Molecular Identification and Phylogeny of Selected Western Himalayan Sedges (Cyperaceae)

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By

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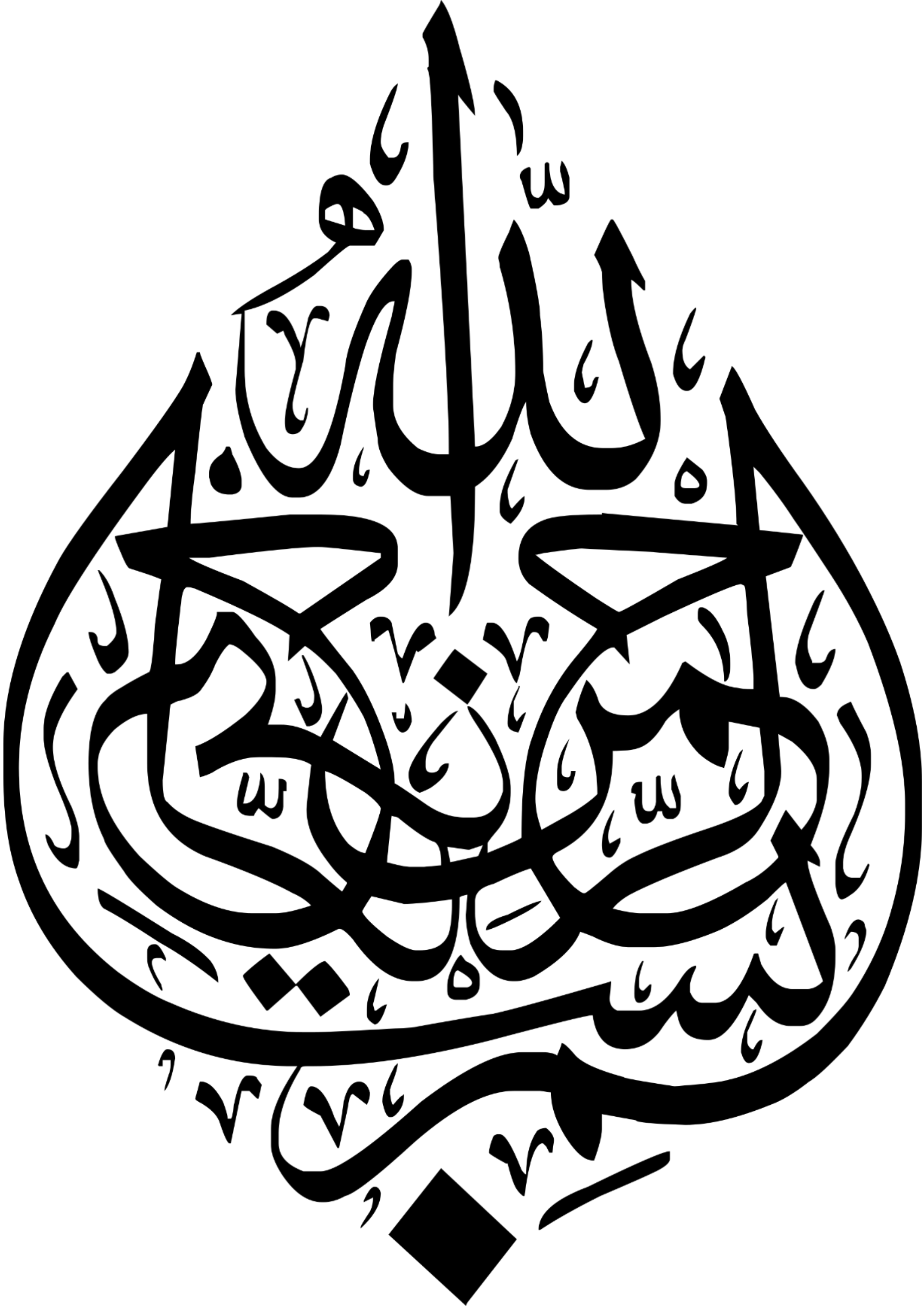
بسم الله الرحمن الرحيم

Dedication

I dedicate this thesis to the women who supported me at every single step and was dear as a mother to me, Mrs. Badria Khanum, Mrs. Batool Akther, Mrs. Naseem Hussain and Mrs. Huma Imran.

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"You shall proclaim the blessing your Lord has bestowed upon you." 93:11. all praises to Allah, without his blessings I was nothing but a piece of meat, and it is because of Him that I am a man and hold my rank in the society. May Allah shower his blessings on Muhammad SAWW and His Progeny (a.s), as it is because of that household that we see another day. I was raised as a single parent child, and never felt emptiness until I met my Supervisor who was a great blessing. And because of him now In future I will feel emptiness and incompleteness as he stood by me as a father. I want to thank my supervisor Dr. Muhammad Qasim Hayat Khan, for giving me the honor and assigning me a whole new project. He has provided me with every possible facility and aid he could give me as a supervisor. I want to thank him because he has pushed me and supported me on every turn. I want to thank him for making my hurdles cross easier. This project was not possible without the help of my lab seniors, I want to thank them, but I don't have words for it. Ms. Saadia Maqbool, Ms. Shabina Ishtiaq, Ms. Shabina Erum, Ms. Alia Sadiq. I want to thank all of the supporting team who helped me at the time of need Ms. Noor-ul-Huda Ghori, Mr. Atif Shafique, Ms. Ambash Riaz Virk, Mrs. Zoya Ghori, Ms. Sandal, Ms. Syeda Hafsa Ali, Ms. Anum Hashmi, Ms. Hafsa, Ms. Huma, Ms Sadia Nasr, Ms Aimen Saleem, Ms. Ufaq Tasneem, Ms. Sumera, and Ms. Sadia. I want to pay my gratitude to Mrs. Hira Hammad and Ms. Tahira khan for helping me in completing the task. I want to thank Mr.Nauman Arshad, Mr. Zeeshan Ali, Mr. Talha Mehmood, Mr. Murtaza Kasi, Mr. Mujtabah Khalid, Mr. Ali Raza and My son Mr. Zohaib Ali for keeping my moral up and advising me through my tough time. I want to thank my peers Mr. Moaz Aqeel, Mr. Muhammad Nouman, Mr. Muneeb Haider, Mr. Sohaib, Mr. Usman Yousuf, and Mr. Hammad Ali for providing me with ample knowledge needed to complete the task. I want to thank all the N.G staff members which relieved me from stress with their fantastic sense of humor and let me refresh my mind.

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List of Acronyms

SOD	Superoxide dismutase
nrDNA	Nuclear ribosomal deoxyribonucleic acid
*rbc*L	Ribulose bisphosphate carboxylase
BC	Before Christ
AD	Anno Domini
rRNA	Ribosomal Ribonucleic Acid
ITS	Internal Transcribed Spacer
ETS	External Transcribed Spacer
Kb	Kilobyte
FISH	Fluorescent in situ hybridization
GISH	Genome in situ hybridization
AT	Adenine Thymine
GC	Guanine Cytosine
DNA	Deoxyribose Nucleic Acid
SOP	Standard Operating Procedure
CTAB	Cetyl trimethylammonium bromide
PVP	Polyvinylpolypyrrolidone
rpm	repeat per minute
M	Molar
PCR	Polymerase Chain Reaction
T.E	Tris- EDTA

T.A.E	Tris-acetate-EDTA
UV	Ultra Voilet
RNA	Ribonucleic Acid
$MgCl_2$	Magnesium Chloride
dNTP	Deoxyribosenucleotide
H_2O	Water
BSA	Bovine Serum Albumin
EXO	Exonuclease
DTCS	Dye terminator cycle sequencing
EDTA	Ethylenediaminetetraacetic acid

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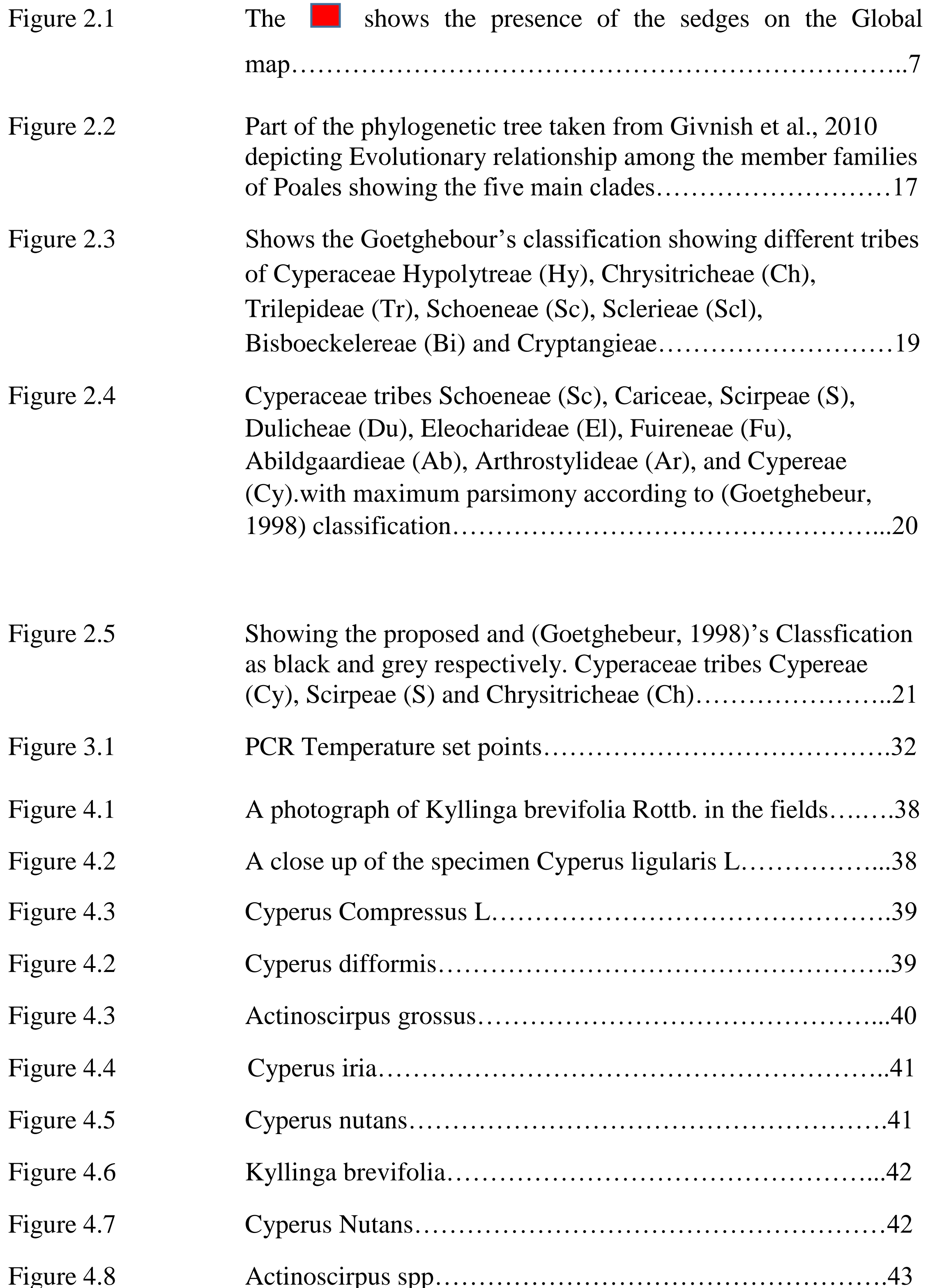

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Abstract

Pakistan hosts rich biodiversity of flora which covers conifer forests to the marsh lands. Pakistan and Kashmir has been reported home for more than 6000 flowering plant species. The vegetation is even richer in sino-himaliyan species. Out of 105 Genera and 5000 families, Pakistan is a home for 22 Genera and 179 species of Cyperaceae. Commonly known as sedges, they are known for their weedy nature in wild and fields, they are found near all type of wetland dominated areas like marshes, fens, bogs, polluted areas and banks of canals. It is because of its huge number of species that it is difficult to identify species on the bases of morphological and diagnostic characters. A few species holds similar morphologically identity that they can't be differentiated unless studied with deep concentration. It was found that some of these species had their ITS sequenced but some were not reported. Different species from family Cyperaceae were collected from the wetlands residing Margalla hills Islamabad. These species were then analyzed and were morphologically diagnosed at first. The markers for molecular identification used are ITS region (ITS1f and ITS4r) which were then BLAST and aligned to the ten close relatives. Due to the presence of polyploidy the results suggests that these gene copies could be transferred from neighboring plant species from the same family but different genus. They were edited manually where necessary. This research also has revealed that family genus Cyperus, Actinoscirpus and Pycreus are polyphyletic in origin. It has also been inferred that they contain double gene copy or different anomalies which can be seen as mutations but close analysis of the sequences and chromatogram revealed the nature of the sequence and proposed the results. The trees of these species are constructed individually on the bases of neighbor joining method with a bootstrap value of 100.

CHAPTER 1

INTRODUCTION

Flowering plants from the family *Cyperaceae*, a monocot family, are also known as sedges in the common world. They are identified as straight, upright more or less grass like herbaceous flowering plants. These plants are usually perennial plants and are rarely annual. Their rooting system is extensive mostly stoloniferous, which can form tubers or bulbs later in the cycle. Three ranked appearance of the leaves is observed, which are commonly basal or sub-basal containing sheaths around them, ligules might be present or absent. Hairless blades are present, hairs are present on apex and margin. Blades are often shortened and divided among many clustered groups. Stem is mostly trigonous (having 3 edges), flat solid, leafless, hairless, others in the row have projections like scales, on their edges. Usually the stem is scaly but it's rarely smooth and cylindrical (Bruhl, 1995). Very diverse inflorescence morphology and very complex to understand. These are transformed into flowers which look like spikes and so are called spiklets. The inflorescence ends up in the group of flowers mostly terms as spiklets (Vegetti, 2003). They vary greatly in height and go up to 5 meters long in height. Their leaves like other monocots are very narrow. Sometimes their leaves are so linear and narrow that they become sheaths only. The most distinct character is their stem. It has three angled cylindrical pure solid stem. Unlike other grasses and rushes there are no joints present in the stem and is not hollow like other grass families. Their flowers are at terminals present as single spikelets or clustered borne on the stem. Their underground rhizomes are richer in starch and forms tubers in some species of genus *Cyperus* (Zohari) Feinbirn Dothan, 1986). *Cyperaceae* is huge family notorious about their weedy nature containing almost 4000-5000 species under 70-105 genera and sub genera (Goetghebeur, 1998). What makes them weedier in nature and adaptive to every environment is that the C3 and C4 photosynthetic pathways are operative which turn them to be more aggressive weeds (Hameed *et al.*, 2012). The two major genera which hold maximum number of species are *Cyperus* and *Carex*. With resemblance to grasses

and rushes it is the taxonomically most difficult family of flowering plants as their stem and sheath resembles to the other type of grasses discussed earlier (RAD and Sonboli, 2008). The family is spread worldwide but majority of its species grow healthily in the temperate or somewhat cold regions (Hafliger et al., 1982). With structural features of that of wetland plants the *Cyperaceae* is rarely found in dry or hot places except a few species which can be found growing on the green belts of hot cities where temperature can go to 48-50 degree centigrade. The utmost important feature of this specie which resembles to the wetland plants is that they possess a great deal of intercellular spaces which make them more adaptive to water logged areas (Bruhl, 1995). Other than their natural habitat near water bodies they have been reported growing on acid soils as well or places which contain high amount of humus (Chopra, 1977). Known for the third largest family in the monocotyledons Cyperaceae are reported to be the prominent part of the wetland ecosystem (Simpson et al., 2003). Likely to adapt in many type of habitats they have adaptions towards certain habitats which are due to their morphological structures that they possess for example some species have water-storing leaf sheaths rhizomes, succulent, tubers or corms. Most of them have now become annuals. Completing their life cycle in very limited time they vanish as soon they are done with their growth and reproducing biology. Sedges carry diversity and versatility when it comes to adaptation to the wild ranging from mangrove forests they can be also found in the plains and near wetlands of plains at higher altitude. The best living example is the specie Cyperus rotundus which is so common that it is found on temperate forests to even hot plains. Most of them however are found in waterbodies like marshes, bogs canal banks, fens etc.

Talking of its importance a few species of the family Cyperaceae has developed a modified root system which turns into a rhizome network containing medicinally important compounds (Ito et al., 2012). It was found from the hydroalchoholic extracts of *Cyperus rotundus* that they contain anticonvulent and antioxidant properties which can enhance SOD and NO levels in mice brain (Azizi, 2011). It was found that the specie of *Cyperaceae* Cyperus esculentus (tiger nut sedge) common among Nigerian people as

a food source was found to have anti-sickling property in-vitro (Monago and Uwakwe, 2009). Cyperus rotundus was known for the control of blood glucose level and was proved experimentally that 500mg/kg extract every day lowered the blood glucose level.

These plants can be found anywhere, even in the drain pipes, sewers or polluted canals (Jakovljević *et al.*, 2013). *Cyperaceae* recycle nutrients and play an important role in food web and are a food source for the many of the grazing animals moreover they are a great source of food of many domesticated animals and wildlife as they live on the seed, foliage tubers and rhizomes, of Cyperaceae (Abad *et al.*, 2002; Miller *et al.*, 1999; Sather and Smith, 1984). Cyperaceae has also proven to be a source of food, drinks, food additives, fibers, animal poisons for human beings. They are also a great source for the industry which include manufacturing of useful items like medicines, mats, boats, clothing, paper, perfumes, shoes, ropes and roofing (Carter *et al.*, 1987). *Cyperaceae* has also played its part in preventing erosion, it has also been used for the betterment of the fertility of soil and vegetation after natural disturbances. *Cyperaceae* as a family has significant economic importance, where members are considered as serious agricultural weeds, there are others which provide fuel, food, and medicines along with weaving, construction and perfumery materials. They're an important constituent in conservation as dominant components of many wetland ecosystems. Moreover they are reliable source of habitat deterioration (Simpson and Inglis, 2001).

Inhabiting in the variety of habitats richly populated species of genus Carex from *Cyperaceae* family (Ball, 1990; Catling *et al.*, 1993), provide unequalled prospects to test evolutionary and ecological hypotheses related to adaptive contamination, niche differentiation, diversification rates and the roles of adaptive processes in community assemblage (Cavender-Bares *et al.*, 2009; Losos, 1996; Sanderson and Donoghue, 1989). To ensure the quality of work the primary work is to be done on the hypotheses which leads to the better classification system (Silvertown *et al.*, 2006). Although all the sub families have been classified on the bases of sub-generic level but uneven distribution of samples among the subfamilies and tribes makes it difficult to understand the relation among tribes, clades and subfamilies. Where the *Cyperaceae* itself makes

its way to the reliable and good source of studying phylogeny; the problem also occurs in recognition and conducting true phylogenetic studies among different genus, sub families and species of the group (Hipp *et al.*, 2006). Systematic studies in recent times have been published only on the bases of regional treatment among single sections or specie complexes (Poczai and Hyvönen, 2010; Simpson *et al.*, 2007). Molecular phylogenetic studies due to their high specificity are the favorite techniques used for classification among scientists these days. Many scientists use more than one set of genes to classify them on the basis of molecular conserved regions. Most commonly used these regions contains internal transcribed spacer region and external transcribed spacer region. Scientists also have used many other gene sets from their mitochondrial DNA and Chloroplast DNA like genes which code for reductase etc. example of these are Yen and Olmstead (Yen and Olmstead, 2000) whom has used two different sets of genes. Among them one set was of a coding region and other was of non-coding region. These genes namely, ndhF and trnL–trnF and with them the trnL intron. The study shows too low diversity, variability among the sequences. The other regions popular among scientists are nuclear ribosomal DNA, which are arranged as tandem repeats, including 26S ITS1, 5.8S subunit, ITS2, 18S sub- unit and the large intergenic spacer (IGS) containing External transcribed spacer region. Problems raised due to evolutionary dynamics of DNA sequences include hybridization, incomplete lineage sorting and recombination within the spacer region these all can occur because of the incomplete concerted evolution. With this the interference of phylogenetic relation is not accurate and not a reliable source of the study (Buckler *et al.*, 1997; Wendel and Doyle, 1998). The use of nrDNA spacers among specie level phylogenetic analyses reduces due to the resolution of non-homogenized paralogous copies within individuals or species because of the fact that it tells about the interference relationship on false facts, so usually a pair or three gens are used specifically for the purpose, particularly if only one of the paralogs is sequenced, as is common in many ITS phylogenetic studies it will interpret a little inadequate but using an ETS region and/or other DNA genes from chloroplast or mitochondria will do that right (Bailey *et al.*, 2000). Others like Starr et al. (1999) has used ITS regions on a small scale research on Phyllostachyae and

closer relatives, the success of which led Starr et al. (2003) to produce the primes for another set of primers which are mire conserved and are my of research the external transcribed spacer (ETS-1f) of the ribosomal gene (18S) repeat. They found variability high enough to have a good resolution along with ITS data in their studies of Carex subgen and Uncinia, and Psyllophora (Starr *et al.*, 1999; Starr *et al.*, 2003; Starr *et al.*, 2004). Gene transformation and uneven crossing-over of the NOR's can leads to the concerted evolution, which other studies have suggested is complete in many pedigrees when there is little or no polymorphism among individuals (Linder and Rudall, 2005). If the paralogue copies keeps on multiplying consistently they can lead to natural existence of non-functional false-genes. In the case of subfamily *Naucleeae* from *Rubiceae* these false-genes from nrDNA have been amplified and used to reconstruct the phylogenetic pedigrees (Razafimandimbison *et al.*, 2004). To study the phylogenetic reconstruction in an individual on specie level it is assumed that all the number of copies of the nrDNA should undergo complete and correct evolution, so that every repeat represents an identical copy. In studies conducted by Bailey et al. (2003) the detection of these false-genes from nrDNA have been discussed. They have also discussed the function of using these copies among individuals to check or determine the rate of polymorphism (Hillis and Dixon, 1991; Small *et al.*, 2004). Studies have proposed phylogenetic and statistical methods to see whether these sequences belong to false-gene by conducting a weighted ratio of replacement rates of the internal transcribed spacer sequence and 5.8S subunit. By use of the former process it is now possible to establish non-parametric bootstrapping to check the strength of each statistic used in the calculations. One of the biggest genus in the family Cyperaceae is Carex. It includes more than 2000 species and has the honor of the largest genus in the sedge family (Reznicek, 1990). For the Family-Level studies mainly the regions like rbcL sequence data are used for the analysis (e.g. Muasya et al., 1998; Simpson et al., 2007), for the sub-familial-level and tribes or clades other important regions like chloroplast, plastid and nuclear regions have been used. Scientists like (Roalson *et al.*, 2001) has also used ITS region along with noncoding chloroplast trnL–trnF–trnT region genes for the classification of *Cyperaceae.* Heindrichs et al. 2004 (Waterway and Starr, 2007) used

ITS data alone and run different analysis like Bayesian and distance methods to generate the phylogenetic relationship among the European Carex (Simpson *et al.*, 2003; Simpson *et al.*, 2007) If Internal transcribed region, external transcribed region and genes from chloroplast are used in the analysis the resolution of result comes out to be better than just single region. But due to lack of time and limited resources I have focused only on the ETS (1F, 18sR). Most of the reported analysis is with GeneBank which is mostly a combined analysis of the two sets of genes rather just one which gives the better resolution of the work. The work is reported on known species of *Cyperaceae* which is again a good step towards better resolution and statistical support

1.1 Objectives

The objective of this study is to explore the unexplored, collecting Cyperaceae from Islamabad and its surrounding. Because of its presence in main crop fields of Pakistan it is of utmost importance. Many of them are still undercover and our research study will uncover the many. Our study will contribute towards its molecular identification so that it would be easier for future studies to identify the weeds from this family. Because of the lack of the information about the identification of certain species and so much ambiguity in already gained information it is the need of time that we need to reevaluate the data and gather new data as we proceed to the digital world. Moreover the changing of a source enriched with beneficial properties and becoming a weed worldwide is a disaster. The need of the time is that we gain much knowledge about the importance about the species which have been used in our daily lives in ancient times. And now in the modern world it is becoming notorious as weed. So we need to reframe the picture. With the findings of anomalies we have to re-name and classify such species which are traditionally or conventionally classified on the bases of their structural morphology.

CHAPTER 2

LITERATURE REVIEW

2.1 Cyperaceae

Cyperaceae is a huge family with over 100 genus and 5000 species. There is no current study on the general population and their relationship among Cyperaceae family. But the most recent evidence is from the study of (Goetghebeur, 1998), he described the division 4 subfamilies and 14 tribe containing 104 genera. Though there are conflicts among scientists which differ a little on it with a few exceptions like the Jerry Bruhl (Bruhl, 1995). Cyperaceae are herbs, mostly annual but some do exist perennial. They exist in very diverse form i.e. they may be cespitose, rhizomatous and soloniferous. In some case they don't exist in these states at all. They have thick fibrous roots which is mainly adventitious. Some of the species are runners. They have a very unique character about their stem. They stem (clum) exists in the trigonous form i.e. it has three distinct edges which can be observed easily. This character separates the entire family from other graminoids. But some exists as terete, compressed, hollow or separate. Usually they are solid and trigonous. They usually have basal leaves but chances are there that they co-exist or separately exist as cauline. They are alternate usually measured as 120° apart. In most of the cases they are 3-ranked but they also exist rarely as 2-ranked or multiple ranking can be seen. They form cylindrical sheath around the stem. Their margins are usually fused. Their junction of sheaths and blades may be with adaxial flapped of tissues or fringes of hairs. Blades are usually absent from basal leaves, rarely from caulin leaves. If they are present they can be either diverging or ascending. They can either be fold or erect. They are linear and terete. Their venation is parallel. For the primary inflorescence or spikelet they have shortened axis, their scales/bracts are glumaceous. Usually they are spirally arranged but sometimes they are positioned 2-ranked. They can be seen as appressed or ascending. All scales are fertile and develop into individual flower. Sometimes proximal and distal scales are empty and lack the capacity. Lateral scales are usually with basal and are empty, with 2-keeled scale. It is

also observed that they are prophyll subtending and enclosing rachilla moreover they bear one pistillate and none to three staminate flowers in addition to empty scales. If we observe the secondary inflorescence we can see that the pinnacles are usually modified to corymb, cyme, spike, raceme, pseudoumbel or captilum. They are rarely in the form of single spikelet. Most commonly they are subtended as foliaceous or sometimes glumaceous bracts. Secondary inflorescence sometime also trigger the spikelet. Flowers are mainly hypogynous. They are mostly bisexual and are present in every genus except a few which includes Carex mainly. There they are unisexual. They are perianth. They can be absent as well or if they occur they can be seen with bristles or scales. They usually fall with fruit. The male part of the flower includes stamens which ae one to three in number. They rarely exceed the more than three in number and they are quite distinct able. The anthers are basifixed. The female part of the flower; pistils are one in number in most cases. If 2 or more than that they are carpulate and fused. Style is rarely undivided. Stigma is occasionally papillate. Fruit are achenes rarely biconvex mostly they are present in the form of trigonous and have thin carp. Seed is free from pericarp and have thin testa embryo has basal positioning and the endosperm is in great quality.

Cyperaceae has been known to man since ages, their usage as food worldwide have been studied (Table 1.) which gives us the information about the use of different parts of the plant from different part of the world as a source of food. The table contain the information from the past which were studied by the biologists and archeologist to examine the use of Cyperaceae in the ancient ages and a little back in history. A few are listed in the following table.

Figure 2.1 The ■ ***shows the presence of the sedges on the Global map.***

Table 2.1. Shows the species used as food purpose worldwide

	Texa	Food Usage	location	Reference
1.	*Actinoscirpus grossus*	Tubers are eaten raw as a source of carbohydrates	Pakistan, India, Malay Peninsula	(Burkill, 1995)
2.	*Bolboschoenus fluviatilis*	The inner portion of the Rhizomes are used as instant source of food.	New Zealand	(Irvine, 1957)
3.	*Bolboschoenus maritimus*	The Rhizomes are used as vegetables and eaten when cooked	Cosmopolitan	(Abbiw, 1990)
4.	*Carex exserta*	Not as a food source but it is used for revegetation.	U.S.A.	(Kala, 2005)
5.	*Carex filicina*	Their nutlets are eaten raw	, India	(Kala, 2005)
6.	*Caustis dioica*	It is used in regetation of mine waste	Australia	
7.	*Courtoisina assimilis*	Source of vegetables eaten raw or cooked	Africa	(Abbiw, 1990)
8.	*Cyperus alternifolius*	Their ash is used to cook leaves of other vegetables.	Africa	(Abbiw, 1990)
9.	*Cyperus amauropus*	Masai people eat bulbous clumes bases raw	Kenya	
10.	*Cyperus articulatus*	Their rhizomes are eaten as a source of fresh and instant food sometimes stored	Pantropical	
11.	*Eleocharis dulcis*	Cooked uncooked, eaten as a source of carbohydrates	China, India, Japan, Philippines, U.S.A., Vietnam	(Navchoo and Buth, 1990)
12.	*Eleocharis sphacelata*	Tubers eaten raw	New Guinea, Australia and New Zealand	(Malik and Naqvi, 1984)

	Taxa	**Food**	**Location**	**Reference**
13.	*Fuirena umbellata*	Tubers are edible moreover burnt and salt is used for cooking	Papua New Guinea Ghana	(Abbiw, 1990; Altschul, 1973)
14.	*Gahnia grandis*	Leaves are eaten fresh	Australia	(Irvine, 1957)
16.	*Kyllinga erecta*	Used for aroma of food, flavoring and sometime eaten fresh rhizomes.	Africa; Nigeria , Ghana	(Abbiw, 1990)
17.	*Kyllinga pumila*	Chewed rhizomes add flavor to the food and medicine	Ghana	(Burkill, 1995)
18.	*Lepidosperma gladiatum*	Edible base of leaves. Eaten fresh.	Australi	(Irvine, 1957)
19.	*Lepironia articulata*	Tubers are edible	Australia	(Irvine, 1957)
20.	*Pycreus nitidus*	Whole plants were burned to ashes and salt was used for cooking purposes.	Uganda	(Burkill, 1995)

Distinguishing features of the family, which separate the *Cyperaceae* from other grasses are some cytological features. For an example their chromosomes are present in clusters with diffused centromere. In some genera the centromere is localized like in *Eleocharis*. They also have post-reductional meiosis. The 3-4 microspores fail to develop as the pollen grains are formed from the tetrad. The former two features are common in the *Junaceae* family. The later feature is also same until the point that *Junaceae* family's all four microspore develop. There is wide variation in the number of chromosome and this is all due to agmatoploidy. Polyploidy is hypothesized because of the fact that it is demonstrated clearly. Due the fact that its vegetation and inflorescence resembles with that of *Poaceae* family they two were closely associated. The cytological features discovered clearly show that the similarity was superficial. In addition to this, the *rbc*L studies show that they are nowhere to be closely related to one another (Duvall *et al.*, 1993; Plunkett *et al.*, 1995). But the relationship between the *Cyperaceae* and *Juncaceae* is supported all over.

Most of the wetland vegetation mainly comprise of the sedges. They contribute enormously in the nutrient cycles and in the formation of the habitat which is the home for many diverse species of animals (Harper, 1992; Chambers et al., 2008). The largest genera among the Cyperaceae family which found growing among wetlands with the specie number of more than 2000 is Carex. They also cover great areas of damp habitats (Govaerts *et al.*, 2007; Waterway *et al.*, 2009). Cyperus, with around 600 species, is another well-known genus that includes important economic and horticultural species (Goetghebeur, 1998; Simpson and Inglis, 2001). Cyperus papyrus is the earliest known

plant used for making paper, in use as early as 3000 BC, and continuing in common use until 200 AD. Papyrus largely replaced animal skin parchments and stone for writing and had a profound impact on human society. World-wide Distribution of Cyperaceae (Australia's Virtual Herbarium xii. 2012) Data have been collected from various articles (Table 2.) showing the usage of some important sedges (Cyperaceae) as medicines worldwide

Table 2.2 Shows the species used as medicine purpose worldwide

	Texa	**Location**	**Medicines**	**Refernce**
1.	*Actinoscirpus grossus*	India, SE Asia	it was used to relive vomiting and diarrhea	(Milliken, 1997)
2.	*Bolboschoenus maritimus*	Cosmopolitan, except for Arctic regions	diuretic & astringent	(Grieve, 1971)
3.	*Bolboschoenus yagara*	East Asia usually wet places.	For reduction in blood clotting	(Harborne *et al.*, 1985)
4.	*Bulbostylis capillaris*	Tropical and subtropical America	Great use for toothache as a mouthwash, also used as blood purifier	(Altschul, 1973)
5.	*Bulbostylis hispidula*	Tropical Africa	The plant was burned along with *Cordia africana Lam* to heal the finger wounds	(Burkill, 1995)
6.	*Bulbostylis junciformis*	Central America, West Indies, tropical South America	Ground paste of leaves and culm used as external bath	(Milliken, 1997)
7.	*Bulbostylis lanata*	Tropical S America, especially Brazil	Boiled paste of whole used to treat fever and headache and applied on skin externally	(Milliken, 1997)
8.	*Bulbostylis puberula*	Tropical Africa and Asia	diuretic,	(T. Lockett, 2000)

9.	*Carex nivalis Boott*	Himalayas; open areas at high altitude	Used as an antiseptics for wounds	(Navchoo and Buth, 1990)
10.	*Cyperus aggregatus*	C and S America	decoction of whole plant used by Tiriy6 as external bath for fevers in children,	(Milliken, 1997)
11.	*Cyperus alternifolius L.,*	Tropical Africa	dried plant ash applied to fresh wounds as disinfectant,	(Burkill, 1995)
12.	*Cyperus articulatus L.,*	Pantropical	tuber used as tonic and stimulant	(Navchoo and Buth, 1990)
13.	*Cyperus bifax*	Australia	culms used for treatment of gonorrhea,	(Osagie and Eka, 1998)
14.	*Cyperus camphoratus*	Tropical America	culm base enhances digestion, culm base used to assist labor	(Tucker, 1986)
15.	*Cyperus compressus L*	Pantropical	roasted tubers made into paste and mixed with coconut oil for killing lice	(Mali *et al.*, 2006)
16.	*Cyperus conglomeratus Rottb*	Africa, Arabia to India	used to treat diarrhea and dysentery	(Abulfatih, 1995; Mali *et al.*, 2006)
17.	*Cyperus corymbosus Rott*	Tropical Africa, Madagascar, India to Indo-	useful in treatment of chest disorders and	(Tucker, 1986)

		China, northern Australia, S America	nasal discharge; blood enricher,	
18.	*Cyperus cyperoides (L.)*	Old World Tropics, W Indies	plant ash used to heal wounds	(Manandhar, 1989)
19.	*Cyperus digitatus*	Pantropical	leaves used to treat coughs	(Abo *et al.*, 2008)
21.	*Cyperus distans*	Pantropical	rhizome used as treatment for gonorrhea	(Burkill, 1995)
22.	*Cyperus esculentus*	Tropical and warm temperate regions worldwide	tubers said to cure constipation	(Abbiw 1990)
23.	*Cyperus exaltatus*	Pantropical	rhizome used to promote milk-flow in nursing mothers	(Burkill, 1995)
24.	*Cyperus fuscus*	Europe and N Africa to Indo-China, N America	used for suppressing flatulence	(Burkill, 1995)
25.	*Cyperus haspan*	Pantropical	whole plant, particularly rhizome, used with other	(Milliken, 1997)

			febrifuge plants by Wayap	
26.	*Cyperus iria*	Pantropical	drunk for fever, ground with C. rotundus tubers	(Tucker, 1986)
27.	*Cyperusjavanicus*	Tropical Africa and Madagascar, India and Sri Lanka to SE China and the Ryukyu Is., Malesia, Pacific Is. and W Australia	inflorescence pounded with coconut oil rubbed on the body as diaphoretic agent in cold, fever and malaria,	(T. Lockett, 2000)
28.	*Cyperus laxus Lam*	India to SE China and Taiwa	whole plant, particularly rhizome, used with other febrifuge plants by Waydipi	(Milliken 1997)
29.	*Cyperus longus L.,*	Europe, Middle East to India, N Africa	rhizome used in enema from for children with stomach problems	(Tucker, 1986)
30.	*Cyperus luzulae*	Tropical and subtropical America	used to maintain and encourage hair growth	(Tournon *et al.*, 1986)

31.	*Cyperus maculatus*	Africa, Madagascar	infusion of leaves and rhizomes put into a preparation for treating 'garli' cattle disease	(Burkill, 1995)
32.	*Cyperus margaritaceus*	Subsaharan Africa	rhizome laxative and purgative	(Burkill, 1995)
33.	*Cyperus odoratus*	Pantropical	antispasmodic and stomachic properties	((Simpson and Inglis, 2001))
34.	*Cyperus papyrus*	Tropical Africa	ash used to prevent spread of malignant ulcers,	(Tredgold, 1986)
35.	*Cyperus platyphyllus*	India	tuber said to have tonic and stimulant properties	(Soudahmini *et al.*, 2005)
36.	*Cyperus prolixus*	C America, tropical and subtropical S America	important medicinal plant in the culture of ancient civilizations	(Mati and de Boer, 2011)
37.	*Cyperus rotundus*	Pantropical	diaphoretic, diuretic, hypotensive and inflammatory due to presence of cyperone	(Saralamp et al. 1996)
38.	*Cyperus rubicundus*	Tropical Africa	infusion of rhizome used to relieve stomach pain	

39.	*Cyperus squarrosus*	Tropical to warm temperateregions worldwide	entire plant used to treat impotence,	(Adjanohoun et al. 1993).
40.	*Cyperus subumbellatus*	Tropical Africa, Mascarenes, W Indies	swollen culm bases used to treat gonorrhea,	(Burkill, 1995)
41.	*Cyperus tenuiculmis*	Old World tropics	unspecified use as a children's medicine,	(Burkill, 1995)
42.	*Eleocharis dulcis*	Pantropical	tubers considered cooling and sometimes used in jaundice	(Nguyen 1993)
43.	*Eleocharis geniculata*	Tropical, subtropical and warm-temperate regions worldwide	infusion of rhizome has tonic properties	(Pittier 1971)
44.	*Fimbristylis aestivalis*	India to northern Australia	may be used in poulticing, along with	(Burkill, 1995)
45.	*Fimbristylis dichotoma*	Pantropical	used to maintain and encourage hair growth, mixed with Genipa americana L. (Rubiaceae) and rubbed on to the hair	(Tournon *et al.*, 1986)

46.	*Fimbristylis dura*	India and Sri Lanka	used after childbirth	(Burkill, 1995)
47.	*Fimbristylis miliacea*	Tropical and subtropical	leaves used for poulticing in fever	(Burkill, 1995)
48.	*Kyllinga brevifolia Rottb*	Pantropical and warm temperate regions	with leaves of Denyung (Cane grass), macerated in cold water and drunk for spleen trouble	(Altschul, 1973)

2.2 Phylogeny

The classification done by the Goetghebeur 1998 is now being supported over many of the tribes from the ongoing studies. *Cyperaceae* when studied thoroughly over the generic or tribal levels has supported the clade and was given intensive DNA phylogenetic at both levels (Muasya *et al.*, 2002). Cypereae, due to the presence of Cyperus-like embryo are characterized on this base. They were also included in the Hellmuthia which previously belonged to the Chrysitricheae (Goetghebeur, 1998; Muasya *et al.*, 2009b).

Previous studies of molecular phylogenetic studies have shown evolution of a new era and has given us the evidence that the clades like Cypereae sensu Goetghebeur were monophyletic but then again the allocations were controversial in the Cypereae (Muasya *et al.*, 2002; Muasya *et al.*, 2009b; Simpson *et al.*, 2003). Lately the studies included the factors which are now not acceptable as the phylogeny character like the presence or absence of spiklets with distichous glumes with reduction, perianthless inflorescence (Muasya *et al.*, 2009b). But this study in the past the led to many misinterpretations in the relationship of lineages of Cypereae. The consequence of which is that a great number of taxa which belonged to other clades like Erioscirous (Palla), Ficinia (Schard),

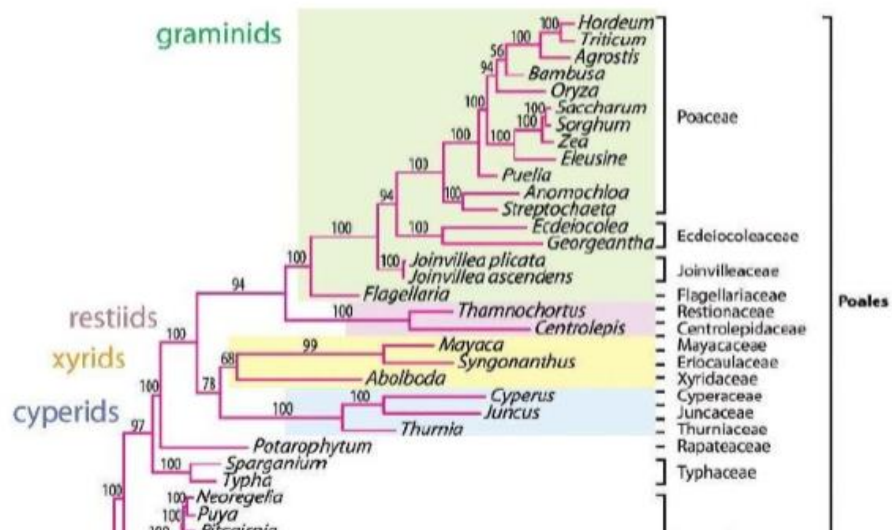

Figure 2.2 Part of the phylogenetic tree taken from Givnish et al., 2010 depicting Evolutionary relationship among the member families of Poales showing the five main clades.

Hellmuthia (Stued), Kyllingiella (R.W. Haines and Lye) have been assigned already present different tribes in *Cyperaceae* (Larridon et al., 2013). Deep studies have revealed that these data on the bases of extensive and intense anatomical, emryographical and molecular phylogenetic studies has shown that these genera are more close to the Genus Cyperus L. (Larridon *et al.*, 2011; Yano *et al.*, 2012). So there was, and there still is a need of the reinterpretation on the bases of molecular phylogeny in the Cypereae. There are two clades recognized in the Cypereae namely Cyperus and Ficinia. The later clade is the smaller one and it includes many genera which are distributed among southern Africa. With more of a ficinoid habitat and mostly spiral glumes (Muasya *et al.*, 2012; Vrijdaghs *et al.*, 2011; Yano *et al.*, 2012). The former and the larger clade of the family includes the distichous glums, with a paraphyletic Cyperus as the core genus of the clade which holds about 12 segregate genera (Alves *et al.*, 2009; Goetghebeur, 1998). Two more species from Androtrichum can be seen in the previous studies at the base of the clade Cyperus but this needs a reconfirmation and verification. However the results from molecular phylogenetic studies has shown us the falling of the clades in the Cyperus. There have been a lot of discussion going on whether to put these clades under Cyperus or not (Larridon *et al.*, 2011; Muasya *et al.*, 2012). New advancements in the molecular phylogenetic study has proven to provide significant and valuable data on the relationship among species and evolutionary patterns. These huge genera provide a new way to discover and gain knowledge about the lineage that have been considered a separate genera up till now (Berry *et al.*, 2005; Miller and Bayer, 2001; Walker *et al.*, 2004). Up till now the study of these huge paraphyletic entities have been challenging as the new classification developed and covered the all aspects of monophyly. When it comes to division of the large paraphyletic taxa into small genera in large number, it has already been proposed (Moore *et al.*, 2011). And here comes our study where a great deal of phylogenetic study is required. Then again there are great challenges up a head which are needed to identify diagnostic character, of course all of them cannot be covered. There lies the controversies about the name

application. (Smith *et al.*, 2006). The other strategy could be making up classification such that the various classified genera are divided in monophyletic but they remain the part of the large entities. Anyhow these strategic movements have been supported as well as strongly opposed (Losos, 1996), but has been strongly opposed by others (Smith and Figueiredo, 2011). The last strategy of dealing with large groups is the grouping of classified segregated genera into border circumscribed genus. But where it has proven to be good it also has a flaw which why it is not acceptable to every study. They negativity about it is that it will become very difficult to interpret the giant genus as a whole. (Stamatakis *et al.*, 2008)

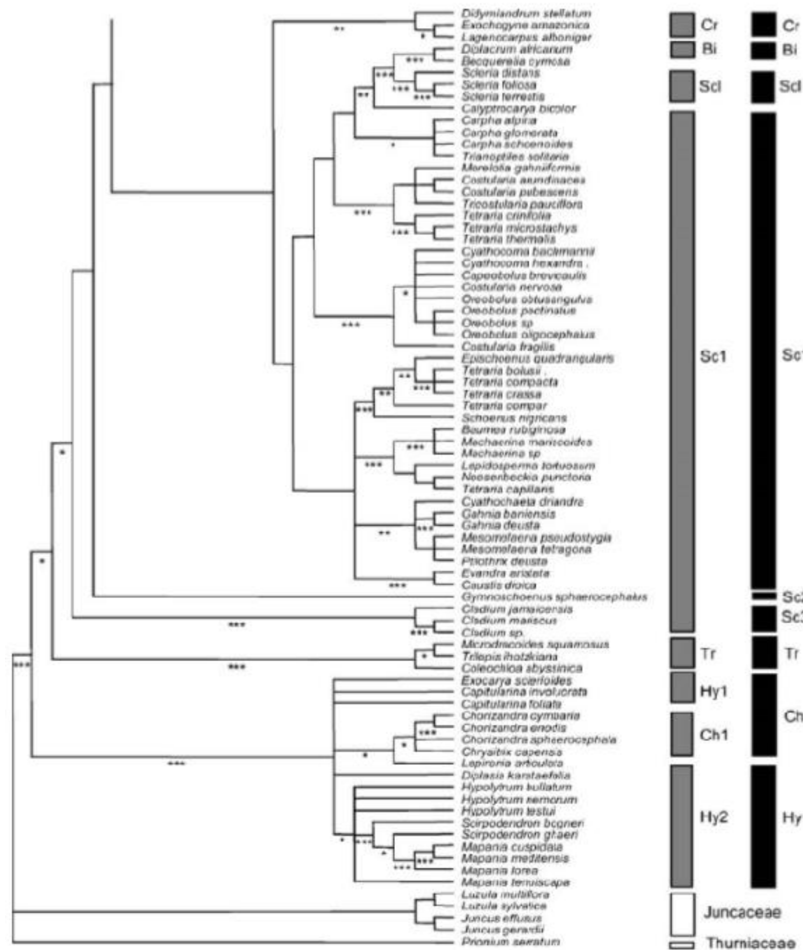

Figure 2.3 is taken from (Muasya *et al.*, 2009a) Shows the Goetghebour's classification showing different tribes of Cyperaceae Hypolytreae (Hy), Chrysitricheae (Ch), Trilepideae (Tr), Schoeneae (Sc), Sclerieae (Scl), Bisboeckelereae (Bi) and Cryptangieae.

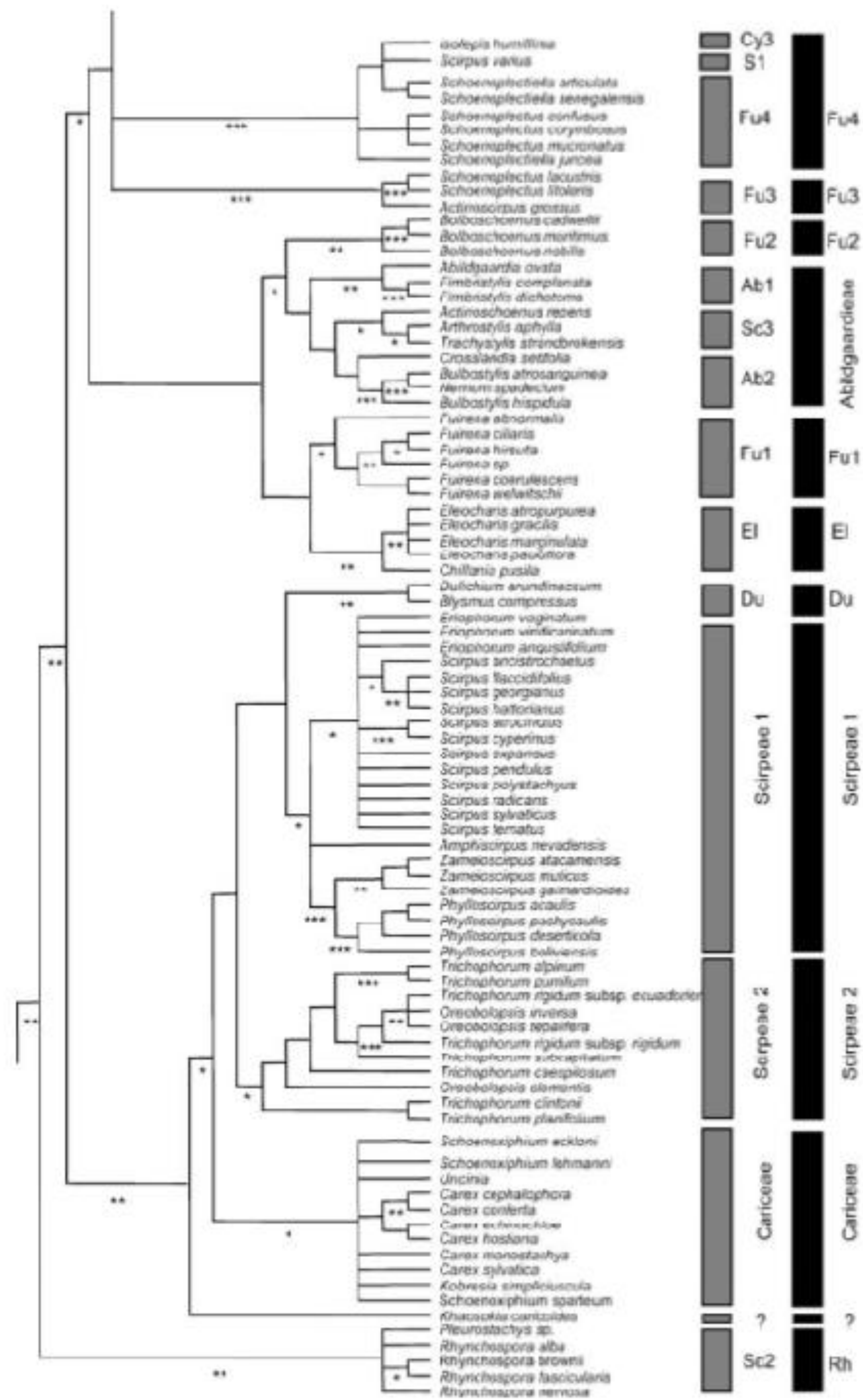

Figure 2.4 (Muasya *et al.*, 2009a) Cyperaceae tribes Schoeneae (Sc), Cariceae, Scirpeae (S), Dulicheae (Du), Eleocharideae (El), Fuireneae (Fu), Abildgaardieae (Ab), Arthrostylideae (Ar), and Cypereae (Cy).with maximum parsimony according to (Goetghebeur, 1998) classification.

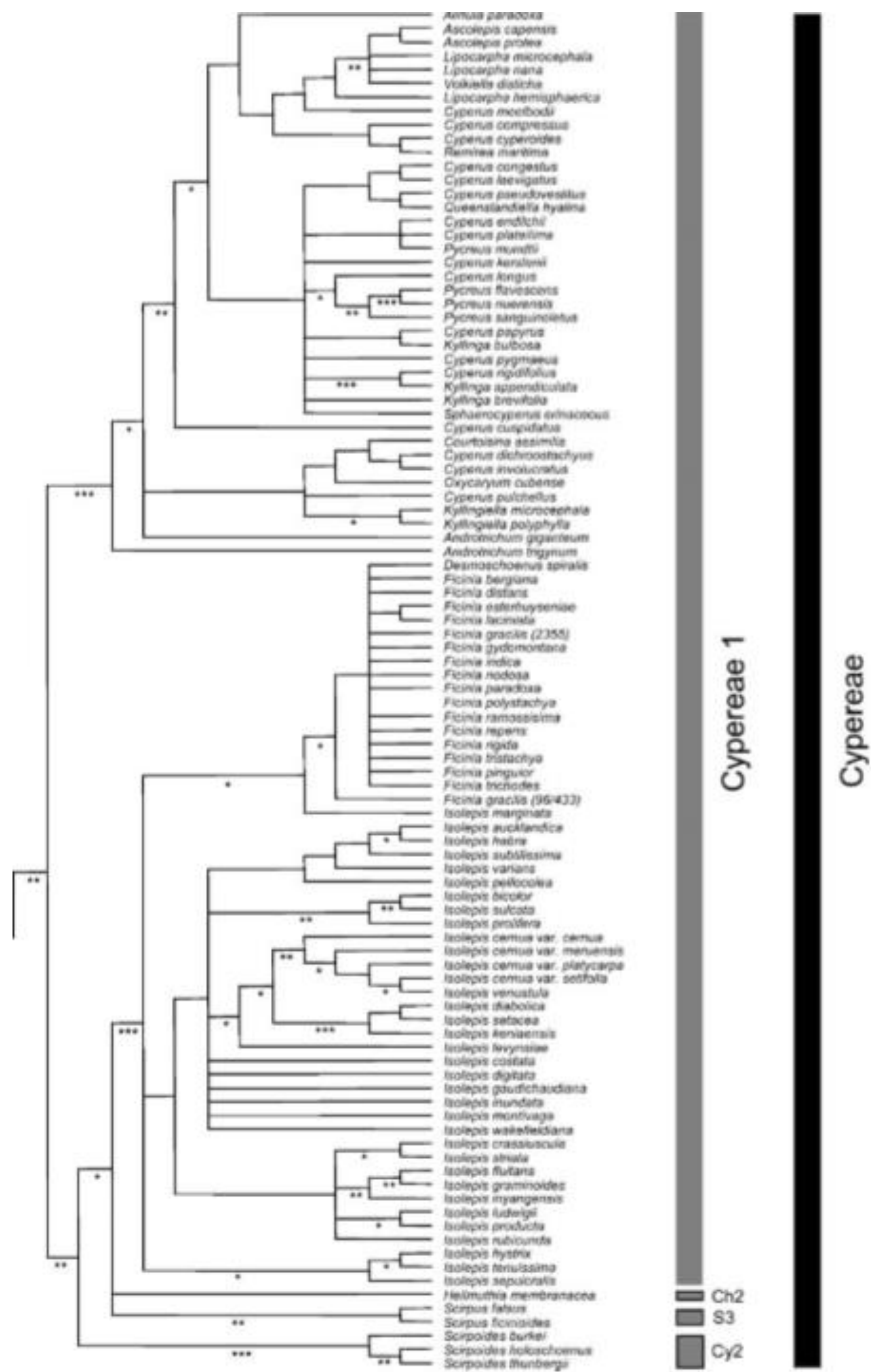

Figure 2.5 taken from (Muasya *et al.*, 2009a) showing the proposed and (Goetghebeur, 1998)'s Classfication as black and grey respectively. Cyperaceae tribes Cypereae (Cy), Scirpeae (S) and Chrysitricheae (Ch)

2.3 Internal Transcribed Spacer region

The ITS region from the nuclear ribosomal DNA has become significant in developing relations among plant phylogenetic studies. This is because of the intactness of the region and very low variation among this region is to be seen (Baldwin *et al.*, 1995). Whereas the External Transcribed spacer region of the nrDNA has contributed more towards the studies and opened gate for the many phylogenetic researchers to make a relation between plants among all angiosperms. As these two regions are lined side by side they may be evolving at compareable rates which is why they are both used in the studies, as they are the part of same transcriptional factor and indicates the similar maturation in the rRNAs (Good *et al.*, 1997; Kim and Mabry, 1991; Musters *et al.*, 1990). The length of the ETS region is way too long even if the ITS1 and ITS2 were combined they would be short as compared to the ETS. Moreover its survival rate is better than the ITS which is based on the restriction site. Its variation is as rapid as that of the ITS (Rogers and Bendich, 1987; Sytsma and Schaal, 1985; Volkov *et al.*, 1996). Very much of the variations in the restriction site among populations and very close species were mapped to the ETS, and very few mapped back on ITS region of the family Krgia (C*ompositae*) (Jorgensen and Cluster, 1988).

Some small sequences from the closely related plants from their external transcribed sequence add significant points to the phylogeny of the plants. About 7-20% of the sequence divergence between plant species of Triticae ETS region was reported and only 3% required the insertion gaps among 38 ends of ETS region in the aligned sites (McIntyre *et al.*, 1988). Looking the earlier mentioned points, we were compelled to use the ETS for our studies as the sequence data analyzer as and not the ITS. Moreover the ETS has its own hurdles like the primary barrier of the region as the lack of highly conserved sequence apart from the transcriptional initiation sequence (Volkov *et al.*, 1996). The molecular sequence data analysis has proven to be a revolutionary way out to define and describe more easily about the phylogeny of a giant family. No matter what the organism is the molecular sequence data or molecular phylogeny in new terms have taken the place and is used in all discipline to identify, track and classify organisms

on a large scale. In the first few days of this great revolution the plants molecular phylogenetic study was based on usually the genome and spacer regions derived from the plastids (Baldwin *et al.*, 1995; Catalán *et al.*, 1997; Olmstead and Palmer, 1994; Soltis and Soltis, 2000)With the developing danger of depending only on the uniparallel inherited sequence data for phylogenetic studies the plant systematic studies has gone wild to take on the inclusions from nuclear markers. . For this reason this nuclear locus has proven to be the most significant move for phylogenetic studies at generic or infrageneric levels in angiosperms and whole plant kingdom (Odorico and Miller, 1997; Rieseberg and Wendel, 1993), The external transcribed spacer region of the 18S nuclear ribosomal is now widely used around the world . its first use came about two decade ago (Baldwin *et al.*, 1995)

To tell the popularity of the this region alone there have been reported papers in which it has described about the intensive use of the marker as a prime source of molecular phylogeny along with other major marker like ITS etc. Also it has been reported that a survey of about 250 papers of plant systematic has been conducted and it has found that most of the paper (60%) includes the ITS region and ETS region alongside for the molecular phylogenetic study (Álvarez and Wendel, 2003; Baldwin *et al.*, 1995).

Why ETS and ITS have gone so popular has a reasons which is presumed advantageous phylogeny inference. And of course its qualities of a proven characterization factor in molecular phylogenetic reconstruction. The most interesting fact about these regions is that they are inherited from two parents and not the single one like in the chloroplast DNA. It makes these regions with more powerful information which can be a great deal when reconstructing phylogeny trees (Kim and Mabry, 1991; Kim and Jansen, 1996; Wendel and Cronn, 2003; Wendel and Doyle, 1998). When the primer sets were obtained for these regions as described by (White *et al.*, 1990), it went viral and it gave more hands on experience to gather data more readily than any other sequence or marker could ever get. Approximately a sequence of 10kb is placed in the nuclear ribosomal DNA of 18S-5.8S-26S. There are many repeats in the DNA of these tandem repeats. Most significant point is that they are isolated easily than any other sequences

present on nuclear loci and require very low experimental experience to amplify them. These sequences can both be of same or unequal lengths in plants usually the ITS region of the plant is about 450bp to some 1000bp (Maggini *et al.*, 2000; Marrocco *et al.*, 1996)

Another very best use of the ITS region is the power of converted evolution which is the homogenization of itself excluding itself from the sequence variation which might be caused due to the crossing over and other mutations. This causes the phylogeny study to restrict towards the clade-specific characteristics to ensure better reconstruction data sets (Park *et al.*, 1998; Rieseberg and Wendel, 1993; White *et al.*, 1990)

The intergenomic variability has led phylogenetecists to work on ETS region because of the view that these are suitable for the reconstruction of phylogeny trees and inference them moreover it is easier to analyze at generic, or even family levels (Baldwin *et al.*, 1995).

2.4 Utility of ITS and its impact on Phylogeny

2.4.1 .More than one nrDNA arrays

In the longer run when we observe and relate to the prehistoric studies of the plants, it was revealed that the 18S-26S nrDNA arrays constitute in nucleolus organizing regions in most of the eukaryotes. These regions vary greatly in size and number and distribution among the nrDNAs. (Bortiri *et al.*, 2002; Gernandt and Liston, 1999). The studies also proved that the NOR arrays were evolutionary liable with the tribes clades and even families (Álvarez and Wendel, 2003; Chen *et al.*, 2002; Simpson and Inglis, 2001). An example of it was observed in *Triticeae,* which gave us the clue of the presence of both the major and minor nrDNA arrays. There chromosomal location varies among species. Two hypothesis ere proposed and the one said it were the minor arrays which formed the major arrays. The other states that it is due to the reduction of major arrays that the minor arrays have arisen due to the deletion of the repeats (Panzera *et al.*, 1996).

The advancements in the techniques which includes FISH (fluorescent in situ hybridization) and GISH (genomic in situ hybridization) has paved paths of nrDNA arrays to be used as source of evolutionary studies (Álvarez and Wendel, 2003; Ma *et*

al., 1997; Soltis and Soltis, 2000). The variation in the minor nrDNA whether it's the number, size or the location is a common observation. This only correlates with the ploidy levels. On the other hand the previous and recent studies also shows that the ancient polyploidization events created two nrDNA sites as a duplication for 18S-26S nrDNA sites following the deletion of 5S but not the 18S-5.8S-26S (Mishima *et al.*, 2002)

The currently situation of the molecular phylogenetic study is intense when it comes to the result analysis. The prime source which is used in the study should be orthologous as compare to or opposed to paralogous. The orthologous sequences of nucleic acid or protein can be defined as the sequences originated from their organismal clade-genesis (Wendel and Doyle, 1998). The use of orthologous sequences can reveal the great deal of divergence events among the taxa. If by any chance there was a duplication of the sequences the result will be a paralogous sequence and will relate back in the tree for the duplication. This can lead to the unintended comparison of orthologous and paralogous study which will result in the phylogenetic incongruence (Wendel and Cronn, 2003)

Because of the nomadic or ancient polyploidization of nrDNA, it cannot be said that all the sequences analyzed are the true copy of the ribosomal orthologous sequences. Rather the resulting sequences can either be the suite of both the orthologous and paralogous sequences amplified by single PCR reaction. And there is a possibility that they are not homogenized satisfactorily through concerted evolutionary process (Buckler *et al.*, 1997; Thomas *et al.*, 2001). When *Quercus* was reviewed some of its species like *Q. ascutissima, Q. ruba and Q. suber* projected long branched trees in a neighbor joining tree. Moreover it showed us the controversial results when reviewed again with the orthologous counterparts (Buckler *et al.*, 1997; Mayol and Rosselló, 2001). It is practically not possible to some extent, to conduct FISH or GISH to describe the presence of the loci. But in some cases this can be overcome by the phylogeny results of ITS sequences (Park *et al.*, 1998)

2.4.2. Concerted evolution

As described earlier the intense and unequal crossing over or very high gene conversions among some specific regions may result into the development of the concerted evolution. Here the sequence variations are reduced to some or all extent and it is quite unrecognizable for the phylogenetic test to identify the variation. This gives rise to a unique kind of sequence among families. Huge data is available on nrDNA genes as the pool in plant kingdom is so huge because of the repeats and the very significant factor that dominates is that all the repeats share the same variation or mutation in the pool of the nrDNA genes. And because of this uniformity in the variation, many of the intergenic sequence homogenization takes place and this phenomena is referred to as the converted evolution (Baldwin *et al.*, 1995).

The accurate phylogenetic reconstructions among the sequences is the attribute of the concerted evolution. It not only eliminates paralogous sequence but also aids in the inference of true homology among taxa. However it cannot be assumed that complete orthologous homology is observed among the amplified sequences. The reason for this is again the pre-historic polyploidization which is the cause of ephemeral coexistence of ITS repeat sequences (Álvarez and Wendel, 2003)

2.4.3. False-Genes (Pseudogenes)

There can be question that if so many copies of the nrDNA are present among plant genome are those at the point are evolutionary functional or if they are just copies and delete themselves as pseudogenes. These genes are swung in by the genome itself until and unless amplified. They evolve independently and at a quite different rate than any other genes do. But somehow they can cause a serious challenges for the phylogenetic analysis. This will be done by completing the intergenic homogenization through the earlier mentioned process. This can lead to the texa resulting in multiplicity in molecular phylogenetic sequence types.

There are large evidence which proves the presence of the widespread pseudogenes. Which also includes the copies of nrDNA (ITS). The proof of this lies in the study

conducted by Buckler and Hotsford 1996 on Zea, Many of these studies show and discuss the phylogenetic consequences of the existence of pseudogenes, and in addition, discuss some of the properties that can lead to their identification. The latter include the obvious indications arising from large indels, but also changes in predicted secondary structure, GC content, sequence divergence, and methylation patterns (Kita and Ito, 2000; Ko and Jung, 2002; Li and Zhang, 2002). The latter authors have also described the unique function of these ETS repeats that they exhibit lower hairpin structure by an increase in AT content through deamination. Bailey has also discussed about the new methods that will allow a new approach using a new tree-based patterns of nucleotide diversification (Bailey *et al.*, 2000).

Nevertheless it is now possible to detect these pseudogenes but what matters is the consequence that will hinder the resultant inference of the phylogeny analysis. For an instance that if the sequences are amplified from same genome they will make the taxa appear at different places in a tree due to the presence of pseudogenes (Buckler and Holtsford, 1996).

The experimental results of two independent researchers have pointed out the flaws, and it is the best example so far now for the nrDNA pseudogenes. On comparison of their work it was found that the GC content of one team of researchers had great deal of length in the sequences. And they also exhibited the lower secondary structure stability. In addition to this the regions like ITS 1 and ITS 2 showed great divergence levels which were 42% for *Q. suber* and 27% *Q. ruba* respectively. It proves the absence of the concerted evolutionary process which other scientists have been presuming for the nrDNA (ETS or ITS). Their studies projected different phylogeny because of the presence of these repeats in the genome (Mayol and Rosselló, 2001). Recent studies of *Zea* by Buckler and Holtsford has also revealed that the sequences amplified and analyzed shows four such sequences that are proven to be pseudogenes, this was because of the presence of low GC content than the other. Out of those sequences were some sequences amplified with DMSO had GC content as expected and not a single pseudo gene was recovered. Similarly in the study of *Quercus* as described earlier, more than

65 genes were sequenced from the region which then turned out to be divided in three families of sequences. It was brought to the information that only one class was function and the other two were pseudogenes. Another study revealed that in *Leucaena* more than 80 sequences from 65 different resources were amplified. From which about 1/3 of the genes proved to be false genes or pseudogenes in general (Hughes *et al.*, 2002)

2.4.4. The conserved regions of nrDNA (ETS and ITS)

When conducting a study on phylogenetic relationship among sequences it was thought that the sequences align on independent positions regardless of any accordance with other sequences. The study suggests the structural assumption of independent positioning can be problematic, which in this case can be of the sequences that code for rRNA (the nrDNA genes). The formation of the secondary structures in the genes is due to the high GC content which leads to the evolution of stem-loop structures in which the loops are the conserved sequences. This suggest the uniqueness of the structures as they are conserved and provide us with great evidence that they exhibit more evolutionary constrains (Baldwin, 1993; Ma *et al.*, 1997; Olmstead and Palmer, 1994). This leads the study to the point that though this feature of nrDNA is better than any other DNA sequence for phylogenetic analysis as there are mutations in the unpaired DNA bases which compensates the mutations and sometimes cause homoplasy, which will be discussed later in the study (Moore *et al.*, 2011; Poczai and Hyvönen, 2010; Yano *et al.*, 2012)

CHAPTER 3

MATERIAL AND METHODS

3.1 Collection of plants

For the collection of plants in this study we surveyed different regions of Margalla Hills and surroundings in the late June. We found more than 20 species of Cyperaceae family rowing near the streams which were running down from the hills near Bari Imam. We took whole plant and sealed them in large sample bags. We then made spate bag for the tissues mainly leaves which were to be used for the DNA extraction purpose. The other whole plants were dried and embossed on the herbarium sheets according to the SOPs of the Pakistan Natural and History Museum Islamabad. The herbarium samples were then stored in the museum for future reference and the voucher number were noted for the reporting purpose.

Tissues which were to be used for the purpose of DNA extraction were then divided into two parts. One of them was stored in -22° Celsius and other were dried using silica beads. The division was made to check whether the frozen or dried give the better results.

GPS (global positioning system) device was also used to note down the position of the plant samples. So that in near future it will be easier for other scientists to track plants.

3.2 DNA Extraction

CTAB extraction method was used with certain modifications. For both type of samples we ground them using mortar and pestle using liquid nitrogen. 4 to five leaves for larger leaves and 7 to 10 leaves for the short leaves. The samples were well ground until fine powder was obtained. The mortar and pestle were well washed and autoclaved, they can be reused by 10% bleach and washed well with distil water. You can use liquid nitrogen or simply grind them but it's better to use liquid nitrogen. Because Cyperaceae is a monocot it has a hard midrib which is really hard to break and ground to pieces, so you need to have more patience and spend time on grinding samples until really fine powder

is obtained. Then we prepared CTAB buffer which is the mixture of three components, the CTAB, the PVP and the beta-β-Marceptoethanol. These three components were well mixed and dissolved prior extraction. The mixture was made according to the following ratio 20ml for CTAB, 0.8g for PVP and 100ul for β-marceptoethanol. Then we took the powder into small Eppendorf tubes and poured 500ul freshly made CTAB buffer. CTAB solution was pre-heated at 50° C. After pouring the CTAB incubate it for 60 minutes on a shaking bath and invert gently twice or thrice depending on the material. More the material more will be the inversions. Then add equal amount of Chloroform-isoamyl alcohol buffer. This buffer was made in the following ratio 24:1. After pouring the solution, tubes were well mixed by shaking gently. The shaking was followed by centrifugation at maximum speed using bench top centrifuge, at 14000 rpm for 10 – 15 minutes. We got three layer out of the sample. The aqueous layer was transferred to the new Eppendorf tube. This step was done quickly because there was a possibility that the layers might mix. Rest was discarded. Then we estimated the amount of the aqueous phase, and we added 7.5 M ammonium acetate and ice-cold isopropanol according to the following table and mixed well.

We stored it for overnight at room temperature. The next morning it was spun at maximum speed for 3 minutes. This time the supernatant was poured off or pipette out by carefully not disturbing the base of the tip which is the DNA we need for PCR. After taking out all of the supernatant we added wash buffer which is usually the 700ul of 70% cold ethanol. We spun it at maximum seed for 1 minute and poured of the liquid very carefully. Keep in mind not to disturb the pallet because it is going to decide your fate. Then again we washed the pellet by adding 95% of ethanol this time. Again we centrifuged it at maximum speed and discarded the pellet. Then we dried our pellet by incubating it 37 degree C incubators. We also spun some samples on vacuum for 20 minutes. After that we added 100ul of T.E buffer and re-suspended the pellet and stored in 55° C for 1 hour or until the pellet was dissolved.

To check whether our DNA was present or not we then run gel electrophoresis or used nano-drop for the quantification of the samples.

3.3 Gel Electrophoresis

Gel electrophoresis is a technique used for the separation or detection of nucleic acid or proteins. We used 1% of gel electrophoresis and we were able to see our presence of the DNA. The gel electrophoresis uses agarose and T.A.E buffer. We take 40 ml of T.A.E buffer. And added 0.4g of agarose. To make it 1% gel. We boiled it until the solution went clear. We then allowed it to cool down a bit. At room temperature. When the solution was cool enough that we could hold it in our hand we added ethidium bromide solution to it which is a dye and stains Nucleic Acid. Then we poured it in the casting tray and put a suitable comb in it and let it solidify. After it solidifies we put the tray into the tank and filled it with 1X T.A.E until the gel dips. Then we prepared our samples for the running by taking 5ul of sample and 2ul of bromophenol blue, which is a loading dye. We mix it on a paraffin tape where we mix them using pipette. Then we took it in pipette and poured in the wells created by the comb. Nice and gently. To check if there is leak in the well we push the gel slightly backward and pour the loading dye, if there is a leak in it the dye will run down. After checking and pouring we then closed the lid and tighten the screws. Then we ran the tank with 80V for 45 minutes. For the visual shots we took the gel under UV gel Doc where we can see the gel. It separated our DNA from the impurities and showed a nice single band on the top of the gel just below the well. That is because the DNA is larger in size it stays up and other impurities like RNA or proteins are shorter and runs down the gel.

3.4 Quantification on Nano-drop

For PCR reactions you cannot use raw DNA extracted sometimes because of the quantity of DNA extracted can be huge which can hinder the PCR reaction because of large size and small space. So we diluted the large quantities of the DNA samples by adding the same solution into it which was T.E in our case. First we wipe the plate with distilled water only and put the 1ul of distilled water in it. Just like weighing balance tare it and then clean it again with tissue. Every time after quantifying we cleaned the plate. The concentration were noted down and diluted accordingly to make it up to 100

ng/µl. Usually 50 to 200 ng/µl works fine. The calculated values and revised concentrations are given below (Table 3.1)

Table 3.1 Ratio of Aqueous Phase, 7.5 M Ammonium Acetate and 2-Propanol

Vol. of Phase	0.08 Vol of 7.5 M Ammonium acetate	0.54 vol of 2-Propanol
100	8	61.2
105	8.4	64.2
110	8.8	67.1
115	9.2	70
120	9.6	72.95
125	10	75.88
130	10.4	78.81
135	10.8	81.74
140	11.2	84.67
145	11.6	87.6
150	12	90.53
155	12.4	93.46
160	12.8	96.39
165	13.2	99.32
170	13.6	102.25
175	14	105.18
180	14.4	108.11
185	14.8	111.04
190	15.2	113.97
195	15.6	116.9
200	16	119.83
205	16.4	122.76
210	16.8	125.69
215	17.2	128.62
220	17.6	131.55
225	18	134.48
230	18.4	137.41
235	18.8	140.34
240	19.2	143.27
245	19.6	146.2
250	20	149.13
255	20.4	152.06
260	20.8	154.99
265	21.2	157.92
270	21.6	160.85
275	22	163.78
280	22.4	166.71
285	22.8	169.64
290	23.2	172.57
295	23.6	175.5
300	24	178.43

3.5 Polymerase Chain Reaction

The quantified DNA was then stored for the next step which was polymerase chain reaction. In this reaction we used external spacer region primers for forward and reverse. All the DNA samples were stored in -21° C. First we tried to optimize our primers and ran PCR reaction using different concentrations, recipe and temperature. Finally after getting our required results we ran the reaction in bulk for 100-150ul of master mix. The master mix contained 10x Taq buffer, $MgCl_2$, DNA taq polymerase, dNTPs, Primers (both forward and reverse), Template DNA and PCR H_2O in the following concentration and volume followed by reaction cycles. The reaction temperature was set as follow:

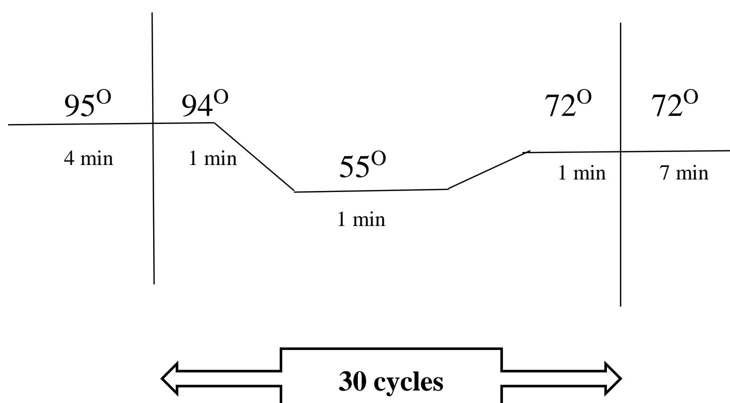

Figure 3.1 Shows the PCR temperature set points

3.6 Product analysis and purification

The PCR product was analyzed through gel electrophoresis and vied on UV gel doc. The resultant PCR product was then by two different techniques was purified. Those which had sharp and bright bands were treated with EXO-Sap. In the PCR tubes we added EXO-sap according to ratio that was for every 5µl, 1ul of exo-sap was added and incubated at 37° for 15 minutes and then at 95° for 15 minutes and stored at 4° C. Then we concentrated the purified product for sequencing.

Table 3.3 shows the recipe used for the reaction

Ingredient	Concentration	Volume for 25µl	Volume for 100µl
10X Taq Buffer	10X	2.5	10
$MgCl_2$	25mM	2.5	10
dNTPs	2mM	1	4
Primer $_f$	50mM	1	4
Primer $_r$	50mM	1	4
Taq-Polymerase	5U/µl	0.2	0.8
DNA template	100ng/µl	1	1+1+1…
BSA		0.5	2
PCR H_2O		15.5	62

3.7 Sequencing

The sequencing was done after the purified products were concentrated well. We also sent our products to Macrogen for forward sequencing for further confirmation of sequences. For the sequencing reaction in our department, we used Sanger Method on Backman Coulter CEQ 8000 Genetic Analysis System. We took 2µl to 3µl of template from each sample and added 4µl of DTCS to it. Everything is done on ice. Then we added 2µl of 10pM primer. Primer was either forward or reverse for one reaction. But for the confirmation we ran 20 reactions of 10 samples for both reverse and forward primers. Then we added 11µl of NF water to make it up to 20µl. This was the recipe for sequencing PCR. Temperature set points were 96^0 C for 20 seconds, 50^0 C for 20 seconds, 60^0 C for 4 minutes and we gave 30 cycles to it and terminated at 4^0 C. After that we added Stop Solution to each PCR tube which contained 2µl EDTA, 2ul Sodium Acetate and 1µl Glygen. Then we incubated it for 2.45 hours. At 37^0 C. Followed by the purification of the product. We take the reaction mixture into new autoclaved Eppendorf tube and add 60ul of 95% ethanol. Then we spin it for 15 minutes at 14000 rpm. Then wash twice with ice-cold chilled 70% ethanol and spun it at maximum speed for 2 to 5 minutes. Then we vacuum dried it with incubation of 37^0 for three to four hours. And left overnight if the pellet is not well dried. The white pallet ensured the presence of PCR product. These results were not satisfying so we sent these PCR products to Macrogen, Korea and retrieve the sequences. The sequences came in a week and were then processed for the phylogenetic and molecular identification.

3.8 Molecular Identification

For this purpose we loaded the sequences in the Geneiuos software version 8.1.6 and chromatograms were analyzed. Their ends were trimmed manually. And were aligned to the ten closest matches. They were aligned according to Geneious alignment. The type was Global Alignment with free ends and gaps, with 65 % similarity 5.0/-4.0. The results were manually edited according to the need and a few ambiguities were removed.

3.9 Phylogenetic tree

Using Geneious tree builder these alignments were formed into trees which showed our species to be placed right where they belonged. So this confirmed the molecular identification was correctly done. The genetic distance model for the trees used was Tamura-Nei according to the method of neighbor joining with no outgroups because of small number of species. The resampling was put ot bootstrap with the value of 100.

CHAPTER 4

RESULTS

4.1 Collection and Preparation of Herbarium sample

The samples were collected and dried in bags with silica beads. The samples were prepared for the herbarium specimen by pressing for a long period of time a day or two. And were treated with $AgCl_2$ so they are not destroyed by the fungus. They were paste to herbarium sheets and were submitted to the Department of Botany, Pakistan Museum of National History. And their voucher numbers were retrieved. Following picture are of the samples collected.

4.2 DNA Extraction.

The DNA was extracted accordingly and run on 1% agarose gel, treated with RNAase A they were purified from contaminations. They were then checked on Nano-drop for concentration calculation. And were diluted according to the needs. The following figure shows the gel eluted picture of the samples and treated with RNAase and not treated with RNAase. And the table shows the value of DNA concentration present.

4.3 PCR results and Purification

PCR products for 9 samples came positive and send to Macrogen Korea, for sequencing. Samples were twice sequenced for forward and once for reverse sequence reaction for reconfirmation.

4.4 Sequence Alignment

Sequences came after one week of arrival and were viewed on bioinformatics tool software called Geneious version 8.1.6 these sequences were then manually edited where necessary and were BLAST to the NCBI gene bank. The ten closest relatives were taken and aligned for the review of mutations different anomalies or double gene presence and ambiguities were removed. Some species were collected twice and because of the lack of morphological and diagnostic character they were considered two different species but then the molecular sequences revealed the maximum identity and

proved reparations. More over the alignments also showed that the morphologically diagnosed species like *Actinoscirpus grossus L. F.* were inferred that this specie fall in the genus Fimbristylis and not in the Actinoscirpus genus. *Cyperus rotundus L.* was also repeated and proved to be correctly diagnosed. It showed maximum identity to already reported sequence of the same species. Similarly *Pycreus flavidus retz.* was diagnosed correctly and repeated sequencing showed the maximum identity to already reported species. The photographs of few species were taken and presented here starting from figure 4.1 to figure 4.15. They contain close ups of few species. The proposed results are given in the figures starting from Figure 4.17 to 4.21 are the results of alignment of ITS sequences of *Actinoscirpus grossus L. F., Actinoscirpus spp. Cyperus nutans vahl. Cyperus rotundus L.* and *Pycreus flavidus retz. respectively.* They have two parts the upper part is the part which shows identity and the lower part shows the sequence and its nucleotide mutation.

4.5 Phylogeny

The consensus trees were built on the bases of neighbor joining and bootstrap value of 100. The results were then BLAST and ten of the closest identity were took to construct a tree. The alignments were made and some sequences were manually edited. The alignments were constructed using neighbor joining preference in Geneious software. The results shows the alignment of our sequences to the closest relatives and the mutation or presence of the double gene or different anomalies. The sequences were analyzed and manually edited where possible. The following figures shows the alignments of sequences. The tree constructed for the specie shows the maximum identity of *Actinoscirpus grossus L. F.* to the *Fimbristylis sieboldii Miq.* with the base value of 91. It shows that this specie might be diagnosed incorrectly or it needs a revision in naming these plant species. Moreover it shows very close relevance to all of the species lie in the genus Fimbristylis and none of the Actinoscirpus specie is present.

Figure 4.22 is the demonstration of the Actinoscirpus specie discussed earlier. We can see that there is not a single specie present from the same genus as diagnosed above. Figure 4.23 is the image representation of the tree *Cyperus nutans,* which was identified

correctly but with very low information about this specie its sequence was not reported elsewhere. The closest ten species were retrieved and trees were constructed on the same measurements as above. Figure 4.23 shows the resulting tree for the specie.

Results in the figure 4.24 for collected specie *Cyperus rotundus* supported that it is the correctly identified and it is further molecularly identified and has a sequence tag, though there were some anomalies which predicted mutations but they were manually edited by reconfirmation of sequencing results. It shows the closet relevance to the *Cyperus rotundus L.* reported back in South Korea and USA. It is the most accurate result we have got and suffice the need of interpretations. In the figure 4.25 the results for the *Actinoscirpus spp.* are shown. It is clear from the results that the specie was not identified correctly. The results shows the maximum identity to the specie from the genus Fimbristylis. And it shows the 100% bootstrap value. The specie was identified morphologically and falls in the genus Actinoscirpus but the results shows that it falls in the Fimbristylis genus. The results of bootstrap are satisfying enough to support that they belong to the Fimbristylis genus.

In Figure 4.26 the results show that the identified specie is correctly identified morphologically and molecularly. *Pycreus flavidus* collected from the streams near Islamabad had sequence identity to those found in South Korea, with a NJ and bootstrap method of value 100 the inferred results shows that they are closely related. Figure 4.20 explains the result out comes.

***Figure 4.12* A photograph of *Kyllinga brevifolia Rottb.* in the fields**

Figure 4.2 a close up of the specimen *Cyperus ligularis L.*

Figure 4.3 Photograph of *Cyperus Compressus L.*

Figure 4.4 Photograph of *Cyperus difformis L.*

Figure 4.5 Photograph of *Cyperus difformis L.*

Figure 4.6 Photograph of *Actinoscirpus grossus L.*

Figure 4.7 Photograph of *Cyperus iria L.*

Figure 4.8 Photograph of *Cyperus nutans Vahl.*

Figure 4.9 Photograph of *Kyllinga brevifolia Rottb.*

Figure 4.10 Photograph of *Cyperus Nutans Vahl.*

Figure 4.11 ***Actinoscirpus spp.***

Figure 4.12 Photograph of *Cyperus rotundus L.*

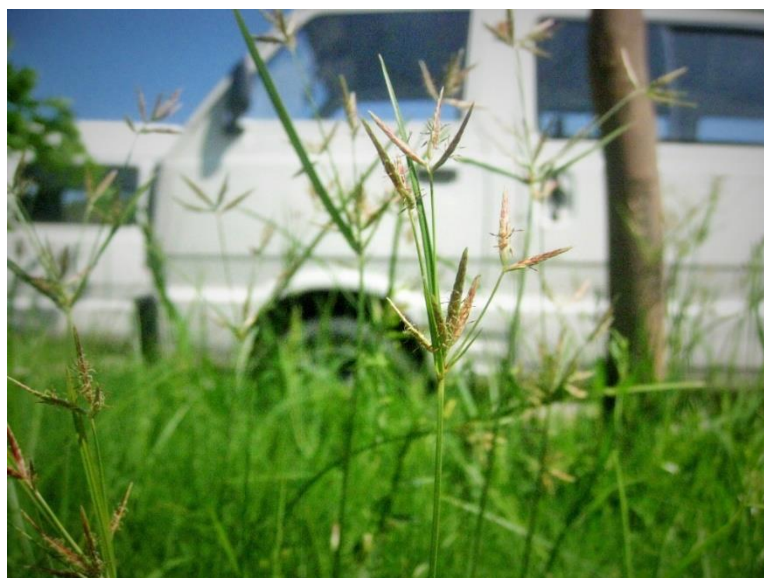

Figure 4.13 Photograph of *Cyperus rotundus L.*

Figure 4.14 Photograph of *Cyperus compressus L.*

Figure 4.15 Photograph of *Cyperus compressus L.*

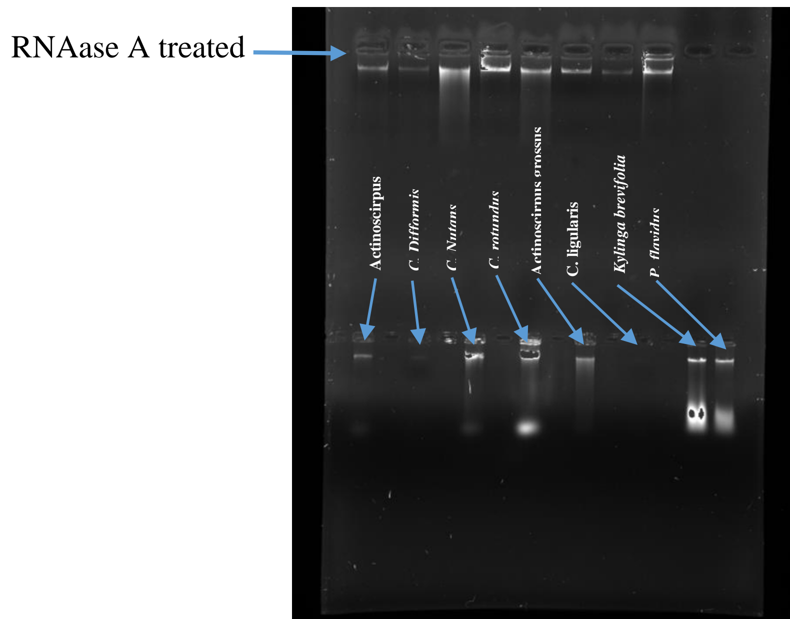

Figure 4.16 DNA extracted and treated with RNAase A eluted on 1% Gel

Table 4.1 The DNA concentrations in ng/µl and average 260/280 and 260//230 values

C. difformis L.	254	217	173	1.7	0.69
Actinoscirpus grossus L. F.	136	117	173	1.57	0.65
Kyllinga brevifolia Rottb.	114	86	10	1.8	0.48
C. ligularis L.	110	191	226	1.62	0.45
P. Flavidus (Retz.)	240	325	306	1.8	0.58
C. nutans Vahl.	107	116	119	1.6	0.7
C. compressus L.	122	187	145	1.56	0.45
C. iria L.	346	377	342	1.6	0.34

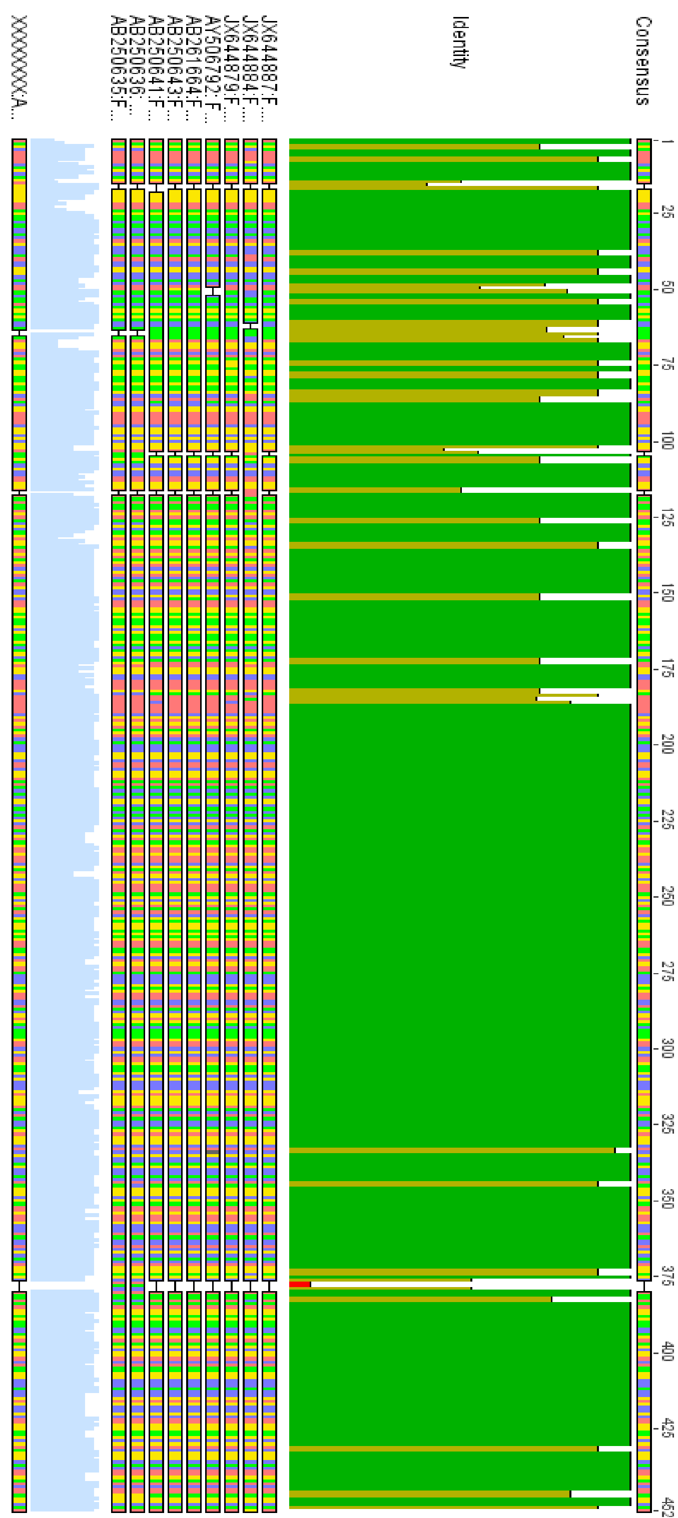

Figure 4.17 Sequence Alignment of ***Actinoscirpus grossus L. of ITS region with other ten close relatives***

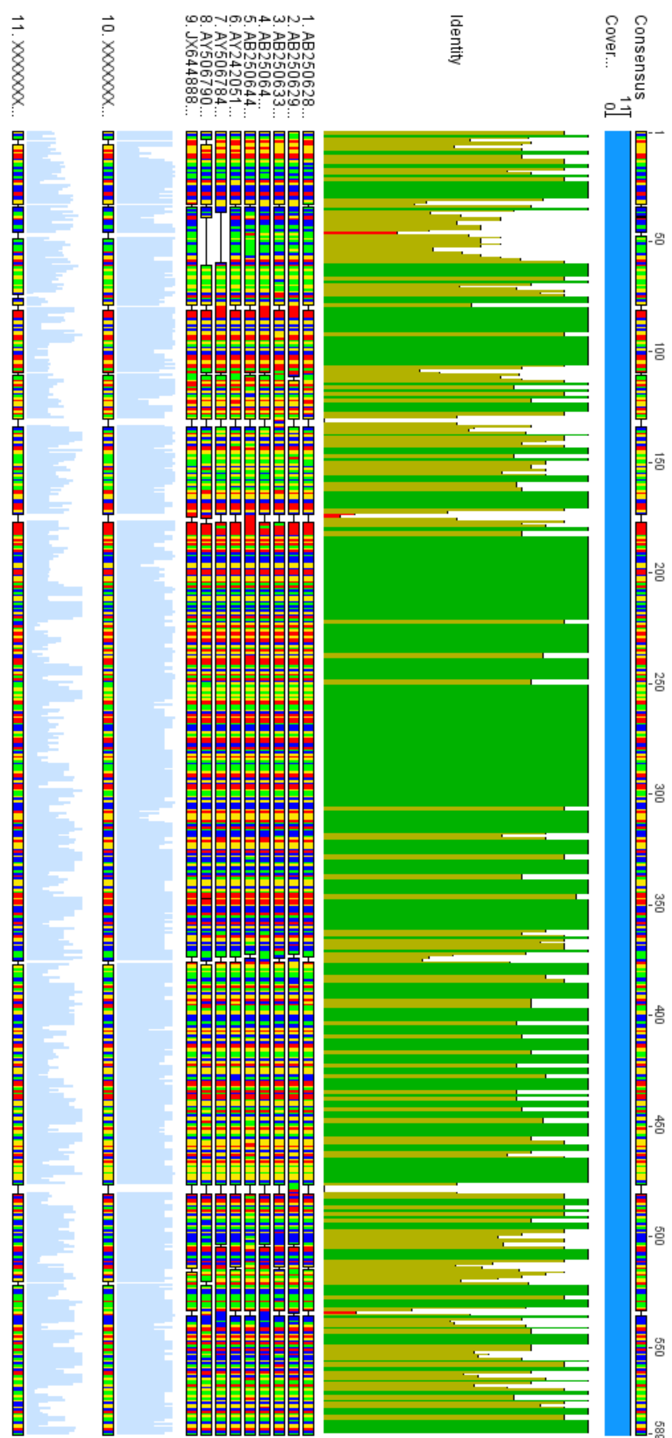

Figure 4.18 Sequence Alignment of *Actinoscirpus spp. of ITS region with other ten close relatives*

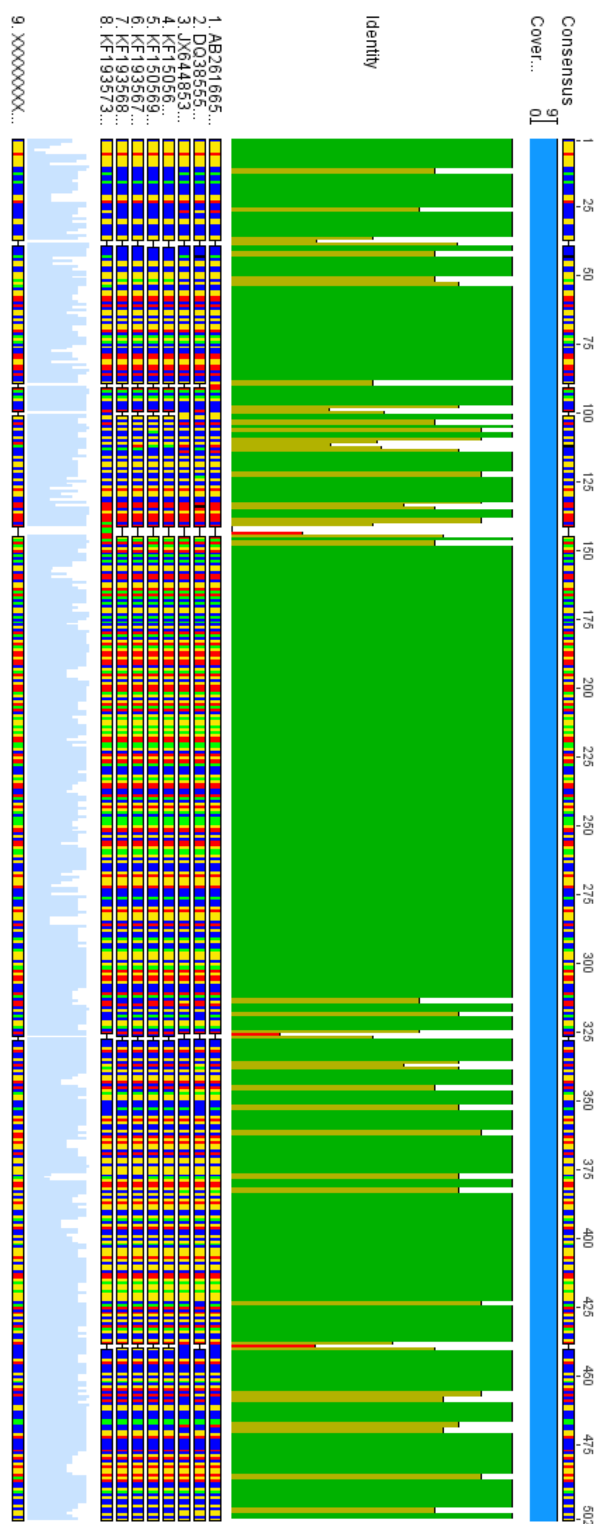

Figure 4.19 Sequence Alignment of ***Cyperus nutans vahl.. of ITS region with other ten close relatives***

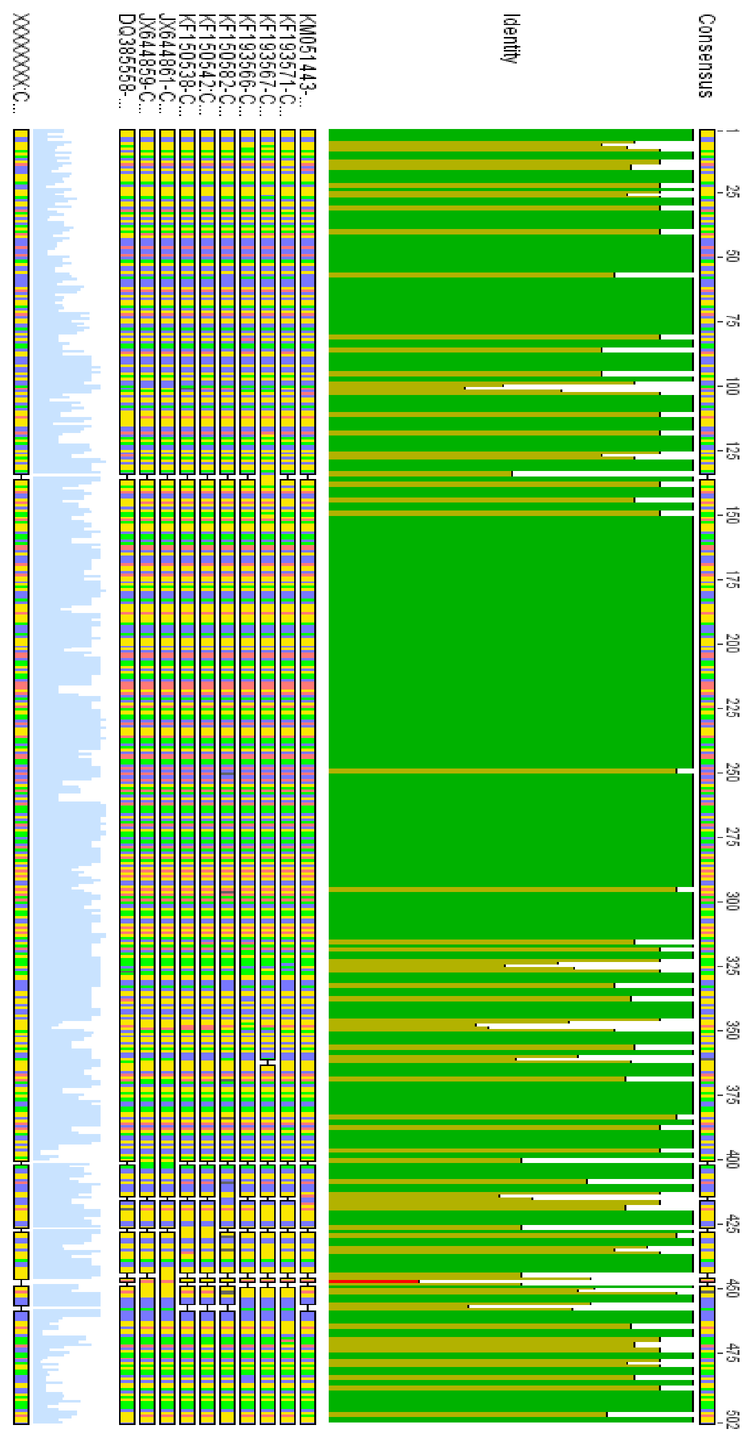

Figure 4.20 Sequence Alignment of *Cyperus rotundus L. of ITS region with other ten close relatives*

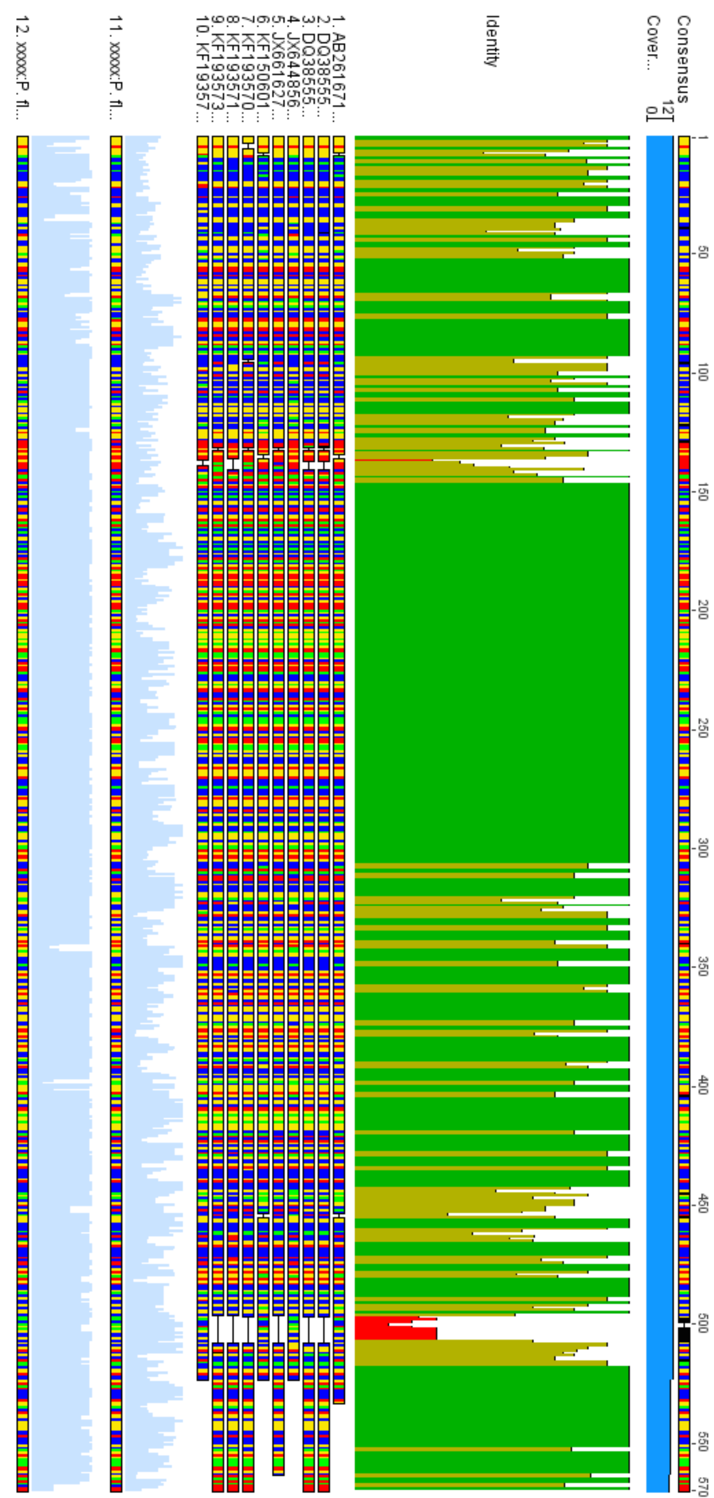

Figure 4.21 Sequence Alignment of ***Pycreus flavidus L. of ITS region with other ten close relatives***

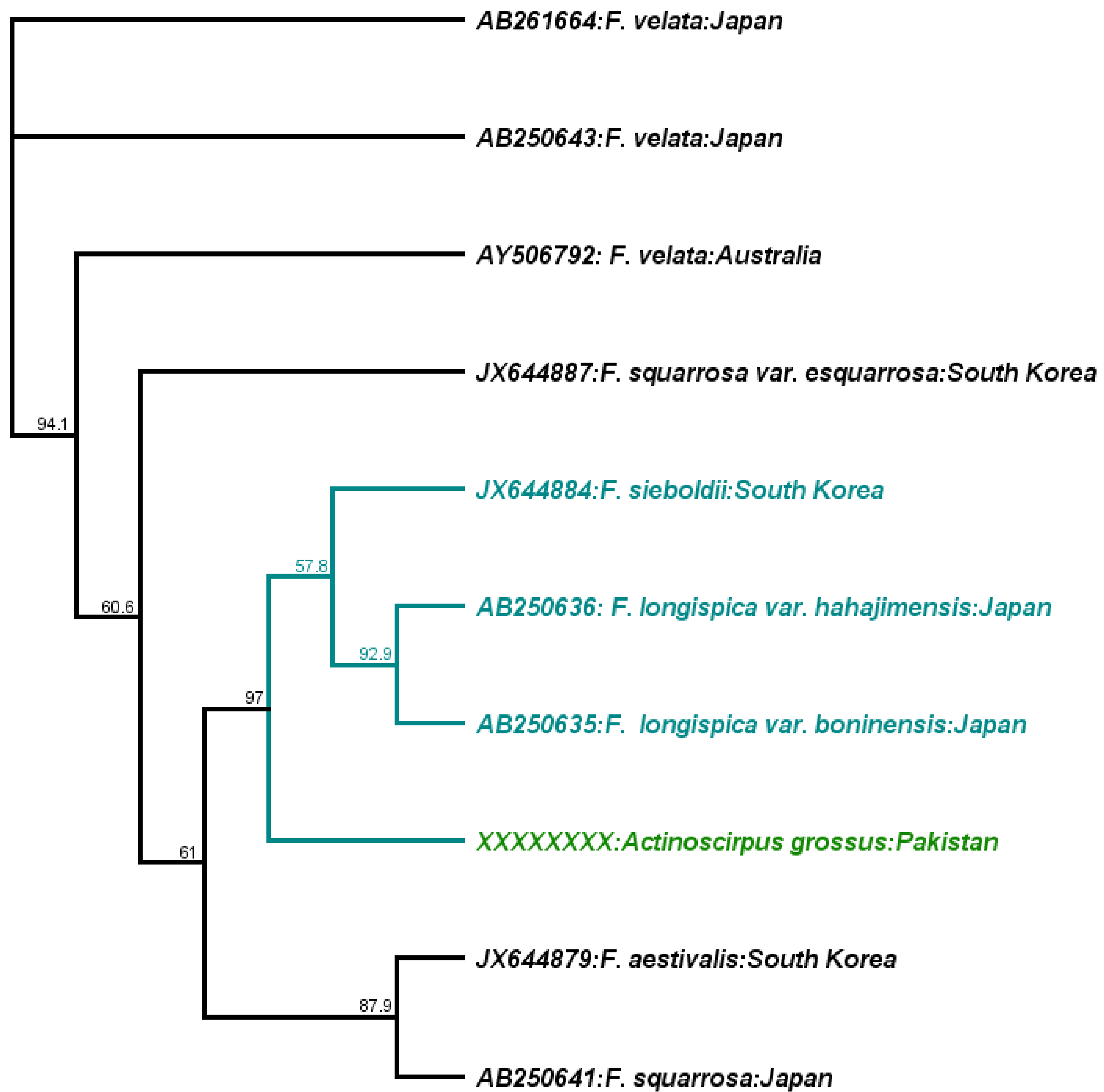

Figure 4.22 Neighbor-Joining consensus tree based on combined ITS sequences of nrDNA of *Actinoscirpus grossus L. F.* with its maximum identity neighbors.

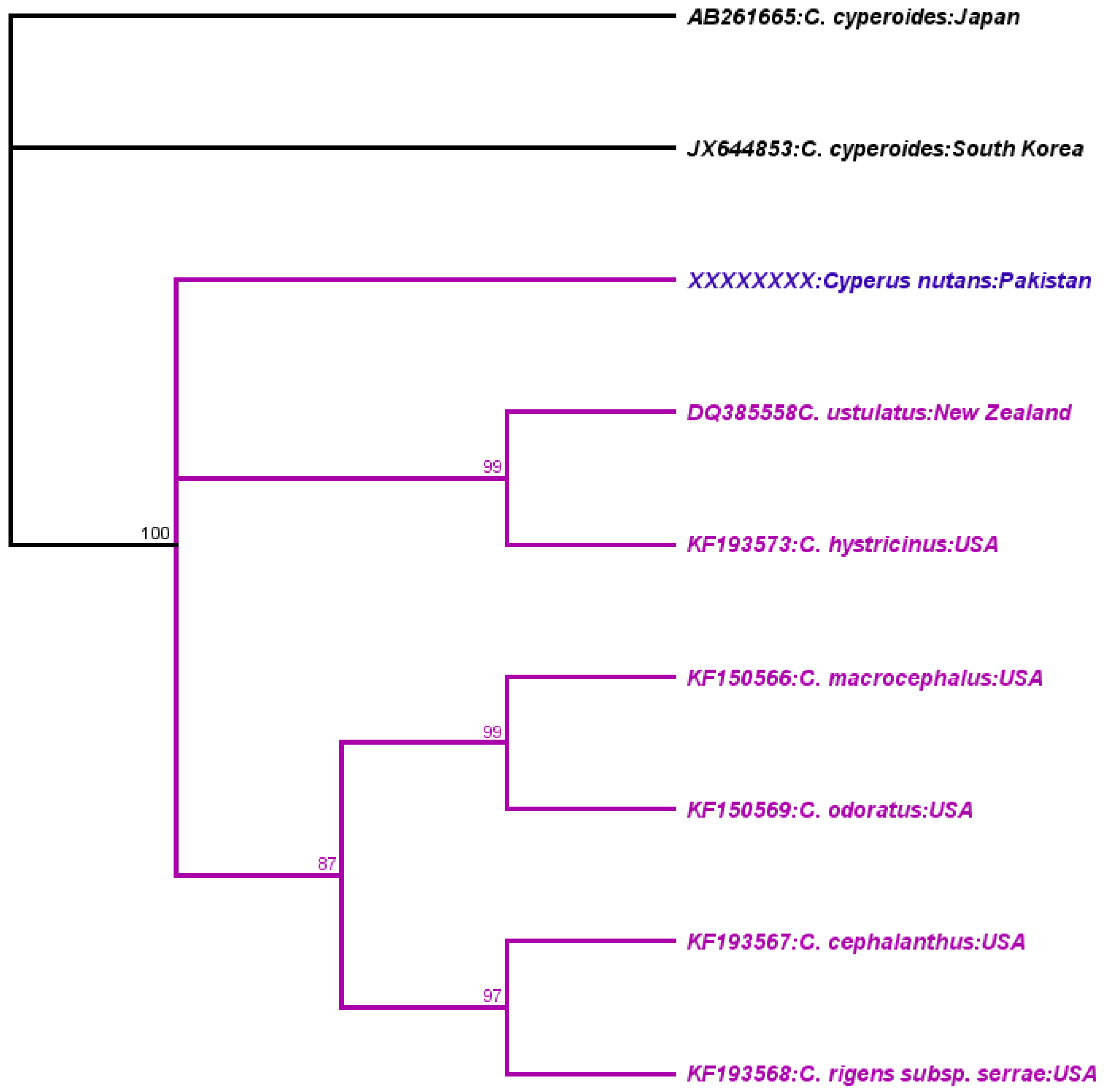

Figure 4.23 Neighbor-Joining consensus tree based on combined ITS sequences of nrDNA of *Cyperus nutans vahl.* with its maximum identity neighbors

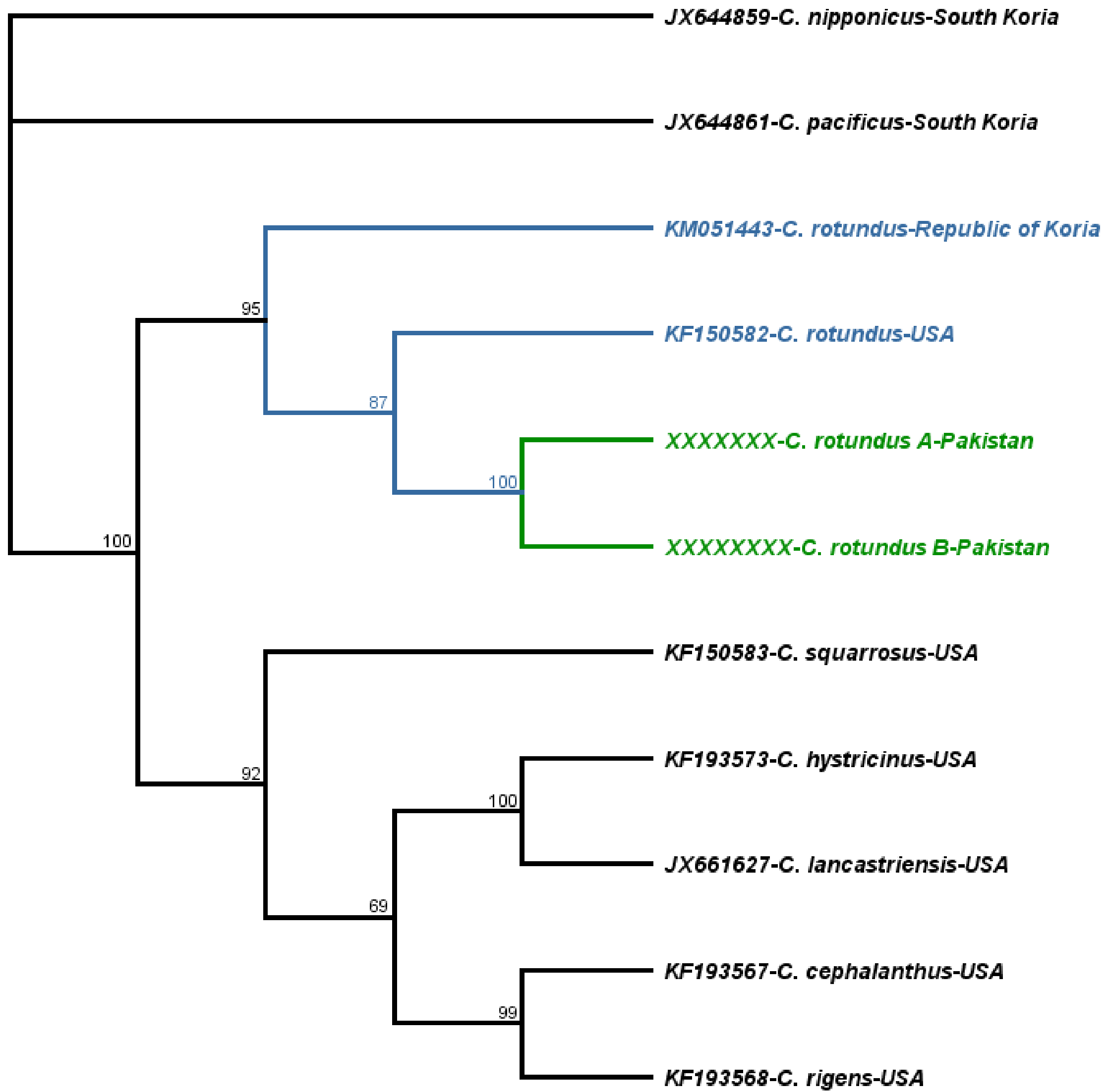

Figure 4.24 Neighbor-Joining consensus tree based on combined ITS sequences of nrDNA of *Cyperus rotundus L.* with its maximum identity neighbors

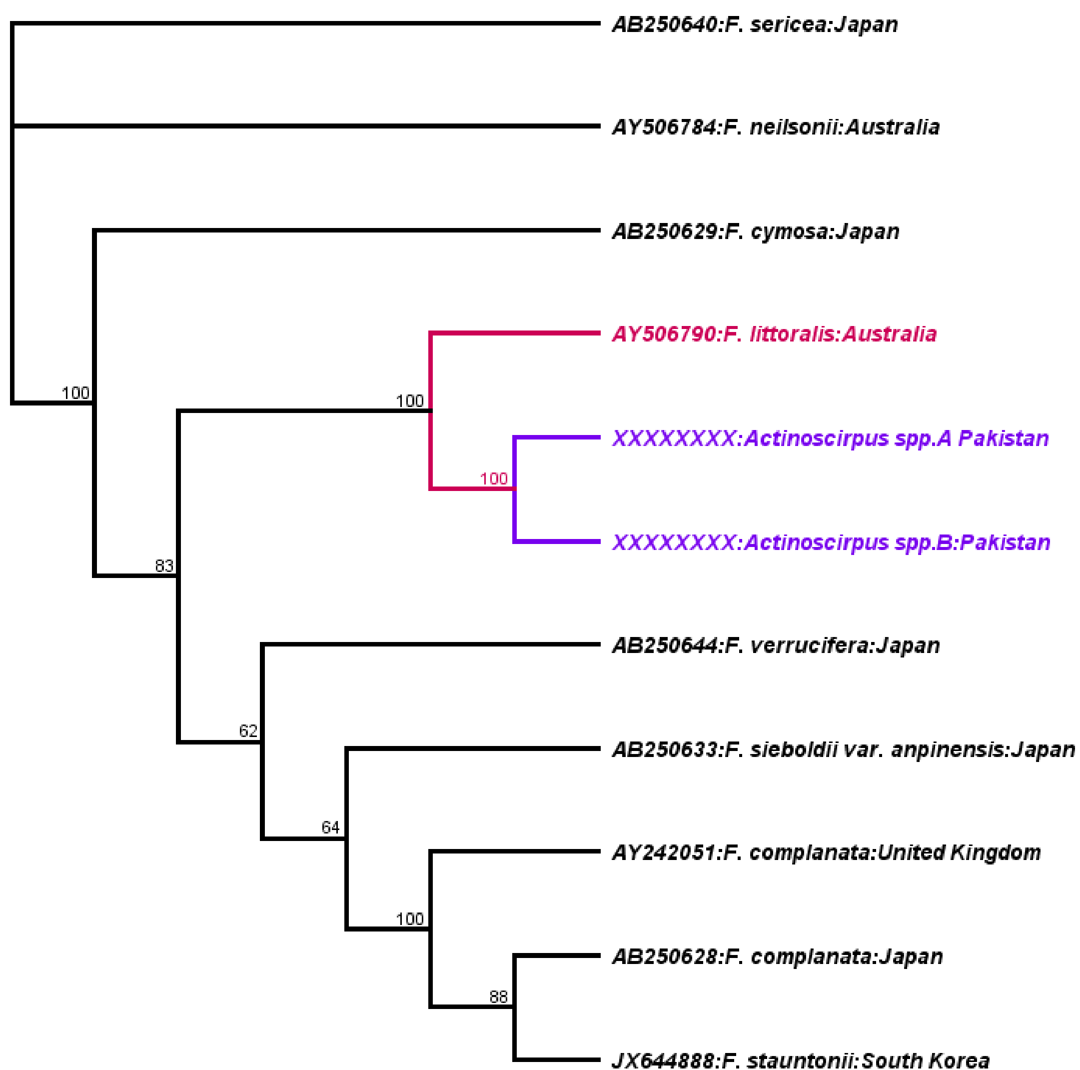

Figure 4.25 Neighbor-Joining consensus tree based on combined ITS sequences of nrDNA of *Actinoscirpus grossus spp.* with its maximum identity neighbors

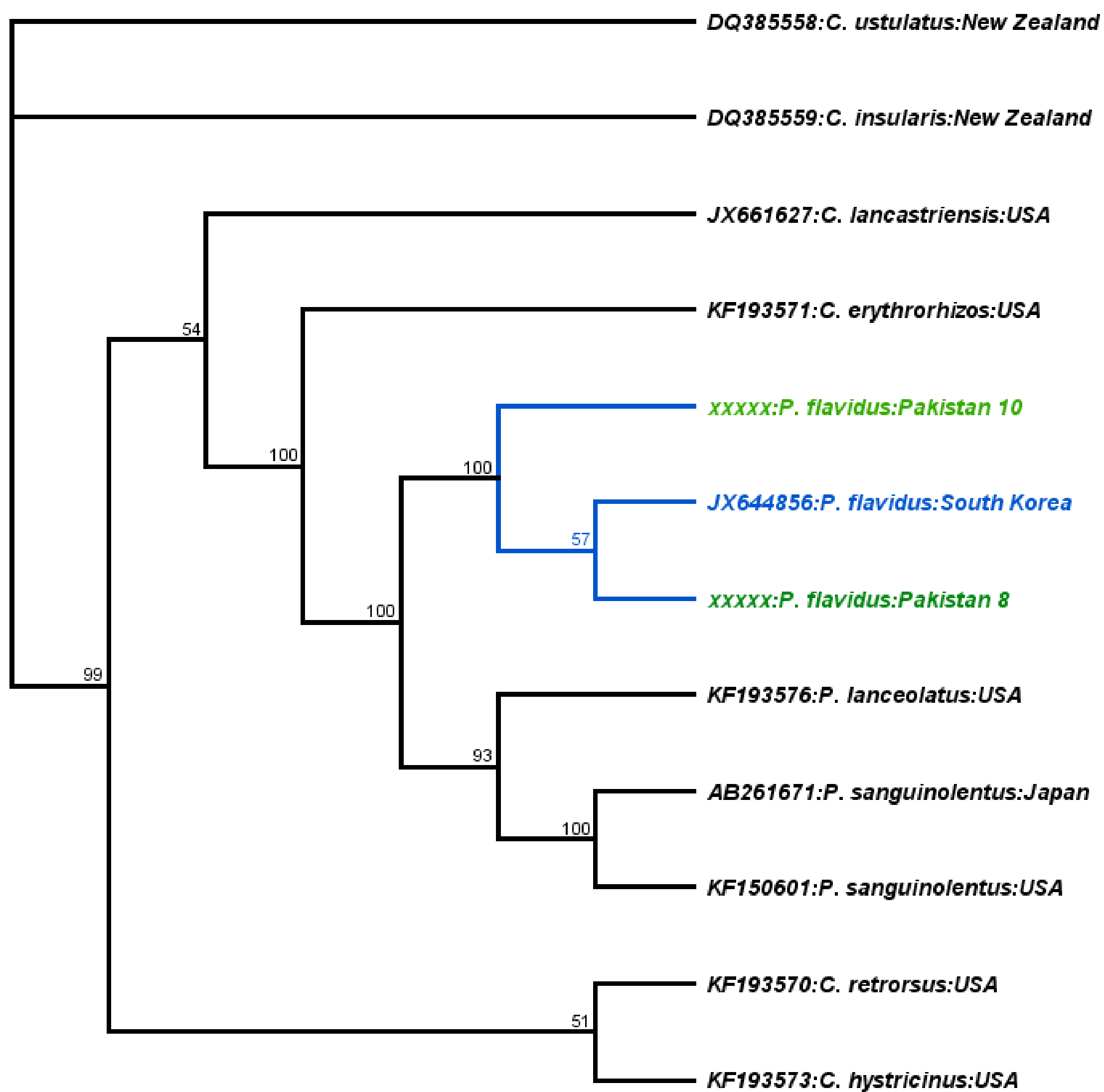

Figure 4.26 Neighbor-Joining consensus tree based on combined ITS sequences of nrDNA of *Pycreus flavidus retz.* With its maximum identity neighbors

CHAPTER 5

DISCUSSION

Cyperaceae is a very huge family, and because of its massive size it is quite impossible to see clearly into the picture and identify each one of them correctly on the bases of simple morphological and diagnostic characters. With the discovery of molecular biology and inventions of new molecular techniques it is devised that the molecular identification of such huge families was a need of the time. It was then that some of the regions were put in such reserved region section and were put under study to scientifically put tags on the identified species. The regions included were from the nrDNA, some chloroplast DNA or mitochondrion DNA. They were amplified with the unique and some universal set of primers like ITS and ETS and reported accordingly.

The problem hindered in the identification of collected plants morphologically and with diagnostic characters. A few species were said to be different but came out to be same. On the other hand some species were identified incorrectly. This was proven by the results that came out from the PCR amplification of the conserved region. In our study it was internal transcribed spacer region that was brought under deep consideration. After sequencing it was also scene that there where peaks that were incorrectly read. Moreover due to the polyploidy which is another a very common factor in plant breeding we get different anomalies. These different anomalies of a single gene can tell you about the false mutation and they can give false phylogenetic analysis resulting in wrong predictions. These reasons should be kept in mind prior performing an experimental study. This study was conducted for a reason and that reason is to highlight and bring this piece of information in to the knowledge that there are many evolutionary processes at organismic level that has the influence on the sequence variation in the internal transcribed spacer repeats in plants.

In our case the plants were first morphologically identified according to diagnostic characters and were supposed to be the right species. Then the identification of these species were further reconfirmed by the amplification of a conserved region ITS using ITS1 as forward and ITS4 as reverse primer. These were then sequenced and from these

sequences it was found that there is a slight difference in identification of some specie. *Actinoscirpus grossus* as identified by the NHMP was showing very close relevance to *Fimbristylis longispica var. boninensis* and the results supported that *A. grossus* is a close relative to the specie furthermore it also suggests that A. grossus lies in the Fimbristylis genus rather than the Actinoscirpus. The bootstrap value and estimation is nearly 100 and neighbor joining results also supports the results. Similarly other plants were also identified morphologically, but the results proposes the specie is too from Fimbristylis genus. The Pakistan Museum of Natural History Islamabad, identified it as specie from Actinoscirpus genus. The bootstrap and neighbor joining results showed the specie falls in the genus Fimbristylis and not in Actinoscirpus.

To understand the possible sequence complexity it can be achieved irrespective of any underlying cause. For this to be conducted it simply requires knowledge of the likelihood of sequence disparity and some simple experimental revisions. It is known by now that the direct sequencing of single PCR of nrDNA can lead to incorrect results and misleading interpretation. This is because of the numerical inequality between the bands of repeat type. As they exists in the genome. For this the solution was reported and they can be done in two ways either PCR bias or PCR selection (Wagner et al., 1994). In general the PCR bias is the occurrence of the amplification favored gene family. This will give us the same product from the selected repeats, under separate reaction but same conditions. The other way is the PCR drift in which random factors affect the selection of the same type of repeat during the first amplification cycles, in which case different products may be obtained in separate reactions (Wagner et al., 1994). These can be removed by the slight modification of the PCR methodology. The use of universal primers lead to the amplification of pseudogenes as well which gave the result of a fungal ITS region in case of one plant species. This also can be removed by using the angiosperm specific primers. The specie which was identified as *Actinoscirpus grossus* is native to Southeast Asia and is a known as the weed in the region (Holm et al., 1979). It is commonly found in the damp places usually where there is a running water like streams, bank of canals marshes and is abundant in lowland areas.

It hosts a fungal specie which is a threat to rice field, *Chilo polychrsus* (Kostermans et al., 1987). On the other hand its sequence identity came out on our molecular results to *Fimbristylis longispica var. boninensis* with a query cover of 89% and identity 98 % more over it showed 97% identity with *Fimbristylis velata* with an identity of 97%. Results supported that the specimen might be identified incorrectly as it shows these results with the species of genus Fimbristylis and not with any specie from the Actinoscirpus. The unrooted tree was formed and it fell in the clade where these two species are rooted.

Another specie which took our attention was also from Actinoscirpus. The specimen also has shown the identity to the Fimbristylis species. With query cover of about 86% it shows identity of 95% with *Fimbristylis littoralis.* This specie is found in most of the tropical and warm regions all over the world. From china all the way to South Asia Middle East Europe it goes to USA (Cook 1996, Verma 2001, Lakshminarasimhan 1996). Moreover it shows 90% identity with *Fimbristylis neilsonii* with a query cover of 87%. Not a single result supported the morphologically identified specie as Actinoscirpus. Moreover its tree also shows the grouping of our specimen with these two species with a bootstrap baseline of 100. For further confirmation we processed its reverse reaction for sequencing but it came out to be the same. The results showed and supported that it is incorrectly diagnosed. This suggests that either our specie was incorrectly diagnosed or it needs to be reviewed again and put in the Fimbristylis genus.

Other species like Cyperus rotundus was correctly identified and shows very strong relation with and identity from its kind. The BLAST shows the results with *Cyperus articulatus* with a query cover of 80% and identity of 97% but it also shows almost 97% identity with a query cover of 79% to the reported *Cyperus rotundus* from South Korea. These species were correctly identified and re-identified by molecular sequencing. Furthermore it also shows that it might contain a gene copy of the ITS region from two different shows. The polyploidy and cross pollination is a common process that takes place in plant’s life so it can happen that it contains regions from two different sources.

However their tree shows that they have been rooted with *C. rotundus* more and not with *C. articulatus.* With the bootstrap of near 100 and neighbor joining alignments it is confirmed that the specie falls in the group. Moreover it was also found that due to the polyploidy there found different anomalies which can be proposed as mutations by infants. But due to the expertise of senior scientists it was not reported as mutation rather presence of double gene was proposed. With the capability of the C. rotundus to grow every in tropical region and even in the sub tropic levels it can assume that it has undergone mutations or it has undergone the exchange of chromatin material with neighboring plant species.

Cyperus nutans vahl. which was identified in diagnostic characters also falls in the genus Cyperus and sub genus Cyperus supported by the molecular results. However it also shows the 96% identity with *Cyperus cyperoides L.* with a query cover of 93%, and it also shows 97% identity with a query cover of *Cyperus cephalanthus L.* Literature on the species *Cyperus nutans and Cyperus cyperoides L.* has revealed the very close relevance morphologically. Both the species are perennial, have sheaths up to 14-15cm blades to 30cm or more Greenish yellow in color sometimes brown. The height of the plants ranges from 50 to 60 cm in rare case it exceeds 80cm in case *of C. Nutans* vahl. Due to its relevance to the morphological characters with these species our specimen put us in doubt. And the sequencing due to the lack of enough information on this specie it's ITS sequence was not reported but its sequence analysis showed and inferred that it falls in the Genus Cyperus and sub genus Cyperus. But due to the lack of molecular knowledge it has not been reported by any other scientific group about the sequence of ITS region of the *C. nutans vahl.* Its close relatives like *Cyperus rotundus* and *Cyperus iria* also shows close relevance in the proposed data. The alignment and tree constructed on the neighbor joining and bootstrap method was put under study. The tree which we built with in the sub genus from where the Cyperus nutans vahl. Belongs, it gives us right information with its relative species. In addition to that it did not give us the results with the *Cyperus cyperoides* in one clade. Rather the *Cyperus cyperoides* was rooted in

totally different clade and Cyperus nutans vahl. Was joined in the clade with *Cyperus ustulalatus and Cyperus hystricinus.* It scored 100 for the bootstrap value.

The *Pycreus flavidus* collected from two different places, it is present side to rivers, rice fields. It is commonly mistaken with its closest relative *P. flavescens*, because of their morphologically identities. The difference lie in the epidermis cells of nuts where *P. flavidus* are isodiametric and *P. flavescens* has much longer (Kern, 1974). The specimen was identified on the bases of their morphological and molecular identifications and fits correctly in the clades. Their identity values to already reported sequences of the same species are satisfying which 98 to 99% in some cases. It was repeated twice for forward and once for reverse and it gives satisfying results.

CHAPTER 6

CONCLUSION

From the study we came to the point that ITS has a region of 5.8S which is very important when we study molecular taxonomy, it is the most conserved part of the ITS region and depending on the identity of this region we can classify things into the groups. Moreover ITS alone cannot be used as a full source for phylogeny as it contains anomalies which can give false inference about the study. Along with the ITS we should use ETS, and other source of DNA like from Chloroplast and Mitochondria.

CHAPTER 7

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