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ORIGINAL ARTICLE

Admixture of ancient mitochondrial lineages in three-spined stickleback populations from the North Pacific

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ABSTRACT

Aim We test the hypothesis that the North Pacific coastline from British Columbia to the Kuril Islands forms a broad region of admixture between two divergent mitochondrial-genome (mtDNA) lineages in three-spined stickleback (*Gasterosteus aculeatus*): the Euro-North American (ENA) and Trans-North-Pacific (TNP or Japanese) clades. We test whether distance is the primary determinant of geographical patterns of haplotype distributions and whether deep-water trenches in the Aleutian and Kuril archipelagos impede gene flow.

Location Coastal marine and freshwater sites from the Kuril Islands (north-western Pacific Ocean) to Oregon (north-eastern Pacific Ocean).

Methods We determined the mtDNA clade for 1327 individuals from 67 locations across 8000 km of the North Pacific using restriction fragment length polymorphism assays of the cytochrome *b* mitochondrial gene. We supplemented this with published clade designations from coastal Pacific populations and applied generalized linear modelling and Mantel tests. We used analysis of molecular variation (AMOVA) to test for significant partitioning of genetic variation by deep-water trenches.

Results The western boundary of the ENA clade was Simushir (Kuril Islands) and the eastern boundary of the TNP clade was British Columbia. Coastline distance from Japan was a significant predictor of TNP abundance. Clade composition variance was high at small geographical scales, in apparent discordance with the broader-scale pattern of admixture. Deep-water trenches were not found to significantly partition genetic variation in the Aleutian and Kuril island chains.

Main conclusions The North Pacific coastline forms a broad region of secondary contact between divergent mitochondrial lineages, but with clear western and eastern boundaries. Patterns of clade abundance in the Kuril and Aleutian islands are similar to those observed in other taxa, suggesting shared biogeographical histories and common barriers shaping species distributions along the North Pacific coast. Overall patterns of clade distributions are likely to be driven by a combination of factors, including geomorphological impediments to migration, ecology, asymmetrical dispersal patterns and differences in timing of population expansions.

Keywords

Admixture, Aleutian Islands, biogeography, Gasterosteidae, *Gasterosteus aculeatus*, introgression, isolation by distance, Kuril Islands, mtDNA, phylogeography.

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INTRODUCTION

Identifying how landscape and the environment shape patterns of genetic diversity improves our understanding of microevolution (Storfer *et al.*, 2006). The distribution of genetic variability at neutral loci has been shown to be associated with geography (e.g. isolation by distance or ecological discontinuities) in marine and freshwater fishes (Jørgensen *et al.*, 2005; Dionne *et al.*, 2008; Jones *et al.*, 2012). However, an open question concerns how well genetic markers are able to recapture deep ancestral divergence over large geographical scales.

A key organism to address this question is the three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) because it is broadly distributed in temperate and subpolar freshwater and marine habitats throughout the Northern Hemisphere and many aspects of its biology have been well studied for decades (for reviews, see Bell & Foster, 1994; Östlund-Nilsson *et al.*, 2006; von Hippel, 2010). A growing body of research on the adaptive evolution of three-spined stickleback to freshwater and marine environments reveals linkages between genetic variants and ecologically important phenotypes (e.g. Peichel *et al.*, 2001; Colosimo *et al.*, 2004, 2005; Cresko *et al.*, 2004; Jones *et al.*, 2012). To reconstruct the biogeographical history of this species and test hypotheses regarding present and historical gene flow, we examined the genetic variation in populations from the North Pacific. This deep history allows us to more fully understand the evolutionary dynamics of adaptive radiation, by enabling the characterization of local, regional and global patterns of genetic variability and the spatial factors that influence the distribution of that variation (e.g. Jones *et al.*, 2012).

Analyses of both nuclear and mitochondrial (mtDNA) genomes reveal distinct lineages in modern populations of three-spined stickleback found in the Atlantic and Pacific basins (Haglund *et al.*, 1992; Ortí *et al.*, 1994; Colosimo *et al.*, 2005; Jones *et al.*, 2012) – the Euro-North American (ENA) and Japanese or Trans-North-Pacific (TNP) clades – named based on assessments of their geographical distribution (Ortí *et al.*, 1994; Johnson & Taylor, 2004). All surveys of standing mtDNA genetic variation reported to date have confirmed that only these two lineages occur in extant freshwater and marine three-spined stickleback populations and each clade has multiple sublineages (Gach & Reimchen, 1989; Haglund *et al.*, 1992; O'Reilly *et al.*, 1993; Ortí *et al.*, 1994; Deagle *et al.*, 1996; Cresko, 2000; Mäkinen *et al.*, 2008; Weigner, 2012).

Divergence between the two mtDNA lineages has been estimated at approximately 0.9–1.3 Ma based on silent-site substitutions in cytochrome *b* DNA sequences, a rate of divergence calibrated from the split between nine-spined stickleback (*Pungitius pungitius*) and three-spined stickleback, the fossil record and Markov chain Monte Carlo approaches to modelling migration rates (Ortí *et al.*, 1994; Nielsen & Wakeley, 2001). The TNP clade is most common in coastal areas of the western Pacific basin and is not found in any surveyed populations from the Atlantic Ocean, whereas the ENA clade is found in the eastern Pacific and in the Atlantic (O'Reilly *et al.*, 1993; Ortí *et al.*, 1994; Mäkinen *et al.*, 2008).

The first global survey of mtDNA diversity revealed that Alaska and British Columbia are regions of clade admixture (Ortí *et al.*, 1994). The observations reported to date point to British Columbia as the eastern extent of the TNP clade members (Ortí *et al.*, 1994; Deagle *et al.*, 1996; Johnson & Taylor, 2004), whereas the ENA lineage has not been reported further west than south-central Alaska. Clade-specific haplotype frequencies have been documented among populations in close proximity in the region of admixture; the proportion of individuals carrying ENA mtDNA haplotypes ranges from 0.03 to 1 in anadromous populations and from 0 to 1 in resident freshwater populations (Deagle *et al.*, 1996; Cresko, 2000; Johnson & Taylor, 2004; Weigner, 2012).

Surveys of mtDNA clade diversity in three-spined stickleback populations from the Pacific have left an unsampled gap, approximately 4600 km long, between south-central Alaska and Japan (Fig. 1). On the western Pacific coast, the northernmost population sampled is on Hokkaido and the westernmost samples from North America originated from Cook Inlet, Alaska (Ortí *et al.*, 1994; Cresko, 2000). Until this report, the distribution and composition of mtDNA clades along the length of the Kuril and Aleutian island chains had not been examined, but we hypothesize that these are likely zones of secondary contact between the mtDNA lineages.

In this study, we test the hypothesis that the North Pacific coastline from British Columbia to the Kuril Islands forms a broad region of admixture between the ENA and TNP clades. We also test whether distance is the primary determinant of geographical patterns of haplotype distributions, and whether deep-water trenches in the Aleutian and Kuril archipelagos impede gene flow. We characterized the distribution of the two mtDNA clades across the North Pacific basin in populations along an approximately 8000-km transect from the Kuril Islands to Oregon (Fig. 1). If the distance from source populations shapes clade composition, then we would expect to see a cline in clade proportions with increasing frequency of TNP haplotypes progressing westwards along the North Pacific Basin toward the source populations. We hypothesize that discontinuities in this cline may represent partial barriers to migration due to present or historical impediments to fish movement, such as deep-water trenches in the Kuril and Aleutian island chains, and/or ecotones.

MATERIALS AND METHODS

Sampling

We determined clade assignment of the mtDNA genome from 1327 individuals originating from 67 sites across

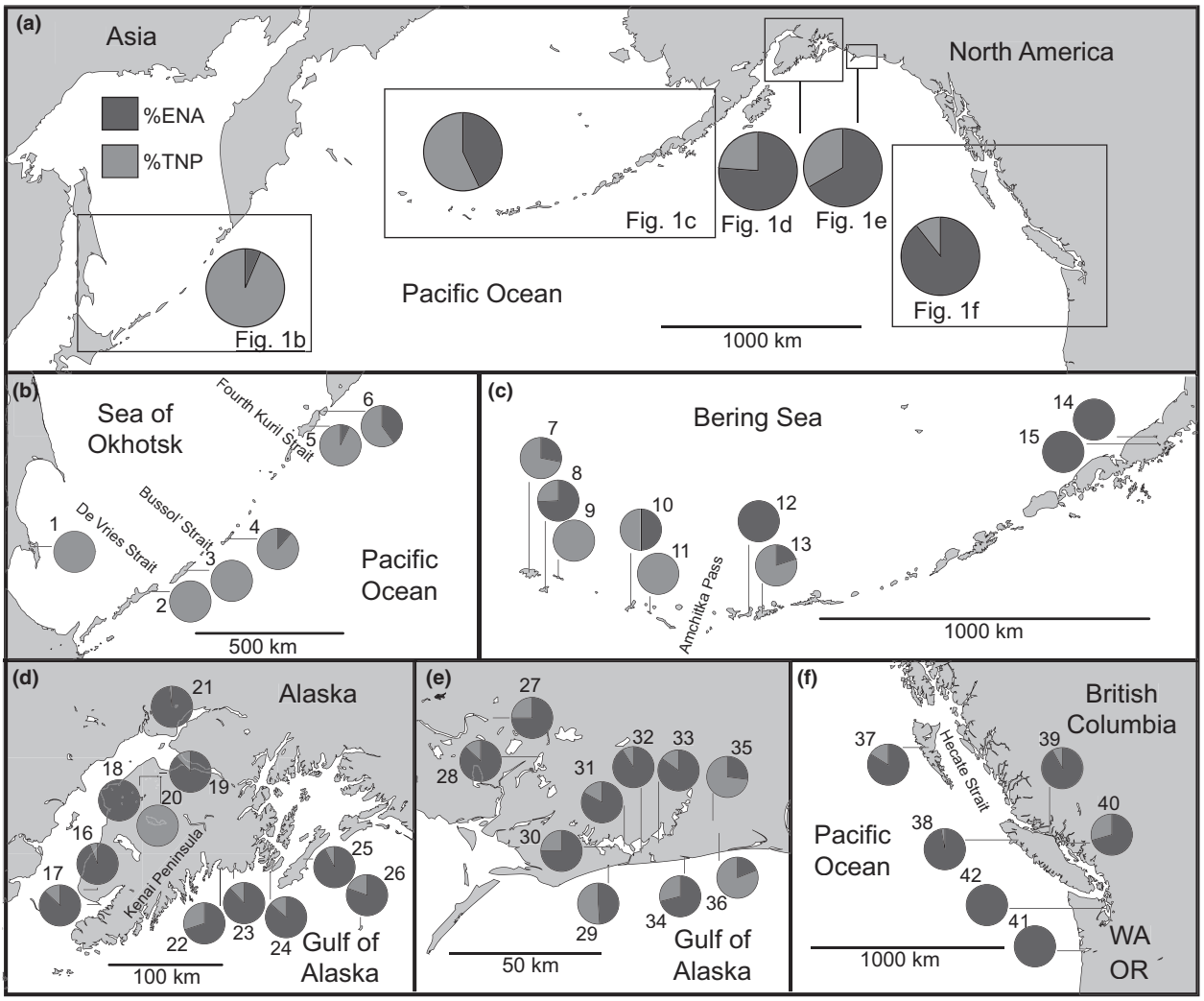

Figure 1 Sampling locations of the three-spined stickleback (*Gasterosteus aculeatus*) with corresponding pie-charts representing clade proportions. (a) North Pacific coast; (b) Kuril island chain; (c) Aleutian island chain and Alaska Peninsula; (d) south-central Alaska; (e) Bering Glacier forelands; and (f) west coast of North America. Numbers of locations correspond to those given in Appendix S1.

approximately 8000 km of the coastal North Pacific from the Kuril Islands to Oregon. Tissue samples of specimens from Washington, the Kuril Islands and Sakhalin were lent by the University of Washington Fish Collection, where the vouchers are archived. We supplemented this dataset with published mtDNA clade frequencies from Cook Inlet, Alaska (Cresko, 2000; E.A.L., unpublished data), Bering Glacier, Alaska (Weigner, 2012) and British Columbia (Deagle *et al.*, 1996; Johnson & Taylor, 2004), resulting in a total dataset of 3682 individuals from 169 locations (Fig. 1, and see Appendix S1 in Supporting Information). Specimens were collected between 1994 and 2011 using minnow traps, beach seines and dip nets. Fish and/or tissue samples were preserved in ethanol. DNA was isolated from fin clips using the Chelex method (Bio-Rad, Hercules, CA, USA) or either DNeasy or Puregene (Qiagen, Valencia, CA, USA) tissue kits following manufacturers' protocols.

Haplotype determination

The following primer pairs were used to amplify a region of the cytochrome *b* gene: Gacu_14459_F (5′-TGATGAAACTT TGGTTCCCTCCTT-3′) and Gacu_15177_R (5′-TTGATGT-GAGGTGGAGTGACTAA-3′), or 14372 (5′-ATGGCAAGCCT ACGAAAAACGCAC-3′) and 15100 (5′-TGCTAGGGATGTA AGGGCAATTAG-3′; Weigner, 2012). Amplification conditions were: 2 min at 94 °C, followed by 28 cycles of 30 s at 94 °C, 15 s at 58 °C and 45 s at 72 °C, and then 7 min at 72 °C, or 6 min at 94 °C, followed by 37 cycles of 60 s at 94 °C, 60 s at 63 °C and 120 s at 72 °C and finally 5 min at 72 °C, for each of the primer pairs respectively.

To assign the amplified cytochrome *b* gene fragments to either the TNP or the ENA clade, we performed restriction digests with *BstXI* and *NlaIII* and size-separated the products on agarose gels. The amplified cytochrome *b* fragment in the

TNP clade lacks *BstXI* recognition sites and includes one *NlaIII* site, whereas descendants of the ENA lineage have one *BstXI* recognition site and two *NlaIII* sites (Ortí *et al.*, 1994). We performed independent digests with each of the two enzymes to increase confidence in clade assignment. All individuals were unambiguously assigned to one lineage based on the diagnostic restriction sites. We also sequenced the amplified gene fragment in a subset of 11 individuals (6 ENA and 5 TNP) from six Alaskan populations to confirm consistent correspondence between restriction-site arrangement and mtDNA clade affiliation.

Biogeographical analyses

To characterize relationships between geography and the distribution of the two mtDNA clades across populations, we first determined direct and coastline distances between each sampling location and Sakhalin, the westernmost sampled population, using Google Earth and ArcGIS 10.1 (ESRI, Redlands, CA, USA). Because direct and coastline distances are highly correlated ($r^2 = 0.99$), we used coastline distances only in our analyses, because oceanic stickleback are thought to move primarily along the coast rather than through open water (Bell & Foster, 1994). In some instances, however, stickleback have been found in offshore regions of the Pacific Ocean (Quinn & Light, 1989; Deagle *et al.*, 1996; Morita *et al.*, 2009; Atcheson *et al.*, 2012a,b).

To test whether distance is the primary determinant of geographical patterns of haplotype distributions, we first fitted a binomial generalized linear model to the relationship between coastline distance and clade proportions. This analysis was initially performed using all populations. We were able to categorize individuals from Alaska, British Columbia and Oregon into ecotypes (freshwater versus oceanic) based on phenotype (high versus low lateral plate counts and body size) or previously published classifications, which allowed us to also perform separate analyses on the two ecotypes from these regions. Outliers were identified through the use of diagnostic plots of residuals to determine which sampling sites deviated from the linear model. Secondly, we used a Mantel test to test for significance between pairwise F_{ST} and geographical distances among populations.

To test whether deep-water trenches impede migration, we tested for significant partitioning of genetic variation using an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992), grouping populations in the Kuril and Aleutian archipelagos by their locations on either side of the Bussol' Strait and Amchitka Pass. Because of our unequal sampling distribution across sites (Appendix S1), we resampled 16 individuals (the mean number of individuals collected across populations) from all admixed sites 1000 times with replacement within each replicate. We then re-ran all analyses with the resampled distributions to ensure that our results were not due to unequal sample sizes. All analyses were performed in R 2.11.1 (R Core Team, 2014) or GenoDive (Meirmans & van Tienderen, 2004).

RESULTS

Overall clade proportions and clade distribution boundaries

Overall, 78% of the surveyed stickleback carried mtDNA from the ENA clade (Fig. 1, Appendix S1), which partly reflects a more intensive sampling of locations and individuals from the eastern Pacific coast. When we resampled our dataset, the ENA haplotype still predominated, but the percentage of individuals decreased to 58%. We found the western distribution limit of the ENA lineage to lie at the island of Simushir in the Kuril chain. The eastern distribution limit of the TNP clade lies along the coast of British Columbia (Johnson & Taylor, 2004; Fig. 1). We found a great deal of heterogeneity in clade proportions among closely spaced populations (Figs 1 & 2), with the Aleutian chain, Kenai Peninsula and Bering Glacier exhibiting the greatest variation.

The newly determined sequences from 11 individuals across Alaska confirm previously published (Ortí *et al.*, 1994; Cresko, 2000; Weigner, 2012) locations of restriction enzyme recognition sites. We did not observe any unexpected variation. These sequences have been deposited in GenBank (accession numbers KM508783–KM508793).

Geographical differences

Results of all statistical analyses on raw and resampled data were not significantly different. We therefore present only the results from the raw data. Coastline distance from Japan was a significant predictor of TNP relative abundance across the North Pacific without taking ecotype into account ($z = -18.62$, $P < 0.001$; Fig. 3). This relationship also held true when considering only freshwater ($z = -15.09$, $P < 0.001$) or oceanic ($z = -3.16$, $P = 0.002$) individuals from Alaska, British Columbia and Oregon. Diagnostic plots of residuals from the overall and freshwater analyses revealed

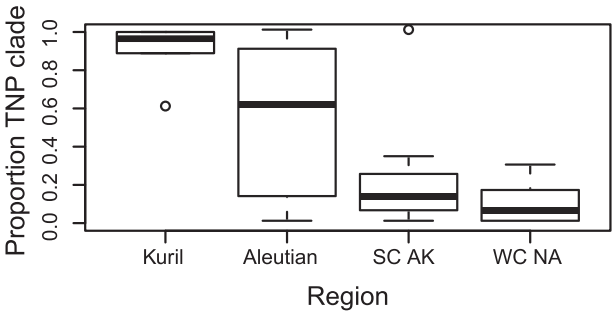

Figure 2 Variation in Trans-North-Pacific (TNP) clade proportions in the three-spined stickleback (*Gasterosteus aculeatus*) from the Kuril Islands (Kuril), Aleutian Islands (Aleutian), south-central Alaska (SC AK) and the west coast of North America (WC NA). Boxplots represent the median, quartiles and outliers for each region.

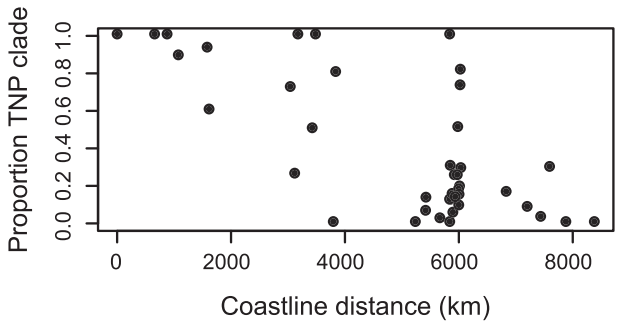

Figure 3 Plot of the proportion of individuals of the three-spined stickleback (*Gasterosteus aculeatus*) belonging to the Trans-North-Pacific (TNP) clade against coastline distance (km) from the westernmost study population (Sakhalin). Each point represents one sampling location along the North Pacific Basin.

two major outliers: Barabara in south-central Alaska and Tashalich River in the Bering Glacier region (see Appendix S2). Barabara was the easternmost population fixed for the TNP haplotype group. The Tashalich River population also had a higher proportion of TNP individuals (51%) than nearby populations. When considering only oceanic individuals, Middleton Island emerged as an outlier, having a greater representation of the TNP (19%) than other south-central Alaskan sites. Because model fits were comparable among freshwater, oceanic and pooled datasets, we performed a Mantel test to confirm significant effects of isolation by distance on only the pooled dataset. Our results were significant (Spearman's $r = 0.353$, $P < 0.001$), supporting the linear model.

The role of deep-water trenches as impediments to migration

The Bussol' Strait represents the breakpoint between the mtDNA clade admixture zone to the north and the zone of TNP lineage fixation to the south (Fig. 1). Similarly, the deep-water Amchitka Pass in the Aleutian Islands also appears to obstruct the movement of stickleback, because islands on either side of the pass are fixed for different clades. Statistical testing does not, however, support significant partitioning of genetic variation. AMOVA revealed that a significant amount of the variation was partitioned among individuals within populations (47.1%) and among populations grouped by location (40.9%; $P = 0.001$), but only 12% of the variation was partitioned across sites separated by trenches ($P = 0.174$).

DISCUSSION

The North Pacific represents an extended zone of admixture between two divergent stickleback mtDNA clades. Mixed populations occur along a transect extending approximately 6000 km from Simushir in the Kuril Islands across Alaska to British Columbia, suggesting widespread movement of oceanic stickleback following Pleistocene deglaciation. Coastline distance from Japan is a significant predictor of the proportion of individuals carrying mtDNA of the TNP haplotype. At the basin-wide scale, the presence of regions where only one mtDNA clade is present adjacent to a broad zone of admixture suggests that dispersal from these eastern and western 'source' populations drives the observed patterns of clade distribution.

The distribution of the two mitochondrial lineages follows an overall pattern of isolation by distance at the basin and regional scales, but at small spatial scales, there is a great deal of mtDNA clade composition heterogeneity among sampled locations. In the west, deep-water trenches in the Kuril and Aleutian island chains may impede stickleback migration (Fig. 1). The Bussol' Strait represents the break-point between the mtDNA clade admixture zone to the north and the zone of TNP lineage fixation to the south (Fig. 1) and has previously been identified as a biogeographical barrier influencing both plant and animal distributions (Barkalov, 2002; Bogatov, 2002; Kostenko, 2002; Lelej *et al.*, 2002; Teslenko, 2002; Pietsch *et al.*, 2003). The apparent absence of individuals carrying mtDNA from the ENA haplotype group south of the Bussol' Strait suggests that north to south movement across the strait is highly restricted or impossible.

Similarly, the deep-water Amchitka Pass in the Aleutian Islands appears to obstruct the movement of stickleback, because islands on either side of the pass are fixed for different clades. This portion of the Aleutian chain has previously been identified as a region of species turnover in skates (*Bathyraja*; Spies *et al.*, 2011), small Pacific Ocean perch (*Sebastes alutus*), Atka mackerel (*Pleurogrammus monopterygius*; Logerwell *et al.*, 2005) and rougheye rockfish (*Sebastes aleutianus*; Gharrett *et al.*, 2005). Our AMOVA results demonstrate, however, that no significant amount of variation is partitioned among groups of populations separated by either of these deep-water trenches, suggesting that mechanisms other than geomorphological barriers may drive the distribution of clade frequencies that we observe across these trenches.

Another potential explanation for the distribution of haplotypes along the Kuril archipelago may be ecological in nature. A species pair of three-spined stickleback is present in the Japanese archipelago and the Sea of Okhotsk – the Japan Sea stickleback and the Pacific Ocean stickleback (Kitano *et al.*, 2007, 2009). Despite being reproductively isolated due to genomic incompatibilities and divergent sex chromosomes, these two species share mitochondrial haplotypes (Yamada *et al.*, 2001). Because these two species differ in ecology, behaviour and genetic architecture (Kitano *et al.*, 2009; Kume *et al.*, 2010), these factors could also explain the distribution of haplotypes in this region. Considering that Japan Sea stickleback are found on Hokkaido and extend into the Kuril Islands, it is possible that some of the discordance in haplotype distributions that we observe is due to ecological differences among habitats rather than, or in addition to, migration barriers caused by deep-water trenches.

In addition, Markov chain Monte Carlo approaches to modelling migration rates using sequences from Ortí *et al.* (1994) are consistent with asymmetrical rates of gene flow across the Pacific Basin. Integrated likelihood surfaces are consistent with a model suggesting ongoing migration from western to eastern populations, but little gene flow in the other direction (Nielsen & Wakeley, 2001). The distribution that we observe may also be due in part to differences in the timing of population expansions. For example, the TNP lineage may have spread more rapidly from Japan, which was not extensively glaciated, than the ENA clade from North America, which was largely glaciated until about 13,000 years ago.

The pattern of alternative fixation of the two mtDNA types suggests a dynamic demographic regime where founder effects, unstable population structure (e.g. stochastic history of colonization and extinction) and colonization barriers (Johnson & Taylor, 2004) may be the primary drivers of local population genetic diversity. Although previous research has not provided evidence of consistent links between mtDNA clade type and morphology (Deagle *et al.*, 1996; Johnson & Taylor, 2004; E.A.L., unpublished data) or nuclear genotype (Cresko, 2000), we cannot rule out a possible role for deterministic evolutionary processes (e.g. selection or genomic co-adaptation) in shaping the observed patterns of mtDNA clade distribution.

This study presents the first densely sampled survey of three-spined stickleback mtDNA clade diversity across the North Pacific. We identified a region of clade admixture spanning approximately 6000 km of coastline from Simushir in the Kuril Islands to British Columbia, with a great deal of spatial heterogeneity in clade proportions among populations. We are the first to report the presence of the ENA haplotype west of south-central Alaska and our findings support previous work (Ortí *et al.*, 1994; Deagle *et al.*, 1996; Johnson & Taylor, 2004) that British Columbia represents the south-eastern extent of the TNP haplotype; the apparent absence of the TNP lineage south of Vancouver Island hints at a unidirectional barrier that impedes southward migrations.

The two extant mtDNA lineages are highly divergent and easily identified because they are separated by 18 nucleotide substitutions that have accumulated over an estimated 1 Myr of divergence (Ortí *et al.*, 1994). Although we were limited to testing for barriers to migration using only one marker, we would predict similar patterns in neutral nuclear markers (e.g. Haglund *et al.*, 1992; Higuchi & Goto, 1996; Peichel *et al.*, 2004; Colosimo *et al.*, 2005; Jones *et al.*, 2012). Loci that experience barriers to gene flow may, however, help us to identify locally adapted gene complexes. A valuable direction for future research would be to examine sequence variation in nuclear loci in populations spanning the North Pacific, to test for significant partitioning of genetic variation by haplotype. This hypothesis has previously been tested using a set of microsatellite markers (Cresko, 2000), with results supporting regular gene flow between the two lineages. Even if individuals containing mtDNA from the two different clades freely interbreed, however, there may be residual influence of this deep divergence on contemporary patterns of nuclear genetic variation at some loci, leading to patterns of cytonuclear disequilibrium, which can be detected using modern genotyping techniques.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Complete list of sampling locations, sample sizes and haplotype determinations.

Appendix S2 Plots of residuals from the generalized linear model fit of coastline distance versus proportion of the Trans-North-Pacific clade. Sites that are labelled were identified as outliers: (a) full data set; (b) freshwater individuals from Alaska, British Columbia and Oregon; and (c) oceanic individuals from Alaska, British Columbia and Oregon.

BIOSKETCH

Emily Lescak is broadly interested in the relative contributions of geography, environment and genetic architecture in shaping phenotypic variation in natural populations. She is a PhD candidate in Fisheries at the University of Alaska Fairbanks.

Author contributions: E.A.L., J.A.L., F.A.v.H. and L.A.K. conceived and designed the study. E.A.L., R.W.M., M.L.S. and J.J.C. collected mitochondrial haplotype data. E.A.L., L.A.K., W.A.C., F.A.v.H. and J.A.L. collected samples. E.A.L. performed analyses. E.A.L. and J.A.L. drafted the manuscript. R.W.M. drafted Fig. 1. R.W.M., L.A.K., W.A.C. and F.A.v.H. contributed to manuscript development and preparation.

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