Armour plate diversity in Japanese freshwater threespine stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

Background: Independent colonization of freshwater habitats by threespine stickleback (*Gasterosteus aculeatus*) offers a great opportunity to investigate the repeatability of phenotypic evolution and the genetic mechanisms underlying parallel evolution. Armour plate reduction occurs repeatedly in North American and European freshwater populations of the threespine stickleback. The repeated fixation of the single-origin freshwater alleles at the *Ectodysplasin* (*Eda*) locus explains the parallel evolution of plate number reduction in these populations. In contrast, we know little about the patterns and genetic basis of armour plate diversification in Japanese freshwater populations.

Questions: Do Japanese freshwater populations show similar patterns of armour plate reduction as the North American and European freshwater populations? Do the same fresh-water alleles underlie plate number reduction in Japanese populations?

Methods: We analysed the armour plate morphology of eleven freshwater populations and one anadromous population of threespine stickleback in Japan. We first classified each fish into one of the three morphs: completely-, partially-, and low-plated morphs. We next measured the heights of armour plates for the completely-plated morph. We also compared the genome sequences at the *Eda* locus among Japanese, North American, and European populations.

Results: Only one Japanese freshwater population was exclusively low-plated. Two freshwater populations were a mixture of low- and partially-plated morphs. One freshwater population was a mixture of partially- and completely-plated morphs. Seven freshwater populations and one anadromous population were exclusively completely-plated, and in four of those freshwater populations, plate heights were reduced compared with those in the anadromous population. Genome sequences at the *Eda* locus of all Japanese freshwater populations were more similar to those of the Japanese, North American, and European anadromous and marine populations than the freshwater alleles of the North American and European populations.

Conclusion: The parallel evolution of plate reduction occurs in some Japanese freshwater populations, but the genetic basis for this phenotypic change differs from that of the North American and European freshwater populations.

Keywords: convergent evolution, East Asia, freshwater adaptation, lateral plate, non-parallel genetic mechanism, scutes.

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INTRODUCTION

Parallel evolution, the evolution of similar phenotypic traits in phylogenetically independent lineages inhabiting similar environments, offers a great opportunity to investigate the repeatability and predictability of evolution (Darwin, 1859; Schluter, 2000; Losos, 2011). Parallelism in trait evolution suggests adaptation plays a role in phenotypic evolution (Schluter, 2000). Although parallel evolution has been widely observed across diverse taxa (reviewed in Elmer and Meyer, 2011; Rosenblum *et al.*, 2014), recent studies have shown variation in the magnitude and directionality of parallelism among different study systems (Kaeuffer *et al.*, 2012; Oke *et al.*, 2017; Stuart *et al.*, 2017; reviewed in Bolnick *et al.*, 2018). This variation can be caused by several factors, such as differences in the microenvironment (Stuart *et al.*, 2017), the age of populations (Berner *et al.*, 2010; Lucek *et al.*, 2014), and the genetic basis of the trait (Leinonen *et al.*, 2012). For example, when two lineages use different genetic mechanisms (i.e. different genes or different mutations), even the same selective pressures can cause different patterns of phenotypic divergence because of different physiological and pleiotropic effects of the causative mutations (Hoekstra and Nachman, 2003; Elmer and Meyer, 2011; Losos, 2011; Leinonen *et al.*, 2012; Rosenblum *et al.*, 2014).

Plate number reduction in the freshwater threespine stickleback (*Gasterosteus aculeatus*) is a textbook example of parallel evolution (Bell, 2001). Glacial cycles created many freshwater habitats during the Quaternary period, into which ancestral marine threespine sticklebacks colonized in multiple regions of the Northern Hemisphere. In North America and Europe, many freshwater populations repeatedly experienced reductions in the number of armour plates (Wootton, 1984; Bell and Foster, 1994). There have been many hypotheses for why the loss of armour plates might be an adaptation to freshwater environments. A reduction in armour could be adaptive by increasing growth through allocation of calcium to somatic growth in calcium-deficient environments (Giles, 1983; Marchinko and Schluter, 2007; Spence *et al.*, 2012), enhancing defensive ability against predatory invertebrates (Reimchen, 1994; Marchinko, 2009), improving burst swimming ability by increasing body flexibility (Bergstrom, 2002), and/or increasing buoyancy by the removal of heavy armour (Myhre and Klepaker, 2009). Previous genetic studies have identified *Ectodysplasin* (*Eda*) as a major gene that explains variation in plate number (Colosimo *et al.*, 2005). All low-plated freshwater threespine stickleback populations in North America and Europe examined thus far have the identical-by-descent freshwater type allele (low *Eda* allele), whereas the ancestral marine or anadromous populations carry a different allele (complete *Eda* allele) (Colosimo *et al.*, 2005; Raeymaekers *et al.*, 2007; Kitano *et al.*, 2008; Bell *et al.*, 2010; Jones *et al.*, 2012; Ravinet *et al.*, 2014; O'Brown *et al.*, 2015). The low *Eda* allele is maintained within marine and anadromous populations at a low frequency by gene flow from freshwater populations, and newly colonized freshwater populations use the pre-existing allelic variant for a rapid reduction in armour plates (Schluter and Conte, 2009).

The evolutionary trajectories of armour plates in freshwater populations if the founder populations did not carry the low *Eda* allele remain largely unknown. One possible answer is the reduction of armour plate size using different genes or mutations. Some European freshwater threespine stickleback populations did not reduce the number of lateral plates; however, they did show a reduction in plate size (Leinonen *et al.*, 2012). The low frequency of the low *Eda* allele in nearby marine populations is thought to constrain the reduction in plate number (Leinonen *et al.*, 2012). Another possible outcome is new mutations at the *Eda* locus, leading to plate number reduction. A Japanese freshwater population from Gifu carries an *Eda* allele similar to the North American and European complete *Eda* alleles (Colosimo *et al.*, 2005); however, these fish are low-plated (Ikeda, 1933; Mori, 1987). Previous complementation tests

and genetic analyses showed that this population likely acquired new mutations at the *Eda* locus (Schluter *et al.*, 2004; Colosimo *et al.*, 2005; O'Brown *et al.*, 2015). Thus, freshwater populations that do not carry the low *Eda* allele offer a great opportunity to investigate how different genetic architectures can influence the patterns of parallel evolution.

Japan is geographically distant from North America and Europe, and more than ten Japanese freshwater threespine stickleback populations have been identified (Ikeda, 1933; Kitano and Mori, 2016). This distinction allows us to examine whether the patterns of parallel armour plate evolution are shared across a species range of threespine sticklebacks. Ikeda (1933) investigated the plate numbers of eight Japanese freshwater populations in addition to several anadromous populations. He reported that Japanese freshwater populations in the Nobi Plain and east coast of Lake Biwa, known as 'Hariyo' in Japan, are low-plated (range = 3–13 plates), whereas other freshwater populations are completely-plated (range = 30–34 plates) or partially-plated (range = 18–33 plates). Although there have been several subsequent studies on the development of the lateral plates in the Japanese populations (Ikeda, 1934; Igarashi, 1964, 1965; Mori, 1987), variations in plate sizes have never been reported. Furthermore, several new habitats have been found since these earlier studies (Kitano and Mori, 2016); however, their plate morphology has never been reported.

In the present study, we first investigated the armour plate morphology of Japanese freshwater threespine stickleback populations and characterized their diversity. Specifically, we determined whether the patterns of armour plate reduction in the Japanese freshwater populations are similar to those in North American and European freshwater populations. Second, we obtained sequences of the *Eda* locus from Japanese freshwater populations and examined their phylogenetic relationships with *Eda* alleles from North American and European populations. Specifically, we tested whether the same freshwater *Eda* alleles underlie plate number reduction in the Japanese freshwater populations.

MATERIALS AND METHODS

Morphological analysis

For morphological analysis, we used eleven freshwater populations and one anadromous population (Table 1). Five sampling locations (Aizu, Gifu, Ono, Shiga_Jizo, and Shiga_Niu) overlap with those reported in Ikeda (1933). The Gifu, Shiga_Jizo, and Shiga_Niu populations are called 'Hariyo' in Japan (Watanabe *et al.*, 2003; Kitano and Mori, 2016). Specimens of two freshwater populations (Towada and Gensui) and one anadromous population (Akkeshi) have been used in previous analyses and the results reported elsewhere (Kitano *et al.*, 2007a; Adachi *et al.*, 2012; Kume *et al.*, 2018). A previous study demonstrated that the Lake Towada population showed rapid morphological and ecological shifts (Adachi *et al.*, 2012); therefore, we analysed samples collected in 1985 and 1997 for this population. For statistical analysis, Lake Towada samples collected in different years were treated as different groups (Towada1985 and Towada1997). Samples from two of the freshwater populations (Nasu and Aizu) were collected by Seiji Igarasi in 1968 and 1969 respectively, and have been stored at the Fukui Prefectural Educational Research Institute. Because these two populations are now considered severely endangered (Kitano and Mori, 2016), we refrained from additional sampling for the present study. The other seven freshwater populations were sampled using minnow traps or hand nets by one of the authors (Table 1; Fig. 1). Because males and females often differ in external morphology (Kitano *et al.*, 2007a), we used only females for the morphological analysis.

Table 1. Plate morph variations

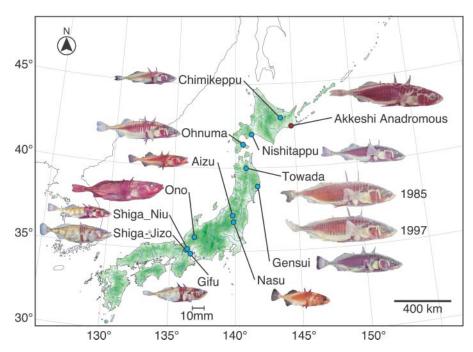| Population	Ecotype	Year	N	Low-plated	Partially-plated	Completely-plated	No. of plates (mean ± SD)
Gifu	Stream	1982	15	15	0	0	5.87 ± 0.74
Shiga_Jizo	Stream	1985	10	7	3	0	6.90 ± 0.88
Shiga_Niu	Stream	1985	8	2	6	0	9.38 ± 1.92
Nasu	Stream	1968	11	0	6	5	28.64 ± 1.91
Aizu	Stream	1969	11	0	0	11	32.09 ± 0.54
Ono	Stream	2002/2006	11	0	0	11	31.36 ± 0.51
Chimikeppu	Lake	2011	30	0	0	30	33.50 ± 0.73
Ohnuma	Lake	1992	15	0	0	15	33.20 ± 0.56
Nishitappu	Stream	2013	19	0	0	19	33.89 ± 0.81
Gensui	Stream	2012	11	0	0	11	33.64 ± 0.51
Towada1985	Lake	1985	15	0	0	15	32.33 ± 1.23
Towada1997	Lake	1997	29	0	0	29	33.31 ± 0.66
Akkeshi	Anadromous	2003	18	0	0	18	32.28 ± 0.57

Fig. 1. Sampling locations in Japan and representative photographs of alizarin red stained specimens. Blue and red circles indicate freshwater populations and an anadromous population, respectively.

All specimens were stained with alizarin red as described previously (Peichel *et al.*, 2001). The lateral plates were counted under a dissecting microscope (SZ61, Olympus, Tokyo, Japan). This was done on the right side of the fish except for one anadromous fish from Akkeshi, which had damage to its right side; thus, the left side was measured. We first classified each fish into one of three morphs: completely-, partially-, or low-plated as described previously (Hagen and Gilbertson, 1972; Bell, 2001; Kitano *et al.*, 2008). Fish with only anterior plates and lacking a keel were classified as low-plated morphs; fish with both anterior plates and a caudal keel with a gap of two or more unplated myomeres were classified as partially-plated morphs; and fish with complete rows of lateral plates with no or just one unplated myomere were classified as completely-plated morphs.

The standard length (SL) of all specimens was measured using a digital calliper with a resolution of 0.01 mm. For completely-plated fish, the heights of plates 8–24 were also measured using the same digital calliper. The heights of the first seven plates, which form a solid structural unit with the pelvic girdle and dorsal spines (Reimchen, 1983), cannot be measured with precision, and thus their heights were not taken into account. For partially-plated fish, we measured the height of the 8th plate: plate height was measured at this position in a previous genetic study of variation in plate height (Colosimo *et al.*, 2004). Both measurements were natural log (ln)-transformed before statistical analysis.

For plate number, we performed an analysis of variance (ANOVA). For plate height, we first performed a principal components analysis using only completely-plated fish to summarize the 17 ln-transformed plate heights. Then, PC1, which explained 97.61% of the variance, was used as a plate height trait (plate height PC1) for subsequent analysis (for component loadings, see evolutionary-ecology.com/data/3158Appendix.pdf). Because plate

height PC1 is correlated with body size, we performed an analysis of covariance (ANCOVA) of PC1 with ln-transformed SL (lnSL) as a covariate to account for the effects of body size on plate height. First, we tested for an interaction between lnSL and plate height PC1. When the interaction was not significant, we tested the effect of population on plate height PC1 with lnSL as a covariate excluding the interaction term. Both the ANOVA and ANCOVA were followed by Tukey-Kramer *post hoc* tests to locate pairs that were significantly different from each other after the correction of multiple comparisons. Next, we similarly conducted ANCOVA for ln-transformed height of the 8th plate using both partially- and completely-plated fish with lnSL as a covariate.

Phylogenetic and SNP analysis of the *Eda* locus

The genomic sequences of the *Eda* locus were obtained from whole genome sequence data (Table 2). For phylogenetic analysis, data for 17 populations of *G. aculeatus* and one population of *G. nipponicus* were obtained from a publicly available database (accession numbers are given in Table 2). We excluded two German freshwater populations whose plate morphs were polymorphic (Feulner *et al.*, 2015). For the Nishitappu, Chimikeppu, Ono, and Gifu populations, sequence libraries of eight individuals per population were prepared using a NEBNext Ultra DNA Library Prep Kit for Illumina (Illumina, San Diego, CA). Whole genome sequencing was performed on three lanes of the Illumina HiSeq X system in 150-base pair (bp) paired-end mode with each individual fish being distinguished by unique index barcodes (NEBNext Multiplex Oligos for Illumina). The obtained reads were deposited in the DNA Data Bank of Japan (DRA007515) (Table 2).

Sequence reads of each fish were mapped to the repeat sequence-masked Broad S1 stickleback reference genome using CLC Genomics Workbench 8.0 (Qiagen, Hilden, Germany) as described previously (Yoshida *et al.*, 2014), or BWA-MEM (Li, 2013) with default settings. SNP calling was performed using samtools 1.7 and bcftools 1.6 (Li *et al.*, 2009), producing vcf files. From the vcf files, we used the VCFtools 0.1.15 software program (Danecek *et al.*, 2011) to extract genomic sequences of the *Eda* locus (Chromosome IV: positions 12,800,220–12,810,446 bp in the Ensembl BROAD S1 coordinate). This region contains all exons and introns of *Eda*. Only sites with eight or more read depths were used. Sites with gaps or low coverage were masked with N for each individual: the average count of N per sequence was 2881 bp. We assigned exons and introns of *Eda* and searched for the best partitioning scheme and the best substitution model for phylogenetic analysis using the PartitionFinder2 software program (Lanfer *et al.*, 2017). The AICc scores between the GTRGAMMA and GTRGAMMAI models were compared; the GTRGAMMAI model had a better AICc score than the GTRGAMMA model. In the best model, the sequence was partitioned into five subsets:

- Subset1 (7818–7988, 9435–9695, and 1–315 bp);
- Subset2 (316–5762 bp);
- Subset3 (8339–8373 and 5763–5862 bp);
- Subset4 (8554–9013, 8374–8456, and 5863–7817 bp); and
- Subset5 (9145–9434, 7989–8338, 9014–9144, and 8457–8553 bp).

RAxML 8.2.4 (Stamatakis, 2014) was then used to construct a maximum likelihood tree using the GTRGAMMAI model. Statistical support of clades was calculated by 1000 bootstrap

Table 2. Populations used for *Eda* sequence analysis

Population	Ecotype	Plate morph	Country	N	Accession #	Reference
Gifu	Stream	Low	Japan	8	DRA007515	This study
Shiga_Edaore	Stream	Low/partial	Japan	1	DRA004954	Yoshida *et al.* (2019)
Nasu	Stream	Partial/complete	Japan	1	DRA004954	Yoshida *et al.* (2019)
Aizu	Stream	Complete	Japan	1	DRA004954	Yoshida *et al.* (2019)
Ono	Stream	Complete	Japan	8	DRA007515	This study
Chimikeppu	Lake	Complete	Japan	8	DRA007515	This study
Nishitappu	Stream	Complete	Japan	8	DRA007515	This study
Towada2010	Lake	Complete	Japan	4	DRA005065	Yoshida *et al.* (2019)
Little Campbell River	Stream	Low	Canada	1	DRA004949	Ishikawa *et al.* (2017)
Bear Paw Lake	Lake	Low	USA	1	Ensembl	Jones *et al.* (2012)
Little Meadow Creek	Stream	Low	USA	1	PRJEB5198	Feulner *et al.* (2015)
Long Lake	Lake	Low	USA	1	PRJEB5198	Feulner *et al.* (2015)
Misty Stream Inlet	Stream	Low	Canada	1	PRJEB5198	Feulner *et al.* (2015)
Misty Lake	Lake	Low	Canada	1	PRJEB5198	Feulner *et al.* (2015)
Malenter Au	River	Low	Germany	1	PRJEB5198	Feulner *et al.* (2015)
Eider	River	Low	Germany	1	PRJEB5198	Feulner *et al.* (2015)
Orraelva	River	Low	Norway	1	PRJEB5198	Feulner *et al.* (2015)
Skogseidvatnet	Lake	Low	Norway	1	PRJEB5198	Feulner *et al.* (2015)
Akkeshi	Anadromous	Complete	Japan	8	DRA001136	Yoshida *et al.* (2014)
Little Campbell River	Anadromous	Complete	Canada	1	DRA004937	Ishikawa *et al.* (2017)
North Sea	Marine	Not described	Denmark	5	PRJEB2954	Feulner *et al.* (2013)
G. nipponicus	Anadromous	Complete	Japan	8	DRA001136	Yoshida *et al.* (2014)
G. wheatlandi	Anadromous	Low/Partial	USA	1	DRA001086	Yoshida *et al.* (2014)
P. pungitius	Brackish	Partial	Japan	1	DRA001085	White *et al.* (2015)

Note: For the Nasu population and *G. wheatlandi*, partially-plated individuals were used for sequencing. The plate morph of a Shiga-Edaore fish used for sequencing is unknown.

replications. A phylogenetic tree was drawn using the Figtree 1.3.1 software program (Rambaut, 2009). A phylogenetic analysis using only sites where all individuals were genotyped (1084 bp) gave rise to qualitatively similar results.

We additionally checked the genotypes at position 12811481 on Chromosome IV in the Ensembl BROAD S1 coordinate, where nucleotide substitution between T and G is suggested to be responsible for plate morph variation (O'Brown *et al.*, 2015), and several nearby sites differing in nucleotides between the low and complete *Eda* alleles (O'Brown *et al.*, 2015) (Chromosome IV: 12800508, 12808630, 12811933, 12813328, 12813394, 12815024, 12815027, 12816201, 12816202, 12816360, 12816402, and 12816464 in the Ensembl BROAD S1 coordinate). A single nucleotide polymorphism at 12808303 of Chromosome IV was analysed previously [position 5 in O'Brown *et al.* (2015)], but was located in the region that was masked in the repeat-masked reference sequence, so we did not analyse this SNP. We also investigated SNPs of two other stickleback species, *G. wheatlandi* and *Pungitius pungitius*, at these loci using previously determined whole genome sequences (Yoshida *et al.*, 2014; White *et al.*, 2015) (Table 2).

RESULTS

Diversity in lateral plate morphology

Only one population, that from Gifu, which provided 15 specimens, was exclusively low-plated (Fig. 2). The Shiga_Jizo population contained a mixture of seven low-plated
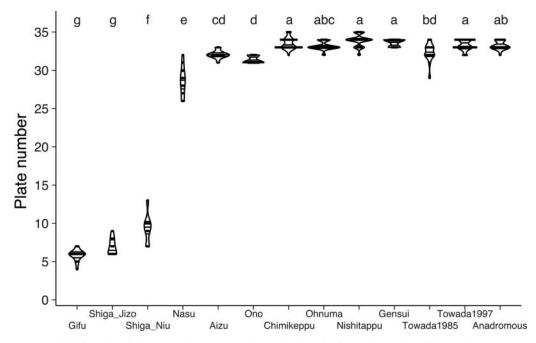

Fig. 2. A violin plot of plate number. Letters above the plots indicate the results of the Tukey-Kramer *post hoc* test. Populations with the same letters indicate there is no significant difference in plate number between them.

individuals (range = 6–7 plates) and three partially-plated individuals (range = 8–9 plates). The Shiga_Niu population also contained a mixture of two low-plated individuals (each with 7 plates) and six partially-plated individuals (range = 9–13 plates). Plate number in the Shiga_Niu population was significantly higher than that in the other two populations (Shiga_Niu vs. Gifu, $P_{adj} < 0.001$; Shiga_Niu vs. Shiga_Jizo, $P_{adj} < 0.001$; and Gifu vs. Shiga_Jizo, $P_{adj} = 0.231$). The Nasu population had a mixture of six partially-plated individuals (range = 26–29 plates) and five completely-plated individuals (range = 29–32 plates). The other seven freshwater populations and one marine population were exclusively completely-plated.

The interaction between population and lnSL was significant for both plate height PC1 and the height of the 8th plate when all populations were included in the ANCOVA (PC1, $F_{9,141} = 4.33$, $P < 0.001$; 8th plate, $F_{9,150} = 2.94$, $P < 0.01$). When we removed the Aizu population, which had a different slope from the other populations (Fig. 3), the interaction became non-significant in the ANCOVA (PC1, $F_{8,132} = 1.46$, $P = 0.177$; 8th plate, $F_{8,141} = 1.72$, $P = 0.099$). For the remaining populations – excluding Aizu – both plate height PC1 and the height of the 8th plate varied significantly among populations (PC1, $F_{8,140} = 51.42$, $P < 0.001$; 8th plate, $F_{8,149} = 27.52$, $P < 0.001$). The Tukey-Kramer *post hoc* test for plate height PC1 showed that four of the freshwater populations (Ono, Chimikeppu, Ohnuma, and Towada1997) had significantly lower plate heights than the anadromous population (Fig. 3A). Of these four populations, the Chimikeppu population had the lowest value (Fig. 3A). The Tukey-Kramer *post hoc* test for height of the 8th plate did not detect any freshwater populations with a significantly lower plate height at this position than the anadromous population (Fig. 3B).

Phylogeny and SNPs of the *Eda* locus

We obtained 9695 bp aligned sequences for the phylogenetic analysis of the *Eda* locus. *Eda* alleles show low-plated populations from North America and Europe make up a different monophyletic group from that containing the North American and European completely-plated marine or anadromous populations (Fig. 4). *Eda* alleles from all Japanese freshwater populations, including those with low-plated morphs, were included in the clade containing the completely-plated anadromous or marine populations of North America, Europe, and Japan (Fig. 4). None of them clustered with the *Eda* alleles from low-plated freshwater populations of North America and Europe.

SNP analysis confirmed that all threespine stickleback populations with low-plated morphs from any region, including Japan (Gifu and Shiga_Edaore), have G at 12811481 of Chromosome IV (box in Fig. 5). Interestingly, other stickleback species examined, including *G. nipponicus*, *G. wheatlandi*, and *P. pungitius*, also had G at this position, although *G. nipponicus* individuals are completely-plated (Kitano *et al.*, 2007b). Other nearby SNPs were divergent between low- and completely-plated populations in North America and Europe. In contrast, all Japanese freshwater populations, including low-plated populations (Gifu and Shiga_Edaore), were homozygotes of marine alleles at all examined sites except position 12811481 (see nucleotides shown in red in Fig. 5).
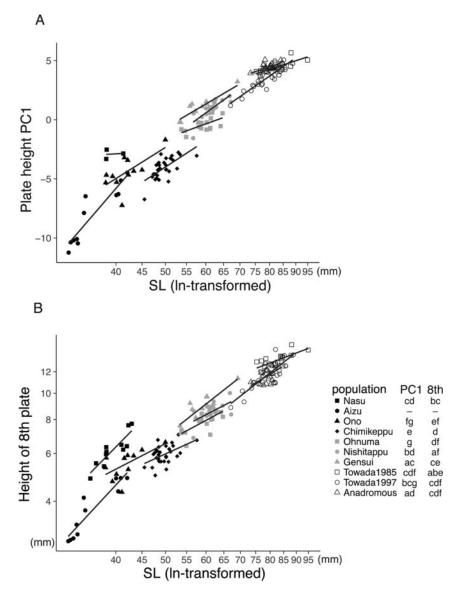
Fig. 3. Plate height variations among populations. Plate height PC1 (A) and ln-transformed height of the 8th lateral plate (B) are plotted against ln-transformed standard length (SL). Letters at the right side of the population names indicate the results of the multiple comparison tests. Populations with the same letters indicate no significant difference in plate height PC1 or the height of the 8th plate between them. Aizu was removed from the ANCOVA because of heterogeneity of slopes.
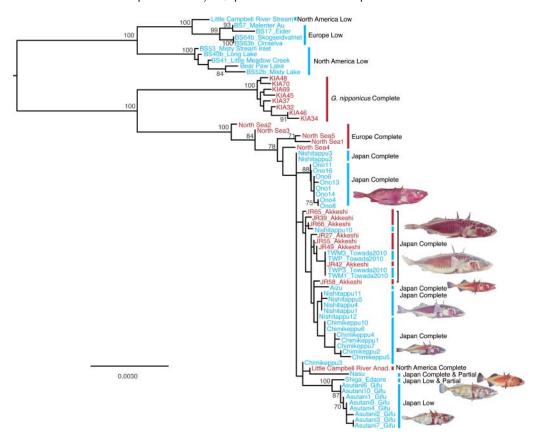
Fig. 4. Maximum likelihood tree of the *Eda* locus based on 9695 bp. Values at the nodes indicate statistical support calculated by 1000 bootstrap replications. Only support values >70% are shown. Blue letters and bars indicate freshwater populations, while red letters and bars indicate marine or anadromous populations. Note that we arbitrarily set a root of the tree between the North American/ European low *Eda* clade and others, so we are unsure whether the low *Eda* allele is older than the split between *G. nipponicus* and *G. aculeatus*.

DISCUSSION

Overall, we observed a reduction in either plate number or plate size in the Japanese freshwater threespine sticklebacks. However, the patterns in plate reduction varied considerably among freshwater populations. Only one population was exclusively low-plated. Three populations were polymorphic, two with low- and partially-plated morphs and one with partially- and completely-plated morphs. The remaining seven populations were exclusively completely-plated, with four populations having a reduction in plate size.

The prevalence of completely-plated morphs in freshwater populations has also been reported from the east coast of Canada (Hagen and Moodie, 1982) and eastern Europe (Münzing, 1963), whereas low-plated morphs are relatively common in freshwater populations of the west coast of North America and southern Europe (Münzing, 1963; Hagen and Gilbertson, 1972; Wootton, 1984). Factors responsible for global variation in the prevalence of low-plated freshwater populations may be both ecological and genetic. Ecologically, based on the global

Population	Plate morph	Counrty	4	6		7	8	9	10	11	12	13	14	15	16
Little Campbell River	Low	Canada	G	A	G	C	T	T	C	C	T	T	G	A	G
Bear Paw Lake	Low	U.S.A.	G	A	G	C	T	T	C	C	T	T	G	A	G
Little Meadow Creek	Low	U.S.A.	G	A	G	C	T	T	C	C	T	T	G	A	G
Long Lake	Low	U.S.A.	G		G	C	T	T	C	C	T	T	G	A	G
Misty Stream Inlet	Low	Canada			G	C	T	T	C	C	T	T	G	A	G
Misty Lake	Low	Canada			G	C	T	T	C	C	T	T	G	A	G
Malenter Au	Low	Germany			G	C	T	T	C	C	T	T	G	A	G
Eider	Low	Germany	A		G	C	T	T	A	T	G	C	T	T	C
Orraelva	Low	Norway		A	G	C	T	T	C/A	C/T	T/G	T/C	G/T	A/T	G/C
Skogseidvatnet	Low	Norway	G	A	G	C	T	T	C/A	C/T	T/G	T/C	G/T	A/T	G/C
Gifu	Low	Japan	A	G	G	G	C	C	A	T	G	C	T		
Shiga_Edaore	–	Japan	A	G	G	G	C	C	A	T	G	C	T	T	C
Nasu	Partial	Japan	A	G	T	G	C	C	A	T	G	C	T	T	C
Aizu	Complete	Japan		G	T	G	C	C	A	T	G	C	T	T	C
Ono	Complete	Japan	A	G	T	G	C	C	A	T	G	C	T	T	C
Chimikeppu	Complete	Japan	A	G	T	G	C	C	A	T	G	C	T	T	C
Nishitappu	Complete	Japan	A	G	T	G	C	C	A	T	G	C	T	T	C
Towada2010	Complete	Japan	A	G	T	G	C	C	A	T	G	C	T	T	C
Little Campbell River	Complete	Canada	A	G	T	G	C	C	A	T	G	C	T	T	C
North Sea	Not described	Denmark			T	G	C	C	A	T	G	C	T	T	C
Akkeshi	Complete	Japan	A	G	T	G	C	C	A	T	G	C	T	T	C
G. nipponicus	Complete	Japan	A	G	G	G	C	C	A	T	G	C	T	T	C
G. wheatlandi	Partial	U.S.A.	A	G	G	G	C	C	A	T	G	C	T	T	C
P. pungitius	Partial	Japan	A	G	G	G	C	C	A	T	T	C	G	C	C

Fig. 5. SNPs in the regulatory region of *Eda* and nearby sites. SNP positions follow O'Brown *et al.* (2015): position 4 (12800508), position 6 (12808630), position 7 (12811933), position 8 (12813328), position 9 (12813394), position 10 (12815024), position 11 (12815027), position 12 (12816201), position 13 (12816202), position 14 (12816360), position 15 (12816402), and position 16 (12816464) on Chromosome IV. A single nucleotide polymorphism located in repetitive sequences (position 5) was not analysed here. The black box indicates the nucleotide substitution between T and G at 12311481 of Chromosome IV, where G is shared by all low-plated threespine stickleback populations examined. Nucleotides shown in blue indicate the low *Eda* allele, while those in red indicate the complete *Eda* allele. Nucleotides that belonged to neither are shown in black. Populations shown in blue indicate freshwater populations, while those in red indicate marine or anadromous populations. A *P. pungitius* fish used for the SNP analysis is brackish water-resident and shown in black. We found heterozygotes at position 12311481 in one North Sea fish and at positions 8–16 in another North Sea fish, which are not shown here.

distribution patterns of completely-plated freshwater populations, Hagen and Moodie (1982) inferred that cold temperatures may be associated with the prevalence of the completely-plated morph, although the physiological mechanisms are unclear. Consistent with this idea, the Japanese low-plated sticklebacks were found only in the southern part of the distribution, where atmospheric temperature is very warm, and sticklebacks occupy only spring-fed habitats with a water temperature of around 10–20°C throughout the year (Kitano

and Mori, 2016). In contrast, all sticklebacks in the northern part of Japan, Hokkaido Island, were completely-plated, a region where the surfaces of lakes and rivers freeze in winter.

In addition to ecological factors, there may be genetic constraints on the evolution of low-plated freshwater morphs. The absence of standing allelic variation at the *Eda* locus in the ancestral marine or anadromous populations could be one potential cause (Leinonen *et al.*, 2012). We observed that all *Eda* alleles in the Japanese populations examined belonged to the group of the complete *Eda* alleles. Furthermore, all 198 anadromous individuals collected in 2006 in eastern Hokkaido (Kitano *et al.*, 2009) were completely-plated. This contrasts with the observation that low- and partially-plated morphs were at low frequencies in North America and Europe (Kitano *et al.*, 2008; Leinonen *et al.*, 2012). These data suggest that the absence of the pre-existing low *Eda* allele in the ancestral populations might constrain the Japanese freshwater populations from reducing their plate number. Thus, it is likely that both eco-logical and genetic factors are responsible for variation in the frequency of differently plated morphs in Japan. Future genotyping of *Eda* alleles of many Japanese Pacific Ocean marine threespine sticklebacks will reveal how much standing variation exists at *Eda* around the Japanese archipelago.

A significant reduction in plate height was observed in four freshwater populations (Chimikeppu, Ohnuma, Ono, and Towada1997) when analysing plate height PC1 scores, whereas no significant reduction was observed in height of the 8th plate in any freshwater population. Although we could not include the Aizu population in our statistical analysis of plate size because of heterogeneity of slopes, this population also appeared to have smaller plates than the anadromous population (Figs. 1 and 3). Populations displaying no reduction in plate size (Nishitappu, Gensui, and Towada1985) are thought to have been introduced only recently. Although the age of freshwater colonization is not clear for the Nishitappu and Gensui populations, the Lake Towada population was established sometime around the 1970s (Adachi *et al.*, 2012; Yoshida *et al.*, 2016). Lake Towada is a crater lake, where no fish were present until the nineteenth century, and the stickleback was first reported in 1979 (Adachi *et al.*, 2012). In 1985, this population did not show any reduction in plate size, but by 1997 it had done so, suggesting that plate size reduction can occur rapidly either due to phenotypic plasticity or genetic changes.

Further research on temporal changes in plate morphology in populations other than the Lake Towada population would help us to understand the ecological mechanisms under-lying plate evolution over contemporary time-scales (Bell *et al.*, 2004; Kitano *et al.*, 2008; Bell and Aguirre, 2013). Two of the Japanese freshwater populations found to display polymorphism in the present study (Nasu and Shiga_Jizo) were also reported to have done so in the 1930s or prior to that time (Ikeda, 1933). This indicates that polymorphism has been maintained for several decades. Disruptive natural selection may be important for the maintenance of polymorphism as reported in a Canadian lake population (Marchinko *et al.*, 2014).

Japanese freshwater stickleback populations are valuable systems to investigate the genetic basis for parallel evolution (Elmer and Meyer, 2011; Losos, 2011; Rosenblum *et al.*, 2014). Our phylogenetic and SNP analysis of the *Eda* locus shows that all *Eda* alleles of Japanese freshwater populations are more closely related to those of completely-plated anadromous or marine populations in Japan, North America, and Europe than to those of the low-plated freshwater populations of North America and Europe. Although previous studies have indicated that *de novo* mutation of *Eda* rather than fixation of the pre-existing low *Eda* alleles is responsible for plate reduction in the Gifu population (O'Brown *et al.*, 2015), and the present study indicates that the Shiga_Edaore population also has the same mutation as the

Gifu population, the genetic mechanisms behind the reduction in plate number in other Japanese freshwater populations remain elusive. Interestingly, *G. nipponicus* also had G at 12311481 of Chromosome IV, despite the fact that *G. nipponicus* is completely-plated (Kitano *et al.*, 2007b; Higuchi *et al.*, 2014). Because *G. nipponicus* displays a lower plate height at the caudal part than sympatric *G. aculeatus* (Kitano *et al.*, 2007b; Higuchi *et al.*, 2014), it is possible that this mutation may play a role in plate size reduction in *G. nipponicus*. The genetic basis for plate size reduction in the North American and European freshwater populations is reported to be caused by transposon insertion at the enhancer region of the *Growth/Differentiation Factor 6* (*GDF6*) gene (Indjeian *et al.*, 2016). Examining whether the *GDF6* locus is also responsible for plate size reduction in the Japanese freshwater populations and whether the same transposons are inserted at the same site will provide further insights into the genetic mechanisms of parallel evolution of plate reduction.

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