

Molecular conformation of *Ziziphus budhensis* and its religious and economic values

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The newly described species, *Ziziphus budhensis* was confirmed as Chinese Jujuba, *Z. xiangchengensis* on the basis of their DNA analyses. *Z. budhensis* was explained as a new species on the basis of some morphological differences in 2015. In the Year 2016, the DNA samples were collected from the type locality of Nepal, and the molecular analyses were carried out. The type specimens and the other available images from the different herbariums were examined. Besides, the protologue and the type images were studied carefully. The result showed that though there were some differences in the habit and the habitat of the plant, the previously described new species, *Z. budhensis* was found to be same as the Chinese species, *Z. xiangchengensis*. This study also showed the importance of the molecular work of *Z. budhensis* and confirmed it morphologically distinct although it was very close to the Chinese species.

Key Words: Molecular conformation, Rhamnaceae, *Ziziphus budhensis*, *Z. xiangchengensis*

The genus, *Ziziphus* is characterized as warm-temperate and subtropical plant. The plants are mostly shrubs or small to medium-sized trees, erect or straggling, often climbing, evergreen or deciduous, often spinose with alternate leaves (Chen and Schirarend, 2007). There are about 100 *Ziziphus* species reported throughout the world (Mabberley, 2008); the number has been reported up to 170 species (Islam and Simmons, 2006). Among them, 17 species are reported from India (Bhandari and Bhansali, 2000), 12 from China (Chen and Schirarend, 2007), 7 from Bhutan (Grierson and Long, 1991) and 7 including *Z. budhensis* are reported from Nepal (Bhattarai and Pathak, 2015; NHPL, 2011; NHPL, 2012). So far, about 275 names (including all taxa) under the genus, *Ziziphus* have been reported (TPL, 2013). Most of the *Ziziphus* fruits are edible (Outlaw Jr. *et al.*, 2002).

The most commonly named “Bodhichitta” or “Buddha Mala”, a new and endemic species to Nepal in 2015 was described as *Ziziphus budhensis*. The name was honored to Lord Buddha, the light of Asia, born in Lumbini, Nepal before 2500 BC. At that time, the species was also compared with some species reported from China. However, due to some morphological differences, the species was explained as a new species. This year, the DNA samples were collected from the type locality of Nepal, and the molecular analyses were carried out. The type images and the other available images together with the specimen from China were re-examined.

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The result showed that though there are some differences in the habit and habitat, the previously described new species was found to be the same as the Chinese species, *Z. xiangchengensis*.

Taxonomic description and distribution of *Z. xiangchengensis*

Ziziphus xiangchengensis Y. L. Chen and P. K. Chou, Bull.Bot. Lab. N. E. Forest. Inst., Harbin 5: 88. 1979.

Ziziphus budhensis Bhattarai and M.L. Pathak. Indian J. Pl. Sci. [Jaipur] 4(2): 73. 2015

Z. xiangchengensis is a spinose 2–3m, sometimes up to 8m tall small trees or shrub. Stem-glabrous; young branches- red-brown, densely pilose whereas old branches- gray-brown, flexuose, glabrous, old branches without spines. Leaves alternate, or 2 or 3 in fascicles; stipular spines 2, both erect or one recurved, 1–1.6cm, slender; petiole 5–8mm, sometimes up to 10mm, sparsely pilose; leaf blade abaxially pale green, adaxially dark green, ovate or ovate-oblong, 2–4cm × 1.5–3cm, papery, abaxially glabrous to clustered hairy in vein axils, adaxially glabrous, 3-veined from base, veins prominent on both surfaces, mid-vein without conspicuous secondary veins, base asymmetric, sub-rounded, margin crenate-serrate, apex obtuse or rounded. Flowers yellow-green, few- to 10, sometimes up to 14, fascicled in axils of leaves, without peduncles. Pedicel 4–5mm, ferruginous pilose. Sepals ovate-triangular, densely ferruginous pubescent, apex acute. Petals 5, creamy white, spatulate, cucullate clawed, ca. 2mm, initiated along with stamen; stamens shorter than petals. Stamens pentandrous extrose, exerted, ca. 2mm, anther 2 lobed cordate dorsally fixed. Ovary globose, glabrous; style 2-fid (rarely 3-fid), drupe yellow-green, globose, generally 1.2 (sometimes 0.6cm)–1.5cm in diam; apex mucronulate, with persistent calyx tube at base; fruiting pedicel 5–7mm, sparsely pilose; mesocarp thin (0.5–1cm), corky; endocarp cartilaginous; stone ca. 4 mm, 2-loculed and with 2 seeds. Seeds compressed, convex on one face, obovoid, ca. 6–10mm, sometimes up to 15mm. Flowering in March–April and fruiting in July–August.

Z. xiangchengensis is rarely distributed in West Sichuan (Xiangcheng) and the Yunnan Province of China (2,200–2,800m) and a few places of central Nepal (1,000–2,000 m). One of the interesting facts about the distribution is that, this plant is reported only in the two places of China, viz. the Sichuan Province and the Yunnan Province, and there are only four specimens (including types) at Chengdu Institute of Biology (CDBI), six specimens at Kunming Herbarium

(KUN) and very few are at National Herbarium of China (PE). The distribution is very limited; however, the presence of this plant in the Tibet Autonomous Region is under investigation. Due to this, the previous fact regarding the evolution of this species is quite mysterious.

Note: The description of the plant is based on the type literature of *Z. xiangchengensis* (Chen and Chou, 1979), the type literature of *Z. budhensis* (Bhattarai and Pathak, 2015) and the Flora of China (2007).

Specimen Examined

- The Holotype and the Isotype of *Z. budhensis*, KATH, C.N. 20701, Kharpakot (1,900m), Kavrepalanchok District, and
- Holotype and Isotype of *Z. xiangchengensis*, CDBI, C.N. 2994, Xiancheng, Sichuan (2,800m), 1979, China,

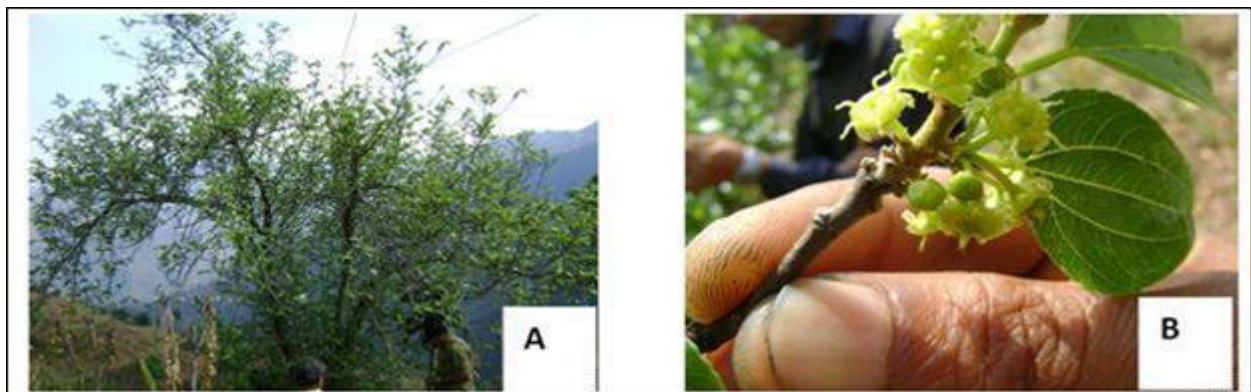
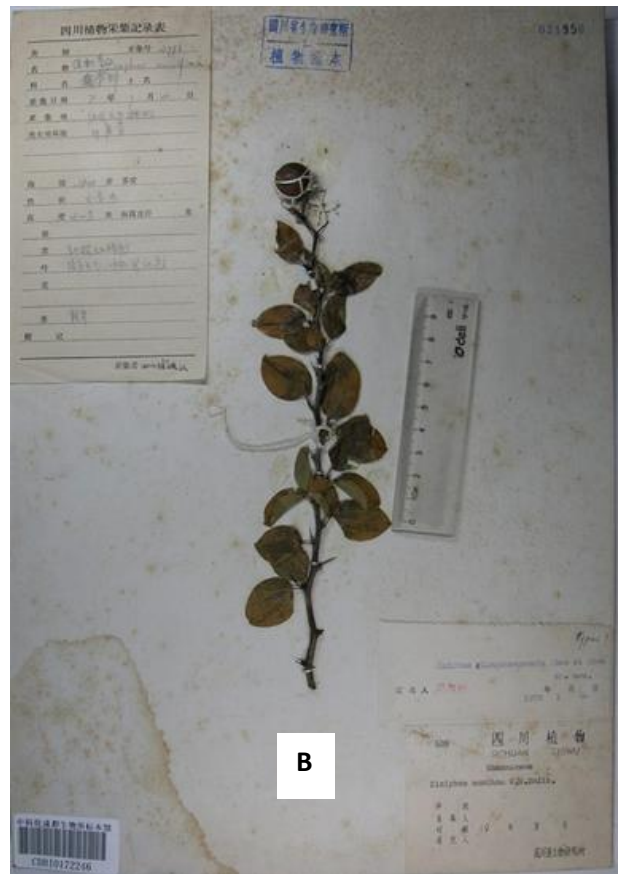


Figure 1: A tree of *Z. budhensis* (A) and its Flowering branch (B), Kharpakot (1,900m), Kavrepalanchok District, Nepal



A



B



C

Fig. 2: The type images of *Z. xiangchengnensis* deposited at the CDBI, Chengdu Institute of Biology, Chengdu, China (A and B) and its three faced-seed (C) (Source: www.cvh.org.cn)

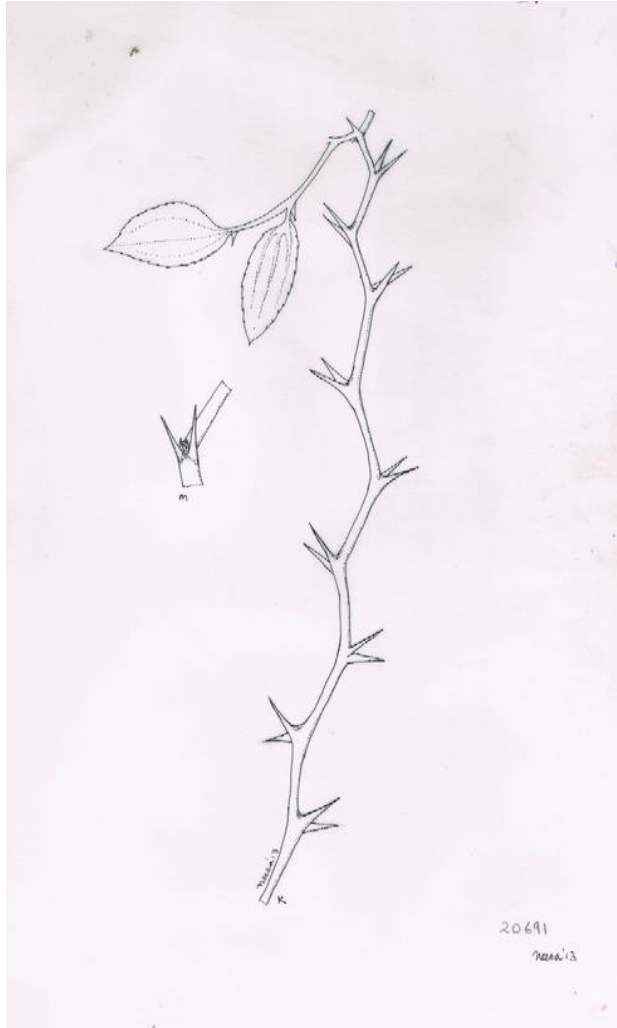
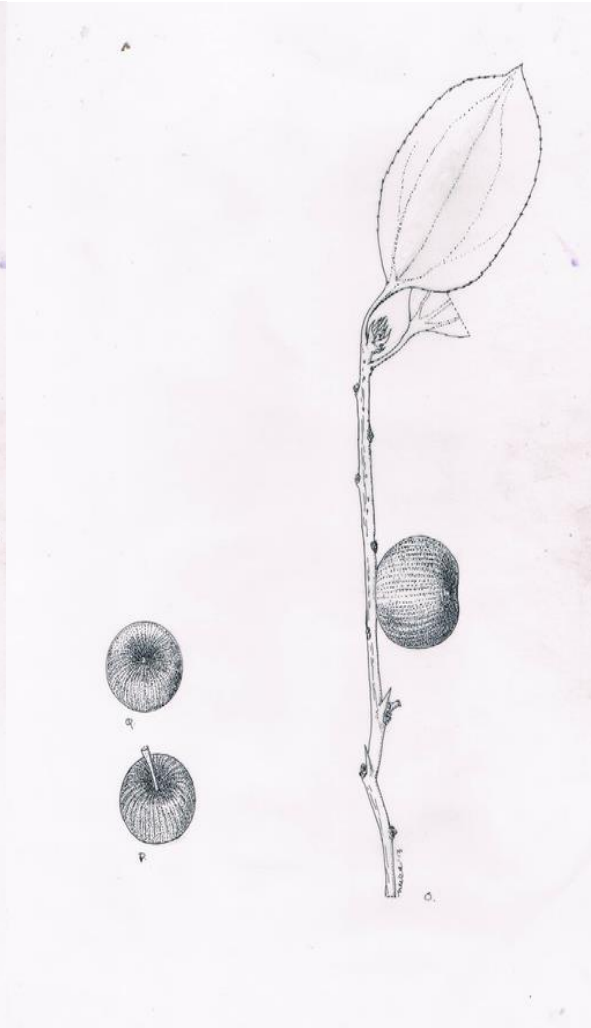


Fig. 3: Sterile branch with spines, X1 C.N.
20691, Timal (2,000m), Kavreplanchok,
Feb.10, 2013, K.R. Bhattarai in KATH



**Fig. 4: Spineless fertile branch with
mature fruit, X1 C.N. 03, Mecksha
(1,800m), Kavreplanchok, Jan. 9,
2010, J. Pradhan in KATH**

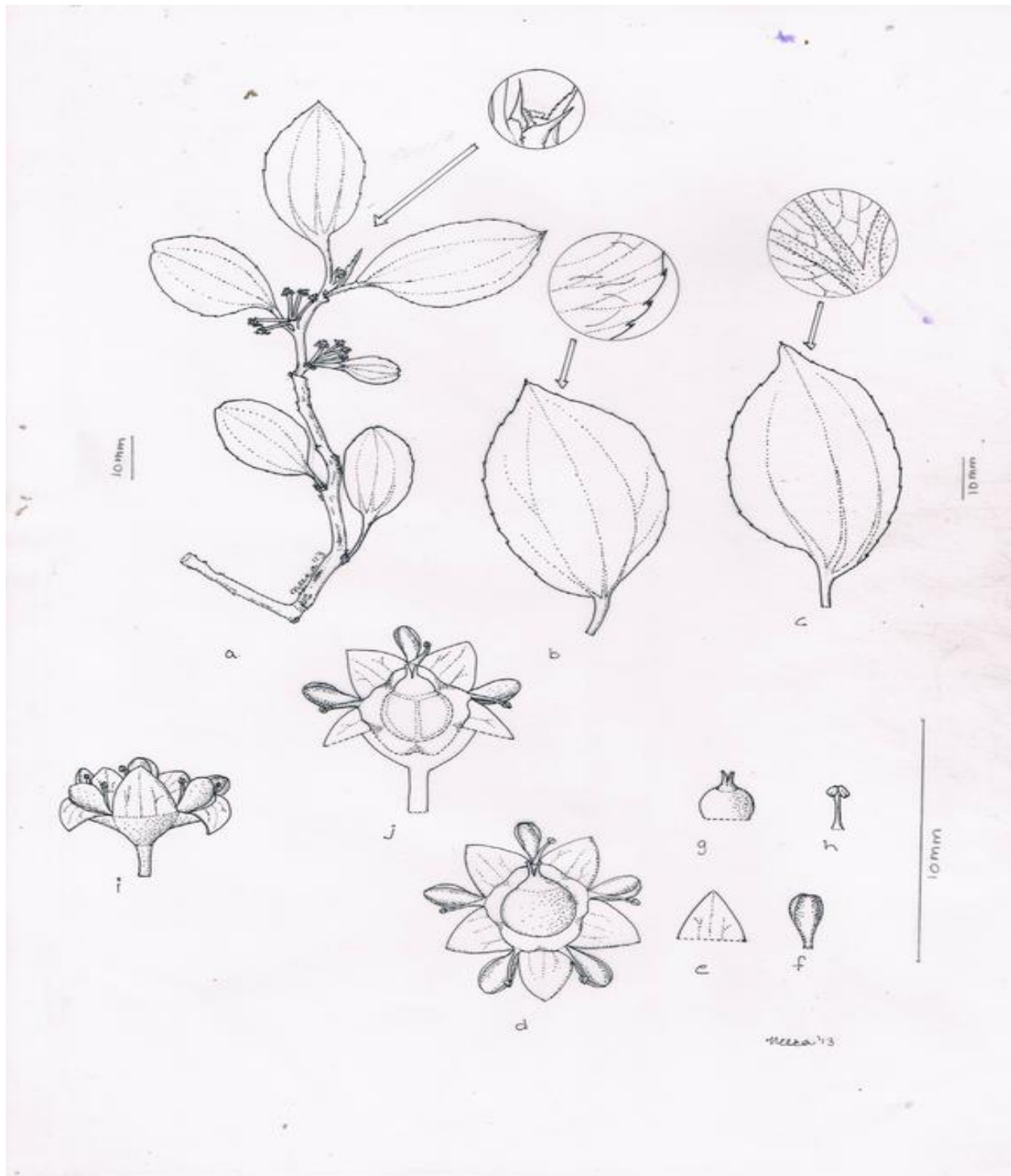


Fig. 5: Fertile branch of *Z. budhensis* with flowers (a); Leaf dorsal side X1 (b); Leaf ventral side X1 (c); Flower X6 (d); Sepal X6 (e); Petal X6 (f); Gynoecium and Ovary X6 (g); Stamen X6 (h); A flower with sepals and petals with stamens x6 (i) and Longitudinal section of a flower X6 (j), KATH Holotype: C.N. 20701 (1,900m), Source: Bhattarai and Pathak (2015)

Methodology

A small piece of young leaves sample was collected from the type locality of *Z. budhensis*, Kabhrepalanchok district of central Nepal (KATH Holotype: C.N. 20701, 1,900m). DNA was extracted from the silica-dried material fragments using the TIANGEN plant genomic DNA extraction kit (TIANGEN Biotech., Beijing, China) following the manufacturers' protocols. The internal transcribed spacer (ITS): ITS-A and ITS-B were used as primer in this study.

PCR was carried out following the same methods as Xu *et al.* (2013). The amplified fragments were purified with the TIAN quick Mini Purification Kit (TIANGEN). The sequencing of the purified PCR products was done by the help of Invitrogen (Shanghai, China). The Sequencer 4.1 (Gene Codes Corp., Ann Arbor, MI, USA) was used to assemble and edit complementary strands. The sequences obtained for each fragment were initially aligned using Clustal X 1.8181.

The DNA sequences obtained from the Company were edited using the Sequencer 4.4. The edited sequence was blasted in ncbi (www.ncbi.nlm.nih.gov) and aligned. For phylogenetic tree, the model suggested by Tamura and Nei (1993) was selected. The aligned sequences were used to make a phylogenetic tree using MWGA6 (Tamura *et al.*, 2013). A maximum likelihood tree (see Figure 6) was constructed, and the maximum parsimony was also observed.

Results

To show the genetic differences, Maximum Likelihood Methods are shown in Figure 6. Table 1, below demonstrates the pair-wise distances among the different *Ziziphus* species. The genetic difference between *Z. budhensis* and *Z. xianchengnensis* was found to be only 0.004. The molecular result was also compared with that of Li *et al.* (2009). The findings revealed that they were almost the same species. Later on, the type specimens of *Z. xianchengnensis* (see Fig. 2: A, B, C) were examined at the Chendgu Institute of Biology (CDBI), which were almost the same as that of *Z. budhensis* from Nepal. When this species was described for the first time, there was no any information about the flower characters. This time, a full description has been provided with flower and fruit characters.

Though there were some differences about the habit, distribution and shape of some flowering parts between the two type descriptions, the important characters- the faces of the seeds and the convex shape on one side of the seeds were remarkable. The DNA analysis showed almost no difference at all while the morphological differences might be due to the different habitats and the other environmental factors. The detail description of the species is given under the taxonomic description.

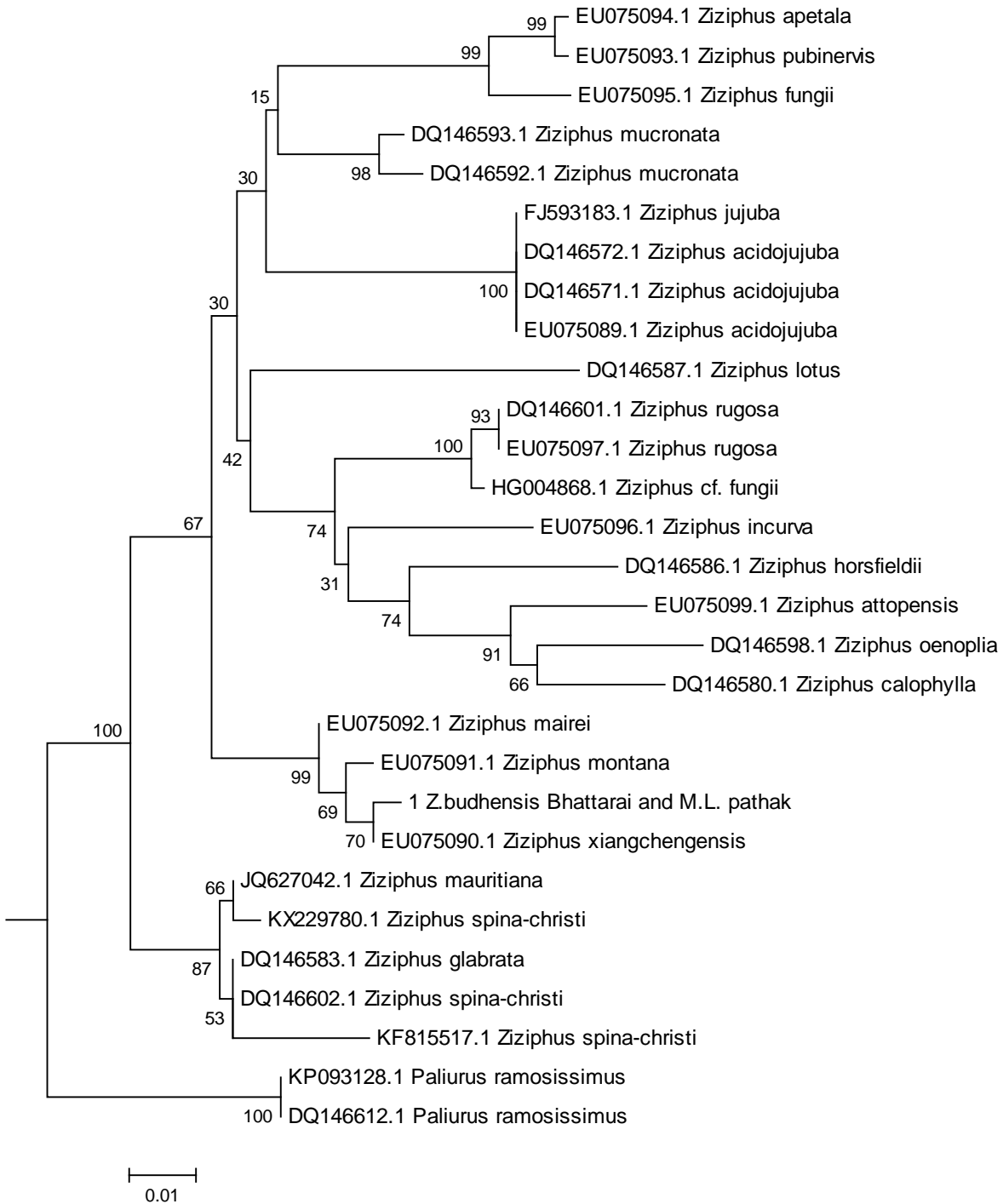


Figure 6: Molecular phylogenetic analysis using the maximum likelihood method

The evolutionary history was inferred by using the Maximum Likelihood Method based on the Tamura-Nei Model [1]. The tree with the highest log likelihood (-2050.2307) is shown. The initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join

and BioNJ Algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 29 nucleotide sequences. All the positions containing gaps and missing data were eliminated. There were a total of 498 positions in the final dataset. The evolutionary analyses were conducted with the help of MEGA6 [2] Software.

Religious and economic values

Although the fruit of *Z. budhensis* is edible, it is not used for eating due to its high-value. The tree is also used as fodder for cattle. The seeds are highly-valued, and are used to make garlands. The garlands made of beads, locally called 'Bodhichita' or 'Buddha Mala' are used by the Buddhist people for the purpose of spiritual peace. Smaller seeds with more faces cost high price than the larger seeds with few faces. A garland of 108 beads costs from US\$ 10.00 to US\$ 5,000.00 (Bhattarai and Pathak, 2015).

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