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RESEARCH ARTICLE

Genetic divergence among threespine stickleback that differ in nuptial coloration

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Abstract

Sexual signals are shaped by their intended and unintended receivers as well as the signalling environment. This interplay between sexual and natural selection can lead to divergence in signals in heterogeneous environments. Yet, the extent to which gene flow is restricted when signalling phenotypes vary across environments and over what spatial scales remains an outstanding question. In this study, we quantify gene flow between two colour morphs, red and black, of freshwater threespine stickleback fish (*Gasterosteus aculeatus*). We capitalize on the very recent divergence of signalling phenotypes in this system to characterize within-species and among-morph genetic variation and to test for levels of gene flow between colour morphs in Oregon and Washington. Despite limited evidence for assortative mating between allopatric red and black populations, we found that black populations are genetically distinct from nearby red populations and that the black morph appears to have evolved independently at least twice in Oregon and Washington. Surprisingly, we uncovered a group of stickleback in one small coastal stream, Connor Creek, which is genetically and morphologically distinct from the red and black colour morphs and from marine stickleback. Historically, both colour morphs have coexisted in this location and sometimes hybridized, raising new questions about the origins and history of these fish, which were first described as anadromous-black hybrids >50 years ago. Understanding how genetic variation is currently partitioned within and among populations and colour morphs in this system should prompt future studies to assess the relative roles of habitat, ecological and pre- and post-reproductive barriers in the genetic divergence and phenotypic patterns we observe in nature.

KEYWORDS
colour morphs, genetic divergence, local adaptation, sexual selection

1 | INTRODUCTION

Understanding the processes that promote or prevent the emergence of new species has been a fundamental goal for evolutionary biologists. Polymorphic species can display multiple, discrete phenotypic variants within a single species and sometimes within a population. Such species provide important insights into the origin and maintenance of biodiversity because the same evolutionary forces (interactions between selection, gene flow and drift) that act in the speciation process can act to generate and maintain

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new morphs within species (Gray & McKinnon, 2007; Hoekstra et al., 2004; Roulin, 2004; Rueffler et al., 2006; Schluter, 2000). In some cases, polymorphism may be a reflection of ongoing, early stages of divergence that may ultimately end in speciation (Hugall & Stuart-Fox, 2012; McLean & Stuart-Fox, 2014). Alternatively, morphological polymorphisms are common and often stable over extended periods of time (Bolnick & Stutz, 2017; Subramaniam & Rausher, 2000), never leading to speciation.

Polymorphisms in traits that act as mating signals, which are typically delivered by males to attract mates, are particularly interesting, in part because these traits are shaped by both sexual and natural selection (Servedio & Boughman, 2017). Animal signals function in communication with conspecific intended receivers, but they are also filtered and shaped by the environment, including transmission properties of the medium through which they travel, before reaching receivers. Mating signals are also often attractive to unintended receivers like predators and parasites that eavesdrop on signallers to locate hosts and prey. Thus, the evolution of mating signals is shaped by intended receivers, signalling environments and unintended receivers (Endler, 1992; Zuk & Kolluru, 1998). The interplay of these selection pressures can lead to divergence of signals in heterogeneous environments (Servedio & Boughman, 2017), possibly over fine spatial scales (Richardson et al., 2014).

In this study, we capitalize on a very recently evolved colour polymorphism in populations of threespine stickleback fish (*Gasterosteus aculeatus*) from the northwest United States. While much threespine stickleback research focuses on divergence between species pairs that have become textbook examples of rapid speciation (e.g. limnetic vs benthic or marine vs freshwater; reviewed in McKinnon & Rundle, 2002), we characterize genome-wide within-species and among-morph variation. In ancestral marine and most derived freshwater locations, male threespine stickleback display a bright red throat during the breeding season, which is strongly preferred by females (hereafter red stickleback; Semler, 1971; Milinski & Bakker, 1990; McKinnon, 1995; Tinghitella et al., 2015). However, in several locations along the Pacific coast of North America, males have lost their iconic red throat, and instead have full-body black breeding coloration (hereafter black stickleback; Hagen & Moodie, 1979; McPhail, 1969; Semler, 1971). The shift from red to black is associated with the light environment—black stickleback are found in tannin-rich waters where their melanic bodies have high contrast in red-shifted light environments (Boughman, 2001; Jenck et al., 2020; Reimchen, 1989; Scott, 2001). The red and black morphs studied here persist in common garden and, unlike benthic stickleback that have been described as 'black' (Boughman, 2001; Brock et al., 2017; Lewandowski & Boughman, 2008), these black fish never express red throats, even in the breeding season and when fed carotenoid-rich diets (C.S. Jenck, W.R. Lehto and R.M. Tinghitella, personal observation). Phenotypic characteristics of the animals beyond colour suggest there is likely genetic isolation between red and black stickleback in WA; the two morphs differ substantially in body shape and lateral plating, for instance (Jenck et al., 2020). The freshwater red and black stickleback morphs are largely allopatric in the Pacific Northwest (WA, OR), but have historically been found in the same freshwater river drainages and even in the same streams during the breeding season (Hagen & Moodie, 1979; McPhail, 1969).

Several new observations and the outcome of multiple sets of mating trials between red and black stickleback from this region raise questions about how much gene flow occurs between the two morphs, if any, and how this polymorphism is maintained in the absence of physical barriers to gene flow. When sexual selection plays a role in speciation, it is thought to do so when female preferences for specific mating signals reduce mating between morphs or populations within a species, initiating reproductive isolation (Boughman, 2001; Panhuis et al., 2001; Seehausen et al., 2008; van Doorn et al., 2009). Yet, in simulated secondary contact in the laboratory, female stickleback from allopatric red and black sites in WA both direct more courtship behaviours towards red than black males (Tinghitella et al., 2015), and multiple studies found no evidence for assortative mating between allopatric populations containing different colour morphs (McPhail, 1969; McKinnon, 1995; R. M. Tinghitella, personal observation). Thus, in regions where red and black fish do encounter one another, female choice may be unlikely to prevent interbreeding between the two morphs. Existing data suggest male competition may be a more important contributor to preventing interbreeding. Stickleback males compete early in the breeding season to establish territories and build nests before attracting females who choose mates and deposit eggs in their nests. In red+black morph assemblages in the laboratory designed to investigate how interactions between the morphs impact male nesting success, black males biased their aggressive behaviours towards red males, but red males had no aggression bias; this results in red males receiving more aggression overall (Tinghitella et al., 2015). This pattern of aggression could allow black males to exclude red males from preferred breeding sites (particularly brighter red males; Tinghitella, Lehto, & Lierheimer, 2018) by increasing habitat use differences and reducing gene flow between the morphs (a mechanism reviewed in Tinghitella, Lackey, et al., 2018). But is this competition bias combined with habitat isolation along an ecological gradient sufficient to generate genetic isolation between colour morphs?

Here, we adopt both broad-scale (among sites) and fine-scale (within a site) approaches to learn the extent to which stickleback morphs that differ in nuptial coloration are genetically distinct from one another. We assess genetic divergence and estimate gene flow across a relatively wide geographic scale that consists of tributaries containing several allopatric red and black sites. We also resample tributaries where red and black morphs are reported to co-occur and interbreed (i.e. Connor Creek; Hagen & Moodie, 1979; McPhail, 1969). Interestingly, we previously found that the fish currently in Conner Creek are distinct phenotypically from both red stickleback and black stickleback from WA, particularly in body shape and size (Jenck et al., 2020). If reproductive barriers have evolved between colour morphs, we expect to find limited gene flow between red and black sticklebacks across these geographic scales. This system has the potential to yield new insights into how phenotypic and genetic differentiation is maintained and whether

evolution within a species can give rise to new species in the absence of assortative mating.

2 | METHODS

2.1 | Samples

Red and black stickleback sites in the Pacific Northwest (WA, OR) are largely allopatric (i.e. occur in distinct drainages), but McPhail (1969) and Hagen and Moodie (1979) documented several places in southwest Washington where red and black stickleback appear to have overlapping breeding grounds and seasons. We sampled stickleback from across this distribution (Figure 1, Table 1), including three allopatric collecting locations where fish had ancestral, red nuptial coloration (Campbell Slough (R1), Wishkah River (R2) and Chehalis River (R3); Figure 1b), and six collecting locations where fish had black nuptial coloration (Vance Creek (B1), Black River (B2), Scatter Creek (B3), McKenzie River (B4), Green Island (B5) and Connor Creek (B6); Figure 1b–d). We also included in our study stickleback provided by W. Cresko from two collecting locations in Oregon that were not glaciated during the Pleistocene epoch (Booth et al., 2003; O'Connor et al., 2001; Figure 1d) to assess the relatedness of black fish across locations that differ dramatically in their colonization times (OR: several million years, WA: ~12 000 years; Catchen, Bassham, et al., 2013; Catchen, Hohenlohe, et al., 2013). Finally, we finely sampled one of the sympatric locations identified by McPhail (1969)—Connor Creek, WA (McPhail, 1969; Hagen & Moodie, 1979; Figure 1c)—along a transect that mimicked McPhail's original survey, nearly

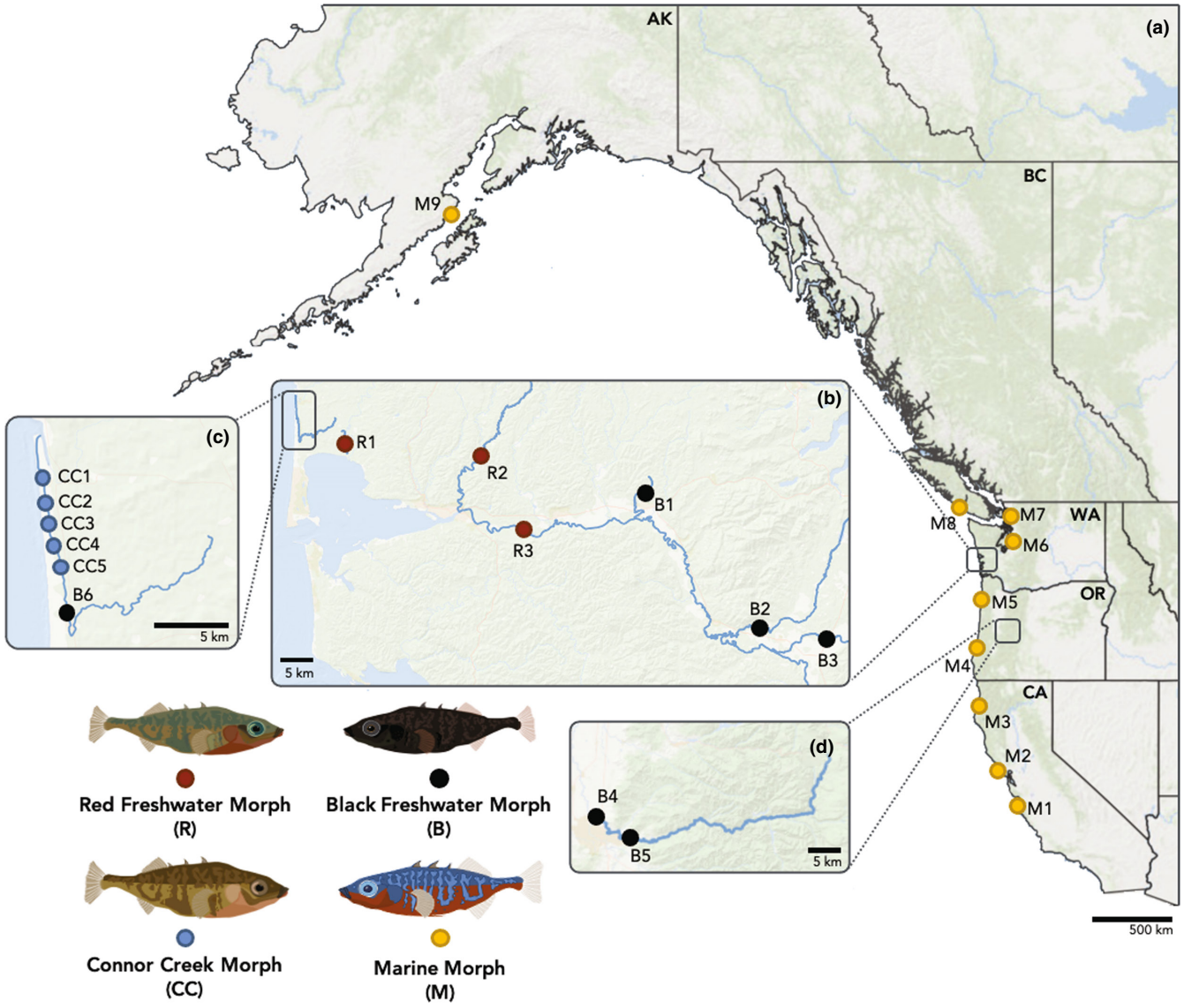

FIGURE 1 Threespine stickleback morphs and collection sites along the Pacific Coast (a), in Washington (b, c), and in Oregon (d). Connor Creek collection sites (c) mirror those of McPhail (1969). Red freshwater stickleback sites are denoted with 'R', black freshwater stickleback sites are denoted with 'B', Connor Creek stickleback sites are denoted with 'CC'. Marine stickleback sites, denoted with 'M', are data extracted from Morris et al. (2018) and Shanfelter et al. (2019)

TABLE 1 Summary of stickleback samples and data included in this study

Collection Site	Abb.	Morph	GPS Coordinates	Year	Seq.	Geno.
Campbell Slough	R1	WA Red	47°2′40″N, 124°3′33″W	2016	39	33
Wishkah River	R2	WA Red	47°0′17″N, 123°48′49″W	2018	20	13
Chehalis River	R3	WA Red	46°56′22″N, 123°18′46″W	2016	38	28
Vance Creek	B1	WA Black	46°59′48″N, 123°24′43″W	2016	27	21
Black River	B2	WA Black	46°49′45″N, 123°8′1″W	2016	35	29
Scatter Creek	B3	WA Black	46°49′20″N, 123°3′11″W	2016	36	27
McKenzie River	B4	OR Black	44°3′41”N, 122°51′11″W	2017	11	9
Green Island	B5	OR Black	44°8′42″N, 123°7′5″W	2017	13	12
Connor Creek	B6	Connor Creek	47°4′11″N, 124°10′5″W	2016	27	26
Connor Creek	CC1	Connor Creek	47°6′55″N, 124°10′52″W	2018	51	49
Connor Creek	CC2	Connor Creek	47°6′26″N, 124°10′45″W	2018	15	14
Connor Creek	CC3	Connor Creek	47°5′57″N, 124°10′39″W	2018	19	18
Connor Creek	CC4	Connor Creek	47°5′29″N, 124°10′30″W	2018	4	4
Connor Creek	CC5	Connor Creek	47°5′12″N, 124°10′20″W	2018	33	28
*Elkhorn Slough	M1	Marine	36°49′45″N, 121°44′07″W	2013	30	29
*Doran Park	M2	Marine	38°18′52″N, 123°1′55″W	2013	30	29
*Arcata Marsh	M3	Marine	40°51′23″N, 124°5′24″W	2013	30	28
*South Slough	M4	Marine	43°17′35″N, 124°19′26″W	2013	30	29
*Tillamook Bay	M5	Marine	45°28′52″N, 123°53′49″W	2013	30	29
*Little Clam Bay	M6	Marine	47°34′32″N, 122°32′43″W	2013	34	23
†Puget Sound	M7	Marine	48°40′58″N, 122°33′42″W	2015	24	24
*Bamfield Inlet	M8	Marine	48°49′55″N, 125°8′17″W	2013	32	31
*Swikshak Lagoon	M9	Marine	58°37′14″N, 153°44′44″W	2012	30	22

Note: Collection sites, morph group found in each site, GPS coordinates, collection year, the number of individuals sequenced and the number of individuals genotyped using the most inclusive data filtering (Dataset 1). Marine stickleback sequences were obtained from * Morris et al. (2018) and † Shanfelter et al. (2019).

Abbreviations: Abb., collection site abbreviation; Geno, number genotyped; Seq, number sequenced; Year, collection year.

50 years later. We paddleboarded a 3.5 km-long transect in Connor Creek, trapping at five locations ~0.9 km apart, beginning near the mouth of the creek (CC1) and moving inland (see Jenck et al., 2020). In addition, we included data from marine sticklebacks sampled from California to Alaska, to compare relatedness among marine and freshwater red and black fish (Morris et al., 2018; Shanfelter et al., 2019) (Figure 1a).

We conducted our sampling between 2016 and 2018, during the breeding season (May to July). At each site, we used non-baited, galvanized steel mesh minnow traps to collect sexually mature, adult sticklebacks. We either fin clipped males in the field or transferred them to the University of Denver (followed by tissue sampling). For fish that were sampled in the field, we took no more than half of the caudal fin and released them immediately after fin clipping. For fish that were transferred to the laboratory, we collected tissue samples consisting of all the caudal fin and muscle tissue up to the posterior end of the ventral fin following their natural death in the laboratory. We stored tissue samples in 90% ethanol until library preparation. All methods were approved by the University of Denver's IACUC (protocol 883302-9), and fish were collected under Washington Department of Fish and Wildlife Scientific Collection permits 16–208, 17–134, and 18–173. In previous work, we quantified phenotypic variation among stickleback from all sites except R2, B4 and B5 (OR black) (Jenck et al., 2020).

2.2 | Library preparation

We used Qiagen DNeasy Blood and Tissue kits to extract genomic DNA and prepared libraries following the double-digest RAD-seq (ddRADseq) protocol from Peterson et al. (2012), with the following modifications. Briefly, we digested DNA with *Eco*RI and *Msp*I restriction enzymes, which were chosen after in silico digestion using *SimRAD* (v0.96; Lepais & Weir, 2014) in R (v4.0.3; R Core Team, 2020) and the *G. aculeatus* genome assembly (Peichel et al., 2017). We divided individuals from each site across four libraries, with five indices in each library (six indices in the fourth library), to avoid batch effects. We ligated up to 19 unique adapter barcodes to digested

samples and pooled samples with unique barcodes. We then size selected 300 ± 24 bp fragments using Pippin Prep size selection at the BioFrontiers Institute Next-Gen Sequencing Core Facility, University of Colorado Boulder. We amplified each pool in three technical replicates to integrate uniquely indexed PCR sequences to all fragments (9 PCR cycles). We pooled the replicates, cleaned the amplified libraries using homemade Sera-Mag SpeedBeads™ (following Rohland & Reich, 2012) and quantified concentrations using the Quantifluor dsDNA System (Promega) and Qubit dsDNA high sensitivity assay (Thermo Fisher). The four final libraries were sequenced on an Illumina NextSeq 500 High Output 75-cycle (SE, 100 bp) at the BioFrontiers Institute Next-Gen Sequencing Core Facility.

2.3 | Data processing

In addition to our ddRADseq data, we included marine stickleback ddRADseq (Morris et al., 2018) and whole genome sequencing (WGS) (Shanfelter et al., 2019) data (Figure 1, Table 1), which allowed us to compare population clustering among marine and freshwater red and black fish. Our ddRADseq libraries shared one restriction enzyme (*Eco*RI) in common with ddRADseq data from Morris et al. (2018), allowing us to include these marine stickleback in our analyses for a subset of SNPs. We processed marine stickleback using the same analysis pipeline, and we analysed our data alone as well as combined with the marine data using several filtering criteria to confirm that combining data and/or restricting the number of markers in our analyses had no qualitative effects on our results.

We demultiplexed and trimmed ddRADseq reads (ours and marine stickleback) separately with *process_radtags* in Stacks (v2.54; Catchen, Hohenlohe, et al., 2013). We discarded reads with a quality score below a 90% probability of being correct (phred score of 10) within a sliding window of 15% the length of the read. We trimmed residual adapter sequences and low-quality regions from the WGS reads using Trimmomatic (v0.39; Bolger et al., 2014), with a 4-base wide sliding window and trimming windows with an average per base quality below 20. We aligned all ddRADseq and WGS processed reads to the revised threespine stickleback genome (Peichel et al., 2017) using BWA-MEM2 (v2.0; Li, 2013). In total, we sequenced individuals from 14 collection sites across four libraries. Of the 788 037 686 total raw reads generated, after filtering we retained 74.80%, 98.15%, 95.80% and 31.31% in each of the four libraries. Using the same parameters, we retained 77.13% of the 180 734 254 total reads in the marine ddRADseq library generated by Morris et al. (2018) and 98.39% of the 1 177 706 520 total reads in the marine WGS library generated by Shanfelter et al. (2019).

We then called single-nucleotide polymorphisms (SNPs) using Freebayes (v1.3.1; Garrison & Marth, 2012) to create a VCF catalogue of 10 547 781 SNPs from all 639 individuals, including marine data. We filtered our SNPs using VCFtools (v0.1.17; Danecek et al., 2011) to include only bi-allelic SNPs, to exclude SNPs that are not present in at least 75% of individuals (across all datasets), to exclude all genotypes with a quality score below 20, to exclude genotypes that do not meet a minimum depth of five and a maximum depth of 200, and to thin SNPs so that no two SNPs are within 100 bp (for discussion of SNP filtering see O'Leary et al., 2018).

With this baseline of SNP filtering, we then analysed our data comparing five different subsets of our population sampling (Table S1). Each of these datasets contained different numbers of SNPs due to variance in coverage across samples and the inclusion or exclusion of certain morphs or geographic regions. Different datasets were required, in part, to restrict our SNP markers to those that overlapped with marine data. In addition, different analyses focused on contrasting different populations (e.g. including or excluding OR sampling), and some analyses required more restrictive datasets with no missing data. We found qualitatively similar results across all filtering combinations and confirmed that the addition of marine data did not change the genetic structure or the patterns we found using only our ddRADseq data. The first dataset (Dataset 1, 555 individuals, 516 SNPs) contained individuals from all collection sites (R1–R3, B1–B6, CC1–CC5 and M1–M9), removing 83 individuals missing more than 60% of loci (See Table 1). The second dataset (Dataset 2, 534 individuals, 516 SNPs) contained the same individuals as Dataset 1, except that OR black were removed (R1–R3, B1–B3, B6, CC1–CC5, M1–M9). The third dataset (Dataset 3, 180 individuals, 1410 SNPs) contained individuals from collection sites only in WA (R1–R3, B1–B3, B6 and CC1–CC5), and we used stricter SNP filtering in this reduced dataset to meet requirements for estimating isolation-by-distance, removing 110 individuals missing more than 10% of loci. The last two datasets contained individuals from only allopatric red and black collection sites and contrasted WA red vs. WA black (Dataset 4, 152 individuals, 716 SNPs) and WA red vs. OR black (Dataset 5, 96 individuals, 716 SNPs), removing 43 individuals missing more than 60% of loci and retaining SNPs closer than 100 bp.

2.4 | Population structure

We first examined population structure using all individuals (Dataset 1) and all individuals except for OR black (Dataset 2). We conducted principal component analyses (PCAs) to identify and display possible genetic clusters across collection sites with *SNPRelate* (v1.24.0; Zheng et al., 2012). To visualize the hierarchical relationships among clusters for all individuals (Dataset 1), we created a dendrogram in *SNPRelate* using hierarchical cluster analysis after standardizing variability among individuals with z-scores. We then used STRUCTURE (v2.3.4; Pritchard et al., 2000) to analyse patterns of genetic structure across collection sites (Datasets 1 and 2). We conducted five replicates for each value of K = 1–10, and each run was performed with a burn-in period of 10 000 followed by 20 000 Markov Chain Monte Carlo (MCMC) repetitions. We used STRUCTURE HARVESTER (v 0.6.94; Earl & vonHoldt, 2012) to identify the value of K that captures the uppermost level of genetic structure (as in Evanno et al., 2005), and we used CLUMPAK (Kopelman et al., 2015) to produce graphical displays of STRUCTURE results. Finally, we

calculated standard population genetic parameters such as heterozygosity and pi, using *populations* in Stacks, as well as pairwise F_{ST} values (Weir & Cockerham, 1984) using *genepop* (v1.1.7; Rousset, 2008) using all individuals (Dataset 1).

We used a restrictive dataset with very little missing data (Dataset 3) to examine genetic structure in a spatially aware context across collection sites in WA only, using *conStruct* (v1.0.4; Bradburd et al., 2018). *conStruct* differs from STRUCTURE in that it jointly models the effects of discrete population structure and continuous isolation-by-distance on sample relationships. We calculated the distance as the shortest distance among sites. We tested both spatial and non-spatial models for each value of K = 1–7, with three independent MCMC chains per K value and 10 000 iterations per MCMC chain. We subsequently compared spatial and non-spatial models over all K values using cross-validation analysis and calculated layer contributions to determine the value of K that best fits the data.

2.5 | Outlier analyses

Finally, we applied two methods, BayeScan (v2.1; Foll & Gaggiotti, 2008) and *pcadapt* (v4.3.3; Privé et al., 2020), to test for loci under selection between red and black individuals. Given that we find evidence of genetic structure between WA black and OR black, we used two different datasets to identify putative outliers between WA red individuals and WA black individuals (Dataset 4), and between WA red individuals and OR black individuals (Dataset 5). We ran each programme using recommended settings. We ran BayeScan with prior odds of neutrality set to 10 under default chain parameters and ran *pcadapt* with K = 2, allowing for a false discovery rate of 0.05 using both methods.

3 | RESULTS

3.1 | Population structure

The greatest axis of genetic divergence across all stickleback in our study was the one that separated Connor Creek stickleback from all others (Dataset 1, 555 individuals, Figure 2a; Dataset 2, 534 individuals, Figure 2b). The remaining stickleback clustered by geographic region (glaciated WA and non-glaciated OR) and by nuptial colour—WA red and marine individuals, which both display the ancestral red throat, formed an overlapping cluster distinct from WA black individuals (Figure 2a). When OR stickleback were removed (Dataset 2), this pattern became clearer, with WA black clearly separated from WA red and marine stickleback on PC2 (Figure 2b). There were two marine and one WA red stickleback that clustered with WA black stickleback and one WA red stickleback that clustered with Connor Creek stickleback; otherwise, clusters were overwhelmingly consistent with morphology. Interestingly, one site on the southernmost stretch of Conner Creek (B6) that was characterized as having morphologically black stickleback by Jenck et al. (2020), consistently clustered with other Conner Creek samples, despite the other Connor Creek samples having 'mixed' coloration and distinct body shape (Jenck et al., 2020). Our hierarchical cluster analysis produced four well-supported groups that parallel the results from the PCAs (Figure 2c). Most notably, we found that there were two major branches in the dendrogram that separated Connor Creek (including B6 black stickleback) from all other WA and OR stickleback.

We found these same general patterns in our STRUCTURE analyses. When including all collection sites, we found that a model using K = 2 was the best fit (Figure 3a), which separated Connor Creek and B6 individuals from WA red, WA black, OR black and marine individuals (Figure 3b; top). While it was not the best supported model, K = 3 provided additional resolution of population structure in which both WA black and OR black individuals were assigned to a distinct cluster (Figure 3b; bottom). When we removed OR black individuals, we found consistent results, with a model using K = 2 as the best fit (Figure 3c,d).

Measures of genetic differentiation (F_{ST}) provided further evidence of high divergence among geographic regions (OR and WA), among colour morphs within each region, and among the three regions where phenotypically black fish are found (OR, WAB1-3, Connor Creek B6; Figure 4). There was one exception; we found overall low divergence among WA red and marine stickleback, on par with the extent of divergence observed within marine stickleback (Figure 4a). The low divergence between marine and freshwater red stickleback is consistent with the lack of differentiation in PCAs and clustering analyses. Pairwise F_{ST} values ranged from 0.0069 to 0.019 among WA red sites, 0.012 to 0.10 among WA black sites, 0.12 between the two OR black sites, and 0.016 to 0.18 among marine sites. Pairwise F_{ST} among Connor Creek collection sites ranged from −0.046 to 0.020, suggesting low differentiation within Connor Creek, even when the geographically adjacent B6—a site containing only the black morph—was included. Average observed heterozygosity and expected heterozygosity ranged from 0.013 at B5 (OR black) to 0.039 at M7 (marine) and 0.015 at B5 (OR black) to 0.037 at R1 (WA red), respectively (Table S2). Average nucleotide diversity (π)ranged from 0.016 at B5 (OR black) to 0.038 at R1 (WA red) and CC2 (Connor Creek).

3.2 | Influence of geography

There is a clear signal of isolation-by-distance across our sampled locations. In our conStruct analyses, the spatial model had higher mean predictive accuracy over all tested values of K and was the preferred model in the cross-validation analysis (Bradburd et al., 2018; Figure 5a). Population structure could be described with K = 3, and adding additional spatial layers beyond K = 3 made negligible contributions to total covariance (Figure 5b). The overall spatial admixture patterns from conStruct were similar to non-spatial STRUCTURE outputs, with three distinct genetic clusters of WA red, WA black and Connor Creek individuals (Figure 5c,d).

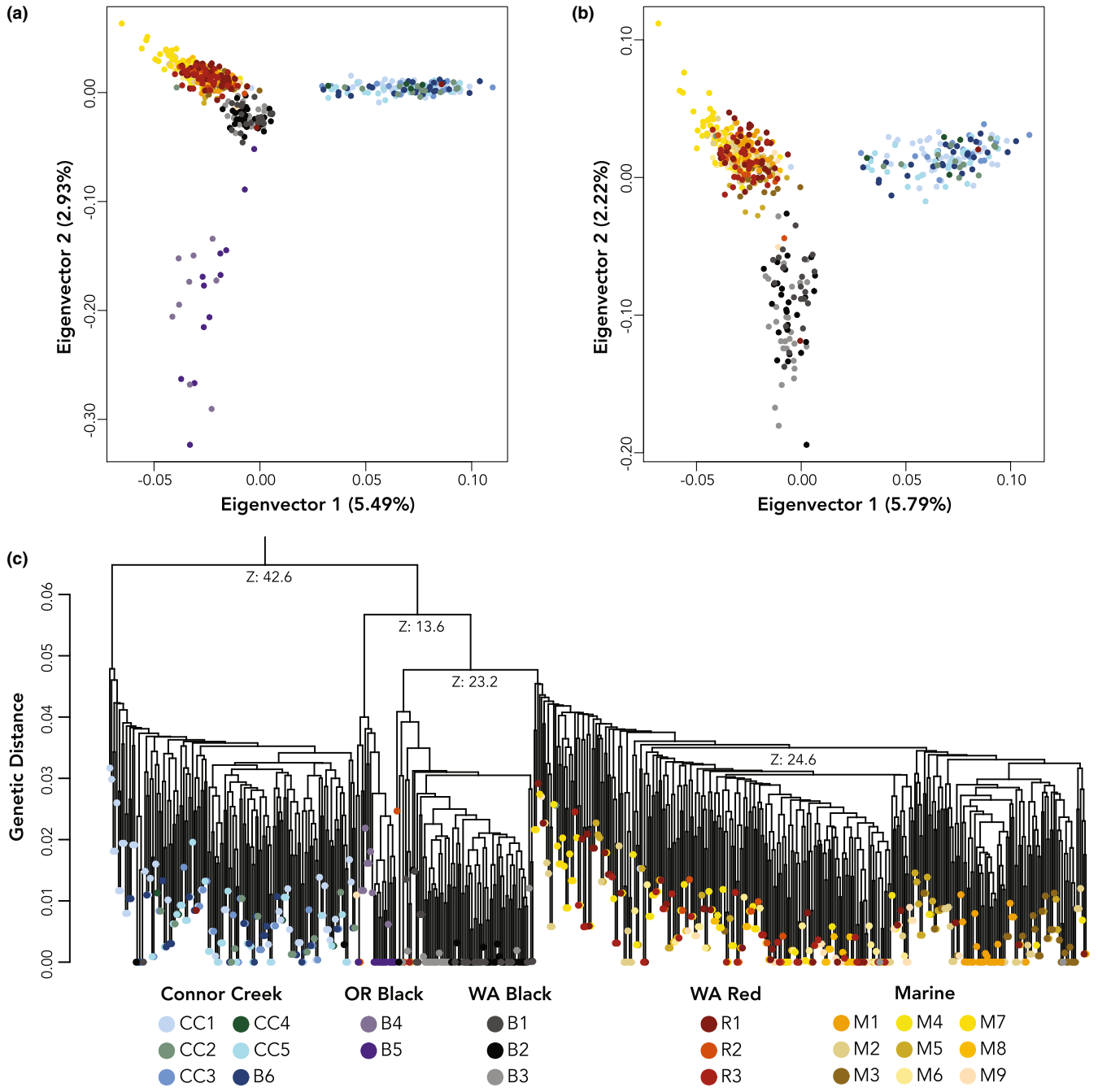

FIGURE 2 Patterns of population structure among marine, red and black stickleback. (a) Principal component analysis (PCA) of individuals from all collection sites (Dataset 1) shows strong clustering of Connor Creek stickleback, followed by clustering by geographic regions (OR black versus marine and WA stickleback). (b) When OR black stickleback are removed from the PCA (Dataset 2), WA black stickleback form a distinct cluster from marine and red stickleback. (c) Dendrogram displaying subgroups of individuals in Dataset 1 determined by hierarchical cluster analysis. The *y*-axis, genetic distance, represents the closeness, or similarity, of individuals and clusters

3.3 | Identifying putative outliers

Based on two approaches for outlier detection, we identified several candidate SNPs that are putatively under selection between red and black stickleback. We used two sets of contrasts, WA red vs WA black (Dataset 4) and WA red vs OR black (Dataset 5). Using two different methods, we identified a total of 89 SNPs that were outliers in at least one comparison (Table S3a). Of these, BayeScan detected three and *pcadapt* detected eight outlier SNPs in common in both red vs. black comparisons. When comparing BayeScan and *pcadapt* outputs for Dataset 4, the two methods identified four SNPs in common, but we found no SNPs in common in Dataset 5. The four SNPs detected by both methods in Dataset 4 were also detected in both datasets by *pcadapt*. There were no outlier SNPs in common across all methods and all comparisons. Several of these putative outliers aligned to annotated regions of the threespine stickleback genome, but none to genes with notable annotation (Table S3b).

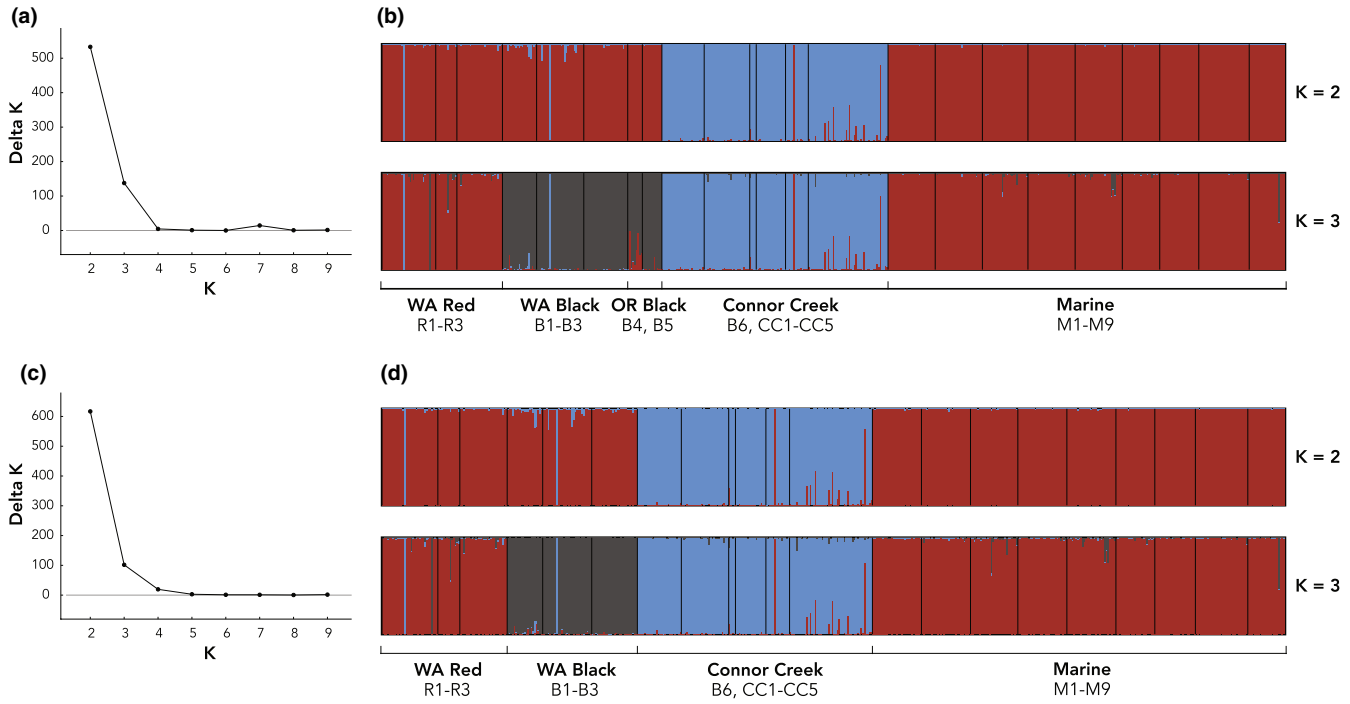

FIGURE 3 STRUCTURE analyses of individuals in Dataset 1 (a, b) and Dataset 2 (c, d). Cluster assignments are based on the two highest calculated delta K values for Dataset 1 (a): K = 2 (top; b) and K = 3 (bottom; b), and for Dataset 2 (c): K = 2 (top; d) and K = 3 (bottom; d). Each coloured column represents a single individual within a collection site, wherein the proportion of each colour represents the likelihood of membership to each cluster. Collection sites are separated by black columns

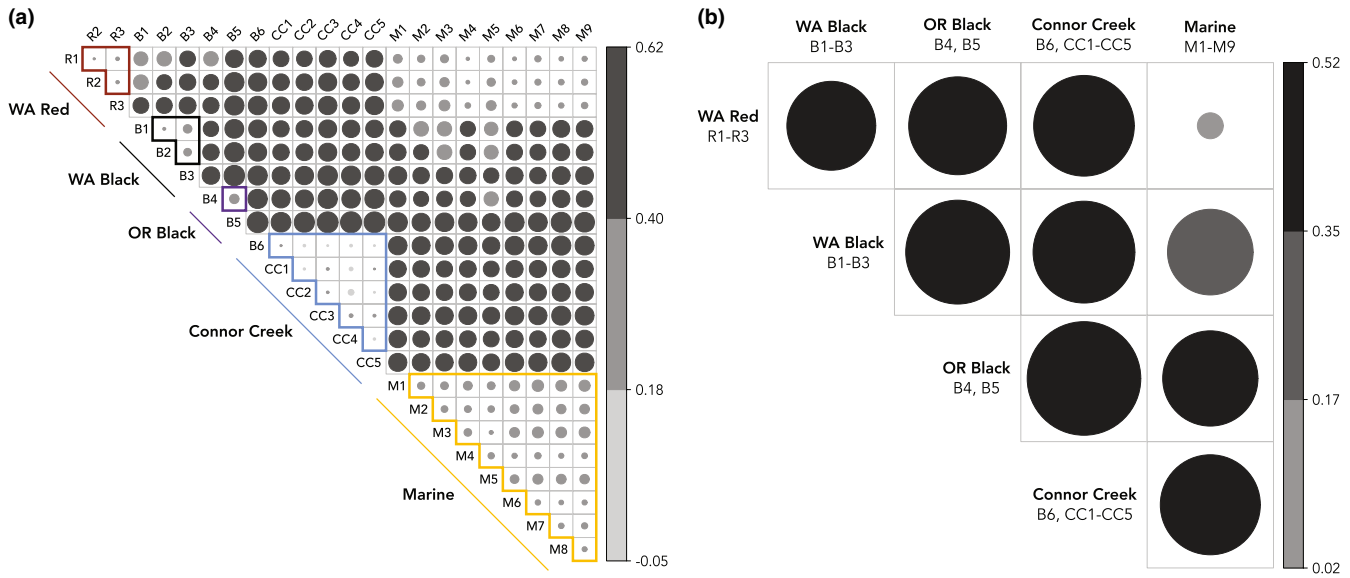

FIGURE 4 Wier and Cockham's F_{ST} pairwise comparisons among all collection sites (a) and among all morph groups (b). The size and degree of grey shading of each circle represents the magnitude of F_{ST} values. Within-morph comparisons are outlined in (a)

4 | DISCUSSION

4.1 | Black stickleback are distinct from ancestral red morphs

Across all of our geographic sampling, black stickleback consistently formed distinct genetic clusters from stickleback with ancestral red nuptial coloration. Some of this genetic divergence is undoubtedly shaped by geographic distance. We sampled WA red and black stickleback from distinct river locations. Moreover, black stickleback populations tend to occur in smaller tributaries that are physically isolated from one another by larger fast-moving rivers with relatively clear water where we find red stickleback (e.g. the Chehalis River, R3, Figure 1). It would not be surprising to find reduced gene flow over

this kind of river topology (Pilger et al., 2017; Thomaz et al., 2016), especially given that patterns of isolation-by-distance have been detected even among stickleback inhabiting contiguous lake and stream habitats (Weber et al., 2018). Our conStruct analyses confirmed that a geographic model that includes isolation-by-distance better explained population structure in WA stickleback, but still clearly identified three distinct clusters of WA black, WA red and Connor Creek stickleback. In addition, marine fish with ancestral red nuptial colour, sampled over a broad geographic range, consistently clustered with WA red stickleback. Unlike benthic sticklebacks that have been similarly described as 'black' and sometimes express red throat coloration (Boughman, 2001; Brock et al., 2017; Lewandowski & Boughman, 2008), we find no evidence that the difference in nuptial coloration between red and black fish in this region is environmentally determined; WA red and black morphs breed true in the laboratory and black fish from the Chehalis and Connor Creek B6 never express red throats, even when fed carotenoid-rich diets (C.S. Jenck, W.R. Lehto and R.M. Tinghitella, personal observation). Additionally, the red, black and Connor Creek (mixed) stickleback morphs in WA described in Jenck et al. (2020) also differ in other non-colour morphological traits including shape, lateral body plating, and in some cases overall body size, supporting our overall finding that black stickleback are a distinct morph from red stickleback (the ancestral breeding colour) in this region.

The red throat of marine and freshwater red stickleback is an iconic sexual signal that is strongly preferred by females (McKinnon, 1995; Milinski & Bakker, 1990; Semler, 1971; Tinghitella et al., 2015), and both natural and sexual selection likely contribute to differences in throat colour among morphs. The shift from red to black coloration is best explained by differences in water colour in multiple geographic regions where it has been investigated, with black fish typically found in water that is red-shifted due to canopy cover and excess decaying matter (Boughman, 2001; Jenck et al., 2020; Reimchen, 1989; Scott, 2001). The mating signals of red stickleback have high contrast in relatively clear water whereas black stickleback have high contrast on a background of

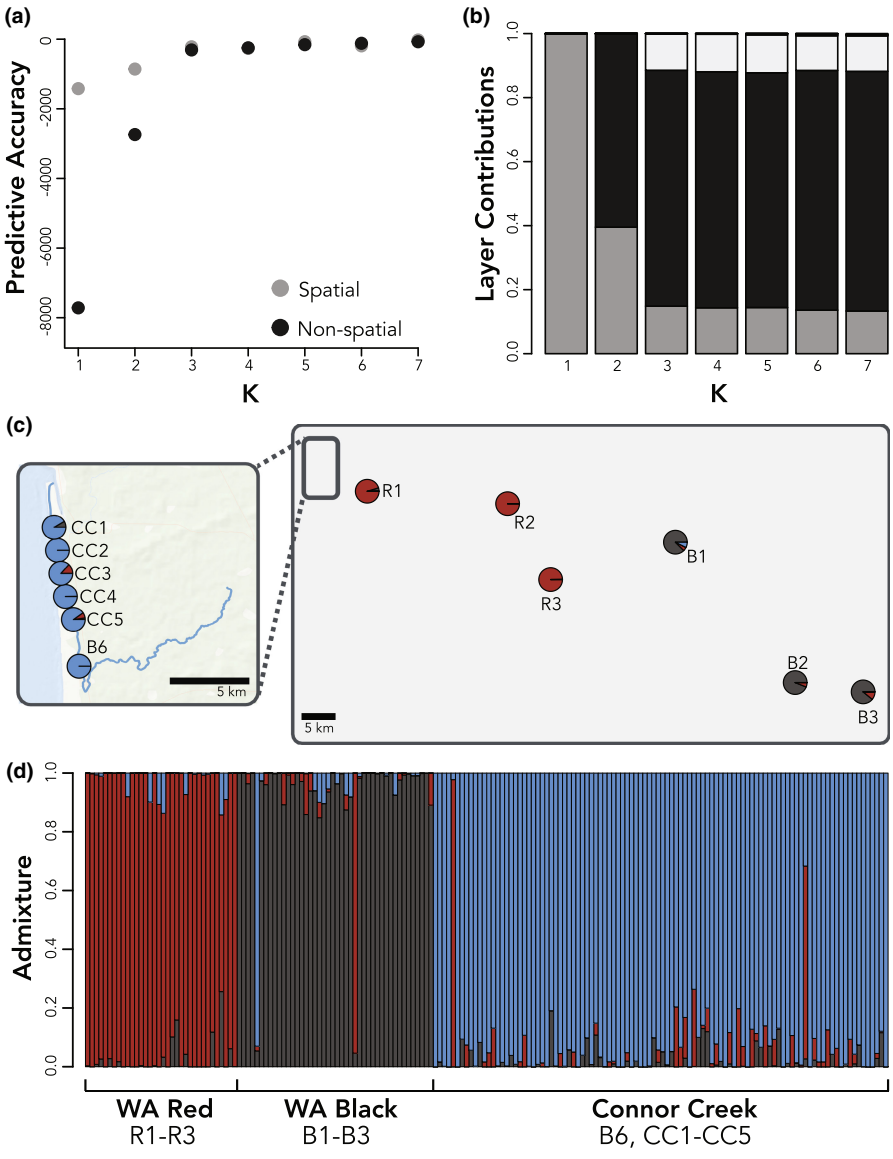

FIGURE 5 Geographic population clusters for WA samples. Cross-validation results comparing spatial and non-spatial conStruct models for K = 1–7 (a). Barplot of the contribution of each layer to parametric covariance for K values 1–7 from the spatial conStruct model. Colours show proportions of different layers. (b). Cluster membership assigned using spatial model in conStruct (c) and STRUCTURE (d) plots of the spatial model for K = 3. Each column represents a single individual and indicates the proportion of shared ancestry within a cluster

red-shifted, tannin-rich water (Boughman, 2001), making each colour morph more visible in their respective environments. In this study, red stickleback were sampled from locations that had higher light transmittance and black stickleback were found in tannin-rich waters, where short wavelength light is filtered (Jenck et al., 2020). When sexual signals shift in response to changes in their transmission environments (sensory drive; Endler, 1992), it can lead to environment-dependent variation in reproductive success among types, ultimately leading to genetic divergence among populations or morphs (Boughman, 2002; Servedio & Boughman, 2017). Our observations suggest that red and black stickleback within WA also inhabit environments that differ in more than just light transmission. Studies that incorporate a broader set of ecological variables (e.g. vegetation, salinity, water depth, flow, substrate, predators and parasites) may further elucidate the selective pressures favouring isolation among freshwater morphs.

It is interesting that the genetic divergence we observed exists despite limited evidence for assortative mating among red and black stickleback in WA. In pioneering work, McPhail (1969) used no choice and choice paradigms to test for assortative mating, finding no pre-mating isolation between colour morphs in no choice scenarios and that females from allopatric red and black populations both strongly preferred red males when given a choice. McPhail (1969) also found, however, that females from one black population immediately contiguous to a purported hybrid zone, mate randomly. Since then, results have continued to be mixed. Two papers have found no evidence for assortative mating (or own morph colour preferences) (McKinnon, 1995; Tinghitella et al., 2015), while a third found that females from Connor Creek prefer black males, and anadromous females from Bowerman Basin near the mouth of the Chehalis River weakly prefer males with the ancestral red coloration (Scott, 2004). While it is unclear at this point whether sexual selection through female choice drives divergence between colour morphs in WA, there is growing evidence that male competition patterns may contribute to divergence (Tinghitella et al., 2015; Tinghitella, Lehto, et al., 2018). In simulated secondary contact black males bias their competitive behaviours towards red males, making red males the recipients of more aggression overall, a pattern that could contribute to habitat isolation between morphs if receiving excessive aggression (red fish) or the energetic costs of nesting near other morphs (black fish) leads the morphs to nest away from one another (competitive exclusion). Such a pattern is thought to contribute to divergence and maintenance of reproductive isolation in several species (reviewed in Tinghitella, Lackey, et al., 2018). More generally, if red and black stickleback come into secondary contact in locations where black sites (B1–B3) meet the Chehalis River (R3), it is possible that ecological selection and male competition work together to yield genetic isolation between red and black stickleback. In situ mate choice and competition experiments conducted under natural (tannin-rich or clear) signalling environments would be particularly illuminating.

The evolutionary switch from red to black nuptial coloration likely occurred in multiple river systems across the Pacific coast (Hagen & Moodie, 1979; McPhail, 1969; Semler, 1971). Here, we show that WA black stickleback are more closely related to WA red stickleback than to OR black (Figure 2a). Stickleback in Oregon represent an older freshwater colonization (Currey & Bassham, 2019). Much of what is presently Oregon was not glaciated during the last glacial maximum, and many aquatic habitats, specifically those inland, are much older than northern or coastal ones (Booth et al., 2003; O'Connor et al., 2001). Stickleback populations in Oregon are estimated to be millions of years older than Washington populations (Catchen, Bassham, et al., 2013), which colonized freshwater habitats following glacial retreat less than 12 000 years ago (McPhail, 1994). This glacial history and the clustering of WA black and WA red stickleback in this study suggests that black stickleback evolved from red freshwater populations at least twice. It would be interesting to know whether current or historical ecological conditions at the OR black sites are similar to those in WA, and whether shared ecology might have promoted parallel evolution of black breeding coloration in OR and WA. Alternatively, the clustering of WA red and WA black may be due to recent and ongoing gene flow. We need additional sampling, particularly of OR red stickleback, to evaluate these alternatives.

4.2 | Unusual patterns of morphological and genetic divergence in Conner Creek

Red and black stickleback have historically been reported to co-occur in Connor Creek, WA (sites CC1–CC5) and even suggested to hybridize there, based on intermediate breeding colours similar to those of laboratory-reared hybrids (McPhail, 1969) and the extent of bony plating (Scott, 2004). Connor Creek has been described as a 'small sluggish, tea-coloured stream' (McPhail, 1969) about 12 km long that meets the Pacific Ocean near the town of Ocean Shores, WA. We previously found differences in both habitat (sandy bottom near the mouth versus highly vegetated habitats inland) and the visual transmission properties of the environment (water from sites further inland in Connor Creek are red-shifted and tannin-rich) along the creek, consistent with the transition from sticklebacks with 'mixed' phenotypic characteristics at CC1–CC5 to characteristically black phenotypes at B6 (Jenck et al., 2020). Contact zones are often found at environmental transitions and across ecological gradients such as this one (Endler, 1986), and given how frequently marine and freshwater environments come into contact, it is not surprising that hybrid zones between freshwater-resident and anadromous sticklebacks are widespread (Hendry et al., 2009; Jones et al., 2006; McPhail, 1994).

We resampled the transect of Connor Creek reported by McPhail (1969), nearly 50 years later. Indeed, stickleback in Connor Creek differ phenotypically from the WA red and black morphs in some important ways; Connor Creek fish are intermediate to the WA red and black colour morphs in shape and colour, larger in size than both red and black fish, and have fewer bony lateral plates than red fish (but still sometimes express 'full' plating) (Jenck et al., 2020). Thus, we hypothesized that Connor Creek stickleback would be

admixed individuals from some combination of black and red or black and marine stickleback. However, we found Connor Creek stickleback (including morphologically black stickleback from B6) were a distinct genetic cluster from all other stickleback we sampled. Red and marine stickleback consistently cluster closer to black morph stickleback—from both WA and OR—than they do to Connor Creek stickleback. This suggests that Connor Creek ancestry may be more complicated than a simple mixing of the populations we have sampled in this study, despite the geographic proximity of our sampled marine, red and black sites to Connor Creek.

This is particularly surprising given that extensive gene flow is likely among all Connor Creek sites including the morphologically black site at B6. Freshwater stickleback can travel up to five kilometres to breeding sites (Snyder & Dingle, 1989); each of our collection sites resides along a 3.5-km-long transect within a single creek, along which we did not observe physical barriers to gene flow. The partitioning of genetic variation within Connor Creek does not exactly mirror the partitioning of phenotypic variation, however. Genetic variation suggests extensive gene flow throughout Connor Creek, yet individuals from the five Connor Creek sites described as mixed in Jenck et al. (2020) (CC1–CC5) were phenotypically different from their upstream neighbour (B6) in both colour and shape. Habitat characteristics are consistent with selection for black coloration at B6 through sensory drive (Jenck et al., 2020). In summary, then, the relationship between Connor Creek and other nearby stickleback populations remains unclear. It is possible that there was some mixing in the past with other species that are no longer in the region or that there was an introduction of stickleback populations from elsewhere into Connor Creek (e.g. unknown refugia elsewhere in the region). Currey and Bassham (2019), for instance, found what appears to be an introduction of marine fish far inland in Oregon, perhaps because stickleback are used for bait during freshwater fishing.

Several outstanding questions remain, including determining the extent of genomic divergence among colour morphs and investigating why red freshwater fish cluster so closely with marine fish. Nuptial colour variation has been mapped in other freshwater stickleback, for instance, in populations where red and black (limnetic and benthic) fish are hybridizing (Malek et al., 2012) and to identify genomic regions responsible for red coloration in female stickleback (Yong et al., 2016). In other systems where colour is the primary axis of differentiation among groups, there are often only a few divergent genomic regions or key genes in colour production pathways (e.g. Funk et al., 2021; Jones et al., 2018; Kim et al., 2019; Nachman et al., 2003; Rosenblum et al., 2010; Toews et al., 2016; Tuttle et al., 2016). WA red and black stickleback may differ from one another in similarly simple ways, although, while colour is the most striking difference between these morphs, they do differ in other morphological traits (Jenck et al., 2020). Further, despite the difference in habitat (freshwater vs marine) between the red WA morph and marine fish, population genetic differences appear minimal (see, e.g.Figures 3b,d and 4b). This is perhaps not surprising, given that some previous work has found little genetic diversity among marine populations (Haenel et al., 2022; Hohenlohe et al., 2010; but see Morris et al., 2018). An intriguing possibility is that the red WA populations sampled in freshwater rivers here are largely anadromous, rather than freshwater resident, despite many of the collection sites being located substantially inland and the morphology of the fish being consistent with published photos of fish referred to as the red-black stream species pair (McKinnon & Rundle, 2002). For instance, the red morph fish that we sampled in the Chehalis River drainage are fully plated, which may either be because they live in fast-moving higher-order streams that contain large fish predators (making retention of plating potentially adaptive; Jenck et al., 2020), or because they are anadromous. Given how far inland we sampled red fish (Figure 1) and the distances that individual stickleback are known to travel (5–10 km; Snyder & Dingle, 1989), it seemed unlikely that these sites would contain anadromous fish. Future work could investigate these alternatives, for instance, by testing for chemical signatures of anadromy.

AUTHOR CONTRIBUTIONS

RMT conceived of the project. RMT, CSJ, ELL, and TWQ designed the research. CSJ, WRL, and RMT performed the research. ELL, KH, and WRL designed, advised on, and contributed to the bioinformatic analyses that were performed by CSJ. CSJ, RMT, ELL, TWQ, and SMM funded the work. All authors contributed to data interpretation, writing and editing the manuscript (with CSJ writing the first draft).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data reported in this paper are available through the National Center for Biotechnology Information Sequence Read Archive under accession number PRJNA842396.

PEER REVIEW

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SUPPORTING INFORMATION

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