The genomic landscape at a late stage of stickleback speciation: high

genomic divergence interspersed by small localized regions of

introgression

Mark Ravinet[1,2], Kohta Yoshida[1,3], Shuji Shigenobu[4], Atsushi Toyoda[5], Asao

Fujiyama[5], Jun Kitano[1,*]

[1]Division of Ecological Genetics, Department of Population Genetics, National

Institute of Genetics, Mishima, Shizuoka, Japan

[2]Centre for Ecological and Evolutionary Synthesis, University of Oslo, Oslo,

Norway

[3]Integrative Evolutionary Biology, Max Planck Institute for Developmental

Biology, Tübingen, Germany

[4]Functional Genomics Facility, National Institute for Basic Biology, Okazaki,

Aichi, Japan

[5]Comparative Genomics Laboratory, National Institute of Genetics, Mishima,

Shizuoka, Japan

[*]jkitano@nig.ac.jp

Short title: High divergence with localized introgression during speciation

Key words: stickleback, migration, speciation continuum, recombination, neo-

sex chromosome

Abstract

Speciation is a continuous process and analysis of species pairs at different stages of divergence provides insight into how it unfolds. Genomic studies on young species pairs have often revealed peaks of divergence and heterogeneous genomic differentiation. Yet it remains unclear how localised peaks of differentiation progress to genome-wide divergence during the later stages of speciation with gene flow. Spanning the speciation continuum, stickleback species pairs are ideal for investigating how genomic divergence builds up during speciation. However, attention has largely focused on young postglacial species pairs, with little known of the genomic signatures of divergence and introgression in older systems. The Japanese stickleback species pair, composed of the Pacific Ocean three-spined stickleback (*Gasterosteus aculeatus*) and the Japan Sea stickleback (*G. nipponicus*), which co-occur in the Japanese islands, is at a late stage of speciation. Divergence likely started well before the end of the last glacial period and crosses between Japan Sea females and Pacific Ocean males result in hybrid male sterility. Here we use coalescent analyses and Approximate Bayesian computation to show that the two species split approximately 0.68-1 million years ago but that they have continued to hybridise at a low rate throughout divergence. Population genomic data revealed that high levels of genomic differentiation are maintained across the majority of the genome when gene flow occurs. However despite this, we identified multiple, small regions of introgression, strongly correlated with recombination rate. Our results demonstrate that a high level of genome-wide divergence can establish in the face of persistent introgression and that gene flow can be localized to small genomic regions at the later stages of speciation with gene flow.

Author summary

When species evolve, reproductive isolation leads to a build-up of differentiation

in the genome where genes involved in the process occur. Much of our

understanding of this comes from early stage speciation, with relatively few

examples from more divergent species pairs that still exchange genes. To

address this, we focused on Pacific Ocean and Japan Sea sticklebacks, which co-

occur in the Japanese islands. We established that they are the oldest and most

divergent known stickleback species pair, that they evolved in the face of gene

flow and that this gene flow is still on going. We found introgression is confined

to small, localised genomic regions where recombination rate is high. Our results

show high divergence can be maintained between species, despite extensive

gene flow.

Introduction

Speciation is a continuous process through which reproductive isolation is

established [1–3]. According to the genic view of speciation [4], when

populations are in contact, gene flow is initially restricted at barrier loci (i.e. loci

underlying reproductive isolation), leading to the emergence of peaks of genetic

differentiation surrounding such barriers; i.e. heterogeneous genomic

differentiation [5,6]. As speciation progresses, this localised build-up of

reproductive isolation spreads to nearby regions due to linkage [4,5,7]. Once a

critical amount of differentiation at multiple barrier loci has accumulated,

reduction of the genome-wide effective migration rate will eventually lead to

divergence across the entire genome [5,7]. This final step of genome-wide

congealing may be a rapid and non-linear phase transition under certain

conditions, such as when isolating barriers have a polygenic basis or a few strong

barrier loci arise [8–10]

Recent empirical genomic studies have revealed regions of high and low

differentiation dispersed throughout the genome at early stages of speciation

[7,11,12]. This empirical data has lent strong support to the genic perspective of

the speciation process [4]. To-date however, the majority of speciation genomic

studies demonstrating heterogeneous genetic differentiation have come from

young species or population pairs with low divergence [7,11,12] with some

exceptions [9,13]. Even for these cases [13,14], it is unclear whether gene flow

has occurred throughout divergence or whether the species pair in question

experienced periods of geographical isolation (see below). A bias towards early

stage divergence has undoubtedly been informative to understand the onset of

the speciation process [15–18]. However, it is limited in its scope for

understanding the factors that eventually lead to the completion of the

speciation process – i.e. the evolution of genome-wide differentiation. When

divergence with gene flow is studied across the speciation continuum in *Timema*

stick insects (including the later stage divergence) the range of genome-wide

differentiation appears to be disjointed with a gap in F_{ST} between 0.3 and 0.7 [8].

Furthermore, literature surveys have also indicated that many species pairs

exhibit a mean F_{ST} between either 0.03-0.27 or 0.70-0.90 when gene flow is

occurring [8,9]. This disjointed distribution of differentiation is consistent with

the idea that progression towards speciation may be non-linear, with a phase

transition due to genome wide congealing [10,19].

In addition to need for more balanced understanding of the extent of

genomic divergence and introgression at later stages of the speciation

continuum, there is a need for studies which account for factors such as

demographic history [12,14]. This is because high genome-wide differentiation

in a single species pair may have evolved via genetic drift and local adaptation

during allopatric isolation, rather than due to divergence with gene flow. In other

cases, heterogeneous genomic differentiation may be due to erosion of genetic

differentiation due to introgression following secondary contact after

geographical isolation. Without a picture of the demographic history, this

scenario may be indistinguishable from primary divergence [20] and will

therefore introduce bias if multiple species pairs are compared. Despite the fact

that the expected pattern of genomic differentiation during speciation is

influenced by the timing and duration of geographical isolation [7], testing

different demographic histories has been somewhat neglected by the field [7,20].

Other factors besides demographic history of a species pair can also

confound patterns of heterogeneous genomic differentiation. For example, local

adaptation or background selection in genomic regions where recombination is

reduced can elevate differentiation measures and be mistaken for barrier loci

[13,21,22]. Similarly, regions of low differentiation may be caused by not only

on-going gene flow but also incomplete lineage sorting (ILS) [21,23] and

mutation rate variation [24]. Distinction between gene flow and ILS is likely

easier in more divergent species pairs [24–26]. Furthermore, the use of multiple

classical and recently developed methods, such as detection of recent hybrid

progeny, ABBA-BABA tests [27,28], model-based inference[29], and comparison

between allopatric and sympatric pairs [22,28] provide a means to distinguish

signatures of gene flow from alternative explanations. Thus, there is a need for

studies which account for factors such as demographic history, recombination

rate variation, and ILS that can confound the interpretation of genome scan data

[7,12].

Three-spined stickleback species pairs (genus *Gasterosteus*) span the

speciation continuum at varying stages of divergence, making them a model

system for speciation research [30,31]. To-date genomic research on speciation

with gene flow in the stickleback complex has largely focused on weakly

divergent species pairs, such as lake-stream ecotypes, with mean genome-wide

F_{ST} values of less than 0.3 [32–34]. Such studies have shown that the genomic

landscape of differentiation between these recently diverged sympatric or

parapatric species pairs is heterogeneous and interspersed with multiple peaks

of high differentiation [16,32,34]. The emerging pattern is consistent with

predictions under the genic concept of speciation – i.e. that reproductive

isolation is localized in the genome at early stages of speciation [4,35]. However,

it remains unclear whether such localized differentiation will eventually

progress toward genome-wide differentiation in the face of gene flow.

Toward the end of the stickleback speciation continuum is a marine

species pair in Japan [36,37]. The Japan Sea stickleback (*G. nipponicus*) is

sympatric with the Pacific Ocean lineage of three-spined stickleback (*G.*

aculeatus) (Fig 1A) in the waters surrounding the Japanese archipelago (Fig 1C)

[36,38]. Divergence time between the two marine species has been estimated to

be 1.5-2 million years based on allozyme and microsatellite data [37,39], making

it much older than postglacial stickleback species pairs. Speciation has been

hypothesized to have occurred as a result of the repeated isolation of the Sea of

Japan during the Pleistocene, but this divergence scenario remains to be

explicitly tested [37,39]. A unique feature of the *G. nipponicus* and *G. aculeatus*

system, relative to postglacial stickleback species pairs, is that a neo-sex

chromosome has arisen due to a fusion between a Y chromosome and a

previously autosomal chromosome IX (chrIX) in the *G. nipponicus* lineage

[36,40]. Furthermore, crosses between Japan Sea females and Pacific Ocean

males show hybrid male sterility [37]. Previous quantitative trait locus (QTL)

mapping identified QTL for courtship behaviour on the neo-X and hybrid male

sterility on the ancestral-X. However, there are other isolating barriers, such as

eco-geographical isolation, temporal isolation, and ecological selection against

migrants [37,41,42]. The combination of these multiple barriers most likely

contributes to the strong reproductive isolation in this system [36,43]. However,

despite such strong divergence, hybrids have been observed where the two

species co-occur in Northern Japan [36] and mitochondrial discordance between

the species suggests some history of introgression during speciation [44,45].

Although the Japanese species pair therefore represents the furthest point of

divergence along the stickleback speciation continuum, speciation remains

incomplete. The evolutionary history and genome-wide patterns of genetic

differentiation and introgression of this strongly divergent species pair therefore

remains an open question

The aim of our study was to address gap in our knowledge; i.e. to quantify

the patterns of genomic differentiation and introgression at a later stage of the

speciation continuum. To this end, we used a whole-genome reseqeuncing data

from the Japanese stickleback species pair to determine their evolutionary

history and characterise patterns of gene flow between them. Our first aim was

to establish how and when divergence took place between *G. nipponicus* and *G.*

aculeatus. Using thousands of genomic loci and a coalescent modelling approach,

we tested a range of divergence scenarios and estimated the timing and duration

of isolation, the extent of gene flow and fluctuations in population size. After

identifying that the two species have indeed diverged in the face of gene flow, we

then used a comparative genome scan approach with an additional *G. aculeatus*

lineage from the Atlantic Ocean [46] as an allopatric control (Fig 1A, Fig S1).

After establishing that gene flow has occurred but that a high level of genomic

differentiation has remained, we used two independent measures of gene flow to

identify where in the genome introgression has left its mark. We tested whether

introgression occurs more frequently in regions of high recombination and

whether it occurs in regions with functionally important genes. Our findings

suggest a high level of genome-wide divergence can be maintained, as

introgression is restricted to small, localized genomic regions.

Results

Ancestral demography and population genomic analyses support

divergence with gene flow

Phylogenetic analysis on 35,666 10 kb non-overlapping genome windows on

autosomes (i.e., excluding chrIX and chrXIX) supports a deep split between *G.*

aculeatus (both Pacific and Atlantic Ocean lineages) and *G. nipponicus* (Japan Sea

stickleback) (Fig 1A). Of all windows, 98.8% support the split between species,

while only 0.51% indicate clustering of fish occurring in Japan (the Japanese

Pacific Ocean *G. aculeatus* and the Japan Sea *G. nipponicus*; Table S1 and Fig 1A).

We calculated genealogical sorting index (*gsi*) [47] on maximum

likelihood phylogenies estimated from non-overlapping sliding windows of 20

kb across the autosome. High *gsi* indicates monophyly, while low *gsi* indicates

mixed ancestry [47]. Genome-wide averages (± SD) of *gsi* were high, but not

complete, for all three *Gasterosteus* lineages with that of the Japan Sea

stickleback being the highest (Atlantic *gsi* = 0.45 ± 0.10, Pacific *gsi* = 0.57 ± 0.09,

Japan Sea *gsi* = 0.72 ± 0.06).

This is in stark contrast to the mitogenome phylogeny where

sticklebacks from both species occurring in Japan fall into a single clade separate

from the clade occurring in the Western Pacific and Atlantic (Fig 1B, Fig S2). A

lack of mitogenome divergence between *G. aculeatus* and *G. nipponicus* from the

Japanese archipelago suggests mitochondrial introgression has occurred where

these lineages overlap (Fig 1C).

Since the consensus autosomal phylogeny suggests a more recent split

between the Pacific and Atlantic *G. aculeatus* lineages, a deeper mtDNA split

based on spatial distribution suggests mitochondrial introgression has likely

occurred from the Japan Sea *G. nipponicus* into the Pacific Ocean *G. aculeatus*.

Divergence time estimates between the mitogenome clades are thus informative

for dating speciation. Bayesian coalescent analysis using a strict clock model in

Bayesian Evolutionary Analysis by Sampling Trees (BEAST) suggests a median

split date of 1.30 million years (0.15-2.41; 95% Highest Posterior Density [HPD]

intervals; Table S2) for the two major mitogenome clades (Fig S2), consistent

with previous estimates [44]. Divergence between Eastern Pacific and Atlantic

haplotypes is more recent at 0.39 million years (0.03-0.74; 95% HPD) but is

older than the Most Recent Common Ancestor (MRCA) of all haplotypes

occurring in Japan (Fig 1B, S2A), suggesting mitochondrial gene flow from *G.*

nipponicus to *G. aculeatus* has occurred in the recent past (i.e. <0.39 million years

BP).

227   Mitochondrial introgression can be driven by large demographic

disparities between populations with gene flow and is more likely to occur from

a larger to a smaller population [48]. To investigate the demographic disparities

between *G. aculeatus* and *G. nipponicus*, we used pairwise sequential Markov

coalescent (PSMC) on all 26 Atlantic Ocean, Japan Sea and Pacific Ocean

resequenced stickleback genomes. Strikingly, *G. nipponicus* experienced a severe

bottleneck around 0.15-0.3 million years before present (BP) (Fig 1D); mean N_e

fell to 26,422 ± 1,191 at its lowest point. Subsequently after 0.1 million years BP,

G. nipponicus underwent a dramatic population size expansion (Fig 1D): mean N_e

rose to 195,974 ± 28,832 (i.e. ~7.5 times increase from the bottleneck) during

the late Pleistocene. In contrast, the Japanese Pacific Ocean *G. aculeatus*

population has remained relatively stable throughout its history (mean N_e ± SD =

118,150 ± 4,330; Fig 1D, see Fig S3 for bootstrap support). Although the Atlantic

(Fig 1D) and Western Pacific lineages of *G. aculeatus* (Fig S4) also experienced

some population growth during the late Pleistocene, their effective population

sizes remained smaller than that of *G. nipponicus*. Genome-wide averages of

Tajima's *D* also support a recent demographic expansion for *G. nipponicus* (mean

± SD of Tajima's *D* = -0.82±0.45) and stable population size in the Pacific Ocean

(mean ± SD of Tajima's *D* = -0.04 ± 0.63). Taken together, these data indicate that

mitochondrial introgression likely occurred during the late Pleistocene, when *G.*

nipponicus N_e was substantially larger than *G. aculeatus* N_e. This is consistent

with the hypothesis that mitochondrial gene flow from *G. nipponicus* to *G.*

aculeatus has occurred <0.39 million years BP (see above).

250   To explicitly test whether divergence between *G. aculeatus* and *G.*

nipponicus occurred in the presence of gene flow, we used an Approximate

Bayesian Computation (ABC) approach with 1,874 2 kb loci randomly sampled

from across the autosome. We tested five divergence scenarios – isolation (I),

isolation with migration (IM), isolation-with-ancient-migration (IAM), isolation-

with-recent-migration (IRM) and isolation-with-ancient-and-recent-migration

(IARM) – i.e. two discrete periods of contact. Since the results of our PSMC

analyses clearly indicate N_e has varied throughout divergence (Fig 1D), we

performed a hierarchical ABC analysis, first selecting the most appropriate

population growth model (i.e. constant size, population growth and a Japan Sea

bottleneck) within each divergence scenario and then performing final model

selection amongst the best supported divergence/growth model scenarios (see

Supplementary Methods for full specification of models, priors, parameters and

extensive sensitivity testing).

264   Using 20 summary statistics (see Supplementary Methods for a full list

of statistics used) and a neural-network rejection method with 1% tolerance of

simulated datasets, the best-supported divergence scenario was a model of IM

(Fig 2A; Table 1). Parameter estimates from the IM model suggest divergence

between *G. aculeatus* and *G. nipponicus* occurred 0.68 million years ago (median

estimate, 0.18-4.17 million years, lower & upper 95% HPD; Fig 2B). A Japan Sea

bottleneck occurred 0.3 million years ago (0.03-2.21 million years 95% HPD),

reducing N_e to about 20% of the contemporary estimate (Fig 2C, Table S3). Mean

migration rates between the two species were low, and migration from the Japan

Sea into the Pacific Ocean lineage was slightly greater (Fig 2D, Table S3).

Contemporary N_e of the Japan Sea lineage is larger than that of the Pacific Ocean,

although the N_e estimates differed in magnitude from those estimated by PSMC

(Figs 1D and 2C, Table S3).

Identifying admixture between species where they co-occur provides

strong evidence of on-going introgression [7,12]. To address this, we used a

RAD-sequencing dataset with a larger sample size of 245 individuals from the

Atlantic, Pacific and Japan Sea lineages, including previously published data from

Pacific -derived populations in North America [49]. Principal component analysis

(PCA) of allele frequencies at 3, 744 high-quality bi-allelic SNPs showed that,

consistent with our whole genome data, the main axis explaining 20% of the

variance was between *G. aculeatus* and *G. nipponicus* (Fig S5). The secondary axis

explaining 9.49% of the variance was mainly between the Atlantic and Pacific

populations (Fig S5). Importantly, PCA shows a single individual is intermediate

between the Pacific and Japan Sea populations occurring in Akkeshi, the

sympatric site in Hokkaido, Japan where our whole genome-sequenced samples

were collected (Fig 1C). A separate Bayesian analysis for admixture using

STRUCTURE [50,51] found greatest support for $K = 2$ in the Japanese populations

and also identified the putative F_1 hybrid plus individuals with possible recent

admixture at this sympatric site (Fig S6).

Taken together, these data indicate that divergence between the

Japanese *G. aculeatus* and *G. nipponicus* is much older and greater compared to

commonly studied postglacial stickleback species pairs. Despite the great extent

of divergence between Japanese stickleback species, parameter estimates and

observational data suggest that gene flow between them is on-going.

High levels of genome-wide divergence with highly localized signatures of

introgression

Genome-wide differentiation was strikingly high between *G. nipponicus* and *G.*

aculeatus regardless of their geographical overlap: both relative (F_{ST}) and

absolute divergence (d_{XY}) were high (Fig 3A & B, Fig 4, and Figs S7 and S8). The

genome-wide average of F_{ST} between the sympatric species was 0.628; this is

higher than all other studied stickleback species pairs [32–34,52] (see Fig 3C).

Despite consistently high divergence, both F_{ST} and d_{XY} values were significantly

lower where the two species occur in contact (Table 2, Figs 3A & B, 4, S7 and S8;

10,000 replicate permutation tests on 10 kb windows: $P < 2.2 \times 10^{-16}$ for both

statistics), consistent with the presence of gene flow in sympatry.

A more fine-scale analysis of genome-wide divergence based on 10 kb

non-overlapping windows revealed that the high baseline divergence between *G.*

nipponicus and *G. aculeatus* is interspersed by regions of low differentiation in

both F_{ST} and d_{XY} genome scans (Figs 4 top two panels, S7 and S8). Processes such

as background selection that alter within-population diversity can bias relative

measures of differentiation such as F_{ST} [7,21,22,53]. Furthermore, absolute

measures like d_{XY} can take a long time to reach equilibrium between populations

following divergence, meaning a lower power for detecting recent introgression

events [7,26]. Therefore, to better identify genomic regions of recent

introgression, we calculated two independent measures of introgression. The

first of these was G_{MIN}, the ratio of the minimum d_{XY} to the average d_{XY} [26].

Under strict isolation, G_{MIN} is the lower bound of divergence time between two

populations, whereas when introgression occurs, G_{MIN} reflects the timing of the

most recent migration [26]. The second measure was f_d, an estimate of the

proportion of introgressed sites in a genome window, calculated using a four

population ABBA-BABA test [54]. G_{MIN} is more effective at identifying recent, low

level gene flow than either F_{ST} or d_{XY} but by definition it is unable to detect

genomic regions where complete introgression has occurred [26], which can

however be detected using f_d. Importantly, both measures are robust to variation

in recombination rate [26,54]. Combining these two statistics therefore allows us

to identify both low-level (G_{MIN}) and strong introgression (f_d).

Focusing on between species comparisons, mean ($\pm$ SD) G_{MIN} measured

from 10 kb non-overlapping windows was greater in allopatry than sympatry

(Japan Sea vs. Atlantic: 0.876 $\pm$ 0.071; Japan Sea vs. Pacific: 0.857$\pm$0.103;

randomization test $P < 2.2 \times 10^{-16}$; Fig 4). Mean f_d was also greater when the

species overlapped (JS vs. AT: -0.0031 $\pm$ 0.0540; JS vs. PO: 0.0039$\pm$0.0328; $P <$

2.2×10^{-16}; Fig 4), and both statistics are more strongly negatively correlated in

sympatry (Fig S9) supporting gene flow between *G. nipponicus* and Japanese

populations of *G. aculeatus*.

Genomic regions of low G_{MIN} (i.e. G_{MIN} valleys) may indicate recent

introgression. We identified genome windows with low G_{MIN} values using a

Hidden-Markov classification model [55] and an outlier approach based on

permutations of variant sites (Fig S10). We then clustered 10 kb outlier

windows occurring within 30 kb of one another into putative G_{MIN} valleys. G_{MIN} in

particular may be susceptible to false positives as a result of ILS. However, lower

d_{XY} and higher f_d in sympatric G_{MIN} valley windows compared to the genomic

background suggests ILS alone does not explain the patterns observed here (Fig

S11; randomization test, $P < 2.2 \times 10^{-16}$ in both cases). These regions of

introgression were more common in the genome when the two species

overlapped, with 637 valleys in sympatry (JS-PO comparison) compared to 337

in allopatry (JS-AT comparison) (randomization test, t = 5.35, P < 2.2 x 10^{-16}) and

a greater number of valleys per chromosome (Fig 5A), although mean valley size

did not differ significantly (77.6 kb and 75.4 kb in sympatry and allopatry

respectively, P = 0.82). Interestingly, 225 valleys were shared between JS-PO and

JS-AT comparisons (Fig. 4). These shared valleys may indicate ILS but they may

also reflect introgression from Pacific Ocean to Japan Sea, where one or a few

Japan Sea individuals carry haplotypes derived from Pacific Ocean and therefore

are also similar to Atlantic Ocean haplotypes too. However, a larger number of

valleys (412 valleys) were unique to the JS-PO comparison, where introgression

might occur from Japan Sea to Pacific Ocean.

A similar geographical comparison of peaks of f_d between species was

not possible, due to the much lower genome-wide distribution of f_d between G.

$nipponicus$ and the Atlantic $G.$ $aculeatus$ (Fig 4). Nonetheless, Hidden-Markov

classification identified 823 f_d peaks occurring between $G.$ $nipponicus$ and Pacific

$G.$ $aculeatus$ (Fig S12). If the f_d peaks are mainly indicate introgression from

Pacific Ocean to Japan Sea, d_{XY} between Japan Sea and Atlantic Ocean is expected

to be lower in these regions compared to the genome background, as Japan Sea

fish carry haplotypes derived from the Pacific Ocean, which in turn are similar to

the Atlantic Ocean haplotypes. While JS-AT d_{XY} was lower in f_d peaks compared to

the genome background (one-tailed permutation test, P < 2.2 x 10^{-16}), this

difference was not very clear (Fig. S13). In contrast, if introgression occurred

mainly from Japan Sea to Pacific Ocean, d_{XY} in the PO-AT comparison should

increase in f_d peaks relative to the genome background, as Pacific Ocean fish

carry Japan Sea-derived haplotypes, which are divergent from the Atlantic Ocean

haplotypes. We clearly observed this pattern ($P < 2.2 \times 10^{-16}$; Fig. S13);

suggesting that introgression from Japan Sea to Pacific Ocean may be more

predominant than the opposite direction.

377   To further investigate the direction of gene flow, we used partitioned D

statistics (an extension of the four population test – see Fig. S14), which tests the

excess of shared derived alleles using five, rather than four populations [56]. To

this end, we added an allopatric Japan Sea population (collected from Lake Shinji,

a brackish lake at the Japan Sea coast of southern Honshu). A positive D_{12}

statistic is proposed to indicate the predominance of introgression from P3 to P2

(Fig. S14) [56]. When P3 was set to Japan Sea (where $P3_1$ is sympatric and $P3_2$ is

allopatric with the Pacific Ocean) and P2 to Pacific Ocean (see Fig. S14B), D_{12} was

significantly positive in f_d peaks (one-tailed permutation test, $P < 2.2 \times 10^{-16}$). In

contrast, when we rotated the populations at the tips – i.e. setting P2 to

sympatric Japan Sea, $P3_1$ to Pacific Ocean, and $P3_2$ to Atlantic Ocean (see Fig

S14C), D_{12} was not positive, consistent with the suggestion that introgression is

occurring mainly from Japan Sea to Pacific Ocean. However, the resolution of

partitioned D statistics has been criticized [57]; positive D_{12} can also be caused

by introgression from the Pacific Ocean (P2) to the common ancestor of the

sympatric and allopatric Japan Sea populations ($P3_1$ & $P3_2$). To overcome this

issue, we calculated D_{FOIL}, which also uses a five-population test but accounts for

all possible introgression events [57]. When P_1 = sympatric Japan Sea, P_2 =

allopatric Japan Sea, P_3 = Pacific Ocean, and P_4 = Atlantic Ocean (Fig. S15), D_{FOIL}

clearly indicated the presence of ancestral introgression (239 out of 4,236 100

kb-windows) between the Japan Sea ancestor (P_{12}) and the Pacific Ocean (P_3)

(see Fig. S15). However, we found only a few windows showing unidirectional

introgression (6 loci in total), precluding conclusion of the predominant

direction of introgression using this analysis (Fig. S15). This low sensitivity may

be due to the fact that structuring in the Japan Sea lineage is low [58] – i.e. recent

divergence time or high intraspecific gene flow.

Characterization of genomic regions of introgression

To investigate whether introgression co-varies with recombination rate, we used

a previously published recombination map from an Atlantic *G. aculeatus* cross

[59] to interpolate genome-wide recombination rate variation (see Methods).

We detected a negative correlation between recombination rate and G_{MIN} and a

positive correlation with f_d (Pearson's correlation, G_{MIN}: r = -0.17, $P < 2.2 \times 10^{-16}$;

f_d: r = -0.08, $P < 2.2 \times 10^{-16}$, Fig S16). Accordingly, mean recombination rate for

putatively introgressed regions was over two times higher than the genome

background (G_{MIN}: valley = 8.98 cM/Mb, non-valley = 3.99 cM/Mb; f_d: peak = 9.64

cM/Mb, non-peak = 4.16 cM/Mb; randomization test $P < 2.2 \times 10^{-16}$ in both cases;

Fig 5B).

415   Sex chromosomes likely played an important role in speciation between

G. aculeatus and *G. nipponicus*. A fusion between Y and chrIX means that chrIX

segregates as a neo-sex chromosome in *G. nipponicus* but not *G. aculeatus* which

only carries the ancestral and shared sex chromosome, chrXIX [36,40]. The

divergent XY (*G. aculeatus*) and X_1X_2Y (*G. nipponicus*) systems means that

recombination is reduced for chrIX and chrXIX in hybrids carrying the neo-Y

[40]. Given this recombination rate reduction and previously identified QTL for

traits involved in reproductive isolation that map to chrIX and chrXIX [36,40], we

tested whether recent introgression (i.e. measured using G_{MIN}) was reduced in

this part of the genome relative to the autosome. For this, we repeated our

analyses using females only (5 Japan Sea and 6 Pacific Ocean). The number and

density of valleys was lowest on the neo-sex chromosome, chrIX (16 valleys or

0.8 valleys per Mb) but not the ancestral sex chromosome (chrXIX, see Table S4).

428   Finally, we investigated the nature of introgression between the two

species. We first asked whether introgression occurs more frequently in genic or

non-genic regions. We identified 3,261 genes occurring in G_{MIN} valleys and 2,958

genes from f_d peaks between sympatric *G. aculeatus* and *G. nipponicus*; 60% of

genes identified were found in both types of introgressed window, whereas 23%

occurred only in G_{MIN} valleys and 15% only in f_d peaks (see Fig S17). Irrespective

of the method used to detect putatively introgressed regions, the number of

genes identified was greater than the number expected by chance ($P < 0.0001$

based on a null distribution generated from 1,000 random samples of the

genome). This suggests that introgression may be more likely in genic regions of

the genome than non-genic regions.

439   To further investigate the functional enrichment of the genes occurring

in regions of introgression, we performed gene ontology (GO) analysis on 2,310

G_{MIN} valley and 2,217 f_d peak genes with orthologs in the human genome.

Enriched GO terms for f_d peaks included immune response, metabolic processes

and chromatin assembly, while enriched GO terms for G_{MIN} valleys included

major histocompatibility complex (MHC) protein and metabolic processes (Table

S5 & S6).

Discussion

448 **Japanese stickleback speciation has occurred in the face of on-going gene**

449 **flow**

450 Determining the demographic and evolutionary history of species pairs is an

451 important first step for understanding how speciation has unfolded in any

452 system [7,12]. Our present study has produced several lines of evidence

453 indicating that divergence between the Japanese sticklebacks has occurred in the

454 presence of gene flow.

455 Firstly, contrasting mitochondrial and nuclear genome phylogenies

456 show that mitochondrial introgression has occurred from *G. nipponicus* into *G.*

457 *aculeatus* at some point in the last 0.39 million years. Our mitogenome

458 phylogeny confirmed previous findings that there is no mitochondrial

459 divergence between the *G. nipponicus* and Japanese populations of *G. aculeatus*

460 [44,45]. This is in contrast to our nuclear autosomal phylogeny which showed

461 that majority of the genome supports a clear split between *G. nipponicus* and *G.*

462 *aculeatus* occurring in Japan and that the latter shares a more recent common

463 ancestor with Atlantic European *G. aculeatus* populations. In short, mitogenome

464 data clusters the *Gasterosteus* lineages by geography, while the nuclear data

465 clusters them by species. Introgression is the most likely explanation for this

466 mitonuclear discordance as the lower effective population size of the

467 mitochondrial genome relative to the autosome makes ILS unlikely, particularly

468 over a 1 million year time-scale of divergence.

469 Disparities in effective population size between lineages are a common

470 cause of unidirectional mitonuclear introgression with introgression likely

471 occurring from a larger to a smaller population [48]. Our reconstruction of

472 temporal variation in effective population size using PSMC showed a rapid

population explosion *of G. nipponicus* during the late Pleistocene that created a

large demographic disparity with the *G. aculeatus* Pacific Ocean lineage, although

it should be noted that admixture can increase effective population size

estimates when using PSMC [60,61]. Reasons for this population growth remain

unclear but it is surprising, particularly since *G. nipponicus* is unable to colonise

freshwater environments [42,58], which might be expected to increase effective

population size due to meta-population dynamics. A possible explanation is that

a lack of dependence on freshwater for spawning [41,62,63] and greater foraging

efficacy on marine prey [42] means *G. nipponicus* is better adapted for exploiting

an abundant marine environment. Unidirectional mitochondrial introgression

might also be caused by female mate choice [64]. Our previous behavioural

studies indicate that Japan Sea females often mate with Pacific Ocean males,

while Pacific Ocean females rarely mate with Japan Sea males [36,37]. Hybrid

females from Japan Sea female and Pacific Ocean male crosses are fertile [37]

and will carry Japan Sea mitochondrial DNA. Backcrossing of these hybrids to

Pacific Ocean males would result in unidirectional mitochondrial introgression

from the Japan Sea to Pacific Ocean.

Secondly, our ABC analysis supported a model of isolation with

migration. Previously, it has been speculated that the Japan Sea stickleback

diverged largely as a result of geographical isolation in the Sea of Japan caused

by sea level fluctuation during the early Pleistocene [37,39]. Using ABC, we were

able to explicitly test several divergence hypotheses in a statistical framework

[65]; our findings suggest that gene flow has likely occurred throughout the

majority of divergence history. It should be noted that ABC and most established

demographic inference methods perform poorly when resolving the timing of

gene flow between lineages [66,67]. Therefore, one caveat to the interpretation

of our ABC results is that we cannot rule out the possibility that the two species

diverged in repeated cycles of contact (i.e. akin to our IARM model which had the

second highest level of support; Table 1), but these periods of contact were

simply too close in time. Nonetheless, the pooled posterior probabilities from the

analysis overwhelmingly support a model of divergence with gene flow

irrespective of the timing or nature of the actual speciation event. The presence

of extant recent hybrids in sympatry also strongly indicates that hybridization is

still on-going. We observed a probable F_1 hybrid in the wild and several other

individuals with evidence of hybrid ancestry in our RAD-seq dataset, consistent

with previous studies that observed wild caught hybrids [36,68]. This provides

direct observation of admixture in the wild.

510   Finally, lower levels of genome-wide divergence (both F_{ST} and d_{XY})

between sympatric pairs compared to allopatric pairs also indicate the presence

of gene flow. Our G_{MIN} and f_d genome scans showed a higher number of

putatively introgressed regions between *G. nipponicus* and Japanese Pacific *G.*

aculeatus than between *G. nipponicus* and Atlantic *G. aculeatus*, suggesting that

introgression has been occurring even after the Atlantic and Pacific stickleback

populations diverged approximately 400,000 years BP. Our partitioned *D*

statistics demonstrated that gene flow from *G. nipponicus* into Japanese Pacific *G.*

aculeatus may be more predominant than the opposite direction in sympatry.

High genomic divergence at a late stage of speciation with gene flow

Compared to young species pairs, less is known about the patterns of genomic

differentiation at more advanced stages of speciation with gene flow. Our study

provides insight on this under-represented stage of divergence. Our ABC

analyses placed the estimated divergence time of *G. aculeatus* and *G. nipponicus*

at 0.68 million years BP. Similarly, our Bayesian coalescent analysis of

mitogenome divergence revealed a 1.3 million year split between the Japanese

and Atlantic-Pacific *Gasterosteus* mitochondrial clades. An older divergence time

is somewhat expected from the mitochondrial genome, given its fourfold lower

effective population size [48]. Because splitting of mitochondrial lineages can

occur by geographical structuring without speciation [69,70], our estimate of

mitochondrial divergence may reflect more ancient geographical structuring that

occurred prior to species divergence. Nonetheless, both mitochondrial and

nuclear split estimates suggest that divergence between *G. aculeatus* and *G.*

nipponicus occurred well before the end of the last glacial period. Therefore the

Japanese stickleback system is older than all other previously examined

postglacial sympatric or parapatric species pairs, which have typically diverged

within the last 20,000 years [30].

The Japanese stickleback system also has a mean genome-wide F_{ST} value

of 0.628, higher than any other sympatric or parapatric stickleback species pair

studied so far (Fig 3C); placing this pair at the furthest end of the speciation

continuum. A recent meta-analysis quantifying genetic differentiation pointed to

a lack of evidence of sympatric or parapatric species pairs with F_{ST} between 0.3

and 0.7[8]. The Japanese species pair is therefore an example of an

underrepresented sympatric species pair that maintains high divergence in the

face of gene flow. Within the stickleback species complex, this high divergence is

in stark contrast to the typical low baseline divergence interspersed with regions

of high differentiation observed in more commonly studied species pairs such as

lake-stream or freshwater-anadromous pairs [16,33,71].

The primary explanation for the observed elevated divergence is most

likely the more ancient divergence time of the Japan Sea-Pacific Ocean species

pair compared to postglacial species pairs [16,72]. However, the results of our

demographic analyses indicate that high divergence is not due to a long period of

allopatric isolation without gene flow, contrary to what has previously been

suggested [37,39]. This is important, as failing to account for variation in

evolutionary history among species pairs placed on a continuum will obscure the

processes leading to higher differentiation as speciation progresses. A further

explanation for the high genomic divergence is the presence of strong isolating

barriers between the Japan Sea and Pacific Ocean sticklebacks. Total

reproductive isolation (0.970) is greater than in all postglacial species pairs

(0.716-0.895) [43] and arises from a combination of habitat [41,42], temporal

[62] and sexual isolation, and hybrid sterility [36,37]. Recent theoretical studies

have shown that selection on many barrier loci in the face of gene flow may

result in a transition from low to high differentiation as a result of 'genome-wide

congealing' [10,19]. It is important to note that we lack evidence that such a

transition might explain the high differentiation we see here relative to the rest

of the stickleback continuum (Fig 3C). However, pervasive selection on multiple

isolating barriers in the Japanese stickleback system does suggest that this

process could have contributed to the high genomic differentiation we observe

[5].

Localized introgression at a late stage of speciation with gene flow

Our study has also demonstrated two important signatures of introgression in

the Japanese sympatric stickleback pair. Firstly, levels of background genome

differentiation between $G.\ aculeatus$ and $G.\ nipponicus$ estimated by F_{ST} were

lower in sympatry compared to allopatry. The higher overall genetic

differentiation between $G.\ nipponicus$ and Atlantic $G.\ aculeatus$ is likely due to

genetic drift and local adaptation and the fact that these two lineages have never

overlapped geographically. Secondly and strikingly, we identified small regions

of localised introgression dispersed throughout the genome when $G.\ nipponicus$

and $G.\ aculeatus$ co-occur in sympatry. These introgression regions were

measured using $G_{MIN,}$ the ratio of minimum d_{XY} to mean d_{XY}, where low values

indicate introgression [26] and $f_{d,}$ the proportion of introgressed sites in a

genome window [54].

Several methodological issues might influence these measures of

introgression. Firstly, both G_{MIN} and f_d are sensitive to sample size; fewer

individuals will mean rare haplotypes have a lower sampling probability.

However by re-conducting our analyses using only females, a much smaller

sample size than our main analysis, we still identified clear signals of

introgression. Secondly, G_{MIN} will be biased downwards if a recently backcrossed

individual is included in the dataset. All Japanese $G.\ aculeatus$ and $G.\ nipponicus$

used in the study were identified as 'pure' individuals with genotyping at

multiple microsatellite loci prior to resequencing [36,40]. Furthermore, we

examined the identity of the two haplotypes producing the lowest value of d_{XY} in

G_{MIN} valleys and confirmed that these came from different individuals in each

case (data not shown). Nonetheless, even a single haplotype segregating in a

population due to ILS can lower the value of G_{MIN}; i.e. ILS could produce small,

interspersed G_{MIN} valleys. However, ILS cannot explain why more introgressed

regions occur in sympatry (Fig 5A) or why there is a clear association with an

increased proportion of introgressed sites (i.e. f_d) and G_{MIN} (Fig S9 & S13).

What then underlies the localised pattern of introgression we observe?

One possible explanation is the fact that many isolating barriers are involved in

reproductive isolation [36,43]. Although the genomic basis of these isolating

barriers remains unknown, it is likely that barrier loci occur throughout the

genome; pervasive selection at multiple loci is expected to limit the extent of

introgression at this scale [73]. The strength and extent of selection at a barrier

locus and genomic regions linked to it is proportional to recombination rate [73].

Recombination determines effective migration rate [74]; when recombination is

high, neutral and adaptive loci linked to the target of negative selection in the

recipient population have a greater probability of escaping removal and so their

probability of introgression is greater [3]. Selection has a higher efficiency in

these high recombination rate regions due to increased effective population size

– therefore deleterious introgression is also more likely to be removed. The

expectation then is that signatures of introgressed neutral or adaptive alleles are

most likely to persist in regions of the genome where recombination rate is

sufficiently high enough, and indeed, the positive association between

introgressed regions and recombination rate we observed supports this (Fig 5B,

Fig S15). Introgression is typically lower on sex chromosomes relative to

autosomes in multiple taxa due to the effects of reduced recombination and

greater exposure to selection in the hemizygous sex [75]. The sex chromosomes

play an important role in the Japanese stickleback system, harbouring QTL for

hybrid sterility and behavioural isolation [36]. Consistent with this, we observed

lower introgression on the neo-sex chromosome (Fig 5E & F), although we

cannot exclude the possibility that the fusion occurred more recently than the

speciation event, so the opportunity for introgression on the neo-sex

chromosomes was simply low relative to the rest of the genome. Taken together,

our findings suggest that strong divergent selection and recombination rate

variation may determine the localised signature of introgression in the genome.

628   The nature of gene flow in the Japanese stickleback system may also

give some clues as to why we observe such highly localised introgression. One

possibility is that a proportion of the introgression we detected is adaptive; i.e. it

is maintained because of either directional or balancing selection. Adaptive

introgression has been detected in a wide range of taxa [76], including humans

[77]. However, the expected signatures of the process remain unclear –

especially when introgression is widespread in the genome, as is possibly the

case here. Our GO analyses suggest an enrichment of immune response genes,

including MHC genes, and metabolism genes in introgressed regions. Immune

genes have been identified as being under balancing selection between

hybridising taxa, particularly plants [78] and birds [79]. Several genes involved

in metabolism are also reported to be under balancing selection in humans [80].

Furthermore, recent analysis suggests that negative frequency dependent

selection might result in introgression of rare MHC alleles between divergent

stickleback ecotypes [81]. Further research is necessary to directly test whether

this process might explain introgression in the Japanese stickleback system.

Conclusion

Much of our knowledge of how genomic differentiation builds along the

speciation continuum is drawn from studies focusing on young, allopatric or

completely reproductively isolated species pairs. Very few examples of species

pairs at a later stage of divergence with on-going gene flow have been

investigated. Here, we have shown that the Japan Sea and Pacific Ocean species

pair exemplifies this under-represented stage of speciation and is situated at the

further end of the stickleback species continuum. The high genomic

differentiation between the species may be due to a more ancient divergence

time than previously studied postglacial species pairs, selection on multiple

isolating barriers or a combination of the two. Despite high differentiation, gene

flow is on-going between the species and we identified localized signatures of

introgression throughout the genome. Although the localized nature of the

introgression remains unclear, selection – either directional or balancing – may

play some role in promoting it. Overall, our study demonstrates that high levels

of genomic divergence can be established and maintained in the presence of

gene flow. Further genomic studies on more species pairs at late stages of

speciation with gene flow will help to understand the generality of the patterns

seen here.

Materials and Methods

Ethics Statement

All animal experiments were approved by the institutional animal care and use

committee of the National Institute of Genetics (23-15, 24-15, 25-18).

Sample collection, whole genome resequencing and RAD sequencing.

Collection and sequencing of all Japanese individuals used for whole genome

resequencing has been described previously [40] except the allopatric Japan Sea

fish. Briefly sympatric populations were captured from the Akkeshi system in

Hokkaido, Japan in 2006 (Fig 1C). The allopatric Japan Sea female was collected

in Lake Shinji in March 2014. The outgroup species, *G. wheatlandi* was captured

from Demarest Lloyd State Park, MA, USA in 2007, as described previously [40].

Libraries were constructed with TruSeq DNA Sample Preparation Kit (Illumina)

and whole-genome 100 bp paired-end sequencing was performed on an Illumina

HiSeq2000 at the National Institute of Genetics (sympatric JS and PO) and

Functional Genomics Facility, NIBB Core Research Facilities (allopatric JS) [40].

Whole genome sequencing of North American marine and stream populations

collected from Little Campbell River, BC, Canada was reported previously

[52,82]. For the six Atlantic *G. aculeatus* individuals (North Sea) included in the

study, we used previously published sequences [46].

Japanese individuals used for RAD sequencing have been previously

described elsewhere [58]. Samples used for RAD sequencing from the Atlantic

lineage were collected from across Ireland in 2009-2011 [70,83]. DNA was

extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA,

USA). Single digest RAD-sequencing was performed using *Sbf*I following a

standard protocol [84]. RAD library preparation and sequencing was conducted

using a 100bp single-end Illumina HiSeq by Floragenex (Oregon, USA).

Accession numbers, sample names and locations for all genome and

RAD-seq samples are listed in Table S6.

Whole genome alignment and variant calling. Sequence reads were mapped

to the stickleback reference genome using CLC Genome 8.0 as described

previously [40]. Alignments were exported as bam files and were sorted and

indexed using samtools 1.2 [85]. Variant calling was carried out in two different

phases. The first phase was used to call consensus bases at all sites (i.e. variant

and invariant) across the genome for all 27 resequenced individuals and the

outgroup (*G. wheatlandi*). Mapped reads from all individuals were piled-up using

samtools *mpileup* and called against the stickleback BROAD reference genome

using the bcftools 1.2 consensus caller without any filters. This consensus call

produced a vcf file with a base call for every position in the genome for all

samples (27 + 1 outgroup). Consensus calls from this phase were used in later

demographic inference using PSMC and ABC. The aim of the second, more

stringent variant calling phase was to produce a subset of high-quality

polymorphic SNPs with which to examine genome-wide differentiation between

the Japan Sea, Pacific and Atlantic Ocean lineages. We used bcftools to filter the

consensus-call vcf for these three lineages, only retaining sites with a Phred

Quality score >10, and with a maximum individual read depth of 200. Additional

downstream filters were applied prior to estimating population genomic

parameters from this subset vcf (see below).

Mitochondrial genome divergence. To estimate divergence times based on

mitochondrial DNA, we performed Bayesian coalescent analysis using BEAST

v2.2.1 [86]. From our resequencing data, we extracted the whole mitochondrial

genome from the 26 Japan Sea, Pacific and Atlantic Ocean individuals. We also

downloaded two *G. wheatlandi* whole mitogenomes as outgroups (NCBI

accession numbers: AB445129 & NC011570). Note that due to poor sequence

coverage across the mitogenome we excluded our own re-sequenced *G.*

wheatlandi individual here. Mitogenomes were aligned using MUSCLE v3.81.3

[87] resulting in a 16,549 bp final alignment.

Although there is a considerable three-spined stickleback fossil record,

it is unfortunately of little use for providing fossil calibration dates for splits

within the *Gasterosteus* genus [88,89]. However biogeographical events can also

be used to calibrate node estimates and as such we used a normal prior (mean =

1.5 million years, SD = 0.75 million years) on the split between the Japan Sea and

Pacific Ocean *G.aculeatus* lineages. We provided a further normal prior on the

split date between the Pacific and Atlantic Ocean mitochondrial lineages (mean =

0.5 million years, SD = 0.25 million years). The latter prior distribution was

intentionally made wide to reflect uncertainty surrounding this estimate. Initial

analyses with BEAST indicated that marginal prior distributions for node ages

did not behave as specified in the model and instead returned extremely recent

divergence times with low likelihood support. This is a common bias in

coalescent divergence time dating and use of a calibrated prior removed this

issue [90,91]. As a result, we performed all further analyses with a calibrated

Yule prior. Incorrect choice of molecular clock model can seriously bias

coalescent estimates of lineage divergence times and so care must be taken to

ensure the appropriate model is chosen [92,93]. We used path-sampling analysis

in BEAST to estimate model marginal likelihoods for three different clock models

– strict, relaxed lognormal and relaxed exponential. For each model, Markov

chain Monte Carlo (MCMC) was run for 5×10^7 with 60 steps and marginal

likelihoods were calculated using BEAST. We then ran the final model using two

10^8 independent MCMC runs. Runs were assessed in TRACER [94] to ensure

convergence and that ESS values > 200 – i.e. the posterior was adequately

sampled. Independent runs were then combined to produce posterior estimates

of divergence times and substitution rates.

Nuclear phylogenetic analysis and genealogical sorting index (*gsi*). To

investigate nuclear phylogenetic discordance, we constructed maximum

likelihood trees from consensus sequences for non-overlapping 10, 50 and 100

kb sliding windows following Martin et al [28]. The best-fit tree was estimated

for each window using RAxML with a 'GTRGAMMA' model and a random number

seed [95]. Trees were classified using a custom R script

(https://github.com/markravinet) that binned trees based on whether they

matched three different topologies; species, geography, ancestral – or were

unresolved. For the species category, all Atlantic, Pacific and Japan Sea

individuals form separate monophyletic groups; for the geography category,

Japan Sea and Pacific Ocean form a monophyletic group separate to the Atlantic

Ocean; trees where the Atlantic Ocean grouped monophyletically with the Japan

Sea were classed as ancestral. Trees that did not fit any of these categories were

classified as unresolved. Following categorisation, trees were then standardised

to ensure equal branch lengths using the *compute.brlen* function from the

Phytools R package [96] and were finally visualised for each gene tree class using

the *densiTree* function in the R package *Phangorn* [97].

767   We additionally used the non-overlapping Maximum Likelihood

phylogenies to calculate genealogical sorting index (*gsi*) [47]. We used a custom

R script to estimate *gsi* across the autosome of 26 resequenced individuals. This

allowed us to compare autosomal signals of introgression with a reduction in *gsi*.

Population size change over time. We used PSMC to estimate fluctuations in

effective population size over time [98]. PSMC uses the density of heterozygote

sites across a single diploid genome to estimate blocks of constant TMRCA that

are split by recombination and then uses these to infer ancestral effective

population sizes (N_e) over time [61,98]. Since PSMC can only analyse a single

genome at a time, we ran the program separately on each of the 26 resequenced

genomes from Japan Sea, Pacific and Atlantic Ocean lineages. We additionally ran

the analyses for a reseqeuenced genome of a marine ecotype fish from Little

Campbell River, Canada as representatives of the Eastern Pacific. Consensus

sequences for each genome were converted to PSMC format - a binary format

indicating the presence/absence of heterozygous sites within a specified

window. We used 100 bp windows, requiring a minimum of 10,000 'good' sites

to be present on a genome scaffold in order for it to be included; heterozygous

sites with a Phred Quality score <20 were removed. We then ran PSMC for 30

iterations with a maximum coalescent time of 15 (measured in units of $2N_0$

where N_0 is ancestral population size). Due to the difficulty of inferring past

effective population sizes across this time, PSMC requires the user to provide

intervals which are combined to produce the same effective population size [98].

Since this method is least accurate for recent (i.e. < 20 kyr BP) and more ancient

periods [98], we estimated N_e for 47 intervals, combining the first four and the

last three using the command "4+19*2+4". To scale our results from coalescent

units, we assumed a generation time of 1 year [99] and used an autosomal

substitution rate of 7.1 x 10^{-9} [100]. Finally, to provide confidence intervals for

our N_e estimates, we performed 100 bootstraps on 500 kb segments for each

analysis.

Approximate Bayesian Computation (ABC). We used ABC to test different

scenarios of divergence between the Japan Sea and Pacific Ocean lineage and to

estimate demographic parameters, such as divergence time and migration rate,

under these scenarios.

To obtain loci suitable for our ABC analysis, we randomly sampled

nuclear loci from the 20 resequenced genomes (sympatric Japan Sea and Pacific

Ocean) using a similar approach to Nadachowska-Brzyska et al [14]. Using a

custom R script, we produced a bed file of reference genome coordinates for 2 kb

loci randomly sampled at 125 kb intervals; resulting in 2,378 loci potential per

individual. Using a custom python script, we called consensus sequences for each

locus from the consensus vcf. This script created two haplotype sequences for

each of the 2 kb loci, randomly assigning heterozygous variants to one of the two

called haplotypes; this step allowed us to use unphased data for demographic

analyses [66,101]. We then further filtered these loci to include only those that

occur on autosomes, with >1,000 bp sequence and a minimum of 30% coverage

(i.e. > 70% bases were called) for all 20 individuals. This resulted in a final

dataset of 1,874 loci. Functions and scripts for generating coordinates and

extracting and filtering consensus sequences are available on GitHub

(https://github.com/markravinet/genome_sampler).

Following Robinson et al [66] we used a custom R-based control script

and msABC [102] to perform simulations, calculate summary statistics and

quantify their distribution across the genome in a single step. This approach

offers considerable flexibility in establishing prior probability distributions for

each of the estimated parameters. Furthermore, given the large size of our

dataset (i.e. approximately 2,000 loci for 20 individuals) each simulation

produces a large amount of data, making storage a challenge. Using R to interface

with msABC allowed us to greatly reduce the required data storage.

For each of the 15 models we performed 10^6 simulations. We used a

combination of GNU Parallel [103] and independent runs across multiple

computing cores to reduce analysis speed to approximately 1 day per model

(scripts and additional instructions available on Github:

https://github.com/markravinet).

We initially ran our simulations to produce all the available summary

statistics that msABC calculates. However since summary statistic choice can

greatly alter the outcomes of ABC analyses [104,105], all post-simulation ABC

analyses were conducted using subsets of 29, 20 and 12 summary statistics.

Following completion of the simulation step, we performed a neural-network

rejection step on log-transformed parameter estimates with a tolerance of 0.01

using the abc function in the R package *abc* [106]. Posterior probability was

estimated for each model using the R *abc postpr* function with multinomial

logistic regression for a range of tolerance values representing 0.1%, 0.5%, 1%

and 3% of the simulated data (i.e. 1,000, 5,000, 10,000 and 30,000 datasets

respectively). In keeping with a hierarchical analysis [14], we performed two

rounds of model selection. We first chose the growth model with the highest

posterior probability within each divergence scenario. Following this, we

performed model selection on the five models with the highest support within

each divergence category.

In order to ensure our ABC approach was reliable, we used pseudo-

observed datasets (PODs) to assess how well we could discriminate between

different divergence scenarios. Essentially, this involves randomly selecting a

series of simulated dataset from a known model (hence pseudo-observed) and

then rerunning the model selection procedure to see whether the true model

could be recovered. For further details of our POD-based sensitivity analysis and

ABC approach, see the *Supplementary methods* section.

Detecting genome-wide divergence and recent introgression. Weir and

Cockerham's F_{ST} [107] was calculated using 10 and 50 kb non-overlapping

windows with VCFtools 0.113 [108]. To calculate haplotype-based statistics such

as d_{XY}, G_{MIN} and f_d, we used a modified version of a python script used by Martin

et al [28]. In addition to our main filters on the dataset (see *Genome Alignment*

and Variant calling), we only calculated these haplotype-based statistics for

windows with >50% of useable bases – i.e. >5,000 sites within a 10 kb sliding

window. For autosomal statistics, all individuals were included in the analyses.

For comparing the ancestral (chrXIX) and neo-sex chromosomes (chrIX) with

autosomes (Fig. 5E and 5F), we re-ran the analyses of all chromosomes using

only females. In addition to 10 kb windows, we also performed analyses for non-

overlapping 50 kb windows to aid visualisation; the results from all analyses

were then combined into a single dataset using custom R scripts.

We calculated recently established statistics, G_{MIN} and f_d, for detecting

introgression between divergent lineages [26,54]. G_{MIN} is particularly suited for

identifying recent, low frequency introgression [26] whereas f_d can also identify

stronger, high frequency introgression events [54]. Importantly, both methods

are robust to variation in recombination rate variation. Initial genome scans

conducted using G_{MIN} revealed a series of 'valleys' present across the genome.

Detection of such valleys, like genomic islands of divergence, presents a variety

of methodological issues. Firstly, how do we determine that G_{MIN} valleys are not

due to stochastic variation in genealogy amongst loci? Secondly, how do we

measure the size and distribution of valleys of introgression? Finally, how can we

determine a null or expected distribution of valleys across the genome to test for

the under- or overrepresentation of valleys?

878   To deal with each of these issues in turn, we first performed

chromosome-specific permutations to identify the null distribution of the value

of G_{MIN}. Specifically, we shuffled the nucleotide sequence of each chromosome

100 times and estimated G_{MIN} for 11 different sliding window sizes (5,000, 5,500,

6,000, 6,500, 7,000, 7,500, 8,000, 8,500, 9,000, 9,500 and 10,000 kb),

representing the distribution of useable sites from the empirical dataset. We

then used the lower 99 percentile of the permutations to determine the mean

value of G_{MIN} below which a window could be classified as a valley. Identifying

the boundaries of divergent genome regions is somewhat subjective and open to

potential bias [109]. To account for this, we used a hidden Markov-model (HMM)

approach to classify windows into two states - i.e. valleys or non-valleys – and to

estimate the probability of state transition. Following Soria-Caracasco *et al.* [55],

we used the R package HiddenMarkov [110] on a logit transformed G_{MIN}

distribution. Transition probabilities between the two states were symmetrical

with an emphasis on it being difficult to transition between states (p = 0.1) but

relatively easy to remain within a state (p = 0.9). Since valleys are relatively rare

in the genome, we set our models to start in the non-valley state and we

provided estimated parameter values for the states based on the empirical

distribution. Our permutation test to find the 99 percentile therefore also

provided an empirical basis for identifying valleys. HMM estimates were run for

both the sympatric and allopatric comparisons using the *baumwelch* function to

estimate parameters using the Baum-Welch algorithm and the *viterbi* function to

estimate the sequence of states using the Viterbi algorithm. We used a similar

approach to identify f_d peaks but we instead performed the analysis using

untransformed f_d values only in the sympatric Japanese *G. aculeatus* and *G.*

nipponicus comparison.

RAD-seq data processing and admixture analysis. RAD sequence reads were

demultiplexed and processed using the *process_radtags* module of Stacks 1.30

[111]. All reads were trimmed to 90 bp and any read where the average Phred

quality score dropped below 10 in a 9 bp sliding window was discarded.

Following filtering, reads were mapped to the Roesti *et al.* [59] build of the *G.*

aculeatus genome using GSNAP [112] allowing a maximum of two indels to be

present in an alignment, reporting no suboptimal hits, allowing a maximum of 8

mismatches and printing only the best alignment. SNPs were then called using

the samtools and bcftools pipeline [113]. Called variants were then filtered using

vcftools to remove all sites with greater than 25% missing data, to include

genotypes only with an individual depth between 15X and 100X, to remove all

sites with a Phred quality score below 20 and with a minor allele frequency

below 0.05. Since common admixture analyses assume independence among

sites (i.e. the absence of linkage disequilibrium) [114], we additionally pruned

our RAD-derived SNP dataset using plink [115] , removing all sites where

pairwise linkage disequilibrium was greater than 0.4.

PCA on allele frequencies from all individuals was conducted using the

glPca function from the R package adegenet [116]. Admixture analysis was

carried out on a subset of samples from the Japanese archipelago using

STRUCTURE [50,51]. For each value of K from 1 to 8, the program was run for 10

iterations with a burn-in of 10,000 steps followed by 20,000 MCMC steps. The

most likely value of K was assessed using STRUCTURE HARVESTER [117].

Detecting the direction of introgression. We investigated the direction of gene

flow between the Japan Sea and Pacific Ocean lineages using partitioned D

statistics [56]. This is conceptually similar to standard four population ABBA-

BABA tests for gene flow but includes a fifth population – an allopatric lineage of

the Japan Sea. This balances the assumed phylogeny (i.e. ((P1, P2), (P3$_1$, P3$_2$), O)

and therefore allows us to rotate the populations used in the analysis – i.e.

testing for an enrichment of gene flow in both directions. We therefore tested

two topologies ((AT, PO), (JS$_S$, JS$_A$), O) and ((JS$_A$, JS$_S$), (PO, AT), O) (see Fig S14).

For either test topology, an excess of the ABBAA (compared to BABAA) or

ABBBA (compared to BABBA) positions in a genome window inflates partitioned

D statistics above zero – indicating gene flow from the P3 into P2.

Given that the partitioned D approach has attracted some criticism, we

also calculated D_{FOIL} statistics [57]. D_{FOIL} is an additional extension of the four

population test but one that incorporates all possible introgression events for a

symmetric four population tree (excluding the outgroup). We used the same test

phylogeny as with the partitioned D statistics (see Figs S14 & S15).

Both partitioned D and D_{FOIL} are based on ABBA/BABA methods – i.e.

where only a single individual is present at the tips of the phylogeny. To account

for this, we extended both methods to account for allele frequency data, meaning

our site pattern counts are weighted by allele frequencies [54]. To calculate both

D and D_{FOIL} statistics, we used a modified version of a python script used by

Martin et al [28].

Characterization of introgression sites. In order to characterize regions of

introgression, we identified candidate regions showing a strong signature of

introgression (i.e. G_{MIN} valleys and f_d peaks) from our genome scan approach. We

then counted the number of unique genes falling within our candidate

valleys/peaks and compared this to a null distribution generated by 1,000

random samples of 10 kb non-valley/non-peak genome windows for the same

number and size range as the valleys or peaks.

We then tested whether genes in introgressed regions were more likely

to have any specific functions. To achieve this, we used gene ontology (GO)

analysis on genes in valleys and 1,000 randomly chosen from across the genome.

GO analysis was performed with the ClueGO plugin [118] for Cytoscape 3.4.0

[119]. Since functional annotations for this analysis were drawn from the human

genome, we first generated a list of human-stickleback orthologous gene IDs

(Ensembl Biomart 86). We then subset our candidate and random gene sets to

include only orthologous genes. Several human genes have multiple stickleback

orthologs; we therefore allowed only a single, randomly chosen occurrence of

each human gene in both sets to prevent pseudo-replication. A hypergeometric

test was conducted for testing enrichment with Benjamini & Hochberg FDR

correction [120].

Acknowledgements

We are grateful to Manabu Kume, Seiichi Mori, and staff at the Aquarium Gobius

for providing samples and Katsushi Yamaguchi for technical assistance. Keisuke

Honda, the Institute of Statistical Mathematics and the DNA Data Bank of Japan

are thanked for their help with running analyses on supercomputers. We are

additionally grateful to Simon Martin, John Robinson and David Marques for

sharing scripts and their advice on analyses. Freddy Chain and Philine Feulner

are also thanked for their assistance with their sequence data. We thank

Cassandra Trier for her assistance and advice with GO analyses. All members of

the Kitano Lab provided invaluable advice throughout the project. We would also

like to thank Mark Kirkpatrick and members of his lab for comments on

manuscript.

References

1.   Mayr E. Animal species and evolution. Cambridge, Massachusetts: Havard

986   University Press; 1963.

987   2.   Coyne JA, Orr HA. Speciation. New York: Sinaeur; 2004.

988   3.   Nosil P. Ecological Speciation. Oxford, UK: Oxford University Press; 2012.

989   4.   Wu C-I. The genic view of the process of speciation. J Evol Biol. 2001;14:

990   851–865.

5.   Feder JL, Egan SP, Nosil P. The genomics of speciation-with-gene-flow.

Trends Genet. Elsevier Ltd; 2012;28: 342–350.

doi:10.1016/j.tig.2012.03.009

6.   Nosil P, Feder JL. Genomic divergence during speciation: causes and

consequences. Philos Trans R Soc London Ser B. 2012;367: 332–342.

doi:10.1098/rstb.2011.0263

7.   Ravinet M, Faria R, Butlin RK, Galindo J, Bierne N, Rafajlović M, et al.

Interpreting the genomic landscape of speciation: finding barriers to gene

flow. J Evol Biol. 2017;in press.

8.   Nosil P, Feder JL, Flaxman SM, Gompert Z. Tipping points in the dynamics

of speciation. Nat Ecol Evol. Macmillan Publishers Limited; 2017;1: 1–8.

doi:10.1038/s41559-016-0001

9.   Riesch R, Muschick M, Lindtke D, Villoutreix R, Comeault AA, Farkas TE, et

al. Transitions between phases of genomic differentiation during stick-

insect speciation. Nat Ecol Evol. Macmillan Publishers Limited, part of

Springer Nature.; 2017;1: 82. doi:10.1038/s41559-017-0082

10.   Feder JL, Nosil P, Wacholder AC, Egan SP, Berlocher SH, Flaxman SM.

Genome-wide congealing and rapid transitions across the speciation

continuum during speciation with gene flow. J Hered. 2014;105: 810–820.

doi:10.1093/jhered/esu038

11.   Seehausen O, Butlin RK, Keller I, Wagner CE, Boughman JW, Hohenlohe P a,

et al. Genomics and the origin of species. Nat Rev Genet. Nature Publishing

Group; 2014;15: 176–92. doi:10.1038/nrg3644

12.   Wolf JB, Ellegren H. Making sense of genomic islands of differentiation in

light of speciation. Nat Rev Genet. Nature Publishing Group; 2017;18: 87–

100. doi:10.1038/nrg.2016.133

13. Burri R, Nater A, Kawakami T, Mugal CF, Olason PI, Smeds L, et al. Linked

selection and recombination rate variation drive the evolution of the

genomic landscape of differentiation across the speciation continuum of

Ficedula flycatchers. Genome Res. 2015;25: 1656–1665.

doi:10.1101/gr.196485.115

14. Nadachowska-Brzyska K, Burri R, Olason PI, Kawakami T, Smeds L,

Ellegren H. Demographic Divergence History of Pied Flycatcher and

Collared Flycatcher Inferred from Whole-Genome Re-sequencing Data.

Payseur BA, editor. PLoS Genet. 2013;9: e1003942.

doi:10.1371/journal.pgen.1003942

15. Via S. Natural selection in action during speciation. Proc Natl Acad Sci.

2009;106: 9939–9946.

16. Marques DA, Lucek K, Meier JI, Mwaiko S, Wagner CE, Excoffier L, et al.

Genomics of Rapid Incipient Speciation in Sympatric Threespine

Stickleback. PLOS Genet. 2016;12: e1005887.

doi:10.1371/journal.pgen.1005887

17. Lawniczak MKN, Emrich SJ, Holloway a K, Regier a P, Olson M, White B, et

al. Widespread divergence between incipient Anopheles gambiae species

revealed by whole genome sequences. Science. 2010;330: 512–4.

doi:10.1126/science.1195755

18. Andrew RL, Rieseberg LH. Divergence is focused on few genomic regions

early in speciation: incipient speciation of sunflower ecotypes. Press.

2013; doi:10.1111/evo.12106.2013

19. Flaxman SM, Wacholder AC, Feder JL, Nosil P. Theoretical models of the

influence of genomic architecture on the dynamics of speciation. Mol Ecol.

2014;23: 4074–4088. doi:10.1111/mec.12750

20. Bierne N, Gagnaire PA, David P. The geography of introgression in a patchy

environment and the thorn in the side of ecological speciation. Curr Zool.

2013;59: 72–86.

21. Cruickshank TE, Hahn MW. Reanalysis suggests that genomic islands of

speciation are due to reduced diversity, not reduced gene flow. Mol Ecol.

2014;23: 3133–3157. doi:10.1111/mec.12796

22. Noor MAF, Bennett SM. Islands of speciation or mirages in the desert?

Examining the role of restricted recombination in maintaining species.

Heredity (Edinb). 2009;103: 439–444.

23. Nater A, Burri R, Kawakami T, Smeds L, Ellegren H. Resolving evolutionary

relationships in closely related species with whole-genome sequencing

data. Syst Biol. 2015;64: 1000–1017. doi:10.1093/sysbio/syv045

24. Rosenzweig BK, Pease JB, Besansky NJ, Hahn MW. Powerful methods for

detecting introgressed regions from population genomic data. Mol Ecol.

2016;25: 2387–2397. doi:10.1111/mec.13610

25. Geneva A, Garrigan D. Population Genomics of Secondary Contact. Genes

(Basel). 2010;1: 124–142. doi:10.3390/genes1010124

26. Geneva AJ, Muirhead CA, Kingan SB, Garrigan D. A new method to scan

genomes for introgression in a secondary contact model. PLoS One.

2015;10: e0118621. doi:10.1371/journal.pone.0118621

27. Meier JI, Marques DA, Mwaiko S, Wagner CE, Excoffier L, Seehausen O.

Ancient hybridization fuels rapid cichlid fish adaptive radiations. Nat

Commun. Nature Publishing Group; 2017;8: 14363.

doi:10.1038/ncomms14363

28.   Martin SH, Dasmahapatra KK, Nadeau NJ, Salazar C, Walters JR, Simpson F,

et al. Genome-wide evidence for speciation with gene flow in Heliconius

butterflies. Genome Res. 2013;23: 1817–28. doi:10.1101/gr.159426.113

29.   Martin CH, Crawford JE, Turner BJ, Simons LH. Diabolical survival in Death

Valley: recent pupfish colonization, gene flow and genetic assimilation in

the smallest species range on earth. Proc R Soc B Biol Sci. 2016;283:

20152334. doi:10.1098/rspb.2015.2334

30.   Hendry AP, Bolnick DI, Berner D, Peichel CL. Along the speciation

continuum in sticklebacks. J Fish Biol. 2009;75: 2000–2036.

31.   McKinnon JS, Rundle HD. Speciation in nature: the threespine stickleback

model systems. Trends Ecol Evol. 2002;17: 480–481. doi:10.1016/S0169-

5347(02)02579-X

32.   Feulner PGD, Chain FJJ, Panchal M, Huang Y, Eizaguirre C, Kalbe M, et al.

Genomics of Divergence along a Continuum of Parapatric Population

Differentiation. PLOS Genet. 2015;11: e1004966.

doi:10.1371/journal.pgen.1004966

33.   Roesti M, Hendry AP, Salzburger W, Berner D. Genome divergence during

evolutionary diversification as revealed in replicate lake-stream

stickleback population pairs. Mol Ecol. 2012;21: 2852–2862.

doi:10.1111/j.1365-294X.2012.05509.x

34.   Roesti M, Kueng B, Moser D, Berner D. The genomics of ecological

vicariance in threespine stickleback fish. Nat Commun. Nature Publishing

Group; 2015;6: 8767. doi:10.1038/ncomms9767

35.   Wu C-I, Ting C-T. Genes and speciation. Nat Rev Genet. 2004;5: 114–22.

doi:10.1038/nrg1269

36.   Kitano J, Ross JA, Mori S, Kume M, Jones FC, Chan YF, et al. A role for neo-

sex chromosomes in stickleback speciation. Nature. 2009;461: 1079–1083.

doi:10.1002/ece3.234

37.   Kitano J, Mori S, Peichel CL. Phenotypic divergence and reproductive

isolation between sympatric forms of Japanese threespine sticklebacks.

Biol J Linn Soc. 2007;91: 671–685. doi:10.1111/j.1095-8312.2007.00824.x

38.   Higuchi M, Sakai H, Goto A. A new threespine stickleback, Gasterosteus

nipponicus sp. nov. (Teleostei: Gasterosteidae), from the Japan Sea region.

Ichthyol Res. 2014; 1–2. doi:10.1007/s10228-014-0403-1

39.   Higuchi M, Goto A. Genetic evidence supporting the existence of two

distinct species in the genus *Gasterosteus* around Japan. Environ Biol

Fishes. 1996;47: 1–16. doi:10.1007/BF00002375

40.   Yoshida K, Makino T, Yamaguchi K, Shigenobu S, Hasebe M, Kawata M, et

al. Sex Chromosome Turnover Contributes to Genomic Divergence

between Incipient Stickleback Species. Zhang J, editor. PLoS Genet.

2014;10: e1004223. doi:10.1371/journal.pgen.1004223

41.   Kume M, Kitano J, Mori S, Shibuya T. Ecological divergence and habitat

isolation between two migratory forms of Japanese threespine stickleback

(Gasterosteus aculeatus). J Evol Biol. 2010;23: 1436–1446.

doi:10.1111/j.1420-9101.2010.02009.x

42.   Ravinet M, Takeuchi N, Kume M, Mori S, Kitano J. Comparative Analysis of

Japanese Three-Spined Stickleback Clades Reveals the Pacific Ocean

Lineage Has Adapted to Freshwater Environments while the Japan Sea Has

Not. Craft JA, editor. PLoS One. 2014;9: e112404.

doi:10.1371/journal.pone.0112404

43.   Lackey AC, Boughman JW. Evolution of reproductive isolation in

stickleback fish. Evolution (N Y). 2017;71: 357–371.

doi:10.1038/hdy.2008.69

44.   Ortí G, Bell MA, Reimchen TE, Meyer A. Global survey of mitochondrial

DNA sequences in the threespine stickleback: evidence for recent

migrations. Evolution (N Y). 1994;48: 608–622.

45.   Yamada M, Higuchi M, Goto A. Extensive introgression of mitochondrial

DNA found between two genetically divergent forms of threespine

stickleback, *Gasterosteus aculeatus*, around Japan. Environ Biol Fishes.

2001;61: 269–284.

46.   Feulner PGD, Chain FJJ, Panchal M, Eizaguirre C, Kalbe M, Lenz TL, et al.

Genome-wide patterns of standing genetic variation in a marine

population of three-spined sticklebacks. Mol Ecol. 2012; no-no.

doi:10.1111/j.1365-294X.2012.05680.x

47.   Cummings MP, Neel MC, Shaw KL. A genealogical approach to quantifying

lineage divergence. Evolution. 2008;62: 2411–22. doi:10.1111/j.1558-

5646.2008.00442.x

48.   Toews DPL, Brelsford A. The biogeography of mitochondrial and nuclear

discordance in animals. Mol Ecol. 2012;21: 3907–30. doi:10.1111/j.1365-

294X.2012.05664.x

49.   Catchen J, Bassham S, Wilson T, Currey M, O'Brien C, Yeates Q, et al. The

population structure and recent colonization history of Oregon threespine

stickleback determined using restriction-site associated DNA-sequencing.

Mol Ecol. 2013;22: 2864–2883. doi:10.1111/mec.12330

50.   Falush D, Stephens M, Pritchard JK. Inference of population structure:

Extensions to linked loci and correlated allele frequencies. Genetics.

2003;164: 1567–1587.

51.   Pritchard JK, Stephens M, Rosenberg NA, Donnelly P. Association mapping

in structured populations. Am J Hum Genet. 2000;67.

52.   Kusakabe M, Ishikawa A, Ravinet M, Yoshida K, Makino T, Toyoda A, et al.

Genetic basis for variation in salinity tolerance between stickleback

ecotypes. Mol Ecol. 2016; doi:10.1111/mec.13875

53.   Charlesworth B, Morgan MT, Charlesworth D. The effect of deleterious

mutations on neutral molecular variation. Genetics. 1993;134: 1289–303.

Available:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1205596&to

ol=pmcentrez&rendertype=abstract

54.   Martin SH, Davey JW, Jiggins CD. Evaluating the use of ABBA-BABA

statistics to locate introgressed loci. Mol Biol Evol. 2014;32: 244–257.

doi:10.1101/001347

55.   Soria-Carrasco V, Gompert Z, Comeault AA, Farkas TE, Parchman TL,

Johnston JS, et al. Stick Insect Genomes Reveal Natural Selection's Role in

Parallel Speciation. Science (80-). 2014;344: 738–742.

doi:10.1126/science.1252136

56.   Eaton DAR, Ree RH. Inferring phylogeny and introgression using RADseq

data: An example from flowering plants (Pedicularis: Orobanchaceae). Syst

Biol. 2013;62: 689–706. doi:10.1093/sysbio/syt032

57.   Pease JB, Hahn MW. Detection and Polarization of Introgression in a Five-

Taxon Phylogeny. Syst Biol. 2015;64: 651–662.

doi:10.1093/sysbio/syv023

58.   Cassidy L, Ravinet M, Mori S, Kitano J. Are Japanese freshwater populations

of threespine stickleback derived from the Pacific Ocean lineage? Evol Ecol

Res. 2013;15: 295–311.

59.   Roesti M, Moser D, Berner D. Recombination in the threespine stickleback

genome - Patterns and consequences. Mol Ecol. 2013;22: 3014–3027.

doi:10.1111/mec.12322

60.   Li H, Durbin R. Inference of human population history from individual

whole-genome sequences. Nature. Nature Publishing Group; 2011;475:

493–6. doi:10.1038/nature10231

61.   Schiffels S, Durbin R. Inferring human population size and separation

history from multiple genome sequences. Nat Genet. Nature Publishing

Group; 2014;46: 919–925. doi:10.1038/ng.3015

62.   Kume M, Kitamura T, Takahashi H, Goto A. Distinct spawning migration

patterns in sympatric Japan Sea and Pacific Ocean forms of threespine

stickleback *Gasterosteus aculeatus*. Icthyological Res. 2005;52: 189–193.

doi:10.1007/s10228-005-0269-3

63.   Kume M, Kuwahara T, Arai T, Okamoto M, Goto A. A part of the Japan Sea

form of the threespine stickleback, Gasterosteus aculeatus, spawns in the

seawater tidal pools of western Hokkaido Island, Japan. Environ Biol

Fishes. 2006;77: 169–175. doi:10.1007/s10641-006-9068-6

64.   Wirtz P. Mother species–father species: unidirectional hybridization in

animals with female choice. Anim Behav. 1999;58: 1–12.

doi:10.1006/anbe.1999.1144

65.   Knowles LL. Statistical Phylogeography. Annu Rev Ecol Syst. 2009;40:

593–612.

66.   Robinson JD, Bunnefeld L, Hearn J, Stone GN, Hickerson MJ. ABC inference

of multi-population divergence with admixture from un-phased

population genomic data. Mol Ecol. 2014;23: 4458–4471.

doi:10.1111/mec.12881

67.   Sousa V, Hey J. Understanding the origin of species with genome-scale

data: modelling gene flow. Nat Rev Genet. Nature Publishing Group;

2013;14: 404–14. doi:10.1038/nrg3446

68.   Yamada M, Higuchi M, Goto A. Long-term occurrence of hybrids between

Japan Sea and Pacific Ocean forms of threespine stickleback, *Gasterosteus*

aculeatus, in Hokkaido Island, Japan. Environ Biol Fishes. 2007;80: 435–

443.

69.   Mäkinen HS, Merilä J. Mitochondrial DNA phylogeography of the three-

spined stickleback (*Gasterosteus aculeatus*) in Europe - Evidence for

multiple glacial refugia. Mol Phylogenet Evol. 2008;46: 167–182.

doi:10.1016/j.ympev.2007.06.011

70.   Ravinet M, Harrod C, Eizaguirre C, Prodöhl P a. Unique mitochondrial DNA

lineages in Irish stickleback populations: cryptic refugium or rapid

recolonization? Ecol Evol. 2013; n/a-n/a. doi:10.1002/ece3.853

71.   Hohenlohe PA, Bassham S, Etter PD, Stiffler N, Johnson EA, Cresko WA.

Population genomics of parallel adaptation in threespine stickleback using

sequenced RAD tags. PloS Genet. 2010;6: e1000862.

72.   Lescak EA, Bassham SL, Catchen J, Gelmond O, Sherbick ML, von Hippel FA,

et al. Evolution of stickleback in 50 years on earthquake-uplifted islands.

Proc Natl Acad Sci U S A. 2015;112: E7204–E7212.

doi:10.1073/pnas.1512020112

73.   Barton NH, de Cara MAR. The evolution of strong reproductive isolation.

Evolution. 2009;63: 1171–90. doi:10.1111/j.1558-5646.2009.00622.x

74.   Barton N, Bengtsson BO. The barrier to genetic exchange between

hybridising populations. Heredity (Edinb). 1986;57: 357–376.

doi:10.1038/hdy.1986.135

75.   Muirhead CA, Presgraves DC. Hybrid Incompatibilities, Local Adaptation,

and the Genomic Distribution of Natural Introgression between Species.

Am Nat. 2016;187: 249–261. doi:10.1086/684583

76.   Hedrick PW. Adaptive introgression in animals: examples and comparison

to new mutation and standing variation as sources of adaptive variation.

Mol Ecol. 2013; doi:10.1111/mec.12415

77.   Racimo F, Sankararaman S, Nielsen R, Huerta-Sánchez E. Evidence for

archaic adaptive introgression in humans. Nat Rev Genet. Nature

Publishing Group; 2015;16: 359–371. doi:10.1038/nrg3936

78.   Castric V, Bechsgaard J, Schierup MH, Vekemans X. Repeated adaptive

introgression at a gene under multiallelic balancing selection. PLoS Genet.

2008;4. doi:10.1371/journal.pgen.1000168

79.   Elgvin TO, Trier CN, Tørresen OK, Hagen, Ingerid J, Lien S, Nederbragt AJ,

et al. The genomic mosaicism of hybrid speciation. Sci Adv. 2017;3.

80.   Sun C, Huo D, Southard C, Nemesure B, Hennis A, Cristina Leske M, et al. A

signature of balancing selection in the region upstream to the human

UGT2B4 gene and implications for breast cancer risk. Hum Genet.

2011;130: 767–775. doi:10.1007/s00439-011-1025-6

81.   Bolnick DI, Stutz WE. Frequency dependence limits divergent evolution by

favouring rare immigrants over residents. Nature. Nature Publishing

Group; 2017;546: 285–288. doi:10.1038/nature22351

82. Ishikawa A, Kusakabe M, Yoshida K, Ravinet M, Makino T, Toyoda A, et al.

Different contributions of local- and distant-regulatory changes to

transcriptome divergence between stickleback ecotypes. 2017; 1–17.

doi:10.1111/evo.13175

83. Ravinet M, Prodöhl PA, Harrod C. On Irish stickleback: morphological

diversification in a secondary contact zone. Evol Ecol Res. 2013;15: 271–

294.

84. Baird N a, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis Z a, et al.

Rapid SNP discovery and genetic mapping using sequenced RAD markers.

PLoS One. 2008;3: e3376. doi:10.1371/journal.pone.0003376

85. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The

Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:

2078–9. doi:10.1093/bioinformatics/btp352

86. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by

sampling trees. BMC Evol Biol. 2007;7: 214–222.

87. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and

high throughput. Nucleic Acids Res. 2004;32: 1792–7.

doi:10.1093/nar/gkh340

88. Bell MA. Paleobiology and evolution of threespine stickleback. In: Bell MA,

Foster SA, editors. The evolutionary biology of the threespine stickleback.

Oxford: Oxford University Press; 1994. pp. 438–471.

89. Bell MA, Stewart JD, Park PJ. The world's oldest fossil threespine

stickleback fish. Copeia. 2009;2009: 256–265.

90.   Heled J, Drummond AJ. Calibrated tree priors for relaxed phylogenetics

and divergence time estimation. Syst Biol. 2012;61: 138–49.

doi:10.1093/sysbio/syr087

91.   Wheat CW, Wahlberg N. Critiquing blind dating: the dangers of over-

confident date estimates in comparative genomics. Trends Ecol Evol.

Elsevier Ltd; 2013;28: 636–42. doi:10.1016/j.tree.2013.07.007

92.   Baele G, Lemey P, Bedford T, Rambaut A, Suchard M a, Alekseyenko A V.

Improving the accuracy of demographic and molecular clock model

comparison while accommodating phylogenetic uncertainty. Mol Biol Evol.

2012;29: 2157–67. doi:10.1093/molbev/mss084

93.   Baele G, Li WLS, Drummond AJ, Suchard M a, Lemey P. Accurate model

selection of relaxed molecular clocks in bayesian phylogenetics. Mol Biol

Evol. 2013;30: 239–43. doi:10.1093/molbev/mss243

94.   Rambaut A, Drummond AJ. Tracer v1.5. 2009.

95.   Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic

analyses with thousands of taxa and mixed models. Bioinformatics.

2006;22: 2688–2690. doi:10.1093/bioinformatics/btl446

96.   Revell LJ. phytools: an R package for phylogenetic comparative biology

(and other things). Methods Ecol Evol. 2012;3: 217–223.

doi:10.1111/j.2041-210X.2011.00169.x

97.   Schliep KP. phangorn: Phylogenetic analysis in R. Bioinformatics. 2011;27:

592–593. doi:10.1093/bioinformatics/btq706

98.   Li H, Durbin R. Inference of human population history from individual

whole-genome sequences. Nature. 2011;475: 493–6.

doi:10.1038/nature10231

99.   Bell MA, Foster SA. Introduction to the evolutionary biology of the

threespine stickleback. In: Bell MA, Foster SA, editors. The Evolutionary

Biology of the Threespine Stickleback. Oxford: Oxford University Press;

1994. pp. 1–27.

100.   Guo B, Chain FJ, Bornberg-Bauer E, Leder EH, Merilä J. Genomic divergence

between nine- and three-spined sticklebacks. BMC Genomics. 2013;14:

756. doi:10.1186/1471-2164-14-756

101.   Mailund T, Halager AE, Westergaard M, Dutheil JY, Munch K, Andersen LN,

et al. A new isolation with migration model along complete genomes infers

very different divergence processes among closely related great ape

species. PLoS Genet. 2012;8: e1003125.

doi:10.1371/journal.pgen.1003125

102.   Pavlidis P, Laurent S, Stephan W. msABC: a modification of Hudson's ms to

facilitate multi-locus ABC analysis. Mol Ecol Resour. 2010;10: 723–7.

doi:10.1111/j.1755-0998.2010.02832.x

103.   Tange O. GNU Parallel - The Command-Line Power Tool. ;login USENIX

Mag. Frederiksberg, Denmark; 2011;36: 42–47. Available:

http://www.gnu.org/s/parallel

104.   Csilléry K, Blum MGB, Gaggiotti OE, François O, Csillery K, Francois O.

Approximate Bayesian Computation (ABC) in practice. Trends Ecol Evol.

2010;25: 410–418. doi:10.1016/j.tree.2010.04.001

105.   Bertorelle G, Benazzo A, Mona S. ABC as a flexible framework to estimate

demography over space and time: some cons, many pros. Mol Ecol.

2010;19: 2609–2625. doi:10.1111/j.1365-294X.2010.04690.x

106.   Csilléry K, François O, Blum MGB. abc: an R package for approximate

Bayesian computation (ABC). Methods Ecol Evol. 2012;3: no-no.

doi:10.1111/j.2041-210X.2011.00179.x

107.   Weir B, Cockerham C. Estimating F-Statistics for the Analysis of Population

Structure. Evolution (N Y). 1984;38: 1358–1370. Available:

http://www.jstor.org/stable/2408641

108.   Danecek P, Auton A, Abecasis G, Albers C a, Banks E, DePristo M a, et al.

The variant call format and VCFtools. Bioinformatics. 2011;27: 2156–8.

doi:10.1093/bioinformatics/btr330

109.   Smadja CM, Canbäck B, Vitalis R, Gautier M, Ferrari J, Zhou J-J, et al. Large-

scale candidate gene scan reveals the role of chemoreceptor genes in host

plant specialization and speciation in the pea aphid. Evolution. 2012;66:

2723–38. doi:10.1111/j.1558-5646.2012.01612.x

110.   Harte D. HiddenMarkov: Hidden Markov Models. R package version 1.8-7.

Wellington: Statistics Research Associates; 2016.

111.   Catchen J, Hohenlohe P a., Bassham S, Amores A, Cresko W a. Stacks: an

analysis tool set for population genomics. Mol Ecol. 2013;22: 3124–3140.

doi:10.1111/mec.12354

112.   Wu TD, Nacu S. Fast and SNP-tolerant detection of complex variants and

splicing in short reads. Bioinformatics. 2010;26: 873–81.

doi:10.1093/bioinformatics/btq057

113.   Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The

Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:

2078–9. doi:10.1093/bioinformatics/btp352

114.   Pritchard JK, Stephens M, Donnelly P. Inference of population structure

using multilocus genotype data. Genetics. 2000;155: 945–959.

115. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, et al.

PLINK: A tool set for whole-genome association and population-based

linkage analyses. Am J Hum Genet. 2007;81: 559–575.

doi:10.1086/519795

116. Jombart T, Ahmed I. adegenet 1 . 3-1️: new tools for the analysis of

genome-wide SNP data. Bioinformatics. 2011;27: 3070–3071.

doi:10.1093/bioinformatics/btr521

117. Earl D a., vonHoldt BM. STRUCTURE HARVESTER: a website and program

for visualizing STRUCTURE output and implementing the Evanno method.

Conserv Genet Resour. 2011;4: 359–361. doi:10.1007/s12686-011-9548-7

118. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al.

ClueGO: A Cytoscape plug-in to decipher functionally grouped gene

ontology and pathway annotation networks. Bioinformatics. 2009;25:

1091–1093. doi:10.1093/bioinformatics/btp101

119. Christmas, Rowan; Avila-Campillo, Iliana; Bolouri, Hamid; Schwikowski,

Benno; Anderson, Mark; Kelley, Ryan; Landys, Nerius; Workman, Chris;

Ideker, Trey; Cerami, Ethan; Sheridan, Rob; Bader, Gary D.; Sander C.

Cytoscape: a software environment for integrated models of biomolecular

interaction networks. Am Assoc Cancer Res Educ B. 2005; 12–16.

doi:10.1101/gr.1239303.metabolite

120. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical

and powerful approach to multiple testing. J R Stat Soc Ser B 1995;57:

289–300. Available: http://www.jstor.org/stable/10.2307/2346101

1364

1365 **Figures**

1366

Fig 1. The Japan Sea stickleback is a separate species. (A) Rooted nuclear

consensus tree for Japan Sea, Pacific Ocean and Atlantic Ocean stickleback

lineages from 10 kb non-overlapping sliding windows across the autosome. Red

trees indicate species clustering; blue trees indicate geographical clustering and

green trees reflect ancestral polymorphism. NB: Only 1,000 subsampled species

trees are shown here to aid illustration. (B) Mitogenome Bayesian consensus

tree shows divergence between two mitochondrial clades – all Japanese

sticklebacks (*G. nipponicus* and *G. aculeatus*) and *G. aculeatus* occurring in Europe

and North America. (C) Present day distribution of *G. aculeatus* (blue) and *G.

nipponicus* (red) around the Japanese archipelago. The two species overlap in

Hokkaido, Northern Japan and samples for this study were collected in

Bekanbeushi River in Akkeshi unless noted. (D) PSMC plot of 26 resequenced

genomes shows a steady population size in the Pacific Ocean lineage (blue) but a

bottleneck around 0.15-0.3 million years before present and a subsequent

increase in the Japan Sea lineage (orange).

1382

Fig 2. ABC analysis supports isolation with gene flow. (A) A model of isolation

with migration and a bottleneck in the Japan Sea lineage is best supported by

ABC analysis using ~2,000 nuclear loci (see Table 1). Posterior probability

densities for model parameters estimated using neural network analysis, a 1%

tolerance rate and 20 summary statistics. Parameters are: T = time of split, m_{12} =

migration from Japan Sea to Pacific Ocean, m_{21} = migration from Pacific Ocean to

Japan Sea; T_G = timing of bottleneck, N_{PO} = Pacific Ocean effective population size,

N_{JS} = Japan Sea effective population size and N_{JSB} = Japan Sea bottleneck effective

population size. Posterior probability density curves for (B) Japan Sea and Pacific

Ocean divergence time and timing of bottleneck in the Japan Sea lineage, (C)

Japan Sea, Pacific Ocean and Japan Sea bottleneck effective population sizes, and

(D) mean migration rates. Figures on each panel are median parameter

estimates.

1396

1397   **Fig 3. Genomic divergence is lower in sympatry than in allopatry between**

1398   **species.** Histograms of (A) relative (F_{ST}) and (B) absolute (d_{XY}) differentiation

1399   measures for each of the species comparisons. (C) Mean genome-wide F_{ST} of the

1400   Japanese species pair compared with those of other stickleback systems taken

1401   from previously published studies [32–34,52].

1402

1403   **Fig 4. Genome-wide distribution of divergence and introgression.**

1404   Divergence was measured using F_{ST} and d_{XY}, while introgression was measured

1405   using G_{MIN} and f_d. Data plotted here is from 50 kb non-overlapping genome

1406   windows. Blue and yellow lines indicates allopatric (Japan Sea vs Atlantic) and

1407   sympatric (Japan Sea vs Pacific Ocean) comparisons, respectively.

1408

Fig 5. Fewer introgression valleys occur on the neo-X chromosome. (A) A

greater number of G_{MIN} valleys occur in sympatry than in allopatry between

species. (B) G_{MIN} valleys and f_d peaks also occur in regions of the genome with a

higher recombination rate. Fewer valleys occur on the neo-X chromosome

(chrIX) compared to autosomes (C), even when chromosome length is taken into

consideration (D); N.B – data for (C) and (D) were measured using females only.

Table 1. Posterior probability values for models for final ABC model

selection using neural network and standard rejection methods. All

estimates produced using a tolerance of 1% and 20 summary statistics. Bold text

indicates the model where posterior probability provides the highest support.

Models are I = isolation, IM = isolation with migration, IAM = isolation and

ancient migration, IRM = isolation and recent migration, IARM = isolation with

ancient and recent migration.

Divergence model	Growth model	Standard rejection	Neural network
IM	bottleneck	**0.265**	**0.511**
I	bottleneck	0.130	0.008
IAM	bottleneck	0.151	0.009
IARM	bottleneck	0.230	0.343
IRM	bottleneck	0.224	0.129

1422

1423

1424 **Table 2. Genome-wide averages for measures of divergence and**

1425 **introgression.** F_{ST}, d_{XY}, G_{MIN}, and f_d for all pairwise comparisons of Japan Sea

1426 (JS), Pacific Ocean (PO) and Atlantic Ocean sticklebacks (AT) are shown. Mean ±

1427 SD and lower and upper limits of the 95% confidence interval (in parenthesis)

1428 are shown. NA, not analysed.

Comparison	F_{ST}	d_{XY}	G_{MIN}	f_d
JS vs PO	0.634 ± 0.122	0.012 ± 0.002	0.857 ± 0.102	0.004 ± 0.054
	(0.333-0.862)	(0.007-0.017)	(0.513-0.942)	(-0.031-0.085)
JS vs AT	0.697 ± 0.116	0.013 ± 0.002	0.876 ± 0.071	-0.003±0.033
	(0.406-0.902)	(0.007-0.018)	(0.666-0.942)	(-0.077-0.029)
PO vs AT	0.215 ± 0.134	0.004 ± 0.002	0.560 ± 0.141	NA
	(0.003-0.539)	(0.001-0.009)	(0.223-0.772)	

1429

Supporting information

S1 Table. Classification of trees. Proportion of trees drawn from 10, 50 and

100 kb windows representing species, ancestral and geographical topologies.

S2 Table. Mitogenome analysis. Marginal log-likelihood values and Bayes

factor comparisons from path sampling of substitution clock models used for

mitogenome phylogeny and divergence time estimation.

S3 Table. Median, L95% and U95% HPD (highest probability densities) for

demographic parameters estimated under IM + bottleneck model.

S4 Table. Number of valleys and valley per Mb for all chromosomes.

S5 Table. Enriched GO terms for genes present in G_{MIN} valleys.

S6 Table. Enriched GO terms for genes present in f_d peaks.

1441

1442 **S7 Table. Sample names, locations and accession numbers.**

1443

S1 Fig. Global species distribution. The global distribution of the Pacific and

Atlantic Ocean lineages of *G. aculeatus* allow sympatric and allopatric

comparisons with *G. nipponicu*; AT = Atlantic Ocean, PO = Pacific Ocean and JS =

Japan Sea.

S2 Fig. Japanese stickleback mitonuclear discordance. (A) Mitogenome

Bayesian tree shows divergence between two mitochondrial clades – the

Transpacific (TPN) and European North American (ENA); asterisks on nodes

indicate appropriate densities shown in (B). (B) Posterior probability densities

for mitochondrial divergence time between *G. aculeatus* and *G. nipponicus* and

between Pacific and Atlantic populations of the ENA clade.

S3 Fig. Bootstrapped PSMC curves for 26 resequenced individuals.

S4 Fig. PSMC profile for all 26 individuals and an additional Eastern Pacific

individual from Little Campbell River, Canada.

S5. Fig. Principal component analysis on RAD-seq data from 295

individuals from across the distribution of all three lineages. The arrow

indicates the presence of an admixed individual occurring in the Akkeshi system.

S6 Fig. STRUCTURE analysis on RAD-seq data from Japanese populations.

Analysis with K = 2 clusters (A), which is supported by likelihood analysis (B),

showed the presence of admixed individuals in the Akkeshi system.

S7 Fig. Genome-wide F_{ST} measured in non-overlapping 50 kb windows for

allopatric and sympatric between and within species comparisons.

S8 Fig. Genome-wide d_{XY} measured in non-overlapping 50 kb windows for

allopatric and sympatric between and within species comparisons.

S9 Fig. Negative association between f_d and G_{MIN} in sympatric (JS vs PO) and

allopatric (JS v AT) between species comparisons.

S10 Fig. Genome-wide G_{MIN} for sympatric between species comparisons.

Black line represents 50 kb non-overlapping window G_{MIN} signature. Points

represent 10 kb windows; grey points are non-valley windows, blue points are

valley windows identified by Hidden Markov Model algorithm.

S11 Fig. Absolute divergence (d_{XY}) is lower and f_d is higher in G_{MIN} valleys

compared to the genome-wide background.

S12 Fig. Genome-wide f_d for sympatric between species comparisons. Black

line represents 50 kb non-overlapping window f_d signature. Points represent 10

kb windows; grey points are non-valley windows, blue points are peak windows

identified by Hidden Markov Model algorithm.

S13 Fig. Comparison of d_{XY} between f_d peaks and non-peaks.

S14 Fig. Analysis of partitioned D statistics. (A) Boxplots comparing

partitioned D statistics between f_d peaks and the autosomal background. Dashed

line at zero indicates a balance between allele patterns indicative of incomplete

lineage sorting. In (B), P1 = Atlantic Ocean (AT), P2 = Pacific Ocean (PO), $P3_1$ =

sympatric Japan Sea (JS_S), $P3_2$ = allopatric Japan Sea (JS_A), and O = *G. wheatlandi*

(WT). In (C), P12 and P3 were swapped. D_1 measures asymmetry between P1

and P2 where the derived allele B is present in $P3_1$ but not $P3_2$, D_2 measures

where allele B is present in $P3_2$ but not $P3_1$, and D_{12} measures where the derived

allele is shared by both $P3_1$ and $P3_2$. If we assume that the derived allele B

occurred at the ancestor of P3, D12 indicates introgression from P3 to P2. See

[56] for a more detailed explanation of these statistics.

S15 Fig. D_{FOIL} statistics. In this statistics, we assume that the divergence time

between P_1 and P_2 is younger than that between P_3 and P_4 and infer all possible

introgressions including ancestral introgression involving P_{12}. The number of

loci (100 kb-window) that show statistically significant introgression are shown.

See [57] for a more detailed explanation of these statistics.

S16 Fig. The relationship between introgression measured as G_{MIN} and f_d

and $\log_{10}$ recombination rate.

S17 Fig. Venn diagram showing overlap between genes occurring in

introgressed regions identified using different measures.

Supplementary methods - ABC analysis.

A

bs28
bs25
bs27
bs26
bs30
bs29

PF_JR65
PF_JR27
PM_JR49
PF_JR42
PO_male
PO_female
PF_JR58
PM_JR39
PM_JR55
PF_JR66

JF_KIA48
JS_male
JM_KIA69
JM_KIA37
JS_female
JF_KIA45
JF_KIA46
JF_KIA31
JM_KIA70
JM_KIA34

G. wheatlandi

Atlantic Ocean
(G. aculeatus)

Pacific Ocean
(G. aculeatus)

Japan Sea
(G. nipponicus)

C

Akkeshi
system

G. nipponicus

Sea
of
Japan

G. aculeatus

Pacific
Ocean

N

B

PF_JR27
JM_KIA37
PM_JR39
JF_KIA45
JM_KIA70
JS_male
PM_JR55
JM_KIA34
JM_KIA69
JF_KIA48
JS_Shinji
PF_JR42
PO_female
PF_JR66
PF_JR58
PF_JR65
PO_male
JF_KIA31
JF_KIA46
PM_JR49
JS_female

All Japanese
stickleback
(G. nipponicus
and
G. aculeatus)

LCM
LCS
bs28
bs30
bs29
bs26
bs25
bs27

European
North American

(G. aculeatus only)

G. wheatlandi 1
G. wheatlandi 2

1.40   1.25   1.0   0.75   0.5   0.25   0.0

D

Japan
Sea

Pacific
Ocean

Atlantic
Ocean

Effective population size (x10^4)

10^4 10^5 10^6

Years (g=1,μ =0.7x10^{-8})

A Isolation with migration + JS bottleneck

N_{JSB}

N_{JS} N_{PO}

m_{12}

m_{21}

T

T_G

B Divergence time T = 0.68 million years

Onset of bottleneck T_G = 0.30 million years

density

Years (millions)

C Pacific Ocean N_e = 22,971

Japan Sea N_e = 69,302

Japan Sea bottleneck N_e = 12,223

density

N_e x 10^5

D JS > PO (m_{12}) = 1.30 x 10^{-6}

PO > JS (m_{21}) = 1.05 x 10^{-6}

density

m

JS vs PO   JS vs AT