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Assembly, Characterization, and Phylogenetic Relationships of Mitogenomes of Two Species of Mexican Trout (*Oncorhynchus chrysogaster* and *O. mykiss nelsoni*)

Angélica Colín [1], Miguel A. Del Río-Portilla [2,*], Fabiola Lafarga-De la Cruz [2], Genoveva Ingle-De la Mora [3] and Francisco J. García-De León [1,*]

[1] Laboratorio de Genética para la Conservación, Centro de Investigaciones Biológicas del Noroeste, Instituto Politécnico Nacional, No. 195, Col. Playa Palo de Santa Rita, La Paz CP 23096, Baja California Sur, Mexico
[2] Departamento de Acuicultura, Centro de Investigación Científica y de Educación Superior de Ensenada, Carretera Ensenada-Tijuana, No. 3918, Zona Playitas, Ensenada CP 22860, Baja California, Mexico
[3] Instituto Nacional de Pesca y Acuacultura, Secretaría de Agricultura y Desarrollo Rural (SADER), Avenida México, No. 190, Col del Carmen, Coyoacán CP 04100, Ciudad de México, Mexico
* Correspondence: mdelrio@cicese.mx (M.A.D.R.-P.); fgarciadl@cibnor.mx (F.J.G.-D.L.)

Abstract: In this study, the complete mitochondrial genomes of the Mexican golden trout, *Oncorhynchus chrysogaster*, and Nelson's trout, *O. mykiss nelsoni*, were assembled and characterized. The mitogenomes were 16,655 bp and 16,661 bp long, respectively, and were composed of 13 protein-coding genes (PCGs), two ribosomal RNA genes, and 22 transfer RNA genes (all with typical 'cloverleaf' secondary structures). The length of the D-loop regions was among the longest found in Salmonids, and mitochondrial synteny in both species was identical to that reported in other Salmonids. Selective pressure analysis in the PCGs indicated that purifying selection, mainly among *cox* and *nd* genes families, likely generated the main differences between the two studied species. Nine tRNA genes showed slight differences relative to other *O. mykiss* subspecies, which were identical between the two study taxa. The origin of the light-strand replication has a loop that was especially large in *O. mykiss nelsoni*. Phylogenetic analysis indicated that *O. chrysogaster* and *O. mykiss nelsoni* are sister species, contrary to the expectation that *O. chrysogaster* would cluster with *O. gilae*. As previous studies have suggested, *O. chrysogaster* and *O. mykiss nelsoni* share common ancestry with North American trout species.

Keywords: Salmonidae; Mexican golden trout; Nelson's trout; mitochondrial genome; taxonomic status; fish

Citation: Colín, A.; Del Río-Portilla, M.A.; Lafarga-De la Cruz, F.; Ingle-De la Mora, G.; García-De León, F.J. Assembly, Characterization, and Phylogenetic Relationships of Mitogenomes of Two Species of Mexican Trout (*Oncorhynchus chrysogaster* and *O. mykiss nelsoni*). *Fishes* **2023**, *8*, 178. https://doi.org/10.3390/fishes8040178

Academic Editor: Joseph Quattro

Received: 19 January 2023
Revised: 24 March 2023
Accepted: 25 March 2023
Published: 27 March 2023

1. Introduction

In past decades, it was common to use short mitochondrial gene fragments to resolve phylogenetic relationships at different taxonomic levels. Recently, whole mitochondrial genome studies have been proposed as a valuable tool for phylogenetic inferences and the modeling of genome evolution [1]. These phylomitogenomic analyses have also been used to resolve a persistent debate about higher-level relationships among teleost fishes or lower-level relationships within species complexes [2–4]. For the family Salmonidae, phylogenetic relationships have been reconstructed to understand the evolutionary history of behavioral, life history, and ecological traits [3,5]. Phylogenies using mitogenomes from each of the genera of the family Salmonidae have recently been published [6–19]. This family includes three subfamilies: Salmoninae (salmons, trouts, and charrs), Coregoninae (ciscoes and whitefishes), and Thymallinae (graylings) [20]. Salmonid fish are commercially important, widely used in aquaculture, and have been introduced in 83 countries [21,22]. Consequently, there is great interest in the genetic features of mitochondrial DNA, which can be informative for stock identification, management, conservation, and population

studies [3,5,23,24]. The genus *Oncorhynchus* and five other genera (*Brachymystax*, *Parahucho*, *Hucho*, *Salmo,* and *Salvelinus*) comprise the subfamily Salmoninae [20].

In Mexico, only the genus *Oncorhynchus* Suckley [25] (Actinopterygii: Salmoniformes: Salmonidae) has native representatives with a wide distribution [26–28]. However, only two species were formally recognized: the Mexican golden trout, *O. chrysogaster*, from the Sierra Madre Occidental (SMO) [26,29]; and Nelson's trout, *O. mykiss nelsoni*. Nelson's trout is the southernmost rainbow trout subspecies, and its distribution is restricted to the Baja California Peninsula. This subspecies is slow-growing, short-lived, rapidly maturing, and reaches a smaller length compared to the subspecies *O. m. mykiss* (used typically in hatcheries). Traditional and genetic data have established Nelson's trout as a subspecies of *O. mykiss* corresponding to a distinct and native genetic group [30–34]. However, comparatively little is known about Mexican golden trout (although see [26,27,29]). The rest of Mexico's native trout biodiversity represents a complex of undescribed species, which contains an undetermined number of new species that inhabit the SMO to the north and south of where the Mexican golden trout is distributed; these taxa are currently referred to by the name of the watershed where they live [26,28,35–46]. Despite the presence of these native species, the rainbow trout *O. mykiss* has been introduced for farming in natural aquatic habitats of the SMO [26–28,39] and most other Mexican mountain ranges [26,28]. In the case of the SMO, such introductions have led to hybridization between Mexican native trout and rainbow trout [40,42,43].

The species radiation of the genus *Oncorhynchus* was promoted by geological activity, climatic changes, and geographic isolation, which led them to evolve in highly dynamic environments [44]. The diversification of this genus has been widely discussed and molecular dating varies depending on the molecular marker employed (SNPs [47], mitochondrial genes [48], nuclear genes [49] and mitonuclear genes [50]). However, there is general agreement that the origin of Pacific salmons and Pacific trouts occurred during the Miocene. The Pacific trouts include *O. mykiss*, *O. clarkii*, *O. gilae*, *O. apache* (now *O. g. apache*) and the SMO's undescribed trout complex [33,50]. Subsequently, radiation into the *O. mykiss* and *O. clarkii* complexes occurred during the Pleistocene [51,52] or possibly earlier during the Late Pliocene [50]. Protein polymorphism analysis established that *O. gilae* and *O. gilae apache* are sister taxa that, together with the Mayo trout (an undescribed northern species from the SMO), are more closely related to the *O. mykiss* complex than to the *O. clarkii* complex, which supports the divergence hypothesis proposed by Loudenslager et al. [53]. In addition, the Yaqui trout from SMO (another undescribed northern species) showed a closer phylogenetic relationship to the *O. mykiss* complex, which included *O. mykiss nelsoni* [54,55]. Lately, Mayden et al. [28] explored these phylogenetic relationships with an increased sampling effort that included the undescribed trout complex of the SMO and the *O. gilae* lineage. They found that only the Southern Conchos trout (on the Atlantic slope) clustered into the *O. gilae* lineage, while the other trout from a different watershed of the SMO (on the Pacific slope) showed multiple divergent lineages that were more closely related to the *O. mykiss* lineage than to the *O. gilae* lineage. For this reason, exploring phylogenetic relationships with a powerful tool like mitogenomes could help to resolve the numerous taxonomic uncertainties in these species complexes.

In this work, we characterized for the first time the complete mitochondrial genome of the two recognized Mexican trout taxa and used only the mitochondrial protein-coding genes (PCGs) to clarify their genetic relationships within the genus *Oncorhynchus*. We have assembled and characterized the whole mitogenome and annotated its genes for *O. chrysogaster* and *O. mykiss nelsoni*. Additionally, we assessed the phylogenetic position of the two species of interest using the mitochondrial PCGs of the *Oncorhynchus* genus available from NCBI GenBank. The information in this work will form the basis of future genetic studies of Mexican trouts and could provide critical information for managing these native species.

2. Methods

Adult *Oncorhynchus chrysogaster* (Needham & Gard [29]) were collected from the Fuerte River, Chihuahua, Mexico using a Smith Root 15-B POW (110V AC) electrofishing rig and kept in captivity at the Aquaculture Center of INAPESCA, Guachochi, Chihuahua, Mexico. An individual of *Oncorhynchus mykiss nelsoni* (Evermann [30]) was collected from a stream segment located between Rancho Mike's Sky and Rancho Garet in San Pedro Mártir, Baja California, Mexico, with a Smith-Root model LR24 backpack electrofisher and transported to Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE, Ensenada, BC, MEX) and kept in recirculating systems until sampling. The fishes analyzed were rapidly and humanely killed. The complete specimen was fixed in ethanol. About 15 mg of fin tissue was used for total DNA extraction with the DNeasy Blood & Tissue Kit (QIAGEN®, Hilden, NRW, DEU) and quantified using a spectrophotometer (Nanodrop 2000®; Thermo Scientific, Wilmington, DE, USA). Total DNA (1000 ng/sample) was sent to the Genomics Center of Georgia (University of Georgia, Athens, GA, USA) for genomic sequencing. The DNA was sheared with a Bioruptor® device by sonication using two rounds of five cycles alternating 30 s of sonication with 30 s without sonication on the high setting. The protocol for library preparation was followed using the Kapa Biosystems® Hyper Prep Kit (KR0961–v4.15), ligating custom adapter and amplifying with 12 cycles of PCR with custom nucleotide indexed primers [56]. Magnetic beads (Speed Beads) were used for Dual-size selection, performed to recover a fragment of ~250–450 bp in size [57]. Libraries were sequenced to produce paired-end 150-nucleotide reads in an Illumina HiSeq 4000 at the Oklahoma Medical Research Foundation Clinical Genomics Center (Oklahoma City, OK, USA).

Fastq files were cleaned by trimming low-quality regions of the reads with CLC Genomics Workbench 10.1.1 software using the default parameters (Trim using quality scores, limit = 0.05, Phred value equivalent to 13.01, and Trim ambiguous nucleotides, number of ambiguities max = 2), and followed by de novo assembly with the following settings: automatic bubble size (50) and word size (20), minimum contig length (200 bp), perform scaffolding (yes), and auto-detect paired distances. The longest contig identified was used as a query to search the nucleotide NCBI GenBank [58] database using the Basic Local Alignment Search Tool (Blast) [59].

To annotate and characterize the complete mitochondrial genome, we followed the recommendations of Baeza [60]. The assembled mitochondrial genomes of *O. chrysogaster* and *O. mykiss nelsoni* were annotated using the web servers MITOS and MITOS2 [61,62] using the mitochondrial vertebrate genetic code. The *in silico* annotated mitochondrial genomes were curated manually, considering start and stop codons corrections in the web server ExPASy [63], and MEGA X [64]. The manually curated annotations were compared with other salmonid mitochondrial genomes in the NCBI nucleotide database (*O. mykiss*-NC_001717, *O. clarkii virginalis*-MW300344, *O. clarkii henshawi*-AY886762, *O. keta*-AP010773, and *O. gilae*-MW300342). The visualizations of the mitogenomes were performed with the web server GenomeVx [65].

Nucleotide composition and codon usage profiles were analyzed for the PCGs in the two mitochondrial genomes. The nucleotide composition was estimated in the software MEGA X for each mitochondrial sequence [64] and compared with the composition of all mitogenomes of the family Salmonidae available in the NCBI nucleotide database [58] (Table S1). Codon usage was estimated in the Sequence Manipulation Suite (SMS) web server using the vertebrate mitochondrial code [66]. Relative synonymous codon usage (RSCU) of concatenated PCGs for each species was visualized using the EZcodon tool in the web server EZmito [67].

The substitution rates of the different PCGs were estimated between closely related species. The values of $K_A = d_N = S_A/L_A$ (the number of non-synonymous substitutions per non-synonymous site), $K_S = d_S = S_S/L_S$ (number of synonymous substitutions per synonymous site) and ω (the K_A/K_S ratio) were evaluated with the software K_AK_S_Calculator2.0 [68]. The ω is a measure of the selective pressures acting on each gene.

Values of $\omega = 1$ indicates neutrality, while $\omega < 1$ indicates negative or purifying selection, and $\omega > 1$ indicates positive or diversifying selection [68]. The ω values were calculated by pairwise comparison between *O. chrysogaster* and *O. masou biwa* (GenBank: EF105342) and between *O. mykiss nelsoni* and *O. masou biwa* (GenBank: EF105342). *Oncorhynchus masou biwa* was selected because it is the congener species that allowed estimation of the substitution rates for almost all the analyzed genes. Other candidate congeners were also tested (*O. clarkii*,
O. gilae and *O. mykiss*), but due to their phylogenetic closeness, it was not possible to estimate non-synonymous substitution rates for any of the genes (results not shown). The γ-MYN model [69] accounted for variable mutation rates at sequence sites. Finally, significant differences in ω between species ($p < 0.05$) were calculated using the $K_A K_S$_Calculator2.0 software to indicate either negative (purifying) selection or positive (diversifying) selection.

Transfer RNAs genes (tRNA) were identified using the software MiTFi [70] implemented in the web server MITOS. The tRNA's secondary structure was visualized for each gene with the tool Forna in the ViennaRNA web server [71].

The control region (CR) was also further examined for the two studied mitochondrial genomes. The putative CR's nucleotide composition was analyze using MEGA X software [64].

PCG-based phylogenetic analysis can be helpful for resolving the phylogenetic relationships among salmonids [13,16]. In addition, an artifact of node overestimation is known to occur when the entire mitogenome is used [13,16]. For this reason, we explored the phylogenetic position of *O. chrysogaster* and *O. mykiss nelsoni* relative to other representative species of the genus *Oncorhynchus* based on their PCGs. The concatenated PCG sequences were aligned using CLUSTAL OMEGA [72], then the best-fitting nucleotide substitution model was selected with JMODELTEST2 [73,74]. Akaike (AIC) and Bayesian (BIC) information criteria were used with default settings. The phylogenetic analysis was conducted using MRBAYES 3.2 [75] with the general time-reversible model (GTR) using a gamma-distributed rate variation among sites (+G) and proportion of invariable sites (+I). Markov chain Monte Carlo simulations were run with 10,000 generations, 100 sample frequencies, and default settings. The phylogenetic tree was visualized using FIGTREE V1.4.4 [76]. This analysis was performed using the two newly sequenced and annotated mitogenomes of *O. chrysogaster* and *O. mykiss nelsoni*, together with those of other species within the genus and five species as outgroups (*Parahucho perryi*, *Salmo salar*, *Salvelinus fontinalis*, *S. leucomaenis*, *S. levanidovi*), which were downloaded from the NCBI web server (Table S2).

3. Results and Discussion

After trimming, 5,578,930 reads were obtained for *O. chrysogaster* with 140 bp of average length, which produced 24,353 contigs with 340 bp of average length (N_{50} = 447). In comparison, 6,614,455 read were obtained for *O. mykiss nelsoni* with 138 bp of average length, which produced 22,913 contigs with 357 bp of average length (N_{50} = 472). The mitochondrial genomes of the Mexican golden trout *O. chrysogaster* (OP902890) and the Nelson trout *O. mykiss nelsoni* (OP902891) were assembled and circularized with a coverage of 21.69 X and 43.97 X, respectively.

The Mexican trouts, *O. chrysogaster* and *O. mykiss nelsoni*, had similar total mitochondrial genome lengths—16,655 bp and 16,661 bp, respectively. In both taxa, the mitochondrial genomes were compact, and few intergenic spaces and overlaps among gene junctions were found (Figure 1, Table 1). In both species, the mitochondrial genome comprised 13 PCGs, two ribosomal RNA genes (*rrnS* [12S] and *rrnL* [16S]), and 22 transfer RNA genes (tRNA) (Table 1). A single long intergenic space was assumed to be the CR, with a length of 1002 bp in *O. chrysogaster* and 1004 bp in *O. mykiss nelsoni*. Three additional short intergenic spaces were observed in the mitochondrial genome, ranging from 14 to 36 bp in each species (21 bp between *tRNA-Val* and *16S*, *ol*-31 bp, 14 bp between *tRNA-Asp* and *cox1*

[*O. chrysogaster*]; 21 bp between *tRNA-Val* and *16S*, *ol*-36 bp, 14 bp between *tRNA-Asp* and *cox1* [*O. mykiss nelsoni*]) (Table 1).

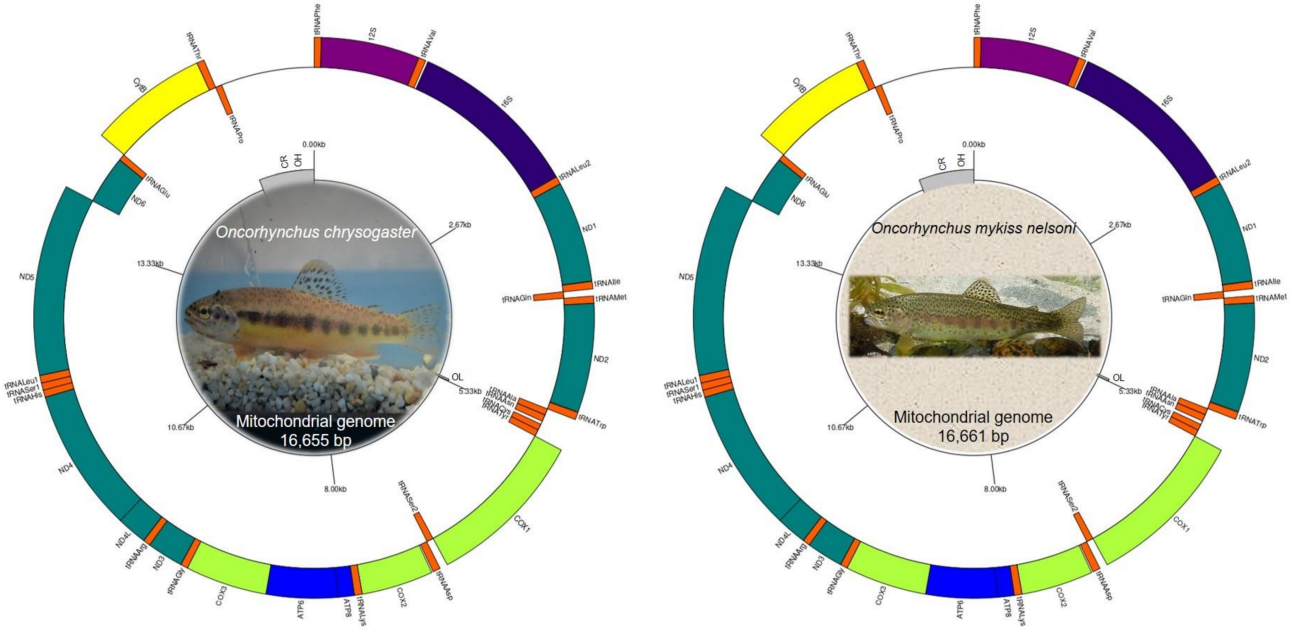

Figure 1. Circular mitochondrial genome maps of the Mexican golden trout, *Oncorhynchus chrysogaster*, and Nelson's trout, *O. mykiss nelsoni*. The annotated maps depict 13 protein-coding genes (PCGs), two ribosomal RNA genes (*rrnS*-12S and *rrnL*-16S) and 22 transfer RNA (tRNA) genes in the external circle. The external or inner positioning in the circle indicates whether the gene was encoded on the heavy strand (+) or the light strand (−), respectively. The putative control region and the origins of replication were indicated in gray in the innermost circle of the map. Photo credit: *O. chrysogaster* photo by FJ García-De León; *O. mykiss nelsoni* photo by Ivan Alejandro Meza Matty (Doi: 10.13140/RG.2.2.36230.86080).

In both species, most (n = 12) of the PCGs, 14 tRNA, and the two rRNA genes were encoded on the heavy strand; only the *nd6* gene and the remaining eight tRNA genes (*tRNA-Gln*, *tRNA-Ala*, *tRNA-Asn*, *tRNA-Cys*, *tRNA-Tyr*, *tRNA-Ser2*, *tRNA-Glu*, *tRNA-Pro*) were encoded on the light strand (Figure 1, Table 1). The same pattern occurs in *Salmo ischchan* [15], hybrids of *O. mykiss* x *Atlantic salmon* [77], and in eight other families of bony fishes (Acipenseridae, Catostomidae, Percidae, Clupeidae, Centrarchidae, Polyodontidae, Cyprinidae, Ictaluridae) [78].

The mitochondrial gene arrangement observed was identical among the 14 species and subspecies of trouts of the genus *Oncorhynchus*, including the study taxa *O. chrysogaster* and *O. mykiss nelsoni*. Furthermore, mitochondrial gene arrangement in this genus was identical to that reported in other Salmonids (e.g., *Brachymystax lenok*-JQ686730, *Coregonus lavaretus*-MK913369, *Hucho bleekeri*-HM804473, *Prosopium cylindraceum*-JQ390062, *Salmo trutta*-MF621763, *Salvelinus albus*-KT266871, *Salvethymus svetovidovi*-MK695627, *Stenodus leucichthys*-JQ390059, *Thymallus thymallus*-MT410870). The only difference in the gene arrangement was in *oh* and *ol* regions, which are frequently not reported. This is similar to findings by Si et al. [79] that the gene content, genes arrangement, and base composition of two Lenoks (*Brachymystax* spp.) were nearly identical to most other teleosts. Similarly, Levin et al. [15] considered the gene arrangement of *Salmo* spp. to be similar to other vertebrates. The mitochondrial gene arrangement in the genus *Oncorhynchus* is therefore not a distinguishing characteristic for differentiation among species, as has been reported in some other fishes, such as *Chirostoma humboldtianum* [80] and *Anoplopoma fimbria* [81], as well as octopuses [82] and some gastropods of the Strombidae family [83]. This contrasts with other taxa, such as

gastropods of the Vermetidae family [83] or anomuran crustaceans [84], in which gene arrangement is relevant for the differentiating species or genera.

Table 1. Gene arrangement of the mitochondrial genome of the Mexican golden trout, *Oncorhynchus chrysogaster*, and Nelson's trout, *O. mykiss nelsoni*.

Name	*Oncorhynchus chrysogaster*						Strand [b]	*Oncorhynchus mykiss nelsoni*					
	Start Position	Stop Position	Length (bp)	Intergene Nucleotides [a]	Start Codon	Stop Codon		Start Position	Stop Position	Length (bp)	Intergene Nucleotides [a]	Start Codon	Stop Codon
tRNA-Phe	1	68	68	0			+	1	68	68	0		
12S rRNA	69	1015	947	0			+	69	1015	947	0		
tRNA-Val	1016	1087	72	21			+	1016	1087	72	21		
16S rRNA	1109	2767	1659	0			+	1109	2766	1658	0		
tRNA-Leu2	2768	2842	75	0			+	2767	2841	75	0		
ND1	2843	3814	972	3	ATG	TAG	+	2842	3813	972	3	ATG	TAG
tRNA-Ile	3818	3889	72	−3			+	3817	3888	72	−3		
tRNA-Gln	3887	3957	71	−1			−	3886	3956	71	−1		
tRNA-Met	3957	4025	69	0			+	3956	4024	69	0		
ND2	4026	5075	1050	1	ATG	TAA	+	4025	5074	1050	1	ATG	TAA
tRNA-Trp	5077	5148	72	0			+	5076	5147	72	0		
tRNA-Ala	5149	5217	69	1			−	5148	5216	69	1		
tRNA-Asn	5219	5291	73	3			−	5218	5290	73	3		
OL	5295	5325	31	0			−	5294	5329	36	0		
tRNA-Cys	5326	5392	67	0			−	5330	5396	67	0		
tRNA-Tyr	5393	5463	71	1			−	5397	5467	71	1		
COX1	5465	7015	1551	0	GTG	TAA	+	5469	7019	1551	0	GTG	TAA
tRNA-Ser2	7016	7086	71	4			−	7020	7090	71	4		
tRNA-Asp	7091	7164	74	14			+	7095	7168	74	14		
COX2	7179	7869	691	0	ATG	T	+	7183	7873	691	0	ATG	T
tRNA-Lys	7870	7943	74	1			+	7874	7947	74	1		
ATP8	7945	8112	168	−10	ATG	TAA	+	7949	8116	168	−10	ATG	TAA
ATP6	8103	8786	684	−1	ATG	TAA	+	8107	8790	684	−1	ATG	TAA
COX3	8786	9571	786	−1	ATG	TAA	+	8790	9575	786	−1	ATG	TAA
tRNA-Gly	9571	9640	70	0			+	9575	9644	70	0		
ND3	9641	9991	351	−2	ATG	TAG	+	9645	9995	351	−2	ATG	TAG
tRNA-Arg	9990	10,059	70	0			+	9994	10,063	70	0		
ND4L	10,060	10,356	297	−7	ATG	TAA	+	10,064	10,360	297	−7	ATG	TAA
ND4	10,350	11,730	1381	0	ATG	T	+	10,354	11,734	1381	0	ATG	T
tRNA-His	11,731	11,799	69	0			+	11,735	11,803	69	0		
tRNA-Ser1	11,800	11,868	69	1			+	11,804	11,872	69	1		
tRNA-Leu1	11,870	11,942	73	0			+	11,874	11,946	73	0		
ND5	11,943	13,781	1839	−4	ATG	TAA	+	11,947	13,785	1839	−4	ATG	TAA
ND6	13,778	14,299	522	0	ATG	TAG	−	13,782	14,303	522	0	ATG	TAG
tRNA-Glu	14,300	14,368	69	3			−	14,304	14,372	69	3		
CytB	14,372	15,512	1141	0	ATG	T	+	14,376	15,516	1141	0	ATG	T
tRNA-Thr	15,513	15,584	72	−1			+	15,517	15,588	72	−1		
tRNA-Pro	15,584	15,653	70	0			−	15,588	15,657	70	0		
OH	15,963	16,468	506				+	15,968	16,474	507			
CR putative	15,654	16,655	1002					15,658	16,661	1004			

[a] Intergene nucleotides indicates the number of nucleotides separating adjacent genes. Negative numbers indicate the number nucleotides that overlap between protein-coding genes. [b] Symbols indicate whether the gene was encoded on the heavy strand (+) or the light strand (−).

The total nucleotide composition of the entire mitochondrial genome in *O. chrysogaster* was as follows: 27.9% A, 26.2% T, 29.0% C, and 17.0% G, resulting in a 54.1% AT-content and a 45.9% GC-content. In *O. mykiss nelsoni*, the composition was: 27.9% A, 26.1% T, 29.0% C, and 17.0% G, for a 54.1% AT-content and a 45.9% GC-content. Overall, the AT-content detected in the two mitochondrial genomes is within the range described for other salmonid fishes. *Thymallus arcticus* exhibits the highest AT content reported, with 55.9%, while *Coregonus autumnalis*, *C. clupeaformis*, *C. lavaretus*, *C. ussuriensis*, and *C. peled*, have the lowest reported AT-content (52.4%) (Table S1).

The PCGs in the mitochondrial genome of *O. chrysogaster* and *O. mykiss nelsoni* comprise 11,430 nucleotides corresponding to 3810 total codons in both cases. For all but one of the PCGs, the start codon was ATG (12 PCGs: *nd1, nd2, cox2, atp8, atp6, cox3, nd3, nd4l, nd4, nd5, nd6, cytb*). The exception was *cox1*, whose start codon was GTG. The most frequent stop codon was TAA (six PCGs: *cox1, atp8, atp6, cox3, nd4l, nd5*); three PCGs used TAG (*nd1, nd3, nd6*); and four PCG presented a truncated stop codon T (*nd2, cox2, nd4, cytb*) (Table 1). The same pattern has been reported for *O. mykiss* [85], *O. tschawytscha* [24], *Hucho taimen* [86], *Brachymystax lenok tsinlingensis* [87], hybrids of *O. mykiss* × *Atlantic salmon* [77], *Thymallus arcticus grubei* [88], and *Salmo ischchan* [15]. The incomplete stop codon, common in the mitogenomes of teleost fishes, is completed later on via posttranscriptional polyadenylation [24,77,85–87].

For the PCGs, the relative synonymous codon usage (RSCU) and amino acid composition are summarized in Figure 2 for *O. chrysogaster* and *O. mykiss nelsoni*. In *O. chrysogaster*, the most frequently used codons (amino acids) were CTA (Leu), which was used 184 times (28%); CTT (Leu), used 152 times (23%); GCC (Ala) used 151 times (44%); and CTC (Leu) used 146 times (22%). The least commonly used codons (amino acids), excluding stop codons, included TGT (Cys) used eight times (30%), followed by CGG and CGT (Arg) used

eight times each (11%), and TCG (Ser) used nine times (4%). Meanwhile, in *O. mykiss nelsoni*, the most frequently used codons were CTA (Leu) used 183 times (28%); GCC (Ala) used 151 times (44%); CTT (Leu) used 151 times (23%); and CTC (Leu) used 150 times (23%). The least common were TGT (Cys) used eight times (3%); and CGG and CGT (Arg) used eight times each (11%). The RSCU and amino acid composition have been reported for PCGs in the salmonids *Brachymystax lenok tsinlingensis* and *Salvelinus malma*, in which the most frequent amino acid was Leu and the least common was Cys [87,89]. CTT was one of the most used codons, similar to other vertebrates; however, unlike other vertebrates, AAG is not the least-used codon [89,90].

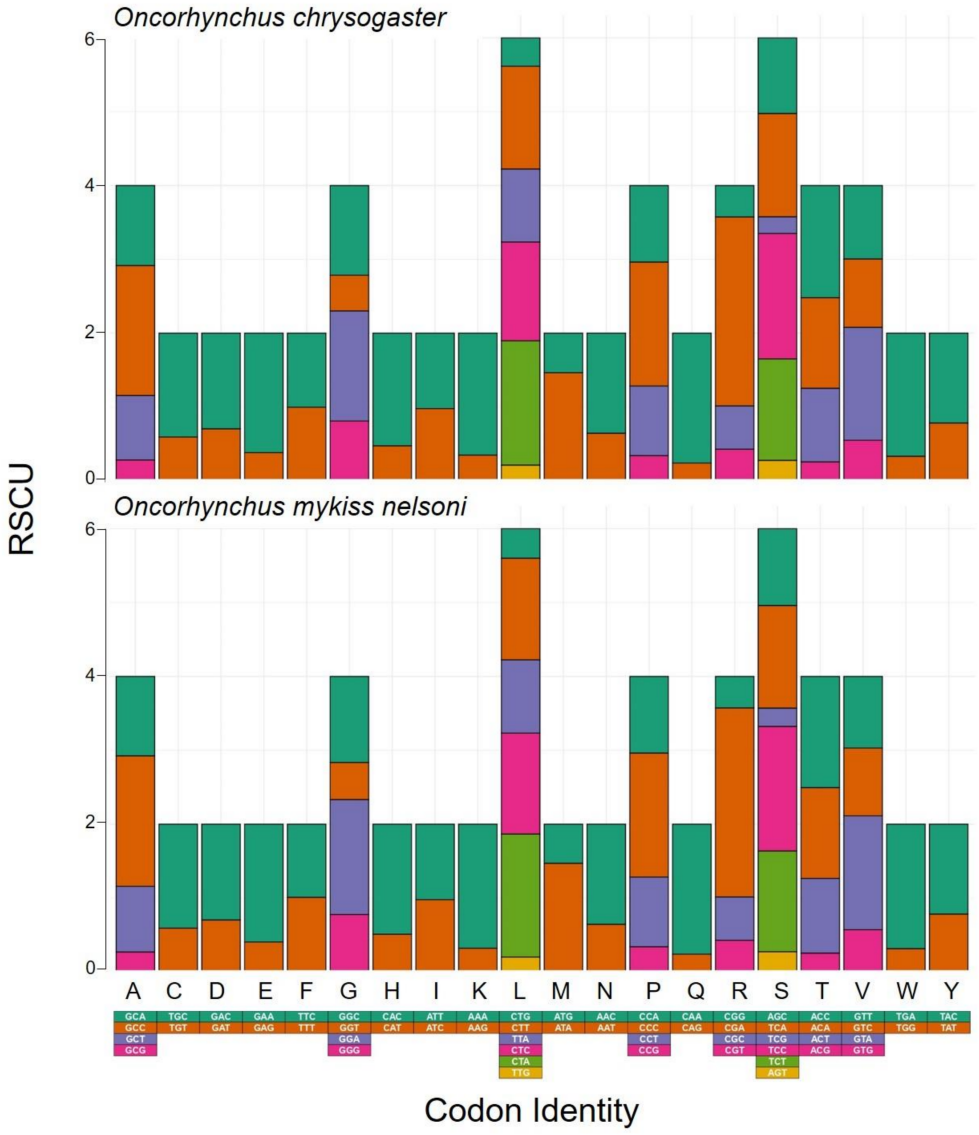

Figure 2. Relative synonymous codon usage (RSCU) in the Mexican golden trout, *Oncorhynchus chrysogaster*, and Nelson's trout, *O. mykiss nelsoni.* Letters represent the single letter abbreviation name of each amino acid.

The mitochondrial PCGs exhibited K_A/K_S ratio values <0.057 (p-value < 0.05 in all cases) for *O. chrysogaster* and *O. mykiss nelsoni*. In *O. chrysogaster*, the *nd6* gene had the highest K_A/K_S value (0.057), followed by the *nd5* and *nd2* genes (0.047 and 0.045, respectively). In contrast, the *atp8, cox* family genes, *nd4* and *cytb* showed zero or near zero values (Figure 3). In *O. mykiss nelsoni*, the *nd5* gene had the highest value (0.054), followed by *nd6* –0.048, *atp8*–0.046 and *nd2*–0.044. In contrast, again, the values for *cox1*, *cox3* and

cytb were closest to zero (Figure 3). The K_A/K_S values in these two species were more similar to those of the freshwater salmonids than to anadromous salmonids [19]. In general, the observed K_A/K_S values were lower than other fishes, such as cichlids (<0.143) [91] or Cobitinae (<0.120) [92]. These results indicate that all mitochondrial PCGs from both trouts analyzed are under purifying selection, which is higher in some cases, such as the *cox* and *cytb* genes. On the other hand, it is common for the *atp* gene to show more relaxed selection than *cox* family genes in vertebrates [93]; for this reason, the *cox* genes are potential barcoding markers [92].

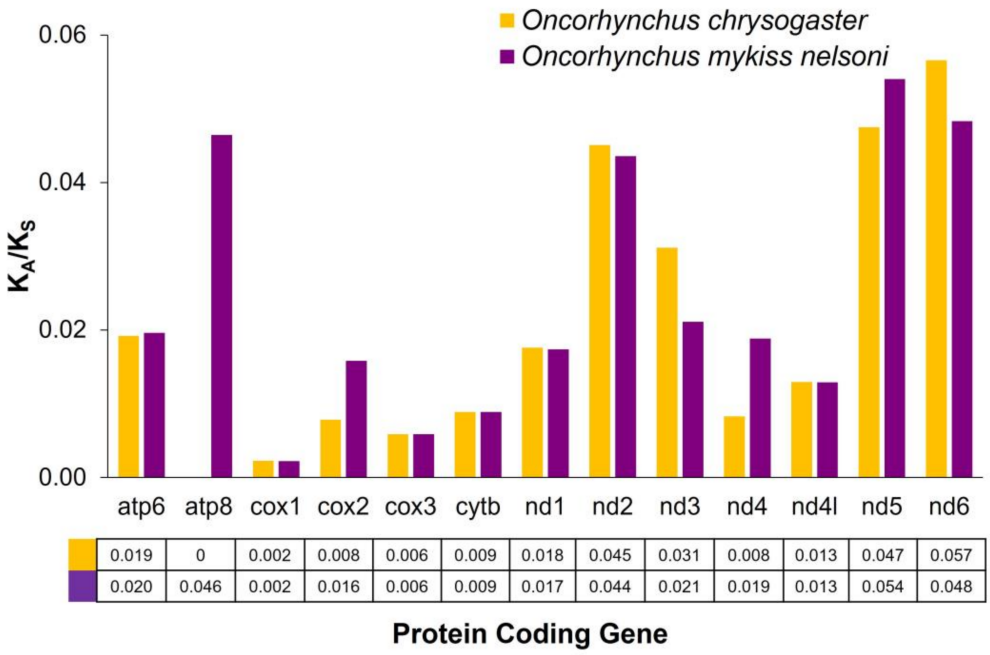

Figure 3. Selective pressure analysis of the protein-coding genes of the Mexican golden trout, *Oncorhynchus chrysogaster*, and Nelson's trout, *O. mykiss nelsoni*.

In the mitochondrial genome of *O. chrysogaster* and *O. mykiss nelsoni*, the length range of 22 tRNA genes was between from 67 bp to 75 bp. The 22 tRNA genes exhibited a typical 'cloverleaf' secondary structure (Figure 4), except the *tRNA-Ser1*, which has a truncated dihydroxyuridine (DHU) loop. This *tRNA-Ser* lost the DHU arm, and the nucleotides formed a simple loop. This loss is a common trait among metazoan mitogenomes, although it is not clear if it is reflected in a modification in their functions; mismatched base pairs are considered as irreversible, evolutionary-derived states, which might be caused by tRNA editing, as reported in Cobitinae and hybridized Salmonids [77,92]. However, *O. mykiss* and *O. tschawytscha* are an exception to this pattern because they have a complete DHU arm [24,85]. A similar pattern was reported in *Gadus morhua* and *Brachymystax lenok tsinlingensis*, except they also present a large anticodon stem [87,94]. Additionally, both trout species studied here showed slight differences from the previously described mitochondrial genome of *O. mykiss* in nine tRNA genes (*tRNA-Arg*, *tRNA-Asp*, *tRNA-Cys*, *tRNA-Glu*, *tRNA-Leu1*, *tRNA-Leu2*, *tRNA-Ser1*, *tRNA-Trp*, *tRNA-Val*). Two tRNA genes had one substitution (a transition and a transversion), four had an insertion of 1–2 nucleotides, and three had deletions of 1–3 nucleotides.

The *12S* rRNA gene length in *O. chrysogaster* and *O. mykiss nelsoni* was 947 pb with an AT-content of 49.5%. The length of the *16S* rRNA gene was 1659 and 1658 pb, with an AT-content of 51.7 and 51.6% in each species, respectively, due to one G deletion in *O. mykiss nelsoni.* Furthermore, as in other Salmonids, they were in the H-strand between *tRNA-Phe* and *tRNA-Leu2* and separated by *tRNA-Val.* For the rRNAs, the nucleotide usage has rarely been reported in Salmonids (AT-content 50.97%) [87], but the

AT-content in these two species was within the range found in other Salmonids (calculated from sequences published in GenBank; see Table S3). The Salmonids with the lowest AT content were *Prosopium cylindraceum* (*12S* rRNA = 48.8%), *Coregonus clupeaformis*, and *Stenodus leucichthys* (*16S* rRNA = 51.0%), while the highest values were found for *O. masou masou* (*12S* rRNA = 50.5%, *16S* rRNA = 52.9%) and *Salmo salar* (*12S* rRNA = 50.5%).

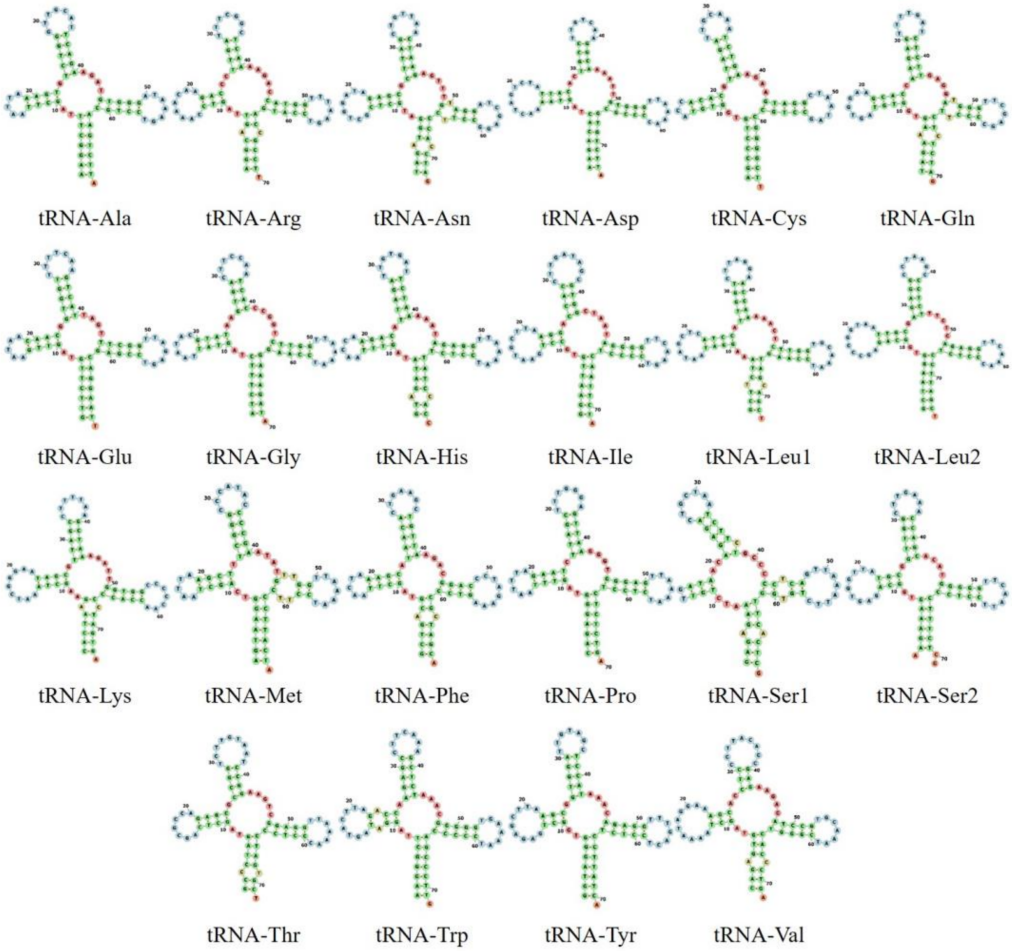

Figure 4. Secondary structures of 22 transfer RNA genes in the Mexican golden trout, *Oncorhynchus chrysogaster*, and Nelson's trout, *O. mykiss nelsoni*. All the secondary structures of the transfer RNA were identical between the two species, so are only shown once.

In *O. chrysogaster* and *O. mykiss nelsoni*, the putative control regions (CR) were 1002 bp and 1004 bp long, respectively, with a high AT content (61.5%). The putative CR was located between the *tRNA-Pro* and *tRNA-Phe* genes (Figure 1); this includes the origin of H-strand replication within it. For *O. chrysogaster*, the CR begins at position 15,654 and ends at position 16,655, while for *O. mykiss nelsoni*, it starts at position 15,658 and ends at position 16,661. The AT content in the CR estimated of other Salmonids, such as *O. masou formosanus* is 60.0% [13], and for *H. taimen* is 61.9% [86]. Our results indicate that the two trouts studied here exhibit an AT content within the range for other species of the family Salmonidae (range: 58.8–66.1% AT, 505–1339 bp long) (Table S4).

Another non-coding region, the origin of light-strand replication (*ol*) was in a cluster of five tRNA genes (the WANCY region) between *tRNA-Asn* and *tRNA-Cys* (Figure 1). The *ol* comprises 51 nucleotides in *O. chrysogaster* and 56 in *O. mykiss nelsoni*; this region can form a secondary structure with a loop of 13 nucleotides in *O. chrysogaster* and 18 nucleotides in *O. mykiss nelsoni*. The stem sequence is conserved among vertebrates, whereas the loop sequence is more variable [24,85,87]. Furthermore, there is a highly conserved motif (5′–GCCGG–3′) which is considered important in transcription and regulation in most

vertebrates [6,7,13,24,85–87]. The rainbow trout *ol* loop is noted for its large size and the presence of a stretch of cytosine residues, which differs from mammalian mtDNA sequences, in which this region presents a T-rich residues. Loss of this sequence during the evolution of vertebrates emphasizes the tendency of mtDNA towards a compact genome size [85].

In the BI tree, *O. mykiss* and *O. mykiss nelsoni* clustered into a single strongly supported clade (100%) that includes hybrids of *O. mykiss* x *Salmo salar* (Figure 5). *Oncorhynchus chrysogaster* clustered into this clade with 100% support. Meanwhile, *O. gilae* and *O. g. apache* grouped together (100%) into a sister clade of *O. mykiss* and *O. chrysogaster*. Interestingly, this is the first time that mitochondrial genomes of *O. gilae* have been included in a phylogenetic analysis. The close relationship between the clades *O. mykiss* and *O. gilae* with the monophyletic clade of *O. clarkii* was strongly supported, consistent with some previous studies using mitochondrial DNA [13,19,48] and other molecular markers [50]. The other cluster includes three clades were monophyletic and highly supported (100 %); *O. masou*; *O. kisutch* and *O. tshawytscha;* and *O. gorbuscha, O. keta and O. nerka*. The position of this last lineage has been less consistent among studies; in some studies, it has been placed as the most basal clade of the *Oncorhynchus* genus [8], while in others, it has been placed as an internal clade, close to *O. kisutch* and *O. tshawytscha* [3,13,14]. These three monophyletic groups, in turn, form a clade with a high support value (99%). Our results are in concordance with some previous studies [19,48,50], but inconsistent with other phylogenies reconstructed with mitochondrial genomes that found these clades to be independent but weakly supported [3,8,13]. Incorporation of the complete mitogenomes of the different species of the *Oncorhynchus* genus (including the Mexican native and undescribed species) and the correct outgroups will surely improve the understanding of the phylogenetic relationships of American salmonids.

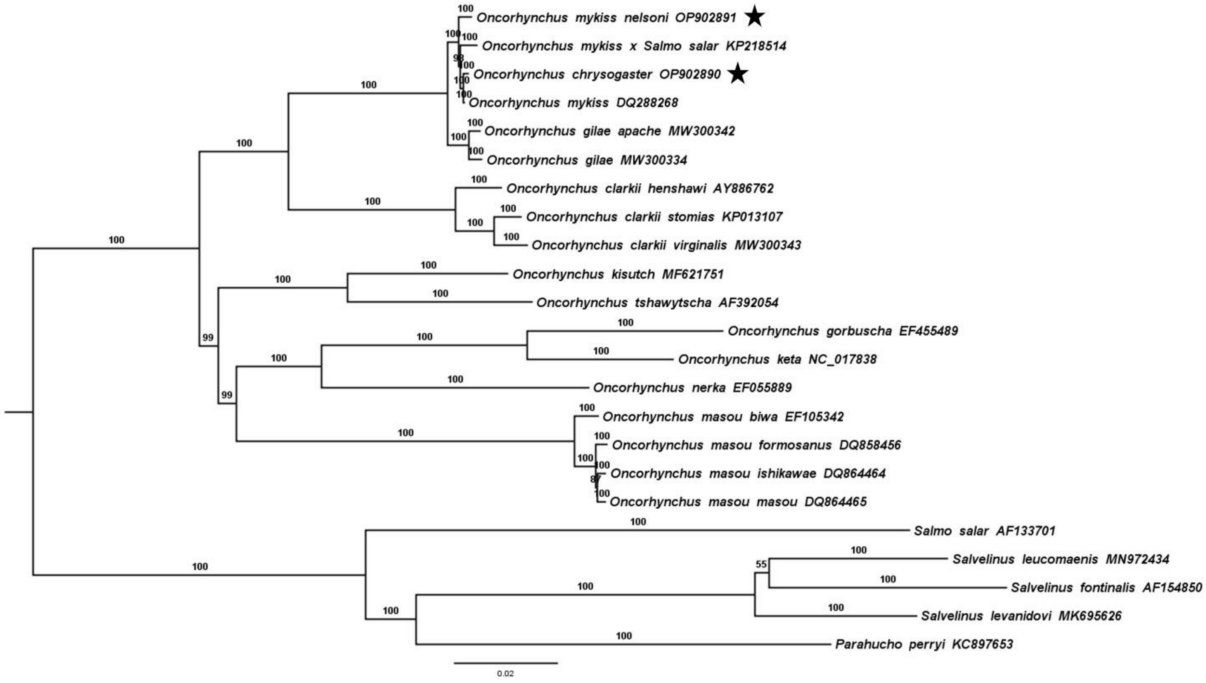

Figure 5. Phylogenetic tree obtained from the Bayesian inference (BI) analysis based on a concatenated alignment of nucleotides of the 13 protein-coding genes present in the mitochondrial genome of the Mexican golden trout *Oncorhynchus chrysogaster*, Nelson's trout *O. mykiss nelsoni*, and other representatives of the genus *Oncorhynchus*. The robustness of the BI tree topology was ascertained by 10,000 generations derived from the best-fit model (GTR+G+I) (percent node support is noted above each bar). The number before each species name is the GenBank accession number. Stars mark the mitogenomes obtained in this study.

The origin and diversification of the Mexican trouts have been subjects of controversy and interest for which there are currently three plausible hypotheses that all revolve around their diversification during the glaciations of the Pleistocene [51,53,95,96]. Behnke's hypothesis [51] sustains that they could be derived from an anadromous trout from the North Pacific that took refuge in the Gulf of California, entering the hydrographic basins that flowed into the Gulf and remaining isolated in the upper areas. This ancestral trout is proposed to have given rise to early radiation, including the taxon that evolved into *O. chrysogaster*, *O. gilae*, *O. apache* (now *O. g. apache*) and the undescribed trout of the SMO. Therefore, these trout share a common ancestor; this has been supported by the presence of primitive morphological characters shared by Rainbow trout (*O. mykiss*) and Cutthroat trout (*O. clarkii* spp.) [51]. A second hypothesis states that an ancestor of the Cutthroat trout moved up the Columbia River basin, then split into two groups. One branch became isolated and evolved into the Yellowstone cutthroat trout, which then moved down the Missouri River, returned, and crossed the Colorado River to the Sierra Mountains. These trout evolved into the California golden trout, which evolved into the Rainbow trout [96]. Finally, the trout forms from Sacramento could then have given rise to the mainland Mexican trout (different from the Rainbow trout group), which shared a common ancestor with the Gila trout inferred by similar characteristics like spotting pattern and coloration [53]. In a third hypothesis, Needham and Gard [29] proposed that Mexican golden, Gila, and Apache trouts could have a hybrid origin from Cutthroat trout and Rainbow trout in the Colorado River basin. However, this hypothesis has been dismissed since it was inconsistent with hybridization experiments [95].

Mayden et al. [28] reported a complete study of Mexican trouts' relationships to their close relatives using nuclear and mitochondrial genes. They found that *O. g. gilae*, *O. g. apache,* and the Mexican trout form a group that is distinct from *O. clarkii*; similar results were reported in another study where mitochondrial and nuclear genes were also used [50], consistent with the previous hypothesis of a common ancestor between these two groups. Our results with the PCGs also indicate a common ancestor between these groups. Mayden et al. [28] also found a sister relationship between *O. g. gilae* and *O. g. apache* and the southern Rio Conchos trout, supporting the close relationship between Gila and the Mexican trout [28]. All these Mexican trouts, which also include the undescribed trouts of the SMO, diverge into multiple independent lineages that are more closely related to *O. mykiss* from hatcheries than to the Gila lineage [28]. However, the relationship between the Gila lineage and the mainland Mexican trout must be clarified. The mitogenomes assembled de novo in this study support the separation of the Gila trout from the Mexican golden trout. However, the lack of a mitogenome of the Conchos trout makes it impossible to determine whether there is a close relationship between the Gila lineages and other SMO trout. Mayden et al. [28] established that the trout lineage from the SMO is native not a hatchery-reared strain or introduced fish and it constitutes a distinct native lineage from the Mexican golden trout. Therefore, our results support the Behnke hypothesis. However, the adding of mitogenomes from undescribed native Mexican trouts could better clarify the phylogenetic relationships of those SMO trouts.

4. Conclusions

Selective pressure analyses indicated that all mitochondrial PCGs are under purifying selection, which was especially evident for some genes (i.e., *cox* and *cytb*). These analyses are rarely conducted on salmonids. A phylogenomic analysis based on a concatenated alignment of nucleotides of PCGs was consistent with *O. mykiss nelsoni* being a true subspecies of the *O. mykiss* lineage. In addition, our phylogeny showed a sister relationship between *O. chrysogaster* and *O. mykiss nelsoni*, which confirms a common ancestor with North American trouts, as was proposed by Behnke [51]. Furthermore, the analysis found a close relationship between *O. mykiss* and the Gila trout, *O. gilae*, although contrary to expectations, *O. chrysogaster* did not cluster into the same clade as the Gila trout. To fully

resolve the relationships and origin of Mexican trouts, mitogenomes of the Conchos trout and others from the SMO will be required.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fishes8040178/s1, Table S1: Nucleotide composition of the complete mitochondrial genome in Salmonids; Table S2: Species used in the phylogenetic analysis; Table S3: Nucleotide composition of the *12S* and *16S* rRNA genes in Salmonids; Table S4: Nucleotide composition of the Control Region in Salmonids.

Author Contributions: A.C.: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, visualization; M.A.D.R.-P.: methodology, resources, data curation, writing—review and editing, supervision, funding acquisition; F.L.-D.l.C.: writing—review and editing, supervision, funding acquisition; G.I.-D.l.M.: writing—review and editing, resources, supervision; F.J.G.-D.L.: conceptualization, resources, writing—review and editing, visualization, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by SAGARPA-INAPESCA project number 2505160167, SAGARPA FIRCO-2012 project number GO/04/05/2012-02, and CICESE internal projects: O0F092 and O0F125.

Institutional Review Board Statement: This article does not contain any studies with human participants. All animals used in this study were collected with the permits to fishing (SGPA/DGVS/02485/13, SGPA/DGVS/02968/14 and SGPA/DGVS/05052/15) and issued by the SEMARNAT General Directorate of Wildlife.

Data Availability Statement: Datasets presented in this study can be found in the NCBI online repository (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov (accessed on 1 March 2023)).

Acknowledgments: The first author thanks the Consejo Nacional de Ciencia y Tecnología (CONACyT) for the fellowship (No. 1193058) granted to conduct postdoctoral research. Many thanks Gorgonio Ruiz Campos and Carmen Paniagua Chávez who helped us in the sampling and maintenance of the fishes. Finally, we thank the anonymous reviewers for their comments that improved the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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