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

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**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. Subgenera *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-protective activity.
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), Torrell 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 inter-specific 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, Absinthium, Seriphidium, 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 infrageneric classifications 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 rps11 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 km². The altitude of this region ranges from ±1400 m to 8611 m. The area is divided into seven main districts, i.e. Gilgit, Hunza-Nagar, Astore, Diamer, Ghizer and Ganche. 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 km² (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-
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 ddH2O, 5 μl 1xPCR buffer, 2 μl deoxyribonucleoside triphosphates (dNTPs), 1 μl of MgCl2, 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 electrophoresis.

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PCR conditions for DNA amplification

PCR amplifications were performed in 50 μl reaction volumes containing: 36 μl ddH2O, 5 μl 1xPCR buffer, 2 μl deoxyribonucleoside triphosphates (dNTPs), 1 μl of MgCl2, 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 electrophoresis.

Table 1 – Historical developments in the infrageneric classification of genus *Artemisia* based on floral morphology.

| Rank          | Infrageneric Taxa          | Reference                  |
|---------------|----------------------------|----------------------------|
| Genera        | *Absinthium*               | Linnaeus (1735)            |
| Genera        | *Abrotanum*                |                            |
| Genera        | *Artemisia*                | Cassini (1817), Lessing (1832) |
| Sections      | *Absinthium*               | Dracunculus (Besser 1829)  |
| Sections      | *Abrotanum*                | Dracunculus (de Candolle 1837) |
| Sections      | *Seriphidium*              |                            |
| Sections      | *Euartermisia*             | Rouy (1903)                |
| Sections      | *Dracunculus*              |                            |
| Sections      | *Seriphidium*              |                            |
| Sections      | *Tridentatae*              |                            |
| Sections      | *Juceum*                   |                            |
| Sections      | *Seriphidium*              |                            |
| Sections      | *Dracunculus*              |                            |
| Genera        | *Artemisia*                | Persson (1974)             |
| Genera        | *Absinthium*               | Rydberg (1916)             |
| Genera        | *Abrotanum*                |                            |
| Genera        | *Seriphidium*              | Polyakov (1961)            |
| Genera        | *Dracunculus*              |                            |
| Genera        | *Seriphidium*              |                            |
| Genera        | *Tridentatae*              |                            |
| Genera        | *Juceum*                   |                            |
| Genera        | *Seriphidium*              |                            |
| Genera        | *Dracunculus*              | Tutin (1976)               |
| Genera        | *Seriphidium*              | Podlech (1986)             |
| Genera        | *Dracunculus*              |                            |
| Genera        | *Absinthium*               | Ling (1991)                |
| Genera        | *Abrotanum*                | Bremer & Humphries (1993)  |
| Genera        | *Seriphidium*              | Ghafoor (2002)             |
| Genera        | *Dracunculus*              |                            |
| Genera        | *Seriphidium*              |                            |

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 “Seriphidium” in Besser (1829), and subsequently as “Seriphidium” (e.g. Besser 1834).
Table 2 – Historical developments in the infrageneric classification of genus *Artemisia* based on molecular data.

| Rank          | Infrageneric taxa | Markers | Reference               |
|---------------|-------------------|---------|-------------------------|
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Seriphidium       | Tridentatae | ITS                      |
|               |                   |         | Kornkven et al. (1998)  |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Seriphidium       | Tridentatae | cpDNA                   |
|               |                   |         | Kornkven et al. (1999)  |
| Groups        | *Artemisia*       |         |                         |
|               | Absinthium        | Seriphidium | Tridentatae | Dracunculus | ITS             |
|               |                   |         | Torrell et al. (1999)  |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Seriphidium       | Tridentatae | ITS1, ITS2              |
|               |                   |         | Watson et al. (2002)    |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Tridentatae       |         |                         |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Seriphidium       | Tridentatae | Dracunculus | ITS, ETS, trn$^S$ UGA, trnF$^M$ GCU, trn$^S$ CAU, trn$^S$ GCU, trn$^C$ GCA | Garcia et al. (2011) |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Tridentatae       | Nebulosae | Filifoliae              |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Tridentatae       | Nebulosae | Filifoliae              |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Seriphidium       | Tridentatae | Dracunculus | Pacifica   |
|               |                   |         | ITIS1, ITIS2, ETS, trn$L$–trn$F$, psbA–trn$H$, rpl32–trn$L$, ndhf–rpl32, trn$T$–trn$L$, rbcL–accD, ndhF–ndhG, trn$F$–ndhC, trn$S$–trn$C$, rps16–trnK, rpl16, trn$S$–trn$M$, rpoB–trn$E$, trn$C$–ycf6 | Riggins & Seigler (2012) |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Seriphidium       | Tridentatae | Dracunculus | Pacifica   |
|               |                   |         | ITIS1, ITIS2, ETS, trn$L$–trn$F$, psbA–trn$H$ | Hobbs & Baldwin (2013) |
| Subgenus      | *Artemisia*       |         |                         |
| Sections      | Seriphidium       | Tridentatae | Dracunculus | Pacifica   |
|               |                   |         | ITIS1, ITIS2, ETS, trn$L$–trn$F$, psbA–trn$H$ | Haghighi et al. (2014) |

**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’t 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 |
|------------------|----------|-----------|----------|--------------------|-------------------------|
| 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-35’54.061 | E-74’12.112 | Naltar valley Gilgit | PMNH-41621 | ----- MH292879 MH330163 |
| A. herba-alba Asso. | N-36’08.967 | E-74’12.762 | Naltar valley Gilgit | PMNH-41618 | 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-41617 | 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 lake 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 Ghizer | PMNH-41614 | MH168384 MH292885 MH330176 |
| A. sp. G** | N-35’16.062 | E-75’38.045 | Manthal Ghizer | 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-35’34.012 | E-74’12.762 | Kargah na Gilgit | PMNH-41602 | MH094656 MH292864 MH330171 |
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 396 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: genthreshforto poterm = 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^-4 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 10000 trees. Posterior probabilities were approximated 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 approximated 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) for final bootstrap and bootstrap analysis respectively.
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 *Pacific* (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 = 63%). 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 capsule 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; Pelllicer 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-
Artemisia 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 Step. 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 subgenus 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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