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On Irish stickleback: morphological diversification in a secondary contact zone

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ABSTRACT

Question: How parallel is adaptive evolution when it occurs from different genetic backgrounds?

Background: Divergent evolutionary lineages of several post-glacial fish species including the threespine stickleback are found together in Ireland.

Goals: To investigate the morphological diversity of stickleback populations in Ireland and assess whether morphology evolved in parallel between evolutionary lineages.

Methods: We sampled stickleback from lake, river, and coastal habitats across Ireland. Microsatellite and mitochondrial DNA data revealed evolutionary history. Geometric morphometrics and linear trait measurements characterized morphology. We used a multivariate approach to quantify parallel and non-parallel divergence within and between lineages.

Results: Repeated evolution of similar morphologies in similar habitats occurred across Ireland, concordant with patterns observed elsewhere in the stickleback distribution. A strong pattern of habitat-specific morphology existed even among divergent lineages. Furthermore, a strong signal of shared morphological divergence occurred along a marine–freshwater axis. Evidently, deterministic natural selection played a more important role in driving freshwater adaptation than independent evolutionary history.

Keywords: ecomorphological divergence, non-parallelism, parallelism, secondary contact, stickleback.

INTRODUCTION

Divergent natural selection between different environments can drive the evolution of habitat-specific phenotypes within diversifying lineages, resulting in an adaptive radiation (Schluter, 1996a, 2000; Losos and Mahler, 2010). Adaptive radiations are relatively common in nature, occurring in a diverse range of taxa including plants (Stebbins, 1970; Baldwin and Sanderson, 1998), birds (Schluter and Grant, 1984; Schluter, 1996a), insects (Nosil *et al*., 2002), lizards (Losos *et al*., 1998), and fish (Schluter, 1996b; Bell and Andrews, 1997; Taylor, 1999; Robinson and Schluter, 2000). The extent selection plays

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in driving such evolutionary diversification, however, is relatively unclear and difficult to examine directly in the wild (Kingsolver *et al.*, 2001; Sobel *et al.*, 2009). Nonetheless, the independent evolution of similar phenotypes in similar environments provides strong evidence for selective determinism (Endler, 1986; Schluter, 2000, Reimchen *et al.*, 2013). Parallel selection between similar environments is a common feature of adaptive radiations (Schluter, 2000) but factors that might constrain selective determinism are relatively understudied. For example, how does parallel phenotypic evolution differ between populations with different genetic backgrounds?

For adaptation to occur, selection must act upon genetic variation that results in phenotypic differences within a population (Endler, 1986). Genetic variation is shaped by the actions of mutation, drift, and selection, thus as lineages experience independent evolutionary histories, these factors can result in the loss of variation (Charlesworth and Charlesworth, 2010). In short, historical contingency can play a role in constraining the trajectory of adaptive evolution (Gould, 1990; Seehausen, 2007). For parallel evolution, standing genetic variation in ancestral populations can increase the rate of adaptation (Barrett and Schluter, 2008). However, when genetic variation is lost between lineages, it has the potential to constrain phenotypic evolution (Berner *et al.*, 2010) and possibly even to prevent adaptive radiation (Cassidy *et al.*, 2013). Alternatively, when parallel phenotypic evolution does occur between lineages experiencing evolutionary independent histories, it suggests selective determinism can overcome such genomic constraints (Losos and Mahler, 2010; Young *et al.*, 2010). Quantifying parallelism and non-parallelism between similar populations with different genetic backgrounds can therefore provide insight into the roles of selective determinism and historical contingency in phenotypic evolution.

The threespine stickleback (*Gasterosteus aculeatus* L.) species complex is one of the most well studied examples of adaptive radiation in northern temperate post-glacial fishes (Bell and Foster, 1994; McKinnon and Rundle, 2002; Bell and Aguirre, 2013; Hendry *et al.*, 2013; Reimchen *et al.*, 2013). Repeated, independent recolonization of freshwater habitat from extant, ancestral marine populations has occurred throughout the circumpolar distribution of this fish (Bell and Foster, 1994; Jones *et al.*, 2012b). Parallel phenotypic evolution is a defining characteristic of the stickleback as a model species with repeated evolution of body shape (Walker, 1997; Walker and Bell, 2000; Leinonen *et al.*, 2006; Aguirre, 2009), anti-predator traits (Moodie and Reimchen, 1976; Wootton, 1976; Reimchen, 1994), and foraging morphology (Hendry and Taylor, 2004; Berner *et al.*, 2008). Furthermore, there is growing evidence to suggest that similar genomic regions are fixed in evolutionary independent populations, thus indicating that this parallel divergence also occurs at the genomic level, most probably due to parallel selection on cryptic standing genetic variation within marine populations (Hohenlohe *et al.*, 2010; Jones *et al.*, 2012b; Bell and Aguirre, 2013).

Parallel morphological divergence in sticklebacks has been well documented within individual catchments (McPhail, 1984, 1992; Aguirre, 2009; Webster *et al.*, 2011) and across regional scales in areas such as Scandinavia (Leinonen *et al.*, 2006), British Columbia (Hendry and Taylor, 2004; Spoljaric and Reimchen, 2007; Kaeuffer *et al.*, 2012; Reimchen *et al.*, 2013), and Cook Inlet, Alaska (Bell *et al.*, 1993; Bell and Ortí, 1994; Walker, 1997; Willacker *et al.*, 2010). In contrast, non-parallel evolution has been largely overlooked in these systems, despite the fact that it can shed light on processes leading to population divergence (Kaeuffer *et al.*, 2012). Studies examining replicate stickleback populations in similar environments have demonstrated that there is a considerable non-parallel component to phenotypic adaptation (Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013). Furthermore, such non-parallelism is matched at the genomic level, with local variation in divergence between replicates (Deagle *et al.*, 2012; Jones *et al.*, 2012; Roesti *et al.*, 2012). Species-poor, post-glacial

landscapes may provide the ecological opportunity to drive rapid phenotypic diversification in stickleback lineages (McPhail, 1994). The characterization of morphological diversification in understudied regions fulfilling these criteria thus presents valuable opportunities to examine the extent of parallelism and non-parallelism, examine the habitat–phenotype association, and provide new insight into stickleback evolution (McKinnon and Rundle, 2002; Reimchen *et al*., 2013).

Located on the western fringe of Northern Europe, the island of Ireland was likely one of the first regions in North Western Europe recolonized by diadromous fishes from the Atlantic Ocean following the Pleistocene deglaciation. Sea level rise during this period rapidly isolated Ireland from Britain and Continental Europe, preventing recolonization by obligate freshwater fish species and leading to a depauperate native freshwater fish fauna, dominated largely by euryhaline species such as salmonids (Griffiths, 1997; Wheeler, 1977). Furthermore, genetic studies of freshwater fish species in Ireland have strongly indicated the region is a zone of secondary contact for evolutionary divergent lineages (Verspoor *et al*., 1999; McKeown *et al*., 2010).

Threespine and ninespine (*Pungitius pungitius* L.) sticklebacks occur throughout the numerous freshwater bodies in Ireland, and fish surveys by Victorian naturalists revealed that morphologically divergent marine and freshwater forms existed in several areas across the region (Thompson, 1841; Thompson *et al*., 1856). To date, however, no comprehensive phenotypic survey of Irish stickleback populations has been conducted. As a post-glacial landscape with a depauperate native fish fauna, the island of Ireland fulfils McPhail's (1994) criteria for regions containing stickleback populations of interest. In addition, genetic evidence indicates Ireland represents a zone of secondary contact for stickleback lineages too (M. Ravinet *et al*., unpublished manuscript). Thus, Ireland presents a useful opportunity both to examine previously unstudied morphological diversity within Northern Europe and to test for the extent of parallelism and non-parallelism in adaptation to freshwater environments between evolutionarily independent lineages.

Given the lack of research on sticklebacks in Ireland to date, this study represents an attempt to redress the balance by characterizing morphological variation across the region. Our first objective was to examine patterns of body shape and anti-predator morphology variation within and among habitat types in Ireland and to assess whether habitat-specific phenotypes were consistent with those observed elsewhere in the stickleback distribution. Taking advantage of the presence of independent evolutionary lineages within Ireland, our second objective was to test whether morphological differences occurred among these lineages. In short, we were interested in examining whether separate lineages in similar habitats would differ morphologically. Finally, combining morphological and genetic data we aimed to quantify the effect size of selective determinism in generating habitat-specific phenotypes. In other words, does parallelism or non-parallelism play a greater role in generating patterns of morphological diversity in Irish stickleback populations?

METHODS

Stickleback sampling

Stickleback ($N = 928$) were sampled from three broad habitat classes – lakes, rivers, and marine environments – from across Ireland between March 2009 and March 2011. In total, 37 sites were sampled using minnow traps, hand-nets, beach seines, and electrofishing. At each site, we attempted to collect a minimum of 30 individuals, although this was not

always possible (see Table 1 for sample sizes). Following capture, individuals were euthanized using an overdose of either clove oil or MS-222 and were then immediately placed in 95% molecular grade ethanol for preservation.

Table 1. Information on sample sites, populations, and sample sizes used in the present study

Site no.	Site	Code	Habitat	Long. (°)	Lat. (°)	CL	LN	N_{IND}	N_{MSAT}	N_{MTDNA}
1	Aibhnin	AIB	M	53.2939	−9.5421	2	EU	8	9	7
2	Annilawn	ANN	L	53.5569	−10.093	2	EU	39	13	7
3	Banagher	BAN	R	53.1823	−7.9981	1	IR	30	8	7
4	Bonet	BON	R	54.2657	−8.2167	1	AD	15	10	7
5	Burren	BUR	R	52.7440	−6.8138	1	IR	13	9	7
6	Blackwater Limerick	BWL	R	52.6756	−8.5773	1	EU	13	8	8
7	Camus Bay	CAM	M	53.2863	−9.5594	2	EU	11	10	10
8	Corofin	CCO	R	53.4476	−8.8587	1	IR	10	5	1
9	Currane	CRN	L	51.817	−10.115	1	AD	40	10	8
10	Curaheen	CUR	R	51.8959	−8.5459	Ad	IR	26	12	10
11	Derriana	DER	L	51.8885	−10.033	1	TA	23	10	3
12	Drongawn	DRG	M	51.8191	−9.8885	2	EU	30	10	8
13	Feeagh	FEE	L	53.9298	−9.5745	3	EU	39	25	7
14	Fern	FER	L	55.0091	−7.3471	1	EU	30	7	4
15	Furnace Tidal	FTO	M	53.9055	−9.5781	2	EU	28	14	10
16	Furnace	FUR	M	53.9055	−9.5781	3	EU	14	28	10
17	Glencar Lough	GCR	L	54.3379	−8.3873	3	EU	13	6	7
18	Gill	GIL	L	52.2599	−10.035	2	EU	40	14	7
19	Glencullough	GLC	L	53.6621	−9.7724	2	EU	40	14	7
20	Glenavy	GLN	R	54.5883	−6.2406	4	EU	29	23	2
21	Glenamoy	GMO	R	54.2432	−9.7008	Ad	EU	40	8	6
22	Lene	LEN	L	53.6613	−7.2303	Ad	IR	16	6	8
23	Lady's Island Lake	LIL	M	52.2029	−6.3933	Ad	AD	10	10	8
24	Neagh	LN	L	54.7106	−6.5293	4	N/A	97	27	N/A
25	Lowery's Stream	LWR	R	53.4714	−9.1010	1	IR	14	12	6
26	Mohra	MHR	L	52.2968	−7.5816	1	N/A	21	5	N/A
27	Marlfield	MRL	L	52.3500	−7.7485	1	N/A	23	9	N/A
28	Namona	NAM	L	51.8786	−10.032	1	TA	15	10	8
*29	North Atlantic Ocean	NAO	M	56.1944	−8.9868	2	TA	N/A	9	8
30	Carlingford	NWR	M	54.1278	−6.3047	4	EU	28	13	6
31	Robertstown Creek	ROB	R	52.5961	−9.0656	Ad	EU	14	7	6
32	Strangford Lough	SFD	M	54.4636	−5.6085	2	AD	20	19	10
33	Swilly	SWI	M	55.1972	−7.6143	4	TA	20	18	8
34	Tacumshin	TAC	M	52.1975	−6.4599	Ad	AD	30	9	8
35	Talt	TAL	L	54.0766	−8.9184	Ad	IR	10	16	13
36	Tully	TUL	R	53.1345	−6.9057	1	IR	14	9	8
37	Tyshe Bridge	TYS	R	52.3398	−9.8196	2	EU	30	8	5
38	Upper Bandon	UBD	R	51.7424	−8.8199	3	TA	57	9	8

Note: CL = microsatellite cluster, LN = mitochondrial lineage, N_{IND} = number of individuals used for morphological analysis, N_{MSAT} and N_{MTDNA} = number of individuals used for microsatellite and mitochondrial analysis respectively. Lineage and cluster codes as follows: EU = European, TA = Trans-Atlantic, IR = Irish, AD = admixed. Habitat codes: M = marine, L = lake, R = river. *No morphological data were available for this population.

Microsatellite and mitochondrial analysis

For a subset of individuals ($n = 257$) from 34 of the populations, the cytochrome B and control region mitochondrial genes were sequenced and combined to provide a composite haplotype (1029 bp). DNA was extracted from caudal fin clips of at least 10 individuals for genetic analysis (mean $n = 11$) from all of the 37 sites and then amplified for nine microsatellite markers (GAC5196, GAC4170, GAC1125, GAC1097, GAC7033, STN18, STN32, STN75, and STN84). Protocols for microsatellite and mitochondria marker amplification are provided in the online appendix (evolutionary-ecology.com/data/2807 Appendix.pdf S2). An additional set of marine fish, captured from the North East Atlantic Ocean, was also included in genetic analyses to represent a purely marine population; however, no morphological data were observed for these individuals (see Table 1). This population was included to assess the possibility that other marine populations in Ireland might group as a single cluster or lineage. Two major mitochondrial lineages, the European and Trans-Atlantic lineages, have previously been described in Northern Europe and both of these are present in British populations (Mäkinen and Merilä, 2008). All haplotypes from these lineages (described in Mäkinen and Merilä, 2008) were downloaded from GenBank (accession numbers EF525391 to EF525449) and the phylogenetic relationship between these haplotypes and those observed in Irish populations were reconstructed using Bayesian approach in mrBayes 3.1.2 (Ronquist and Huelsenbeck, 2001). Since phylogenetic methods may not resolve relationships between contemporary populations with extant haplotypes in high frequencies, we additionally constructed a haplotype network (Posada and Crandall, 2001; Pfenniger and Posada, 2002). In addition to the European and Trans-Atlantic lineages, we detected a third, monophyletic, putative Irish lineage. An Approximate Bayesian Computation approach suggested that this Irish lineage likely arose post-glacially following recolonization and then isolation due to ice sheet retreat and advance during the Late Pleistocene (M. Ravinet *et al.*, unpublished manuscript). The frequency of lineage-specific haplotypes within each population sample was then calculated: where the total frequency of haplotypes from a lineage exceeded 0.65, the sample was assigned to that evolutionary lineage. It should be noted, however, that the majority of assigned populations (90%, 26 of 29; see 2807Appendix.pdf S1) exhibited frequencies above 0.8 for haplotypes from a given lineage. In admixed samples, no assignment could be made, thus these were classified as admixed in the analyses.

To identify regional groupings of populations sharing gene flow and/or ancestry, Bayesian population assignment based on microsatellite data was conducted using STRUCTURE 2.3.3 (Pritchard *et al.*, 2000). Under this approach, STRUCTURE uses a Bayesian algorithm to assign individuals to K populations where populations are assumed to be in Hardy-Weinberg equilibrium and are characterized by a set of allele frequencies (Pritchard *et al.*, 2000). The lambda parameter was first estimated from the data to improve model performance, and then a total of 10 iterations for each estimate of K (1–39) were run. For each iteration, a burn-in of 100,000 was used to ensure independence in the parameter estimates and the MCMC was run for a further 100,000 steps. Upon completion, the most probable value of K was assessed using the delta K method (Evanno *et al.*, 2005). Given the large number of geographically distinct samples within the dataset and thus the presence of hierarchical genetic structure, the first value of K with an increase in the value of delta K was of interest, i.e. that representing the highest level of shared ancestry/gene flow. Assignment values from across each of the runs were averaged in CLUMPP using the full search method (Jakobsson and Rosenberg, 2007) and visualized using DISTRUCT (Rosenberg, 2004).

Morphological analysis

(Data at evolutionary-ecology.com/data/2807Dataset.csv)

To characterize body shape variation within Irish populations, individuals were photographed on the left flank using a CANON EOS 1000D Digital SLR camera with a macro lens and the CANON EOS utility remote operating software. A subset of 17 landmarks based on configurations used by Albert *et al.* (2008) were placed on each image using tpsDig2 (Fig. 1A). Preliminary analysis indicated that specimen bending contributed strongly to shape variation, a common phenomenon in fish shape analysis (Valentin *et al.*, 2008). To account for this, the 'unbend specimens option' of tpsUtil v1.46 was used; an additional three landmarks were placed on the lateral line of each specimen (Fig. 1A) to facilitate this correction but these were removed prior to subsequent analysis. Following digitization, Procrustes coordinates were generated in MorphoJ (Klingenberg, 2011) and size-corrected using multivariate regression with centroid size from unbent specimens as an independent variable. To summarize the major axes of shape variation among populations with no *a priori* grouping variables, a principal components analysis (PCA) was performed on size-corrected Procrustes residuals, referred to as PC_{SHAPE} herein.

To provide an alternative means of characterizing phenotypic variation among Irish populations, eight linear trait measurements were made following Leinonen *et al.* (2006): body depth (BD), head length (HDL), jaw length (JWL), head depth (HDD), dorsal fin length (DSL), caudal peduncle length (CDL), caudal peduncle depth (CDD), and eye diameter (EYD). All measurements were obtained using inter-landmark distances generated using PAST (Hammer *et al.*, 2001) (Fig. 1B). Following this, PCA was conducted on size-corrected linear traits (see Statistical analysis below), referred to as PC_{TRAIT}. In addition, three anti-predator traits – 1st dorsal spine (DS1), 2nd dorsal spine (DS2), and pelvic spine (PS) length – were measured from photographs of the left flank of the body using imageJ (Abramoff *et al.*, 2004); thus pelvic spine was measured from one side only. These anti-predator traits were subsequently summarized using PCA, referred to as PC_{AP}. Finally, all lateral plates were counted on the left and right sides of individuals using an OLYMPUS SZX10 dissecting microscope at 6.3× magnification. The average of these two counts provided a mean plate number (MPN) per individual. All individuals with a standard length of less than 30 mm were removed from the dataset to prevent the inclusion of juveniles lacking fully developed lateral plate armour (Hagen and Gilbertson, 1972).

Statistical analysis

Prior to analysis, all linear trait measurements (morphological and anti-predator) were first size-corrected using the common-within population relationship to account for among-population differences in allometry (Reist, 1986; McCoy *et al.*, 2006; Berner, 2011). As it provides a meaningful estimator of body size, landmark-based centroid size was used for this correction (Berner, 2011). To test for shape differences among assigned mitochondrial lineages, microsatellite-inferred clusters, and habitat types, principal component scores were used as response variables in general linear models (GLMs) and general linear mixed models (GLMMs). The latter class of models was used to account for population as a random factor when examining differences among habitat classes within evolutionary lineages. To ensure the correct model structure, we used analysis of variance (ANOVA) and the Akaike Information Criterion (AIC) (Zuur *et al.*, 2007). To examine whether percentage of lineage

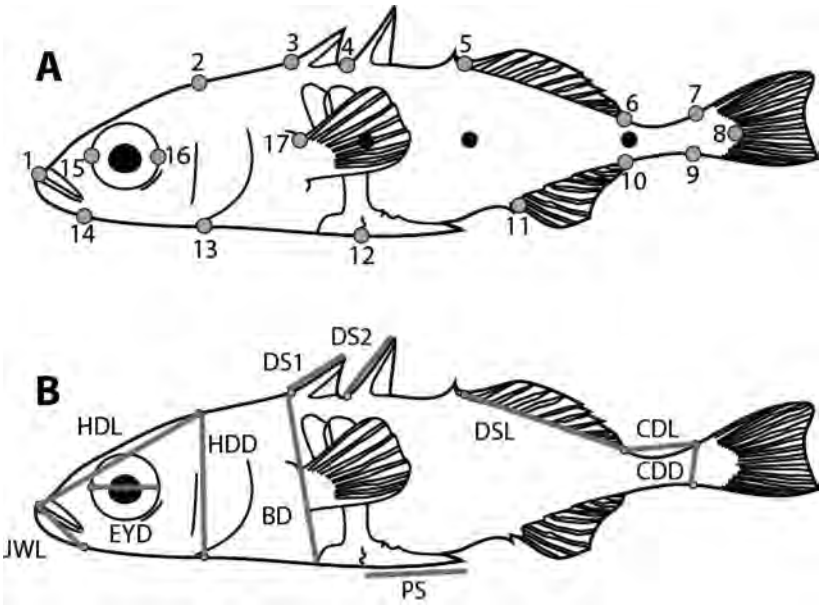

Fig. 1. (A) The landmark configuration for morphometric analysis (grey circles denote homologous landmarks for defining body shape, black circles indicate landmarks used to correct specimen bending); landmarks are (1) anterior extent of premaxilla, (2) posterior extent of supraoccipital, (3) anterior insertion of first dorsal spine, (4) anterior insertion of second dorsal spine, (5) anterior insertion of dorsal fin, (6) posterior insertion of dorsal fin, (7) dorsal insertion of caudal fin to caudal peduncle, (8) posterior extent of caudal peduncle, (9) ventral insertion of caudal fin to caudal peduncle, (10) posterior insertion of anal fin, (11) anterior insertion of anal fin, (12) insertion point of pelvic spine to pelvic girdle, (13) posterioventral extent of preopercular, (14) anterioventral extent of preopercular, (15) anterior extent of orbit, (16) posterior extent of orbit, (17) anteriodorsal insertion of pectoral fin. (B) Anti-predator and linear body measurements (HDL = head length, JWL = jaw length, EYD = eye diameter, HDD = head depth, BD = body depth, DS1 = 1st dorsal spine, DS2 = 2nd dorsal spine, PS = pelvic spine, DSL = dorsal fin length, CDL = caudal peduncle length, CDD = caudal peduncle depth).

ancestry in populations influenced phenotypic variation, we also performed correlation tests between haplotype frequencies and the mean values for body shape, measured traits, and anti-predator traits. A significant correlation between these traits and percentage ancestry for a given lineage would indicate a trend towards specific phenotypes within lineages. All statistical analyses were conducted using R 2.15.1 (R Development Core Team, 2012).

Quantifying parallelism and non-parallelism

The putative parallel and non-parallel nature of morphological divergence among environments can be quantified using a multivariate approach (Langerhans and DeWitt, 2004; Berner *et al*., 2010; Kaeuffer *et al*., 2012). To quantify the extent to which adaptation to freshwater habitats has occurred in parallel among evolutionarily independent lineages in Ireland, we used the multivariate analysis of variance (MANCOVA) approach developed by Langerhans and DeWitt (2004). As a multivariate ordination approach, MANCOVA produces orthogonal

axes, independent of one another – thus allowing the estimation of both the parallel (i.e. among habitat types) and non-parallel (i.e. among lineage) components of trait variation (Langerhans and DeWitt, 2004). The interaction term from these models can be interpreted as a response from a given lineage to freshwater adaptation. The method was applied to three datasets: body shape (size-corrected Procrustes coordinates), measured linear and anti-predator traits. MANCOVA was first performed separately for the river and lake habitat types. In each case, the model used a two-level habitat factor (i.e. the habitat of interest versus marine) and mitochondrial lineage or microsatellite cluster as an additional factor. The analysis was then repeated with habitat as a three-level factor (lake, river, marine). For each model, Wilks' η^2 was calculated, providing a percentage value measuring the proportion of explained variance for each axis (Langerhans and DeWitt, 2004). It should be noted that the sum of η^2 for a given model can exceed 1.

RESULTS

Genetic structure and evolutionary lineages

Populations were assigned to one of three mitochondrial lineages present in Ireland – European, Trans-Atlantic or Irish. Two of these have been previously described in Northern Europe and Britain (Mäkinen and Merilä, 2008). The third, a putative Irish lineage, appears to have diverged in isolation in this region (M. Ravinet *et al.*, unpublished manuscript). Five of the 35 populations with sequence data could not be assigned, as haplotypes from a single lineage did not exceed a frequency of 0.65. Three of these admixed populations were marine populations (LIL, population 23; SFD, population 32; TAC, population 34; see Table 1) and one was a lake in close proximity (<1 km) to the Atlantic Ocean (CRN, population 9).

Assessing STRUCTURE output using posterior probability values and ΔK revealed that $K = 4$ (Fig. 2C) was the most supported small K value, representing the first hierarchical level. Support was greater for high values of K (i.e. $K = 23$) but $K = 4$ was chosen as it better represented shared ancestry over a larger regional scale (Figs. 2A and 2B). Of the 39 populations, seven could not be assigned to a cluster (i.e. population q-values < 0.5). As with populations admixed for mitochondrial DNA, five of these were marine or in close proximity to the sea (CUR, population 10; GMO, population 21; ROB, population 31; LIL, population 23; TAC, population 34). Only two populations were admixed for both mitochondrial lineages and microsatellite clusters (LIL, population 23; TAC, population 34) and both of these were marine populations. Southern and central populations grouped largely in cluster 1, while cluster 2 included northern and western populations in close proximity to the coast. Cluster 3 lacked a clear geographical association, while cluster 4 contained freshwater populations situated in the north. Within clusters and lineages, the frequency of habitat types occupied by populations did not differ significantly from that expected by chance (lineages: $\chi^2 = 10.26$, d.f. = 8, $P = 0.11$; clusters: $\chi^2 = 12.16$, d.f. = 8, $P = 0.14$).

Morphological variation within Ireland

Principal components analysis of Procrustes residuals revealed the first three eigenvectors accounted for 46.7% of the total variance in size-corrected body shape ($PC_{SHAPE}1 = 20.6\%$, $PC_{SHAPE}2 = 15.3\%$, $PC_{SHAPE}3 = 10.9\%$ respectively). Variation along $PC_{SHAPE}1$ largely represented a change in body depth, with individuals from riverine and marine habitats

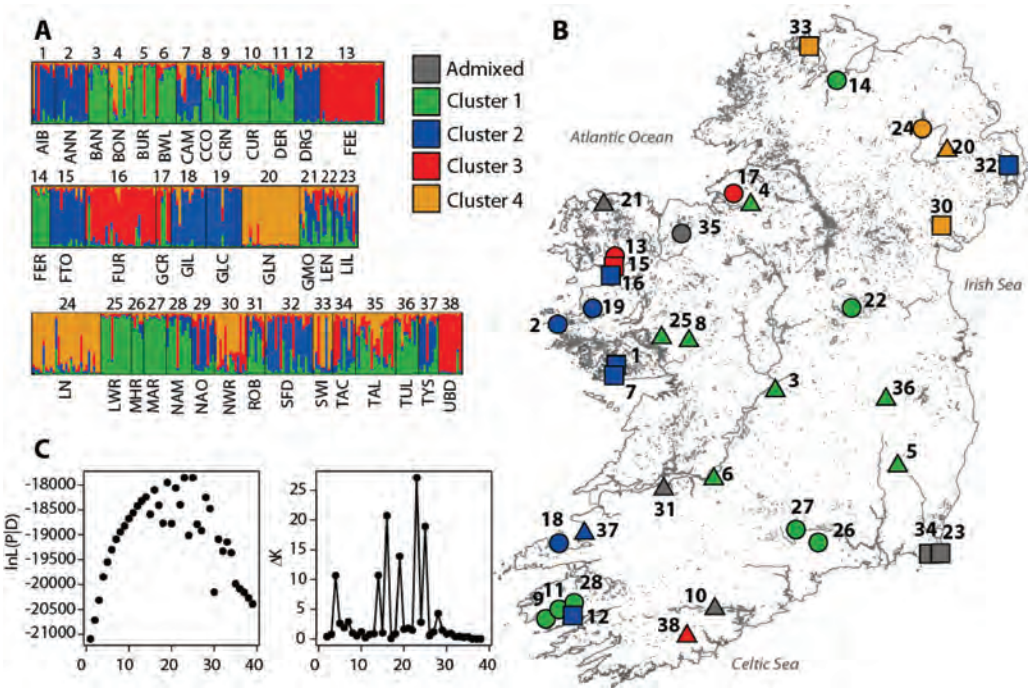

Fig. 2. (A) Microsatellite-inferred population clusters estimated using STRUCTURE; population codes are the same as in Table 1. (B) Map showing distribution of clusters across Ireland (symbols denote habitat class, with circles, triangles, and squares representing lakes, rivers, and marine habitats respectively). (C) Posterior probability and ΔK values for STRUCTURE runs.

having a deeper body and shorter caudal peduncle than their lacustrine counterparts (Fig. 3). $PC_{SHAPE}2$ largely explained divergence among populations within habitat classes and was characterized by an increase in eye size, a shortening of the snout, and an increase in head size (Fig. 3). GLMMS (with population as a random factor) revealed that $PC_{SHAPE}1$ ($R^2 = 0.53$, $F_{2,36} = 5.95$, $P = 0.006$) and $PC_{SHAPE}2$ ($R^2 = 0.42$, $F_{2,36} = 4.38$, $P = 0.019$) differed between habitat types. Body shape varied considerably among population samples within habitat classes too (Fig. 3; 2807Appendix.pdf S3).

Multivariate analysis of measured anti-predator traits captured 90.7% of the variance in a single principal component ($PC_{AP}1$). Trait loadings revealed that variation along this axis was driven by an increase in spine length (Table 2); thus high $PC_{AP}1$ values represent longer spines. $PC_{AP}1$ values differed significantly among habitat types (GLMMs with population as a random factor: $R^2 = 0.76$, $F_{2,36} = 14.63$, $P < 0.0001$). Specifically, marine populations had larger spines than both lake and river populations (Tukey's HSD, $P < 0.0001$, Fig. 4A); however, there was no observable difference between the two freshwater habitat types. As with body shape, univariate GLMs revealed considerable differences among populations within habitat classes (see 2807Appendix.pdf S2). $PC_{AP}1$ was also positively correlated with both body depth and $PC_{SHAPE}1$ (body depth: $r = 0.33$, $t = 9.48$, d.f. = 746, $P < 0.0001$; $PC_{SHAPE}1$: $r = 0.24$, $t = 6.89$, d.f. = 749, $P < 0.0001$).

PCA on measured body traits produced three significant PC axes (i.e. more than 10% variance explained (PVE)), cumulatively explaining 73.3% of the total variance in the eight

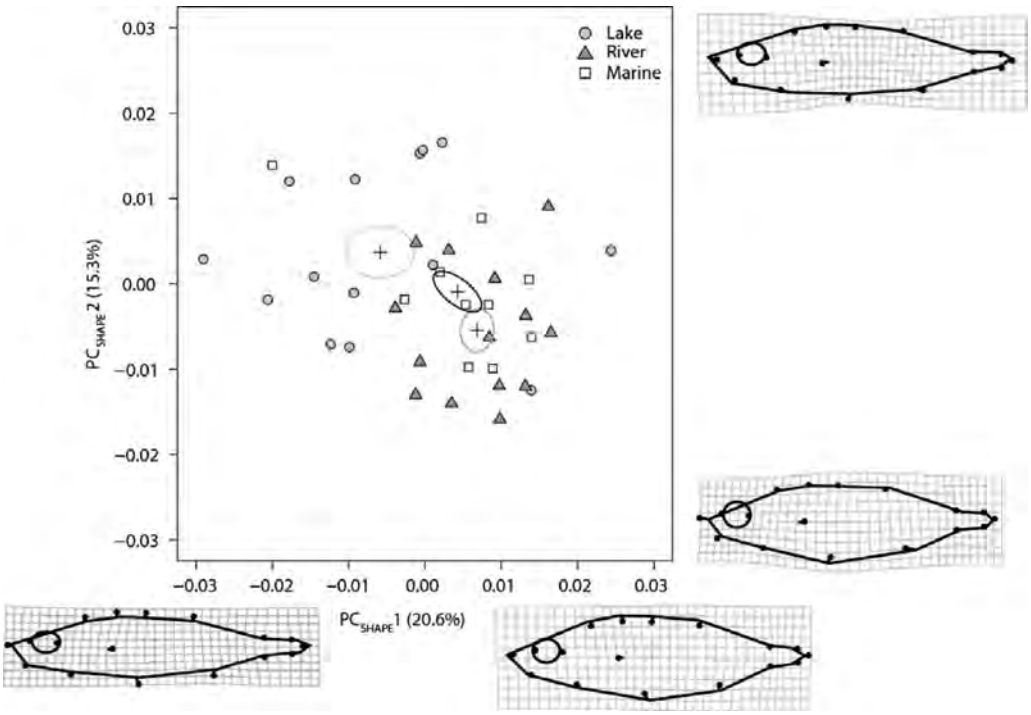

Fig. 3. Principal components analysis of shape variation (PC_{SHAPE}) represented by Procrustes captured using 17 landmarks. Symbols indicate population means grouped by habitat type; ellipses represent bivariate spread around the mean for habitat classes, denoted by crosses. Deformation grids, scaled to 1.5× to aid visualization, represent major shape variation along axes.

Table 2. Percentage variance explained (PVE), cumulative variance explained (CVE), and trait loadings from principal component analyses performed on anti-predator and measured body traits

	$PC_{AP}1$	$PC_{TRAIT}1$	$PC_{TRAIT}2$	$PC_{TRAIT}3$
PVE	90.72	35.61	19.69	18.01
CVE	90.72	35.61	55.30	73.31
Loadings				
1st dorsal spine	0.96	—	—	—
2nd dorsal spine	0.96	—	—	—
Pelvic spine	0.94	—	—	—
Body depth	—	0.54	−0.34	0.53
Head length	—	0.78	0.30	−0.25
Jaw length	—	0.68	0.31	−0.14
Head depth	—	0.90	0.08	0.16
Dorsal length	—	0.26	−0.70	−0.49
Caudal length	—	−0.39	0.75	0.31
Caudal depth	—	0.61	0.04	0.47
Eye diameter	—	0.26	0.45	−0.70

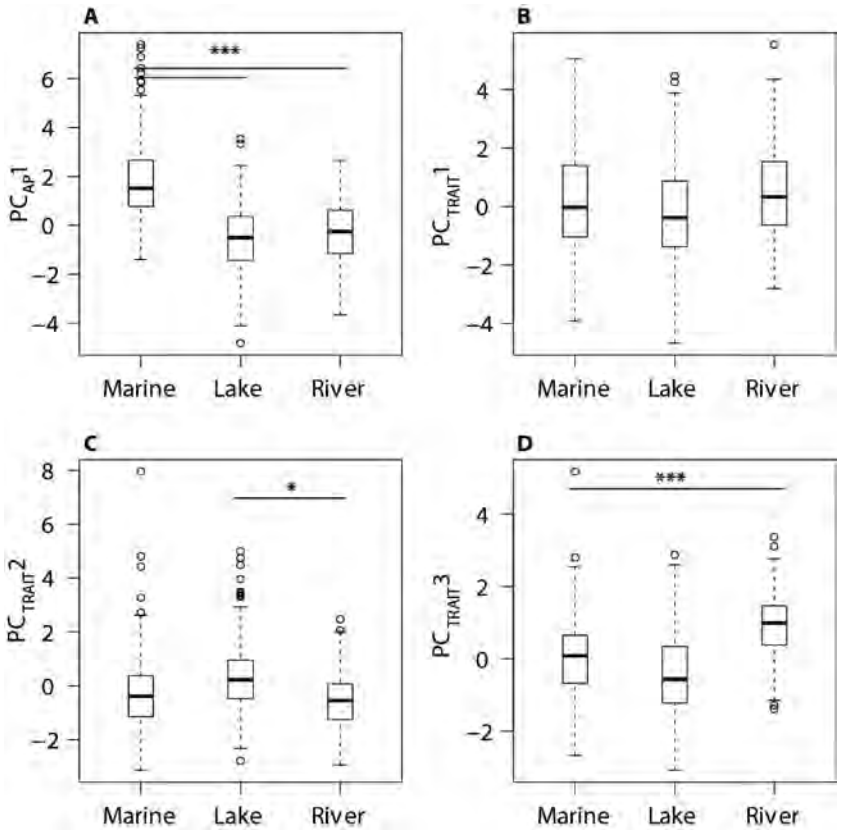

Fig. 4. Boxplots showing variation in (A) $PC_{AP}1$, (B) $PC_{TRAIT}1$, (C) $PC_{TRAIT}2$, and (D) $PC_{TRAIT}3$ between habitat classes. Asterisks denote significance in pairwise comparisons (Tukey's HSD) following detection of significant difference between habitat groups using GLMMs. $***P < 0.0001$, $**P < 0.01$, $*P < 0.05$.

traits. Variation in $PC_{TRAIT}1$ was dominated by increasing head size, shorter but deeper caudal peduncles, and a deepening body (Table 2). However, unlike geometric morphometrics, there was no clear pattern of variation among habitats (GLMM with population as a random factor: $P = 0.22$, Fig. 4B). In contrast, $PC_{TRAIT}2$, driven largely by variation in caudal peduncle length, did vary among habitats ($R^2 = 0.39$, $F_{2,36} = 3.55$, $P = 0.039$), largely between lake and river environments (Fig. 4C). $PC_{TRAIT}3$ varied among habitats ($R^2 = 0.49$, $F_{2,36} = 18.49$, $P < 0.0001$) where river fish had a deeper body and caudal peduncle than their marine or lake conspecifics (Fig. 4D). Univariate models revealed measured body traits on all three PC_{TRAIT} axes varied considerably among populations within habitat classes (2807Appendix.pdf S4).

Lateral plate morphology

Mean lateral plate number differed considerably among habitats (GLMM with population as a random effect: $R^2 = 0.80$, $F_{2,36} = 10.24$, $P < 0.0001$; Fig. 5). Mean number of lateral plates was positively correlated with spine length ($PC_{AP}1$: $r = 0.50$, $t = 15.64$, d.f. = 748,

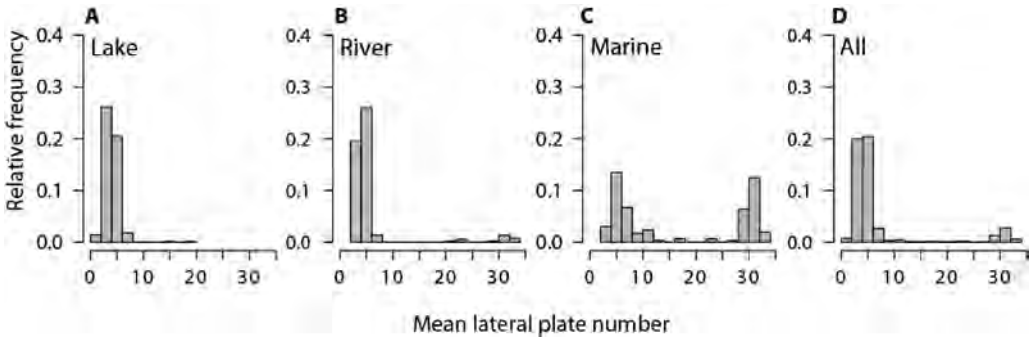

Fig. 5. Frequency density histograms showing mean lateral plate number of Irish populations in different habitat classes: (A) lake ($n = 424$), (B) river ($n = 185$), (C) marine ($n = 149$), and (D) all populations combined ($n = 758$).

$P < 0.0001$). Populations from marine habitats had the most lateral plates (17.1 ± 12.1) and differed significantly from all other habitats ($P < 0.0001$), while lake and river populations were typically low plated (4.3 ± 1.4 and 6.1 ± 6.2 respectively) but did not differ from one another. In general, Irish lake populations did not vary greatly in terms of mean lateral plate number (Fig. 5A), especially compared with river environments.

Morphological differences among evolutionary lineages

Mean shape, trait, and anti-predator values did not differ among mitochondrial lineages ($P > 0.10$ in all cases), suggesting similar variation within evolutionarily independent groupings. Similarly, no significant correlations between haplotype frequency and shape, measured traits, and anti-predator values were detected ($P > 0.05$ in all cases: 2807Appendix.pdf S5). The majority of mean trait values also overlapped among microsatellite clusters ($P > 0.10$ in these cases). However, a significant difference in anti-predator traits among microsatellite clusters was observed ($R^2 = 0.78$, $F_{4,30} = 4.87$, $P = 0.004$). *Post-hoc* comparisons revealed that this was driven mainly by clusters 1 and 2 ($P < 0.001$) where the latter had a higher mean $PC_{AP}1$ value, indicating longer spine length in populations within this cluster (2807Appendix.pdf S6).

Parallelism and non-parallelism in morphological freshwater adaptation

All MANCOVA models quantifying parallel and non-parallel morphological divergence exhibited highly significant habitat, lineage (or cluster), and interaction terms ($P < 0.0001$ in all cases, see Table 3). Parallel morphological divergence between marine and freshwater habitat had the highest PVE in all cases (Table 3), although the magnitude of PVE by habitat varied considerably among models (31.6–59.6%). In contrast to divergence between marine and freshwater environments, morphological divergence among lineages and clusters was limited (5.8–43.6% PVE). Interaction terms also accounted for a considerable proportion of variance in each model (2.0–42.1% PVE), particularly in body shape. Indeed, the combined variance explained by the lineage and interaction terms for body shape exceeded that for the habitat term in all MANCOVA models (Table 3). The axis of parallel morphological divergence between marine and freshwater environments was consistent with

Table 3. Results of MANCOVA analysis on shared and unique components of body shape, measured traits, and anti-predator morphology divergence between habitats (marine–lake, marine–river, marine–freshwater) and lineages or clusters

	Lake					River					All				
	Wilks' λ	*F*	d.f.	*P*	**PVE**	Wilks' λ	*F*	d.f.	*P*	**PVE**	Wilks' λ	*F*	d.f.	*P*	**PVE**
Body shape															
Habitat	0.45	17.13	1	<0.0001	**54.52**	0.43	17.33	1	<0.0001	**56.97**	0.23	24.93	2	<0.0001	**51.67**
Lineage	0.23	9.22	3	<0.0001	**39.17**	0.27	7.11	3	<0.0001	**35.17**	0.42	7.90	3	<0.0001	**25.29**
Habitat × Lineage	0.48	6.41	2	<0.0001	**30.95**	0.50	5.46	2	<0.0001	**29.44**	0.23	8.06	5	<0.0001	**25.64**
Measured traits															
Habitat	0.62	31.20	1	<0.0001	**37.67**	0.63	22.70	1	<0.0001	**37.40**	0.39	43.20	2	<0.0001	**37.42**
Lineage	0.58	10.38	3	<0.0001	**16.69**	0.53	9.07	3	<0.0001	**19.19**	0.72	8.44	3	<0.0001	**10.44**
Habitat × Lineage	0.61	14.71	2	<0.0001	**22.18**	0.62	10.11	2	<0.0001	**21.02**	0.48	11.45	5	<0.0001	**13.54**
Anti-predator morphology															
Habitat	0.44	174.77	1	<0.0001	**55.64**	0.54	87.90	1	<0.0001	**46.05**	0.47	89.78	2	<0.0001	**31.60**
Lineage	0.72	16.54	3	<0.0001	**10.49**	0.73	11.39	3	<0.0001	**9.84**	0.84	12.03	3	<0.0001	**5.79**
Habitat × Lineage	0.71	25.90	2	<0.0001	**15.68**	0.75	15.67	2	<0.0001	**13.21**	0.60	21.57	5	<0.0001	**15.51**
Body shape															
Habitat	0.46	16.82	1	<0.0001	**54.11**	0.41	18.37	1	<0.0001	**58.50**	0.23	25.51	2	<0.0001	**52.30**
Cluster	0.10	11.07	4	<0.0001	**43.63**	0.14	8.21	4	<0.0001	**38.59**	0.20	11.45	4	<0.0001	**32.94**
Habitat × Cluster	0.34	10.37	2	<0.0001	**42.11**	0.22	8.54	3	<0.0001	**39.58**	0.15	8.82	6	<0.0001	**27.41**
Measured traits															
Habitat	0.62	32.19	1	<0.0001	**38.46**	0.64	20.93	1	<0.0001	**35.67**	0.40	42.49	2	<0.0001	**37.11**
Cluster	0.42	12.71	4	<0.0001	**19.62**	0.37	10.71	4	<0.0001	**21.89**	0.52	13.07	4	<0.0001	**15.26**
Habitat × Cluster	0.54	18.73	2	<0.0001	**26.67**	0.49	10.18	3	<0.0001	**21.15**	0.46	10.07	6	<0.0001	**12.10**
Anti-predator morphology															
Habitat	0.40	205.09	1	<0.0001	**59.60**	0.55	84.03	1	<0.0001	**45.09**	0.45	96.42	2	<0.0001	**33.24**
Cluster	0.58	20.87	4	<0.0001	**16.50**	0.64	12.49	4	<0.0001	**13.87**	0.60	27.25	4	<0.0001	**15.64**
Habitat × Cluster	0.96	2.92	2	<0.0001	**2.06**	0.93	2.66	3	<0.0001	**2.52**	0.90	3.34	6	<0.0001	**3.33**

Note: PVE = partial variance explained calculated as η^2 (Langerhans and DeWitt, 2004).

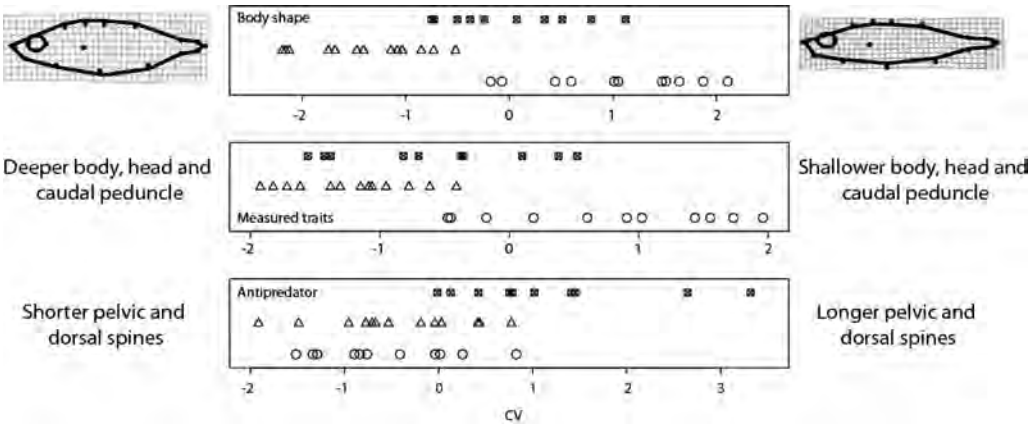

Fig. 6. Mean values for populations along main axes of shared divergence between marine and freshwater environments for body shape, measured traits, and anti-predator traits. Axis of shared divergence represents parallel divergence between marine and freshwater environments, i.e. it is the first orthogonal axis from our MANCOVA approach. Here this axis is represented as the distribution canonical variates extracted from the first term of the MANCOVA performed on morphological traits. Deformation grid scaled to 1.5× and axis for measured traits inverted to aid interpretation. Symbols denote habitat type: open circles = lake, triangles = river, and squares = marine.

the trait variation among habitats described using other methods (Fig. 6). For example, lake fish tended towards a shallower body with an elongated caudal peduncle. Furthermore, both lake and river fish showed a reduction in defensive spine length (Fig. 6; 2807Appendix.pdf S7).

DISCUSSION

In the first extensive survey of stickleback populations across the island of Ireland, we identified a considerable degree of morphological diversity that has evolved since the region became ice-free ~17 kyr B.P. (Clark *et al.*, 2012). Like elsewhere in the distribution of the three-spine stickleback, considerable habitat-specific phenotypic evolution is present in Irish populations, supporting the existence of strong differential selection for morphological adaptations among freshwater environments. Genetic analysis confirmed that Ireland is a zone of secondary contact for independent evolutionary lineages of sticklebacks. Thus, we were able to quantify the extent of parallelism and non-parallelism in morphological freshwater adaptation. While the extent of shared divergence differed depending on the traits examined, parallel divergence along the marine–freshwater axis accounted for a large proportion of morphological variance (mean PVE = 41%). In contrast, there was little evidence to suggest that evolutionary lineage could account for phenotypic variation among populations, thus selective pressures within habitat classes probably played a major role in driving evolutionary diversification in Irish populations. Nonetheless, significant non-parallel divergence among habitats and lineages was apparent for traits such as body shape, suggesting an important role for non-parallel processes too.

Morphological divergence among habitats

Our findings indicate that repeated evolution of habitat-specific freshwater phenotypes is a feature of Irish stickleback populations. Furthermore, our results demonstrate that although considerable phenotypic variation exists within habitat categories, habitat-specific phenotypes are present. For example, lake populations generally exhibited a shallow-bodied, more elongate and fusiform body shape, in contrast to the deeper bodied, stouter morphology of river fish. Phenotypic divergence between lake and river sticklebacks has been observed within a single catchment in Ireland, despite ongoing gene flow (Ravinet *et al*., 2013), thus phenotypic divergence between allopatric lake and river populations is also likely. Marine fish were phenotypically similar to river fish, although they had a bulkier, stouter morphology with a more compressed caudal peduncle. Irish populations therefore undergo body shape diversification between habitats in a similar pattern to that observed elsewhere in North America (Walker and Bell, 2000; Spoljaric and Reimchen, 2007; Reimchen *et al*., 2013) and Europe (Leinonen *et al*., 2006).

Body shape divergence along a limnetic–benthic axis is common within the stickleback species complex, occurring both within (i.e. lakes) and between (i.e. lake–stream) habitat types (Hendry and Taylor, 2004; Aguirre, 2009; Willacker *et al*., 2010). Variation along such an axis is generally represented by a shift in body depth and thus corresponds well to the $PC_{SHAPE}1$ axis determined in our study. The deep body of benthic fish appears to improve manoeuvrability in structurally complex environments, potentially aiding foraging on benthic macroinvertebrate prey (Taylor and McPhail, 1986; Walker, 1997; Hendry *et al*., 2011). In contrast, the fusiform, shallower body shape of limnetic fish allows for greater sustained swimming, a potential fitness advantage when feeding on pelagic prey items (Walker, 1997; Hendry *et al*., 2011). The strong divergence among the three habitat classes and the large extent of shared parallel divergence in Irish populations provides strong evidence for deterministic selection as a result of habitat characteristics (Reimchen *et al*., 2013; Hendry *et al*., 2013).

Within the three habitat categories, river populations exhibited the lowest level of variation along $PC_{SHAPE}1$, while lake populations differed considerably (see Fig. 2). Although we lack data on environmental variation among the population samples surveyed, it is likely that diversity in habitat characteristics facilitates greater phenotypic variation. Stickleback trophic and anti-predator morphology can vary with characteristics in lake size (Hagen and Gilbertson, 1972; Nosil and Reimchen, 2005). Since Irish lakes are ecologically heterogeneous (Reynolds, 1998), we suspect that this drives greater phenotypic variation in lake fish; in contrast, river environments may be less variable. Again, the lack of data on environmental variation among habitats is a limitation of our study, thus there is a need for future research in Ireland to take this into account, as has been done extensively in other regions (Reimchen *et al*., 2013). In addition, it should be noted that we did not identify the sex of the fish in our study. Sexual dimorphism is widespread in stickleback populations and can account for considerable morphological differences within marine and freshwater ecotypes (Kitano *et al*., 2007, 2012; Aguirre *et al*., 2008). It is possible then that sex differences could account for at least part of the variation within habitat classes.

The considerable phenotypic variation observed in Irish marine stickleback populations is surprising, since other researchers have typically reported low body shape diversity in anadromous stickleback (Leinonen *et al*., 2006; Spoljaric and Reimchen, 2007; Aguirre, 2009). The ancestral marine stickleback phenotype is highly conserved, having remained stable throughout the Pleistocene and possibly since the mid-Miocene (Bell, 1994; Bell *et al*., 2009). Marine populations

in our study showed some variation along $PC_{SHAPE}1$ and furthermore overlapped with both lake and river populations. It is unlikely, however, that Irish marine stickleback have uniquely evolved greater phenotypic diversity. A more plausible explanation focuses on how habitats are categorized; Ireland, for instance, has a large number of sheltered marine systems and tidal inlets that act as transitional habitats between freshwater and marine environments (e.g. Strangford Lough and Lough Swilly, populations 32 and 33; Fig. 2). Variation in shape polymorphism within marine habitats may occur as a result of gene flow between abutting environments or as a result of parapatric overlap between freshwater and anadromous populations. This is also a likely explanation for the greater variation in lateral plate phenotype in Irish marine stickleback populations, although we cannot rule out the possibility that these populations are genuinely polymorphic (Klepaker, 1996). These findings again highlight the need to quantitatively characterize habitat using measured environmental characteristics (Reimchen *et al.*, 2013) or to use long-term indicators of habitat use such as stable isotope analysis (Harrod *et al.*, 2005) and otolith microchemistry (Arai *et al.*, 2003). Assigning populations to discrete, qualitatively determined habitat categories ignores variation in selective forces within and among these categories (Klepaker, 1996; Berner *et al.*, 2008).

Anti-predator morphology in Irish populations

Irish marine populations exhibited greater dorsal and pelvic spine length than freshwater populations. This has been commonly reported in other stickleback populations (Leinonen *et al.*, 2006; Aguirre, 2009) and is consistent with the hypothesis that marine stickleback populations experience greater predation from pelagic piscivorous predators. A positive correlation between spine length and body depth detected among Irish populations further supports this idea; increased distance between spines as a function of body depth can impede capture by gape-limited predators (Reimchen, 1988, 1991; Reimchen *et al.*, 2013). However, the lack of divergence in spine length between lake and river environments is surprising. Longer pelvic spines are typically observed in lake environments with considerable piscivorous predation, while shorter spines are expected when benthic macroinvertebrate predation is high (Reimchen, 1994; Nosil and Reimchen, 2005; Marchinko, 2009; Mobley *et al.*, 2013). Again it is likely that variation within a habitat category is overlooked when habitats are classified discretely, as local selective forces can differ between apparently similar environments (Berner *et al.*, 2008; Kaeuffer *et al.*, 2012). Thus, by characterizing environments by predator assemblages, we might gain a better understanding of what drives anti-predator divergence among populations (*sensu* Reimchen *et al.*, 2013). This is especially pertinent given the large number of invasive freshwater fish species in Ireland (Griffiths, 1997); non-native piscivorous predators such as Northern pike (*Esox lucius* L.), for example, may alter phenotypic evolution in some stickleback populations.

The majority of lake and river populations in Ireland exhibit the low plate phenotype (0–9 plates), with freshwater populations in the region being relatively invariant for lateral plate number. Low plate number is predominant in freshwater stickleback populations throughout the species' distribution (Hagen and Moodie, 1982; Bell, 1984; Colosimo *et al.*, 2005). However, several regional surveys of lateral plate number in both Europe and North America have identified isolated freshwater populations dominated by the completely plated phenotype (Reimchen, 1994; Klepaker, 1995; Lucek *et al.*, 2010; Reimchen *et al.*, 2013). No such populations were observed in Ireland, although a small number of individuals with high lateral plate numbers occurred in river environments (Fig. 5B). This likely reflects the fact that several of the rivers sampled

for this study represented secondary contact zones between divergent freshwater and anadromous forms, a common phenomenon in rivers flowing to the sea (Hagen, 1967; McPhail, 1994; Jones *et al*., 2006).

Shared and unique features of morphological divergence

As a caveat to further discussion, we note that some evolutionary biologists do not feel that terms such as parallel and convergent evolution are fit for purpose when describing phenotypic traits (Arendt and Reznick, 2007; Wake *et al*., 2011). Seemingly parallel phenotypes may arise as a consequence of convergent evolution at the genomic level, i.e. different genetic architecture is involved (Wake *et al*., 2011). Furthermore, convergent evolution of similar phenotypes between distantly related species may actually arise from very similar genomic mechanisms (Arendt and Reznick, 2007). Thus some authors argue that the term 'convergent' should be used in place of 'parallel' when discussing repeated evolution of similar phenotypes (Arendt and Reznick, 2007; Losos, 2011). In contrast, we feel that the term parallel is suitable in the context of stickleback adaptation, because the stickleback species complex represents a closely related set of populations, without full reproductive isolation (Hendry *et al*., 2009). Furthermore, standing genetic variation appears to play a major role in freshwater stickleback adaptation, leading to similar genomic architecture underlying similar phenotypes (Colosimo *et al*., 2005; Jones *et al*., 2012b). Since a lack of such standing variation may play a role in constraining freshwater adaptation in some cases (Berner *et al*., 2008; Leinonen *et al*., 2012; Ravinet *et al*., 2013), we feel the term parallel is most appropriate in the context of this study.

A further point should be noted regarding the lineages and clusters examined in our study. To some extent, it is difficult to distinguish the effects of geography on evolutionary history, thus it is possible that our analysis does not necessarily represent different lineages or clusters but rather groups with relatively distinct evolutionary history. This seems unlikely to be the case for lineages based on inference from mitochondrial data. First, two of the three lineages observed in Ireland have been recorded elsewhere in Europe and North America (Mäkinen and Merilä, 2008). Second, associations between lineage and geography are not uncommon, particularly when ice-sheet movement is responsible for vicariance events leading to divergent evolutionary history (Hewitt, 1996, 2001). Although it is possible that geographical patterns can arise by chance as a result of coalescent stochasticity (Knowles and Maddison, 2002), phylogenetic analysis and an Approximate Bayesian Computation approach support the hypothesis that a third lineage has arisen in Ireland (M. Ravinet *et al*., unpublished manuscript). For microsatellite-based clusters, it is possible that contemporary gene flow obscures evolutionary independence among groups. Certainly, a wider analysis of stickleback populations in the British Isles suggests that gene flow mediated via marine basins can account for clustering patterns in this region (M. Ravinet *et al*., unpublished manuscript). Nonetheless, we argue that our use of a higher hierarchical value of K more likely reflects shared ancestry than contemporary admixture (Rosenberg *et al*., 2002).

We found little evidence of noticeable morphological differences among evolutionary lineages and microsatellite-inferred clusters. In nearly all cases, phenotypic traits did not differ between habitat classes within genetic groupings once variation at the population level was accounted for. This is consistent with similar large-scale stickleback morphology studies; for example, Spoljaric and Reimchen (2007) found no difference in freshwater habitat adaptation among stickleback lineages in Haida Gwaii, Canada. We, however, did detect some differences in anti-predator traits among population clusters, where a single cluster

had longer dorsal and pelvic spines. It is likely that such a difference may be a unique component of marine–freshwater adaptation; as previously discussed, anti-predator morphology varies with predator assemblage and thus habitats inhabited by this cluster may experience higher predation. Furthermore, cluster 2 consisted largely of populations on the western coast of Ireland, in close proximity to the Atlantic Ocean. It is possible that contemporary gene flow with marine sticklebacks has helped maintain more developed anti-predator traits in these populations. Alternatively, these habitats may be more marine-like than those observed elsewhere, potentially explaining why anti-predator morphology in these populations is more similar to the ancestral form. The results of our MANCOVA analyses suggest a sizeable proportion of non-parallel anti-predator divergence is accountable by clusters (15% PVE); however, since we lack reliable data on predator assemblages or environmental variation, we cannot test this hypothesis directly.

Taking advantage of the fact that Ireland is a secondary contact zone for divergent evolutionary lineages, our results demonstrate that morphological divergence along the marine–freshwater axis has a strong parallel component. Thus, the deterministic nature of selection in similar habitats results in parallel phenotypic evolution (Langerhans and DeWitt, 2004; Berner *et al.*, 2008; Reimchen *et al.*, 2013). Parallel phenotypic adaptation is characteristic of stickleback evolution and similar genomic architecture underlies major features of freshwater–marine divergence (Colosimo *et al.*, 2005; Miller *et al.*, 2007; Chan *et al.*, 2010). Recent studies making use of next generation sequencing technology have also demonstrated that multiple genomic regions are shared between evolutionarily independent freshwater populations, suggesting that deterministic selection on cryptic genetic variation in marine populations drives parallel adaptation to freshwater (Hohenlohe *et al.*, 2012; Jones *et al.*, 2012a, 2012b).

Genomic variation unique to specific freshwater populations or localized to geographical regions demonstrates non-parallel divergence at the genomic level (DeFaveri *et al.*, 2011; Jones *et al.*, 2012a). Our study demonstrates that a biologically significant proportion of freshwater morphological adaptation is attributable to non-shared components (>5%), concordant with this genomic pattern. Non-parallel divergence appears to be most pronounced for body shape, where the combined lineage and interaction terms in our MANCOVA models accounted for a greater proportion of phenotypic variance than divergence among habitats. High body shape diversity within habitat categories is most likely driven by ecological variation but this is difficult to assess without appropriate environmental data (Kaeuffer *et al.*, 2012; Reimchen *et al.*, 2013). This emphasizes the point that considering habitat as discrete overlooks environmental variation that alters selective forces within habitats (Berner *et al.*, 2008; Kaeuffer *et al.*, 2012). Non-parallel morphological evolution in Irish populations may also arise from historical contingency at the genomic level. For example, genomic constraints, limited to specific evolutionary lineages, may act to constrain adaptation in freshwater habitats (Leinonen *et al.*, 2012). Thus, the pattern of lower divergence observed in European lake–stream stickleback populations may arise because such populations lack the allelic variants responsible for stronger lake–stream stickleback divergence in North America (Berner *et al.*, 2010; Ravinet *et al.*, 2013). Genomic constraint may also act at a higher level, as standing genetic variation in marine populations within lineages bias responses to selection (Hohenlohe *et al.*, 2010; Jones *et al.*, 2012a). There is also a possibility that functionally equivalent phenotypes allow populations to evolve towards alternative adaptive peaks, leading to non-parallel divergence (Schluter, 2000; Kaeuffer *et al.*, 2012). This appears to have occurred in some European stickleback populations that have evolved smaller lateral plates as an alternative to reduced body armour (Leinonen *et al.*, 2012). Since morphological diversity is high among Irish populations,

additional work is required to identify and distinguish the processes that result in both parallel and non-parallel adaptation.

CONCLUSION

As a post-glacial landscape with a depauperate freshwater fish fauna and numerous water bodies, Ireland fits all the criteria outlined by McPhail (1994) as a region of potential interest for stickleback research. Our study thus represents the first attempt to extensively characterize stickleback populations in Ireland, developing a baseline for future stickleback research in the region. Furthermore, as a secondary contact zone for divergent evolutionary lineages in Europe, Ireland offers an excellent opportunity to quantify the respective roles of parallelism and non-parallelism in freshwater adaptation. As our results show, there appears to be little morphological divergence among these lineages; instead, deterministic natural selection is an important driver of freshwater adaptation in these populations, resulting ultimately in a strong pattern of parallel phenotypic evolution.

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