Phenotypic divergence and reproductive isolation between sympatric forms of Japanese threespine sticklebacks

JUN KITANO[1], SEIICHI MORI[2], and CATHERINE L. PEICHEL[1,*]

[1]*Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA*

[2]*Biological Laboratory, Gifu-keizai University, Ogaki, Gifu 503-8550, Japan*

*Corresponding author. E-mail: cpeichel@fhcrc.org

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ABSTRACT

The threespine stickleback (*Gasterosteus aculeatus*) species complex is well suited for identifying the types of phenotypic divergence and isolating barriers that contribute to reproductive isolation at early stages of speciation. Here, we characterize the patterns of genetic and phenotypic divergence, as well as the types of isolating barriers that are present between two sympatric pairs of threespine sticklebacks in Hokkaido, Japan. One sympatric pair consists of an anadromous and a resident freshwater form and shows divergence in body size between the forms, despite the lack of genetic differentiation between them. The second sympatric pair consists of two anadromous forms, which originated before the last glacial period and are currently reproductively isolated. These two anadromous forms have diverged in many morphological traits as well as in their reproductive behaviors. Both sexual isolation and hybrid male sterility contribute to reproductive isolation between the anadromous species pair. We discuss the shared and unique aspects of phenotypic divergence and reproductive isolation in the Japanese sympatric pairs, as compared with postglacial stickleback species pairs. Further studies of these divergent species pairs will provide a deeper understanding of the mechanisms of speciation in sticklebacks.

ADDITIONAL KEYWORDS: body size - courtship behavior - mate choice - sexual isolation – hybrid male sterility - speciation – species pair

INTRODUCTION

Speciation often includes two correlated processes, phenotypic divergence and the establishment of reproductive isolation (Dobzhansky, 1937; Mayr, 1942). The study of phenotypic divergence and reproductive isolation between sympatric pairs of closely related, but reproductively isolated populations is crucial for understanding the mechanisms underlying speciation (Mayr, 1942; Schluter, 2001; Coyne & Orr, 2004). The threespine stickleback (*Gasterosteus aculeatus*) species complex provides a great model system for speciation research because there are multiple, independent pairs of phenotypically and ecologically divergent forms, so-called "species pairs", that come into contact with each other and are reproductively isolated in nature (McPhail, 1994; McKinnon & Rundle, 2002). Recent speciation research in sticklebacks has been conducted mostly on Canadian species pairs that were established after the last glacial recession, approximately 12,000 years ago (McPhail, 1994; McKinnon & Rundle, 2002). These studies have revealed the types of phenotypic divergence as well as the types of isolating barriers present at early stages of speciation in sticklebacks. However, the relative order in which these different types of phenotypic divergence and isolating barriers have occurred has not yet been investigated, because most of the species pairs studied thus far are of postglacial origin and have similar evolutionary histories.

In Japan, there are two sympatric pairs of threespine sticklebacks with distinct evolutionary histories from the postglacial pairs (Higuchi & Goto, 1996; McKinnon & Rundle, 2002; Goto & Mori, 2003). The first sympatric pair consists of two anadromous forms of sticklebacks: the Japan Sea form

and the Pacific Ocean form. These two forms are thought to have diverged when the Sea of Japan was geographically isolated from the Pacific Ocean, which has occurred several times during the last 3 Myr (Fig. 1A; Nishimura, 1974; Higuchi & Goto, 1996). Allozyme data covering a broad range of global populations revealed that the threespine stickleback species complex could be divided into two lineages: the Japan Sea lineage and the Pacific/Atlantic Ocean lineage (Haglund et al. 1992; Buth and Haglund 1994). Currently, threespine sticklebacks of the Japan Sea lineage are confined to coastal areas around the Sea of Japan, are relatively uniform in morphology, and are exclusively anadromous (Higuchi, 2003). In contrast, threespine sticklebacks of the Pacific/Atlantic Ocean lineage occupy diverse aquatic habitats and have undergone extensive adaptive radiation mainly through postglacial dispersal in the past 12,000 years (Bell & Foster, 1994). After the last glacial period, threespine sticklebacks of the Pacific Ocean and Japan Sea lineages were brought into secondary contact, and anadromous fish of the two lineages can be found in overlapping habitats in eastern Hokkaido, Japan (Fig. 1B; Higuchi & Goto, 1996). While allozyme data indicate that there is reproductive isolation between these sympatric forms (Higuchi & Goto, 1996), there has not yet been a systematic investigation of the types of phenotypic divergence and isolating barriers present between the Japan Sea and Pacific Ocean forms found in sympatry.

A second sympatric pair in Japan consists of an anadromous and a resident freshwater form of the Pacific Ocean lineage. In eastern Hokkaido, Japan, several lakes have originated from coastal lagoons or inner bays within the last 2,000-3,000 years (Kumano *et al.*, 1990; Okazaki & Yamashiro, 1997). Anadromous sticklebacks migrate from the ocean via streams into these lacustrine systems to

breed, where they overlap with the resident freshwater forms (Mori, 1990; Arai, Goto & Miyazaki, 2003; Kume & Kitamura, 2003). Reflecting the recent origin of these coastal lakes, a previous allozyme study found no genetic differentiation between the sympatric anadromous and resident freshwater forms of the Pacific Ocean lineage in eastern Hokkaido (Higuchi, Goto & Yamazaki, 1996). However, genetic markers such as microsatellites are more sensitive than allozymes for the detection of recent population divergence in sticklebacks (Reusch, Wegner & Kalbe, 2001; Raeymaekers et al. 2005).

In this study, we first used microsatellite markers to analyze the patterns of genetic differentiation between sympatric forms of Pacific resident freshwater (PF), Pacific anadromous (PA) and Japan Sea anadromous (JA) threespine sticklebacks in Japan. Second, we examined phenotypic divergence in body size and courtship behavior, because these traits are proposed to be involved in sexual isolation between the postglacial species pairs (McPhail, 1994; Schluter, 2001; McKinnon & Rundle, 2002). Third, we performed female mate choice experiments to determine whether sexual isolation exists between the sympatric PA and JA forms. Finally, we performed crosses between JA and PA fish to determine whether any intrinsic postzygotic reproductive isolation exists between these sympatric forms.

MATERIAL AND METHODS

FISH

Sympatric threespine sticklebacks of the Pacific Ocean anadromous form (PA) and the Japan

Sea form (JA) were collected using stationary nets in Akkeshi Bay and Lake Akkeshi, Hokkaido Island, Japan (Fig. 1B; Kume *et al*., 2005) in May 2003 and 2005. Sympatric threespine sticklebacks of the Pacific anadromous (PA) and Pacific resident freshwater (PF) forms were collected with casting nets and minnow traps from Hyotan Pond, a small pond connected to Akkeshi Bay by a short stream, the Shiomi River (Fig. 1B; Kume & Kitamura, 2003). Fish collected in 2003 were used for analyzing genetic, morphological, and behavioral divergence, while samples collected in 2005 were used for mate choice experiments. The PA forms collected from Akkeshi Bay, Lake Akkeshi and Hyotan pond were analyzed together because the PA forms collected from different adjacent freshwater systems in eastern Hokkaido have been shown to be morphologically and genetically indistinct from one another (Mori, 1990; Higuchi *et al.*, 1996).

Three morphological forms were first distinguished by visual inspection, based on body size and lateral plate morphology (Fig. 2A). After behavioral studies, right pectoral fins were clipped from anaesthetized fishes and preserved in ethanol for DNA analysis. Fishes were then preserved in 10% buffered formalin for morphological analysis. Our initial classification was confirmed by retrospective microsatellite and morphological analysis (see Results). We identified a single possible hybrid among 93 fish by microsatellite analysis, but excluded this individual from our subsequent analyses.

GENETIC ANALYSIS

To amplify microsatellite loci, genomic DNA isolation and polymerase chain reaction (PCR)

were performed as previously described (Peichel *et al.*, 2001). To investigate the genetic differentiation between different morphological forms of threespine sticklebacks, we first used a random number table to choose 25 microsatellite loci. Eight of these loci (*Stn67*, *Stn159*, *Stn233*, *Stn238*, *Stn323*, *Stn330*, *Stn389*, and *Stn390*) gave robust PCR products in all three morphological forms, were in Hardy-Weinberg equilibrium, and met the stepwise mutation model (Nei & Kumar, 2000); these eight loci were used for our analyses. Deviation from the Hardy-Weinberg equilibrium was tested with an exact test using Genepop (Raymond & Rousset, 1995a; Raymond & Rousset, 1995b). We used Arlequin software (Schneider, Roessli & Excoffier, 2000) to test for genetic differentiation between forms using both allele lengths, R_{ST} (Slatkin, 1995), and allele frequencies, θ (Weir & Cockerham, 1984). Genetic distance $(\delta\mu)^2$ (Goldstein *et al.*, 1995) and Nei's genetic distance (D) (Nei & Kumar, 2000) were calculated with Populations software (http://www.pge.cnrs-gif.fr/bioinfo/populations/index.php). Divergence time was estimated from $(\delta\mu)^2$ as described previously (McKinnon *et al.*, 2004) except we used a mutation rate of 10^{-4} (Feldman, Kumm & Pritchard, 1999), which is a reasonable estimate based on previous estimates of microsatellite mutation rates in fish (Shimoda *et al.*, 1999). Effective population size (N_e) and migration rate (m) were calculated using the maximum likelihood coalescent program MIGRATE (Beerli & Felsenstein, 1999) as described previously (Gow, Peichel & Taylor, 2006).

To find lineage-specific markers, we first performed PCR with 197 microsatellite primer sets on genomic DNA pools from each of the morphologically classified forms. Differences in allele frequencies were found between the JA and PA forms in over 90% of loci analyzed. Six loci had

non-overlapping allele repertoires between the JA and PA forms, with the exception of a few individuals that were assumed to carry a signature of introgression. These loci were used to discriminate fish from the Pacific Ocean and Japan Sea lineages.

MORPHOLOGICAL ANALYSIS

We measured standard length, head length, body depth, first dorsal spine length, second dorsal spine length, pelvic spine length, pelvic girdle length, snout length, gape width, eye diameter, and upper jaw length from the left side of formalin-fixed samples with a vernier caliper (Mori, 1990; Schluter & McPhail, 1992). For the analysis of lateral plates and gill rakers, fish samples were stained with alizarin red to visualize the bony structures. Gill raker number was counted on the first right gill arch. As most morphological traits were sexually dimorphic (J. Kitano, unpublished data), we used only the male morphological measurements for our analysis.

BEHAVIORAL ANALYSIS

Behavioral experiments were performed as previously described (Ishikawa & Mori, 2000), with several modifications. In order to observe horizontal movement as well as fish body curvature during courtship behavior, we recorded the behaviors from above. Each 60 L plastic tank (40 cm width x 75 cm length x 20 cm depth) was divided into a larger male compartment (40 cm width x 60 cm length x 20 cm depth) and a smaller female compartment (40 cm width x 15 cm length x 20 cm depth) by a

transparent partition. Curtains were arranged so that the tank was lit and accessible by a digital video camcorder, but the rest of the laboratory, including the experimenter, was hidden from the tested fish. A randomly chosen male threespine stickleback with red nuptial coloration was placed in the male compartment together with 10 g of boiled palm fibers for nest materials and a nesting dish filled with sand. We confirmed nest completion by observing that the male performed fanning and/or creeping-through before or during the courtship experiments, since these behaviors are typically observed after the late nest-building phase of threespine sticklebacks (van Iersel, 1953). Then, a randomly chosen gravid female of the same form was placed in the female compartment and shown to the nesting male. After the male started the first approach toward the female, the courtship behaviors of 13 PA males, 21 PF males, and 13 JA males were monitored for 15 min each.

During our initial behavioral observations, we noticed the males trying to dorsal prick the females through the glass (Wilz, 1970; Wootton, 1984). To observe the dorsal pricking behavior of a subset of the males tested above (five PA males, nine PF males, and seven JA males), the female was put into the male tank after the initial 15 min of indirect interaction, and the direct interaction was recorded for 20 min or until the female inspected the nest entrance. If the females never responded to the male either by showing a head-up posture or by following the male (Tinbergen, 1951; Wootton, 1984), we repeated the experiments with another gravid female at least 30 min later.

Images were captured at the speed of 30 frames per second and recorded on mini-digital videotapes. Male approaches were analyzed in slow motion replay or frame-by-frame on a computer.

Male approaches were classified into zigzag, C-form, rolling, and straight approaches. Slow-motion replay of several typical zigzag approaches revealed that the zigzag approach consists of two components: a sudden change of direction with C-form body bending and fast undulatory swimming with an S-form body shape (Fig. 3A). Therefore, an approach composed of at least one C-turn and at least one undulation with an S-form was counted as a zigzag approach. An approach composed only of C-form and not an S-form was counted as a C-form (Fig. 3A). The Japan Sea males rolled onto their sides while slowly approaching females (Fig. 3A). This behavior was termed the rolling approach. An approach that contains neither zigzag, rolling nor C-form was counted as a straight approach. For 13 PA males, 21 PF males, and 13 JA males, we calculated the frequency of each type of approach per male per 10 minutes. Male biting behavior was frequently observed during male courtship and also counted.

The intensity of male dorsal pricking was determined by measuring the distance that the male snout moved during dorsal pricking using NIH image software (http://rsb.info.nih.gov/nih-image/; Fig. 3B). The start of dorsal pricking was defined as the time when the male suddenly stops or slows down swimming and rolls onto his side, while the end was defined as the time when the male stops rolling and restarts fast forward swimming. The distance of female movement during dorsal pricking was similarly measured. The five PA males, nine PF males, and seven JA males for which we recorded direct interaction with a female were used for analysis of dorsal pricking.

FEMALE MATE CHOICE EXPERIMENT

We performed a female choice experiment to investigate sexual isolation between the sympatric PA and JA forms. Each 360 L aquarium tank (43 cm width x 180 cm length x 50 cm depth) was divided into two compartments of equal size with a removable partition composed of an opaque board and a transparent column (Fig. 4) and filled with water to the depth of 30-40 cm. To avoid the effects of male dominance on female choice, each male's territory within the tank was larger than a usual stickleback territory size (Mori, 1993; Mori, 1995). In addition, we put artificial weeds in each corner of the tank to stimulate the males to make nests behind the weeds and to visually separate the two males (Semler, 1971). Curtains were arranged so that other fish tanks and experimenters were hidden from the test fish. One PA male and one JA male were randomly chosen from a communal tank and put into each compartment. Once both males made nests, we put a gravid female of the PA or the JA form in the removable transparent column. Females were confined within the transparent column and allowed to observe both males for 15 minutes after they both started courtship behaviors towards her. The partition was then lifted with a string by the experimenter behind the curtains to allow the female to interact directly with the males. The partition was only lifted 15 cm during the experiment to keep the territory boundary apparent to both males and to avoid antagonistic interactions as much as possible.

Behaviors were monitored by a digital camcorder through small holes located on the curtains and recorded on videotapes. During the initial 15 minute period when the female was confined, we measured the time that the female spent oriented to either male with the head-up posture (Hay & McPhail,

1975; Rowland, 1994). We also counted the number of zigzag and rolling dances performed by the PA and the JA males, respectively. After the female was allowed to interact directly with the males, behaviors were observed for 60 minutes or until the female followed a male to his nest entrance. Final mate choice was determined by female nest inspection (Luttberg *et al.*, 2001; McKinnon *et al.*, 2004), and the female was removed before she spawned her eggs in the male's nest. When the female did not follow either male, we repeated the experiment with another female after at least one day.

Eight PA females and ten JA females were tested with pairs of PA and JA males. Each pair of males was used to test a single PA female and a single JA female. The type of female tested first with each pair was randomly decided, and a retrospective analysis with Fisher's exact test did not detect an order effect (for both PA and JA females, $P = 1.0$). Individual females were only tested once in order to avoid pseudo-replication (Kroodsma *et al.*, 2001).

STATISTICAL ANALYSIS OF MORPHOLOGICAL AND BEHAVIORAL DATA

For comparisons of morphological and behavioral traits between forms, we used a Mann-Whitney *U*-test. Prior to principal component analysis (PCA) of morphological traits, data were normalized by natural-log transformation (Sokal & Rohlf, 1995). PCA was based on a correlation matrix. In all multiple pairwise comparisons, statistical significance was corrected using sequential Bonferroni correction ($\alpha < 0.05$; Rice, 1989).

For the female mate choice tests, the amount of time a female spent orienting towards the two

males was compared with the Wilcoxon signed rank test. To examine whether the probability of the final female choice was significantly different from 0.5, a two-tailed binomial test was used. A significant deviation from 0.5 was taken as evidence of assortative mating by form. The frequencies of the courtship dances toward conspecific and heterospecific females were compared with a Mann-Whitney *U*-test.

TESTIS HISTOLOGY OF HYBRID MALES

We analyzed the testes of two F1 hybrid males generated by crossing a JA female and a PA male, two F1 hybrid males generated by crossing a PA female and a JA male, two males generated by crossing a PA female and a PA male, and two wild-caught JA males. These males were all reproductively mature because they displayed nuptial coloration, built a nest, and performed courtship behavior toward a female in an experimental tank. The males were collected from the tank before fertilization occurred, so their testes should contain sperm. The fish were sacrificed in MS-222 and fixed in 10% buffered formalin, and the fixed testes were dissected at a later time. Fixed testes were embedded in paraffin, processed into four μm sections, and stained with hematoxylin and eosin.

RESULTS

GENETIC STRUCTURE OF THE SYMPATRIC FORMS

Genetic differentiation is significant between the Pacific Ocean and the Japan Sea forms, using

tests based both on allele length (R_{ST}) and allele frequency (θ; Table 1). The genetic distances $(\delta\mu)^2$ and Nei's D between the Japan Sea and the Pacific Ocean forms are 299.9-314.8 and 0.732-0.735, respectively, which are larger than the previously reported $(\delta\mu)^2$ values of 3.765-5.178 and Nei's D of 0.193-0.359 for sympatric pairs within the Pacific/Atlantic Ocean lineage (McKinnon *et al.*, 2004). Using these data, we calculate a divergence time of approximately 1.5 Myr between the Pacific Ocean and Japan Sea forms.

Six loci were identified that could discriminate between the Pacific Ocean and the Japan Sea lineages (Appendix 1). Using these markers, we found one adult hybrid in a sample of 93 wild-caught fish from Akkeshi (1.08%). The migration rate (m) was symmetrical and estimated as 5×10^{-4} between the PA and JA forms. The effective population size of these two anadromous forms is similar, with N_e = 4625 for the PA form and N_e = 5000 for the JA form.

No genetic differentiation was found between the anadromous and resident freshwater forms of Pacific Ocean sticklebacks (Table 1). The genetic distances, both $(\delta\mu)^2$ and Nei's D, between the Japanese PA and PF forms were smaller than the previously reported genetic distances between sympatric pairs of anadromous and freshwater forms from Canada and Alaska (McKinnon *et al.*, 2004).

MORPHOLOGICAL DIVERGENCE

There are significant differences in standard length between the three forms. The PA fish were larger in standard length (75.88 ± 0.71 mm, N = 14) than the PF fish (57.06 ± 0.74 mm, N = 25; U =

0; $P < 0.001$) and the JA fish (61.47 ± 0.37 mm, $N = 38$; $U = 0$; $P < 0.001$). The JA fish were larger than the PF fish in standard length ($U = 143$, $P < 0.001$). Comparison of gill raker number, a good indicator of trophic ecology (Schluter & McPhail, 1992), revealed that JA fish have more gill rakers (25.1 ± 0.2, $N = 38$) than both the PA (21.4 ± 0.3, $N = 14$; $U = 12$; $P < 0.001$) and the PF forms (22.2 ± 0.3, $N = 25$; $U = 52.5$; $P < 0.001$). In contrast, there was no significant difference in gill raker number between the PA and PF fish ($U = 112.5$; $P = 0.067$).

Principal component analysis of 14 morphological traits identified two significant principal components (PCs; Appendix 2). A scatterplot of PC1 and PC2 revealed three non-overlapping clusters (Fig. 2B), confirming the existence of three morphological forms in the coastal area of Akkeshi. The first principal component (PC1) represents overall body size, while PC2 represents gill raker number and dorsal spine length. The JA form has significantly larger values of PC2 than both the PA ($U = 0$, $P < 0.001$) and the PF forms ($U = 0$, $P < 0.001$), while no significant differences were found in PC2 between the PA and the PF forms ($U = 74.0$, $P = 0.082$). Therefore, body size differences can explain most of the morphological divergence in the measured traits between the resident freshwater and the anadromous forms of the Pacific Ocean lineage, while there are additional morphological differences between the Japan Sea and the Pacific Ocean forms.

DIVERGENCE OF COURTSHIP BEHAVIOR

During courtship behavior, the PA and the PF males frequently performed zigzag approaches

and no significant differences were found in their approach patterns (Table 2; Fig. 3). The JA males rarely performed the zigzag approach (Table 2): only a single approach (1/215) performed by a single JA male was classified as a zigzag approach. Instead, the JA males frequently performed the rolling approach (Fig. 3), which was never observed in either the PA or the PF males (Table 2). No significant differences were found in the frequency of C-form approach, straight approach, or biting behaviors between forms (Table 2).

We next analyzed divergence in dorsal pricking between forms. The Japan Sea males performed more frequent dorsal pricking than the Pacific Ocean males (Table 3). In addition, during dorsal pricking, JA males moved backward for a longer distance than the PA and PF males (Table 3). Female movements during dorsal pricking were quite similar to the male movements (Table 3): the female was pushed back for a longer distance during dorsal pricking by Japan Sea males than by Pacific Ocean males. No significant differences were found between the PA and PF forms in dorsal pricking behaviors (Table 3).

ASYMMETRIC SEXUAL ISOLATION

We performed female mate choice experiments to examine sexual isolation between the sympatric PA and JA forms. First, analysis of female orientation time revealed that PA females oriented towards PA males (4.15 ± 3.26 min / 15 min, $N = 8$) more frequently than towards JA males (1.63 ± 2.91 min / 15 min, $N = 8$; $Z = -2.10$, $P = 0.0357$), while JA females oriented towards JA (5.61 ± 2.24 min / 15 min, $N = 10$) and PA males with similar frequencies (4.68 ± 2.34 min / 15 min, $N = 10$; $Z = -0.66$, $P =$

0.663). Then, the females were allowed to interact with both males. PA females chose PA males in all mate choice tests (8/8; $P = 0.008$), suggesting that PA females have a strong preference for conspecific males. In contrast, JA females did not have a preference for conspecific males over heterospecific males, as only six of the ten JA females tested chose JA males ($P = 0.754$).

We compared the frequency of the male courtship dance toward the conspecific and heterospecific females during the indirect experiment. The PA males performed the zigzag dance toward the JA females (0.91 ± 0.14 / min, $N = 10$) as frequently as toward the PA females (1.00 ± 0.39 / min, $N = 8$; $U = 34.0$, $P = 0.594$). In contrast, the JA males performed the rolling dance less frequently toward the PA females (0.28 ± 0.16 / min, $N = 8$) than toward the JA females (0.73 ± 0.13 / min, $N = 10$; $U = 17.0$, $P = 0.041$).

ASYMMETRIC HYBRID MALE STERILITY

The testes of F1 males resulting from a cross between a JA female and a PA male lack mature sperm (Fig. 5), while the testes of F1 males resulting from a cross between a PA female and a JA male contain mature sperm, as do the testes of both PA and JA males (Fig. 5).

DISCUSSION

PATTERNS OF GENETIC DIVERGENCE IN STICKLEBACKS

Our microsatellite analysis estimated that the sympatric Pacific Ocean and Japan Sea forms

have been diverging for approximately 1.5 Myr, suggesting that these two anadromous forms originated before the most recent glacial period. The evolutionary history of the Japanese anadromous pair is thus quite different from other known stickleback species pairs, many of which are found in habitats that were only available after the end of the last glacial period approximately 12,000 years ago (Schluter & McPhail, 1992; McPhail, 1994; McKinnon & Rundle, 2002). Using six lineage-diagnostic markers, we identified only one hybrid among 93 individuals (1.08%), which is lower than the frequency of hybrids (~5%) identified by microsatellite analysis in two benthic-limnetic species pairs (Gow *et al.*, 2006). The migration rate between the Pacific Ocean and Japan Sea forms was 5×10^{-4}, which is close to or an order of magnitude lower than was found between a Canadian lake-stream pair (2.7×10^{-3} to 4.8×10^{-4}; Hendry, Taylor & McPhail, 2002) and an order of magnitude lower than those calculated for two benthic-limnetic species pairs (1.5×10^{-3} to 3.6×10^{-3}; Gow *et al*, 2006). These data are consistent with a longer divergence time and greater levels of reproductive isolation between the Japan Sea and Pacific Ocean forms than between the postglacial species pairs. There is also a sympatric pair in Japan that is of more recent origin than the postglacial species pairs. Although the Pacific anadromous and Pacific resident freshwater forms can be distinguished morphologically (Fig. 2), they are not genetically differentiated by our microsatellite analyses, consistent with the geological history of the region and previous allozyme analysis (Higuchi *et al.*, 1996)

We found body size divergence in both the old and young Japanese sympatric pairs. Body size divergence occurs in several other sympatric pairs of threespine sticklebacks with a wide variety of divergence times (Hagen, 1967; Blouw & Hagen, 1990; McPhail, 1994; McKinnon & Rundle, 2002; McKinnon *et al.*, 2004), suggesting that body size divergence can occur rapidly and may be a common feature of population differentiation in the threespine stickleback species complex. Although body size in threespine sticklebacks is greatly influenced by rearing environment (Mori & Nagoshi, 1987; Wootton, 1994; McKinnon *et al.*, 2004), there is also a heritable component to variation in body size (McPhail, 1977; Snyder & Dingle, 1989; Snyder & Dingle, 1990; Snyder, 1991; Colosimo, *et al*. 2004). At this time, we cannot exclude the possibility that PF and PA forms may represent different age classes of breeding fish. However, anadromous fish are generally larger than freshwater fish at 1 year of age (Baker, 1994), and a similar divergence in body size is observed between a young sympatric pair of anadromous and resident freshwater forms in Alaska (von Hippel & Weigner, 2004). Body size divergence may be an adaptation for the exploitation of divergent resource environments (Bentzen & McPhail, 1984; Schluter, 1993) or for survival under divergent predation regimes (Moodie, 1972; Reimchen, 1991). Furthermore, body size divergence is an important component of sexual isolation between sympatric stickleback species pairs (Nagel & Schluter, 1998; McKinnon *et al*, 2004). Thus, body size divergence can occur rapidly and may play an important role during the early stages of population differentiation in sticklebacks.

Divergence in gill raker number is also common in stickleback species pairs (McPhail, 1994;

McKinnon & Rundle, 2002). Our preliminary study of feeding ability in the Japanese anadromous pair suggests that the PA forms, which have fewer gill rakers, are better at feeding on benthic foods than the JA forms (J. Kitano, unpublished data), which have more numerous gill rakers. These results are consistent with previous findings in postglacial sympatric pairs (Bentzen & McPhail, 1984; Schluter, 1993). Divergence in both body size and gill raker number suggests that the two anadromous forms of threespine sticklebacks may exploit divergent ecological resources in sympatry, supporting the idea that ecological divergence is an important process in stickleback speciation (McPhail, 1994; Schluter, 2001; McKinnon & Rundle, 2002).

In contrast to the shared features of morphological divergence between the Japanese sympatric pairs and the postglacial pairs, we found evidence for a unique behavioral divergence in the Japanese anadromous pair. During male courtship behavior, PA males perform the zigzag dance, while the JA males perform the rolling dance. Although quantitative variation in courtship behaviors, such as the frequency of the zigzag dance, has been found in postglacial species pairs (McPhail & Hay, 1983; Ridgway & McPhail, 1984; Blouw & Hagen, 1990), virtually all threespine sticklebacks in the Pacific/Atlantic Ocean lineage do perform the zigzag dance (Bell and Foster, 1994). Taken together with a previous study of an allopatric population of the Japan Sea lineage (Ishikawa & Mori, 2000), our data suggest that the complete absence of the zigzag dance and the acquisition of the rolling dance is unique to the Japan Sea lineage. Although the origin of the rolling dance in the Japan Sea lineage is unknown, it is interesting to note that the body movements during rolling resemble the body movements

during dorsal pricking, and JA males do more intense and frequent dorsal pricking (Table 3). Thus, this behavior may have evolved through the co-option of an existing behavior into the male display behavior (Foster, 1995).

The unique divergence of courtship behaviors in the Japanese anadromous pair may reflect the relatively long divergence time (1.5-2 Myr) between the Japan Sea and the Pacific/Atlantic lineages. Although courtship behaviors are relatively stable within the Pacific/Atlantic lineage of threespine sticklebacks, several courtship behaviors that are not observed in the threespine stickleback are found in both the blackspotted stickleback (*Gasterosteus wheatlandi*; McInerney, 1969; Reisman, 1986; McLennan, Brooks & McPhail, 1988) and the ninespine stickleback (*Pungitius pungitius*; McLennan *et al.*, 1988), which have diverged from the threespine stickleback within the past 3.5-10 Myr and 7-16 Myr ago, respectively (Hudon & Guderly, 1984; Bell & Foster, 1994; Buth & Haglund, 1994; Nei & Kumar, 2000). These data suggest that in the stickleback family it may take longer to alter the genetic and neural circuitry that underlies male courtship behavior than to alter the genetic circuitry that underlies life history or morphological traits, which can diverge in less than 10,000 years (Bell & Foster, 1994).

PATTERNS OF REPRODUCTIVE ISOLATION IN STICKLEBACKS

We found both prezygotic (sexual isolation) and postzygotic (hybrid male sterility) isolating barriers between the sympatric PA and JA forms. Sexual isolation is asymmetric: PA females exclusively choose PA males, while JA females do not have a preference for conspecific males. Hybrid

male sterility is also asymmetric: only crosses between JA females and PA males yield sterile sons. Although neither of these isolating barriers alone would be sufficient to maintain reproductive isolation, these two mechanisms may work in combination to prevent extensive hybridization between the Japan Sea and Pacific Ocean forms in sympatry. These data further suggest that female hybrids resulting from crosses between JA females and PA males may be the main contributors to introgression between the forms. These results are consistent with a previous mitochondrial DNA analysis of these sympatric populations, which revealed that the Japan Sea and Pacific Ocean forms have similar mitochondrial DNA (Yamada, Higuchi & Goto, 2001). Asymmetric sexual isolation is expected to result in unidirectional introgression of the maternally transmitted mitochondrial DNA between the two species, such that hybrids are likely to have only a single type of mitochondrial DNA (Wirtz, 1999).

Asymmetric sexual isolation is widely observed in many animal species, although the reasons for it are controversial (Watanabe & Kawanishi, 1979; Kaneshiro, 1980; Moodie, 1982; Arnold, Verrell & Tilley, 1996; Bordenstein, Drapeau & Werren, 2000; Shine *et al.*, 2002; Coyne & Orr, 2004). The asymmetric pattern of sexual isolation in the Japanese anadromous pair may result from divergence in both female and male mate choice. In addition to the divergence we observed in female mate choice between the PA and JA females, we also found evidence for divergence in male mate choice between these forms. The JA males performed the rolling dance less frequently towards PA females than towards JA females, while the PA males performed the zigzag dance towards JA females as frequently as toward PA females. Further experiments are required to understand the types of male and female mating

signals that are important for sexual isolation between the Japanese anadromous pair, for example by using dummy models and/or video animation (Rowland, 1994; Kunzler and Bakker, 1998).

Among the many threespine stickleback species pairs, hybrid male sterility has only been reported in the Japanese anadromous pair (McKinnon and Rundle, 2002). A previous *in vitro* fertilization experiment had shown that male hybrids resulting from crosses between sympatric JA females and PA males are sterile, while male hybrids resulting from crosses between PA females and JA males are fertile (Yamada and Goto, 2003). Hybrid females resulting from crosses in both directions are fertile (Yamada & Goto, 2003; J. Kitano, unpublished data). We have extended the findings of the previous study and have shown that hybrid male sterility is due to impaired spermatogenesis (Fig. 5). Because threespine sticklebacks have genetic sex determination, with XX females and XY males (Peichel *et al*., 2004), hybrid male sterility in the Japanese stickleback anadromous pair is consistent with Haldane's rule, which states that the heterogametic sex is more likely to suffer from hybrid incompatibilities (Haldane, 1922).

The establishment of genomic incompatibility usually follows the evolution of sexual isolation in animals (Prager & Wilson, 1975; Coyne & Orr, 1989; Coyne & Orr, 1997; Grant & Grant, 1997; Mendelson, 2003). In sticklebacks, this pattern also appears to hold true. In contrast to the hybrid male sterility observed between the Japan Sea and Pacific Ocean forms, viable and fertile hybrids can be produced in the laboratory by *in vitro* fertilization between virtually any pair of threespine sticklebacks within the Pacific/Atlantic Ocean lineage (McKinnon & Rundle, 2002), despite the presence of sexual

isolation between populations (McPhail, 1994; McKinnon & Rundle, 2002). Complete hybrid inviability has been observed in reciprocal crosses between threespine sticklebacks and blackspotted sticklebacks (*G. wheatlandi*; C. L. Peichel, unpublished data), which diverged 3.5-10 Myr ago (Hudon & Guderly, 1984; Buth & Haglund, 1994; Nei & Kumar, 2000). Furthermore, reduced viability was observed in a cross between threespine and ninespine sticklebacks (*Pungitius pungitius*; Leiner, 1940; Kobayashi, 1959), which diverged 7-16 Myr ago (Bell & Foster, 1994). Taken together, these data suggest that sexual isolation has evolved faster than intrinsic genomic incompatibility in sticklebacks.

CONCLUSIONS

Our studies demonstrate that there are two unique pairs of sticklebacks found in regions of sympatry in Japan. The first is a young anadromous-resident freshwater pair that is not yet genetically differentiated but shows divergence in body size. The second is an older pair, consisting of two lineages of anadromous threespine sticklebacks. We have shown that this Japanese "species pair" is both genetically and phenotypically divergent, and that both sexual isolation and intrinsic postzygotic isolation contribute to reproductive isolation between the species. These unique Japanese stickleback sympatric pairs provide a valuable resource for further analysis of the genetic and ecological factors that underlie patterns of speciation in sticklebacks.

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FIGURE LEGENDS

Figure 1. (A) Map showing the hypothetical coastal lines around the Japanese archipelago and the Sea of Japan during the late Pliocene through the early Pleistocene (left panel) and during the last glacial period (right panel). Solid lines indicate the hypothetical coastal lines, while the broken lines indicate current coastal lines. Map data were taken from Nishimura (1974). (B) Map showing the collection site in Akkeshi, Hokkaido, Japan. Threespine sticklebacks of the PA and JA forms were collected in Akkeshi Bay and Lake Akkeshi, while threespine sticklebacks of the PA and PF forms were collected from Hyotan Pond. Scale bar in lower panel = 2 km.

Figure 2. (A) Alizarin red-stained male threespine sticklebacks of the PA, PF, and JA forms. In the JA form, the heights of the lateral plates decrease dramatically after the arrow. Scale bar = 1 cm. (B) Principal component analysis of morphological divergence between Japanese sympatric forms. Open circles, open triangles, and filled circles indicate PA, PF, and JA males, respectively. A scatterplot of PC1 and PC2 indicates that there are three mutually separable clusters of morphological forms in Akkeshi.

Figure 3. (A) Representative image of body movement during a zigzag approach of a PA male (upper panel), a rolling approach of a JA male (middle panel), and a C-form approach of a JA male (lower panel). Body shapes were moved a little vertically to visualize every body shape in the upper panel. S, C, and

St indicate S-form, C-form, and straight body shapes, respectively. Time in seconds (s) of the start and the end of the representative approach is shown in each panel. Arrowheads in the middle panel indicate the direction of the dorsal side of the male. Scale bar = 10 cm. (B) Difference in dorsal pricking between forms. Schematics of the start (*S*) and the end (*E*) of dorsal pricking for a PA (upper panel), a PF (middle panel), and a JA male (lower panel). Arrows indicate the traces of snouts of the male (gray) and the female (white). Scale bar = 20 mm.

Figure 4. Tank used for female mate choice experiment.

Figure 5. Representative image of testes histology from males generated by crossing a PA female and a PA male (PP), a wild-caught JA male (JJ), an F1 hybrid male generated by crossing a PA female and a JA male (PJ), and an F1 hybrid male generated by crossing a JA female and a PA male (JP). The PP, JJ, and PJ testes were filled with mature sperm (arrowhead), while the JP male testes were empty and contained no mature sperm. Scale bar, 0.1 mm.

Table 1. Genetic differentiation between Japanese sympatric forms. Twenty-eight PA individuals, 33 PF individuals, and 25 JA individuals were analyzed with eight randomly chosen microsatellites.

	PA - PF	PA - JA	PF - JA
R_{ST}	-0.0078	0.5905*	0.6039*
θ	0.0048	0.1605*	0.1545*
Genetic distance $(\delta\mu)^2$	1.3070	299.853	314.777
Genetic distance (Nei's D)	0.0370	0.7345	0.7319

*$P < 0.001$.

Table 2. Comparison of male approach patterns between different morphological forms. The approaches of 13 PA males, 21 PF males, and 13 JA males were analyzed.

	Mean frequency (± S.E.) / 10 min			Mann-Whitney *U*-test (*P*)		
	PA	PF	JA	PA-PF	PA-JA	PF-JA
Zigzag approach	4.31 (2.62)	3.27 (0.70)	0.05 (0.05)	0.190	< 0.001*	< 0.001*
Rolling approach	0	0	5.18 (1.41)		< 0.001*	< 0.001*
C-form approach	0.21 (0.16)	0.79 (0.40)	1.08 (0.50)	0.607	0.166	0.348
Straight approach	2.46 (1.09)	5.59 (1.25)	3.59 (0.97)	0.074	0.191	0.446
Biting	0.62 (0.22)	1.56 (0.71)	2.26 (0.96)	0.559	0.522	0.818

*Significant after sequential Bonferroni correction.

Table 3. Comparison of dorsal pricking behavior. The frequency of dorsal pricking (DP), distance of male backward swimming (BS) and the distance the female was pushed back (PB) during dorsal pricking was analyzed for five PA males, nine PF males, and seven JA males. Means (± S.E.) are shown.

	PA	PF	JA	Mann-Whitney *U*-test (*P*)		
				PA-PF	PA-JA	PF-JA
Frequency of DP (/10 min)	5.6 (1.8)	11.8 (3.2)	31.3 (10.3)	0.206	0.006*	0.061
Male BS (mm)	12.5 (8.1)	1.0 (2.5)	66.9 (6.0)	0.624	0.006*	< 0.001*
Female PB (mm)	3.8 (8.5)	-2.6 (2.1)	63.0 (8.3)	0.178	0.006*	< 0.001*

*Significant after sequential Bonferroni correction.

APPENDIX 1

Identification and characterization of lineage specific markers. Thirty PA individuals, 33 PF individuals, and 30 JA individuals were analyzed.

Locus name	Range of allele size in base pairs (most common allele size) Observed/expected heterozygosity (H_O/H_E)		
	PA	PF	JA
Stn46	232-238 (232)	232-238 (236)	240-280 (244)
	0.63/0.69	0.73/0.66	0.83/0.92
Stn215	153 (153)	153 (153)	139-151, 155-169 (157)*
	0.06/0.06	0/0	0.59/0.77
Stn273	226-232 (228)*	226-230 (228)*	238-258 (246)
	0.20/0.38	0.27/0.37	0.83/0.90
Stn383	172-186 (176)	172-186 (176)	162-170 (170)
	0.80/0.69	0.64/0.60	0.93/0.79
Stn384	118-134 (130)	116-132 (130)	96-110 (106)
	0.83/0.72	0.58/0.64	0.69/0.68
Stn385	210 (210)	210 (210)	206-208, 212-220 (214)
	0/0	0.06/0.06	0.69/0.73

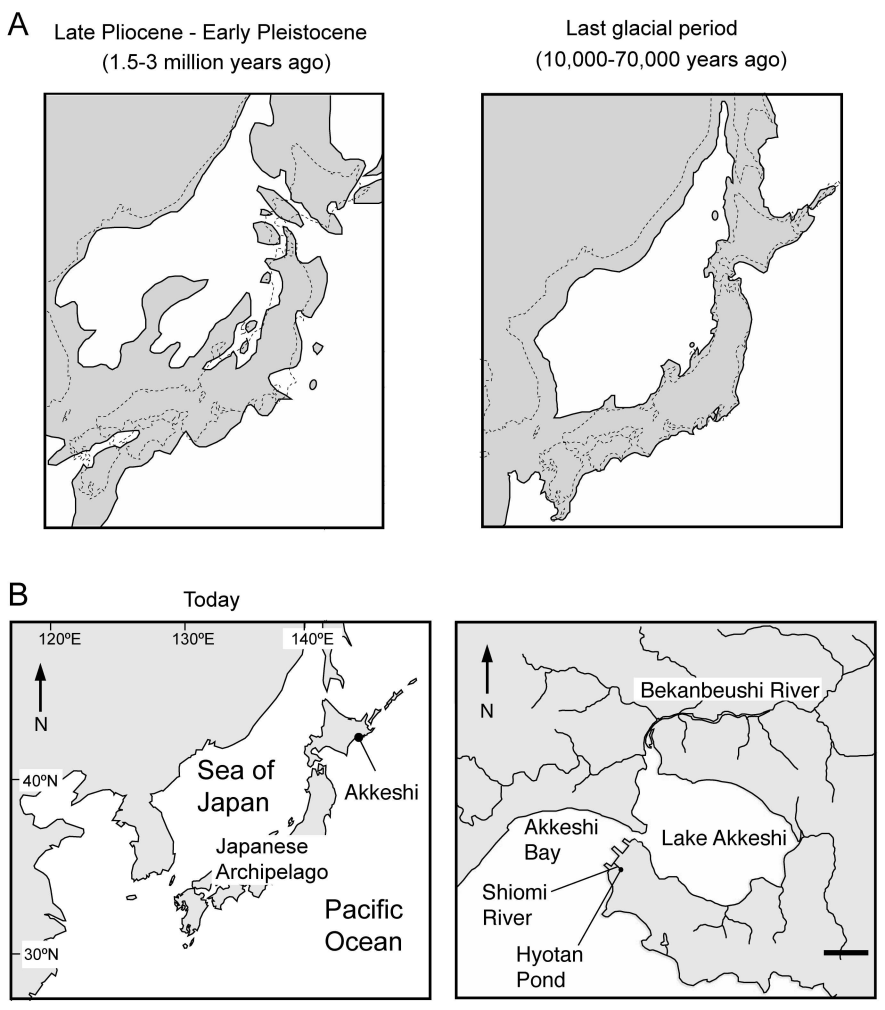
* Deviation from Hardy-Weinberg equilibrium within form after sequential Bonferroni correction.

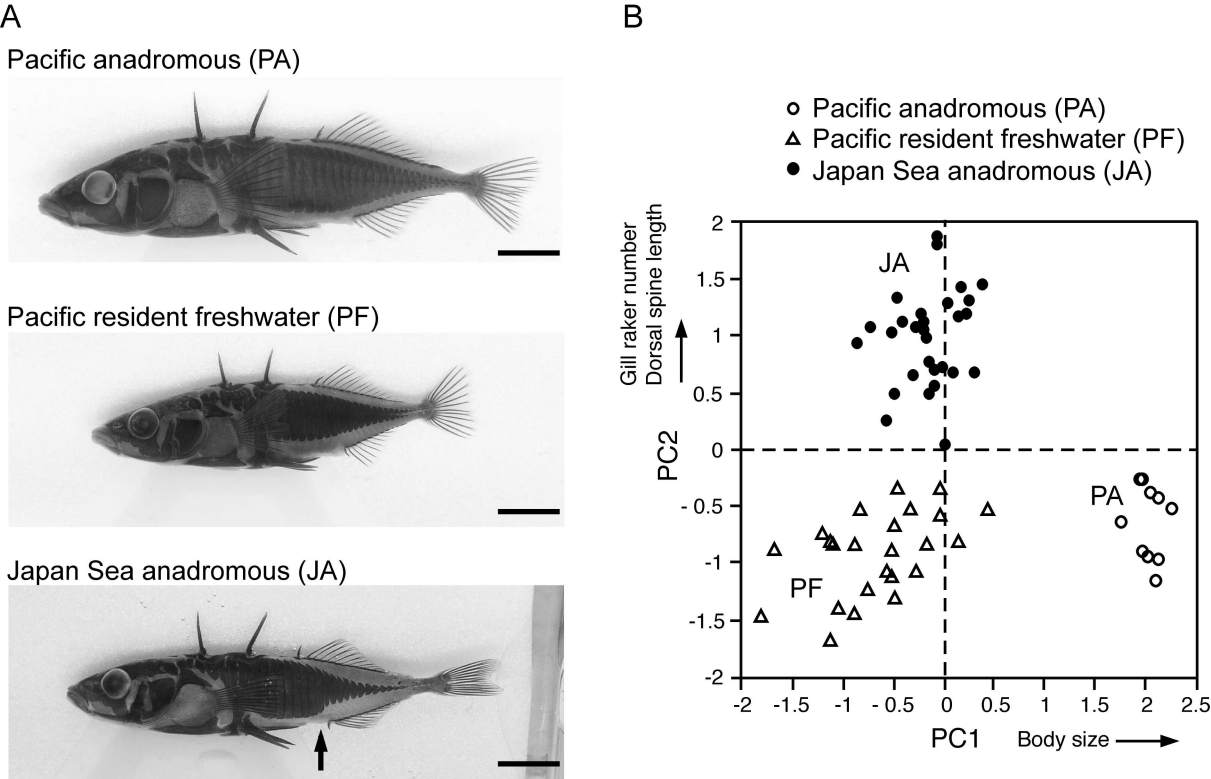
APPENDIX 2

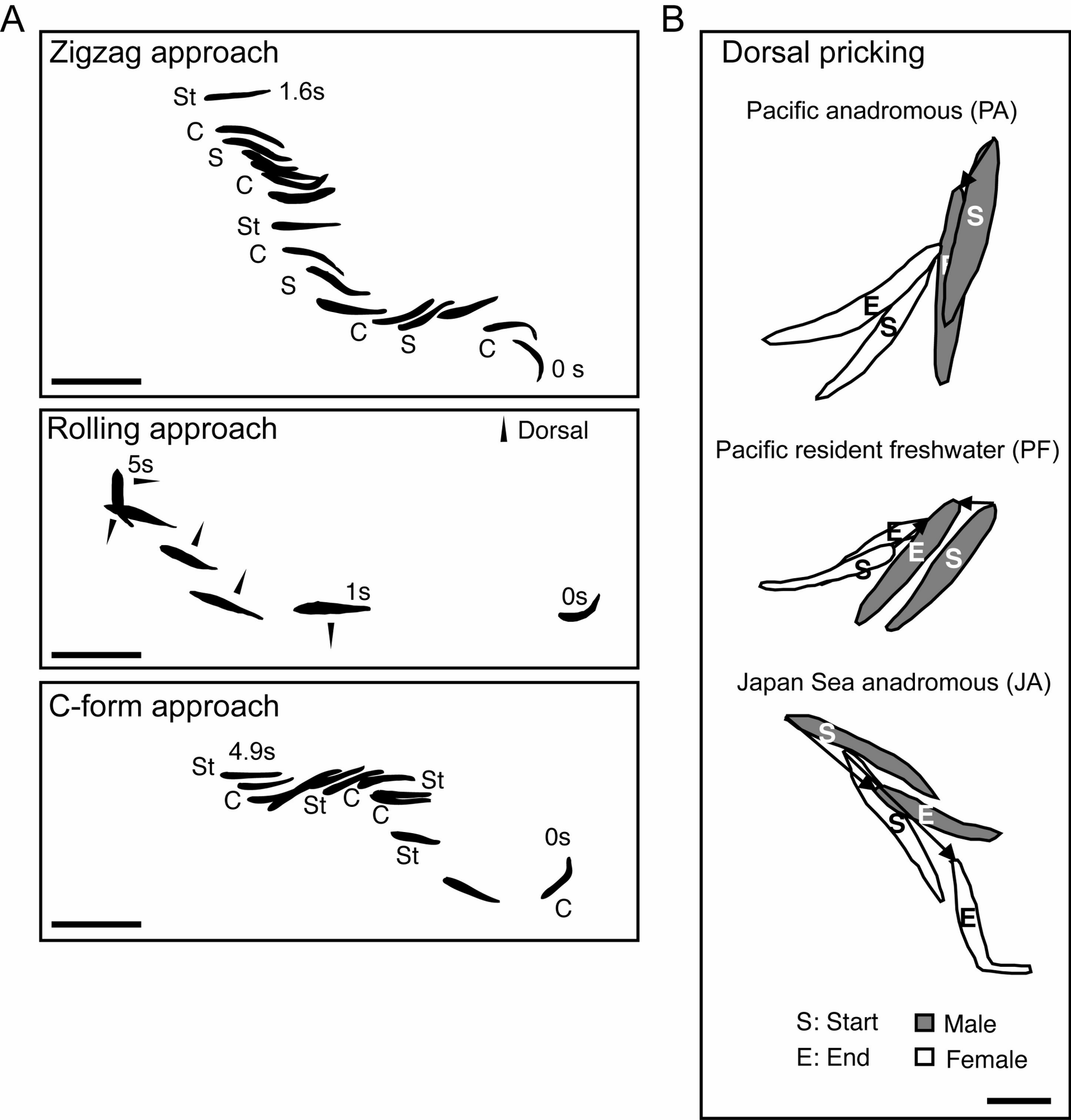
Principal component analysis of morphology.

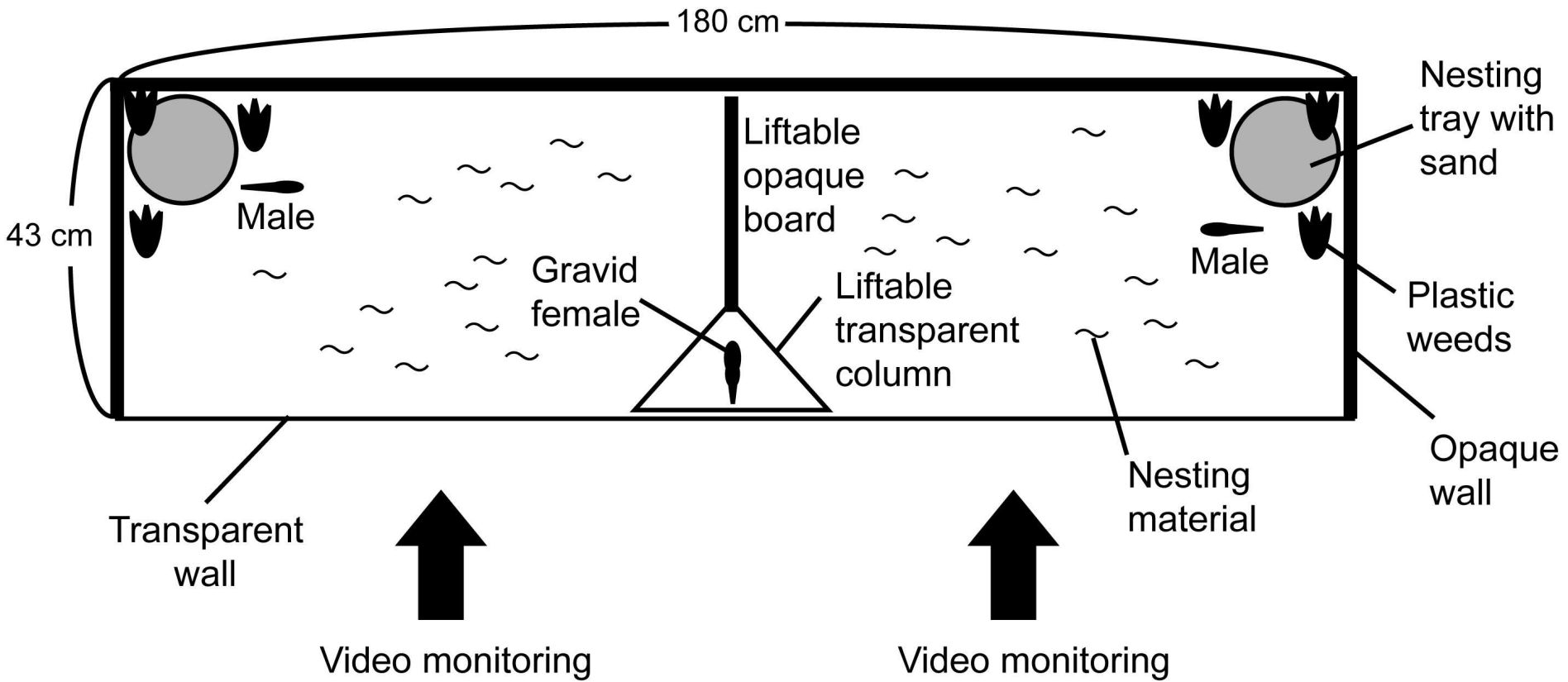
	PC1	PC2
Component loadings		
Standard length	0.972*	0.020
Head length	0.947*	-0.247
Body depth	0.952*	-0.143
First dorsal spine length	0.765*	0.542*
Second dorsal spine length	0.783*	0.513*
Pelvic spine length	0.889*	0.165
Pelvic girdle length	0.923*	-0.063
Snout length	0.938*	-0.132
Gape width	0.804*	-0.213
Eye diameter	0.809*	-0.082
Jaw length	0.931*	-0.071
Gill raker number	-0.140	0.907*
% Variance explained	72.1	13.1

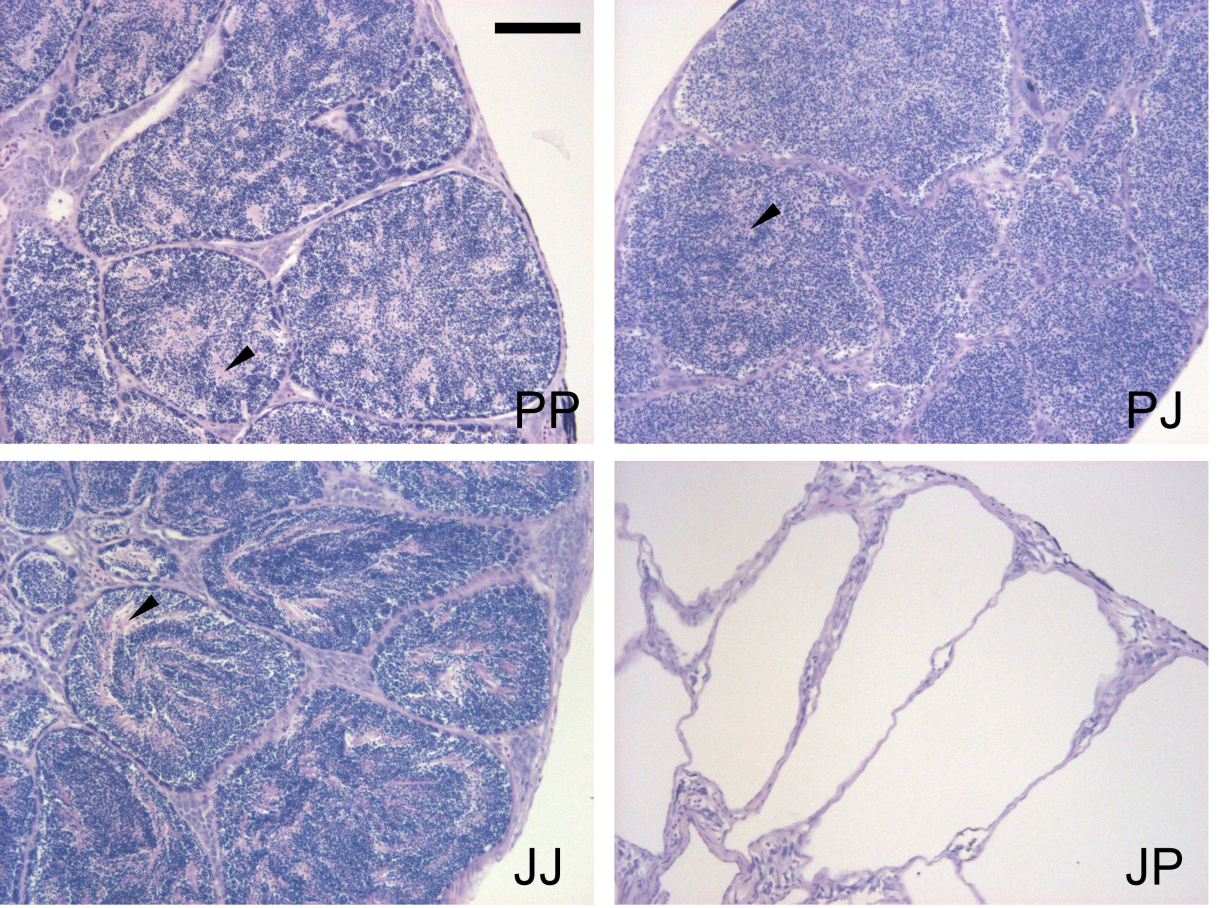
*Morphological traits whose component loadings exceed 0.5.


A
Late Pliocene - Early Pleistocene
(1.5-3 million years ago)
Last glacial period
(10,000-70,000 years ago)
B
Today
120ºE
130ºE
140ºE
N
40ºN
30ºN
Sea of
Japan
Akkeshi
Japanese
Archipelago
Pacific
Ocean
N
Bekanbeushi River
Akkeshi
Bay
Lake Akkeshi
Shiomi
River
Hyotan
Pond


A
Pacific anadromous (PA)
Pacific resident freshwater (PF)
Japan Sea anadromous (JA)
B
Pacific anadromous (PA)
Pacific resident freshwater (PF)
Japan Sea anadromous (JA)
Gill raker number
Dorsal spine length
PC2
PC1
Body size
JA
PA
PF
2
1.5
1
0.5
0
- 0.5
-1
-1.5
-2
-2
-1.5
-1
- 0.5
0
0.5
1
1.5
2
2.5


A
Zigzag approach
St
1.6s
C
S
C
St
C
S
C
S
C
0 s
Rolling approach
Dorsal
5s
1s
0s
C-form approach
St
4.9s
C
St
C
C
St
St
0s
C
B
Dorsal pricking
Pacific anadromous (PA)
S
E
S
Pacific resident freshwater (PF)
E
E
S
S
Japan Sea anadromous (JA)
S
S
E
E
S: Start
E: End
Male
Female


180 cm
43 cm
Male
Gravid female
Liftable opaque board
Liftable transparent column
Male
Nesting tray with sand
Plastic weeds
Opaque wall
Nesting material
Transparent wall
Video monitoring
Video monitoring


PP
PJ
JJ
JP