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COMPARATIVE MITO-GENOMIC ANALYSIS OF DIFFERENT SPECIES OF GENUS CANIS BY USING DIFFERENT BIOINFORMATICS TOOLS

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ABSTARCT

In the current study, the mitochondrial gene sequences for COI, Cyt b and 16s rRNA of seven species found in different parts of the world were retrieved from NCBI Gene Bank. While, PCR products of COI, Cyt b and 16s rRNA genes obtained from *C. lupus* and *C. aureus* present in Pakistan were sequenced and aligned for comparison. The phylogenetic analysis of mitochondrial COI, Cyt b and 16s rRNA genes showed almost the same results for the extant species of genus Canis. 16s rRNA revealed *C. latrans*, *C. lycaon* and *C. simensis* showed close phylogenetic relation with each other. While, COI and Cyt b showed wider genetic distance. Cyt b and 16s rRNA analysis indicated *C. mesomelas* and *C. adustus* as sister taxa, while COI also proved the same phylogenetic association. COI and 16s rRNA revealed the least genetic distance between *C. aureus* and *C. lycaon* while Cyt b showed more genetic distance. COI, 16s rRNA analysis determined close phylogenetic relations between *C. lupus* and *C. lycaon* while Cyt b showed greater phylogenetic distinction between these two species. Cyt b and 16s rRNA revealed no genetic distance between *C. latrans* and *C. lycaons* while COI analysis identified both as genetically distant. Finally, COI, 16s rRNA successfully determined the phylogenetic association between *C. aureus* and *C. adustus* but Cyt b clarified more with higher genetic distance. In conclusion, 16s rRNA revealed least genetic distance while Cyt b showed higher genetic distance which suggests its potential use for species delineation, especially for mammals.

Keywords: carnivore, Cyt b, CO Ⅰ, rRNA, phylogenetic

INTRODUCTION

Genus Canis belongs to family Canidae, order Carnivora, class Mammalia and phylum Chordata. The nine different species of Genus Canis i.e. *C. aureus* (golden jackal), *C. rufus* (red wolf), *C. mesomalas* (black backed jackal), *C. adustus* (side striped jackal), *C. lycaon* (Eastern wolf, timber wolf), *C. latrans* (coyotes), *C. lupus* (gray wolf), *C. simensis* (Ethiopian wolf), and *C. anthus* (African golden wolf) are distributed in different parts of the world (Duleba et al., 2015; Koepfli et al., 2015). However, only two species i.e. golden jackal (*C. aureus*) and gray wolf (*C. lupus*) are reported in Pakistan (Robert, 1997).

The gray wolf (*C. lupus*) occurs in almost any type of habitat but generally it avoids thick natural forest regions. It exists in all the mountainous regions of Pakistan from Baluchistan up to Chitral, and Gilgit Baltistan. The body of gray wolf (*C. lupus*) is of a large size. Male usually measures up to 65cm at shoulder height. First upper molars are not distinct from outer cingulum. The golden jackal (*C. aureus*) is of medium

size. Height measures up to shoulder are about 40 cm. First upper molars are distinct from outer cingulum (Robert, 1997).

Recent molecular phylogenetic studies further narrowed the century old debate on gray wolf taxonomy by advent of oldest gray wolf lineage from Indo-Himalayan region. Now Himalayan wolf (*C. lupus chanco*) and Peninsular Indian gray wolf (*C. lupus pallipes*) has been reclassified as *C. indica* and *C. himalayensis* respectively, and diverged from worldwide wolf-dog clade 400,000 and 800,000 years ago. Morphologically and genetically both of these wolf species are different from all wolf and dogs of the world and each represents a distinct species. While the third wolf *C. lupus chanco* (Tibetan Wolf) is related genetically to worldwide wolf-dog clade (Aggarwal et al., 2003, 2007; Sharma et al., 2004; Habib et al., 2013).

The mitochondrial genome is the most effective single locus marker because it has smaller population size than the nuclear genome, so it increases the similarity between the gene trees and the underlying species tree (Lou et al., 2011). The DNA sequence of mitochondrial genome has been determined from a large number of organisms and individuals (including some organisms that are extinct) and a comparison of those DNA sequences represents the pillar of phylogenetics; it allows biologists to elucidate the evolutionary relationships among species (Duleba et al., 2015).

Mitochondrial Cytochrome Oxidase subunit I, 16s ribosomal RNA and Cytochrome b genes are used for identification and classification of animals and plants (Hebert et al., 2003). Mitochondrial COI gene is a well-known international standard used for DNA barcoding to identify the different unknown specimen to either existing species in the data base or flagged to explore further to assign a new species etc. (Smith et al., 2008). Mitochondrial 16s ribosomal RNA and Cyt b genes are used successfully for the taxonomic or phylogenetic resolution in many related taxa (Aggarwal et al., 2007; Rueness et al., 2011).

The current study was designed to compare the three extensively used mitochondrial genes COI, Cyt b and 16s ribosomal RNA to evaluate the evolutionary history and position of different species of genus Canis on phylogenetic trees. For this purpose, the sequences of COI, Cyt b and 16s ribosomal RNA of seven extant species belonging to other parts of the world were retrieved from NCBI gene bank. Sequences for *C. lupus* (gray wolf) and *C. aureus* (golden jackal) mitochondrial genes COI, Cyt b and 16s rRNA were extracted and amplified by utilizing available samples in Centre for Bio-Resource Research laboratory and sequenced by Sanger's method to calculate the genetic distance, compare phylogenetic position and to determine major phases of diversification among member species of genus Canis.

MATERIALS AND METHODS

Sample Collection

For detailed comparative analysis of mito-genome, the hair and skin samples of *C. lupus* and *C. aureus*, inhabiting Pakistan were obtained from CBR (Center for Bio-Resource Research, Islamabad) and were further processed.

Polymerase Chain Reaction (PCR)

The skin samples of *C. lupus* and *C. aureus* were processed to extract DNA for subsequent analysis for getting the gene sequences. The chloroform/phenol protocol defined by Sambrook & David (2001) with some modifications was used for DNA extraction. For confirmation of extracted genomic DNA, 1% agarose gel was used

and electrophoresis was done on horizontal gel Electrophoresis apparatus (Labnet. International, Inc USA). Gel was then visualized by using Gel Documentation System (Alpha Digi Dog, Innotech, Taiwan). After confirmation of genomic DNA, the extracted DNA was further processed to PCR for amplification of targeted regions i.e. Cytochrome oxidase subunit I (COI), Cytochrome b (Cyt b) and 16s ribosomal RNA (16srRNA).

Details of the primers are given in Table 1.

Table 1: The primer pairs used in the study to amplify COI, Cyt b and 16s rRNA genes of mitochondria from species of genus Canis.

Primer	Primer Sequence	Target gene	TM* (◦C)	Amplicon size (bp)	Length	References
VF1D	TTCTCAACCAACCAC AARGAYATYGG	COI	57.6	650	26	Ivanova et al., 2006
VRID	TAGACTTCTGGGTGG CCRAARAAYCA		59.2		26	
16SAR-L	CGCCTGTTTATCAAA AACAT	16s rRNA	48.9	550-580	20	Palumbi et al., 1991
16SBR-H	CCGGTCTGAACTCAG ATCACGT		60.1		22	
L14841F	AAAAAGCTTCCATCC AACATCTCAGCATG ATGAAA	Cyt b	61.6	332	35	Kocher et al., 1989
H15149R	AAACTGCAGCCCCTC AGAATGATATTTGTC CTCA		64.5		34	

** TM Melting Temperature.*

Sequencing

After amplification for further analysis, the PCR product was sequenced by using Sanger's method (Sanger et al., 1977). The sequences obtained after sequencing of PCR products were analyzed by Bio Edit Software (Version 3.2) to convert them into FASTA format. The sequences with fine peaks were selected for the further analysis while sequences with noise in peaks were sequenced again. The sequence peaks after proofreading were subjected to BLAST at National Center for Biotechnology Information (NCBI) Gene Bank web portal to identify the species of these sequences. For the rest of extant species of genus Canis from other parts of the world, the sequences were retrieved from NCBI gene bank database. The sequences of their COI, Cyt b and 16s rRNA genes were downloaded in FASTA format.

Phylogenetic Analysis

The software MEGA 7 (Tamura et al., 2003) was used for Phylogenetic analysis of obtained sequences. Retrieved sequences of COI, Cyt b and 16s rRNA genes were aligned by using CLUSTAL W bioinformatics tool www.clustal.org/omega/ (Li, 2003). The genetic distances based on above mentioned three genes was determined by entering the aligned sequences of different species of genus Canis in the distance option of the MEGA 7 (Tamura et al., 2013). The different aligned sequences were entered in the phylogeny option of the MEGA 7 (Tamura et al., 2013) and different phylogenetic trees like maximum Likelihood tree, Maximum parsimony tree and UPGMA trees were obtained to show the phylogenetic relations of different species of genus Canis.

RESULTS

The genetic distances of different species of genus Canis estimated by MEGA 7 (Tamura et al., 2013) based on COI gene, Cyt b and 16s rRNA are given in Table 2, 3, 4 respectively.

Table 2: COI based Genetic distance of different species of genus Canis

Species	1	2	3	4	5	6	7	8
C.aureus								
C.lupus	0.015							
C.adustus	0.061	0.065						
C.latrans	0.617	0.622	0.632					
C. mesomelas	0.619	0.624	0.634	0.032				
C.lycaon	0.019	0.018	0.054	0.618	0.620			
C.anthus	0.638	0.642	0.642	0.019	0.042	0.628		
C.simensis	0.637	0.637	0.635	0.020	0.035	0.637	0.025	

Table 3: Cyt b based Genetic distance of different species of genus Canis

Species	1	2	3	4	5	6	7	8	9
C.aureus									
C.adustus	19.541								
C.latrans	19.018	0.144							
C.lupus	0.125	18.120	17.67						
C.mesomelas	19.098	0.125	0.086	17.739					
C.rufus	19.018	0.150	0.004	17.670	0.081				
C.simensis	19.073	0.115	0.041	17.694	0.071	0.037			
C.anthus	19.045	0.132	0.027	17.693	0.076	0.023	0.023		
C.lycaon	19.018	0.144	0.000	17.670	0.086	0.004	0.041	0.027	

Table 4: 16s rRNA based genetic distance of different species of genus Canis.

Species	1	2	3	4	5	6	7	8
C. aureus								
C. adustus	3.277							
C. latrans	3.172	0.058						
C. lupus	2.408	2.170	2.132					
C. mesomelas	3.127	0.071	0.050	2.153				
C. simensis	3.083	0.065	0.026	2.099	0.055			
C. anthus	3.067	0.075	0.035	2.157	0.055	0.030		
C. lycaon	3.172	0.058	0.000	2.132	0.050	0.026	0.035	

COI based genetic distance

The phylogenetic distances estimated shows that *C. aureus* was most closely related to *C. lupus* while distantly related to *C. anthus*. *C. aureus* showed 0.617 relatedness to *C. latrans* while 0.169 relatedness to *C. simensis*. *C. simensis* had the same genetic distance 0.637 from *C. aureus* and *C. lupus*. The *C. simensis* showed the least distance i.e. 0.020 from *C. latrans*. *C. anthus* showed maximum genetic diversity from *C. aureus* while minimum distance from *C. latrans*. *C. lycaon* was genetically more distant from *C. mesomelas* and closely related to *C. lupus*. *C. latrans* was genetically more distant from *C. aureus*, *C. lupus* and *C. adustus*. The genetic distances of different species of genus Canis are given in Table 2.

Cyt b based genetic distance

The phylogenetic distance estimated showed that *C. adustus* was distantly related to C. aureus while *C. aureus* was closely related to *C. lupus*. *C. latrans* showed 19.018 relatedness to *C. aureus* and *C. mesomelas* showed 19.098 relatedness to *C. aureus.* There was no phylogenetic difference between *C. latrans and C. lycaon* i.e. 0.000. *C. lupus* was more genetically distant from *C. mesomelas*, *C. rufus*, *C. simensis*, *C. anthus,* and *C. lycaon*. *C. anthus* had same genetic distance from *C. rufus* and *C. simensis* i.e. 0.023. *C. lycaon* was more closely related to *C. rufus* and *C. rufus* was also more closely related to *C. latrans*. The phylogenetic distances are given in Table 3.

16s rRNA based genetic distance

C. adustus was genetically more distant from *C. aureus*. The *C. lupus* was less genetically distant from *C. aureus* than any other species. There was no genetic distance between *C. lycaon* and *C. latrans*. The genetic distance of *C. simensis* from *C. latrans* was least i.e. 0.026. Similarly, the genetic distance of *C. lycaon* from *C. simensis* was 0.026. *C. lycaon*, *C. latrans,* and *C. simensis* were more closely related to each other. The phylogenetic association of *C. latrans* and *C. simensis* was also mentioned in Systemic of Golden jackal, based on mtDNA Cyt b and control region analysis. *C. lupus* was genetically distant from *C. latrans* by 2.132. *C. latrans* and *C. lycaon* had the same genetic distance from *C. aureus* i.e. 3.172. The genetic distances are given in table 4.

Comparison of genetic distance between different species of genus Canis on the basis of COI, Cyt b and 16s rRNA revealed that *C. lycaon* and *C. latrans* were more closely related to each other. The genetic distance also revealed the phylogenetic association between *C. aureus* and *C. lupus*.

Maximum Likelihood Tree

The maximum likelihood trees constructed by MEGA 7 (Tamura et al., 2013) based on COI, Cyt b and 16s rRNA gene are shown in Figure 5.

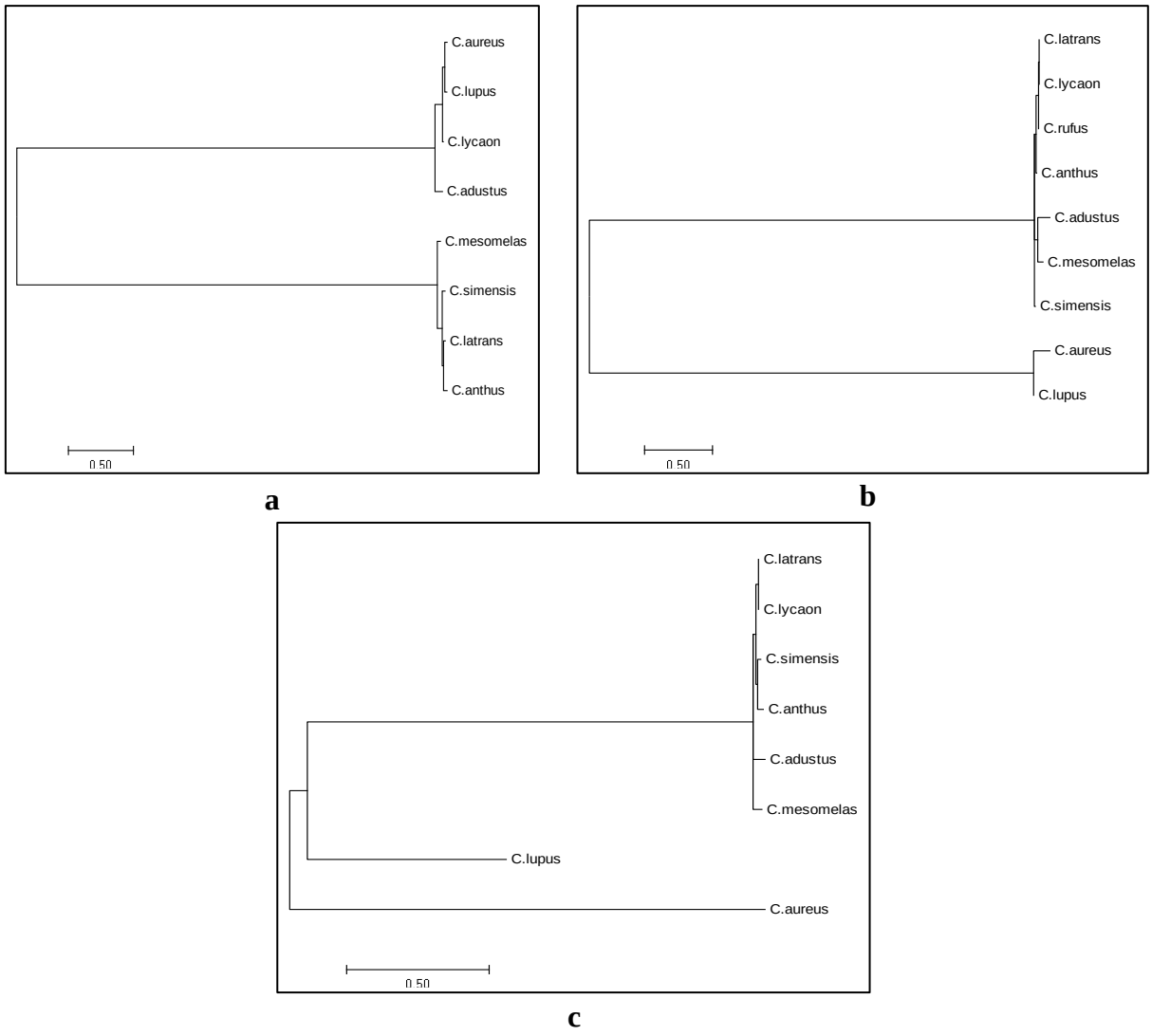

Figure 5: **Maximum Likelihood Tree constructed by MEGA 7 using (a) COI gene (b) Cyt b gene and (c) 16s rRNA gene sequence.**

Comparison of maximum likelihood tree by using COI, Cyt b and 16s rRNA gene revealed that *C. aureus* and *C. lupus* were closely related. Similarly, *C. mesomelas* and *C. simensis* showed phylogenetic relation and *C. latrans* and *C. lycaon* were closely related.

Maximum Parsimony Tree

The maximum Parsimony trees constructed by MEGA 7 (Tamura et al., 2013) based on COI, Cyt b and 16s rRNA gene are shown in Figure 6.

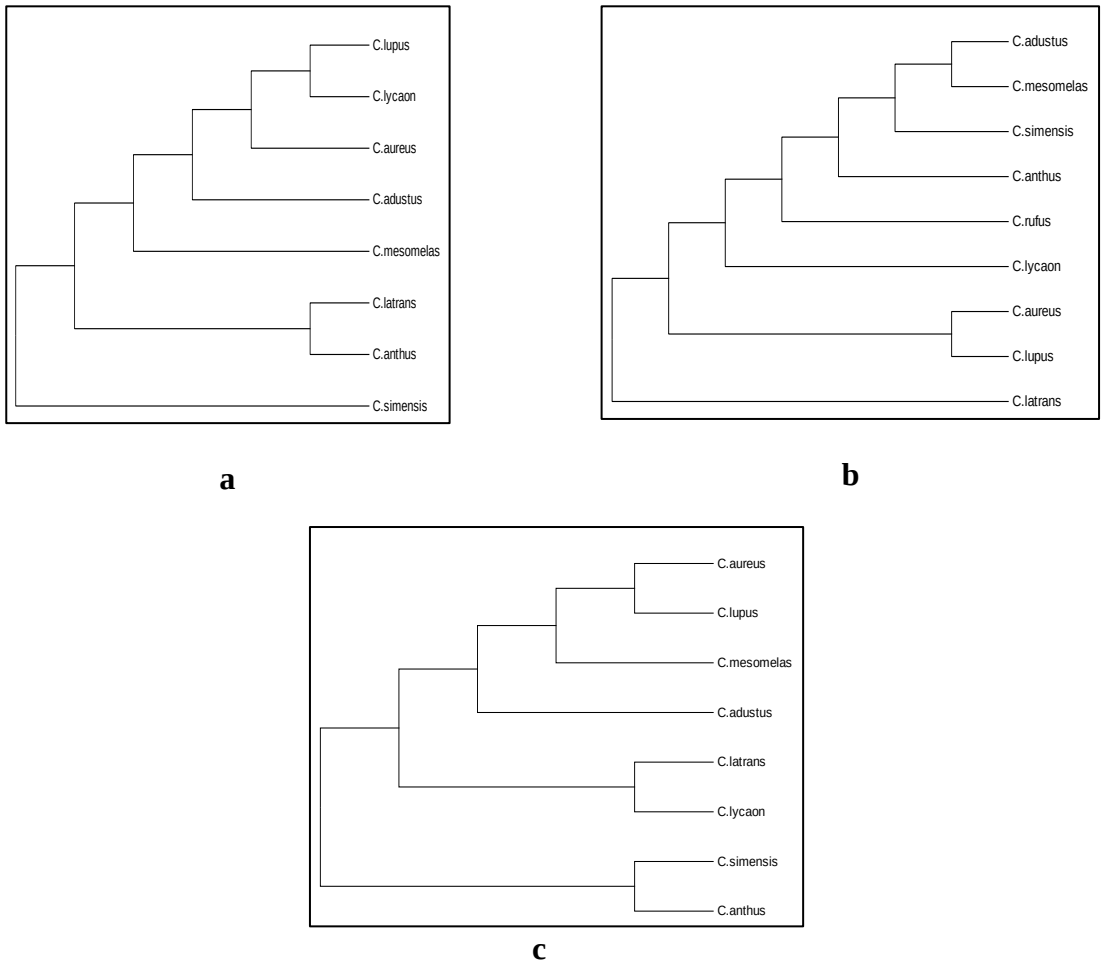

Figure 6: Maximum Parsimony Tree constructed by MEGA7 using (a) COI gene sequence (b) Cyt b gene sequence (c) 16s rRNA gene sequence.

Comparison of Maximum Parsimony trees on the basis of COI, 16s rRNA and Cyt b gene determined that *C. aureus* and *C. lupus* were closely related to each other. *C. adustus* and *C. mesomelas* were phylogenetically related and *C. simensis* and *C. anthus* showed association with each other.

Unweighted Pair Group Method with Arithmetic Mean

The UPGMA trees constructed by MEGA 7 (Tamura et al., 2013) based on COI, Cyt b and 16s rRNA gene are shown in Figure 9.

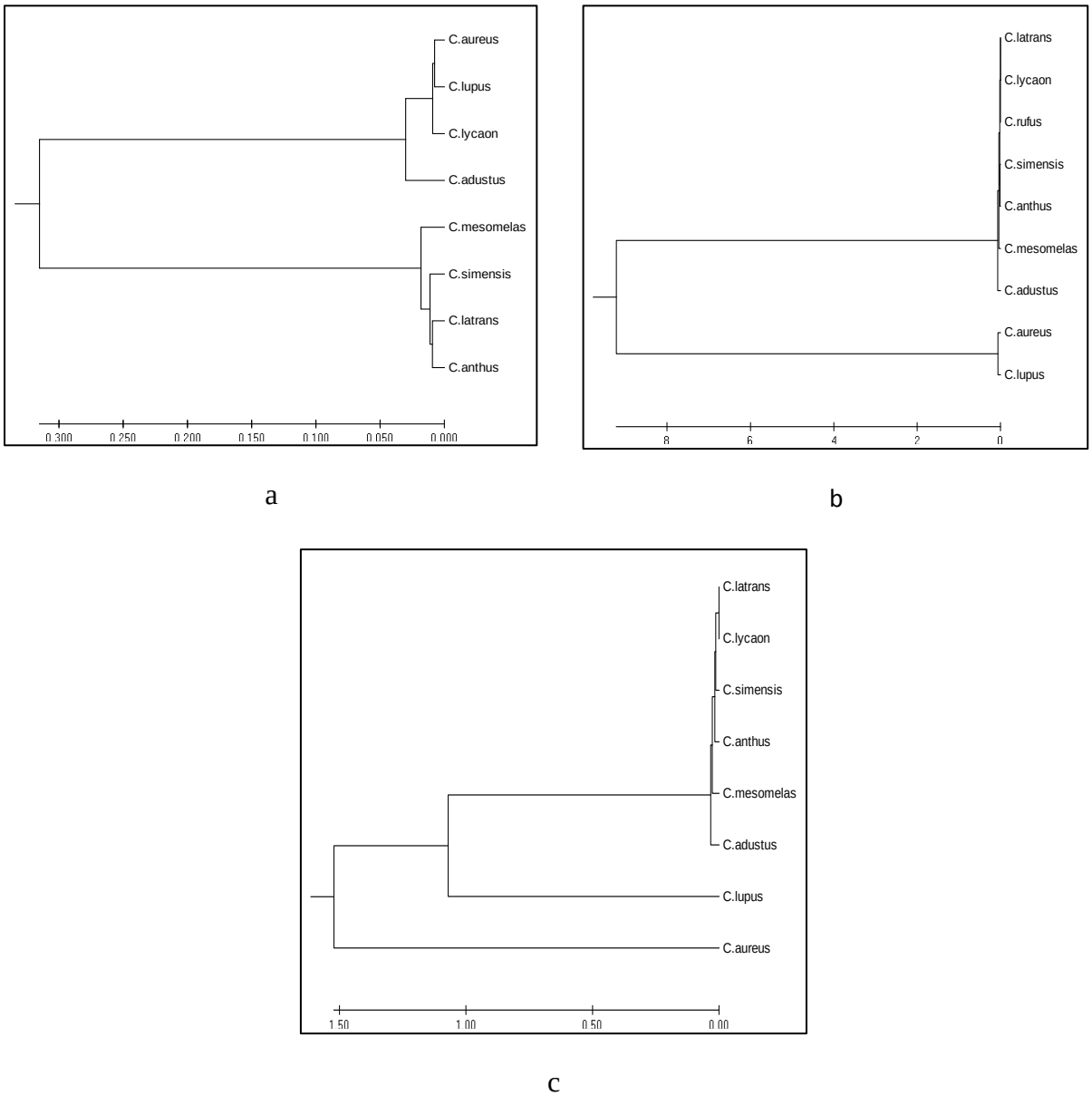

Figure 9: UPGMA Tree constructed by MEGA7 by using (a) COI gene sequence (b) Cyt b gene sequence (c) 16s rRNA gene sequence.

Comparison of UPGMA tree on the basis of COI, 16s rRNA and Cyt b for different extant species of genus Canis revealed the relatedness of *C. aureus* and *C. lupus*. Similarly, *C. simensis* and *C. anthus* were phylogenetically associated and *C. mesomelas* and *C. simensis* were related to each other.

DISCUSSION

According to mitochondrial COI gene analysis, *C. lupus* and *C. aureus* were closely related while *C. aureus* was distantly related to *C. anthus*; same results were noticed by the Koepfli et al. (2015). The Cyt b gene analysis revealed that *C. latrans* and *C. rufus* were phylogenetically related to each other as noticed by Wilson et al., (2000). The mitochondrial 16s rRNA analysis revealed that *C. aureus* showed phylogenetic association to coyotes and wolves, same results have been indicated by Nowak (1999). However, Nowak's work showed that *C. aureus* shows phylogenetic association to coyotes and wolves; it also demonstrated some morphological convergence with *C. adustus* and *C. mesomelas*. The *C. aureus*, *C. adustus*, and *C. mesomelas* were considered as phylogenetically associated as the same results were determined by Bardeleben et al. (2005) on combined analysis of Cyt b, COI and CO II mitochondrial genes.

In the current study, the phylogenetic maximum likelihood tree for COI and 16s rRNA gene analysis revealed the close similarity between *C. lupus* and *C. aureus* as noticed by Koepfli et al. (2015); Vila et al. (1999). The maximum likelihood tree based on COI tree also showed that *C. anthus* and *C. latrans* were closely related as the same results were noticed by Koepfli et al. (2015). Koepfli et al. (2015) also determined similarity between *C. anthus* and *C. latrans* by phylogenetic tree based on nuclear sequence. The similar phylogenetic association between *C. lupus* and *C. lycaon* was also verified by Pollinger et al. (2011) based on single nucleotide polymorphism. The Cyt b gene analysis showed phylogenetic similarities among *C. latrans*, *C. lycaon*, *C. rufus*, and *C. anthus*. The similarities between these species has also been determined by Rustledge et al. (2010). Similar results were reported by Wilson et al. (2000) that there exists phylogenetic association between *C. lycaon*, *C. latrans* and *C. rufus*. The mitochondrial 16s rRNA gene revealed *C. aureus* as the ancestor of *C. lupus* as determined by Bardeleben et al (2005).

The maximum parsimony tree constructed in the current study, according to mitochondrial COI analysis showed the close phylogenetic association between *C. latrans* and *C. anthus* (Koepfli et al., 2015) based on six nuclear loci. The phylogenetic tree also revealed the relatedness of the *C. lupus* and *C. lycaon* which concurs with findings of Pollinger et al. (2011) based on single nucleotide polymorphism. The Cyt b and 16s rRNA analysis revealed phylogenetic association between *C. aureus* and *C. lupus* as determined by Koepfli et al. (2015) on the basis of mitochondrial Cyt b sequence. The maximum parsimony tree according to 16s rRNA gene analysis determined that *C. latrans* and *C. lycaon* were phylogenetically associated with each other, as was also found by Rustledge et al. (2010).

The UPGMA tree constructed in the present study based on mitochondrial COI gene and 16s rRNA analysis revealed the close similarity between *C. lupus* and *C. aureus* same as noticed by Wayne (1992); Vila et al. (1999); Koepfli et al. (2015). This tree also revealed the phylogenetic association between *C. anthus* and *C. latrans*. Koepfli et al. (2015) also reported the close phylogenetic association between

C. anthus and *C. latrans*. The Cyt b analysis revealed the distant relation of *C. aureus* and *C. adustus* as the same results have been shown by Bardeleben et al. (2005) based on combined analysis of Cyt b, CO1 and COII. The 16s rRNA revealed the association between *C. aureus* and coyotes and the same results have been indicated by Nowak (1999). The tree also demonstrated some morphological convergence with *C. adustus* and *C. mesomelas* and the same phylogenetic similarity between these species has also been determined by Rustledge et al. (2010).

CONCLUSION

The phylogenetic analysis of mitochondrial COI, Cyt b and 16s rRNA genes showed almost the same results. The incorporation of sequence data to genetics and evolution has revolutionized phylogenetics over the past two decades. With the advancement of whole-genome sequencing projects, some researchers have even advocated the use of entire genomic sequences for phylogenetic inference. The selection of COI, 16s rRNA and Cyt b has a great potential to distinguish phylogenetic relations and evolutionary history of species at molecular level. The results of the current study supported that 16s rRNA revealed least genetic distance while Cyt b showed higher genetic distances which suggest its potential use for species delineation especially for mammal while COI proved useful to delineate the species as barcode.

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