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Ancient three-spined stickleback (*Gasterosteus aculeatus*) mtDNA lineages are not associated with phenotypic or nuclear genetic variation

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Historic population divergence can shape contemporary patterns of genetic variation, but the extent to which this happens in natural populations is unclear. We tested whether ancient mitochondrial DNA (mtDNA) divergence was associated with phenotypic and nuclear genetic variation in three-spined stickleback fish (*Gasterosteus aculeatus*) from two geographic regions in Alaska where individuals from ancient mitochondrial clades are found in sympatry. We tested the hypotheses that historic evolutionary divergence led to reproductive barriers that persist between fish carrying different mtDNA lineages in contemporary populations, or in the absence of such barriers, that epistatic interactions among loci in the mitochondrial and nuclear DNA created non-random associations between these genomes. We found no relationship between mtDNA lineage and armour and body shape traits. We also did not detect global nuclear genetic differentiation or genomically localized patterns of variation between fish with alternative mtDNA lineages. Thus, we find that the divergence that is still evident in the mtDNA of contemporary stickleback populations appears to have no residual influence on patterns of phenotypic or nuclear genetic variation at any scale.

ADDITIONAL KEYWORDS: morphological divergence – phenotypic variation – phylogeography – population genomics.

INTRODUCTION

Population genetic variation and genomic structure can be shaped by both historic and contemporary divergence (e.g. Scribner, Page & Bartron, 2000; Gokcumen *et al.*, 2013). At the extreme, historic mitochondrial DNA (mtDNA) divergence can result in reproductive barriers that persist in contemporary populations or, more subtly, patterns of variation due to epistatic interactions between mitochondrial and nuclear DNA (Dasmahapatra *et al.*, 2002). In *Drosophila*, for example, patterns of cytonuclear epistasis have been supported by functional genetic analyses of effects on metabolic processes due to the OxPhos pathway (Rand, Clark & Kann, 2001; Rand, Fry & Sheldahl, 2006).

Individuals across the present distribution of three-spined stickleback fish (*Gasterosteus aculeatus*) contain one of two lineages of ancient mtDNA clades that diverged during a period of allopatry estimated to range between 90–260 Kya and 1 Mya (Ortí *et al.*, 1994; Nielsen & Wakeley, 2001; Kitano *et al.*, 2009). The Euro North

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American Clade (ENAC) is found in both the Atlantic and Pacific basins, while the Trans North Pacific Clade (TNPC) is restricted to the Pacific basin (Ortí *et al.*, 1994; Johnson & Taylor, 2004). The North Pacific Basin is a region of widespread clade admixture (Lescak *et al.*, 2015b). Previous analyses have tested the hypothesis that the distribution of the two clades in this region has been influenced by both post-glacial colonization and lake elevation and that clade identity is associated with armour phenotypes (Johnson & Taylor, 2004). In addition, a small but statistically significant proportion of nuclear genetic variation in *Schistocephalus solidus*, a common stickleback parasite, corresponds with host mtDNA lineage, suggesting the potential for some degree of specificity between this parasite and its host (Strobel *et al.*, 2016). An open question remains, however, as to whether historic mtDNA divergence influences contemporary patterns of phenotypic and nuclear genetic variation in subtle ways that can only now be detected with more extensive genome-wide data provided by next-generation sequencing.

Population-level associations between reduced bony armour and presence of the TNPC have been documented in British Columbia (O'Reilly *et al.*, 1993; Deagle, Reimchen & Levin, 1996) and Japan (Higuchi & Goto, 1996). However, an association between morphology and clade was not detected in subsequent analyses of additional populations from British Columbia or Alaska (Johnson & Taylor, 2004). Analysis of variation in mtDNA clade and phenotype at the individual level is required to determine whether previously documented population-level associations are correlational or causative. Furthermore, a dense set of nuclear genetic markers is required to test whether the two lineages are reproductively isolated or if more subtle mtDNA-nuclear genome associations remain that indicate a form of cytonuclear epistasis that could residually affect physiology and fitness, as has been documented in other organisms (e.g. Rand, Haney & Fry, 2004; Schmidt *et al.*, 2011).

We predicted that if historic divergence in stickleback mtDNA lineages resulted in reproductive isolation that is still maintained in extant populations, we would observe genome-wide nuclear genetic, and probably attendant phenotypic, differences that correspond with mtDNA clade. If the two lineages are not reproductively isolated, mtDNA divergence could still influence contemporary evolutionary patterns through epistatic interactions that originated during the ancient period of allopatry between the mitochondrial and nuclear genomes. Alternatively, clade divergence could be neutral, leaving a signature only in the non-recombining mtDNA. We generated data on genome-wide single-nucleotide polymorphism (SNP) markers, phenotype, and mtDNA clade from multiple populations to test these nested hypotheses. We tested for associations between mtDNA clade and phenotype (both armour morphology and body shape) in stickleback from the Cook Inlet Basin and Middleton Island, Alaska, which are regions of clade admixture (Lescak *et al.*, 2015b). In the Middleton Island populations, we also tested for significant partitioning of nuclear genetic variation by clade and sought to identify nuclear genomic regions of significant divergence between fish from alternative mitochondrial lineages, using a dense set of over 100 000 SNP markers across the stickleback genome.

METHODS

Field collections

Stickleback were collected between 2005 and 2011 using 0.32- and 0.64-cm mesh minnow traps set near shore and left overnight. Fish were euthanized with an overdose of neutral pH MS-222 anaesthetic and preserved in 95% undenatured ethanol. We sampled six resident freshwater populations from the Cook Inlet Basin and 11 resident freshwater and two oceanic populations from Middleton Island (Fig. 1; Supporting Information, Table S1). Freshwater and oceanic habitats were determined based on salinity.

Sample preparation

Caudal and pectoral fins were clipped for DNA extraction. Each set of fins and corresponding body was assigned a unique identification number for association of genotype with phenotype, except for the Two Island and Sportfish Lake samples, for which we gathered morphological and genetic data from different individuals. Bodies were fixed in 10% neutral buffered formalin for at least 48 h, bleached in a 0.05% hydrogen peroxide solution, stained in a 0.1% Alizarin red S solution, destained in 1% KOH and preserved in 70% undenatured ethanol. DNA was extracted from clipped fins using a Qiagen DNeasy kit and samples were normalized to 25 ng/µL (or 10 ng/µL in populations with low yields).

Clade identification

The following primer pair was used to amplify a region of the cytochrome *b* gene: 14 372 (5′-ATGGCAAGCCTACGAAAAACGCAC-3′) and 15 100 (5′-TGCTAGGGATGTAAGGGCAATTAG-3′; Lescak *et al.*, 2015b). To assign the amplified cytochrome *b* gene fragments to either the TNPC or ENAC, we digested the amplicons with BstXI and NlaIII and size separated the products on agarose gels. The amplified cytochrome *b* amplicon in the TNPC lacks BstXI recognition sites and includes one NlaIII site, while descendants of the ENAC have one BstXI recognition site and two NlaIII sites (Ortí *et al.*, 1994).

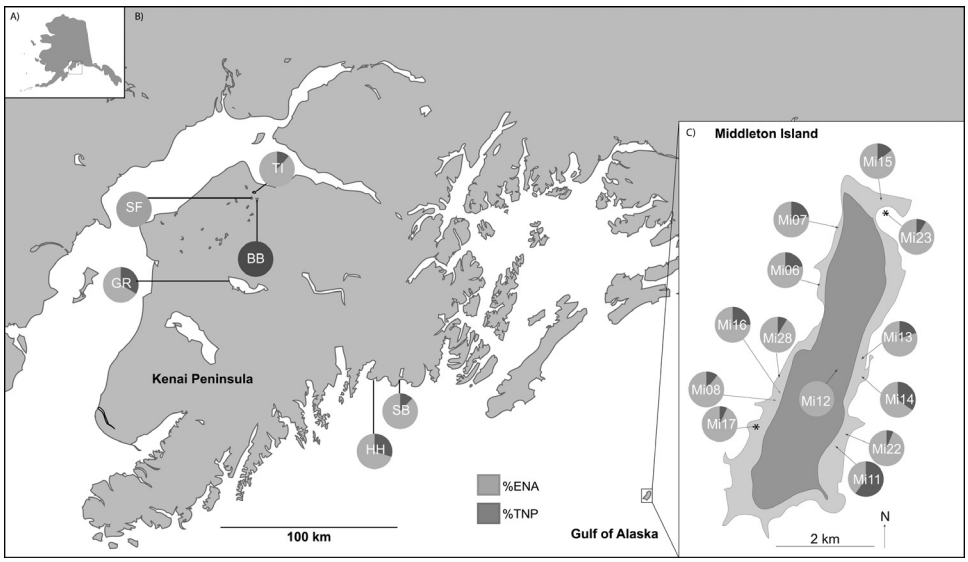

Figure 1. The extensive admixture of two mtDNA lineages was found in populations sampled from Cook Inlet and Middleton Island. (A) Map of Alaska with box indicating sampling region. Middleton Island is a small island in the lower right corner. (B) Kenai Peninsula. TI = Two Island, SF = Sportfish, BB = Barabara, GR = Grant, HH = Horsehead, SB = Salmonberry. (C) Middleton Island. Shading represents the pre-1964 size of the island. Asterisks indicate the two oceanic sites.

We performed independent digests with each of the enzymes to unambiguously assign each individual to one or the other lineage. We also sequenced the amplified gene fragment in 11 individuals total (6 ENAC and 5 TNPC) from six populations to confirm consistent correspondence between restriction site arrangement and mtDNA clade affiliation (Lescak *et al.*, 2015b; GenBank accession numbers KM508783–KM508793).

Armour analysis

Pelvic scores were assigned according to methods described in Bell *et al.* (1993) and lateral plates were counted from photographs. Pelvic spines were measured from the anterior-most point of the articulation with the pelvic girdle to the tip of the spine. All remaining linear measurements were made using ImageJ v1.43r. Dorsal spines were measured from the anterior side of the articulation to the tip of the spine. The most anterior supporting plate that articulated with the second pterygiophore was measured for maximum height and total area. However, in 12 samples, no plates met this criterion, so the plate that came closest to articulation was measured. Morphometric data were size standardized following Berner *et al.* (2011; Supporting Information, Fig. S1).

We performed Pearson correlations between trait means and proportion TNPC to compare our results to previous findings (Johnson & Taylor, 2004). These analyses were performed with and without the samples from Barabara Lake because this population was an outlier (Figs. 1, 2). We also analysed variation at the level of the individual with a principal components analysis (PCA) using the pcaMethods package (Stacklies *et al.*, 2007) in R (R Development Core Team, 2014). Because principal components (PCs) are by definition orthogonal, separate analyses of variance (ANOVAs) were performed on the first three PCs to test for significant differences in phenotype between clades, sexes and regions (Cook Inlet mainland vs. Middleton Island), and among populations. Regions were analysed collectively and independently to account for potential variation due to their different spatial and temporal scales; Cook Inlet freshwater populations were originally colonized ~10 Kya (Bell & Foster, 1994), while populations from Middleton Island were colonized less than 60 years ago (Lescak *et al.*, 2015a). Tukey's *post hoc* tests were used to determine which populations significantly differed. If mtDNA clade had a significant effect on phenotypic variation, we expected to see relationships reflected in the ordination, with the two clades clustered separately along one of the major axes of variation.

Body shape analysis

Body shape was characterized using geometric morphometrics (Zelditch *et al.*, 2004). Nineteen homologous landmarks were identified on 712 individuals (Supporting Information, Fig. S1) and placed on photographs of the left lateral view of the fish using TPSdig v. 2.16 (Rohlf, 2010). Prior to geometric morphometric analysis of shape, the 'unbend specimens' algorithm in TPSutil v. 1.47 (Rohlf, 2010) was used to remove non-biological shape differences due to specimen

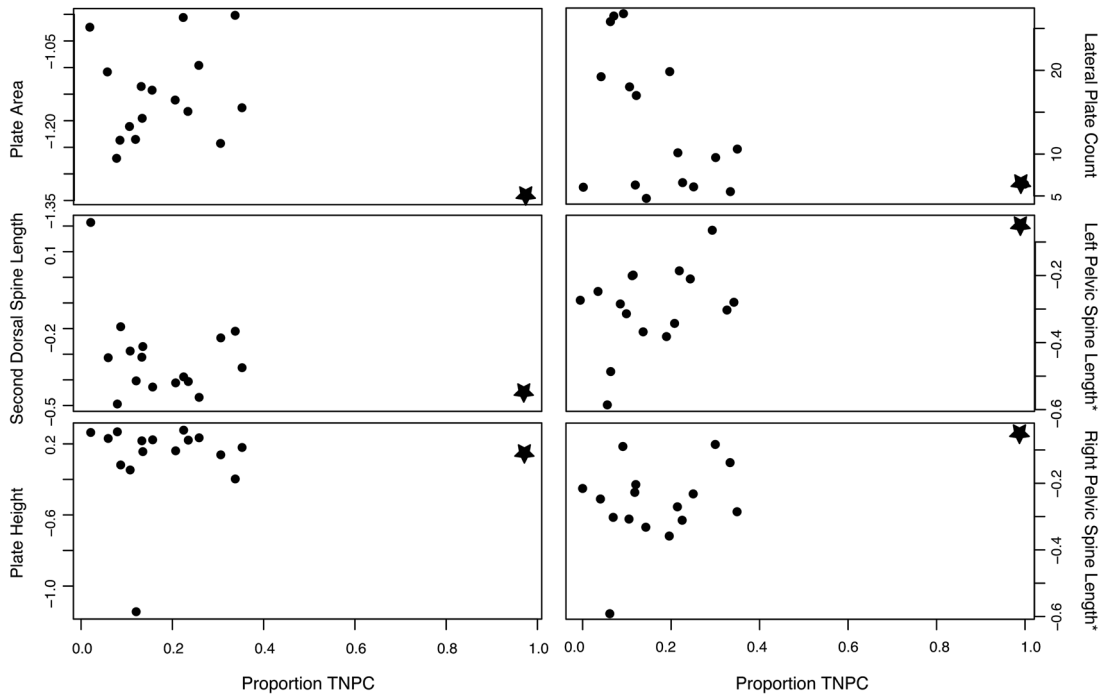

Figure 2. mtDNA clade was not significantly correlated with most phenotypic traits. Mean trait values for each population are plotted against the proportion of individuals belonging to the TNPC. Both left and right pelvic spine lengths have significant positive correlations with TNPC proportion in analysis of the full data set, but these relationships are no longer significant when the Barabara population (indicated by a star) is removed.

bending. Landmark coordinates were adjusted such that landmarks 1, 8 and 19 (Supporting Information, Fig. S1) fell along a straight line, as they would naturally.

Using MorphoJ v. 1.05f (Klingenberg, 2011), generalized Procrustes analysis superimposition was performed on all specimens to obtain adjusted landmark coordinates that were independent of size, rotation and translation (Zelditch *et al.*, 2004). We used canonical variates analysis (CVA) to examine shape differences between regions, sexes and clades, and among populations. Prior to CVA, shape differences due to body size (measured as centroid size, the sum of the squared distances between each landmark and the landmark configuration centroid) were removed using pooled within-group regression to account for possible slope heterogeneity among populations (Reist, 1986). Residuals from this regression were then used in CVA.

ANOVAs on CV scores determined whether shape differed between regions, ecotypes, sexes and clades, and among populations nested within regions. All factors except population were included as fixed factors in ANOVA models. Population was included as a nested (within region) random factor. Two separate CVAs (and subsequent ANOVAs) were performed. The first included all populations and determined whether shape differed between regions, ecotypes and sexes, and among populations. The second CVA also included clade as a factor and omitted the two populations for which clade was not associated with individuals (Two Island and Sportfish Lakes).

Genetic sex determination

Stickleback that could not visually be assigned to a sex were genotyped using a PCR assay (Griffiths *et al.*, 2000). The PTC-200 thermocycler (Bio-Rad) denatured the reactions at 94 °C for 3 min and then performed 36 cycles of 94 °C for 45 s, 44 °C for 45 s and 72 °C for 45 s before a final extension at 72 °C for 10 min. All individuals were unambiguously assigned to a sex.

Library preparation and sequence analysis

We used the same Middleton Island restriction site-associated DNA sequencing (RAD-seq) data that we reported in Lescak *et al.* (2015a; Supporting Information, Table S2), which yielded 130 000 SNPs. F_{ST} was calculated at every SNP among populations using the populations program in *Stacks* (Catchen *et al.*, 2011, 2013). These calculations were used to generate an F_{ST} scan to determine whether any SNP position was associated with mitochondrial lineage and, if so, to identify its genomic location. This analysis was performed for all Middleton Island individuals

pooled, as well as separately for three sites containing comparable numbers of each clade. To analyse population structure using multilocus genotypes, we used the populations program in *Stacks* to output filtered SNP data from all RAD loci across all populations into a file formatted for *STRUCTURE*. As in Lescak *et al.* (2015a), we randomly chose three subsets of 1000 SNPs each to complete the analysis, which based on the literature, is a more than adequate sample size for determination of population structure (e.g. Morin, Martien & Taylor, 2009). These subsets were used in *GenoDive* (Meirmans & van Tienderen, 2004) to identify the major axes of genetic variation using PCA and to test for significant partitioning of variation using an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992).

RESULTS

Alaskan stickleback populations contain a mixture of mtDNA clades and significant nuclear genome variation. While the ENAC predominated over the TNPC overall (77–23%), TNPC proportions ranged from 0 to 100% among populations (Fig. 1). Populations from Middleton Island significantly differed in scores from genetic PC 1 ($F_{12, 717}$ = 115.2; P < 0.001), PC 2 ($F_{12, 717}$ = 655.7; P < 0.001) and PC 3 ($F_{12, 717}$ = 75.4; P < 0.001; Supporting Information, Fig. S2).

Phenotypic variation was not consistently associated with mtDNA clade. At the population level, correlations between mean armour traits and TNPC proportion were not significant, with the exception of right and left pelvic spine length in the analysis of the full data set (Fig. 2; Supporting Information, Tables S3, S4). These associations were no longer significant if we imposed a Bonferroni correction or removed the Barabara population from the analysis, which is an outlier because it is the only sampled population apparently fixed for the TNPC and also the only one with pelvic reduction, typified by extreme diminution or loss of parts of the pelvic apparatus, including the spines (Bell *et al.*, 1993).

At the individual level, ANOVAs of scores from PC 1 to 3 in the analysis of armour traits revealed significant differences between clades (Fig. 3). However, when the two regions were analysed separately, there were no significant differences between sexes or clades in individuals from Cook Inlet (Fig. 3), but there were significant differences among populations (P < 0.001; Supporting Information, Fig. S3). Individuals from Middleton Island significantly differed in scores from phenotypic PC 1 to 3 based on sex and population (P < 0.001; Supporting Information, Fig. S3). Middleton phenotypic PC 3 scores significantly differed between clades (Fig. 3), but this PC accounted for less than 1% of the overall variation; any differences between clades are likely to be driven by clade by population interactions. When lateral plates were removed from the analysis because they had the highest loading on PC 1 (Supporting Information, Table S5), no significant differences were found between clades in scores from PC 1 to 3 in individuals from Cook Inlet or Middleton Island.

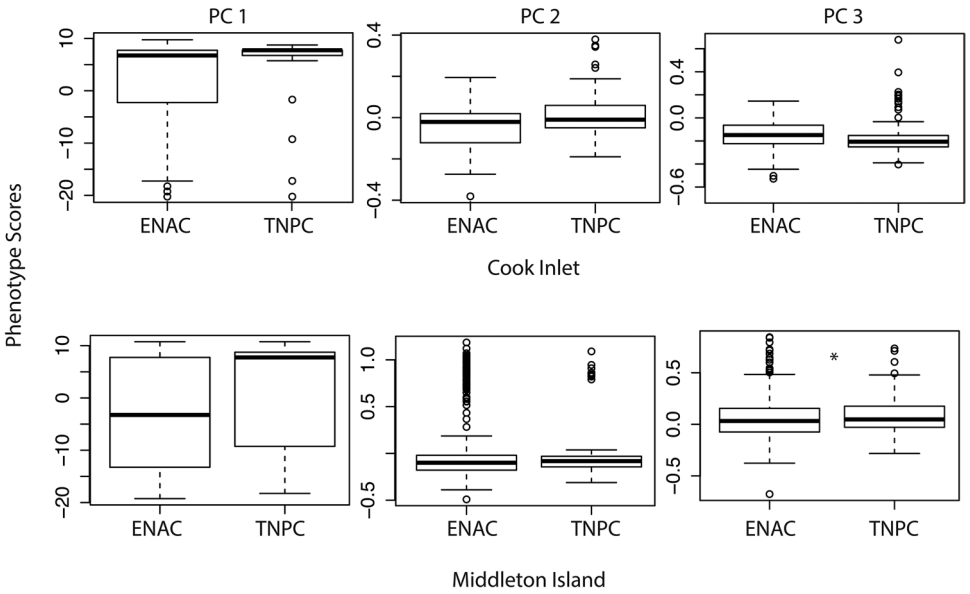

Figure 3. Individuals from Cook Inlet and Middleton Island belonging to the two lineages do not vary in PC scores of phenotype, with the exception of PC 3 in the Middleton Island data set.

In the analysis of body shape, significant differences in CV 1 were found between regions and sexes and among populations ($P < 0.001$; Supporting Information, Fig. S4; Supporting Information, Table S6). Significant differences in CV 2 were found among populations and between ecotypes and sexes ($P < 0.001$; Supporting Information, Fig. S4; Supporting Information, Table S6). When we removed Two Island and Sportfish Lakes due to a lack of corresponding genetic and phenotypic data, significant differences in CV 1 were still detected between regions and sexes and among populations ($P < 0.001$; Supporting Information, Fig. S4; Supporting Information, Table S6) and significant differences in CV 2 scores were detected among populations and between ecotypes, sexes and clades ($P < 0.001$; Fig. 4, Supporting Information, Fig. S4; Supporting Information, Table S6).

There was little evidence for epistasis between mtDNA and the nuclear genome. The two mtDNA lineages differed significantly in scores from genetic PC 1 ($F_{2, 717} = 11.0$; $P < 0.001$), but not PC 2 ($F_{2, 717} = 0.51$; $P = 0.599$) or PC 3 ($F_{2, 717} = 0.27$; $P = 0.766$). Differences in PC 1 scores were driven by a significant population by clade interaction ($F_{23, 717} = 3.49$; $P < 0.001$; Fig. 5), due to the large variation in clade frequencies among populations (Fig. 1). The three subsets of the data are concordant based on ANOVA of PC 1 scores ($F_{2, 16} = 0.066$; $P = 0.936$), so analyses are shown for only one data set. Global pairwise F_{ST} between the two lineages was 0.011. Intra-population analyses of Mi7, Mi14 and Mi16, which contained comparable proportions of each clade, revealed low levels of genetic divergence among individuals based on clade identity (pairwise F_{ST} between the two lineages ranged from 0.001 to 0.070; Supporting Information, Table S7). Most of the variation was partitioned at the level of the individual, with less than 2% attributed to differences between clades (Supporting Information, Table S8). Scans of F_{ST} across the genome did not identify any genomic regions that were highly divergent between the two lineages in inter- or intra-population analyses (Fig. 5).

DISCUSSION

Southcentral Alaska is a region of admixture for divergent mtDNA clades. Our fine-scale sampling revealed that individuals from both ancient clades are present in allopatry and sympatry across habitat types. In isolated water bodies, lineage sorting would probably result in one haplotype eventually becoming fixed. However, this process may occur over a longer time frame than the age of the populations that we examined (~10 000 years for the Cook Inlet Basin populations and less than 60 years for those on Middleton Island; Bell & Foster, 1994; Gelmond, von Hippel & Christy, 2009; Lescak *et al.*, 2015a) and is also dependent on effective population size, which is unknown. Since the lakes in our study are coastal and low-lying, it is also possible that these populations have experienced multiple invasions (Johnson & Taylor, 2004; Lescak *et al.*, 2015a).

Typically, about 5% of anadromous stickleback in southcentral Alaska belong to the TNPC (Cresko,

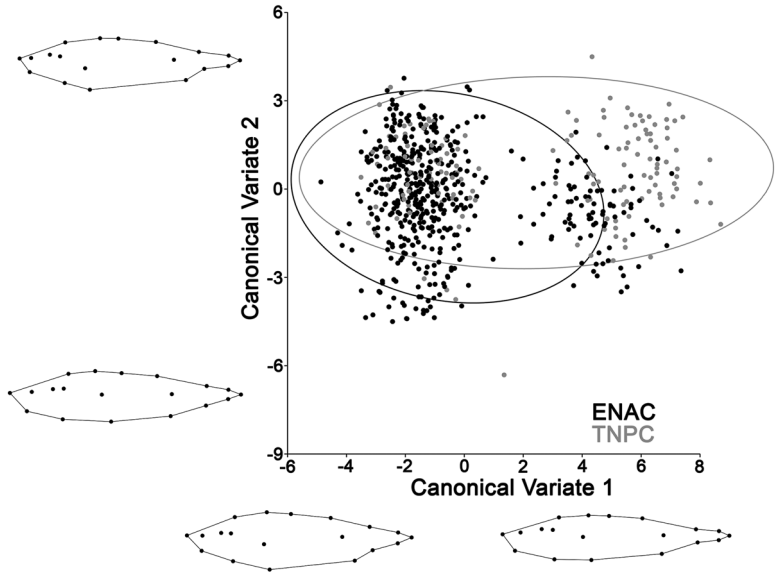

Figure 4. The plot of CV 1 vs. CV 2 from two CVAs. Individuals from the two mtDNA clades differ in CV 2, but not CV 1 scores of body shape. Equal frequency ellipses contain 90% of the observations. Wireframe diagrams represent shapes of fish at CV values of ±6.0.

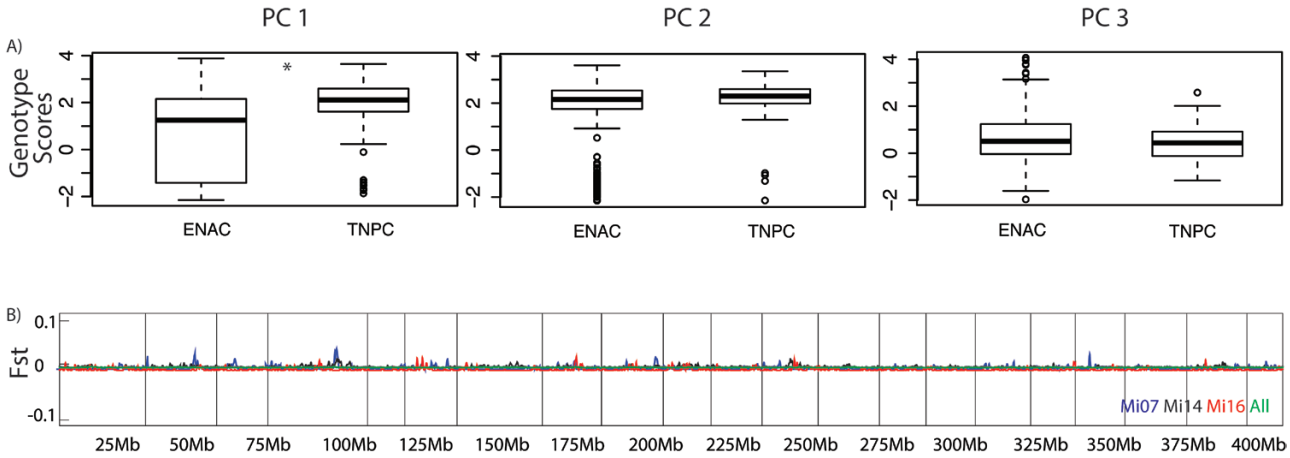

Figure 5. Associations between mtDNA clade and nuclear genetic variation. (A) Distributions of PC scores in analyses of nuclear markers in Middleton Island populations. Asterisks indicate significant differences at $P < 0.05$. Differences in PC 1 scores were driven by a significant population by clade interaction ($F = 3.49$, $P < 0.001$), due to the large variation in clade frequencies among populations. (B) Average F_{ST} comparisons across the genome do not reveal nuclear regions that significantly differ between individuals belonging to the two mtDNA lineages. Vertical lines separate linkage groups.

2000; Drevecky, 2011; Weigner, 2012; Lescak *et al.*, 2015b). Because oceanic fish founded the freshwater populations, the expectation is that freshwater clade proportions would be comparable to those seen in oceanic populations. However, the Barabara, Horsehead and Grant Lake freshwater populations show substantially higher proportions of the TNPC than the oceanic populations, similar to what has been observed in the Bering Glacier region of Alaska (Weigner, 2012). The variation we observed in clade proportions between ecotypes is probably the result of neutral processes, such as gene flow or genetic drift (Johnson & Taylor, 2004).

The two mtDNA clades do not define cryptic species. Previous population-level analyses correlated TNPC proportions with armour reduction (O'Reilly *et al.*, 1993; Deagle *et al.*, 1996). However, similar to Johnson & Taylor (2004), we did not detect morphological differences between clades when Middleton Island and Cook Inlet were analysed separately. Additionally, reduced armour is also a common phenotype in resident freshwater populations from the Atlantic Basin, where the TNPC lineage is absent (Hendry *et al.*, 2013). We should note that while body armour shows strong global parallelism, heritability in body shape differs among stickleback populations (e.g. Baumgartner, 1995; Berner *et al.*, 2011; Leinonen *et al.*, 2011; Lucek & Seehausen, 2015), and therefore may not have a strong genetic basis in the populations we studied. The population from Barabara Lake, which is apparently fixed for the TNPC, was not morphologically similar to stickleback from other TNPC-dominated populations (Horsehead and Grant), supporting the hypothesis that local environmental conditions, rather than mtDNA clade, are probably the major driver of phenotypic variation (Bell & Foster, 1994; Johnson & Taylor, 2004). While ecological data were not collected for each of our sample sites, it is well documented that predation regime and water chemistry influence the morphology of stickleback populations throughout their distribution (e.g. Giles, 1983; Reimchen, 1994; Lescak & von Hippel, 2011; Lescak *et al.*, 2012; Zeller *et al.*, 2012; Hendry *et al.*, 2013; Voje *et al.*, 2013). Alternatively, the absence of gene flow could cause the Barabara population to be morphologically distinct (DeFaveri *et al.*, 2013; Lucek & Seehausen, 2015).

mtDNA divergence does not affect contemporary patterns of population structure. Concordant patterns of mitochondrial, nuclear and phenotypic variation have been observed in congeneric butterflies *Anartia fatima* and *Anartia amathea* within their hybrid zone, supporting assortative mating within taxa and epistasis between mitochondrial and nuclear loci (Dasmahapatra *et al.*, 2002). However, discordant patterns of genetic divergence between mtDNA and nuclear DNA have been reported in other species, including the barnacle *Notochthamalus scabrosus* (Zakas, Jones & Wares, 2014), Pacific herring (*Clupea palasii*; Liu *et al.*, 2012) and rock pocket mouse (*Chaetodipus intermedius*; Hoekstra, Krenz & Nachman, 2005). In our study, the low levels of nuclear genetic divergence between the two mtDNA clades and the lack of localized regions of divergence across the genome, coupled with the absence of a relationship between mtDNA lineage and microsatellite variation (Cresko, 2000), suggest that if the nuclear genome diverged concurrently with the mitochondrial genome ~90 Kya–1 Mya, the signal of divergence has been lost, probably due to

recombination in the nuclear genome and a prolonged period of secondary contact between the two clades.

CONCLUSION

We tested the hypotheses that historic evolutionary divergence during an ancient period of allopatry led to reproductive barriers that persist between fish with different mtDNA lineages in contemporary populations, or that epistatic interactions among loci in the mitochondrial and nuclear genomes would maintain non-random associations among these genomes. Although ancient genetic divergence has affected contemporary patterns of population genetic and phenotypic diversification in some taxa, we did not find strong evidence for it in our studied populations of three-spined stickleback. Rather, the two mtDNA clades in this species represent ancient variation that has probably been largely reorganized in the nuclear genome due to recombination and widespread secondary contact, but is still segregating in the mtDNA. While there appear to be no reproductive barriers between the two lineages, this hypothesis would have to be explicitly tested through attempts to identify pre- and post-zygotic isolating mechanisms.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sample collection data, including site name, coordinates, habitat type based on water chemistry (FW = freshwater, OC = oceanic), and whether nuclear genetic and/or phenotypic data were collected.
Table S2. Per sample read statistics after cleaning and demultiplexing.
Table S3. Means of morphological measurements for individuals from each population (BB = Barabara, GR = Grant, HH = Horsehead, SB = Salmonberry, SF = Sportfish, TI = Two Island, Mi = Middleton). LP: left side lateral plate count, SDS: second dorsal spine length (mm), SPh: second supporting plate height (mm), SPa: second supporting plate area (mm^2), PS: pelvic score, LPS: left pelvic spine length (mm), RPS: right pelvic spine length (mm), SL: standard length (mm).
Table S4. Pearson correlations of each trait mean vs. proportion Trans North Pacific Clade.
Table S5. Loadings for phenotypic principal components (PCs) 1–3.
Table S6. Univariate tests of significance, effect sizes and powers for canonical variates (CV) 1 and 2 for the entire data set and the analysis excluding Sportfish (SF) and Two Island (TI).
Table S7. Heat map of pairwise FST in TNPC and ENAC individuals from Mi07, Mi14 and Mi16.
Table S8. Comparison of analyses of molecular variance on the Middleton Island dataset using three replicates of 1000 nuclear loci.
Figure S1. Armour and body shape measurements. (A) Armour measurements and (B) locations of landmarks for geometric morphometric analysis. Landmarks 1–23 are from McGuigan *et al*. (2011). Landmark 24 was added to unbend the fish.
Figure S2. Significant differences among Middleton Island populations in PC scores from analysis of 1000 nuclear loci. Letters represent sites that significantly differ at $P < 0.05$.
Figure S3. Significant differences in phenotypic principal component (PC) scores between sexes and regions and among populations. Distributions of PC scores are shown for (A) sex, (B) region, (C) Cook Inlet and (D) Middleton Island in the analysis of armour phenotypes. Asterisks in panels (A) and (B) represent differences at $P < 0.05$. Letters in panels (C) and (D) indicate population-level differences at $P < 0.05$ based on Tukey's *post hoc* tests.
Figure S4. Significant differences in body shape between regions and sexes and among populations. Plots are shown of CV 1 vs. CV 2 from two canonical variates analyses. Panels (A)–(D) depict results from a single analysis in which CVs were computed to distinguish between regions (Middleton Island vs. Cook Inlet), sexes, ecotypes and among populations. Colours in each panel highlight different groupings within the same analysis: (A) regions, (B) sexes, (C) ecotypes, (D) populations. In (A)–(C), ellipses are equal frequency ellipses containing 90% of the observations in respective groups, whereas ellipses in panel (D) are 95% confidence ellipses of population means. Wireframe diagrams in panel A represent shapes of fish at CV values of ±6.0.