Phylotaxonomic Investigation of *Carex caucasica* and *Cyperus iria* from Bannu, Khyber Pukhtunkhwa, Pakistan

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A thesis submitted to the National University of Sciences and Technology Islamabad in partial fulfillment of the requirements for the degree of Master of Science in Plant Biotechnology

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Dedicated to my beloved Parents for their love, support, and encouragement in every step of my life.

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Ijaz ul haq

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List of Abbreviations

µl	Microlitter
^{0}C	Degree Celsius
BSA	Bovine Serum Albumin
CTAB	Cetyltrimethylammonium bromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic
ETS	External Transcribed Spacer
IAA	Isoamyl alcohol
ITS	Internal Transcribed Spacer
matK	Maturase K
MgCl2	Magnesium chloride
ML	Maximum Likelihood
NCBI	National Center for Biotechnology Information
NJ	Neighbor-Joining

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Abstract

Cyperaceae (sedges) is one of the most common flowering plant family. It is tenth largest family among angiosperms, with over 5500 species distributed among 109 genera all over the world. The trigonous stem with typically longer bracts and terminal flowers are the main differentiating traits of the family members. Sedges are herbs that live for a long time. These plants have a part in a variety of bioactivities, as well as having ethnobotanical significance since a variety of plants are used to cure illnesses organically. Species of cyperaceae may be found in a variety of habitats, including waterways, marshlands, bogs, and grasslands. The taxonomy of this family is quite complicated due to the separate inflorescence and multifarious form. The biosystematic examination of two Cyperaceae species from Bannu, Khyber pukhtunkhwa is the focus of this work. Initially, stereomicroscope is used to examine various components of the inflorescence, including the utricle, anthers, pollens, and glumes, in order to reveal morphological characteristics. The micro-morphological characteristics are shown using a scanning electron microscope (SEM). Herbarium specimens were then submitted to PMNH, Islamabad. Molecular analysis is used to validate the identity. DNA is extracted using a 2 percent CTAB DNA extraction procedure, amplified using a polymerase chain reaction and sequenced using two marker genes: ITS and ETS. These sequences were eventually submitted to the National Center for Biotechnology Information (NCBI). For phylogenetic analysis, Geneious prime was utilized. To reveal the phylogeny, three trees (Bayesian, NJ, and maximum likelihood) were created. This computer investigation revealed a close link between species and taxa that are similar. According to the findings, ETS are good marker for identifying these sedge species, whereas additional study is needed to establish that rbcL and matK are suitable markers for relevant species.

1. Introduction

The sedge family (Cyperaceae) is the third-largest family of monocotyledons and the eleventh-largest family of angiosperms, with more than 5600 species now known. Cyperaceae-assignable plants have been known since Antiquity, indicating that at least some of the family's members have long been thought to be noteworthy. Although Cyperaceae are sometimes seen of as being plants of little practical relevance, especially when compared to Poaceae Barnhart, a more thorough examination revealed that this perception is incorrect and that they are both commercially and environmentally significant (Govaerts et al., 2007). There are really many other historical or modern applications of Cyperaceae by man, according to (*Simpson D. A., 2008. Frosted curls to tiger nuts:... - Google Scholar*, n.d.). Sedges have given materials to domesticated animals or even humans (Cyperus papyrus L. is an excellent example) or food (e.g., the edible rhizome corms of Eleocharis dulcis (Burm. f.) Hensch.). Some species are useful in horticulture as attractive plants, while others are used to revegetate very infertile locations or to consolidate soils that are at risk of eroding. Furthermore, a number of Cyperaceae species, such as Cyperus rotundus L. and C. esculentus L., are problematic agricultural weeds. Beyond the merely utilitarian considerations, Cyperaceae play a critical function in plant communities and whole ecosystems. Family members inhabit a variety of environments, from the tropics to the arctic, and they often dominate marshes (David A. Simpson et al., 2003). On the other hand, many Cyperaceae species are less able to compete, are confined to fragile environments, are thus uncommon, endangered, and significant from the perspective of nature conservation. Cyperaceae are often helpful as phytoindicators of site features for ecologically oriented research since many species in the family have very small

ecological amplitudes in relation to environmental parameters like soil acidity or water chemistry (David A. Simpson et al., 2003).

Inconspicuous and decreased generative organs, in particular, characterise Cyperaceae and make it challenging to accurately identify species (Bruhl, 1995). (Abraham M. Muasya et al., 1998). It is difficult to circumscribe taxa due to the perceived lack of characteristics and the enormous variety of morphological forms in the Cyperaceae family. And it may not come as a surprise that one of the main foci of plant biosystematic study is the attempt to understand the patterns and causes of biological diversity in the Cyperaceae.

1.1. Outline of the Family

Cyperaceae are graminoid monocotyledonous plants with reduced, often wind-pollinated flowers and vaginate leaves that are frequently grouped in three rows as opposed to the two-ranked leaves of seemingly related Poaceae and Juncaceae Juss (Meirelles, 2016). Almost no morphological characteristic can be stressed as a complete synapomorphy of the family due to the vast number of species. According to (Bruhl, 1995), there is intertaxon diversity in life duration, growth form, vegetative morphology, and floral traits. For example, while the stems are often triquetrous, they may also be terete, compressed, or multangular; Inflorescences may be unbranched or branched to different orders; leaves can be two-ranked or bladeless; numerous kinds of prophylls can be generated; flowers can be unisexual or bisexual; perianths can be present or entirely absent; and styles can be whole, bifid, trifid, or quadrifid. Instead of the pollen tetrads characteristic of the Juncaceae family, all Cyperaceae have pollen pseudomonads or monads; only one of the four microspores generated during meiosis of a pollen mother cell matures into a pollen grain, with the other three aborting (David A. Simpson et al., 2003).

(A Muthama Muasya et al., 2009; Abraham M. Muasya et al., 1998; David A. Simpson et al., 2003) corroborated Cyperaceae's monophyly and its sister connection to Juncaceae in phylogenetic analyses based on sequences of plastid DNA. The infrafamiliar Cyperaceae classification was revisited by the molecular research, and it was recommended that two of the five monophyletic subfamilies now recognized—Mapanioideae C. B. Clarke and Cyperoideae Suess—be distinguished.

Less than 200 species make up the tiny, entirely tropical, and subtropical group known as Mapanioideae. The distinctive complex inflorescences of the subfamily serve as an identifying feature. The terminal inflorescence branches have imbricately organised glumes that are grouped in compact spike-like formations (bracts). Male reproductive units with an aborted gynoecium or bisexual reproductive units with one distal pistil and proximal stamens coated in scales (bracts) are both covered with fertile glumes (Richards et al., 2006).

All of the main genera of the Cyperaceae family are included in the Cyperoideae, which has a global distribution and more than 5000 species. Recent research demonstrates that all members of the subfamily share an early ontogeny of generative components that includes the development of stamen, gynoecium, and perianth bristles (i.e., perfect hermaphroditic flowers; as these are typical for the genus Scirpus L. among others, the term "scirpoid ontogenetic pattern" was coined). Then, many other cyperoids, particularly the largest genus Carex L., which have unisexual or perianth-free flowers, are thought to have descended from the scirpoid pattern (Vrijdaghs et al., 2009).

Cyperaceae is a widely widespread and very diversified family that may be found in Africa, Australia, northern America, the neotropics, and Asia (Govaerts et al., 2007). Sedge species thrive in all-natural (Egorova et al., 1999; Jiménez-Mejías et al., 2011)

environments from lower sea levels to higher mountainous locations, making the cyperaceae family biologically varied (Egorova et al., 1999). They thrive in moist, aquatic environments such as marshes, streamsides, and marches (Ueno et al., 1992). Sedge species play an important part in the vegetation of wetlands. It is the third biggest monocotyledonous family (Abraham M. Muasya et al., 1998) and the tenth largest angiosperm family (Abraham M. Muasya et al., 1998). (Abraham M. Muasya et al., 1998). Swamps, shorelines, swamps, and wetlands are the most common sedge habitats. Although sedges thrive in maritime and wet habitats (Ueno et al., 1992), several species may also be found in dry places and are classified as grassland ecosystem components (Stock et al., 2004). With about 960 species, the genus Cyperus L. is most often found in the tropical area. The temperate zone is home to the genus Carex L, which has around 2000 species.

On morphological grounds, it is difficult to discern connections in Cyperaceae due to compact inflorescence and diminished blooms. To prevent ambiguous homologies and diverse interpretations, several subfamilial classifications have been suggested. Among the suggested sub familial categorization, there are two complete groups based on characteristics. The first is based on whether the inflorescence is hermaphrodite or unisexual, while the second is based on how a broad range of floral features and inflorescence are interpreted. Some taxa, such as the mapaniod genera, have been classified differently depending on floral features at the conclusion of this classification. Two recent systems, (Abraham M. Muasya et al., 1998) and BRtmL, have been suggested based on a wide variety of anatomical, phytochemical, morphological, physiological, and embroyological features (1995).

1.1.1. Genus Carex

The Cyperaceae family's biggest genus, Carex, has over 2000 species, making it the fourth largest genus of angiosperms overall. It is found practically everywhere, with the exception of Antarctica, where it is totally absent. The tropical lowlands and subtropical deserts have rather limited representation. The majority of Carex species are found in the temperate, boreal, and arctic regions of the northern hemisphere, with North America and East Asia having the highest species diversity (Ball, 2011). There are 222 Carex species in Europe (Koopman et al., n.d.).

A modified prophyll with connate borders covering the ovary and a totally closed perigynium (utricle) are the distinctive morphological features of the Carex genus. Currently, it is understood that the utricle does not contain a single female flower (reduced to an ovary), but rather a reduced spikelet that was formerly bisexual but now only contains a single female bloom and a remaining axis known as the rachilla (Standley, 1985). With the exception of C. microglochin Wahlenb., which is one of the exceptions with a lengthy rachilla projecting from the perigynium, the rachilla in Carex virtually never surpasses the edge of the perigynium (Reznicek, 2011). Uncinia Pers. and Cymophyllus Mackenzie, two genera that are allied to Carex, also include closed 8 perigynium. Uncinia differs from Carex in that it has a long, well-developed rachilla; Cymophyllus, a monotypic genus, has a short rachilla but differs from Carex in that it has vegetative morphology (very broad, flat leaves), an inflorescence that always has just one androgynous spike, and the only method of pollination is by entomophily (David A. Simpson et al., 2003). The tribe Caricae Dumort, which includes the genera Kobresia Willd. and Schoenoxiphium Nees (Reznicek, 2011), Carex, Uncinia, and Cymophyllus, has open perigynia with partly connate or free edges. The most comprehensive infrageneric categorization of Carex based on morphological

characteristics was given by (Koopman et al., n.d.), with four different subgroups. (1) The subgenus Psyllophora (Degl.) Peterm. (syn. Primocarex Kük) is used to classify unpicate plants (with inflorescence consisting of one spike only, either bisexual or unisexual). (2) There are several sessile, bisexual spikes in the subgenus Vignea (P. Beauv.) Nees plants that lack cladoprophylls (tubular bracts enclosing the base of lateral inflorescence axes). (3) Carex L., a subgenus of plants, has cladoprophylls and many pedunculate unisexual spikes; a lesser number of species in the subgenus sometimes contain both bisexual and unisexual (female) spikes in one inflorescence. (4) The bases of the paracladia of members of the subgenus Vigneastra Tuck (syn. Indocarex Baill) exhibit numerous tubular cladoprophylls. Additionally, they have several pedunculate lateral inflorescence units, each of which has several bisexual spikes with a prophyll that resembles a perigynium at the base. Significant changes were made to the other supraspecific taxa of the tribe Cariceae as well as the morphologically recognised subgenera of Carex using a molecular phylogenetic technique. These studies, which were based on the nuclear and chloroplast DNA sequences, revealed that some of the morphologically limited supraspecific taxa belonging to the family Caricae comprise natural, monophyletic lineages whereas others do not. According to (Vrijdaghs et al., 2009), the whole tribe Cariceae and the genus Uncinia both seem to be monophyletic entities (Starr et al., 2009).

On the other hand, the unispicate sedges (Psyllophora) are abundant across the Cariceae and are 9 obviously polyphyletic (Starr et al., 2009; Waterway et al., 2007). When compared to Uncinia, Cymophyllus, and Kobresia (which also seems to be a polyphyletic group), certain Psyllophora species were shown to be more closely linked to Schoenoxiphium (Starr et al., 2009; Waterway et al., 2009). So, according to the conventional morphological delimitation, the genus Carex is paraphyletic. Carex has

no monophyletic subgenera found in the Kükenthal. Although the subgenus Vignea is polyphyletic, it might become monophyletic by excluding certain Psyllophora species (such as Carex dioica L.) and removing a few morphologically dissimilar tristigmatic species (such as Carex baldensis L. and C. curvula All). (Starr et al., 2009). While several Psyllophora species and the subgenera Carex and Vigneastra seem to form a monophyletic group, the subgenus Vigneastra is unquestionably polyphyletic (Starr et al., 2009).

The Carex subgenera's many sections are further categorised according to morphological traits. Molecular phylogenetic analysis revealed that some of the sections were monophyletic, while many others were discovered to be arbitrary groupings (Starr et al., 2009). One of the largest in the genus and one of the 90 species that make up Section Phacocystis Dumort is Carex nigra (L.) Reich (Dragon et al., 2009). Despite appearing to be non-monophyletic (David A Simpson et al., 2007a), the section differs significantly morphologically from the other members of the subgenus Carex (especially by the combination of unbranched unisexual spikes, reduced sheaths of inflorescence bracts, dorsiventraly compressed utricles, and bifid styles) and is occasionally treated in a separate subgenus Kreczet. Despite being a frequent subject of systematic research (Faulkner, 1972; FAULKNER, 1973; Jiménez-Mejías et al., 2011; Standley, 1985; Volkova et al., 2008), the section Phacocystis can still be considered to be taxonomically critical, and the delimitation of some species within the section is questionable.Carex is the biggest genus in the cyperaceae family (>2000 recognised species), accounting for 40% of species diversity and ranking third among angiosperm genera. Except for Antarctica, species of this genus may be found all over the globe, although they are most frequent in cold and temperate climates in both hemispheres. This clade is thought to have started about 37 Mya in south eastern Asia,

and its worldwide spread was the consequence of a series of expansions and dispersals from its lineage (Więcław et al., 2021). The pecular chromosomal structure of this genus is shared by all species, and it is thought to be the driving force for diversification (Shane & Lambers, 2005). Cyperus is the second biggest cyperaceae genus, with over 600 species endemic to the tropics (A. Muthama Muasya et al., 2002). Fimbristylis (300 species), Scleria (250 species), Rhynchospora (250 species), Bulbostylis (100 species), and Schoenus (100 species) are some of the other important Cyperaceae genera (Govaerts et al., 2007).

Sedges are grass-like species that may grow into shrubs, lianas, or herbs. They are generally annual or perennial, and their height can vary from 3 cm (Isolepis inconspicua) to >12m (Scleria boivinii) (Semmouri et al., 2019). Plants may be helophytic, however they are most often terrestrial, with the remainder being epiphytic or aquatic (Goetghebeur, 1998). Cyperaceae plants are morphologically similar to poaceae (grasses), however phylogenetic study shows that they are more closely related to juncaceae (rashes) (Abraham M. Muasya et al., 1998). Sedges belong to the order Poales, which has a species diversity of 23%. (Christensen et al., 2013a). On the basis of morphological study, the family Cyperaceae was formerly split into three subfamilies: Sclerioideae, Caricoideae, and Mapanioideae (Goetghebeur, 1998). (*Simpson D. A., 2008. Frosted curls to tiger nuts:... - Google Scholar*, n.d.) defined the Cyperoideae and Mapanioideae subfamilies.

Sedges have scapose trigonous stems in most cases, while other species have angular or terete stems (Kern, 1972; Xu et al., 2017). Sedges are rhizomatous by nature, although they may also be bulbiferous or stoloniferous (Xu et al., 2017). Leaf blades are usually basal, have parallel venation, and are spirally connected to the stem (Goetghebeur, 1998). Some sedge species contain ligule, and by decreasing their leaves,

they may occasionally produce a closed leaf sheath (Goetghebeur, 1998; Xu et al., 2017). Sedges have terminal inflorescences that are covered by bracts and may be paniculodium, cephalodium, stachyodium, anthelodium, or corymbodium (Reutemann et al., 2012). Their flowers are monoecious, unisexual, or bisexual, and are encased in the spikelet of glumes (Xu et al., 2017). (Goetghebeur, 1998). Their fruit is an achene or nut that comes in a variety of colours, sizes, and shapes (Arnstein Lye et al., 2004).

Sedges are important medicinally, economically, horticulturally, and ecologically. Except for Antarctica, the family's species can be found in every ecosystem on the planet. Sedges have a vital ecological function in erosion control, habitat development, and the rehabilitation of contaminated wet ground (Starr et al., 2009). Sedeges have been exploited economically since ancient Egypt, when enormous sedges (Cyperus paprus) were employed for riparian resources (Pacini et al., 2018). By producing various commercial goods like as bags, braids, rugs, table decorations, fans, chairs, and baskets, these species may contribute significantly to the development of local communities (Báez-Lizarazo et al., 2017; *Simpson D. A., 2008. Frosted curls to tiger nuts:... - Google Scholar*, n.d.). The tiger nut, Cyperus esculentus, is used as a food by humans and animals all across the globe. Sedges are known to have a significant function in tropical and subtropical climates in maintaining a balance between agricultural output and population increase (Léveillé-Bourret et al., 2018). Sedges have a significant ethnobotanical function in the treatment of a variety of ailments across the globe. Sedges have anti-inflammatory, anti-tumor, anti-malarial, antibacterial, antiallergic, antioxidant, neuroprotective, anti-feedant, and hepatoprotective qualities, among others. Some of the taxa have been classified as noxious weeds. Nearly 168 species pose a major danger to agricultural output (*Simpson D. A., 2008. Frosted curls to tiger nuts:... - Google Scholar*, n.d.).

Because of its global distribution, richness in species, extended ecological inclinations, diverse phenotypes, huge variance in lineage diversity (Escudero et al., 2013), holocentric chromosome presence (Roalson et al., 2021), and multiple origins of C4 photosynthesis, the family Cyperaceae is an ideal family to study evolutionary biology (Besnard et al., 2009).

1.1.2. 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 (Blaxter, 2003; Hebert et al., 2005; Tautz et al., 2002), several studies have reported the application of COI in a wide range of animal taxa (Hebert et al., 2005; Smith et al., 2005; Vences et al., 2005; Ward et al., 2005). 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 (David A Simpson et al., 2007a). Following regions are suggested for plant DNA barcoding: ITS and psbA-trnH (Taberlet et al., 2007); psbA-trnH and rbcL (Kress et al., 2007); psbA-trnH (Shaw et al., 2007); trnLUAA (Taberlet et al., 2007); matK and psbA-trnH (Lahaye et al., 2008), matK and rbcL (CBOL et al., 2009) matK, rbcL and trnH-psbA (Kress et al., 2009) and ITS2 (Gao 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 (COBL et al.,2009).

Former studies were focused on plastids region for plant barcoding. (Kress et al., 2005; David A. Simpson et al., 2003) 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 (Chase et al., 2005(Kress et al., 2005; Pacini et al., 2018)

1.1.3. Pakistani Cyperaceae

There are 179 species of Cyperaceae in Pakistan's flora, divided into 22 genera (Starr et al., 2015). These species came from practically every corner of the nation. They may be found all across Pakistan, from the lower sea level to extremely high mountains of more than 5000 metres in the north (Ullah et al., 2015). Sedges of various sizes may be found in various pools and bogs around the nation (Bellemain et al., 2007).

The majority of sedge species are prevalent weeds in Pakistan, wreaking havoc on many of the country's most vital crops. Cyperus, Bulboschoenus, Eleocharis, Pycreus, Schoenoplectus, and Fimbristylis are some of the most common weed genera. One of the most significant cereals affected by Cyperaceae is rice. Fimbristylis, Cyperus, Schoenoplectus, and Pycreus are some of the most common rice weeds (Khalid, 2014). Cyperus iria, Cyperus rotundas, Cyperus difformis, Echinochloa crusgalli, and Echinochloa colona are common rice weeds in Punjab (Khalid, 2014). Wheat is another key crop damaged by Cyperus difformis, Cyperus rotundus, Cyperus alalutus, Cyperus iria, and Fimbristylis ferruginea, among other cyperaceae weeds (Ullah et al., 2015). Cotton weeds Cyperus rotundus and Cyperus difformis are the most common (Ullah et al., 2015).

Sedges like Cyperus iria, Cyperus fuscus, Cyperus strigosus, Cyperus brevifolius, and Fimbristylis bisumbellata are generally hydrophytes since they are usually associated with water supplies (Fawad Khyber et al., n.d.). Some species, such as Fimbristylis dichotoma and Schenoplectus juncoides, which can thrive in salinity-affected regions, have the potential to help the country's salinity-affected areas recover (Zahoor et al., 2012).

Sedges are also useful in a variety of fields, including economics and ethnobotany. Animal fodder includes Cyperus iria, Kyllinga brevifolia, and Cyperus niveus (Zahoor et al., 2012). In various parts of the nation, certain species are utilised to cure a variety of ailments. In Cholistan, Cyperus conglomeratus infusions and decoctions are used to treat a variety of digestive disorders such as hyperacidity, constipation, and dyspepsia. Cyperus rotundus tubers are used to cure burning, inflammation, and diabetes (Farrukh Nisar et al., 2014). Eye problems, wound healing, fistula drying, cancer, and ulcers are all treated with Cyperus papyrus in Bahawalpur.

1.2. Morphology of cyperaceae

Cyperaceae species are annual or perennial herbs with small blooms that are pollinated by the wind. Sedges are typically having straight aerial culms, bracts on the terminal inflorescence, and horizontally rhizomatous (Takhtajan, 1980). The majority of the species have rhizomes; however some are stoloniferous (Xu et al., 2017). The culms or stems are typically upright and originate centrally or laterally; some may scatter radiantly, and each stem contains a terminating inflorescence (Kern, 1972). Mostly, stems are trigonous or teiquetrous, although others are rounded, subrounded, or terete (Xu et al., 2017). The stems are typically compact but can be empty, and the bottom is bulbous and surrounded with leaf sheaths (Kern, 1972). The scapose stem, which can contain one to several nodes, is another key characteristic of Cyperaceae stems. The stems are seldom crushed and typically smooth, with the exception of a few that may have hair or papillae (Rajbhandari et al., 1988).

Cyperaceae leaves are generally basal, although a few are cauline, and are straight with straight venation and spirally distributed around the stem. Terete leaf blades are seen in a few Cyperaceae species, and blades of leaves can be shortened in some plants (Rajbhandari et al., 1988). The blades of the leaves can be smooth or scabrous , however

under moist circumstances, the scabrousness is dropped (Lee et al., 2010). At the base of the stem, the leaves may form sheaths that are generally closed but a few are open (Rajbhandari et al., 1988). A ligule may be found on the border between the leaf blade and the leaf sheath in most species, and even on the opposite end of the leaf blades(*Achene epidermis in the Carex flava complex (Cyperaceae) studied by scanning electron microscopy on JSTOR*, n.d.; Xu et al., 2017). In a few species, the leaf blades have a well-known midrib that may be squeezed (Karpana et al., 2021).

Sedges have one or more leaf-like involucral bracts (Xu et al., 2017). Some genera have foliar and bracteate bracts in addition to the basic involucral bracts (Ellison, 1989). The bracts are generally the same form as the leaf blades and have the similar appearance(Xu et al., 2017).

Sedges have a terminal inflorescence that may have a variety of forms (Xu et al., 2017). Cephalodium, Anthelodium, Corymbodium, Paniculodium, Stachyodium and Sciadodium are examples of inflorescences (Reutemann et al., 2012).Paniculodium, also known as panicula of spikelets, is a cone - shaped unspecified inflorescence with a terminal spikelet and long branches that hide the short branches, whereas Anthelodium, also known as Anthela of spikelets, is an undefined inflorescence with a terminal spikelet and long branches that cover up the short branches (Raole et al., 2011). The corymbodium is an indeterminate inflorescence with spikelets arranged in a pattern similar to corymb flowers.Similarly, sciadodium, often known as umbelliform inflorescence, is an ill-defined inflorescence with spikelets that look like Umbel flowers (Reutemann et al., 2012). Cephalodium, also called Capituliform inflorescence, is an undefined inflorescence that looks like a head, whereas stachyodium contains stalkless spikelets on the major axis (Reutemann et al., 2012).

Spikelet is the inflorescence's major unit, often known as the structure, that bears sessile real flowers (Karpana et al., 2021). Cyperaceae's inflorescence is made up of spikelets with different numbers of glumes that hold the flower, and the glumes can be grouped in spiral pattern or in two ranks (Karpana et al., 2021; Xu et al., 2017).

Cyperaceae flowers can be unisexual or bisexual, although they are predominantly monoecious (Xu et al., 2017). An ovule, three stamens, Perianth, and a single pistil with three carpels are the most common components of flowers (Goetghebeur et al., 1984). The fruit is an achene or nut, which is a sessile or free nut that can be covered by modified components like perigynium (Kern, 1972). The achenes come in a wide range of colours. shapes, and sizes. The form of nuts varies from ovate to elliptic, while some have circular and square nuts. White, black, grey, and brown nuts are the most prevalent, however greenish to bluish nuts may also be found in some family members (Jiménez-Mejías et al., 2011).

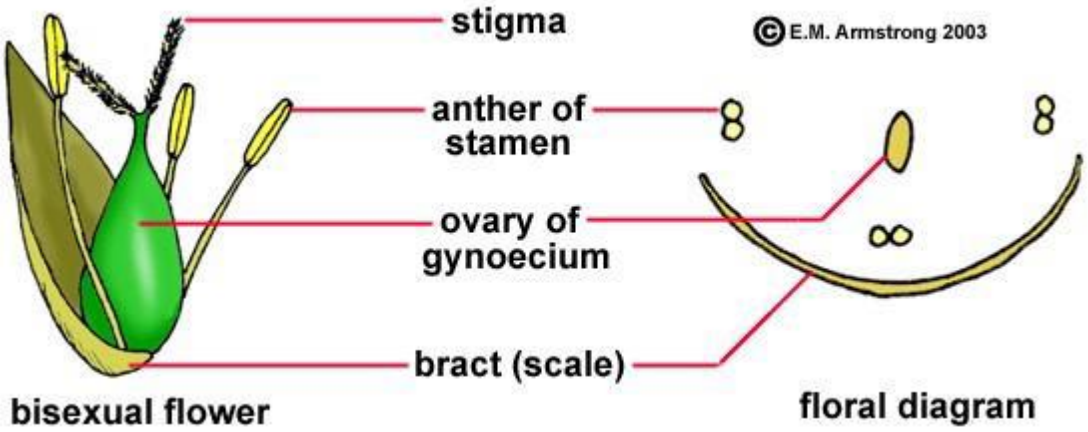

Figure 1.1. General structure of flower of Cyperaceae (adapted from http://www2.palomar.edu/users/warmstrong/termfl3.html)

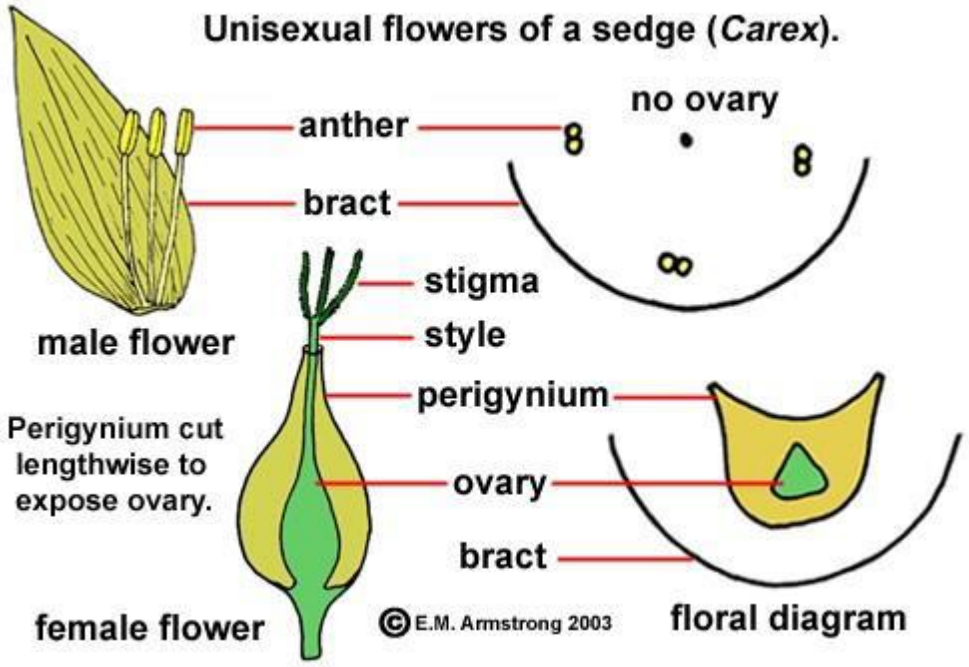

Figure 1.2. Male and Female flower of genus Carex of Cyperaceae (adapted from http://www2.palomar.edu/users/warmstrong/termfl3.html)

1.2.1. Micromorphology of Cyperaceae

In systematic investigations on Cyperaceae, micromorphological features, particularly those connected with reproductive structures, are regarded an essential criterion (Pignotti et al., 2004). Perigynia, Achene, epidermal cells of achenes, perianth bristles, glume surface, pollen morphology, and pericarp architecture are some of the micro morphological features used in systematic investigations on Cyperaceae (Karpana et al., 2021; Pignotti et al., 2004).

The nut has the following characteristics: Shape, colour, size, epidermal, and ornamentation microstructures of sedges are all different. These characteristics are more consistent, and so have a high level of dependability in Cyperaceae taxonomic research. In the Cyperaceae family, nut shapes range from obovate to ovate to elliptic to sharply triangular in section. Within a genus, the nut form is almost same; for

example, in the genus Cyperus, obovate to elliptic nuts are prevalent. Similarly, Eriophorum achenes are narrowly to widely obovoid and moderately to weakly trigonous, while achenes with a rounded to virtually square form are common in many Rhynchospora species. The achenes of the genus Fimbristylis, on the other hand, range from lenticular to trigonous to various morphologies such as obovoid, obovate, linear-oblong, or orbicular(Goetghebeur, 1998; Tucker et al., 1990). Based on these micromorphological study, (Xanthos et al., 2015) determined Schoenoplectiella lateriflora to be identical with Schoenoplectiella erecta. The structure of the cells, the features of the buttresseare, the properties of anticlinal walls, and the presence of silica bodies within the cell are all taxonomically significant elements of the achene epidermis (Wronska-Pilarek et al., 2010). The structure of the achene differs between species and at the infra-specific level. The morphology of the achene surface ranges from smooth to verruculose to tubercle, and some may be reticulate, smooth, or ornamented with puncticulate, verrucose, pitted, trabeculate, tessellate or zonate ornamentations. Bulbostylis achenes have a button-like structure generated by the persisting style base, whereas Scleria achenes have a disc-like or cup-like hypogynium. According to studies, Carex achene micromorphology is usually constant at the species level in most groups, although it has demonstrated to be of limited value in identifying taxa or separating related species in some groups(J. B.-A. J. of Botany et al., n.d.).

The pattern of leaf epidermal cells is quite constant across the family's several groupings. Leaf epidermal patterns in Cyperaceae are similar within genera, subgenera, and sections, according to (*Pollen Morphology and Plant Taxonomy : Soil Science*, n.d.) The stomata on the abaxial surface are paracytic, with two conspicuous guard cells and parallel oriented subsidiary cells in the Luzulae group of Cyperaceae (*Achene epidermis in the Carex flava complex (Cyperaceae) studied by scanning electron microscopy on JSTOR*,

n.d.). Similarly, on the adaxial surface of the leaves of Kobresia, there are many rows of paracytic stomata that are significantly thicker and have papillae (Rajbhandari et al., 1988). Sinuous anticlinal walls with raised or flat interlocking on both surfaces of the leaf have been seen in numerous additional genera of the family, including Fuirena, Scirpus, Carex, Eriophorum, and Cyperus (Rajbhandari et al., 1988). Stomata on cyperaceous leaves are often paracytic and organised in longitudinal lines parallel to the long axis of leaves. Many Mapanioideae have tetracytic stomata (which are irregularly dispersed in other taxa), as well as certain Schoeneae, Cryptangieae, and Trilepideae (Goetghebeur, 1998).

The arrangement of cells in the glumes can also be used to differentiate between genera. Trichophorum members, for example, feature glume surfaces with oblong, smooth cells with strongly wavy borders. In the same way, the Schoenoplectus genus is known for its glumes with oblong, nearly rectangular cells and virtually linear edges. The glumes of Scirpus feature a lot of cells with rough stripes in the perimedian area, which makes the glumes look ribbed ((Pignotti et al., 2004)).

Plant pollens are extremely useful in identifying and distinguishing between various plant species (Ellison, 1989). Features like as pollen sculpturing, aperture number, pollen shape, and proportion of viable pollens can be used to distinguish species based on pollen (Butt et al., 2018). The pollen characteristics reflect the changes in inflorescence structure. The pollen morphology correlates to the classification of the Carex genus into subgenera, according to (Wronska-Pilarek et al., 2010)(Vignea and Carex). Pollen feature variations have also been detected at the sub-familial level. Pollen grains of the Mapanioideae family have just one distal ulcus, although pollen grains in the Sclerioideae and Caricoideae families are comparable (mostly one distal ulcus and three lateral pores).Pollen from the Cyperoideae family comes in a variety of

forms(van Wichelen et al., 2010). According to (Reutemann et al., 2012), Cyperaceae has two types of pollen patterns: Cyperus and Carex. One has a distinct form of colpus (Cyperus type), while the other has one ulceroid aperture on the thick end and three laterals with a weakly defined poroid or elongated aperture on the lateral end (Carex type). The genus Cyperus has mainly elongated heteropolar pollen as shown in Cyperus rotundus, Cyperus alternifolius, Cyperus digitatus, subsferoid as in Cyperus bulbosus, Cyperus flavidus, Cyperus flavescens, pumpkin-shaped represent in Cyperus compressus, Cyperus pumilatus, and Cyperus iria (Butt et al., 2018).

1.3. Ecological importance

Sedges (Cyperaceae) play a significant role in various ecosystems all over the globe. Sedge ecology is seldom investigated, and there isn't a thorough analysis of it.

1.3.1. Chemistry and medicinal properties

The chemical composition of Lepidosperma tissues has not received much attention. Three Western Australian Lepidosperma species were examined by (Céspedes A et al., 2006), who discovered that none of them contained any alkaloids. L. concavum contained six distinct flavonoid compounds, albeit the specific components were not identified (Kukkonen, 1971). L. tortuosum contains quercetin, according to a study of the flavonoid chemistry in Cyperaceae (Williams et al., 1977). Later studies (B. Harborne et al., 1985) tested 16 species of Lepidosperma for the presence of flavonoids and found that flavonols, which include certain yellow aurone pigments and an anthocyanin-type pigment, carexidin, were present in large amounts in Lepidosperma species. Lepidosperma may be recognised chemically by the presence of tricin, quercetin, and isorhamnetin, although luteolin and aurones are often missing, with the latter present seldom in certain species, according to (B. Harborne et al., 1985). Pigmentation may be crucial for photoprotection, which is particularly true for animals

living in xeric settings (Steyn et al., 2002). Plants with flavonoids perform particular defence mechanisms that often deter herbivores from eating the plants (Morimoto et al., 2005). The discovery of two potentially allelopathic chemicals (cyperotundone and -cyperone) in Cyperus rotundus was explored by (Morimoto et al., 2005), although it is unknown if these substances are more prevalent in the Cyperaceae or whether they may be present in Lepidosperma species.The principal source of new pharmaceutical medications is still naturally derived products, and current medical research continues to place a high priority on the investigation of novel chemicals originating from plants (Allan et al., 1978). Aboriginal people in South Australia have reportedly used the stem bases of L. gladiatum and L. viscidum traditionally to treat colds (Carter et al., 2005). Researchers (Palombo et al., 2001; Semple et al., 1998) examined the antibacterial properties of these species and found that extracts of L. gladiatum and L. viscidum were both efficient against a range of Gram-positive bacteria. (Palombo et al., 2001) conducted further research on the most active species, L. viscidum, and found minimal effectiveness against vancomycin-resistant Enterococcus and methicillin-resistant Staphylococcus aureus (MRSA) (VRE). (Meilak et al., 2008) investigated L. viscidum for potential activity against Mycobacterium fortuitum and M. smegmatis, but no observable effect was found. The identification of stilbenes, including chemical analogues of resveratrol, has been examined in a variety of Carex species, including Scleria holoschoenus (Abdel-Mogib et al., 2001; González-Sarrías et al., 2011). Red wine's well-known component resveratrol has been linked to advantages for lowering the risk of heart disease and has been proven to have anti-cancer and anti-aging characteristics (González-Sarrías et al., 2011). The potential medicinal advantages of new stilbenes, which were recently discovered from a species of Lepidosperma in South

Australia, are the topic of continuing study (*Climate Change, Ecology and Systematics - Google Books*, n.d.).

1.3.2. Nutrient acquisition

The capacity of Cyperaceae to colonise a wide variety of environments is well known. Their capacity to draw phosphorus from nutrient-poor soils may help to explain why they are so common in arid shrublands and forests in Australia. Cyperaceae's cluster roots were the first to be described by (Renner, n.d.). (Wronska-Pilarek et al., 2010) observed the formation of these specialised root structures in Carex and mistook them for mycorrhizal structures. Further defining the root type as "dauciform roots" and characterising their capacity to improve nutrient absorption in Cyathochaeta, Gahnia, Lepidosperma, and Schoenus are (B. Lamont, 1982; LAMONT, 1974; B.B. Lamont, 1984; Byron B. Lamont, 1983). Recent research has shown the ability of many genera and species in the Cyperaceae tribes Schoeneae (including Lepidosperma) and Cariceae to develop such dauciform roots, and they may be present in all species in the tribe Schoeneae (Shane, Dixon, et al., 2005; Shane & Lambers, 2005; *SOIL AMINO ACID UTILIZATION AMONG SPECIES OF THE CYPERACEAE: PLANT AND SOIL PROCESSES - Raab - 1999 - Ecology - Wiley Online Library*, n.d.).

Fire and Disturbance Response

Persistency and reproduction are the main factors that determine sustainability in ecosystems that are vulnerable interruption or environmental changes. Plants that can resprout often invest more resources in below-ground biomass than in sexual reproduction after disturbances like fire or grazing, with lower seedling recruitment rates but longer generation durations (Bond et al., 2003). The vast majority of Lepidosperma species exhibit these traits. All Lepidosperma species are perpetual

clones, and the author has seen the majority of them in the wild. These findings suggest that clones may vary in size from L. inops, which can be 5 to 10 cm, to L. gladiatum, which can be over 10 m. These clones provide a robust supply that can withstand interruptions and dry periods. Although there is no information on the exact age of individual clones, a comparison of clone size to yearly growth rates indicates that most species of plants presumably survive for at least fifty years, and some clones are thought to be older than hundred years (Nuytsia et al., n.d.). Because of their lengthy lifespans and resistance to disturbance, Lepidosperma species may survive in harsh habitats with extremely low recruitment rates. They may become a highly important and stable part of the ecosystems in which they are found due to their tenacity.

1.3.3. Germination and Dormancy

The ‘seeds' of Cyperaceae are really nutlets, which are fruits with several carpels but only one seed; nevertheless, since they are so little and have only one seed, they are frequently mistakenly called achenes (Bruhl, 1995). A perianth is what these clones provide a robust supply that can withstand interruptions and dry periods, according to (J. C.-E. Botany et al., n.d.). Inflorescence development is the initial prerequisite for nutlet formation. Lepidosperma's inflorescences begin in the winter with the development of immature buds, go through a period of stasis throughout the summer, and then mature and blossom a few weeks after the first fall rains (or rarely after severe summer rainfall) (Nuytsia et al., n.d.). Western Australian species exhibit this pattern strongly, although eastern Australian species' timing is less reliable (Bruhl, 1995). The majority of Lepidosperma species grow many basal sterile bracts, a functionally male bloom, and a bisexual flower at the end of their spikelets, which are hermaphrodite and bisexual (Bruhl, 1995). In protogynous spikelets, the stigma of the bisexual bloom develops first, then the lower functionally male flower, whereas the bisexual flower's

stamens may open later or remain partially developed (Nuytsia et al., n.d.). In a few species, like L. jacksonense, each spikelet has two bisexual blooms (Nuytsia et al., n.d.). It is believed that all species are wind pollinated, and population genetic studies show that outcrossing is the primary means of reproduction for all species (M. D. Barrett and M. Wallace, unpubl. data).

1.3.4. Nutlet Removal and Dispersal

Lepidosperma nutlets' persistent hypogynous scales act as an elaiosome for the dispersal of ants(*Barton: The perianth and dispersal in Cyperaceae - Google Scholar*, n.d.; J. C.-E. Botany et al., n.d.). Ants, which gather dropped nutlets, and birds, who pick nutlets straight from the inflorescence, are likely responsible for local distribution, with birds most likely to be responsible for long-distance dispersal. According to (J. C.-E. Botany et al., n.d.), myrmechory is a crucial factor in the dispersion of these species and the preservation of habitat variety. According to (*Edgar: Indigenous Tracheophyta: Monocotyledones Except... - Google Scholar*, n.d.; Thorsen et al., 2009), L. australe's hypogynous scales at the base of its nutlets may become very spongy. which may help the nutlets be dispersed by water (hydrochory).

1.4. Economic Importance

There is little question that weeds inflict significant economic losses, but estimating their global effect, particularly in natural or non-agricultural regions, is challenging. More than $138 billion in economic damage is attributed to invasive species (pathogens, animals, and plants) each year in the United States (Pacini et al., 2018). Economic losses arise from agricultural and forest disruption or competition, as well as the price of pesticides, fuel, equipment, labour, cultural management methods, extra irrigation, and fertiliser (D. A. Simpson et al., 2001). Weeds, particularly sedges, incur

significant indirect economic losses globally, but it is more difficult to assess the increased expenditures for both human and animal health (i.e., allergies and toxins).

The significance of an agricultural weed may rely on a variety of factors, including soil type, climatic circumstances, the amount of viable propagules in the seed bank, pesticide and cultural management regimens, the abundance of the weed within the crop, and other variables (Báez-Lizarazo et al., 2017). Some weeds may be numerous and noticeable in crops without interfering with the development or production of the crops, such as winter annuals that germinate, emerge, blossom, and set seeds early enough to have no impact on summer crops' growth or productivity. In the southern United States, reduced-tillage cotton and soybean (Glycine max (L.) Merr.) often have high populations of Isolepis carinata Hook. & Arn. ex Torr. (Báez-Lizarazo et al., 2017). I. carinata has no negative effects on crop development and production since it completes its life cycle and passes away early in the growing season. The most significant weeds in agriculture are those that are hard to manage, compete with crops for nutrients, water, light, and space (David A Simpson et al., 2007b), lower the quality of seed and lint (Carter et al., 2005), or interfere with crop harvest efficiency. The most significant agricultural weeds in the world are listed in (*World Weeds: Natural Histories and Distribution - LeRoy Holm, Jerry Doll, Eric Holm, Juan V. Pancho, James P. Herberger - Google Books*, n.d.). The Composite List of Weeds from the Weed Science Society of America (WSSA, 1989) and Bayer AG's Important Crops of the World and Their Weeds (Schomäcker et al., 1992) are two organisations that keep lists of weeds. (Schomäcker et al., 1992) is a more extensive global list that contains over 5000 scientific names of crops and weeds, while the WSSA only identifies roughly 2000 weeds that are found only in the United States and Canada. The rights to the five-digit

"Bayer codes" for weeds have been sold to the European Plant Protection Organization since the publication of (Schomäcker et al., 1992), second edition.

The family Cyperaceae is widely known for its role in horticulture, ethnobotany, and the economy (*Simpson D. A., 2008. Frosted curls to tiger nuts:... - Google Scholar*, n.d.). Numerous sedges are used to make paper, perfumes, medicines, mats, boats, garments, shoes, ropes, and roofs, as well as foods, food additives, beverages, fibres, and animal poisons (A Muthama Muasya et al., 2009). Sedges are an essential source of food and fodder for both wild and domesticated animals (Dragon et al., 2009) Additionally, Cyperaceae are used to amend and enhance soil, reduce erosion, and restore vegetation following natural disturbances (A Muthama Muasya et al., 2009). Sedge species have characteristics that make them valuable for stabilising soil and preventing erosion, yet they are also weeds.

Justification of study

Cyperaceae is among largest families of monocots. Members of this family have very complex morphology because of its very small inflorescence. Many other plant family's members closely resemble to this family. So, because of these reasons, taxonomical study of cyperacea family is very challenging. This family is cosmopolitan.

Details characteristics of this family is available in Flora of Pakistan. However, many things are missing in Flora of Pakistan. Like many morphological features of its different members are missing in Flora of Pakistan. Phylogenetic studies based on molecular data is also limited in Pakistan. So, there is a need to conduct study on this family based on both its molecular markers and on its morphology as well.

The present study is focused on two species of cyperaceae that are *Carex caucasica* and *Cyperus iria*. They are collected from different areas of Pakistan.This study involves the imaging of morphological features and sequencing of molecularmarkers.

That will be very helpful to fill the missing gaps in Flora of Pakistan and formation of updated Flora of Pakistan which includes all the characteristic features and images of this family members. This will also helpful in improvement of Herbarium specimen of PMNHand sequence availability on NCBI database related to this family.

1.5. Objectives

The current study is focused on the morphological and molecular identification of both species Cyperaceae family; *Carex caucasica* and *Cyperus iria*

Objectives of the current study are;

Morphological and molecular identification of ***Carex caucasica*** **and** ***Cyperus iria***

1. Mounting of Herbarium Specimens and submission to Pakistan Museum of Natural History.
2. Light Microscope (LM) & Scanning electron microscope (SEM) Imaging of micro morphological characters.
3. Amplification of ITS, ETS, *rbcL* and *matK* genes for phylogenetic analysis

2. Review of the Literature

Cyperaceae comprises more than 5500 of its species in more than 100 genera and have almost cosmopolitan distribution (Govaerts et al., 2007). Almost 35% genera of the family are monotypic, 26% encompasses two to five species, and seven genera (6% of the family) with over 200 species, the largest being the *Cyperus* with about 700 species after Carex with 1757 species (A Muthama Muasya et al., 2009). Cyperaceae have a exterior resemblance to grasses and both are principally wind pollinated. On the other hand, molecular phylogenetic studies using plastid DNA sequencing demonstrate that Cyperaceae have a sister group relationship with Juncaceae (rushes) while Poaceae are more distantly related (Abraham M. Muasya et al., 1998). On the basis of morphological study, the family Cyperaceae subdivided into fourteen tribes and four subfamilies: Mapanioideae, Cyperoideae, Sclerioideae and Caricoideae (Goetghebeur, 1998); however, Simpson *et al.* (2007) change the division of subfamily on the basis of molecular phylogenetic studies utilizing plastid gene *rbcl* and put all the tribes into two subfamilies: Mapanioideae and Cyperoideae.

At the end of middle age, Tragus (1552) also known as one of 'German Fathers of Botany', applied Latin polynomial names to the Carex species but placing under grasses. Similarly, Lobelius (1576) described several Carex species under Latin polynomial naming. John Gerard (1597) was an English botanist famous for his book, Herbal (1633) about botany in which he described Carex as grass-like plant which is distinct from grasses for the very first time. Gerard described and illustrated several Carex species in Latin polynomial naming for instants: Carex flava L. as Gramen palustre echinatum, Carex diandra Schrank as Gramen cyperoides parvum, Carex leporina L. as Gramen sylvaticum minuls, Carex hirta L. as Gramen exile hirsutum, Carex panicudata L. as Gramen palustris cyperoides, Carex nigra L. Reichard as

Gramen cyperoides angustifolium najus, Carex pallescens L. as Gramen 8 cyperinum nemorosum and Carex rostrata Stokes as Gramen cyperoides. Towards the beginning of 17th century, John Ray provided the significant contribution in the study of Carex, by dividing plants into trees, herb, shrub, and then classified into monocot and dicot and further divided based on floral and leaf characteristics. He grouped 32 Carex species isolated from grass species as a major first attempt mentioned in his book, Historia Plantarum. In 1700, Toumefort first introduced the concept of genera and treated most of Carex species under genus Cyperoides. Two German and Italian botanists, Ruppius (1726) and Micheli (1729) first applied true sense of genus Carex to the species but mainly to distigmatic Carex. However, Linnaeus first designate binomial system to the genus and species in his book Fundamenta Botanica (1736). Linnaeus (1753) recorded 29 species in the genus Carex L. mentioned in the first edition of his book, Species Plantarum which were further increased to thirtyseven in second edition. Initially, Carex was divided into five sections based on inflorescence structure, which are: 1) single-spike, 2) androgynous-spike, 3) spikes either completely pistillate or staminate with pistillate spikes sessile, 4) spikes either completely pistillate or staminate with pistillate spikes peduncle, and 5) spikes either completely pistillate with many staminate spikes. In later treatment, these five sections were classified into three groups: monostachyae (including section 1), homostachyae (including section 2) and heterostachyae (including section 3-5). The first Carex species described by Linnaeus (1753) were Carex squarrosa L. and Carex folliculata L. which were collected by his student Peter Kalm during his visit to North America in 1749. Later on, Walter (1788), Lamarck (1789) and Michaux (1803) collected and described 11, 62 and 22 Carex species from North America, respectively. 9 By the end of the 18th century, Jussieu (1789) designated name Cyperaceae for Sedge family, and subsequently, number of

Carex species grew rapidly. Later on, Wahlenberg (1803) described 142, Schkuhr (1801) published 105 in his book part I and in part II (1806) 220, Agardh (1823) 328 species, Sprengel (1826) 267, Kunth (1837) 440, Steudel (1840) 524, and Boott (1840-1856) 550 species of Carex. Firstly, Kunth placed Carex in Caricineae (=Cariceae) which was the sixth tribe of family Cyperaceae. Further, systematic treatment of Carex included division at subgenus level by Schweinitz and Torrey (1825) into subgenus Vignea (distigmatic) and subgenus Carex (tristigmatic). Again, Kunth introduced 13 sections under subgenus Vignea and 20 sections under subgenus Carex. With the addition of large number of species, which indicates extreme diversity, the classification of Carex become much complex and was the challenge. Therefore, in the same regards, Tuckerman (1843) introduced with natural classification of Carex that opposed Linnaean system. He proposed five sections of Carex: I. Vignea, II. Psyllophorae, III. Vigneastrea, IV. Legitimae and V. Leptantherae, with some subsections. This classification was nearly accepted and still in use for instance sections Dioeceae, Nardinae, Pauciflorae, etc under these sections in the modern systematics. Drejer (1844) was the influencer of Lamarckian, and devised a natural classification system for Carex. Therefore, he was widely recognized as an evolutionary theorist since he proposed that the monostachyous are reduced from the heterostachyous. By the middle of 19th century, the work on systematic classification was progressing and in this regards, Miquel (1865-66), Boott (1858-67), Bockeler (1875-76), Maximowicz (1886), Franchet (1884-98), Hance (1871), and Leveille and Vanoit (1901-1904 mainly focus from Asia. Most of the work was done on systematics of Carex species derived from only two continents, European and North American. Pax (1887) recognizing this deficiency and started working on Carex classification from all major continents, and followed the Linnaean 10 proposed three major groups, viz. Monostachyae,

Homostachyae and Heterostachyae. Later on, it was Kükenthal (1892-1922) who contributed hugely in the history of caricology and published his work as Cyperaceae-Caricoideae in Engler's book, Pflanzenreich. Kükenthal (1909) proposed four subgenera: Primocarex (=Psyllophora), Indocarex (=Vigneastra), Eucarex (=Carex) and Vignea. He also recognized basically four genera: Carex, Kobresia, Schoenoxiphium and Uncinia. He believed that the subgenus Primocarex and genus Uncinia were descended through intermediate genus Kobresia from genus Schoenoxiphium and the rest of three subgenera (Indocarex, Eucarex and Vignea) were derived from Primocarex. Mackenzie described >500 species collected from North American and generally, agreed to Kükenthal, but discarded subgenera system of classification. Kükenthal classified 793 species based on gender distribution, inflorescence structure, number of spikes and branching and placed them under genus Carex (=tribe Cariceae). Bruhl (1995) provided more daring new classification and added fifth monotypic subgenus Vesicarex Steyerm. (Carex collumanthus Steyerm.) in the tribe Cariceae. By the end of 20th century, there were > 2000 Carex species collected all around the world (Goetghebeur, 1998). Kükenthal's classification was criticized by many caricologists (Hamlin, 1959; Kreczetovicz, 1936; Kern, 1958; Koyama, 1961; Nelmes, 1952; Ohwi, 1936), due to his treatment to unispicate species which he believed were distinct from the rest of species in the family. However, Kükenthal's classification was continued to modify (Chater, 1980; Haines and Lye, 1983; Egorova, 1999; Dai and Liang, 2000; Ball, Reznicek and Murray, 2002; Luceño et al., 2008; Dai et al., 2010; Hoshino et al., 2011) and finally, subgenus Psyllophora (Degl.) Peterm. (= subgenus Primocarex Kük.), subgenus Vignea (P. Beauv ex T. Lestib.) Peterm., subgenus Vigneastra (Tuck.) Kük. (= subgenus Indocarex Baill. Kük.) and subgenus 11 Carex (= subgenus Eucarex Peterm.) grouped in tribe Cariceae. This

traditional treatment at subgeneric level was mentioned and used in all the flora of genus Carex.

Larridon *et al.* (2021) said that Sedges are a perfect model family to study evolutionary biology, because of their diversity of species, widespread distribution, significant differences in lineage diversity, wide range of ecological preferences, and adaptations like multiple origins of C4 photosynthesis and holocentric chromosomes. The most current thorough categorization at tribal and generic levels was supplied by Goetghebeur's major work on Cyperaceae, published in 1998. It was based on a morphological analysis of Cyperaceae inflorescence, spikelet, flower, and embryo features, as well as anatomical and other information. Sanger sequencing data has now been used in a number of family-level molecular phylogenetic analyses that have been published. We provide the first family-wide phylogenomic research of Cyperaceae based on targeted sequencing utilising the Angiosperms353 probe kit surveying 311 accessions here, more than 20 years after the last thorough taxonomy of the family. Additionally, 62 GenBank-accessible accessions were searched for overlapping data and included to the phylogenomic analysis. A new categorization for the family at the tribal, subtribal, and generic levels is suggested in light of this backbone phylogeny. We provide evidence for the tribe Cryptangieae as a clade including the genus Koyamaea for the first time, in addition to the bulk of previously identified suprageneric groupings. We provide a taxonomic treatment that includes identification criteria and diagnoses for the 24 tribes, 10 subtribes, and 2 subfamilies, as well as fundamental details on the 95 genera. Anthelepidinae, Caustiinae, Gymnoschoeninae, Lepidospermatinae, and Oreobolinae are five new subtribes that are included in the taxonomy for the tribe Schoeneae.

Glon *et al.* (2017) determined that Fuireneae is unnatural group and paraphyletic in nature as compared to Cypereae on the basis of molecular phylogenetic studies performed by maximum parsimony, maximum likelihood and Bayesian analyses by using five marker gene; ITS, *trnL* intron/ *trnL-trnF* spacer, *ndhF, rbcL* and *matK. Isolepis humillima* was also transfered to *Schoenoplectiella humillima* after the analysis. Villaverde *et al* (2017) confirmed *Schoenoxiphium* clade as monophyletic and suggested to place this clade in *Carex* sect. *Schoenoxiphium Baillon* on the basis of phylogenetic reconstruction executed by maximum likelihood and Bayesian analyses using four marker region, IT'S, ETS, *matk* and *rps16*. Wangwasit *et al.* (2017) did both molecular (using ITS region) and morphological phylogenetic analysis on the tribes of subfamily Cyperoideae to position newly found species *Fimbristylis fusiformis* in Thailand. The superficially similarity was discovered between *Fimbristylis fusiformis* and *Fimbristylis pauciflora* due to morphological convergence as these species are not present in sister related clades in tree.

Jiménez-Mejías *et al.* (2016) globally classified genus *Carex* on the basis of molecular phylogenetic studies by using combine analysis of three marker region; ITS, ETS and *matk* to overcome the difficulties of morphological classification. The trees were constructed by using maximum likelihood analysis based on 996 (50.23%) of the 1983 accepted species of *Carex*. It was concluded that the polyphyly and paraphyly are the key reason of the global diversity of *Carex.* Ito *et al.* (2016) conformed the non-monophyly of three varieties of *Isolepis fluitanssensu; Isolepis fluitanssensu* var. *fluitans* from Africa and Europe, *Isolepis fluitanssensu* var. *lenticularis* from New Zeeland and *Isolepis fluitanssensu* var. *nervosa* from Ethiopia on the basis of molecular phylogenetic studies. Maximum parsimony, maximum likelihood and Bayesian analyses were performed by using four marker gene three from plastid DNA; *rbcL,*

rps16 and *trnL*, and one from nuclear DNA; ITS to construct a tree. It was concluded that the species are not monophlytic instead illustrated close relationship to their respective geological species. Musili *et al.* (2016) conducted molecular phylogenetic analysis based on 195 species of tribe Schoeneae to attest the polyphyly of genus *Schoenus*. Maximum parsimony and Bayesian inference were carried out for tree building by using nuclear DNA region, IT'S sequence data.

Villaverde *et al.* (2015) verified that the bipolar distribution of *Carex arctogena* (*Carex* sect. *Capituligerae*, Cyperaceae) is foremost reason of long distant dispersal by birds using molecular phylogenetic analysis. There were 55 population of *C. arctogena* and 36 population of *C. capitata* included in the study and tree was obtained by using combination of maximum likelihood and Bayesian inference. The sequence data of three plastid DNA region; *atpF-atpH, matk* and *rps 16* and one nuclear DNA region ITS were utilized with combination of achmatic data for all the species used in study. Starr *et al.* (2015) executed maximum parsimony and Bayesian analysis to present evidence for three lineages: Hypolytroides Clade sister related to the Siderostictae Clade, and for Dissitiflora Lineage and Small Core Carex Clade as consecutive sisters related to roughly 1400 species in the Core Carex Clade. It was verified the polyphyly of subg. *Vigneastra*. The DNA marker gene of ITS, ETS-1f, *rps16, matk*, and *ndhF* sequence datasets were employed for study. Gebauer *et al.* (2015) estimated molecular phylogenetic relationship of *Carex* sect. *Racemosae* using maximum likelihood, maximum parsimony and Bayesian inferences. The combine nuclear and plastid DNA region; ITS, ETS, *trnK-matK* and *rps16* sequence datasets were exploited to create tree. The analysis also attested the non-monophyly of sect. *Racemosae*. Castro *et al.* (2015) performed dated molecular phylogeny to uncover the origin of Cyperus *esculentus*. The tree was acquired on 70 sample by employing sequence datasets of DNA marker region;

ndhF, rbcl, rps16 and ETS-1f. The possible ancestral ranges of *Cyperus esculentus* were reconstructed by statistical dispersal vicariance (S-DIVA) and Bayesian binary method (BBM) analyses.

Léveillé-Bourret *et al.* (2014) observed Trichophorum clade as sister clade of Cariceae by using plastid gene *matK* and *ndhF* for phylogeny. The tree was constructed by maximum parsimony analysis and covered 55% species other than Cariceae. The reduction and proliferation of inflorescence were examined throughout the core clade Cariceae Dulichieae-Scirpeae (CDS) while the chromosome numbers were increased in *Scirpus* and *Eriophorum* clade. Mausya *et al.* (2014) did both morphological studies of nutlet and molecular analysis of three chloroplast genes; *rbcl, trnL-trnF* and *rps16,* and two nuclear DNA genes; ETS and ITS sequence datasets. Tree was built by Bayesian inferences. The 140 years old mystery that nutlet do not have gynophore was solved and *Cyperus clandestinus* was placed in C4 *Cyperus* clade near to *Remirea maritima* and *Cyperus cyperoides* on the basis of their study. Reid *et al.* (2014) studied molecular systematics of genus *Cyperus* by exploiting ITS marker on 95 samples covering 85 taxa. The clade of C4 photosynthetic group was recuperated and the paraphyly of genus *Cyperus* was confirmed other than *Cyperus* subgenus *Diclidium* which is monophyletic in nature. Maximum likelihood and Bayesian analysis were applied to draw tree. Sheils *et al.* (2014) estimated the phylogenetic relationship of genus *Schoenoplectus* and *Schoenoplectiella* by using ITS, *trn, trnl-trnF* and *ndhF* sequence datasets. Maximum parsimony, maximum likelihood and Bayesian analysis were exercised to assemble tree. It was concluded that the genus *Schoenoplectus* is monophyletic in nature and sisterly related to genus *Actinoscirpus* whereas genus *Schoenoplectiella* is paraphyletic in nature and sisterly related to genus *Pseudoschoenus*. Escudero *et al.* (2014) declined hybrid origin hypothesis of *Carex* on

the basis of molecular phylogenetic analysis using GBS (Genotyping by Sequencing) and SNPs. The tree was constructed by applying maximum likelihood and Bayesian inference. The D-statistic test for historical introgression and partitioned RAD (Restriction-site Associated DNA) was exploited for comparison of analysis. Britton *et al.* (2014) did topography on the basis of morphological and molecular phylogenetic analysis through Bayesian inferences. In the high elevation sedge *Tetraria triangularis,* the non-ecological' speciation driver role was observed and five cryptic or semi-cryptic lineages of Miocene-Plioceneages which succeeds as evolutionary species were found. Minimally three of the said, sustain their differentiation in sympatry, distinguishing as a biological species.

Escudero and Hipp (2013) estimated threefold overall swing raise in the rate of diversification of sedges and the species richness was associated with climate change via dated molecular phylogeny. The sequence datasets of three marker region of plastid DNA; *rbcl, trnl*, and *trnl-trnf* were utilized to assemble tree by Bayesian inferences. The study illustrated around 33% variation in diversity of Cyperaceae species as in angiosperms. Jiménez-Mejias and Martinetto (2013) characterized fossil fruit of Carex for taxonomy on the basis of morphological analysis. The characters of both live and fossil sample of achenes were exploited to draw tree by means of unweighted pair group method with arithmetical mean (UPGMA). The study determined that *Carex* paleotaxonomy was exercised to categorize genus *Carex* into subgenus or sections. Jung and Choi (2013) pointed out major groups of subfamily Cyperoideae on account of molecular phylogeny which are valuable for establishing the major lineage of family. The molecular datasets of ITS, 5.8S, *rbcl, trnL* and *trnL-trnF* were brought into play to obtain robust tree by the practice of maximum parsimony and Bayesian inference. The 81 genera and 426 species of sedges were analyzed in the study. Martin-Bravo *et al.*

(2013) evaluated the taxonomic status of *Carex rainbowii* found in shady places and positioned it in *Carex* sect. *Sylvaticae* as it was closely related to *Carex Sylvaticae* on the basis of molecular and morphological analysis. Three molecular datasets; trnK, ETS and ITS were exercised to acquire tree by Bayesian inferences while the morphological analysis was based on qualitative and quantitative characters.

Zahidullah *et al.* (2013) uncovered the phylogentic relationship within species of genus *Carex* found in Pakistan at subgeneric and sectional level. Wild samples of 11 species of *Carex* L. and 2 species of *Kobresia* as outgroup (Table 2.1) were collected from the concerned area of taxa and scrutinized to acquire trees (Figures 2.1, 2.2, 2.3 and 2.4). The chloroplast marker region *matk* was amplified for each sample and utilized in the study. The sequence length of each amplification were varied from 753 to 1360 bp with the 15.08% of nucleotides variation rate. The obtained sequences were aligned and analyzed by maximum parsimony, maximum likelihood, neighbor joining and UPGMA (unweighted pair group method with arithmetical mean). The 15 to 27.5% pairwise divergence of sequence were found among *Kobresia* and *Carex* whereas 8.6% within genus Carex. The pattern of nucleotides substitution was estimated (Table 2.2) and the frequencies of change were 25% for each. All the trees were compared and it was concluded the significance of molecular datasets of *matk* gene for the classifications and tackling systematic problems.

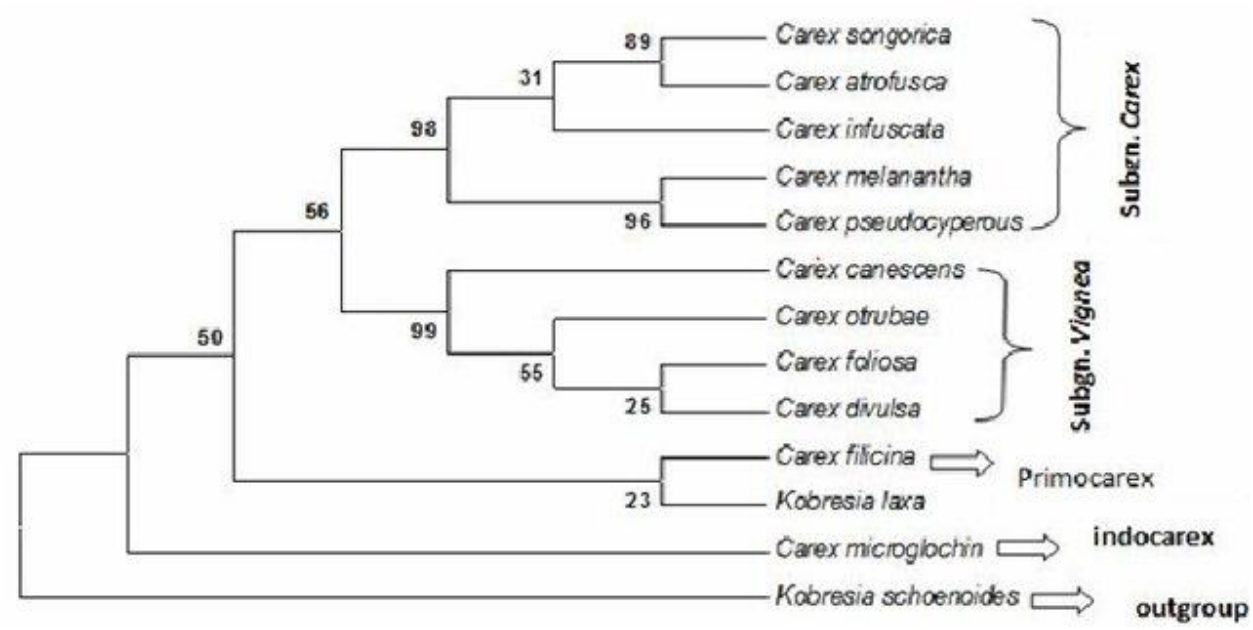

Figure 2.1: Strict consensus parsimonius tree (Zahidullah et al. 2013).

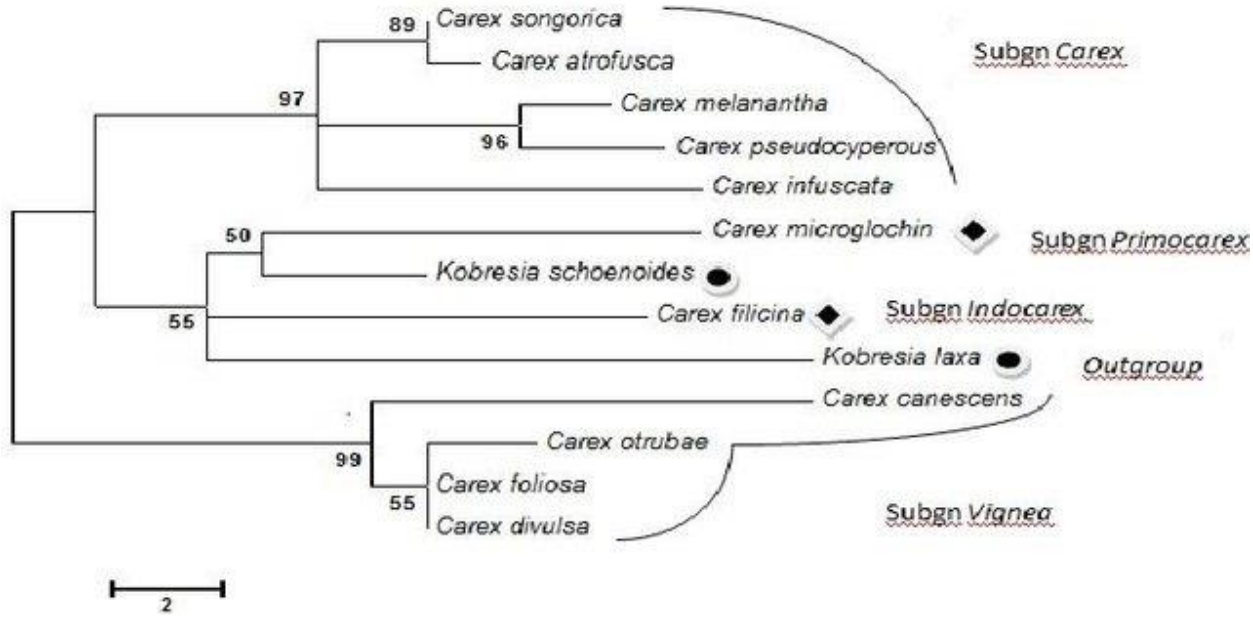

Figure 2.2: Tree Formed by Maximum parsimony method (Zahidullah et al. 2013).

Larridon *et al.* (2013) classified C4 Cyperus genus by means of molecular datasets of ETS-1f, *rpl32-trnl* and *trnH-psbA*. The 107 specimens were analyzed to construct tree

which demonstrated all the phylogenetics information (Figure 2.2) by using maximum likelihood and Bayesian analysis methods. The study used for the revise of classification and characters evaluations of genus *Cyperus*. Larridon *et al.* (2011) performed both molecular and morphological phylogeny for taxonomical changes in C3 Cyperus and discovered new clade of C4 Cyperus as well.

Muasya M.A *et al.* (2009) claimed that during the last 10 years, attempts to rebuild the Cyperaceae's suprageneric phylogeny have increased. We analyse 262 taxa from 93 genera and 15 tribes that were sequenced for the plastid rbcL and trnL-F genes (intron and intergenic spacer). Mapanioideae and Cyperoideae are the two clades that make up the monophyletic Cyperaceae, and the general topology is consistent with findings from earlier research. While Cryptangieae, Bisboeckelerieae, and Sclerieae are resolved within Schoeneae within Cyperoideae, Trilepideae is sister to the other taxa. Resolution of Cladium, Rhynchospora, and Pleurostachys into clades sister to the remainder of the Schoeneae provides evidence in favour of the classification of these species as distinct tribes. In the meantime, we continue to include these species in the Schoeneae genus. This research presents the first evolutionary positions of 40 species in 21 genera, explaining their positions in the Abildgaardieae (Trachystylis), Cryptangieae (Didymiandrum, Exochogyne), Cypereae (Androtrichum, Volkiella), Eleocharideae (Chillania), and Schoeneae families (Calyptrocarya, Morelotia). To clarify connections between Fuireneae and Schoeneae, additional sampling effort (more taxa and the use of more quickly changing markers) is required.

SIMPSON A.D *et al.* (2007) said that Significant new data have been released since the Monocots II conference in 1998, expanding our systematic understanding of the Cyperaceae family. The family's phylogenetic investigations have also made steady progress. In this work, all presently known rbcL sequences for the Cyperaceae family,

including information for two new taxa, were used in a parsimony analysis. Seven of the 14 tribes (Bisboeckelereae, Cariceae, Cryptangieae, Dulichieae, Eleocharideae, Sclerieae, Trilepideae) and one of the four subfamilies (Caricoideae) are monophyletic. If Hellmuthia is regarded as a member of Cypereae, as suggested by the data, then subfamily Mapanioideae and tribe Chrysitricheae are monophyletic. Other aspects of our analysis include well-supported clades for Trilepideae and Sclerieae-Bisboeckelereae, a potential close relationship between Cryptangieae and Schoeneae, polyphyletic tribes for Schoeneae and Scirpeae, the presence of Cariceae within the Dulichieae-Scirpeae clade, and a clade representing Cyperus and related genera Such patterns agree with findings from earlier research using DNA sequence information. One result might be the identification of only two subfamilies, Mapanioideae and Cyperoideae. Researcher activities must be properly organised and require a great deal of additional labour. The investigation should concentrate on gathering morphological and molecular information for each of the family's genera.

Simpson *et al.* (2003) found put the phylogenetic relationship using both morphological and molecular analysis of *trnL-trnF* and *rps16* and in (2006) exploiting *rbcL* gene carried out the basic classification of Cyperaceae by analyzing 167 species representing all 14 tribe. All tribes parted into two subfamilies instead of four on the basis of study. Maximum parsimony methods was applied for the construction of mega tree.

Starr *et al.* (1999) using ITS, (2003 and 2006) employing ETS and ITS, (2007) via *rbcL* and (2009) by *rbcL* and *matk* did phylogenetic analysis of genus Carex to resolve the problems of the classification of sedges.

Muasya *et al.* (2001, 2002, 2006 and 2009) exploited *rbcl, rps16, trnl and trnl-trnF* genes for the classification and phylogeny of Cyperaceae.

First time, Muasya *et al.* (1998) used molecular phylogenetic analysis for Cyperaceae. The 80 species from 40 genera were analyzed by using chloroplast DNA region rbel. Tree was drawn through maximum parsimony analysis method.

Phylogeny of Cyperaceae

Cyperaceae was previously thought to be related to the Poaceae family, however molecular phylogenetic analyses revealed that it is monophyletic with Juncaceae (Plunkett et al., 1995). These two groups are classified as Cyperales based on these findings (Starr et al., 2015). The ancestral prerequisite for the divergence of the Cyperaceae and Juncaceae families is Prionium from the Thurniaceae family (Plunkett et al., 1995).

(Goetghebeur, 1998) divides Cyperaceae into three subfamilies: Sclerioideae, Mapanioideae, and Caricoideae. Sclerioideae is divided into Cryptangieae, Tribe Fuireneae, Trilepideae, Tribe Cypereae, Eleocharideae, Schoeneae, Cryptangieae, Caricoideae, Bisboeckelereae, Trilepideae, Sclerioideae, and Sclerieae. The subfamily Mapanioideaes' morphological data is used to classify the species. (A. Muthama Muasya et al., 2002), on the other hand, divided the Cyperaceae family into two subfamilies: Mapanioideae and Cyperoideae. Mapanioideae is monophyletic with Cyperaceae, however non-monophyletic Cyperoideae and Sclerioideae are classified in a different group called Caricoideae.

According to (Waterway et al., 2007), the Cariaceae tribe demonstrates strong monophyly together with two additional monophyletic lineages, Carex subgen. Carex and Vigneastra and subgen. Vignea, although the Carex, sect. Hymenochlaenae, and sect. Physocarpae are polyphyletic. However, the phylogeny of the Cyperaceae's major genera has long been perplexing and uncertain. The finding of little sister tibe

Sumatroscirpeae, which explains the patterns of inflorescence, diversification, and biogeography of Carex, revealed the missing link in the evolutionary connection of Carex (Léveillé-Bourret et al., 2018).

Based on morphological examination of Kranz anatomy paired with genetic data based on rbcL, rps16 intron, trnL intron, and trnL-F markers, the second biggest genus Cyperus has been separated into two subgenera: Cyperus and Anosporum. These subgenera's origins were shown to be polyphyletic(A. Muthama Muasya et al., 2002). The Cyperus genus includes the polyphyletic C3 Cyperus and the monophyletic C4 Cyperus, as well as nine other C4 Cyperus genera: Alinula, Ascolepis, Lipocarpha, Kyllinga, Pycreus, Queenslandiella, Remirea, Sphaerocyperus, and Volkiella, which are mostly monophyletic and can form a subgenera in the Cyperus (Larridon et al., 2013a; Vrijdaghs et al., 2009).

Fimbristylis belongs to the Abildgaarddieae tribe, while the species Abildgaardia is connected to the Bulbostylis and Nemum genera (A. Muthama Muasya et al., 2009). The tribes Arthrostylideae and Abildgaardieae are closely related, however Bulbostylis is not related to Abildgaardieae (Ghamkhar et al., 2007). The genus Schoenoplectus is monophyletic in nature and sisterly connected to the genus Actinoscirpus, according to genetic study based on various markers, however the genus Schoenoplectiella is paraphyletic in nature and sisterly related to the genus Pseudoschoenus (Shiels et al., 2014). Based on molecular analyses, the genus Scirpus is classed as a polyphyletic genus (Christensen et al., 2013a).

According to genetic, morphological, and embryological evidence, the genus Cladium forms a distinct lineage from tribe Schoeneae and is classified in tribe Cladieae (Semmouri et al., 2019).

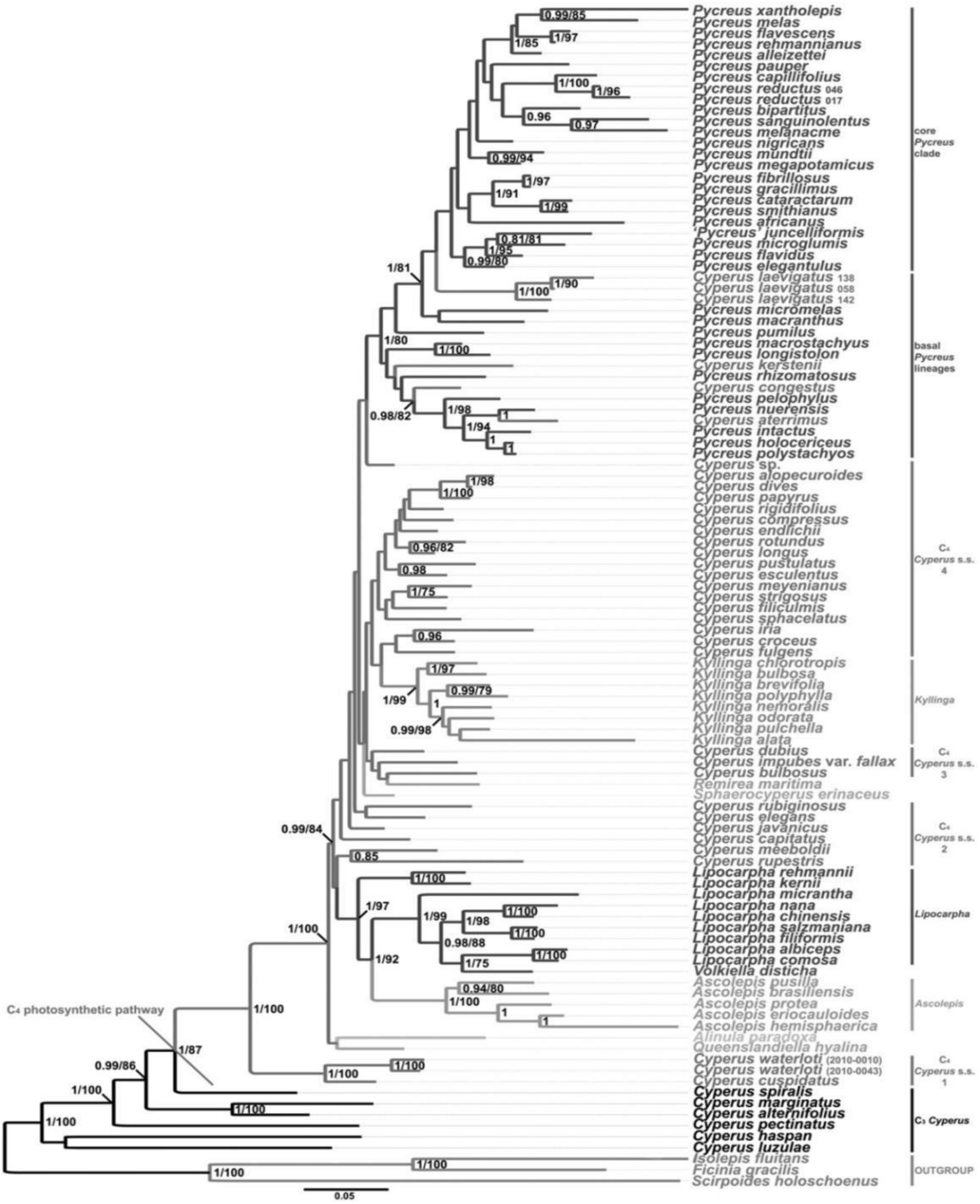

Figure 2.3: Phylogenetic analysis of genus Cyperus clade with C3 and C4 species and further species from the nine other genera within Cyperaceae (revised from (Larridon et al., 2013a))

3. Material and Methodology

One of the significant plant families is the Cyperaceae, or sedge family. Sedge species may be found all over the globe, although they are particularly common in tropical and subtropical areas. This essay will concentrate on 2 species that were gathered from various regions of Bannu, Pakistan. This research examines the morphological and molecular characteristics of the gathered species as well as their phylogeny.

3.1 Sample collection

Sample collection was performed from June to September 2021. Some species were collected in their early flowering season while some were collected at their maturity season. These samples were collected from the peaks of mountainous area and along water channels. Species were collected from Kurma River and Thughul khel village of District Bannu, Khyber Pukhtunkhwa. Soil digger was used to dug out complete plant along with their roots, inflorescence and leaves were separately placed in a zipper bag for morphological and molecular analysis. Location of the collected species were noted with the help of GPS device.

3.2 Preparation of Herbarium specimens

After collection, samples from various sites were packed in rough drawing charts for moisture absorption and then compressed between two hardwood sheets to prevent fungus attack. The Pakistan Museum of Natural History (PMNH), in Islamabad, followed normal methodology for the preservation of the samples that were obtained. After using sticking glue to mount the preserved species on herbarium sheets and obtaining an accession number for each species for future use, the conserved species were then sent to PMNH.

3.3 Analysis of Morphological characters

light and stereomicroscope Morphological examination was carried out in the lab using the IRMECO Models (IM-900 and IM-SZ-500). With the use of needles, the inflorescence was dissected on a glass plate to separate the various components, including the glume, nut, anther, pollen, and perigenium. After examining under a microscope, permanent slides of the different inflorescence components were made using Canada- Balsam for further usage. For identification, the physical characteristics were compared to the Pakistani Flora.

3.4 Analysis of Micro-Morphological characters

The inflorescence's many components were subjected to a micro-morphological study using a scanning electron microscope (SEM). Forceps were used to transfer certain inflorescence components, such as the glume, anther, nut, and perigenium, to the aluminium stubs, whereas pollen was directly stabbed. Incubator or ultrasonicator were used to remove moisture from the surface, while pollen glume and anthers were directly stabbed on the aluminium stabs. Different methods were used to remove dust particles from the surface of different parts of the inflorescence, such as 70% ethanol or chloroform for surface sterilisation of nuts and utricles (Cyperaceae et al., 1983). Following gold coating, samples were fed into a SEM apparatus to examine the surface patterns of various components, and a TESCAN-VEGA3 (LMU) camera was used to take photos at various scales.

3.5 DNA Extraction

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

3.5.1 Reagents

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

3.5.2 Procedure

CTAB method with slight modifications was used extract DNA from collected samples. 1-2 dried leaf samples were used and grind with liquid nitrogen in the autoclaved mortar and piston. Liquid nitrogen is frequently used for grinding dried leaves because it helps in cell wall rapturing and helps to make fine powder of the sample. Pre-heated CTAB along with 10% beta marceptaethanol (denatures different phenolic compounds) was added to grinded sample in the mortor and allowed it to melt. After melting, CTAB with powder sample was transferred to 1.5 ml Eppendorf and kept in shaker incubator or incubator with regular shaking after 5 minutes for 30 minutes at 65^{o}. Equal concentration of 24:1 chloroform: isoamyl alcohol was added after cooling for 3-5 minutes. After mixing for 5 minutes, centrifuged it at 13000 rpm for 10 minutes. After centrifuging, three layers were formed and carefully removed the tubes from the centrifuge so that the layer should not mix. The upper clean layer (supernatant) was picked with the help of pippete and transfer it to new autoclaved Eppendorf. White threads of DNA were visible upon the addition of ice chilled isopropanol and stored over night at -20^{o} at this step. Centrifuged the precipitated DNA at 13000 rpm for 10 minutes next day. A thin pallet of DNA was formed at the bottom of Eppendorf. Removed the supernatant and add 700ul of 70% ethanol to it, suspended the pallet in 70% ethanol for 30 minutes. After 30 minutes, centrifuged at 10000 rpm for 5 minutes. Removed the supernatant and invertly kept the eppendrof on a clean surface for 30 minutes to completely dry the pallet. After drying, add 50-100 ul TE buffer or distilled

water and spined it to dissolve the pallet completely by using minicentrifuged and then stored at -20^{o} for further use (Yoon et al., 2008).

3.6 DNA Quantification

Quantification of DNA was done with thermo Fisher Scientific Nanodrop 2000.

3.6.1 Procedure

On the lower optical pedestal of the sample retention system of NanoDrop, lul of clean distal deionized water was added and closed the lever arm in way that the upper surface was come in contact with water. The lever arm was lifted and both the surfaces were cleaned with clean and dry lab wipe. NanoDrop software was opened and the "Nucleic Acid" application was selected. The Iul of TE buffer (the same buffer used to suspend DNA) was dispensed on the lower optical pedestal and lever arm was closed. The "Blank" option was selected to calibrate blank measurement for sample and the constant for dsDNA was selected. The lower and the upper optical surfaces were cleaned by new clean and dry lab wipe. Again lul of clean distal deionized water was added and closed the lever arm in way that the upper surface was come in contact with water. The lever arm was lifted and both the surfaces were cleaned with new clean and dry lab wipe. The lul of sample was dispensed on the lower optical pedestal and lever arm was closed. The "Measure" option was selected in the application and the reading was noted. The lower and the upper optical surfaces were cleaned by new clean and dry lab wipe. Again lul of clean distal deionized water was added and closed the lever arm in way that the upper surface was come in contact with water. The lever arm was lifted and both the surfaces were cleaned with new clean and dry lab wipe (Desjardins et al., 2010).

The DNA with higher concentration was diluted with TE buffer to make concentration 100ng/ml.

3.7 PCR Amplification

Four markers (ITS, ETS, rbcL, and matK) were amplified using PCR using sets of universal barcoding primers. The reaction mixture was made in the traditional manner by combining all the components. The reaction mixture was diluted to a final volume of 25 ul, and amplification was carried out using a BioRad thermocycler. The PCR conditions varied a little for each marker and species. The first heating for ITS was 4 minutes at 94oC, followed by 45 seconds of DNA denaturation at 940oC. For all the plant species studied, the 45s primer annealing temperature was 50oC. The elongation took 1 minute at 720C, while the final termination took 5 minutes at 720C. The reaction was repeated for 35 times. The first heating for ETS was 5 minutes at 94oC, followed by 30 seconds of DNA denaturation at 94oC. The primer annealing temperature is 30s, ranged from 51 to 52^0C. The extension was done at 72^oC for 60 seconds, and the ultimate termination was done at 72^oC for 5 minutes. For 35 cycles, the reaction was carried out. The first heating for rbcL was 4 minutes at 94°C, followed by 30 seconds of DNA denaturation at 94°C. For all species, the 30s primer annealing temperature was 50^oC. The extension took 60 seconds at 72 degrees Celsius, while the final termination took 5 minutes at 72^0 C. For 32-35 cycles, the reaction was carried out. matK was initially heated to 95°C for 1 minute prior to getting denaturated at 95°C for 45 seconds. For all species, the 45s primer annealing temperature was 55^0C. The elongation took 60 seconds at 72^0C, while the ultimate termination took 3 minutes at 72^0C. A total of 35 cycles were used to complete the reaction.

3.8 Gel electrophoresis

Gel electrophoresis techniques were used for the PCR results detection. In most of situations 2 % gel was used usually, however in a few, a 1 % gel was also used. For a

2 % gel, 2.0 g and 2 g of commercially available agarose powder were mixed to 100 ml of TAE buffer. The TAE buffer containing agarose was heated for 1:30 seconds. 7ul of ethidium bromide was added to the gel mixture when it had cooled, and it was then placed into the casting tray with combs fastened. After the gel had formed, it was added to the gel tray that contained the buffer. The same amount of sample and the loading dye were mixed, and 4ul of sample and 100bp of DNA ladder were added to the wells. The gel was run for 27 minutes at 120V. The gel was examined using a UV trans illuminator and a gel documentation system.

3.9 PCR products purification

The PCR products were purified using ExoSAP IT, a purification enzyme that is available for purchase. This enzyme eliminates any residual single-stranded primers and excess single-stranded DNA from the PCR product. 2ul of ExoSAP IT and 5ul of PCR product were heated at 37°C for 15 minutes before the enzyme was inactivated at 80°C for 15 minutes.

Table 3.1: Primers along with their sequences used for PCR amplification

Region	Primer	Sequence	Reference
ITS	Forward (17SE-f) Reverse (ITS-4r)	5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3' 5'-TCCTCCGCTTATTGATATGC-3'	(Sun *et al.*, 1994)
ETS	Forward (ETS-1f) Reverse (18S-r)	5'-CTGTGGCGTCGCATGAGTTG-3' 5'-AGACAAGCATATGACTACTGGCAGG-3'	(Starr *et al.*, 2003)
rbcL	Forward (rbcL-af) Reverse (rbcL-ar)	5'-ATGTCACCACAAACAGAGACTAAAGC-3' 5'-CTTCTGCTACAAATAAGAATCGATCTC-3'	(Kress and Erickson, 2007)
matK	Forward (matK-2.1f) Reverse (matK-5r)	5'-CCTATCCATCTGGAAATCTTAG-3' 5'-GTTCTAGCACAAGAAAGTCG-3'	(Starr *et al.*, 2009)

Table 3.2: Volume and concentration of reagents used for PCR reaction

Sr.	**Reagent**	**Concentration Volume (added in mastermix)**	**Volume (added in mastermix)**
1	10X buffer (-$MgCl_2$)		2.5ul
2	MgCl2	50mM	1.5ul
3	dNTPs	2mM	2.5ul
4	Forward Primer	10 µM	0.5ul
5	Reverse Primer	10 µM	0.5ul
6	BSA		1ul
7	DMSO		1ul
8	Taq Polymerase		0.2ul
9	DNA Template	100 ng/ml	1ul
10	PCR water		14.03ul
Total volume			25ul

3.10 Sequencing of PCR products

Purified PCR products were delivered to Macrogen Inc. Korea for sequencing, and the sequences were used in further research.

3.11 Phylogenetic analysis

The phylogenetic analysis was performed using Geneious version R10.

3.11.1 Data collection

The sequences obtained after sequencing were compared to sequences in the GenBank database using the Blast programme. The sequences with the greatest similarity were retrieved for future use. The sequences were given the new names:

Accession number-Species name-Country name

3.11.2 Alignment of sequences

The renamed sequences were aligned using the alignment tool in the Geneious programme. The following inclusion/exclusion criteria were used to choose the sequences:

i. Short enough sequences were removed.
ii. We cut long sequences.

The nearest genera of the species under study were selected as the outgroup. It was decided to use Cyperus iria as the outgroup for Kyllinga brevifolia (Larridon, Bauters et al. 2013). For Pycreus sanguinolentus and Pycreus flavidus, Cyperus rotundus, Kyllinga appendiculata, and Cyperus laevigatus were selected as the outgroups for the ITS, rbcL, and ETS markers, respectively (Christensen et al., 2013b; Larridon et al.,

2013b). Fimbristylis bisumbellata was selected as the outgroup for Eleocharis atropurpurea.

3.11.3 Phylogenetic tree construction

The Maximum Likelihood trees were made using Geneious' PhyML software plugin. The bootstrap analysis made use of 100 replicates. The Neighbour Joining trees were made using the Geneious programme. The bootstrap analysis made use of 100 replicates. The Geneious plugin was used to do the Bayesian analysis.

4 Results

4.1 Carex caucasica

Locality: Tughul Khel Bannu

Voucher Specimen Number:

4.1.1 Morphological Identification

The morphological characters of Carex caucasica are explained in table 4.1.

4.1.2 Micro-morphological Analysis

The micro-morphology of Carex caucasica was done by using scanning electron microscopy (Figure 6, 7).

4.1.3 Molecular Identification

The molecular identification of Carex caucasica was done by using ExternalTranscribed Spacer (ETS), Internal Transcribed Spacer (ITS genes amplification.

4.1.4 Phylogenetic Analysis

Phylogenetic analysis was performed on basis of the sequences obtained from sequencing. The obtained sequences were aligned with the sequences of same genus. Phylogenetic analysis is based on Bayesian, Neighbor Joining, and Maximum Likelihood. The alignment of Carex caucasica ETS region sequences with other sequences of same genus is given in Figure 8 respectively. Then the trees were constructed on bases of this alignments. Firstly, trees were constructed for

the species identification purpose based on ETS region. (Figure respectively). Carex caucasica appeared in the same clade with the other species of Carex caucasica collected from the different areas of the world. Clades for ETS are well supported because they are showing the bootstrap valueof 77 and posterior probability 1. Then trees were constructed for confirmation ofspecies geographical location like what is exactly the origin of species. To confirm either the specie is native of Pakistan or migrated from some other region. For ETS regions geographical trees are given in (Figure 9, 10, 11 respectively). Clades for ETS are well supported because they are showing the posterior probability of 1 and bootstrap value of 77.

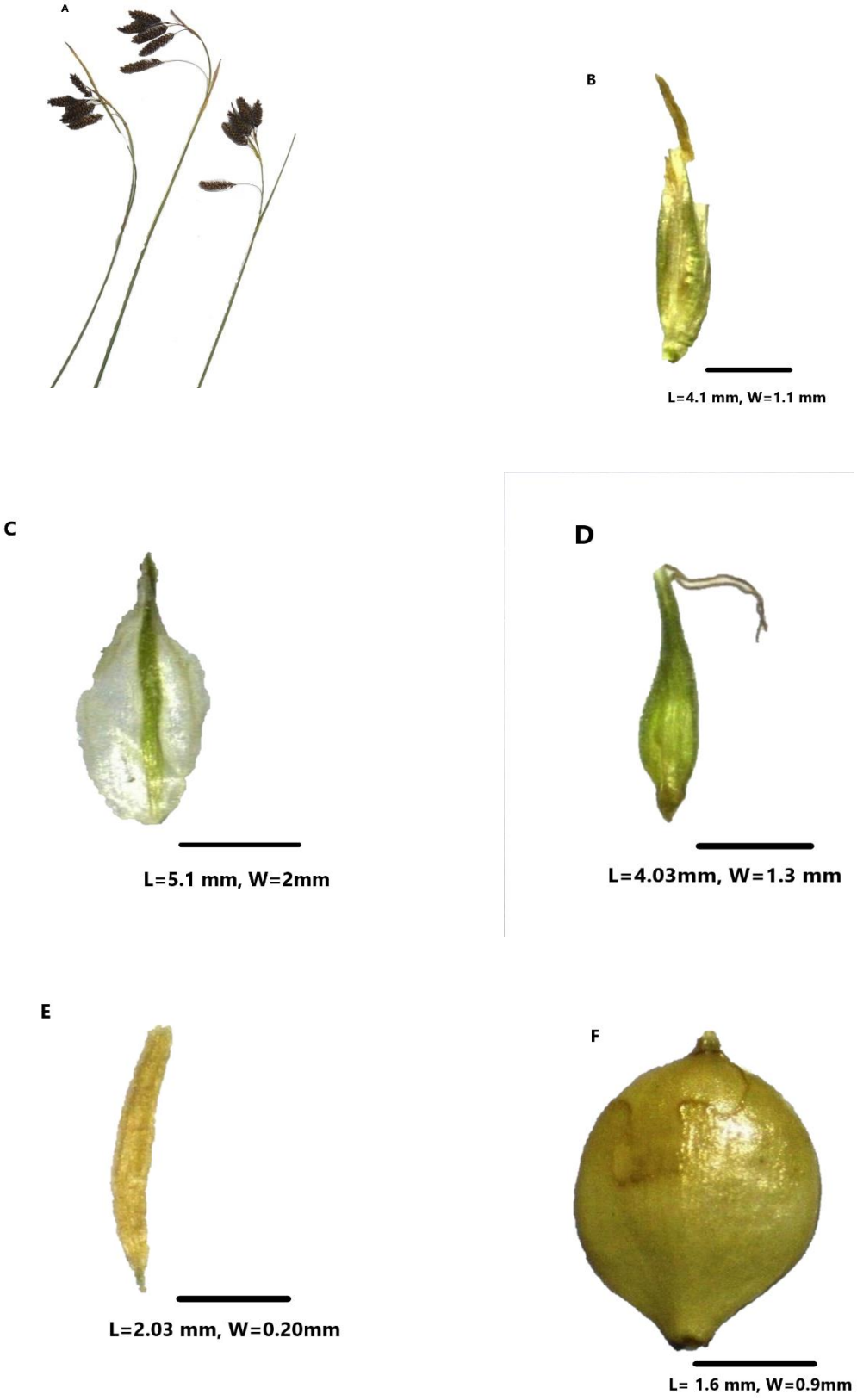

Figure 4.1: Illustration of newly explore flora, Carex caucasica in detail. First picture is inflorescence (A) Male Flower; (B) Female glume; (C) Utricle; (D) Anther; (E) Nut

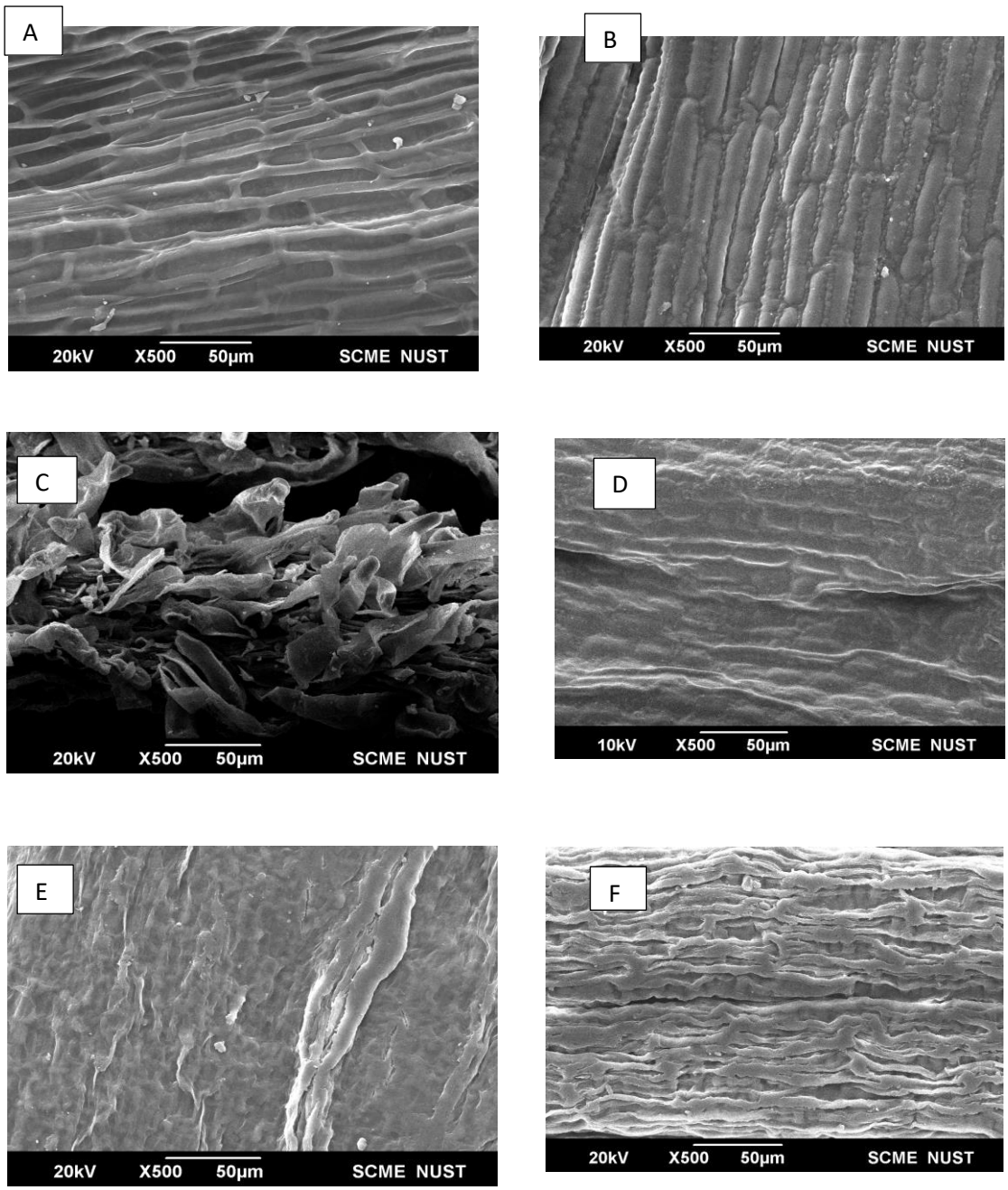

Figure 3: Micromomorpholgical features of Carex caucasica (A) Female glume (B) Male glume (C) Stigma (D) Utricle (E) Nutlet (F) Anther (G) Pollen (Scale: A-E;50µm g;10µm)

Table 4.1: Morphological & Micromorphological features of C*arex caucasica* Linnaeus

Collection Area	Kurram River
Synonyms	
Common name	White Sedge
Habitat	Wetlands (inland), Forest, Shrubland, Grassland
Voucher number	043883
Characters	**Observed Character State**
Plant Height	20-45 cm
Rhizome	
Rhizome	Short
Root length	12 cm
Appearance	Fibrous
Stem	
Appearance	Papillose and scabrous
Shape	Sharply trigonous
Diameter	
Color	Greyish green,
Leaves	
Leaf sheaths	soft, grey, margin of scarious side concave

Number	
Length	sheaths to 90 mm
Color	White
Appearance	Smooth, Shorter than stem
Shape	Straight
Margins	Smooth
Leaf blades	Wide, carinate, grey-green, papillose
Bracts	
Appearance	Green, narrow
Size	Much longer than its spike
Ligule	
Ligule	Present (0.5 mm)
Inflorescence	
Shape	3-7 gynecandrous spikes
Inflorescence Length	25-50 mm
Spikes Length	5-8.5 x 3-4 mm
Flower	Unisexual
Glume	

Male glume	1.5-2.3 x 1.5-1.7 mm Midnerve green, scarious (Figure 4.3 B)
Female glume	1.5-2 x 1.3-1.5 mm, cymbiform, acute, midnerve green or yellow (Figure 4.3 C)
Utricle	
Shape	ellipsoid to ovoid, plano-convex (Figure 4.3 D)
Size	4.3 x 1-1.3 mm
Color	Green to Yellow (Figure 4.3 D)
Stigma	2 stigmas (Figure 4.3 D)
Beak	obscure, conical, with scabrous
Anther	2.03 x 0.20 mm yellowish color, single lobbed (Figure 4.3 E)
Nut	1.3-1.6 x c. 0.9 mm, ovoid, lenticular, apiculate, light brown to brown, finely striate papillose (Figure 4.3F)

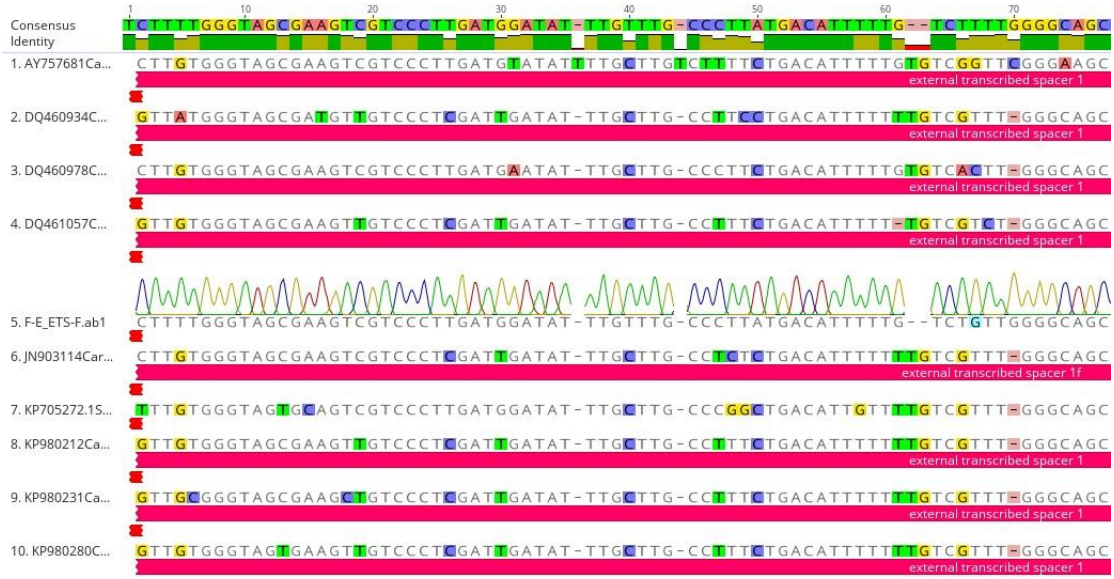

Figure 4.3: Sequence Alignment of ETS sequence of *Carex caucasica*

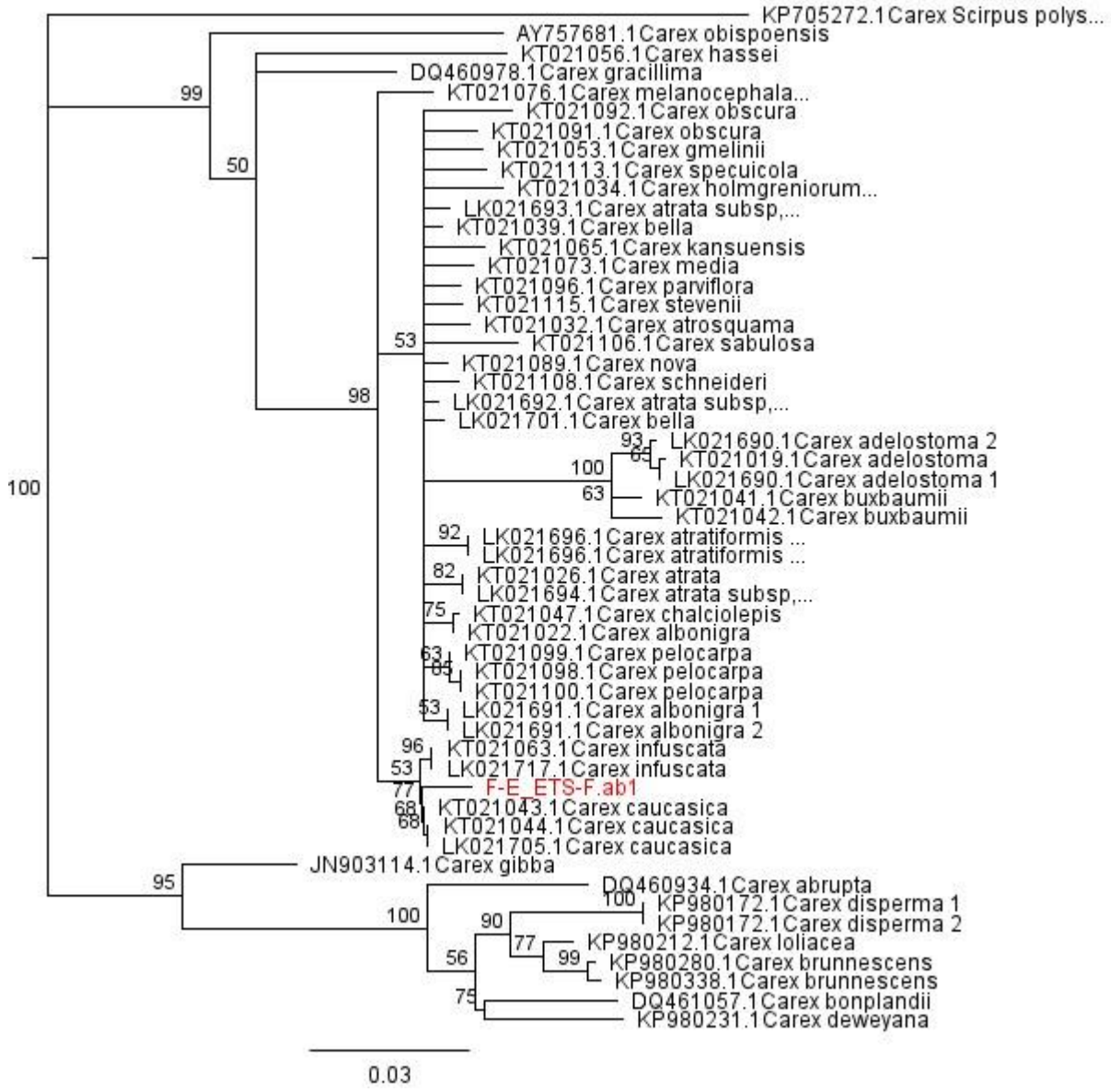

Figure 4.4: Neighbor joining tree of Carex caucasica based on ETS region by using geneious prime software

Figure 4.5: Maximum likelihood tree of *Carex caucasica* based on ETS region by using Rx x ML algorithm in geneious prime software

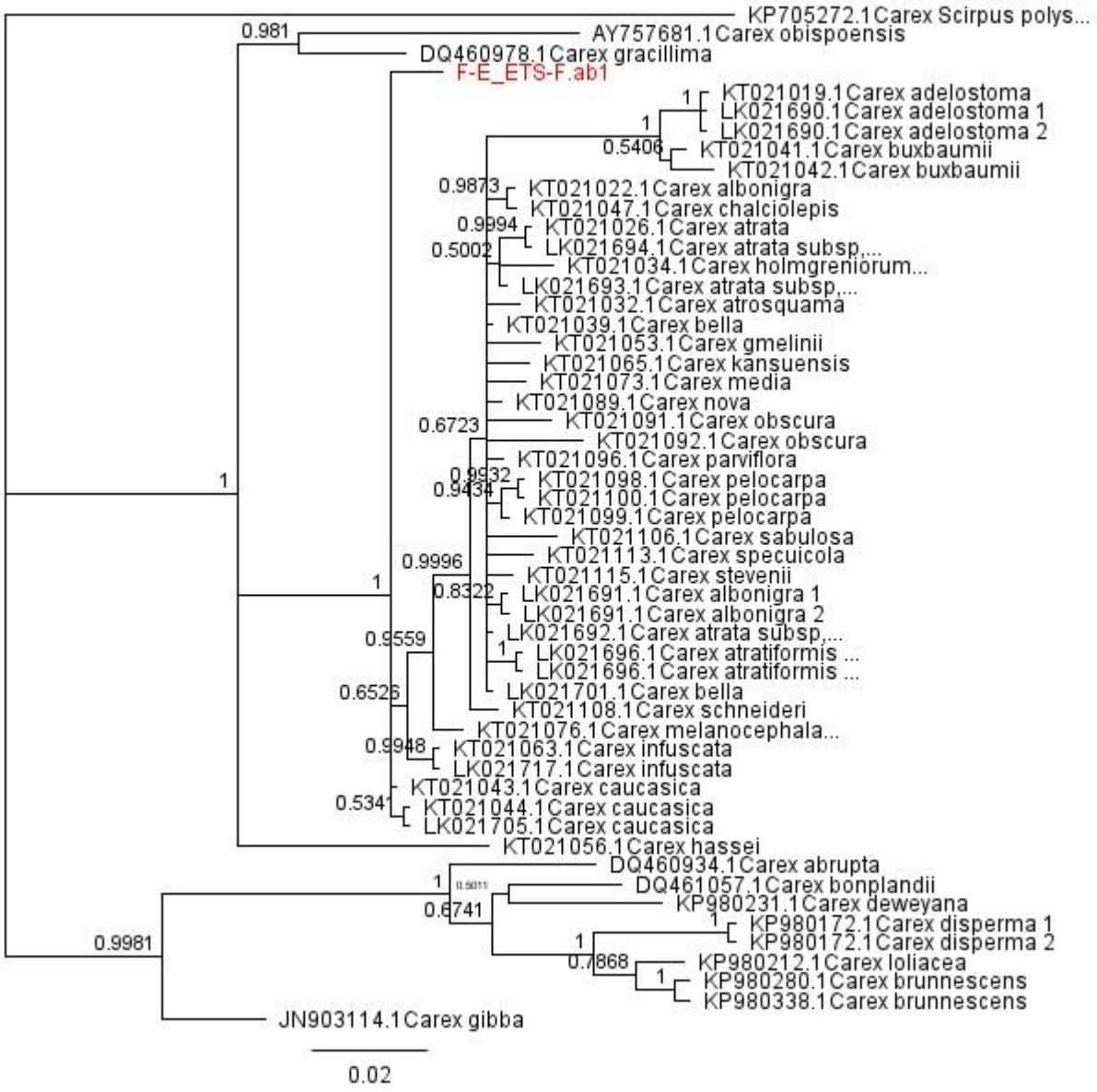

Figure 4.6: Bayesian tree of Carex caucasica based on ETS region by using Mr. Bayes algorithm in geneious prime software

4.2 Cyperus iria

- **Other Names:** Rice flat sedge and rice flatsedge
- **Locality:** Tughul khel Bannu

Voucher Specimen Number: -

4.2.1 Morphological Identification

The morphological characters of Cyperus iria are explained in Table 4.1 (Figure 4.3).

4.2.2 Micro-morphological Analysis

The micro-morphology of Cyperus iria was done by using scanning electron microscopy (Figure).

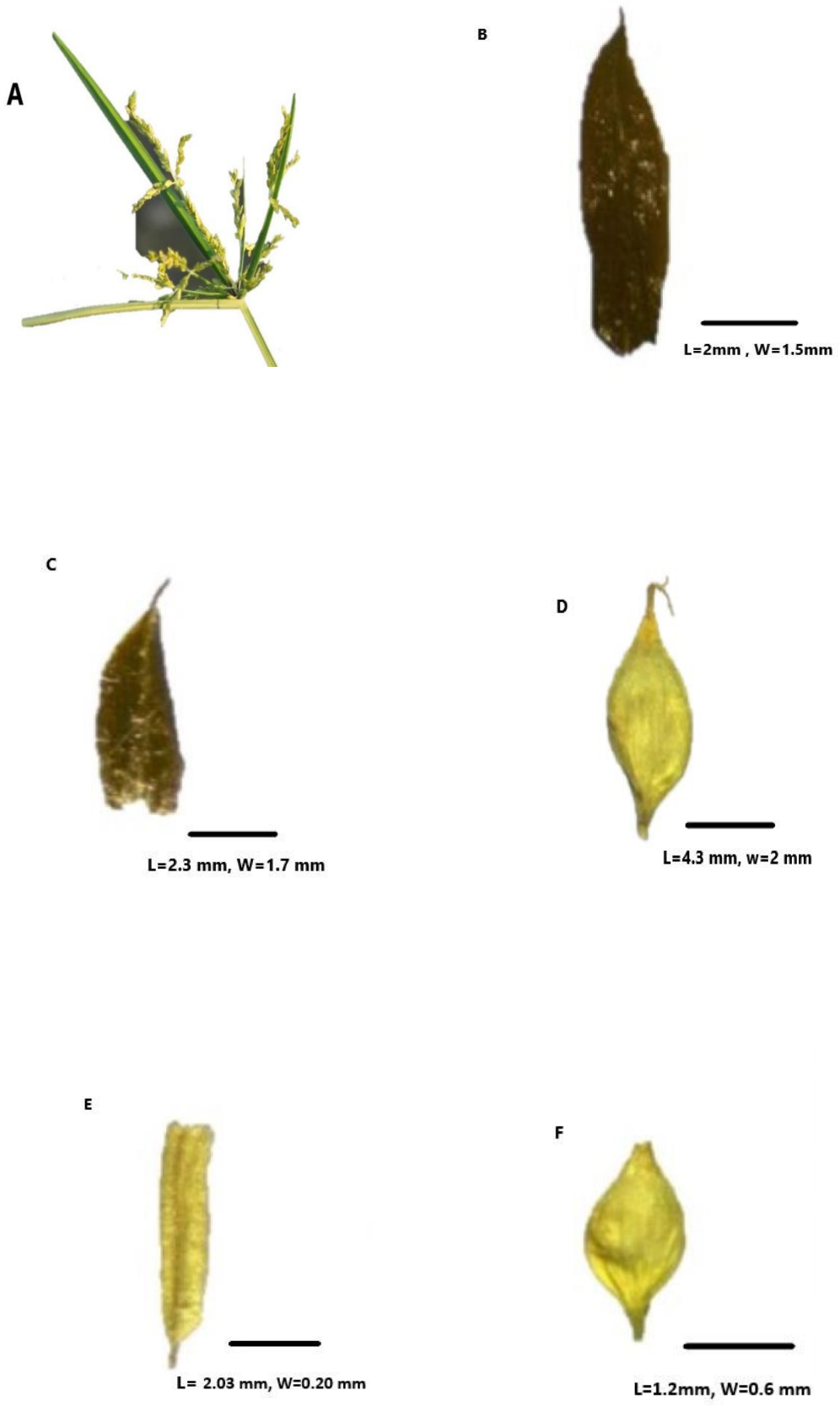

Figure 4.7: (A) Infloresence, (B) Female Glume, (C) Male Glume, (D) Utricle, (E) Anther, (F) NUT

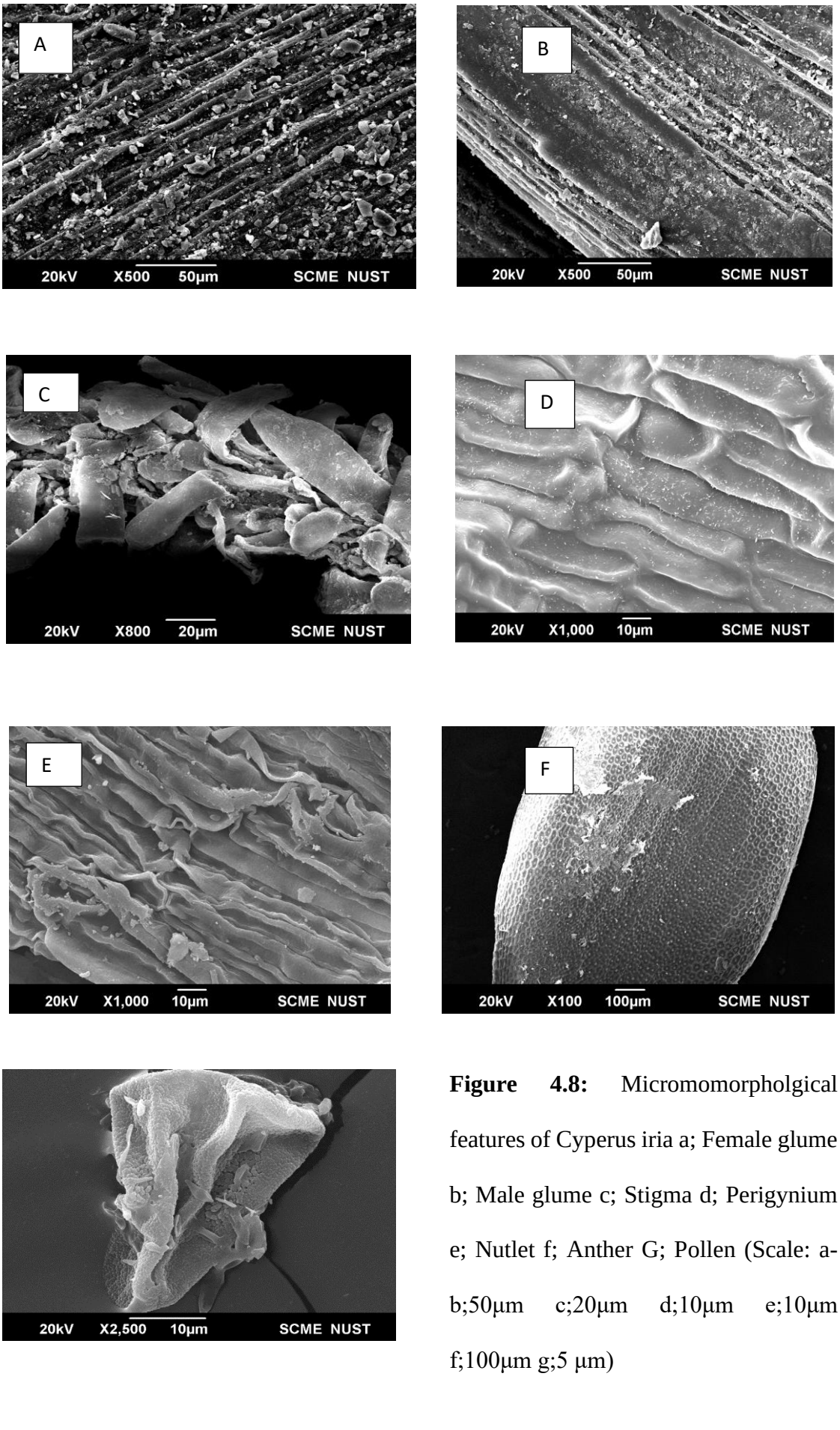

Figure 4.8: Micromomorpholgical features of Cyperus iria a; Female glume b; Male glume c; Stigma d; Perigynium e; Nutlet f; Anther G; Pollen (Scale: a-b;50µm c;20µm d;10µm e;10µm f;100µm g;5 µm)

Table 4.2: Morphological & Micromorphological features of Cyperus *iria* Linnaeus

Collection Area	Tughul khel Bannu
Synonyms	Rice flat sedge and rice flatsedg.
Common name	Grasshopper's Cyperus, Ricefield Flatsedge, Rice Flatsedge, Rice Flat Sedge, Umbrella sedge
Habitat	Wetlands (inland), Forest, Shrubland, Grassland
Voucher number	---
Characters	**Observed Character State**
Plant Height	20-70 cm
Rhizome	
Rhizome	Small
Root length	12 cm
Appearance	Fibrous
Stem	
Appearance	Papillose and scabrous
Shape	Trigonous, smooth
Diameter	2-3 mm diam
Color	Greyish green,
Leaves	
Leaf sheaths	Soft, Yellow brown, sometimes reddish tinted, finely brown dotted, Mouth margin straight

Number	
Length	sheaths to 20 cm
Color	White
Appearance	Smooth, Shorter than stem
Shape	Straight
Margins	Margins slightly revolute, margins and keel towards the apex scabrous, apex acute, scabrous
Leaf blades	wide, flat, keeled, green or greyish green
Bracts	
Appearance	Green, narrow
Size	Much longer than its spike
Ligule	
Ligule	0
Inflorescence	
Shape	compound anthelodium, 5-20 cm
Inflorescence Length	25-50 mm
Spikes Length	3.5-5 x c. 2 mm
Flower	Unisexual
Glume	

Male glume	1.5-2.3 x 1.5-1.7 mm glume-like prophyll scarious, bi-nerved (Figure 4.3 B)
Female glume	1.5-2 x 1.3-1.5 mm, cymbiform, acute, midnerve green or yellow (Figure 4.3 C)
Utricle	
Shape	ellipsoid to ovoid, plano-convex (Figure 4.3 D)
Size	4.3 x 1-1.3 mm
Color	Green to Yellow (Figure 4.3 D)
Stigma	3 stigmas (Figure 4.3 D)
Beak	obscure, conical, with scabrous
Anther	2.03 x 0.20 mm yellowish color, single lobbed (Figure 4.3 E)
Nut	Nut c. 1.2 x 0.6 mm, rather sharply trigonous, ellipsoid, brown or dark brown, finely papillose. (Figure 4.3F)

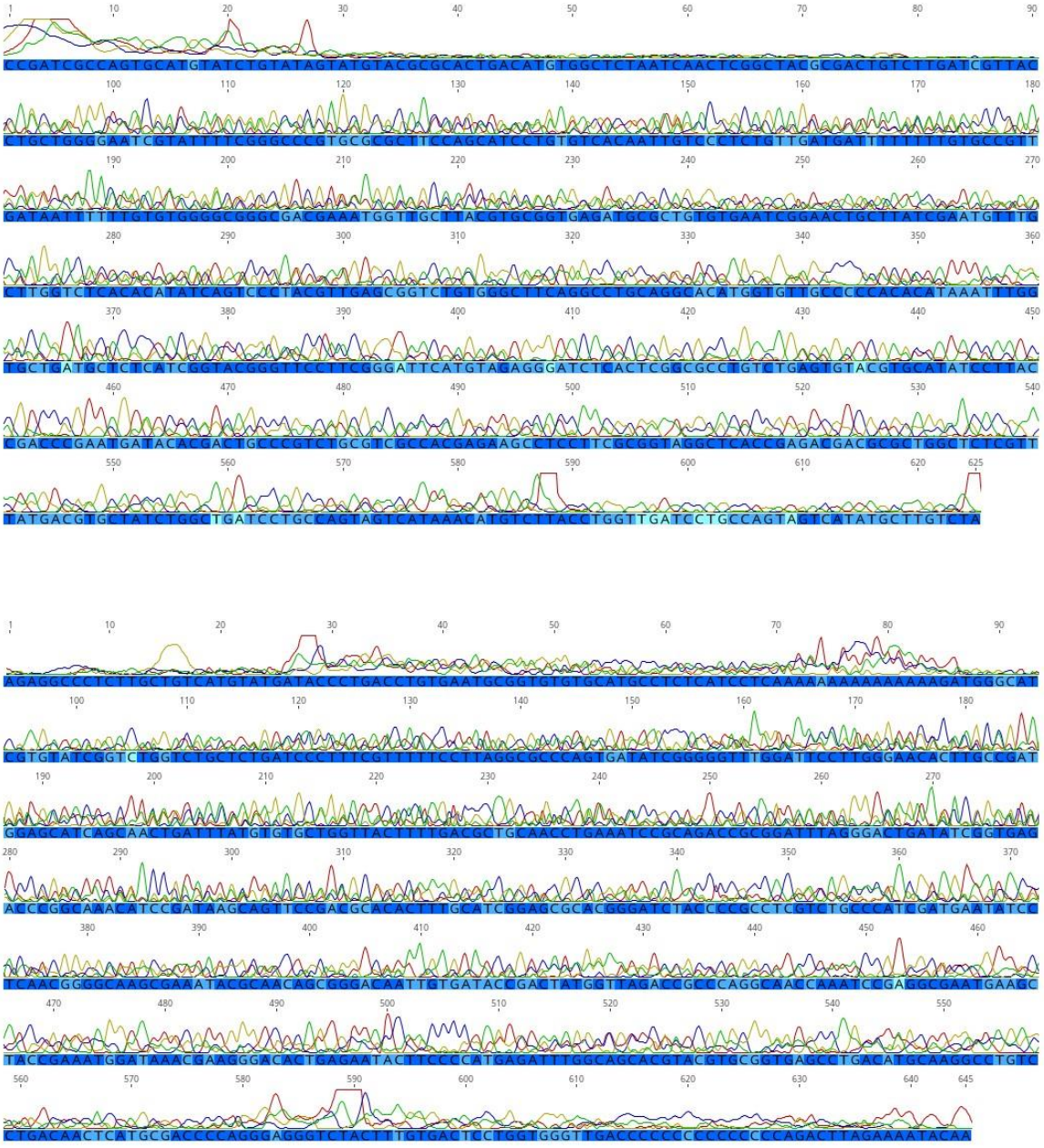

Figure 4.9: Sequence Alignment of ITS sequence of *Cyperus iria*

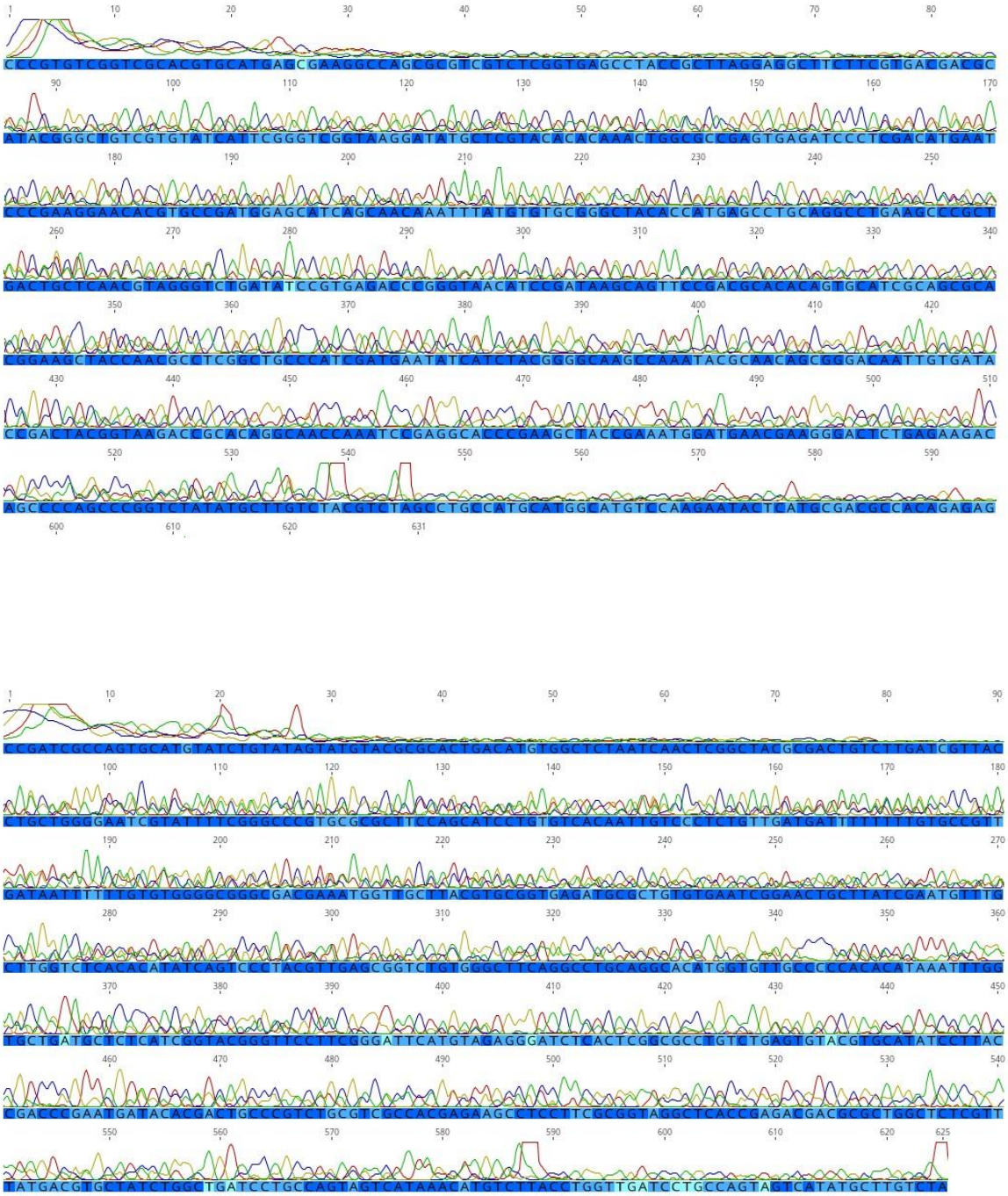

Figure 4.10: Sequence alignment of ETS sequence of *Cyperus iria*

5 Discussion

Cyperaceae (Sedges) are found all over the world. It is a monophyletic family. Among monocots it is the third largest family after Poaceae and Orchidaceae, and seventh among angiosperms (A Muthama Muasya et al., 2009). Cyperaceae comprise of 104 genera and 104 genera contain around 5000 species (Goetghebeur, 1998). Cyperaceae distribution is cosmopolitan, but they are concentrated in tropical areas. Carex genera, containing about 2000 species. The Global Carex Group 2015, 2016). In reference to distribution Carex is one of the world's largest genera. And it has great significance in seasonally damp habitats and wetlands around world (Goetghebeur, 1998). Present study was focused on three species of Cyperaceae carex caucasica and Cyperus iria. In this study species were analyzed on molecular and morphological bases. In morphology, morphological and micromorphological characters were observed by using compound microscope and scanning electron microscope respectively. Herbarium specimens were also prepared for two species. Specimen of carex caucasica were submitted to PMNH and accession number were obtained. In molecular studies 2 genes ITS, ETS were amplified. PCR products were sequenced by sending PCR products to Macrogen. Sequence of carex caucasica were submitted to NCBI and accession number were obtained. Plant specimens were collected from different areas of Pakistan from where they were not already reported. Carex caucasica was collected from Kurram river and Cyperus iria was collected from Tughul khel. These species were not already reported from these areas of Pakistan. So, these two species were new to flora of Pakistan and Pakistan Museum of Natural History (PMNH).

5.1 Analysis of Morphological Characteristics

The morphological characters of Carex caucasica and Cyperus iria are in accordance with the Flora of Pakistan. Some of features are not available in Flora of Pakistan. The features that were not available in Flora of Pakistan they were compared with Flora of China. Colored illustration for this family is not available before but will be available after this study.

Micromorphology of glume, nut, anther, utricle and pollen were also studied their surface structures. Glumes are the least studied feature of this family. Glumes of both species have oblonged cells. Carex caucasica stomata was present in both glume and utricle. In Cyperus iria stomata was present in glumes.

5.2 Phylogenetic Analysis

Phylogenetic analysis of 4 genes were performed by using Bayesian, Maximum Likelihood, Neighbor Joining analysis. Phylogenetic analysis was performed for ETS region of Carex caucasica. In this study ITS, ETS regions were amplified. ITS and ETS regions were easily amplified. They both are nuclear DNA region. The rbcL region and matK was not amplified for single species. These both rbcL and matK are chloroplast regions. The reason behind their hard amplification is maybe they are chloroplast regions and chloroplast DNA are not get extracted well. So, these regions are not amplified. After sequencing of amplified PCR product trees are constructed on bases of ETS sequence. Trees are constructed on bases of Neighbor Joining, Maximum Likelihood and Bayesian analysis. Two types of trees are constructed one are for molecular identification of species and another one is for biogeographical identification of species. Molecular identification

trees for Carex caucasica ETS regions are confirming that this species because after aligning Carex caucasica ETS sequences with other closely related sequences and constructing trees on bases of these alignments, our Carex caucasica from Pakistan was in same clade with other Carex caucasica. Bootstrap value of this clade is 77 and posterior probability of 1 for ETS regions. So, ETS gene are confirming that this is Carex caucasica. In case of biogeographical trees our Carex caucasica is making clades with Carex caucasica of Finland, Siberia, and Russia with bootstrap of 77 and posterior probability of 1. So, these are well supported clades for ETS. Molecular Identification trees of Carex caucasica, Carex caucasica is appearing in the same clades with the other Carex caucasica and showing the bootstrap of 77 and posterior probability of 1. So, clades for ETS region are well supported. So, these are confirming Carex caucasica specie.

Conclusion

Cyperaceae family is in one of largest families of monocotyledons. Taxonomical study of this family is very difficult because of its such a small inflorescence. In this present study focused was on two cyperaceae species, Carex caucasica and Cyperus iria. There morphological features were studied in detail with the help of stereo microscope and scanning electron microscope. The features were in accordance with the flora of Pakistan. Molecular analysis was also performed. ITS, ETS regions were also amplified by using universal plant primers. Then on bases of these amplified regions sequences phylogenetic analysis was performed to study these species evolutionary lineage. Both morphological and molecular studies confirmed Carex caucasica and Cyperus iria species. Herbarium specimens of these species were submitted to PMNH as reference for future studies. Sequence of amplified regions of carex caucasica specie were submitted to GenBank for future studies while sequence of Cyperus iria is negative.

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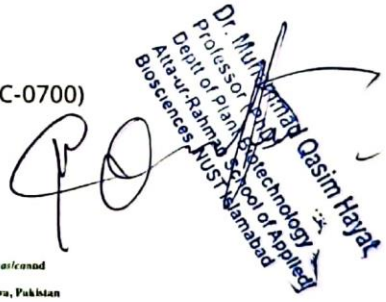

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