NEW DISTRIBUTION REPORT ONTHE ALIEN SPECIES*Artemisiaverlotiorum*LAMOTTE(ASTERACEAE-ANTHEMIDEAE) FROM GILGIT-BALTISTAN REGION OF PAKISTAN

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DOI: https://doi.org/10.28941/pjwsr.v26i2.826

ABSTRACT

Artemisia verlotiorum Lamotte *(Asteraceae) is a species of Artemisia described from Europe but native to East Asia. It is an alien and/or invasive species that has become naturalized in many European regions, Australia, South America, New Zealand, North and South Africa and Western Asia.In continuation of our work on Northeastern Pakistani Artemisia, we report the first local occurrence of* Artemisia verlotorum *Lamotte from Gilgit-Baltistan region of Pakistan by investigating its geographical distribution and phylogenetic analysis. To date, we have observed this species in stony landscapes of Ghizer district of Gilgit-Baltistan. Phylogenetic analysis of* A. verlotiorum *with maximum likelihood approach using its ETS (External transcribed spacer)and ITS (Internal transcribed spacer) of nrDNA sequences showed its resemblance with other Artemisia species reported from the world. This species needs to be included in the rare plant species list in the flora of Pakistan following the criteria of IUCN.*

Keywords*:* *Artemisia verlotiorum* Lamotte, Asteraceae, Gilgit-Baltistan, rare species, Pakistan

Citation: Hussain, A., M. Q. Hayat and S. A. I. Bokhari. 2020. New Distribution Report on The Alien Species *Artemisia Verlotiorum* Lamotte (Asteraceae-Anthemideae) From Gilgit-Baltistan Region 0f Pakistan. Pak. J. Weed Sci. Res., 26(2): 167-178.

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INTRODUCTION

The Asteraceae is one of the largest families of angiosperms containing~1,700 genera and ~24,000 species with a global distribution excluding the Antarctica (Funk *et al*., 2009).*Artemisia*, a member of the tribe *Anthemideae* with nearly ~500 species including both herbs and shrubs (Vallès and McArthur, 2001) and is taxonomically challenging. Some *Artemisia* species are economically significant because of their reported antispasmodic, antiseptic, antimicrobial, antitumor, antimalarial, antirheumatic and hepato-protective properties(Terra *et al*., 2007; Hussain *et al*., 2017). Species from this genus grow mostly in the northern hemisphere, but a feware also present in the southern hemisphere (Ling, 1994; Oberprieler *et al*., 2009). *Artemisia* originated from Central Asia. Microfossils of *Artemisia* are known from the Eocene (Zaklinskaja, 1957) and Miocene radiation in China (Wang, 2004).

Most *Artemisia* species are perennial, while some are annual or biennual (Valles *et al*., 2003). Many chemicals extracted from *Artemisia* species showed promising therapeutic attributes. For example, *A. annua* contains Artemisinin, which is a very important component of the drugs used to treat malaria in Artemisinin combined therapy(ACT) (Mannan *et al.,* 2010; Nageeb*et al*., 2013).

Artemisia verlotiorum Lamotte is an invasive species reported from European regions(Verlot 1875, 1876) and also described by Lamotte(1877). Brenan (1950) reported detailed comparative morphology and anatomy of *A. vulgaris* and *A. verlotiorum* in Florence Britain. From Europe, James and Wurzell (2000) again revised the morphology of *A. verlotiorum*, *A. vulgaris* and their hybrid viz *Artemisia* x *wurzellii* (C. M. lames & Stace hybr. Nov). According to Brenan (1950) *A. verlotiorum* is rhizomatous plant. Its primary leaves have narrow lobes with serrated secondary lobes. Segments of the aerial leaves are conspicuously elongate, linear-lanceolate to linear. Capitula are usually ovoid-ellipsoid 3.5-4.2 x 2.4-3.0 mm of length and breadth. Stamens have 5 bisexual flowers 0.4-0.5 mm in length. Receptacle of *A. verlotiorum*is low-domed. The chromosome number of *A. verlotiorum* reported is 2n = 50, 52 (James and Wurzell, 2000).

Ghafoor(2002) included 25 species in his account of *Artemisia* for the Flora of Pakistan, but recent studies have reported occurrence of more species from this country (Hussain *et al*., 2019 a, b). In this study, we provide the first evidence that *Artemisia verlotorum* grows in Pakistan and show its phylogenetic position on the basis of ETS (External transcribed spacer)and ITS (Internal transcribed spacer) sequences of nrDNA.

MATERIALS AND METHODS

Plant Specimen

During field surveys for our research project on *Artemisia* from Gilgit-Baltistan Pakistan, an *Artemisia* species was collected in district Ghizer of Gilgit-Baltistan with GPS coordinates N-3608.543’ and E-7351.721’. The subsequently collected samples of the same plant were pressed and mounted on herbarium sheets with proper labels. The herbarium specimens were then brought to the Pakistan Museum of Natural History Islamabad (PMNH) where it was identified by the plant taxonomist. All the duplicate specimens were deposited in the PMNH her barium under a specimen numberPMNH-41684.

Phylogenetic Analysis

The samples from the same plant in the form of herbarium specimens were also submitted to the Plant Sciences Department, University of California Davis USA. Samples were taken from herbarium specimens and employed for molecular phylogenetic analysis in Prof. Daniel Potter’s laboratory, Plant Sciences Department, University of California, Davis, USA.

Genomic DNA extraction and quantification

The leaf material was first washed with 70% ethanol and then extraction of genomic DNA was performed with plant DN easy kit (QIAGEN). The extracted DNA was quantified by measuring A260/280 in a spectrometer ND-2000 (Nano drop

Technologies, Wilmington DE USA) (Urreizti *et al*., 2012). 1.5% agarose gel electrophoresis was used to visualize quality of extracted DNA.

PCR for DNA amplification

Two set of primers were used for the PCR amplifications of ITS9-6 and ETS regions as given in Table-1. The PCR conditions for amplification of these regions were carried out following Hussain *et al.*(2019a).

Nucleotide sequencing and data assemblage

Sequencing of both the amplified ETS and ITS regions of *A. verlotiorum* was performed in the core sequencing facility at UC Davis, USA in a capillary electrophoresis genetic analyzers (ABI 3730) with Big Dye terminator version 3.1 cycle sequencing (ABI) from both strands. The details of ITS (ITS6 and ITS9) and ETS (18SETS and ETS-AST-1) primers employed for sequencing are given in Table-1. Subsequently, the raw sequenced data were assembled with softwares like BioEdit version 7.1.9 (Hall, 1999) and Sequencher version 5.4.6 (Gene codes Co.).

Multiple sequence alignments and phylogenetic tree construction

Phylogenetic analysis was carried out with sequenced data of ETS and ITS sequences of nrDNA to circumscribe the relationship of Northeastern Pakistani *A. verlotiorum.* For the phylogenetic analysis, previously published ETS and IT S sequences of *A. verlotiorum* and other species of genus *Artemisia* were obtained from Genbank. *Chrysanthemum indicum* and *Ajania fastigiata* were included as out groups using their ETS and ITS sequences from Gen Bank. The ETS and ITS sequences of Northeastern Pakistani *A. verlotiorum* were deposited in the Gen Bank under the accession numbersMH100668 for ETSandMH292872 for ITS. These sequences were used to produce multiple sequence alignments (MSAs) with other retrieved sequences of *Artemisia* from the Gen Bank. We generated two multiple sequence alignments (MSAs) for ETS and ITS sequenced data of Pakistani *A. verlotiorum* with those of sequences retrieved from GenBank; nrDNA-ETS (n = 50) and nrDNA-ITS (n = 50).These MSAs were then individually analyzed with maximum likelihood to check the relationship of *A. verlotiorum* from Northeastern Pakistani with other *Artemisia* species. The maximum Likelihood (ML) analysis was carried out with 1000 bootstrap replicates using software MEGA-7 as given by Kumar *et al*.(2016). The resulting trees were then visualized in Fig Tree (2018)software version 1.4.3.

Table-1. Set of primers used to amplify ETS and ITS regions of nr DNA in *A. verlotiorum* from Gilgit-Baltistan Pakistan

Primer	Sequence	Base length	Reference
ITS-forward primer	ITS-9: 5′-GGAAGGAGAAGTCGTAACAAGG-3′	22	Potter *et al*. (2007)
ITS-reverse primer	ITS-6: 5′-TCCTCCGCTTATTGATATGC-3′	20	
ETS-forward primer	AST-1: 5′-CGTAAAGGTGCATGAGTGGTGT-3′	22	Markos and Baldwin (2001)
ETS-reverse primer	18S-ETS: 5′ACTTACACATGCATGGCTTAATCT-3′	24	Baldwin and Markos (1998)

RESULTS AND DISCUSSION

The identification of specimen PMHN-41684 as *Artesmisa verlotiorum* represents the first record of the species from Pakistan. It was collected from one site in District Ghizer, Gilgit-Baltistan, Pakistan. It may be more widespread,

not just in Gilgit-Pakistan but in other parts of Pakistan and adjacent countries as well. Its morphology is shown in Figs. 1 and 2. So far, the species is known only from the one site where it grows in grassy and stony landscapes. The phylogenetic trees (Figs. 3 and 4) placed *A. verlotiorum* (BS= > 50%) in a clade that included some of the available species of the polyphyletic subg. *Artemisia* from Gen Bank.

A. verlotiorum is said to be native to East Asia, especially to China (Sanz *et al.*, 2004).This species is currently naturalized in many African, western Asian, European, Australian, New Zealand and South American regions (Pampanini, 1923, 1933; Brenan, 1950; Bangerter, 1978; Webb *et al*., 1988; Esler, 1987; Leonova, 1994; Ariza, 1997; Thompson, 2007; Verloove, 2013; Ling *et al*., 2011; Kurşat and Civelek, 2011; Mosyakin *et al*., 2018) and other parts of the world (Gams, 1929; Mosyakin, 1990; Dubovik and Mosyakin, 1991; Gabrielian and Vallès 1996; Mosyakin 2006; Boiko, 2013; Mamchur *et al*., 2017; Mosyakin *et al*., 2019)

Gams (1929) reported the first occurrence of *A. verlotiorum* in Ukraine and Eastern Europe. According to Gams (1929), he observed this species as a weed in the Nikita Botanical Garden. Nevertheless, his study was abandoned and the species was not reported and considered in Floras and other publications. Studies of Mosyakin(1990), Dubovik and Mosyakin(1991) and Mosyakin(2006) then confirmed Gams (1929)report and acknowledged the occurrence of *A. verlotiorum* in Nikita of Ukraine. Some other investigations disagreed and argued that *A. verlotiorum* reported was actually misidentified and considers it as *A. vulgaris* (Boiko, 2009). Investigations of Gabrielian and Vallès (1996) reported *A. verlotiorum* from the Caucasus and Armenia with and investigated the Armenian plants for their chromosome number.

Some studies propose that the species is also present in North America, where for many years its cryptic invasion was unnoticed totally due to them isperception with Eurasian *A. vulgaris* L. Mosyakin, (1990, 1991, 1992, 2006), Dubovik and Mosyakin (1991) and Boiko(2009, 2013) addressed numerous questions concerning the distribution, morphology, taxonomy and nomenclature of *A. verlotiorum* and other species of *A. vulgaris* complex that tend to grow together in Ukraine and other eastern European countries such as Latvia, Belarus, parts of Russia and Lithuania. They found that *A. verlotiorum* flowers later than members of the *A. vulgaris* complex, *A. verlotiorum* under different climatic conditions. Its shoots start to grow and develop in the mid May and its first inflorescences may not appear until the late August and seeds are not developed until October (Mosyakin *et al*., 2019).

Growing pattern of this plant suggests that it usually grows from seeds which are brought by wind from other nearby populations, not from rhizomes or rhizome fragments even though *A. verlotiorum* normally develops many vegetative shoots and, under favorable conditions, these shoots may become established. Because of this wind dispersal of seed, further spreading of the species to different regions of the world is possible(Boiko, 2009). In many countries, *A. verlotiorum* is declared as rare and relict species and in some countries, it is protected under different Biodiversity Acts. Based on this study, we suggest that *A. verlotiorum* be added to the rare species list of the flora of Pakistan following the criteria of IUCN (IUCN 2001, 2003), at least until it is shown to be more widespread once its occurrence in the country and its distinguishing features are known.

Fig.1. *Artemisia verlotiorum* Lamotte.collected from district Ghizer of Gilgit-Baltistan Pakistan. a) Habit, b) Aerial part with leaves, c) Inflorescence, d) Roots. Collectors: Adil Hussain and Amar Abbas. Photographs by: Adil Hussain

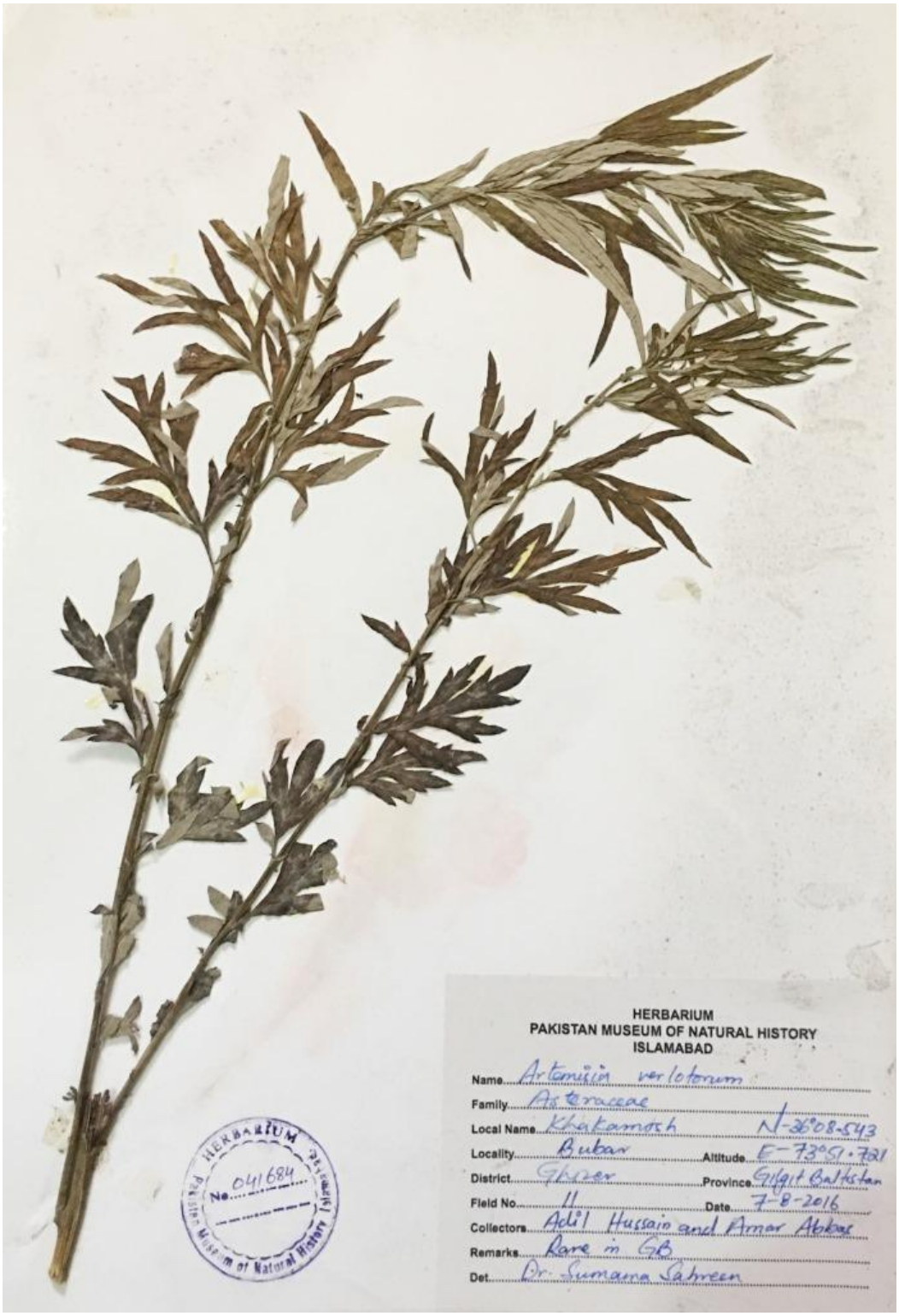

Fig. 2. Herbarium voucher specimen (PMNH-41684) of *A. verlotiorum* Lamotte deposited in the Pakistan Museum of Natural History (PMNH) Islamabad Pakistan.

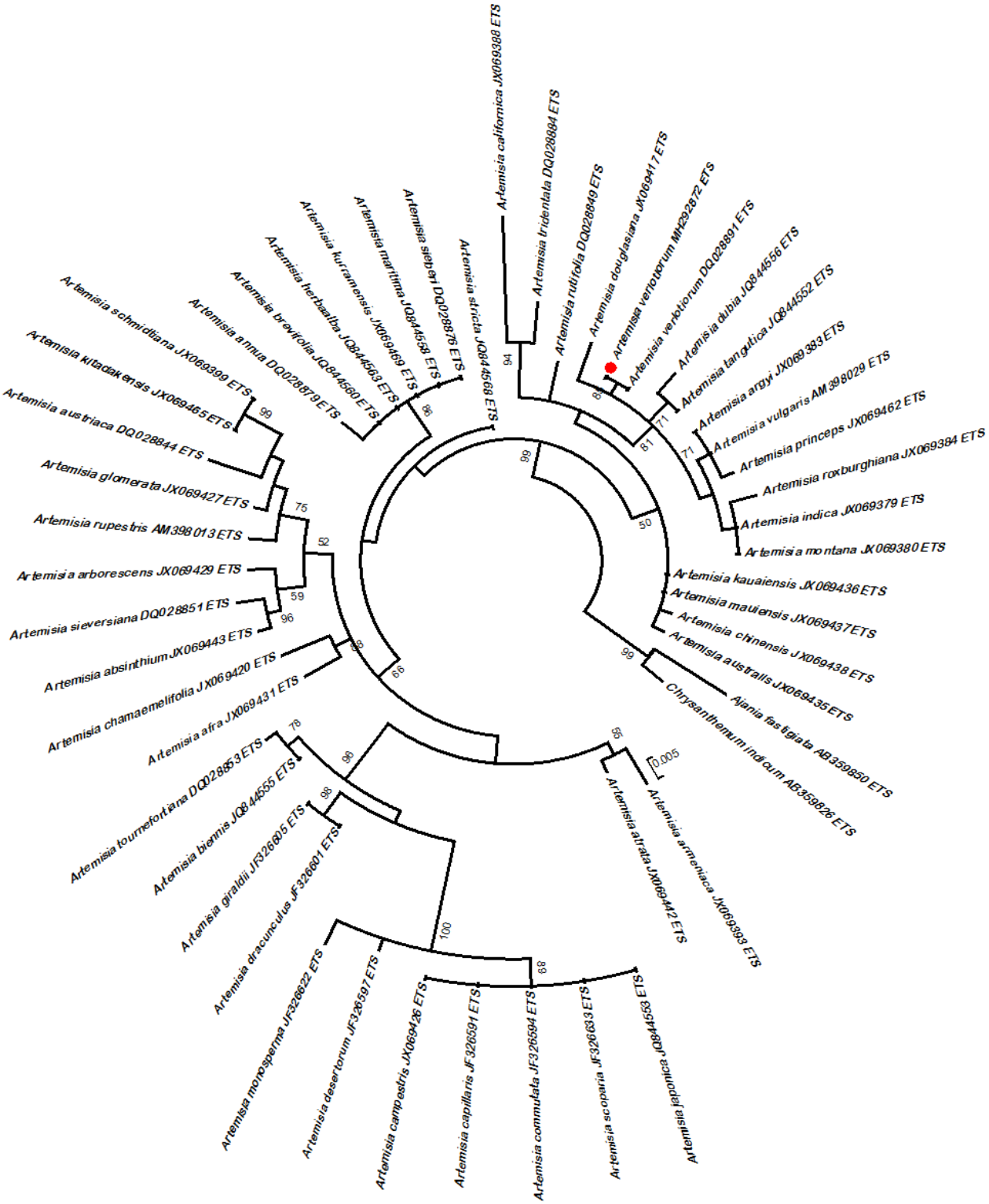

Fig. 3. Maximum likelihood (ML) consensus tree of ETS sequences of *Artemisia* species*.* The values shown along branches are the bootstrap support achieved from ML analysis with 1000 replicates. The red colored circle specifies corresponding Northeastern Pakistani *A. verlotiorum* with species of polyphyletic sub genus *Artemisia.*

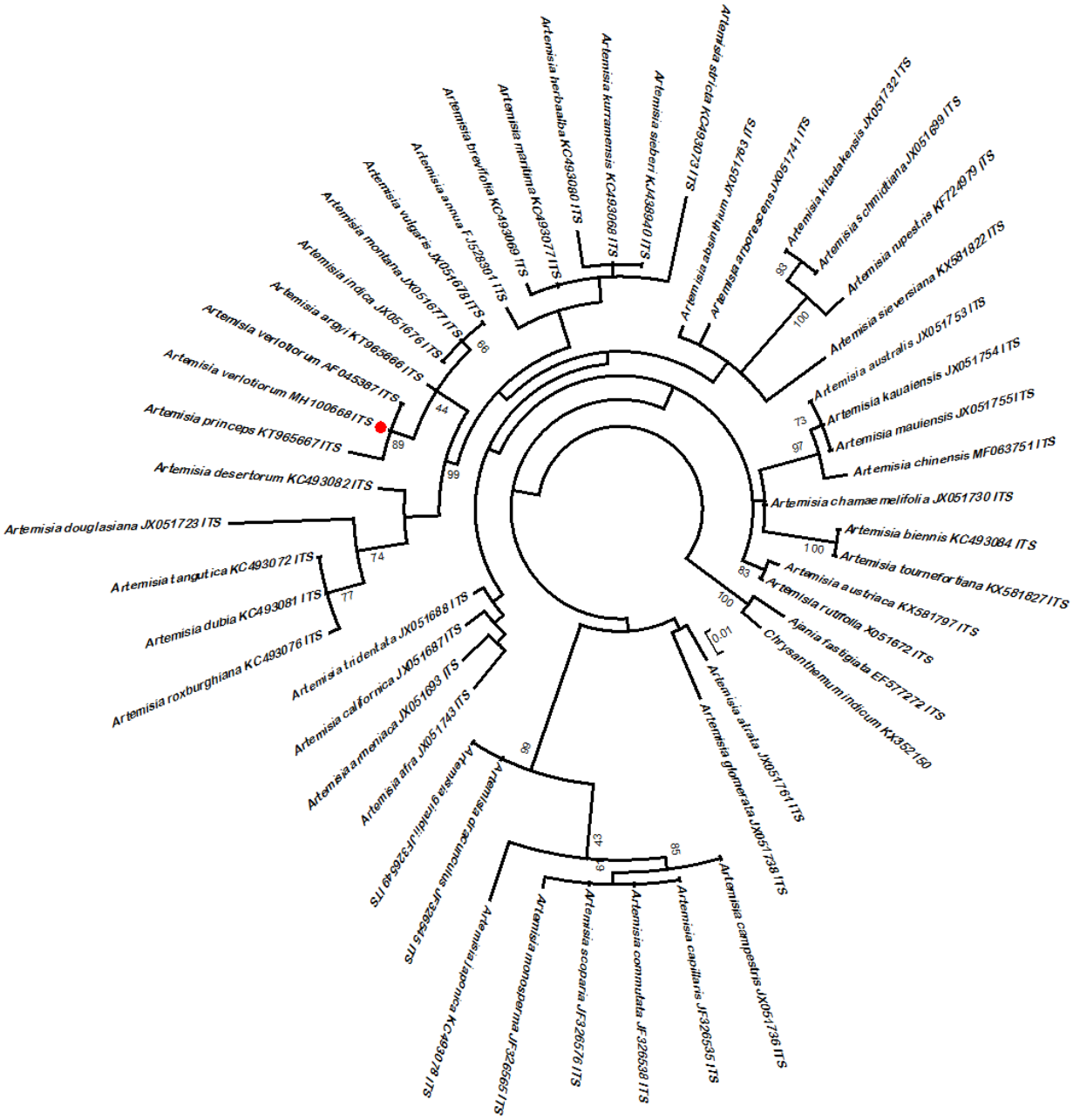

Fig. 4. Maximum likelihood (ML) consensus tree of ITS sequences of *Artemisia* species. The values shown along branches are the bootstrap support achieved from ML analysis with 1000 replicates. The red coloured circle specifies corresponding Northeastern Pakistani *A. verlotiorum* with species of the polyphyletic sub genus *Artemisia.*

CONCLUSION AND RECOMMENDATIONS

The occurrence *A. verlotiorum* in Gilgit-Baltistan Pakistan reported here requires further extensive survey from other regions of Gilgit-Baltistan Pakistan. It is possible that it is more widespread in the Northeastern region of Pakistan and adjacent countries like China, but has so far gone overlooked on Pakistan. There is likelihood of its extensive distribution and occurrence in other temperate and high altitude regions of Northern (Gilgit-Baltistan) Pakistan. This inquiry recommends that *A. verlotiorum* should be added in the rare plant species list in the flora of Pakistan.

ACKNOWLEDGEMENTS

We are thankful to the International Islamic University Islamabad (IIUI) Pakistan and Plant

Sciences Department, University of California Davis, CA, USA for providing research facilities. We are grateful to Prof. Daniel Potter of Department of Plant Sciences, UC Davis, CA, USA for providing assistance in phylogenetic analysis. We thank Dr. Sumaira Sahreen, Associate Curator, Pakistan Museum of Natural History Islamabad (PMNH) for her assistance in herbarium specimen preparation and deposition.

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