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REGULAR PAPER

Molecular phylogeny of *Artemisia* (Asteraceae-Anthemideae) with emphasis on undescribed taxa from Gilgit-Baltistan (Pakistan) based on nrDNA (ITS and ETS) and cpDNA (*psbA-trnH*) sequences

Adil Hussain[1,*], Daniel Potter[2], Sangtae Kim[3], Muhammad Q. Hayat[4] & Syed A.I. Bokhari[1]

[1]Department of Biological Sciences, International Islamic University Islamabad, 44000 Pakistan
[2]Department of Plant Sciences, College of Agricultural and Environmental Sciences, University of California Davis, 95616 USA
[3]School of Biological Sciences and Chemistry, Sungshin Women's University, Korea
[4]Department of Plant Biotechnology, Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology Islamabad, 44000 Pakistan
*Corresponding author: adil.phdbt31@iiu.edu.pk

Background – Gilgit-Baltistan, the Northeast region of Pakistan, is well known for its floristic diversity, including members of the genus *Artemisia*. *Artemisia* is a large, taxonomically complex genus including ~500 species of both herbs and shrubs. This study was conducted to determine the phylogenetic position of ten undescribed *Artemisia* taxa from northern Pakistan, using nrDNA internal transcribed spacer (ITS), external transcribed spacer (ETS) and cpDNA intergenic spacer (*psbA-trnH*) regions.
Methods – The phylogenetic relationships of 28 taxa of *Artemisia* using separate and combined data sets of sequences of three markers (ITS, ETS and *psbA-trnH*) were analysed with maximum parsimony, maximum likelihood, and Bayesian approaches.
Key results – The results resolve northeastern Pakistani *Artemisia*, which represent five morphologically defined subgenera, into ten major clades. *S*ubgenera *Artemisia* and *Absinthium* are shown to be polyphyletic, while *Dracunculus*, *Pacifica* and *Tridentatae* appear monophyletic. All species of subgenus *Seriphidium* are retrieved in a single clade that also includes annual species from subgenus *Artemisia*. In the Flora of Pakistan, *Seriphidium* is described as a separate genus but in this study, *Seriphidium* fell within the genus *Artemisia*. In addition, on the basis of phylogenetic analysis, we present evidence that ten as-yet undescribed taxa are present in northeastern Pakistan based on newly recognized three groups (Groups I, II and III) of taxa within the genus *Artemisia*. One undescribed taxon from group I was placed within the subgenus *Dracunculus* clade and the remaining nine taxa from groups II and III were placed in the subgenus *Absinthium* clade. Morphological studies coupled with modern molecular techniques may lead to a new infrageneric classification of the genus *Artemisia.* It will also clarify and characterize the undescribed taxa reported in this study.

Keywords – *Artemisia*; Asteraceae; nrDNA; cpDNA; molecular phylogeny; undescribed taxa; Gilgit-Baltistan; Pakistan.

INTRODUCTION

The genus *Artemisia* (family Asteraceae; tribe Anthemideae) is a large taxonomically challenging group that includes ~500 species of both herbs and shrubs (Martin et al. 2003). Several species from this genus have a noteworthy economic status because they exhibit antispasmodic, antiseptic, antitumor antimicrobial, antimalarial, antirheumatic and hepato-protec-

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tive properties (Terra et al. 2007; Hussain et al. 2017). The genus is distributed primarily in the northern hemisphere's temperate zones; a few *Artemisia* species are also found in the southern hemisphere (Oberprieler et al. 2009). The centre of diversity for *Artemisia* is Central Asia. The earliest microfossils of the genus are known from the Miocene radiation (Wang 2004) and the Eocene end (Zaklinskaja 1957).

Since many years, the infrageneric classification of *Artemisia* has offered a challenge for researchers dealing with taxonomy. These historical studies were well acknowledged in the previous revelations of Torrell et al. (1999) and Vallès & McArthur (2001). From the studies of Tournefort (1700) to Bremer (1994) and Ghafoor (2002), all investigations regarding the classification and taxonomy of *Artemisia* were based on capitulum morphology. They documented four subgenera in the genus *Artemisia* (s. lat.) i.e. *Artemisia*, *Absinthium*, *Seriphidium* and *Dracunculus* as shown in table 1. During the course of this period, the position of *Seriphidium* as a separate genus or a subgenus of *Artemisia* (s. lat.) persisted and was a subject of discussion among taxonomists. For example, the generic recognition was implemented by Ling (1982), Bremer & Humphries (1993), Bremer (1994), Ling (1995) and Ghafoor (2002), whereas subgeneric status was followed by Kornkven et al. (1998, 1999), Torell et al. (1999), Watson et al. (2002) and D'Andrea et al. (2003).

Kornkven et al. (1998) provided a pioneering molecular phylogenetic study of *Artemisia* based on nrDNA internally transcribed spacer (ITS) with the aim of resolving its interspecific associations. In their study, they supported the North American origin of *Tridentatae*. They concluded that *Tridentatae* could be restricted as a monophyletic group with the omission of *A. palmeri* A.Gray and *A. bigelovii* A.Gray. Subsequently, Torrell et al. (1999) revealed the phylogeny of genus based on ITS sequences, in which they found support for five subgenera of *Artemisia*: *Artemisia*, *Seriphidium*, *Absinthium*, *Dracunculus* and *Tridentatae*. These results were additionally confirmed by Watson et al. (2002) and followed by numerous other molecular phylogenetic revisions. The detailed history of molecular phylogenetic efforts on the genus is provided in table 2. These works here surveyed proposed numerous taxonomic reorganizations for the subgeneric classification based on molecular data and related the latter's outcomes with the traditional morphology-based classifications (table 1). However, the infrageneric classification has not yet been entirely fixed. This is because of the inconsistent assignments of some taxa during phylogenetic examinations with respect to the classification based on morphology.

In the flora of Pakistan, Ghafoor (2002) treated *Artemisia* (s. lat.) by two separate genera, *Artemisia*, with 25 species, and *Seriphidium*, with 13 species. All these 38 species are recorded from the arid and semi-arid areas of Baluchistan, Khyber Pakhtunkhwa, North Punjab and the temperate areas of Gilgit-Baltistan and Kashmir territory (Ghafoor 2002). Within Pakistan, the centre of diversity for the genus is the western Himalayan region (Hayat et al. 2009).

Hayat (2011) initiated the phylogenetic study of Pakistani *Artemisia* using ITS and ETS sequences of nrDNA and found support for uniting the two genera. Malik et al. (2017) further confirmed this finding, treating *Seriphidium* as a subgenus of *Artemisia*. Mahmood et al. (2011) carried out a molecular phylogenetic study of *Artemisia* species collected from different localities of Pakistan based on restriction fragment length polymorphism of the chloroplast *rps*11 gene. They provided evidence that hybridization occurred at an infrageneric level during the evolutionary process, due to which the natural classification of the genus is still a challenging problem.

Here, we determine the phylogenetic position of ten undescribed *Artemisia* taxa from northern Pakistan, using nrDNA internal transcribed spacer (ITS), external transcribed spacer (ETS) and cpDNA intergenic spacer (*psbA-trnH*) regions.

MATERIALS AND METHODS

Study area

Gilgit-Baltistan is a northeastern region of Pakistan situated between 74°–77.5°E and 34.6°–37.4°N, covering an area of about 45 224 km^2. The altitude of this region ranges from ±1400 m to 8611 m. The area is divided into seven main districts, i.e. Gilgit, Skardu, Hunza-Nagar, Astore, Diamer, Ghizer and Ghanche. This region includes world-renowned mountain ranges like the Karakorum, Hindu Kush and the Himalayas. There are several peaks with heights above 7000 m, including Godwin Austin (K-2, 8611 m), Rakaposhi (7788 m) and Deran peak (7268 m). The world's largest glaciers are also found in this region, such as Baltoro Glacier, which extends for about 62 km with an area of 529 km^2 (Anonymous 2003). This area is well known for a great diversity of plants (Shinwari 2010) and is a centre for traditional medicinal herbs (Shinwari & Gilani 2003).

Plant collection and sampling

The plant samples employed for molecular phylogenetic analysis were taken from both herbarium specimens and silica gel dried samples collected during expeditions to various parts of Gilgit-Baltistan region of Pakistan as already given in our preceding paper (Hussain et al. 2019). Provenance of the different populations of *Artemisia* studied from Northern Pakistan, with their collection details are listed in table 3. Thus, covering all the Northeastern Pakistani endemic *Artemisia* taxa representing five subgenera of the genus *Artemisia*, including *Artemisia*, *Absinthium*, *Dracunculus*, *Pacifica* and *Seriphidium*, were included, except the North American endemic *A. tridentata* Nutt. of which we could not get the material.

Voucher specimens were deposited in the herbarium of Pakistan Museum of Natural History (PMNH) and the details are given in table 3. Earlier published ITS (Internal transcribed spacer), ETS (External transcribed spacer) of nrDNA and *psbA-trnH* (Intergenic spacer) of cpDNA sequences representing all subgenera of the genus *Artemisia* were retrieved from GenBank (supplementary file 1).

Nucleotide sequences for all the collected *Artemisia* species were newly determined for this study. *Chrysanthemum indicum* L., *Dendranthema mongolicum* (Y.Ling) Tzvelev and *Ajania fastagiata* (C.Winkl.) Poljakov were included as outgroups using their internal transcribed spacer (ITS), ex-

Table 1 – **Historical developments in the infrageneric classification of genus *Artemisia* based on floral morphology.**
Sources: Bremer (1994), Kornkven et al. (1999), Haghighi et al. (2014). * as "*Scriphidium*" in Besser (1829), and subsequently as "*Seriphidium*" (e.g. Besser 1834).

Rank	Infrageneric Taxa					Reference
Genera	*Absinthium*	*Abrotanum*	*Artemisia*			Tournefort (1700)
Genus	*Artemisia*					Linnaeus (1735)
Genera	*Artemisia*				*Oligosporus*	Cassini (1817), Lessing (1832)
Sections	*Absinthium*	*Abrotanum*	*Seriphidium**		*Dracunculus*	Besser (1829)
Sections	*Absinthium*	*Abrotanum*	*Seriphidium*		*Dracunculus*	de Candolle (1837)
Subgenera	*Euartemisia*		*Seriphidium*		*Euartemisia*	Rouy (1903)
Subgenera	*Absinthium*	*Abrotanum*	*Seriphidium*		*Dracunculus*	Rydberg (1916)
Sections			*Seriphidium*	*Tridentatae*		
Subgenera	*Artemisia*		*Seriphidium*		*Dracunculus*	Polyakov (1961)
Sections			*Seriphidium*	*Juceum*		
Subgenera	*Absinthium*	*Artemisia*	*Seriphidium*		*Dracunculus*	Persson (1974)
Sections	*Artemisia*				*Dracunculus*	Tutin (1976)
Subgenera	*Artemisia*		*Seriphidium*	*Tridentatae*	*Dracunculus*	McArthur et al. (1981)
Subgenera	*Artemisia*		*Seriphidium*		*Dracunculus*	Podlech (1986)
Genera	*Artemisia*			*Seriphidium*		Ling (1991)
Subgenera	*Absinthium*		*Dracunculus*			
Genera	*Artemisia*			*Seriphidium*		Bremer & Humphries (1993)
Genera	*Artemisia*			*Seriphidium*		Ghafoor (2002)

ternal transcribed spacer (ETS) and intergenic spacer (*psbA-trnH*) sequence records from GenBank (supplementary file 1).

Genomic DNA extraction and quantification

After the leaves were cleaned up with ethanol (70%), genomic DNA was extracted from dried leaves by using CTAB method (Doyle & Doyle 1990) and when necessary, the plant DNeasy kit (QIAGEN) was used. Quantification of extracted genomic DNA was done on the basis of measuring A260/280 using a ND-2000 spectrometer (Nanodrop Technologies, Wilmington DE USA) as given by Urreizti et al. (2012). The visual quality of extracted DNA was checked with 1.5% agarose gel electrophoresis.

PCR conditions for DNA amplification

PCR amplifications were performed in 50 µl reaction volumes containing: 36 µl ddH_2O, 5 µl 1xPCR buffer, 2 µl deoxyribonucleoside triphosphates (dNTPs), 1 µl of $MgCl_2$, 1.5 µl of forward and reverse primers for ITS (ITS9 and ITS6), ETS (ETS-AST1 and 18SETS) and chloroplast *psbA-trnH* (psbA3'f and trnHf) (table 4). 1–1.5 µl of 20–50 ng of template DNA, 1 µl DMSO, 0.5 µl of 5 units Taq polymerase (Thermo Scientific, Maxima Hot Start) and 21 µl deionized water in an ABI thermo-cycle.

PCR conditions for the amplification of nuclear ITS9-6 region were: pre-denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 1 minute or 55°C for 30 seconds, and extensions at 72°C for 1 minute, with final extension at 72°C for 5 minutes. PCR conditions for the amplification of nuclear ETS region were: pre-denaturation at 97°C for 2 minutes, followed by 36 cycles of denaturation at 97°C for 2 seconds, annealing at 55°C for 30 seconds, and extensions at 72°C for 30 seconds, with final extension at 72°C for 7 minutes. PCR conditions for chloroplast *psbA-trnH* region were: pre-denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1.5 minute, with the final extension at 72°C for 7 minutes. The electrophoresis of PCR products was carried out at 100 voltages for 45 min in a 1.5% agarose gel prepared in 1xTBE (Trisborate-ethylenediaminetetraacetic acid) buffer and finally checked under the trans-illuminator with ultra violet light. The size of PCR product was observed on the gel by means of 1kb DNA standard size markers (N-3232L, Biolabs Company). After visualizing its size, the gel extraction of PCR product was performed with QIAquick gel extraction kit (QIAGEN) following the standard protocol.

Table 2 – **Historical developments in the infrageneric classification of genus *Artemisia* based on molecular data.**

Rank	Infrageneric taxa								Markers	Reference
Subgenus	*Artemisia*		*Seriphidium*			*Dracunculus*			ITS	Kornkven et al. (1998)
Sections			*Seriphidium*	*Tridentatae*						
Subgenus	*Artemisia*		*Seriphidium*			*Dracunculus*			cpDNA	Kornkven et al. (1999)
Sections			*Seriphidium*	*Tridentatae*						
Groups	*Artemisia*	*Absinthium*	*Seriphidium*	*Tridentatae*		*Dracunculus*			ITS	Torrell et al. (1999)
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*			*Dracunculus*			ITS1, ITS2	Watson et al. (2002)
Sections				*Tridentatae*						
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*	*Tridentatae*		*Dracunculus*			ITS1, *trnL*-F	Vallès et al. (2008)
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*			*Dracunculus*			ITS, ETS	Sanz et al. (2008)
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*	*Tridentatae*		*Dracunculus*			ITS, ETS	Pellicer et al. (2010)
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*			*Dracunculus*			ITS, ETS, $trnS^{UGA}$, $trnfM^{CAU}$, $trnS^{GCU}$, trn_C^{GCA}	Garcia et al. (2011)
Sections			*Tridentatae*	*Nebulosae*	*Filifoliae*					
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*	*Tridentatae*		*Dracunculus*			ITS1, ITS2, *psbA–trnH*, *rpl32–trnL*, *ndhF–rpL32*, *trnT–trnL*, *rbcL–accD*, *ndhI–ndhG*, *trnV–ndhC*, *trnS–trnC*, *rps16–trnK*, *rpL16*, *trnS–trnfM*, *rpoB–trnE*, *trnC–ycf6*	Riggins & Seigler (2012)
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*	*Tridentatae*		*Dracunculus*	*Pacifica*		ITS1, ITS2, ETS, *trnL–trnF*, *psbA-trnH*	Hobbs & Baldwin (2013)
Sections				*Nebulosae*	*Filifoliae*					
Sections	*Artemisia*		*Seriphidium*			*Dracunculus*			ITS, *psbA-trnH*	Haghighi et al. (2014)
Subgenus	*Artemisia*	*Absinthium*	*Seriphidium*	*Tridentatae*		*Dracunculus*	*Pacifica*		ITS, ETS, *rpl*32-*trn*L, *ndh*C-*trn*V	Malik et al. (2017)

Nucleotide sequencing and alignment

The amplified DNA regions were sequenced in both directions in the core UC Davis sequencing facility using capillary electrophoresis genetic analysers (ABI 3730) with BigDye terminator version 3.1 cycle sequencing (ABI) from both strands, using the primer set ITS (ITS9 and ITS6), ETS (ETS-AST-1 and 18SETS) and *psbA-trnH* (psbA3'f and trnHf) (table 4). The raw sequenced data from studied taxa were assembled using BioEdit version 7.1.9 (Hall 1999) and Sequencher version 5.4.6 software (Gene codes Co.).

A total of four multiple sequence alignments (MSAs) generated from three markers for newly sequenced data of 28 *Artemisia* species from northern Pakistan with those of retrieved carefully from GenBank were nrDNA-ETS (n = 79) (supplementary file 2), nrDNA-ITS (n = 78) (supplementary file 3), and cpDNA-*psbA-trnH* (n = 65) (supplementary file 4). One multiple sequence alignment (MSA) was generated by concatenating these three markers with maximum species coverage but with missing data (CAT79; n = 79) (supplementary file 5). The details of MSAs generated are given below.

MSA1 = nrDNA-ETS (n = 79) (28 new sequences + 48 GenBank sequences + 3 Outgroup sequences)

MSA2 = nrDNA-ITS (n = 78) (27 new sequences + 48 GenBank sequences + 3 Outgroup sequences)

Table 3 – Collection details of *Artemisia* species from Gilgit-Baltistan region of Pakistan with latitude, longitude, location, voucher specimen and GenBank accession numbers of ITS, ETS and *psbA-trnH* markers.
The voucher numbers have been obtained from Pakistan Museum of Natural History (PMNH) Islamabad Pakistan. Collectors: Adil Hussain, Tanseer Hussain and Amar Abbas. * Rare *Artemisia* species; ** Undescribed taxa reported first time in this study from Northeast (Gilgit-Baltistan) region of Pakistan

***Artemisia* spp.**	**Latitude**	**Longitude**	**Location**	**Voucher specimen no**	**GenBank Accession Number**		
					ITS nrDNA	**ETS nrDNA**	***psbA-trnH* cpDNA**
A. annua L.	N-35′54.949	E-74′18.508	Barmas paen Gilgit	PMNH-41582	MH091335	MH257318	MH330156
A. arborescens (Vaill.) L.*	N-35′26.758	E-74′47.990	Hacho paen Astore	PMNH-41702	MH161334	MH292877	MH330157
A. argyi H.Lév. & Vaniot.*	N-35′54.951	E-74′18.503	Barmas paen Gilgit	PMNH-41583	MH091340	MH257319	MH330175
A. austriaca Jacq.*	N-36′01.609	E-74′33.255	Bagrote valley Gilgit	PMNH-41643	MH100692	MH292878	MH330170
A. biennis Willd.	N-36′09.387	E-74′11.941	Naltar valley Gilgit	PMNH-41622	MH161338	MH292883	MH330179
A. campestris L.	N-36′08.708	E-74′12.397	Naltar valley Gilgit	PMNH-41619	MH095575	MH292866	MH330162
A. chamaemelifolia Vill.*	N-36′09.622	E-74′11.622	Naltar valley Gilgit	PMNH-41630	MH100697	MH292867	MH330180
*A. chinensis**	N-35′26.585	E-75′27.011	Shangrilla Skardu	PMNH-41722	MH101881	MH292876	MH330169
A. gmelinii Weber ex Stech.	N-36′08.967	E-74′12.112	Naltar valley Gilgit	PMNH-41621	-----	MH292879	MH330163
A. herba-alba Asso.	N-35′54.061	E-74′12.762	Kargah nala Gilgit	PMNH-41599	MH113802	MH292882	MH330172
A. indica Willd.	N-36′15.250	E-73′24.240	Yasin Ghizer	PMNH-41694	MH100676	MH292873	MH330167
A. maritima L.	N-35′56.694	E-74′30.184	Bagrote valley Gilgit	PMNH-41639	MH161339	MH292863	MH330160
A. rutifolia Steph. ex Spreng.	N-36′08.708	E-74′12.397	Naltar valley Gilgit	PMNH-41618	MH092832	MH292865	MH330161
A. scoparia Waldst. & Kit.*	N-35′26.665	E-75′26.960	Kachura lake Skardu	PMNH-41714	MH100678	MH292875	MH330168
A. sieberi Bess.*	N-35′54.785	E-74′18.591	Barmas bala Gilgit	PMNH-41591	MH091348	MH292862	MH330159
A. tournefortiana Rachb.	N-35′25.493	E-75′44.507	Shigar valley Skardu	PMNH-41704	MH161337	MH292868	MH330173
A. verlotiorum Lamotte*	N-36′08.543	E-73′51.721	Bubar Ghizer	PMNH-41684	MH100668	MH292872	MH330166
A. vulgaris L.	N-36′20.508	E-74′52.277	Shishkat Hunza Nagar	PMNH-41646	MH107243	MH292876	MH330174
A. sp. AD-H**	N-35′55.133	E-74′18.487	Barmas paen Gilgit	PMNH-41586	MH094666	MH257320	MH330158
A. sp. A**	N-36′09.612	E-74′12.042	Naltar valley Gilgit	PMNH-41631	MH102419	MH292869	MH330164
A. sp. B**	N-36′09.122	E-74′12.045	Naltar valley Gilgit	PMNH-41632	MH104610	MH292870	MH330183
A. sp. C**	N-36′20.550	E-74′51.278	Gojal shishkat Hunza	PMNH-41649	MH102417	MH292871	MH330165
A. sp. D**	N-36′07.436	E-73′52.341	Thingdas Ghizer	PMNH-41680	MH168383	MH292886	MH330181
A. sp. E**	N-35′25.463	E-75′44.366	Shigar valley Skardu	PMNH-41707	MH102420	MH292880	MH330182
A. sp. F**	N-35′52.680	E-74′26.123	Minawar Gilgit	PMNH-41614	MH168384	MH292885	MH330176
A. sp. G**	N-35′16.062	E-75′38.045	Manthal Skardu	PMNH-41710	MH102418	MH292874	MH330177
A. sp. H**	N-35′26.764	E-74′47.998	Hacho paen Astore	PMNH-41700	MH102416	MH292881	MH330178
A. sp. I**	N-35′54.012	E-74′12.762	Kargah nala Gilgit	PMNH-41602	MH094656	MH292864	MH330171

Table 4 – **Set of primers utilized for amplifying ITS, ETS of nrDNA and *psbA-trnH* regions of cpDNA in *Artemisia* species.**

Primer	Sequence	Base length	Reference
Forward primer for ITS	ITS9: 5'-GGAAGGAGAAGTCGTAACAAGG-3'	22	Potter et al. (2007)
Reverse primer for ITS	ITS6: 5'-TCCTCCGCTTATTGATATGC-3'	20	Potter et al. (2007)
Forward primer for ETS	AST-1: 5'-CGTAAAGGTGCATGAGTGGTGT-3'	22	Markos & Baldwin (2001)
Reverse primer for ETS	18SETS: 5'ACTTACACATGCATGGCTTAATCT-3'	24	Baldwin & Markos (1998)
Forward primer for *psbA-trnH*	psbA3'f: 5'-GTTATGCATGAACGTAATGCTC-3'	22	Sang et al. (1997)
Reverse primer for *psbA-trnH*	TrnHf-05: CGCGCATGGTGGATTCACAATCC-3'	23	Tate & Simpson (2003)

Table 5 – **PCR amplified region length and summary statistics from the nrDNA (ITS & ETS) and the cpDNA (*psbA-trnH*) dataset of genus *Artemisia*.**
The numbers shown between brackets specify ingroup results.

Marker genes	ITS	ETS	*psbA-trnH*	ITS+ETS+*psbA-trnH*
Length	~700 bp	~500 bp	~450 bp	
No. of samples	78 (75)	79 (76)	65 (62)	79 (76)
No. of sites	657	397	396	1450
No. of informative sites	322(307)	137(129)	56(54)	515(490)

MSA3 = cpDNA-*psbA-trnH* (n = 65) (28 new sequences + 34 GenBank sequences + 3 Outgroup sequences)

MSA4 = nrDNA-ETS + nrDNA-ITS + cpDNA-*psbA-trnH* (n=79) (28 new sequences + 48 GenBank sequences + 3 Outgroup sequences)

These sequences were each aligned separately using MAFFT version 7.272 (Katoh & Standley 2013) (options: linsi) followed by manual adjustments.

Model selection and phylogenetic analysis

At first, *Artemisia* ITS, ETS and *psbA-trnH* sequences were examined independently with the aim of evaluating congruence among the markers. Then, the sequences from the three regions were aligned separately (ITS with 657 characters, ETS with 397 characters and *psbA-trnH* with 396 characters) and concatenated (Haghighi et al. 2014; Holzmeyer et al. 2015) in the final data matrix of 1450 characters (table 5). This concatenated nuclear ribosomal and chloroplast dataset was scrutinized with maximum likelihood, maximum parsimony algorithms and Bayesian inference analyses to check the taxonomic relationships within the genus *Artemisia*. The best base substitution models were determined for the MSAs of each individual marker (ETS, ITS, and *psbA-trnH*) and were used for phylogeny reconstruction with ML and Bayesian approaches. In all cases the best models were predicted using jModelTest version 2.1.7 (Darriba et al. 2012) (options: -f -g 4 -i -s 203 -S BEST -t ML). The best model was designated on the basis of Bayesian information criterion (BIC). The estimated model was then passed on to GARLI version 2.0.1 (Zwickl 2006) to generate a maximum likelihood tree. GARLI was executed under default conditions except for the following options (options: genthreshfortoposterm = 100000, significanttopochange = 0.00001, treerejectionthreshold = 50.0). Parameters values were estimated by GARLI. Four parallel searches have been performed to get rid of choosing a tree lodged on local optimum. Branches with length less than $1x10^{-8}$ substitution/site were collapsed. Bootstrap analysis was conducted with 1000 replicates. For the concatenated tree, region for each marker was partitioned and treated independently.

MrBayes version 3.2.1 software (Ronquist et al. 2012) was used for BI analyses for ITS, ETS and *psbA-trnH* substitution parameters estimated in different partitions for the pooled data. With four Metropolis Coupled Chains, two autonomous Markov Chain Monte Carlo (MCMC) analyses were run for 5 million generations, sampling every 100 groups (Malik et al. 2017). The best fitting DNA substitution model for BI analyses was nominated with Mr.Modeltest version 2.3 (Nylander 2004), GTR+I+G for the combined data set as well as for the individual cpDNA and nrDNA data sets was done. After the validation of average standard deviation of split frequencies to < 0.0, the first 25% trees were discarded as 'burn in' and 1.0 potential scale reduction factor was approached for all factors. The samples left were merged to construct a 50% majority rule consensus trees for posterior probabilities.

For the ETS, ITS, and *psbA-trnH* sequences, jModelTest predicted HKY+G, 012030+I+G with equal equilibrium base frequencies, and 012010+G as the best model respectively. For CAT79, the best model for the portions representing ETS, ITS, and *psbA-trnH* were HKY+I+G, 012010+G with equal equilibrium base frequencies, and 012010+G respectively.

ML and MP analysis were performed with MEGA-7 (Kumar et al. 2016) and RAxML-HPC version 8 (Stamatakis

2014). The final tree was checked using the software FigTree (2018) version 1.4.3. All the sequenced data of collected *Artemisia* species were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the obtained accession numbers are presented in table 3.

RESULTS

Data on the lengths of amplified DNA regions, raw sequences, MSAs and the numbers of informative characters for sequences of nuclear ribosomal (ITS and ETS) and chloroplast (*psbA-trnH*) DNA for all investigated samples of *Artemisia* are provided in table 5. All trees attained from independent ML, MP and Bayesian analyses of *psbA-trnH*, ITS and ETS regions recovered similar topologies with no significant conflicts. Some discordance involving clades with lesser support were observed, which could be taken as soft incongruences. When the data from three different markers were concatenated, the Bayesian, maximum likelihood and maximum parsimony approaches of the combined dataset exhibited slightly different phylogenetic reconstructions (supplementary file 6). Nevertheless, the ML and Bayesian tree provides greater resolution than the tree attained with MP. Only a consensus tree with BS and BI values from ML, MP and Bayesian tree is provided in fig. 1.

The inclusion of subgenus *Seriphidium* within the genus *Artemisia* is evident and strongly supported (PP = 1.00; ML-BS = 100%, MP-BS = 100%). In the resulting trees, maximum backbone nodes revealed better support (PP > 0.80; BS > 50%) except few lineages displayed poorly determined nodes.

The clades comprising all subgenera of the genus *Artemisia* including *Seriphidium* species were fully supported. All species of subgenus *Seriphidium* appeared in a single clade (PP = 1.00; ML-BS = 84%, MP-BS = 97) that includes annual species from subgenus *Artemisia* (PP = 1.00; ML-BS = 100%, MP-BS = 97%). The subgenus *Dracunculus* (PP = 1.00; ML-BS = 96%, MP-BS = 80%), subgenus *Pacifica* (PP = 1; ML-BS = 100%) and subgenus *Tridentatae* (PP = 1; ML-BS = 97%, MP-BS = 88%) species were placed in separate monophyletic groups. The analysis revealed a polyphyletic nature of subgenus *Artemisia* in the resulting ML tree (PP = 0.88; ML-BS > 88%) and the polyphyletic state of subgenus *Absinthium* is also evident (PP = 0.88; ML-BS > 88%).

We also observed ten new undescribed taxa of *Artemisia* from the Northeast (Gilgit-Baltistan) region of Pakistan. On the basis of our phylogenetic analysis, these undescribed taxa were categorized as new groups (Groups I, II & III). All clades which comprise undescribed taxa were also fully supported. One undescribed taxon (*Artemisia* sp. AD-H) (fig. 2) was placed in Group I. Four undescribed taxa of *Artemisia* (*Artemisia* sp. A, *Artemisia* sp. B, *Artemisia* sp. C and *Artemisia* sp. E) were placed in Group II (fig. 3) and five undescribed taxa (*Artemisia* sp. D, *Artemisia* sp. F, *Artemisia* sp. G, *Artemisia* sp. H and *Artemisia* sp. I) were positioned in Group III (fig. 4).

One undescribed taxon within Group I was found under the clade of subgenus *Dracunculus* with *Artemisia japonica* Thunb. and *A. desertorum* Spreng. (PP = 1.00; ML-BS = 83%, MP-BS = 100%). Four undescribed taxa from group II were found within subgenus *Absinthium* with *Artemisia rutifolia* Steph. ex Spreng. (PP = 1.00; ML-BS = 98%, MP-BS = 76%). Five undescribed taxa from group III were also found in the subgenus *Absinthium* with *Artemisia sieversiana* Ehrh. ex Willd. (PP = 1.00; ML-BS = 62%, MP-BS = 65%). The subgeneric classification of the genus is indicated by coloured symbols as shown in fig. 1.

DISCUSSION

The data presented in the ML tree (fig. 1) based on ITS, ETS and *psbA-trnH* marker genes shows the dispersion of northeastern Pakistani *Artemisia* throughout the clades corresponding to the subgenera. The tree indicated that all sampled species of genus *Artemisia* form a well-supported monophyletic group (PP = 1; ML-BS = 100%, MP-BS = 100%). From this study, some primary conclusions about the inclusion of *Seriphidium* within *Artemisia* genus and appearance of some undescribed taxa (Groups in fig. 1) can be made on the emerging pattern of the resultant phylogeny.

In the combined ITS, ETS and *psbA-trnH* phylogeny, two subgenera of genus *Artemisia* were not resolved as monophyletic. Subgenus *Absinthium* appeared as polyphyletic forming two major clades. One clade appeared separately (PP = 1; ML-BS = 70%, MP-BS = 60%), while the other clade appeared with species of subgenus *Artemisia* (PP = 1; ML-BS = 98%, MP-BS = 76%). Subgenus *Absinthium* is different morphologically from other subgenera due to the hairy receptacle.

Subgenus *Artemisia* was also not supported as monophyletic and appeared as polyphyletic with its species placed in four major clades corresponding to subgenera *Absinthium*, *Artemisia*, *Dracunculus* and *Seriphidium*. Morphologically, subgenus *Artemisia* is different from the other subgenera on the basis of plesiomorphies (heterogamous, disciform capitula with pistillate ray florets and fertile disk florets) and this subgenus needs to be recircumscribed. In previous findings, the two subgenera like *Absinthium* and *Artemisia* both were previously pooled as subgenus *Artemisia* (Gray 1984; Watson et al. 2002; Shultz 2009). But some studies based on molecular data separated them as distant subgenera. Apparently, in this study, these two formed a clade. So, it requires further investigation with more species to decide whether these two could be merged within a single subgenus *Artemisia* or not. However, in their study, Gray (1884) and Watson et al. (2002) united these two subgenera in a single subgenus *Artemisia*.

The taxonomic status of subgenus *Seriphidium* is unresolved; it has sometimes been treated as a separate genus (Ling 1982; Bremer 1994; Bremer & Humphries 1993; Ling 1995). The ITS, ETS and *psbA-trnH* phylogenies placed *Seriphidium* among the annual *Artemisia* species supporting its reunion within *Artemisia.* The reunion of *Seriphidium* with genus *Artemisia* is strongly supported (PP = 1; ML-BS = 84%, MP-BS = 97%) in complete agreement with previous studies (Kornkven et al. 1999; Torell et al. 1999; Watson et al. 2002; D'Andrea et al. 2003; Pellicer et al. 2010; Garcia et al. 2011; Hayat 2011; Riggins & Seigler 2012; Hobbs & Baldwin 2013; Malik et al. 2017) and is not in agreement

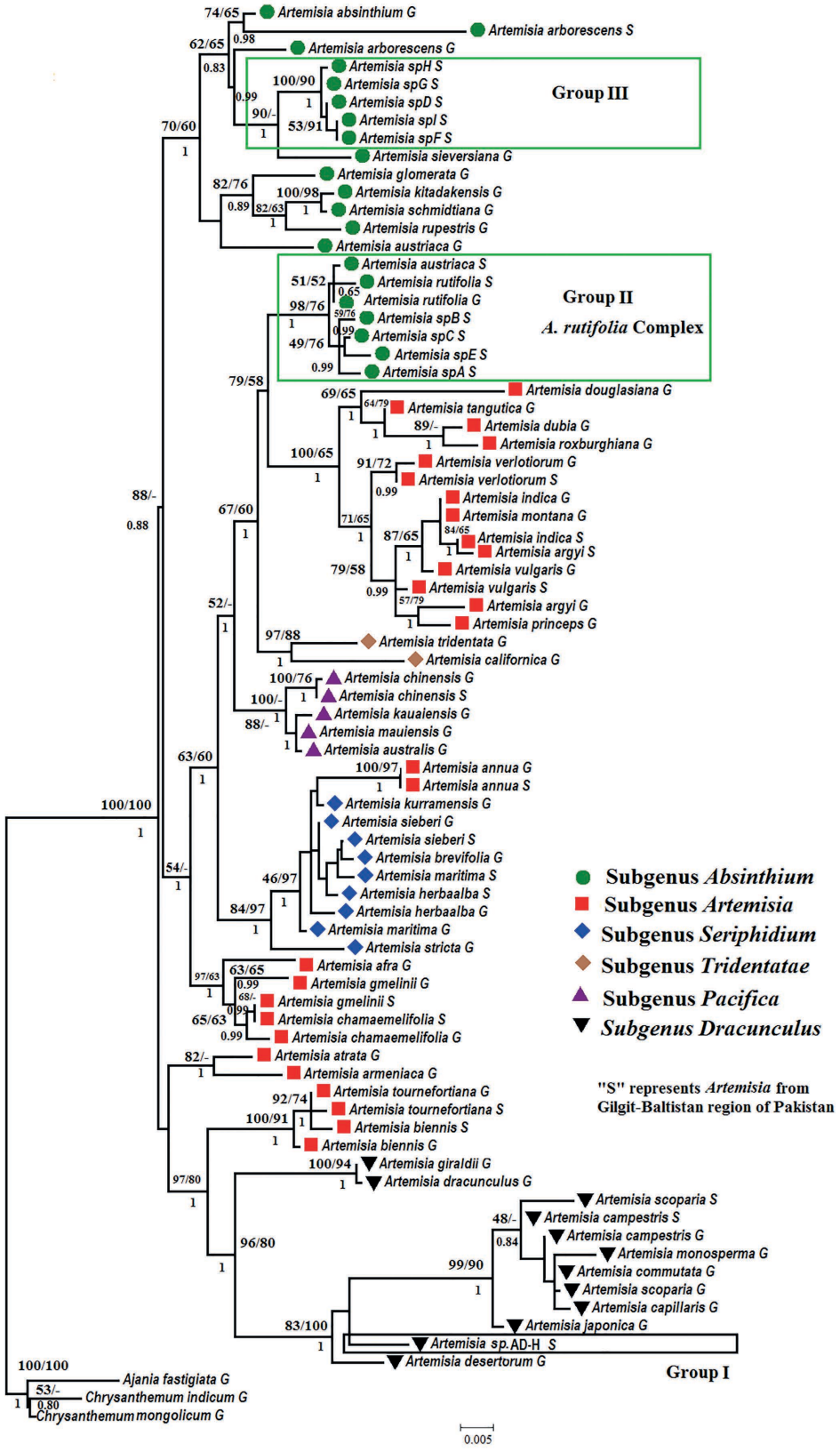

Figure 1 – Maximum likelihood (ML) consensus tree of combined ITS, ETS and *psbA-trnH* sequences of *Artemisia.* The values indicated above branches are the Bootstrap values (> 50%) obtained from ML and MP analysis with 1000 replicates. The values below branches indicate posterior probability (PP) values. The coloured shapes specify traditional subgeneric classification of the genus *Artemisia.* "S" represents the new sequences of corresponding species from the Gilgit-Baltistan region of Pakistan and "G" the ones from GenBank.

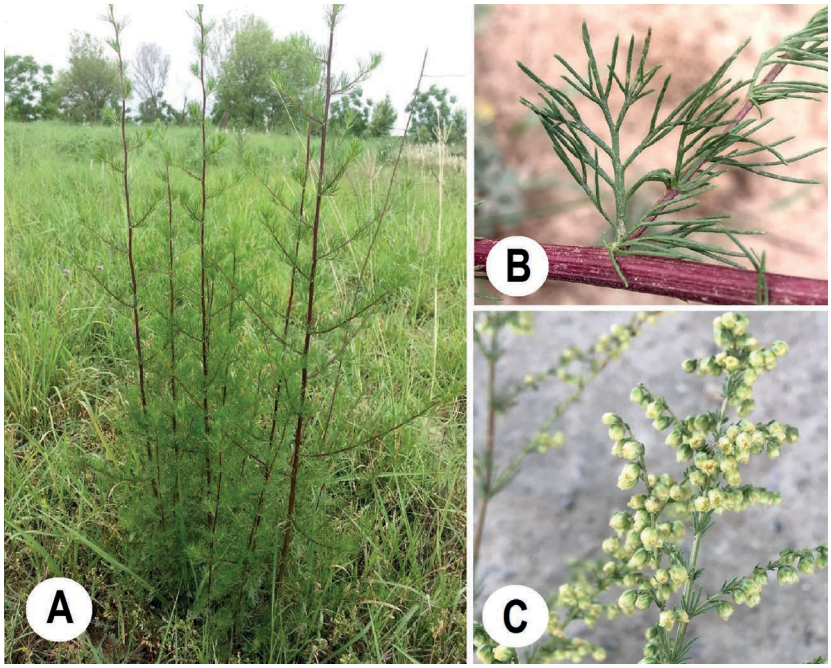

Figure 2 – Habit and synflorescence of an undescribed *Artemisia* taxon (*Artemisia* sp. AD-H) in group I. **A**. Plant. **B**. Leaves. **C**. Inflorescence.

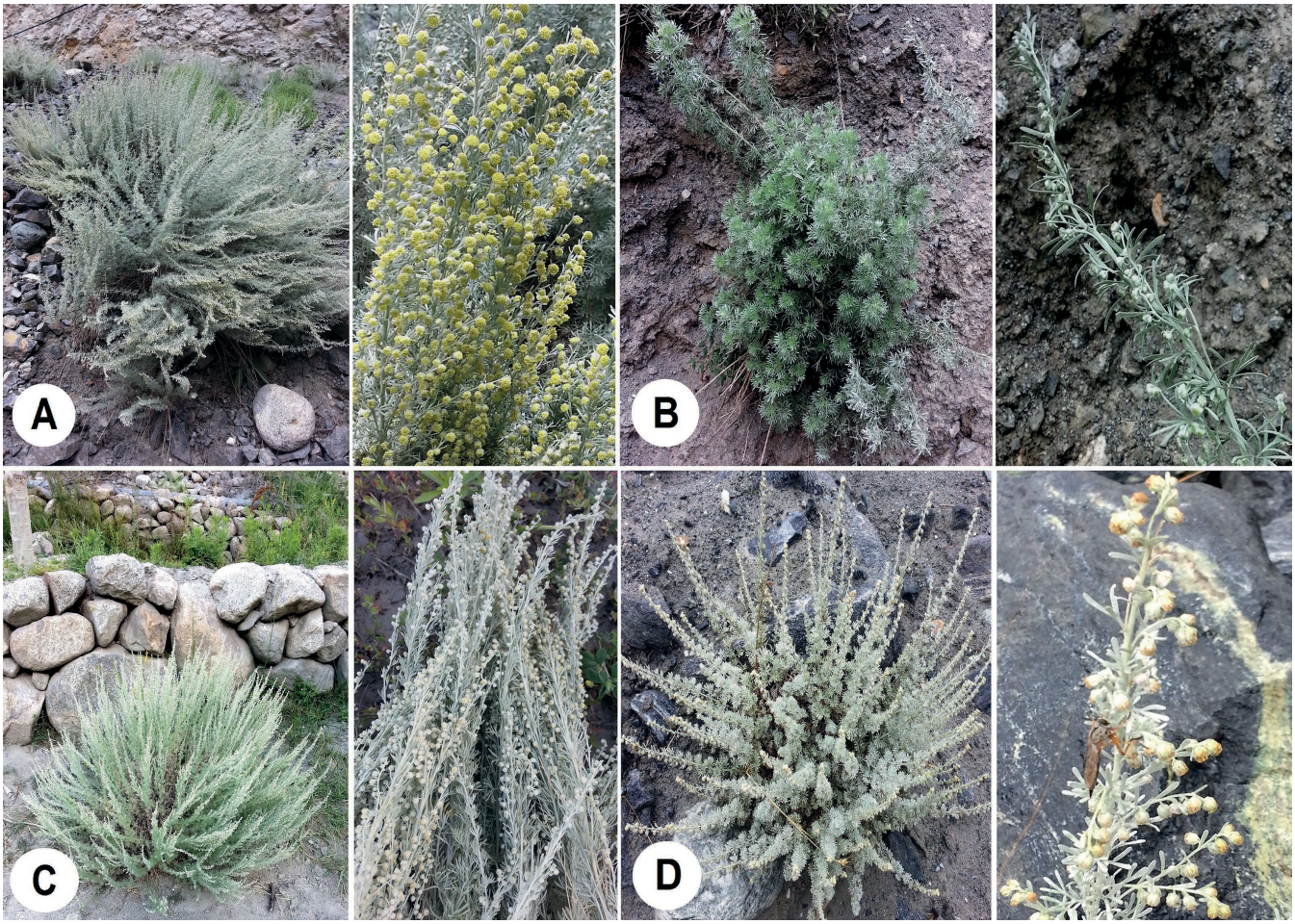

Figure 3 – Habit and synflorescence of undescribed taxa of *Artemisia* in group II. **A**. *Artemisia* sp. A. **B**. *Artemisia* sp. B. **C**. *Artemisia* sp. C. **D**. *Artemisia* sp. E.

with Ling (1982), Bremer (1994), Bremer & Humphries (1993), Ling (1995) and Haghighi et al. (2014). Our phylogenetic reconstruction, showed *Seriphidium* species forming a single clade with annual *Artemisia* species. Nevertheless, Malik et al. (2017) showed *Seriphidium* species in two clades suggesting that this subgenus is not monophyletic. They corroborated that one large monophyletic group corresponded to the formerly recognized subgenus *Seriphidium* and that a second small clade was phylogenetically distant. Morphologically, the subgenus *Seriphidium* is different from other subgenera by discoid homogamous capitula with bisexual disc florets and no ray florets.

Species from the subgenus *Dracunculus* formed a strongly supported clade (PP = 1; ML-BS = 96%, MP-BS = 80%) that is sister to a clade comprising two species of subgenus *Artemisia*, viz. *A. biennis* Willd. and *A. tournefortiana* Rchb. Watson et al. (2002) retained *Dracunculus* as a subgenus of the genus *Artemisia* but our study found subgenus *Dracun-*

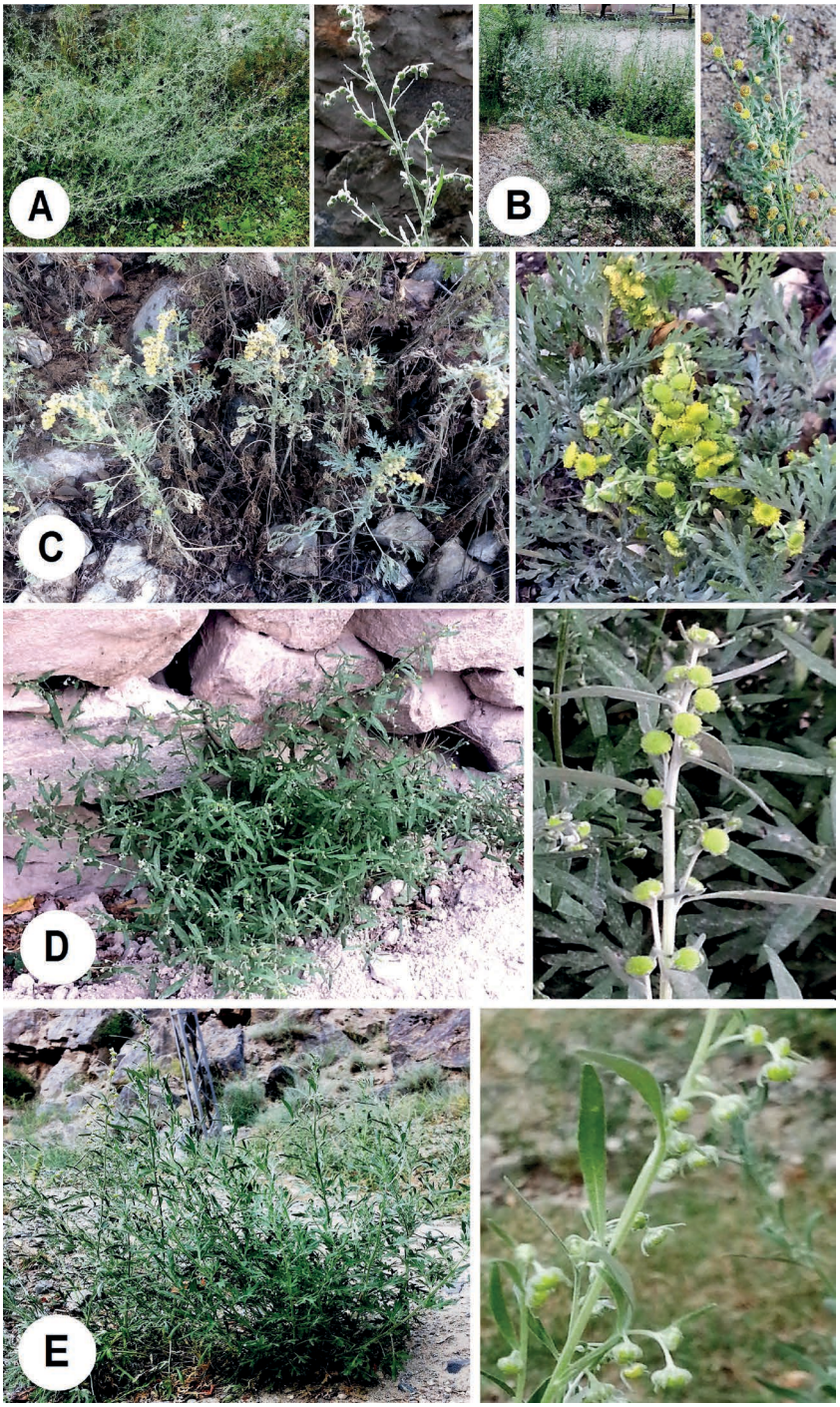

Figure 4 – Habit and synflorescence of undescribed taxa of *Artemisia* in group III. **A**. *Artemisia* sp. D. **B**. *Artemisia* sp. F. **C**. *Artemisia* sp. G. **D**. *Artemisia* sp. H. **E**. *Artemisia* sp. I.

culus forming sister clade with subgenus *Artemisia* groups. Morphologically, subgenus *Dracunculus* possesses heterogamous flower heads with pistillate outer florets and sterile inner florets.

The subgenus *Tridentatae* formed a monophyletic group with strong support (PP = 1; ML-BS = 97%, MP-BS = 88%) in the ML tree obtained from combined sequenced data of the three markers. But, the monophyly of subgenus *Tridentatae* was not consistent in the trees generated with separate sequenced data. The monophyly of subgenus *Tridentatae* is confirmed in many previous studies (Kornkven et al. 1998, 1999; Torrell et al. 1999; Vallès et al. 2008).

Species from subgenus *Pacifica* also formed a strongly supported monophyletic group (PP = 1; ML-BS = 100%); its monophyly is confirmed, in agreement with Hobbs & Baldwin (2013) and Malik et al. (2017) retaining it as a subgenus. More studies of the diverse and large genus *Artemisia* (s. lat.) are crucial for the further unravelling of the phylogeny of the genus.

Besides the infrageneric classification of *Artemisia*, our phylogenetic investigation observed and placed some undescribed taxa of *Artemisia* as three unique groups (Group I, II & III) from the Northeast (Gilgit-Baltistan) region of Pakistan (fig. 1).

One undescribed taxon (*Artemisia* sp. AD-H) (group I) appeared with high supporting values (PP = 1; ML-BS = 83 %, MP-BS = 100 %) within subgenus *Dracunculus.* Four undescribed taxa appeared as Group II with high supporting values (PP = 1; ML-BS = 98 %, MP-BS = 76 %) in the second clade of subgenus *Absinthium.* The undescribed taxa within Group II were placed with the *A. rutifolia* Steph. ex Spreng. lineage. This clade was therefore named "*A. rutifolia* complex". In the genus *Artemisia*, previous workers have already reported taxonomic complexes, for example the *A. vulgaris* complex, described in detail by Kaul & Bakshi (1984) and again reported by Sanz et al. (2008). A detailed morphological study of extensive sampling coupled with modern molecular techniques might resolve the taxa delimitation in the *A. rutifolia* complex, possibly leading to identification of new species.

In the first clade of subgenus *Absinthium*, five undescribed taxa were placed in Group III with strong PP support and moderate ML and MP support (PP = 1; ML-BS = 62%, MP-BS = 65%). If we compare a minimum branch length before the terminal node in a clade then it is clear that the five taxa are different from each other. This is because the branch lengths are too long in case of Group III. This is also the case for the sample observed as undescribed taxon in Group I.

The new groups of undescribed taxa of *Artemisia* shown in this study might represent putative new species. Koloren et al. (2016) observed two new haplotypes within *Artemisia* samples including both rare and common ones from the Ordu province of Turkey. In their resulting phylogenetic trees, the two haplotypes were placed with *A. argyi* H.Lév. & Vaniot, *A. sylvatica* Maxim., and *A. verlotiorum* Lamotte of subgenus *Artemisia.* Additionally, we agree with the conclusions made by Koloren et al. (2016) that the grouping of all new *Artemisia* haplotypes disjointedly from each other requires further multiple approach taxonomic examinations. Such inquiries must include an extensive number of samples in order to confirm and characterize potential new species or subspecies.

CONCLUSION

This study reports for the first time, molecular phylogeny of *Artemisia* from the northeastern region (Gilgit-Baltistan) of Pakistan using nrDNA (ITS and ETS) and cpDNA (*psbA-trnH*) sequences. The results confirmed polyphyletic appearance of subgenus *Artemisia* and *Absinthium.* Other subgenera including *Tridentatae*, *Pacifica* and *Dracunculus* were found to be monophyletic. Species of subgenus *Seriphidium* formed a single clade with annual species of subgenus *Artemisia*. The undescribed *Artemisia* taxa from Northeast region of Pakistan were placed in three groups within the resulting phylogenetic tree. One observed new group belongs to the subgenus *Dracunculus*, and the other two belongs to the subgenus *Absinthium*. Within these new groups, one undescribed taxon of *Artemisia* in group I was found with *A. japonica* and *A. desertorum* lineages. Four undescribed taxa within group II were designated with *A. rutifolia* lineage. Five undescribed taxa within group III were found in the same lineage with *A. sieversiana*. Based on the current data and all available in literature, it is concluded that the morphological studies coupled with modern molecular techniques may lead to the clear infrageneric classification of the genus *Artemisia.* It will also clarify and characterize the undescribed taxa reported in this study.

SUPPLEMENTARY FILES

Six supplementary files are associated to this paper:

(1) List of specimens included in the phylogenetic analysis with Genbank references (pdf)
https://doi.org/10.5091/plecevo.2019.1583.1901

(2) Multiple sequence alignment generated from ETS marker. nrDNA-ETS (n = 79) (Nexus file)
https://doi.org/10.5091/plecevo.2019.1583.1903

(3) Multiple sequence alignment generated from ITS marker. nrDNA-ITS (n = 78) (Nexus file)
https://doi.org/10.5091/plecevo.2019.1583.1905

(4) Multiple sequence alignment generated from psbA-trnH. cpDNA-*psbA-trnH* (n = 65) (Nexus file)
https://doi.org/10.5091/plecevo.2019.1583.1907

(5) Multiple sequence alignment generated by concatenating sequences of three markers. nrDNA-ETS + nrDNA-ITS + cpDNA-*psbA-trnH* (n = 79) (Nexus file)
https://doi.org/10.5091/plecevo.2019.1583.1909

(6) Phylogenetic trees based on ITS, ETS and *psbA-trnH* sequences of *Artemisia* with different methods. (pdf)
https://doi.org/10.5091/plecevo.2019.1583.1911

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