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Mitochondrial DNA for Phylogeny Building: Assessing Individual and Grouped mtGenes as Proxies for the mtGenome in Platyrrhines

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ABSTRACT

Phylogenetic trees are analytic tools used in primate studies to elucidate evolutionary relationships. Because of its relative ease to sequence and rapid evolution compared to nuclear genomes, mitochondrial DNA is frequently used for phylogeny building. This project evaluated the effectiveness of using individual or grouped mitochondrial genes (mtGenes) as a proxy for the mitochondrial genome (mtGenome) in phylogeny building within two nested primate datasets, Cebidae and Platyrrhini, with differing divergence dates. mtGene utility rankings were determined based on congruence values to the mtGenome tree. mtGenes trees were also assessed on tree resolution and ability to sort nested clades. We found that most individual mtGenes, including ribosomal genes (12S and 16S), COX genes, most ND genes, and D-Loop are not appropriate for use as proxies for the mtGenome when tree building in either the Cebidae or Platyrrhini set. On average, grouped mtGenes outperformed individual mtGenes in both sets, and mtGene and grouped mtGene rankings varied between sets. Pairing CYB and COX3 together or pairing ND2 and CYB worked well in both the Cebidae set and the Platyrrhini set. We also found that nucleotide diversity is not a predictor of mtGene performance. Instead, it may be that unique mtGene or mtGene system evolutionary history impacts mtGene performance.

1 | Introduction

The mitochondrial genome is a popular genetic source for answering research questions across primate clades (Table 1). The mitochondrial genome (mtGenome) is a circular genome found within mitochondria that contains 13 protein-coding genes (12 on the heavy strand and 1, ND6, on the light strand), 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes encoding for all 20 amino acids, and 1 noncoding region (NCR) referred to as "D-Loop." All protein-coding genes relate to the oxidative phosphorylation pathways and are functionally and generally structurally conserved across animals (Desalle 2017). Mitochondrial DNA (mtDNA) has several practical advantages over nuclear DNA for phylogenetic inference. mtDNA has a faster rate of evolution due to the smaller effective population size, making mitochondrial genes (mtGenes) more informative than nuclear genes of the same length when investigating relationships within clades with young divergence times, such as at the species, genus, or potentially family level depending on the age of the clade (Desalle 2017). Due to the matrilineal inheritance of mitochondria, mtDNA analyses can provide alternative insight into demography and evolutionary history compared to nuclear DNA. Additionally, the whole mtGenome is small (~16.5 kb) compared to the nuclear genome, and as such, genome sequencing and data processing are more accessible.

Despite the surge in publicly available mtDNA, most platyrrhine species do not have available mtGenomes. Out of 171 recognized platyrrhine species (Estrada et al. 2017), we were

Summary

- From raw UCE sequence data, we extracted, annotated, and assembled 61 mitochondrial genomes of *Sapajus* and *Cebus* individuals under GenBank accession codes PP454502–PP454561.
- Phylogenetic inference using sets of mtGenes usually, but not always, produced phylogenies with greater congruence with the entire mitochondrial genome, indicating single mtGene derived phylogenies may not be reliable.
- Differences in the performance of mtGenes in approximating the mtGenome indicate researchers should choose which mtGenes to sample depending on the research question and population information.

only able to access mtGenomes for 85, including new sequences made available as part of this study. mtGenomes from wild individuals, from across conspecific populations, or between subspecies are even rarer to access, leaving many primatologists needing to sequence their own samples. Though the cost of sequencing the entire mtGenome has plummeted since its inception, the cost still poses a significant barrier to those with constrained resources. This might include researchers without access to high-income funding or who include molecular data as only a small aspect of their research question. Due to this, it is still common to see research using partial mtGenome data published today (see Table 1 for examples). Partial mtDNA usage is not only relevant to those who pursue it directly, but also to those who consume it: it is common to see citations of support for hypotheses that rely on partial mtDNA phylogenies. When partial mtDNA phylogenies are being used as evidence for the matrilineal evolutionary history, it becomes necessary to evaluate if such DNA can appropriately proxy the mtGenome.

mtDNA can have disadvantages within certain phylogenetic contexts. For older divergence times, its faster rate of evolution can lead to sequences becoming oversaturated with back or parallel mutations, potentially leading to sequence alignments that are difficult to interpret and model (Desalle 2017). This oversaturation additionally makes mtGenes susceptible to long-branch attraction, where two paraphyletic taxa are parsimoniously grouped together as sister taxa as a function of convergent evolution (Bergsten 2005). Long-branch attraction is typically thought to occur only at very deep divergences, but also has been implicated in primate infraorder phylogenetic studies (Schmitz et al. 2002), especially in regions with rapid evolution. Conversely, if a region has too few differences—either due to too short of a sequence or a slow rate of evolution—then the opposite problem may occur where there is not enough information to model an evolutionary history. This may result in trees with low resolution and not in congruence with currently accepted species' evolutionary histories (see Ruiz-García et al. 2019 for example).

Using sequences with slower rates of evolution may lead to a more accurate phylogenetic reconstruction by reducing the likelihood of homoplasy (Wu et al. 2000). Another approach may be to selectively use genes that have evolutionary characteristics tailored to the clade of interest. Though all mitochondrial genes are related to cellular oxidative phosphorylation and share characteristics from being located on the mitochondrial genome, each gene is differentiated in structure, role, and pathway involvement. This is even true across genes with similar functional roles, such as the COX genes (COX1, COX2, and COX3), which have different dN/dS ratios and overall nucleotide substitution rates and interact and coevolve with different nuclear genes (Wu et al. 2000). As such, the evolutionary constraints on mtGenes in a dataset may differ, and some may be more appropriate for certain analyses. This could be as simple as using a constrained less-diversified gene for groups with deep divergences (such as at the level of infraorder), or a rapidly changing gene for groups with younger divergences (such as within genera or species).

In the primate literature, some research has analyzed the appropriateness of specific mtGenes to particular applications. Researched topics include analyses of nucleotide substitution patterns (Zhao et al. 2012), of coevolution between mtGenes and nuclear genes (Wu et al. 2000), and of positive selection in mtGenes (Menezes et al. 2013). Outside of primates, variation and incongruency of mtGenes to mtGenomes have been investigated previously, such as in birds (Meiklejohn et al. 2014), bats (Zhang et al. 2021), and salamanders (Weisrock 2012). When considered alongside research that demonstrates differences between mtGenes across animal clades, such as in evolutionary rates and intragenomic heterogeneity levels (Wu et al. 2000; Morón-López et al. 2022), these studies highlight how clade differences can result in conflicting performance evaluations of the same mtGene. These conflicts prompt the need to robustly evaluate mtGene performance in phylogeny building, specifically within primates.

The main goals of this study are as follows: (1) to assess if individual genes or small subsets of the mtGenome can produce resolved trees (i.e., trees with confident bifurcation) at different divergence depths and (2) to assess the congruence between trees produced from individual and concatenated mtGenes to trees produced from entire mtGenomes. Our findings will aid in evaluating if past phylogenetic work in platyrrhines using single or grouped mtGenes as a proxy for the mtGenome is appropriate and give suggestions for future work using mtDNA samples for phylogenetic reconstruction. Additionally, our methods presented here can be used as a guideline for investigating questions of congruence in other types of DNA or clades, where we use a novel approach to evaluate gene performance through using multiple congruence metrics, assessing family sorting (in the Platyrrhini set), and considering gene tree resolution. We are also interested in determining if the paraphyly and poor congruence of mtDNA trees to morphological species hypotheses for Cebidae in previous papers (Lynch Alfaro et al. 2012, 2015; Lima et al. 2017; Ruiz-García et al. 2015, 2016, 2019) are due to the use of relatively short sequence lengths of mtDNA in these studies, or if the mtGenome similarly recovers paraphyly among several hypothesized species, indicating instead that hybridization or introgression better explains the relationships across these taxa even with full information from the mtGenome.

TABLE 1 | Examples of mtDNA genes used for phylogenetic analyses in primates, with a focus on Platyrrhini.

Group	mtGenes included	Citation
Strepsirrhines	CYB	Yoder et al. (1996)
	COX3, ND3, ND4, ND4L, tRNA	Pastorini et al. (2001)
	CYB, D-Loop	Penna et al. (2023)
	COX1, CYB, D-Loop, ND4	Blair et al. (2023)
Platyrrhines	16S	Horovitz and Meyer (1995)
	D-Loop, CYB	Jacobs et al. (1995)
	ND1	Tagliaro et al. (2005)
Atelidae	ATP6, ATP8, CYB	Cortés-Ortiz et al. (2003)
	COX1, COX2	Ruiz-García et al. (2014)
	CYB	Viana et al. (2015)
	COX1, COX2, CYB, ND4, ND5, tRNA	Ruiz-García et al. (2020)
	CYB	Povill et al. (2023)
Callitrichidae	16S	Araripe et al. (2008)
	CYB	Cezar et al. (2023)
	CYB, D-Loop	Porter et al. (2023)
Cebidae	CYB	Casado et al. (2010)
	12S, CYB	Lynch Alfaro et al. (2012)
	CYB, D-Loop	Mercês et al. (2015)
	CYB, D-Loop	Lynch Alfaro et al. (2015)
	D-Loop, COX1, COX2, CYB	Ruiz-García et al. (2016)
	CYB, D-Loop	Martins-Junior et al. (2018)
	COX1, COX2	Ruiz-García et al. (2019)
	COX1, COX2	Penedo et al. (2021)
	COX2, CYB, D-Loop	Szynwelski et al. (2024)
Pitheciidae	16S, COX1, CYB	Carneiro et al. (2020)
	COX1, CYB	Byrne et al. (2021)
	CYB	Ennes Silva et al. (2022)
	COX1, CYB	Carneiro et al. (2023)
Cercopithecoids	Protein mtGenes (except ATP8)	Wang et al. (2019)
Colobinae	ND3, ND4, ND4L, tRNA	Wang et al. (1997)
Papionini	COX1, COX2	Burrell et al. (2009)
Hylobatidae	ND3, ND4	Takacs et al. (2005)
	D-Loop, COX1	Trivedi et al. (2021)
Primates	tRNA, ND4, ND5	Hayasaka (1988)
	12S, 16S, COX2, CYB, ND3, ND4, ND4L	Chatterjee et al. (2009)

To determine how well different individual or concatenated mtGene trees represent the whole mtGenome phylogenetic tree at different scales of analysis, this study utilizes two nested clades, Platyrrhini and Cebidae, with different ranges of divergence dates. The crown platyrrhine infraorder is estimated to have diverged approximately 24 Mya (Beck et al. 2023), with the five crown families diverging approximately 21 Mya to 12 Mya (Beck et al. 2023). The crown Cebidae family, which includes the *Cebus*, *Sapajus*, and *Saimiri* genera, is estimated to have diverged between approximately 19.2 Mya and 14 Mya (Beck et al. 2023), and Cebinae (*Sapajus* and *Cebus*) are estimated to have diverged approximately 6 Mya (Lynch Alfaro et al. 2012). Within-genus divergences range from 3 Mya to < 1 Mya in cebines (Lynch Alfaro et al. 2012; Perez et al. 2013) and from 1.5 Mya to < 1 Mya in squirrel monkeys (Chiou et al. 2011).

2 | Methods

Overview: Sixty-one whole mtGenomes from Cebidae were assembled based on raw data from Lima et al. (2018), and

157 additional platyrrhine mtGenomes were downloaded from GenBank. We ran two analysis sets: the Cebidae set and the Platyrrhini set. The Cebidae set was used to evaluate the mtGene approximation of the mtGenome in tree building in more recently diverging sequences, whereas the Platyrrhini set was used to evaluate older divergences. Individual mtGenes and selected groupings of concatenated mtGenes were extracted from the genomes, aligned, partitioned, and then used to generate individual and grouped mtGene trees ("mtGene trees" and "grouped mtGene trees," respectively). mtGene and grouped mtGene trees were compared to the entire mtGenome tree ("mtGenome tree") using 11 metrics from Goluch et al. (2020) for determining rooted tree dissimilarity to evaluate the effectiveness of using individual or small groups of genes as a proxy for the entire mtGenome when tree building.

2.1 | Mitochondrial Sequences

We obtained 217 unique platyrrhine mtGenomes for phylogenetic reconstruction. Sixty-one whole mitochondrial genomes from *Cebus* and *Sapajus* individuals were sequenced through the Ultraconserved Elements project (Lima et al. 2018). The contigs from all individuals in this project were assembled and annotated in Geneious Prime v. 2023.1.2 (Biomatters Ltd.) using two reference individuals, *Sapajus xanthosternos* (Accession no. KC757410) for *Sapajus* samples and *Cebus albifrons* (Accession no. AJ309866) for *Cebus* samples. One sample with poor read quality was discarded. Genomes are available on Genbank under accession codes PP454502–PP454561. One hundred and fifty-seven assembled primate sequences were downloaded from GenBank, including one sample of *Theropithecus gelada* (Accession no. FJ85426) selected as an outgroup for the Platyrrhini set. Samples were divided into a Platyrrhini set ($n = 132$; 82 species including outgroup *T. gelada*) and a Cebidae set ($n = 120$; 22 species including outgroup *Mico argentatus*). Thirty-seven of the Cebidae sequences were used in both the Platyrrhini set and the Cebidae set. Sample information is included as Supporting Information at https://doi.org/10.5061/dryad.q2bvq83w8. Sequences were selected based on genome completeness and for a balanced representation of primate genera in the dataset. Primate taxonomy follows Brcko et al. (2022), Ennes Silva et al. (2022), Rylands and Mittermeier (2023), and Spironello et al. (2024). To avoid biasing results within the Platyrrhini set toward genes that produce similar within-species arrangements to the mtGenome phylogeny, only two individuals from each available species were included in the Platyrrhini set. This ensured that we were not considering within-species relationships in the Platyrrhini analyses, as the purpose of the Platyrrhini set analysis was to evaluate the effectiveness of using mtGenes as a proxy for the mtGenome in phylogeny building in deep between-family divergences, not recent divergences within species. In the Cebidae dataset, we opted to include all available good-quality sequences for the mtGenome phylogeny, even when that included sequences from multiple individuals from the same locality; this increased the number of polytomies in the dataset but allowed for the most complete sample of Cebidae mtGenomes available for comparison to other previously published mtDNA phylogenies of this taxonomic group.

2.2 | Alignment

To create individual mtGene alignments from known full mtGenome sequences, we isolated each of the 15 mtGenes (13 coding regions and 2 rRNAs) and D-Loop region from each of the mtGenome sequences. MtGene sequences were extracted from the mtGenomes as guided by their annotations. In cases where annotations did not include the same nucleotides across samples, the sequences were translated and reannotated to start at methionine and end at a stop codon for protein-coding mtGenes or trimmed or extended to match the majority of annotations for nonprotein-coding mtGenes.

For both sets, alignments were performed using Geneious Prime v. 2023.1.2 (Biomatters Ltd.) and are available in the Supporting Information at https://doi.org/10.5061/dryad.q2bvq83w8. We implemented the most appropriate algorithm available for each mtGenome region to ensure an accurate alignment that required little adjustment. Entire mtGenomes, rRNA genes ($n = 2$), and D-Loop were aligned using the Clustal Omega v.1.2.2 (Sievers et al. 2011) alignment feature. Protein coding genes ($n = 13$) were aligned using the Muscle v.5.1 (Edgar 2022) alignment feature. All gene alignments were additionally checked by eye and realigned if necessary. We were unable to confidently align the D-Loop sequences across the Platyrrhini set, as expected given its high rate of evolution and saturation in mammals (Pesole et al. 1999), so D-Loop was removed from the Platyrrhini analysis. Pairwise identity (identity of bases at the same position for sequence pairs over all possible pairings) and position identity (percent identity of all sequences at a position) were computed for each alignment using the Geneious Prime multiple sequences statistics tool. Both metrics are useful for understanding the level of genetic diversity across alignments, where higher position and pairwise identity indicate a higher proportion of conserved regions.

2.3 | Partitioning

To determine the best-fit model for tree generation, aligned sequences were partitioned based on the estimated similarity of evolutionary processes acting on nucleotide sites. Protein-coding genes were partitioned according to codon position, and rRNA mtGenes were partitioned based on stem and loop regions. Stems and loops were determined using ViennaRNA (v.2.5.1; Lorenz et al. 2011). The mtGenome alignments were partitioned by (1) gene; (2) partitions within the gene, such as codon position; (3) tRNA; and (4) a small number of introns. Best-fit nucleotide substitution models for all alignments were selected using PartitionFinder2 (Lanfear et al. 2017) using PhyML (Guindon et al. 2010) and the MrBayes models set, AICc model selection, and greedy (Lanfear et al. 2012) scheme search.

2.4 | Gene Groupings

We also concatenated several gene pairs or groups to construct trees, as many studies using mtDNA for phylogenetic tree building have done. This project examined eight mtGene groupings: (1) "rRNA," made up of 12S and 16S, the two rRNA genes on the mtGenome; (2) "Shortest," made up of the five shortest genes

(ATP8, COX2, ND3, ND4L, and ND6); (3) "COX_genes," made up of COX1, COX2, and COX3; (4) "ND_genes," made up of ND1, ND2, ND3, ND4, ND4L, ND5, and ND6; (5) "CYB_DLOOP"; and (6) "ND4_ND5," selected due to their past combined usage in primate phylogenetic studies (see Table 1); (7) "CYB_COX3"; and (8) "ND2_CYB." (7) and (8) were concatenations of the two highest ranking genes using rank averages in the preliminary analyses in the Cebidae and Platyrrhini sets, respectively. CYB_DLOOP was only run in the Cebidae set as D-Loop was unable to be aligned in the Platyrrhini set. Grouped mtGenes were formed by concatenating individual mtGenes; then, the concatenated sequence set was partitioned and modeled using the same parameters for its mtGene components.

2.5 | Tree Building

Three categories of trees were built: (1) mtGenome trees generated using the mtGenome alignments; (2) mtGene trees generated from individual mtGene alignments; and (3) grouped mtGene trees generated from concatenated mtGenes. All trees were generated using MrBayes on XSEDE (v.3.2.7a; Ronquist et al. 2012) via the CIPRES Science Gateway (V.3.3; Miller et al. 2011). All MrBayes analyses had two runs with 4 chains for the Metropolis-coupled Markov chain Monte Carlo analyses, with the chains sampled every 250 trees and the first 20% of cold-chain samples discarded (burn-in). The number of iterations varied between 4,000,000 and 30,000,000 depending on alignment size. For all analyses, the two runs converged using the recommended threshold of an average standard deviation of split below 0.01. Consensus trees from MrBayes were visualized using FigTree (v1.4.4; Rambaut 2018) and TreeGraph 2 (v.2.15.0-887 beta; Stöver and Müller 2010). Using TreeGraph 2, nodes were collapsed based on posterior probability node support, creating trees with a minimum support of 0.50, 0.75, and 0.90 to consider multiple levels of confidence. The number of polytomies at a given posterior probability threshold (PPT) was determined by counting the number of nodes with support lower than the PPT for each tree.

2.6 | Tree Comparisons

To evaluate the similarity of each mtGene tree and grouped mtGene tree to the mtGenome tree, mtGene trees were iteratively compared to the whole mtGenome tree using 11 rooted metrics (Table 2; modified from Goluch et al. 2020) available through Visual TreeCmp (Goluch et al. 2020). mtGene and grouped mtGene trees were ranked within each metric by dissimilarity score, and an overall ranking was established using the average z-score across all metrics. The highest-ranking genes had the lowest dissimilarity score, indicating high similarity between the mtGene or grouped mtGene tree and the mtGenome tree.

3 | Results

3.1 | Nucleotide Diversity

The mtGenome for Cebidae and Platyrrhini varied in percent pairwise identity and percent identity with additional variance between the Cebidae and Platyrrhini sets (Table 3). Percent pairwise identity ranged from 81.6 to 94.6 among Cebidae

TABLE 2 | Rooted distance metrics from Visual TreeCMP.

Metric	Summary	Citation
Triples	Number of three-taxa subtrees that differ between two trees	Critchlow et al. (1996)
Robinson–Foulds based on clusters (RF metric)	Number of data partitions that differ between two trees, thus the number of operations needed to transform one tree into another	Robinson and Foulds (1981)
Matching pair	A matching metric and extension of the RF metric based on pairs	Bogdanowicz and Giaro (2017)
Nodal split with L2 norm	Differences based on pathways from taxa in a pair to the LCA of the pair	Cardona et al. (2010)
Matching cluster	A matching metric and extension of the RF metric based on clusters	Bogdanowicz and Giaro (2013)
Rooted maximum subtree distance (MAST)	Finds agreement or "intersection" on the maximum number of species between trees	Farach and Thorup (1995)
Cophenetic with L2 norm	Compares cophenetic values, or LCA depth measurements, between trees	Cardona et al. (2013)
Weighted geodesic (BHV) rooted	Measures the shortest pathway between two trees in a continuous tree space	Owen and Provan (2011)
Weighted Robinson–Foulds based on clusters	See above, considers edge weights	Robinson and Foulds (1979)
Weighted nodal split with L2 norm	See above, considers edge weights	Cardona et al. (2010)
Weighted cophenetic with L2 norm	See above, considers edge weights	Cardona et al. (2013)

TABLE 3 | Percent pairwise identity and percent identity.

	Cebidae			Platyrrhini		
Gene	*Length*	*Percent pairwise identity*	*Percent identity*	*Length*	*Percent pairwise identity*	*Percent identity*
12S	963	94.6	80.6	978	88.3	55.8
16S	1561	92.7	72.2	1643	84.1	50.9
ATP6	681	89.8	60.2	681	81.4	39.9
ATP8	201	88.7	52.7	208	78.3	24.0
COX1	1558	91.5	67.5	1572	85.1	56.9
COX2	696	93.0	69.4	696	86.3	51.3
COX3	784	91.1	64.3	784	83.8	51.3
CYB	1140	89.5	58.0	1141	82.4	45.6
D-Loop	1192	81.6	36.5	—	—	—
ND1	957	90.8	62.6	957	83.5	46.7
ND2	1041	89.5	59.6	1042	81.5	36.6
ND3	348	88.6	54.9	346	80.8	38.2
ND4	1375	89.7	58.9	1378	81.5	41.1
ND4L	297	88.1	56.2	297	81.5	41.1
ND5	1809	89.6	59.3	1815	81.2	39.3
ND6	534	90.3	62.5	543	83.4	41.1

mtGenes and 78.3 to 88.3 among Platyrrhini mtGenes. Position identity ranged from 36.5 to 80.6 among Cebidae and 24.0 to 56.9 within the Platyrrhini set. The D-Loop region displayed the lowest percent pairwise identity and percent identity measure within Cebidae, whereas ATP8 was the lowest for the Platyrrhini set in both statistics. The 12S mtGene tree displayed the highest percent pairwise identity and percent identity within the Cebidae set and the highest percent identity within the Platyrrhini set. COX1 had the highest Platyrrhini percent pairwise identity. As expected, percent pairwise identity and percent identity were lower for all gene alignments in the Platyrrhini set than in the Cebidae set.

3.2 | Resolution of Trees

The mtGenome tree within the Cebidae set was not fully resolved, with 26 polytomies present (14 polytomies at a 0.50 PPT). Within *Cebus*, two polytomies were present at node heights 1 and 3. Within *Sapajus*, 22 polytomies were present, with most occurring within *Sapajus apella*, *Sapajus macrocephalus*, and *Sapajus cay*. Within *Saimiri*, two polytomies were present in a clade of only *Saimiri cassiquiarensis* samples. Individual mtGene trees showed poor resolution (0.50 PPT: $X = 61.7$; R: 24–86; 0.90 PPT: $X = 77.6$; R: 51–102), whereas grouped mtGene trees showed higher resolution (0.50 PPT: $X = 42.5$; R: 19–60; 0.90 PPT: $X = 54.0$; R: 39–73; see Figure 1A).

The mtGenome tree within the platyrrhine set was fully resolved at all PPTs except for a four-branched polytomy unable to resolve the relationships among *Sapajus flavius*, *S. macrocephalus*, *S. cay*, and *S. apella* individuals. Platyrrhini mtGene trees (0.50 PPT: $X = 20.1$; R: 11–43; 0.90 PPT: $X = 42.7$; R: 23–77) and Platyrrhini grouped mtGenes (0.50 PPT: $X = 7.3$; R: 3–11; 0.90 PPT: $X = 19.3$; R: 10–25) were well-resolved (Figure 1B).

3.3 | Congruence With the mtGenome Tree

Dissimilarity z-scores across three PPTs (0.50, 0.75, and 0.90; Supporting Information) revealed that mtGenes and grouped mtGene trees vary in how well they match to the mtGenome trees (0.50 and 0.90 PPT displayed in Figure 2, see Figure 3 for example). In Cebidae, six out of eight grouped mtGenes (ND2_CYB, ND_genes, ND4_ND5, Shortest, COX_genes, and CYB_COX3) produced trees largely congruent to the mtGenome tree. Among individual mtGene trees, ND5, ND2, and CYB displayed the lowest average dissimilarity z-scores. Calculating dissimilarity at different PPT thresholds affected average z-score, and thus gene rankings, due to differences in edge weights (for weighted metrics) and how metrics consider polytomies. However, the rank position was relatively constant across PPTs. Using the triples metric, a metric most useful for evaluating tree similarity where most variation occurs near the tips, such as in the Cebidae set, at a PPT of 0.90, the ND5 gene tree showed a 94.5% congruency rate with the mtGenome tree and ND2 showed a 95.0% congruency rate. These rates are competitive with the highest performing Cebidae grouped genes, ND2_CYB and ND_genes, which showed congruency rates of 98.9% and 97.7%, respectively, at 0.90 PPT. The lowest-ranking gene using the triples metric (12S) showed an 85.7% congruency rate. We found that mtGene length was a significant predictor of average mtGene incongruency to the mtGenome tree in the Cebidae set ($\beta = -0.0007$, $R^2 = 0.31$, $p = 0.025$), indicating a statistically significant negative relationship between gene length and incongruency. In the grouped

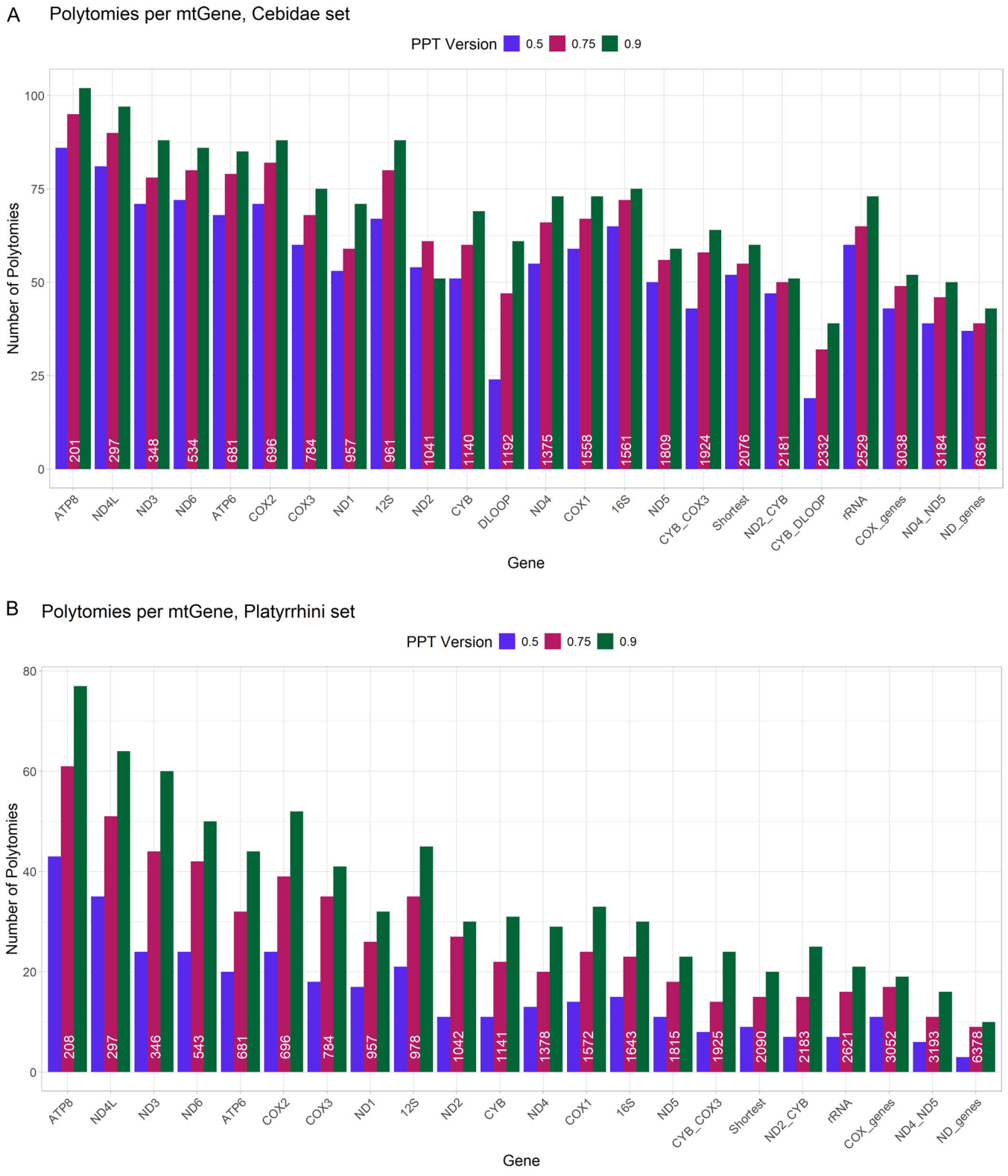

FIGURE 1 | Polytomies per mtGene, Cebidae (A) and Platyrrhine (B) sets. The number of polytomies (calculated as the number of nodes with support lower than the given PPT) plotted against mtGene. mtGenes are ordered by length of nucleotides, with length indicated horizontally on bars.

mtGenes, length did not predict incongruency ($\beta = -0.0002$, $R^2 = 0.16$, $p = 0.332$).

In Platyrrhini, there was considerable variation in how different genes organized the families, subfamilies, and genera (see Figure 4 for example). Most uncertainties in the Platyrrhini set occurred at deep divergences, such as in family sorting or placement of *Aotus*, with phylogenies placing *Aotus* as the sister group to Cebidae (mtGenome, ND2_CYB, and ND2), as the most basal platyrrhine (12S), as the sister group

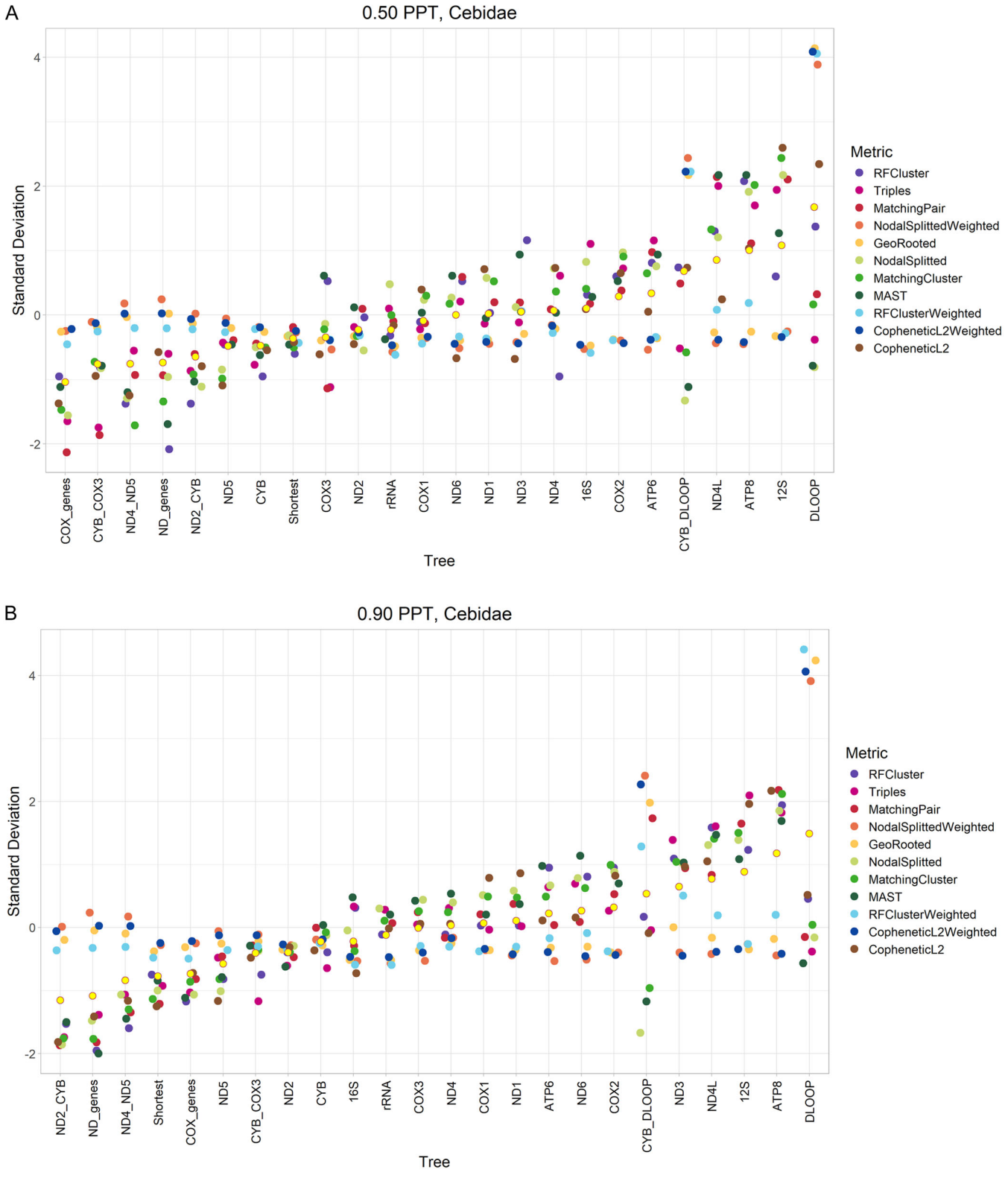

FIGURE 2 | Dissimilarity *z*-scores across metrics for each individual and grouped mtGene tree within Cebidae (A and B) and Platyrrhini (C and D) at 0.50 and 0.90 PPT. mtGenes ordered per chart from lowest average *z*-score (highest congruency with the mtGenome tree) to highest average *z*-score. The average *z*-score is indicated by a yellow circle with a red outline. PPT: posterior probability threshold.

to Pitheciidae (COX3 and ND1), as a sister to Callitrichidae (ATP6 and COX2), as a sister to Atelidae (ATP8, CYB, and ND6), or in other unique places (16S, COX1, ND3, ND4, and ND5).

Only nine mtGenes or grouped mtGenes produced trees that correctly placed all taxa from the known platyrrhine families within their respective clades (Table 4); of these, only CYB, CYB_COX3, and ND2_CYB were able to sort

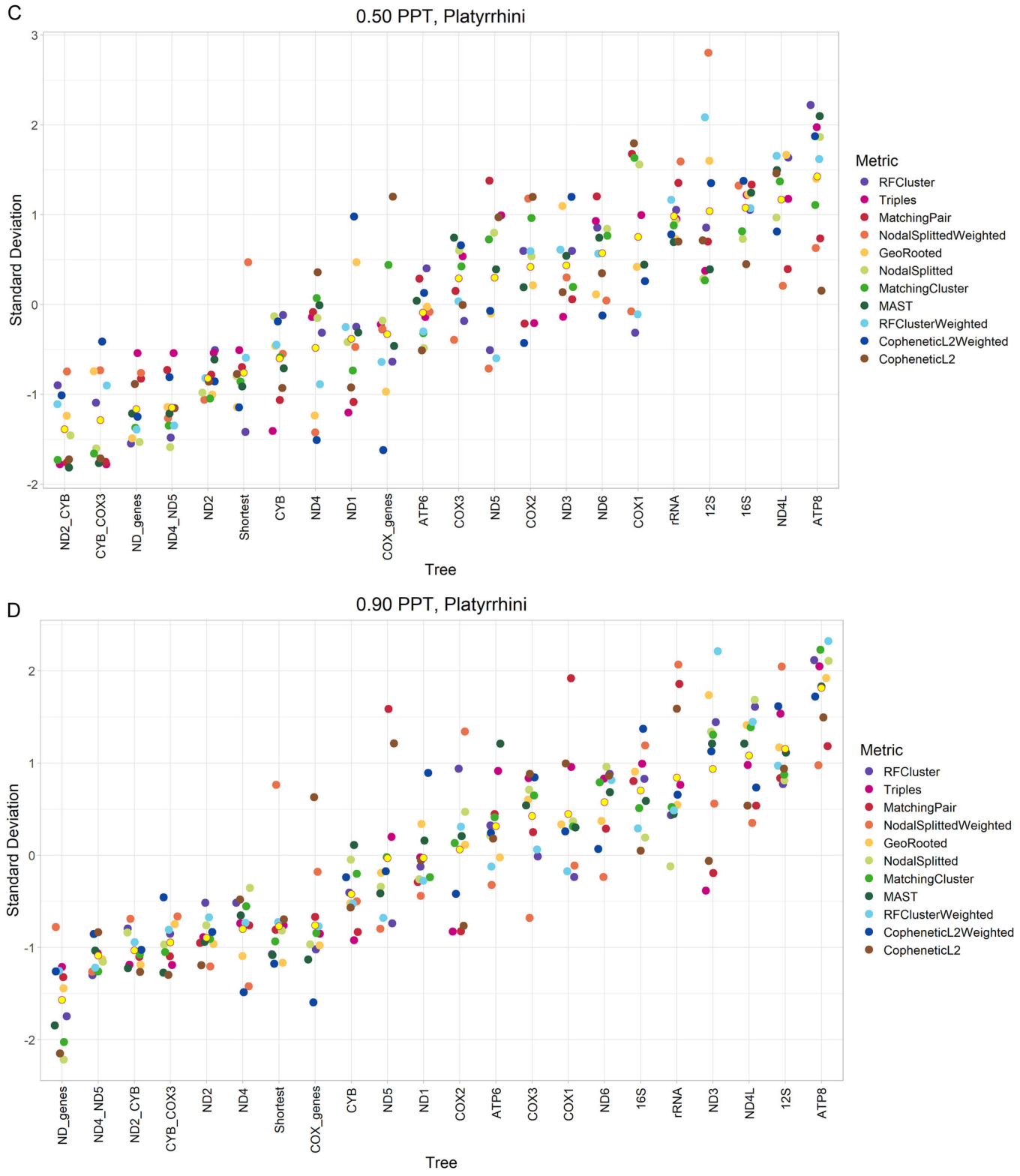

FIGURE 2 | (Continued)

genera within each subfamily as congruent with the entire mtGenome phylogeny: (Pitheciidae, (Atelidae, (Cebidae, Callitrichidae))). COX3 sorted families as (Atelidae, (Cebidae, (Callitrichidae, Pitheciidae))); ND1 sorted families as (Pitheciidae, (Cebidae, (Atelidae, Callitrichidae))); and ND_genes, Shortest, ND4_ND5, and ND2 sorted families as (Atelidae, (Pitheciidae, (Cebidae, Callitrichidae))). This does not include the placement of Aotidae due to difficulties in placement (see Section 4).

Though CYB did produce a tree with congruent topology in the Platyrrhini set, the posterior probabilities were slightly

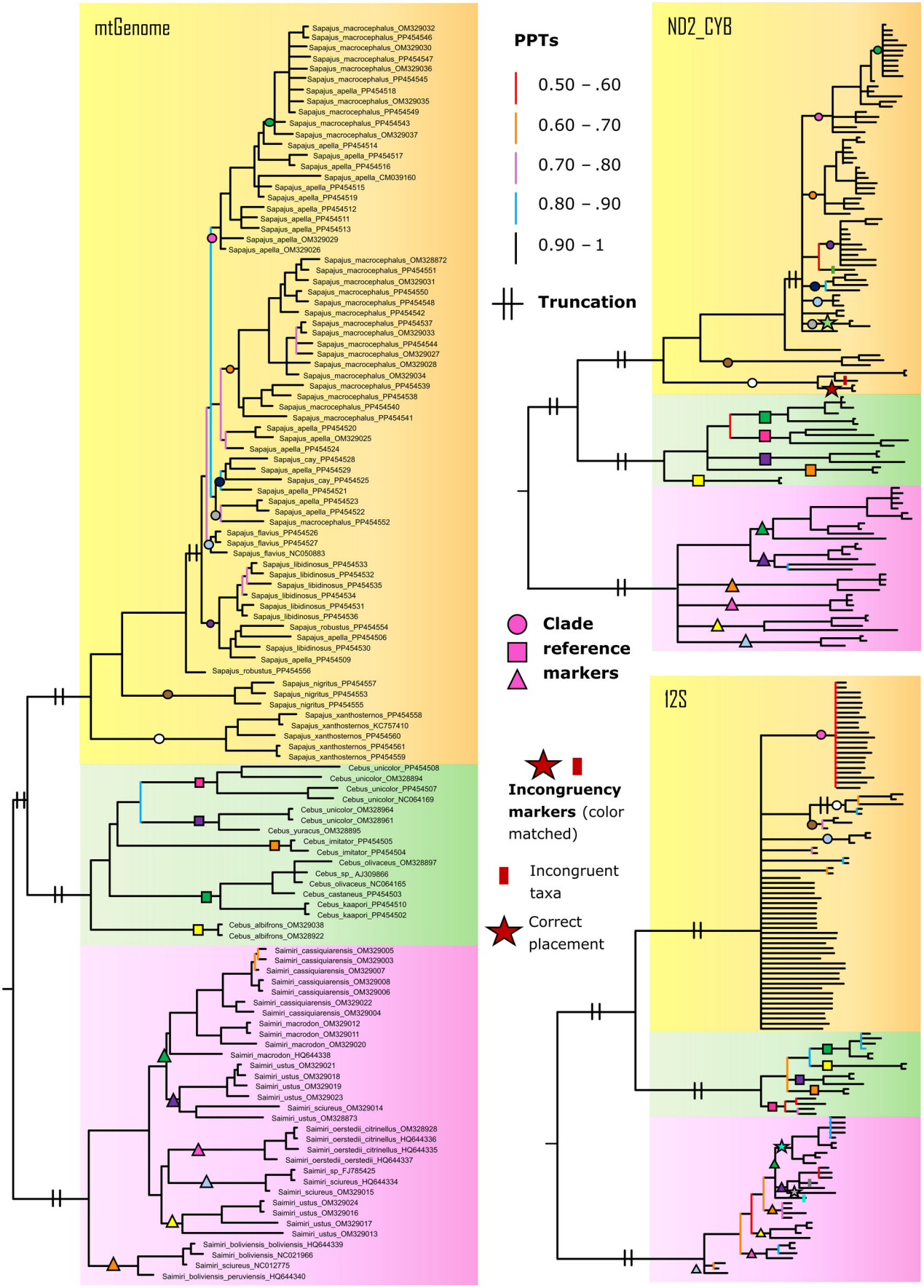

FIGURE 3 | Legend on next page.

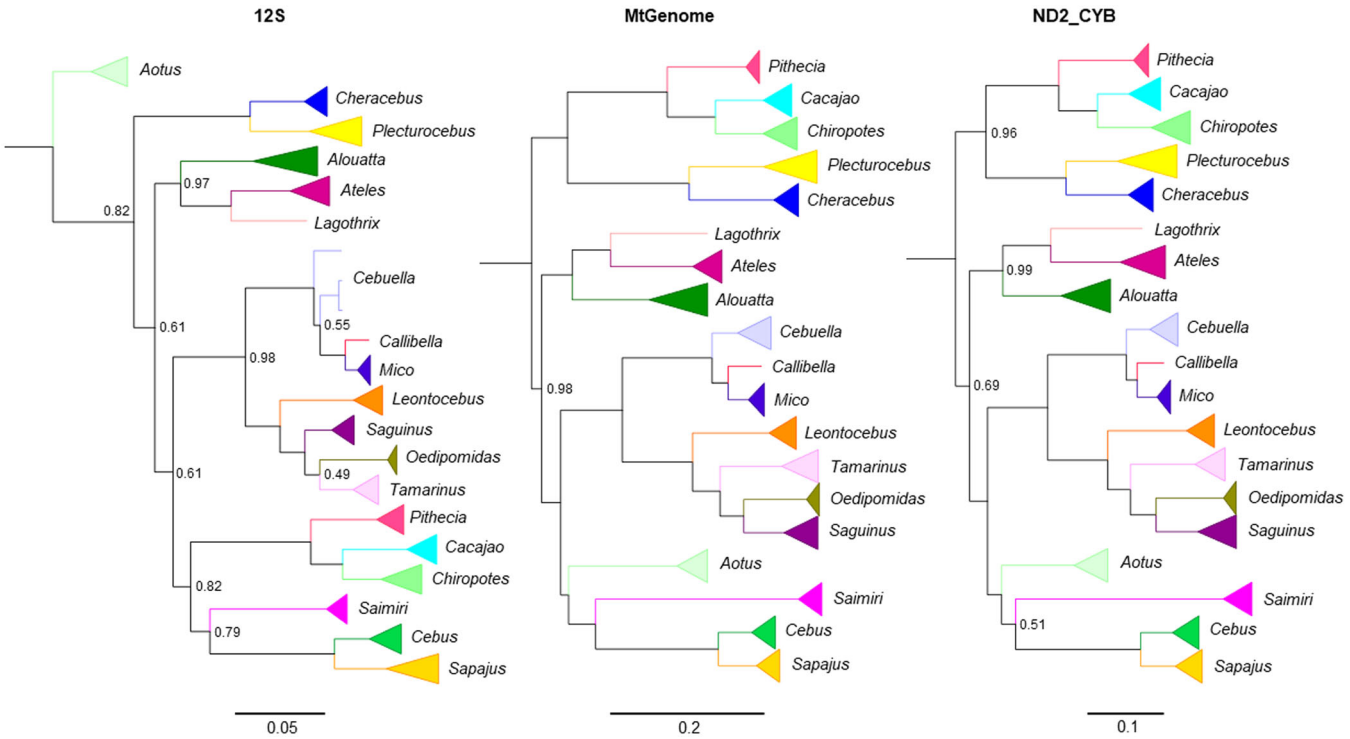

FIGURE 4 | Three Platyrrhini mtDNA Trees. The mtGenome tree, a mtGene tree (12S), and a grouped mtGene tree (ND2_CYB). Nodes with a posterior probability of less than one are displayed. Scale bars indicate genetic distance. ND2_CYB represents a mtGene tree with high congruence to the mtGenome tree topology; 12S represents a mtGene tree with poor congruence.

lower than the probabilities for both CYB_COX3 and ND2_CYB trees at all nodes. Additionally, the species topology in the CYB_COX3 and ND2_CYB trees were more similar to the mtGenome tree than the CYB tree was to the mtGenome tree, as indicated by the Visual TreeCMP dissimilarity scores. CYB performed better than all other individual mtGene trees and many of the grouped mtGene trees in family sorting, and ND2 outperformed all other mtGenes when considering all metrics and average z-score. Using the triples metric at a 0.50 PPT, ND2 and CYB showed an 81.3% (76.5% at 0.90 PPT) and 94.3% (76.9% at 0.90 PPT) congruency rate, respectively. Combined as ND2_CYB, the congruency rate increased to 99.99% at a 0.50 PPT and 80.6% congruency rate at a 0.90 PPT. We found that mtGene length was a moderate predictor of average mtGene congruency to the mtGenome tree in the Platyrrhini set at a 0.90 PPT ($\beta = -0.0008$, $R^2 = 0.27$, $p = 0.048$), indicating a statistically significant but weak negative relationship between gene length and incongruency. No significant correlation was found between the grouped mtGenes and length ($\beta = -0.0002$, $R^2 = 0.18$, $p = 0.341$). Overall, the mtGene groups ND_genes, ND4_ND5, ND2_CYB, and CYB_COX3, Shortest, as well as the individual mtGene ND2 ranked as most similar to the mtGenome tree in the Platyrrhini set. Like in Cebidae, slight differences in z-score averages arose across PPTs, but mtGene rankings were relatively constant.

4 | Discussion

4.1 | Gene Selection in mtGene Phylogeny Building

The low resolution of the individual Cebidae mtGenes in comparison to the mtGenome tree suggests that most individual mtGenes are not appropriate for phylogeny building, especially within the Cebidae set. On average, the individual Cebidae gene trees had 51.6 more polytomies than the entire mtGenome phylogeny, and the grouped Cebidae genes had 28.5 more polytomies at a 0.90 PPT (47.7 and 28.0 at a 0.50 PPT, respectively). For this reason, we recommend using more than one mtDNA gene to achieve a tree with higher confidence.

Some individual genes in the Cebidae set, such as ND5, ND2, 16S, and CYB, produced topologies mostly congruent with the mtGenome phylogeny. However, in Cebidae, when these mtGenes were combined, the resulting grouped mtGenes almost always outperformed the individual mtGenes in having a lower number of polytomies and higher congruence, the only exception being the rRNA set, where that set ranks lower than 16S alone at a 0.90 PPT, likely due to the inclusion of 12S, which is largely incongruent with the mtGenome tree. Together, these results suggest that using more than one gene will generally

FIGURE 3 | Cebidae mtGenome tree, ND2_CYB tree, and 12S tree. ND2_CYB is an example of a highly congruent tree, and 12S is an example of a highly incongruent tree. Colors indicate corresponding genera. Clade markers indicate where clades in the mtGenome tree appear in the mtGene trees if they appear. Incongruency markers highlight taxa or clades sorted incorrectly, with expected placement from mtGenome indicated by a color-matched star. Nodes with posterior probabilities less than 0.50 collapsed. Truncated branches shortened to fit. PPTs: posterior probability thresholds.

TABLE 4 | Sorting four families as clades in generated phylogenies.

Gene	Callitrichidae	Cebidae	Pitheciidae	Atelidae
12S	Y[1]	Y	N[2]	Y
16S	Y	N	N[2]	Y
rRNA	Y	N	N[2]	Y
ATP6	Y	Y	N[2]	Y
ATP8	Y[1]	N	N[2]	Y[3]
COX1	Y	N	Y	Y
COX2	N	N[4]	Y	N[5]
COX3	Y	Y	Y	Y
COX_genes	Y	Y	Y	N[5]
CYB	Y	Y	Y	Y
CYB_COX3	Y	Y	Y	Y
ND1	Y	Y	Y	Y
ND2	Y	Y	Y	Y
ND2_CYB	Y	Y	Y	Y
ND3	Y	N[3,4]	Y	N
ND4	Y	N	Y	N
ND4L	N	N	Y	N
ND5	Y	N	Y	Y
ND4_ND5	Y	Y	Y	Y
ND6	Y	N	Y	Y
ND_genes	Y	Y	Y	Y
Shortest	Y	Y	Y	Y

Abbreviations: N: No, taxa are not in a clade; Y: Yes, taxa are in a clade.
[1]Indicates genera incongruently sorted.
[2]In Pitheciidae, family is not in a clade but subfamilies (Pitheciinae and Callicebinae) are.
[3]*Aotus* included in the clade.
[4]In Cebidae, *Sapajus* as a clade within *Cebus*.
[5]*Lagothrix* as basal platyrrhine.

increase the resolution and accuracy of resultant phylogenies when looking at evolutionary relationships. Preferable genes for use include ND2, ND5, CYB, 16S, or COX3 due to their adequate congruency scores compared to other mtGenes. However, it may not be valuable to increase kb over ~3 kb, as after reaching a length of ~3 kb, the rate of change of the number of polytomies decreased and we did not see length as a predictor of grouped mtGene incongruency in either the Platyrrhini or Cebidae set. Like Meiklejohn et al. (2014), a similar study that investigated mtDNA in Galliformes, we found that while length was a predictor of mtGene performance in both the Cebidae set and the Platyrrhini set, the longest mtGenes were not necessarily the most informative for phylogeny building.

As found in the Cebidae set analyses, some mtGenes (ND2, ND4, and CYB) performed well in the Platyrrhini set but were generally outperformed by grouped mtGenes. On average, Platyrrhini mtGene trees had 40.7 more polytomies than the mtGenome tree, while grouped mtGene trees had 17.3 more polytomies than the mtGenome tree at a 0.90 PPT (20.1 and 7.3 at 0.50 PPT, respectively). On average, grouped mtGenes outperformed individual mtGenes in congruency. Additionally, many mtGenes misplaced the Aotidae family. However, this is not unexpected, as determining the exact phylogenetic placement of *Aotus* has been a long-standing question that appears to be unresolvable even when combining large genomic and morphological datasets (Valencia et al. 2018; Di Fiore et al. 2023). The best-supported hypothesis is that *Aotus* is more closely related to cebids than to pitheciids or atelids (Di Fiore et al. 2023), indicating that the mtGenome, ND2_CYB, and ND2 are most congruent with the evolutionary history of platyrrhines including *Aotus*.

Though CYB, ND2, and ND4 ranked well in mtGenome congruency as individual mtGenes in comparison to the other mtGenes in the Platyrrhini set, we suggest they are not appropriate for phylogeny building when used alone. CYB was able to sort families accurately to the mtGenome but failed to have congruence with the mtGenome genera relationships beyond that. ND2 and ND4, the overall best-performing mtGene trees across metrics within the Platyrrhine set, were highly congruent with the mtGenome tree between genera and species but failed to sort families. Notably, they inaccurately sorted Atelidae, instead of Pitheciidae, as the earliest diverging platyrrhine family. When combined, ND2 and CYB were able to accurately sort both the relationships between families and genera to the mtGenome tree with a 99.9% congruence rate.

4.2 | Comparisons to Similar Studies

As expected, when comparing our results to those of similar studies, we found that the mtGene rankings within platyrrhines and cebids were not congruent with the ranking orders reported elsewhere. However, studies that focused on taxa more closely related to primates tended to show more similar gene rankings to ours. In a genus of bats, Zhang et al. (2021) found that the best-performing mtGenes at approximating the mtGenome were CYB, ND4L, ND5, and ND6. AT6 and COX3 performed moderately, and all other mtGenes performed very poorly, unable to accurately recover even one major clade recovered by the entire mtGenome. This is somewhat consistent with our findings in Cebidae, where we also found that some genes like ND5 and CYB performed better than average, but that most individual mtGenes perform comparatively poorly. In Galliformes (Meiklejohn et al. 2014), like platyrrhines, ND2 and ND4 performed well. However, ATP6 and COX2 performed well in Galliformes but poorly in both groups in our analysis, while CYB, which performed poorly in Galliformes, performed well here. This may be due to clade-level variations, but also because the crown Galliform clade is approximately 70 million years old, much older than the platyrrhine clade. In annelids (Seixas et al. 2016), mtGenes CYB, COX1, COX3, ND1, ND6, and ND4L performed well. Except for CYB, these rankings were inconsistent with our findings in Platyrrhini. This is likely due to the ancient divergence between protostomes and deuterostomes leading to different evolutionary rates and constraints on mtGenes and that even the "shallow" nodes of annelids likely diverged in the Mesozoic, much older than the platyrrhine clade (Parry et al. 2014). Both factors together make it very unlikely that our rankings would align.

Taking phylogenetic similarity into account, then, it may be reasonable to use the Platyrrhini mtGene rankings to inform which mtGenes to sequence for use in phylogeny building in non-platyrrhine primates with deep divergences and to use the Cebidae mtGene rankings when phylogeny building in other platyrrhine families or in primate clades with a similar divergence date. We expect that our results may be applicable across platyrrhine families as they share commonalities in divergence and mtDNA evolution. Out of all mtGenes, only COX2 shows evidence of positive selection in platyrrhines (Menezes et al. 2013), and divergence times (Ma) for other crown platyrrhine families are not significantly different from that of Cebidae (14–19.2): Pitheciidae (18–24.6); Atelidae (13.5–18.6); Callitrichidae (8.4–14.9; Beck et al. 2023). To further test if the best-ranking gene combinations we found here are applicable to other primate clades, it would be useful to repeat our analyses in Malagasy strepsirrhines to compare to the Platyrrhini results and in other platyrrhine families (when a significant number of mtGenomes become available) to compare to the Cebidae results.

4.3 | mtGene Nucleotide Diversity Does Not Predict Congruence

The presented diversity data here are consistent with earlier findings. Nucleotide diversity, and therefore the level of evolutionary constraint, did not predict congruence to the mtGenome tree. Similarly, Morón-López et al. (2022) found that genetic distance within mtGenes did not necessarily lead to congruence with the mtGenome tree, especially for D-Loop. In this study, rRNA had the lowest total nucleotide diversity, followed by COX genes and CYB, and the highest nucleotide diversity occurred in ATP genes, ND genes, and D-Loop. D-Loop and ATP genes ranked relatively low in Cebidae across all PPTs, despite our prediction that a faster rate of change would be able to capture species differentiation. Instead, CYB and ND2, genes with moderate-to-low diversity, ranked well across PPTs in both analysis sets. ND5 performed well in the Cebidae set, and ND2 performed well in the Platyrrhine set, indicating set differences despite similar nucleotide diversity. When combined as a grouping, the conserved COX gene system consistently performed well within the Cebidae set, but not in the Platyrrhine set. rRNA, another highly conserved gene system, consistently performed poorly within the Cebidae and Platyrrhine sets.

Instead of nucleotide diversity, it may be that unique evolutionary changes within mtGenes and mtGene systems affect congruence with the mtGenome. In the family sub-tree analysis in the Platyrrhine set, some ND gene segments (ND4_ND5, ND5, and ND6) were able to sort the four families as clades except in Cebidae, where *Saimiri* and Cebinae were separated from each other. It may be that these ND genes in Cebidae have evolved in such a way that has altered the effectiveness of using ND genes in phylogeny building with cebid taxa. The rRNA genes and the ATP genes experienced a similar issue with the Pitheciidae clade, separating Pitheciinae and Callicebinae. It may be helpful in the future to repeat these analyses with a focus on other mammalian families to elucidate mtGene system evolution.

4.4 | Advances in Understanding Cebidae Phylogeny: Species Relationships and Introgression

An ongoing debate within Cebidae taxonomy includes the number of species recognized and the degree of introgression that has occurred across species; one limitation to this research has been the use of a small number of mtGenes for phylogeny building in many of the phylogenetic studies of Cebidae. Here, we present the most extensive mtGenome phylogeny for Cebidae to date. Even using all the mtGenome data (~16.5 kb), we did not recover monophyletic clades for any of these species: *Sapajus apella*, *S. macrocephalus*, and *S. cay*. Instead, we recovered several well-supported clades, each containing a mix of samples from more than one of these species (see Figure 3). The absence of clear phylogenetic lineages in our dataset is not unexpected, as trees built using nuclear DNA (Lima et al. 2018; Martins et al. 2023) and mtDNA (Lynch Alfaro et al. 2012; Lima et al. 2017; Ruiz-García et al. 2015, 2016, 2019; Martins-Junior et al. 2018) are unable to fully resolve species-level divergences within *Sapajus*, which suggests it may be appropriate to group *S. apella*, *S. macrocephalus*, and *S. cay* into one species. However, some studies focusing on morphology continue to support these lineages as distinct taxa (Cáceres et al. 2014), and a genome-wide sampling study uses species delimitation analyses to argue for retaining at least eight species of *Sapajus* (Martins et al. 2023). Our study identified that even the mtGenome tree for Cebidae does not resolve all currently recognized *Sapajus* species into distinct clades and verified that the species relationships in the mtGenome tree are not congruent with those in tree topologies found using nuclear genomic data, such as

Lima et al. (2018) using Ultraconserved Elements and Martins et al. (2023) using ddRAD genomic sampling.

Species in *Cebus* were generally well-resolved in the mtGenome tree topology, though *Cebus unicolor* was recovered as paraphyletic, with some individuals grouping in a clade with *Cebus yuracus* (as found in Boubli et al. 2012 using CYB and D-Loop markers). There are still several species of *Cebus* for which mtGenomes are unavailable. For *Saimiri*, as found in the CYB + D-Loop mtDNA phylogeny in Lynch Alfaro et al. (2015), *S. ustus* and *S. macrodon* are both polyphyletic; the rest of the mtGenome topology is highly congruent to the CYB + D-Loop topology, but in strong contrast to the nuclear phylogeny based on ddRAD sequences (Mercês et al. 2020), suggesting substantial introgression, gene swamping, and incomplete lineage sorting have occurred in the short evolutionary history of extant *Saimiri* diversification. This study may help to determine whether some morphotypes within Cebidae that are currently considered to be species should be accommodated as junior synonyms. It is likely there is a limit to the possible resolution of Cebidae mtGenome phylogenies, in part due to the very recent diversification of some morphologically distinct taxa, as even genome-wide SNP markers lead to inconsistent species groupings, perhaps indicating uncertainty in within-genus level divergences, which may account for this sorting (Martins et al. 2023).

4.5 | Polytomies and Gene Rankings

Certain metrics, such as the Robinson–Foulds metric and other pairwise comparative metrics, penalize the presence of polytomies by marking the internal branch of the resolved tree and the ambiguous multifurcating branch as incongruent. Thus, a higher distance value may be calculated between a resolved tree and an ambiguous tree than between two resolved trees with conflicting branching structures (Simmons et al. 2023). Therefore, increasing or decreasing the threshold at which to collapse nodes into polytomies influences the overall similarity score between gene trees and the entire genome tree, where trees with high confidence but low topological congruence may rank higher than trees with uncertainty but more accurate topological congruence. Relatedly, increasing the threshold at which to collapse nodes of the reference tree may lead to a higher congruence value by increasing the number of polytomies, which in turn leads to nodes with low resolution in a comparison tree able to be calculated as identical and, therefore, congruent. For this reason, and because some mtGene trees performed very similarly (such as in the top-ranking mtGene trees in Cebidae), some genes showed movement in the rankings as PPTs were adjusted.

A similar issue where congruency scores may be affected in undesirable ways arising from polytomies can occur when a gene tree is more resolved than the reference tree, which is possible if the reference tree uses a collection of genes that have conflicting gene histories, such as from incomplete lineage sorting, differing recombination rates, or a significant introduction of noise. Because the Cebidae mtGenome tree had a high number of polytomies, this was a potential concern, though it did not occur as a problem in our analyses—there were no mtGene or grouped mtGene trees that showed higher resolution than the reference tree. While many mechanisms that result in conflicting gene histories in nuclear genes do not affect mitochondrial DNA, hybridization and introgression can, and if investigating these types of questions, it is generally advisable to use the entire mitochondrial genome. We highlight the importance of considering multiple congruency metrics when determining which trees best match a reference tree, as relying on one metric or one class of metrics (e.g., pair comparison, LCA distance, and cluster analysis) may produce different results depending on how they treat polytomies and other tree features.

4.6 | Conclusions

This project was primarily aimed at determining appropriate mtGenes as proxies for the entire mtGenome within platyrrhines. However, the topology of trees based on entire mtGenomes does not always correspond to an accurate evolutionary history of taxa diversification. For this reason, it is important to recognize that the genes presented here as the "best proxies" are proxies only for the mtGenome and do not necessarily reflect the true evolutionary history, especially as the mtGenome is matrilineally inherited and has a different history than nuclear DNA. This is especially relevant when looking within species, where we cannot corroborate genealogical relationships without knowledge of within-species relationships, or in cases where it is unclear if a species is missorted or mislabeled or has matrilineal ancestry in another species due to past hybridization. In the future, it may be interesting to compare different mtGenes to a known extended pedigree—such as within long-standing captive populations—or to genetically characterized geographic populations (i.e., through microsatellites) to assess different mtGenes' comparative ability to serve as population identification tools. For instance, we might assess the ability of D-Loop to inform within-species population structure (such as in Szynwelski et al. 2024) across different primate taxa.

Except regarding the phylogenetic placement of Aotidae (Di Fiore et al. 2023), most recent studies using a plethora of evidence agree on a platyrrhine family phylogeny of (Pitheciidae, (Atelidae, (Callitrichidae, Cebidae))) (Beck et al. 2023; Shao et al. 2023), which was also the topology that we recovered in the platyrrhine mtGenome phylogeny. Additionally, the genus-level topology presented here is consistent with that presented in Kuderna et al. (2023) using whole nuclear genome sequences. That is, this study was able to assess not only the accuracy of genes as proxies to the entire mtGenome but also the accuracy of the mtGenome tree topology as reflecting the accepted evolutionary history at the levels of genus, subfamily, family, and infraorder in platyrrhines.

Author Contributions

Natalie Finnegan: data curation (equal), formal analysis (lead), investigation (lead), methodology (lead), visualization (lead), writing – original draft (lead), writing – review and editing (equal). **Marcela G. M. Lima:** conceptualization (equal), data curation (equal), writing – review and editing (equal), contribution of 61 original mitochondrial genome reads for use in this project. **Jessica W. Lynch:** conceptualization (lead), methodology (supporting), supervision (lead), writing – original draft (supporting), writing – review and editing (equal).

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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

The data that supports the findings of this study are available in the Supporting Information of this article. Assembled genomes are available on GenBank under accessions PP454502–PP454561. Supporting Information can be found at https://doi.org/10.5061/dryad.q2bvq83w8.

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