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Phylogenetic analysis of camel complete mitochondrial DNA sequences

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Abstract

One-humped and two-humped camels diverged 4-5 million years ago, however, they produce fertile hybrids. We are thus interested in the phylogenetic relationship of mitochondrial DNA sequences of these camels to detect any introgression between these two camel species. We conducted a phylogenetic analysis of complete mitochondrial DNA (mtDNA) sequences of 26 and 156 individuals of one-humped camels (*Camelus dromedarius*) and two-humped camels (*C. bactrianus* and *C. ferus*), respectively, as well as those of other camelid species. MAFFT was used for multiple alignment, and the neighbor-joining and maximum likelihood methods were used for phylogenetic tree construction. Seven camel mtDNA sequences from Iran, Kazakhstan, and Russia had statistically significant heterogeneity of evolutionary rates using Tajima's test, though mtDNA sequences of closely related species are expected to be under a molecular clock. We found that these problematic sequences had

certain mapping problems. After remapping of short-read sequences, we obtained six mtDNA sequences different from previously reported ones. Two of these re-determined mtDNA sequences were originated from morphologically two-humped camels. However, their mtDNA sequences were very similar to those of one-humped camels. We conclude that these two camel species have been hybridizing for some time, because introgression of mtDNAs were inferred in this study.

Keywords: mtDNA, bactrian camel, dromedary, introgression, short-read sequences

Introduction

Family Camelidae belongs to Cetariodactyla, and their lineage (Tylopoda) diverged from the common ancestor of Ruminantia and Cetanodonta more than 60 million years ago (Zurano et al., 2018). Camelidae includes humped type *Camelus* and non-humped type *Lama* and *Vicugna*. *Camelus* are distributed in the Old World (Eurasia and Africa), while *Lama* and *Vicugna* are distributed in South America. There are three extant species of genus *Camelus; C. bactrianus* (bactrian camel), *C. ferus* (wild camel), and *C. dromedarius* (dromedary). *C. bactrianus* and *C. ferus* are two-humped camels, and are distributed from East to Central Asia, including Kazakhstan (Imamura et al., 2017). *C. dromedarius* is a one-humped camel distributed in Central Asia, South Asia, North Africa, and Australia (Burgher et al., 2019). *C. bactrianus* and *C. dromedarius* are domesticated, but *C. ferus* remains wild. Camelidae in South America include domesticated (alpaca and llama) and wild (guanaco and vicuna) populations. The divergence time between one-humped and two-humped camel lineages was estimated to be 4.4 (confidence intervals 1.9-7.2) million years ago based on whole nuclear genome sequence data (Wu et al., 2014).

Nowadays, many complete mtDNA sequences were determined for Camelidae. Saitou and Shokat (2017) collected 37 complete mtDNA sequences of camelids available at that time from the DDBJ/ENA/GenBank International Nucleotide Sequence Database. These mtDNA sequences included those of now-extinct *Camelops*, which were distributed in North America (Heinzman et al., 2015). Saitou and Shokat (2017) conducted phylogenetic analyses of these camelid mtDNA sequences, and inferred evolution of Camelidae with special reference to genus *Camelus*.

Recently many more camel mtDNA data were published, especially by Ming et al. (2020), who determined whole-genome sequences of 128 camels. We thus conducted phylogenetic analyses of complete camelid mtDNA sequences currently available, and would like to suggest a possibility of frequent introgression of mtDNA between *C. bactrianus* and *C. dromedarius* in Eurasia.

Materials and Methods

Data sources

Camelid complete mtDNA genome sequences were obtained from the DDBJ/ENA/GenBank International Nucleotide Sequence Database. We found that NCBI presents three camel species mtDNA sequences only in their genome database under NCBI-specific IDs (NC_009628, NC_009629, and NC_009849). All these sequences are listed in Supplementary Table 1 (http://idarwin.org/docs/vol2_suppl_files_1/Suppl_Table_1.pdf). There are a total of 191 complete mtDNA sequences; 32 individuals of *C. ferus* from Mongolia, 124 individuals of *C. bactrianus* (35 from Mongolia, 56 from China, 7 from Kazakhstan, 10 from Russia, 14 from Iran, 1 from Austria, and 1 from Japan; see the map shown in Supplementary Figure 1A (http://idarwin.org/docs/vol2_suppl_files_1/Suppl_Fig_1.pdf), 26 individuals of *C. dromedarius* (14 from Iran, 3 from Saudi Arabia, 3 from UAE (Dubai), 1 from Qatar, 1 from Kenya, 1 from Sudan, 1 from Morocco, 1 from Pakistan, and 1 from Austria; see the map shown in Supplementary Figure 1B (http://idarwin.org/docs/vol2_suppl_files_1/Suppl_Fig_1.pdf), 3 individuals of extinct *Camelops* from North America, 4 individuals of Lama and 2 individuals of Vicugna from South America. *Bos taurus* (cow), *Ovis aries* (sheep), and *Pantholops hodgsonii* (Tibetan antelope) were used as the outgroup. Table 1 shows a summary of the camelid sequence data used in this study.

Phylogenetic analyses

Multiple alignment of these 191 camelid mtDNA sequences and the three outgroup species was conducted using MAFFT version 7 (Katoh and Standley, 2013), and pairwise numbers of nucleotide substitutions were estimated by using Tamura and Nei's (1993) method. The neighbor-joining method (Saitou and Nei 1987) and a maximum likelihood method implemented in MEGAX (Kumar et al., 2018) were used for construction of phylogenetic trees. The reliability of the branches in the tree was evaluated by the bootstrap method with 1,000 replications. The "complete deletion" option was used for excluding all positions containing gaps and missing data. These evolutionary analyses were conducted by using MEGAX. Tajima's (1993) branch length heterogeneity test was also conducted using MEGAX.

Additionally, mtDNA sequences were extracted from whole-genome Next Generation Sequencing (NGS) raw files. Paired-end FASTQ files were downloaded from the NCBI BioProject database (accession number PRJNA383081) reported by Ming et al. (2020). Quality control, adapter trimming and quality filtering were conducted using fastp tool (Chen et al., 2018).

Table 1. List of mtDNA complete sequences of camelid species
Numbers in parentheses after region names are number of camel mtDNA sequences from that region.

(A) *Camelus dromedarius*
Austria (1): KU605076 Iran (14): KX554931-KX554934, MH109998-MH110007[1] Kenya (1): KU605078
Morocco (1): JN632608[2] Pakistan (1): KU605080 Qatar (1): KU605072
Saudi Arabia (3): KU605073-KU605075 Sudan (1): KU605079 UAE (3): EU159113, KU605077, NC_009849**
(B) *Camelus bactrianus*
Austria (1): KU666460 China: EF212037[3], MH109872-MH109911[2], MH109931-MH109945[2]
Iran (14): KX554925-KX554930, MH109990-MH109997[2] Japan (1): AP003423
Kazakhstan (7): KU666461, MH109984-MH109989[2]
Mongolia (59): EF507798[3], EF507799[3], KU666462-KU666465, MH109946-MH10997[2], NC_009628**
Russia (10): MH109974-MH109983[2]
(C) *Camelus ferus*
Mongolia (32): EF212038[3], EF507800[3], EF507801[3], KU666451-KU666459, MH109912-MH109930[2], NC_009629**
(D) Cameridae (extinct) from Canada
Camelops sp. (3): KR822420-KR822422[4]
(E) Cameridae from South America
Lama glama (1): AP003426 *Lama guanicoe* (1): EU681954[5] *Lama pacos* (2): AJ566364[6], Y19184[7]
Vicugna pacos (1): KU168760 *Vicugna vicugna* (1): FJ456892

** NCBI-specific IDs
References: 1) Ming et al. (2020), 2) Hassanin et al. (2012), 3) Ji et al. (2009), 4) Heinzman et al. (2015), 5) di Rocco et al. (2010), 6) Arnason et al. (2004), 7) Ursing et al. (2000)

Trimmed reads were aligned to the mitochondrion complete reference genome of *C. ferus* (NC_009629), *dromedarius* (NC_009849) and *bactrianus* (NC_009628) using the mem-algorithm of the BWA (Li et al, 2009). The mapped reads were sorted and indexed using SAMtools (Li et al., 2009) and Picard tools (https://broadinstitute.github.io/picard/) was used to mark and remove PCR duplicates from the sorted BAM files. Single-nucleotide variants (SNVs) and indels were called using BCFtools (http://samtools.github.io/bcftools/). VCF format files were converted into FASTA format files by using vcf-consensus option in VCFtools (https://vcftools.github.io/index.html) developed by Danecek et al. (2011).

Results

We performed a multiple alignment for complete mtDNA sequences listed in Supplementary Table 1, and eliminated the positions with gaps and missing data. There were a total of 15,739 nucleotide positions in the final dataset (data not shown). We then constructed a neighbor-joining tree of 191 mtDNA sequences (Supplementary Figure 2; http://idarwin.org/docs/vol2_suppl_files_1/Suppl_Fig_2.pdf). As the evolutionary distances among many sequences were very small, we chose only representative 31 sequences, and generated another neighbor-joining tree (Figure 1). Camelid mtDNA sequences are classified into three clusters in this tree; cluster 1 contains one-humped camel (*C. dromedarius*) and two-humped camel (*C. ferus* and *C. bactrianus*), extinct *Camelops* forms the second cluster, and South American non-humped camelids (genus *Lama* and *Vicugna*) formed the third cluster. These three clusters are consistent with the phylogenetic tree shown in Figure 1 of Saitou and Shokat (2017).

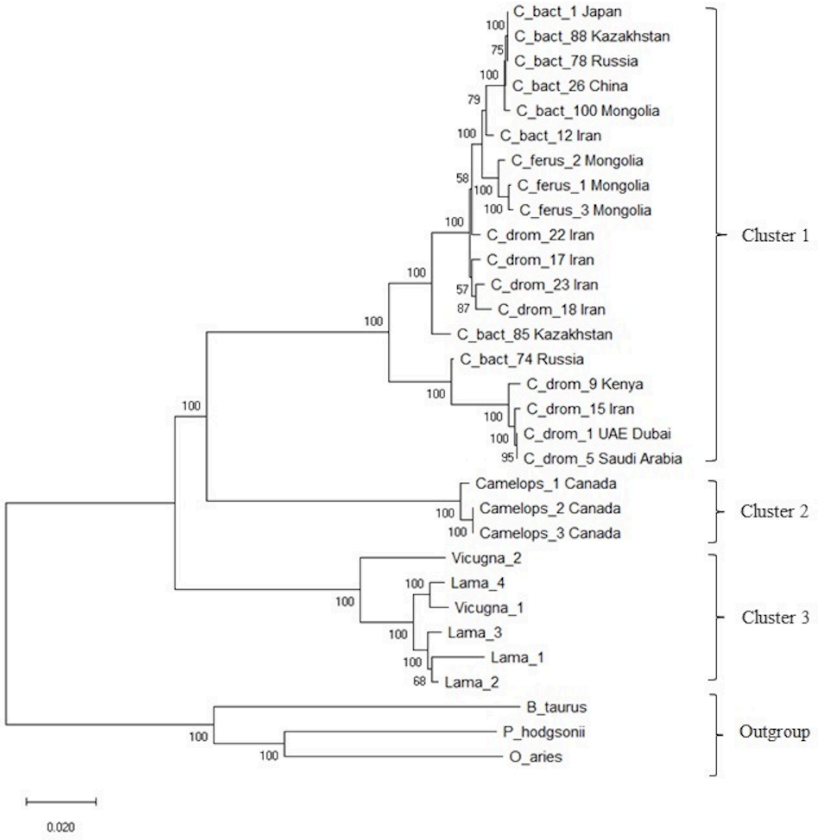

Figure 1. A neighbor-joining tree of representative 31 sequences.

We focused our phylogenetic analysis on 19 mtDNA sequences of cluster 1 in Figure 1. The neighbor-joining tree of these 19 sequences is shown in Figure 2. The root position follows that of cluster 1 in Figure 1 with the mid-point rooting. There are two clusters, A and B, in Figure 2. Cluster A contains mtDNA sequences of seven *C. bactrianus*, three *C. ferus*, and four *C. dromedaries*, and Cluster B contains mtDNA sequences of one *C. bactrianus* and four *C. dromedaries*. We also constructed an ML tree (Figure 3) for these sequences, and their branching patterns with high bootstrap values were very similar to those in Figure 2.

Both one-humped camels (*C. dromedaries*) and two-humped camels (*C. bactrianus*) are included in these two clusters.

This pattern is quite different from Figure 2 of Saitou and Shokat (2017) in which *C. dromedaries* and *C. bactrianus* formed two distinct monophyletic clusters. The divergence time of one-humped and two-humped camels was estimated to be 4.4 million years ago (Wu et al., 2014). We expect a clear-cut cluster for mtDNA sequences of these two camel species as shown by Saitou and Shokat (2017).

Ming et al. (2020) constructed a phylogenetic tree of mtDNA sequences using a maximum-likelihood method (see their Figure 3C). They suggested independent introgression events in two *C. bactrianus*

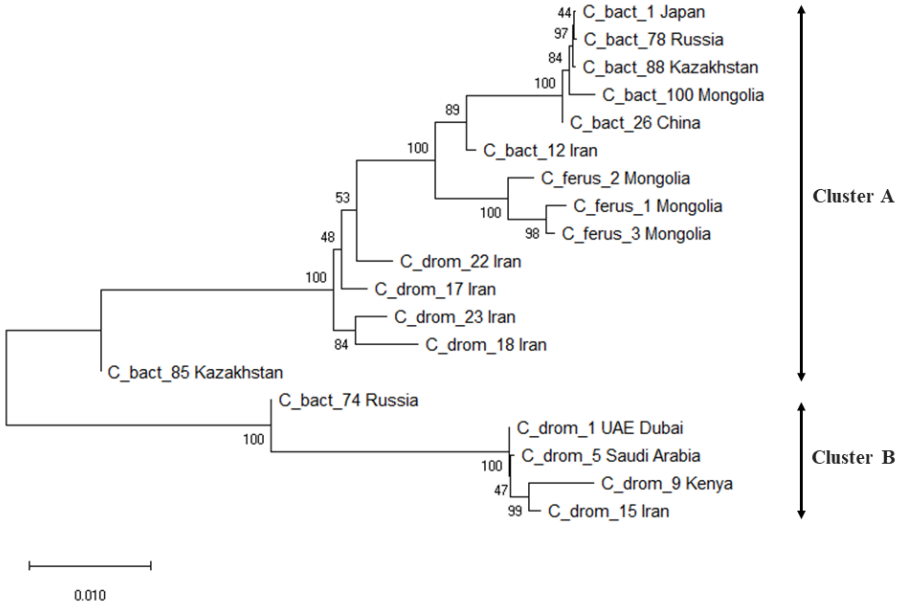

Figure 3. A maximum likelihood tree using the same mtDNA sequences used for the neighbor-joining tree in Figure 2.

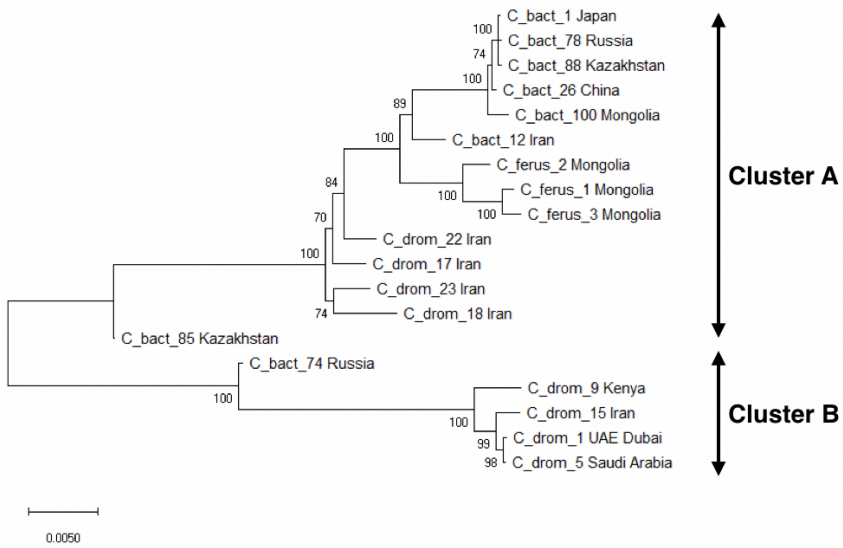

Figure 2. A neighbor-joining tree of 19 complete mtDNA sequences of camels. Sequence IDs are the same with those of Supplementary Table 1.

mtDNA sequences that are included in our Figure 2; C_bact_85 Kazakhstan (accession number MH109985) and C_bact_74 Russia (accession number MH109974). Interestingly, these two sequences have considerably shorter branches from the root than the other sequences (see Figures 2 and 3). The evolutionary rate of mtDNA among congeneric species is expected to be roughly the same under the neutral evolution (e.g., Saitou [2018]). Therefore, some unexpected situation may exist for these two sequences.

We thus used Tajima's (1993) test for these two sequences

(C_bact_85 Kazakhstan and C_bact_74 Russia) as well as five more mtDNA sequences (C_bact_12, C_drom_17, C_drom_18, C_drom_22, and C_drom_23) fromIran, whose branch lengths are somewhat shorter than the remaining 12 sequences (see Figures 2 and 3). The result is shown in Table 2. All the seven sequences were statistically shorter than control sequences. This result is inconsistent with the expected pattern for mtDNA sequences with the putatively same evolutionary rates.

Table 2. Result of Tajima's (1993) test for seven sequences

Set ID	Short branch sequence	Control sequence*	Outgroup sequence*	Chi-square value	p value
1	C_bact_74 Russia	A	B	244.98	6.36E-54
2	C_bact_85 Kazakhstan	B	A	383.79	4.58E-84
3	C_drom_17 Iran	B	A	78.54	8.82E-18
4	C_drom_18 Iran	B	A	45.96	1.05E-10
5	C_drom_22 Iran	B	A	73.93	8.84E-17
6	C_drom_23 Iran	B	A	73.48	1.11E-16
7	C_bact_12 Iran	B	A	17.61	1.50E-04

* Sequence A = C_drom_5_Saudi Arabia, Sequence B = C_bact_1 Japan

Ming et al. (2020) used *C. ferus* as the reference sequence of mtDNA for morphologically bactrian (two-humped) camel. We thus remapped all short read data reported by Ming et al. (2020) to the reference dromedary (one-humped camel) mtDNA sequence (NC_009849), and obtained mtDNA sequences. Somehow, C_bact_12 short read data did not exist in the database, and we omitted this individual from mapping. Mitochondrial DNA sequences of six camels were quite different from those reported by Ming et al. (2020). Then we tried mapping of the short read data of the six camels onto mitochondrial sequences of *C.*

bactrianus and *C. dromedarius*. The resulted sequences were all close to *C. dromedarius* whichever reference was used. The vcf files of the mapping also showed that variant calls were the least when *C. dromedarius* was used as the reference (supplementary vcf files 1 – 18; http://idarwin.org/docs/vol2_suppl_files_1/Suppl_vcf_files.pdf). This means that these six camels are morphologically two-humped like *C. ferus* or *C. bactrianus* but their mitochondria are close to one-hump camel, *C. dromedarius*, probably because of maternal hybridization in the past. Therefore, we employed mitochondrial sequences mapped onto *C. dromedarius* for these six camels. These re-determined mtDNA sequences were deposited to DDBJ as TPA (third party annotation), as listed in Table 3.

Table 3. A list of 6 TPA entries for remapped camel mtDNA sequences

Camel	Short read	Assembly	Morphology	mtDNA lineage
C_bact_74 Russia	SRR5563521	BR001658	Two-hump	One-hump
		MH109974		Cluster B (one-hump)
C_bact_85 Kazakhstan	SRR5563543	BR001659	Two-hump	One-hump
		MH109985		Cluster A (one/two mix)
C_drom_17 Iran	SRR5563504	BR001657	One-hump	One-hump
		MH110001		Cluster A (one/two mix)
C_drom_18 Iran	SRR5563498	BR001654	One-hump	One-hump
		MH110002		Cluster A (one/two mix)
C_drom_22 Iran	SRR5563499	BR001655	One-hump	One-hump
		MH110006		Cluster A (one/two mix)
C_drom_23 Iran	SRR5563500	BR001656	One-hump	One-hump
		MH110007		Cluster A (one/two mix)

The "assembly" column shows the DDBJ/ENA/GenBank accession numbers of mitochondrial DNA assembled by us (above) and by Ming et al. (below). The "mtDNA lineage" column indicates the clusters our sequences belonged to in Figure 2 (above) and that of the Ming's sequences in Figure 1 (below).

We used these new six mtDNA sequences determined by us for phylogenetic tree construction (Figure 4). All the six mtDNA sequences are now very close to typical dromedary mtDNA sequences (C_drom_1, C_drom_5, C_drom_9, and

C_drom_15), and formed a clear-cut cluster. Although morphologically two-humped, our new mtDNA sequences of C_bact_74

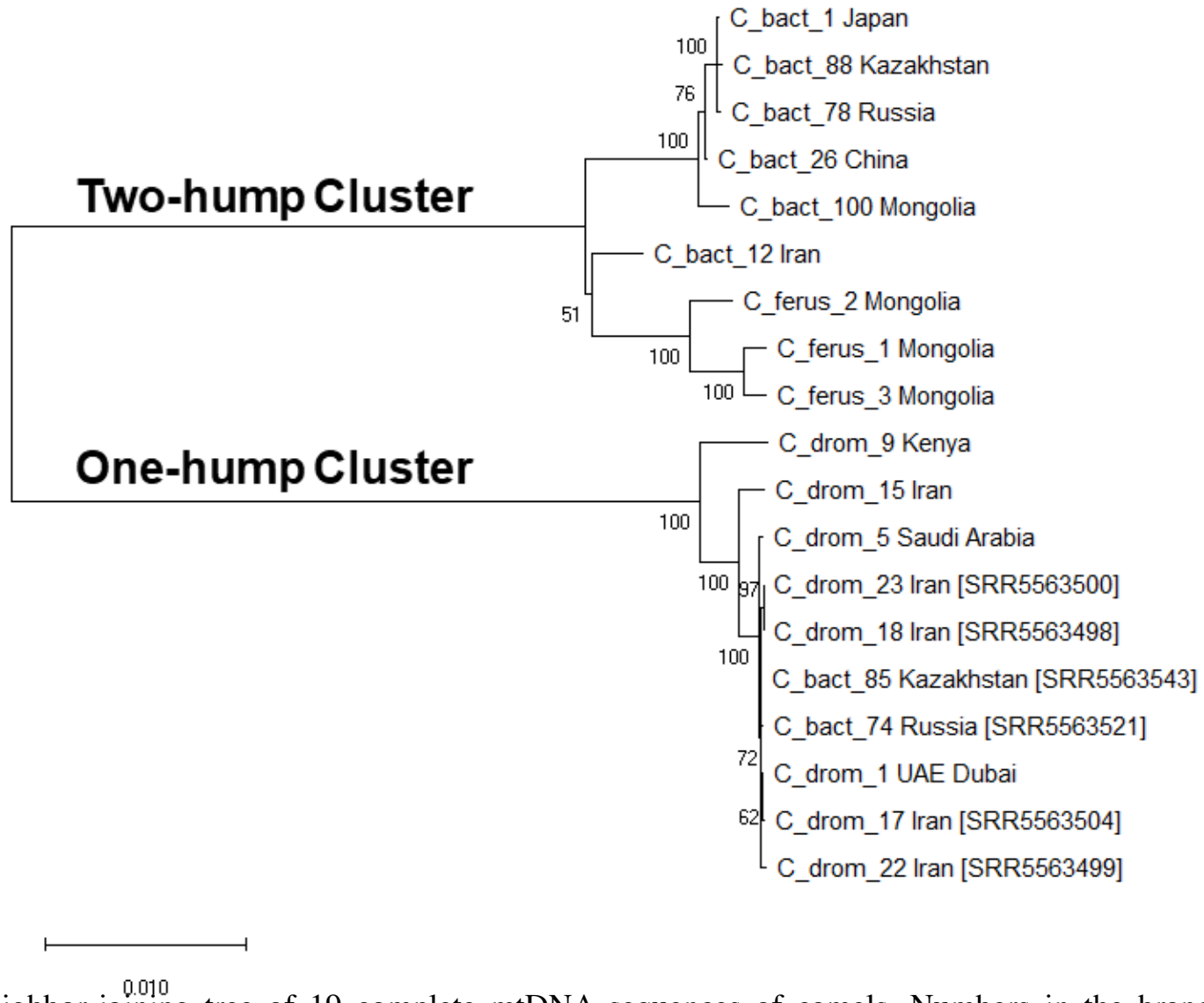

Figure 4. A neighbor-joining tree of 19 complete mtDNA sequences of camels. Numbers in the branches represent the bootstrap values (%) from 1,000 replicates. Two clusters are named as Two-hump Cluster and One-hump Cluster. Six sequences re-determined from short read data (SRR IDs are given to them) are all belonging to One-hump Cluster.

and C_bact_85 from Russia and Kazakhstan, respectively, are very similar to mtDNA sequences of one-humped camels. We named this cluster as "One-hump Cluster" in Figure 4. The other cluster contains ferus and bactrian mtDNA sequences, and we named this cluster as "Two-hump Cluster" in Figure 4. The external branch for the C_bact_12 sequence is still somewhat shorter than other sequences in this cluster. An ML tree obtained for the same 19 sequences (Figure 5) had an identical branching pattern with that for the NJ tree (Figure 4) for branches with high bootstrap values.

There is one possibility that the noncoding region (so-called D-loop) is the major cause of error made by Ming et al. (2020). We thus eliminated the noncoding region and constructed phylogenetic trees. Supplementary Figure 3 (http://idarwin.org/docs/data/vol2_suppl_files_1/Suppl_Fig_3.pdf) shows four trees and they correspond to those of Figure 2 (Supp. Fig. 3A), Figure 3 (Supp. Fig. 3B), Figure 4 (Supp. Fig. 3C), and Figure 5 (Supp. Fig. 3D). In all cases, phylogenetic trees using complete mtDNA sequence data (Figures 2-5) and those without the noncoding region (Supp. Figs. 3A-3D) are more or less the same. Therefore, sequence errors of Ming et al. (2020) are not focused on the noncoding region.

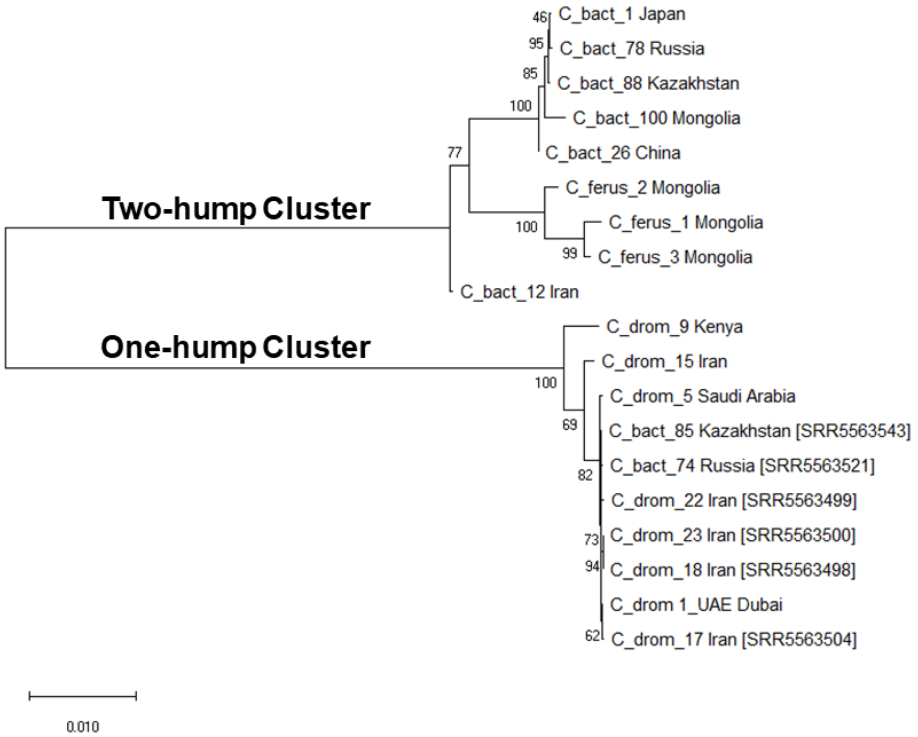

Figure 5. A maximum likelihood tree using the same mtDNA sequences used for the neighbor-joining tree in Figure 4.

Discussion

When we constructed phylogenetic trees for camel mtDNA sequences, we found some unexpected patterns, namely, violation from the approximate constancy of the evolutionary rates among closely related species mtDNA. Ming et al. (2020) presented a

phylogenetic tree of mtDNAs, but they did not show branch lengths, and all sequences are located at the same vertical line. This clearly indicates the importance of branch lengths for phylogenetic analyses in general.

We reanalyzed short read data of these camel sequences submitted to NCBI BioProject database (accession number PRJNA383081), and found mtDNA sequences different from those reported by Ming et al. (2020). Two of these re-determined mtDNA sequences, one from Russia (SRR5563521) and the other from Kazakhstan (SRR5563543), were originated from morphologically two-humped camels. However, their mtDNA sequences were very similar to those of one-humped camels (see Figure 2). This suggests that these two camel species have been hybridizing for a long time. Ming et al. (2020) also noticed mtDNA introgression from dromedary to bactrian camels.

If we consider repeated back-crossing to bactrian camels after creating F1 hybrid between C. dromedaries (one-humped camel) and C. bactrianus (two-humped camel), it is possible to obtain this discrepancy. In fact, Lado et al. (2020) who examined nuclear genome sequences of many dromedary individuals reported that some dromedaries from Iran and Kazakhstan were found to have considerable proportion of Bactrian nuclear DNAs. In any case, we have to be careful to choose reference sequences to determine mtDNA sequences from NGS-generated short read data.

The main point of study is to indicate the erroneously reported camel mtDNA sequences. Unfortunately, camel DNA researchers do not realize this problem, and just use reported sequences (e.g., Ming et al. [2021]). We thus think this report is useful for camel mtDNA sequence analyses.

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