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Genomic Connectivity and Adaptation Signals of the Freshwater Sponge *Ephydatia muelleri* Across Its Distribution

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ABSTRACT

Aim: Genetic connectivity and local adaptation were examined across the distribution of the freshwater sponge *Ephydatia muelleri*. Because it occupies an exceptional breadth of freshwater environments across a broad geographic range, this species offers key insights into biogeographic processes shaping genetic structure and adaptation in inland waters.

Location: Freshwater habitats across North America, Europe and Asia.

Taxon: Freshwater sponges (Porifera, order Spongillida).

Methods: A total of 106 individuals were sampled from 11 localities across three continents. Double-digest RADseq was used to generate genome-wide SNP data, resulting in 3114 putatively neutral SNPs for analyses of population structure and connectivity and 115 candidate SNPs potentially under selection for assessing signatures of local adaptation to environmental variables such as light and temperature.

Results: Neutral loci revealed low connectivity and strong genetic differentiation among regions, with two major genetic clusters corresponding to North America and Eurasia. Loci under selection indicated polygenic adaptation to environmental gradients of light and temperature and evidence of selection on gene regulatory processes. Patterns of genetic structure are consistent with the monopolisation hypothesis, suggesting that historical processes, particularly range shifts and barriers associated with the Last Glacial Maximum, have had a stronger influence on current genetic structure than ongoing gene flow.

Main Conclusions: *Ephydatia muelleri* populations show limited dispersal and strong historical imprints on genetic structure but also exhibit adaptive responses to local environmental variation. These findings highlight how monopolisation and historical climatic events shape freshwater invertebrate biogeography. Our study provides new insights into dispersal pathways, genetic plasticity and the resilience of freshwater sponges, with implications for the conservation of freshwater ecosystems.

Laura de la Cruz-Castillejo and Robert Cassidy are co-first authors and contributed equally to this work.

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1 | Introduction

Freshwater ecosystems are highly biodiverse (Strayer and Dudgeon 2010) and provide essential services to humans (Vári et al. 2022). However, freshwater species face higher extinction risks than their terrestrial or marine counterparts (Tickner et al. 2020), with distribution ranges that are highly threatened, primarily due to climate change (Dudgeon et al. 2006; Haase et al. 2023; Geist 2011). Given the current pressures on freshwater ecosystems, as well as their fragmented nature and strong environmental heterogeneity, these habitats are also excellent study models for fundamental research in evolutionary biology. Natural barriers between populations and marked seasonal variation in these systems have repeatedly resulted in unique local adaptations, strong population genetic structure and adaptive radiations (Thomaz et al. 2016). These characteristics can lend insight into how physical and ecological dynamics interface with biological mechanisms such as phenotypic plasticity, development, reproduction and dispersal to produce evolutionary change (Bohonak and Jenkins 2003; De Meester et al. 2002). Indeed, in freshwater ecosystems, evolutionary change has been found to closely follow ecological change over relatively short timespans (De Meester and Pantel 2014), placing these ecosystems at the intersection of fundamental evolutionary theory and pressing conservation priorities. Given this context, the use of genomic data in population-level studies of evolutionary history, connectivity, adaptation and demographic structure is critical for biodiversity research and conservation in freshwater habitats. While this area of research has been investigated extensively in a selection of popular 'model' taxa, such as cichlid fishes and *Daphnia* water fleas (e.g., Keller et al. 2013; Lynch et al. 2017), the literature on many taxonomic groups and on other freshwater invertebrates in particular, is scarce.

Sponges (Porifera) are among the most common and abundant invertebrates in aquatic ecosystems, but among them, only the order Spongillida has successfully transitioned to freshwater ecosystems (Manconi and Pronzato 2008; Van Soest et al. 2012), sometime around the Permo-Carboniferous period ≈300 million years ago (Pronzato et al. 2017). Spongillids have a global distribution (except Antarctica) and diversified significantly during the Paleogene, allowing adaptation to diverse climates (Manconi and Pronzato 2008, 2016a; Pisera et al. 2016; Pronzato et al. 2017). Spongillida currently includes seven families, 47 genera and 236 species worldwide, with high endemism except for certain widespread genera, that is, *Spongilla* and *Ephydatia* (Manconi and Pronzato 2002, 2016b). To disperse over large distributions, freshwater sponges rely on asexual reproduction through the production of specialised, dormant gemmules, which are resilient structures consisting of totipotent cells encased in a protective layer (Manconi and Pronzato 2002, 2016b; Ungemach et al. 1997) that survive adverse conditions in a cryptobiotic state until conditions improve (Kenny et al. 2020; Manconi and Pronzato 2016a). Gemmules possess structural adaptations, including spiny spicules and a spongin-based pneumatic layer that enhance their potential for attachment and flotation, facilitating dispersal by both abiotic and biotic vectors such as water currents, wind, mammals, fish and birds (Carballo et al. 2024; Maldonado and Riesgo 2009; Pronzato and Manconi 1994). For example, buoyant gemmule-bearing fragments have been observed rafting during flood events and intact gemmules of *Ephydatia fluviatilis* have been recovered from the pellets of piscivorous birds, indicating the potential role of endozoochory in long-distance transport (Carballo et al. 2024). These adaptations presumably allow freshwater sponges to inhabit and disperse across strong environmental and geographic barriers, including discontinuous water bodies, mountain ranges and extreme temperature changes. However, such presumed dispersal capabilities through gemmule transport strongly contrast with the limited gene flow observed for the few freshwater sponges studied to date (Li et al. 2018; Lucentini et al. 2013). From this perspective, while hardy gemmule formation and adapted life strategies facilitate survival in freshwater environments in Spongillidae, it is not clear what processes allow cosmopolitan species to colonise waters with dramatically different environmental conditions while other species are limited to narrow ranges with clear environmental limits. A possible explanation could arise from the known rich community of microscopic symbionts (microbes and microalgae) present in freshwater sponges (Hustus et al. 2023; Sugden et al. 2022), which contributes to increase their plasticity and play an essential role for the function and survival of sponges (Thomas et al. 2016). Understanding the adaptive processes that contribute to this remarkable plasticity and wide ecological niche can allow us to anticipate how this widely distributed species will adapt to changing environmental conditions, but can also lend insights into the origins of general adaptive processes of freshwater organisms.

Another adaptation of freshwater sponges to the extremely variable conditions of continental waters is a life cycle containing asexual cyclic phases (including parthenogenetic), which for freshwater invertebrates (including sponges) that are purely aquatic usually show strong genetic structure and limited gene flow (e.g., Li et al. 2018; Lucentini et al. 2013; Okamura and Freeland 2002; Schröder et al. 2022). In this context, local adaptation has a prominent role in the survival of the species and has been largely linked to the phenomenon known as the monopolisation hypothesis (De Meester et al. 2002). This hypothesis explains the paradoxical population structure observed in groups such as rotifers, bryozoans and *Daphnia* water fleas, which display low gene flow and high population structure despite high dispersal capabilities by pointing to rapid adaptation to local conditions and the monopolistic colonisation of resources by large quantities of asexual propagules that arrive first and act as effective barriers to colonisation by later generations (Declerck et al. 2001; De Meester et al. 2002; Orsini et al. 2013). Genetic evidence supporting the monopolisation hypothesis has also been found in the freshwater sponge *Ephydatia fluviatilis* through the use of microsatellite markers (Lucentini et al. 2013; Li et al. 2018). However, these genetic markers constrain the depth of insight into underlying adaptive processes. To date, no study has used genome-scale data to examine local adaptation in the context of the monopolisation hypothesis. Such high-resolution datasets are essential for differentiating neutral population structure from putatively adaptive patterns, whether shaped by natural selection, epigenetic mechanisms, or other drivers of genetic variation, such as structural variants. Furthermore, this data also enables reconstruction of historical demographic

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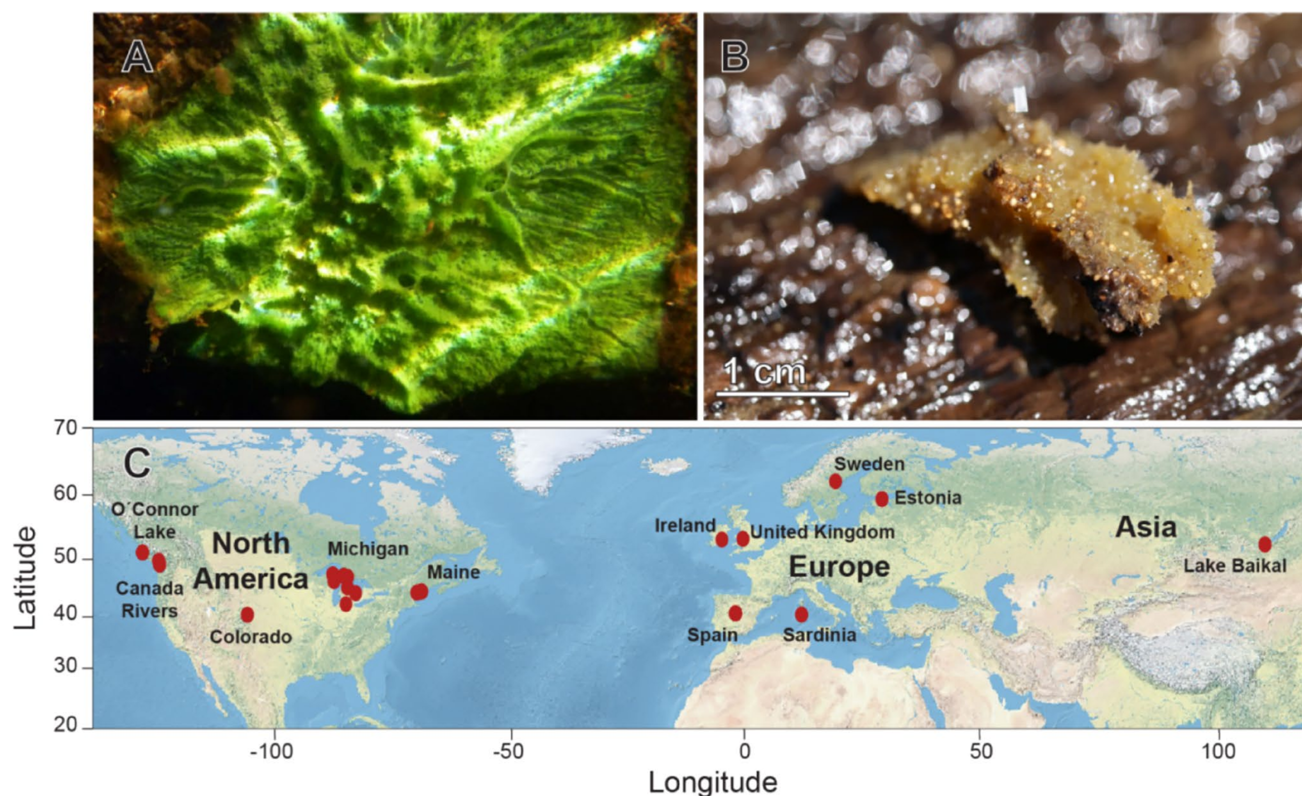

FIGURE 1 | *Ephydatia muelleri* from (A) Sooke River, Canada (photograph by Sally Leys, original image) and (B) Embalse de los Morales reservoir, Spain, showing gemmules within the body tissue (photograph by Ana Ramón-Laca, original image). (C) Map showing the location of sampled sites, generated by the authors in R using publicly available geographic data.

processes that influence present-day genetic diversity and connectivity (Galià-Camps et al. 2025; Hotaling et al. 2018; McGuigan et al. 2021; Savolainen et al. 2013).

Here, by using a genomic approach using ddRADseq on 106 *Ephydatia muelleri* (Figure 1A) specimens across its whole distribution range, we explore the current population structure, genetic connectivity, coancestry patterns, demographic history and adaptive mechanisms of this unique freshwater sponge species. *Ephydatia muelleri* is a strong candidate for assessing these processes for numerous reasons. Firstly, *E. muelleri* is a freshwater demosponge that is widely distributed across temperate regions of the Holarctic, including Europe, North America, Asia and Iceland (Cárdenas et al. 2012; Hall et al. 2021; Manconi and Pronzato 2016a). Moreover, *E. muelleri* exhibits a cyclical life cycle alternating between sexual and asexual reproduction through gemmules (Figure 1B) (Mukai 1990; Sallin 1995), which enhance the species' ability to persist, disperse and colonise a wide array of freshwater habitats (Frost 1991; Manconi and Pronzato 2002; Sallin 1995), aligning with predictions of the monopolisation hypothesis. Furthermore, *E. muelleri* is found in highly disparate types of freshwater habitat, ranging from massive, deep lakes to narrow, shallow channels and from the warm, dry Mediterranean region to frequently frozen water bodies in Siberian climates (Pronzato and Manconi 1994), allowing sampling across diverse environmental conditions. Finally, the available high-quality reference genome (Kenny et al. 2020) of *E. muelleri* makes it an ideal experimental model for examining genetic variability, local adaptation and evolutionary dynamics using high-resolution genomic data. Given its widespread distribution, reliance on durable asexual propagules and exposure to strong environmental heterogeneity, we hypothesize that *E. muelleri* will exhibit pronounced genetic structure driven by limited effective gene flow and rapid local adaptation, consistent with predictions of the monopolisation hypothesis.

2 | Materials and Methods

2.1 | Sample Collection and Preservation

A total of 106 specimens of *E. muelleri* were collected from 11 sites across the species' distribution range in Canada, United States of America, Ireland, Spain, the United Kingdom, Italy, Sweden, Estonia and Russia (Table 1; Figure 1C; Appendix S3). Samples were gathered manually from lakes, rivers and reservoirs at depths up to 40 cm. Specimens were preserved in 96% ethanol, DNA/RNA shield reagent (Zymo Research) or RNAlater (Invitrogen) and stored at −20°C for further analysis. All specimens were preliminarily identified based on the morphology of spicules.

2.2 | DNA Extraction, Barcoding and Haplotype Networks

DNA barcoding was performed on 98 specimens to confirm their identification as the target species. Although 106 specimens were collected, the remaining eight samples repeatedly failed to yield PCR amplicons of sufficient quality despite

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TABLE 1 | Population genetic statistics for *Ephydatia muelleri*. Samples grouped in eight areas and at the global level.

Sampling group	N	Private alleles	Private alleles/N	H_e	H_o
O'Connor lake (Canada)	10	84	6.46	0.044 ± 0.002	0.047 ± 0.003
Nanaimo and Sooke river (Canada)	13	132	13.2	0.075 ± 0.002	0.063 ± 0.003
Colorado	4	29	7.25	0.043 ± 0.002	0.080 ± 0.005
Michigan	21	555	26.42	0.105 ± 0.003	0.076 ± 0.002
Maine	13	268	20.61	0.087 ± 0.003	0.071 ± 0.003
Spain	30	52	1.73	0.018 ± 0.001	0.027 ± 0.003
Europe without Spain	10	409	40.9	0.084 ± 0.003	0.058 ± 0.002
Baikal	5	139	27.8	0.058 ± 0.003	0.062 ± 0.003
Total	106	—	—	0.108 ± 0.002	0.050 ± 0.001

Abbreviations: H_e, expected heterozygosity; H_o, Observed heterozygosity; N, number of samples.

multiple optimisation attempts and thus could not be included in the barcoding analyses. Genomic DNA was extracted from approximately 1 cm^3 of each sample using the Speedtools Tissue DNA extraction kit. DNA concentration was measured with the Qubit 1× dsDNA High Sensitivity assay kit (Thermo Fisher), and quality was assessed using a NanoDrop spectrophotometer (Thermo Fisher). The Internal Transcribed Spacer (ITS1) region was amplified using the primers FS-ITSF 5′-TACACACCGCCCGTCGCTACTA-3′ and FS-ITS-R 5′-CTYYGACGTGCCTTTCCAGGT-3′ (Itskovich et al. 2008) and the PCR protocol was: 94°C/2 min, (94°C/1 min, 55°C/1 min, 72°C/1 min) × 40 cycles, 72°C/8 min. Each PCR reaction was conducted in a 16.5 μL volume, which included 12.5 μL of 2× PCRBIO Taq Mix Red (PCR Biosystems, UK), 1 μL of each primer (10 μM) and 1 μL of DNA. The amplified samples were then sequenced at Macrogen. Overlapping sequence fragments per each individual were assembled and trimmed into consensus sequences using the software *Geneious* Prime (http://www.geneious.com, Kearse et al. 2012). Consensus sequences were checked for contamination using *BLAST* (Altschul et al. 1997) and aligned with the inbuilt *MAFFT* v.7.309 (Katoh and Standley 2013), using the Q-INS-I option. The resulting alignment was implemented in *PopArt* (Leigh et al. 2015) for the construction of TCS haplotype networks.

2.3 | ddRADseq Library Preparation and Sequencing

ddRADseq genomic libraries were prepared for all samples following the protocol of Peterson et al. (2012) with modifications according to Combosch et al. (2017), as described in Taboada et al. (2023). In short, genomic DNA was digested using the restriction enzymes EcoRI and BfaI (New England Biolabs). The resulting fragments were cleaned with Agencourt AMPure beads (Beckham Coulter) and their concentration quantified using a Qubit dsDNA HS assay. Fragments were posteriorly ligated to adaptors with unique barcodes for each sample. After adaptor ligation, up to 12 samples were pooled into libraries. The libraries were cleaned by manual pipetting using AMPure beads and size-selected (200–400 bp) using a Blue Pippin Prep system (Sage Science). Libraries were amplified by PCR using library-specific primers to enable library multiplexing. The resulting PCR products were cleaned by manual pipetting using AMPure beads, quantified with a Qubit dsDNA HS assay, and the fragment size pattern was checked on a Tapestation 2200 (Agilent Technologies). Finally, the library concentrations were normalised and pooled for sequencing in a single run on an Illumina NovaSeq 6000 (2 × 150 bp) at Novogene Europe (Cambridge, UK).

2.4 | Assembly and Filtering of ddRADseq Loci

Following sequencing, quality filtering and loci assembly were conducted using *Stacks* v2.57 (Catchen et al. 2013) following the same strategy as in Taboada et al. (2023). The 'process_radtags' module was used to filter raw reads by removing low-quality sequences, those missing barcodes, or with incomplete restriction sites. RAD-tags with minimal divergence, such as adapter mismatches (allowing up to two mismatches with the parameter --adapter_mm = 2). Reads were trimmed to 145 bp to enhance SNP detection accuracy. After filtering, 43,651,303 sequences were retained from an initial 44,103,246, averaging 4,270,730 sequences per individual (range: 206,006 to 30,804,006; Appendix S4).

High-quality sequences were aligned to the *E. muelleri* reference genome (Kenny et al. 2020) using *Bowtie2* v2.5.4 (Langmead and Salzberg 2012). Alignments were conducted with paired-end sequence files specified via the '-1' and '-2' parameters, along with the '--very-sensitive-local' setting to enhance alignment sensitivity, accommodating partial matches when reads diverged from the reference. This approach ensured accurate alignments, even for reads with significant variations. A bash command was used to extract alignment percentages from output files, averaging 80% for the dataset, indicating high coverage and optimised alignment. Following alignment, the *Stacks* tool 'ref_map.pl' was employed to process the aligned data. A population map ('—popmap') and sample directory ('—samples') were provided, and the *Stacks* module 'gstacks' was run to assemble loci and identify SNPs based on read alignment positions.

The 'populations' tool in Stacks was used to extract SNPs present in at least 80% of individuals (r = 0.8). To reduce linkage

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disequilibrium (LD), the first SNP from each RAD-tag was selected with the '--write_single_SNP' parameter. Following Roesti et al. (2012), only SNPs with a minor allele frequency (--min_maf) above 0.05 were retained. SNPs failing Hardy–Weinberg equilibrium ($p \leq 0.05$) in at least two areas or showing excess heterozygosity ($H_o > 0.5$) were also removed (Hohenlohe et al. 2021). Further filtering was performed using *PLINK v1.9* (Purcell et al. 2007) with the '--indep-pairwise 50 10 0.5' command. This step removed highly correlated SNPs by recalculating LD across 50-SNP windows every 10 SNPs, ensuring uncorrelated data for downstream analyses. The number of SNPs retained after each filtering step is summarised in Appendix S5. Note that filters reducing LD (i.e., '--write_single_SNP' parameter in *Stacks* and *PLINK* analysis, resulting in a LD-pruned SNP dataset) were not applied to run *fineRADstructure* as linked regions may capture insightful information regarding coancestry. The resulting matrix containing 3182 SNPs was visualised with the *Matrix Condenser* interface (de Medeiros, 2024), revealing global missing data of 10.05%, ranging from 4.39% to 18.29% per individual. These values supported retaining all 106 individuals for further analyses. The SNPs inferred to be under selection were later filtered (see Section 2.5) from the neutral ones and the remaining SNPs were subsequently used for the population connectivity and migration/demographic approaches (see Sections 2.6 and 2.7).

2.5 | Detection of Putative SNPs Under Selection and Functional Assignment

In order to examine possible mechanisms of local adaptation in populations of *E. muelleri* in a way that was robust for diverse types of natural selection, we analysed our dataset for SNPs under selection using a variety of both Genome Scan (GS) and Genome-Environment Association (GEA) methods. GS techniques used included *BayeScan* v 2.1 (Foll and Gaggiotti 2008), *Arlequin* v 3.5.2.1 (Excoffier and Lischer 2010) and *Baypass* v2.4 (Gautier 2015), while GEA methods included LFMM and pRDA analyses using the *vegan* v2.6–8 (Oksanen et al. 2019) and *lfmm* v1.1 (Caye et al. 2019) packages in *R*, respectively.

For *Arlequin*, which detects outlier SNPs based on F-statistics, we set the missing data threshold per locus to 0.05 and used a non-hierarchical island model, which assumes that populations are partially isolated from each other like islands. We then ran 100,000 simulations and 100 demonstrations per group, and we adjusted the resulting *p*-values using the '*p*.adjust' function in R with the 'BH' method by Benjamini and Hochberg (1995) to control the false discovery rate (FDR) and generate *q*-values for putative SNPs. *BayeScan*, which follows a Bayesian approach using allele frequency differences to identify SNPs under selection based on a multinomial-Dirichlet model, was run according to default parameters. SNPs with *q*-value > 0.05 were considered outliers for *Arlequin* and *BayeScan*. *Baypass*, which incorporates XtX statistics and allele frequency differentiation to calculate outlier SNPs, was run first on the LD-pruned SNP dataset with default parameters in order to create an omega matrix accounting for linkages and population structure. Because *Baypass* outputs can vary when runs are repeated, the resulting omega matrix was then applied in five consecutive runs of the *Baypass* program on a SNP dataset that had been filtered for HWE, H_o and minimum allele frequency according to previously described thresholds, but had not been LD pruned. Of these five runs, the output with the median average XtX value was selected for analysis. Following the recommendations of Gautier (2015), XtX thresholds were then calculated by simulating a neutral SNP distribution and running *Baypass* on the simulated genotypes and were then applied as FDR cut-offs to identify outlier SNPs in the real dataset ($\text{FDR} < 0.5$).

For GEA methods, a pRDA and LFMM approaches were chosen for their ability to account for population structure and identify associations between SNPs and environmental variables. Freshwater environmental variables with potential biological significance for *E. muelleri* were selected from the Copernicus DynQual (Jones et al. 2023) and GeoFresh (Domisch et al. 2024) databases. We extracted 49 environmental variables related to water quality and temperature of the sampled site and topography, soil characteristics and land cover variables at the subcatchment level (see details in Appendix S1–S5) from our sampling sites using the *R* packages *sf* v1.0–19 (Pebesma 2018), *raster* v3.6-30 (Hijmans 2024) and *ncdf4* v1.23 (Pierce 2024). We assessed multicollinearity between variables with the *psych* package v2.4.6 in *R* (Revelle and Revelle 2015) and filtered out collinear variables (Pearson's $\text{rho} > 0.6$), resulting in the following 14 environmental variables: mean temperature seasonality (MTS); precipitation seasonality (PS); elevation (ELEV); tree cover, broad-leaved deciduous (TCBD); tree cover, needle-leaved deciduous (TCND); tree cover, mixed leaf type (TCML); tree and shrub cover (TS); shrubland (SL); grassland (GL); tree cover, flooded, fresh/brackish (FBFW); bare areas (BA); water bodies (WB); water temperature (TEMP); and faecal coliform concentration (cf 100 mL-1) (FC) (Appendix S1–S5). Then, the same set of filtered SNPs used for *Baypass* analysis was loaded (i.e., filtered for HWE, H_o and minimum allele frequency, but not LD-pruned) and missing data was imputed with the most common genotype data for each SNP in the dataset. Next, a PCA was performed on the LD-pruned SNP dataset and without potential outlier SNPs identified by genome scan methods in order to best characterise the neutral structure of the sample populations; this was done using the first three PCs, as they captured the majority of the variance according to an eigenvector plot. This structure was run as a condition in a pRDA with the scale-transformed environmental factors using the *rda* function in *vegan*. Then, ANOVA, VIF and R^2 analyses were run, and outlier SNPs were calculated based with $z = 3$.

For LFMM analysis, in order to maintain an optimal genome inflation factor (GIF) profile, the dataset of LD-pruned SNPs was used. A PCA was performed using the SNP data and PC1 and PC2 were selected for respective LFMM analyses because they explained similar levels of variance. Seven latent factors ($K = 7$) were assigned according to the populations identified in the DAPC and STRUCTURE analyses (see Section 2.6), and the *lfmm_ridge* and *lfmm_test* functions of the *lfmm* package were used to run latent factor mixed models for each PC. *p*-values were then converted to *q*-values according to the FDR procedure and using the *qvalue* package by Benjamini and Hochberg (1995), with *q*-values < 0.05 considered to be significant.

A total of 115 SNPs were detected to be under selection using the aforementioned methods. In order to determine possible

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functions of these SNPs, we extracted the sequences containing these SNPs using *Stacks* and blasted them against the annotated *E. muelleri* genome using *blastn* function from *BLAST* with *e*-value 0.005 as threshold (Altschul et al. 1997). The resulting gene identities were implemented in *shinyGO* in order to obtain enriched gene ontologies, using the human genome for comparison and the *E. muelleri* genome as background (Ge et al. 2020). Then, GO terms were run in *REVIGO* to visualise categorisations using treemaps (Supek et al. 2011).

2.6 | Population Differentiation and Structure

For population differentiation and structure analyses, the same neutral dataset of 3114 SNPs was used after filtering out FST outlier SNPs derived from Arlequin and Bayescan. Loci yielded by GEA analyses were not filtered out because they had already been derived by pre-correcting for neutral structure based on these FST outliers and would not meaningfully impact neutral structure. Here, samples were grouped into eight regions: O'Connor Lake (Canada), Nanaimo and Sooke River (Canada), Colorado, the Great Lakes (Michigan), Maine, Spain, Europe excluding Spain (comprising the United Kingdom, Ireland, Estonia, Sardinia and Sweden) and Asia (Lake Baikal). This grouping was selected not only to reduce the initial 11 sites due to low and uneven sample sizes in certain locations, thereby ensuring more robust parameter estimations, but also following a preliminary analysis performed on the dataset. This preliminary analysis consisted of a Discriminant Analysis of Principal Components (DAPC) with less stringent criteria to guide the delineation of populations for subsequent analyses. This preliminary analysis demonstrated that population differentiation could be enhanced by effectively separating lake and river populations in Canada and distinguishing populations in Spain from those in the rest of Europe. Genetic diversity indices, including private alleles, observed heterozygosity (H_o) and expected heterozygosity (H_e)—commonly considered as a measure of genetic diversity—were calculated using the '*populations*' module in *Stacks*.

Population structure was evaluated using three methods: *STRUCTURE* v2.3 (Pritchard et al. 2000), Discriminant Analysis of Principal Components (DAPC) from the *adegenet* v2.1.10 package in *R* (Jombart and Ahmed, 2011) and *fineRADstructure* v0.3.2 (Malinsky et al. 2018). *STRUCTURE* was run with 200,000 MCMC iterations under the admixture model, with a burn-in of 100,000 iterations, evaluating *K* values from 1 to 8 with 15 replicates for each *K* value. *CLUMPP* v 1.1.2 (Jakobsson and Rosenberg 2007) was used to identify the most probable number of clusters and to average the ancestry proportions of each individual across *K* replicates.

Population structure in DAPC was assessed with the *snapclust* function using the genetic clustering mode *snapclust.choose.k* in the *adegenet* package in *R*. Optimal genetic clusters were determined using Akaike (AIC), Bayesian (BIC) and Kullback (KIC) information criteria combined with the *k*-means algorithm, allowing up to 16 clusters and 100 iterations. The cluster number with the lowest AIC, BIC and KIC values was selected. Once the optimal number of clusters was selected, the optimal number of principal components (PCs) for each *k* was identified using the *xvalDapc* validation function with 1000 replicates, minimising the mean squared error (MSE) while retaining maximum variance. Individual assignment probabilities and cluster scatterplots were visualised using *assignplot* and *scatter.plot*, respectively.

We also used *fineRADstructure* to assess shared ancestry among the analysed individuals of *E. muelleri* and to provide further support to the *STRUCTURE* and DAPC analyses. This program leverages the information from all SNPs present in RAD-tags to provide high-resolution results on coancestry between individuals. The analysis included all individuals and was based on approximately 43,000 SNPs, obtained by running 'populations' in *Stacks* with 3114 neutral SNPs. This was done using the LD-unpruned dataset while deselecting the '--write_single_SNP' option. The *fineRADstructure* analysis was run with default values: 100,000 for *-x*, 100,000 for *-y* and 1000 for *-z*, assigning individuals to populations and 10,000 for *-x* in tree construction. The results were graphically interpreted using the 'Finestructure R Library' and the *fineRADstructurePlot.R* script, both provided in the *fineRADstructure* package for *R*.

In addition to the genetic structure analyses described above, a molecular variance analysis (AMOVA) was also performed using *Arlequin* to evaluate the significance of differentiation between the samples from two regions: Eurasia and North America. Eurasian samples included Spain, Europe (United Kingdom, Ireland, Estonia, Sardinia, Sweden) and Lake Baikal, while the ones from North America included O'Connor Lake (Canada), Nanaimo and Sooke River (Canada), Colorado, the Great Lakes (Michigan) and Maine. The AMOVA analysis was conducted using the standard AMOVA option, with the missing data level per locus set at 1 and 20,000 permutations, resulting in 3114 usable loci. The samples for each of the regions were grouped into the eight populations aforementioned to increase the statistical power of the comparisons in the analysis.

Finally, pairwise F_{ST} values were estimated to measure genetic differentiation between the eight sample groupings using *Arlequin*. The analysis was configured to allow up to 1% missing data per locus and 20,000 permutations were performed.

2.7 | Demography and Migration Patterns

The neutral SNPs dataset was transformed into folded Site Frequency Spectrum (SFS) files with the software *easySFS.py* (https://github.com/isaacovercast/easySFS) for demographic purposes. We used the neutral SNPs dataset as adaptive loci might generate biases in the resulting demographic history. We modelled the historical demographic events with the software *Stairwayplot2* (Liu and Fu 2020). Recent studies using RADseq data with this software have shown that drastic changes in effective population size can mirror colonisation and bottleneck processes (Galià-Camps et al. 2025). We used a single SFS file including all the individuals, regardless of the locality or genetic cluster, and kept 158 haplotypes to generate the species SFS file. We established a generation time of 1 year since most of continental sponge species have an annual cycle (Gugel 2001), and a mutation rate per site and generation of 1 × 10e-9, as spongillids

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are known to have low mutation rates in comparison to other sponge taxa (Maas et al. 2023). We ignored singletons as recommended by the software developer (Liu and Fu 2020), generated 200 input files to estimate the demographic patterns and visualised the results with *ggplot2*.

To identify current patterns of gene flow in the study area, the *Nm* method was used with bootstrapping = 1000 to estimate contemporary migration between geographic areas, employing the *divMigrate* function from the *R* package *diverRsity* v1.9.90 (Keenan et al. 2013). Additionally, a filtering threshold of 0.3 was selected for the analysis, meaning that only gene flows with values above this threshold were considered. This value was chosen to ensure that detected migrations reflected relevant genetic connections between the studied geographic areas.

3 | Results

3.1 | Population Structure and Connectivity Using ITS and Neutral SNPs

We obtained six ITS haplotypes that corresponded to North American populations (three haplotypes) and Eurasian populations (three haplotypes). We detected a single main haplotype in North America shared among all populations in this region, and two private haplotypes in Nanaimo River (one individual) and Colorado (two individuals) separated by one mutational step from the main shared haplotype. In samples from Eurasia, we detected a shared haplotype present in all the European populations, separated by three mutational steps from the main haplotype from North America. We also detected two Eurasian haplotypes separated from the previous one by one mutation step: one in most of the individuals from Spain ($n = 30$) and the other shared in all individuals from Baikal ($n = 5$) and three individuals from Estonia (Figure S1–S5).

Genetic structuring was evident across the *E. muelleri* SNPs dataset, as shown by STRUCTURE and DAPC analyses (Figure 2). STRUCTURE identified the optimal number of clusters, as $K = 8$, following the Pritchard criterion, and $K = 2$ based on the Evanno criterion (Figure S2). In addition to these two clustering groupings, we also plotted $k = 6$ given the results obtained in the DAPC analysis (see below). The K2 clustering showed clear differentiation between North American and Eurasian sites (Figure 2A), while the K6 and K8 showed subclustering into smaller groups of samples, some of them showing variable degrees of introgression (Figure 2B,C). In the K6 clustering, within the Canadian sites, O'Connor Lake samples were entirely assigned to the orange genetic cluster, while Nanaimo and Sooke River individuals were predominantly assigned (> 90%) to the green cluster, though with a varying degree of slight introgression from the orange cluster (Figure 2B). The North American sites were broadly assigned to different genetic clusters (Colorado = green, Michigan = blue and Maine = purple), with Michigan individuals exhibiting a varying degree of genetic admixture from multiple clusters, including blue, orange and purple, and some individuals from Maine (the ones from DP) showing a slight introgression from the green and blue clusters (Figure 2B). Samples from Spain were assigned to the yellow cluster, while the rest of European samples and the ones from

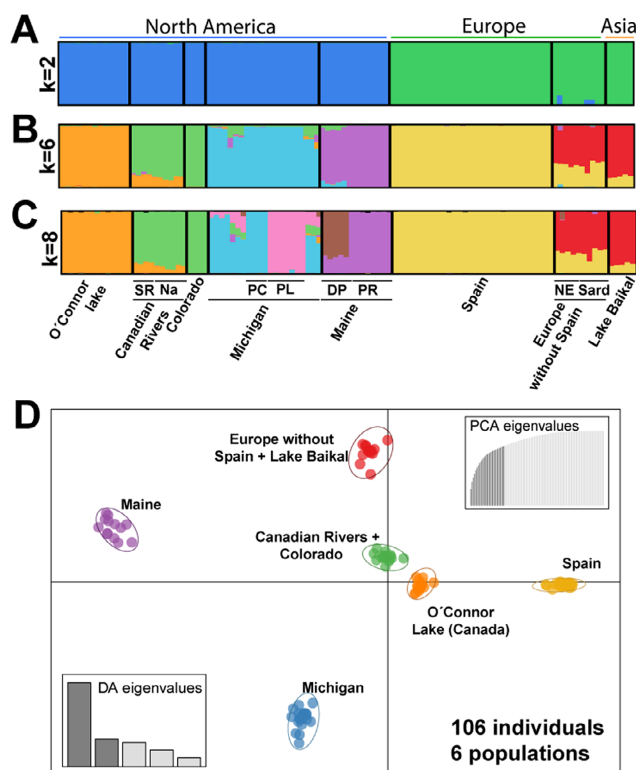

FIGURE 2 | Population structure in *Ephydatia muelleri*. (A) STRUCTURE plot for $K = 2$. (B) STRUCTURE plot for $K = 6$. (C) STRUCTURE plot for $K = 8$. Locations are abbreviated as follows: DP (Dundee Pond), Sard (Sardinia), Na (Nanaimo River), NE (North Europe: Including United Kingdom, Ireland, Estonia and Sweden), DK (Dead Kelsey), Ma (Manistee), M (Menominee), PC (Pendills Creek), PL (Perch Lake), P (Pigeon-Wiscoggin), SJ (St. Joseph), PR (Pemaquid River) and SR (Sooke River). (D) DAPC analysis showing the samples grouped into six genetic clusters.

Lake Baikal were predominantly assigned to the red cluster, but showing remarkable levels of introgression from the yellow genetic cluster in European samples (≈20%–30%), while a smaller degree of introgression in the Asian ones (≈10%) (Figure 2B). In K8 clustering, groups were similar to those described for K6, with the exception of some samples from Michigan and Maine, which showed a higher degree of genetic differentiation. In Michigan, all PC samples were exclusively assigned to the blue cluster, whereas all PL samples were mostly assigned to the pink cluster. Moreover, four individuals from DK were primarily assigned to the blue cluster, exhibiting a minor introgression from the pink cluster (≈5%). Additionally, one individual from DK and all individuals from Ma, M, P and SJ exhibited a similar genetic signature mostly assigned to the blue cluster but with a smaller contribution of the pink and other less relevant genetic clusters. In Maine, DP samples clearly differed from the PR ones, since they were mostly assigned to the brown cluster (ca. 70% sample assignation) with a varying degree of assignation to the purple cluster (Figure 2C).

The DAPC analysis identified six distinct genetic clusters ($k = 6$), corresponding to Spain, Europe together with Baikal, O'Connor Lake (Canada), Nanaimo and Sooke Rivers (Canada), Colorado, Michigan and Maine, with 51.76% of the variation explained on the x-axis and 17.19% on the y-axis (Figure 2D). Although

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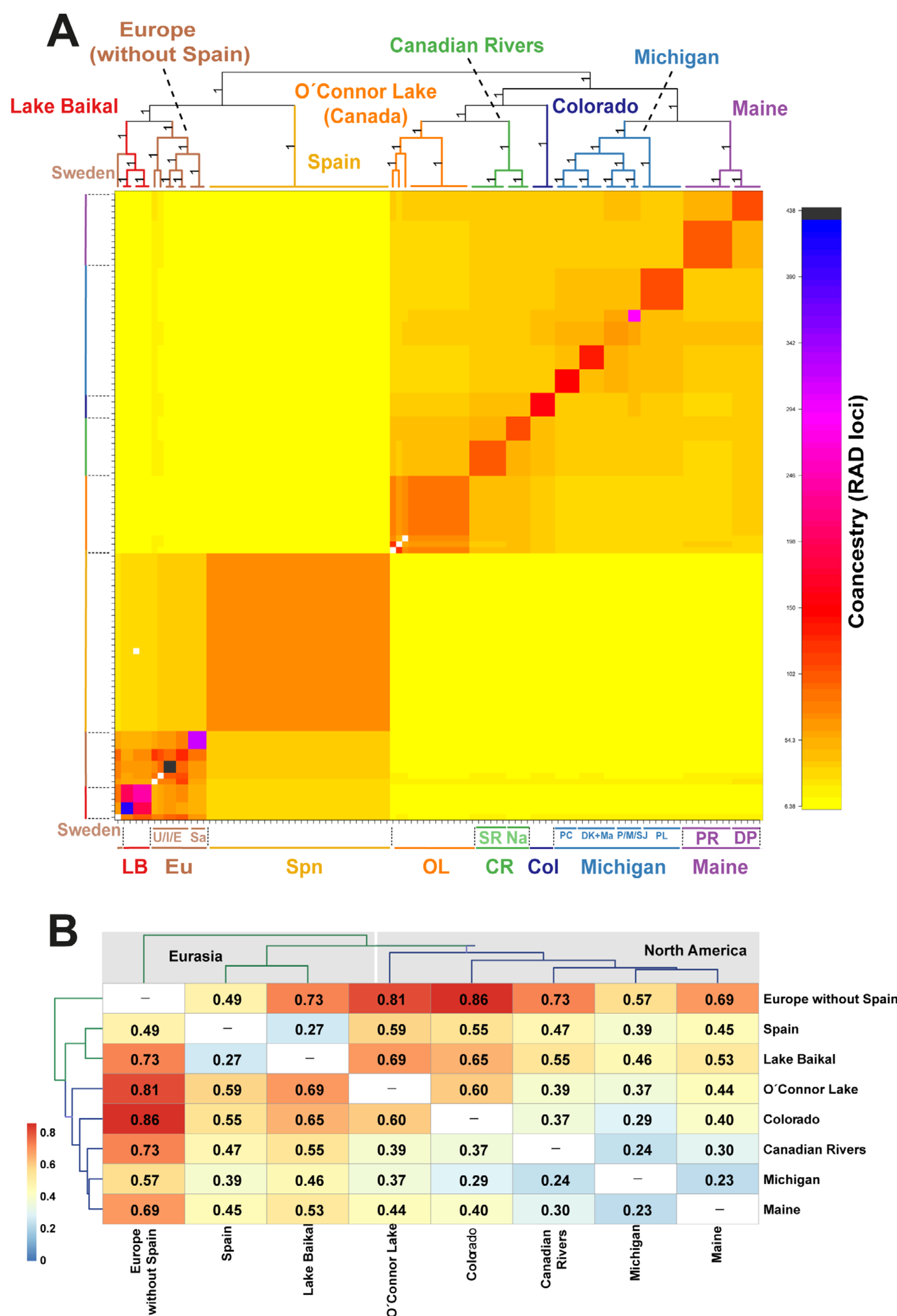

FIGURE 3 | Genetic substructure and isolation in *E. muelleri*. (A) Coancestry matrix derived from the *fineRADstructure* analysis. Locations are abbreviated as follows: Col (Colorado), CR (Canadian Rivers), DK (Dead Kelsey), DP (Dundee Pond), E (Estonia), Eu (Europe), I (Ireland), Sa (Sardinia), LB (Lake Baikal), M (Menominee), Ma (Manistee), Na (Nanaimo River), OL (O'Connor Lake, Canada), PC (Pendills Creek), PL (Perch Lake), PR (Pemaquid River), SJ (St. Joseph), SR (Sooke River), U (United Kingdom) and Pigeon-Wiscoggin (P). (B) Heatmap of pairwise F_{ST} values between sampling groups.

the greatest separation among populations was between Maine and Spain, we did not detect a clear separation between samples from North America and Eurasia.

In line with the *STRUCTURE* results, fineRADstructure revealed a distinct genetic separation between North America and Eurasia (Figure 3A). Within Eurasia, we observed a further subdivision, with a clear separation between European and Asian (Lake Baikal) populations, except for the Swedish individual, which clustered with the Baikal group. In Europe, the samples exhibited a lack of strict geographic structuring and appeared largely intermixed, with the exception of the Italian population, which formed a distinct cluster. In North America, a genetic split was observed between Canadian samples (including those from the Nanaimo and Sooke Rivers and O'Connor Lake) and those from Colorado, Michigan and Maine. Within these groups, additional subgroups were identified, with each location forming a distinct cluster separate from the others.

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Pairwise F_{ST} comparisons among the eight *E. muelleri* groups were all significant, with values ranging from 0.23 to 0.86 (Figure 3B), indicating substantial genetic differentiation between groups. The highest F_{ST} values were observed when comparing Eurasian versus North American regions (0.39 to 0.86), indicating greater genetic divergence. Within Eurasia, pairwise F_{ST} comparison of Spain and Baikal showed lower values (0.27) than when comparing Baikal and Europe excluding Spain (0.73). North American populations generally showed lower pairwise F_{ST} values, with the lowest observed between Michigan and Maine (0.23).

Population genetic parameters for the eight *E. muelleri* sample groups obtained with neutral SNPs are summarised in Table 1. Private allele counts varied widely, with Colorado showing the fewest number (29) and Michigan the highest (555). Private alleles per individual ranged from 1.73 in Spain to 40.9 in Europe excluding Spain. The overall expected heterozygosity (H_e) was relatively low (0.108 ± 0.002) (mean ± SE), with values ranging from 0.018 ± 0.001 in Spain to 0.105 ± 0.003 in Michigan. Observed heterozygosity (H_o) was 0.050 ± 0.001, ranging from 0.027 ± 0.003 in Spain to 0.080 ± 0.005 in Colorado.

AMOVA results when grouping the samples into two major regions (North America vs. Eurasia), revealed significant genetic differences between these two groups (Table 2). Significant genetic variation was also observed between regions within continents and between sites within regions. A remarkable amount of the total variance was attributed to comparison between continents and the variance attributed to differences between regions within each of the continents (ca. 28% of the comparisons), although most of the observed variance was attributed to differences between sites within each of the regions (ca. 44%).

3.2 | Demography and Migration Patterns

Our migration analysis using the eight regions did not detect contemporary migration between regions from North America and Eurasia (Figure 4A). In North America, Colorado was identified as the present-day source population for the remaining North American populations, with the highest migration rates directed towards Michigan. A remarkable migration from Michigan to O'Connor Lake (Canada) was also detected. In

TABLE 2 | Results of the AMOVA analysis for *Ephydatia muelleri*.

Source of variation	d.f.	Sum of squares	Fixation index	% variation	*p*
Between continents (North America vs Eurasia)	1	6.74663	F_{CT}: 0.27955	27.96	**0.017**
Between regions within continents	6	6.87169	F_{SC}: 0.38777	27.94	**0.000**
Between sites within regions	204	15.10013	F_{ST}: 0.55892	44.11	**0.000**

Note: Significant *p*-values in bold. The regions refer to the original eight study areas: O'Connor Lake (Canada); Nanaimo and the Sooke River (Canada); Colorado (USA); the Great Lakes region (Michigan, USA); Maine (USA); Spain; the rest of Europe excluding Spain (including the United Kingdom, Ireland, Estonia, Sardinia and Sweden); and Asia (represented by Lake Baikal).
Abbreviation: d.f., degrees of freedom.

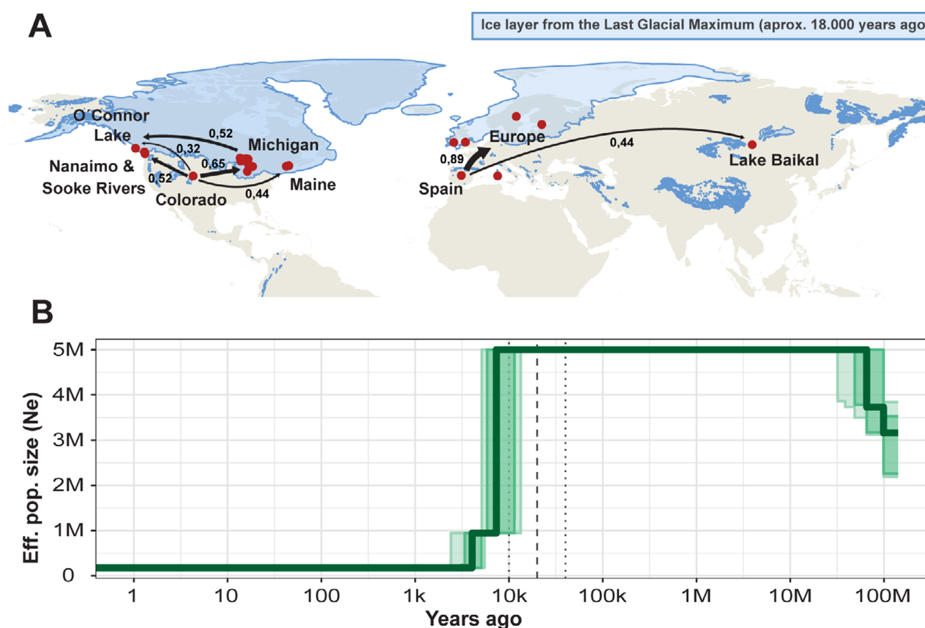

FIGURE 4 | (A) Observed contemporary migration patterns of *E. muelleri* among the eight geographic regions, showing the relative migration values between regions. The migration map also illustrates areas covered by ice and those that remained ice-free during the Last Glacial Maximum. Data provided by Esri, USGS, FAO, NOAA, USGS and the University of Koeln. (B) Demographic history of *E. muelleri* using StairwayPlot2. The bold dashed vertical line indicates the Last Glacial Maximum (20,000 years ago), while the thin dotted lines represent the estimated beginning (40,000 years ago) and end (10,000 years ago) of the Last Glacial Period.

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Eurasia, Spain was identified as the present-day source population for the rest of the populations in this region, with the highest migration rates directed towards the rest of the European countries.

The demographic analysis revealed the dynamics of effective population size (*Ne*) over time, spanning from 100 million years ago to the present. Initially, the population size was notably high, peaking ≈5 million individuals in the past (Figure 4B). Over time, an abrupt decline in *Ne* was observed ≈10,000 years ago, coinciding with the end of the Last Glacial Maximum (LGM). This trend likely reflects significant historical changes in environmental conditions, genetic diversity and ecological pressures associated with that era. Following this sharp decline, effective population sizes did not return to their earlier maximum levels.

3.3 | Adaptation Patterns Detected Using SNPs Under Selection

Of the genome scan (GS) methods used to detect SNPs under selection, BayeScan identified six SNPs, Arlequin identified 67 and Baypass identified 73, producing a set of 139 unique SNPs. Of the GEA methods, pRDA identified 185 potential adaptive SNPs, while LFMM identified 499, for a total of 601 unique SNPs under selection between the two methods. All combined methods (GS plus GEA) used to identify SNPs under selection identified 709 unique loci, with 115 of them appearing in at least two different analyses. Notably, all these SNPs were found to have relatively even distributions across the genome, with no visible islands of adaptation (Figure 5A, Figure S3).

Our ANOVA analyses ran to test the significance of the pRDA axes and showed that relationships were highly significant ($Pr(>F)=0.001$). Additionally, we found R^2 and adjusted R^2 values of 0.31 and 0.26, respectively. Outlier SNPs calculated at $z=3$ were not evenly distributed across RDA axes: RDA1 yielded eight SNPs, while RDA2 and RDA3 yielded 131 and 82 SNPs, respectively, with 36 SNPs identified more than once across the axes. While not all populations were strongly correlated with environmental variables, several populations, such as Lake Baikal, Spain, Sardinia (Italy) and Maine, showed clear separation along associations with environmental variables (Figure 5B,C). Linear regressions were also run with the 14 environmental factors and the 185 pRDA-identified outlier SNPs to identify the most strongly correlated variable with each SNP. All the strongest correlations were represented by only six environmental factors: tree and shrub cover (TS), tree cover, needle-leaved, deciduous (TCND), faecal coliform concentration (FC), water temperature (TEMP) and grassland cover (GL). Of these, the most common environmental factor was TS, which was the most highly correlated factor with 117 of the 185 SNPs.

As with the neutral SNP dataset, a DAPC analysis was run on the 115 SNPs identified by at least two outlier SNP analyses (Appendix S2) under the same parameters mentioned previously. Here, AIC, BIC and KIC models all grouped the dataset into four clusters ($k=4$), identifying samples in the following groups: North America, Lake Baikal, Spain and Europe without Spain (Appendix S4). We mapped the 115 SNPs to the genome of *E. muelleri* and recovered 30 different genes, of which 29 had functional annotations (Appendix S2). From the genes functionally annotated, we assessed the GO enrichment for relevant Biological Process categories (Figure 5E; Appendix S2), and found: inorganic anion transport (associated with the gene CLCN6), cellular oxidant detoxification (associated with the gene PXDN), cold-induced thermogenesis (associated with the gene OMA1), bradykinin catabolic process (associated with the gene ECE1), regulation of mitotic centrosome separation (associated with the gene KIF11) and heterochromatin organisation (associated with the gene TET1). Finally, an adaptive-enriched RDA (i.e., RDA of only the SNPs identified as being under selection) showed that SNPs corresponding with these annotated genes were most strongly associated with environmental variables representing vegetation cover (e.g., TS, SL, TCND, TCBD) and temperature (Figure 5D).

4 | Discussion

4.1 | Population Structure Driven by Neutral SNPs in *Ephydatia muelleri*

It is widely assumed that freshwater sponges with a wide distribution may be connected across their distribution thanks to a combination of biological and physical factors that include riverine connections and the hardy, cyclically produced gemmules of freshwater sponges (Manconi and Pronzato 2016a, 2016b). In fact, our globally distributed sampling sites for *E. muelleri* coincide with major avian migration routes, including the Mississippi and American Atlantic Flyways in North America, as well as the East Atlantic and Black Sea/Mediterranean Flyways in Eurasia and northern Africa (Pronzato and Manconi 1994; Maldonado and Riesgo 2009; Veen et al. 2005). However, despite the apparent presence of all the necessary tools for dispersal, our findings revealed highly structured populations, with marked genetic differentiation between populations and limited gene flow despite presumably well-connected study sites. This paradoxical genetic structure is pervasive in freshwater invertebrates (e.g., Okamura and Freeland 2002) and highly consistent with the monopolisation hypothesis (De Meester et al. 2002). The monopolisation hypothesis builds on the persistent founder effects hypothesis (Boileau et al. 1992) and predicts high genetic differentiation and limited gene flow across populations of continental aquatic species, passively dispersing cyclical parthenogens due to founder effects amplified by barriers to migration caused by local adaptation and large, resting propagule populations (De Meester et al. 2002).

The first major structure detected in our data was the division in two large, distinct clusters: one corresponding to populations in North America and the other to those in Eurasia. This differentiation is likely a result of large oceanic barriers that limit gene flow and promote local adaptation for freshwater species (e.g., Lande 1980; Rahel 2007). However, beyond the broad genetic divergence between North American and Eurasian populations, our analyses also uncovered marked genetic substructure within each continent, with some populations forming distinct genetic entities that reflect considerable genetic isolation. These findings suggest a pattern that aligns with those observed in other passively dispersed aquatic organisms, such as bryozoans, cladocerans and rotifers (Freeland et al. 2000; De Meester et al. 2002).

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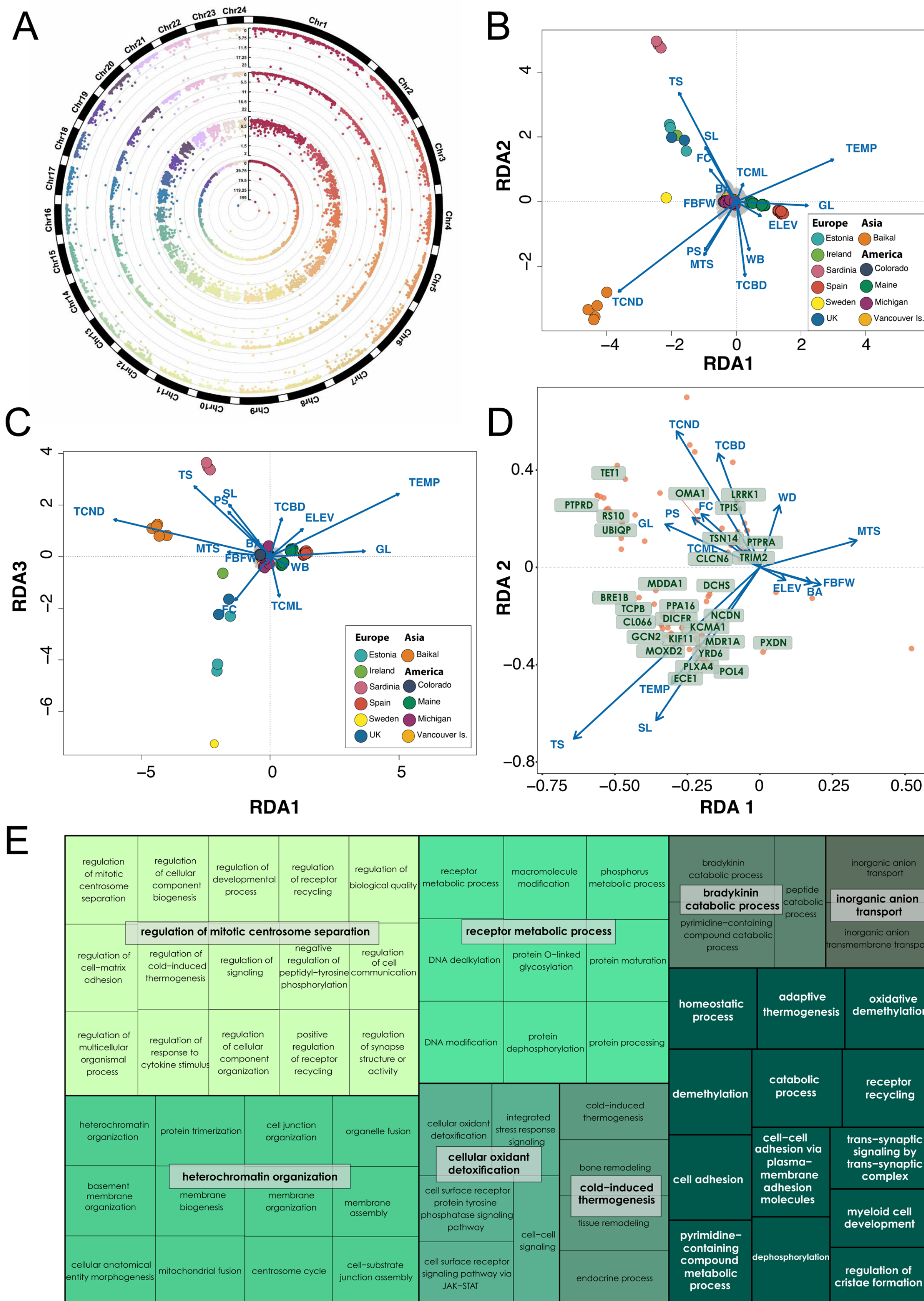

FIGURE 5 | Legend on next page.

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FIGURE 5 | (A) Manhattan plots of p-values for loci under selection derived from the analyses: LFMM (PC1), LFMM (PC2), Baypass, Arlequin (in order from the outer circle to the centre, respectively). (B, C) pRDA (axes 1,2 and 1,3, respectively) showing associations of SNP population datasets with environmental factors (Appendix S1–S5). Environmental factors are abbreviated as follows: BA (bare areas), ELEV (elevation), FBFW (tree cover flooded fresh/brackish), FC (faecal coliform concentration), GL (grassland), MTS (mean temperature seasonality), PS (precipitation seasonality), SL (shrubland), TCBD (tree cover broad-leaved deciduous), TCML (tree cover mixed leaf type), TCND (tree cover needle-leaved deciduous), TEMP (water temperature), TS (tree and shrub cover), WB (water bodies). (D) DAPC analysis of putative outlier SNPs, showing groupings for $K=4$. (E) Treemap showing the enriched Gene Ontologies assigned to the loci under selection that coincided in two of the five adaptive SNP analyses (i.e., LFMM, pRDA, Baypass, BayeScan, Arlequin).

The observed genetic structure suggests that historical colonisation events have had a more profound influence on shaping large-scale genetic patterns than contemporary dispersal mechanisms. In contrast, current dispersal routes, such as migratory bird pathways, appear to have nowadays minimal impact on genetic connectivity, at least when considering the samples we analysed. Notably, our demographic analysis revealed a bottleneck event estimated to have occurred approximately 10,000–20,000 years ago, which coincides with significant environmental shifts associated with the retreat of glaciated regions following the LGM. The impact of this bottleneck on the current population structure of *E. muelleri* is further substantiated by observations in other freshwater invertebrate species, where demographic events such as bottlenecks have been associated with a reduction in effective population size (Ne), as well as with elevated pairwise F_{ST} values and restricted gene flow in the post-bottleneck phase (Hartl and Clark 2007; Orsini et al. 2013). Our results also showed significant migration from the populations in Spain to the other European sites. This pattern suggests that Spain likely served as a glacial refuge for *E. muelleri* during the LGM, since most of the European sites studied here, with the exception of Italy, were covered by ice during this period (Figure 4A). STRUCTURE analyses further indicated the presence of a dominant genotype in Spain, with European and Baikal populations resulting from the admixture of the Spanish genotype with another distinct genetic lineage. These results align with well-established biogeographical patterns of post-glacial expansion from refugia in the Iberian, Italian and Balkan Peninsulas in Europe (Schmitt 2007; Taberlet et al. 1998), strongly indicating that modern populations in central and northern Europe emerged from two primary glacial expansion routes: one originating from the Iberian Peninsula and the other potentially from an unsampled refuge in another Southern area. Interestingly, Lake Baikal exhibited migration and admixture patterns similar to those of central European populations, suggesting colonisation from European-derived groups. While Lake Baikal was not directly covered by glaciers during the LGM, previous studies have documented the absence of sponge life due to ecological collapse in the lake during this period (Karabanov et al. 2004), thereby supporting the hypothesis that the lake was subsequently colonised by admixed European populations.

In North America, genetic analyses of *E. muelleri* revealed significantly lower F_{ST} values among populations, indicating reduced genetic differentiation and smaller genetic distances compared to those observed in Eurasia. This pattern is likely the result of the rapid northward expansion of populations from southern glacial refugia during interglacial periods, as suggested by Hewitt (2000). Moreover, the south-to-north migration pattern may help explain the genetic flow we observed for *E. muelleri* from Colorado to other areas, as well as the resulting genetic differentiation between the Colorado population and those in other regions. Our results thus suggest that the Colorado population may represent one of the principal refuges for *E. muelleri* during the LGM. We also identified a migration pattern from Michigan to O'Connor Lake, something that has already been suggested for populations of quaking aspen migrating from refugia in eastern North America towards the northwest (Ding et al. 2017). In any case, the limited number of phylogeographic studies on North American species, particularly in comparison to the more extensively studied areas of Eurasia, hinders the ability to draw definitive conclusions. This would definitively enhance regional understanding and contribute to broader insights into biogeographic patterns (Hewitt 2000).

Apart from the relatively low heterozygosity observed in the presumed founder populations of *E. muelleri* in both Colorado and Spain, they showed a notable reduction in genetic diversity and a lower number of private alleles. This finding could be explained by either historical events that caused significant reductions in population size, leading to bottlenecks that reduced heterozygosity and resulted in allele loss (Hundertmark and Van Daele 2010); or alternatively, it is possible that the original refugia in these regions have not yet been identified. Interestingly, a similar pattern has been recently observed in a study on freshwater snails, where lower genetic diversity was detected in populations from tributaries connected to the main rivers (Redak et al. 2021). In any case, further sampling in larger aquatic systems would be necessary to explore the potential presence of original refugia.

4.2 | Environmental Variation Drives Adaptation in *Ephydatia muelleri*

DAPC analysis of SNPs under selection showed groupings that were clearly distinct from the neutral population structure, instead segregating into the four groups of Lake Baikal, Spain, Europe excluding Spain and North America, indicating that adaptation is likely occurring in response to local selective pressures. Furthermore, the genome-wide distribution of selective SNPs supports the segregation of populations indicated in the neutral population structure, as the lack of adaptive islands throughout the genome is consistent with a lack of gene flow that homogenises linked, non-selective loci and leads to island-shaped adaptive signatures (Tigano and Friesen 2016). This even distribution of selective SNPs also suggests either that there are multiple adaptive traits being selected for, or that the adaptive traits are polygenic.

Our pRDA revealed that most outlier SNPs detected along the RDA axes were most strongly correlated with several

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environmental factors. Among those, the association with temperature was significant but weaker with respect to others, a surprising finding given the high variability in mean annual temperature across our sampling sites and its relevance for ice formation, which is presumably a selective process. Nonetheless, signatures of selection related to water temperature could be linked with adaptive traits at the gemmule stage, as the hatching period has previously been shown to vary according to water temperature (Benfey and Reiswig 1982). Additionally, some of the genes we identified as under selection were involved in processes related to cold-shock and cold adaptation. *oma1* (*metalloendopeptidase OMA1*) was annotated under the GO category 'cold-induced thermogenesis' (Figure 5E; Appendix S2) and plays an essential role in mitochondrial quality control for regulating lipid metabolism as well as for developing the appropriate adaptive response to different metabolic stressors such as cold-shock (Quirós et al. 2012). We also found *ece1* (*Endothelin-converting enzyme 1*) to be under selection in *E. muelleri* (Figure 5E; Appendix S2), which is a molecule involved in response to acute cold in mice and is able to regulate thermogenesis as well (Xiao et al. 2023). The selection of cold-inducible thermogenic factors in sponges is intriguing since their main role is to increase mitochondrial-based energy expenditure and heat production, largely relying on fat as a fuel source in homeothermic animals (Qiu et al. 2023; Wu et al. 2023), a process that is unknown in poikilotherms (Wu et al. 2023), but our results open up an exciting avenue for research in this topic.

Besides temperature, other environmental factors were strongly correlated to the genomic makeup of *E. muelleri*, such as tree and shrub cover, tree cover (needle-leaved, deciduous), faecal coliform concentration and grassland cover. This correlation with tree cover is most likely associated with adaptive responses to the local availability of light. Previous studies have also shown that tree canopy can substantially affect algal communities in rivers and streams by altering the amount of light that penetrates to the water (Atkinson and Cooper 2016; Halliday et al. 2016; Roberts et al. 2004). For instance, in the congeneric *E. fluviatilis*, such differences in light can have dramatic effects on the growth of sponge hosts due to changes in distribution and development of their algal symbionts (Gost et al. 2023).

Notably, we found selection signals for the *moxd2* and *upiqp* genes (Figure 5D), which have previously been demonstrated to be upregulated during uptake of symbiotic algae in a previous study of *E. muelleri* (Geraghty et al. 2021), thereby providing support for the importance of adaptation to local algal communities in these populations. Microalgal endosymbionts of *E. muelleri* can be transferred vertically from adults to gemmules through fragmentation, while horizontal acquisition of symbionts is more prevalent in sexually produced larvae (Hustus et al. 2023). Algal symbionts may play an important part in determining differences in fit to the environment between gemmules and sponges of local origin and arriving gemmules, as algal communities have previously been found to adapt to local light conditions in freshwater systems (Roberts et al. 2004). Fragmented gemmules that have inherited symbionts which were previously acquired from the local environment by the parent adult may therefore have an inherent advantage over arriving gemmules carrying symbionts derived from other localities, as is consistent with the monopolisation effect hypothesis. Given the previously established associations between the microbiomes of *E. muelleri* and its algal symbionts (Hustus et al. 2023), microbial regulation may also play a role in this dynamic and also be reflected in this adaptive genetic profile. It is therefore likely that local adaptation in this system, rather than being solely a result of sponges' response to the environment, may actually result from the coevolution of the respective components of the sponge-algae-microbiome holobiont.

4.3 | Evidence for Transgenerational Adaptive Responses to Environmental Conditions

Beyond symbiosis-associated genes, we also found signatures of selection on the *ptpra*, *ptprd* and *Dicer* genes, which have previously been found to hold a broad diversity of roles in the post-translational regulation of biological processes across different lineages (Byrum et al. 2006; Chen et al. 2017; de Jong et al. 2009; Hu et al. 2023; Iwama and Moran 2023). The *ptpra* and *ptprd* genes code for protein tyrosine phosphatase receptors, which are involved in post-translational protein modification and processes such as cell differentiation, adhesion and growth (Byrum et al. 2006; Chen et al. 2017). *Dicer* genes, on the other hand, have been extensively studied for their importance in RNA interference (RNAi), innate immunity and epigenetic inheritance (de Jong et al. 2009; Iwama and Moran 2023). Interestingly, both of these gene groups have been found to be expanded in sponges as compared to other vertebrates (Chen et al. 2017; de Jong et al. 2009).

Notably, the *Dicer* gene has been studied extensively across both vertebrate and invertebrate models for its role in the regulation of gene expression and has been demonstrated to act as a developmental switch that facilitates environmental determination of polyphenisms in development in *Caenorhabditis elegans* and in aphid model systems (Grishok et al. 2001; Srinivasan and Brisson 2012), contributing to transgenerational adaptation in these organisms. This leads us to speculate that *Dicer* may be a key epigenetic mechanism contributing to phenotypic plasticity in competence for the variable environments inhabited by *E. muelleri*. In this scenario, it is possible that *Dicer* may serve as a molecular switch in *E. muelleri*, regulating processes that respond to certain thresholds of variation. Possible candidates for such processes might include timing of gemmulation, association with symbionts, or metabolic rates, given these traits' previously described variation in sponges in response to environmental variation (Calheira et al. 2019; Carballo et al. 2006; Morley et al. 2016).

Our hypothesis is particularly compelling considering the importance of light availability for adaptive fit to the local environment in *E. muelleri*, as shown by both our results and previous work on the relationship between gemmule hatching and environmental conditions (Benfey and Reiswig 1982). In other sponges, the impact of light on gemmule hatching, larval settlement and development is speculated to result from polyphenism in development maintained through polymorphisms caused by natural selection on key regulatory loci (Degnan and Degnan 2010). Our results support this proposal, as we detected selection acting on genes demonstrated to be involved in regulation of polyphenic traits in other metazoans (i.e., *Dicer, ptpra,*

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ptprd). While epigenetics is not mentioned within the framework originally proposed by De Meester et al. (2002), it has been observed that epigenetic marks determined by environmental conditions can be passed to the next generation following asexual production in sponges and other invertebrates (Vogt 2022, 2024). Given the preliminary support for selection on genes associated with phenotypic plasticity found here, we propose that local adaptation within the monopolisation hypothesis framework may also be attributed to rapid genome evolution induced by genetic assimilation or canalisation of environmentally induced polyphenisms under diverging environmental conditions. Such polyphenism in *E. muelleri* may be associated with light and development, although further experimental research is needed to confirm this.

Author Contributions

A.R. and S.T. conceived and designed the study, and S.P.L., A.R., S.T. and A.L.H. obtained funding. Sample collection was performed by all authors. Laboratory work was carried out by K.M., C.G.-S. and A.Á.F. L.C.-C., R.C., C.G.-C., A.B.-L. and S.T. conducted the analyses. L.C.-C. and R.C. wrote the first draft of the manuscript. All authors contributed to data interpretation, manuscript revision and approved the final version.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The ddRADseq data underlying this article are publicly available in the ENA database at https://www.ebi.ac.uk/ena/ under the project identifier PRJEB91114.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1–S5:** jbi70142-sup-0001-AppendixS1-S5.zip.

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