*, 2007).

In addition to the two anadromous forms, numerous freshwater populations of threespine stickleback with divergent morphological and life-history traits also occur in the northern parts of the Japanese archipelago (Mori, 1987, 2003). However, the evolutionary history of Japanese freshwater populations remains unclear. Previous attempts to resolve the phylogeography of Japanese stickleback populations have provided conflicting evidence of ancestry. Allozyme analysis demonstrated that two clearly distinct lineages occurred across the region (Higuchi and Goto, 1996). A strong correlation between genetic data and morphological measurements (caudal plate height and caudal keel ossification) supports the hypothesis that these two lineages correspond to the JA and PA forms. Furthermore, all of the freshwater populations surveyed in this study grouped clearly within the PA lineage (Higuchi and

Goto, 1996). These data suggest that the PA rather than the JA lineage might be the ancestor of the Japanese freshwater populations. However, a further study using mitochondrial DNA on similar populations did not detect such clear genetic divergence (Yamada *et al.*, 2001, 2007). Instead, all Japanese populations were grouped in a monophyletic lineage consistent with the Japanese clade revealed by a global survey of threespine stickleback mitochondrial DNA (Ortí *et al.*, 1994). This suggests that introgression of mitochondrial haplotypes between PA and JA has likely occurred on secondary contact (Yamada *et al.*, 2001, 2007). Given conflicting results from different marker types, further evidence from nuclear loci is required.

In light of the complicated evolutionary history of Japanese stickleback populations, we investigated the genetic relationships between marine and freshwater populations using microsatellite markers. In short, we wished to examine whether the Japanese freshwater populations are derived from the Pacific Ocean lineage, the divergent Japan Sea lineage or both.

MATERIALS AND METHODS

Sampling and fish collection

Threespine stickleback were collected from 22 sampling locations across Japan (Fig. 1, Table 1). This included nine freshwater habitats, eight JA stickleback habitats, and five PA stickleback habitats. All freshwater populations analysed were sampled from habitats that were landlocked or located above dams and falls. JA and PA forms were collected from the estuary and first classified into JA and PA forms on the basis of external morphology: PA fish have a larger caudal plate height and a larger body size than JA fish (Higuchi and Goto, 1996; Kitano *et al.*, 2007). Ten individuals from each population were sampled, with the exception of the Gifu population from which only eight samples could be obtained. DNA was isolated from the pectoral fin using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as described previously (Adachi *et al.*, 2012).

Microsatellite marker amplification

Fish were genotyped using 12 microsatellite markers (Stn170, Stn215, Stn233, Stn64, Stn76, Stn159, Stn46, Stn90, Stn120, Stn278, Stn332, and Stn384), chosen because they are located on different threespine stickleback linkage groups (Peichel *et al.*, 2001), are not linked to sex (Peichel *et al.*, 2004; Kitano *et al.*, 2009), and were observed to be polymorphic in many populations previously analysed (Kitano *et al.*, 2008a, 2009; Adachi *et al.*, 2012). Forward primers were labelled with HEX, NED or FAM and the 5′-ends of the reverse primers were tailed with GTTTCTT to increase the accuracy of fragment analysis (Ballard *et al.*, 2002). Markers were combined into three different multiplexes based on dye colour and were then amplified using the KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems, Woburn, MA, USA). After 3 min of 95°C, 30 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s were performed, followed by 10 min of 72°C. Amplified fragments were analysed by BEX Co. Ltd. (Tokyo, Japan). Allele lengths were then determined and scored using Peak Scanner Software (Life Technologies, Grand Island, NY, USA). Eleven of the 12 primer pairs amplified microsatellite fragments in all populations, whereas one marker (Stn76) failed to amplify in JA populations. Alleles at this locus were therefore private to PA and freshwater populations and thus were excluded from genetic diversity and distance analyses.

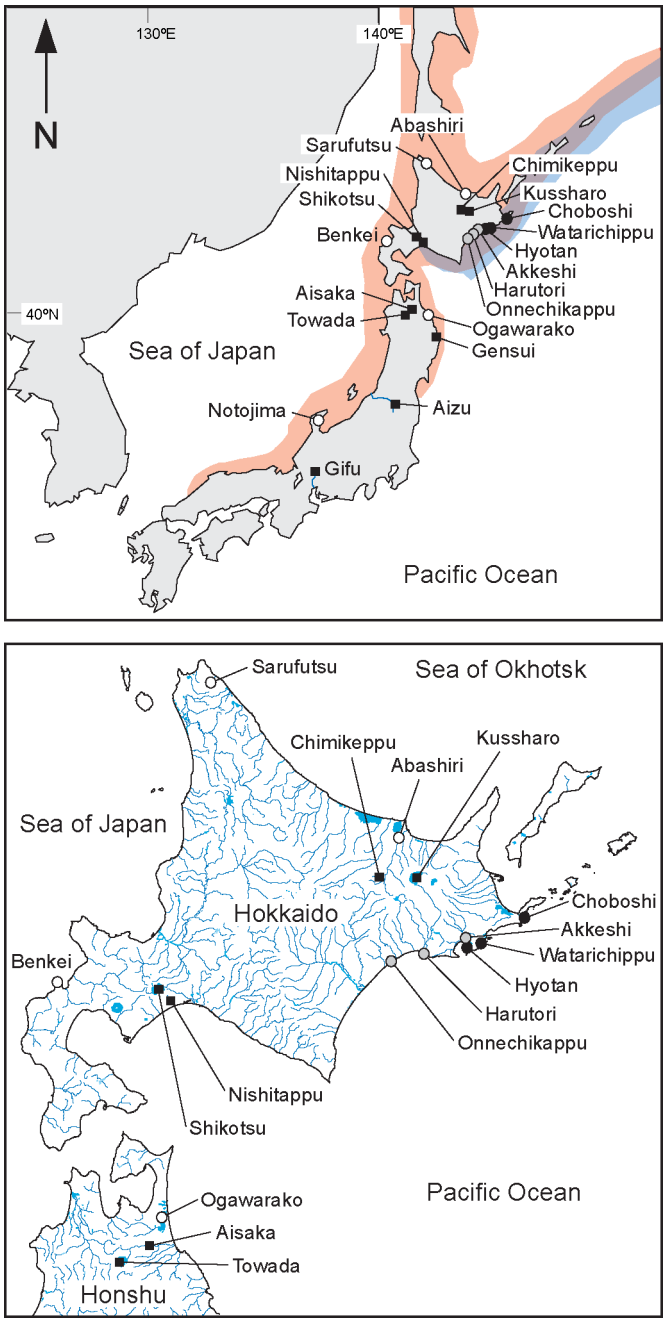

Fig. 1. Map showing the sampling sites. Open circles and solid circles denote the allopatric JA and PA sampling sites, respectively. Grey circles denote the sympatric PA/JA sampling sites. Squares denote freshwater populations. In the upper panel, proposed distributions of the JA and PA lineages are shown in red and blue respectively. In the lower panel, the sampling points of Hokkaido Island and the northern parts of Honshu Island are shown.

Table 1. Information relating to the sampling sitess

Population	Forms	Latitude	Longitude	Collection date	Sample size
Aisaka	Freshwater	40.592	141.221	October 2010	10
Aizu	Freshwater	37.511	139.866	May 2008	10
Chimikeppu	Freshwater	43.629	143.885	June 2011	10
Gensui	Freshwater	39.365	141.897	August 2011	10
Gifu	Freshwater	35.394	136.620	May 2007	8
Kussharo	Freshwater	43.600	144.348	May 2003	10
Nishitappu	Freshwater	42.646	141.476	September 2010	10
Shikotsu	Freshwater	42.775	141.400	September 2010	10
Towada	Freshwater	40.445	140.842	June 2011	10
Hyotan	Allopatric PA	43.032	144.844	June 2010	10
Watarichippu	Allopatric PA	43.0364	145.0532	June 2011	10
Choboshi	Allopatric PA	43.2579	145.556	May 2012	10
Akkeshi PA	Sympatric PA	43.105	144.891	June 2006	10
Harutori PA	Sympatric PA	42.969	144.396	June 2006	10
Abashiri	Allopatric JA	43.960	144.200	May 2008	10
Benkei	Allopatric JA	42.825	140.188	June 2008	10
Notojima	Allopatric JA	37.127	137.044	May 2007	10
Ogawarako	Allopatric JA	40.840	141.372	March 2002	10
Sarufutsu	Allopatric JA	45.255	142.239	June 2005	10
Onnechikappu	Sympatric JA	42.966	144.116	June 2001	10
Akkeshi JA	Sympatric JA	43.068	144.884	June 2006	10
Harutori JA	Sympatric JA	42.969	144.396	June 2010	10

However, the marker was used as prior information for STRUCTURE analysis of population structure (see below).

Genetic diversity, distance, and population clustering

Populations were tested for linkage disequilibrium and Hardy-Weinberg equilibrium (HWE) using Genepop (http:genepop.curtin.edu.au/) (Raymond and Rousset, 1995). GenoDive was used to estimate levels of genetic diversity (H_o, observed heterozygosity; H_e, expected heterozygosity) and effective number of alleles (A_E) within populations (http:www.bentley drummer.nl/software/Home.html) (Meirmans and Van Tienderen, 2004). To indicate genetic differentiation between populations, pairwise Weir and Cockerham's (1984) F_{ST} and Jost's D values (Jost, 2008) were estimated using the diveRsity R package (Keenan, 2012). General linear mixed-models (GLMMs) were used to test for differences in A_E, H_o, and H_e between freshwater and marine populations. All statistical analyses were conducted in R 2.15.1 (R Development Core Team, 2012).

To investigate population structure, we used a Bayesian clustering method implemented in STRUCTURE (Pritchard *et al.*, 2000). The number of assumed populations (K) was set from 2 to 23. The model allowed for admixture and prior information was provided, based on private alleles at the Stn76 locus, which amplified in PA populations and freshwater populations only. Without including the prior information, we obtained similar results. For each run of the model, a burn-in of 50,000 steps was used, followed by 500,000 iterations. For each value of K, the model was repeated 10 times to ensure consistent results. We

evaluated the most probable value of K using log posterior probabilities and Evanno's ΔK method (Evanno *et al.*, 2005) implemented in STRUCTURE Harvester (Earl and vonHoldt, 2012). Independent runs were grouped using CLUMPP (Jakobsson and Rosenberg, 2007) and displayed using DISTRUCT (Rosenberg, 2004).

To further examine the relationships between populations, we analysed the distribution of alleles across all loci using the program ADZE (Szpiech *et al.*, 2008). Prior to analysis, populations were grouped as being Pacific Ocean, Japan Sea or freshwater. Following this, we used ADZE to estimate the mean number of alleles per locus in each group and then the mean number of private alleles. Unlike other rarefaction methods (Petit *et al.*, 1998; Kalinowski, 2004), the ADZE method calculates private alleles as those occurring private to all populations within a grouping. Furthermore, ADZE conducts this analysis at different sample sizes (g), therefore demonstrating the effect of sample size on these estimates; for the present analysis, the value of g was set to vary from 2 to 50. Finally, we used ADZE to estimate the mean number of private alleles for all possible pairwise combinations of the Pacific Ocean, Japan Sea, and freshwater groups. Following Szpiech *et al.* (2008), we hypothesized that more closely related groups would retain a higher proportion of private alleles than those distantly related.

Microsatellite phylogenetic analysis

Two phylogenetic trees were generated, based on different measures of genetic distance. The first was Nei's D_A (Nei *et al.*, 1983), which is based solely on allele frequencies. The other was stepwise weighted genetic distance (DSW) (Shriver *et al.*, 1995), which takes into account the amplified microsatellite nucleotide length. DSW is considered more suitable for estimation of deeper phylogenetic split than Nei's D_A (Goldstein *et al.*, 1995; Terazaki and Nei, 1996). Using these two genetic distances, phylogenetic trees were created using neighbour-joining methods (Saitou and Nei, 1987) with 3000 bootstrap replications in Poptree2 software (http:www.med.kagawa-u.ac.jp/~genomelb/takezaki/poptree2/index.html) (Takezaki *et al.*, 2010).

RESULTS

Genetic diversity and distance

All populations were in HWE for at least nine of the 11 loci following Bonferroni corrections (i.e. $P > 0.005$; see Appendix 1) and no linkage disequilibrium was detected between any microsatellite loci ($P > 0.05$). Tests for genetic diversity showed that freshwater stickleback populations have lower numbers of alleles and lower levels of heterozygosity than marine populations (GLMMs with population as a random effect; A_E: $R^2 = 0.73$, $F_{1,19} = 35.81$, $P < 0.0001$; H_o: $R^2 = 0.99$, $F_{1,19} = 33.82$, $P < 0.0001$; H_e: $R^2 = 0.68$, $F_{1,19} = 44.05$, $P < 0.0001$; see Table 2). Within the marine populations, JA populations appeared to have a higher mean number of alleles than their PA counterparts (mean $A_E \pm$ S.D.: JA = 6.27 ± 0.83; PA = 3.63 ± 0.13), although this was not statistically significant ($R^2 = 0.76$, $F_{1,1} = 47.94$, $P = 0.09$). In terms of heterozygosity, H_o did not differ between the two forms ($P = 0.18$) whereas H_e was higher in JA fish ($R^2 = 0.83$, $F_{1,1} = 1524.5$, $P = 0.02$).

Pairwise measures of genetic differentiation ranged from 0.00 to 0.70 (F_{ST}) and 0.95 (Jost's D; see Table 3). The highest differentiation was present between freshwater populations and between freshwater and marine populations. For example, differentiation between JA fish from Akkeshi and the freshwater population in Aisaka was 0.30 and 0.82

Table 2. Number of alleles (N_A), effective number of alleles (Effective N_A), and expected heterozygosity (H_e)

Population	N_A	Effective N_A	H_e
Freshwater			
Aisaka	2.818	2.310	0.509
Aizu	1.727	1.386	0.198
Chimikeppu	3.909	2.842	0.475
Gensui	3.273	2.128	0.478
Gifu	2.636	2.008	0.360
Kussharo	5.182	3.614	0.716
Nishitappu	3.545	2.468	0.566
Shikotsu	3.182	2.356	0.535
Towada	2.273	1.758	0.392
Mean	3.172	2.319	0.470
Japan Sea			
Abashiri	9.000	6.344	0.849
Akkeshi JA	8.700	6.352	0.841
Benkei	8.400	6.220	0.852
Harutori JA	10.00	7.195	0.860
Notojima	7.000	4.598	0.804
Ogawarako	7.600	5.830	0.858
Onnechikappu	9.600	7.259	0.872
Sarufutsu	8.700	6.377	0.847
Mean	8.625	6.272	0.848
Pacific Ocean			
Akkeshi PA	6.182	3.642	0.713
Choboshi	6.000	3.726	0.677
Harutori PA	5.909	3.590	0.725
Hyotan	5.727	3.774	0.722
Watarichippu	5.000	3.421	0.715
Mean	5.764	3.631	0.710

(F_{ST} and Jost's D, respectively). In contrast, much lower differentiation occurred between JA populations (Table 3). Between groups of populations, genetic differentiation was highest between JA and freshwater populations ($F_{ST} = 0.18$, Jost's $D = 0.66$) and between pooled JA and PA populations ($F_{ST} = 0.17$, Jost's $D = 0.65$). For both measures, divergence was markedly lower between freshwater and PA populations ($F_{ST} = 0.04$, Jost's $D = 0.08$).

Population structure

$K = 4$ was determined as the most probable number of populations by using the mean log P(K), Evanno's ΔK, and the q-values at each probable K (see Fig. 2). ΔK values indicated that $K = 10$ might be the true K, and several freshwater populations started to appear as independent clusters. At both $K = 4$ and $K = 10$, JA and PA fish formed two distinct clusters. Because we wanted to determine whether the freshwater populations are genetically similar to the JA or PA, we analysed the genetic structure at $K = 2$. At $K = 2$, two clusters

Table 3. Pairwise F_{ST} and Jost's D values between populations (values below the diagonal are Weir and Cockerham's (1984) estimated F_{ST}; values above the diagonal are estimated Jost's D)

	AIS	AIZ	CHI	GEN	GIF	KUS	NIS	SHI	TOW	ABA	AKJ	AKP	CHO	HRJ	HRP	HYO	SAR	WAT	BEN	NOT	OGA	ONN
AIS	—	0.484	0.402	0.175	0.811	0.186	0.239	0.201	0.034	0.918	0.824	0.166	0.196	0.757	0.116	0.129	0.805	0.191	0.857	0.709	0.891	0.769
AIZ	0.504	—	0.573	0.364	0.788	0.429	0.491	0.541	0.482	0.902	0.852	0.532	0.459	0.924	0.524	0.520	0.910	0.592	0.911	0.946	0.937	0.809
CHI	0.371	0.563	—	0.398	0.924	0.264	0.340	0.255	0.560	0.695	0.781	0.304	0.306	0.726	0.234	0.384	0.715	0.319	0.886	0.834	0.832	0.759
GEN	0.219	0.475	0.388	—	0.755	0.081	0.152	0.165	0.213	0.821	0.779	0.168	0.165	0.812	0.210	0.240	0.788	0.180	0.785	0.603	0.823	0.732
GIF	0.513	0.671	0.566	0.520	—	0.753	0.770	0.767	0.842	0.826	0.843	0.759	0.775	0.833	0.832	0.813	0.724	0.850	0.765	0.896	0.801	0.828
KUS	0.140	0.358	0.243	0.102	0.370	—	0.079	0.146	0.277	0.776	0.682	0.082	0.023	0.730	0.071	0.064	0.734	0.048	0.762	0.708	0.807	0.633
NIS	0.211	0.463	0.272	0.192	0.470	0.065	—	0.052	0.329	0.796	0.766	0.121	0.063	0.803	0.274	0.216	0.781	0.171	0.861	0.711	0.874	0.741
SHI	0.200	0.482	0.250	0.196	0.481	0.102	0.070	—	0.315	0.807	0.831	0.187	0.176	0.719	0.236	0.238	0.735	0.243	0.832	0.590	0.822	0.796
TOW	0.082	0.609	0.456	0.311	0.598	0.228	0.330	0.330	—	0.934	0.774	0.238	0.281	0.775	0.225	0.226	0.802	0.280	0.889	0.843	0.944	0.777
ABA	0.303	0.440	0.312	0.307	0.344	0.179	0.253	0.266	0.373	—	0.001	0.661	0.687	0.000	0.757	0.756	0.016	0.730	0.001	0.206	0.000	0.002
AKJ	0.295	0.442	0.316	0.303	0.346	0.176	0.257	0.266	0.352	0.000	—	0.557	0.573	0.000	0.726	0.669	0.005	0.608	0.003	0.134	0.001	0.000
AKP	0.127	0.389	0.216	0.137	0.376	0.041	0.095	0.117	0.210	0.184	0.178	—	0.004	0.642	0.009	0.002	0.705	0.001	0.751	0.733	0.742	0.605
CHO	0.157	0.390	0.227	0.135	0.407	0.021	0.060	0.117	0.247	0.199	0.199	0.020	—	0.632	0.049	0.014	0.563	0.085	0.723	0.667	0.655	0.415
HRJ	0.282	0.437	0.305	0.296	0.339	0.169	0.250	0.250	0.343	0.000	0.000	0.171	0.193	—	0.723	0.618	0.001	0.709	0.002	0.149	0.016	0.000
HRP	0.132	0.386	0.192	0.136	0.388	0.057	0.138	0.138	0.223	0.185	0.182	0.012	0.049	0.176	—	0.020	0.785	0.011	0.737	0.607	0.794	0.670
HYO	0.105	0.389	0.248	0.162	0.390	0.048	0.116	0.136	0.215	0.187	0.186	0.014	0.041	0.174	0.041	—	0.736	0.051	0.653	0.630	0.741	0.605
SAR	0.284	0.443	0.309	0.300	0.334	0.171	0.248	0.251	0.344	0.008	0.002	0.178	0.190	0.000	0.183	0.181	—	0.699	0.043	0.135	0.005	0.000
WAT	0.164	0.416	0.220	0.140	0.399	0.044	0.130	0.130	0.231	0.187	0.177	0.013	0.056	0.175	0.019	0.051	0.175	—	0.781	0.677	0.807	0.659
BEN	0.294	0.454	0.329	0.309	0.337	0.174	0.262	0.266	0.367	0.001	0.000	0.184	0.203	0.008	0.183	0.180	0.005	0.184	—	0.184	0.000	0.000
NOT	0.296	0.471	0.337	0.297	0.376	0.187	0.265	0.249	0.376	0.048	0.045	0.197	0.211	0.047	0.188	0.195	0.044	0.188	0.047	—	0.123	0.144
OGA	0.304	0.463	0.333	0.313	0.348	0.184	0.268	0.271	0.380	0.006	0.009	0.189	0.206	0.024	0.185	0.189	0.015	0.190	0.000	0.036	—	0.015
ONN	0.264	0.421	0.295	0.273	0.325	0.147	0.226	0.239	0.330	0.000	0.000	0.150	0.162	0.000	0.153	0.152	0.000	0.156	0.000	0.035	0.007	—

Note: Population abbreviations are as follows: AIS = Aisaka, AIZ = Aizu, CHI = Chimikeppu, GEN = Gensui, GIF = Gifu, KUS = Kussharo, NIS = Nishitappu, SHI = Shikotsu, TOW = Towada, ABA = Abashiri, AKJ = Akkeshi JA, AKP = Akkeshi PA, CHO = Choboshi, HRJ = Harutori JA, HRP = Harutori PA, HYO = Hyotan, SAR = Sarufutsu, WAT = Watarichippu, BEN = Benkei, NOT = Notojima, OGA = Ogawarako, ONN = Onnechikappu.

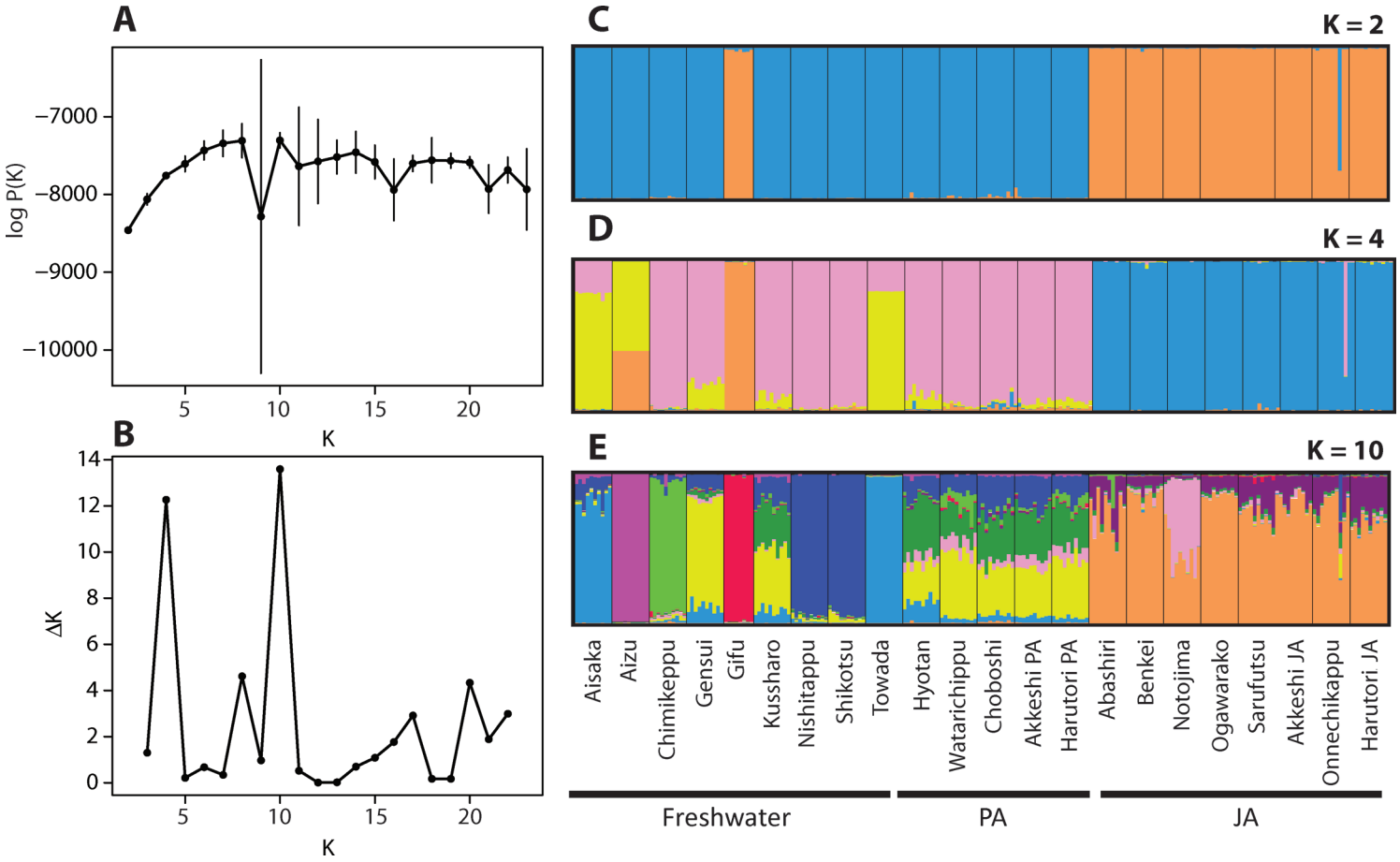

Fig. 2. Results of STRUCTURE analysis. Analysis of posterior probabilities with mean log P(K) ± S.D. (A) and ΔK (B) indicating that $K = 4$ or $K = 10$ is the true K; STRUCTURE output for $K = 2$, i.e. JA and PA lineages (C), $K = 4$ (D), and $K = 10$ (E).

represented grouping into the JA and PA populations (Fig. 2C). Interestingly, all freshwater populations, with the exception of the Gifu population, grouped with the PA cluster. In contrast, all individuals from Gifu clustered with JA fish.

Shared private alleles between forms

Analysis of allelic distribution revealed that the number of mean distinct alleles per locus ($\pm$ S.E.) was greater for JA populations (14.53 ± 2.05) than the pooled PA or freshwater populations (8.90 ± 1.41 and 8.22 ± 1.32 respectively; Fig. 3A). A similar pattern was seen for private alleles (Fig. 3B). As expected, both measures of allelic distribution increased as a function of sample size, although this was less pronounced for private alleles shared between populations within a group (Fig. 3B). Tests of all pairwise combinations of populations revealed the highest proportion of shared private alleles was between PA and freshwater populations, while the lowest proportion occurred between JA and freshwater populations (see Table 4).

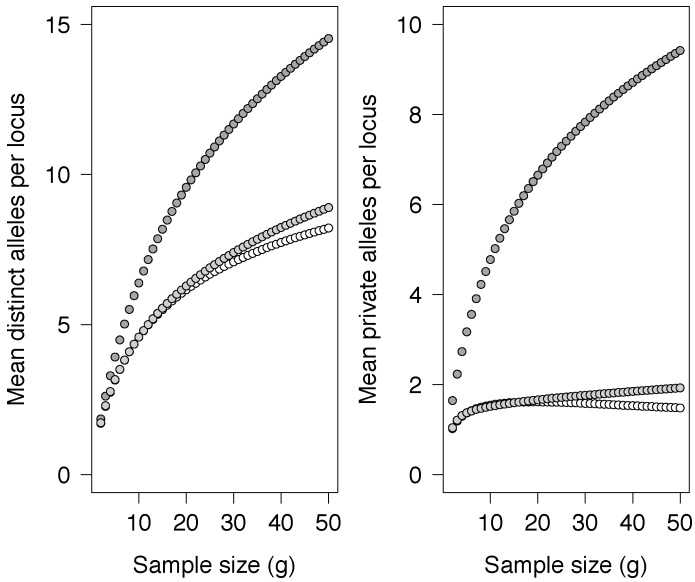

Fig. 3. Results of Allelic Diversity AnalyZEr (ADZE) analysis for (A) mean distinct alleles per locus and (B) mean private alleles per locus, each with increasing sample size (g). Dark grey circles denote pooled Japan Sea populations, light grey circles indicate Pacific Ocean populations, and open circles indicate freshwater populations.

Table 4. Mean number of private alleles per locus for all pairwise combinations of Japan Sea, Pacific Ocean, and freshwater populations ($g = 50$)

Combination	Mean private alleles per locus	S.E.
Pacific Ocean (PA) and freshwater	2.72	2.38
Pacific Ocean (PA) and Japan Sea (JA)	1.10	1.89
Japan Sea (JA) and freshwater	0.86	0.83

Microsatellite phylogeny

In the tree of Nei's D_A (Fig. 4A), JA populations were monophyletic. All of the freshwater populations were genetically similar to the PA populations. The tree also indicates that a freshwater population from Gifu was located close to the root of the Japanese threespine stickleback populations, suggesting that this Gifu population might have diverged earlier than the other freshwater populations. In the tree of DSW (Fig. 4B), JA populations were again monophyletic and all freshwater populations were clustered with the PA populations. The DSW tree also demonstrated that the Gifu population might have diverged earlier than the other freshwater populations. Furthermore, both trees do not indicate a monophyletic origin of freshwater populations, but rather support multiple colonizations of fresh water in the Japanese Archipelago.

DISCUSSION

Our microsatellite data confirm that the Japan Sea and Pacific Ocean anadromous stickleback forms are genetically divergent across their distribution, as previously suggested by allozyme analysis (Higuchi and Goto, 1996). Therefore, the discrepancy between nuclear DNA data and mitochondrial DNA data is likely due to the introgression of mitochondrial DNA. Despite the fact that this suggests gene flow throughout the evolutionary history of this species pair, reproductive isolation persists where the forms occur in sympatry. At least five types of sympatric/parapatric species pairs at varying stages of divergence have evolved throughout the stickleback distribution (Hendry *et al.*, 2009). Of these, the JA–PA species pair lies at the far end of the stickleback speciation continuum. The Japanese species pair is unique in that it is the only known example of near-complete reproductive isolation and divergence between anadromous populations within the stickleback species complex (McKinnon and Rundle, 2002; Hendry *et al.*, 2009). Hybrid males produced in crosses between JA females and PA males are sterile with attenuated sperm production (Kitano *et al.*, 2007). The two forms also exhibit divergent courtship behaviours, which act as a prezygotic isolating barrier when the forms occur in sympatry (Kitano *et al.*, 2007, 2008b, 2009). In addition, JA and PA sticklebacks are divergent in body size and ecologically relevant traits such as number of gill rakers, diet, spawning habitat choice, and migration patterns (Kume *et al.*, 2005, 2010; Kitano *et al.*, 2007, 2009). Divergent ecological selection between different spawning habitats and foraging regimes appears to exist between them (Kume *et al.*, 2010; M. Ravinet and J. Kitano, unpublished data). Therefore, a combination of these multiple isolating barriers likely prevents these species from hybridization in sympatry (Kitano *et al.*, 2009).

We also found that all freshwater populations analysed were genetically similar to the PA populations, suggesting that they are likely derived from the PA lineage rather than the JA lineage. Disentangling the relative contributions of shared ancestral polymorphism (i.e. incomplete lineage sorting) and recent gene flow to genetic differentiation is a major challenge in population genetics research (Nielsen and Wakeley, 2001; Duthiel and Hobolth, 2012). In short, low genetic differentiation may represent recent divergence, recent gene flow or both. For Japanese stickleback, it is therefore possible that some freshwater populations were originally established by the JA lineage but these have subsequently become isolated from JA but not PA populations, leading to high and low differentiation respectively. We consider this scenario an unlikely explanation for the genetic differentiation values estimated in this study. For example, the higher proportion of private alleles across loci shared between

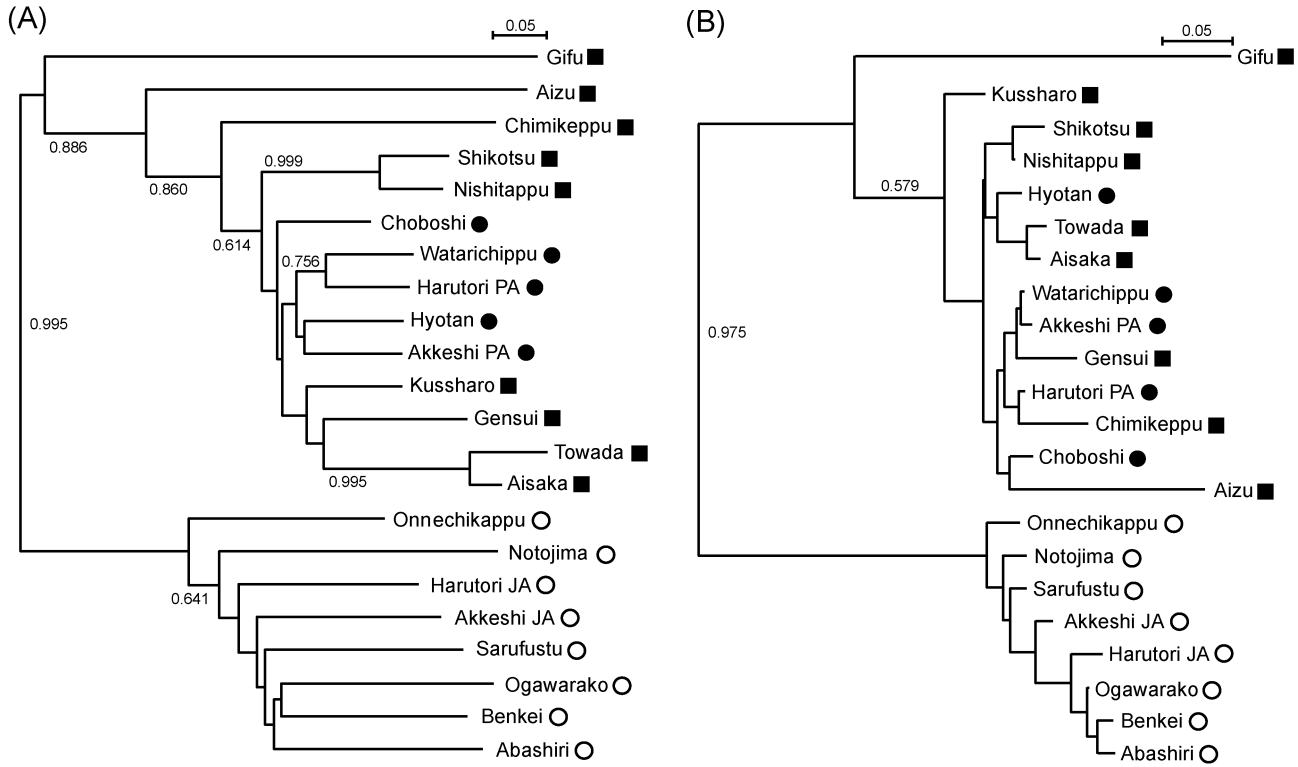

Fig. 4. Phylogenetic tree based on Nei's D_A (A) and DSW (B). Bootstrap values larger than 0.5 are shown at the branch. Solid circles, open circles, and solid squares denote PA, JA, and freshwater populations, respectively.

PA and freshwater populations suggests that the PA lineage is ancestral to freshwater populations. Furthermore, differential amplification at the Stn76 locus also provides evidence of shared ancestry between freshwater and PA fish. Stn76 failed to amplify in all of the JA populations analysed, but successfully amplified in all others. Based on the whole genome sequences of an Akkeshi Japan Sea fish, the failure of amplification is likely due to the existence of two nucleotide substitutions within the reverse primer sequence (K. Yoshida and J. Kitano, unpublished data). As non-amplification of Stn76 is a shared characteristic common to all JA fish in this study, such polymorphism probably occurred soon after the split between JA and PA fish.

Nonetheless, a single freshwater population, Gifu, did group with the Japan Sea lineage when $K = 2$ in the STRUCTURE analysis. However, there are several reasons we think the Gifu population is likely derived from the PA lineage. First, genetic divergence between the Gifu and JA populations is higher than between the Gifu and PA populations (Jost's $D = 0.84$ and 0.76 with Akkeshi JA and PA respectively). Furthermore, although in the tree based on Nei's D_A, the Gifu population was located very close to the root of the Japanese threespine stickleback, the tree based on DSW clearly demonstrated that the Gifu population is more genetically similar to the PA rather than to the JA populations. DSW generally performs better than Nei's D_A for the resolution of the genetic relationships of distantly related species (Goldstein *et al.*, 1995; Terazaki and Nei, 1996). Because previous mitochondrial DNA data indicate that the Gifu population likely diverged well before most contemporary freshwater populations (Watanabe *et al.*, 2003), a phylogenetic tree based on DSW might be appropriate to resolve the relationships between the Gifu population, the JA lineage, and the PA lineage. In addition, the analysis of private alleles also supports the hypothesis that the Gifu population is genetically similar to the PA populations. Furthermore, our recent whole genome sequence data support the hypothesis that the Gifu population is genetically more similar to the PA than the JA populations (K. Yoshida and J. Kitano, unpublished data). Therefore, the Gifu population is also likely derived from the PA lineage.

Since none of the Japanese freshwater populations surveyed to date are derived from the JA lineage, this leads us to ask what factors might prevent freshwater colonization in this lineage. First, ninespine stickleback (genus *Pungitius*) inhabit freshwater lakes and rivers connected to the Sea of Japan (Ikeda, 1933; Takahashi *et al.*, 2001; Tsuruta and Goto, 2006). This contrasts with the Pacific Ocean coast, where ninespine stickleback do not occur in freshwater environments except on Hokkaido. Therefore, the ninespine stickleback may have occupied these freshwater environments earlier than the threespine stickleback, precluding their colonization. Second, JA fish may lack the key traits important for survival in freshwater environments. For example, previous studies demonstrated that JA fish kept in fresh water had low survival rates in contrast to higher rates in PA fish (Honma, 1975; Yamada, 2003). Differences in the survival rate in freshwater environments might be due to differences in the ability of osmoregulation and fatty acid synthesis (A. Ishikawa and J. Kitano, unpublished data).

Understanding the factors that facilitate and constrain adaptive radiations is one of the central challenges in evolutionary ecology. Although novel niches are thought to promote adaptive radiation, not all lineages are able to seize the ecological opportunity. Following the last deglaciation, rising sea levels and shifting ice mass revealed multiple freshwater environments. Several lineages of stickleback, smelts, and salmonids colonized these newly formed lakes and rivers, resulting in impressive adaptive radiations (Bernatchez *et al.*, 1999; Taylor, 1999; Schluter, 2000; Kinnison and Hendry, 2004). In contrast, some lineages have failed to diversify. The Japanese anadromous species pair provides a good demonstration of this. While

the PA lineage has repeatedly colonized freshwater environments, resulting in increased ecological, morphological, and genetic diversity, the JA lineage has not radiated to any substantial extent. Further comparative studies focusing on the PA and JA lineages will provide important insight into the factors that facilitate and constrain adaptive radiation. For example, further studies on the genetic and genomic mechanisms underlying the divergence in freshwater adaptation between the PA and JA lineages and genomic comparison of standing genetic variation between the two lineages will help to answer why some lineages could colonize and utilize vacant niches to achieve adaptive radiation, while others could not.

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APPENDIX 1: Hardy-Weinberg equilibrium (HWE) test

Results of exact tests for HWE across loci in all sampled populations

Population	Stn170	Stn233	Stn64	Stn76	Stn159	Stn46	Stn90	Stn120	Stn278	Stn332	Stn384
Aisaka	1.000	0.115	0.862	0.391	0.019	0.479	0.306	0.833	NP	0.307	0.170
Aizu	0.085	NP	0.021	NP	0.019	NP	NP	NP	NP	1.000	NP
Chimikeppu	1.000	0.747	**0.001***	0.017	0.383	NP	NP	1.000	NP	1.000	0.478
Gensui	0.346	1.000	0.819	0.680	0.185	1.000	1.000	1.000	NP	1.000	0.416
Gifu	1.000	1.000	0.258	0.200	0.164	NP	NP	NP	NP	0.711	NP
Kussharo	0.956	0.668	0.111	0.863	0.847	1.000	0.313	0.235	0.490	0.816	0.219
Nishitappu	0.967	0.325	0.862	0.520	0.900	1.000	1.000	0.158	1.000	0.241	1.000
Shikotsu	0.728	0.217	0.087	1.000	0.854	0.100	1.000	0.332	NP	1.000	0.641
Towada	1.000	0.221	0.046	1.000	1.000	0.573	1.000	0.306	NP	NP	1.000
Abashiri	0.583	0.738	0.500	NA	**0.001***	0.778	**0.000***	0.089	0.278	0.633	0.479
Akkeshi JA	0.144	0.676	0.779	NA	**0.008**	0.129	**0.007**	0.641	0.548	**0.001***	1.000
Akkeshi PA	0.215	0.814	0.959	0.550	0.301	0.534	0.773	0.093	1.000	1.000	0.984
Choboshi	0.071	0.559	0.805	0.883	0.215	0.183	0.713	1.000	1.000	0.254	0.740
Harutori JA	0.424	0.169	0.546	NA	0.625	0.425	**0.000***	0.963	0.436	**0.014**	1.000
Harutori PA	0.324	0.190	0.766	0.028	0.274	0.629	0.408	1.000	1.000	0.034	0.273
Hyotan	0.998	0.748	0.349	0.411	0.287	0.183	0.908	1.000	1.000	0.152	0.338
Sarufutsu	0.169	0.755	0.815	NA	0.408	0.939	0.019	0.970	0.826	0.414	1.000
Watarichippu	0.218	0.189	0.642	1.000	0.855	1.000	0.723	0.876	0.753	0.544	0.625
Benkei	0.029	0.335	0.741	NA	1.000	0.181	0.044	0.465	1.000	0.788	0.122
Notojima	0.172	0.099	0.260	NA	**0.016**	0.870	**0.004***	0.221	0.587	**0.014**	1.000
Ogawarako	0.442	0.190	0.355	NA	0.373	1.000	**0.000***	0.476	0.467	1.000	0.729
Onnechikappu	0.081	0.044	0.721	NA	0.455	1.000	**0.005**	0.165	0.023	0.148	0.183

Note: Bold text denotes loci significant after false discovery rate (B-Y method) has been accounted for; asterisk denotes loci significant after Bonferroni correction ($\alpha = 0.05$, $\alpha_{FDR} = 0.0165$, $\alpha_{BF} = 0.005$). NA, microsatellites were not amplified (null allele). NP, not polymorphic.