MOLECULAR IDENTIFICATION OF *Juniperus* GENOTYPES FROM GILGIT-BALTISTAN USING CHLOROPLAST DNA BARCODES

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ABSTRACT

DNA barcoding is a molecular identification technique that utilizes a standardized region of the genome to distinguish organisms at the genus and species levels. The current study aims to confirm and identify *Juniperus* species using chloroplast DNA Barcodes. A total of 16 samples were collected from various biodiversity-rich locations in the Hunza Valley, Gilgit-Baltistan (GB), Pakistan. Among them, 4 samples were selected based on variable morphological traits for molecular confirmation. A total gDNA was isolated by CTAB method and confirmed through gel electrophoresis. Chloroplast markers (*mat*K and *rbc*L) were used to amplify and sequence the targeted regions to confirm homology and resolving taxonomic uncertainties. Both the selected markers showed good results in amplification, and sequencing. While in molecular data analysis, various types of sites and domains were recorded in the form of conserved, variable, parsimony-informative and singleton sites. In phylogenetic tree analysis, the query species sequences shared a clade with *Juniperus chinensis, Juniperus communis, Juniperus excelsa,* and *Juniperus squamata* with bootstrap values greater than 90%. In conclusion, this study provides one of the first molecular characterizations studies of *Juniperus* species specifically from the high-altitude zones of Hunza, GB. The findings contribute to accurate species identification and conservation efforts for these ecologically important *Juniperus* species.

Keywords: Genetic diversity; Himalayan flora; Conifer identification; Chloroplast genome.

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INTRODUCTION

The genus *Juniperus* L. (family Cupressaceae) comprises approximately 60–70 species of evergreen conifers distributed widely across the Northern Hemisphere, from the Arctic regions to subtropical and mountainous zones of Africa, Asia, and North America (Adams, 2011; Adams & Schwarzbach, 2011; Bejenaru *et al*., 2024). The members of this genus are major ecological constituents of forest habitats and are critical for maintaining ecosystem stability (Rahmonov *et al*., 2017, 2021; Uckele *et al.,*2026). They serve as vital soil stabilizers in arid and semi-arid environments, act as significant sinks for soil organic carbon, and provide essential shelter and habitat for a diverse range of fauna (Charro *et al*., 2017; Rostamikia *et al*., 2024). Beyond their ecological roles, *Juniperus* species are economically significant and widely utilized for their fragrant wood, therapeutic essential oils, and diverse medicinal applications (Tufail *et al.,* 2023; Bozyel *et al.,* 2024). Taxonomic differentiation within the genus remains difficult due to high morphological plasticity, similar phenotypic traits, and the effects of environmental factors on growth patterns (Knyazeva & Hantemirova, 2020).

In Pakistan, *Juniperus* species are mainly found in the north and west mountainous parts, such as Gilgit-Baltistan, Khyber Pakhtunkhwa, and Baluchistan. *Juniperus* forests of Ziarat, Baluchistan, and Gilgit-Baltistan are some of the oldest-known coniferous forests in the region, and the trees have been estimated to be more than 3,000 years old (Sarangzai *et al*., 2012; Shaheen *et al*., 2017). These forests also support the hydrological cycle, as well as counteracting the effects of climate change in the high-altitude ecosystems besides enhancing biodiversity conservation. Despite being ecologically and economically important, now these *Juniperus* populations are under the pressure of excessive overexploitation, grazing, habitat fragmentation, and climate stress which lead to a precipitous decrease in rates of regeneration and forest health (Gauquelin *et al*., 1999; Redmond *et al*., 2023).

Correct identification of *Juniperus* species is a fundamental requirement for successful design of conservation and management practices (Shaheen *et al*., 2017; Irfan *et al*., 2021). Traditionally *Juniperus* taxonomic classification has been based on morphological features such as the morphology of leaves, cones, and seeds (Vasek, 1966; Köroğlu *et al*., 2018). However, these features have a high level of overlap among species as well as they are open to environmental forces and, consequently, morphological recognition under most circumstances cannot be relied upon (Knyazeva &

Hantemirova, 2020). This has caused a great deal of taxonomic complexity, particularly in such complexes as *J. excelsa, J. polycarpos* and *J. seravschanica* which morphologically resemble but differ genetically (Douaihy *et al*., 2012).

Over the past decades, molecular techniques have revolutionized plant systematics and taxonomy and provided plausible techniques of species identification and phylogenetic inference (Pyron, 2015; Zaman *et al*., 2025). One such technique is DNA barcoding, which has proven to be an effective tool for differentiating closely related taxa based on short and standardized DNA sequences (Hebert *et al*., 2003; Li *et al*., 2015). The concept of DNA barcoding is to have some loci, usually in the chloroplast genome in plants, which vary sufficiently to identify species but are maintained across species (Dong *et al*., 2012). Two of the most popular chloroplast markers to use in plant DNA barcoding are the *matK* (*maturase K*) and *rbcL* (*ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit)* genes (Wattoo *et al*., 2016; Irfan *et al*., 2024). Their choice is motivated by their universality, high amplification success and good discriminatory power in a wide taxonomic group of plants (Group *et al*., 2009; Dormontt *et al*., 2018). Although these markers can face resolution challenges in *Juniperus* due to frequent hybridization and incomplete lineage sorting (Adams *et al*., 2016; Uckele *et al.,* 2021), they remain the primary standardized tools for establishing a baseline molecular database for the Himalayan flora. The congruence between *matK* and *rbcL* results across our studied genotypes further justifies their application, demonstrating their reliability for consistent species-level identification within the specific geographic context of Gilgit-Baltistan (Song *et al.,* 2019; Irfan *et al.,* 2024).

Several previous studies have stressed the usefulness of chloroplast DNA markers in clarifying phylogenetics and taxonomy on uncertainties between the conifers, especially *Juniperus* (Almerekova *et al*., 2024). Indeed, a whole chloroplast genome sequence of Pakistan populations of *J. polycarpos* confirmed the apparent genetic dissimilarity to justify its separation of other species close to it, such as *J. excelsa* and *J. seravschanica* (Irfan *et al*., 2021). Similarly, molecular analysis based on *matK* and *rbcL* sequencing has been successfully used to circumscribe *J. oxycedrus* subspecies and estimate genetic diversity of *J. oxycedrus* Mediterranean species (Aggag, 2020). All these studies testify to the effect that DNA barcoding used together with phylogenetic studies provide a sound basis of disclosing evolutionary associations amid morphologically congruent species (Almerekova *et al*., 2024; Irfan *et al*., 2024).

Gilgit-Baltistan (GB), which is located at the intersection of the Himalaya, Karakoram, and Hindu Kush mountains, is one of the most diverse regions of Pakistan (Abbas *et al.,* 2022). The topography, altitude gradients, and climatic variability are unique to it, and these features support excellent distribution of plant biodiversity including different endemic and relict species (Abbas *et al*., 2019; Abbas *et al*., 2022). Despite this, *Juniperus* species of this region and their evolutionary relationships have little molecular data and little understanding of the evolutionary relationships. An ordered molecular study is therefore required to determine the right *Juniperus* genotypes and be aware of their phylogenetic location in the genus. The objective of the current research was to determine the molecular identification of *Juniperus* species sampled in the under-explored Hunza Valley of GB using two chloroplast DNA barcodes of *matK* and *rbcL*. While 16 genotypes were initially collected to assess field-level morphological variation, four representative specimens were strategically selected for molecular analysis to provide a robust taxonomic verification across the observed phenotypic spectrum. This targeted selection ensures that the molecular data captures the core genetic identity of the population while avoiding redundancy, serving as a reliable baseline for the *Juniperus* stands of the region (Song *et al.,* 2019; Aggag, 2020; Sajjad *et al*., 2024). Through the use of DNA barcoding for molecular identification, this study aims to resolve taxonomic ambiguity and provide baseline genetic information for the conservation and sustainable management of *Juniperus* forests in the Himalayan region of Pakistan.

MATERIALS AND METHODS

Study Area and samples collection: Gilgit-Baltistan is a region located in the northernmost part of Pakistan, situated at the crossroads of India, China, and Afghanistan. The region has a total area of approximately 72,971 km 2 (27,188 square miles) and is between the latitude of 37°04 to 43°04 and 72°30 to 77°50. Geographically, GB is a special trans-Himalayan area with a complicated topography and diverse geological layers and with specific climatic regimes. Some of the mountain ranges on the planet are the highest and include the Karakoram, Hindu Kush, and the Western Himalayas that characterize the region (Fig. 1; Table 1). In contrast to the cis-Himalayan areas under monsoon precipitation control, GB receives little monsoonal rainfall and is otherwise dominated by arid to semi-arid climatic regimes (Dani, 2001; Khan, 2012). For this study, 16 plant samples were gathered from different sites of Hunza valley, GB. The gathered specimens were at once put inside sterile polyethylene bags to avoid desiccation and contamination. Each sample was properly labeled, recorded, and shipped to the Molecular Genetics Laboratory, Department of Biotechnology and Genetic Engineering, for further molecular analyses. Out of 16 samples collected, 4 representative genotypes were selected based on distinct morphological variations (leaf shape, cone size) to cover the maximum taxonomic diversity of the region (Fig. 2).

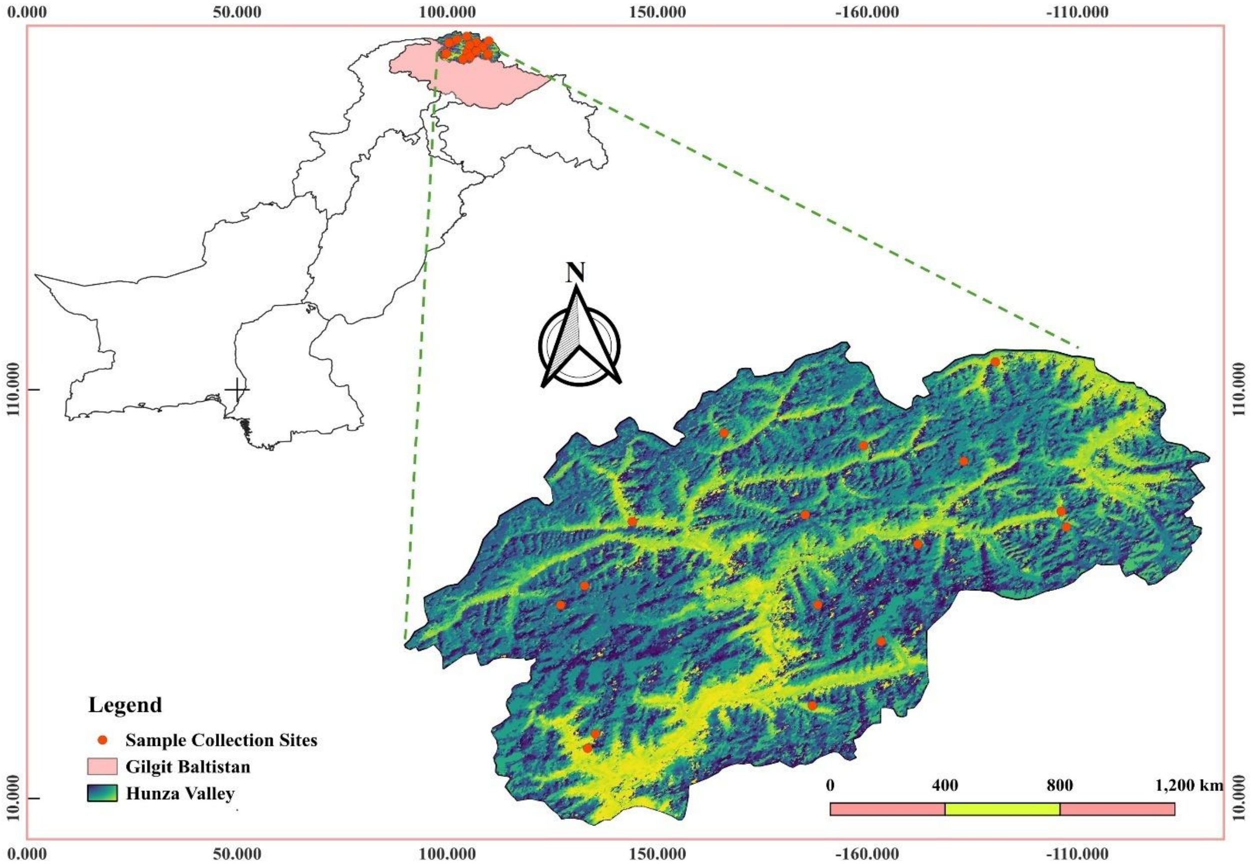

Figure 1; Map of the study area, red dots showing the sample collection sites in Hunza, GB.

Table 1. Detailed description of collected species, with GPS coordinates and collection sites of different regions of study area.

S. No	Collected genotypes	Collection sites	Latitude N	Longitude E
1	*Query-R1*	Hone mountain Karimabad, central Hunza Gilgit-Baltistan	35.4222 ^{O}N	74.0966 ^{O}E
2	*Query-R2*	Makhra Shogran	34.6716 ^{O}N	73.0184 ^{O}E
3	*Query-R3*	Shisparnala Hasanabad Hunza Distt	34.5431 ^{O}N 34.6912 ^{O}N	73.3858 ^{O}E 72.9537 ^{O}E
4	*Query-R4*	Shisparnala Hasanabad Hunza Distt	34.5427 ^{O}N 34.6913 ^{O}N	73.3856 ^{O}E 72.9536 ^{O}E

Figure 2; Morphological comparison of *Juniperus* species collected from Hunza. The figure displays representative branches and foliage of: (A) *Juniperus excelsa*, showing mature scale-like leaves on elongated shoots; (B) *Juniperus communis*, displaying dense, prickly needle-like foliage; (C) *Juniperus chinensis*, highlighting scale leaves and a high density of mature seed cones (galbuli); and (D) *Juniperus squamata*, characterized by its distinctively "flaky" and clustered needle-like leaves.

DNA extraction: Genomic DNA was isolated by the Cetyltrimethylammonium Bromide (CTAB) procedure with some modifications (increased incubation time and additional wash steps) (Doyle & Doyle, 1987). Dried leaf materials were finely crushed to powder in a sterile mortar and pestle. For every 100 mg of powdered material, 800 µL of pre-heated 2% CTAB extraction buffer (kept at 65°C) was added, followed by vigorous vortexing. The homogenate was incubated for 2 hours at 65°C. After incubation, 600 µL of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added, and the contents were centrifuged at 13,000 rpm for 20 minutes. The obtained supernatant was pipetted out to another tube, combined with 500 µL of ice-cold isopropanol, and kept overnight at –20°C to precipitate DNA. Samples were then centrifuged at 13,000 rpm for 20 minutes, and the supernatant was discarded. The pellet of DNA was then washed three times with 70% ethanol, with each washing followed by centrifugation at 13,000 rpm for 3 minutes. Following the removal of remaining ethanol through air-drying of the inverted tubes, the pellet of DNA was dissolved in 80 µL double-distilled water (ddH$_2$O). The integrity and quality of isolated genomic DNA were evaluated by 1% agarose gel electrophoresis.

PCR amplification: The most extensively studied and best-characterized plant chloroplast DNA primers were selected for amplification (Table 2). DNA amplification was performed via polymerase chain reaction (PCR) using experimentally optimized conditions. Every 25 µL PCR reaction mixture was set up in a 200 µL PCR tube with the following components: 14 µL of double-distilled water (ddH$_2$O), 2 µL of genomic DNA template, 2.5 µL of 10× PCR buffer, 2 µL of MgCl$_2$, 2 µL of dNTPs, 0.5 U of Taq DNA polymerase, and 2 µL each of forward and reverse primers. Amplification was carried out using an Applied Biosystems 2720 Thermal Cycler with the following thermocycling protocol: 94°C for 5 minutes of initial denaturation, and then 35 cycles of 30 seconds at 94°C of denaturation, 40 seconds at 52°C of annealing, and 35 seconds at 72°C of extension, with a final 10-minute extension at 72°C. The amplified PCR products were examined by 1% agarose gel electrophoresis in TAE buffer to ensure successful amplification.

Table 2. DNA barcoding makers selected for the evaluation of *Juniperus* genotypes in the study area along with its forward and reverse primers.

DNA barcodes	Primers	5' to 3' sequences	References
matK	*matK* F *matK* R	5'CGATCTATTCATTCAATATTTC 3' 5'TCTAGCACACGAAAGTGAAGT 3'	(Cuénoud *et al*., 2002)
rbcL	*rbcL* F *rbcL* R	5'ATGCCACAAACAGAGACTAAAGC3' 5'GTAAAATCAAGTCCACCRCG3'	(Kress & Erickson, 2007)

Nucleotide Sequencing and Analysis: Resultant PCR amplified products were sequenced through the Sanger sequencing method from Macrogn Inc. South Korea. And after successful sequencing, to ensure resulting sequences, accuracy and to recover numerous defined sequences were subjected to BLAST analysis in the NCBI GenBank database. Sequences of composed data were arranged via BioEdit version 7.2.5 (Hall, 1999) and Multiple sequence alignment was performed using MUSCLE and ClustalW. The resultant data was processed to compute the Kimura-2-parameter (K2P) distances for each region by MEGA version X (Kumar *et al.*, 2018).

RESULTS

Molecular Identification of *Juniperus* species Using *matK* and *rbcL* Markers: In the current study, 4 out of 16 collected *Juniperus* samples from the various locations of the study area (GB) were evaluated through *matK* and *rbcL* markers to confirm their molecular identity using reference sequences databases. Genomic DNA extraction yielded high-quality and high-concentration templates (Figure 3A). Successful PCR amplification of the target chloroplast markers, *matK* and *rbcL*, was confirmed by the presence of distinct bands of the expected size (Figure 3B and C). The amplification and sequencing success rate were 100% while, the species identification success rate of selected DNA markers was greater than 90%.

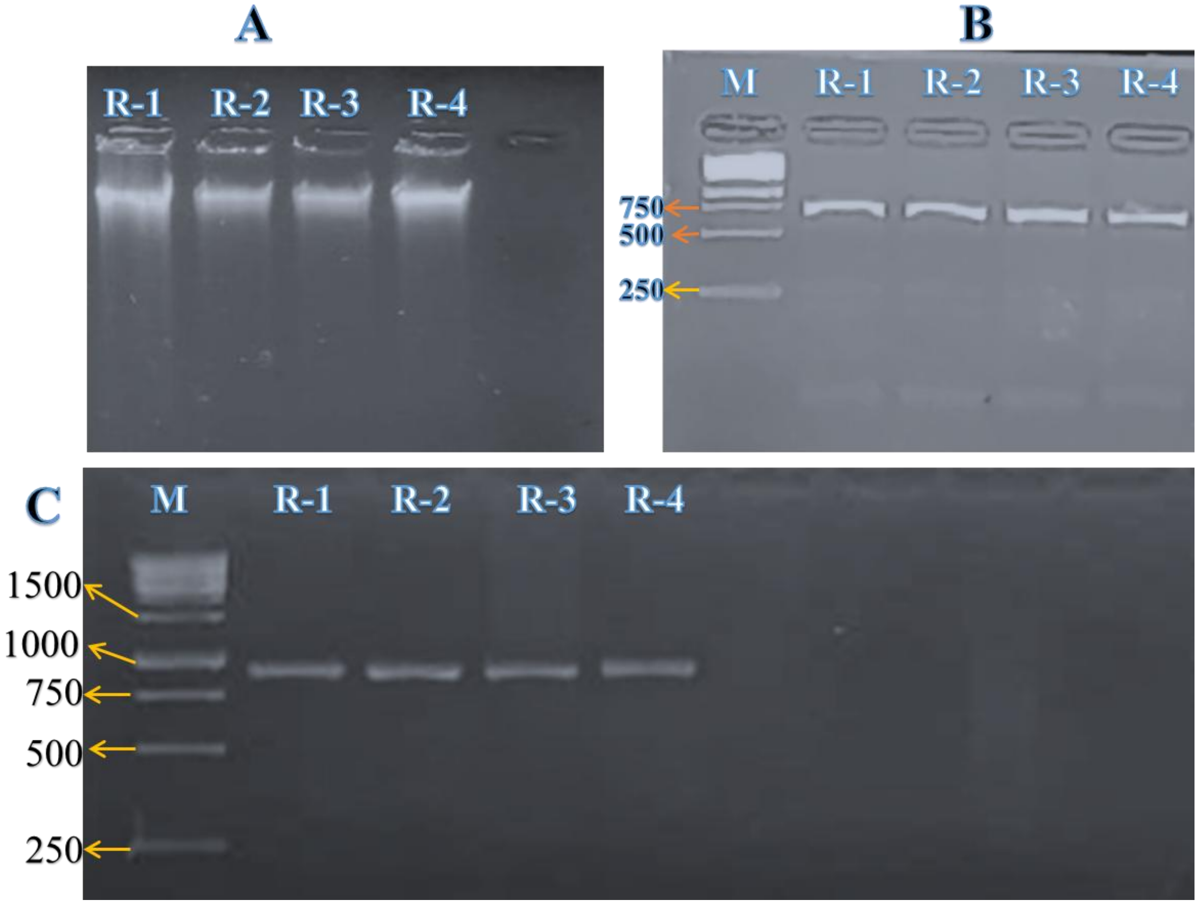

Figure 3; Gel documentation of the genomic DNA and PCR of the selected *Juniperus* Genotypes. A) Genomic DNA, B) PCR results of matK marker, C) PCR results of rbcL marker. M=1kb DNA ladder, R1-R4 selected genotypes.

Nucleotide BLAST analysis of candidate barcodes: BLAST analysis confirmed high homology between the query sequences and existing NCBI database records, enabling the identification of closely related reference sequences for phylogenetic comparison. The acquired sequence, made up of the entire nucleotide length, displayed a high level of similarity with reference sequences and exhibited very high percent identity and query coverage values. Percent identity was calculated, and an E-value of 0.0 was reflected, pointing to an extremely significant alignment. The final sequence alignment yielded a high-quality dataset of the expected length, providing a robust basis for subsequent phylogenetic characterization (Table 3).

Table 3. Detailed description of query species, its successful sequence length, and BLASTn results characterizations.

Species	Total seq length	Align seq length	Query cover	E-Value	Identities	Barcodes
J. chinensis	753	715	99	0.0	99	*matK*
J. chinensis	862	838	99	0.0	98	*rbcL*
J. communis	749	723	98	0.0	99	*matK*
J. communis	861	828	97	0.0	99	*rbcL*
J. excelsa	743	721	99	0.0	98	*matK*
J. excelsa	859	826	98	0.0	99	*rbcL*
J. squamata	742	716	99	0.0	98	*matK*
J. squamata	851	814	99	0.0	97	*rbcL*

Molecular characterizations of DNA barcodes: The final aligned dataset, comprising both query and reference sequences, exhibited high sequence homology and was utilized for subsequent phylogenetic reconstruction. The MEGA analysis comprised total aligned sequence length, conserved sites, variable sites, parsimony informative sites, and singleton sites in both the query species sequences and retrieved database sequences (Table 4).

Table 4. Descriptive information of DNA barcode sequences of both query sequences along with references database sequences in Molecular and evolutionary analysis.

DNA barcode	Total characters	Conserved sites	Variable sites	Parsimony informative	Singleton
Matk	743	459	284	283	1
rbcL	862	608	254	252	2

DNA barcode-based similarities indexes: Based on molecular characterizations, the monophyletic clade of query sequences was confirmed with database references sequences using Mega (x) software. The evolutionary history was inferred using different methods like blast similarities and percent identities. The optimal tree is shown in different clade with bootstrap support. The bootstrap percentages (based on 1000 replicates) show the percentage of replicate trees in which the given taxa were found to be together. Evolutionary distances were computed using the Kimura 2-parameter (K2P) model and the number of base substitutions per site. The analysis comprised both query and reference nucleotide sequences, and all positions with less than full data were eliminated using the complete deletion option. In the resulting tree of phylogeny, the query sequences showed a strong evolutionary relationship with the corresponding reference sequences pulled from the database (Table 5).

Table 5. A descriptive table showed the evaluated DNA barcodes in collected genotypes, their blast sequences similarities along with species authorities, Genebank Accession and geographic location of submitted sequences.

DNA Barcodes	Collected species/accession no	Neighbour species with spp authorities and reported years	Genebank Accessions, submission date and authorities, and geo-locations
matK	*J. chinensis* PZ049342.1	*J. chinensis,* Roxb. (1838) *J. chinensis,* Roxb. (1838) *J. chinensis,* Roxb. (1838)	MH659306.1, Xu *et al.,* 2018. China LC404753.1, Crisp *et al.,* 2018. Japan JQ512420.1, Shin, 2012. Korea
rbcL	*J. chinensis* PZ049346.1	*J. chinensis,* Roxb. (1838) *J. chinensis,* Roxb. (1838) *J. chinensis,* Roxb. (1838)	MH657749.1, Xu *et al.,* 2018. China PP356738.1, Liu, 2024. China OQ184424.1, Dan, 2023. China
matK	*J. communis* PZ049345.1	*J. communis,* Thunb. (1784) *J. formosana,* Hayata. (1908) *J. rigida,* Desf. (1829)	MH121052.1, Zhu *et al.,* 2018. USA HQ245897.1, Yang *et al.,* 2010. China NC062083.1, Wang, 2022. USA
rbcL	*J. communis*	*J. communis,* Thunb. (1784)	NC035068.1, Wu *et al.,* 2017. USA

	PZ049347.1	*J. communis,* Thunb. (1784) *J. communis,* Thunb. (1784)	OQ644239.1, Almerekova *et al.,* 2023. Kazakhstan OQ644240.1, Almerekova *et al.,* 2023. Kazakhstan
matK	*J. excelsa* PZ049343.1	*J. excelsa,* Griff. (1854) *J. polycarpos,* K. Koch. (1849)	HM024025.1, Mao *et al.,* 2010. China HM024045.1, Mao *et al.,* 2010. China
rbcL	*J. excelsa* PZ049348.1	*J. excelsa,* Griff. (1854)	MN662645.1, Boukhdoud *et al.,* 2019 Lebanon
matK	*J. squamata* PZ049344.1	*J. squamata,* Buch. Ham. (1824) *J. procera,* Hochst. (1847)	NC044076.1, Xie, 2019. USA HM024046.1, Mao, 2010. China
rbcL	*J. squamata* PZ049349.1	*J. squamata,* Buch. Ham. (1824) *J. squamata,* Buch. Ham. (1824)	NC044076.1, Xie, 2019. USA OQ184437.1, Dan, 2023. China

Phylogeny of *Juniperus* species based on *matK:* Initially the *matK* sequences of all the query species along with references database sequences were utilized to construct the phylogeny. In the phylogenetic tree the query species showed evolutionary relationship with reference database sequences reported from the different parts of the world with strong bootstrap support (Annexure 1). The top most similar sequences from the database along with 4 query species sequences were further used to construct combined phylogenetic tree showing the evolutionary relationship among query species and available references database species. In the phylogenetic tree, query species (PZ049341.1-*J. chinensis*) showed close relationships with (MH659306.1-*J. chinensis*), (LC404753.1-*J. chinensis*), and (JQ512420.1- *J. chinensis*), and (NC065035.1-*J. procumbens*) with bootstrap support of 96. Similarly, the query species (PZ049345.1-*J. communis*) shared a clade with (MH121052.1, *J. communis*), (MK925622.1-J. *communis*), and (HQ245897.1-*J. formosana*). On the other hand, the query species (PZ049344.1- *J. squamata*) showed a close relationship with (NC044076.1-*J. squamata*) and (NC062329.1-*J. przewalskii*) with bootstrap value of 96. While, the query species (PZ049343.1-*J. excelsa*) shared clade with (HM024025.1- *J. excelsa*), and (HM024045.1- *J. polycarpos*) with bootstrap of 100. The outgroup species (HE966910.1, HM023994.1-*Cupressus sempervirens*) sequences make a separate clade from the query species sequences along with references database sequences in the phylogenetic tree (Fig. 4).

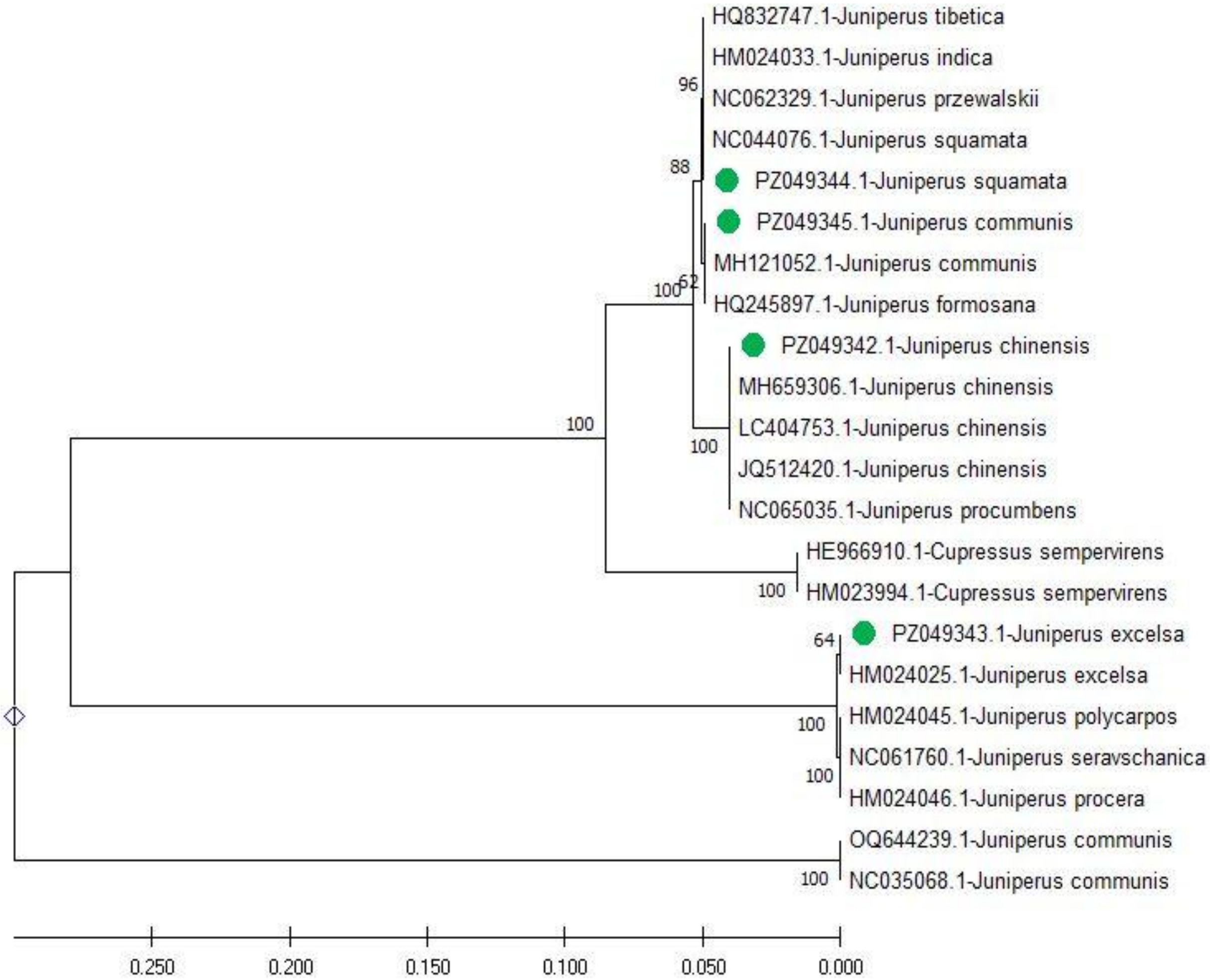

Figure 4: A representative NJ tree showed the phylogenetic relationship of query genotypes sequences with references database sequences, tree confirms the monophyly of query *Juniperus* species such as *J. chinensis, J. communis, J. excelsa*, and *J. squamata* with bootstrap of 64, 88, and 100.

Phylogeny of *Juniperus* species based on *rbc*L: Initially the *rbc*L sequences of all the query species along with references database sequences were utilized to construct the phylogeny. In the phylogenetic tree the query species showed evolutionary relationship with reference database sequences reported from the different parts of the world with strong bootstrap support (Annexure 1). In the neighbour-joining tree, the query species (PZ049346.1-*J. chinensis*) showed a close relationship with (MH657749.1-*J. chinensis*)*,* (PP356738.1-*J. chinensis*)*,* and (OQ184424.1-*J. chinensis*). Similarly, the query specie (PZ049347.1-*J. communis*) shared clade with *(*PP374792.1-*J. communis*)*.* On the other hand, the query specie (PZ049349.1-*J. squamata*) makes a close relationship with (NC044076.1-*J. squamata*), and (OQ184437.1-*J. squamata*). In the same tree, the query specie (PZ049348.1-*J. excelsa*) shared clade with (MN662645.1-*J. excelsa)*, (AY380894.1-*J. procera*), and MN662648.1-*Juniperus polycarpos*) with bootstrap of 64. While the outgroup genus species (JX571816.1, JHE963440.1-*Cupressus sempervirens*) makes a separate clade in the phylogenetic tree (Fig. 5).

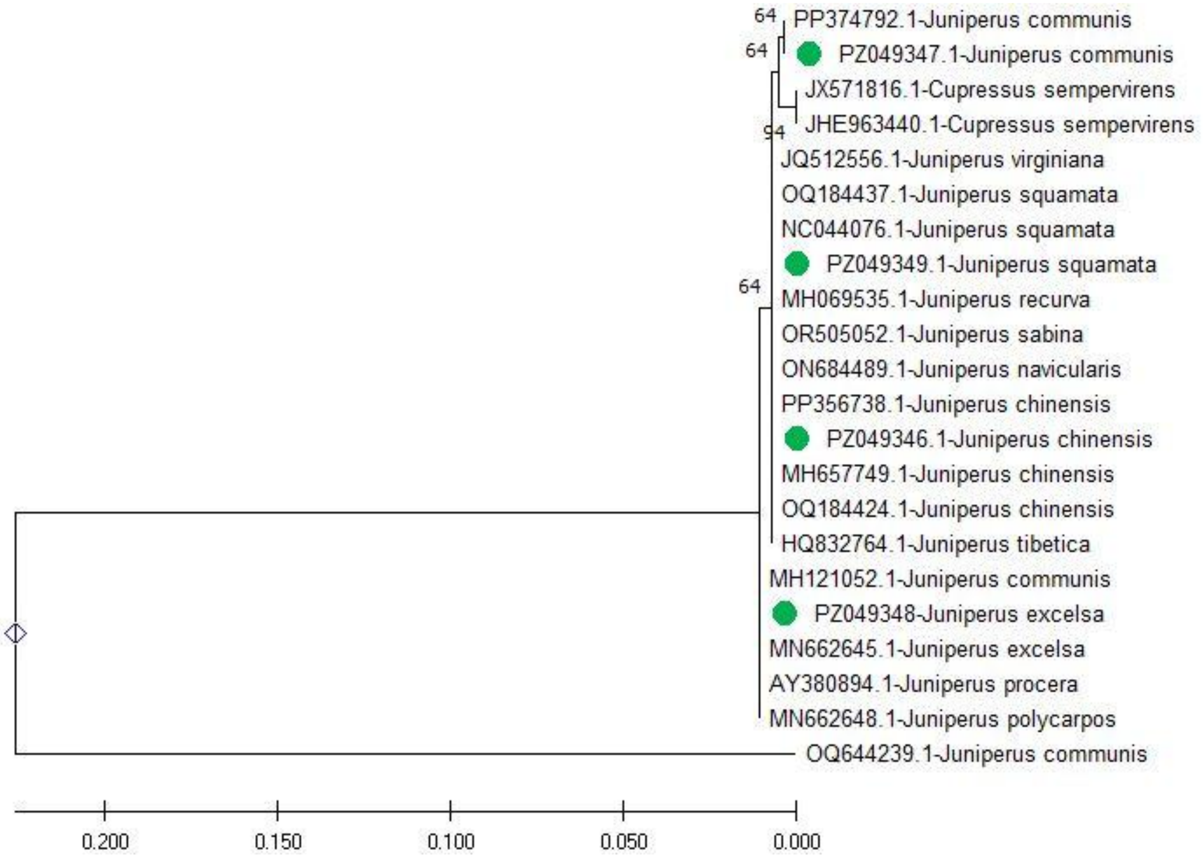

Figure 5: A representative NJ tree showed the phylogenetic relationship of query genotypes sequences with references database sequences, tree confirms the monophyly of the query genotypes *Juniperus* species such as *J. chinensis, J. communis, J. excelsa*, and *J. squamata* with bootstrap of 64, and 94.

DNA Barcode and QR-code of the Query species sequences: The DNA barcode from the *matK* and *rbcL* sequence was generated by DNA barcode generator (Bio-Rad) software (v 1.3) for their exact identification (Fig 6), and QR-code was generated online through bioinformatic tools (Fig 6).

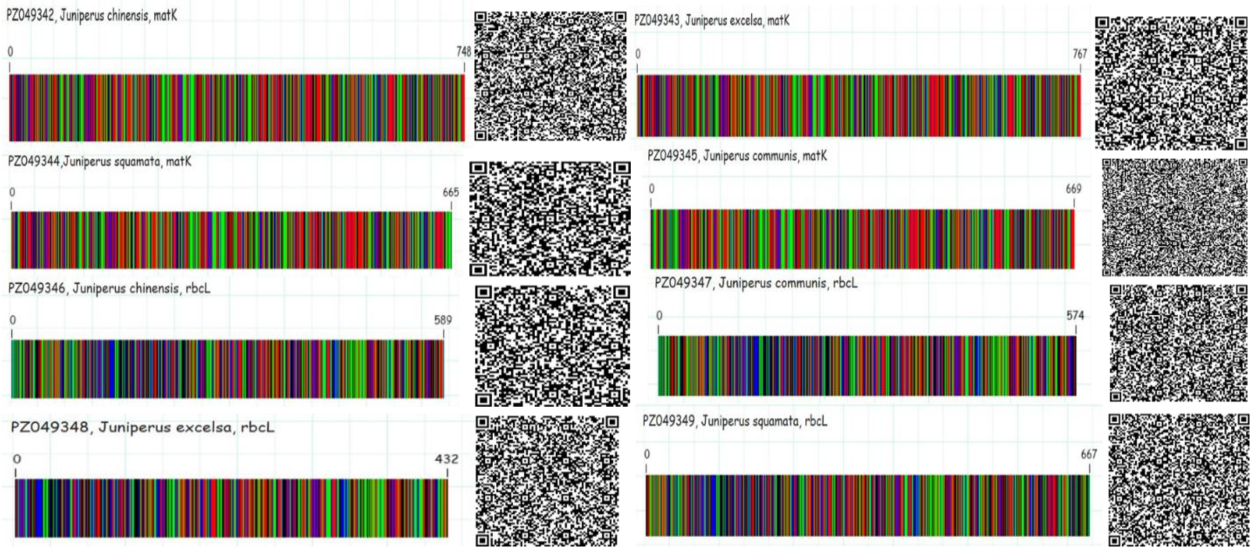

Figure 6; Visual representation of DNA barcodes and corresponding QR codes generated from the sequences of the query species.

DISCUSSION

The present study utilized two chloroplast DNA barcodes, *mat*K and *rbcL*, to identify and establish the molecular identity of *Juniperus* genotypes collected from different regions of Gilgit-Baltistan. Both markers demonstrated high amplification and sequencing success, reflecting their reliability and universality for DNA barcoding in conifers. The findings revealed that all query genotypes shared high sequence similarity (>98%) with reference *Juniperus* species deposited in the NCBI GenBank database, confirming the accuracy of molecular identification.

The *matK* and *rbcL* genes gave consistent and complementary data in circumscribing *Juniperus* species. The *matK* locus had greater interspecific variation than *rbcL*, consistent with previous findings which revealed that *matK* has greater nucleotide substitution rate and thus offers improved discrimination among closely related taxa (Irfan *et al*., 2021). On the other hand, the *rbcL* region, as being more conserved, is nonetheless applicable in resolving higher-level relationships due to its high universality and amplification efficiency. Similar findings were reported by (Aggag, 2020), further noting that the combination of both markers has increased species-level identification accuracy and phylogenetic resolution of morphologically similar plant groups.

The BLAST and phylogenetic results unambiguously verified that the investigated genotypes represent four *Juniperus* species: *J. chinensis*, *J. communis*, *J. excelsa*, and *J. squamata*. These were well-supported by high bootstrap values (>90%) in the neighbor-joining trees, verifying their monophyletic origin and evolutionary proximity with reference species. The clustering pattern described here aligns well with earlier phylogenetic estimations of *Juniperus*, in which respective groupings were documented using chloroplast and nuclear markers (Mao *et al.,* 2010; Adams & Schwarzbach, 2012, 2013; Adams *et al.,* 2016). The same clustering pattern validates the reliability of *matK* and *rbcL* in reconstructing phylogenetic relations within the genus.

The high level of sequence conservation in the *rbcL* and a relatively larger proportion of variable sites in *matK* is evidence of the evolutionary bottlenecks operating differently on those loci (Wattoo *et al.,* 2016). The *rbcL* gene is functionally important in photosynthesis and thus puts the gene under strong purifying pressure, resulting in a reduced rate of variability, whilst *matK*, which encodes a maturase enzyme that splices RNA, evolves at a quicker rate and has more substitutions (Wolfe & DePamphilis, 1998; Barthet & Hilu, 2007; Christin *et al*., 2008). Such variation in the rate of substitution allows *matK* to be more useful in identifying the recent events of speciation and in discriminating closely related *Juniperus* species (Adams & Schwarzbach, 2012; Aggag, 2020; Irfan *et al*., 2021).

Interestingly, phylogenetic trees that were derived during the current study depicted clear difference of the four *Juniperus* species of Gilgit-Baltistan into separate clades which were related to their morphological and ecological variations. As an example, *J. chinensis* and *J. communis* were closely related to the East Asia accessions, whereas *J. excelsa* and *J. squamata* were closely related to the Central Asian and Himalayan populations. This pattern of biogeography can be attributed to the historic paths of dispersal and the effect of glacial-interglacial cycles on *Juniperus* distribution over the trans-Himalayan area. Other Himalayan and Karakoram coniferous taxa have had similar biogeographic associations (Sarangzai *et al*., 2012; Irfan *et al.,* 2021).

Beyond simple taxonomic clustering, the identification of these four *Juniperus* species carries significant weight regarding the local ecology and genetic distinctiveness of the GB region. GB is a high-altitude, orogenic, confluence of Karakoram, Hindu Kush and Himalayan ranges. The genetic similarity of our samples to Central Asian and Himalayan accessions indicates that these populations might have served as glacial refugia, where unique genetic variations were formed as a result of the excessive environmental pressures and long-term seclusion in deep mountain valleys (Irfan *et al.,* 2021; Abbas *et al.,* 2022). Such *Juniperus* species are key taxa in the sub-alpine cold deserts of GB in which they are important in making soil stable and preserving moisture in an otherwise weak ecosystem (Redmond *et al*., 2023).

The strong bootstrap and proximity to the authenticated database sequences confirm the validity of molecular data to be used in taxonomic confirmation. DNA barcoding is therefore an effective approach to the shortcomings of morphological identification that may be unraveled by environmental change or hybridizations (Miller, 2007; Sheth & Thaker, 2017). The findings of the study emphasize the utility of molecular barcoding in conjunction with morphological observation to effectively resolve taxon ambiguities within *Juniperus*, also found in other conifers such as *Cedrus* and *Cupressus* (Aggag, 2020).

From a conservation perspective, the molecular verification of these genotypes is a critical step toward protecting the dwindling forest cover in northern Pakistan. The *Juniperus* forests in GB are extremely slow growing and their life span is very high, so they are very susceptible to anthropogenic impacts, including overgrazing, illicit logging to get fuel and changes in climate leading to habitat alteration (Irfan *et al.,* 2021). Our results give a requisite ground upon which we can define Evolutionarily Significant Units (ESUs). Through the validation of the genetic identity of those species, conservationists can now formulate specific management plans that would then focus on preserving these particular high-altitude populations which might have specific local adaptations to be able to survive in the midst of the fast-paced climate change in the Trans-Himalayan region (Van, 2014; Shaheen et al., 2017).

In addition, amplification of chloroplast markers on field-collected material indicates that experiments can be done on herbarium or preserved samples, which is advantageous in areas like GB where sampling and access is hard. The current results thus offer a useful genetic background on future genetic studies on population genetics, phylogeography and conservation of *Juniperus* species in Pakistan. On the whole, this paper confirms that *matK* and *rbcL* markers can be used to authenticate species and examine evolutionary history of *Juniperus*. The resulting molecular evidence does not only help to clear the taxonomic ambiguity among the sampled genotypes but also helps to understand *Juniperus* diversity and distribution in the Himalayan region at large. The further integration of DNA barcoding, chloroplast genome sequencing, and morphological data will further enable species demarcation and conservation measures of these economically and ecologically vital conifers.

Although the phylogenetic tree produced superior bootstrap values on the recognized clades, the derivation of the results is treated with caution as far as strict monophyly is concerned. Considering the logistical difficulties and the inaccessibility of the high-altitude terrain of GB, the present study is a first step in molecular characterization on the basis of localized sampling attempt. This clustering is therefore seen as a sign of intimate evolutionary relationship as opposed to a conclusive proof of monophyletic relationship of these taxa. These results offer a general genetic context of the *Juniperus* species of the region but the evolutionary histories of the area and their intricate genetic origins need to be further researched with the inclusion of extended geographic coverage and more genomic signatures to clarify the diverse evolutionary histories of the genus *Juniperus*.

Conclusion: The present study established the molecular identity and taxonomic classification of *Juniperus* genotypes from the Gilgit-Baltistan region (Hunza valley) based on chloroplast DNA barcode (*matK* and *rbcL*). The two markers showed high amplification efficiency and could distinctly separate the different genotypes into four species namely *J. chinensis*, *J. communis*, *J. excelsa* and *J. squamata*. The *matK* was more varied and showed better species resolution as compared to *rbcL*. Phylogenetic analyses identified supported monophyletic clades in line with evolutionary connections recorded in world databases. The findings are a valid source of molecular data in proper species identification and it also forms a point of reference in future research on genetic diversity and conservation of *Juniperus* in the Himalayan region of Pakistan. However, the study is limited by its exclusive geographical focus on the Hunza region and the use of only two chloroplast markers (*matK* and *rbcL*); therefore, future research should involve broader sampling across the entire Gilgit-Baltistan region and incorporate nuclear markers or high-throughput sequencing to more comprehensively capture the genetic diversity and evolutionary complexity of these species.

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Author contributions: RN, SG, AU and IU conceived and designed the study. RN, IU, SI, S and MH performed the experiments. RN, SI, HA, MH and S contributed to data collection and analysis. RN, IU, HA and S assisted in manuscript drafting and reviewing. SG, AU, HA and IU provided supervision and critical revisions. All authors have read and agreed to the submitted version of the manuscript.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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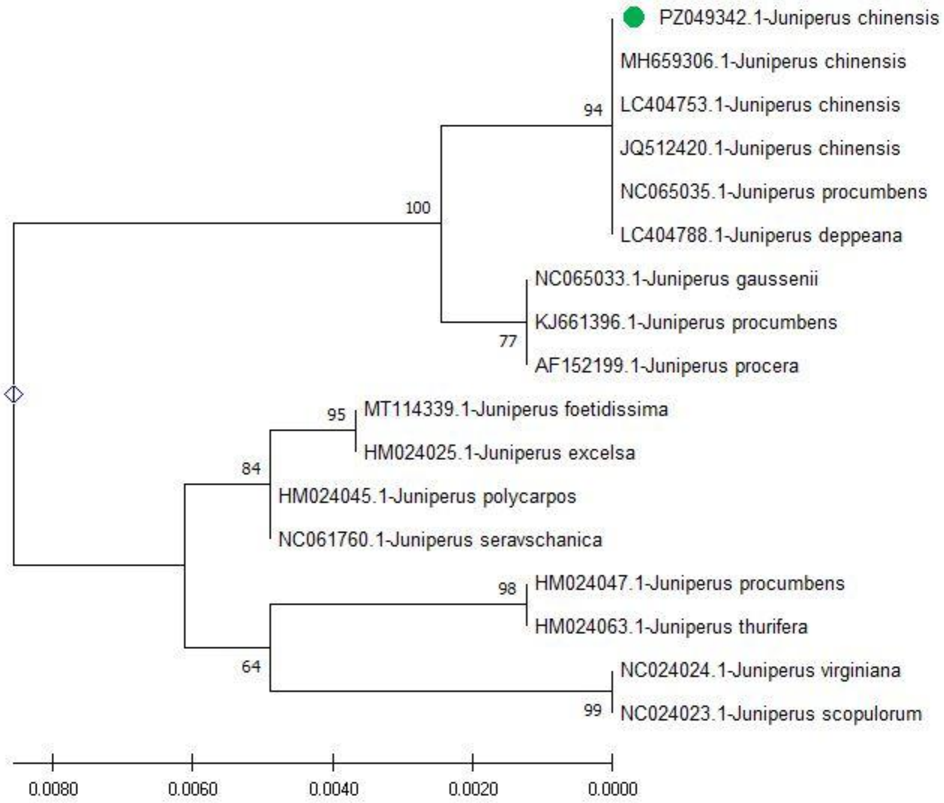
Annexure 1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LC404788.1-Juniperus deppeana
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KJ661396.1-Juniperus procumbens
AF152199.1-Juniperus procera
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HM024025.1-Juniperus excelsa
HM024045.1-Juniperus polycarpos
NC061760.1-Juniperus seravschanica
HM024047.1-Juniperus procumbens
HM024063.1-Juniperus thurifera
NC024024.1-Juniperus virginiana
NC024023.1-Juniperus scopulorum
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0.0040
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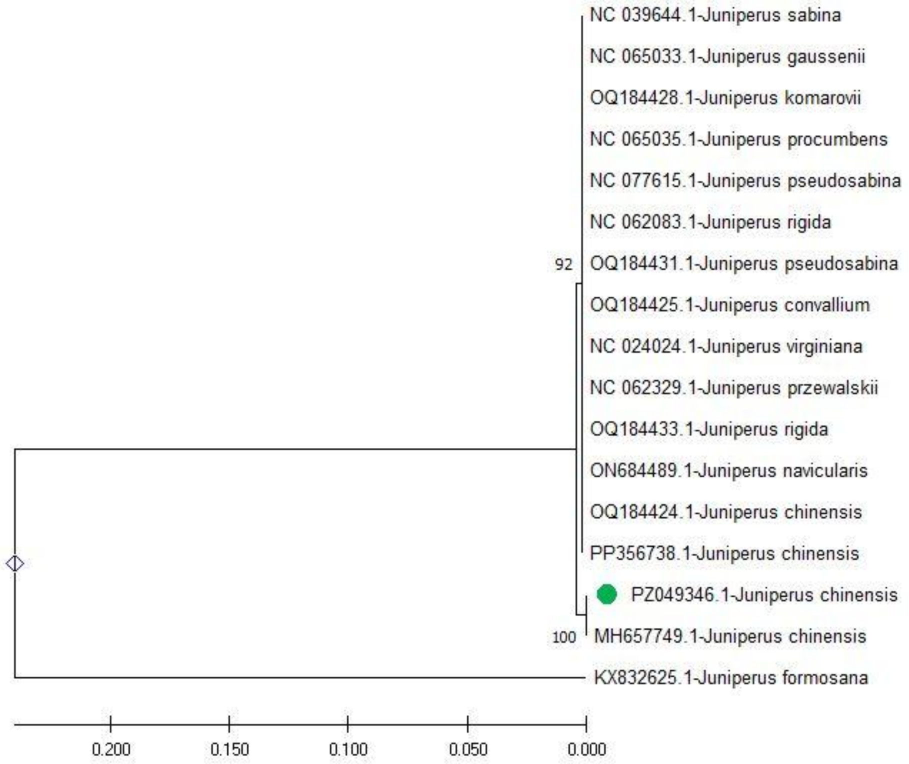
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NC 024024.1-Juniperus virginiana
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ON684489.1-Juniperus navicularis
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PP356738.1-Juniperus chinensis
● PZ049346.1-Juniperus chinensis
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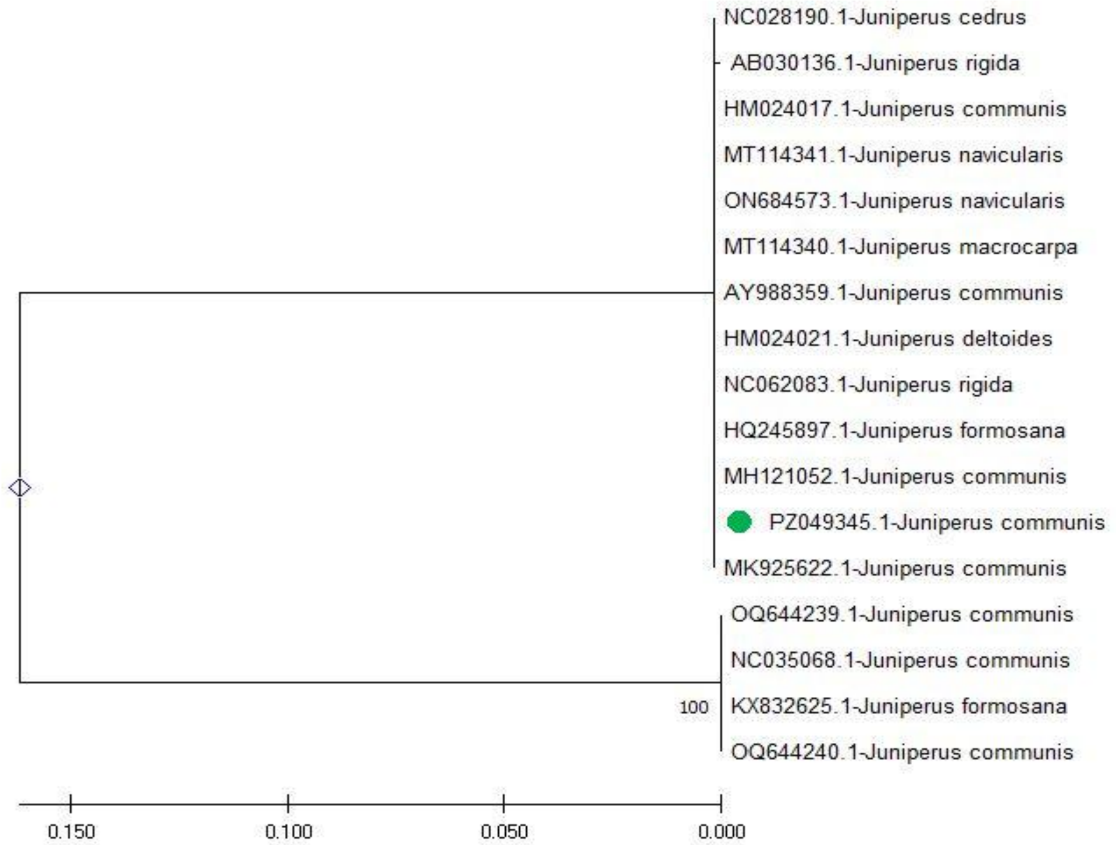
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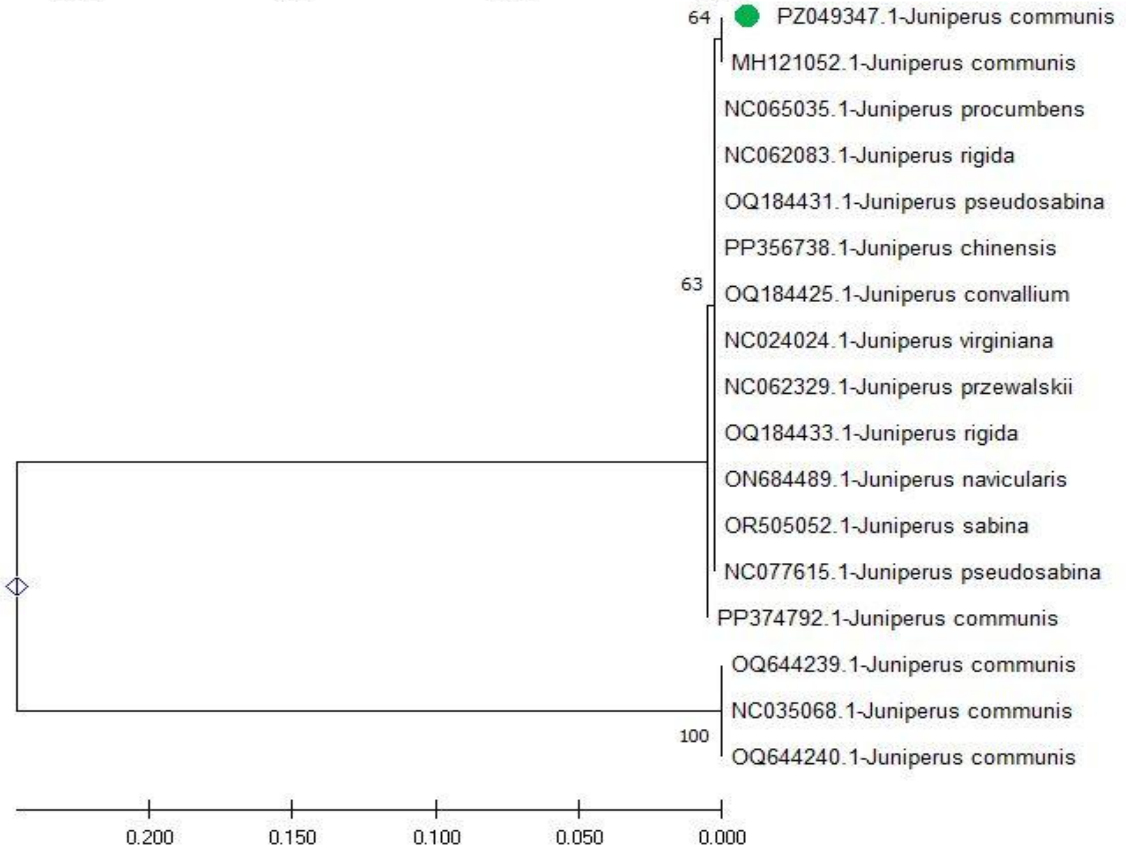
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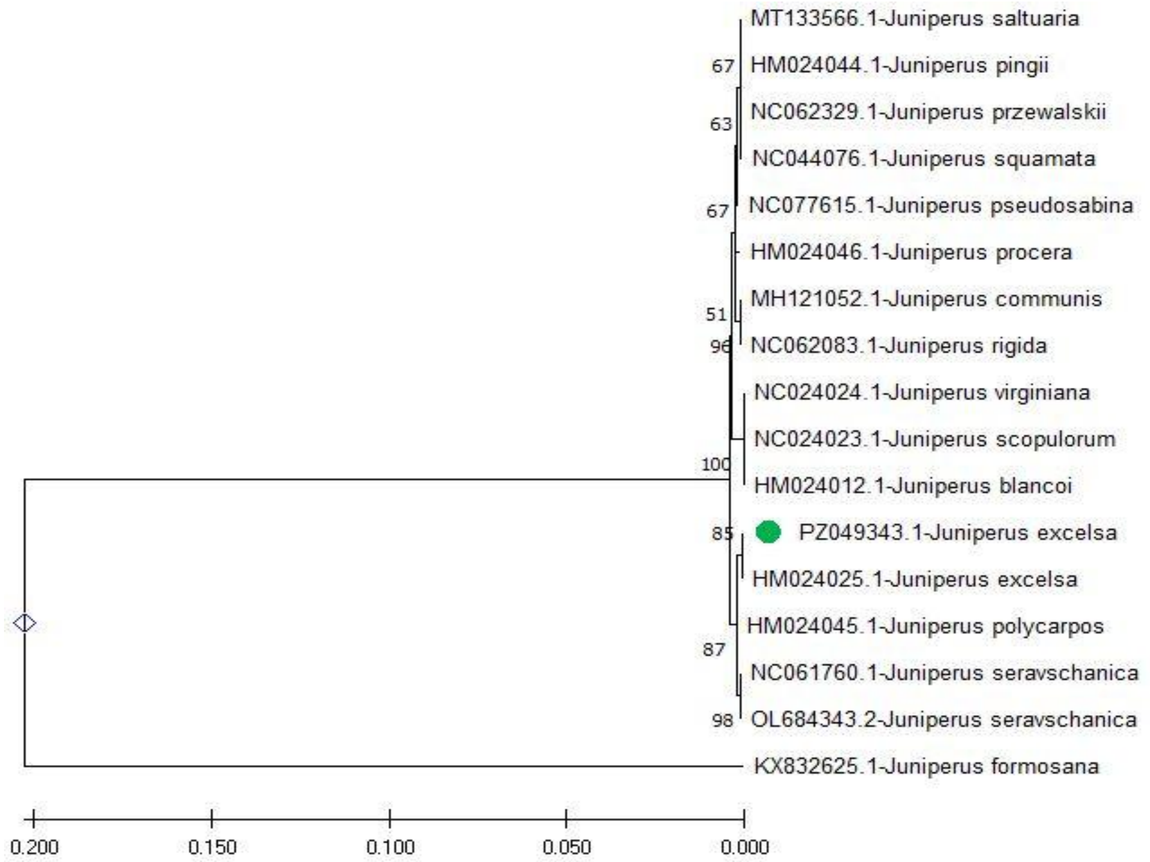
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HM024045.1-Juniperus polycarpos
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NC061760.1-Juniperus seravschanica
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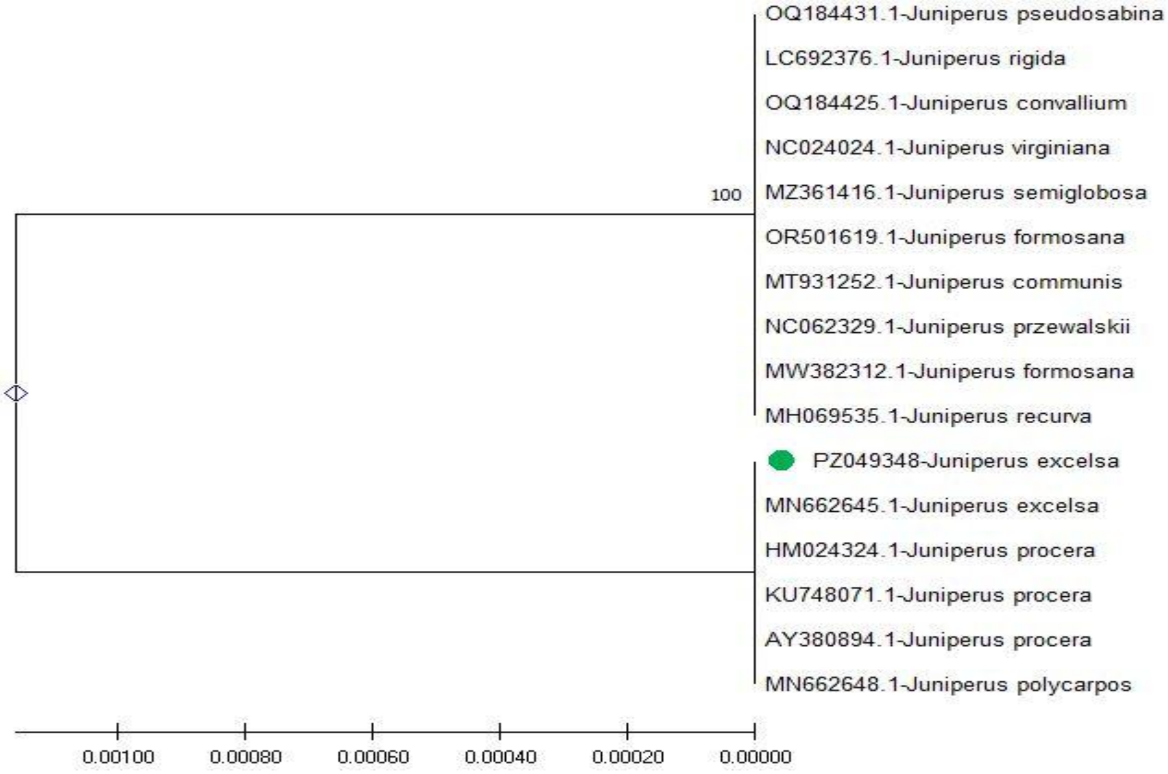
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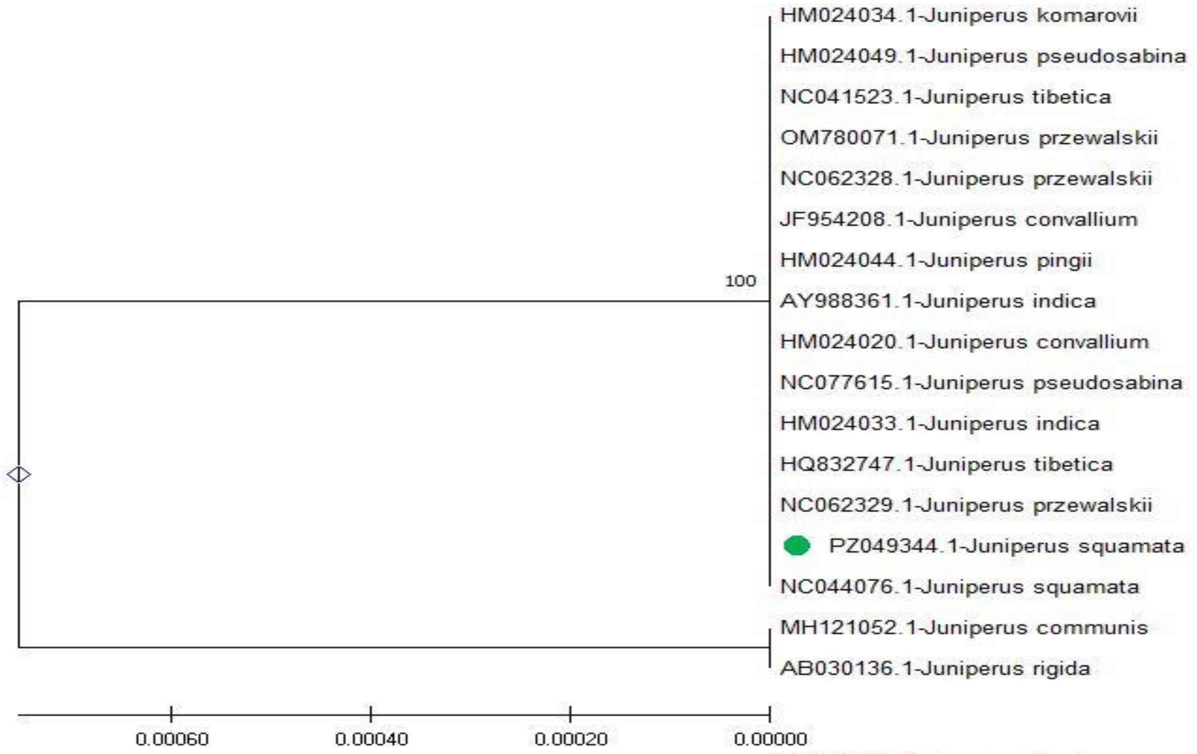
KU748071.1-Juniperus procera
AY380894.1-Juniperus procera
MN662648.1-Juniperus polycarpos
0.00100
0.00080
0.00060
0.00040
0.00020
0.00000


HM024034.1-Juniperus komarovii
HM024049.1-Juniperus pseudosabina
NC041523.1-Juniperus tibetica
OM780071.1-Juniperus przewalskii
NC062328.1-Juniperus przewalskii
JF954208.1-Juniperus convallium
HM024044.1-Juniperus pingii
100
AY988361.1-Juniperus indica
HM024020.1-Juniperus convallium
NC077615.1-Juniperus pseudosabina
HM024033.1-Juniperus indica
HQ832747.1-Juniperus tibetica
NC062329.1-Juniperus przewalskii
● PZ049344.1-Juniperus squamata
NC044076.1-Juniperus squamata
MH121052.1-Juniperus communis
AB030136.1-Juniperus rigida
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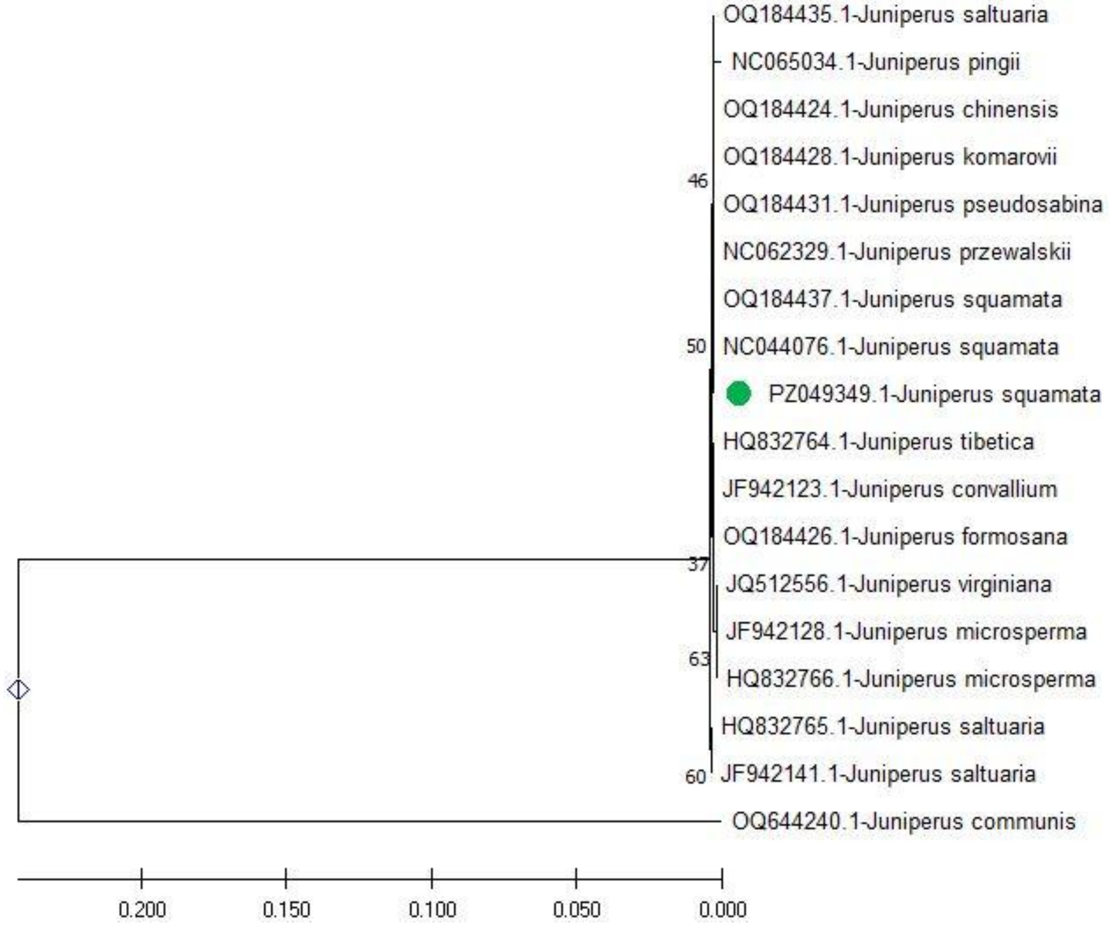
0.00040
0.00020
0.00000


OQ184435.1-Juniperus saltuaria
NC065034.1-Juniperus pingii
OQ184424.1-Juniperus chinensis
OQ184428.1-Juniperus komarovii
46
OQ184431.1-Juniperus pseudosabina
NC062329.1-Juniperus przewalskii
OQ184437.1-Juniperus squamata
50
NC044076.1-Juniperus squamata
● PZ049349.1-Juniperus squamata
HQ832764.1-Juniperus tibetica
JF942123.1-Juniperus convallium
OQ184426.1-Juniperus formosana
37
JQ512556.1-Juniperus virginiana
JF942128.1-Juniperus microsperma
63
HQ832766.1-Juniperus microsperma
HQ832765.1-Juniperus saltuaria
60
JF942141.1-Juniperus saltuaria
OQ644240.1-Juniperus communis
0.200
0.150
0.100
0.050
0.000