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Original Research

Phylogenetic Analysis Between *Puma concolor* and the Genus *Panthera* within the Family Felidae based on COI Gene

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Abstract

Puma concolor exhibits morphological similarities to large cats of the genus *Panthera*, which often lead to ambiguity in assessing its phylogenetic relationships. This study aimed to analyze the phylogenetic position of *Puma concolor* within the Family Felidae using the mitochondrial gene *Cytochrome Oxidase Subunit I* (COI). Phylogenetic analyses were conducted on 28 Felidae species with three outgroup species using MEGA4 and PAUP*. The results showed that *Puma concolor* is clearly separated from the *Panthera* clade and occupies an independent phylogenetic position, supported by bootstrap values as well as Consistency Index and Retention Index values greater than 0.50. These findings confirm that the morphological similarities between pumas and large cats are the result of convergent evolution and support the classification of *Puma concolor* within the Subfamily Felinae.

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Introduction

The Felidae family is a family in the Carnivora order that includes all cat species, from domestic cats to large wild cats such as tigers and lions. Biologically, felidae are characterized by a slender and flexible body, digitigrade feet with retractable claws, sharp carnivorous teeth, and excellent night vision, making them obligate carnivores and efficient predators. Recent genetic analysis based on the complete mitogenomes of 37 species shows that Felidae is divided into two main clades/subfamilies, namely Pantherinae (big cats) and Felinae (small-medium cats). The morphology between species is relatively conservative, making it difficult to distinguish them based on physical appearance alone (Yu et al., 2025).

Puma concolor, commonly known as the puma or cougar, is one of the members of the Felidae family with the widest geographical distribution in the Americas, ranging from Canada to South America (Culver et al., 2000). This species exhibits unique genetic diversity and complex evolutionary history patterns due to Pleistocene biogeographic dynamics, including recolonization events and megafauna extinctions that affected its distribution and genetic structure (Barnett et al., 2005). *Puma concolor* is a large carnivore native to the Americas, with the widest distribution of any

terrestrial mammal on the continent, from Canada to South America, and has an evolutionary history influenced by biogeographic dynamics during the Pleistocene (Culver et al., 2000). Although taxonomically classified as a member of the subfamily Felinae, the puma exhibits a number of morphological characteristics similar to members of the genus *Panthera*, such as a large body and ambush hunting patterns, similar to lions, tigers, or leopards (O'Brien & Johnson, 2007). These similarities are largely the result of convergent evolution, in which similar forms and behaviors develop without close genetic relationships (Johnson et al., 2006).

However, various phylogenetic studies reveal that *Puma concolor* consistently belongs to the *Puma* lineage along with the cheetah (*Acinonyx jubatus*) and jaguarundi (*Herpailurus yagouaroundi*), which separated from the *Panthera* lineage approximately 6–10 million years ago (Johnson et al., 2006). The main difference between the two is also seen in the structure of the hyoid bone. The *Panthera* genus has a partially ossified hyoid bone, allowing it to roar, while the puma has an ossified hyoid bone and can only purr like other small cats (Weissengruber et al., 2002). Furthermore, morphological studies of the skull show that the mandibular structure, jaw shape, and skull proportions of *Panthera* are much more robust than those of the puma, reflecting substantial evolutionary differences between the two (Christiansen, 2008). Therefore, although pumas share some external similarities with big cats, consistent genetic, vocal, and anatomical traits place them in the subfamily Felinae and explain why *Puma concolor* is not classified in the genus *Panthera*. Understanding these differences and similarities is crucial for supporting further phylogenetic research, particularly analyses based on mitochondrial genes such as Cytochrome c Oxidase Subunit I (COI), which are useful for evaluating phylogenetic relationships within the Felidae family (Luo et al., 2004).

Molecular data based phylogenetic approaches such as DNA sequencing are considered more precise and stable than conventional morphological analysis. The reason is that all inherited and expressed morphological traits have been fully encoded in DNA molecules. This method utilizes DNA markers, namely short DNA sequence fragments from standardized genomic regions (Hebert et al., 2003), to accurately identify species.

When morphological characters are considered unreliable for distinguishing closely related species or species with overlapping characteristics, a genetics-based identification approach allows unknown samples to be compared with a reference database of target gene sequences (Hsieh et al., 2001). This approach makes extensive use of mitochondrial DNA (mtDNA) loci, which are known to exhibit interspecific variation and are therefore informative for species separation (Tobe, Kitchener, & Linacre, 2010). One of the most frequently used mitochondrial genes as a genetic marker in species identification is cytochrome b, due to its level of variability being suitable for interspecies resolution (Verma & Singh, 2003).

The main advantage of mitochondrial markers is the large number of mtDNA copies per cell, which increases the chances of successful DNA recovery from small or damaged samples compared to nuclear DNA (Budowle et al., 2003). This makes mtDNA markers particularly useful in biodiversity studies and forensic contexts where DNA quality is often compromised (Parson & Bandelt, 2007). In addition to the cytochrome b gene, advances in DNA barcoding technology have also shown that the cytochrome oxidase subunit I (cytochrome oxidase I or COI) mtDNA gene has the potential to be a widely used barcoding marker for most animal groups (Hebert et al., 2003). The use of COI has become increasingly reliable when supported by the existence of global reference databases such as BOLD, which integrates sequences and taxonomic information for species identification purposes (Ratnasingham & Hebert, 2007).

Method

2.1. Materials

This study used secondary data obtained from the National Center for Biotechnology Information (NCBI) (Table 1). The data used were nucleotide sequences of the Cytochrome Oxidase I (COI) gene from mitochondrial DNA. The ingroup specimens consist of *Puma concolor* and twenty-

eight species from the Felidae Family consisting of the Subfamilies Felinae and Pantherinae. The Felinae Subfamily is represented by species from the genera Ly*nx, Prionailurus, Leopardus, Leptailurus, Catopuma, Puma, Otocolobus, Felis, Acinonyx, Herpailurus, Caracal, Profelis*, and *Leopardus*. Meanwhile, the Pantherinae Subfamily is represented by the genera *Panthera* and *Neofelis*. Three outgroup species were selected from two Families, namely Canidae and Ursidae. These species include *Canis lupus*, *Urocyon cinereoargentius* and *Ursus maritimus*. The use of secondary data was chosen because the available sequences have gone through a verification process so they can be reused for molecular analysis.

Table 1. Database of Sample Species from the Family Felidae and Outgroups (Ursidae and Canidae)

Subfamily/Family	Species	Accession Number
Felinae	*Leopardus geoffroyi*	KF297758
	Profelis aurata	KJ192801
	Prionailurus bengalensis	KF297757
	Prionailurus viverrinus	KF297768
	Lynx canadensis	JF443254
	Lynx rufus	JF443256
	Caracal caracal	JF444289
	Puma concolor	JF443379
	Herpailurus yaguarondi	KF297727
	Acinonyx jubatus	KF297759
	Felis silvestris	KJ192803
	Felis silvestris lybica	JN311871
	Felis catus	JQ735460
	Felis margarita	KF297765
	Felis chaus	KJ634464
	Felis nigripes	KF297746
	Otocolobus manul	KF297805
	Catopuma temminckii	KF297741
	Leptailurus serval	KJ192804
	Leopardus wiedii	KT236259
	Leopardus tigrinus	KF297732
	Prionailurus rubiginosus	KF297735
	Lynx lynx	MK040984
Pantherinae	*Neofelis nebulosa*	KF367744
	Panthera leo	KF297751
	Panthera pardus	MZ099218
	Panthera onca	KF297767
	Panthera uncia	KX859291
Ursidae	*Ursus maritimus*	JF499382
Canidae	*Canis lupus*	KX156591
	Urocyon cinereoargenteus	JF443521

2.2. Methods

2.2.1. DNA Sequencing

The COI gene sequence was downloaded from the NCBI database in FASTA format. The taxonomic identity of each sequence was verified through publication metadata to ensure specimen validity. In addition, the identity of each sequence was verified using BLAST on NCBI to ensure species matching and avoid sequence misidentification. The DNA sequences were then stored in Notepad software using the following format: Accession Number_Species Name_Genetic Marker_Source (Figure 1).

```
>KF297758_Leopardus_geoffroyi_Cytochrome_Oxidase_1_NCBI
TACTCTCTACCTCTTATTTGGTGCTTGAGCTGGTATAGTAGGAACCGCTCTCAGTCTCTTGATTCGGGCC
GAACTAGGCCAACCTGGCACACTACTAGGAGATGACCAGATTTACAATGTAATCGTCACTGCTCATGCCT
TCGTAATAATTTTCTTCATAGTGATGCCTATTATGATCGGAGGATTCGGAAACTGATTGGTCCCATTAAT
AATTGGAGCTCCTGACATAGCGTTCCCTCGAATGAACAATATGAGCTTTTGACTTCTTCCCCCCTCCTTT
TTACTCCTACTTGCTTCATCTATGGTAGAGGCCGGAGCAGGGACTGGGTGAACAGTATATCCGCCTCTAG
CTGGTAATCTAGCTCATGCAGGAGCATCCGTAGATCTAACTATTTTTCACTACATCTAGCAGGTATTTC
CTCAATCTTGGGTGCTATTAATTTTATCACCACTATTATCAACATAAAACCCCCTGCCATATCTCAATAT
CAAACACCTCTCTTCGTCTGATCTGTCTTAATTACTGCTGTTTTGTTGCTCCTATCACTTCCAGTTTTAG
CAGCAGGAATCACCATATTATTAACAGACCGAAACCTAAACACTACATTTTTTGATCCCGCTGGGGGAGG
AGACCCCATCTTATATCAGCATCTGTTC

>KJ192801_Profelis_aurata_Cytochrome_Oxidase_1_NCBI
TACTCTTTACCTTCTATTTGGTGCCTGAGCTGGTATGGTGGGGACTGCTCTCAGTCTCCTAATCCGAGCC
GAACTGGGCCAGCCCGGCACACTACTAGGGGATGATCAGATTTATAATGTAATTGTCACTGCTCATGCTT
TCGTAATAATCTTCTTTATAGTGATGCCTATTATAATTGGAGGATTCGGAAACTGATTGGTTCCACTAAT
AATTGGAGCCCCTGACATAGCATTTCCCCGAATAAATAATATGAGCTTCTGACTTCTTCCTCCATCCTTC
CTACTTTTACTCGCCTCATCTATGGTGGAGGCCGGAGCAGGAACTGGATGAACAGTATATCCTCCCTTAG
CCGGTAACCTAGCTCATGCAGGAGCATCCGTAGACCTAACTATTTTCTCACTACACCTAGCAGGTGTCTC
TTCAATTTTAGGTGCTATTAATTTTATTACTACTATTATTAATATAAAACCTCCTGCCATATCTCAATAT
CAAACGCCCCTATTTGTTTGATCAGTTTTAATTACTGCCGTCCTACTACTCTTATCACTCCCAGTTTTAG
CAGCAGGAATCACTATGCTACTAACGGATCGAAATTTAAACACCACATTCTTTGATCCCGCTGGAGGAGG
AGATCCTATCTTATATCAACACTTATTC
```

Figure 1. DNA sequence samples in FASTA format in Notepad software

2.2.2. Multiple Alignment of DNA Sample Sequences

Multiple alignment of all COI sequences obtained from GenBank was performed using Clustal-X software to identify nucleotide similarities between species (Figure 2). This process yielded information on sequence homology and the presence of gaps at specific positions reflecting mutations in the form of insertions or deletions.

The alignment results were then trimmed using MEGA4 and PAUP* by removing nucleotide positions that were not aligned, contained excessive gaps, or were non-informative. This step aimed to obtain conserved and relevant sequence regions for phylogenetic analysis, thereby minimizing potential bias due to suboptimal alignment.

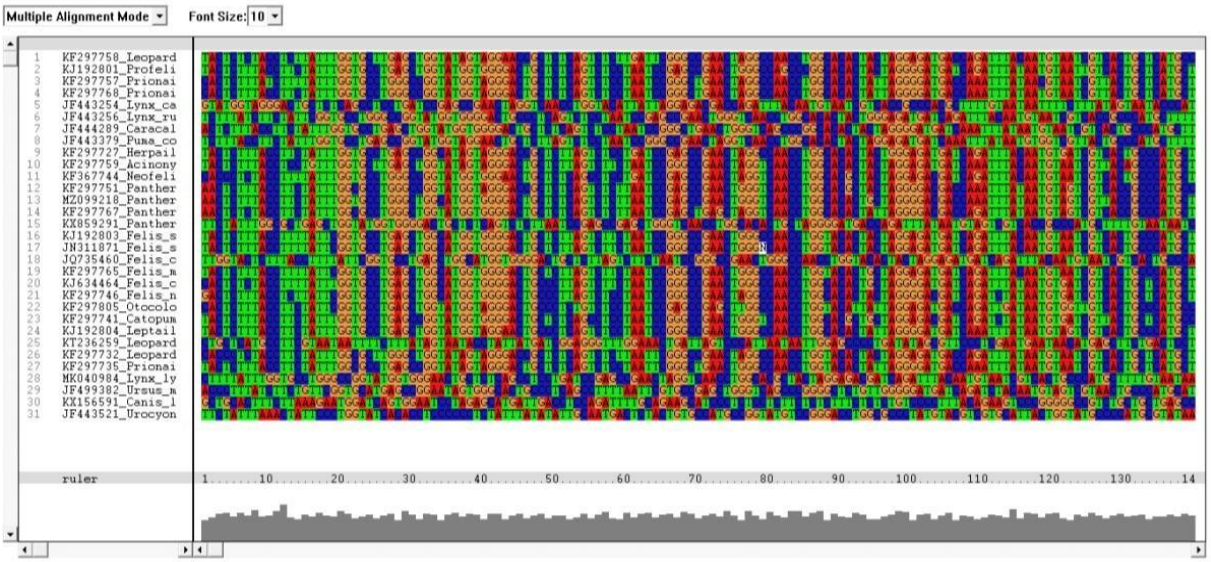

Figure 2. Results of multiple alignment of DNA sequences using Clustal-X software

2.2.3. Phylogenetic Tree Construction

The aligned sequence data were then analyzed to construct a phylogenetic tree using MEGA4 and PAUP* software as comparators (Figure 3 and 4). Tree reconstruction was performed using the Maximum Parsimony method to determine the pattern of relationships between species in the Felidae family. This analysis also included the calculation of the Consistency Index (CI) and Retention Index (RI) to evaluate the level of homoplasy and the quality of the resulting tree topology. In addition, three outgroup species were used to root the tree and help determine the direction of character evolution in the COI dataset.

2.2.4. Genetic Distance Analysis

The interpretation of kinship relationships was based on clade structure and branch position to compare the genetic proximity of *Puma concolor* with other members of the Pantherinae and Felinae subfamilies.

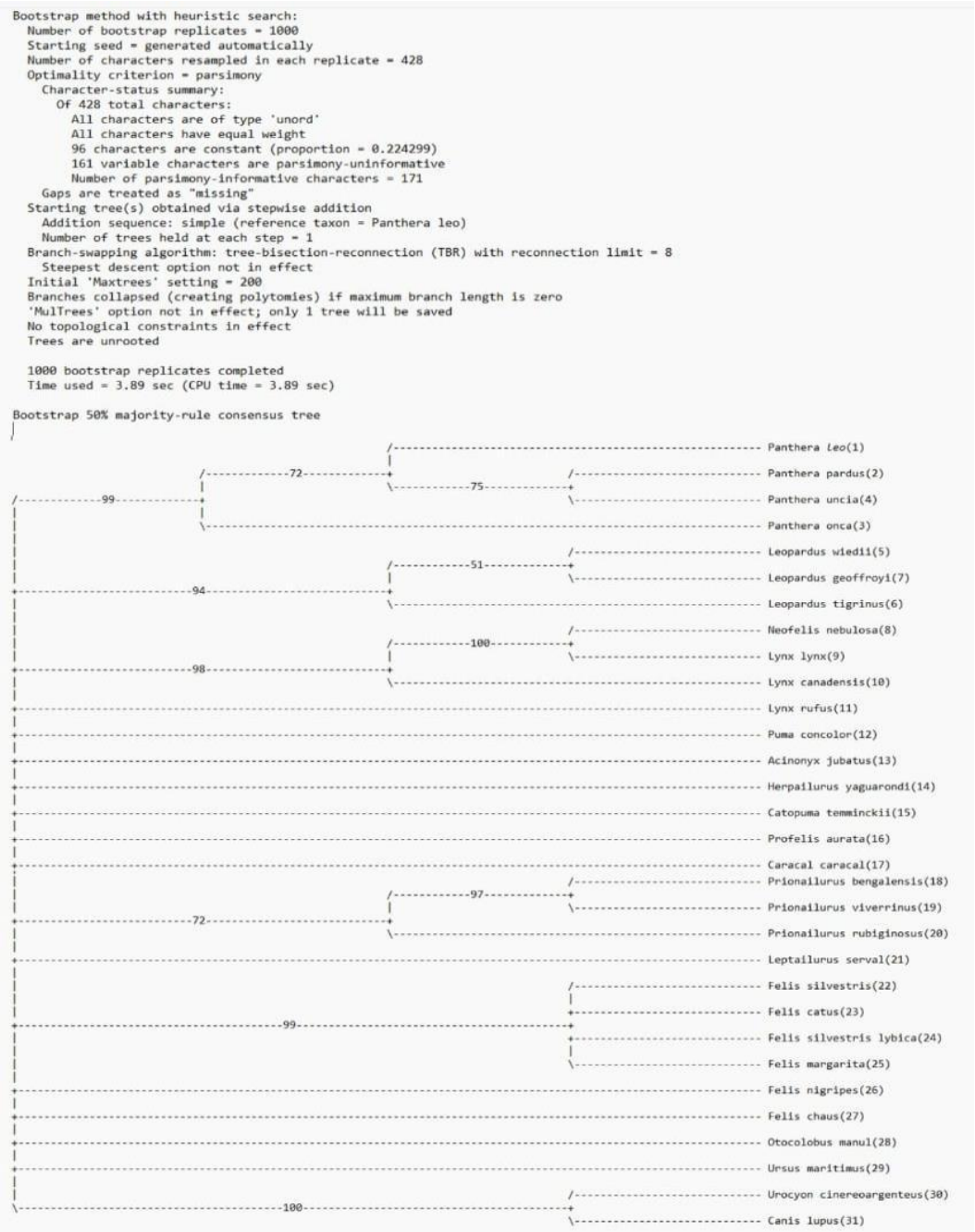

Figure 3. The process of constructing phylogenetic trees using PAUP* software

```
->Method : Maximum Parsimony
->Phylogeny Test and options : Bootstrap (500 replicates; seed=64238)
->Search Options : CNI ( level = 1) with initial tree by  Random addition (10 reps)
Include Sites : ==============================
->Gaps/Missing Data : Complete Deletion
->Codon Positions : 1st+2nd+3rd+Noncoding
No. of Sites : 361
No Of Bootstrap Reps = 500
CI = 0.516919
RI = 0.543550
RCI = 0.280972(for all sites)
iCI = 0.380240(for parsimony informative sites)
iRI = 0.543550(for parsimony informative sites)
iRCI = 0.206679(for parsimony informative sites)
```

Figure 4. MEGA4 Consistency Index (CI) and Retention Index (RI) Values

Results and Discussion

Phylogenetic analysis of *Puma concolor* in the Cytochrome Oxidase Subunit I (COI) dataset using 28 Felidae species, represented by 23 species of the Felinae subfamily and 5 species of the Pantherinae subfamily, as well as 3 outgroups from the Ursidae and Canidae families, was conducted using two software programs, MEGA4 and PAUP*, and showed consistent results (Figure 5). The trees generated by both methods displayed similar topologies, where all species of the genus *Panthera* (such as *P. leo, P. tigris, P. pardus*, and others) formed a separate clade from *Puma concolor* with sufficient bootstrap support. Bootstrap is a measure of confidence in the branches of a phylogenetic tree, calculated from how often the same branch reappears after the data is repeatedly resampled (Rohlf, 2004).

In both trees, all species of the genus *Panthera* (*P. leo, P. tigris, P. pardus*, and their relatives) form a monophyletic clade that is clearly separated from *Puma concolor*, with adequate bootstrap support >50%. These bootstrap values indicate branch stability and provide a sufficient level of confidence in the separation of clades (Rohlf, 2004). These findings are consistent with Yu et al. (2025) research, which found that although pumas share morphological similarities with big cats, their genetic relationships do not indicate a direct affinity with *Panthera*.

The subfamily Felinae clade (including *Felis catus, Lynx lynx*, and others) also exhibits a monophyletic structure. *Puma concolor*, on the other hand, appears as an independent branch that does not merge directly with either the *Panthera* clade or the main Felinae clade. This position is consistent across both analysis methods and suggests that pumas represent a separate evolutionary lineage, as suggested by Barnett et al. (2005) in the *Puma* lineage with *Acinonyx jubatus* and *Herpailurus yagouaroundi*. Modern phylogenomic approaches incorporating complete mitogenome data also confirm that these three species form a monophyletic *Puma* linage, separated from the larger *Panthera* clade and other Felinae groups at a deep evolutionary level, thus supporting the independent position of *P. concolor* as a distinct evolutionary lineage within the Felidae (Li et al., 2016).

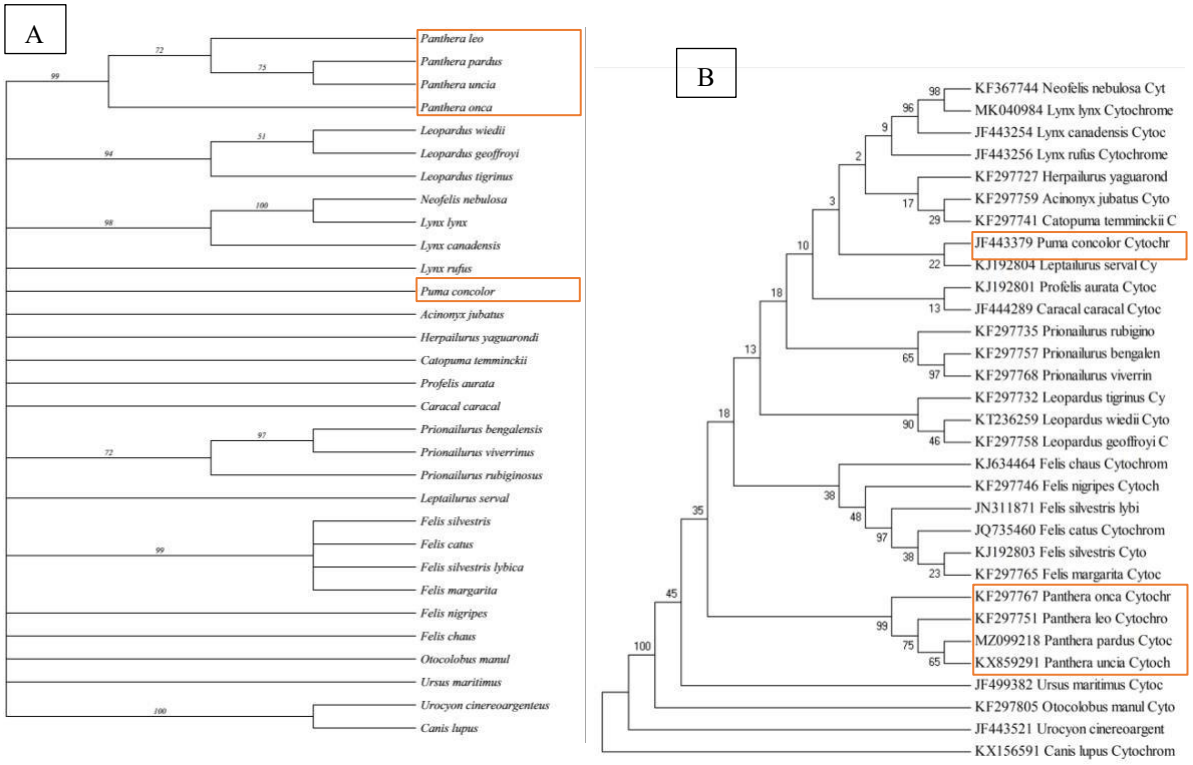

Figure 5. Results of the phylogenetic tree construction for *Puma concolor*. (A) Using PAUP* software. (B) Using MEGA4 software.

Tree quality analysis using the Consistency Index (CI) and Retention Index (RI) values after trimming showed improvement, with both values >0.50. This indicates a relatively low level of homoplasy and that the analyzed genetic characters provide a strong phylogenetic signal. Thus, the resulting topological structure can be considered representative of the evolutionary history of Felidae, including the relative basal position of *Puma concolor*. Furthermore, interspecific genetic distances show that the distance between *Puma concolor* and other *Panthera* species is quite large, while the distances to some members of Felinae are relatively small, supporting the evolutionary separation of puma from the *Panthera* group.

The findings of this study are consistent with a comprehensive phylogenetic study by Johnson et al. (2006), which used a combination of mitochondrial and nuclear markers to reconstruct the evolution of the Felidae and placed *Puma concolor* outside the *Panthera* clade, with an estimated divergence time of approximately 6–10 million years ago. The alignment of these results with previous studies strengthens the conclusion that the classification of pumas within the subfamily Felinae has a strong genetic basis, in line with previously reported anatomical differences such as hyoid bone structure and skull morphology (Christiansen, 2008).

Furthermore, research by Kitchener et al. (2017) emphasized that the discrepancy between morphological similarity and genetic relatedness in Felidae is a common phenomenon influenced by convergent evolution and morphological conservatism. In this context, the position of *Puma concolor*, which morphologically resembles big cats, but is genetically separate from *Panthera*, reflects adaptive evolutionary patterns related to hunting strategies and body size, rather than phylogenetic closeness. This finding is in line with the results of the COI analysis in this study, which consistently places the *Puma* outside the *Panthera* clade with stable statistical support.

The use of three outgroup species from the Canidae and Ursidae families proved effective in rooting the tree without producing significant long-branch attraction artifacts. This ensures that the phylogenetic position of *Puma concolor* as an independent evolutionary line is not a methodological artifact, but rather a reflection of its actual evolutionary history. In tree construction, the most

informative outgroup in phylogenetic tree reconstruction is the group that acts as the sister group of the taxon under study. In other words, the outgroup has a more distant relationship with the taxon than the relationships among members within the taxon itself (Brown, 2002).

Conclusion

Phylogenetic analysis based on the mitochondrial COI gene shows that *Puma concolor* is clearly separated from the *Panthera* genus clade and represents an independent evolutionary line within the Felidae family. The consistency of results obtained using the MEGA4 and PAUP* methods, supported by bootstrap values and CI and RI > 0.50, confirms that the morphological similarities between pumas and big cats are the result of convergent evolution, not genetic relatedness. These results support the classification of *Puma concolor* in the subfamily Felinae.

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