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Molecular Phylogeny Of Mouse Hare (*Ochotona*: Lagomorpha) Using Cytochrome b Gene As A Phylogenetic Marker

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Abstract: ***Mouse hare (Ochotonidae) is small, short and rounded ears, small limbs and non-ostensible tail. In present study total, 402 bp sequences of mitochondrial DNA cytochrome b gene have been taken for 31 species of Ochotona family. In each sequence having length 402bp coded 134 amino acids, and initial codon is ATG in maximum species. In present analysis Neighbor-joining, minimum evolution and maximum likelihood methods strongly indicated five major groups. The first group comprises fourteen animals (the surrounding Qinghai–Tibet Plateau group) includes present study animal (Ochotona spp.) with O. macrotis, O. roylei, O. koslowi, O. iliensis, O. rutila, O. himalayana, O. forresti, O. rufescens, O. ladacensis, O. erythrotis, O. gloveri, O. muliensis, and O. brookei. The second (the northern group) is composed of eight animals i.e. O. collaris, O. princeps, O. mantchurica, O. hyperborea, O. coreana, O. turuchanensis, O. pallasi, and O. alpine. The third group (the Huanghe group) comprises only one species, O. huangensis. The fourth group comprises seven animals included O. daurica, O. thibetana, O. thomasi. O. annectens, O. cansus, O. curzoniae and O. nubrica. The fifth group the Central Asia comprises only one species, and is O. pusilla.***

Keywords: Mouse hare, *Ochotona,* Phylogeny, Cytochrome b, Lagomorpha.

1. Introduction

The extant mouse hare is endemic to the Holarctic realm and consist of a monotypic genus, *Ochotona*. The species of *Ochotona* occur in the open Gobi in Eurasia, at western Chinese highlands (Qinghai-Tibet Plateau) and the Himalayas, as well as at their vicinities, in America distributed in the northwest alpine areas. Most mouse hare lives at high altitudes (between 2000 to 8000 meters above sea level) cold and moist climatic conditions [1]. Although all species of mouse hare (*Ochotona*) show notable consistency in general morphology, so that the number of recognized species and phylogenetic relationships among mouse hare are not stable throughout. Systematically, the mouse hare is novel living representatives of *Ochotona* form a monotypic genus within the family Ochotonidae, which was clearly differentiated from the other lagomorphs as early as the Oligocene [2]. In addition, all species of *Ochotona* are remarkably homogeneous in external morphology and body mass, so that constraints imposed on comparative data by allometric relationships are reduced [3]. Mouse hares are also a very poorly known group of mammals. The morphological similarities among species which make them ideal for comparative studies have presented major obstacles to systematists. In addition, most species of mouse hare occur almost exclusively in remote settings, thus biological data needed to support early attempts to classify the genus have generally been lacking. Till date, the number of recorded species varies from 18 to 30 [4,5]. The phylogeny of mouse hare is also questionable. Luo and Feng, [6] first described the phylogeny of genus *Ochotona* using fossil data, but their phylogenetic tree was not accurate and the relationships of many species were unresolved. Weston, [7] made a comprehensive effort to examine the relationship of the genus Ochotona, but Smith, et al., [4] pointed out that her revision was a phonetic analysis and not useful for inferring phylogenetic relationships. Yu, et al., [8] reported the phylogeny of genus *Ochotona* using mitochondrial DNA sequences of 19 species and recommended that *Ochotona* could be divided into three subgenera. Later on in 2004, Niu et al., [5] evaluate 27 species within the genus *Ochotona* using 402 base-pair cytochrome b protein coding gene and proposed five major species groups: the northern group, the surrounding Qinghai-Tibet Plateau group, the Qinghai-Tibet Plateau group, the Huanghe group, and the Central Asia group. However, many known species [4] were not examined in their study, so it could not provide full phylogenetic relationships among mouse hare, and their deduction on evolutionary processes of *Ochotona* is also incomprehensible. In order to resolve the phylogenetic issues, we employed sequence data from the mitochondrial cytochrome b gene, which is adequate for studying taxed at low taxonomic levels like intrageneric or intraspecific relationships [9]. This report is the first international attempt to compare and analyzed a 402 base-pair region of the cytochrome b protein coding gene of the 31 species of *Ochotona* included one sample analyses from Garhwal Himalaya, India (Table 1). The goals are to present a parsimonious systematic treatment of mouse hare that will prove useful for their conservation and management; to summarize the important elements of the biology of each species; to define clearly the conservation and management issues concerning mouse hare.

2. Methodology

Tissue of mouse hare has been collected from the high altitude areas of Tungnath Himalaya Uttarakhand, India (attitudinal 3290 m above sea level, situated between latitudes 30.23 N to longitude 79.22 E) during field survey in session 2007-2009 and preserved in 95% (v/v) ethanol at 4°C. High molecular weight DNA was isolated by protein

digestion, phenol: chloroform extraction (24:1 v/v) and ethanol precipitation using standard phenol/chloroform method [10] with partial modifications. The DNA stock sample was quantified using a Nanodrop spectrophotometry and purity of DNA was checked by Agarose 0.8% (w/v) gel electrophoresis. Electrophoresis was carried out at 80V for 30 min at room temperature. The DNA was amplified by Gradient PCR (Touch-Gene model manufacture by TECHNE and supplied by AXYGEN) using Forward Primer, L14724 and Reverse Primer H15274 [5] in the departmental laboratory, HNB Garhwal University, Srinagar Garhwal, Uttarakhand, India. PCR amplification was performed in a 50µl reaction volume mixture, which contained 10 mM Tris-HCl (pH9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, each dNTP at 200 µM, 1.25 U of *Taq* DNA polymerase, each primer at 200 nM, and 3–6 µl of the purified DNA as a template [5]. The cycling parameters were initial denaturation at 95°C for 10 min (1 cycle); denaturation at 94°C for 1 min; annealing for 1.5 min at 45°C–52°C; extension at 72°C for 1.5 min (42 cycles), and a final extension at 72°C for 10 min (1 cycle). The amplified PCR amplicon having molecular size of 575 bp was purified using Exo-SAP enzyme as per manufacturers guidelines, and further used for sequencing reaction. Sequencing was carried out by Xcelris Labs Limited, Ahemadabad using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturer's instructions. Full length sequences have been made from forward and reverse strands and aligned using the Clustal W program using all Databases included GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences) 9,213,241 sequences; 28,392,641,000 total letters and total 575 bp sequence obtained was submitted to GENBANK database and assigned Genebank ID No "GQ442628"

In a present study one sample analyses from Garhwal Himalaya, India and other 30 animal sequence were retrieved from GenBank database. The extent of sequence difference between species was calculated by averaging pairwise comparisons of sequence difference across all individuals. The pairwise evolutionary distance among haplotype was determined by the Kimura 2-Parameter method [11] using the Molecular Evolutionary Genetics Analysis software program MEGA4 [12]. The first phylogenetic tree was constructed by the Neighbor-Joining method [13] based on bottom-up clustering and distance between each pair of taxa and interior branch test. The second phylogenetic tree was inferred by Minimum Evolutionary methods based on character values observed for each species, rather than the distances between the sequences. Branch lengths generally not obtained for each topology; the sequences at each node inferred to be those that require the least number of changes to give each of the two immediately descendant sequences [14]. Bootstrap values were included to test the reliability of inferred trees [11] and the estimation of evolutionary divergence between sequences was computed. All pairwise distances were analyzed using the Kimura 2 Parameter (K2P) method in MEGA4 and the maximum - likelihood tree was calculated via quart puzzling search method using PAPU software programs [16].

3. Results and Discussion

In the present study, total 402 bp sequences of mitochondrial DNA cytochrome b gene have been taken for 31 species of *Ochotona* family including one from Garhwal Himalaya, India. Each sequence having length 402bp coded 134 amino acids, and initial coding is ATG in maximum species. In present study nucleotides composition revealed the fewest guanines (14.0%) among four nucleotides. The degree of bias is depending upon the codon composition 21.6 % G in the first position, 16.4 % in second position and 3.9 % in third position. The first position is rich in adenines (30.2 %), the second position is rich in thymines (38 %), and third position is rich in cytidines (47.2 %). The base composition bias for each position is 0.0665, 0.1720 and 0.4200 which is similar to the reported values for other mammal groups [5,8]. In the present analysis recorded 248 Conserved sites, 154 variable sites, 31 singleton sites and 123 parsimony informative in 402 sequence sites. The estimates of average codons based evolutionary divergence sequence pair is 0.377 (SE 0.030). The nucleotide frequencies are 0.285 (A), 0.277 (T/U), 0.289 (C), and 0.148 (G). The transition / transversion rate ratios are k1 = 2.876 (purines) and k2 = 6.461 (pyrimidines) and overall transition/transversion bias is R = 2.278 in final data bases.

In the present analysis, 31 species of *Ochotona* have been evaluated using neighbor-joining, minimum evolution and maximum likelihood analysis method, indicated five major groups. The first groups comprise fourteen animals (the surrounding Qinghai–Tibet Plateau group) includes present study animal (*Ochotona* spp.) with *O. macrotis, O. roylei, O. koslowi, O. iliensis, O. rutila, O. himalayana, O. forresti, O. rufescens, O. ladacensis, O. erythrotis, O. gloveri, O. muliensis, and O. brookei.* The second group (the northern group) composed of eight animals i.e. *O. collaris, O. princeps, O. mantchurica, O. hyperborea, O. coreana, O. turuchanensis, O. pallasi, and O. alpine*. In the northern group recognized in both Minimum Evolution and Neighbor-Joining trees test (bootstrap values more than 60%); the third group (the Huanghe group) comprises only one species, *O. huangensis*. The fourth group comprises seven animals included *O. daurica, O. thibetana, O. thomasi. O. annectens, O. cansus, O. curzoniae and O. nubrica*. In this group, three sub-groups are observed in Minimum Evolution, Neighbor-Joining and Maximum-likelihood trees. The fifth group the Central Asia comprises only one species, and is *O. pusilla*.

In current revision the surrounding Qinghai-Tibet Plateau group comprises a group of rock-talus-dwelling species (*O. macrotis, O. roylei, O. illiensis, O. himalayana, O. rutila, O. erythrotis, O. gloveri, O. brookei and O. muliensis*) and intermediate types between talus and steppe dwelling (*O. ladacensis, O. rufescens, O. forresti, and O. koslowi*) [4,17]. Present data *O. roylei* and *O. macrotis* form a sister taxon, in which sequence divergence is very low. Our data strongly imply that these four species (*O. erythrotis, O. gloveri, O. brookei and O. muliensis*) originated from a common ancestor, and that differentiation has occurred recently. The sister taxa pair between *O. ladacensis* and *O. koslowi* [8] not supported by our study. In previous studies, phylogeny of 14 species derived from morphological studies suggested that the steppe-dwelling species (*O. curzoniae* and *O. daurica*)

and the shrub-dwelling species (*O. huangensis, O. thibetana, O. cansus,* and *O. thomasi*) were very distinct, and constructed the base and top branches of the phylogenetic tree respectively [17]. However, subsequent molecular data indicated that relationships between the steppe and shrub species were very close [8]. The northern group consists of eight species, and placed in a single group and can be divided into two subgroups, North Palearctic (*O. collaris, O. princeps, O. mantchurica, O. hyperborea, O. turuchanensis*) and Nearctic subgroup (*O. pallasi, O. Alpine, O. coreana*). The former distributed at high latitudes, and occupy rock-talus areas (*O. alpina* and *O. hyperborea*) or intermediate areas between talus and steppe (*O. pallasi*), but the latter is a group of typical rock and talus-dwelling mouse hare [4]. The comparative cytogenetic analysis also revealed the high level chromosomal divergence between North Palearctic groups with a low diploid chromosome number: 2N=38-42 and Nearctic species with a high diploid chromosome number 2N=68 [1]. In this study, the shrub-dweller *O. huangensis* constructs a distinct group (The Huanghe group), and the topology is stable in terms of methods. Our result indicates that these species have a common ancestor, and the differences in habitat choice reflect the adaptability to environment changes, which are considered as a major element in the evolutionary processes of mouse hare [8]. Similar to some species within the Qinghai-Tibet Plateau group, the steppe mouse hare *O. pusilla* is a characteristic burrowing steppe-dweller [4]. The present study also supported on the base of phylogenetic trees, and represents that *O. pusilla* is very distinct an ancient species. According to environmental alteration can often produce strong discriminatory pressures that can result in rapid morphological diversification [18]. During the Pleistocene, *O. pusilla* diversified considerably and inhabited the vast plains of Europe and Asia, spreading widely with the steppe. However, during postglacial time until the Holocene, the reestablishment of forest and grassland restricted the range of *O. pusilla*. Recently, it only inhabited the steppe areas of Central Asia [19]. On the basis of fossil remains, the structure of cheek teeth, and number of chromosomes, *O. pusilla* is often considered as a relic of the Late Pliocene [20], on the basis of chromosomal & taxonomical data the same diploid numbers of chromosomes and the similar archaic feature in teeth morphology, the Nearctic pikas are considered to be much closer to *O. pusilla*. There is a hypothesis that the ancient pusilla-like taxon probable ancestral form of *O. pusilla*, *O. princeps* and *O. collaris* migrated from Asia to the North America at the end of Late Pliocene and the beginning of Pleistocene, and became distributed widely to southeastern America [1] The sequence data and geographic study also indicates that separation between O. princeps in the south and *O. collaris* in the north is probably the result of the Wisconsinan glaciation [21]. According to taxonomical data, the *O. princeps* consists of 36 subspecies, which may be due to the strong isolation of these post pleistocene events [22]. However, speciation events within the North Palearctic subgroup are few because of its comparatively stable habitats [8]. The Qinghai-Tibet Plateau geological studies indicate that three large-scale uplifting of the Qinghai-Tibet Plateau occurred strongly and frequently, accompanied by the large glaciers in the Northern Hemisphere. However, glaciers developed only in major mountain chains, and no unified ice sheet covered the Qinghai-Tibet Plateau during the Quaternary Ice Age [23], so a large-scale biotic extinction did not happen, and ancestral pikas survived. Species of the surrounding Qinghai-Tibet Plateau group, the Qinghai-Tibet Plateau group, and the Huanghe group are just distributed in the Qinghai-Tibet Plateau and adjacent mountains. Obviously, differentiations of the three groups are closely related to the uplifting of the Qinghai-Tibet Plateau and subsequent climatic changes. The species within the surrounding Qinghai-Tibet Plateau group is typical plateau and high altitude adapted species, which have undergone a rapid radiation since the Early Pleistocene. The molecular data also indicate that radiation of this group may be multi-diverse. The species within the Qinghai-Tibet Plateau group are young and the dominant inhabitants of the plateau. As previously reported [8], the steppe dwellers and shrub dwellers are interspersed within the group of the phylogenetic trees (figure one to three), and the sequence divergence between them is very low, but that between the steppe dwellers or the shrub dwellers are high. This result implies that the divergence event between the two dwellers may have happened recently. The morphological similarities within steppe dwellers or shrub dwellers was probably due to convergent evolution, apparently because the morphological characters have tracked the environment and resulted in adaptive modification in structure that increase the probability of survival [24]. The convergent event may also have happened between the Huanghe group and the Qinghai-Tibet Plateau group, now that the former shares the identical habitat with the shrub dwellers of the latter.

4. Conclusion

Mouse hare (family Ochotonidae) is small; with comparatively short and rounded ears, small limbs and a visibly non-ostensible tail. According to different systematic studies, 30 living species of *Ochotona* are known. In present study Neighbor-joining , minimum evolution and maximum likelihood analysis strongly indicated five major groups. The first group comprises fourteen animals present at the surrounding Qinghai–Tibet Plateau group. The second (the northern group) is composed of eight animals. The third group (the Huanghe group) comprises only one species, *O. huangensis*. The fourth group comprises seven animals included present at Qinghai–Tibet Plateau. The fifth group the Central Asia comprises only one species, and is *O. pusilla*.

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Table 1: The Genbank accession numbers of sequences analyzed in the present study.

S.No.		**Species Name**	**GenBank Number**
1.	The Surrounding Qinghai–Tibet Plateau Group	*Ochotona iliensis*	AY191824
2.		*Ochotona koslowi*	AF272993
3.		*Ochotona macrotis*	AF273010
4.		*Ochotona roylei*	AF272988
5.		*Ochotona rutila*	AY056601
6.		*Ochotona sp.*	GQ442628
7.		*Ochotona himalaya*	AF272997
8.		*Ochotona forresti*	AF272998
9.		*Ochotona rufescens*	AJ132206
10.		*Ochotona erythrot*	AF272999
11.		*Ochotona gloveri*	AY056602
12.		*Ochotona muliensi*	AF421884
13.		*Ochotona brookei*	AY056600
14.		*Ochotona ladacens*	AF272992
15.	The Northern Group	*Ochotona collaris*	AF176578
16.		*Ochotona princeps*	U58940
17.		*Ochotona coreana*	EF567060
18.		*Ochotona mantchur*	DQ335518
19.		*Ochotona hyperbor*	AF272994
20.		*Ochotona turuchan*	DQ335488
21.		*Ochotona pallasi*	AY056607
22.		*Ochotona alpina*	AF273009
23.	The Huanghe Group	*Ochotona huangens*	AF272995
24.	Qinghai–Tibet Plateau Group	*Ochotona dauurica*	DQ335519
25.		*Ochotona thomasi*	AF272987
26.		*Ochotona thibetan*	AF272986
27.		*Ochotona annecten*	AF273008
28.		*Ochotona cansus*	AF273003
29.		*Ochotona curzonia*	AF273001
30.		*Ochotona nubrica*	AF272991
31.	Central Asia Group	*Ochotona pusilla*	AY260744
32.	Outer Group	*Ocryctolagus cuniculus*	CU07566

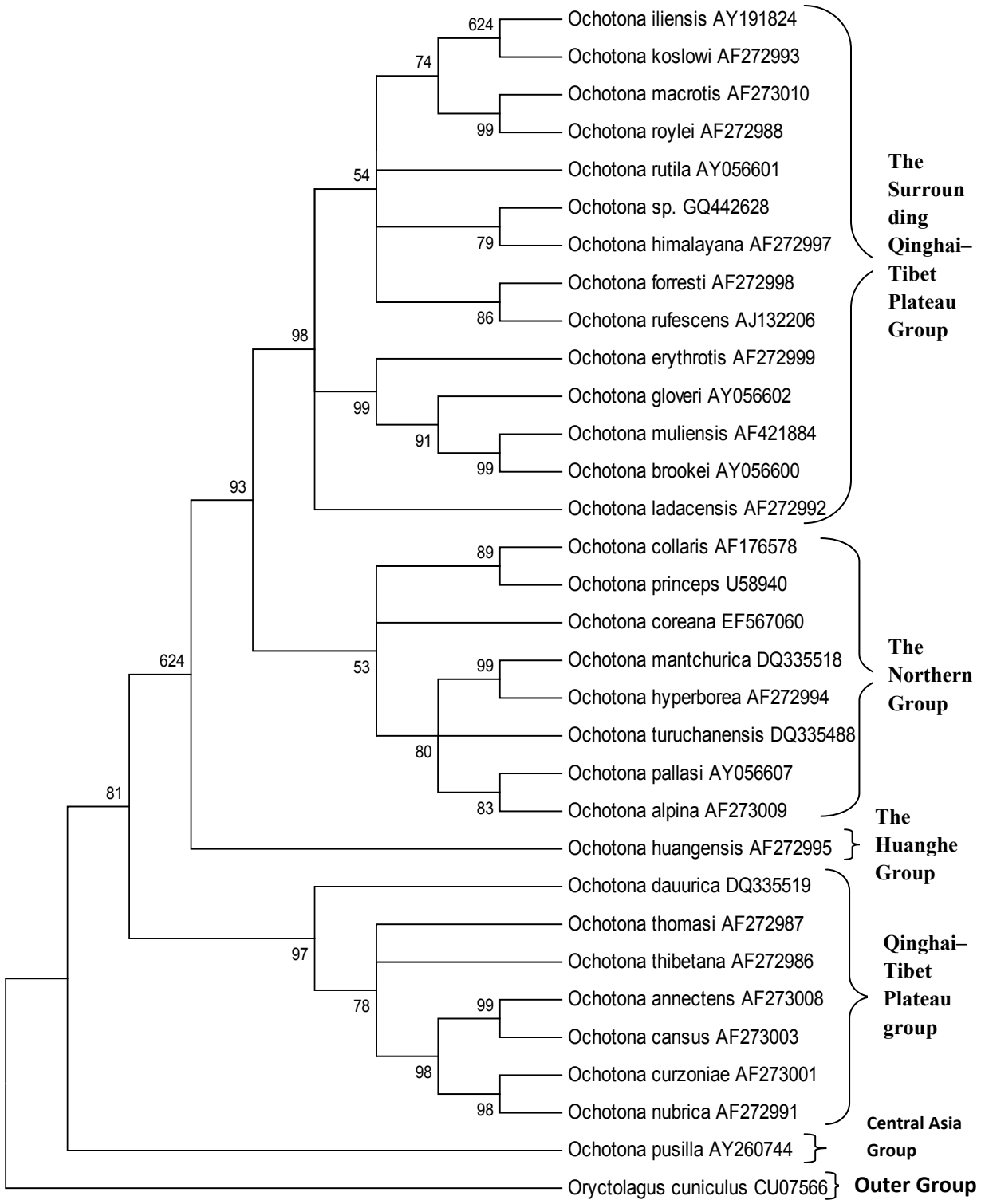

Figure 1: Neighbor-Joining distance tree with distance estimated by Kimura two parameter method Score derived from bootstrap analysis with 1000 replications respectively are shown above the branches.

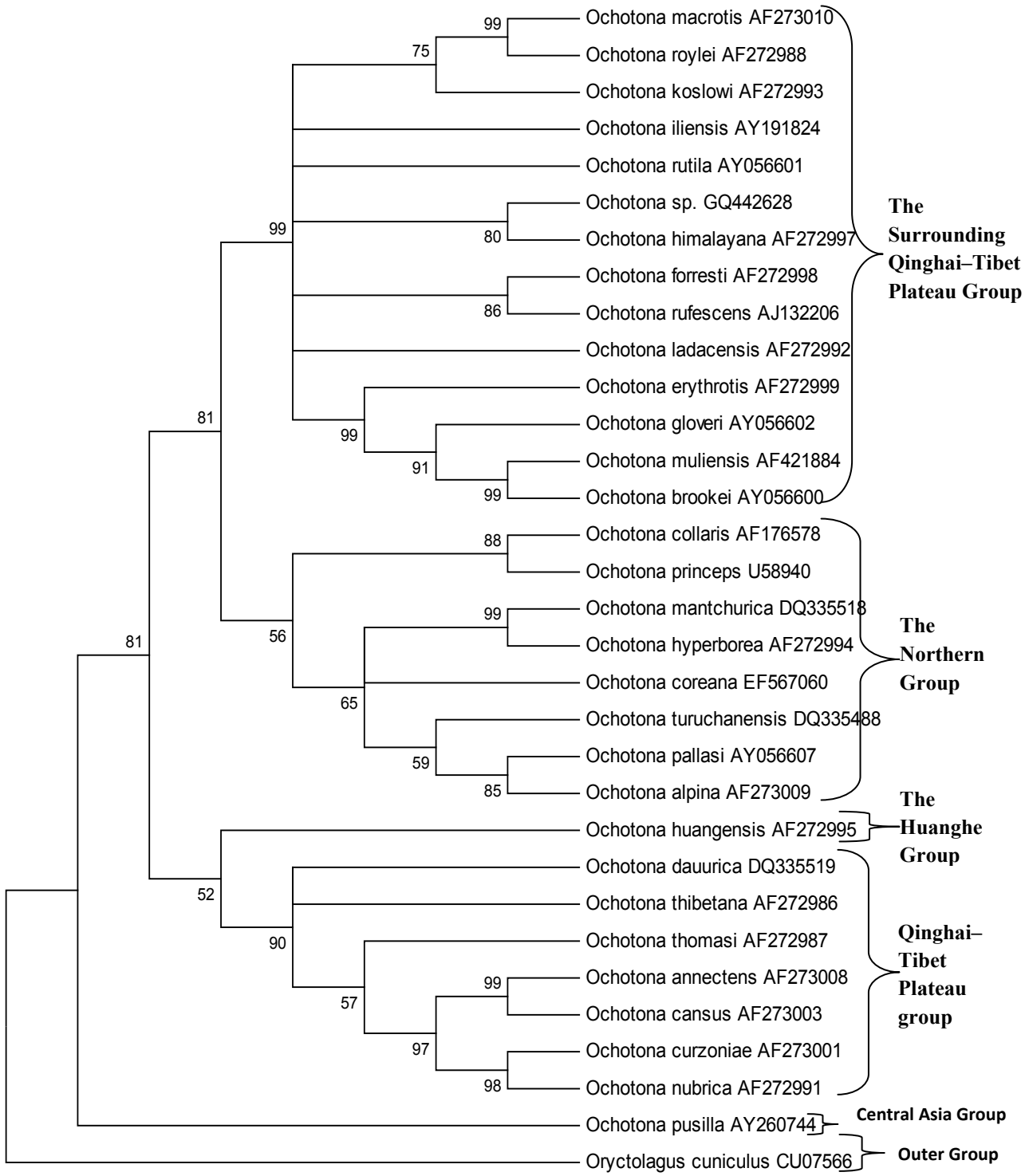

Figure 2: Minimum Evolution tree with distance estimated by Kimura two parameter method Score and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1.

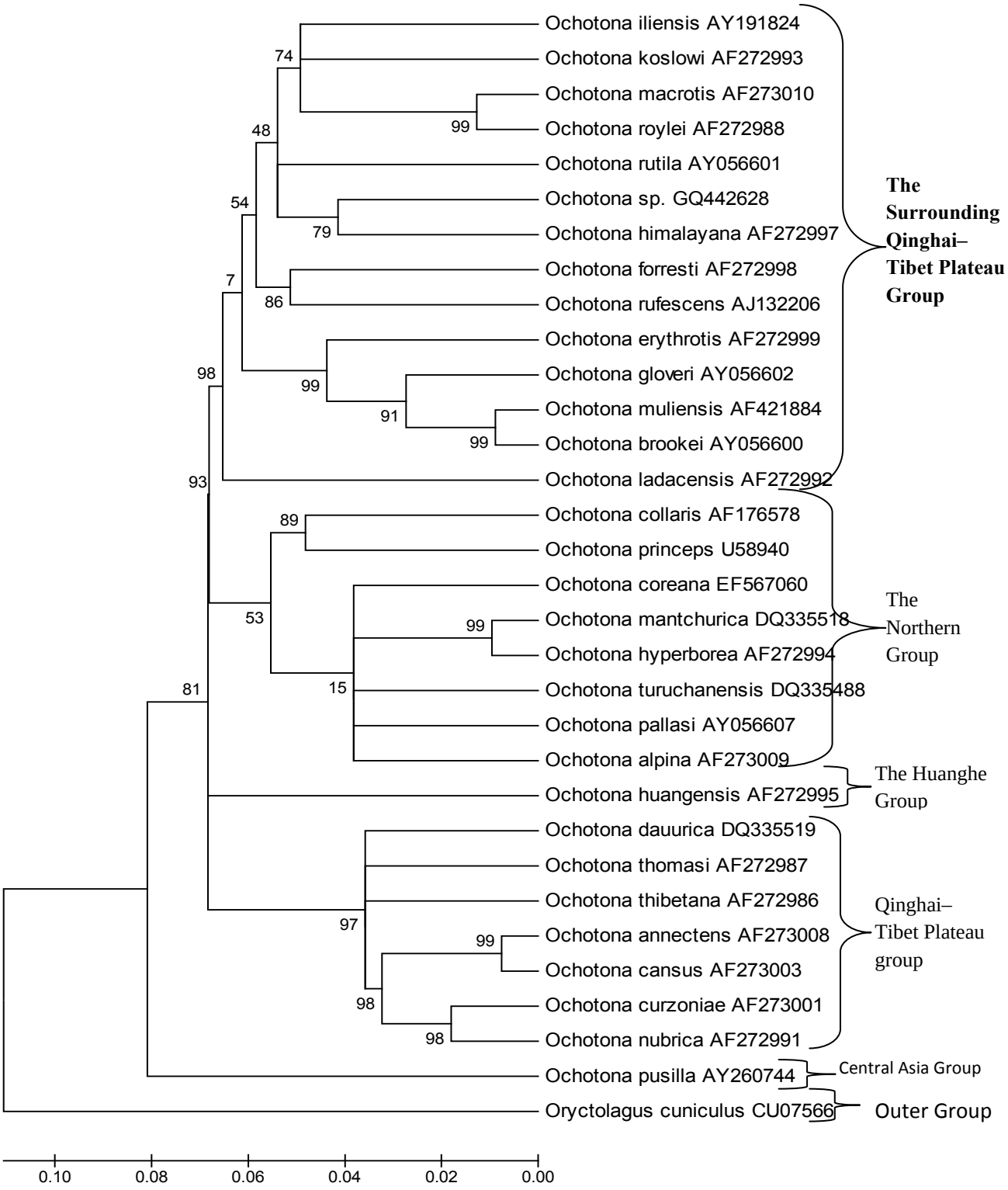

Figure 3: Maximum-likelihood tree obtained using quartet-puzzling analysis using a 2:1transition: transversion ratio. The numbers above the branches are puzzling scores.