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Phylogeny of *Arbacia* Gray, 1835 (Echinoidea) Reveals Diversification Patterns in the Atlantic and Pacific Oceans

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ABSTRACT

Aim: The aim of the current study is to conduct a comprehensive phylogenetic analysis of the genus *Arbacia* to elucidate the evolution and phylogenetic relationships among all extant species and reevaluate the presence of geographic structure within species that have wide, fragmented distributions.

Location: Specimens of *Arbacia* were collected from 34 localities spanning the Atlantic and Pacific Oceans, and the Mediterranean Sea.

Methods: We obtained sequences from three mitochondrial markers (COI, 16S and the control region and adjacent tRNAs) and two nuclear markers (28S and 18S; the latter ultimately excluded from the final analyses). Phylogenetic trees were constructed using maximum likelihood and Bayesian inference approaches. A time-calibrated phylogenetic tree was inferred using a relaxed Bayesian molecular clock and three fossil calibration points.

Results: Our analysis supports the monophyly of the genus *Arbacia*, including the species *Arbacia nigra* (previously assigned to the monotypic genus *Tetrapygus*). The new phylogenetic topology suggests an alternative biogeographic scenario of initial divergence between Atlantic and Pacific subclades occurring approximately 9 million years ago. The dispersal and subsequent diversification of the Pacific subclade to the southeast Pacific coincides with the onset of glacial and interglacial cycles in Patagonia. In the Atlantic subclade, the split between *A. punctulata* and *A. lixula* occurred 3.01–6.30 (median 3.74 million years ago), possibly associated with the strengthening of the Gulf Stream current connecting the western and eastern Atlantic. Our study also reveals significant genetic and phylogeographic structures within both Atlantic species, indicating ongoing differentiation processes between populations.

Main Conclusion: Our study provides valuable insights into the evolutionary history and biogeography of the genus *Arbacia* and highlights the complex interplay between historical climate changes and oceanic currents in shaping the distribution and diversification of echinoids in the Atlantic and Pacific Oceans.

1 | Introduction

The gradual closure of the Isthmus of Panama during the Neogene played a crucial role in re-shaping the pattern of oceanic circulation between the Atlantic and Pacific basins, contributing to important climate changes at the scale of the entire northern hemisphere (Schneider and Schmittner 2006). Terrestrial connectivity between North and South America also had major biogeographic impacts, resulting in either significant biotic interchanges or barriers to the dispersal of land and marine fauna, respectively (Schneider and Schmittner 2006; Lessios 2008; Bacon et al. 2015; O'Dea et al. 2016; Lima et al. 2020). Populations of marine organisms isolated by land elevation embarked on distinct evolutionary pathways in response to diverse intrinsic or extrinsic drivers (Lessios 1998, 2008). In particular, the closure of the Isthmus of Panama had strong regional impacts on echinoid diversity and biogeography, playing a significant role in driving speciation across diverse clades, including the genera *Eucidaris*, *Tripneustes*, *Diadema*, *Lytechinus* and *Echinometra* (Lessios 1998; Lessios et al. 1999; McCartney, Keller, and Lessios 2000; Lessios, Kessing, and Pearse 2001; Zigler and Lessios 2004; Lessios 2008, 2011).

The echinoid genus *Arbacia* comprises six extant species with highly contrasting latitudinal ranges and distribution patterns across shallow waters of the Pacific and Atlantic Oceans (Figure 1; Lessios et al. 2012). The amphi-Atlantic *Arbacia lixula* (Linné 1758) displays a broad and discontinuous distribution that includes the Brazilian coast in the West Atlantic; the Gulf of Guinea, the Azores and the eastern Atlantic Islands in the East Atlantic, extending into the Mediterranean Sea. In the western Atlantic Ocean, *Arbacia punctulata* (Lamarck 1816) also shows a wide distribution, that encompasses several biogeographic provinces, as is known from the northeastern United States (Massachusetts) to French Guiana (Mortensen 1935; Durand 1959). Three species of *Arbacia* are distributed along the Pacific coasts of North and South America: *Arbacia stellata* (Blainville 1825) is found from California to southern Peru while *Arbacia spatuligera* (Valenciennes 1846) and *Arbacia nigra* (Molina 1782) occur from northern Peru to Puerto Montt in south-central Chile. Finally, *Arbacia dufresnii* (Blainville 1825) is distributed along the Pacific and Atlantic coasts of Southern South America, spanning from Puerto Montt, Chile to Buenos Aires, Argentina, including the Falkland Islands and Tristan da Cunha in the southern Atlantic Ocean (Mortensen 1935; Lessios et al. 2012).

Metz, Gómez-Gutiérrez, and Vacquier (1998) were the first to publish a phylogeny that included *A. stellata*, *A. punctulata*, *A. dufresnii* and *A. lixula*. The study used two mitochondrial markers (cytochrome *c* oxidase subunit I, COI and ATPase subunit 6, ATPase) and one nuclear gene (*bindin*). The Neighbour Joining topology obtained showed an early split between Atlantic species (*A. lixula* and *A. punctulata*) and Pacific species (*A. dufresnii* and *A. stellata*), followed by additional speciation events occurring within each oceanic region. In addition, the level of COI divergence (average distances ranging from 9% to 13%) suggested that extant species of *Arbacia* diverged between four and eight million years ago, before the final closure of the Isthmus of Panama (estimated at 3 Ma; O'Dea et al. 2016).

Subsequently, Lessios et al. (2012) published the first extensive phylogenetic study of the genus, using partial COI and *bindin* sequences. Using *Tetrapygus niger* as the outgroup, their phylogenetic reconstruction suggested a different biogeographic history involving early speciation events in *Arbacia* along southern South America, with diversification first along the Pacific coasts and subsequently in the west and east Atlantic, where the mid-Atlantic barrier induced further isolation. Contrary to Metz, Gómez-Gutiérrez, and Vacquier (1998), Lessios et al. (2012) calibrated the split between the Pacific and Atlantic subclades to coincide with the closure of the Isthmus of Panama. The divergence times obtained indicated that the extant species of the genus *Arbacia* likely diverged relatively recently, within the last 5 myr. However, the recent taxonomic revision of the genus *Tetrapygus* by Courville, Poulin, et al. (2023) reassigned *Tetrapygus niger* to the genus *Arbacia*, as *Arbacia nigra*, which is now considered the sister species of *A. dufresnii*. This revision calls for a reassessment of the phylogeny and biogeographic scenario proposed by Lessios et al. (2012).

The aim of the current study is to conduct a comprehensive phylogenetic analysis of the genus *Arbacia* to elucidate the evolution and phylogenetic relationships among all extant species and reevaluate the presence of geographic structure within species that have wide, fragmented distributions. To achieve this, we obtained sequences from three mitochondrial markers (COI, 16S and the control region and adjacent tRNAs, hereafter termed 'CRA') and two nuclear markers (18S, 28S) from 34 populations across the entire geographical range of the clade. Our results prompt a reassessment of the biogeographic scenarios regarding the origin of the genus and help evaluate the importance of the closure of the Isthmus in its evolutionary history.

2 | Materials and Methods

2.1 | Taxon Sampling

Over two hundred specimens of *Arbacia* were collected from 34 localities spanning the Atlantic and Pacific Oceans, and the Mediterranean Sea. Fourteen of these localities were previously sampled by Lessios et al. (2012), while 20 represent new localities (see Supporting Information 1 for details). Wherever material was available, a collection of five to ten specimens was obtained. Specific sampling localities along with the total number of specimens within each are depicted in Figure 1. Specimens were identified following Kroh, Madeira, and Haring (2011) and Smith et al. (2006). Sequences were obtained from both newly collected material and specimens deposited in museum collections (Appendix S1).

Given the current assignment of *Tetrapygus niger* to *Arbacia*, two species of the expected sister group *Coelopleurus* L. Agassiz 1840, member of the Arbaciidae, were also used in the phylogeny (Mongiardino Koch et al. 2018; Smith et al. 2006): *Coelopleurus floridanus* A. Agassiz 1872 and *Coelopleurus undulatus* Mortensen 1934. The public availability of a complete mitochondrial genome for the deep-sea Arbaciidae *Pygmaeocidaris prionigera* A. Agassiz 1879 (GenBank accession number MW354512; Na, J., Cheng, H., Wang, C. and Zhang, D.), further provided opportunities to test the monophyly of *Arbacia*
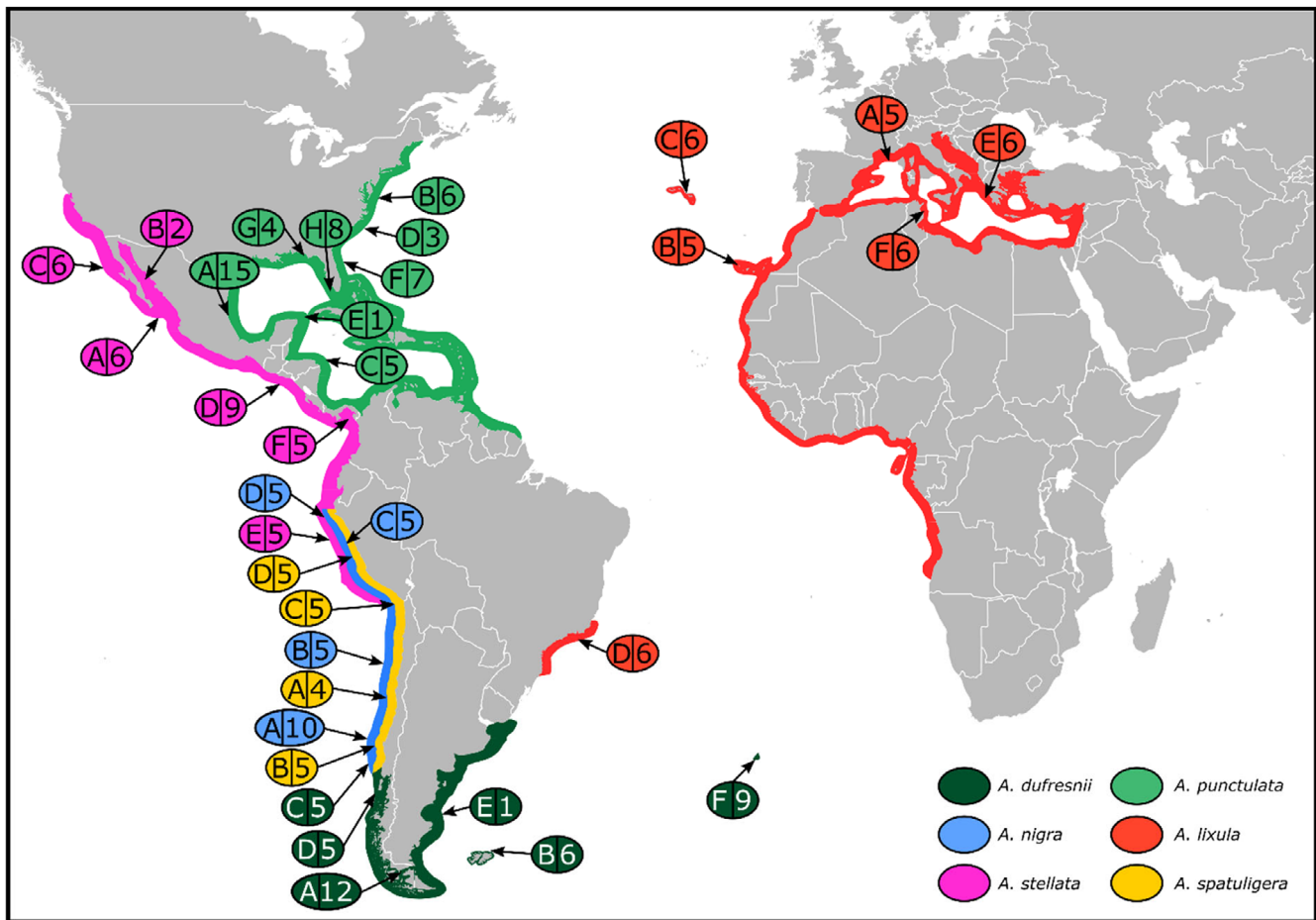
FIGURE 1 | Sampling localities of specimens used in this study. Colours indicate species, letters indicate localities and numbers represent sample sizes. Dark green: *A. dufresnii*—A, Punta Arenas, Chile; B, Falkland Islands, (Malvinas); C, Los Molinos, Chile; D, Puerto Montt, Chile; E, San Jorge Gulf, Argentina; F, Tristan da Cunha. Blue: *A. nigra*—A, Concepción, Chile; B, Playa Hippie, Chile; C, Puerto Inglés, Peru; D, Chiclayo, Peru. Pink: *A. stellata*—A, South Baja California, Mexico; B, North Gulf of California, Mexico; C, Guerrero Negro, Mexico; D, Acajutla, El Salvador; E, Pisco, Peru; F, Bay of Panama, Panama. Light green: *A. punctulata*—A, Veracruz, Mexico; B, Virginia, USA; C, Cayos Cochinos, Honduras; D, Beaufort, North Carolina, USA; E, Cancún, Mexico; F, Fort Pierce, Florida, USA; G, Panama City, Florida, USA; H, Key West, Florida, USA. Red: *A. lixula*—A, Marseille, France; B, Gran Canaria, Islas Canarias, Spain; C, Faial, Azores, Portugal; D, Rio de Janeiro, Brazil; E, Ionian Sea, Greece; F, Tunis, Tunisia. Yellow: *A. spatuligera*—A, Coquimbo, Chile; B, Arica, Chile; C, Concepción, Chile; D, Pisco, Peru.

in relation to *Coelopleurus*. The COI, 16S and CRA markers used in this study were extracted from the *P. prionigera* full mitochondrial genome.

2.2 | DNA Extraction and Sequencing

The DNA extraction, PCR and preparation for Sanger sequencing were performed at Biogéosciences Lab (SC2B molecular service), University of Burgundy, Dijon, France; LEMAS, Universidad de Magallanes, Punta Arenas, Chile; Molecular Ecology Lab, Universidad de Chile, Chile and in NAOS, Smithsonian Tropical Research Institute, Balboa, Panama. All DNA extractions performed in France and Chile were made using the DNeasy Blood & Tissue Kit by QIAGEN. In NAOS, the extraction method used in Lessios et al. (1996) was employed. All amplifications were carried out with Accustart II Taq DNA polymerase (ref. 95141–250), following recommended volumes, along with the Accustart II PCR ToughMix (ref. 95142–800) from Quantabio (Table 1 in Appendix S2). The first pool of COI

sequences was sequenced in both forward and reverse directions. The high quality of the sequences allowed us to sequence the rest of the samples using only the forward direction, an approach also taken for 16S. Sequences for CRA were obtained for both forward and reverse reads due to the repeated portion in the middle of the targeted sequence. 28S and 18S were amplified using internal primers and sequenced in forward reads. Further details on PCR procedures can be found in Appendix S2. Sanger sequencing was performed at the NAOS Lab (Panama) and MACROGEN (Korea and Chile).

Sequence editing and alignment were performed using ProSeq v2.91 (Filatov 2002). To avoid pseudogenes (Bensasson et al. 2001; Williams and Knowlton 2001), sequences with ambiguous chromatograms were discarded and the rest were translated to amino acids to check the absence of stop codons using MEGA 11 (Tamura, Stecher, and Kumar 2021). Loci were manually aligned, and several datasets were prepared for analysis, including (i) COI; (ii) 16S; (iii) CRA; (iv) concatenated COI + 16S + CRA + 28S genes. The 18S data were excluded

from the combined mtDNA and nuDNA datasets due to their insufficient resolution (see Section 3). For each marker, we calculated standard diversity indices such as the number of haplotypes (Nh), number of polymorphic sites (Np), haplotype diversity (h) and nucleotide diversity (π) using DnaSP v6.12.03 (Rozas et al. 2017). PartitionFinder2 (Lanfear et al. 2017) was used to find the best partitioning scheme. The best substitution model for each gene was determined based on the lowest Bayesian information criterion (GTR + I for COI; GTR + I + G for 16S; K80 + I + G for CRA; GTR for 28S). Model corrected (K2P; Kimura 1980) pairwise COI distances between all specimens were calculated using MEGA 11.

2.3 | Phylogenetic Reconstruction

Analyses of all datasets (i–iv) were performed using both Bayesian inference (BI) and maximum likelihood (ML) approaches. For the first of these, we used the program MrBayes v3.2.7 (Ronquist, Teslenko, et al. 2012). As MrBayes only allows a single outgroup, we selected *Pygmaeocidaris prionigera*. The program was run in 6 chains for 3×10^7 steps, sampling every 3×10^3 steps. The average standard deviation of split frequencies was < 0.01, and the potential scale reduction factor was 1.00 for all parameters. Convergence was also determined in two runs, which produced the same topology. The first 25% of the genealogies were discarded before combining the rest in a 50% majority consensus tree. Node support was estimated using posterior probabilities. ML analyses were performed using the program IQ-TREE v2.0.3 (Minh et al. 2020) and estimating support with at least 1000 replicates of ultrafast bootstrap (Hoang et al. 2018). Nodes exhibiting bootstrap support values ≥ 70% and posterior probability values ≥ 0.95 are considered well-supported.

In addition, a time-calibrated phylogenetic tree was inferred with BEAST v1.10.4 (Suchard et al. 2018) using a relaxed Bayesian molecular clock and three fossil calibration points. We employed the concatenated dataset and implemented a birth–death tree prior with default parameter values and an uncorrelated log-normal clock (Drummond et al. 2006). An exponential prior was used for the clock rate, with a mean set to the value obtained by dividing the mean tree height of the uncalibrated BI analysis (after pruning *Pygmaeocidaris*) by the mean prior age set on the divergence between *Arbacia* and *Coelopleurus* (i.e., 0.00464; Ronquist, Klopfstein, et al. 2012). Fossil calibration points were incorporated using offset log-normal priors, using hard minimum and soft maximum bounds that left 5% of prior probability beyond maxima (Yang and Rannala 2006; Ho and Phillips 2009) as follows:

1. The divergence between *Arbacia* and *Coelopleurus* must have occurred prior to the oldest fossil attributed to either genus. The oldest known fossil *Arbacia* (*A. abiquaensis*) is from the Upper Oligocene (Linder, Durham, and Orr 1988), while the oldest records of *Coelopleurus* include several species from the much older Middle Eocene Castle Hayne Limestone (Osborn, Mooi, and Ciampaglio 2016). Therefore, a hard minimum bound was placed on the divergence between these genera at the end of the Lutetian, Middle Eocene (41.2 Ma). A relatively uninformative maximum softbound was placed at the start of the Eocene

(56.0 Ma), incorporating a broad uncertainty that even allows for an origination at, or before, the Palaeocene (offset mean = 3.925, SD = 10.0).

2. The node representing the divergence between *A. punctulata* and *A. lixula* was constrained based on middle to upper Pliocene occurrences of *Arbacia improcera* (Conrad 1843). This fossil species shares numerous features with *A. punctulata*, and occurs in the same region (Kier 1972; Courville, Mooi, et al. 2023). For these reasons, it is here assumed that *A. improcera* is nested within the clade defined by *A. punctulata* and *A. lixula*, pending an explicit phylogenetic analysis. Parameters were set to establish a minimum age for the divergence between *A. punctulata* and *A. lixula* at 3.0 Ma and a soft maximum age at the base of the Pliocene that is, 5.33 Ma (offset mean = 0.728, SD = 1.0).

3. The node separating *A. stellata* from the clade of *A. spatuligera*, *A. nigra* and *A. dufresnii* was calibrated to predate the uppermost Pliocene taxa, *A. larraini* and *A. terraeignotae*, both of which were recently described from Chile (Courville, González, et al. 2023). The morphology of these two extinct species displays characters that are diagnostic of this clade of South American species (e.g., insert ocular plates; similar pattern of ambulacral and interambulacral tuberculation; Courville, González, et al. 2023). These extinct species are likely nested within the aforementioned extant clade, and we thus take the most conservative approach of using their occurrences to constrain the age of its oldest node. Parameters were set to establish a minimum age for the divergence at 2.58 Ma, and a soft maximum age at the base of the Pliocene, at 5.33 Ma (offset mean = 0.9785, SD = 1.0).

Two independent chains were run for 100 M generations, sampling every 10,000. After confirming stationarity, convergence and adequate sample sizes (ESS values > 200) had been attained using Tracer v.1.7.1 (Rambaut et al. 2018), the first 10% of generations were discarded as burn-in, and runs were combined and summarised using a maximum clade credibility tree with median heights.

2.4 | Genetic Structure

Additional analyses were conducted on COI sequences to assess the level of genetic differentiation among geographic locations (populations) when high nucleotide diversity was observed within a single taxonomic unit. This involved the calculation of pairwise Φ_{ST} and conventional F_{ST} between all groups using Arlequin v3.5.2.2 (Excoffier and Lischer 2010). The Φ_{ST} statistic was calculated based on K2P distances and 10,000 permutations were performed to test for significance. In addition, we calculated S_{NN} (nearest-neighbour statistic; Hudson 2000) among populations, with DnaSP v6.12.03 (Rozas et al. 2017). S_{NN} estimates how frequently similar pairs of sequences originate from the same population, offering enhanced statistical power compared to other tests of genetic differentiation, particularly when dealing with small sample sizes (Hudson 2000). Median-joining haplotype networks were constructed for the three mtDNA datasets (COI, 16S, CRA) using PopArt (Leigh and Bryant 2015).

3 | Results

3.1 | Genetic Diversity Indices

COI gene sequences were successfully obtained from 166 individuals encompassing the six extant species of *Arbacia* and two species of *Coelopleurus*. The truncated alignment resulted in a final length of 655 bp and 91 haplotypes.

Amplification and sequencing of other loci were equally successful across species, except for the CRA loci among specimens of *A. spatuligera*. The 18S data exhibited limited phylogenetic informativeness, showing few substitutions that were unique to individual specimens, and none shared by all conspecific specimens, unlike the mutations observed in the 28S data. As a result, the 18S data were excluded from subsequent analyses. Detailed diversity indices for all loci are provided in Tables 1–5 of Appendix S3.

3.2 | Genetic Distances

COI distances are presented in Table 1. Consistent with the findings reported by Courville, Poulin, et al. (2023), *A. dufresnii* and *A. nigra* are identified as the most closely related species, exhibiting a genetic divergence of less than 2%, and consistent with the generic reassignment of the latter to the genus *Arbacia*. The genetic distances between *A. spatuligera* and *A. dufresnii/A. nigra* are around 6%, whereas *A. stellata* displays higher pairwise values (10%–13%) when compared to other Pacific species. In the case of the Atlantic species, *A. lixula* and *A. punctulata* exhibit distances from each other (11%) comparable to those observed against Pacific species (12%). The genetic distances separating the *Arbacia* and *Coelopleurus* species are close to 22%.

Within the Pacific species of *Arbacia*, intraspecific distances are consistent, ranging from 0.03% (*A. spatuligera*) to 0.35% (*A. dufresnii*). These findings concur with prior work on *A. nigra* (Haye et al. 2014) and *A. spatuligera* (Millán et al. 2019). In contrast, Atlantic species exhibit much higher intraspecific distances, with values of 0.83% for *A. lixula* and 1.31% for *A. punctulata*.

3.3 | Phylogenetics Relationships

All methods of phylogenetic inference produced topologies congruent with each other. BI topologies are presented along with posterior probabilities and ultrafast bootstraps percentages for each node (Figure 2). Most clades are strongly supported.

The phylogeny based on the concatenation of four genes supports the reciprocal monophyly of *Arbacia* and *Coelopleurus* (Figure 2). *Arbacia* is divided into two main clades from two distinct biogeographic regions: clade I encompass species from the Pacific region (*A. nigra*, *A. dufresnii*, *A. spatuligera*, *A. stellata*), while clade II includes both Atlantic species (*A. lixula* and *A. punctulata*). All analyses consistently point to a deep split between the clades inhabiting the Atlantic and Pacific Oceans.

Along the Pacific coast of North and South America, clade I is further divided into two lineages. The first of these includes all *A. stellata* specimens, mainly inhabiting the Tropical East Pacific province (Spalding et al. 2007). The other includes the three remaining Pacific species *A. spatuligera*, *A. nigra* and *A. dufresnii*, from the Temperate Southeastern Pacific and Magellanic provinces. All species of clade I exhibit low intraspecific genetic variation (nucleotide diversity ranging from 0.00032 to 0.035). Interestingly, despite the large geographic distance separating the Malvinas and Tristan da Cunha from the South American mainland, island populations of *A. dufresnii* do not exhibit isolation from those found in Argentina and Chile.

Clade II includes the two species of *Arbacia* from the Atlantic Ocean and the Mediterranean Sea, which are consistently recognised as separate monophyletic taxa. *A. punctulata* ranges from the warm-temperate to cold-temperate provinces of the Northwest Atlantic as well as the Tropical Northwestern Atlantic provinces. The amphi-Atlantic species *A. lixula* is characterised by a disjunct distribution including the Tropical Southwestern Atlantic, the West African Transition, the Lusitanian and the Mediterranean Sea provinces. The *A. lixula* clade is split into two well-supported subclades. The first group includes all individuals from Brazil, whereas the second comprises all remaining specimens originating from the eastern Atlantic Ocean and the Mediterranean Sea. Both subclades are monophyletic and are restricted to separate biogeographic areas. The *A. punctulata* clade also exhibits internal phylogenetic structure, with well-supported monophyletic assemblages represented by all specimens from both the Caribbean Sea (Honduras) and the Gulf of Mexico situated in a large polytomy of specimens from the Atlantic coast of North America (Figure 2). The identification in some cases of robust phylogenetic subclades (such as the populations of *A. lixula* from Brazil and *A. punctulata* from Honduras) prompted a more thorough exploration of genetic

TABLE 1 | Intraspecific (diagonal) and interspecific (below diagonal) model-corrected COI genetic divergences (K2P distances) among nominal *Arbacia* species.

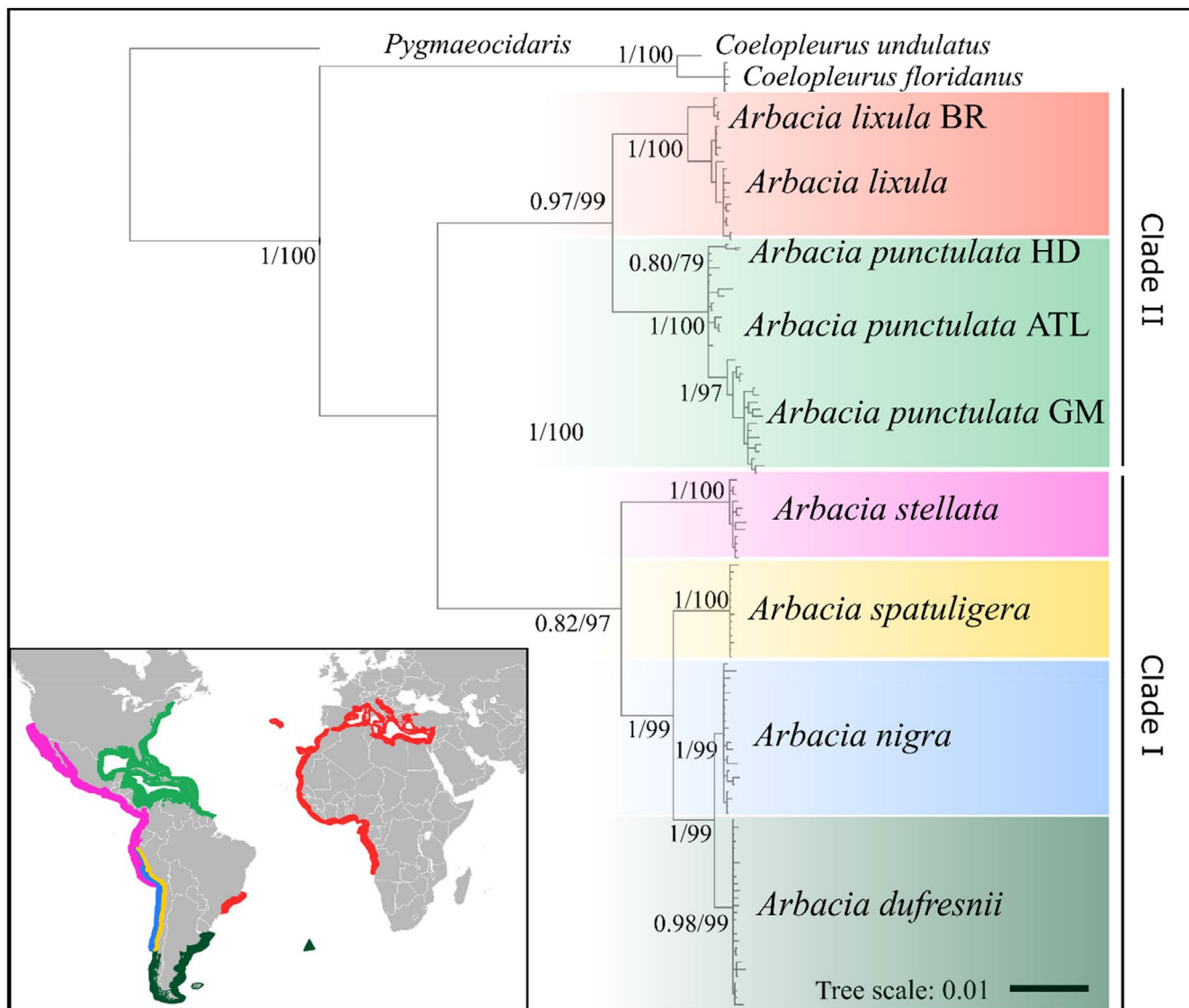|	*A. dufresnii*	*A. nigra*	*A. spatuligera*	*A. stellata*	*A. lixula*	*A. punctulata*
A. dufresnii	0.35%					
A. nigra	1.86%	0.24%				
A. spatuligera	6.52%	5.39%	0.03%			
A. stellata	9.55%	9.49%	10.4%	0.32%		
A. lixula	10.63%	10.45%	10.33%	12.72%	0.83%	
A. punctulata	12.92%	11.8%	11.17%	12.02%	11.08%	1.31%

FIGURE 2 | Phylogeny of *Arbacia* and extant species distribution based on Figure 1. Bayesian phylogenetic tree based on the concatenated dataset (COI, 16S, CRA, 28S) of 1948 bp obtained from 138 sequences generated during this study. HD = Honduras, ATL = Atlantic coast, GM = Gulf of Mexico, BR = Brazil. Branch support values are given from the BI (posterior probabilities) and ML (bootstrap percentage).

diversity, aimed at assessing the phylogeographic structure of these putatively distinctive evolutionary units.

3.4 | Divergence Time Estimation

BEAST estimated a split between *Coelopleurus* and *Arbacia* at 42.16 Ma (41.20–47.06 Ma, 95% highest posterior density [HPD interval]). The divergence between the Atlantic and Pacific clades (clades I and II of Figure 2, respectively), which corresponds to the last common ancestor of extant *Arbacia*, was estimated to have occurred at 8.88 Ma (95% HPD: 3.86–19.00; Figure 3). Despite the large uncertainty associated with dating this node, the entirety of the 95% HPD precedes the estimated final closure of the Panama Isthmus (O'Dea et al. 2016). Inferred divergence times between populations of the two Atlantic species are relatively deep and comparable to those separating established species of the Pacific clade. For example, the split between the population of *A. punctulata* from Honduras and

the remaining specimens of this species is dated to 1.59 Ma (95% HPD: 0.44–2.73 Ma), and that between *A. lixula* from Brazil and the eastern Atlantic clade to 1.40 Ma (95% HPD: 0.72–2.81 Ma). In comparison, node age for the last common ancestor of *A. nigra* and *A. dufresnii* is estimated to be only 1.01 Ma (95% HPD: 0.50–1.67 Ma).

3.5 | Intraspecific Genetic Structure

The computed K2P distances for COI reveal a mean divergence of 1.59% between specimens of *A. lixula* from Brazil and those from the East Atlantic and Mediterranean Sea. At the intra-regional level, genetic distances decrease to 0.49% in the East Atlantic and Mediterranean Sea and 0% in Brazil, where all specimens share the same haplotype (Figure 4).

Haplotype networks of *A. lixula* based on all three mitochondrial loci (Figure 4a–c), consistently reveal a similar genetic pattern,
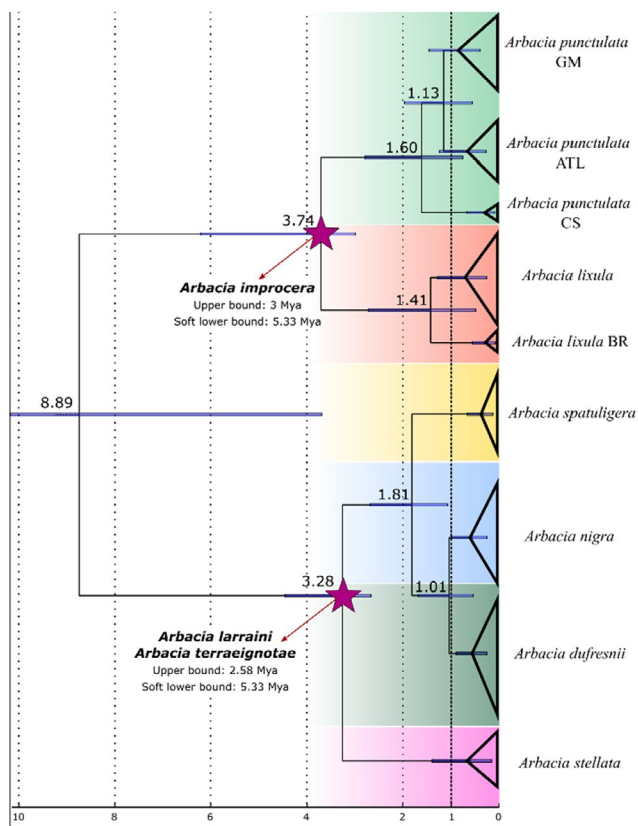
FIGURE 3 | Estimated divergence times among major clades of *Arbacia* (species and highly divergent populations) along with the fossil calibration points used in this study. Node heights are median estimates (also noted on some nodes), and blue bars denote 95% of highest-posterior density intervals (truncated for the basalmost node, see main text). The stars indicate two calibration points used. Scale bar in millions of years. Outgroups are not shown. The scale bar is in millions of years.

in which all individuals from Brazil have distinct haplotypes from those of the eastern Atlantic. In particular, the haplotype network based on 16S reveals a minimum of 11 mutations between individuals from Brazil and those from the East Atlantic. Otherwise, there is no prominent geographical structure among specimens from the Mediterranean Sea (Tunisia, Greece, France) and those from eastern Atlantic islands (Azores, Canary Islands), all of which share several haplotypes.

All Φ_{ST} and F_{ST} values for COI are highly significant between populations of Brazil and those from other populations (both Mediterranean Sea/eastern Atlantic islands; Table 2). In support of this, the S_{NN} index (Hudson 2000) is nearly 1 ($S_{NN} = 0.808$), signifying highly differentiated populations. Comparisons between populations from the Mediterranean Sea and eastern Atlantic revealed much lower Φ_{ST} and F_{ST} values. Nevertheless, some degree of genetic structure between both populations is likely given significant COI F_{ST} values (although Φ_{ST} were non-significant), but are non-significant for other genes.

In the case of *A. punctulata*, the most significant genetic difference occurs between individuals inhabiting the Caribbean Sea (Honduras) and individuals from the Gulf of Mexico, resulting in a mean K2P distance of 2.49%. Interestingly, this intra-specific

distance surpasses that between the two species *A. nigra* and *A. dufresnii* (1.86%). Genetic distances between specimens from the Atlantic Ocean and the Caribbean Sea, as well as between those from the Atlantic Ocean and the Gulf of Mexico, fall within a moderate range of 1.66% and 1.59%, respectively. Within each population, mean distances are 0.25% (Caribbean Sea), 0.53% (Atlantic Ocean) and 0.74% (Gulf of Mexico).

The haplotype networks based on the three mitochondrial markers (Figure 4d–f) consistently reveal a similar genetic pattern, with all individuals from Honduras forming a distinct and separate haplogroup. Individuals from the Gulf of Mexico and the North American Atlantic coast also exhibit a clear geographic structure, yet do not form separate haplogroups that are specific to their respective geographical regions (Figure 4).

All F_{ST} and Φ_{ST} values for COI between populations are significant (Table 3). The S_{NN} index (Hudson 2000) is also nearly 1 ($S_{NN} = 0.905$).

4 | Discussion

Our phylogenetic results support the monophyly of the genus *Arbacia* including *A. nigra* (formerly *Tetrapygus niger*) and suggest that the genus *Coelopleurus* is a credible extant sister-group of *Arbacia*. Within the Arbaciidae family, only three genera inhabit littoral to mesophotic environments: *Arbacia*, *Coelopleurus* and the monospecific genus *Arbaciella* (Mortensen 1910). The validity of the genus name *Arbaciella* has been questioned in the literature, this monotypic genus being considered as either a juvenile form of *Arbacia lixula* (López et al. 2013) or a juvenile of the African variety of *Arbacia lixula* (Kroh, Madeira, and Haring 2011). Other extant members of the Arbaciidae are abyssal and morphologically very distinct from *Coelopleurus* and *Arbacia*. This suggests that the two genera are likely deeply divergent sister clades, which harbour genetic distances close to 22%, and have diverged during the mid-Eocene. In contrast, genetic distances within the genus *Arbacia* span between 1.86% and 13%, in agreement with intrageneric values measured across Echinoidea (Ward, Holmes, and O'Hara 2008).

Our new phylogeny supports the existence of two main clades within *Arbacia* that correspond with the Atlantic and Pacific. Our new rooting with *Coelopleurus* contrasts with the most recent biogeographic hypothesis for the genus (Lessios et al. 2012), which had used *Tetrapygus* as an outgroup. The revision of the taxonomic status of *A. nigra* (Courville, Poulin, et al. 2023), and its recognition as a species nested within the genus *Arbacia*, was necessary to reveal an initial split between Atlantic and Pacific taxa, a biogeographic scenario that aligns more closely with prevalent patterns observed in other genera that occur on both sides of the Isthmus of Panama (Collins and Coates 1999; Budd 2000; Allmon 2001). Estimated divergence times between these two clades imply that the initial divergence between Atlantic and Pacific species of *Arbacia* occurred approximately 9 Ma (95% HPD: 3.86–19 Ma), earlier than estimates for the closure of the Isthmus of Panama (approx. 3 Ma). Interestingly, a previous study by Metz, Gómez-Gutiérrez, and Vacquier (1998) had arrived at similar biogeographic and evolutionary conclusions, despite a much smaller taxonomic and molecular sampling.
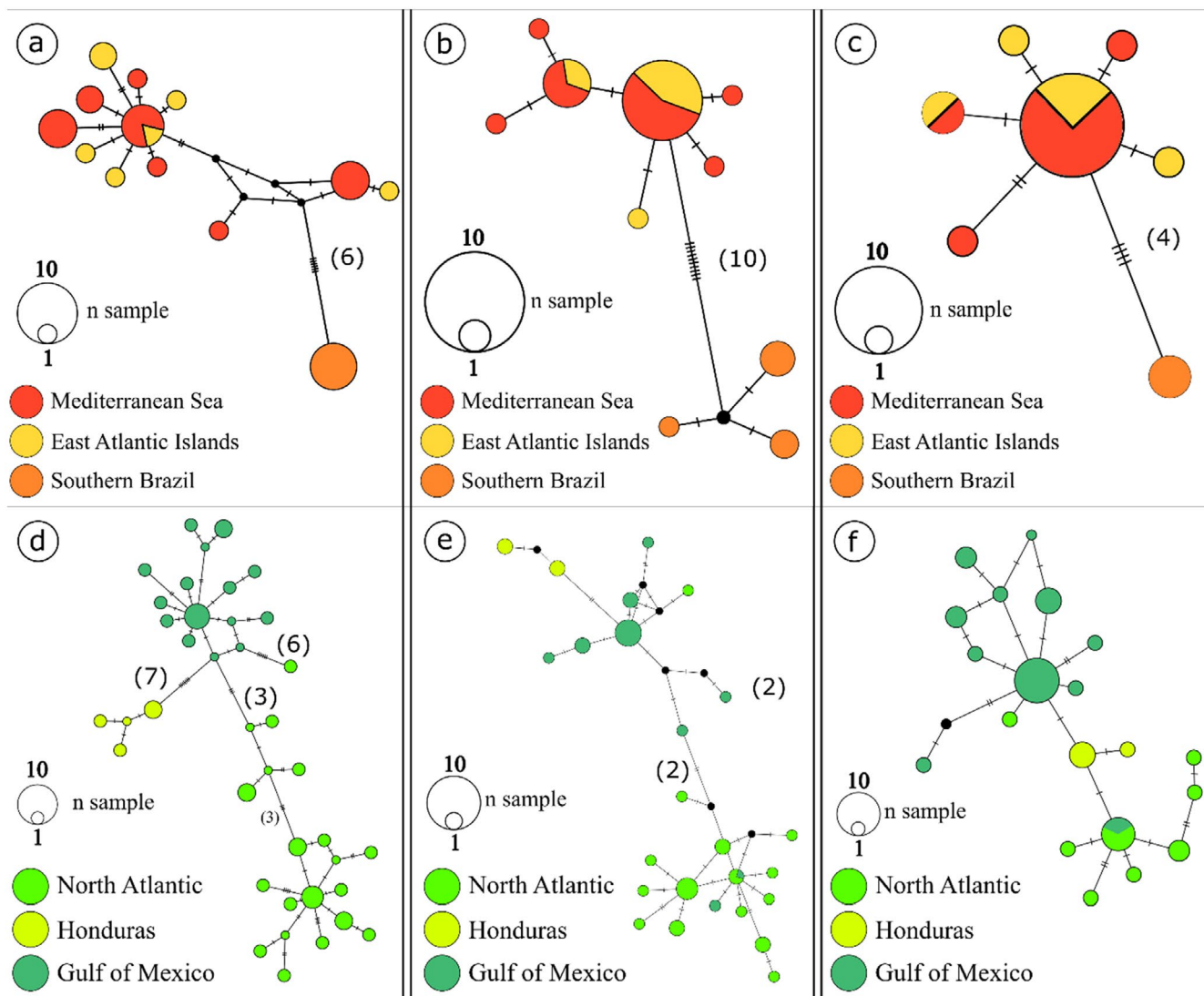
FIGURE 4 | (a–c), Median-joining haplotype network of mitochondrial sequences of *Arbacia lixula*. (a) COI haplotype network based on 30 sequences (655 bp); (b) 16S haplotype network based on 32 sequences (540 bp); (c) CRA haplotype network based on 20 sequences (242 bp). (d–f), Median-joining haplotype network of mitochondrial sequences of *Arbacia punctulata*. (d) COI haplotype network based on 39 sequences (655 bp); (e) 16S haplotype network based on 39 sequences (540 bp); (f) CRA haplotype network based on 38 sequences (242 bp). Colour code as in Figure 1. Number of haplotypes is indicated by size of the circular symbol; bars show a number of substitutions between nodes; small black circles indicate hypothetical haplotypes.

TABLE 2 | Pairwise values of Φ_{ST} and conventional F_{ST} based on COI among *Arbacia lixula* populations.

Populations of *Arbacia lixula*	Mediterranean Sea		Eastern Atlantic Islands	
	Φ_{ST}	F_{ST}	Φ_{ST}	F_{ST}
Eastern Atlantic	0.062	0.123*		
Brazil	0.780*	0.480*	0.829*	0.476*

*Significant ($p < 0.05$).

Based on mitochondrial divergence between sister species on either side of the isthmus, some studies suggest divergences as old as 23 Ma (Bacon et al. 2015). Potential biases such as incomplete taxonomic sampling may have led to significant overestimates (Marko, Eytan, and Knowlton 2015; Lessios 2015). Based on over 100 marine species, Lessios (2008) suggested a divergence time between 1 and 5 Ma for 62 species, and between 6 and 10 Ma for 29 species, which is in line with our results for *Arbacia*. The emergence of the Isthmus of Panama was a protracted process that likely started in the late Miocene (i.e., as early as 13 Ma; Bacon et al. 2015; Lessios 2008). The initial divergence between Pacific and Atlantic clades of *Arbacia* was likely a consequence of poorly understood oceanographic changes and biogeographic barriers that commenced before the complete

TABLE 3 | Pairwise values of Φ_{ST} and conventional F_{ST} based on COI among *Arbacia punctulata* populations.

Populations *of Arbacia punctulata*	Gulf of Mexico		North Atlantic	
	Φ_{ST}	F_{ST}	Φ_{ST}	F_{ST}
North Atlantic	0.659*	0.038*		
Honduras	0.762*	0.082*	0.760*	0.091*

*Significant ($p < 0.05$).

uplift of the Isthmus of Panama (Collins et al. 1996; Leigh, O'Dea, and Vermeij 2014; O'Dea et al. 2016; Huguet, Jaeschke, and Rethemeyer 2022).

Future work based on a broader sampling of individuals and populations, as well as incorporating a larger number of genetic markers, will likely improve upon our estimates of phylogeny, population structure and divergence. Nonetheless, hereafter we present a biogeographic interpretation of our results that we hope will serve as a working hypothesis and guide future research. In the Pacific clade, an initial divergence occurred around 3.28 Ma (95% HPD: 2.67–4.50 Ma) between the tropical species *A. stellata* and the remaining southeast Pacific group. This aligns with a biogeographic pattern well-known among extant temperate East Pacific marine faunas. Subsequent diversification in more southern regions has been documented for gastropods, foraminifera and fish during the late Miocene to Pliocene (Landini et al. 2002; Naughton et al. 2014; Bean and Vermeij 2016; González-Wevar et al. 2018; Vermeij et al. 2019). According to Vermeij et al. (2019), the observed pattern of a North Pacific center of origin and subsequent dispersal across the tropics towards the south could have been facilitated by either cool water 'stepping stones' in the tropics, upwelling areas or long-distance dispersal. We hypothesize that the complex and multi-phased establishment of the Isthmus of Panama as a biogeographic barrier—between Pacific and Atlantic Oceans—may have also influenced north–south connectivity of shallow-water organisms through multiple dispersal–vicariance events, as suggested by studies on the Gibraltar Strait (Mendes et al. 2017). Successive narrowing and widening between North and South American shallow waters likely facilitated repeated dispersals to the south, with subsequent re-openings triggering population differentiation between the two subcontinents.

The southeast Pacific group of *Arbacia* diversified in colder temperate waters along the Pacific coasts of South America. Divergence of the lineage of *A. spatuligera* from the clade of *A. dufresnii* and *A. nigra* can be dated to about 1.81 Ma (95% HPD: 1.04–2.69 Ma), followed by the later split between *A. dufresnii* and *A. nigra* at 1.01 Ma (95% HPD: 0.49–1.67 Ma). These recent events could be associated with the onset and intensification of recurrent glacial and inter-glacial cycles that are reported to have shaped marine biodiversity in Patagonia (Fernandez Iriarte et al. 2020). Glacial and inter-glacial cycles intensified during the Pleistocene (Rabassa 2008; Clague et al. 2020), with ice caps and glaciers covering the Pacific margin of the Magellan province, from Chiloe Island (42° S) to Cape Horn (56° S) to varying degrees during periods of glacial maxima (Fernandez Iriarte et al. 2020). This is expected to have strongly affected near-shore sheltered and hard-substrate habitats in the Pacific Magellanic region. The habitats would have become greatly diminished or even eliminated, accordingly reducing the persistence of numerous near-shore marine species. These multiple glacial cycles were associated with significant eustatic variations that strongly limited the extension of continental shelves, particularly along the Pacific South American coast (Fraser et al. 2012; Fernandez Iriarte et al. 2020), contributing to habitat fragmentation, restricting gene flow among populations and promoting speciation. These repeated habitat contractions and expansions of marine organisms could have greatly influenced the distribution and evolutionary dynamics of populations of *Arbacia* in the late Pliocene—Pleistocene. *A. dufresnii* is known from greater depths, up to 300 m in Patagonia (Mortensen 1910). The species occurrence at depth, along with larval stages that have high dispersal capacity (Gianguzza 2020), may have significantly mitigated the impact of habitat fragmentation during late Pleistocene glacial cycles, with successive recolonization events from refugia resulting in limited intraspecific genetic divergence. This contrasts with what is known in the gastropod *Siphonaria lessonii*, a strictly littoral benthic species of Patagonia, for which glacial cycles led to persistent phylogeographic structuring (Fernandez Iriarte et al. 2020).

Within the Atlantic clade, a split estimated to be at 3.74 Ma (95% HPD: 3.01–6.30 Ma) occurred between the lineages of *A. punctulata* and *A. lixula*. In both Atlantic species, there are signs of ongoing diversification, as evidenced by marked phylogeographic structuring among populations. In the early Pliocene (during the Zanclean, 4.6–4.2 Ma), a significant acceleration of the Gulf Stream and North Atlantic Current has been posited (Haug and Tiedemann 1998; Auderset et al. 2019) in relation to the formation of the Isthmus of Panama. This could have increased connectivity between the western and eastern Atlantic Ocean, facilitating the eastward dispersal of *Arbacia* larvae and their settlement in the Azores, Canary Islands and along the African coasts, in advance of much more recent colonisation of the Mediterranean Sea (Wangensteen et al. 2012). Finally, dispersal towards the southern coast of Brazil could have occurred later, given that Brazilian haplotypes are believed to derive from eastern Atlantic populations (Wangensteen et al. 2012). The dispersal towards the southern coast of Brazil could have been promoted by transoceanic dispersal between the Gulf of Guinea, and Brazil in the Southern South Equatorial Current (Joyeux et al. 2001; Pinho et al. 2021). However, our results do not provide strong support for this idea, given our findings concerning tree topology and haplotype characterisation.

In *A. punctulata*, previous studies (Metz, Gómez-Gutiérrez, and Vacquier 1998; Lessios et al. 2012) suggested geographical structuring among populations from the Gulf of Mexico and Woods Hole (Massachusetts, USA). A phylogenetic study performed by Lessios et al. (2012) also highlighted

geographic structuring between samples from the Caribbean Sea (Honduras and Belize) and those from the Atlantic coast of Florida and North Carolina. In the present work, COI sequences of *A. punctulata* display substantial genetic differences among geographical populations and a phylogeographic structuring into three subclades. The three subclades are not found in the two other, slower evolving markers (16S, CRA), suggesting a recent diversification process. This pattern is expected, as certain genes reflect separation more quickly during divergence, supporting the presence of recent subclades. The monophyly of the Caribbean subclade is well-supported, while the subclades from the North Atlantic (NA) and the Gulf of Mexico (GM) share several 16S and CRA haplotypes. Interestingly, similar patterns were encountered in various phylogeographic studies of marine invertebrates over the last decades (Young et al. 2002; Lee and Foighil 2004, 2005; Mathews 2006; Dennis and Hellberg 2010). For example, the distribution of the mussel *Brachidontes exustus* (Linné 1758) is very similar to the one of *A. punctulata*, with populations present along the North American Atlantic coast, the Gulf of Mexico and the Caribbean Sea (Lee and Foighil 2004, 2005). Using COI, 28S and ITS markers, Lee and Foighil (2005) demonstrated that lineages from the Gulf of Mexico and the Atlantic formed well-supported sister groups and were genetically distinct from populations in the Caribbean Sea. The peninsula of Florida has often been considered a geographical barrier to gene flow between the Gulf of Mexico and the North Atlantic coast (Hedgpeth 1953; Marcus 1977), with a well-established phylogeographic break in planktonically dispersed coastal species at Cape Canaveral (Avise 1994; Pelc, Warner, and Gaines 2009). In addition, morphological variation within *A. punctulata* has been well-documented since the early 20th century (Jackson 1912, 1927; Clark 1923; Mortensen 1935). This suggests morphological and ecological differentiation, in addition to the genetic distances observed here, and prompts further investigation into the phylogeography and detailed morphology of *A. punctulata*.

In *A. lixula*, the structure of haplotype networks based on mitochondrial markers as well as genetic and differentiation indices, suggest recent divergence between populations from Brazil and those from Eastern Atlantic Islands and the Mediterranean Sea. These distinct subclades diverged approximately 1.40 Ma (95% HPD: 0.45–2.73 Ma), comparable, and potentially even older than the split between pairs of currently recognised species of the genus, such as *A. nigra* and *A. dufresnii*. As a result, we here regard the population from the southwestern Atlantic coast of Brazil as a separate evolutionary unit. The disjunct distribution of *A. lixula*, its absence from deep environments, and the time it would require the larvae to cross the Atlantic on the prevailing currents have contributed to the geographic and genetic isolation of the Brazilian population. As proposed by Wangensteen et al. (2012) and Pérez-Portela et al. (2019), a broader sample that includes specimens from the African coast is needed for a better understanding of the phylogeographic structure and demographic processes at play, as well as to determine whether this evolutionary unit should be recognised as a distinct species.

The study by Lessios et al. (2012) highlighted an unusual biogeographic pattern in *Arbacia* contrasting with the common trend where marine species typically originate in the tropics and then diversified into temperate regions. However, following the taxonomic reassignment and phylogenetic position of the species *Tetrapygus niger* to the genus *Arbacia* by Courville, Poulin, et al. (2023), the genus *Arbacia* appears to revert to a more conventional biogeographic pattern. Although increasing the number of studied specimens and sequenced genes would provide deeper insights into more recent events such as the phylogeographic structure within *A. lixula* and *A. punctulata*, as well as improve divergence time estimates among species, this study indicates that the diversification of the genus *Arbacia* has been primarily, and originally conditioned by the closure of the Isthmus of Panama, while preceding its final closure; a hypothesis originally proposed by Metz, Gómez-Gutiérrez, and Vacquier (1998). These findings highlight the need for accurate taxonomic and phylogenetic frameworks for the inference of biogeographic events.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in Zenodo at https://doi.org/10.5281/zenodo.13830674, and all the sequence data have been submitted to the GenBank databases under accession number PQ505255-PQ505419, PQ507187-PQ507452, and PQ513224-PQ513359.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.