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Full Length Article

Pattern of Diversity among Pistillate Scales of the Western Himalayan *Carex* spp. (Cyperaceae): Micromorphological and Molecular Inferences

Uzma[1,2,3], Betty Strack[2], Zahid Ullah[4], Andrew L. Hipp[2,3], Rabia Amir[1*] and Muhammad Qasim Hayat[1*]
[1]*Plant Systematics and Evolution Laboratory, Department of Plant Biotechnology, Atta-Ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Sector H-12, Islamabad, Pakistan*
[2]*The Field Museum, Chicago, USA*
[3]*The Morton Arboretum, Lisle, USA*
[4]*Center for Plant Sciences and Biodiversity, University of Swat, KP, Pakistan*
[*]For correspondence: rabi.amir@hotmail.com; mqasimhayat@hotmail.com

Abstract

Carex L., representing approximately 2,100 species, is one of the world's largest angiosperm genera and nearly cosmopolitan in distribution. However, limited studies are conducted on this genus in the Western Himalayas, from both a molecular and a morphologic perspective. In this study, *Carex* species were explored across the Western Himalayan range to identify *Carex* species previously unknown from the region and to contextualize collected plants in a large barcoding dataset generated by the Global *Carex* Group. Phylogenetic relationships among collected specimens were estimated using maximum likelihood (ML) analysis of three barcoding genes (the external and internal transcribed spacer regions of nuclear ribosomal DNA, ETS and ITS respectively, and the plastid *mat*K gene). Micromorphological traits including epidermal features of the pistillate scale were inspected using light and scanning electron microscopy. Eighteen *Carex* species were sampled from the Western Himalayan region of Pakistan. Among these, *Carex simpliciuscula* Wahlenb. (1803: 141), previously with doubtful record in the *Flora of Pakistan*, was confirmed for the region. Further, 12 range extensions for *Carex* species were identified in the Western Himalayas. Additionally, ten variable micro- and macromorphological characters of pistillate scale were evident that may serve useful for future taxonomic work and identification of the genus in the region; combinations of hooks, prickles, papillae and various trichomes under the scanning electron microscope. These results have great implications for understanding taxonomic and floristic scope of *Carex* species in the Western Himalayas region particularly in global climate change and its associated impacts.

Keywords: Western himalayas; *Carex*; Micromorphology; Molecular phylogeny; Pistillate scale; Scanning electron microscopy (SEM)

Introduction

Carex Linnaeus (*Cyperaceae* Juss.) is nearly cosmopolitan in distribution and the second vast angiosperm genus after *Astragalus* L. (Zarre and Azani, 2013), with *ca.* 2100 species (Reznicek, 1990; Starr *et al.*, 2009; Global *Carex* Group, 2016; Léveillé-Bourret *et al*., 2018). The genus has a center of distribution in the northern temperate region (Starr *et al*., 2009). The inflorescence of *Carex* (construed broadly, including the previously segregated genera *Kobresia* Willd., *Cymophyllus* Mack., *Uncinia* Pers., and *Schoenoxiphium* Nees; Global *Carex* Group, 2015) consists of unisexual flowers arranged in bisexual spikes (spikes unisexual in very few species). Each pistil is enclosed by a sac-like structure called the perigynium or utricle (Jiménez-Mejías *et al*., 2016), which is subtended by a glume or scale. (Hereinafter used perigynium and pistillate scale in lieu of utricle and glume). The pistillate scale exhibits numerous variable morphological characters that are useful for species-level identification, but likely not for higher-level classification. While a number of studies have characterized the morphology of the highly diverse grass floral scales (Brullo *et al*., 2009; Lizarazu *et al.*, 2011; Nobis, 2013; Nobis *et al.*, 2016; Ren *et al*., 2016; Kumar *et al.*, 2017), comparable studies are much rarer for the sedge pistillate scale.

In Pakistan, *Carex* is represented by 73 species, of which 11 are referred to the genus *Kobresia* (Kukkonen, 2001; Ullah *et al.*, 2013), the latter is now included in *Carex s.l.* (Global *Carex* Group, 2015; Table 1). The genus forms a dominant component of the alpine and northern temperate ecosystem across Pakistan (Ullah *et al.*, 2013). Despite this fact, *Carex* is poorly studied in the region due to difficulty of collection and the reduced morphology and challenging taxonomy of the genus (Ullah *et al.*, 2013). The present study contributes to our understanding of the Himalayan *Carex* flora by (1) adding new sampling areas

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Table 1: *Carex* names from the *Flora of Pakistan* (Kukkonen, 2001) updated to names utilized in Global *Carex* Group (2015, 2016)

Old treatment of *Carex* species mentioned in *Flora of Pakistan*	New treatment of *Carex* species provided by Global *Carex* Group
Kobresia laxa Nees	*Carex pseudolaxa* (C.B.Clarke) O. Yano & S. R. Zhang
Kobresia royleana (Nees) Boeckeler	*Carex kokanica* (Regel) S.R.Zhang
Kobresia humilis (C.A.Mey. ex Trautv.) Serg.	*Carex alatauensis* S.R.Zhang
Kobresia schoenoides (C.A.Mey.) Steud.	*Carex deasyi* (C.B.Clarke) O.Yano & S.R.Zhang
Kobresia capillifolia (Decne.) C.B.Clarke	*Carex capillifolia* (Decne.) S.R.Zhang
Kobresia nitens C.B.Clarke	*Carex ovoidispica* O.Yano
Kobresia duthiei C.B.Clarke	*Carex clavispica* S.R.Zhang
Kobresia pygmaea (C.B.Clarke) C.B.Clarke	*Carex parvula* O.Yano
Kobresia nepalensis (Nees) Kük.	*Carex unciniiformis* Boeckeler
Kobresia esenbeckii (Kunth) Noltie	*Carex esenbeckii* Kunth
Kobresia simpliciuscula (Wahlenb.) Mack.	*Carex simpliciuscula* Wahlenb.
Carex foliosa D.Don	*Carex wallichiana* Spreng.
Carex decaulescens V.I.Krecz.	*Carex popovii* V.I.Krecz.
Carex duthiei C.B.Clarke	*Carex gracilenta* Boott ex Boeckeler
Carex chitralensis Nelmes	*Carex koshewnikowii subsp. chitralensis* (Nelmes) Dickore
Carex royleana Nees	*Carex breviculmis var. breviculmis.*
Carex griersonii Noltie	*Carex rufulistolon* T.Koyama
Carex schlagintweitiana Boeckeler	*Carex setigera var. schlagintweitiana* (Boeckeler) Kük.
Carex lateralis Kük.	*Carex oxyphylla* Franch.
Carex serotina Mérat	*Carex oederi var. oederi*.

for *Carex* of the region, refining our understanding of species' distribution in the Himalayas and adjoining mountain ranges within Pakistan, (2) using molecular phylogenetic methods to compare samples from the region with a global sampling of the genus based on three barcoding genes (the external and internal transcribed spacer regions of nuclear ribosomal DNA, ETS and ITS respectively; and the plastid *mat*K gene) and (3) characterizing patterns of pistillate scale variation within and among major *Carex* clades. This work may thus have broader relevance to studies of *Carex* taxonomy as a reference for pistillate scale morphology in the genus.

Materials and Methods

Field Survey and Taxa Collection

Fifty three specimens of 18 *Carex* species were collected from different parts of Pakistan, particularly in the Western Himalayas, during the years 2011–2016 (Uzma *et al.*, unpublished). Plant specimens are deposited in the Pakistan Museum of Natural History, Islamabad, Pakistan and The Morton Arboretum, Lisle, Illinois, USA (Table 2).

Molecular Study

Genomic DNA was isolated from 20–30 mg of silica-dried leaf tissue from each specimen using DNeasy Plant Mini Kits (QIAGEN, Valencia, California, USA, catalog # 69106), following manufacturer's protocols, in the laboratory at The Morton Arboretum, Lisle, USA. The extracted DNA was used as a template for the amplification of ETS, ITS and *mat*K (following protocols in Global *Carex* Group, 2016), followed by amplicon purification using ExoSap and capillary sequencing on an ABI Prism 3730 in Pritzker Laboratory at The Field Museum, Chicago, USA (Uzma *et al.*, unpublished).

Sequences from the three regions were edited manually and aligned automatically in Geneious version 9.0.4 (Biomatters LTD, Aukland). Alignments were then concatenated after trimming ends (32, 37 and 78 characters including gaps, respectively from ITS, ETS and matK region alignments) to get equal number of molecular characters for every sequence. The datasets were constructed included all collected samples aligned with one exemplar per species retrieved from the Global *Carex* Group (2016) dataset (http://dx.doi.org/10.5061/dryad.k05qb), which was curated by a global group of researchers, to correlate our sequences with validated sequences by the global *Carex* community. Maximum likelihood phylogenetic analyses on combined dataset were conducted in RAxML HPC-PTHREADS-SSE3 version 8.2.4 (Stamatakis, 2006) using the general time reversible nucleotide substitution model with the CAT rate categories approximation (the GTRCAT model). Analysis was performed using 100 fast bootstrap (BS) searches, followed by full maximum likelihood search (the –f a search option in RAxML). *Trichophorum alpinum* and *T. caespitosum*, from tribe *Scirpeae*, were designated as outgroups following Léveillé-Bourret *et al.* (2014).

Micromorphological Study

Ten characters were analyzed, six under stereo microscope (scale color, scale shape, arista presence, hyaline margin, mid-rib color and mid-rib width), three under SEM (arista surface, mid-rib surface and scale surface) and one under compound light microscope (epidermal cell types). For morphometric study of the pistillate scales, one representative individual was selected from each of the 18 species collected. Plant material was taken from herbarium vouchers deposited at The Morton Arboretum (MOR).

Table 2: Collecting information of samples presented in this study

Carex Spp.	Sample collecting ID	*MOR Voucher	*PMNH voucher	Locality	New records of locality	Elevation (meter)	Coordinates	Mountain ranges	Plant Type	Sampling month & year
C. atrofusca	KJB-01	183078	42672	Khunjerab, Gilgit-Baltistan	+	4724	36° 50.919' N; 75° 25.682' E	Karakoram	Aquatic	Sept, 2015
C. atrofusca	KJB-05	183077	42677	Khunjerab, Gilgit-Baltistan	+	4724	36° 50.919' N; 75° 25.682' E	Karakoram	Aquatic	Sept, 2015
C. brunnea	BGH-02	183095	42664	Bagh, Kashmir	+	1446	34° 01.928' N; 73° 45.952' E	Himalayas	Terrestrial	Sept, 2015
C. brunnea	BGH-04	183093	42665	Bagh, Kashmir	+	1446	34° 01.928' N; 73° 45.952' E	Himalayas	Terrestrial	Sept., 2015
C. brunnea	HRG-01	183087	42657	Harighal, Kashmir	+	1719	33° 55.581' N; 73° 43.329' E	Himalayas	Terrestrial	Sept.,2015
C. brunnea	MUR-02	183089	42682	Ghora gali, Murree	-	1782	33° 52.813' N; 73° 20.901' E	Himalayas	Lithophyte	August2015
C. brunnea	RWT-09	183090	42695	Banjosa Lake, Rawalakot	+	1775	33° 48.602' N; 73° 48.325' E	Himalayas	Terrestrial	July, 2015
C. brunnea	RWT-11	183056	42660	Piona, Kashmir	+	1722	33° 51.328' N; 73° 43.521' E	Himalayas	Terrestrial	Sept.,2015
C. brunnea	RWT-12	183094	42655	Banjosa Lake, Rawalakot	+	1797	33° 48.690' N; 73° 49.779' E	Himalayas	Terrestrial	Sept.,2015
C. brunnea	RWT-14	183088	42661	Piona, Kashmir	+	1722	33° 51.328' N; 73° 43.521' E	Himalayas	Terrestrial	Sept.,2015
C. brunnea	RWT-15	183096	42662	Piona, Kashmir	+	1722	33° 51.328' N; 73° 43.521' E	Himalayas	Terrestrial	Sept.,2015
C. brunnea	RWT-19	183092	42658	Piona, Kashmir	+	1722	33° 51.328' N; 73° 43.521' E	Himalayas	Terrestrial	Sept.,2015
C. brunnea	RWT-20	183091	42656	Piona, Kashmir	+	1722	33° 51.328' N; 73° 43.521' E	Himalayas	Terrestrial	Sept.,2015
C. canescens	FMS-05	183104	42671	Fairy Meadows, Gilgit-Baltistan	+	3342	35° 22.380' N; 74° 34.706' E	Karakoram	Aquatic	Sept., 2015
C. canescens	FMS-08	183103	42674	Fairy Meadows, Gilgit-Baltistan	+	3312	35° 22.943' N; 74° 34.779' E	Karakoram	Aquatic	Sept., 2015
C. cardiolepis	SAD-07	183054	42694	Miandam Swat	-	2500	35°38.33' N, 72° 18.33' E	Hindu Kush	Terrestrial	July, 2012
C. filicina	BGH-01	183074	42663	Bagh, Kashmir	-	1446	34° 01.928' N; 73° 45.952' E	Himalayas	Terrestrial	Sept., 2015
C. filicina	RWT-08	183073	42696	Banjosa Lake, Rawalakot	-	1775	33° 48.602' N; 73° 48.325' E	Himalayas	Terrestrial	July, 2015
C. filicina	RWT-17	183065	42659	Banjosa Lake, Rawalakot	-	1797	33° 48.690' N; 73° 49.779' E	Himalayas	Terrestrial	Sept., 2015
C. fedia	SAD-01	183064	xxx	Matta Swat	+	1200	35°38.33' N, 72° 18.33' E	Himalayas	Terrestrial	April, 2014
C. infuscata	FMS-06	183057	42675	Fairy Meadows, Gilgit-Baltistan	+	3342	35° 22.380' N; 74° 34.706' E	Karakoram	Aquatic	Sept., 2015
C. infuscata	FMS-07	183079	42673	Fairy Meadows, Gilgit-Baltistan	+	3312	35° 22.943' N; 74° 34.779' E	Karakoram	Aquatic	Sept., 2015
C. infuscata	SAD-05	183080	42693	Naran	-	2543	35°38.33' N, 72° 18.33' E	Himalayas	Terrestrial	July, 2011
C. infuscata	SAD-10	183058	42692	Saiful Malook, Naran	-	2543	34° 54.460' N; 73° 39.727' E	Himalayas	Terrestrial	July, 2011
C. nubigena	KSH-07	183082	42687	Arang Kel, Kashmir	+	2380	34° 48.419' N; 74° 20.955' E	Himalayas	Aquatic	June, 2015
C. nubigena	TLP-01	183085	42667	Tolipeer, Kashmir	+	2600	33° 53.430' N; 73° 54.613' E	Himalayas	Terrestrial	Sept., 2015
C. nubigena	TLP-03	183086	42669	Tolipeer, Kashmir	+	2600	33° 53.430' N; 73° 54.613' E	Himalayas	Terrestrial	Sept., 2015
C. nubigena	TLP-04	183081	42668	Tolipeer, Kashmir	+	2600	33° 53.430' N; 73° 54.613' E	Himalayas	Terrestrial	Sept., 2015
C. nubigena	TLP-05	183084	42666	Tolipeer, Kashmir	+	2600	33° 53.430' N; 73° 54.613' E	Himalayas	Terrestrial	Sept., 2015
C. nubigena	TLP-06	183083	42670	Tolipeer, Kashmir	+	2600	33° 53.430' N; 73° 54.613' E	Himalayas	Terrestrial	Sept., 2015
C. pamirica	SAD-06	183076	42691	Deosai, Skardu	-	2,226	35°18.00′N; 75° 37.00′ E	Himalayas	Terrestrial	June, 2011
C. pseudofoetida	KJB-04	183071	42678	Khunjerab, Gilgit-Baltistan	-	4724	36° 50.919' N; 75° 25.682' E	Karakoram	Aquatic	Sept., 2015
C. pseudofoetida	KJB-06	183072	42676	Khunjerab, Gilgit-Baltistan	-	4724	36° 50.919' N; 75° 25.682' E	Karakoram	Aquatic	Sept., 2015
C. pseudolaxa	KGN-01	183109	42653	Saif ul maluk, Naran	-	2543	34° 54.460' N; 73° 39.727' E	Himalayas	Aquatic	Sept.,2015
C. pseudolaxa	KGN-02	183107	42654	Saif ul maluk, Naran	-	2543	34° 54.460' N; 73° 39.727' E	Himalayas	Aquatic	Sept.,2015
C. pseudolaxa	KSH-01	183106	42688	Taobat, Kashmir	-	2273	34° 43.589' N; 74° 42.759' E	Himalayas	Aquatic	June, 2015
C. pseudolaxa	KSH-04	183108	42689	Taobat, Kashmir	-	2263	34° 43.610' N; 74° 43.099' E	Himalayas	Aquatic	June, 2015
C. pseudolaxa	RKP-01	183112	42679	Rakaposhi, Gilgit-Baltistan	+	2295	36° 14.370' N; 74° 29.308' E	Karakoram	Aquatic	Sept., 2015
C. pseudolaxa	RKP-04	183111	42681	Rakaposhi, Gilgit-Baltistan	+	2295	36° 14.370' N; 74° 29.308' E	Karakoram	Aquatic	Sept., 2015
C. pseudolaxa	RKP-05	183110	42680	Rakaposhi, Gilgit-Baltistan	+	2295	36° 14.370' N; 74° 29.308' E	Karakoram	Aquatic	Sept., 2015
C. pseudolaxa	SAD-04	183105	42690	Deosai, Skardu	-	2,226	35°18.00′ N; 75° 37.00′ E	Himalayas	Terrestrial	June,2011
C. psychrophila	SAD-12	183055	42685	Miandam Swat	+	2400	35°38.33' N, 72° 18.33' E	Hindu Kush	Terrestrial	May, 2013
C. sanguinea	SAD-08	183075	42686	Miandam Swat	-	2000	35°38.33' N, 72° 18.33' E	Hindu Kush	Terrestrial	May, 2011
Carex schlagintweitiana	SAD-11	183068	42683	Malamjaba Swat	+	2500	35°38.33' N, 72° 18.33' E	Hindu Kush	Terrestrial	April, 2011
C. wallichiana	RWT-03	183066	xxx	Banjosa Lake, Rawalakot	-	1775	33° 48.602' N; 73° 48.325' E	Himalayas	Terrestrial	July, 2015
C. wallichiana	SAD-14	183067	42684	Miandam Swat	-	2400	35°38.33' N, 72° 18.33' E	Hindu Kush	Terrestrial	May, 2013
C. dimorpholepis	8	184272	xxx	Miandam Swat	-	2400	35.0541° N, 72.5648° E	Hindu Kush	Terrestrial	May, 2013
C. brunnea	D-2	184278	xxx	Dunga Gali, Galyat	-	2,500	34.0528° N, 73.4070° E	Himalayas	Lithophyte	April, 2016
C. diandra	4	184275	xxx	Miandam Swat	+	2400	35.0541° N, 72.5648° E	Hindu Kush	Terrestrial	June, 2015
C. schlagintweitiana	Shogran-8	184274; 184273	xxx	Shogran, Kaghan	-	2,362	34.6398° N, 73.4607° E	Himalayas	Lithophyte	April, 2016
C. cardiolepis	D-4	184271	xxx	Dunga Gali, Galyat	+	2,500	34.0528° N, 73.4070° E	Himalayas	Lithophyte	April, 2016
C. simpliciuscula	KJB-02	183069	xxx	Khunjerab, Gilgit-Baltistan	+	4724	36° 50.919' N; 75° 25.682' E	Karakoram	Aquatic	Sept., 2015
C. simpliciuscula	KJB-03	183070	xxx	Khunjerab, Gilgit-Baltistan	+	4724	36° 50.919' N; 75° 25.682' E	Karakoram	Aquatic	Sept., 2015

*MOR = Morton Arboretum, Lisle, USA

*PMNH= Pakistan Museum of Natural History, Islamabad, Pakistan

*+ New collection site

*- Already reported collection site

Characters were observed and measured using a Nikon SMZ-745T stereo microscope using a ruler and ocular micrometer. The same individual per species was also inspected under SEM. Dry pistillate scales were mounted on aluminum stubs using double-sided copper tape (12–7 mm), coated with gold using an Denton Vacuum Desk IV Sputter Coater and imaged at high vacuum and high voltage (20 KVa) using a Carl Zeiss EVO 60 Scanning Electron Microscope at The Field Museum, Chicago, USA. Epidermal cells were inspected using a compound light microscope (OPTIKA B-150 at 100x objective lens) at National University of Sciences and Technology, Islamabad. Specimens were mounted using Canada balsam solution in a drop for staining specimens and observed slide under light microscope using an oil immersion. The terminologies for microscopic characters studied under SEM were followed the studies by (Gaglioti *et al.*, 2010; Nobis, 2013; Nobis *et al.*, 2016), which were focused on morphometric study of leaves from Poaceae and Cyperaceae, and lemma (scale-like structure) of species from Poaceae.

Results

New Flora and Distribution of Taxa

One species collected, *Carex simpliciuscula* Wahlenb. (1803: 141), which was previously treated as doubtful in the *Flora of Pakistan* (Kukkonen, 2001), was confirmed from the Karakoram range (KJB-02 and KJB-03). The species was identified using Flora of North America (Ball and Reznicek, 2002) and confirmed using molecular data (Fig. 1), where it falls sister to *C. pseudolaxa* and strongly aligned with another sequence of *C. simpliciuscula* from Tibet, China (100% BS; Fig. 1). Flora of China (Dai *et al.*, 2010) also lists this species. *C. simpliciuscula* is illustrated in Fig. 2 as a guide to future searches for the species.

Out of 18 collected species, 12 were found from collection sites not previously mentioned in Flora of Pakistan (Fig. 3 and Table 2). *C. atrofusca* and *C. pseudolaxa* were previously known from the Himalayan range: our collections extend their distribution to the Karakoram range. Similarly, *C. diandra, C. schlagintweitiana, C. psychrophila*, previously known from the Himalayan range, were based on our collections also known from the Hindu Kush range. *C. nubigena* was also collected from Tolipeer and Arang Kel (Azad Kashmir) at slightly higher altitude (2600 m) than previously reported (PatniTop, Jammu Kashmir). These findings highlight the connectivity between the floras of the Himalayan, Karakoram and Hindu Kush ranges.

Molecular and Micromorphological Inferences

The molecular datasets for ETS, ITS and *mat*K were represented by 62, 59 and 56 sequences respectively (including outgroup), with alignment length of 590, 667 and 522 sites after trimming ends (Supplementary files S1, S2 and S3). The concatenated matrix comprised 62 sequences of 1779 aligned sites (Supplementary file S4). The ML analysis is strongly resolved (Fig. 1) and demonstrates that the Western Himalayan species fall into three strongly supported clades; i) Vignea, ii) Unispicate and iii) core Carex. This result is congruent with previous findings (Global *Carex* Group, 2016). As previously demonstrated (Global *Carex* Group, 2015), all species within the Vignea clade have traditionally been classified in subgenus *Vignea*, while species of the Unispicate and core Carex clades have traditionally included members of former genus *Kobresia* (along with other genera in other regions of the world) and subgenera *Vigneastra* and *Carex*. This updates the only previous work on the phylogeny of Pakistani *Carex* species (Ullah *et al.*, 2013), which was based on one genomic marker (*mat*K).

Ovate scales are most common in the flora while oblong and lanceolate scales are less common (Fig. 4). The mid-rib is smooth in all taxa except six (*C. brunnea, C. cardiolepis, C. pseudolaxa, C. sanguinea, C. schlagintweitiana, C. wallichiana*), which are barbed

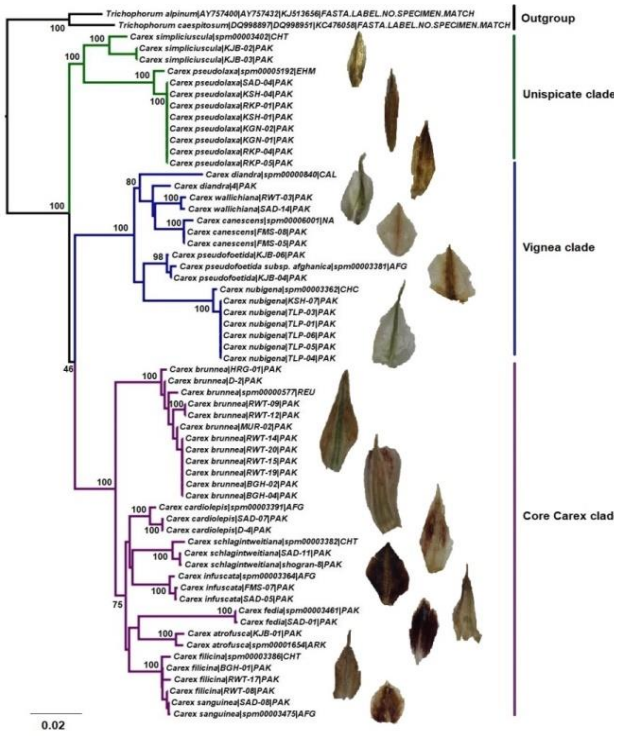

Fig. 1: Phylogenetic relationships of 18 Western Himalayan *Carex* species based on ML analysis of ITS, ETS and *mat*K with Global *Carex* Group dataset (2016). Numbers above branches are bootstrap values based on 100 fast bootstrap replicates. Tip labels are in the form species|collector number or specimen number|country of collection (coded according to three-letter TDWG geographical code). Each tip also represent respective species's pistillate scale (collected from the Western Himalayas), which appear to show variation among species and within clades

along the mid-rib. Most species have hyaline margins. Electron microscopy of upper surface of pistillate scale revealed prickles, hooks, trichomes and papillae (Gaglioti *et al.*, 2010; Nobis, 2013; Nobis *et al.*, 2016) in various combinations on all studied species while some species show only smooth surface (Fig. 5k) without any characters on scale surface such as *C. diandra, C. infuscata, C. pseudofoetida* and *C. simpliciuscula*. Pistillate scale surface patterns based on SEM-studied characters are: 1) prickles (*e.g., C. sanguinea, C. fedia*), 2) hooks (*e.g., C. pamirica*), 3) trichome (*e.g., C. canescens*), 4) hooks and prickles (*e.g., C. wallichiana, C. brunnea*), 5) papillae and trichomes (*e.g., C. atrofusca*), 6) hook and trichomes (*e.g., C. nubigena*), 7) papillae and prickles (*e.g., C. dimorpholepis*), 8) prickles, hooks and papillae (*e.g., C. cardiolepis*), 9) hooks, prickles and trichomes (*e.g., C. pseudolaxa*), 10) papillae, hooks, prickles and trichomes (*e.g., C. schlagintweitiana, C. psychrophila, C. filicina*) (Fig. 5a–j). However, variation in surface morphology viewed using SEM contrasts with the relative homogeneity of epidermal cell shape across species. Epidermal cells were mainly rectangular with sinuous outlines (*e.g.*, Menapace, 1991; Jin *et al.*, 2012) except in *C.*

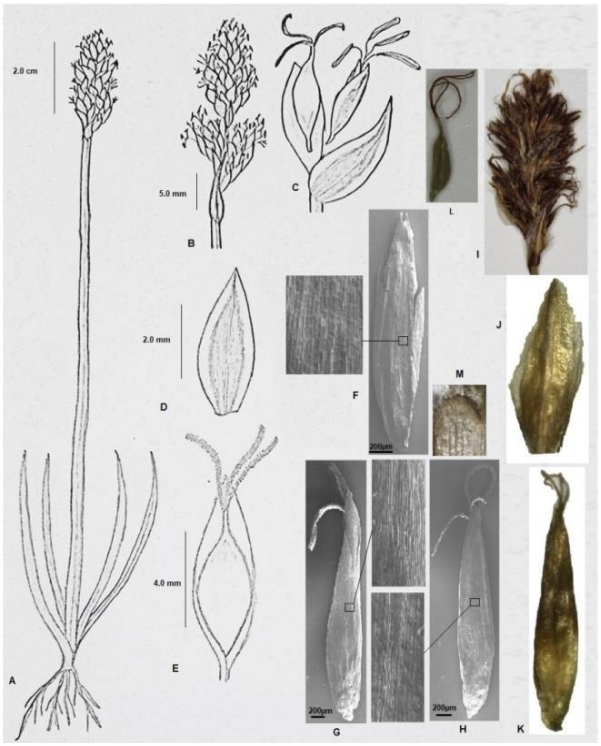

Fig. 2: Illustration of newly explore flora, *Carex simpliciuscula* Wahlenb in detail; A: complete plant of 20-25 cm with 2 cm long inflorescence and basal leaves, B: inflorescence with lowest bract of 5 mm in length which has brown color and hyaline margin and ovate-oblong in shape, C: female spikelet with 3-4 stigmas and male spikelet with 1-2 mm long anthers, D: scale 2 mm in length, E: prophyll with nutlet 4 mm long, F: scale or scale with upper surface smooth under SEM, G: adaxial face of prophyll with nutlet, H: abaxial face of prophyll with nutlet, I: compound inflorescence, brown in color and ovate-oblong in shape, J: scale or scale in brown color with hyaline margin and ovate in shape, K: prophyll with nutlet, oblong in shape, brown in color and smooth, L: nutlet 2-3 mm long, M: leaf ligule with round arch. F, G, H under SEM while I, J, K, L, M under stereo microscope

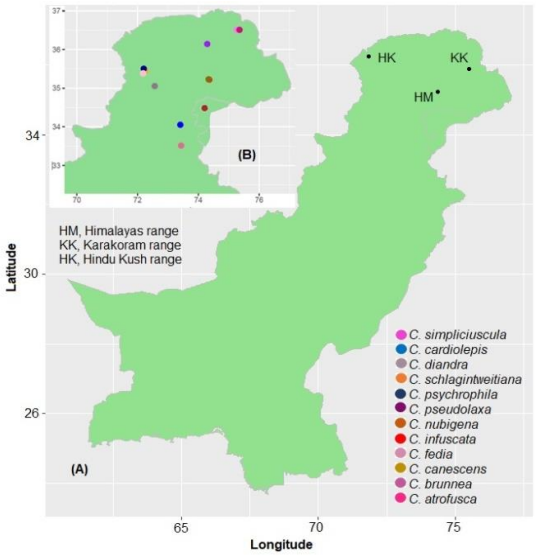

Fig. 3: The map of study (A): presenting collection sites covering three ranges: Himalayas (HM), Karakoram (KK) and Hindu Kush (HK), (B): shows the new sites of collection for the *Carex* species designated with different colors on the map (horizontal axis shows longitude while vertical axis displays latitude), which are not reported previously in the *Flora of Pakistan* (Kukkonen, 2001)

wallichiana, *C. schlagintweitiana*, *C. infuscata*, *C. fedia*, *C. dimorpholepis*, *C. diandra* and *C. atrofusca*, in which cells are rectangular but without sinuous outline (Fig. 6). Despite the lack of intraspecific sampling and the fact that sampled only 18 of the estimated 73 species of the Western Himalayas, this study suggests that different species generally do not share the exact same combination of character states for the ten studied characters, pointing to the potential utility of the scale in identifying species in the region.

Discussion

The majority of morphological studies in *Cyperaceae* (*e.g.*, Menapace, 1991; Vrijdaghs *et al.*, 2006; Larridon *et al.*, 2011; Shalabi and Gazer, 2015; Semmouri *et al.*, 2018) and *Carex* in particular (*e.g.*, Wujek and Menapace, 1986; Saarela and Ford, 2001; Zhang, 2004; Jin *et al.*, 2014; Molina *et al.*, 2015; Hoffmann and Gebauer, 2016; Jiménez-Mejías *et al.*, 2017) have focused on the perigynium, achene and/or leaf morphology. The present study thus contributes a focus on the pistillate scale as a potential source of characters for identifying *Carex*. All of the pistillate scales of Western Himalayan species appear to show variations that may be useful for distinguishing species, though given our sampling, this inference is suggestive rather than conclusive. Perhaps as striking as the variation among species is the variation within clades, for none of the characters inspected demonstrated strong morphological coherence within major clades (Fig. 1). In the Vignea clade, two species *C. nubigena* and *C. wallichiana* exhibit very long arista at the apex and looked superficially similar under stereo microscope compared to other three species sampled from the clade; however, these differed in other characters such as scale, arista and mid-rib surfaces and epidermal cell type, studied under SEM and compound microscope. In the Unispicate clade, the two species sampled, *C. pseudolaxa* and *C. simpliciuscula*, resemble each other in all noted characters except in scale shape, surface and mid-rib appearance. On the other hand, in the core Carex clade, high variation observed in almost all characters (Table 3). This is in keeping with the high species and ecological diversity of core Carex (Waterway *et al.*, 2009; Global *Carex* Group, 2016; Waterway *et al.*, 2016).

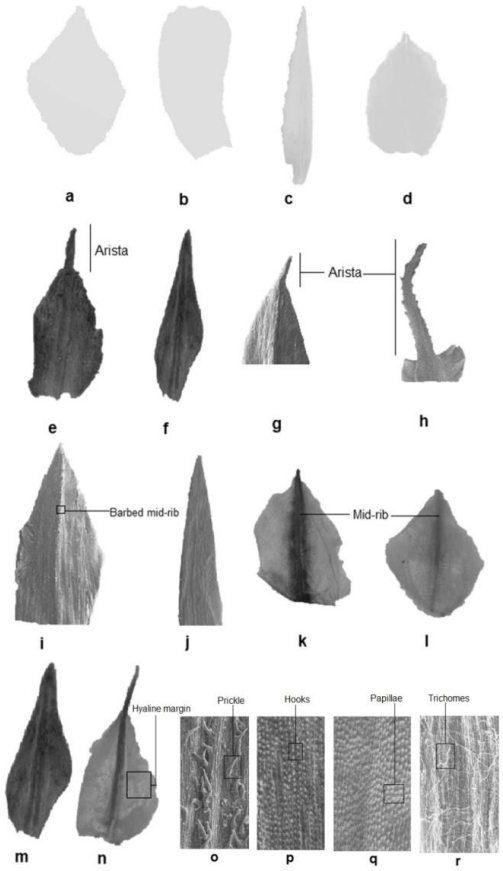

Fig. 4: Characters of pistillate scale listed in Table 1. a) ovate, b) oblong, c) lanceolate, d) cymbiform, e) scale with arista, f) scale without arista, g) smooth arista, h) barbed arista, i) scale with barbed mid-rib, j) scale with smooth mid-rib, k) broaden mid-rib, l) narrow mid-rib, m) scale without hyaline surface and n) scale with hyaline, o) prickles, p) hooks, q) papillae, r) trichomes. o, p, q, r on pistillate scale surface under SEM (scale in 100 μm)

Ten overarching surface patterns were identified among the species inspected that were distinguishable from one another using SEM (Fig. 5), excluding four species—*C. simpliciuscula*, *C. diandra*, *C. pseudofoetida* and *C. infuscata*—that exhibited a smooth surface without any trichomes, prickles or papillae. Some species show only one character on their scale surface; *C. pamirica*, for example, exhibits hooks evenly over the scale surface, *C. canescens* has trichome over scale surface, while scales of *C. sanguinea* and *C. fedia* are covered with prickles (Fig. 5). The remaining species demonstrated a mixture of papillae, trichomes, hooks and prickles overall in different combinations (Table 3). However, none of the clades follow a particular pattern of character combinations on pistillate scale. Perhaps not surprisingly, the cell type under light microscope resembled leaf epidermal cells of *Carex* species (Jin *et al.*, 2012). This scale surface pattern will provide an important source of taxonomic characters for species identification in the genus, with additional study.

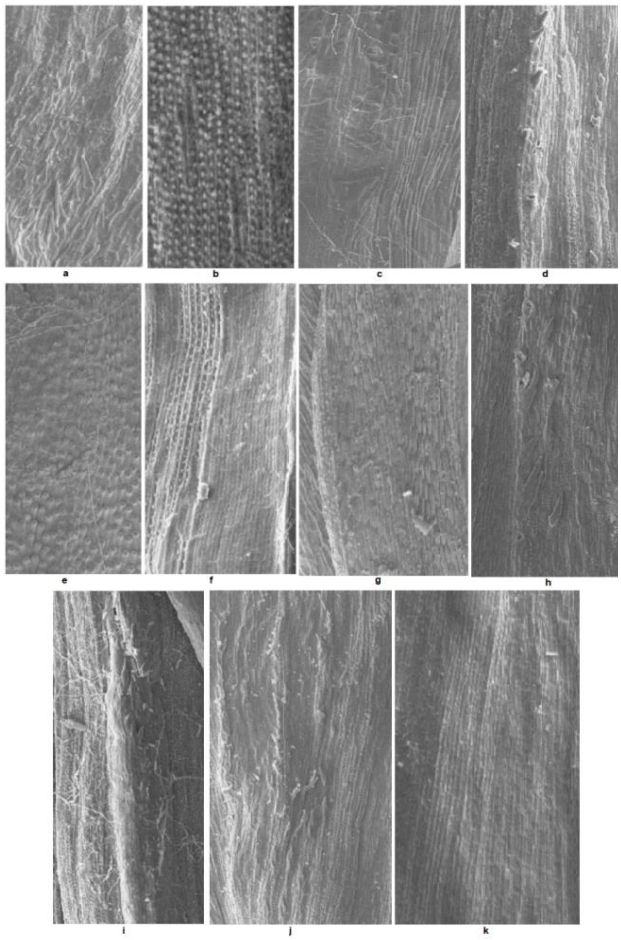

Fig. 5: Surface patterns of pistillate scale under SEM. Scale in 100 μm. a) prickles (*Carex fedia*), b) hooks (*Carex pamirica*), c) trichomes (*Carex canescens*), d) hooks and prickles (*Carex brunnea*), e) papillae and trichome (*Carex atrofusca*), f) hooks and trichome (*Carex nubigena*), g) papillae and prickles (*Carex dimorpholepis*), h) prickles, hooks and papillae (*Carex cardiolepis*), i) hooks, prickles and trichome (*Carex pseudolaxa*), j) papillae, hooks, prickles and trichome (*Carex schlagintweitiana*), k) smooth (*Carex simpliciuscula*)

Conclusion

A significant biodiversity is to be discovered in the genus *Carex* to explore a new species for the region (*C. simpliciuscula*) and numerous new collection localities. Moreover, micromorphological characters of pistillate scale studied particularly under SEM seemed to contribute in identify lying species for the region. Therefore, our findings encourage further research on micro- and macromorphological studies of pistillate scales in the genus.

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Table 3: Characters of pistillate scale of each species. Characters measured under stereoscope: scale color, scale shape, arista presence, hyaline margin, mid-rib color and mid-rib width. Character measured under compound scope: epidermal cell types. Characters measured under SEM: arista surface, mid-rib surface and scale surface

Carex Spp.	Scale color	Scale shape	Arista*	Arista surface	Hyaline margin*	Mid-rib surface	Mid-rib color	Mid-rib width	Scale surface	Cell type of upper epidermis
C. atrofusca	Blackish Brown	Ovate	-	No	-	Smooth	Whitish	Narrow	Papillae and trichomes	Large rectangular interlocking cells
C. brunnea	Yellowish Brown	Ovate	-	No	-	Barbed	Green	Broad	Prickle and hooks	Small and large rectangular cells with sinuous outline
C. canescens	Yellowish Brown	Ovate	-	No	+	Smooth	Brown	Broad	Trichomes	Large rectangular with sinuous outline around cells
C. cardiolepis	Pale to Reddish Brown	Oblong	+	Barbed	+	Barbed	Yellow Green	Broad	Prickles, hooks and papillae	Small and large rectangular cells with sinuous outline
C. diandra	Brown	Ovate	-	No	+	Smooth	Pale	Narrow	Smooth	Large rectangular interlocking cells
C. dimorpholepis	Pale	Lanceolate	+	Barbed	+	Smooth	Brown	Broad	Papillae and prickles on apex	Large rectangular interlocking cells
C. fedia	Yellowish Brown	Lanceolate	+	Barbed	+	Smooth	Green	Broad	Prickles	Large rectangular cells
C. filicina	Pale Brown	Lanceolate	+	Barbed	-	Smooth	Pale	Narrow	Prickles, hook, papillae and trichomes	Small and large rectangular cells with sinuous outline
C. infuscata	Blackish Brown	Ovate	+	Barbed	-	Smooth	Pale	Broad	Smooth	Large rectangular interlocking cells
C. nubigena	Pale Green	Ovate	+	Smooth	+	Smooth	Green	Broad	Hook and trichomes	Large rectangular cells with sinuous outline
C. pamirica	Reddish Brown	Lanceolate	-	No	+	Smooth	Pale	Narrow	Hooks	Large rectangular cells with sinuous outline
C. pseudofoetida	Brown	Ovate	+	Barbed	+	Smooth	Brown	Narrow	Smooth	Large rectangular cells with sinuous outline
C. pseudolaxa	Reddish Brown	Lanceolate	-	No	+	Barbed	Green	Narrow	Hooks, prickles and many trichomes	Large rectangular cells with sinuous outline
C. psychrophila	Reddish Brown	Ovate	+	Smooth	+	Smooth	Green	Broad	Papillae, hooks, prickles and trichome	Large rectangular cells with sinuous outline
C. sanguinea	Brown	Cymbiform	+	Barbed	+	Barbed	Pale	Narrow	Prickles	Large rectangular cells with sinuous outline
C. schlagintweitiana	Brown	Ovate	-	No	+	Barbed	Pale	Narrow	Papillae, hooks, many prickles and trichomes	Large rectangular cells
C. simpliciuscula	Brown	Ovate	-	No	+	Smooth	Pale	Narrow	Smooth	Large rectangular cells with sinuous outline
C. wallichiana	Pale Green	Ovate	+	Barbed	+	Barbed	Green	Broad	Hooks and prickles	Large rectangular cells

***-** Absent, + Present

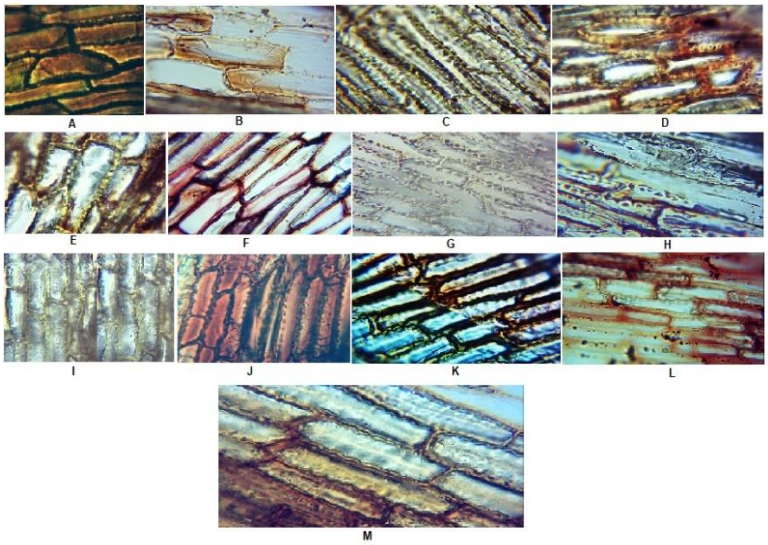

Fig. 6: Upper epidermal cells under compound microscope at 100x objective lens for the species with unique pattern given on SEM. A) *Carex atrofusca*, B) *C. brunnea*, C) *C. canescens*, D) *C. cardiolepis*, E) *C. filicina*, F) *C. infuscata*, G) *C. nubigena*, H) *C. pamirica*, I) *C. pseudofoetida*, J) *C. psychrophila*, K) *C. sanguinea*, L) *C. schlagintweitiana*, M) *C. simpliciuscula*

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