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Complete Mitochondrial Genomes Recovered From Environmental Metagenomics Samples Can Facilitate Non-Intrusive Population Genetic/Genomics Studies in the South American Sea Lion *Otaria byronia*

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ABSTRACT

The abundance of many marine mammals is declining due to local, regional, and global climate stressors that characterize the Anthropocene. Long-term monitoring is crucial for understanding how these declining populations respond to further environmental stress, and developing non-invasive genetic sampling strategies is needed to guide their recovery effectively. The aim of this study was to test if complete mitochondrial genomes can be assembled from environmental DNA (eDNA) metagenomics scat samples taken non-invasively using the South American Sea Lion *Otaria byronia* as a model and examine if the retrieved mitochondrial genomes can facilitate non-intrusive population genetic studies. Complete mitochondrial genomes of *O. byronia* were assembled from each of a total of 30 eDNA samples with coverages greater than 40× using a "target-restricted-assembly" bioinformatics strategy. The AT-rich mitochondrial genomes contained 13 protein coding genes, 22 transfer RNA genes, two ribosomal RNA genes, and a control region. Mitochondrial gene order in *O. byronia* was identical to that reported for all other cofamilial species. An Analysis of Molecular Variance and pairwise Φ_{ST} tests using a 228 bp fragment of the CR demonstrated statistically significant genetic dissimilarity among the sampled population and others in the Pacific and Atlantic basin. This study demonstrates that complete mitochondrial genomes can be assembled from eDNA metagenomics scat samples, with which insights into metapopulation genetics can be achieved. Mitochondrial genomes assembled from eDNA metagenomics scat samples can support non-disruptive biomonitoring of this iconic marine mammal across its distribution.

1 | Introduction

Marine mammals play a crucial role in the functioning of natural ecosystems (Bossart 2011), yet their abundance, especially that of large-bodied species, has declined during the last decades due to local, regional, and global stressors characteristic of the Anthropocene (Luypaert et al. 2020; Pimiento et al. 2020). One of the most important but challenging goals in conservation biology is to restore the natural abundance of these and other mammals in ecosystems that are often altered due to the direct or indirect impact of human-related activities (Seddon et al. 2016; Lotze 2021). Long-term monitoring is of utmost relevance for understanding the response of declining populations to pervasive environmental insult, and

subsequently, for the efficient development and implementation of conservation measures. Unfortunately, in situ wildlife monitoring strategies and the collection of biological samples for genetic and genomic research are often difficult to implement due to logistical challenges and ethical concerns, particularly in threatened species that have become elusive and have short- and/or long-term predicted decaying population trajectories (Bradshaw et al. 2020). Such limitations impact conservation efforts, and traditional in situ and "invasive" bioprospecting and biomonitoring strategies that can disturb and/or stress individuals and populations that are already facing major anthropogenic impact (Bradshaw et al. 2020) might need to be replaced by other non-invasive strategies, if possible.

During the last decade, environmental DNA (eDNA) has materialized as a reliable substitute or complement to invasive and/or intrusive sampling strategies for the biomonitoring of imperiled species both in marine and non-marine (terrestrial and freshwater) environments (Beng and Corlett 2020; Didaskalou et al. 2022). The term "eDNA" refers to the DNA found in environmental samples including air, water, sediment, and feces, among a few others (Li 2023), and eDNA research has already significantly improved our ability to identify and track the presence, distribution, and abundance of numerous species without the need for direct sampling and/or observation (Ficetola et al. 2019; Parsons et al. 2018; Rourke et al. 2022). eDNA can be particularly useful in situations in which invasive sampling is logistically problematic (i.e., in rare or highly aggressive [predator] species) and/or when it collides with ethical concerns (i.e., in at-risk-of-extinction species with only a few surviving individuals) (Beng and Corlett 2020; Didaskalou et al. 2022).

Importantly, eDNA retrieved from fecal material has been increasingly used for bioprospecting of endangered species without requiring direct physical contact or inducing mortality in the target organisms (Baeza et al. 2023; Beng and Corlett 2020; Foote et al. 2012; Parsons et al. 2018), and recent studies have demonstrated that complete and nearly complete mitochondrial genomes, as well as full or almost complete bacterial genomes, can be bioinformatically recovered from fecal matter-isolated genomic DNA (Cappozzo and Perrin 2009; Cárdenas-Alayza 2018; Heckel and Schramm 2021). To the best of our knowledge, no studies have yet leveraged the retrieval of complete mitochondrial genomes for examining connectivity among closely or distantly located populations.

In this study, we explored if eDNA in the form of scat samples can be used to retrieve, bioinformatically, complete mitochondrial genomes that can be used then for population genetics/genomics studies. We have focused on the retrieval from eDNA scat samples of complete mitochondrial genomes as these molecules are speedily becoming markers of choice for examining phylogenetic relationships at multiple taxonomic levels in both marine and terrestrial taxa (Chak et al. 2020; Veldsman et al. 2020; Chak et al. 2021; Ennis et al. 2021; Gutiérrez et al. 2023; Baeza et al. 2024, among many others) and have chosen as a model species an iconic marine mammal from the southeastern Pacific Ocean currently impacted by direct and indirect anthropogenic environmental stressors, the South American sea lion *Otaria byriona*.

Otaria byronia is a monotypic species distributed across the Atlantic and Pacific coasts of South America, from Peru to southern Brazil (Cappozzo and Perrin 2009), however, a few sporadic records of this species exist for Ecuador and Colombia (Reeves et al. 2002). *Otaria byronia* is also present in the Islas Malvinas/Falkland Islands (Cárdenas-Alayza 2018). This pinniped exhibits considerable sexual dimorphism; females typically reach a total length of up to 2 m and weigh approx. 150 kg, while males can grow up to 3 m in total length and weigh up to 350 kg (Cárdenas-Alayza 2018). These large-bodied mammals breed from mid-December to early February, with timing differences observed according to location and latitude (Cárdenas-Alayza et al. 2016; Heckel and Schramm 2021). Their diet primarily consists of pelagic, demersal, and benthic species of fish, cephalopods, and crustaceans, with dietary preferences differing between the sexes and changing according to prey availability (Cárdenas-Alayza 2018; Mondragón-Martiínez et al. 2021). The global population of *O. byronia* is estimated at approximately 425,000 individuals based on the 2015 International Union for the Conservation of Nature (IUCN) assessment (Cárdenas-Alayza et al. 2016). Sea lions are currently impacted by human activities, including fisheries, aquaculture, and tourism (Sepúlveda et al. 2007). For instance, sea lions have been shown to be highly vulnerable to incidental entanglement in fishing gear or to experience collisions, often fatal, with vessels (Heckel and Schramm 2021). Conflict between sea lions and commercial fishing operations also occurs (Foote et al. 2012), exemplifying the complex dynamics at the interface of marine mammal conservation and fisheries management, highlighting the multifaceted challenges in maintaining marine biodiversity (Borja et al. 2016).

In *O. byronia*, the first genetic studies based on the mitochondrial protein coding gene (PCG) *cob* and the mitochondrial control region (CR) plus nuclear microsatellites revealed that Atlantic and Pacific populations were genetically dissimilar, suggesting prolonged isolation among populations inhabiting different ocean basins and the presence of two distinct Evolutionarily Significant Units (ESUs), one on each side of the South American subcontinent (Pont et al. 2016; Túnez et al. 2007). Furthermore, along the Atlantic coast, the genetic data suggested two distinct breeding clusters of *O. byronia*: in Uruguay and Patagonia (Túnez et al. 2007). Interestingly, in disagreement with earlier studies, more recent analyses that have included a larger number of populations have confirmed the existence of gene flow between the Pacific and Atlantic basins in *O. byronia*, suggesting that the species constitutes a single ESU across its range of distribution. The same study suggested the existence of five Conservation Units in *O. byronia* (Peralta et al. 2021). Due to the multiple threats that *O. byronia* faces across its distribution, it is critical to advance non-invasive genomic resources and strategies for monitoring their populations and guiding conservation and management initiatives for this iconic species in South America. This study assesses the feasibility of assembling complete mitochondrial genomes from eDNA metagenomic samples obtained from fecal matter and examines if the retrieved mitochondrial genomes can facilitate non-intrusive population genetic studies. If successful, this genomic resource could facilitate population genetic/genomics studies in this iconic marine mammal.

Additionally, it could enable biomonitoring and bioprospecting efforts by leveraging cost-effective molecular markers and streamlined bioinformatics workflows.

2 | Material and Methods

2.1 | Sampling of Fecal Samples and Sequencing of eDNA Metagenomic Datasets

Fresh (soft) fecal samples ($n = 30$) were collected from off the ground in a rocky outcrop that served as a rookery for *O. byronia* in the Comau Fjord (42°S), Chile, during the year 2019. We made every possible effort to collect samples from different sea lions each time. However, due to sampling time constraints and other logistical issues (rough seas, aggressive animals in the rocky outcrop), we cannot discard that two or more scats were produced by the same individual. Immediately after collection, samples were frozen in an ice chest for transportation to the dry laboratory, where a 250 mg aliquot was taken from each fecal sample using a sterile spatula. This step was conducted under biosafety level 2 (BSL2) biohood. Next, gDNA was extracted from each aliquot using the Qiagen PowerFecal DNA extraction kit following the developer's instructions. Shotgun genomic libraries were prepared with the Hyper Library construction kit from Kapa Biosystems (Roche) and sequenced on an Illumina NovaSeq 6000 platform (150 bp paired-end [PE] reads). Between 10,580,467 and 56,688,820 short reads were generated per sample, and the totality of the reads per sample was used for mitochondrial genome assembly in each sample. We note that in a previous study, we have used this data to explore the gut metagenome in *O. byronia* (Guajardo-Leiva et al. 2023).

2.2 | Assembly of Mitochondrial Genomes From eDNA Metagenomic Datasets

We attempted to assemble mitochondrial genomes from scat samples of *O. byronia* following the protocol detailed in Baeza et al. (2023). In short, we used the program GetOrganelle v1.2.3 (Kim et al. 2021) that employs a "target-restricted-assembly" strategy and a "seed-and-extend" algorithm to assemble mitochondrial genomes from the different samples. We used as a "seed" sequence the mitochondrial genome of the same species available in GenBank (MK654904—Kim et al. 2021). All the different runs, for a total of 30 metagenomics datasets each belonging to a different sample (total $n = 30$), used k-mer sizes of 21, 55, 85, and 115. Reads were not quality trimmed before the assembly step following the developer's suggestions (Kim et al. 2021). Next, the software Bandage (Tian et al. 2021) was used to visualize the assembly graph produced by GetOrganelle. Finally, as in Baeza (2022), assembled contigs (if any) were compared to the nucleotide non-redundant database in NCBI's GenBank to determine if the aforementioned contigs matched the mitochondrial genome of the target species available in GenBank. If the strategy above was successful in assembling the complete mitochondrial genome of the target species from eDNA metagenomic datasets, then we predicted that a circular sequence ~17,000–19,000 bp long would be observable in the program Bandage and that the same sequence would be a close match to the mitochondrial genome of *O. byronia* available in GenBank.

2.3 | Annotation of Assembled Mitochondrial Genomes

The newly assembled mitochondrial genomes were first annotated *in silico* using the pipeline MITOS2 (Donath et al. 2019) as implemented in the Galaxy platform (Jalili et al. 2020), using the vertebrate genetic code (code 2). Nucleotide composition of each assembled mitochondrial genome plus other 12 mitochondrial genomes from other species belonging to the family Otariidae available in NCBI's GenBank was calculated using the software MEGA X (Kumar et al. 2018). The 12 additional mitochondrial genomes were all the sequences available in GenBank that belonged to other species in the family Otariidae other than *O. byriona* and permitted us to compare our results with those from other confamilial species. Codon usage profiles of all concatenated PCGs in each new mitochondrial genome were analyzed using the vertebrate mitochondrial code in the web server Codon Usage (https://www.bioinformatics.org/sms2/codon_usage.html—Stothard 2000) and visualized with the web server heatmapper (https://heatmapper.ca/—Babicki et al. 2016). Relative Synonymous Codon Usage (RSCU) was also examined using the tool EZcodon in the web server EZMito (Cucini et al. 2021; Lee 2018).

We examined selective pressure in selected mitochondrial PCGs. For this purpose, we used three relatively dissimilar newly assembled mitochondrial genomes: samples SRR14857231, SRR14857244, and SRR14857254. We note that we named newly assembled mitochondrial genomes depending upon the GenBank accession code for the raw reads. The PCG from samples SRR14857231, SRR14857244, and SRR14857254 was aligned with orthologous sequences from three different confamilial species. Specifically, "outgroups" used during the analysis of selective pressure for sample SRR14857231 were *Zalophus japonicus* (NC_058016), *Arctocephalus pusillus* (NC_008417), and *Phocarctos hookeri* (NC_008418). Outgroups for specimen SRR14857244 included *Zalophus wollebaeki* (NC_062331), *Arctocephalus townsendi* (NC_008420), and *Neophoca cinerea* (NC_008419). Lastly, sample SRR14857254 was compared to *Zalophus californianus* (NC_008416), *Arctocephalus australis* (NC_063561), and *Arctocephalus forsteri* (NC_004023). Next, we used the software KaKs_calculator v. 2.0 (Wang et al. 2010) with the γ-MYN model to estimate Ka, Ks, and Ka/Ks values. When $Ka/Ks = 1$, < 1, and > 1, then PCGs are exposed to neutral, purifying (negative), or diversifying (positive) selection (Wang et al. 2010), respectively. We have used a set of different outgroups that represent species less and more closely related to our focus species to detect any effect of phylogenetic relatedness in our selective pressure Ka/Ks estimates.

Transfer RNA (tRNA) genes were detected, and their secondary structure was predicted in each studied mitochondrial genome using the software MITFI (Jühling et al. 2012), as implemented in MITOS2. Visualization of each tRNA secondary structure in each studied mitochondrial genome was conducted in the web server Forna (Kerpedjiev et al. 2015).

Lastly, the CR of each mitochondrial genome was examined in detail. First, we used the web server microsatellite repeat finder (https://insilico.ehu.es/mini_tools/microsatellites/—Bikandi et al. 2004) to detect microsatellites in this non-coding region. Then, the web server Tandem Repeat Finder v. 4.09.1 (https://tandem.bu.edu/trf/trf.html—Benson 1999) was used to detect short tandem repeats. Lastly, secondary structure predictions of the CR in each studied mitochondrial genome were modeled using the RNA structure Web Server tool v. 6.4 (https://rna.urmc.rochester.edu/RNAstructureWeb/—Bellaousov et al. 2013, 2018) with default options. Conserved elements in each analyzed CR were also identified after aligning each newly assembled mitochondrial genome to that of *Arctocephalus australis* (available in GenBank, MK616240) using the program ClustalW (Thompson et al. 1994) as implemented in the software MEGA X.

2.4 | Population Genetics of *Otaria byronia*

We examined the population genetics of *O. byronia* along its geographic distribution and tested if the population from which eDNA samples were retrieved was genetically dissimilar from other populations in the coast of the Eastern Pacific and Western Atlantic basins using a fragment of the CR of each newly assembled mitochondrial genome plus 164 other shorter fragments available in GenBank. First, we downloaded all CR sequences belonging to *O. byronia* available in GenBank ($n = 214$), aligned them using the program ClustalW (Thompson et al. 1994) as implemented in the software MEGA 11 (Tamura et al. 2021), and manually trimmed the sequences resulting in a final alignment 228 bp long that included 244 sequences. Samples were pooled together over large geographical regions (i.e., Peru, North Chile, Central Chile, South Chile, Comau Fjord, Falkland Islands, Southern Argentina, Central Argentina, Northern Argentina, Uruguay, Brazil), knowing that previous studies have not detected genetic dissimilarities among geographically close locations and allowing us to increase the sample size per contrasted localities in this study (De Oliveira et al. 2017; Peralta et al. 2021). The final trimmed data matrix permitted us to analyze population genetics in this species with the longest possible alignment and for at least 13 specimens per population/region other than Central Chile, South Chile, and Central Argentina that only included each 7, 6, and 8 sequences, respectively. Then, nucleotide diversity (π), haplotype diversity (h), the number of polymorphic sites (S), and the neutrality (D) for each population and for the entire dataset were calculated using the software DnaSP 5.10.01 (Librado and Rozas 2009). Genetic distances among and within examined populations were calculated in the software MEGA X. Genetic variability among and within populations was examined using an Analysis of Molecular Variance (AMOVA), with 10,000 permutations, and with the statistic Φ_{ST}, both estimated in the program ARLEQUIN 3.0 (Excoffier et al. 2005). We also conducted pairwise population comparisons with Φ_{ST} recalibrated with statistically significant values corrected using the Bonferroni correction (Excoffier et al. 2005). The software Arlequin was also used to estimate gene flow (Nm, interpreted as the effective number of migrants exchanged between demes per generation, *sensu* Wright 1969) among geographical regions. Lastly, genealogical relationships among haplotypes divided according to geographical region were assessed with a haplotype network constructed with the method Median-Joining in the program Network 10.2.0.0. (Bandelt et al. 1999).

3 | Results and Discussion

3.1 | Assembly and Annotation of Mitochondrial Genomes From eDNA Metagenomic Datasets

Complete (circularized) mitochondrial genomes were bioinformatically recovered (de novo assembled) from each of the 30 environmental metagenomics samples belonging to the sea lion *O. byronia*. Average coverage (per nucleotide) varied between 40.1× and 2817.4× in samples SRR14857241 and SRR14857251, respectively (Table S1). We note that we named newly assembled mitochondrial genomes depending upon the GenBank accession code for the raw reads and that only 4 of the newly assembled mitochondrial genomes were identical to other mitogenomes assembled in this study (SRR14857235 = SRR14857256, SRR14857245 = SRR14857246, SRR14857252 = SRR14857260, and SRR14857253 = SRR14857258). The length of the newly assembled mitochondrial genomes varied between 16,533 bp (SRR14857231) and 16,618 bp (SRR14857244) (Table S1). Previously reported mitochondrial DNA length in the family Otariidae ranges from 16,310 bp in *Eumetopias jubatus* (Ryu et al. 2010) to 17,154 bp in *Callorhinus ursinus* (Lee et al. 2018). All the mitochondrial genomes comprised 13 PCGs, 22 transfer RNA genes, 2 ribosomal RNA genes, and a relatively long non-coding CR that varies in length between 1074 and 1158 bp (Figure 1). Gene order in the newly assembled mitochondrial genomes was identical to each other, to that reported before for *O. byronia*, and to that reported for all other representatives of the family Otariidae (Lee et al. 2018; Tian et al. 2019).

All newly assembled mitochondrial genomes were AT-rich with AT values ranging between 58.77% (SRR14857237) and 58.82% (SRR14857231) (Table S2–S31). The observed range in AT values is similar to that previously reported for *O. byronia* (58.8%—Tian et al. 2019), and nucleotide composition is within the range reported for closely related species. In the family Otariidae, AT values range between 58.75% in *Phocarctos hookeri* (NC_008418—Arnason et al. 2006; Rawlence et al. 2016) and 59.69% in *Callorhinus ursinus* (NC_008415—Lee et al. 2018).

In all mitochondrial genomes analyzed, all PCGs started with conventional codons (ATG and ATA) and 11 PCGs ended with complete and conventional stop codons (TAA, TAG, and AGA), while *cox3* and *nad4* ended with an incomplete stop codon (T). Truncated stop codons are often observed in vertebrates, including pinnipeds (Arnason et al. 2006; Austin et al. 2023; Kim et al. 2021; Ryu et al. 2010), and are thought to be completed through post-transcriptional polyadenylation (Mofayezi et al. 2024).

The most frequently used codon in the PCGs of the studied mitochondrial genomes was AT-rich and included CTA, ATA, and ATC. In turn, other than stop codons, codons with the lowest observed frequencies were GC-rich and included CGG, CCG,

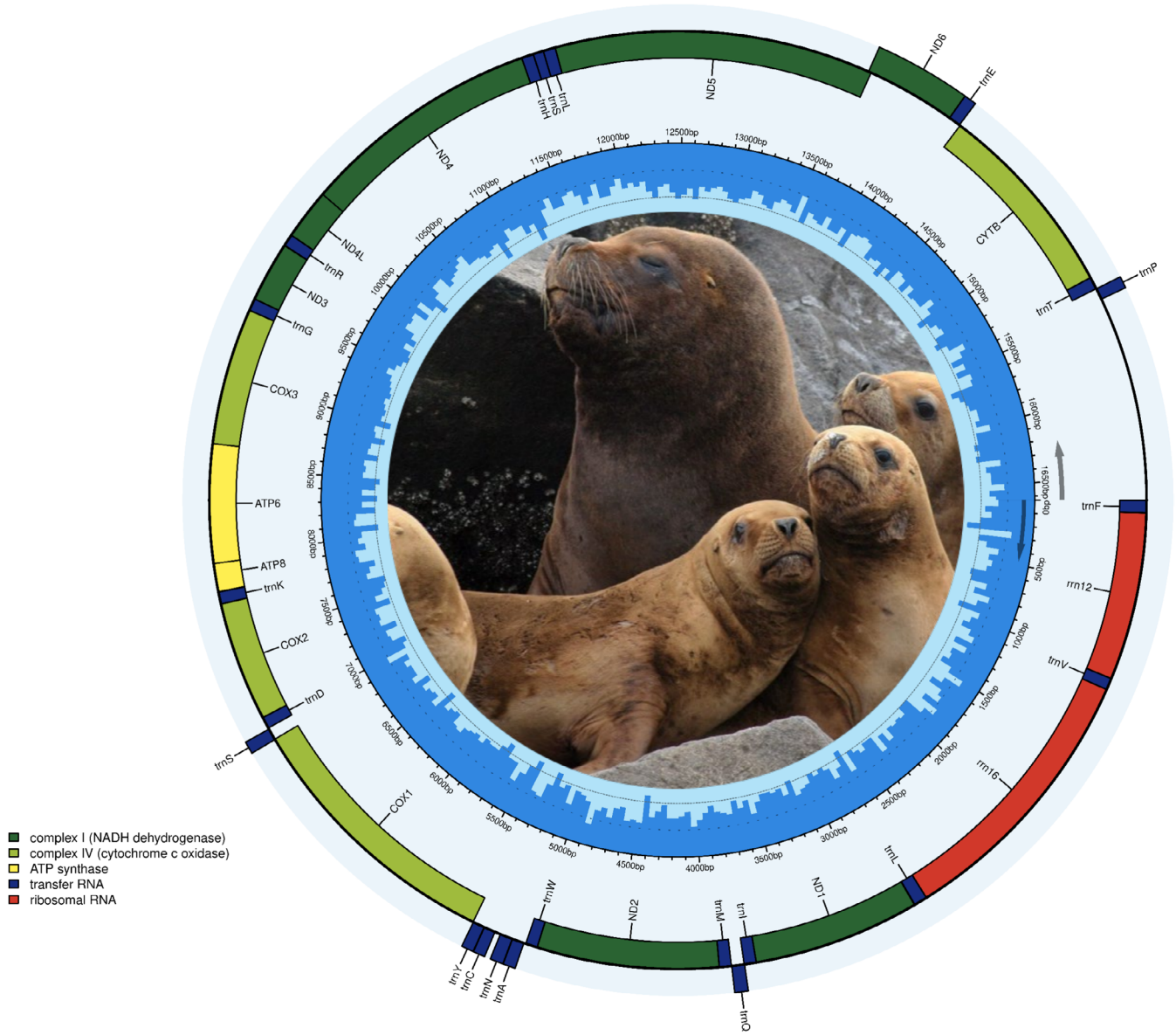

FIGURE 1 | Circular map of the mitochondrial genome of South American Sea Lion *O. byronia* assembled from environmental metagenomic samples. Bars in the inner circle depict GC-content, the external dotted line represents 0.25 GC-content and the continuous line represents 0.5 GC-content. Photograph by Nestor Galina, used with permission.

TGT, and CGT, among a few others (Figure 2). Overall, codons were used disproportionately in all the newly assembled mitogenomes.

The analysis of selective pressure indicated that all 13 mitochondrial PCGs have experienced purifying selection given that for each of the PCGs in each of the three studied mitochondrial genomes, Ka/Ks values were <1 (Figure 3). *Atp8* was the gene with the highest Ka/Ks ratio ($1 > Ka/Ks > 0.3$; $p < 0.05$), indicating weak purifying selection affecting this compared to other PCGs. In turn, the Ka/Ks ratios estimated for *cox1*, *nad1*, and *nad6* (Ka/Ks <0.0112; $p < 0.05$) were low, suggesting stronger purifying selection and evolutionary constraints in these compared to the remainder of the studied PCGs. This is the first analysis of selective pressures conducted in a representative of the family Otariidae, and the observed purifying selection testifies to the importance of these genes for energy production (Bernt et al. 2013).

In the newly assembled mitochondrial genomes, tRNA genes varied in length from 59 (trnS1) to 76 bp (trnL2). All tRNA exhibited a "cloverleaf" secondary structure, except for trnS1, which lacked the dihydrouridine (DHU) arm (Figure 4; Figure S1), in line with that reported before in the same species (Tian et al. 2019) and in the few other confamilial species in which tRNA secondary structure has been examined: *Arctocephalus australis* (Tian et al. 2021), *Zalophus japonicus* (Kim et al. 2021), and *Eumetopias jubatus* (Ryu et al. 2010). Truncated mitochondrial trnS1 genes appear to be functional, as suggested by in vitro experiments in which the truncated tRNA gene adopts a conformation that is suitable for ribosomal interaction and activity (Krahn et al. 2020).

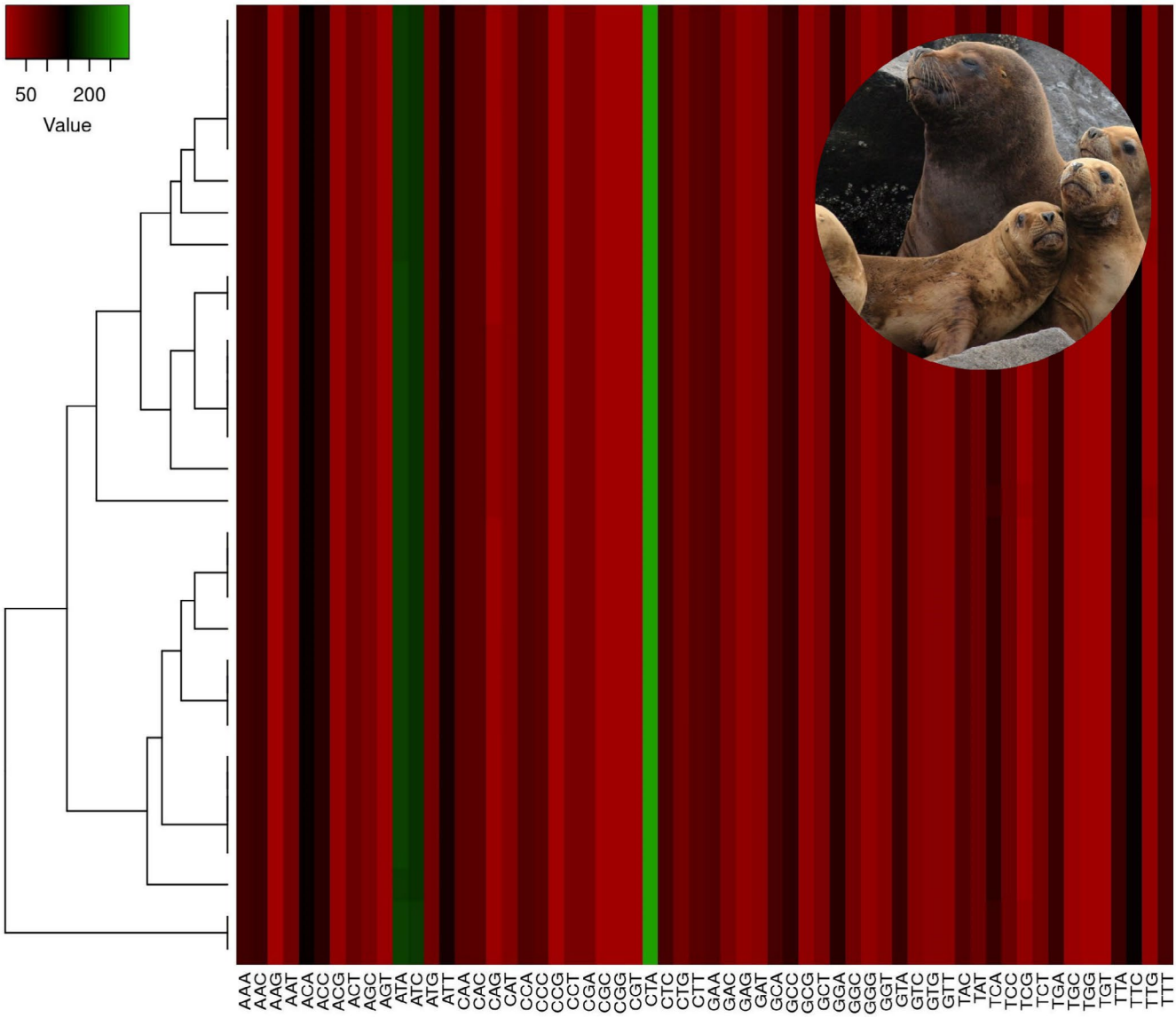

FIGURE 2 | Codon usage profile of protein coding genes in the mitochondrial genome of South American Sea Lion *O. byronia* assembled from environmental metagenomic samples. Green colors indicate more frequently used codons, red indicate less frequently used codons, and in between, codons with moderate use are represented in black. Photograph by Nestor Galina, used with permission.

The two ribosomal RNA genes in the assembled mitochondrial genomes are encoded on the heavy (H) strand and exhibited identical lengths across all samples (rrnS (12S) and rrnL (16S) gene length = 961 and 1578 bp, respectively). The 12S rRNA gene was flanked by tRNAPhe and tRNAVal. In turn, the 16S rRNA was flanked by tRNAVal and tRNALeu. The two genes exhibited a high A + T content (Tables S32 and S33), in line with that reported for the few other confamilial species in which this parameter has been calculated; 12S AT-content ranges from 58.75% in *Phocarctos hookeri* to 59.69% in *C. ursinus*, and 16S rRNA AT-content varies from 61.01% in *A. pusillus* to 62.38% in *C. ursinus*, based on our calculations (Tables S34, S35).

The length of the CR in the studied mitochondrial genomes varied between 1074 (SRR14857231) and 1159 (SRR14857244) and exhibited an AT-skewed nucleotide composition with AT values ranging from 55.4% (SRR14857250) to 56% (in SRR14857231 and SRR14857251) (Table S36), which is slightly lower than the AT content reported for *Arctocephalus australis* (59.2%—Tian et al. 2021). The CR length in *O. byronia* was slightly shorter than that reported for other confamilial species, including *A. australis* (1121 bp—Tian et al. 2021), *Eumetopias jubatus* (1182 bp—Ryu et al. 2010), *Callorhinus ursinus* (1695 bp—Lee et al. 2018), including that previously reported for *O. byronia* 1186 bp (Tian et al. 2019).

A multiple sequence alignment of the studied mitochondrial genomes with those of other confamilial species, including *A. australis* (Tian et al. 2021), permitted us to detect the presence of the three functional domains reported in mammals: extended termination associated sequence (ETAS), central, and conserved sequence block (CSB) (Figure 5; Figures S31, S32). Blocks within ETAS, Central (F, E, C, D, and B Box), and CSB domains (1–3) were also revealed by our analysis. In the studied mitochondrial CRs, five ETAS haplotypes were found, all different from the motif reported before for *A. australis* (Tian et al. 2021). In turn,

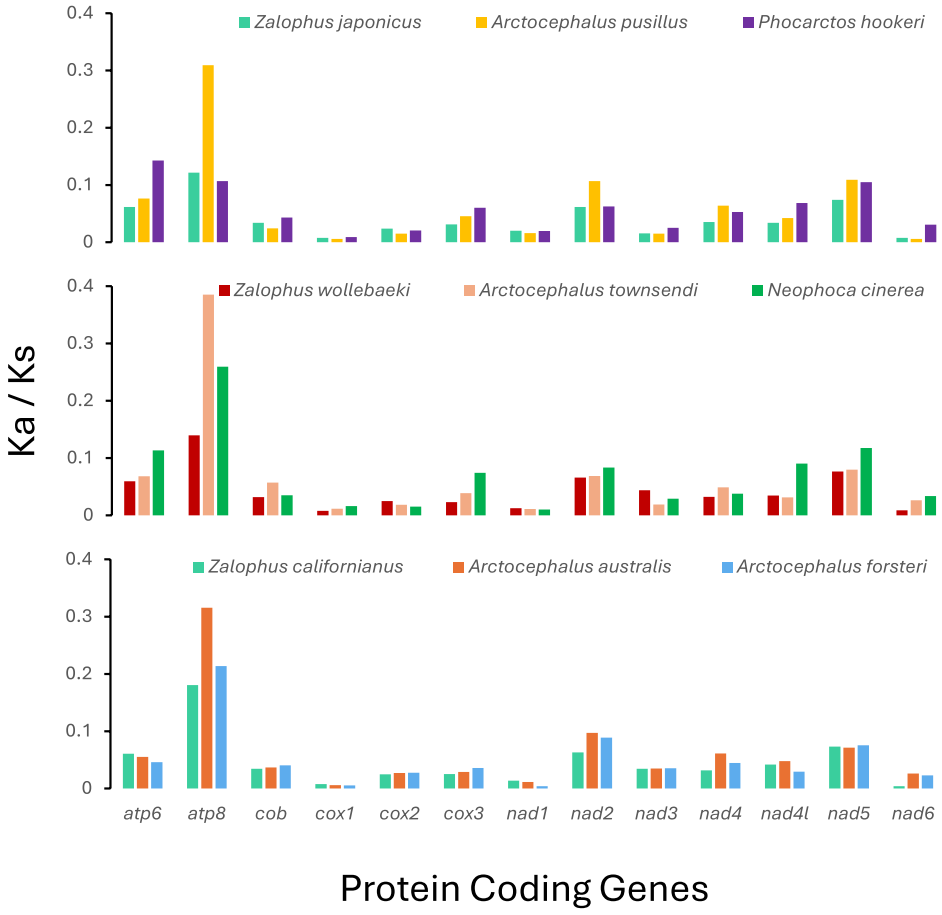

FIGURE 3 | Selective pressures affecting protein coding genes in the mitochondrial genome of South American Sea Lion *O. byronia* assembled from environmental metagenomic samples. The analysis was conducted in three samples.

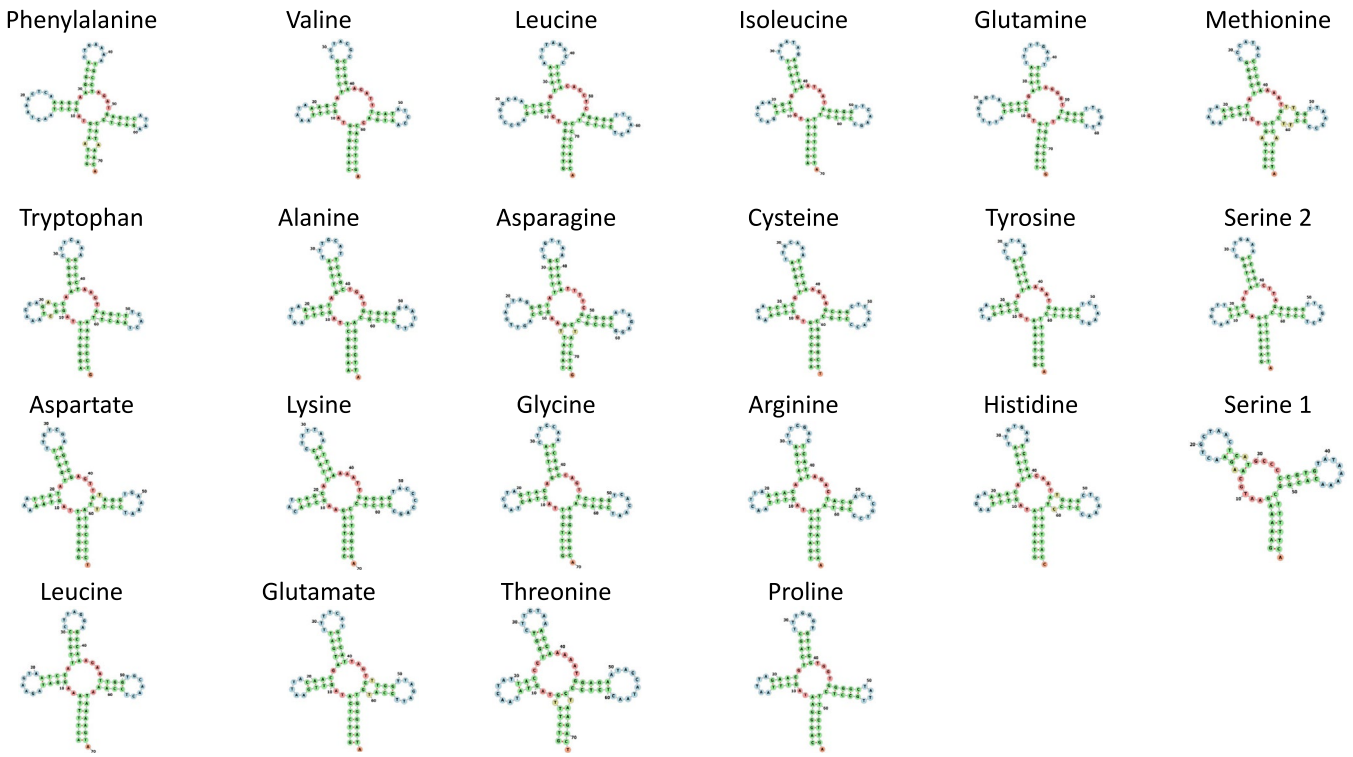

FIGURE 4 | Secondary Structure of tRNA genes in the mitochondrial genome of the South American Sea Lion *O. byronia* assembled from environmental metagenomic sample SRR14857250.

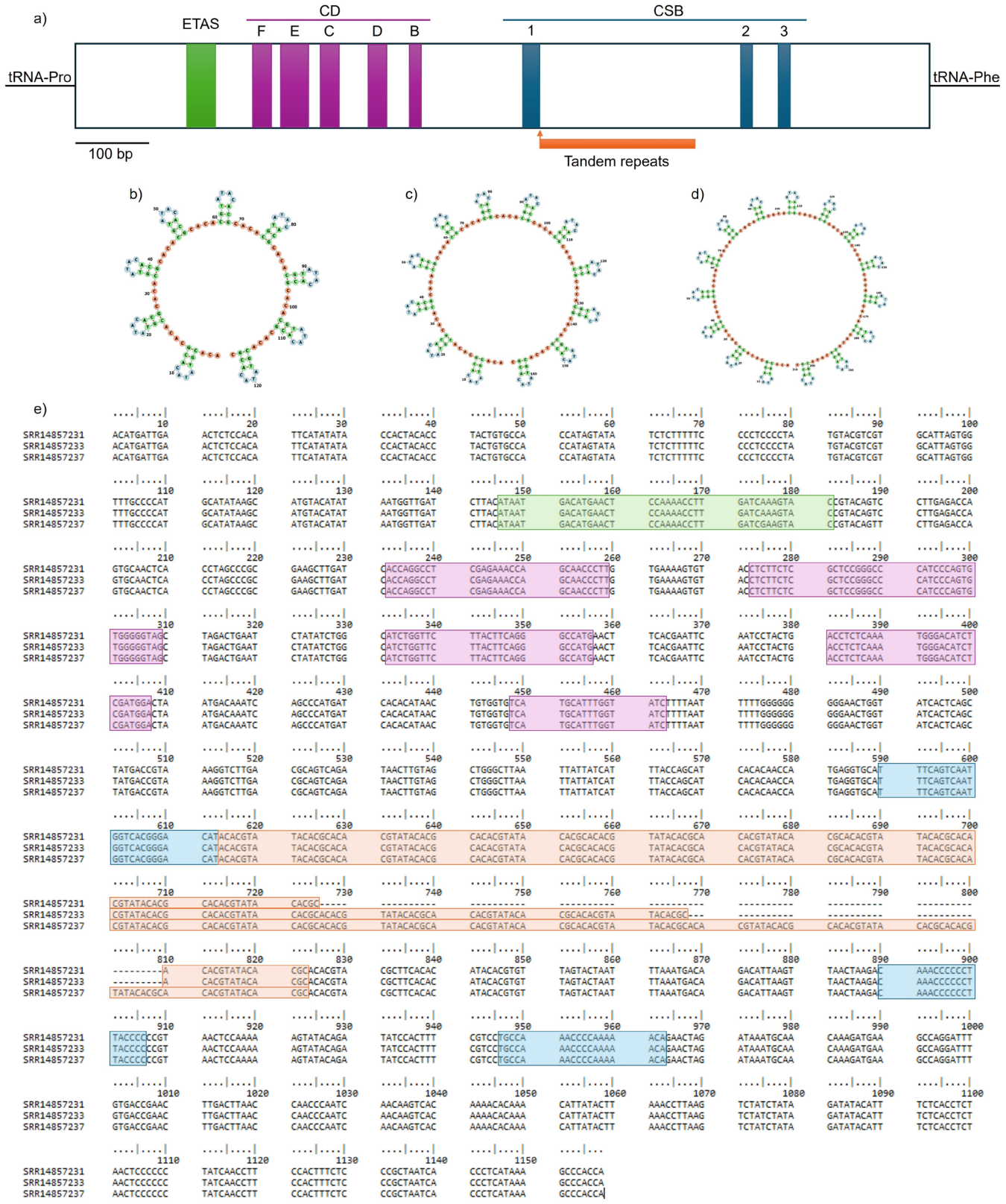

FIGURE 5 | Organization of the control region (CR) in the mitochondrial genome of the South American Sea Lion *O. byronia* assembled from environmental metagenomic samples. The location of conserved domains: Extended termination associated sequences (ETAS), central domains (CD), and conserved sequence blocks (CSB) are shown and the position of short tandem repeats is indicated in three selected mitochondrial genomes are shown in (a). Secondary structures of the tandem repeats are shown in (b), (c), and (d). Alignment of the CR of three samples are depicted in (e) and conserved domains are identified with different colors, (ETAS = pink, CD = green, CSB = yellow) also the tandem repeats are shown (blue).

in the central domain, the F, D, and B boxes were identical while the remaining boxes were very similar when compared among each other and with the corresponding boxes in *A. australis* and exhibited no more than two single nucleotide variants. Finally, in the CSB domain, three single nucleotide variants were observed in the CSB-1 box with respect to *A. australis*, while CSB-2 and CSB-3 were identical when compared among each other and with the corresponding boxes in *A. australis*. In this CSB domain, a short tandem repeat was present (Figure 5; Figures S31, S32) between boxes CSB-1 and CSB-2, which coincides with that reported for the confamilial *A. australis* and other mammals (Choi et al. 2010; Silva et al. 2011; Tian et al. 2021; Barrera et al. 2023). This short tandem repeat (motif: 5′-[ACA CGT ATA CAC GC]$_{9.5–15.5}$—3′f) was repeated between 9.5 and 15.5 times depending upon the studied samples and all exhibited a secondary structure featuring multiple "hairpins" or "stem-and-loops" whose number increased with the number of times that the motif was repeated (Figure 5; Figures S31, S32). Tandem repeats have also been reported in the CR CSB domain of the closely related *C. ursinus* (Lee et al. 2018). Also in the studied CRs, between seven and eight microsatellites were detected, all of them dinucleotide motifs, most of them GC-rich, that were repeated between three and five times (Table S37).

3.2 | Population Genetics of *Otaria byronia*

The CR used in the population study did not show evidence to reject the hypothesis of neutral evolution for this gene in each of the populations or for the total data used (Table 1). The studied sequences ($n = 244$, 288 bp long when aligned) contained between 4 and 40 polymorphic sites (Table 1) that defined a total of 116 haplotypes with an average nucleotide diversity (π) of 0.030 ± 0.001 and an average haplotype diversity (h) of 0.979 ± 0.004 (Table 1). The geographic region with the lower nucleotide diversity was Brazil ($\pi = 0.006$), while the region with the higher nucleotide diversity was Uruguay ($\pi = 0.028$). In turn, the lower and higher haplotype diversity was found in Brazil (h = 0.821) and Central Argentina (h = 1.000), respectively (Table 1). The observed average diversity values were somewhat higher than those previously reported for some sea lion populations in the Atlantic Ocean ($\pi = 0.008–0.021$, h = 0.841–0.987—Feijoo et al. 2011), Falkland Islands ($\pi = 0.015$, h = 0.864—Hoffman et al. 2016), Peru to Brazil ($\pi = 0.005–0.019$, h = 0.830–0.950—De Oliveira et al. 2017; $\pi = 0.015$, h = 0.869—Peralta et al. 2021), and the Brazilian coast ($\pi = 0.006$, h = 0.670—Artico et al. 2010).

A first AMOVA indicated statistically significant differences among the compared geographic regions ($\Phi_{ST} = 0.404$, $p < 0.001$), although the greatest source of variation (59.6%) was found within regions (Table 2). Similarly, a second AMOVA in which we compared genetic dissimilarity between the Atlantic and Pacific basins also indicated statistically significant differences ($\Phi_{CT} = 0.411$, $p = 0.002$) (Table 2). Finally, when analyzing each basin independently, we observed that the Pacific presents very low levels of differentiation between populations ($\Phi_{ST} = 0.092$, $p < 0.001$) while in the Atlantic, a more marked genetic differentiation is observed ($\Phi_{ST} = 0.295$, $p < 0.001$) (Table 2). Our results are in line with previous studies indicating divergence between populations inhabiting the Atlantic and Pacific basins (Tunez et al. 2010; Artico et al. 2010; De Oliveira et al. 2017). Previous studies have also demonstrated genetic dissimilarities among populations within each ocean basin, but with lower Φ_{ST} values (Tunez et al. 2010; Feijoo et al. 2011; Hoffman et al. 2016; De Oliveira et al. 2017; Peralta et al. 2021).

All pairwise population comparisons of Φ_{ST} values indicated high genetic differentiation among geographic regions with statistically significant p values after Bonferroni correction (Table 3), except those between the Peruvian region and Central and Southern Chile. When all populations both in the Atlantic and Pacific basins are compared, the lower Φ_{ST} value (0.320) was observed between Central Chile and Southern Argentina, while the higher Φ_{ST} value (0.796) was observed between Brazil and the Comau Fjord,

TABLE 1 | Summary of the genetic diversity for *Otaria byronia* from Pacific and Atlantic Oceans according to the control region (CR) of mitochondrial DNA for all databases (sequences from this study and from GenBank). The sample size (N), number of haplotypes, haplotype diversity (h), nucleotide diversity (π), polymorphic sites (S), and Tajima's index (D) are shown.

Region	Abbreviation	*N*	Haplotypes	h	π	S	D
Peru	PE	17	13	0.926	0.012	14	−1.2
Northern Chile	NC	25	19	0.970	0.016	21	−1.3
Central Chile	CC	7	5	0.857	0.018	13	−1.3
Southern Chile	SC	6	5	0.933	0.021	13	−1.0
Fjord (this study)	Stu	30	9	0.830	0.010	11	−0.6
Falkland Islands	FI	29	20	0.973	0.019	23	−1.0
Southern Argentina	SA	42	24	0.947	0.024	27	−0.4
Central Argentina	CA	8	8	1.000	0.017	11	−0.9
Northern Argentina	NA	54	36	0.977	0.022	40	−1.5
Uruguay	UR	13	12	0.987	0.028	15	−1.0
Brazil	BR	13	5	0.821	0.006	4	−0.3
Total		244	116	0.979	0.030	63	−1.1

Chile (Table 3). In turn, when our comparison is restricted to localities from the Pacific basin only, the lowest Φ_{ST} value (0.027) was observed between Peru and Southern Chile, while the higher Φ_{ST} value (0.205) was observed between Peru and Comau Fjord, Chile (Table 3). Lastly, if we compare localities restricted to the Atlantic Ocean, the lower Φ_{ST} value was found between Central and Southern Argentina (0.084), while the higher Φ_{ST} value was observed between Uruguay and Brazil (0.564). Interestingly, in the Atlantic, Uruguay had the highest Φ_{ST} value compared to the rest of the geographic regions (0.433–0.564) (Table 3).

TABLE 2 | Results of analysis of molecular variance among regions using control region fragment based on pairwise distance.

Structure tested	% Variance	*F*-statistics	*p* value
One group (all locations)			
Among populations	40.4	$\phi_{ST}=0.404$	<0.001
Within populations	59.6		
Two groups (Pacific vs Atlantic)			
Among groups	41.06	$\phi_{SC}=0.263$	<0.001
Among populations within groups	15.5	$\phi_{ST}=0.566$	<0.001
Within populations	43.44	$\phi_{CT}=0.411$	0.002
One group (Pacific locations)			
Among populations	9.16	$\phi_{ST}=0.092$	<0.001
Within populations	90.84		
One group (Atlantic locations)			
Among populations	29.47	$\phi_{ST}=0.295$	<0.001
Within populations	70.53		

Migration estimates indicate limited gene flow between Pacific and Atlantic basins (Nm = 0–1) and between geographical regions located in the Atlantic Ocean (Nm = 0–5) but relatively high gene flow between geographical regions in the Pacific Ocean (Nm = 2–18) (Table 3). It is important to mention that due to the uniparental and non-recombinant nature of mitochondrial DNA, the results of genetic structure and migration may present a bias that can be corrected by integrating nuclear data in future studies.

A haplotype network analysis did not demonstrate the existence of obvious (well-defined) haplogroups in *O. byronia*, although a weak clustering of sequences belonging to each the Pacific basin, Atlantic basin, and Uruguay + Northern Argentina was observed (Figure 6). Importantly, only three haplotypes were shared between Pacific and Atlantic basins, and 76 unique haplotypes were identified in the three poorly defined haplotype clusters. These observations are in agreement with our AMOVA analyses. Lastly, the haplotypes present in the Islas Malvinas/Falkland Islands tend to form a distinct cluster, yet Islas Malvinas/Falkland Islands shared haplotypes with continental Argentina and other geographical regions in the Pacific basin (Figure 6).

Overall, our results indicate that the South American Sea Lion *O. byronia* exhibits an overall pattern of higher genetic connectivity within than among ocean basins, with an overall signature of genetic structuring across its distribution in South America, in particular between the Pacific and Atlantic meta-populations. We note that previous studies suggest philopatric behavior by female sea lions (Tunez et al. 2010; Feijoo et al. 2011) affecting population genetic structuring that we

TABLE 3 | Pairwise Φ_{ST} estimates between geographic regions for control region sequences (below diagonal) and migration values (upper diagonal). Significant values after Bonferroni correction are in bold ($\alpha<0.05$) and values greater than one migrant per generation are in bold and italics. The abbreviations are shown in Table 1.

	PE	NC	CC	SC	Stu	FI	SA	CA	NA	UR	BR
PE	—	***2.35***	***11.48***	***17.94***	***1.94***	0.52	0.92	0.31	0.62	0.32	0.17
NC	**0.176**	—	***5.04***	***4.89***	***7.09***	0.32	0.55	0.19	0.40	0.19	0.15
CC	0.042	**0.090**	—	***12.35***	***4.30***	0.66	***1.06***	0.37	0.68	0.33	0.22
SC	0.027	**0.093**	**0.039**	—	***3.65***	0.47	0.79	0.31	0.53	0.28	0.19
Stu	**0.205**	**0.066**	**0.104**	**0.121**	—	0.37	0.61	0.24	0.42	0.26	0.13
FI	**0.491**	**0.612**	**0.433**	**0.514**	**0.573**	—	***2.42***	***1.13***	***2.61***	0.43	***1.39***
SA	**0.353**	**0.475**	**0.320**	**0.388**	**0.451**	**0.171**	—	***5.48***	***5.24***	0.48	***1.81***
CA	**0.618**	**0.722**	**0.574**	**0.619**	**0.672**	**0.306**	**0.084**	—	***3.36***	0.41	0.62
NA	**0.448**	**0.555**	**0.423**	**0.485**	**0.543**	**0.161**	**0.087**	**0.130**	—	0.65	***4.81***
UR	**0.609**	**0.725**	**0.599**	**0.642**	**0.656**	**0.535**	**0.509**	**0.548**	**0.433**	—	0.39
BR	**0.746**	**0.767**	**0.698**	**0.722**	**0.796**	**0.264**	**0.216**	**0.447**	**0.094**	**0.564**	—

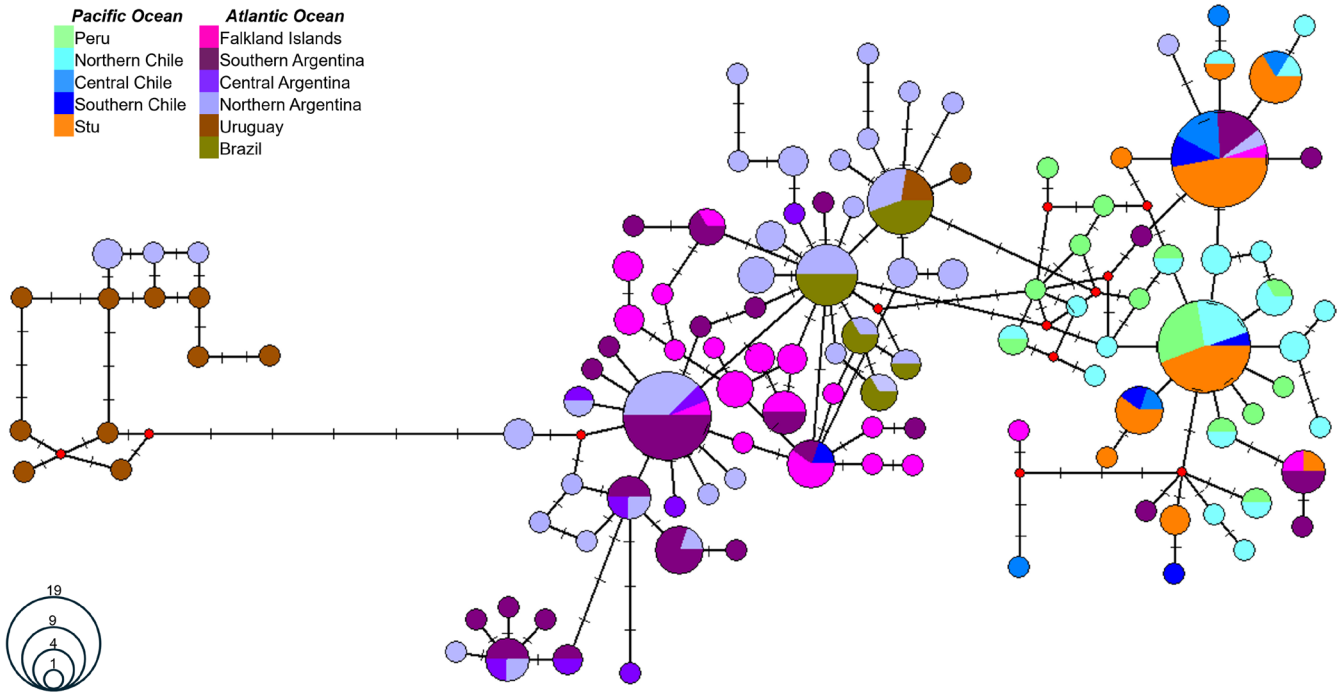

FIGURE 6 | Haplotype network estimated using a fragment of the control region in the South American Sea Lion *O. byronia*. The area of each circle corresponds to the number of haplotypes it represents, and different colors represent different geographic regions where specific haplotypes are found. Stu, study site, Comau Fjord.

are not able to examine due to the nature of our sampling strategy.

4 | Conclusion

In this study, we have demonstrated that it is possible to assemble complete mitochondrial genomes from field-collected scat (eDNA) metagenomic samples belonging to the South American Sea Lion *O. byronia* and used a partial fragment of the CR to examine if and tested if the population from which eDNA samples were retrieved was genetically dissimilar from other populations on the coast of the Eastern Pacific basin. The annotation and detailed description of the assembled mitochondrial genomes indicate that these assemblies recovered from the eDNA are accurate. The bioinformatic protocol we have used is forthright and can be replicated by other research teams at lower computational/bioinformatics costs. Our approach represents a strategy that can contribute to the biomonitoring and population genomics of this and other imperiled marine vertebrates of conservation concern in which sampling is logistically complicated using indirect surveillance strategies. Additional non-intrusive sampling of scats across the southern coast of South America, from Peru to Brazil, is needed to continue improving our understanding of population genetic structure and the genomic foundation of adaptability to climate change and anthropogenic activities (i.e., historical harvesting) in this iconic marine mammal.

Author Contributions

J.A.B. conceived and designed the experiments. J.A.B., A.C., M.D., and S.B. performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. E.C.-N. provided resources, authored or reviewed drafts of the paper, and approved the final draft.

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Disclosure

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All raw reads are available at NCBI's GenBank as part of Bioproject PRJNA735411. All assembled mitochondrial genomes are available in GenBank with accession numbers PV358026-PV358055.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** edn370213-sup-0001-TableS1-S37-FigureS1-S32.pdf.