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RAD Sequencing Highlights Polygenic Discrimination of Habitat Ecotypes in the Panmictic American Eel

Scott A. Pavey,[1,*] Jérémy Gaudin,[1] Eric Normandeau,[1] Mélanie Dionne,[2] Martin Castonguay,[3] Céline Audet,[4] and Louis Bernatchez[1]

[1]Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, QC G1V 0A6, Canada
[2]Ministère des Forêts, de la Faune et des Parcs, Direction de la Faune Aquatique, QC G1S 4X4, Canada
[3]Maurice Lamontagne Institute, Fisheries and Oceans Canada, Mont-Joli, QC G5H 3Z4, Canada
[4]Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, Rimouski, QC G5L 3A1, Canada
*Correspondence: scott.pavey.1@ulaval.ca
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SUMMARY

The two primary ways that species respond to heterogeneous environments is through local adaptation and phenotypic plasticity. The American eel (*Anguilla rostrata*) presents a paradox; despite inhabiting drastically different environments [1], the species is panmictic [2, 3]. Spawning takes place only in the southern Sargasso Sea in the Atlantic Ocean [1]. Then, the planktonic larvae (leptocephali) disperse to rearing locations from Cuba to Greenland, and juveniles colonize either freshwater or brackish/saltwater habitats, where they spend 3–25 years before returning to the Sargasso Sea to spawn as a panmictic species. Depending on rearing habitat, individuals exhibit drastically different ecotypes [4–6]. In particular, individuals rearing in freshwater tend to grow slowly and mature older and are more likely to be female in comparison to individuals that rear in brackish/saltwater [4, 6]. The hypothesis that phenotypic plasticity alone can account for all of the differences was not supported by three independent controlled experiments [7–10]. Here, we present a genome-wide association study that demonstrates a polygenic basis that discriminates these habitat-specific ecotypes belonging to the same panmictic population. We found that 331 co-varying loci out of 42,424 initially considered were associated with the divergent ecotypes, allowing a reclassification of 89.6%. These 331 SNPs are associated with 101 genes that represent vascular and morphological development, calcium ion regulation, growth and transcription factors, and olfactory receptors. Our results are consistent with divergent natural selection of phenotypes and/or genotype-dependent habitat choice by individuals that results in these genetic differences between habitats, occurring every generation anew in this panmictic species.

RESULTS

Genome-wide Association and Data Verification

We collected genetic samples from yellow and silver eel life stages at eight locations each of freshwater and brackish/saltwater habitats that have known phenotypic differences (Figure 1) in the Atlantic Canada and St. Lawrence River regions (Figure S1). We then performed a high-resolution genome-wide association study (GWAS) with restriction-site-associated DNA markers (RAD tags) and used a multivariate approach to reveal genetic variation association with these ecotypes. Overall, we found a subtle genetic basis for the differences between the ecotypes in the form of co-varying allele frequencies in many genomic regions.

Out of the 42,424 SNPs initially considered (Table S1), 331 SNPs in 325 different scaffolds were found to be significantly associated with rearing phenotype in a random forest analysis (Figure 2; Table S2). We performed this analysis on a subset of 15,331 markers that were most variable by sampling site (see the Supplemental Experimental Procedures for details). The "out-of-bag" correct assignment was 89.6%. Nothing close to this percentage was achieved when individuals were randomly assigned to ecotype (200 datasets, mean correct assignment: 48.4%; Figure S2). Moreover, using a jackknife procedure, we predicted the individuals of the excluded sampling site with a mean accuracy of 91.2% ± 6.9% (Figure 3). There was significant genetic differentiation when only the 331 random forest SNPs were considered (analysis of molecular variance [AMOVA]; Fct = 0.017; $p < 0.001$), which is in contrast to the absence of significant differentiation between ecotypes when considering all markers (AMOVA; Fct < 0.001; $p = 0.317$), confirming panmixia as previously reported [2]. Yet, the allele frequency differences at each of the co-varying 331 random forest SNPs were modest (Δp mean ± SD = 0.0342 ± 0.0022), as expected by quantitative genetics theory for differences between polygenic traits [11].

Of the 331 associated markers, 55% (n = 182) are nearly fixed in one ecotype. This occurred in the freshwater locations with 137 markers (mean freshwater minor allele frequency [MAF] = 0.0027 ± 0.0083) and in the brackish/saltwater locations for 45 markers (mean freshwater MAF = 0.0016 ± 0.0061). We refer to these subsets as freshwater and brackish/saltwater modules, respectively. The fact that the freshwater module is three times larger than the brackish/saltwater one suggests that more genes

Figure 1. Phenotypic Differences between Freshwater and Brackish/Saltwater Ecotypes

Two sexually maturing female American eels were captured in the St. Lawrence River during their spawning migration en route to the Sargasso Sea. The large eel is representative of the slow-growing, late-maturing (>20 years) ecotype that characterizes the Lake Ontario-Upper St. Lawrence River, the numbers of which are in steep decline. The small eel is representative of the brackish/saltwater ecotype, which is fast growing and early maturing (about 5 years) and this individual is the result of a transplant of young eels from the Atlantic coast to Lake Ontario in an attempt to mitigate the decline of eels in that region. Contrary to conservation goals, the transplanted individuals did not exhibit the phenotype that characterizes the receiving region. Photo by Guy Verreault, used with permission.

are influenced by intra-generational directional selection and/or genotype-dependent habitat choice in this ecotype.

Functional Annotation

Of the 331 SNPs most important in discriminating the ecotypes, 99 SNPs were associated with 101 annotated protein-coding genes (exon or interior intron) from the American eel genome (S.A.P., unpublished data) that blasted to Swissprot and were associated with unique gene IDs (Table S2). Of these, seven were in exons, one occurred in the 3′ UTR (30S ribosomal protein S18; rs18), and the rest were in interior introns. Of the seven mutations that occurred in exons, five were non-synonymous. The remaining unique 91 divergent SNPs were in interior introns and most likely involved or linked with *cis*-regulation [12]. One of the five SNPs that caused a non-synonymous mutation was Myosin light chain kinase 3 (Mylk3). It was completely fixed in the freshwater ecotype (e.g., a minor allele not found in a single freshwater individual). This gene has been demonstrated to be important for early heart development in vertebrates [13]. The polymorphism is found in the eighth exon, downstream of the conserved ATP binding and active sites. Another non-synonymous mutation was found in an olfactory receptor (O52D1) [14]. It has been suggested that olfaction plays a role in migration for both American and European eel, especially during migration to rearing areas [15, 16].

Based on the Gene Ontology (GO) analysis of the protein-coding regions of these 331 SNPs, there is a pronounced over-representation of developmental GOs: respiratory system development (GO: 0060541; p = 0.003), cardiac muscle tissue development (GO: 0048738; p = 0.008), and limb bud formation (GO: 0060174; p < 0.001) (Table 1). There is a wealth of migration and locomotion differences between these ecotypes. In the extreme case of the Lake Ontario-Upper St. Lawrence River, these freshwater rearing have more than 1,300 additional kilometers to travel during their migrations in both directions. Also, many freshwater individuals need to swim against the current to reach their rearing areas, whereas brackish/saltwater eels can rely more on selective tidal transport [17]. This suggests that energetic and locomotion costs that differ greatly between ecotypes are reflected in the genome and GO terms that define the genetic differences.

The freshwater module subset is characterized by enrichment of transcription factors (GO: 0033276; transcription factor TFTC complex p = 0.0008) and calcium ion binding (GO: 0005509; p = 0.0031) (Table 1). Specifically, the Urinary transporter 2 (Ut2) gene is a possible adaptation to the transition from freshwater back into saltwater during the spawning migration. Though most fish excrete ammonia directly through the gills, Ut2-mediated urea transport may be essential for the freshwater-to-saltwater transition, and the gene has been found to be highly expressed in gill tissue under these conditions in the American eel [18]. The SNP found in this gene was fixed in the freshwater ecotype, which would be the only group assured to experience such a fresh to saltwater transition.

The brackish/saltwater module subset is enriched in growth factor receptor binding (GO: 0070851; p = 0.001), positive

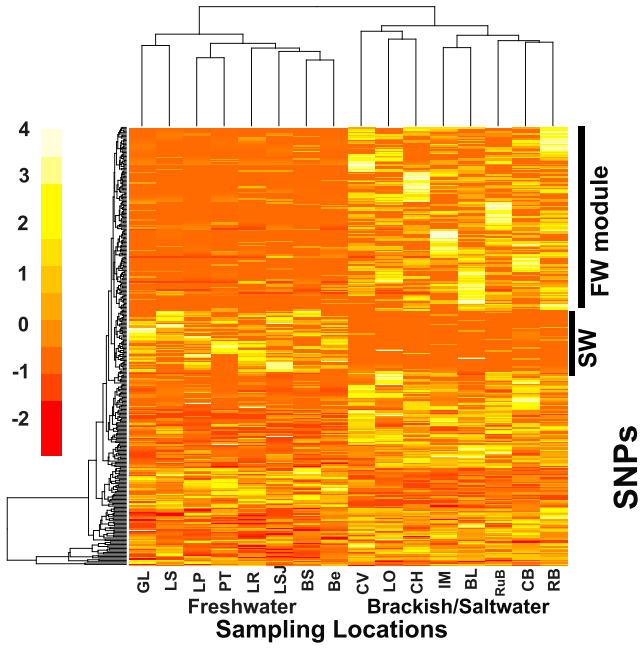

Figure 2. Sample Location Allele Frequencies for the 331 Most Important SNPs to Distinguish Eel Ecotypes

This heatmap illustrates the allele frequencies for all 16 study sampling sites. Each row represents a specific SNP, and each column represents a sampling site. Sampling site acronyms are defined in the map (Figure S1).The colors represent normalized (by row) allele frequencies. Half of the markers are nearly fixed in one ecotype and comparatively variable in the other. We designate SNPs exhibiting this pattern as either freshwater (FW; 137 SNPs) or brackish/saltwater (SW; 45 SNPs) modules and consider them separately. The LO location is freshwater, but we considered it to be a brackish/saltwater on the map because it is the result of brackish/saltwater-transplanted individuals. See also Figure S1.

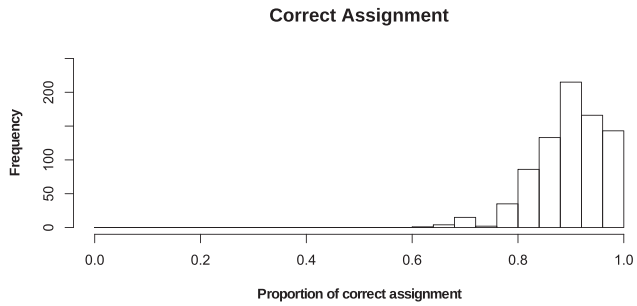

Figure 3. Proportion of Correct Ecotypic Assignment in the Jack Knife Procedure

For each iteration, all individuals from a single sampling location were excluded from the random forest analysis of the remaining 15 locations. Then, the results were used to predict the excluded individuals' ecotype. The success rate was 91.2% ± 6.9%. See also Figure S2.

regulation of chemotaxis (GO: 0050920; p = 0.0005), and respiratory system development (GO: 0060541; p = 0.003) (Table 1). One specific gene of interest in this module, vascular endothelial growth factor A (*VEGFA*), is essential for blood vessel formation (both vasculogenesis and angiogenesis). It has also been found to play a role in red blood cell formation in zebrafish [19].

DISCUSSION

Causes of Parallel Genetic Differences Despite Panmixia

We found consistent genetic differences that correlate with habitat ecotypes in the American eel. Though there has been conflicting evidence of panmixia versus subtle population structure in the European eel [3, 20–23], panmixia in the American eel is definitive with both nuclear and mitochondrial markers [2, 3]. This begs the question, which mechanisms could be acting in each generation that would result in consistent genetic difference between habitat ecotypes that are sufficient for 90% successful blind assignment? We propose that two possible mechanisms are (1) genotype-dependent habitat choice and (2) intra-generational spatially variable selection.

There is empirical evidence that European eel (*A. anguilla*) glass eels (young juvenile life stage) make choices based on salinity differences in controlled choice experiments [24]. In addition, a recent study with the American eel found that glass eels did make choices based on salinity in a controlled setting (migrated from brackish water to either salt or freshwater when given a choice between saltwater, freshwater, or remaining in brackish water) [9]. However, the proportion of choice groups did not vary by the two origins tested, the St. Lawrence (the most upstream glass eels known, thus more likely to become the freshwater ecotype) versus Canadian Maritimes (proximate to abundant marine ecotype eels), and there was no difference in growth among choice groups. There were, however, growth differences between origins independent of salinity choice [9]. Also, in an effort to restore Lake Ontario-Upper St. Lawrence abundance, glass eels from the Maritimes were transplanted to these locations [25]. This forced movement resulted in the transplanted individuals growing fast, with a substantial proportion becoming males compared with the historically slow growth and near absence of males [26], as well as assessed natural upstream migrants to the area [8]. Thus, eels do have the capacity to choose salinity habitats, and if these choice groups in nature are genetically different, this mechanism has the potential to result in the genotype-habitat associations that we observe.

The second mechanism that could result in the observed pattern is spatially variable selection. Indeed, selection has now been empirically demonstrated to be associated with a latitudinal and temperature gradients in the American eel [27–29], as well as in the sister species, the European eel [30]. Thus, the empirical evidence indicates that spatially variable selection occurs in both species of Atlantic eel. In contrast to the clinal variation associated with these studies, the drastic differences in salinity, biotic interactions, and flow regime in our studied ecotypes may represent stronger selection, making spatially varying selection acting on the freshwater-saltwater axis even more plausible. Mathematical modeling efforts also indicate that within-generation selection can result in differences in quantitative traits, even in panmixia (see the Supplemental Discussion for more details) [31–33]. In the large-scale transplant (see above), the eels grew fast, matured early, and out-migrated at a young age [34]. Given that eels from that area have the longest migration back to the Sargasso Sea, it is unknown whether the transplanted young eels would have the energy reserves for the spawning migration. Thus, their fitness cannot be evaluated.

Although we cannot rule out or definitively support either of the two hypotheses regarding the mechanism (or their interaction), we do demonstrate that there are polygenic genetic differences between the ecotypes that are sufficient enough to correctly reassign them blindly to their habitat of origin. This is complementary to the phenotypic plasticity known to occur in the species [35] and other recent studies indicating differences in reaction norms to salinity levels among sampling locations differs [8, 9]. We cannot rule out or support the presence of sex-specific strategies. The mechanism of sex determination is unknown in *Anguilla* sp. but is thought to partially or completely involve plasticity [8, 36, 37]. Similar to all GWAS approaches, the genetic differences are only correlated with the ecotypes. Moreover, the 331 SNPs are certainly not comprehensive, as we used RAD-tag sequencing, which is a reduced representation of the genome. Also, some quantitative genetic difference may be too subtle to detect with any current method [11].

More generally, assuming that these associated genetic differences underlie the phenotypic difference between the ecotypes, these findings illustrate theoretical expectations that the genetic basis of quantitative phenotypic traits is manifested as polygenic at the genomic level [11]. Despite the emphasis on examples containing genes of major effect accounting for phenotypic variation in nature [38], quantitative traits are expected to involve many genes of minor effects; thus, subtle shifts in allele frequency should be the expected mechanism underlying polygenic selection. This has recently been demonstrated for salmon survival at sea [39] and coral thermal tolerance [40], but more strikingly with height in humans, where the cumulative total effects of identified outliers (univariate approach) only explain 5% of variation, as opposed to a polygenic approach that explained 45% of the phenotypic variation [41]. This demonstrates the inherent difficulty in detecting quantitative genetic differences with traditional outlier approaches.

Table 1. GO Enrichment for the 331 Random Forest SNPs that Differentiate Freshwater from Brackish/Saltwater American Eel

GO ID	Ref.	SNPs	p Value	Term
Entire Set of 331 SNPs with a p Value <0.01 and Containing at Least Two SNPs				
0060174	4	2	0.0004	limb bud formation
0004683	5	2	0.0006	calmodulin-dependent protein kinase activity
0015026	6	2	0.0009	coreceptor activity
0043114	8	2	0.0016	regulation of vascular permeability
0040036	9	2	0.0021	regulation of fibroblast growth factor receptor signaling pathway
0060541	74	4	0.0025	respiratory system development
0051701	37	3	0.0029	interaction with host
0052126	11	2	0.0031	movement in host environment
0033276	12	2	0.0038	transcription factor TFTC complex
0030532	13	2	0.0044	small nuclear ribonucleoprotein complex
0070851	45	3	0.0051	growth factor receptor binding
0019059	15	2	0.0059	initiation of viral infection
0005669	16	2	0.0067	transcription factor TFIID complex
0048738	51	3	0.0072	cardiac muscle tissue development
0048286	17	2	0.0075	lung alveolus development
0005104	17	2	0.0075	fibroblast growth factor receptor binding
0050839	18	2	0.0084	cell adhesion molecule binding
0043535	18	2	0.0084	regulation of blood vessel endothelial cell migration
0006094	18	2	0.0084	gluconeogenesis
0044403	55	3	0.0088	symbiosis, encompassing mutualism through parasitism
0048646	310	7	0.0098	anatomical structure formation involved in morphogenesis
Subset Representing the Freshwater Module of 137 SNPs with a p Value <0.005				
0010927	48	3	0.0006	cellular component assembly involved in morphogenesis
0033276	12	2	0.0008	transcription factor TFTC complex
0005669	16	2	0.0015	transcription factor TFIID complex
0006094	18	2	0.0018	gluconeogenesis
0019319	23	2	0.003	hexose biosynthetic process
0005509	286	5	0.0031	calcium ion binding
Subset Representing the Brackish/Saltwater Module of 45 SNPs with a p Value <0.005				
0050920	31	2	0.0005	regulation of chemotaxis
0050795	40	2	0.0009	regulation of behavior
0070851	45	2	0.0011	growth factor receptor binding
0003779	209	3	0.0014	actin binding
0005126	56	2	0.0017	cytokine receptor binding
0060541	74	2	0.003	respiratory system development
0048878	283	3	0.0033	chemical homeostasis
0008092	313	3	0.0043	cytoskeletal protein binding
0001666	90	2	0.0045	response to hypoxia
0019058	92	2	0.0046	viral infectious cycle
0070482	93	2	0.0047	response to oxygen levels

99 of these SNPs were within a gene. The columns represent the GO identifier enriched, the number of genes implicated for that term in the entire annotation for the genome (Ref.), the number of genes implicated in that term for the random forest SNPs most important in distinguishing the ecotypes (SNPs), and the name of the term. Terms from both the biological process and molecular function and only terms enriched with at least two SNPs were included.

Implications for Management and Beyond

There is great conservation concern for the freshwater ecotype of the American eel. The most extreme case is individuals in the Lake Ontario-Upper St. Lawrence River, where individuals are 99.9% female and can reach lengths exceeding 1 m and ages exceeding 20 years before maturing (Figure 1). Notably, these individuals also have the longest spawning migration requiring an abundance of energy reserves [5]. Namely, due to hydroelectric dams, overfishing, and pollution, abundance in the Lake Ontario-Upper St. Lawrence River, which is exclusively freshwater habitat, has declined by 99% in the past 40 years [42]. This is especially alarming because this area is nearly exclusively composed of large females and has historically represented a large percentage of fecundity for the entire species [26]. In contrast, Atlantic Canada includes a diversity of habitats (fresh, brackish, and saltwater), but commercial fishing primarily occurs in brackish and saltwater [43]. This ecotype sustains the fishery in that region and has been relatively stable over the same time period [43]. Given that the species is panmictic, managers have assumed that the divergent phenotypes were 100% the result of phenotypic plasticity in contrasted environments. Our results demonstrate the presence of a genetic component to the divergent ecotypes and help explain why transplanted young eels from abundant rearing areas fail to exhibit the freshwater ecotype [44].

These findings are most relevant for the management practices of *Anguilla* sp. Genetic diversity must be conserved in eel contingents associated with the different ecotypes. Mitigation efforts (fish ladders, transporting individuals that are naturally migrating safely around dams) should be maintained for the rarefying individuals of the Lake Ontario and Upper St. Lawrence River. Indeed, these individuals are homozygous,

or nearly so, for the many alleles resulting in this most extreme example of the freshwater ecotype. However, the genetic diversity found in this depleted contingent is also present in other freshwater-rearing groups (correct allelic combinations for freshwater) and is even contained in the brackish/saltwater groups, albeit not in the correct allelic combinations for freshwater in this generation. Management should continue to support the robust numbers in many coastal populations in order to conserve genetic diversity in the panmictic species that is essential for the intra-generational mechanisms to continue. Our results thus bring strong support to the hypothesis that ecotypic differences between eels occupying different habitats is not the sole effect of plasticity but may also be caused by functional genetic differences stemming from intra-generational spatially varying selection and/or genotype-dependent habitat choice (or both) of ecologically divergent habitats.

Furthermore, despite a lack of genetic subdivision, these mechanisms would occur within each generation to result in divergent ecotypes associated with distinct habitat use. Thus, the commonly held assumption that plasticity is the only reason for phenotypic differences in systems with weak population subdivision (such as marine species with planktonic dispersal) must be re-evaluated. Similar patterns have been found in recent studies of divergence that have sufficient resolution or design to detect such subtle genomic changes [39, 45, 46].

ACCESSION NUMBERS

The contigs containing the 331 random forest SNPs and raw genotypes for all samples are archived in the Dryad repository at http://dx.doi.org/10.5061/dryad.n1mn9.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Discussion, Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.04.062.

AUTHOR CONTRIBUTIONS

S.A.P. analyzed the data and wrote the paper. J.G. performed the lab work, analyzed the data, and wrote the methods. E.N. analyzed the data. M.D., C.A., M.C., and L.B. conceived and planned the study. L.B. co-wrote the paper, and all authors contributed substantially to revisions.

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