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Research Article

Occurrence of *Artemisia chinensis* (L.) plant (Asteraceae) in the Northeastern (Gilgit-Baltistan) Pakistan: Evidence From Molecular Phylogeny of nrDNA and cpDNA Sequences

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Abstract: *Artemisia chinensis* L. referred as *Crossostephium chinensis* (L.) Makino in the Flora of China is a rare and conceivably threatened plant species with an unclear origin in Asia. The species has been acknowledged so far from some islands of Taiwan. However, as it is extensively cultivated for ornamental and medicinal purposes in Japan, China, and the Philippines, it is still challenging to delimit its native range. This study confirms the presence of *A. chinensis* from Northern (Gilgit-Baltistan) Pakistan using molecular phylogenetic analysis and by assessing its distribution. The species were found in one site in the Skardu District of GB Pakistan and phylogenetic analysis indicated a close resemblance of the collected *A. chinensis* from the Skardu region with species of subgenus Pacifica of the genus *Artemisia* reported globally. According to the outcomes of the present study, it is proposed that broader field surveys should be conducted to acknowledge the distribution of *A. chinensis* plant from other districts of GB and cities of Pakistan as well. It is proposed that *A. chinensis* plant is present in North Pakistan and this plant should be mentioned and retained as rare species in the flora of Pakistan.

Keywords: Genus *Artemisia,* Asteraceae, *Artemisia chinensis* L., nrDNA and cpDNA Phylogeny, Gilgit-Baltistan, Pakistan.

1. INTRODUCTION

Artemisia L. is the largest plant genera of the Anthemideae tribe from the family Asteraceae with ~500 species frequently distributed in the Northern Hemisphere [1-4]. Based on floral and capitular characteristics, this genus was traditionally classified into 5 major subgenera-like subg. *Artemisia*, subg. Absinthium, subg. *Dracunculus* subg. *Seriphidium* and subg. Tridentatae [3]. Nevertheless, some inconsistencies were found in this traditional subgeneric classification due to incongruence with the latest molecular investigations on the genus *Artemisia* [5]. However, this classification is still extensively used and cited while dealing with the taxonomy and classification of this genus. Species of the *Artemisia* genus are very significant from both medicinal and economic points of view.

Plentiful secondary metabolites from extracts of *Artemisia* species have been reported for the treatment of certain health-related issues including anxiety, depression, epilepsy, insomnia, irritability, stress, and psychoneurosis [6]. *Artemisia* species hold crucial biological activities. The most prominent are antibacterial, antirheumatic, anthelmintic, antispasmodic, antimalarial, antitumor, antiseptic, hepato-protective [7-10], antidiabetic [11], antioxidant, and cytotoxic activities [12-14].

Additionally, an active drug Artemisinin [15-17] obtained from annual *Artemisia* species like *A. annua* is specifically used to cure malaria [18] and other deadly diseases [19-20]. Artemisinin discovery from *A. annua* was so for considered a noteworthy achievement in the field of ethnopharmacology and Physiology or Medicine category in 2015, this plant has been awarded a Nobel Prize [21]. Recently, the activity of *A. annua*

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against diseases COVID-19 and SARS-CoV-2 is under investigation [22]. *A. chinensis* is one of the rare species that belongs to the genus *Artemisia*.

This species was first described by Linnaeus [23] and is not in the *Crossostephium* Makino which is considered to be a monotypic genus and where this plant was frequently retained [24]. *A. chinensis* in Asian regions has been recognized as dispersed naturally in Taiwan, adjacent to southernmost Ryukyu, Orchid, Bonin, and the Islands of Okinawa. However, Ling et al. [25] questioned the native position of *A. chinensis* along with the Fujian, Zhejiang, and Guangdong coasts, but the suitability of climatic there was anticipated by the Maxent analysis. In China, Japan, and the Philippines, due to its extensive cultivation for ornamental and medicinal purposes, the origin or indigenous position of this plant is unclear and problematic. Hobbs and Baldwin, [24] believed that the distribution of *A. chinensis* plant is restricted to tropical regions because this plant lacks tolerance against cold.

The *Artemisia* genus was formally acknowledged with nearly 25 species [26] from Pakistan. An extensive taxonomic study by Hayat et al. [27, 28] on the genus *Artemisia* reported more species from the arid regions of Pakistan. However, the latest inquiries on *Artemisia* from Pakistan documented more species, especially from Northern Pakistan and the *Artemisia* genus now characterizes nearly 60 species from all subgenera from Pakistan except for the subgenus Tridentatae [29] *Tridentatae* species of the genus *Artemisia* are believed to be endemic only to North America and there is no evidence of the occurrence of subgenus *Tridentatae* species from other regions including Pakistan [27, 28]

This inquiry is the first attempt to report the presence of a medicinally important *Artemisia* species (*A. chinensis*) from the far-flung Skardu district of Gilgit-Baltistan Pakistan. This study also investigated the phylogenetic association of *A. chinensis* from Northeastern Pakistan with other *Artemisia* plants based on internal and external transcribed spacer (ITS and ETS) sequences of nrDNA and intergenic sequences (*psbA-trnH*) of cpDNA that sanctioned its taxonomic identity.

2. MATERIAL AND METHODS

2.1 Specimen Collection

Gilgit-Baltistan is situated in the Northeast of Pakistan between 34.6°–37.4°N, and 74°–77.5°E with an area of 45, 224 km^2. The maximum range of

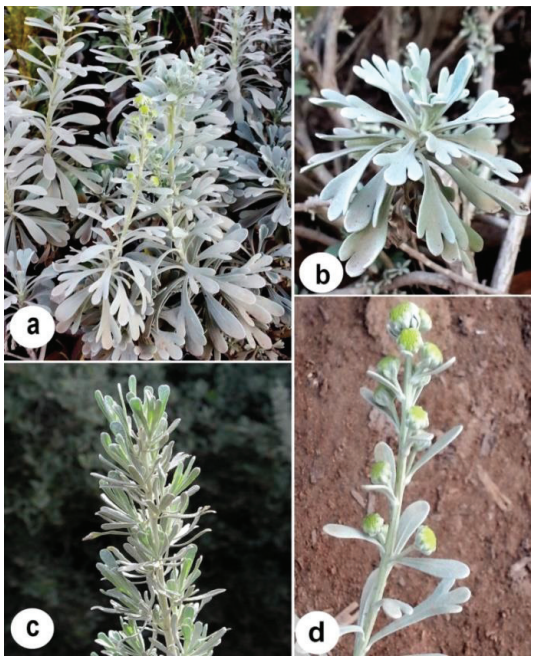

Fig. 1. Morphology of *A. chinensis* obtained from Skardu Gilgit-Baltistan Pakistan. a) Habit of the plant b) Basal leaves with lanceolate or spatulate 3 lobed blades, c) Aerial part with aggregated leaves, d) Inflorescence. Plant collection and original photographs by Adil Hussain and Tanseer Hussain

altitude of this region is 8611 m and the minimum is 1400 m. Gilgit-Baltistan has many districts and the major ones include Astore, Gilgit, Diamer, Ghizer, Ghanche, and Skardu Hunza-Nagar [30]. It has a temperate climate that is suitable for great plant diversity. In the course of the field samplings for the *Artemisia* project from the study area in 2016 and 2017 [31, 32], the occurrence of *A. chinensis* (Figure 1) was noticed in the Skardu District of Gilgit-Baltistan at a latitude of N-35′26.585 and longitude E-75′27.011. The sample was collected and the herbarium was consequently arranged (Figure 2). The specimen was deposited in the herbarium of Pakistan Museum of Natural History (PMNH) Islamabad Pakistan under the accession number PMNH-41722.

2.2 Morphology of Collected Plant

After assessing morphological characters and based on a BLAST search of molecular data, the species was recognized as *A. chinensis*. Various morphological characters of *A. chinensis* were evaluated in the dissecting and compound microscope with 4X, 10X, and 20X magnifications.

2.3 Phylogenetic Analysis

After collection, herbarium preparation, and morphological assessment of various features, the herbarium specimen of *A. chinensis* was transferred to the University of California Davis USA. From the exported herbarium specimens, leaf samples were taken to extract the total genomic DNA and to perform PCR for molecular phylogenetic analysis. The overall experimentation and data analysis were performed in the laboratory of Prof. Dr. Daniel Potter at the Department of Plant Sciences, University of California Davis CA, United States of America.

2.4 Extraction and Quantification of Genomic DNA

The leaf specimen was cleaned up with 70 % ethanol and the extraction of genomic DNA was done with a plant DNeasy kit (QIAGEN). After extraction, quantification of genomic DNA was done with the measurement of A260/280 values in a nanodrop spectrometer (ND-2000, Nanodrop Technologies USA) following Urreizti et al. [33]. 1.5% agarose gel was used and electrophoresis was performed to visualize the quality of extracted genomic DNA.

2.5 PCR Condition for the Amplification of ITS, ETS, and psbA-trnH regions

PCR was performed in the ABI thermo-cycle with 50 µl reaction volumes with ddH2O (36 µl), de¬oxyribonucleoside triphosphates (2 µl), 1xPCR buffer (5µl), MgCl2 (1µl), 1.5 µl both primers for ETS (18SETS and ETS-AST1), ITS (ITS9 and ITS6) and chloroplast *psbA-trnH* (trnHf and psbA3'f) (Table 1), template genomic DNA (1 to 1.5 µl of 20 to 50 ng), 0.5 µl of 5 units Taq polymerase and DMSO (1 µl). The optimized PCR amplification of ITS was achieved at 2 minutes pre-denaturation at 95 °C following 35 cycles with denaturation of 30 seconds at 95 °C, 1 minute annealing at 50 °C or 30 seconds annealing at 55 °C, and 72 °C, 1 minute extensions with the final extension of 5 minutes at 72 °C. The optimized PCR amplification of ETS was achieved at pre-denaturation for 2 minutes at 97°C with 36 cycles following denaturation for 2 sec at 97 °C, annealing at 55 °C for 30 seconds, and extensions at 72 °C for 30 seconds. A final extension was performed for 7 minutes at 72°C for ETS region amplification. The amplification of *psbA-trnH* sequence was performed at pre-denaturation of 5 minutes at 94 °C, following 30 cycles of denaturation for 1 minute at 94 °C, annealing of 1 minute at 55 °C, extension for 1.5 minutes at 72 °C. A final extension was achieved for 7 minutes at 72 °C.

The PCR products amplified were visualized and quantified in the electrophoresis containing agarose gel (1.5 %) arranged in 1xTBE with a voltage of 100 for 45 min in a buffer of Trisborate-ethylenediaminetetraacetic acid. The gel was then visualized under ultraviolet light in the trans-illuminator. During electrophoresis, PCR product size was perceived in comparison to the 1kb DNA ladder of standard size (Biolabs Company, N-3232L). The extraction of PCR product from the gel was carried out using a QIAGEN QIA-quick gel extraction kit with standard protocol.

2.6 Sequencing of PCR product and Multiple Sequence Alignment of Sequenced Data

The amplified DNA regions were then sequenced at the University of California Davis CA USA in a Big dye terminator version 3.1 cycle sequencing (ABI)

with capillary electrophoresis genetic analyzers (ABI 3730) using ETS, ITS, and *psbA-trnH* primers from both strands. The raw data of sequences of *A. chinensis* were assembled with software Sequencher. Total of four alignments (MSAs) were generated from ETS, ITS and *psbA-trnH* for new *A. chinensis* sequence and GenBank reference *Artemisia* species sequences were nrDNA-ITS (n=36), nrDNA-ETS (n=36), and cpDNA-psbA-trnH (n=36). One multiple sequence alignment (MSA) was obtained by combining ITS, ETS and *psbA-trnH* sequences (CAT-36; n=36). The details of multiple sequence alignments produced are given as;

MSA-1= ETS nrDNA (n=36) (1 new sequence+34 Gen¬Bank reference sequences+1 Outgroup sequence)
MSA-2= ITS nrDNA (n=36) (1 new sequences+34 Gen¬Bank reference sequences+1 Outgroup sequences)
MSA-3= *psbA-trnH* cpDNA (n = 36) (1 new sequences + 34 GenBank reference sequences+1 Outgroup sequences)
MSA4 = ETS nrDNA + ITS nrDNA + psbA-trnH cpDNA (CAT=36) (1 new sequence+34 GenBank sequences+1 Out¬group sequences)

Using the software MEGA-7 [34], these sequences were each aligned disjointedly followed by manual adjustments.

2.7 ML Phylogenetic Tree Construction

Primarily, 3 separate alignments were generated for ITS, ETS and psbA-trnH sequences (ETS with 390 characters, ITS with 653 characters, and psbA-trnH with 392 characters), and then the sequences of these three markers were combined [35, 36] to obtain final data matrix of 1435 characters. These separate and combined data matrices were analyzed using the maximum likelihood algorithm to find out the relationship of *A. chinensis* with other *Artemisia* species. The MEGA-7 software [34] was used to perform a maximum likelihood (ML) analysis and to visualize the final tree. *A. chinensis* sequenced

Fig. 2. *A. chinensis* herbarium specimen deposited at Pakistan Museum of Natural History (PMNH) Herbarium in Islamabad Pakistan (PMNH-41722

data with ITS, ETS, and *psbA-trnH* markers were submitted in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession number MH101881 for nrDNA ITS, MH292876 for nrDNA ETS and MH330169 for cpDNA *psbA-trnH*.

3. RESULTS AND DISCUSSION

The documentation of specimen PMHN-41722 (Figure 2) as *A. chinensis* characterizes the first record of this rare *Artemisia* species from Northern Pakistan. This species was recovered from one site in the district Skardu, Gilgit-Baltistan, Pakistan. The species was so for is recorded only near the Kachura/Shangrilla site in Skardu region of Gilgit-Baltistan Pakistan where it grows in grassy areas.

Morphological analysis showed the shruby nature of *A. chinensis* which is 10-40 cm tall (Figures 1 and 2). It has sometimes trailing branches with sessile leaves, aggregated at the top of the branches. It has densely gray-white pubescent. The blades of leaves are narrowly lanceolate or spatulate 2 to 4×0.4 to 0.5 cm thickly gray or white pubescent surfaces, cuneate-attenuate base, entire margin, apex 3 to 4 lobed and thick sometimes.

Small many disciform capitula with a diameter of 7 mm in a frondose raceme laterally branch. Hemispheric involucres and phyllaries are present in 3 rows where the outer and middle ones are elliptic, equal, and herbaceous. There is densely gray-white pubescent abaxially. The apex is acute or obtuse, the inner ones small, oblong, and subglabrous abaxially and the margin is scarious broadly. Female marginal florets are present in a single row which is tubular (ca. 1.5 mm), gland-dotted from outside with 2 or 3 denticulate apexes. Disk florets are many, tubular and 5-lobed, densely gland-dotted from the outer side. Fruits are conspicuously 5-ribbed and pappus is sometimes present with irregular teeth 0.5 mm, coroniform of small scales. The length of amplified DNA regions, raw generated sequences, multiple sequence alignments, and the numbers of informative sites for sequences of nuclear ribosomal DNA regions (ETS and ITS) and chloroplast DNA regions (*psbA-trnH*) for the species *Artemisia* are given in Table 2.

The data presented in the ML trees (Figures 3-6) based on ITS, ETS and *psbA-trnH* markers displays the dispersal of *A. chinensis* from Skardu district Gilgit-Baltistan Pakistan all through the clades corresponding to the other *Artemisia* species. All trees obtained from both independent and combined ML ETS, ITS, and *psbA-trnH* regions mended related topologies without any substantial conflicts. In the tree from independent data sets of each marker, *A. chinensis* from Skardu Gilgit-Baltistan was entirely supported which appeared in a single clade (ITS ML-BS = 97 %, ETS ML-BS= 64 %, *psbA-trnH* ML-BS= 82 %) covering species of the subgenus Pacifica including the previously reported *A. chinensis* from other parts of the world (ITS ML-BS = 100 %, ETS ML-BS= 86 %, *psbA-trnH* ML-BS= 99 %) as shown in Figures 3-5.

In a tree from combined data set of ITS, ETS, and *psbA-trnH* markers, *A. chinensis* from Skardu Gilgit-Baltistan was also completely supported in one clade (ML-BS = 100 %) with *A. chinensis* and other *Artemisia* species in a subgenus *Pacifica* clade reported from other parts of the world (ML-BS= 99 %) as shown in Figure 6. Consequently, the appearance of Pakistani *A. chinensis* in a clade (BS > 50 %) containing other *Artemisia* species, especially *A. chinensis* lineage from subgenus *Pacifica* of the genus *Artemisia* endorses its taxonomic identity. The plant species which are rare in nature have intrinsic, political, and ecological values that lead conservationists and land managers to ensure the protection of these plants. Keeping the rarity status of plant species, reserves are established in those areas where larger rare plant species are present [37-39]. It helps in the protection of the biodiversity contributions and the development of conservation strategies for rare plant species of the area [40].

A lot of studies reported the occurrence and native status of *A. chinensis* from different regions of the world [24, 46-48]. Studies concerning the presence of important phytochemicals having promising biological activities were also reported [49-52] *A. chinensis* was first described by Linnaeus [23] which is a monotypic genus, *Crossostephium* Makino in the flora of China and is a very popular traditional Chinese medicinal herb in Taiwan [48].

A. chinensis in Asian regions is thought to be distributed naturally in Taiwan adjacent to southernmost Ryukyu, Orchid, Bonin, and the Islands of Okinawa. However, Ling et al. [25] questioned the native position of *A. chinens*is along

Table 1. Details of three primers used for the amplification of nrDNA ITS, ETS, and cpDNA *psbA-trnH* sequences of the species of *Artemisia*

Markers	Marker name and Sequence	Length of base	References
ETS-forward primer	(AST1) 5′-CGTAAAGGTGCATGAGTGGTGT-3′	22	[41]
ETS-reverse primer	(18SETS) 5′ACTTACACATGCATGGCTTAATCT-3′	24	[42]
ITS-forward primer	(ITS9) 5′-GGAAGGAGAAGTCGTAACAAGG-3′	22	[43]
ITS-reverse primer	(ITS6) 5′-TCCTCCGCTTATTGATATGC-3′	20	
psbA-trnH-forward primer	(psbA3′f) 5′-GTTATGCATGAACGTAATGCTC-3′	22	[44]
psbA-trnH-reverse primer	(TrnHf-05) 5′CGCGCATGGTGGATTCACAATCC-3′	23	[45]

Table 2. Length of the PCR products and summary statistics of nrDNA ETS, ITS and cpDNA *psbA-trnH Artemisia* datasets. The numbers that appeared in the brackets specify the outcomes from the ingroup.

DNA Markers	ITS (nrDNA)	ETS (nrDNA)	*psbA-trnH* (cpDNA)	ITS+ETS +*psbA-trnH* (nrDNA + cpDNA)
Length of the PCR amplified region	~700 bp	~500 bp	~450 bp	
Samples number	36 (35)	36 (35)	36 (35)	36 (35)
Total number of sites	653	390	392	1435
Total number of informative sites	157(136)	96(83)	44(41)	300(257)

with the Fujian, Zhejiang, and Guangdong coasts, but the suitability of climatic there was anticipated by the Maxent analysis.

The monotypic *C. chinensis* was considered as a distant genus from the genus *Artemisia* [53]. It is believed that this genus is different from *Artemisia* due to the coroniform pappuspresence, however, it was formerly positioned with *Artemisia californica* by Rydberg [54] and Gray [46] on a deeply ribbed cypselas basis.

Gray [46] formerly put forwarded a close relationship of Hawaiian *A. australis* and *A. chinensis* earlier to the account of additional Hawaiian species. Conferring to Watson et al. [47] ITS phylogenetic analysis, the *C. chinensis* (*A. chinensis*) is allied with Old World Seriphidium and numerous species of subgenus *Artemisia* advocates that *C. chinensis* (*A. chinensis*) is unified within *Artemisia*.

Molecular phylogenetic study of Hobbs and Baldwin, [24] displayed a robust relationships resolution of the South Asian *A. chinensis* and Hawaiian *Artemisia*. They proposed a clade containing Hawaiian *Artemisia* and *A. chinensis* a new subgenus called *Artemisia* subgenus *Pacifica*.

This new subgenus Pacifica contains *A. chinensis* from littoral habitats in Southeast Asia, for example, Okinawa, Taiwan, Bonin, and Ryukyu islands, and 3 species viz *A. kauaiensis, A. australis,* and *A. mauiensis* of subalpine to littoral habitats in Hawaiian Islands [24]. Species of this subgenus *Pacifia* appeared monophyletic in the phylogenetic investigation of Malik et al. [55] concerning the taxonomy and classification of the subg. Seriphidium of the genus *Artemisia*

Hobbs and Baldwin [24] displayed that the nuclear ribosomal DNA and chloroplast DNA sequences support the hypothesis that the Southeast Asian *A. chinensis* is closely allied to the Hawaiian *Artemisia* taxa, which also make a clade. Results of this study also showed a close relationship between *A. australis* and *A. chinensis* under the subgenus Pacifica clade from the Northeastern Skardu region of Pakistan.

Presently, *A. chinensis* has been declared as possibly threatened in the Flora of China. Based on the rarity and less population, *A. chinensis* reported here from Northeastern Pakistan should also be declared a rare and possibly threatened species in the flora of Pakistan.

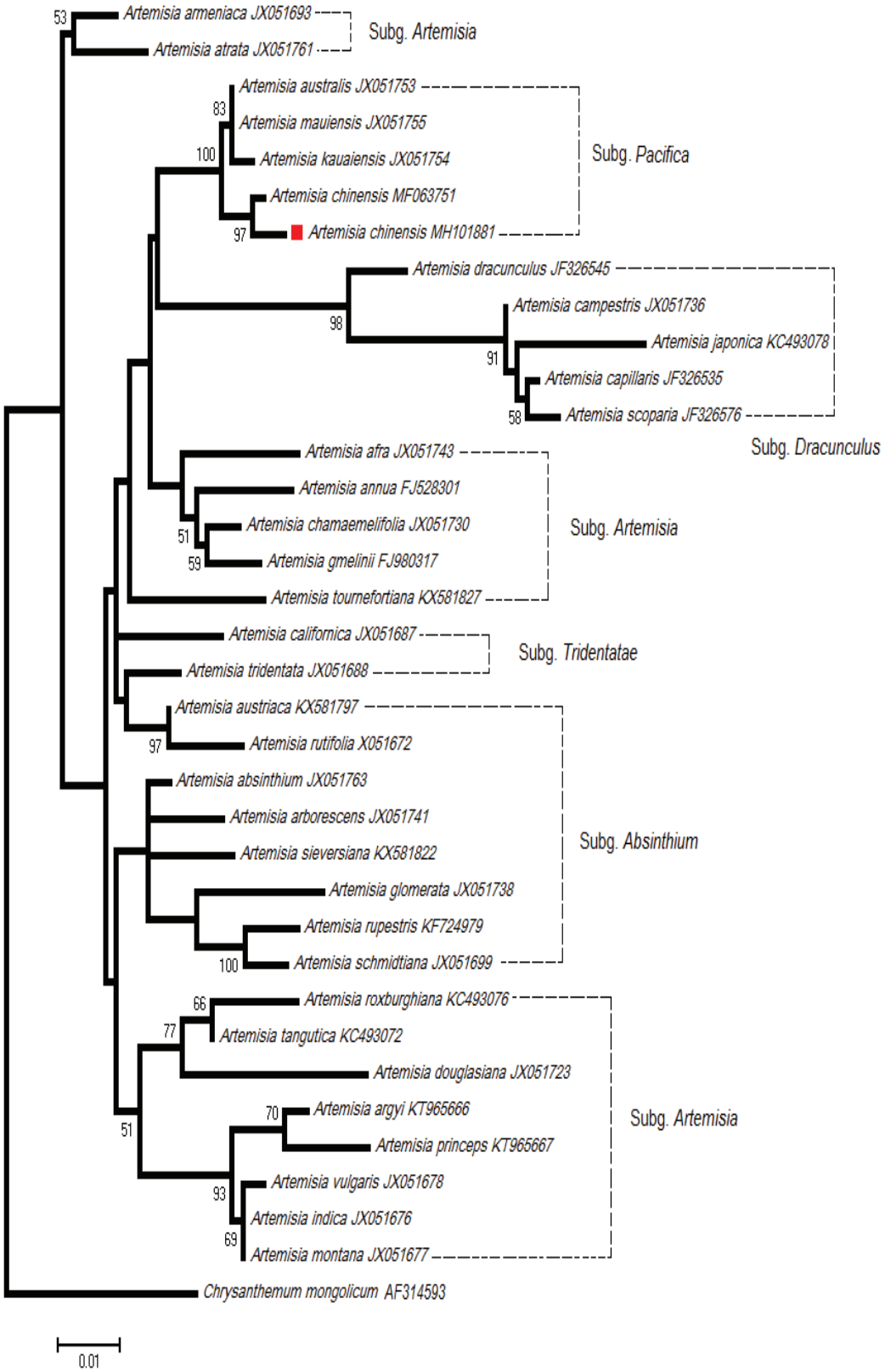

Fig. 3. Maximum likelihood phylogenetic tree constructed based on nrDNA ITS sequences of *Artemisia.* Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al*. [1], Valles *et al*.[57], Sanz *et al*. [2], Garcia *et al.* [4], Pellicer *et al.* [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik *et al*. [55] is indicated with vertical bars.

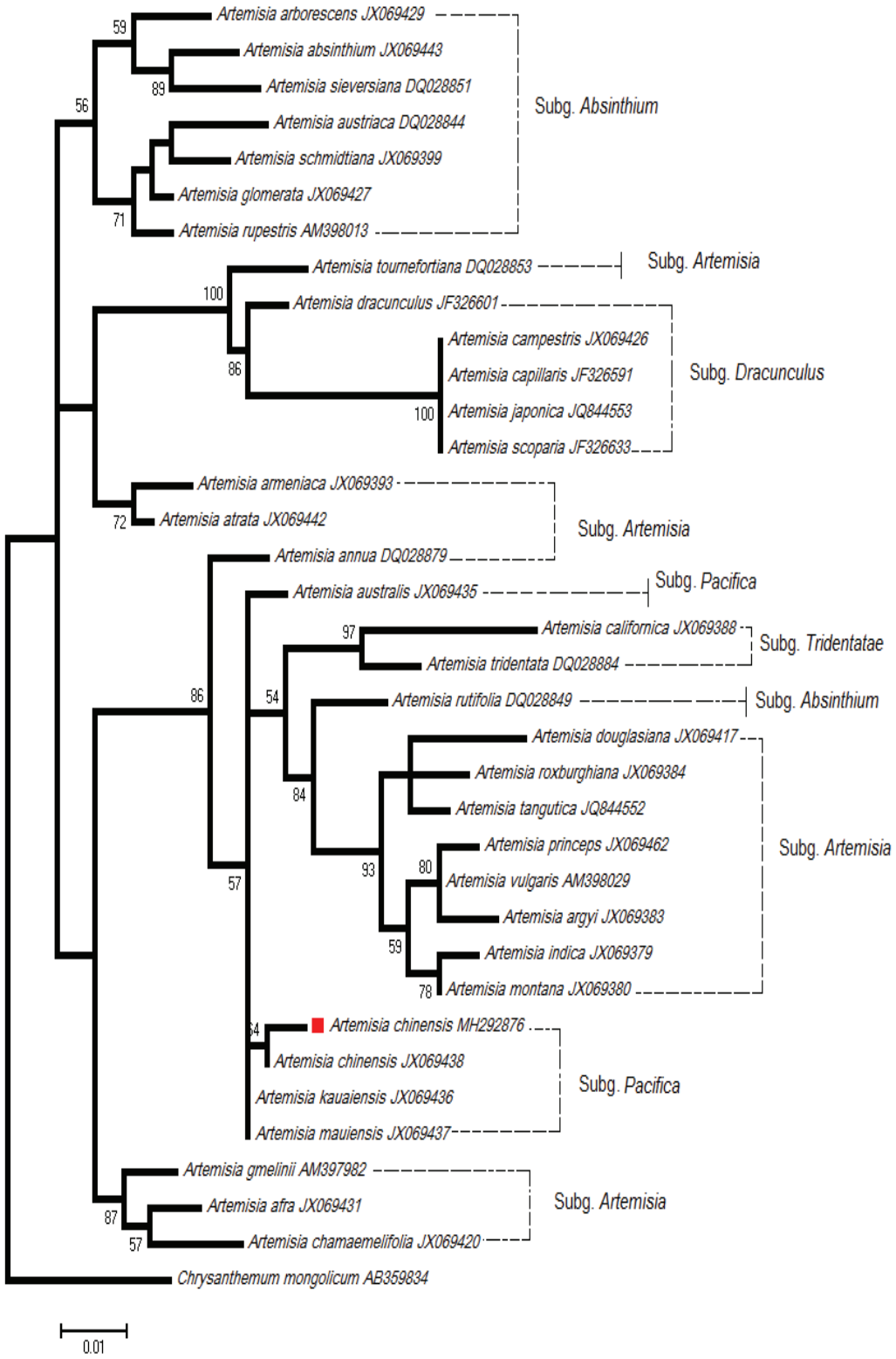

Fig. 4. Maximum likelihood phylogenetic tree constructed based on nrDNA ETS sequences of *Artemisia*. Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al.* [1], Valles *et al.*[57], Sanz *et al.* [2], Garcia *et al.* [4], Pellicer *et al.* [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik *et al.* [55] is indicated with vertical bars

Fig. 5. Maximum likelihood phylogenetic tree constructed based on cpDNA *psbA-trnH* sequences of *Artemisia*. Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al*. [1], Valles *et al*.[57], Sanz *et al*. [2], Garcia *et al*. [4], Pellicer *et al*. [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik *et al*. [55] is indicated with vertical bars.

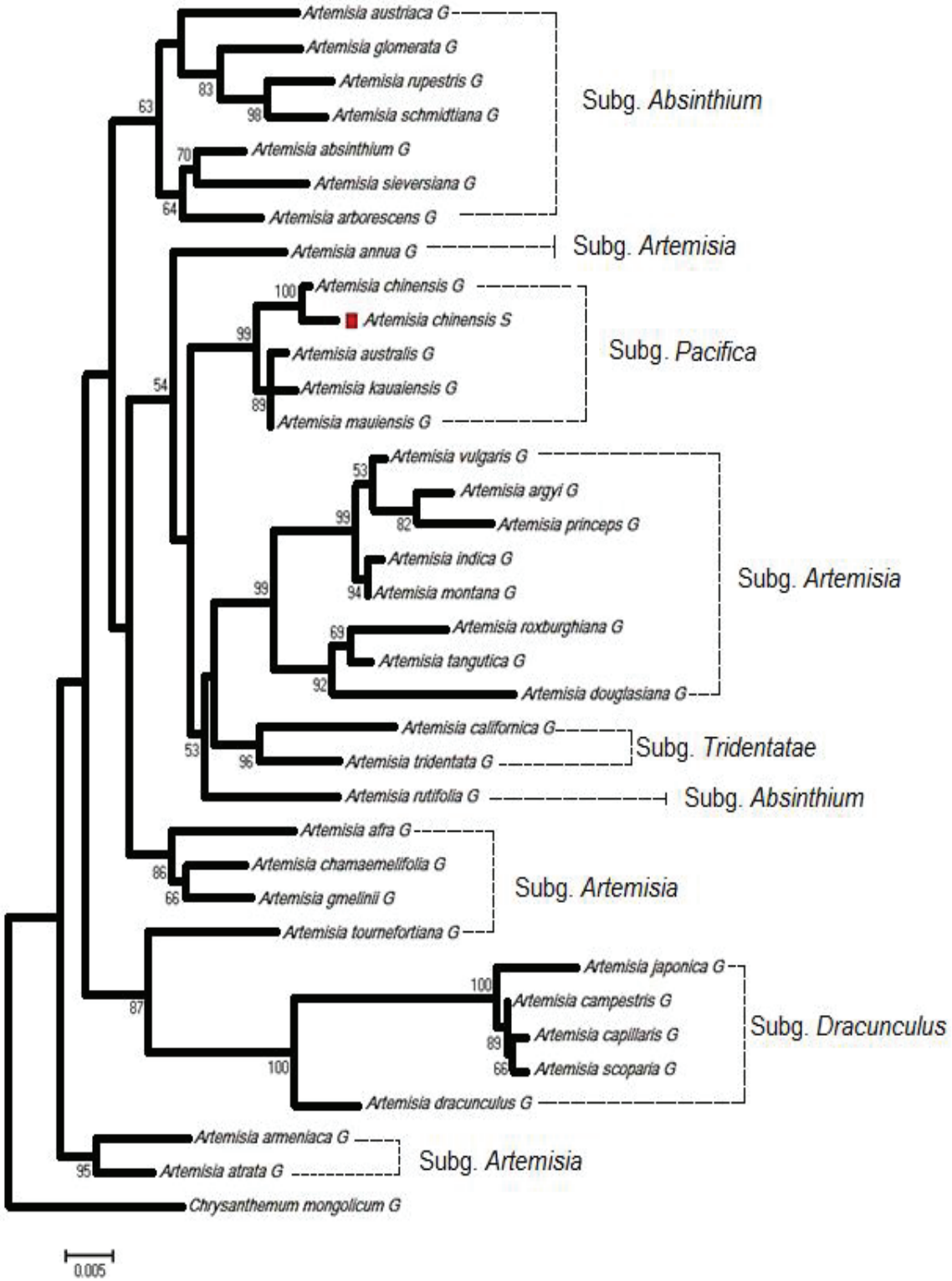

Fig. 6. Maximum likelihood phylogenetic tree constructed based on nrDNA+cpDNA (ITS+ETS+*psbA-trnH*) sequences of *Artemisia.* Values displayed above branches are the bootstrap values acquired from ML analysis with 1000 replicates. Colored shape denotes *A. chinensis* sequence from the Skardu region of Gilgit-Baltistan Pakistan. The subgeneric classification of the genus *Artemisia* following Bremer [56], Torrell *et al*. [1], Valles *et al*.[57], Sanz *et al*. [2], Garcia *et al*. [4], Pellicer *et al*. [58], Riggins and Seigler [59], Hobbs and Baldwin [24], Malik et al. [55] is indicated with vertical bars.

4. CONCLUSION

The occurrence of *A. chinensis* in the Skardu GB region of Pakistan stated in the present study permits the necessity of wide-ranging sampling to confirm its distribution not only from other sites of the Gilgit-Baltistan region but across Pakistan. It is assumed that *A. chinensis* plant may be also present in other parts of the GB, but not been reported by researchers and its presence could also be evident in other localities of Pakistan as well. Based on the outcomes of this study, it is proposed that *A. chinensis* plant is present in North Pakistan and this plant should be retained as rare species in the flora of Pakistan.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The author declare no conflict of interest.

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