Phylogenetic study of *Cyperaceae* species: *Carex flaviformis*, *Carex stenantha* & *Cyperus iria* from Pakistan, using Morphological and Molecular approaches.

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Dedication

“I dedicate this work to my Parents who are my support system in every walk of life, my younger brothers who stood by me in every thick and thin, and my colleagues for their eternal love, support, and constant help, motivation and encouragement and my supervisor, without his inspiration, coaching, and guidance none of this would have been possible.”

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Muhammad Ammar Ashar

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Abbreviations

µl	Microliter
^{0}C	Degree Celsius
BBM	Bayesian binary method
bp	Base pair
BSA	Bovine Serum Albumin
CDS	Cariceae-Dulichieae-Scirpeae
CO_2	Carbon dioxide
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic
ETS	External Transcribed Spacer
GBS	Genotyping by Sequencing
GPS	Global Positioning System
IAA	Isoamyl alcohol
ITS	Internal Transcribed Spacer
KPK	Khyber Pakhtunkhwa
matK	Maturase K
MgCl2	Magnesium chloride
NCBI	National Center for Biotechnology Information
NJ	Neighbor Joining
NUST	National University of Sciences and Technology

PCR	Polymerase chain reaction
pH	Potential Hydrogen
PMNH	Pakistan Museum of Natural History
PVP	Polyvinylpyrrolidone
rbcL	Ribulose-1,5-bisphosphate carboxylase large subunit
RAD	Restriction-site Associated DNA
rpm	Revolutions per minute
S-DIVA	Statistical Dispersal-Vicariance Analysis
SNPs	Single nucleotide polymorphisms
TAE	Tris, Acetic acid, EDTA
TE	Tris EDTA
Tris	Trisaminomethane
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetical mean
USA	United States of America

Abstract

Cyperaceae is one of the common groups of blossoming plants and the top 10th group of angiosperms containing around 5500 species scattered in 109 genera from one side of the planet to the other. The relatives are by and large known as sedges. Fundamental distinctive elements of the relatives are having the trigonous stem with generally bract longer than and terminal inflorescence. The sedges are sorted as lasting spices. These plants play a part in various bioactivities and furthermore have ethno-herbal significance in light of the fact that different plants are utilized for treatment of problems normally. Cyperaceae is appropriated from streams, marshlands, lowlands to grasslands. Because of discrete inflorescence and multi-layered morphology scientific classification of this family is exceptionally intricate. This exploration accentuations on the biosystematic investigation of three Cyperaceae species; Fimbristylis littoralis, Cyperus difformis and Carex setigera var. schalgintwietiana. These examples were gathered from Northern spaces of Pakistan. First and foremost, for disclosing the morphological characters stereomicroscope is utilized to notice different pieces of inflorescence: utricle, anthers, dusts and glumes. Checking electron magnifying instrument (SEM) is utilized to disclose the miniature morphological characters. Herbarium examples were submitted to PMNH. To additionally affirm recognizable proof atomic investigation is performed. DNA extraction is performed utilizing 2% CTAB DNA extraction convention, enhanced through polymerase chain response utilizing four marker qualities ITS, ETS, rbcL and mat-K and sequenced. These groupings were subsequently submitted to NCBI. Geneious prime were utilized for phylogenetic examination. Three trees (Bayesian, NJ and most extreme probability) were built to uncover the phylogeny. This computational work showed cozy relationship of specie with the same taxa. From this exploration, it was summed up that ITS and ETS are amazing markers to recognize these three individuals from sedges while more examination is expected to affirm rbcL and matK are acceptable markers for important species.

INTRODUCTION

INTRODUCTION

Cyperaceae (Sedges) are tracked down everywhere. It is a monophyletic family. Among monocots it is the third biggest family after Poaceae and Orchidaceae, and seventh among angiosperms (Muasya et al., 2009). Their beginnings are in the late Cretaceous (Escudero & Hipp, 2013) in what is presently South America (Spalink et al., 2016). Sedges obviously take after surges or grasses, Juncaceae is sister gathering of cyperaceae (Jones et al., n.d.).

1.1Cyperaceae

Cyperaceae involve 109 genera and 109 genera contain around 5000 species (Goetghebeur, 1998). Cyperaceae conveyance is cosmopolitan, yet they are gathered in tropical regions. Carex genera, containing around 2000 species (The Global Carex Group 2015, 2016). Carex has incredible importance in occasionally clammy environments and wetlands (Govaerts et al., 2007). Schoeneae clan of cyperaceae contains numerous species that found in dry environments, that main clammy occasionally for example wellbeing networks, forest. (Goetghebeur, 1998). These sorts of environment are extremely strange in this family in light of the fact that the greater part of the individuals from this family found in clammy regions and wetlands. Cyperaceae (sedges) species normally involve wide range of territories, similar to, bogs, wetlands, swamps, pounds, riverbanks, and shoal conditions (Goetghebeur, 1998), however they are likewise found in dry regions in numerous sorts of vegetation, including xerophytic clean. Sedges has an incredible commitment in natural surroundings development and supplement reusing in wetland environments (Chambers et al., 2007). Cyperus is another notable sort of cyperaceae family, containing around 600 species, it incorporates species that are significant, green and financially (Simpson and Inglis, 2001). One of the soonest known plant of Cyperaceae family is Cyperus papyrus, in advanced ages (3000BC) it is utilized for paper making. It supplanted old composing material like materials it played extraordinary part the advancement of proficient and viable correspondence frameworks (Parkinson et al., 1995).

Cyperaceae relative's significance is regularly at nearby or local and this relative assumes a fundamental part in numerous neighborhood economies. These are the plants of conservative significant most likely as a result of their confined use (Simpson & Inglis, 2001). The majority of the cyperaceae species have an extraordinary ecological significance (e.g., as food and

territory for wild species and as soil stabilizers) and many have affordable and ethno natural significance (Bye, 1979; Ludlow-Wiechers et al., n.d.). A few animal categories are among the world's most exceedingly awful weeds, e.g., *C. esculentus* L., *Cyperus rotundus* L., and *Fimbristylis miliacea* (L.) Vahl (Carter, 2008) Other cyperaceae species are utilized as food, as the tuberous knobs present on the rhizomes of Eleocharis dulcis (Burm. f.) aTrin. ex Hensch., and *Cyperus esculentus* or the Schoenoplectus (Rchb.) Palla, three species' delicate youthful shoot, that were utilized as food by Native Americans (Rink and Licher 2015). Among the ornamentals are *C. involucratus* Rottb. *Cyperus alternifolius* L., and *C. Papyrus* L. A few types of Schoenoplectus and Cyperus L., Eleocharis R. Br. Plays part in phytoremediation (Rice et al., 1997). There is wide variety in chromosome number of this family ($2n= 4$ to $2n \geq 200$) (Hipp et al., 2006). The chromosomal advancement is more powerful in the cyperaceae family than in some other group of blooming plants and this blessing fast expansion and development and a significant degree of endemism in certain gatherings (Hipp et al., n.d.).

This different family has no such broad element which can be applied to all of its members. Cyperaceae and Poaceae has discernable life structures from one another on bases of intracellular space volume, which is more prominent for both C4 and C3 types of Cyperaceae. Thus, on account of this distinction both these families have diverse biological appropriation. (Soros & Dengler, 1998). A few types of cyperaceae display Kranz life systems.

1.2 Genus Carex

Flowers in Carex are unisexual and perianth is absent. Male flower has 3 stamens covered by glume and directly attached to the axis. The female of carex are enclosed in organ which is sac-like and called perigynium or utricle. Carex Cymphyllus and carex Uncinia have distinguished morphology because of presence of closed perigynium in contrast to other Cariceae genera (Schoenoxiphium and Kobresia) who have partially or completely opened perigynium (Fennici & 1994, n.d.; Snell, 1936).

1.3 DNA Barcoding in Cyperaceae

Use of DNA short sequences for species identification is called DNA barcoding. Since its inception as an approach for large scale species identification (Herbert et al., 2003; Blaxter,

2003; Tautz et al., 2002), several studies have reported the application of COI in a wide range of animal taxa (Blaxter, 2003; Tautz et al., 2002; Ward et al., n.d.). However, many attempts had made for identification of single locus in plants which can be used for their identification, but they are unsuccessful (Pennisi, 2007; Rubinoff et al., 2006).

For plant barcoding it is suggested that there is need of more than one locus for plant barcoding (Chase et al., 2007). Following regions are suggested for plant DNA barcoding: ITS and psbA-trnH (Taberlet et al., n.d.); psbA-trnH and rbcL(Kress et al., 2009); psbA-trnH (Shaw et al., 2007) ; trnLUAA (Taberlet et al.,2007); matK and psbA-trnH(Lahaye et al., n.d.), matK and rbcL ; matK, rbcL and trnH-psbA (Kress et al., 2009) and ITS2 (Chen et al., 2010).In the recent studies conducted on plant barcoding, have suggested that, the coding regions rbcL and matK are the prime candidates for DNA barcoding of plants (Kress et al., 2009). Consortium for the Barcoding of Life (CBOL) suggested that trnH-psbA is not good for barcoding because of consistent errors in bidirectional sequence reads.

Former studies were focused on plastids region for plant barcoding. (Chase et al., 2007) and (Kress et al., 2009) recovered that nrITS region has highest sequence divergence for barcoding. Sometimes ITS is not considering favorable for plant barcoding because of its paralogs in several plants. But in some other studies ITS has been uses as successful marker for plant identification (Edwards et al., 2008; Kress et al., 2009).

1.4 Justification of work

The species exist in nature but, in plant taxonomy, the species exists if:

- Its herbarium specimen is there in the herbarium.
- Its morphological identification is available in the Flora.
- Imaging of its characters is accessible.
- Its marker gene sequences are submitted and are available in the Genbank.

Although the portrayal and herbarium specimen of some species of Pakistan sedges are accessible, yet there are some gaps in the literature that needs to be filled. This study would work to fill those gaps for *Carex flaviformis*, *Carex stenantha* and *Cyperus iria*. Besides, the morphological classification of sedges on the basis on microscopic inflorescence is troublesome and goes through numerous revisions as the progression

in technology. This study likewise finds the situation of *Carex flaviformis*, *Carex stenantha* and *Cyperus iria* in phylogentic trees by utilizing ETS, ITS, matk and rbcL regions.

1.5 OBJECTIVES

The prime objective of this study is to do the phylogenetic study using morphological and molecular approach or identification while to fulfil the main objective the sub-objectives are:-

1. Preparation and submission of the herbarium specimen to Pakistan Museum Natural History.
2. Imaging of morphological and micromorphological characters using Light Microscope & Scanning Electron Microscope, respectively.
3. Molecular identification using selectable marker genes; ETS, ITS, rbcL, and matK for phylogenetic analysis.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Cyperaceae (Sedges) is third largest family among monocots and seventh among angiosperms, with 5,500 species and 109 genera (Muasya et al., 2009). Very closely resembles rushes and grasses (Jones et al., n.d.). Cyperaceae is distributed worldwide. Carex is the largest genus with 2000 species and Cyperus is second largest genus with 600 species (Simpson & Inglis, 2001). *Fimbristylis* with 300 species, Scleria & Rhynchospora with 250 species, Pycreus, Schoenus and Bulbostylis with 100 species (Goetghebeur, 1998).

Sedges are flowering and resemble grasses, have linear leaves with parallel venation and small leaves. Flowers are mostly pollinated by wind. Their distinguishing feature is their triangular stem, but some like for example Eleocharis has rounded stem.

They are found in variety of habitats ranging from dry to wet land. They can occur in marshes, swamps, ponds, sandbanks and riverbank environments (Goetghebeur, 1998). Most the species have great ecological, economical and ethnobotanical importance (Bye, 1979; Simpson & Inglis, 2001).

2.1 Ecological Impotance of *Cyperaceae*

The greater part of the cyperaceae species play incredible natural part. They play part in living space development. A large number of the cyperaceae species seeds, tubers and leaves are utilized as food hotspot for the two people and creatures. They likewise go about as soil stabilizers. They give control of soil disintegration by developing on waterway banks (Bye, 1979). Cyperaceae colonize the different scope of territories. They play extraordinary part in supplement obtaining. Their quality in dry forests and shrublands might be a direct result of their phosphorous extraction capacity, from soils. Bunches establishes are found in Carex genera species, on account of this root type they have capacity to take-up more supplements. They have long haul endurance in environment (Bond & Midgley, 2015).

2.1.1 Flood Tolerance

A few types of cyperaceae additionally have flood resilience like for instance Carex remota. Delayed flooding establishes anaerobic climate for roots. In anaerobic condition aerenchyma arrangement begins in cortex(Armstrong, 1980). Through these association oxygen's dissemination happens from shoot to roots to keep up with the vigorous condition in the underground plant parts where anaerobic condition was made due to flooding. Aerenchyma development in cortex is fundamentally thing that is giving flood resilience (DREW & C., 1991). Leaves have showed same life structures when flood conditions, yet roots had showed a few changes in morphology due to anaerobic pressure. Gas occupied space was expanded in the root in view of aerenchyma development in anaerobic condition (Beals 1917).

These recently framed aerenchyma permit oxygen trade between staying living cells (Drew, 1997). Development of aerenchyma is exceptionally relied upon a chemical, ethylene (Bradford et al., n.d.) ,which is delivered, because of anerobic stress (Jackson et al., 1985). Capacity of arrangement of these aerenchyma cells are needed for the *Cyperaceae* species in flooding regions.

2.1.2 Sedges in Wetlands

Sedges are significant piece of food web. They give food to numerous wild species. They likewise give living space to faunal variety. They play extraordinary part in supplement reusing and utilize these supplements for photosynthesis and creation of biomass that is utilized by essential shoppers as food source. They likewise go about as obstruction for soil disintegration by developing on streams furthermore stream banks. They likewise play part in bioremediation. They have capacity eliminate harmful mixtures from water (*Sather, J. H., & Smith, R. D. (1984). An Overview... - Google Scholar*, n.d.). They play major useful part in wetland biological systems. Sedges give energy to the numerous types of vertebrates and birds during basic time of their life cycle. They give rearing, settling, escape, taking care of, organizing living space for Cranes, waterfowls, and for transitory birds.

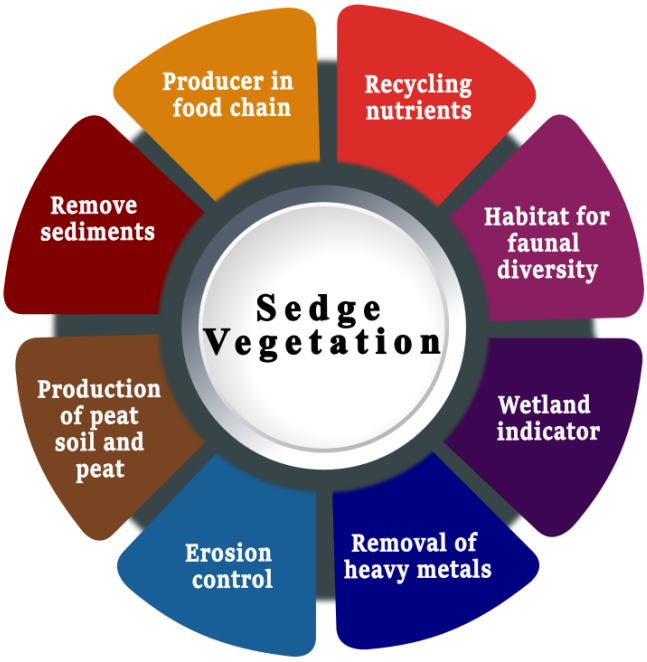

Figure 1.1: Role of Sedge in Wetlands

(Adapted from Mishra *et* al., 2015)

2.1.3 Food Source

Different species of sedges used as food source in different parts of world. Two species of Cyperaceae are used as crop plants. *Cyperus esculentus* commonly known as tiger nut/Chufa and *Eleocharis dulcis* commonly known as water chestnut these both species tubers are edible. They are used as food source since the time of Paranthropus boisie (Nutcracker Man). It is also reported that *Cyperus esculentus* was cultivated in the Egypt in very ancient era (Zohary & Hopf, 2000).

2.1.4 Environmental Cleaner

Urbanization and industrialization have created many environmental problems. They are source of pollutants in environment (Marchand et al., n.d.). The contamination of heavy metal severally effecting our natural ecosystem, and this is the problem of great concern (Kumar et al., 2014). These heavy metals accumulate in ground water surface water plants and substrates (Everard et al., n.d.; Maine et al., n.d.). Toxic solid wastes and liquids are excreted in our aquatic ecosystems from industrial, agricultural (Valipour et al., 2009). This not only problem of this era but was also of middle ages (Lodeninuse, 1991). Domestic sewage is also a problem for mankind (Veeken & Hamelers, 1999). Study of accumulation, distribution, detoxification and uptake of heavy metal in water ecosystem is very importance because wetlands are considered as kidney of the earth.

Wetlands have vital function in natural eco system. One of their functions is phytoremediation. In recent era plants have been used as environmental cleaner. Sedges are dominant in wetland. They are grown on the river, steam banks and they have ability to uptake organic inorganic contaminants from water (Mclncyre,2003).

Sedges are one of the best options for bioremediation because they have ability to grow on both environments like dry land and wet land so they are semi aquatic and biomass production is low. So, they are easy to handle as compared to aquatic plants.

Some Cyprus genera species are aquatic weeds and have fast growing habit. They have ability to grow in harsh environment and they can tolerate stress factors like for example the salt stress, heat stress and cold stress (Schonbeck, M. 2013). So, they are the good option for bioremediation. One of the Cyprus specie known as umbrella sedge have ability to remove 65 to 90% of heavy metals like copper, nickel, chromium, zinc and cobalt from water bodies (Soda et al., n.d.).

2.2 Medical and Ethno-botanical importance of *Cyperaceae*

Cyperaceae handles solid ethnobotanical and therapeutic significance, in light of the fact that these sedges are tremendously utilized for treatment of problems normally. In Pakistan, *Cyperus rotundus* is utilized to countless sicknesses like (Rheumatic torment, dermatitis, the runs, body achne and epilepsy (Peerzada et al., n.d.). In Pakistan and India *Cyperus difformis* are utilized to treat seveal stomach related framework grievances (Simpson & Inglis, 2001). *Scleria pterota* are essentially used to treat snake chomps all around since it plays a part as antiophidian (Soares et al., n.d.). From writing survey, it is presently clear that sedges assume a crucial part in treatment of sicknesses since it has bountiful natural properties expounded in (Table 2.1). These sedges likewise work with climate explained in (Table 2.2).

Table 2.1 Medicinal and Ethno-botanical importance of Genus (Cyperus and Carex)

Sr #	Plant species	Location	Part used	Uses	References
1	*Cyperus difformis*	subtropical and in the warm temperate regions.	Rhizomes	Crushed rhizomes are used as aphrodisiac.	(Tournon et al., n.d.).
2	*Cyperus subumbellatus*	Africa, West Indies, seasonally wet areas.	Rhizomes, Culms	Rhizomes used to cure ringworms or skin diseases and Culms are also used to treat gonorrhea.	(Abbiw, 1996)
3	*Cyperus exaltatus*	Pantropical in distribution.	Rhizomes	Rhizomes are used on diseased skin swellings, and for covering over scarifications.	(Burkill 1985).
4	*Cyperus haspan*	Pantropical and wet areas.	Whole plant	Treat infections	(Milliken, 1997).

5	*Cyperus iria*	Pantropical and wet areas.	Ground with plant C. rotundus.	Used to treat nervous system illnesses.	(Miller & Morris, 1990).
6	*Cyperus javanicus*	Africa, Madagascar, India and China.	Inflorescence.	Inflorescence pulverized with the coconut oil scrubbed on body as (diaphoretic) agent in cold.	---
7	*Cyperus malaccensis*	Middle East, India and China.	Rhizomes	Used to treat the urinary disorders.	(Nguỹen, 1991).
8	*Carex nivalis*	Middle East, India and China.	Leaves	Powdered leaves paste are pragmatic as (antiseptic) on the open wounds in India.	(Navchoo and Buth 1992).
9	*Carex bella*	Located in moist woods and near streams in USA.	Whole plant	Used as important forage sedge in USA.	(Hermann, 1970).
10	*Carex siderosticta*	Asia and China. Also located in shady areas.	Leaves	In India it is used for unspecified medical disorders.	(Cauis and Banby 1935).

2.3 Morphology of *Cyperaceae*

Cyperaceae (Sedges) have multi-layered morphology, the individuals from this family have decreased and wind-pollinated bloom, these blossoms are organized in type of spikelet to frame different scopes of inflorescence, for example, (most normal anthelodium inflorescence which are moved by numerous sedges, other is panicle or umbellate sort). State of their inflorescences not entirely settled by their expanding designs(Reutemann et al., 2012). The majority of the sedges are rhizomatous and new from roots yet some are stoloniferous likewise (Xu et al., n.d.).

Inflorescence are of two sorts (Branched inflorescence and Un-spread inflorescence), in any case, inflorescence wherein fanning doesn't exist and contains just 1 spike are known as Unispikate inflorescence. Blossoms can be either unisexual or maybe bisexual (Goetghebeur, 1998; Guarise & Vegetti, 2008). During the development of spikes, the morphology and shade of glume change.

Stems of sedges are for the most part trigonous yet in certain individuals stems are adjusted, or

sub-adjusted. (Trigonous stems are typically strong yet adjusted stems are empty and protected by thick leaf sheaths (Kern, 1972).

Leaves of sedges are by and large basal however not many of them had direct leaves with equal venation. Scabrous or generally smooth leaf edges are available however in some cases scabourness of leaf edges are lost when dampness is available in air (Fennici & 1994, n.d.). Leaf-like design are available called as "bracts" or now and again these constructions are longer than the inflorescence (Xu et al., n.d.).

Parienth is available in sedges here and there however in many sedges perianth is totally missing. The separation between two nearest families (Cyperaceae and Juncaceae) are based on dusts either Monads or Psuedo-monads, these dusts are shared component of individuals from family Cyperaceae. On premise of monophyly, phylogenetic investigation uncovered that Juncaceae is a related group of Cyperaceae (Starr et al., 2003).

Fruit of sedges are known as "achene" likewise called "Nutlets" which are either sessile or free, somtimes encased by "perigynium" a particular design (Kern, 1972). There is colossal variety in design, and surfaces of achenes of sedges they shift from elipsoid, trigonous to warty in surface. Shading varieties are likewise tracked down goes from earthy, greenish to rich in various individuals from sedge.

2.4 Micromorphology of Cyperaceae:

Micromorphological investigation are utilized for the investigation of various characters of inflorescence parts that shouldn't be visible with unaided eye, for example, achene designs and their spidermal cells, state of utricle and nut, also stoamata present on glumes surface furthermore morphology of anthers and their dusts (Ghosh and Maiti, 2016). These micromorphological charismas are used for the precise investigation of Cyperaceae.

Nut shape in class Cyperus, goes from obovate formed to elipsoidal achenes. achenes in variety *Fimbristylis* goes from lenticular in shape to trigonous most yet ovoid formed are likewise known (Tucker et al., n.d.).

Glume edpidermal cells and their examples are the unmistakable component for the separation among comparable genera. Glumes comprises of rectangular, harsh, smooth or then again ribbed epidermal cells (Pignotti & Mariotti, 2004).

Pollens in sedges are generally essential for separation between comparative genera. The separation depends on (state of pollens, their surface, and on number of fruitful pollens. Cyperus type pollens are those which have bulbous colups also generally are hetero-polar sort.

2.5 Phylogenetic analysis of Cyperaceae:

Phylogenetic examination is the most crucial device to break down the most firmly related genera or then again species. It is fundamentally used to concentrate on the parent gathering of obscure specie. Notwithstanding, (Cyperaceae) family was assessed with the assistance of sub-atomic phylogenetic examination, observed that this family is sister group of monophyletic family (Juncaceae) whose individuals are additionally called as "rushes". It was considered already that Cyperaceae is related with (Poaceae) family (Plunkett, et al., 1995).

DNA extraction and sequencing of these nucleotides has been an imperative device for the sub-atomic systematics and for transformative investigation (Boysen et al., 1996). For phylogenetic examination, ITS and ETS districts of atomic ribosomal DNA are of outrageous significance (Gardes and Bruns 1993).

ITS district repetitively present between various subunits (18S, 5.8S and the 28S) nrDNA quality and ITS is extremely rationed district and this locale is amplified with the help of markers and easily by weakened DNA tests or even little focus or from corrupted DNA's. ETS is likewise very notable district for the phylogeny investigation preferably it is viewed as more significant over ITS area for phylogenetic investigations.

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Sample Collection

For the samples collection, we visited different regions of KPK during growing season i.e., from April to October. *Carex flaviformis* was collected from Kalam, Khyber PakhtunKhwa, *Carex stenantha* and *Cyperus iria* was collected from Dera Ghazan Khan of District Haripur, Khyber PakhtunKhwa with GPS reading. The whole plants with roots were collected, pressed and labelled for the herbarium specimens. The leaves of the plants under study were used for DNA extraction were gathered in zip bag and dried with silica gel beads.

3.2 Mounting of Herbarium Specimens

The pressed plants were poisoned using mercuric chloride (HgCl2) by the authority of Pakistan Museum of Natural History Islamabad and mounted on the herbarium sheets. The mounted specimens were then submitted there in PMNH for future use and the accession number were assigned for the reference.

3.3 Microscopy

The inflorescence was analyzed under stereomicroscope of IRMECO and various pieces of bloom were seen under 10X focal point. The pictures of all pieces of the bloom were taken and the estimation was sone by TCapture programming. The trigonous state of stem was likewise seen under magnifying lens. The forceps and meager needles were utilized to isolate the piece of bloom and sharp cutting edge was utilized to cut the cross segment of stem. The extremely durable slides of the relative multitude of parts of blossom were ready by utilizing Canada resin for future references (ISCapture Instruction Manual).

3.4 Morphological Identification

The morphological identification of collected sample was done on the basis of inflorescence (Goetghebeur, 1998) and characters describe by Kukkonen in Flora of Pakistan.

3.5 DNA Extraction

For DNA extraction, the CTAB DNA extraction protocol was used.

3.5.1 Reagents

2X CTAB buffer, PolyVinylPolyPyrrolidine, β mercaptoethanol, Cholorofromisoamyl alcohol (24:1), Isopropanol, 70% ethanol (wash buffer) and TE buffer.

3.5.2 Procedure

The little pieces of leaves were granulated into fine powder by the pestle and mortar. If necessary, a spot of autoclaved sand was additionally added during granulating. The granulated material was moved into 1.5ml Eppendorf tube. The 1ml of preheated at 650°C cradle of CTAB/mercaptoethanol and a touch of PVP were added to each example. The example was shaken delicately and brooded at 650̊ C in water shower for 30 min. The example was blended twice during brooding. After brooding, the Eppendorf tube was permitted to chill off to ordinary temperature and 500µl of chloroform/IAA arrangement was added. The Eppendorf tube was blended delicately by reversing for 10-20 min. The example was centrifuged for 10 min. at 13000rpm. The upper layer was moved into new Eppendorf cylinder and rehash the centrifugation subsequent to adding 500µl of chloroform/IAA arrangement. The upper layer was moved into new eppendorf cylinder and DNA was hastened by adding 600µl of chilled isopropanol and was left for the time being at - 200° C. DNA precipitation was centrifuged at 13000rpm for 10 min. to get bed and the supernatant was disposed of. The bed was washed with 70% ethanol and dried for 5 min. The bed was broken up in 100µl of TE cushion and put away at - 200°C (Clark and Hollingsworth, 2008).

3.6 Quantification of DNA

For the quantification of DNA, the NanoDrop 2000 of Thermo Fisher Scientific was used.

3.6.1 Procedure

On the lower optical platform of the example maintenance arrangement of NanoDrop, 1µl of clean distal deionized water was added and shut the lever arm in a way that the upper surface was interacted with water. The lever arm was lifted, and both the surfaces were cleaned with perfect and dry lab wipe. NanoDrop programming was opened, and the "Nucleic Acid" application was chosen. The 1µl of TE support (a similar cradle used to suspend DNA) was administered on the lower optical platform and switch arm was shut. The "Clear" choice was chosen to align clear estimation for test and the consistent for dsDNA was chosen. The lower and the upper optical surfaces were cleaned by new spotless and dry lab wipe. Again, 1µl of clean distal deionized water was added and shut the lever arm in way that the upper surface was interacted with water. The lever arm was lifted, and both the surfaces were cleaned with new spotless and dry lab wipe. The 1µl of test was apportioned on the lower optical platform and lever arm was shut. The "Action" choice was chosen in the application and the reading was noted. The lower and the upper optical surfaces were cleaned by new perfect and dry lab wipe. Again, 1µl of clean distal deionized water was added and shut the lever arm in a way that the upper surface was interacted with water. The lever arm was lifted, and both the

surfaces were cleaned with new perfect and dry lab wipe (Desjardins and Conklin, 2010). The DNA with higher fixation was weakened with TE buffer to make concentration 100ng/ml.

3.7 PCR Amplification

The four markers: two nuclear DNA (ITS and ETS) and two chloroplast DNA (matK and rbcL) locales were intensified by sets of widespread barcoding groundworks. The diverse concentration of reagents (Table 3.1) was utilized in every response. The final volume of every response was kept up with 25µl and BioRad thermocycler was utilized. After initial heating at 94°C for 2 min., 30 patterns of DNA denaturation at 94°C for 30 sec, 30sec of groundwork toughening at 50°C for ETS, ITS and rbcL while 54°C for matK, DNA expansion at 72°C for 60sec and last end at 72°C for 5 min. were practiced for every response.

Table 3.1 Volume and concentration of reagents used for PCR reaction.

Sr. #	REAGENTS	CONCENTRATIONS	VOLUME (added in master mixture)
1	PCR Water		14.03µl
2	10X Buffer ($MgCl_2$)	50mM	2.5µl
3	dNTPs	2mM	2.5µl
4	Forward Primer	10µM	0.5µl
5	Reverse Primer	10µM	0.5µl
6	DMSO		1µl
7	BSA		1µl
8	Taq Polymerase		0.2µl
9	DNA Template	100ng/ml	1µl
10	$MgCl_2$		1.5µl
			25µl

3.8 Gel electrophoresis

Gel electrophoresis technique is utilized for the evaluation of PCR items and 1% agrose gel is ready for electrophoresis.

3.8.1 Reagents

Agarose, Ethidium bromide, Bromophenol blue (loading dye) and 1X TAE buffer

3.8.2 Procedure

The 0.5g of agrose was added in 50ml of TAE buffer and boiled. After cooling down to normal temperature, 5µl of ethidium bromide was added and poured in casting tray. After the solidification, the gel was transferred into gel tank. The 3µl of sample was mixed in 3µl of loading dye and loaded into the well. The 100bp ladder was also loaded alongside the sample. The gel was run at 80V for 50 min. and images were taken in Gel Doc (Clark and Hollingsworth, 2008).

RESULTS

RESULTS

4.1 *Carex flaviformis*

- **Other Names**: Yellow sedge
- **Locality**: Kalam, Khyber Pakhtunkhwa
- **Voucher Specimen Number**: 045424

4.1.1 Morphological Identification

The morphological characters of ***Carex flaviformis*** are elaborated in table 4.1

4.1.2 Molecular Identification

The molecular identification of ***Carex flaviformis*** was done by using External Transcribed Spacer (**ETS**) region and Internal Transcribed Spacer (**ITS**) region 4.1

4.1.2 Phylogenetic Analysis

Phylogenetic examination was performed on premise of the groupings got from sequencing. The acquired groupings were lined up with the arrangements of same variety. Phylogenetic investigation depends on Bayesian, Neighbor Joining, and Maximum Probability. The tree(s) were built on bases of the arrangements of ***Carex flaviformis*** ETS and ITS area groupings with different groupings of same sort. ***Carex flaviformis*** showed up in a similar clade with the different types of ***Carex flaviformis*** gathered from the various region of the world. Clades for the two ITS and ETS are very much upheld on the grounds that they are showing the bootstrap esteem of 91 and back likelihood 0.951. Then, at that point, trees were built for affirmation of species topographical area like what is actually the beginning of species. To affirm either the specie is local of Pakistan or relocated from another area. For the two ITS what's more ETS locales topographical trees are surrendered. Clades for the two ITS and ETS are all around upheld in light of the fact that they are showing the back likelihood of 0.986 and bootstrap worth of 68.

Table 4.1: Morphological features of *Carex flaviformis*

Characters	**Observed Character State**
Stem	Smooth, trigonous
Leaves	Wide, initially, margins scabrid towards the tip, close-set teeth
Ligule	Absent
Inflorescence	Yellow-green, sessile, densely crowded spikes forming a head
Spike	Androgynous
Bracts	Subtending inflorescence leaf-like, often overtopping the foliage leaves
Glume	Oblong-ovate, obtuse, membranous, white
Anther	Single lobbed, yellowish green
Nut	Trigonous with thickened angles
Utricles	Inflated or subtrigonous, ovoid, rather bright yellow-green
Stigmas	3

Figure 4.1: Herbarium specimen of *Carex flaviformis*

Figure 4.2: Morphological Characters of *Carex flaviformis*:

A: **Inflorescence:** densely crowded spikes forming a head B: **Spike**: Androgynous C: **Glume:** membranous glumes are present in this species D: **Utricle:** This part is Inflated E: **Nut:** It appears to be trigonous with thickened angles F: **Anther:** This part of flower is single lobbed and yellowish green.

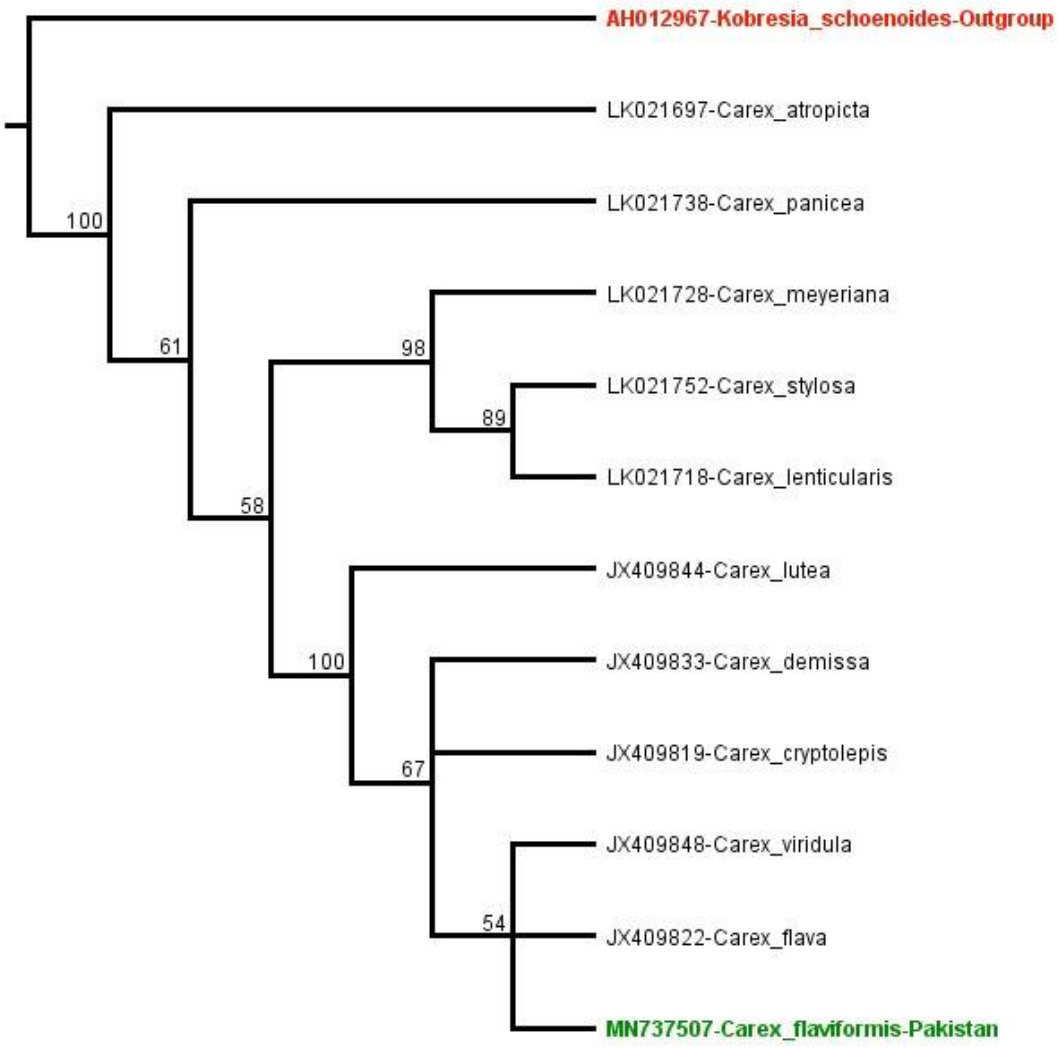

Figure 4.3: Neighbor Joining (NJ) tree of *Carex flaviformis* constructed via sequences of ETS region.

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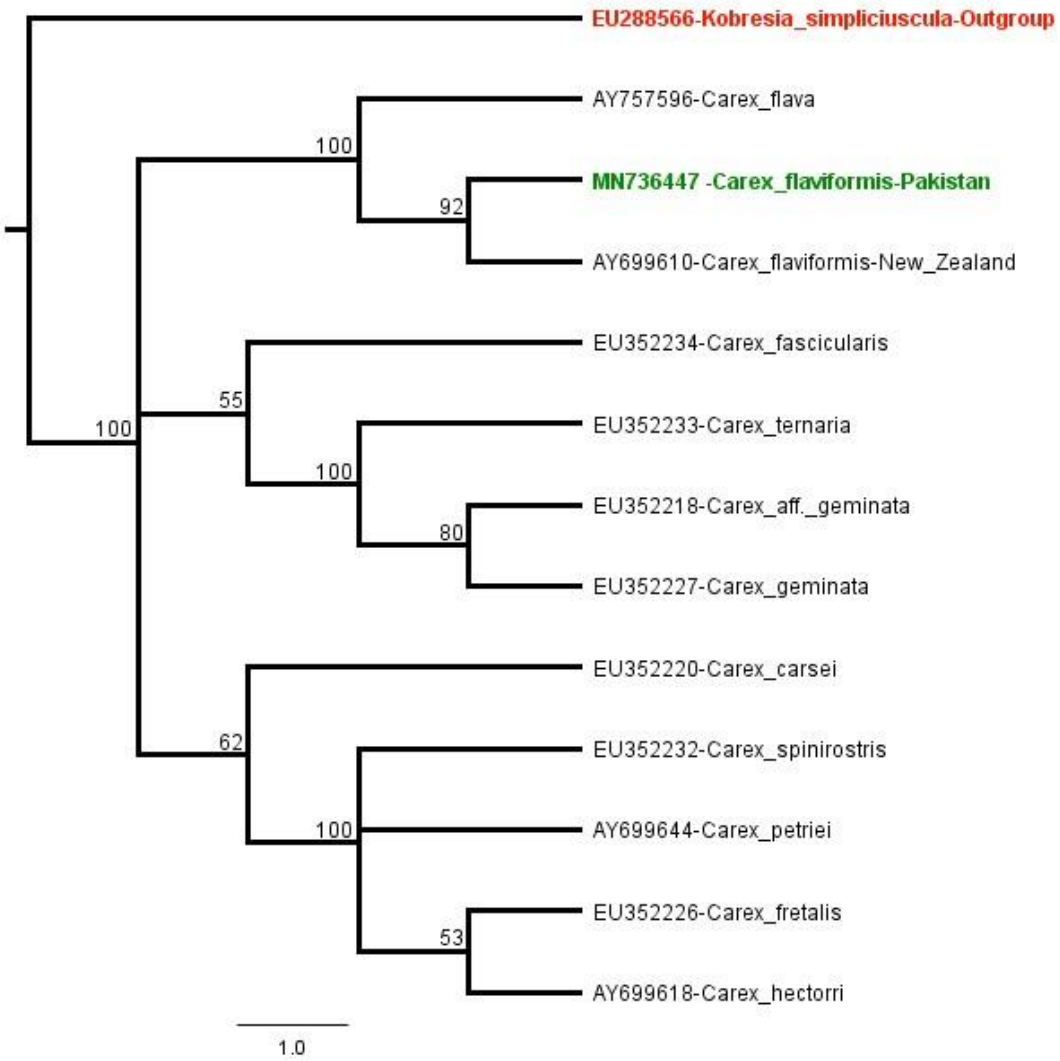

Figure 4.4: Neighbor Joining (NJ) tree of *Carex flaviformis* constructed via sequences of ITS region.

4.2 *Carex stenantha*

- **Other Names**: Yellow sedge
- **Locality**: Kalam, Khyber Pakhtunkhwa
- **Voucher Specimen Number**: 045424

4.2.1 Morphological Identification

The morphological characters of ***Carex flaviformis*** are elaborated in table 4.2

4.2.2 Molecular Identification

The molecular identification of ***Carex flaviformis*** was done by using External Transcribed Spacer (**ETS**) region and Internal Transcribed Spacer (**ITS**) region. (Table)

4.2.3 Phylogenetic Analysis

Phylogenetic examination was performed on premise of the groupings got from sequencing. The acquired groupings were lined up with the arrangements of same variety. Phylogenetic investigation depends on Bayesian, Neighbor Joining, and Maximum Probability. The tree(s) were built on bases of the arrangements of ***Carex flaviformis*** ETS and ITS area groupings with different groupings of same sort. ***Carex flaviformis*** showed up in a similar clade with the different types of ***Carex flaviformis*** gathered from the various region of the world. Clades for the two ITS and ETS are very much upheld on the grounds that they are showing the bootstrap esteem of 100. Then, at that point, trees were built for affirmation of species topographical area like what is actually the beginning of species. To affirm either the specie is local of Pakistan or relocated from another area. For the two ITS what's more ETS locales topographical trees are surrendered. Clades for the two ITS and ETS are all around upheld in light of the fact that they are showing the back likelihood of 0.986 and bootstrap worth of 100.

Table 4.2: Morphological features of *Carex stenantha*

Characters	**Observed Character State**
Rhizome	Shortly creeping
Stem	flower bearing stalk, 20-30 cm long
Leaves	Up to 1 mm wide
Leaf Sheaths	Brown, fibrillose
Leaf Blades	Scabrous
Spike	Terminal spike staminate, linear cylindrical, long pedunculate Lateral spikes pistillate
Bracts	Leaf-like, with short sheaths
Glume	Pale brown, apex shortly awned
Anther	Single lobbed, yellowish green
Utricles	Longer than glume, lanceolate, base shortly stipitate, long beaked, apical margins serrulate, mouth obliquely bidentate
Stigmas	3

Figure 4.5: Herbarium specimen of *Carex stenantha*

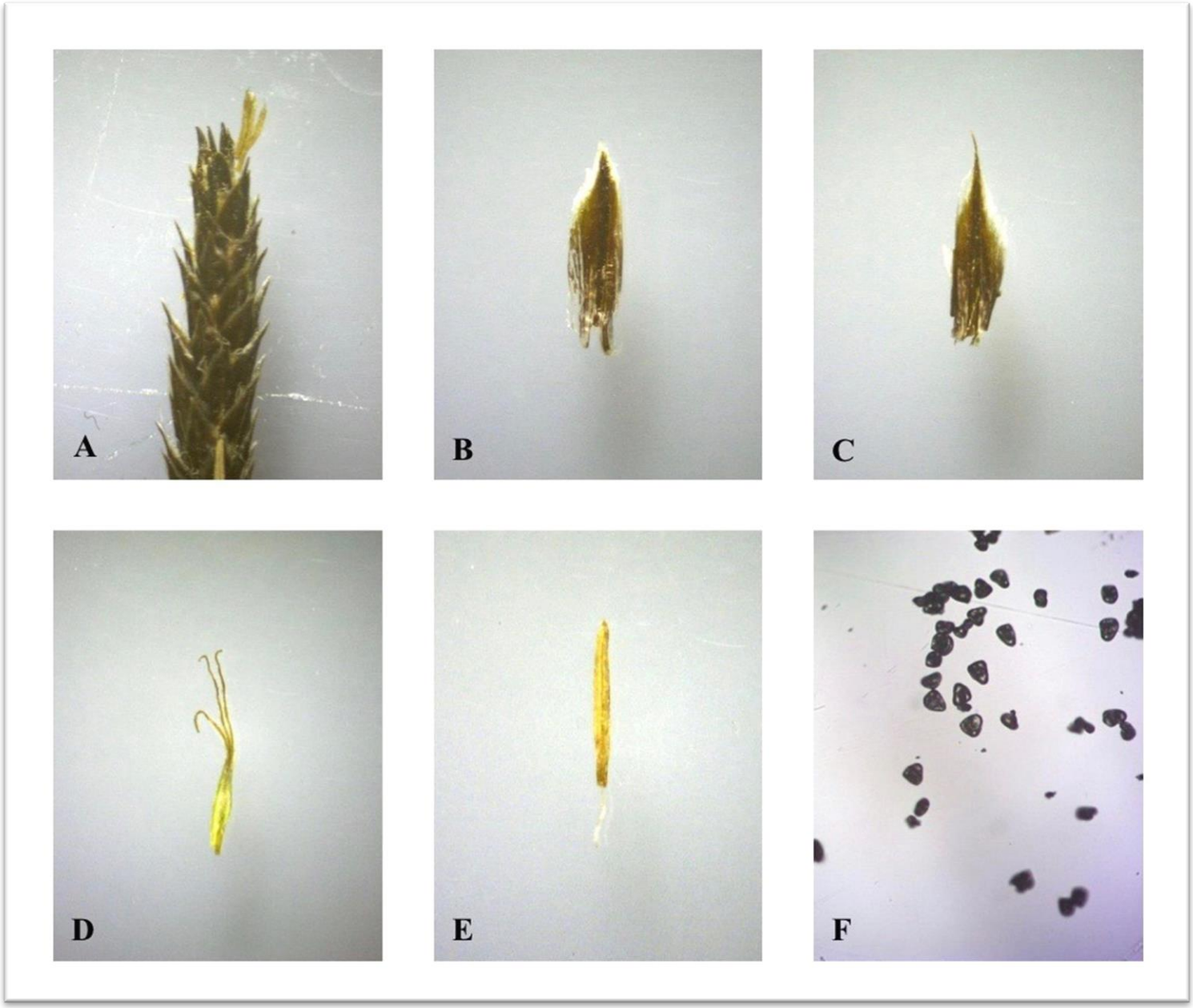

Figure 4.6: Morphological Characters of *Carex stenantha*:

A: **Spike:** long pedunculate spikes are present at theses species. B: **Male Glume:** apex of male glumes is shortly awned C: **Female Glume:** These are Pale brown in color D: **Utricle** E: **Anther:** It also has single lobbed and yellowish green anthers F: **Pollen:** These are triangular shaped.

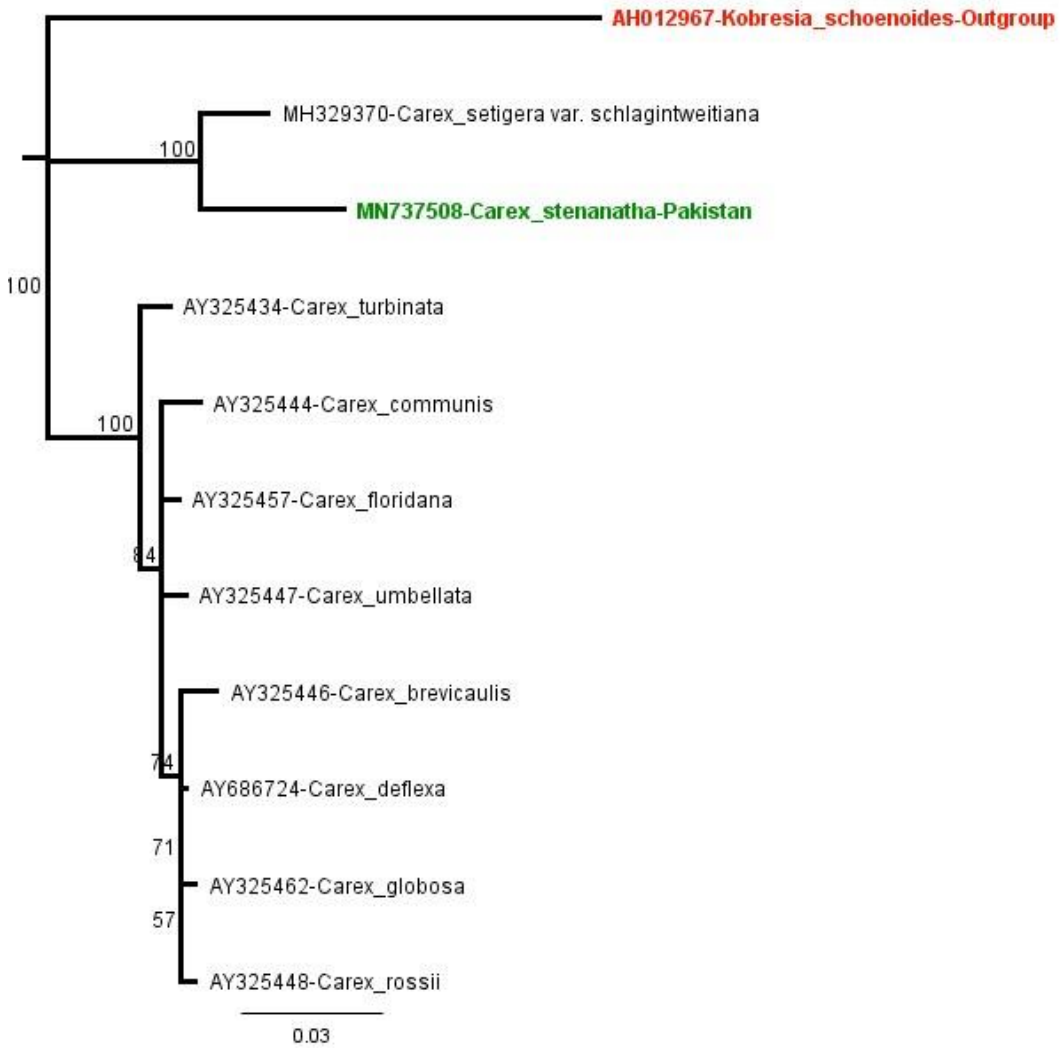

Figure 4.7: Neighbor Joining (NJ) tree of *Carex stenantha* constructed via sequences of ETS region.

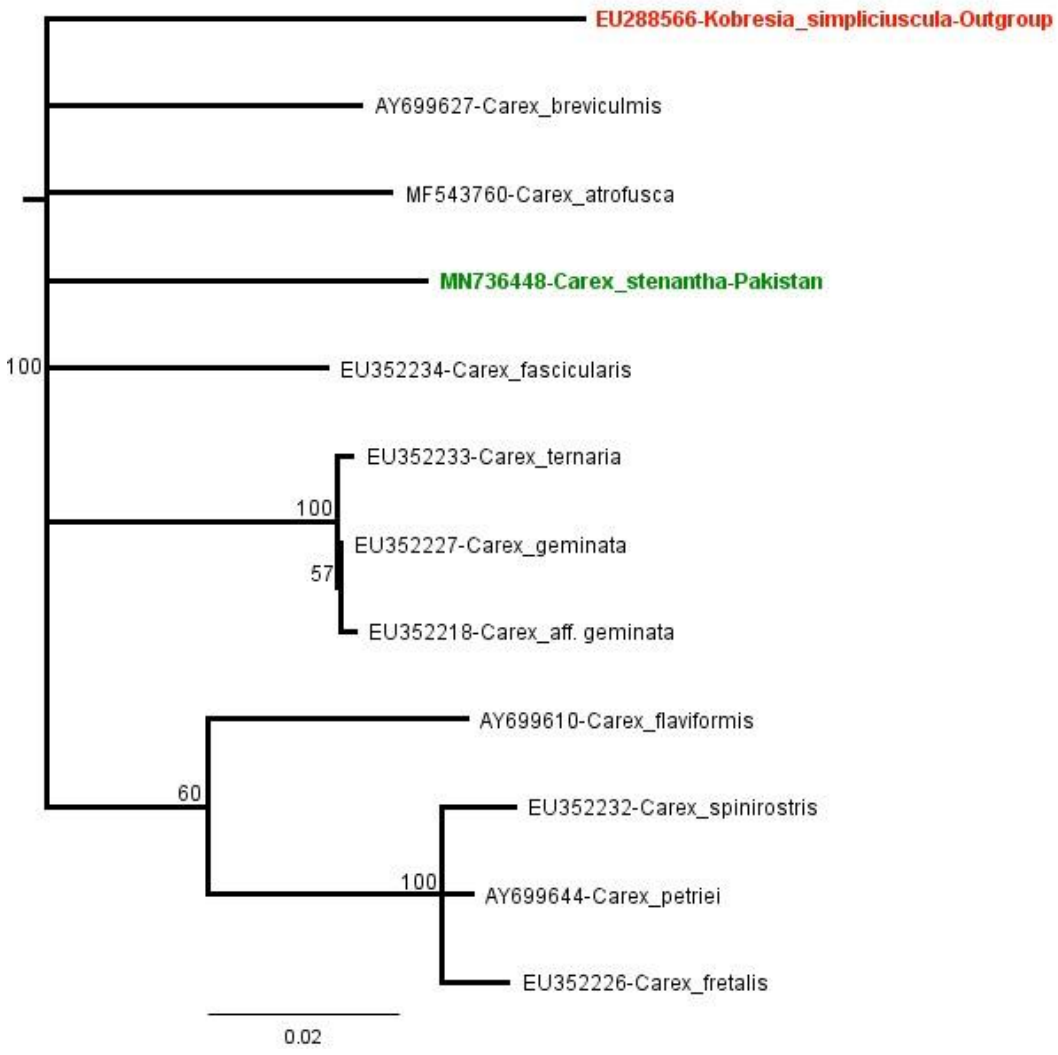

Figure 4.8: Neighbor Joining (NJ) tree of *Carex stenantha* constructed via sequences of ITS region.

4.3. *Cyperus iria* L.

- **Other Names:** *Chlorocyperus iria* (L.) Rikli, *Cyperus chrysomelinus* Link, *Cyperus diaphaniria* Steud.
- **Common Name:** Umbrella sedge
- **Voucher Specimen Number:** 042315

4.3.1. Morphological identification of *Cyperus iria*

The morphological characters of *Cyperus iria* were explained in table 7.13 (Figure 7.48).

4.3.2. Micro-morphological Analysis (SEM)

The micro-morphology of *Cyperus iria* is done by using Scanning Electron Microscopy (Figure 7.49).

Table 4.3: Morphological features of *Cyperus iria*

Character	Observed character state
Roots	Fibrous
Stem	Slender to slightly stout, trigonous, smooth
Leaves	Up to equaling stem
Leave blade	Slightly folded, flat, 15 to 25cm, lower most 2 or 3 longer then stem
Leave sheaths	Sheaths 10 to 20cm, soft, yellow brown, mouth margin straight
Ligule	Absent
Inflorescence	Compound anthelodium, each ray contains 5 to 10cm
Spike	Cluster of spikes, compressed
Bract	Glume like bract, glume like prophyll
Glume	Yellow to straw colored, broadly obovate, margin white hyline, 3-5 veined
Nutlet	Dark brown, 3 sided, densely prominently, obovoid to sub-ellipsoid

Figure 4.9: Herbarium specimen of *Cyperus iria*

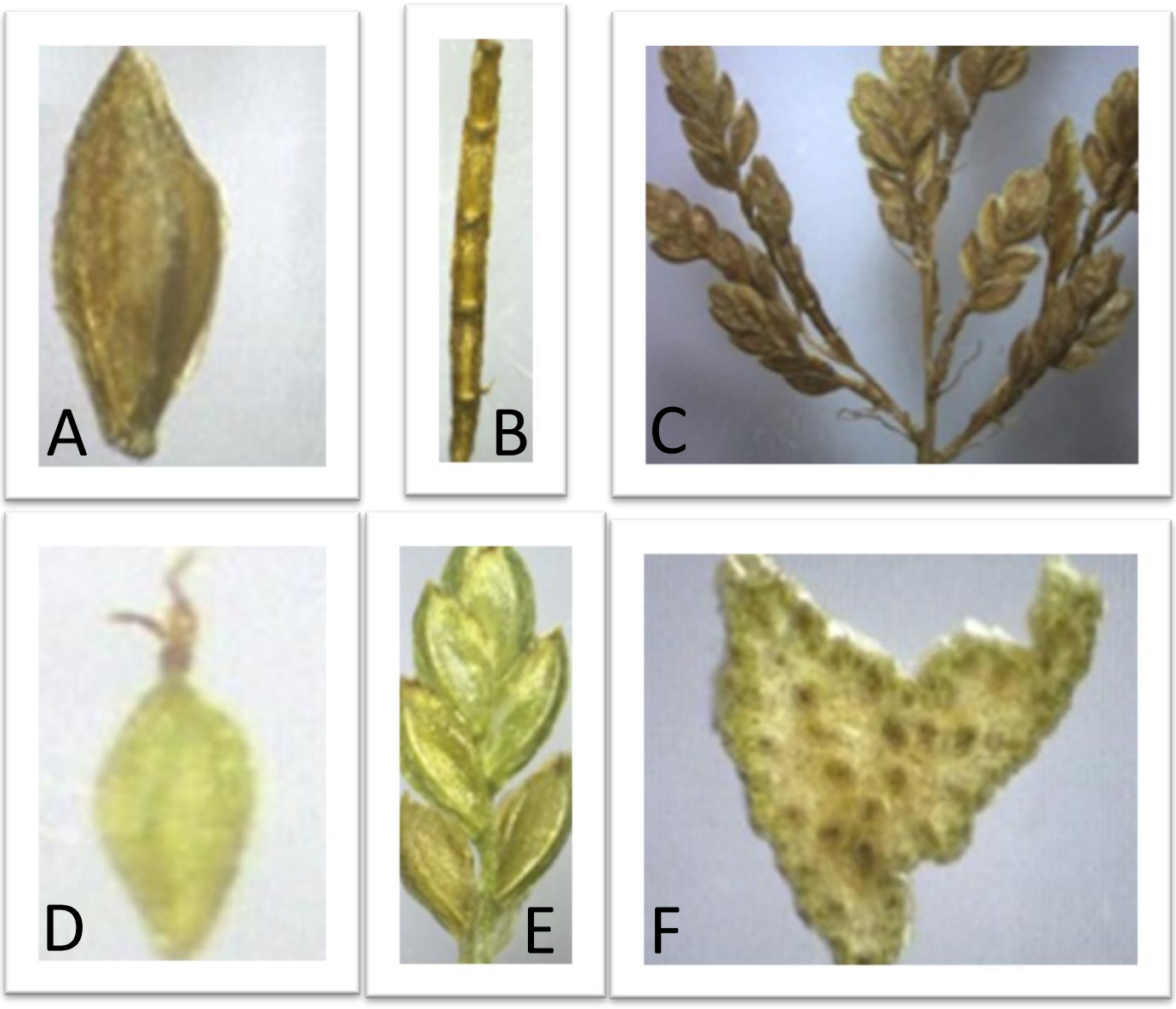

Figure 4.10: Morphological Characters of *Cyperus iria*:

A: **Glume:** Yellow to straw-colored glumes are present which are broadly obovate. B: **T.S. of Stem:** Slender to slightly stout and trigonous stem is present. C: **Spike:** Cluster of spikes are present which are compressed. D: **Nut**: Pear shaped nut is present. E: **Flower:** compound inflorescence is present F: **Ovary with anthers:** Anthers are present in large number attached to the ovary.

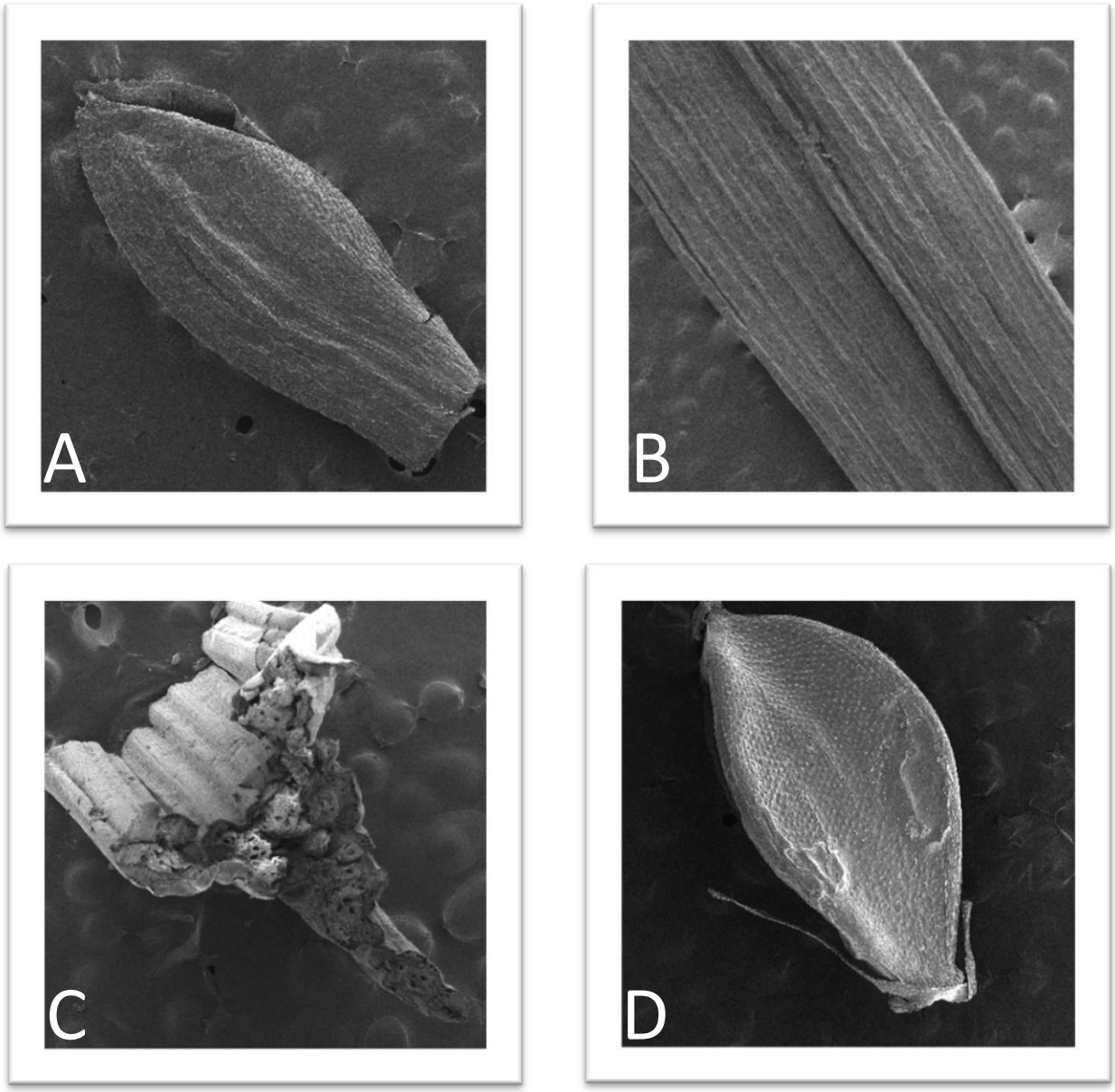

Figure 4.11: Scanning Electron Micrographs of *Cyperus iria*:

A: **Glume** B: **Leaf** C: **Stem** D: **Nut**

Table 4.4: *Carex flaviformis, Carex stenantha* and *Cyperus iria* successful amplification and sequencing results with voucher information.

Sr. No	Species Name	Voucher Number	GenBank Accession Number		
			ITS	ETS	Mat-K
01	*Carex flaviformis*	PMNH 045424	MN736447	MN737507	---
02	*Carex stenantha*	PMNH 045423	MN736448	MN737508	---
03	*Cyperus iria*	PMNH 042315	---	---	---

--- Not Amplified

DISCUSSION

DISCUSSION

Cyperaceae, Poales) is the most assorted and generally appropriated family which contains around 5600 species in 109 genera with cosmopolitan conveyance in Australia, Asia, Africa, towards Northern America and in the Neotropics (Govaerts et al., 2007). The individuals from Cyperaceae otherwise called sedges while these sedges are extremely assorted in environment on the grounds that these species fills in ocean levels, every single normal environment, and on the highest point of high mountains (Egorova, 1999) and furthermore circulated from cold tundra to tropical rainforest (Muasya et al., 1998).

The current review zeroed in on biosystematics examination of three Cyperaceae species; *Carex flaviformis*, Carex stenantha *Cyperus iria* , and from Pakistan. Micromorpholgical investigation was performed to disclose variety of different pieces of their inflorescence like utricle, glume, anther and dusts and pictures were taken. The herbarium examples of these three species was submitted to PMNH for future reference. This concentrate moreover covered the phylogentic investigation utilizing intensification of marker ITS, ETS, matK furthermore rbcL qualities. The increase quantities of these species were acquired from NCBI data set by presenting the sequences. Plant assortment of these specific species was performed from various areas of Pakistan that were not detailed in Flora of Pakistan (Fennici & 1994, n.d.) beforehand. The plant sample, for example, *Cyperus iria* has been gathered from various areas of Pakistan yet are not revealed from Haripur Hamlet beforehand. Comparably *Carex Stenantha* plant is gathered from numerous areas yet not revealed from Kumrat, Khyber Pakhtunkhwa beforehand. The third specie *Carex flaviformis* is the new specie revealed from Kalam, Kyber Pakhtunkhewa, as there are no successions of this species in Pakistan, hence, this species is the new record to Pakistan Museum of Natural History (PMNH).

5.1 Analysis of Morphological Characters

The characters of species are comparative as portrayed in Flora of Pakistan however the imaging of these characters were likewise taken which were not accessible already. The imaging of these characters will help for the exact recognizable proof of these species based on morphology in future. The glumes of *Cyperus flaviformis* have green base with green mid nerve (Figure 5.1) which is the one of a kind person of this species and recently was not depicted in Greenery of Pakistan. Albeit the length of stem was portrayed in a reach as a recognizable person yet it relies upon the climate

and environment. Assuming that the conditions are good for development, the length of stem might increment to more than meter in any case may breaking point to not many centimeters. In addition, the outer layer of stem of Fuirena pubescens likewise have nerves and scored which beforehand were not depicted in Flora of Pakistan (Fennici & 1994, n.d.)

Figure 5.1: Glume of *Carex*

5.2 Analysis of Micromorphological Characters

Micromorphological investigation was perform under Scanning electron magnifying lens to reveal the examples of cells or epidermal dividers on different parts of inflorescence like utricle, achenes, dusts and glumes. In this review, surface morphology of these parts was examined. In *Carex flaviformis*, achene's epidermal cells are askew direct or straight and to some degree oblong, however at the surface membranous sheath found securing the scattered projections that are as per past investigations by Pignotti and Mariotti (Pignotti et al., 2004). In *Carex flaviformis* utricle noticed are exceptionally thorned with middle mouth, nettle or then again achene cells are not separated, ornamented and raised. Glume surface morphology is the least concentrated on character and restricted writing is accessible in Pignotti and Mariotti (Pignotti et al., 2004). The glumes of these species under study had membranous sheaths and oval to huge rectangular cells as in *Carex flaviformis*.

5.3 PCR Amplification and Sequencing

The widespread preliminaries, typically utilized for barcoding of angiosperms, were worked out for the intensification of four marker area (Two ribosomal DNA locale ETS and ITS, and two plastid DNA area matK and rbcL) in light of the fact that only one marker locale isn't enough for phylogenetic examination. The enhancement of ITS area, if there should arise an occurrence of

Carex flaviformis, by utilizing (17SE-f and ITS-4r) set of preliminary was fizzled and the enhancement interaction was rehashed three time with same conditions while the positive control and *Carex stenantha* effectively gave groups. In the event of *Carex stenantha*, the ETS locale gave vague intensification with ETS band and the test was likewise rehashed three time albeit the positive control also *Carex flaviformis* was effectively intensified with (ETS-1f and 18S-r) set of groundwork. The *Cyperus rotundus* was utilized as a positive control as this specie is effectively recognizable and found all over the place, in addition, the groupings of all marker districts of this specie were accessible in NCBI with the exception of ETS. All the enhanced area were effectively sequenced and later submitted on NCBI. *Cyperus iria didn't* give result with any of the used markers, and the amplification is still in progress.

5.4 Phylogenetic Analysis

Phylogenetic investigation of four marker qualities ITS, ETS, rbcL and matK was done by utilizing the Neighbor joining (NJ) approach. Utilizing ITS locale the phylogenetic tree for *Carex flaviformis* developed, that showed the shut relationship with comparable species gathered from New Zealand. Results were comparable utilizing three tree approaches which showed that it is all around upheld clade. In any case, utilizing ETS district, Neighbor joining tree showed resembling result as that of the ITS region. The phylogenetic work on *Carex stenantha* utilizing ITS district showed that gathered specie is firmly connected with same species gathered from the South Korea. Results were comparative for all investigation and comparable revealed by (Larridon et al., n.d.). Since the *Carex flaviformis* ITS groupings were not accessible in NCBI data set, and this may be the new species detailed in Pakistan based on atomic information. The phylogenetic investigation utilizing ETS locale likewise showed *Carex stenantha* as in confined clade with close relationship with its neighbor *Carex setigera var. Schlagintweitiana* and *Carex atrofusca*. Successions for ETS locale was additionally not accessible on NCBI information base.

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