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Shifts in morphology and diet of non-native sticklebacks introduced into Japanese crater lakes

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Abstract

An increasing number of exotic animals are causing ecological problems. Therefore, for better ecosystem management, it is important to understand how exotic species colonize and adapt to novel environments. The threespine sticklebacks (*Gasterosteus aculeatus*) can be a good vertebrate model system to explore the ecological and genetic mechanisms of adaptation not only in natural populations, but also in non-native populations. Although morphological changes have been documented in several introduced populations of stickleback, little is known about the dietary changes during colonization into novel environments. Here, we investigated the morphological and dietary changes of exotic threespine stickleback populations introduced into three Japanese crater lakes (Lake Towada, Lake Kussharo, and Lake Shikotsu). Sticklebacks were introduced into the crater lakes likely along with salmonids transplanted for aquaculture. The stickleback population in Lake Kussharo had multiple mitochondrial haplotypes and had larger phenotypic variances than other crater lake stickleback populations that had only one mitochondrial haplotype. Compilation of historical data on the morphology and stomach contents of the Lake Towada stickleback population showed that substantial shifts in body size and stomach contents occurred after colonization. Some of these changes may be related to an outbreak of the *Schistocephalus* parasite. These results suggest that sticklebacks can change their morphology and trophic ecology when they colonize novel environments. Therefore, extreme care should be taken when salmonids are transported between watersheds for aquaculture and that long-term monitoring of exotic species is essential for ecosystem management. In addition, further genetic studies on phenotypic changes in crater lake sticklebacks would help elucidate the genetic mechanisms underlying the adaptation of exotic fishes to novel environments.

Introduction

An increasing number of animals have been transplanted from native habitats to novel environments (Elton 1958; Sakai et al. 2001). Because exotic species are causing many ecological problems, it is important to understand how exotic species invade, colonize, and adapt to novel environments for better management of ecosystem functions (Pejchar and Mooney 2009). Exotic species have been intentionally introduced for specific reasons, such as aquaculture and sports, or unintentionally, such as when they contaminate the transplanted stocks of other organisms (Jeschke and Strayer 2005, 2006). Meta-analyses of biological invasions have demonstrated that human affiliates, such as domesticates, pets, human commensals, have high invasion success (Jeschke and Strayer 2005, 2006) and the success rate of invasion increases when humans intend their establishment (Ruesink 2005). Therefore, it is important to identify the dispersal pathways of exotic species to prevent their further colonization.

Figure 1. Threespine stickleback (*Gasterosteus aculeatus*) in a crater lake, Lake Kussharo.

Furthermore, the mechanisms through which exotic species adapt to novel environments should be elucidated. There are several empirical examples of the introduced populations changing their phenotypes after the colonization of novel environments (Hendry and Kinnison 1999; Reznick and Ghalambor 2001; Bossdorf et al. 2005; Huey et al. 2005). Such phenotypic changes can occur by both phenotypic plasticity and genetic changes (Hendry et al. 2011). When genetic changes are crucial, the genetic variances of introduced populations can influence the success rate of colonization (Willi et al. 2006; Hendry et al. 2011). For example, multiple introductions or hybridizations between different populations may increase genetic variances and promote adaptation (Kolbe et al. 2004; Facon et al. 2005; Lockwood et al. 2007). In addition, even in cases where phenotypic plasticity plays an important role in adaptation, increased genetic variances may improve the plasticity of exotic species, because phenotypic plasticity is usually a heritable trait (De Jong 1990; Scheiner 1993; Pigliucci 2005). Therefore, genetic studies of introduced species and investigation of the genetic mechanisms of adaptation are crucial for better management of exotic species (Hendry et al. 2011).

The threespine sticklebacks (*Gasterosteus aculeatus*) can be an excellent vertebrate model system to explore the ecological and genetic mechanisms of adaptation not only in natural populations, but also in non-native populations (Fig. 1). The ecological and genetic mechanisms underlying adaptive evolution during the last postglacial dispersal have been extensively investigated in sticklebacks (Schluter 2000; Shapiro et al. 2004; Colosimo et al. 2005; Miller et al. 2007; Chan et al. 2010; Hohenlohe et al. 2010). Ancestral marine sticklebacks have colonized various freshwater environments in many coastal regions of the Northern Hemisphere within the last 12,000 years, resulting in the evolution of diverse ecotypes (Bell and Foster 1994; Schluter 2000; McKinnon and Rundle 2002). In addition, previous studies have characterized phenotypic changes in exotic populations introduced into freshwater ponds, lakes, and lagoons (Klepaker 1993; Kristjánsson et al. 2002; Aguirre et al. 2004; Bell et al. 2004; Lucek et al. 2010). Although these studies have demonstrated substantial phenotypic changes in foraging traits, which might occur as adaptation to dietary shifts, they have not directly tested dietary changes of the introduced populations. Furthermore, genetic structures of the introduced populations have not been investigated except in a case of a Swiss population (Lucek et al. 2010).

The present study aimed to characterize phenotypic and dietary changes of exotic populations of threespine stickleback that were introduced into three crater lakes. We also investigated genetic structures of the introduced crater lake populations. Crater lakes provide an interesting ecosystem for investigating the invasion by exotic species, because these lakes were formed by volcanoes, so they were originally depauperate (Barluenga et al. 2006; Barluenga and Meyer 2010; Elmer et al. 2010). Depauperate environments with vacant niches often provide a stage for biological diversification and have been extensively investigated in evolutionary ecology (MacArthur and Wilson 1967; Diamond 1974; Whittaker and Fernández-Palacios 2007). The colonization of organisms into crater lakes occurs through multiple routes, such as immigration through newly formed rivers and introduction by hurricanes, animals, and human release (Hikita and Taniguchi 1959; Tokui 1964; Ban and Suzuki 2003; Elmer et al. 2010). Thus, the investigation of nascent ecological communities in crater lakes will provide a window into the early stages of community assembly. Furthermore, the investigation of impacts of exotic species on the crater lake ecosystem is important for management of ecosystem services provided by crater lakes, because crater lakes are often used for aquaculture and sightseeing and are important financial resources for local communities (JFRCA 2004a, b).

The threespine sticklebacks have been found in three crater lakes in Japan (Fig. 2A; Table 1). The most plausible route of colonization into crater lakes is human release. Although no fish were found in Lake Towada during the 19th century (Hikita and Taniguchi 1959; Tokui 1959), landlocked forms of *Oncorhynchus nerka* (kokanee) have been stocked in the lake since the early 1900s. Several other exotic fishes, such as carp (*Cyprinus carpio*), smelt (*Hypomesus transpacificus nipponensis*), masu salmon (*Oncorhynchus masou*), char (*Salvenilus leucomaenis*), threespine stickleback, nine-spined stickleback (*Pungitius sinensis*), and loach (*Misgurnus anguillicaudatus*) can be also found in Lake Towada today (JFRCA 2004b). Although the lake has an outflow into the Pacific Ocean (via the Oirase River), this river has so many rapid rifles and falls that anadromous fishes are unlikely to have naturally colonized the lake (JFRCA 2004b). Threespine sticklebacks were first

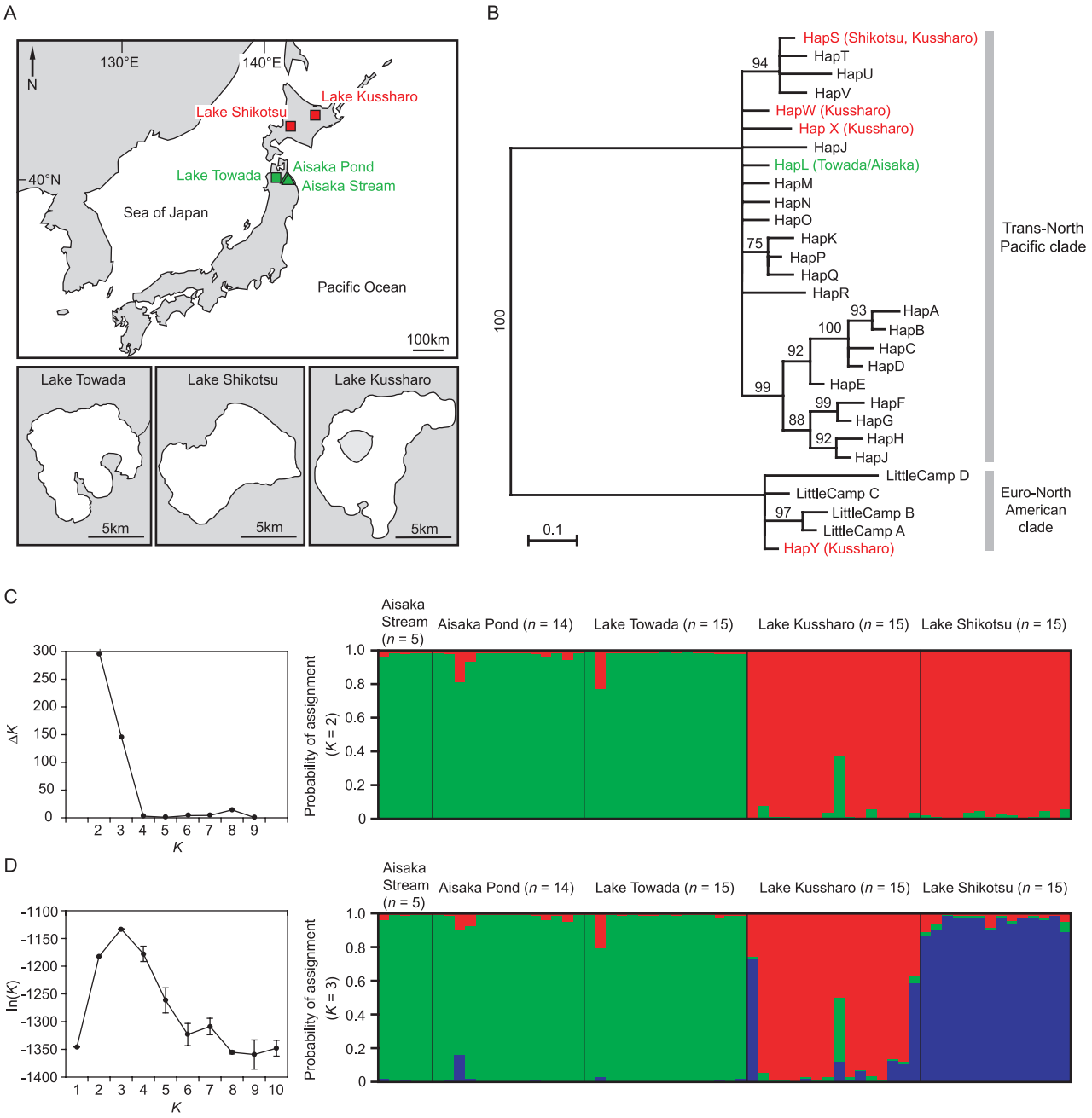

Figure 2. (A) Map showing the collection sites. Squares indicate the locations of the crater lakes (Lake Towada, Lake Kussharo, and Lake Shikotsu) where threespine sticklebacks were found. The triangles indicate Aisaka Pond and Aisaka Stream, where potential ancestral populations of the Lake Towada population were collected. Different colors indicate the different genetic clusters in Fig. 4C. (B) Bayesian phylogenetic tree based on the 3′-terminal region of the mitochondrial cytochrome *b* gene. Haplotype names (HapA–HapV) are based on Watanabe et al. (2003). New haplotypes were named HapW-Y and LittleCamp A-D. The probability of each clade is shown on the branch. The scale indicates the expected substitution rate per site. (C) STRUCTURE analysis of crater lake sticklebacks with $K = 2$. The left panel indicates the ad hoc statistic ΔK, which has a peak at $K = 2$. In the right panel, each individual is indicated by a single vertical line. The probability of assignment to each genetic cluster is indicated by the length of the colored bar. (D) STRUCTURE analysis of crater lake sticklebacks with $K = 3$. The left panel indicates the mean ± SD of Ln(K). Ln(K) has a peak at $K = 3$. The right panel is shown as in Fig. 2C.

Table 1. Characteristics of three crater lakes.

	Altitude (m)	Surface area (km^2)	Average depth (m)	Maximum depth (m)
Lake Towada	400	59.8	80.0	326.8
Lake Kussharo	121	79.3	28.4	117.0
Lake Shikotsu	247	78.4	265.4	363.0

captured in 1979 and have been constantly caught since 1985 (JFRCA 2004b). For two other crater lakes, Lake Shikotsu and Lake Kussharo, detailed historical records are unavailable, but salmonids have often been released into these lakes for aquaculture (Tokui 1964; Ban and Suzuki 2003). In addition, these two lakes do not have direct connections to the ocean, and no threespine sticklebacks were caught in streams or lakes near these two lakes. Therefore, anthropogenic introduction is a probable route of colonization.

In the present study, we first investigated the genetic structures of crater lake sticklebacks to elucidate the invasion routes and identify the potential ancestral populations. Second, the morphological traits of the three crater lake populations were compared to characterize morphological variation. Third, by compiling historical data of the Lake Towada stickleback, we investigated 25 years of phenotypic and trophic changes in that population.

Materials and Methods

Genotyping

Fish for mitochondrial DNA analysis were collected from Lake Towada (40.445N, 140.841E; western side of the lake near Mount Namari) in October 2008 ($n = 12$) and June 2010 ($n = 36$), Lake Kussharo (43.602N, 144.350E; eastern side of the lake) in May 2003 ($n = 30$), Lake Shikostu (42.775N, 141.400E; northeastern side of the lake) in October 2010 ($n = 16$), Aisaka Stream (a tributary of the Oirase River) in the city of Aisaka (40.594N, 141.224E) in March 2002 ($n = 5$), Aisaka Pond (a pond connected to the Aisaka Stream) (40.592N, 141.221E) in October 2010 ($n = 19$), and the Harutori River (marine ecotype; 42.968N, 144.396E) in June 1999 ($n = 2$). For an outgroup, we used fish collected from the Little Campbell Estuary (49.015N, 122.779W) in British Columbia, Canada, in June 2004 ($n = 4$) (Kitano et al. 2010). Fish were collected using minnow traps or gill nets and stored in ethanol until use. Genomic DNA was isolated from caudal or pectoral fins by phenol/chloroform extraction (Kitano et al. 2008, 2009, 2010) or using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). The 3′-half of mitochondrial cytochrome *b* gene was analyzed because this region is known to exhibit great sequence variation among Japanese stickleback haplotypes (Watanabe et al. 2003). This region was amplified by polymerase chain reaction (PCR) using KAPA HiFi DNA polymerase (KAPA Biosystems, Boston, MA, USA). A forward primer (5′-CCTACCAGGACTTTAACCAGGACTA-3′) and a reverse primer (5′-CCGGCGCTCTGGCGCTGAGCTACTTT-3′) were designed based on the sequences in the Ensembl database (http://asia.ensembl.org/Gasterosteus_aculeatus/Info/Index) to cover the regions reported in Watanabe et al. (2003). Reactions were run on a Verti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following thermal cycling conditions: after 95°C for 5 min, 35 cycles at 98°C for 20 sec and 65°C for 15 sec and 72°C 30 sec, followed by 72°C 5 min. All PCR products were separated on 2% agarose gels. The PCR products were extracted from the gel fragments using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in the Sequencing Department of Operon Biotechnologies (Tokyo, Japan) using the forward primer used for PCR and another sequencing primer (5′-TTGACAACGCCACCTTAACA-3′). We determined 1081-bp sequences of cytochrome *b*.

Sequences were first aligned with previously reported haplotypes of the Japanese threespine stickleback populations (Watanabe et al. 2003) using ClustalX 2.0.3 (Larkin et al. 2007). Watanabe et al. (2003) analyzed 18 populations, including the Lake Towada population collected in June 1996, and all fish form Lake Towada ($n = 4$) had Haplotype L (see HapL in Fig. 2B). No insertion or deletion polymorphisms were found. Three new haplotypes (HapW, HapX, and HapY) were found in the Lake Kussharo population, and four new haplotypes (LittleCamp A–D) were found in the Little Campbell River marine population. Sequences of novel haplotypes are available from the DNA Data Bank of Japan (DDBJ accession numbers AB678412–AB678418). A haplotype phylogenetic tree was constructed using MrBayes 3.1.2. (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). The Markov chain Monte Carlo search was run with two chains for 800,000 generations, sampling the Markov chain every 10 generations with the first 25% generations discarded, as described previously (Kitano et al. 2010).

For microsatellite analysis, we used nine microsatellite markers that are located on nine different linkage groups (Table 2). Fish collected from Lake Towada in June 2010 ($n = 15$), Lake Kussahro in May 2003 ($n = 15$), Lake Shikostu in October 2010 ($n = 15$), Aisaka Stream in March 2002 ($n = 5$), and Aisaka Pond in October 2010 ($n = 14$) were used. The forward primers were labeled with either HEX, NED or FAM. The 5′ end of the reverse primers were tailed with GTTTCTT to increase the accuracy of fragment analysis (Ballard et al. 2002). Three differently colored primer sets were combined and the genomic DNA was amplified using the KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems). The amplified fragments were analyzed in the genotyping

Table 2. Allelic richness/gene diversity of each microsatellite locus.

Marker	LG	Lake Towada ($n = 15$)	Lake Kussharo ($n = 15$)	Lake Shikotsu ($n = 14$)	Aisaka Pond ($n = 12$)	Aisaka Stream ($n = 5$)
Stn384	1	1.95/0.41	2.85/0.65	2.75/0.62	2.00/0.52	2.00/0.45
Stn46	4	1.99/0.48	4.53/0.82	3.52/0.73	3.49/0.73	3.76/0.73
Stn332	5	1.47/0.13	3.44/0.73	3.41/0.73	1.50/0.14	2.00/0.25
Stn64	6	2.72/0.62	4.09/0.79	2.89/0.66	3.21/0.70	2.98/0.68
Stn76	7	1.00/0	2.33/0.55	1.00/0	1.00/0	1.00/0
Stn90	8	3.66/0.76	3.01/0.54	2.18/0.38	3.35/0.33	3.00/0.70
Stn278	11	1.87/0.33	3.84/0.77	2.67/0.61	1.87/0.33	2.00/0.55
Stn170	15	3.06/0.65	5.40/0.87	3.86/0.75	3.34/0.67	2.00/0.42
Stn233	16	1.87/0.33	3.06/0.56	1.84/0.3	1.90/0.36	2.00/0.58
Average (S.E.)		2.18 (0.29)/0.41 (0.09)	3.62 (0.34)/0.70 (0.05)	2.68 (0.32)/0.53 (0.09)	2.41 (0.33)/0.46 (0.09)	2.30 (0.29)/0.48 (0.08)

LG, linkage group.

center of BEX Co. Ltd (Tokyo, Japan). None of the marker pairs exhibited significant linkage disequilibrium in any populations after sequential Bonferroni correction when tested with Genepop software (Raymond and Rousset 1995). None of the markers significantly deviated from the Hardy–Weinberg equilibrium after sequential Bonferroni correction when tested with Genepop software (Raymond and Rousset 1995).

These data were analyzed using the STRUCTURE, which uses Markov chain Monte Carlo simulations to identify groupings that minimize Hardy–Weinberg and linkage disequilibrium within cluster groups (Pritchard et al. 2000). Three simulations were run for each cluster number (K) from $K = 1$ through $K = 10$. We estimated the number of clusters by finding the K value with the highest log likelihood Ln(K) as well as the K value with the highest ad hoc statistic ΔK, which is based on the rate of change in Ln(K) between successive K values (Evanno et al. 2005). Parameters were estimated after 200,000 iterations, following a burn-in of 25,000 iterations. Allelic richness (number of alleles per locus corrected for sample size) and gene diversity were calculated using the FSTAT 2.9.3 software (Goudet 1995).

Morphological analysis

For morphological comparison among populations, only females were used because significant sexual dimorphism exists in most morphological traits (Kitano et al. 2007a) and because more female specimens were available than male specimens. Although all historical specimens were not preserved in the same fixative, the external morphological traits analyzed in this study are the skeletal structures that are less likely to change substantially during fixation. Specimens collected from Lake Towada in June 2010 ($n = 38$), Lake Kussharo in May 2003 ($n = 15$), Lake Shikostu in October 2010 ($n = 20$), and Aisaka Pond in October 2010 ($n = 14$) were preserved in ethanol. Specimens collected from Aisaka Stream in June 1993 ($n = 19$) and the Harutori River in 1984 ($n = 19$) were preserved in formalin.

The following 11 external morphological traits were measured using vernier calipers as described previously (Kitano et al. 2007a, b): standard length, head length, body depth, upper jaw length, snout length, gape width, eye diameter, first dorsal spine length, second dorsal spine length, left pelvic spine length, and pelvic girdle length. In this study, plate morph was not analyzed because all of the sticklebacks analyzed here were completely plated. All traits were natural log-transformed and subjected to principal component (PC) analysis. The F-test was used for comparing variances between populations (Whitock and Schluter 2009). Because the slope of regression of PC1 against standard length differed among groups (data not shown), PC1 may include some shape traits, so our conclusion of PC analysis is tentative. In order to confirm divergence in a foraging trait, a gape width, we conducted analysis of covariance (ANCOVA) of log-transformed gape width with log-transformed standard length as a covariate. First, interaction between population and log-standard length was tested. When the interaction was not significant, we excluded the interaction term and tested the effect of population on log-transformed gape width with log-transformed standard length as a covariate. When the interaction was significant (i.e., slope heterogeneity was found), we did not conduct further statistical tests.

Changes in body size, trophic ecology, and catch rate

Long-term data on the standard length, foraging rate (weight of stomach content divided by body weight) and percentages of each food item in the stomach contents of the Lake Towada threespine sticklebacks were obtained from the Annual Reports of the Akita Prefectural Department of Fisheries (1986–2008). These fish were sampled from the southern end of Lake Towada (40.415N, 140.868E) with stationary seine nets. Data obtained from a sample size of minimum three

fish were used (Table S1). Because fish were sampled at different times of the year, we used data obtained only during the breeding season of the sticklebacks (May–July). In order to confirm our findings based on the fish collected from the southern side of the lake (see above), we also analyzed matured adult fish collected from the western side of the lake during the breeding season (Table S2) and examined the changes in standard length.

For stomach content analysis of ancestral populations, fish collected from Aisaka Stream (June 1993, $n = 8$) and Aisaka Pond (July 2010, $n = 10$) were analyzed as described in the Annual Reports of the Akita Prefectural Department of Fisheries. In brief, the stomach of each fish was dissected. After whole stomach was weighed, the stomach contents were removed and the stomach was weighed again. By calculating the difference in the weight of the stomach before and after the removal of the stomach contents, we could measure the weight of the stomach contents. The stomach contents were then classified into Cladocera, Copepoda, Amphipoda, Chironomidae, snails, fish eggs, and others under dissecting microscopes. The percentage of the volume of each food item was calculated and multiplied by the stomach content weight (Table S2).

Data on the amounts of plankton collected by plankton nets were obtained from the Annual Report of the Akita Prefectural Department of Fisheries. Plankton were collected from 10 different sites in the lake at the depth of 16 m to the water surface using vertical tows with a conical plankton net (diameter 30 cm; mesh size 0.1 mm) in June. The catch rate data (catch per unit effort; CPUE) of stickleback at the southern end of the lake in May–July were kindly provided by the Aomori Prefectural Industrial Technology Research Center. All of the compiled data would be available in Table S1.

To visualize the temporal change, non-parametric cubic spline curve fitting was used (Schluter 1988) with the R statistical packages (R Development Core Team 2010). Smoothing was conducted by generalized cross-validation. Body length and body weight were natural log-transformed and analyzed using a linear regression model (Whitock and Schluter 2009). The stomach content and CPUE data were analyzed with a generalized linear model based on the mixed Poisson-gamma (Tweedie) distributions with logit as a link function, because the count data may follow Poisson distributions, while the amounts of each item may follow gamma distributions (Willmot 1986; Shono 2008; Hansan and Dunn 2010). When plankton data were analyzed, the collection site was also included in the model, although it was not a significant predictor of the plankton data.

Analysis of parasite infection

The presence or absence of a tapeworm *Schistocephalus* in the stickleback body cavity was observed in the fish collected from Lake Towada in June 2008 ($n = 22$) and June 2010 ($n = 90$), Aisaka Stream in June 1993 ($n = 19$) and March 2002 ($n = 5$), and Aisaka Pond in October 2010 ($n = 19$). Data on the parasite infection in the Lake Towada population in 1985 and 1995 were obtained from published literature (Mori 1999). For the fish collected in June 2010, the effects of parasite infection on the natural log-transformed standard length were analyzed by analysis of variance (ANOVA).

To identify the *Schistocephalus* species, DNA was isolated from *Schistocephalus* isolated from the body cavity of the sticklebacks collected from Lake Towada in June 2011 ($n = 5$). We amplified the internal transcribed spacer 2 (ITS2) of the ribosomal RNA genes with a forward primer (5′-GTTACCACCTGGCGGAATAA-3′) and a reverse primer (5′-TCAGTAGCTGCCACCACAAC-3′), which were designed based on the reported *Schistocephalus solidus* ITS2 sequence (AY549509.1) (Logan et al. 2004). The amplified fragments were sequenced with PCR primers, and all sequences of the five parasites were identical (GeneBank Accession number AB685170). The ITS2 sequence of the Lake Towada tapeworm was aligned with ITS2 sequences of diphyllobothriid tapeworms reported in Logan et al. (2004). A phylogenetic tree was constructed with MrBayes 3.1.2. (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). The Markov chain Monte Carlo search was run with two chains for 1,500,000 generations, sampling the Markov chain every 10 generations with the first 25% generations discarded, as described previously (Kitano et al. 2010).

Results

Genetic analysis of crater lake sticklebacks

Mitochondrial DNA analysis revealed that the three crater lake populations have different origins (Fig. 2B). In the Lake Towada population, only one haplotype of cytochrome *b* (HapL in Fig. 2B) was found ($n = 4$ for 1996, $n = 12$ for 2008, and $n = 36$ for 2010). This haplotype was also found in sticklebacks collected from Aisaka Pond ($n = 19$) at a trout farm and the adjoining Aisaka Stream ($n = 5$), which is approximately 25 km east of Lake Towada (Fig. 2A). This haplotype has never been observed in any other populations that have been examined (Watanabe et al. 2003). Because kokanee were often transplanted into Lake Towada from the trout farm (Hikita and Taniguchi 1959), contamination of salmonids with sticklebacks was a possible route for the introduction of sticklebacks into Lake Towada. Aisaka Pond is a small pond (approximately 500 m^2 with a depth of 0.3–0.4 m) fed by cold spring water. Aisaka Stream is an adjoining shallow stream less than 1-m wide and located between Aisaka Pond and agricultural fields. Thus, the habitats of the potential ancestral populations differ greatly from those of crater lakes.

All Lake Shikotsu sticklebacks ($n = 16$) had a common haplotype (HapS in Fig. 2B), which was the same as that widely found in various marine and coastal populations in Japan (Watanabe et al. 2003), suggesting that sticklebacks in Lake Shikotsu probably originated from some other Japanese coastal populations. In contrast to Lake Towada and Lake Shikotsu populations, four distinct haplotypes were found in the Lake Kussharo population. The most common haplotype was HapS, which was found in 24 of 30 fish. This is not surprising because kokanee had been transplanted from Lake Shikotsu to Lake Kussharo, although we cannot exclude the possibility that some coastal populations were directly introduced into Lake Kussharo. In addition, three unique haplotypes (HapW, HapX, and HapY) were found in the Lake Kussharo population. Two haplotypes (HapW and HapX) were similar to those found in other Japanese populations, while one haplotype (HapY) found in one fish among 30 was considerably different from other Japanese haplotypes (Fig. 2B). This different haplotype was rather similar to the North American haplotypes (LittleCamp A–D in Fig. 2B). Since salmonids were transplanted from North America to several lakes and rivers in Hokkaido (Tokui 1964), juvenile salmon or trout might have been contaminated with sticklebacks, which were accidentally brought into Japan across continents. Thus, the three crater lake populations have different origins.

The results of genetic analysis with nuclear microsatellite markers were consistent with the mitochondrial data. Evanno's ΔK analysis indicated that two main genetic clusters ($K = 2$) exist among the crater lake populations (Fig. 2C), whereas the peak of Ln(K) indicated that $K = 3$ may be optimal (Fig. 2D). In any case, the probability plot of assignment to distinct genetic clusters revealed that the Lake Towada population was genetically similar to the Aisaka Pond and Aisaka Stream populations (Fig. 2C and 2D).

Although the Lake Shikotsu and Lake Kussharo populations are genetically similar, they could be distinguished when $K = 3$ (Fig. 2D). The gene diversity and allelic richness of the Lake Kussharo population was the highest among the analyzed populations (Table 2); this finding is consistent with the mitochondrial data indicating that the Lake Kussharo population may have originated from multiple genetic sources.

Morphological comparison among crater lake sticklebacks

Greater genetic variances can increase phenotypic variances. As expected, variance of body length was larger in the Lake Kussharo population (mean ± SD of female standard length = 62.9 ± 19.7 mm, $n = 16$) than in the Lake Towada population (mean ± SD of female standard length = 58.7 ± 12.7 mm in 2010, $n = 39$, F-test, $F_{1,51} = 39.1$, $P < 0.001$) or the Lake Shikotsu population (mean ± SD of female standard length = 51.2 ± 6.6 mm, $n = 20$, F-test, $F_{1,33} = 48.4$, $P < 0.001$). To further investigate the difference in phenotypic variances, we compared variances in body shape among the three crater lake stickleback populations. Two potential ancestral populations (Aisaka Stream and Aisaka Pond) and one Japanese marine population (Harutori River) were also included in this analysis. By using PC analysis (Table 3), we first excluded the body size effect (PC1) and compared PC2 and PC3 among these populations. Variances of PC2 and PC3 of the Lake Kussharo population are significantly larger than those of the Lake Towada population (F-test, $F_{1,50} = 10.3$, $P = 0.002$ for PC2; $F_{1,50} = 13.2$, $P = 0.001$ for PC3) and slightly larger than those of the Lake Shikotsu population (F-test, $F_{1,33} = 3.1$, $P = 0.088$ for PC2; $F_{1,33} = 2.2$, $P = 0.151$ for PC3).

Table 3. Principal component (PC) analysis of morphological traits.

	PC1	PC2	PC3
Component loadings			
Standard length	0.987	−0.003	−0.036
Head length	0.988	0.057	−0.091
Body depth	0.971	−0.018	−0.136
Jaw length	0.944	−0.018	−0.213
Snout length	0.968	−0.104	−0.135
Eye diameter	0.884	0.373	−0.069
Gape width	0.763	0.570	0.271
First dorsal spine length	0.909	−0.238	0.269
Second dorsal spine length	0.922	−0.283	0.160
Left pelvic spine length	0.938	−0.229	0.147
Pelvic girdle length	0.974	0.010	−0.089
% Variance explained	87.2	6.1	2.7

PCs explaining more than 2% of phenotypic variances are shown.

A scatter plot of PC2 and PC3 also revealed that contemporary Lake Towada and Lake Shikotsu populations are relatively similar in PC2 (Fig. 3A) ($F_{1,56} = 0.06$, $P = 0.804$), although they differ in PC3 ($F_{1,56} = 4.7$, $P = 0.035$). The Lake Towada population significantly diverges from the ancestral populations in PC2 (for Aisaka Pond, $F_{1,50} = 120.3$, $P < 0.001$; for Aisaka Stream, $F_{1,55} = 349.4$, $P < 0.001$), but not in PC3 (for Aisaka Pond, $F_{1,50} = 1.33$, $P = 0.254$; for Aisaka Stream, $F_{1,55} = 3.89$, $P = 0.054$), suggesting that the Lake Towada stickleback has a smaller gape width and a smaller eye diameter than the ancestral populations.

To confirm the divergence in gape width between the ancestral and the derived populations, we analyzed the gape width with standard length as a covariate (Fig. 3B). This analysis demonstrated that the Lake Towada population has a significantly smaller gape width than the Aisaka Pond population (ANCOVA, $F_{1,49} = 141.8$, $P < 0.001$). Because the slope of the regression line differed between the Lake Towada and the Aisaka Stream populations (ANCOVA, $F_{1,53} = 5.72$, $P = 0.020$), we did not test the difference in gape width

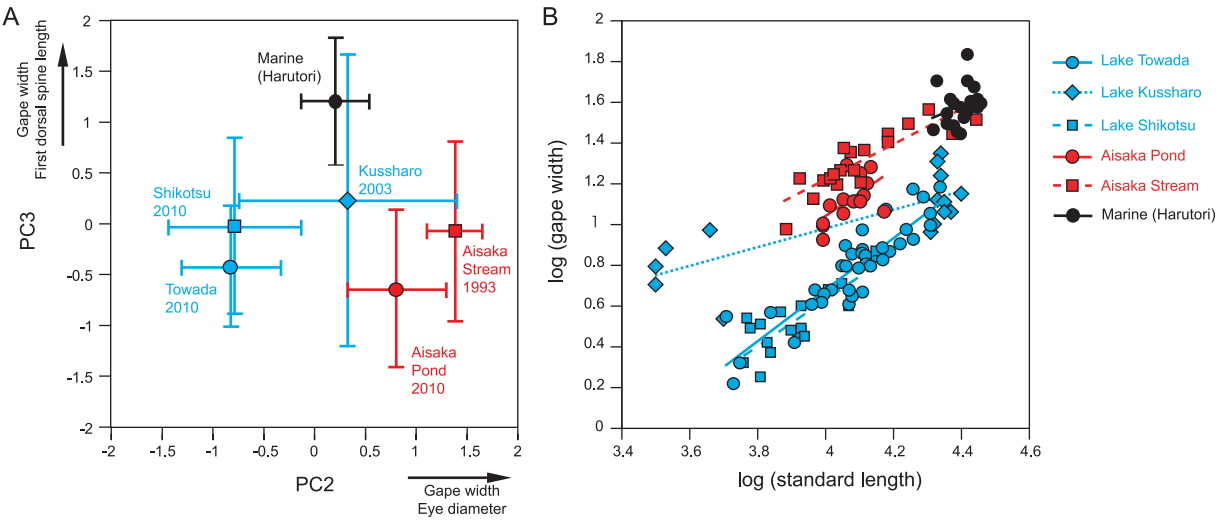

Figure 3. (A) A scatter plot of PC2 and PC3 of morphological traits. Means ± SD are shown. A Japanese marine population was collected from Harutori River. (B) Correlation between log-transformed standard length and log-transformed gape width.

between them, but the gape width seems larger in the Aisaka Stream population than in the Lake Towada population (Fig. 3B). The Lake Shikostu population and the Lake Towada population did not differ in gape width (ANCOVA, $F_{1,55} = 2.6$, $P = 0.115$). Interestingly, the allometric relationship between the standard length and gape width in the Lake Kussharo population significantly differed from that of the Lake Towada population (ANCOVA, $F_{1,49} = 32.2$, $P < 0.001$) and that of the Lake Shikotsu population (ANCOVA, $F_{1,31} = 9.2$, $P = 0.005$).

Shifts in body size and trophic ecology in a crater lake population

Because the Lake Towada population diverged from the ancestral populations (see above), we investigated the phenotypic and ecological shifts in the Lake Towada population over the last 25 years. Compilation of body length data from the fish collected from the southern side of the lake revealed that body size has changed considerably. In the 1980s, mean standard length was larger than 90 mm (Fig 4A), which is much larger than that of the ancestral populations (Aisaka Stream in 1993, mean ± SD = 61.2 ± 9.7 mm, $n = 18$; Aisaka Pond in 2010, mean ± SD = 58.5 ± 3.4 mm, $n = 14$). However, the body length decreased over the last 25 years (Fig. 4A). Similar results were obtained for the adult fish collected from the western side of the lake (Table S2).

We next investigated whether the population density of sticklebacks has changed, because body size might become smaller at a higher population density (Kitano et al. 2007a). Contrary to expectations, the relative catch rate (CPUE) has been declining (Fig. 4B) (Tweedie generalized linear model, $N = 50$, $b = -0.149 \pm 0.03$, Wald $\chi^2 = 31.3$, $P < 0.01$). Although CPUE is not a direct measure of the population size, these data do not suggest that body size reduction is caused by the increased population size.

To investigate the potential factors that drive body size shifts, we next investigated shifts in trophic ecology. The foraging rate (percentage of stomach content weight divided by body weight) of sticklebacks has significantly declined during the last 25 years (Fig. 4C; Tweedie generalized linear model, $N = 20$, $b = -0.023 \pm 0.11$, Wald $\chi^2 = 4.03$, $P = 0.045$). The percentages of fish with empty stomach also tend to increase (Fig. S1). In particular, a decrease was observed in the proportion of benthos in the stomach contents (Fig. 5), including both Chironomidae (Tweedie generalized linear model; $N = 20$, $b = -0.083 \pm 0.028$, Wald $\chi^2 = 8.89$, $P = 0.003$) and Amphipoda (Tweedie generalized linear model, $N = 20$, $b = -0.908 \pm 0.321$, Wald $\chi^2 = 8.02$, $P = 0.005$). Instead, the proportions of Cladocera significantly increased (Tweedie generalized linear model; $N = 20$, $b = 0.116 \pm 0.049$, Wald $\chi^2 = 5.56$, $P = 0.018$), while that of Copepoda did not change significantly (Tweedie generalized linear model; $N = 20$, Wald $\chi^2 = 0.024$, $P = 0.887$). The Aisaka Stream and Aisaka Pond populations almost exclusively preyed upon benthos (Table S3). Thus, the Lake Towada sticklebacks initially preyed upon benthos, but recently shifted to planktivory.

To examine whether this trophic shift was related to the overall changes in the abundance of plankton in the lake, we analyzed changes in the amount of Cladocera collected by plankton nets. Cladocera significantly increased in number during the last 25 years (Fig. 6; Tweedie generalized linear model, $N = 220$, $b_{year} = 0.082 \pm 0.011$, Wald $\chi^2 = 58.4$, $P < 0.001$). This data suggests that overall changes in the lake

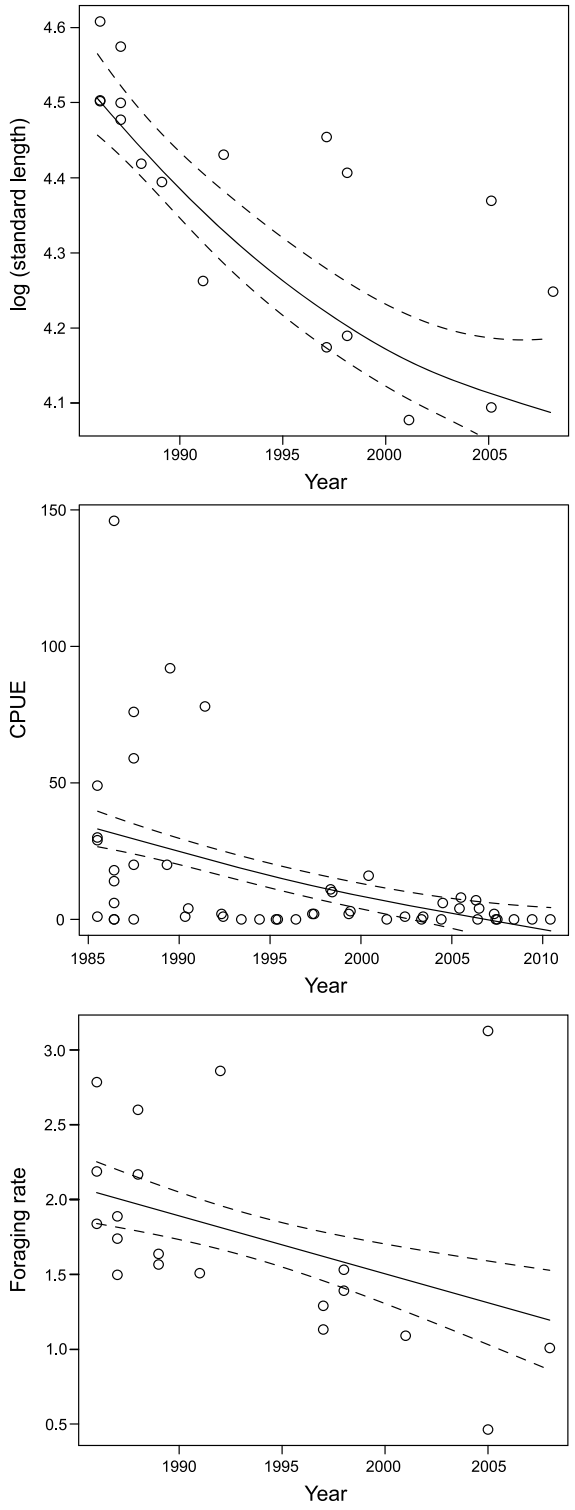

Figure 4. Temporal change in the natural log-transformed standard length, catch per unit effort (CPUE), and foraging rate (percentage of stomach content weight divided by body weight) of the Lake Towada stickleback population. Estimated values (±SE) were determined by nonparametric cubic spline method.

ecosystem might have partially contributed to some of the trophic changes in the sticklebacks.

Parasite outbreak

Parasite infection was another potential factor that drove reduction in body size and trophic shifts. Infection with a tapeworm, *Schistocephalus solidus*, can reduce the growth rate of sticklebacks (Barber and Scharsack 2009), and parasitic infection may favor early maturation, thereby driving the evolution of smaller body size at its maturity (Maccoll 2009). Furthermore, sticklebacks infected with *Schistocephalus solidus* showed reduced foraging efficiency, particularly for chironomids (Cunningham et al. 1994) or large-sized prey (Millinski 1984).

Neither the Asiaka Stream (0/19) nor the Aisaka Pond stickleback populations (0/24) were infected with any tapeworms. In 1985, only one stickleback among 124 fish (0.8%) of Lake Towada was infected with a tapeworm (Mori 1999). However, sticklebacks of Lake Towada have been heavily infected with a tapeworm since the 1990s: 59% of fish (39/66) in 1995 (Mori 1999), 63.7% of fish (14/22) in 2008, and 32.2% of fish (29/90) in 2010 were infected with a tapeworm. All of the ITS2 sequences of the tapeworms collected from five different individuals of the Lake Towada stickleback were identical. The ITS2 phylogenetic tree revealed that the tapeworm in the Lake Towada stickleback is *Schistocephalus solidus* (Fig. 7A). Thus, the outbreak of *Schistocephalus solidus* occurred in Lake Towada between the late 1980s and 1990s.

Significant effects of *Schistocephalus solidus* infection on the standard length of breeding fish collected in June 2010 were found in females (ANOVA, $F_{1,61} = 4.6$, $P = 0.035$), but not in males (ANOVA, $F_{1,25} = 0.1$, $P = 0.779$). Parasitized females (mean ± SD = 56.7 ± 9.9 mm) were smaller than nonparasitized females (mean ± SD = 61.8 ± 7.5 mm), while parasitized males had approximately the same standard length (mean ± SD = 41.5 ± 7.6 mm) as nonparasitized males (mean ± SD = 40.9 ± 9.0 mm). The data in females is consistent with the idea that infection with *Schistocephalus solidus* can reduce the growth rate (Barber and Scharsack 2009). Interestingly, however, even nonparasitized females were much smaller than the fish in the late 1980s (Fig. 4A). These results suggest that the direct negative effect of *Schistocephalus solidus* on growth is not the only factor responsible for reduction in body size.

Discussion

Multiple events of introduction

Multiple events of introduction are responsible for the presence of the threespine sticklebacks in crater lakes in Japan. Although threespine sticklebacks have been intentionally introduced into Canadian lakes as forage prey for salmonids

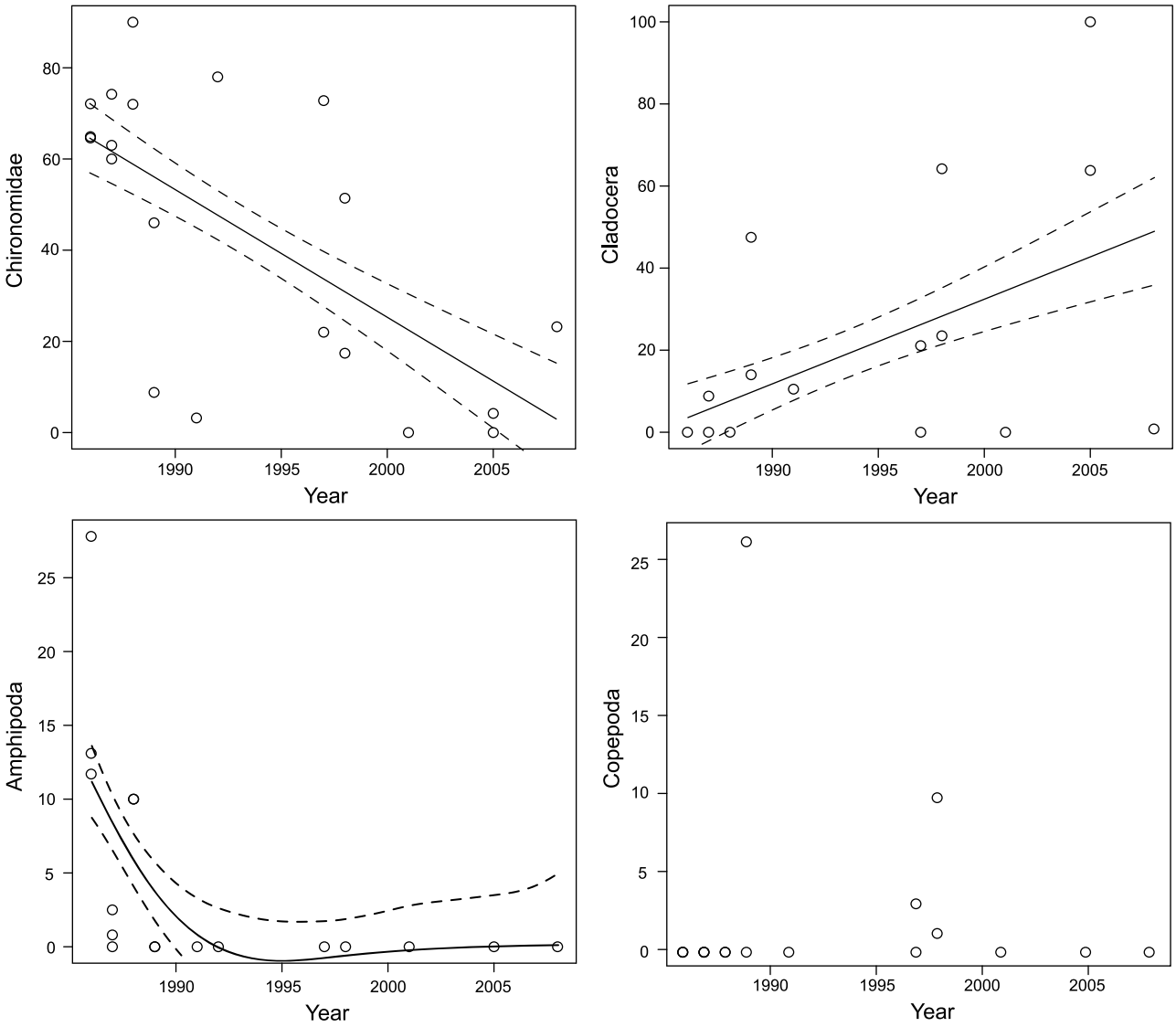

Figure 5. Temporal change in the percentages of Chironomidae, Amphipoda, Cladocera, and Copepoda found in the stomach contents of the Lake Towada stickleback population. Estimated values (±SE) were determined by non-parametric cubic spline method. Copepoda was found in the stomach content only in four years, so curve fitting was not conducted.

(van Zyll de Jong et al. 2004), there is no record of intentional introduction of threespine sticklebacks into these three Japanese crater lakes. Therefore, contamination of salmonids with sticklebacks is the most likely route of introduction. Although salmonids had been frequently introduced into Lake Towada since early 1900s, sticklebacks were first caught in 1979. Such time lags often exist between initial introduction and population growth in many cases of invasive species, although the duration of lag times varies between cases (Crooks 2005).

In Lake Kussharo, even a haplotype belonging to the Euro-North American Clade was found. Mitochondrial haplotypes of global threespine stickleback populations were classified into the Euro-North American Clade and the Trans-North Pacific Clade (Ortí et al. 1994; Johnson and Taylor 2004). Because none of the natural populations of Japanese threespine stickleback examined have haplotypes belonging to the Euro-North American Clade (Watanabe et al. 2003), introduction of sticklebacks from foreign countries is a likely route of introduction. From the 1950s to 1990s, sockeye salmon and rainbow trout were frequently transplanted from the North America into several lakes and rivers in Hokkaido (Tokui 1964; Hosoya 1993; Yamamoto et al. 2011). Along with rainbow trout from the North America, *Daphnia* was also

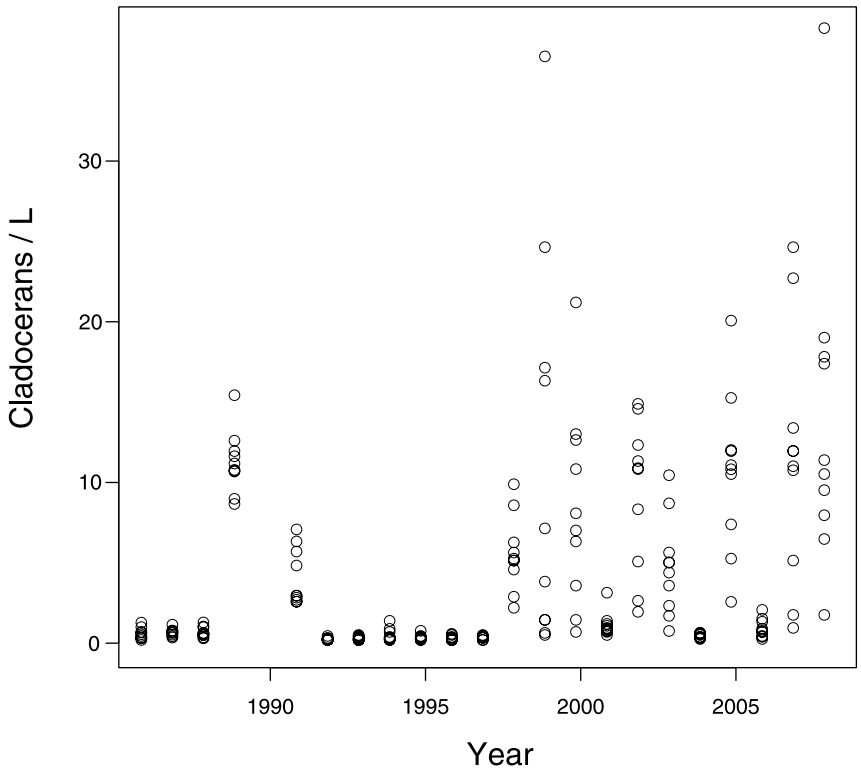

Figure 6. Temporal change in the number of Cladocerans caught by plankton nets in Lake Towada.

introduced to a Japanese lake on Honshu Island (Ishida and Taylor 2007). Therefore, transplantation of fishes for aquaculture should be conducted with extreme caution, because this process can result in the transplantation of fishes that were not originally intended to be moved. Several endangered unique stickleback populations that are genetically and phenotypically divergent from the North American and European populations are found in Japan (Mori 1990; Watanabe et al. 2003; Kitano et al. 2007b; Kitano et al. 2009). To conserve these invaluable biological resources, efforts should be made to prevent further dispersal of exotic sticklebacks.

Multiple introduction events are generally thought to promote successful colonization of exotic species by increasing their genetic and phenotypic variances (Kolbe et al. 2004; Facon et al. 2005; Lockwood et al. 2007), and this might be the case with sticklebacks (Lucek et al. 2010). Our samples were collected in different years and the sample sizes were relatively small for some of the specimens due to logistical constraints. Therefore, we cannot exclude the possibility that some of the genetic variances might be due to the differences in the sampling year or any sampling bias. However, all of the mitochondrial data, microsatellite data, and morphological data demonstrated that the Lake Kussharo stickleback has larger genetic and phenotypic variances than other crater lake populations. Although we do not know how much of the phenotypic variance in the Lake Kussharo population is heritable, previous studies on other stickleback populations have demonstrated that most of the morphological traits in sticklebacks have substantial genetic basis (McPhail 1994; Peichel et al. 2001; Albert et al. 2008; Sharpe et al. 2008; Leinonen et al. 2011).

Morphological and ecological shifts in a crater lake population

Substantial shifts in body size were observed in the Lake Towada population over the last 25 years. Mean standard length was above 90 mm in 1985, which is larger than any reported mean lengths of other Japanese populations (Ikeda 1933; Mori 1987, 1990; Kitano et al. 2007a, b): the largest mean standard lengths of Japanese threespine sticklebacks were reported in the Pacific Ocean marine threespine sticklebacks, which are 80.7–82.9 mm in females and 74.4–75.9 mm in males (Kitano et al. 2007a, b). Island theory predicts that release from enemy and competitor would increase the body size of small organisms following colonization of islands (Lomolino et al. 2010). Sticklebacks are relatively small organisms and become larger in environments with reduced risk of predation and interspecific competition (Herczeg et al. 2009). Thus, the observed increase in stickleback body size may be consistent with the island theory.

However, the sizes of sticklebacks in Lake Towada became smaller in the 1990s. Multiple factors may have been responsible for this phenotypic change. First, *S. solidus* infection may directly reduce the growth rate of sticklebacks. The

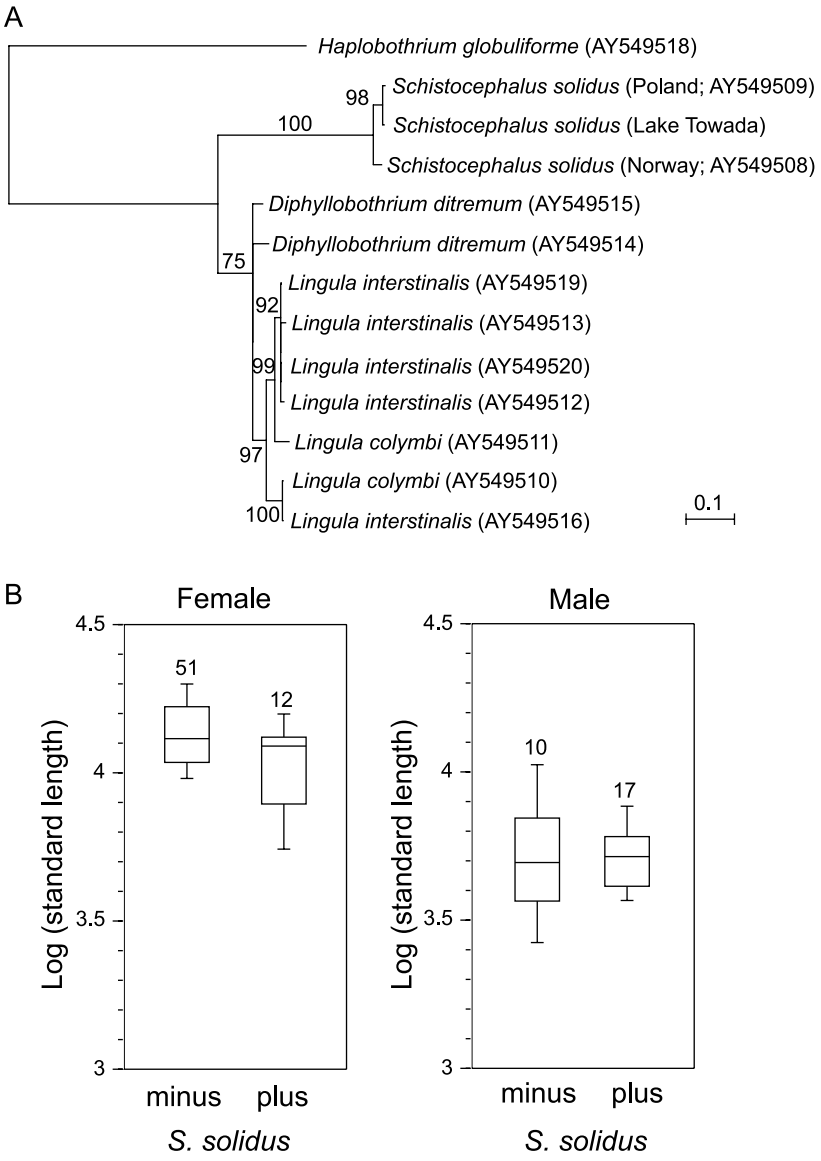

Figure 7. (A) Bayesian phylogenetic tree of the ITS2 sequences. The probability of each clade is shown on the branch. The scale indicates the expected substitution rate per site. (B) Effects of infection with *Schistocephalus solidus* on the body length of adult threespine sticklebacks collected in June 2010. Box plots of natural log-transformed standard length of nonparasitized females ($n = 51$), parasitized females ($n = 12$), nonparasitized males ($n = 10$), and parasitized males ($n = 17$) are shown.

significant effect of parasitic infection on the female stickleback body size was consistent with this idea. However, the effect of parasite was not significant in males. In addition, even nonparasitized females were smaller than the fish in the late 1980s. Thus, additional factors, such as adaption to parasite and/or planktivory, may be responsible for the body size reduction. Parasitic infection may favor early maturation, and it may have driven the evolution of smaller body size at maturity (Maccoll 2009). In addition, smaller body size is generally favored in planktivorous sticklebacks (McPhail 1994). In order to further investigate the proximate causes of the body size shifts, aging of fishes using otoliths or spine rings is required (Reimchen 1992; Taylor et al. 2011).

Smaller gape width in the Lake Towada population might be also an outcome of adaptations to the trophic shifts from benthivory to planktivory, because planktivorous sticklebacks usually have a smaller gape (McPhail 1994), although smaller eye size is a characteristic of benthic sticklebacks (McPhail 1994). Currently, we do not know whether these morphological changes are plastic responses or evolutionary changes. However, previous studies revealed substantial heritable components in body size variation in sticklebacks (McPhail 1977; Snyder and Dingle 1989; Colosimo et al. 2004; Kitano et al. 2009). Because the genetic and genomic tools are available for sticklebacks (Peichel et al. 2001; Cresko et al. 2006; Kingsley and Peichel 2007), these crater lake sticklebacks would be an excellent model system for determining the genetic basis of the adaptation of exotic species.

Contrary to the cases of threespine sticklebacks introduced into freshwater ponds and lagoons in North America and

Europe (Klepaker 1993; Kristjánsson et al. 2002; Bell et al. 2004), reduction in armor plate was not observed and all sticklebacks in the Japanese crater lakes were completely plated. Interestingly, in Japan, only freshwater sticklebacks collected in the central region of Honshu Island are low plated, whereas all freshwater-resident sticklebacks in northern Japan are completely plated (Ikeda 1933; Mori 1987, 1990; Kitano et al. 2007b). Even low plated populations in central Honshu are likely to have mutations at a major armor plate locus (*Eda* gene) different from other North American and European sticklebacks (Schluter et al. 2004; Colosimo et al. 2005). Therefore, the Japanese sticklebacks might lack the standing allelic variation at the *Eda* locus, which is prevalent in European and North American populations.

Our data demonstrate that sticklebacks can change their diets when they are introduced into novel environments, although we do not know whether the sticklebacks have changed the preference for particular items of prey. Empirical studies have demonstrated that the trophic ecology of sticklebacks can influence the ecosystem functions (Pont et al. 1991; Harmon et al. 2009; Ingram et al. 2011). For example, the presence of planktivorous sticklebacks may lead to a decline in zooplankton populations (Pont et al. 1991). Because zooplankton graze on phytoplankton, the increase in the number of planktivorous sticklebacks can subsequently increase algal growth, resulting in an increase in water turbidity (Jakobsen et al. 2003, 2004). Therefore, how introduction and dietary changes of exotic sticklebacks can influence the crater lake ecosystem should be studied for better ecosystem management in crater lakes.

Conclusion

Our results demonstrate that sticklebacks can change their morphology and trophic ecology when they colonize novel environments. Intricate relationships among exotic fishes, plankton, and parasites may underlie these ecological and phenotypic changes. In addition, larger phenotypic variances in the Lake Kussharo population than other crater lakes might be caused by introduction of multiple populations into Lake Kussharo. Because further genetic studies will be possible in genetically tractable sticklebacks, these crater lake sticklebacks will be a good vertebrate model system for elucidating both ecological and genetic mechanisms underlying rapid phenotypic changes in exotic species.

Data Accessibility

DNA sequences were deposited in DNA Data Bank of Japan (DDBJ accession numbers AB678412–AB678418).

Acknowledgments

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Supporting Information

Additional Supporting Information may be found online on Wiley Online Library.

Figure S1. Increase in the frequency of stickleback with empty stomach (ANOVA, $F_{1,17} = 11.7$, $P = 0.003$).
Table S1. Data from annual reports of the Akita Prefectural Department of Fisheries.
Table S2. Change in female standard length in the Lake Towada population collected on the western side of the lake.
Table S3. Stomach content analysis.

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