Studies for Identification and Conservation of *Cyperus* spp. using DNA Barcoding

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In

AGRICULTURE
Molecular Biology and Biotechnology

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Jawaharlal Nehru Krishi Vishwa Vidyalaya
Jabalpur, Madhya Pradesh

2017

CERTIFICATE – I

This is to certify that the thesis entitled **"Studies for identification and conservation of *Cyperus* spp. using DNA barcoding"** submitted in the partial fulfillment of the requirement for the degree of **DOCTORATE of PHILOSOPHY in Molecular Biology and Biotechnology** of Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur is a record of the bonafied research work carried by **Mrs. Keerti Tantwai** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

All the assistance and help received during the course of the investigation has been acknowledged by him.

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Date: 04.02.2017

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ABSTRACT

Cyperus is the second largest genus of the sedges or Cyperaceae (Cyperae) family, the plants of which are identified as one of the most common agricultural weeds. In present study, a total of 82 sedge plants were collected from 21 different places covering 7 states of India. The plant species and morphological variations among different species were determined based on overall plant growth characteristics. The samples were identified as belonging to 17 different species of *Cyperus* and related genera on the basis of UPGMA cluster analysis using Jaccard and Simple Matching coefficients. The mantel test coefficient of 0.97169 between these two similarity coefficients was found significant. Several morphological traits were assessed for identification of plants up to species level, among those spikelet was found the best to be used for the identification of sedge species.

DNA barcoding of the collected sedge plant samples was done using 4 plastid based barcode loci viz., *rbcL*, *matK, trnH-psbA* and *ycf1(b)*. Barcode loci *rbcL* generated 36 barcodes of length 314-347bp, *matK* in 17 samples of length 540-826bp, *trnH-psbA* in 14 samples of length 856-883bp, while *ycf1(b)* in only three samples of length 688-793bp. All the barcode sequences were deposited in GenBank and given accession numbers. Similarity based searches by BLASTn and BOLD plant identification tools revealed 17 unique species specific barcodes generated in present study which has not been reported in the GenBank for the same locus and species. In present study, for the first time, barcode of *ycf1(b)* has been generated for *Cyperus* spp. Similarity analysis in TaxonDNA program using DNA barcode sequence data for *rbcL*, *matK*, *trnH-psbA* and the concatenated dataset for *rbcL+matK*, *rbcL+trnH-psbA*, *matK+trnH-psbA* and *rbcL+matK+trnH-psbA* led to the correct identification rate of 67% by best close match method for the combined barcode *rbcL+matK*. Based on UPGMA and Maximum parsimony analysis *rbcL+matK* was found the best barcode loci followed by *matK* and *trnH-psbA* resolving monophyletic cluster for all the individual species. Barcode gap analyses based on inter and intraspecific divergence revealed *trnH-psbA* as best barcode locus exhibiting barcode gap. The *rbcL+matK* was found the best barcode loci for DNA barcoding in *Cyperus* spp.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
°C	Degree Centigrade
µg/ml	microgram per milliliter
Acc No.	Accession Number
accD	acetyl coenzyme A carboxylaseD
BLAST	Basic Local Alignment Search Tool
BLASTn	BLASTnucleotide
BOLD	Barcode of Life Data System
bp	base pair
CBOL	Consortium for the Barcode of Life
CG	Chhattisgarh
CI	Consistency index
cm	Centimetre
CTAB	Cetyl-tri-methyl Ammonium Bromide
DNA	Deoxyribose nucleic acid
etc.	et cetera
Fig	Figure
GJ	Gujarat
iBOL	International Barcode of Life Project
in	inch
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
J	Jaccard
m	metre
matK	Maturase K
$MgCl_2$	Magnesium Chloride
min	minute
mm	millimetre
MMDBD	Medicinal Materials DNA Barcode Database
MP	Madhya Pradesh
MP analysis	Maximum Parsimony analysis
ndhJ	NADH-quinone oxidoreductase
nm	nanometre
nrDNA	Nuclear ribosomal DNA
№	Number
p	Probability
PCR	Polymerase chain reaction
p-distances	Pairwise-distance
Pi	Parsimony informative
pM	Pico mole
PPi	pyrophosphate
rbcL	rubilose-1,5-bisphosphate carboxylase/oxygenase Large subunit
RCI	Rescaled Index for all sites
RI	Retention index
RJ	Rajasthan
rpoB/C1	RNA polymerase beta subunit B/C1
*rps*16	Ribosomal Protein S16
s/ sec	second

Seq.	Sequence
SM	Simple Matching
TN	Tamil Nadu
trnH-psbA	*trnH-psbA* intergenic spacer region
UK	Uttarakhand
UP	Uttar Pradesh
UPGMA	Unweighted Pair Group Method with Arithmetic Averages
UV	Ultra violet
ycfa/b	*ycf*1 plastid gene a/b
λ	Lambda
μl	microliter
μM	micromole

INTRODUCTION

About 50 million plants and animals are living on earth, out of which less than 2 million have been identified. In recent years, new ecological approach called DNA barcoding has been proposed to identify species and used for further ecological research. DNA barcoding is a system for fast and accurate species identification which has made ecological system more accessible. DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. DNA barcoding first came to the attention of the scientific community in 2003 when Paul Hebert’s research group at the University of Guelph in Ontario, Canada, published a paper titled "Biological identifications through DNA barcodes". It differs from molecular phylogeny in that the main goal is not to determine patterns of relationship but to identify an unknown sample in terms of a preexisting classification. Although barcodes are sometimes used in an effort to identify unknown species or assess whether species should be combined or separated, the utility of DNA barcoding for these purposes is subject to debate. A desirable locus for DNA barcoding should be standardized (so that large databases of sequences for that locus can be developed), present in most of the taxa of interest and sequenceable without species-specific PCR primers, short enough to be easily sequenced with current technology, and provide a large variation between species yet a relatively small amount of variation within a species. In plants, several candidate DNA barcodes have attracted the attention of many researchers such as *trnH-psbA*, *rbcL*, *matK*, *rpoB*, *rpoC1*, *ndhJ*, *accD*, *ycfa/b* in the plastid genome and rDNA ITS in the nuclear genome to classify the plants. Consortium for the Barcode of Life (CBOL) Plant Working Group (2009) has recommended the core 2-locus combination of *rbcL+matK* as the plant barcode in order to provide a universal framework for the routine use (Rai et al, 2012).

There are an estimated 300,000 plant species in the world but relatively few of these can be identified based on traditional plant identification methods (IUCN, 2012). Accurate classification and identification of this large number of species remains a significant challenge even for specialist

taxonomists. The emergence of DNA barcoding has had a positive impact on biodiversity classification and identification (Gregory, 2005).

Cyperus spp. is the most invasive weeds known, having spread out to a worldwide distribution in tropical and temperate regions. *Cyperus* spp. are sedge plants belonging to the family Cyperaceae. It is the third largest monocotyledonous family, with a considerable economic and conservation importance. The family comprises nearly 104 genera and more than 5000 species world-wide. The *Cyperus* species are herbaceous plants and mostly used as fodder and in particular purple nut sedge (*C. rotundus*) and yellow nut sedge *C. esculentus* are used as medicinal herb. They are commonly found as a perennial weed with slender, scaly creeping rhizomes, bulbous at the base which is about 1-3 cm. long. They are useful for bowel disorder and inflammatory diseases as well as uterine relaxation in both pregnant and non-pregnant women and relieving pain. *C. rotundus* is a traditional herbal medicine used widely as antimalarial, analgesic, sedative, and treating stomach disorder etc. Anti-bacterial effect is also found in rhizome extract of *C. rotundus* (Kabbashi et al, 2015).

Cyperus rotundus has been identified as an important medicinal plant found in Madhya Pradesh state (Shukla et al, 2010). It has a wide range of medicinal and pharmacological applications and has now been identified as a source of several pharmacologically important active substances such as α-cyperone, β-selinene, cyperene, cyperotundone, kobusone, isokobusone etc. (Mehta et al, 2013). Other pharmacological and biological activities of *Cyperus rotundus* include anti-inflammatory, antimicrobial, anti-pyretic, antioxidant, etc. have been also reported (Sivapalan, 2013). Silver nanoparticles have been synthesized from *C. rotundus* plant extract as reductant and capping agent. The reduction of silver ions and stabilization of Ag nanoparticles may be due to tannins and flavonoids present in *C. rotundus*. This biosynthesis may help in reducing toxicity to environment (Siva et al, 2014). Due to similar properties, *C. rotundus* has been identified as a substitute for Himalayan species, Ativisha (*Aconitum heterophyllum*) in Ayurvedic texts (Venkatasubramanian et al, 2010). Dried rhizome powder of *C. rotundus* is commercially marketed by companies as 'Musta root powder'. Barbosa et al (2011) observed that extract of *C. rotundus* leaves has a toxic

effect on pest *Diabrotica speciosa* which can be a good biopesticide for pest control in agroecological systems. Antimalarial, anticancer, antimicrobial activities in essential oil from the aerial parts of *Cyperus kyllingia* has also been reported (Khamsam et al, 2011). *Cyperus involucratus* is planted as an ornamental plant. *Cyperus iria* is used as fodder and its stem is woven into mats. Leaf and tuber parts of *C. iria* are used as tonic, stimulant, stomachic and astringent (Ambasta, 2016). *Kyllinga odorata* is known for diaphoretic and diuretic properties (Lindley, 1847). *Kyllinga brevifolia* is a Paraguayan folk medicine and used as sedative and tonic for nervous system (Hellión-Ibarrola et al, 2014). *Fimbristylis miliacea* has been found to play an important role in phytoremediation to absorb heavy metals and zinc in waste water treatment (Liu et al, 2007).

Biological species are authenticated traditionally according to their morphological features which usually requires the expertise of an experienced professional taxonomist. In the case if the diagnostic morphological trait of a specimen is lacking, it becomes difficult even for the specialists to recognize it correctly. Authentication using DNA barcode overcomes these problems. The short DNA sequence is taken from standard region of genome to generate DNA barcode. Even non experts can identify a plant species correctly by using DNA barcode from small, damaged or industrially processed material. Further, identification can be done with small tissue samples from virtually any part of the organism, does not require reproductive material, and the identification process is in general fast and reproducible. The technique can also prove valuable for accurate species identification as the important first step in conservation plans for threatened species. Apart from identifying target species, DNA barcoding can also be applied in floristics. Constructing a DNA barcoding reference library of entire local floras can allow for fast and effective floristic analyses without expert knowledge, or even be a method of estimating species richness in a taxonomically poorly known flora. There is also a huge potential for application of DNA barcoding to the vast collections at herbaria and Natural History Museums, which could serve as excellent reference databases and help identify the many unidentified specimens present in most collections. This would contribute significantly to improved knowledge about distribution patterns of each species. DNA barcoding thus has the potential of

becoming an important supporting tool for conservation and biodiversity assessments in areas with a high number of plant species, a shortage of expert taxonomists, and limited descriptions of the flora (Hartvig et al, 2015).

The loci to be verified or developed as DNA barcode for plants could be from the nuclear or chloroplast genome. These, may be from either coding or non-coding regions.DNA barcoding studies in plants have proposed *rbcL*,*matK*,*trnH-psbA*, *rpoB*, *rpoC1* and nr*ITS* as promising candidates.Core 2-locus combination of *rbcL*+*matK* and multi locus barcode i.e. 3-locus combinations, *matK*+*rpoB*+*rpoC1* or *matK*+*rpoC1*+*trnH-psbA*have also been proposed. Recently, a chloroplast region *ycf1(b)* has been suggested as the most promising plastid DNA barcode of land plants. In Cyperaceae, DNA barcoding related studies have mostly done with taxonomic view for placing a species or genus at correct level based on phylogenetic studies (Muasya et al 1998; 2001; 2002; 2009). Currently, DNA barcode for identification of plants of only few *Cyperus* spp. are available. Hence, It is pertinent to generate DNA barcode for correct identification of commonly found plants of *Cyperus* spp. in India.

Keeping in view, the agro-ecological, economical and medicinal importance of *Cyperus* spp., present study was formulated with the following objectives:

Objectives

1. Collection and morphological characterization of *Cyperus* species.
2. DNA barcoding of the *Cyperus* spp.
3. To assess the most efficient barcoding loci for the identification of *Cyperus* spp.

REVIEW OF LITERATURE

Cyperaceae is the third largest monocotyledon family, with considerable economic and conservation importance. Plants of the family Cyperaceae are grass-like herbaceous plants found in wet regions throughout the world. *Cyperus* having about 650 species, is the second largest genus after *Carex* in the Cyperaceae family. Members of the Cyperaceae (sedge family) resemble the Gramineae (grass family) but are distinguished by three-ranked leaves with one-third phyllotaxy and leaves that have closed leaf sheaths, usually solid stems, absence of a ligule and each flower subtended by a single glume or scale (Wills, 1998). All *Cyperus* have a uniform embryo type and a relatively consistent morphology. Morphologically all the *Cyperus* species have common characteristics of leaves that are shiny, light to dark green, three-ranked and corrugated in cross section. Leaf initiation terminates with the formation of seed bearing culm. The culm that grows through the center of the leaf fascicle is erect, simple, smooth and triangular in cross section. The culm supports a terminal inflorescence which is a simple or slightly compound loose umbel subtended by two or four leaf-like bracts (Wills, 1998).

The *Cyperus* species are herbaceous plants and mostly used as fodder. In particular, *C. rotundus* and *C. esculentus* are used as medicinal herb. They are also called as purple nut sedge and yellow nut sedge, respectively. They are commonly found as a perennial weed with slender, scaly creeping rhizomes, bulbous at the base which is about 1-3 cm. long. They are useful for bowel disorder and inflammatory diseases as well as uterine relaxation in both pregnant and non-pregnant women and relieving pain. *C. rotundus* is a traditional herbal medicine used widely as antimalarial, analgesic, sedative, and treating stomach disorder etc. Anti-bacterial effect is also found in rhizome extract of *C. rotundus* (Mehta et al 2013; Kabbashi et al, 2015). Silver nanoparticles have been synthesized from *C. rotundus* plant extract as reductant and capping agent. The reduction of silver ions and stabilization of Ag nanoparticles may be due to tannins and flavonoids present in *C. rotundus*. This biosynthesis may help in reducing toxicity to environment

(Siva et al, 2014). Barbosa et al (2011) observed that extract of *C. rotundus* leaves has a toxic effect on pest *Diabrotica speciosa.* It can be a good biopesticide for pest control in agroecologic systems. Antimalarial, anticancer, antimicrobial activities in essential oil from the aerial parts of *Cyperus kyllingi* has also been reported (Khamsam et al, 2011). *Cyperus involucratus* is planted as an ornamental plant. *Cyperus iria* is used as fodder and its stem is woven into mats. Leaf and tuber parts of *C. iria* are used as tonic, stimulant, stomachic and astringent (Ambasta, 2016). *Kyllinga odorata* is known for diaphoretic and diuretic properties (Lindley, 1847). *Kyllinga brevifolia* is a Paraguayan folk medicine and used as sedative and tonic for nervous system (Hellión-Ibarrola et al, 2014). *Fimbristylis miliacea* has been found of an important role in phytoremediation to absorb heavy metals and zinc in waste water treatment (Liu et al, 2007).

Identification of the plant species of a potential use is not always easy in the absence of knowledge of the most preferred morphological trait to look upon on site of collection. It is also not feasible always to apply costly and sophisticated molecular techniques to identify plants which are otherwise regarded as weeds only. Whereas their correct identification not only helps to formulate their control strategy but to explore their potential uses too. Morphological parameters are commonly used as tools for investigating genetic relatedness and diversity among plant populations. It is pertinent to study variations in morphological traits among species to determine how plant genotype and diverse environmental conditions could influence the plant morphology of sedges under uniform conditions.

2.1 Characteristic morphological traits of commonly found sedge plants in India

***Cyperus compressus* Linn.:** Annual, Erect herb with numerous, fine tufted root, plant greenish or grayish-green, 5-10 cm high, Stem 3-gonous, Leaves basal, as long as or longer than the stem, Inflorescence umbellate, Spikelets condensed linear or oblong, grey-green or straw-color, Anthela simple, lax, glumes ovate, mucronate, strongly keeled, Glumes green on the sides winged, Stamens 3, shortly apiculate, dark brown to blackish, Nuts broadly

triquetrous, dark brown (Oomachand and Shrivastava 1996; Verma et al 1984).

Cyperus cyperoides **Linn.:** A slender grass like sedge, 30-75 cm high. Stems several on a short creeping rhizome covered with the remains of old leaf-sheath. Leaves often as long as or exceeding stem. Spikelets closely spirally arranged in cylindric pedunculate spikes in simple terminal umbel. Fruiting spikelet after falling consists of 2 sub-equal glumes, outer lanceolate oblong, muticous many nerved, inner cymbiform with curved keel. Nuts brown, curved oblong, triquetrous (Shrivastava, 1976).

Cyperus esculentus **Linn.:** Stem at base erect, stolons lateral long very slender bearing tubers, leaves and bracts long, spikelets yellow or yellow-brown, glumes over nearly their whole breadth plicate striate (otherwise as C. rotundus) [Hooker, 1894].

Cyperus involucratus **Roxb.:** Clump-forming perennial herb to 2 m tall with bare, triangular stems (culms), fibrous roots and hardened rhizomes. Leaves reduced to sheaths at base of stem. Inflorescences large, with 12-25 distinct, leaf-like bracts to 25 cm long and approximately equal in length, arranged in a flat, umbrella-like pattern beneath 15- to 27-stalked spikelet clusters (rays). Rays 5-12 cm (2-5 in) long, each ray bearing 8-20 shiny brown clusters of tiny flowers (spikelets). Fruit a tiny, dark brown, 3-angled achene (Langeland et al, 2008).

Cyperus iria **Linn.:** Erect, glabrous annual variable in size and form. Stems striate, 3-quetrous. Leaves basal, blade linear, with papery sheaths and blades scabrid towards apex, sheath brown. Spikelets brown-yellow. Rachilla glabrous, wingless. Glumes obovate. Nuts obovate-elliptic, exceeding the glumes when mature (Oomachand and Shrivastava, 1996).

Cyperus kyllingia **Endl.:** An erect, glabrous sedge up to 30 cm tall, with well-developed rhizomes. Stem slender, covered with leaves linear, acuminate, rough on midrib and margin bracts 3 to 4, unequal. Spikes splitary, sub-globose white; spikelets obliquely lanceolate, elliptic, containing on flower. The lowest glume hyaline, second glume narrower; third glume boat-shaped

with 3 well marked nerves on each side of the prominent winged keel; fourth glume slightly narrower; stamen 2-3. Nut elliptic-ovoid flattened (Shrivastava, 1976).

***Cyperus microiria* Stud.:** It resembles the rice-field flatsedge (*C. iria*), but has smaller achenes and spikelets. The inflorescence is at the tip of the plant and branched. Inflorescence a compound or decompound anthela; rays 5-9, mostly to 13 cm, unequal in length. Spikes ovoid, broadly ovoid, with many spikelets. Spikelets slightly laxly arranged, linear to very narrowly linear-ovoid, obliquely spreading to spreading, 8-24 flowered; rachilla wings white, narrow, straight, hyaline; successive glumes on rachilla 1-1.5 mm apart. Glumes straw-colored to pale, lax, broadly obovate, keel abaxially extended beyond apex into a mucro, apex rounded (http://www.efloras.org).

***Cyperus nutans* Vahl.:** Large size, spikelets recemose (i.e. loosely spicate) ripe suberect, glumes somewhat remote often minutely mucronate. Usually 2-3.5 feet. Umbel primary rays often 8-12 inch spikes bowing on the ultimate rays. Spikelets in ripe fruit collapsing in a tassel (Hooker, 1894).

***Cyperus odoratus* Linn.:** Annual, Herb, Culms trigonous, Cylindric to subcylindric spikelets in which the corky rachilla of the mature spikelet disarticulates at the base of each scale. The mature spikelet breaks into segments each consisting of a scale and an internode of the rachilla clasping the achene with its corky wings (http://www.efloras.org).

***Cyperus retrorsus* Chapm.:** Herbs, perennial, shortly rhizomatous. Culms trigonous, glabrous. Inflorescence: spike 1, densely oblong-ovoid often with small basal branches, rachilla deciduous, wings persistent, Spikelets 40–120, oblong-lanceoloid, subterete, distal spikelet spreading or ascending; floral scales persistent (http://www.efloras.org).

***Cyperus rotundus* Linn.:** Erect, perennial herb with a woody, stoloniferous rhizome, which is clothed with fibrous. Stems nodose at base leaves radical, shorter than the stem, linear. Inflorescence a compound umbel of short spikes. Spikelets pale yellow or brown often with reddish tinge, glume ovate,

straw-colored. Nuts broadly obovoid, greyish-black (Oomachand and Shrivastava, 1996).

***Cyperus tenuispica* Stud.:** Erect, annual, glabrous sedge. Stems tufted, 15-20 cm tall. Leaves shorter or longer than the stem, linear acute. Umbels compound or decompounds. Spikelets 3-6 mm linear lanceolate. Glumes 0.8 mm long, brown, oblong, rounded. Stamen one. Nuts globosely obovoid (Shrivastava, 1976).

***Fimbristylis autumnalis* (L.) Y. Vahl.:** Inflorescence: anthelae compound, mostly diffuse, mostly turbinate, as broad as long, ascending-branching; scapes filiform to linear, distally variously compressed, bract usually 1, blade exceeding or exceeded by anthela. Spikelets red-brown or brown, mostly narrowly lanceoloid to narrowly ellipsoid, fertile scales lanceolate, keeled, narrowly acute, glabrous, midrib excurrent as mucro (http://www.efloras.org).

***Fimbristylis littoralis* Gandich.** A glabrous, leafy annual. Stems 15-60 cm long, slender, obtusely angled below and triquetrous above. Leaves shorter than the stem, tapering to a firm point and with nearly smooth margins. Umbels decompounds rays unequal, suberect or spreading, filiform bearing may scattered very small pedicellate spikelets. Spikelets sub-globose or sub-cylindric, obtuse, brown; rachilla stout, pitted, not winged. Glumes closely imbricate, ovate, stamens 1-3. Nuts obovoid, obtusely trigonous (Shrivastava, 1976).

***Fimbristylis miliacea* (Linn.) Vahl.:** An erect, tufted, annual sedge. Stems angular. Leaves basal, longer or shorter than the stems scabrid on the margins and midrib beneath. Spikelets very numerous, small, ellipsoid or oblong lanceolate, Spikelets brown, Inflorescence decompound umbels. Glumes ovate; keel 3-nerved, slightly excurrent. Nuts globosely obovoid, pale brown or whitish, minutely tuberculate (Oomachand and Shrivastava 1996; Shrivastava 1976).

***Kyllinga brevifolia* Rottb.:** A glabrous, 8-20 cm high, slender sedge with horizontally running rhizomes. Stems covered towards the base with usually brown leaf sheath. Leaves few erect, 2-8 cm long, sometimes exceeding the

stems, bracts very similar to the leaves. Spikes green oblong cylindrical; Spikelets lanceolate or ovate-lanceolate, containing one flower. The lowest glumes empty and sub-equal; third glume boat-shaped, acuminate, 2 nerves on either side of the keel; fourth glume slightly larger; stamen 2. Nuts obovate ellipsoid, strongly compressed laterally; styles with two filiform arms (Shrivastava, 1976).

***Kyllinga odorata* Vahl.:** Erect, tufted, rhizomatous perennial; culms 12-29 cm tall, triquetrous. Leaves few, linear, acute, scabrid on upper margins; sheaths, pale brown. Inflorescence a head with 1-3 spikes, whitish-green; central spike cylindrical; lateral spikes globose, much shorter than the central spike; leafy bracts 3-5. Spikelets elliptic, flattened. Glumes 4, broadly ovate, folded with green, smooth keel. Nuts brown to black oblong, apiculate, biconvex.

The first crucial step in conserving and managing plant species is correct identification of the target species that traditionally relies on morphological characters of especially reproductive parts, such as flowers and fruits, which is not accessible throughout the year. Accurate identification in species-rich or taxonomically complex groups also typically requires expert knowledge, which is not always available. DNA barcoding is a potential method to meet these identification challenges (Hartvig et al, 2015).

2.2 DNA barcoding technique

DNA barcoding is the identification of species by a short universal DNA sequence that exhibits a sufficient level of variation to discriminate among species (Hebert et al, 2003). The key advantage of DNA barcoding is that once a solid reference database has been established, the method does not require expert taxonomic knowledge in order to identify specific samples. Further, identification can be done with small tissue samples from virtually any part of the organism, does not require reproductive material, and the identification process is in general fast and reproducible. DNA barcoding, on the basis of its initial success in lepidopteron insects, fishes and birds (Hebert et al, 2003) was projected as a powerful technique for proper identification of all eukaryotes. The short DNA sequence proposed as the universal barcode was a 658 bp long region of *CO1* gene, commonly known as 'Folmer' region.

Subsequently, various DNA barcode programmes were initiated to generate species specific molecular signatures for identifying animals and plants. Shortly, it was realized that this region of the *CO1* gene, suggested as universal barcode for all organism, might not work in plants, except in some macro algae, as *CO1* sequences in land plants are highly invariant (Chase et al 2005; Kress et al 2005). Moreover, in plants the events of hybridization, introgression and allopolyploids are more pronounced than in animals and the species identification based on one locus was considered to be insufficient (Chase et al, 2005).

Hence, for DNA barcoding in plants, it has proven necessary to use a combination of regions to obtain sufficient discrimination success (Kress and Erickson 2007; Fazekas et al 2008, 2009; Hollingsworth et al 2009). However, since the introduction in 2003, the method is now widely applied in plant studies. First of all DNA barcoding can be used as a tool for identifying species that are difficult to identify based on morphological characters, or be used as a supporting taxonomic tool in delimitation and description of problematic species (Newmaster and Ragupathy, 2009). The technique can also prove valuable for accurate species identification as the important first step in conservation plans for threatened species (Hartvig et al, 2015). Apart from identifying target species, DNA barcoding can also be applied in floristics. Constructing a DNA barcoding reference library of entire local floras can allow for fast and effective floristic analyses without expert knowledge (Burgess et al, 2011), or even be a method of estimating species richness in a taxonomically poorly known flora. There is also a huge potential for application of DNA barcoding to the vast collections at herbaria and Natural History Museums, which could serve as excellent reference databases and help identify the many un-identified specimens present in most collections, as well as identify new collections. This would contribute significantly to improved knowledge about distribution patterns of each species. DNA barcoding thus has the potential of becoming an important supporting tool for conservation and biodiversity assessments in areas with a high number of plant species, a shortage of expert taxonomists, and limited descriptions of the flora (Hartvig et al, 2015).

2.3 DNA barcoding loci

DNA barcoding studies in plants have proposed a few plastid coding as well as non-coding regions, such as *rbcL* (Newmaster et al, 2006) *rbcL* and *trnH-psbA* (Kress et al 2005; Kress and Erickson 2007), *matK*, *rpoB*, *rpoC1* and *trnH-psbA* (Chase et al, 2007) and *atpF/H*, *matK*, *psbK/I* and *trnHpsbA* (Hollingsworth et al, 2011) as promising candidates. However, the slow evolving coding regions of plastid genomes might not possess enough variation to discriminate closely related plant species and this could lower their potential as effective barcodes (Pettengill and Neel, 2010). This can be overcome by analyzing the selected loci either individually or in combination (Chase et al 2005; Kress and Erickson, 2007). Recently evolved nuclear region, i.e. nuclear internal transcribed spacer from ribosomal gene (nrITS) has also been proposed as potential barcodes (Chen et al, 2010). Kress et al (2005) recommended the use of nuclear ribosomal *ITS* and *trnH-psbA* spacer from the plastid genome for discriminating plant species. Chase et al (2005) using *in silico* approach tested the utility of *rbcL* and *ITS* sequences available in GenBank for species identification using BLAST method and suggested that multi-locus combination with regions from plastid genome and one from nuclear genome would suffice as universal barcode for plants. Core 2-locus combination of *rbcL+matK* was recommended as the plant barcode in order to provide a universal framework for the routine use (CBOL Plant Working Group 2009). Chase et al (2007) again proposed multi locus barcode and recommended that either of the two 3-locus combinations, *matK+rpoB+rpoC1* or *matK+rpoC1+trnH-psbA*, may be used as universal barcodes for plants. Taberlet et al (2007) proposed that the *trnL* (UUU) intron and its shorter P6 loop (10-143bp) could be a suitable plant barcode. Though this region provided low species resolution (67.3%), because of highly conserved primers and very robust amplification it was considered the most suitable barcode to be used in applications other than taxonomy (forensic science, biotechnology and food industry, animal diet). Mattia et al (2011) evaluated four candidate barcode regions (*rpoB, rbcL, matK and trnH-psbA*) in different genus of Lamiaceae family. Barcoding loci *rbcL, matK and trnH-psbA* in combination with a nuclear transcribed spacer ITS was evaluated to discriminate *Dalbergia*

Species and *matK* alone or in combination with *rbcL* has been suggested as potential barcodes (Bhagwat et al, 2015). Recently, a chloroplast region *ycf1(b)* has been suggested as the most promising plastid DNA barcode of land plants (Dong et al, 2015).

The loci to be verified or developed as DNA barcode for plants could be from the nuclear or chloroplast genome. These, may be from either coding or non-coding regions. However, to prove to be suitable as a DNA barcode this locus should possess certain pre-defined characteristics (Kress et al, 2005). An ideal barcode sequence needs to have conserved flanking regions and should be short, so that it can be amplified by universal primers and sequenced routinely in single pass sequencing (Kress et al, 2005). Moreover, the primers used should not be prone to non-specific annealing that result in production of double bands or amplification of loci other than the targeted one (Kress et al 2005; Ford et al 2009). Most importantly, they should be capable of generating comparable data which enable species to be distinguished from one another. It should have an adequate sequence variations among the species with no or low divergence values within the species to provide a distinct barcode gap (Hebert et al 2003; Lahaye et al 2008). It should be easy to align and could be recovered from dry herbarium samples and parts/fragments (http://www.kew.org/barcoding/ rationale.html).

Among the plastid genes, *rbcL* is the best characterized gene sequence. Therefore, most of the investigating groups tested its suitability in barcoding. It encodes the large subunit of rubilose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO). As RUBISCO is a critical photosynthetic enzyme, *rbcL* was the first gene that was sequenced from the plants (Zurawski et al, 1981). The *rbcL* locus is also the most suitable gene for studying phylogeny of various plant groups; therefore, a huge number of *rbcL* sequences are already available in the public databases. Due to its high recoverability and ease of sequencing, *rbcL* frequently appears as one of the core loci in different suggested barcode locus combinations (Kress and Erickson 2007; Fazekas et al 2008; CBOL Plant Working Group 2009; China Plant BOL Group 2011). The barcode locus *matK* has been attributed with

higher variability of the *matK* region especially in monocots (Chase et al 2007; Fazekas et al 2008). Therefore, it was suggested by Hollingsworth et al (2011) that to increase the *matK* recovery rates either taxonomic group specific primers or modified universal or a cocktail of primers should be used for cost effective and efficient plant barcoding. However, from different studies, it appears that finding a universal barcode or even a barcode at family level is difficult and it may be possible to establish a discriminating barcode only at genus level (Riaz et al, 2011).

2.4 DNA barcoding studies in family Cyperaceae

The locus *rbcL* had been a marker of choice for the phylogenetic studies in family Cyperaceae (Muasya et al 1998, 2001, 2002). Cladistic analysis of *rbc*L sequence data of 80 species in 40 genera revealed the family as monophyletic and derived from a juncaceous grade as the *Oxychloe* (*Juncaceae*) showed moderate support as the sister taxon to *Cyperaceae* (Muasya et al, 1998). Cladistic analysis of *rbcL* and *trnL-F* sequence data from 69 species in 26 genera of Cyperaceae revealed that the genus *Isolepis* was not monophyletic (Muasya et al, 2001). The phylogeny of *Cyperus* and allied genera was reconstructed using cladistic analysis of *rbcL* gene, *rps16* intron, *trnL* intron, and *trnL-F* intergenic spacer sequence data in 40 species of tribe Cypereae (Muasya et al, 2002). DNA sequence data using the *trnL-F* and *rps16* regions from 25 genera and 35 species of Cyperaceae, Juncaceae, and Thurniaceae revealed two types of pollen, Mapania-type and pseudomonad (Simpson et al, 2003).

Hendrichs et al (2004) evaluated phylogenetic relationships in Carex, subgenus Vignea based on ITS sequences of 58 species by Neighbor Joining (NJ) and Markov chain Monte Carlo (MCMC) methods. Parsimony analysis carried out using all *rbcL* sequences available for Cyperaceae revealed that one of the four subfamilies (Caricoideae) and seven of the 14 tribes (Bisboeckelereae, Cariceae, Cryptangieae, Dulichieae, Eleocharideae, Sclerieae, Trilepideae) were monophyletic (Simpson et al, 2007). The relationships and limits of tribes Abildgaardieae and Arthrostylideae and their genera were evaluated across 49 representative species using parsimony and

maximum likelihood analyses of ITS (nuclear ribosomal) and *trn*L–F (plastid) DNA sequence data separately and combined. *Bulbostylis* was found monophyletic and clearly separated from *Fimbristylis* (Ghamkhar et al, 2007).

Hirahara et al (2007) investigated the phylogeny of Japanese Cyperaceae using chloroplast *ndhF* sequence data from 37 species in 27 genera. ITS and ETS 1f sequence data were used to estimate the phylogeny of 19 taxa of Japanese *Fimbristylis* (Yano and Hoshino, 2007). Phylogenetic relationships of Juncaceae and related families of Cyperales were investigated using DNA sequence data from the plastid *rps16* intron, *trn*L intron, and *trn*L–F intergenic spacer. Results using parsimony analysis of the aligned matrices found Juncaceae and Cyperaceae to be monophyletic families (Jones et al, 2007). The *matK* barcode was found most suitable for species discrimination in *Carex* genus in the family Cyperaceae from a subset of plant barcoding loci *matK*, *rbcL*, *rpoC1*, *rpoB* and *trnH-psbA* (Starr et al, 2009).

Muasya et al (2009a) reported an analysis of 262 taxa representing 93 genera of the Cyperaceae family, sequenced for the plastid *rbcL* and *trnL-F* (intron and intergenic spacer). Plastid DNA (rbcL gene, rps16 intron, trnL intron and trnL-F intergenic spacer) sequence matrix for 50 species in 19 genera of Cypereae analysed using the maximum parsimony algorithm of PAUP revealed two major groups as Ficinia and *Cyperus* clades. There are no unambiguous characters separating C3 and C4 *Cyperus* species (Muasya et al, 2009b). Phylogenetic analyses based on nuclear ITS and chloroplast *rbcL* sequences using maximum parsimony, maximum likelihood, and Bayesian method revealed the polyphyly of Korean *Scirpus* s.l. with five distinct clades (Jung and Choi, 2010). Plant DNA barcoding for distinguishing species of *Carex* and *Kobresia* in the Canadian Arctic Archipelago resulted in *matK* resolving the greatest number of species (95%), and when combined in a two-locus barcode (*matK*+*atpFH*), it provided 100% species resolution (Clerc-Blain et al, 2010).

Erioscirpus (Cyperaceae) was established as a distinct genus using plastid and nuclear DNA sequence data (Yano et al, 2012). Genealogical

relationships among four wild type genotypes of *Cyperus esculentus* were inferred by using plastid DNA haplotype and nuclear ribosomal (nr) DNA ribotype sequences corresponding to the loci *rbc*L, *ndh*F, *rps*16 intron and ETS1f, for 70 specimens either collected in the field or obtained from herbaria (De Castro et al, 2015). Bauters et al (2016) provided molecular phylogenetic evidence for placing the genus Scleria in the monotypic tribe Sclerieae using three molecular markers: the coding chloroplast marker *ndhF*, the chloroplast intron *rps16* and the nuclear ribosomal region *ITS* through Bayesian inference and maximum likelihood approaches.

A review of the DNA barcode sequences of family Cyperaceae available in the GenBank database till 31st December 2016 has been presented in Table 2.1. While there is abundance in the barcode sequences of *rbcL*, the sequences for *matK* and *trnH-psbA* are not reported in many species. However, barcode sequence has not been reported for *ycf1(b)* locus in *Cyperus* spp.

2.5 DNA barcoding for authentication of plants or plant products

Natural sources of medicinal plants are often unable to meet demand for popular herbal products. Populations of many species have limited distribution in their natural habitats, requiring conservation strategies for protection. In the absence of a desired first choice medicinal herb, classical Ayurveda recommends use of a functionally similar substitute. Post 16th century Ayurvedic texts and lexicons give specific examples of possible substitutes. Ayurvedic substitution pair: Musta (*Cyperus rotundus*, Cyperaceae), for the rare Himalayan species, Ativisha (*Aconitum heterophyllum*, Ranunculaceae) has been reported. Despite being taxonomically unrelated, the first choice, but relatively unavailable (Abhava) plant, *A. heterophyllum*, and its substitute (Pratinidhi) *C. rotundus*, are not only similar in Ayurvedic pharmacology (Dravyaguna) profile, but also in phytochemical and anti-diarrheal properties (Venkatasubramanian et al, 2010). Dried rhizome powder of *C. rotundus* is commercially marketed by companies as ‘Musta root powder’ (Cheema, 2015).

Traditional plant identification is based on morphological characteristics, which can be problematic especially for medicinal plants. Medicinal plants cover a broad range of taxa, which may be phylogenetically less related but morphologically very similar. Such similarity between species may lead to misidentification and inappropriate use (Chen et al, 2010). This is of high concern as it may cause fatalities especially given that several medicinal plants are poisonous (Watt and Breyer-Brandwijk, 1962; Bruni et al, 2010). The safety of medicinal plants largely depends on the ability to eliminate all forms of mis-identification. Verification at the DNA level assure greater reliability in comparison to RNA and Protein because DNA is a steady macromolecule that is not influenced by environmental effect and is found in all tissues. Hence, advancement in DNA-based markers is critical for identification of plants as well as confirmation of herbal ingredients in products.

Seethapathy et al (2014) authenticated Ativisha (*Aconitum heterophyllum*) and Musta (*Cyperus rotundus*) using nrDNA *ITS* sequence based SCAR markers and when market samples were examined it was found that SCAR primers (Cyr-FP and Cyr-RP) could identify plant tissue in sample containing 750 µg to 4.76 mg/100 mg of Musta in complex mixtures of DNA extracted from commercial herbal drugs and Ativisha was not found to be identified through SCAR markers confirming that this authentic species was not used to prepare herbal drugs despite its being labeled as one of the ingredients in formulations.

The ability to separate and identify individual elements in botanical mixtures using DNA barcoding has strengthen the possibility of its uses to prove cases in law courts based on forensic botany (http://www.promega.com). The potential of DNA barcoding to identify a plant species from minute leaf fragments and pollen grains have increased its acceptance and use by forensic botanists because often botanical trace evidence does not contain the necessary morphological features that would allow one to identify a plant at the genus or species level (Coyle et al, 2001).

DNA barcoding has the potential to be used in maintaining and ensuring the security of a nation’s biological diversity. Bio security encompasses protecting against any risk through ‘biological harm’, not least being the economic impact from the spread of pest insects (Armstrong and Ball, 2005). The ability to control invasive species depends much on the proper identification of the species involved and how fast this can be done. Invasive species are mostly alien species, experts are most often not available, and above all the eggs and larvae of these species are first found making morphological identification very difficult (http://www.genemetrix.net). Most often these stages lack features that enable their morphological identification, hence they have to be reared till they grow to adult stage before identification. These slow processes give alien species ample time to establish themselves in their new environment. DNA barcoding is therefore an appropriate tool for identifying alien species due to its ability to identify the organism at any stage of its life cycle. According to (Hebert et al, 2003), the emergence of DNA barcoding as a means of species identification has the potential to address all the shortcomings in morphological and other molecular forms of identification.

2.6 Challenges and limitations of DNA Barcoding

Molecular marker technology though is superior has certain drawbacks. One requirement is of high quality DNA while analyzing samples, which might be a problem for dried or processed materials. During drug-processing, there could be a change in temperature and pH that may lead to degradation (fragmentation) of the DNA, rendering PCR analysis difficult. However, depending on the degree of degradation of DNA some methods can still be used in processed materials. Again, even low content of secondary metabolites (polysaccharides, tannins, essential oils, phenolics, alkaloids, etc.) may inhibit PCR or might affect DNA isolation. As secondary metabolite content increases with the age and this becomes severe as the material gets older. Such contaminations are problematic, either they stop or minimize the activity of many enzymes, such as polymerases, ligases, and restriction endonucleases. The market samples might be contaminated with endophytic

fungi that could shatter the results of dominant markers like RAPD, AFLP and ISSR and might also influence DNA sequencing; however, this can be overcome with a plant-specific primer design. DNA barcoding primers based on short DNA sequences may be applied for sheared and low quality DNA. These primers are useful for the amplification of contaminated DNA because of their specificity.

DNA barcoding is a perfect tool that can lessen the hard work and hurdles in identification and discovery of new species. It must therefore be acceptable for use in the core areas of science that deals with identification, verification and authentication of species such as the Food and Agriculture industries, Quarantine Services, Food and Drugs Authority, Standards Authority, Pharmaceutical industries, Herbal medicine industries among others.

2.7 Availability of data

It is desirable to have access to a single barcode library for all plants. Currently, however several barcode libraries are freely accessible.

(i) BOLD: The barcode of life data system (http:// www.barcodinglife.com) was created and is maintained by the University of Guelph in Ontario. It offers researchers a way to collect, manage, and analyze DNA barcode data. The goal is, over the next 20 years, to provide a barcode library for all eukaryotic life.

(ii) CBOL: Consortium for the barcode of life (http:// www.barcodeoflife.org/) is a public reference library of species identifiers which could be used to assign unknown specimens to known species. CBOL was founded in 2004 and promotes barcoding through working groups, networks, workshops, conferences, outreach, and training. CBOL has 200 member organizations from 50 countries and operates from a Secretariat Office located in the Smithsonian Institution’s National Museum of Natural History in Washington, DC.

(iii) iBOL: International Barcode of Life project (http://www.ibol.org/) consists of a group of hundreds of scientists from 25 nations working together to

construct a DNA barcode reference library that will be the foundation for a DNA-based identification system for all multi-cellular life.

(iv) The GenBank online genetic sequence database available at http://www.ncbi.nlm.nih.gov/genbank/ (Benson et al, 2013) is possibly one of the most important repositories of genetic information. GenBank contains over 108 million entries for over 260,000 named organisms and is one of the most frequently used databases for genomic authentication (Hennel et al, 2012). With the BLAST (Altschul et al, 1990) program an unknown DNA sequence can be rapidly and accurately compared to known and well characterized sequences.

(v) MMDBD (Medicinal Materials DNA Barcode Database) (http://137.189.42.34/mherbsdb/index.php) is a website that includes DNA sequences and information and key references of the medicinal materials recorded in the Pharmacopoeia of the People's Republic of China, American Herbal Pharmacopoeia and other related references. This database, updated in May 2012 with 1658 species and 31,468 sequences available, provides information material for distinguishing medicinal materials (plant, animal, and fungi) from their common substitutes and adulterants (Lou et al, 2010).

Table 2.1 DNA barcode sequences of Cyperaceae available in the GenBank database (till date 31st December 2016)

Species	*rbcL*		*matK*		*trnH-psbA*		*ycf1(b)*	
	Acc No.	Seq. length	Acc No.	Seq. length	Acc No.	Seq. length	Acc No.	Seq. length
Cyperus rotundus	KJ773433.1	1307	KJ772708.1	645	HQ705831.1	836	-	-
	KU556619.1	644			KX405810.1	868		
	JX644667.1	1295			KX405809.1	875		
	GQ436645.1	703			KR108259.1	879		
	GQ436644.1	703						
	KU569193.1	415						
	HM849942.1	685						
	AM999813.1	1342						
Cyperus esculentus	KJ773425.1	1318	KJ772700.1	651	HQ705828.1	851	-	-
	HM849937.1	1363	HM850854.1	819	KX405753.1	855		
	KT695561.1	497			KX405754.1	876		
	JQ591227.1	552			KX405755.1	857		
	JQ591226.1	552						
	JQ591225.1	552						
	LK029904.1	1277						

	LK029903.1 LK029902.1 LK029901.1	1277 1277 1277						
Cyperus iria	KJ773427.1 KR869866.1 AB369948.1 AM999807.1	1317 1307 1408 1342	KJ772703.1	648	KX405772.1 KX405771.1 HE993908.1	865 872 811	-	-
Cyperus microiria	JX644662.1 KR869868.1	1295 1309	-	-	-	-	-	-
Cyperus retrorsus	KJ773432.1	1318	KJ772707.1	651	KX405805.1 KX405803.1 KX405802.1	876 877 879	-	-
Cyperus compressus	AF449506.1	1408	KJ772696.1	651	KX405735.1 KX405734.1 HE993902.1	871 877 837	-	-
Cyperus odoratus	KJ773430.1 AB369949.1	1323 1408	KJ772706.1	651	KX405788.1 KX405787.1 KX405786.1	803 792 789	-	-

Cyperus tenuispica	JX644669.1	1295	-	-	-	-	-	-
Cyperus nutans	-	-	-	-	-	-	-	-
Cyperus cyperoides	AB369947.1 AF449509.1 JX644655.1 KR737528.1 KR737433.1 KR736830.1 KR736614.1	1408 1408 1295 553 525 527 553	KR735147.1 KR735079.1 KR734665.1 KR734519.1	490 439 449 419	-	-	-	-
Cyperus involucratus	GU135273.1 GU135246.1 HM849939.1 JX644653.1 Y12967.1	567 525 1363 1295 1408	GU135106.1 GU135083.1	827 827	GU135444.2 GU135417.2	869 834	-	-
Cyperus kyllingia	HM849934.1 AF449515.1	658 681	-	-	-	-	-	-

	(*K. brevifolia*)							
Kyllinga monocephala	AM999845.1	1342	-	-	-	-	-	-
Kyllinga nemoralis	-	-	-	-	HQ705832.1	849	-	-
Kyllinga odoratus	-	-	KJ772880.1	651	-	-	-	-
Fimbristylis miliacea	JX644692.1	1295	-	-	-	-	-	-
Fimbristylis littoralis	AM999834.1	1342	-	-	-	-	-	-
Fimbristylis autumnalis	KJ773510.1 JX644686.1	1097 1295	KJ772787.1	657	-	-	-	-

MATERIALS AND METHODS

The present study entitled "Studies for identification and conservation of *Cyperus* spp. using DNA barcoding" was conducted at Biotechnology Centre, Jawaharlal Nehru Agricultural University, Jabalpur.

3.1 Materials

3.1.1 Source of Biological Materials

A total of eighty two sedge plant samples were collected from several places located in seven states of India namely Chhattisgarh (CG), Gujarat (GJ), Madhya Pradesh (MP), Rajasthan (RJ), Tamil Nadu (TN), Uttarakhand (UK) and Uttar Pradesh (UP) in the year 2015. Plant samples were named according to their places of collection. Details of plant samples, their collection sites and date of collection are given below in table 3.1 and Fig 3.1.

Table 3.1 List of plant samples and their collection sites used for DNA barcoding

S.N.	Place and Sample ID	Latitude_Longitude	Collection date
1	Chabi-2-MP	22.825720 N 80.700650 E	27-Aug-2015
2	Chennai-2-TN	12.990738 N 80.185958 E	20-Nov-2015
3	Jabalpur-1-MP	23.2203 N 79.9638 E	15-July-2015
4	Jabalpur-2-MP	23.2095 N 79.9533 E	15-July-2015
5	Kundam-2-MP	22.845746 N 81.075466 E	26-Aug-2015
6	Mandla-1-MP	22.611542 N 80.372824 E	27-Aug-2015
7	Mandla-3-MP	22.601269 N 80.378423 E	27-Aug-2015
8	Niwas-2-MP	23.035570 N 80.437791 E	27-Aug-2015
9	Amarkantak-2-MP	22.677726 N 81.758913 E	27-Aug-2015
10	Amarkantak-4-MP	22.682933 N 81.748302 E	27-Aug-2015
11	Dindori-1-MP	22.938539 N 81.080024 E	27-Aug-2015
12	Kapildhara-1-MP	22.700877 N 81.705569 E	27-Aug-2015
13	Sagartola-1-MP	22.941793 N 81.076845 E	27-Aug-2015
14	Garjiya-1-UK	29.462150 N 79.153404 E	30-Sep-2015
15	Garjiya-3-UK	29.468026 N 79.146967 E	30-Sep-2015
16	Jabalpur-6-MP	23.2095 N 79.9533 E	15-July-2015
17	Jabalpur-13-MP	23.2203 N 79.9638 E	15-July-2015

18	Corbett National Park -3-UK	29.5486 N 78.9353 E	30-Sep-2015
19	Garjiya-2-UK	29.466382 N 79.145765 E	30-Sep-2015
20	Anand-2-GJ	22.569384 N 72.931226 E	15-Oct-2015
21	Anand-4-GJ	22.56451 N 72.92887 E	15-Oct-2015
22	Jabalpur-3-MP	23.2203 N 79.9638 E	15-July-2015
23	Jabalpur-12-MP	23.2095 N 79.9533 E	15-July-2015
24	Kapildhara-4-MP	22.700877 N 81.705569 E	27-Aug-2015
25	Jabalpur-23-MP	23.2203 N 79.9638 E	20-Nov-2015
26	Jabalpur-5-MP	23.2203 N 79.9638 E	18-July-2015
27	Jabalpur-11-MP	23.2095 N 79.9533 E	15-July-2015
28	Jabalpur-14-MP	23.2203 N 79.9638 E	15-July-2015
29	Jabalpur -21-MP	23.233164 N 79.967455 E	30-Aug-2015
30	Jagdalpur-8-CG	19.075409 N 82.012864 E	10-Sep-2015
31	Lucknow-2-UP	26.8429 N 80.9544 E	01-Jan-2014
32	Niwas-4-MP	23.035570 N 80.437791 E	27-Aug-2015
33	Amarkantak-3-MP	22.677726 N 81.758913 E	27-Aug-2015
34	Dindori-2-MP	22.938539 N 81.080024 E	27-Aug-2015
35	Jagdalpur-9-CG	19.075409 N 82.012864 E	10-Sep-2015
36	Amarkantak-1-MP	22.677726 N 81.758913 E	27-Aug-2015
37	Jabalpur-16-MP	23.2203 N 79.9638 E	15-July-2015
38	Kapildhara-2-MP	22.700877 N 81.705569 E	27-Aug-2015
39	Pantnagar-1-UK	29.025347 N 79.477147 E	20-Sep-2015
40	Chabi-1-MP	22.825720 N 80.700650 E	27-Aug-2015
41	Jabalpur-4-MP	23.2095 N 79.9533 E	15-July-2015
42	Jabalpur-7-MP	23.2203 N 79.9638 E	15-July-2015
43	Jabalpur-8-MP	23.2203 N 79.9638 E	18-July-2015
44	Jabalpur-9-MP	23.2095 N 79.9533 E	18-July-2015
45	Jagdalpur-2-CG	19.075409 N 82.012864 E	10-Sep-2015
46	Jagdalpur-4-CG	19.078032 N 82.004904 E	10-Sep-2015
47	Jagdalpur-5-CG	19.078722 N 82.006148 E	10-Sep-2015
48	Kundam-1-MP	22.845746 N 81.075466 E	26-Aug-2015
49	Niwas-1-MP	23.035570 N 80.437791 E	27-Aug-2015
50	Niwas-3-MP	23.04752 N 80.44701 E	27-Aug-2015
51	Shahpura-1-MP	23.185804 N 80.703249 E	26-Aug-2015
52	Lucknow-1-UP	26.8429 N 80.9544 E	01-Jan-2015

53	Kapildhara-3-MP	22.700877 N 81.705569 E	27-Aug-2015
54	Kundam-3-MP	22.845746 N 81.075466 E	26-Aug-2015
55	Chennai-3-TN	12.990738 N 80.185958 E	20-Sep-2015
56	Kausani-1-UK	29.846996 N 79.604959 E	30-Sep-2015
57	Rewa-1-MP	24.536571 N 81.272274 E	20-July-2015
58	Amarkantak-5-MP	22.681982 N 81.753827 E	27-Aug-2015
59	Anand-1-GJ	22.569384 N 72.931226 E	15-Oct-2015
60	Dindori-3-MP	22.938539 N 81.080024 E	27-Aug-2015
61	Jabalpur-10-MP	23.2203 N 79.9638 E	15-July-2015
62	Jabalpur-19-MP	23.208857 N 79.955460 E	18-Aug-2015
63	Jabalpur -20-MP	23.233164 N 79.967455 E	30-Aug-2015
64	Jagdalpur-1-CG	19.075409 N 82.012864 E	10-Sep-2015
65	Jagdalpur-6-CG	19.078032 N 82.004904 E	10-Sep-2015
66	Corbett National Park-1-UK	29.5486 N 78.9353 E	30-Sep-2015
67	Corbett National Park-2-UK	29.52744 N 78.77467 E	30-Sep-2015
68	Pantnagar-3-UK	29.025347 N 79.477147 E	20-Sep-2015
69	Anand-3-GJ	22.569384 N 72.931226 E	15-Oct-2015
70	Chennai-1-TN	12.990738 N 80.185958 E	20-Nov-2015
71	Jaipur-1-RJ	26.9000 N 75.8000 E	30-Oct-2015
72	Jabalpur-18-MP	23.208857 N 79.955460 E	18-Aug-2015
73	Jabalpur-22-MP	23.233164 N 79.967455 E	30-Aug-2015
74	Mandla-4-MP	22.601269 N 80.378423 E	27-Aug-2015
75	Mandla-2-MP	22.611542 N 80.372824 E	27-Aug-2015
76	Pantnagar-2-UK	29.025347 N 79.477147 E	20-Sep-2015
77	Jagdalpur-7-CG	19.075409 N 82.012864 E	10-Sep-2015
78	Jabalpur-17-MP	23.2203 N 79.9638 E	15-July-2015
79	Jabalpur-15-MP	23.2203 N 79.9638 E	15-July-2015
80	Karanjia-1-MP	22.710416 N 81.639093 E	27-Aug-2015
81	Karanjia-2-MP	22.710550 N 81.638962 E	27-Aug-2015
82	Jagdalpur-3-CG	19.075409 N 82.012864 E	10-Sep-2015

3.1.2 Chemicals

Chemicals used for extraction of DNA and Agarose gel electrophoresis were procured from Himedia Biosciences Chemicals Pvt. Ltd., India, and DNA ladder (100bp and 1kb) from Thermo Scientific Pvt. Ltd.

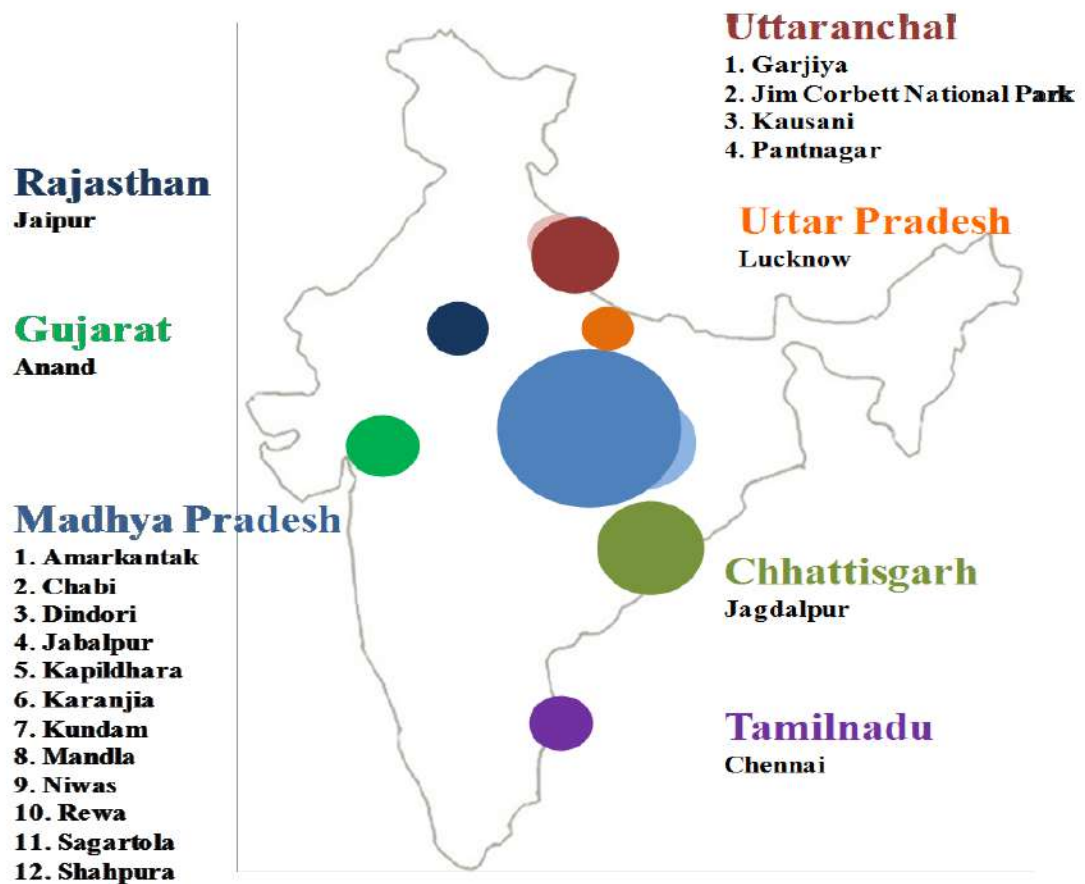


Fig 3.1 Collection sites of plant samples: Location coordinates plotted on the map

3.1.3 Molecular markers

The details of four DNA barcode loci selected for present study is given below in table 3.2. The PCR primers specific for the DNA barcode loci were synthesized from Integrated DNA Technology, USA as per the sequences given below in table 3.3.

Table 3.2 DNA barcode loci selected for present study

Barcode loci	Plastidial gene	Protein product
matk	*matk* "maturase K gene"	Maturase K
rbcL	*rbcL*2 "ribulose-1,5-bisphosphate carboxylase / oxygenase large subunit gene"	Ribulose-1,5-bisphosphate carboxylase / oxygenase
ycf1(b)	*ycf*1 plastid gene	Protein of 1800 amino acids, Tic214 component of the *Arabidopsis* TIC complex
trnH-psbA	*trnH-psbA* intergenic spacer region	-

Table 3.3 DNA barcoding primers and their sequences

Primer	Forward 5'-3'	Reverse 5'-3'	Reference
matk	CGTACAGTACTTTT GTGTTTACGAG	ACCCAGTCCATCT GGAAATCTTGGTTC	CBOL Plant Working Group (2009)
rbcL	GACAACTGTGTGG ACCGATG	CCACCGCGAAGAC ATTCATA	CBOL Plant Working Group (2009)
trnH-psbA	CGCGCATGGTGGA TTCACAATCC	GTTATGCATGAACG TAATGCTC	Kress et al (2005)
ycf1(b)	TCTCGACGAAAATC AGATTGTTGTGAAT	ATACATGTCAAAGT GATGGAAAA	Chen et al (2010) Dong et al (2015)

3.2 Method

3.2.1 Morphological observations

Morphological observations were recorded at various stages of growth at the site of collection as well as from some of the collected plants grown in pots till flowering that occurs in rainy season in India (July-October).

Observations were recorded for each plant sample on various morphological traits including root, rhizome, nut, stem or culm, leaf, inflorescence, spike, spikelet, floret, glume etc enlisted in table 3.4. For each trait, different observations were tabulated in Microsoft excel sheet and a qualitative data was generated by recording 0 and 1 for the absence and presence of the trait, respectively. These traits were divided into 22 groups and their 186 subgroup in a particular plant sample.

Table 3.4 Morphological traits observed in collected plant samples

SN	Group	Subgroup
1	Plant Type	herb
2	Life cycle	perennial
		annual
3	Plant colour	green
		greyish green
4	Root	fine
		fibrous
		creeping
		yellowish red
		reddish purple
5	Stem	at base Erect,
		at base creeper
		trigonous
		glabrous (Smooth)
		clump-forming, solid
		striate, 3-quetrous
		slender, covered with leaves
		triangular, nodos at base leaves
		angular, four angled, flattened
		tufted, tall
		slender, obtusely angled below, triquetrous above
6	Leaves	basal, as long as or longer than the stem
		linear, 3 ranked
		flanged V- or inversely W-shaped
		slender and bracts long

		shorter than culm leaf blade 6-12 mm wide, flat, margin scabrous
		distichous
		wide and long
		erect, sometimes branched, smooth, triquetrous, linear
		linear, acute, scabrid on upper margins; flate to slightly folded
7	Inflorescence	anthelae compound, terminal compound
		usually open, terminal
		umbellate
		compound umbel
		terminal simple
		ovoidglobose to ellipsoid
		anthelodium
		multi-umbel, diffuse compound
		decompounds umbels
		capitate oblong cylindrical, simple
		head with 1-3 spikes, whitish-green
		head tip of the plant and branched, compound or decompound anthela
		head densely oblong-ovoid often with small basal branches
8	Spikelets	condensed/linear/oblong
		grouped into small cylindrical heads
		flattened, 2-ranked
		clusters of tiny flowers
		erect-spreading, crowded
		obliquely lanceolate, elliptic
		spreading, compressed, linear
		linear to narrowly linear-ovoid. recemose
		linear-oblong to narrowly linear, cylindric to slightly flattened
		spreading
		spreading or ascending; consist a long stalk
		linear lanceolate/narrowly linear, stellately spreading
		mostly narrowly lanceoloid to narrowly ellipsoid
		sub-globose or sub-cylindric, obtuse
		numerous, small, ellipsoid or oblong lanceolate, globose, slender pedicelled

		numerous, lanceolate or ovate-lanceolate, containing one flower
		long, elliptic, flattened, ovate to ovate lanceolate
9	Spikelet colour	green
		grey-green or straw-color
		yellow or yellow-brown
		shiny brown
		brown-yellow, golden to yellowishgreen
		initially pure white, later rusty brown
		laterally brown to golden brown, medially green, 5 ribbed
		light brown to reddish-brown
		Green turn dark brown at maturity
		pale yellow or brown often with reddish tinge
		green or reddish
		red-brown or brown
		brown/ rusty-brown
10	Spikes	umbellate
		cluster
		leaf-like bracts
		either sessile or long peduncles
		splitary, sub-globose white
		ovoid, broadly ovoid, or subconic
		cylindric
		loosely to densely cylindric, ovoid, or pyramidal
		short
		sessile, compressed
		solitary
		central spike long, cylindrical; lateral spikes globose, whitish
11	Glumes colour	green on the sides winged
		2 sub-equal
		pale yellow to light brown
		straw-colored to pale
		yellowish on both surfaces and rusty brown striate
		brown, straw colour
		reddish brown, broadly white-hyaline on margins

		green
12	Glumes shape	Ovate/mucronate
		outer lanceolate oblong, muticous many nerved, inner cymbiform
		long, deciduous, concave, nerved
		the florets each have a bract
		obovate
		lowest glume hyaline, 2nd glume narrower; 3rd glume boat-shaped, 4th glume slightly narrower
		lax, broadly obovate
		lax, elliptic
		ovate
		ovtate-oblong, rounded
		closely imbricate, ovate, membranus, obtuse or acute
		lowest glumes empty and sub-equal; 3rd glume boat-shaped, 4th glume slightly larger
		4, braodly ovate, folded
13	Keel	keeled
		strong
		smooth
		curved
		hardly
		prominent winged
		abaxially extended beyond apex
		reddish brown
		minutely scabrous, often minutely notched at tip
		2 nerves on either side of the keel
		3-nerved keel ending below apex, broad
		3-nerved, slightly excurrent
14	Scales	long, obvious overlapping scales
		sterial basal pair scale when ripe
		floral scales persistent
		floral scales, whitish
15	Culms	scattered
		1 to several, stout, smooth, glabrous, triangular, the angles rounded
		trigonous, glabrous

		triangular longer than basal leaves
		slender
		tall, triquetrous, smooth
16	Bracts	involucral bracts 4-8, leaflike, basal 3/4 usually longer than inflorescence
		ascending at 45°, flat to broadly V shape, scabridulous along margins only
		dry, membranous oval shape
		few, 1-3 leafy bracts
		leaf bracts short
17	Rhachilla	broadly winged, persisting
		hardened
		glabrous
		wingless
		flexuose, wings white and hyaline, cymbiform
		corky, hyaline or thickened bronze, carmine, or yellowish
		deciduous, wings persistent
		rachis 3-angled
		stout, pitted, not winged
18	Achene	3-angled
		wings clasping
		seed, white to yellowish, three angled sugar coated
		reddish brown to dark brown
		pale brown, trigonous-obovoid
		flowering and fruiting throughout the year
		compound anthela
		brown, reddish brown, or black, stipitate, narrowly ellipsoid to oblong
		brown, oblong-ellipsoid
19	Fruit	tiny, dark brown
		three angled
		brown, slightly stipitate, obovoid, small
		3–sided ellipsoid, black, slightly beaked at tip
		straminous brownish
20	Rhizome	short creeping
		oval, woody rhizomes

		well-developed, slender, creeping
		successive glumes on rachilla 1-1.5 mm apart
		short, hardened
		shortly rhizomatous
		woody, fibrous, stoloniferous rhizome, initialy white covered with scaly modified leaves
		slender sedge with horizontally running rhizomes, creep, covered with scaly leaves
		short, thick, rhizomatous
21	Nut colour	dark brown
		yellow
		brown
		black
		shiny dark brown to black
		greyish-black
		becoming whitish when quite ripe or yellowish
		pale brown or whitish, straw or ivory colour
22	Nut shape	broadly obovoid
		broadly triquetrous
		trigonous, elongated
		small, hard, spherical
		3-angled, tiny
		obovate-elliptic
		elliptic-ovoid flattened
		oblong to obovoid-oblong, trigonous
		globosely obovoid, spherical obovoid, obtusely trigonous
		obovoid, obtusely trigonous
		globosely obovoid
		obovate ellipsoid, strongly compressed laterally
		oblong, apiculate, biconvex

3.2.2 Morphological data analysis

The morphological trait data recorded in Excel sheet was subjected to Unweighted pair group method with arithmetic averages (UPGMA) based cluster analysis and dendrogram was generated using NTSYSpc version 2.02e software program (Rohlf, 1997). The data analysis was performed adopting Jaccard (Jaccard, 1901) and Simple Matching (Sokal and Michener, 1958) similarity coefficients. Clustering method and similarity coefficients were tested by applying SIMQUAL, SAHN, TREE procedures in NTSYSpc program. For the comparison of original matrices generated by implementing Jaccard (J) and Simple Matching (SM) similarity coefficients, Mantel test (Mantel, 1967) was applied in the option of MXCOMP in NTSYSpc program. The plant samples clustered in a group were identified up to species level by comparing the recorded morphological traits with various documented texts such as 'Flora of Jabalpur' (Oomachand and Shrivastava, 1996), 'Flora of British India' (Hooker, 1894), 'Flora of India' (Verma et al, 1984), 'Flora of Gorakhpurensis' (Shrivastava, 1976), 'Hand Book on Weed Identification' (Naidu, 2012) including 'eFloras' (www.efloras.org).

3.2.3 Plant sampling for DNA barcoding

The individuals, branches or tissues of the collected plant species were brought to the laboratory in their native state. These were preserved under the conditions where the DNA damage was minimal. The plants or their parts were wrapped in aluminium foil, sealed in plastic bags and stored at -80°C in a deep freezer to minimize the degradation of DNA in the samples and to preserve the same until DNA extraction.

3.2.4 DNA extraction and Quantification

Genomic DNA was isolated from plant samples using a modified cetyl-tri-methyl ammonium bromide (CTAB) method (Saghai–Maroof et al, 1984). Isolated DNA was quantified by measuring the absorbance at 260nm and 280nm on a UV-spectrophotometer. 50 µg/ml concentration of double

stranded DNA showed an absorbance of 1 at 260nm. Concentration of DNA samples was calculated using following formula:

$$\frac{O.D._{260\ nm}\ X\ 50\ \mu g\ DNA/ml\ X\ Dilution\ factor}{1000}$$

The quantity, quality and integrity of isolated DNA were also checked by gel electrophoresis. 2 µl of DNA samples were electrophoresed in 0.8% agarose gel along with the *λ* uncut standard DNA (50 ng/µl) at 80 volts for 90 min. The DNA was stained by ethidium bromide and observed under UV-Trans-illuminator. The amount of fluorescence was proportional to the total mass of DNA.

3.2.5 Dilution of DNA

The quantified DNA was diluted according to the DNA quantity in each sample for PCR amplification in molecular biology grade water. Dilutions were carried out according to the following formula

$$Dilution = \frac{Required\ concentration\ of\ DNA\ (ng/\mu l)\ X\ Total\ volume\ required\ (\mu l)}{Available\ concentration\ of\ DNA\ (ng/\mu l)}$$

3.2.6 PCR amplification and Sequencing

Each PCR amplification reaction for DNA barcode primers contained 1µl DNA template (25 ng), 1µl 10X reaction buffer, 0.7µl $MgCl_2$ (50 µM), 0.2µl dNTPs mix (10 mM), 0.5 µl each forward primer (10 µM), 0.5 µl each reverse primer (10 µM), 0.2µl *Taq* polymerase (5 U/µl) and the final volume 10µl was adjusted with molecular biology grade water (Table 3.5).

The PCR reactions were initiated with a heated lid at 94 °C for 5 min, followed by a total of 35 cycles of 94 °C for 30 s, with annealing temperature of 55 °C for barcode primers *matk*, *rbcL*, *trnH-psbA* and 58 °C for *ycf1(b)* (according to the Tm of the primers) for 45 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min, and hold at 4 °C (Table 3.6). PCR reactions were carried out using thermal cycler (Agilent Technologies).

Table 3.5 List of components with their concentrations used for PCR

S. No.	Components	Concentrations
1.	10X PCR buffer	1 x
2.	25mM $MgCl_2$	2.5 mM
3.	10mM dNTPs	100µM
4.	Forward Primer	5pM
5.	Reverse Primer	5pM
6.	*Taq* Polymerase (5 Unit/µl)	1 unit
7.	Nuclease free H_2O	For volume making
8.	DNA	25ng

Table 3.6 Temperature profile used in PCR amplification of Barcode Primers

Steps	Temperature (°C)	Duration	Cycles	Activity
1	94	5 min	1	Initial denaturation
2	94	30 sec	↑	Denaturation
3	55-58	45 sec	35	Annealing
4	72	30 sec	↓	Elongation
5	72	10 min	1	Final elongation
6	4	∞		Storage

The PCR amplification efficiency was verified by 1.5% agarose gel electrophoresis. After verification of PCR amplification success the reaction volumes were increased up to 50µl for sequencing. The final PCR products were sequenced by outsourcing on an ABI 3730XL sequencer (Applied Biosystems Inc.) using the amplification primers.

3.2.7 Sequence alignment

Sequence alignments were performed using ClustalW (Thompson et al, 1994) and alignments were subsequently adjusted manually using BioEdit (Hall, 1999).

3.2.8 Data analysis

3.2.8.1 Sequence similarity searches

Each and every barcode sequence generated from each barcode primer was subjected to similarity based searches in the freely available online tool BLASTn (Altschul et al, 1990) and online Plant barcodes identification tool available at BOLD Systems v3 (www.boldsystems.org/) for preliminary identification of the plant species. Reference sequences available for each barcode locus and outgroup sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov/).

3.2.8.2 Generating a concatenated dataset

Datasets for the combination of barcode loci viz., *rbcL+matk*, *rbcL+trn*H-*psb*A, *matk+trnH-psbA* and *rbcL+matk+trnH-psbA* was done by concatenating the alignments for the three barcode regions in software program Mesquite (Maddison and Maddison, 2014).

3.2.8.3 Species identification through species identifier program of TaxonDNA

The sequence datasets for all the seven barcodes viz., *rbcL*, *matk*, *trn*H-*psb*A, *rbcL+matk*, *rbcL+trn*H-*psb*A, *matk+trnH-psbA* and *rbcL+matk+trnH-psbA* were analyzed in the SpeciesIdentifier program of TaxonDNA (Meier et al, 2006) for identification of the species based on best match, best close match and all species barcode methods. The pair wise intraspecific and interspecific distances were also calculated by the species identifier program uncorrected *p*-distance method.

3.2.8.4 Phylogenetic analysis for the clustering based identification of plant species by UPGMA method

In order to identify the sedge plant species based on evolutionary relationship, the barcode sequences of *rbcL, matk, trnH-psbA* and *ycf1(b)* were subjected to UPGMA based phylogenetic analysis along with all the available reference species specific sequences from GenBank. The evolutionary distances were computed using the Maximum Composite Likelihood method in the units of the number of base substitutions per site

involving codon positions 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. The UPGMA evolutionary analyses were conducted in MEGA7 program (Kumar et al, 2015).

3.2.8.5 Tree based analyses by Maximum parsimony method

In order to assess the most efficient barcoding loci for the identification of sedge plant species and to infer on the taxonomy of the sampled sedge plants, phylogenetic analysis based on maximum parsimony (MP) method was conducted on all the seven datasets including reference sequences with or without outgroup species in MEGA7 program. The tree was constructed by the bootstrap method in 1000 replications using the Tree-Bisection-Regrafting (TBR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences in 10 replicates. The tree was drawn to scale, with branch lengths calculated using the average pathway method and in the units of the number of changes over the whole sequence. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. If the species were recovered as monophyletic with each barcode it was considered resolved and identified as the same species.

3.2.9 Barcode generation

The flow diagram of steps followed for barcode generation has been shown in Fig 3.2. For visual depiction and to obtain an illustrative barcode to identify similarities, differences, and nucleotide compositions of each sequence, barcodes for each primer were generated using software PAK (Aguiar, 2013).

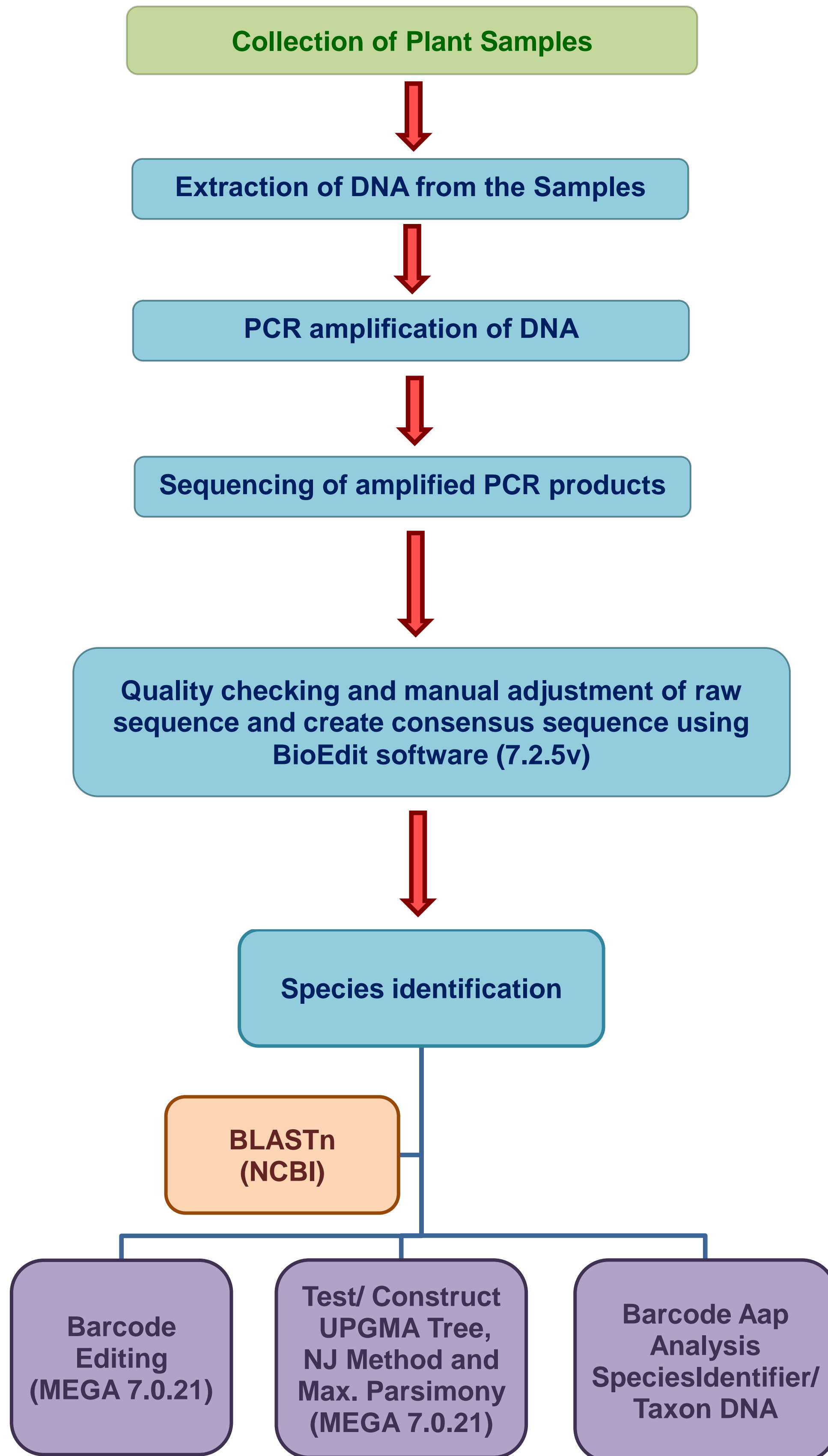


Fig 3.2 Flow chart of various steps involved in DNA barcoding

RESULTS

Present study was carried out with an aim to establish a robust and reproducible system for the identification of the sedge plants belonging to *Cyperus spp*. A gamut of methods including morphological identification, molecular marker or DNA barcode based identification and software system based identification approaches were applied to fulfill the desired aim of this study. The results of the present study are presented under the different headings mentioned below.

4.1 Species identification based on morphological traits

4.1.1 Clustering based on morphological data

Morphological descriptors characteristic to a particular sedge plant species were identified (Table 4.1) and the observations were recorded for all the 82 sedge plant samples collected from various places of the country. The observations recorded in a matrix were subjected to statistical analysis using the software NTSYSpc. Cluster analysis based on recorded morphological traits in all the eighty two sedge plant samples resulted in to grouping of plant samples in to a total of 17 clusters in case of both the clustering methods based on Jaccard (J) and Simple Matching (SM) similarity coefficients. The dendrogram generated for both the similarity coefficients has been presented in Fig 4.1 A & B. Each cluster was found to represent a group of plants of a single species. The plant samples in the 17 clusters were identical in the groups made by both the clustering methods. Both the methods had shown the plant samples Jagdalpur-3-CG, Jabalpur-15-MP, Jabalpur-17-MP, Jabalpur-22-MP, Jabalpur-23-MP and Rewa-1-MP as groups with single species. However, the differences were observed in the major clusters containing various groups of plants. As first major cluster from top of the dendrogram (Fig 4.1 A & B) made from J coefficient showed only two individual sample groups of Jagdalpur-3-CG and Jabalpur-23-MP while the first major cluster made from SM coefficient showed individual sample groups of Jabalpur-15-MP, Jabalpur-17-MP, Rewa-1-MP and Jagdalpur-3-CG.

Table 4.1 Morphological traits of different species of sedge plants

SN	Species and its Morphological Characters
1	***Cyperus rotundus*** **Linn.** Erect, perennial herb with a woody, stoloniferous rhizome, which is clothed with fibrous. Stems nodose at base leaves radical, shorter than the stem, linear. Inflorescence a compound umbel of short spikes. Spikelets pale yellow or brown often with reddish tinge, glume ovate, straw-colored. Nuts broadly obovoid, greyish-black (Oomachand and Shrivastava, 1996) Plate 1A.
2	***Cyperus esculentus*** **Linn.** Stem at base erect, stolons lateral long very slender bearing tubers, leaves and bracts long, spikletets yellow or yellow-brown, glumes over nearly their whole breadth plicate striate (otherwise as *C. rotundus*) Hooker (1894) Plate 1B.
3	***Cyperus iria*** **Linn.** Erect, glabrous annual variable in size and form. Stems striate, 3-quetrous. Leaves basal, blade linear, with papery sheaths and blades scabrid towards apex, sheath brown. Spikelets brown-yellow. Rachilla glabrous, wingless. Glumes obovate. Nuts obovate-elliptic, exceeding the glumes when mature (Oomachand and Shrivastava, 1996) Plate 1C.
4	***Cyperus microiria*** **Stud.** It resembles the rice-field flat sedge (*C. iria*), but has smaller achenes and spikelets. The inflorescence is at the tip of the plant and branched. Inflorescence a compound or decompound anthela; rays 5-9, mostly to 13 cm, unequal in length. Spikes ovoid, broadly ovoid, with many spikelets. Spreading spikelets linear to ovoid shape bearing 8-24 flowers; straight or hyaline white winged rachilla, straw-colored to pale glumes on rachilla, Glumes broadly obovate, rounded apex, keel abaxially extended into a mucro beyond apex (http://www.efloras.org) Plate 1D.
5	***Cyperus cyperoides*** **Linn.** A slender grass like sedge, 30-75 cm high. Stems several on a short creeping rhizome covered with the remains of old leaf-sheath. Leaves often as long as or exceeding stem. Spikletes closely spirally arranged in cylindric pedunculate spikes in simple terminal umbel. Fruiting spikelet after falling consists of 2 sub-equal glumes, outer lanceolate oblong, muticous many nerved, inner cymbiform with curved keel. Nuts brown, curved oblong, triquetrous (Shrivastava, 1976) Plate 2E.
6	***Cyperus compressus*** **Linn.** Annual, Erect herb with numerous, fine tufted root, plant greenish or grayish-green, 5-10 cm high, Stem 3-gonous, Leaves basal, as long as or longer than the stem, Inflorescence umbellate, Spikelets condensed linear or oblong, grey-green or straw-color, Anthela simple, lax, glumes ovate, mucronate, strongly keeled, Glumes green on the sides winged, Stamens 3, shortly apiculate, dark brown to blackish, Nuts broadly triquetrous, dark brown (Oomachand and Shrivastava 1996; Verma et al 1984) Plate 2F.

7	***Cyperus involucratus* Roxb.** Perennial plant of height about 2 m with fibrous root, hard rhizomes, triangular stem with leaf sheaths at stem base. Large umbrella-like Inflorescence with 12-25 leaf-like bracts beneath 15 to 27stalked spikelet clusters (rays). A ray has 8-20 shiny brown clusters of tiny flowers (spikelets). Fruit small dark brown triangular achene (Langeland et al, 2008) Plate 2G.
8	***Cyperus nutans* Vahl.** Large size, spikletes recemose (i.e. loosely spicate) ripe suberect, glumes somewhat remote often minutely mucronate. Usually 2-3.5 feet. Umbel primary rays often 8-12 inch spikes bowing on the ultimate rays. Spikletes in ripe fruit collapsing in a tassel (Hooker, 1894) Plate 2H.
9	***Cyperus retrorsus* Chapm.** Herbs, perennial, shortly rhizomatous. Culms trigonous, glabrous. Inflorescence: spike 1, densely oblong-ovoid often with small basal branches, rachilla deciduous, wings persistent, Spikelets 40–120, oblong-lanceoloid, subterete, distal spikelet spreading or ascending; floral scales persistent (http://www.efloras.org) Plate 3I.
10	***Cyperus odoratus* Linn.** Annual, herb, culms trigonous, spikelets cylindric to subcylindric, corky rachilla disarticulated at base of scale. Segmented mature spikelet consisting of a scale and an internode of the rachilla, achene clasped into corky wings of rachilla (http://www.efloras.org) Plate 3J**.**
11	***Cyperus tenuispica* Steud.** Erect, annual, glabrous sedge. Stems tufted, 15-20 cm tall. Leaves shorter or longer than the stem, linear acute. Umbels compound or decompounds. Spikelets 3-6 mm linear lanceolate. Glumes 0.8 mm long, brown, oblong, rounded. Stamen one. Nuts globosely obovoid (Shrivastava, 1976) Plate 3K.
12	***Cyperus kyllingia* Endl.** An erect, glabrous sedge upto 30cm tall, with well-developed rhizomes. Stem slender, covered with leaves linear, acuminate, rough on midrib and margin bracts 3 to 4, unequal. Spikes splitary, sub-globose white; spikelets obliquely lanceolate, elliptic, containing on flower. The lowest glume hyaline, second glume narrower; third glume boat-shaped with 3 well marked nerves on each side of the prominent winged keel; fourth glume slightly narrower; stamen 2-3. Nut elliptic-ovoid flattened (Shrivastava, 1976) Plate 3L.
13	***Fimbristylis miliacea* (Linn.) Vahl.** An erect, tufted, annual sedge. Stems angular. Leaves basal, longer or shorter than the stems scabrid on the margins and midrib beneath. Spikletes very numerous, small, ellipsoid or oblong lanceolate, Spikletes brown, Inflorescence decompound umbels. Glumes ovate; keel 3-nerved, slightly excurrent. Nuts globosely obovoid, pale brown or whitish, minutely tuberculate (Oomachand and Shrivastava 1996; Shrivastava 1976) Plate 4M.
14	***Fimbristylis littoralis* Gandich.** A glabrous, leafy annual. Stems 15-60 cm long, slender, obtusely

	angled below and triquetrous above. Leaves shorter than the stem, tapering to a firm point and with nearly smooth margins. Umbel compounds rays unequal, suberect or spreading, filiform bearing may scattered very small pedicellate spikeletes. Spikletes sub-globoseor sub-cylindric, obtuse, brown; rachilla stout, pitted, not winged. Glumes closely imbricate, ovate, stamens 1-3. Nuts obovoid, obtusely trigonous (Shrivastava, 1976) Plate 4N.
15	***Fimbristylis autumnalis* (L.) Y. Vahl.** Tiny sedge grows in clusters, 5-8 cm tall plants with thread-like leaves, larger leaves are wider than 1.0 mm. Small egg-shaped spikes (3-7 mm long) borne in clusters on top of the flat stems. anthelae compound and mostly diffuse, filiform to linear scapes, usually single bract, blade exceeded by anthela. Mostly narrowly lanceoloid to narrowly ellipsoid brown or red-brown spikelets, fertile keeled lanceolate scales (http://www.efloras.org) Plate 4O.
16	***Kyllinga brevifolia* Rottb.** A glabrous, 8-20 cm high, slender sedge with horizontally running rhizomes. Stems covered towards the base with usually brown leaf sheath. Leaves few erect, 2-8 cm long, sometimes exceeding the stems, bracts very similar to the leaves. Spikes green oblong cylindrical; Spikelets lanceolate or ovate-lanceolate, containing one flower. The lowest glumes empty and sub-equal; third glume boat-shaped, acuminate, 2 nerves on either side of the keel; fourth glume slightly larger; stamen 2. Nuts obovate ellipsoid, strongly compressed laterally; styles with two filiform arms (Shrivastava, 1976) Plate 4P.
17	***Kyllinga odorata* Vahl.** Erect, tufted, rhizomatous perennial; culms 12-29 cm tall, triquetrous. Leaves few, linear, acute, scabrid on upper margins; sheaths, pale brown. Inflorescence a head with 1-3 spikes, whitish-green; central spike cylindrical; lateral spikes globose, much shorter than the central spike; leafy bracts 3-5. Spikelets elliptic, flattened. Glumes 4, braodly ovate, folded with green, smooth keel. Nuts brown to black oblong, apiculate, biconvex. (http://keralaplants.in/keralaplantsdetails.aspx?id= Kyllinga_odorata_ssp._cylindrica) Plate 4Q.

4.1.2 Testing the significance of the two similarity coefficients J and SM

The Mantel test correlation coefficient value 0.97169 between the two similarity coefficients J and SM was found to be significant at $p < 0.05$, shown in graph (Fig 4.2) which suggests that the dendrograms constructed from J and SM coefficients were highly correlated. The observed correlation between similarity matrix and phenetic trees indicated the goodness of fit of cluster analysis in accordance with the similarity matrix.

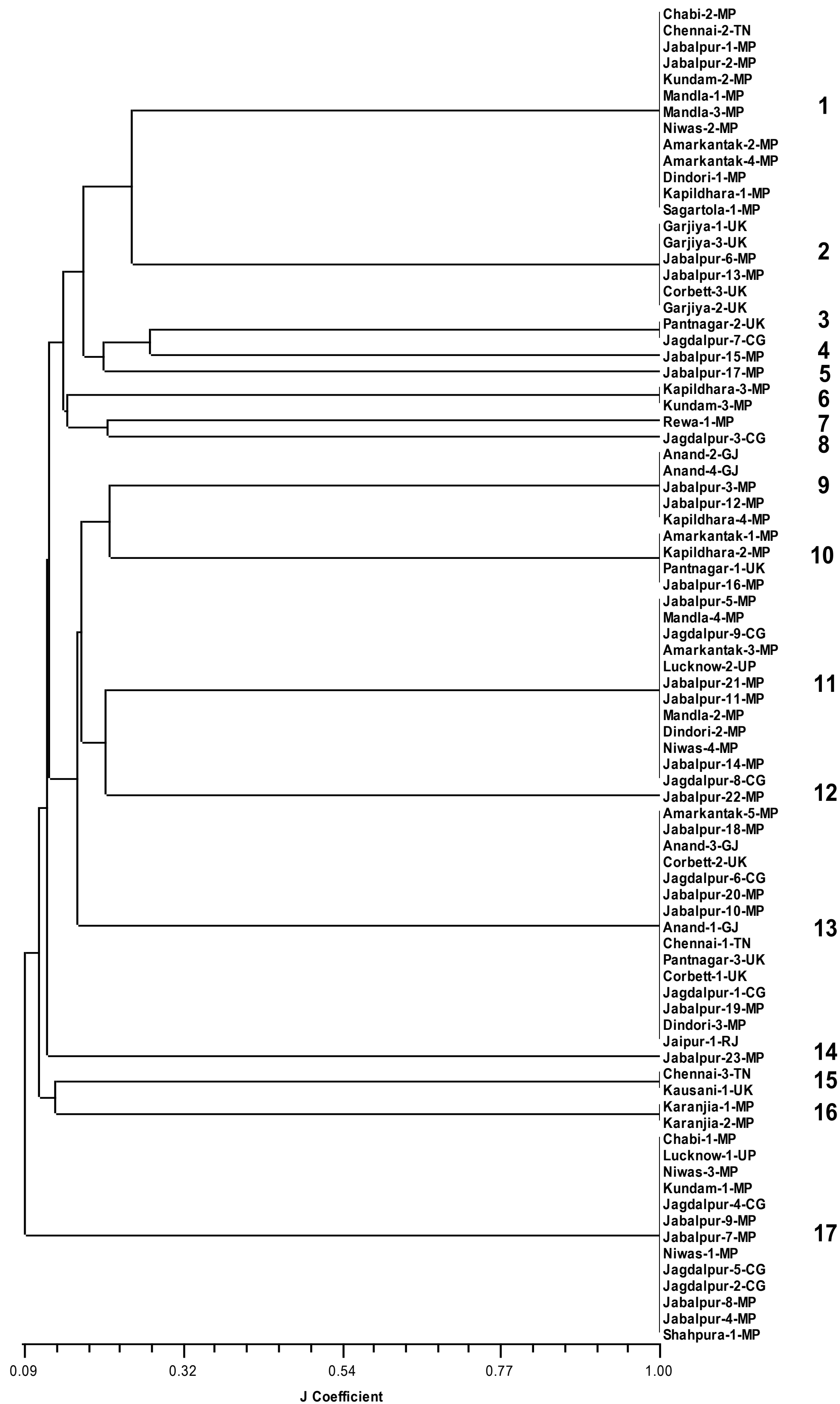


Fig 4.1 (A) UPGMA clustering based on Jaccard similarity coefficient on morphological data of 82 samples

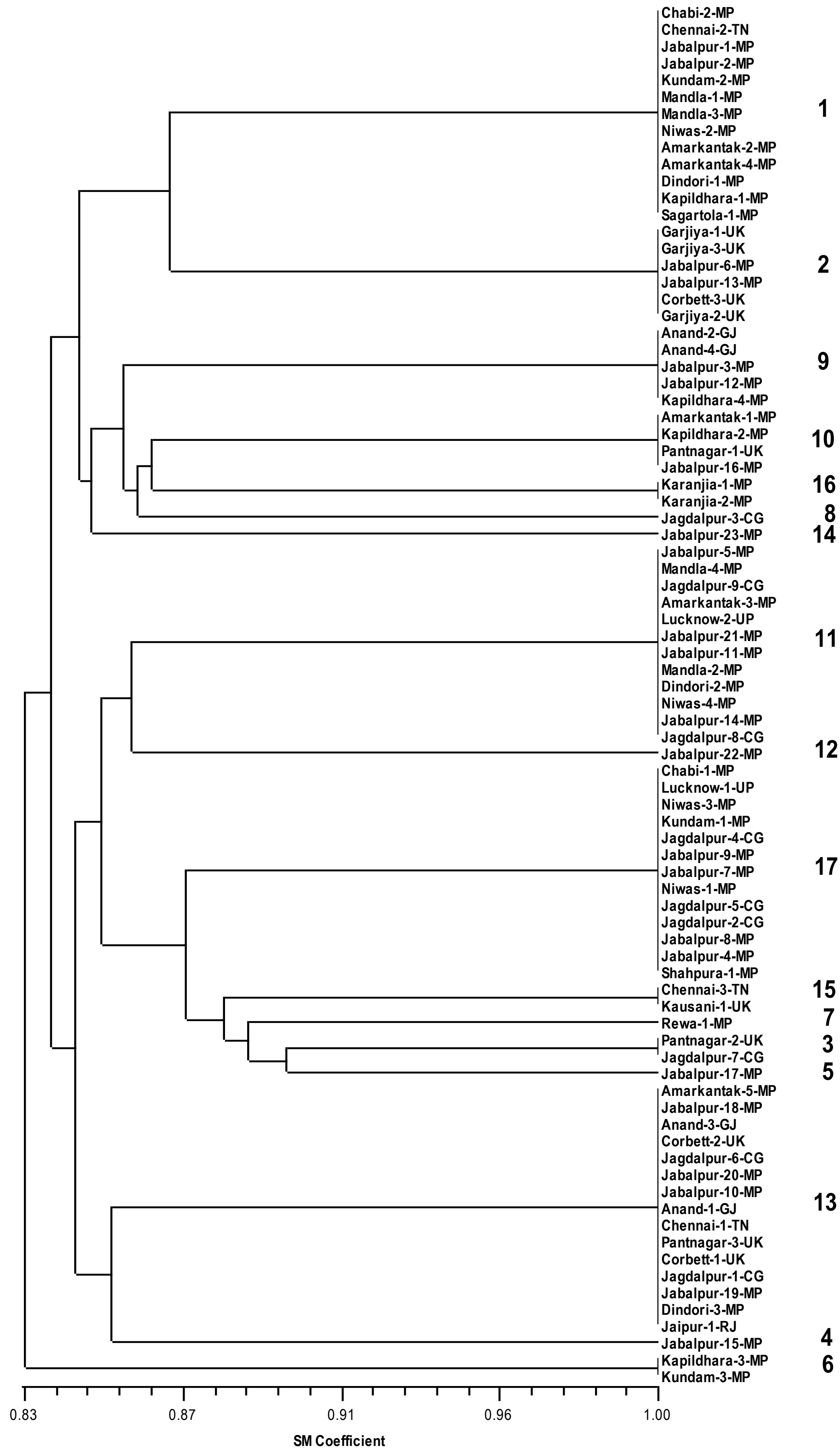


Fig 4.1 (B) UPGMA clustering based on Simple Matching similarity coefficient on morphological data of 82 samples

In Fig 4.1 A and B each cluster group represents a species: 1. *Cyperus compressus,* 2. *Cyperus cyperoides*, 3. *Fimbristylis autumnalis*, 4. *Fimbristylis milliacea*, 5. *Fimbristylis littoralis*, 6. *Cyperus nutans*, 7. *Cyperus retrorsus*, 8. *Kyllinga brevifolia*, 9. *Cyperus esculentus*, 10. *Cyperus kyllingia*, 11. *Cyperus iria*, 12. *Cyperus tenuispica*, 13. *Cyperus rotundus*, 14. *Cyperus involucratus*, 15. *Cyperus odoratus*, 16. *Kyllinga odorata*, 17. *Cyperus microiria*

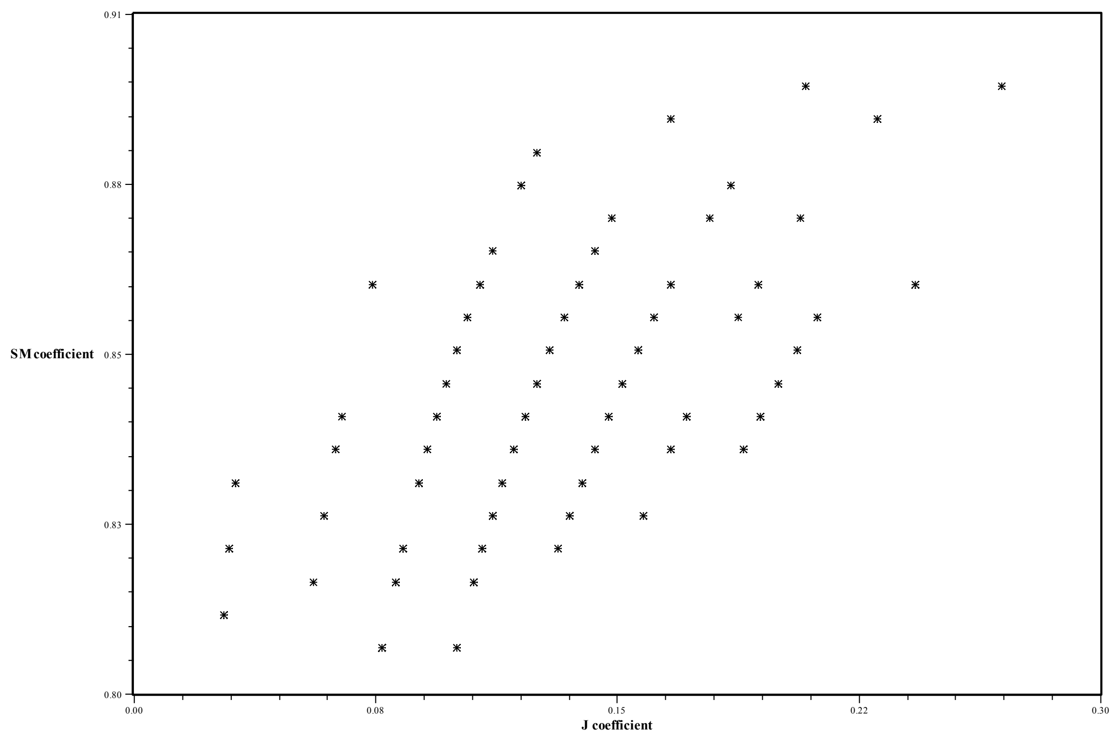


Fig 4.2 Mantel test graph showing correlation between the two similarity coefficients J & SM at $p < 0.05$.

4.1.3 Identification of the species of the sedge plant samples

The sedge plant samples of each group in different clusters were identified to the species level under family Cyperaceae on the basis of comparing and matching the recorded morphological trait observations with the identified morphological descriptors as per the available documented flora texts. They were identified as belonging to 17 different species (Table 4.2) among which twelve species were from genus *Cyperus* namely *C. compressus, C. cyperoides, C. esculentus, C. involucratus, C. iria, C.*

kyllingia, C. microiria, C. nutans, C. odoratus, C. retrorsus, C. rotundus and C. tenuispica. Three species were belonging to genus *Fimbristylis* namely *F. autumnalis, F. littoralis and F. milliacea* and two were from genus *Kyllinga* of species *K. brevifolia and K. odoratus* (Plates 1-4). Different morphological structures of the sedge plant species from the genus *Cyperus, kyllingia* and *Fimbristylis* from different locations around India are presented in Table 4.1. While the collected sedge plant samples varied from 83-100% (Fig 4.1), the visible appearances of plant samples of a particular species did not vary in different locations throughout India, indicating that all possessed several traits common within the species leading to no location specific morphological variations among sedge plants of a particular species in India. However, the interspecific variations were prominently observed for all the collected plant samples.

Table 4.2 Species identification according to morphological analysis

S.N.	Place and Sample ID	Species Identified
1	Chabi-2-MP	*Cyperus compressus* Linn.
2	Chennai-2-TN	*Cyperus compressus* Linn.
3	Jabalpur-1-MP	*Cyperus compressus* Linn.
4	Jabalpur-2-MP	*Cyperus compressus* Linn.
5	Kundam-2-MP	*Cyperus compressus* Linn.
6	Mandla-1-MP	*Cyperus compressus* Linn.
7	Mandla-3-MP	*Cyperus compressus* Linn.
8	Niwas-2-MP	*Cyperus compressus* Linn.
9	Amarkantak-2-MP	*Cyperus compressus* Linn.
10	Amarkantak-4-MP	*Cyperus compressus* Linn.
11	Dindori-1-MP	*Cyperus compressus* Linn.
12	Kapildhara-1-MP	*Cyperus compressus* Linn.
13	Sagartola-1-MP	*Cyperus compressus* Linn.
14	Garjiya-1-UK	*Cyperus cyperoides* Linn.
15	Garjiya-3-UK	*Cyperus cyperoides* Linn.
16	Jabalpur-6-MP	*Cyperus cyperoides* Linn.
17	Jabalpur-13-MP	*Cyperus cyperoides* Linn.
18	Corbett National Park -3-UK	*Cyperus cyperoides* Linn.
19	Garjiya-2-UK	*Cyperus cyperoides* Linn.

20	Anand-2-GJ	*Cyperus esculentus* Linn.
21	Anand-4-GJ	*Cyperus esculentus* Linn.
22	Jabalpur-3-MP	*Cyperus esculentus* Linn.
23	Jabalpur-12-MP	*Cyperus esculentus* Linn.
24	Kapildhara-4-MP	*Cyperus esculentus* Linn.
25	Jabalpur-23-MP	*Cyperus involucratus* Roxb.
26	Jabalpur-5-MP	*Cyperus iria* Linn.
27	Jabalpur-11-MP	*Cyperus iria* Linn.
28	Jabalpur-14-MP	*Cyperus iria* Linn.
29	Jabalpur -21-MP	*Cyperus iria* Linn.
30	Jagdalpur-8-CG	*Cyperus iria* Linn.
31	Lucknow-2-UP	*Cyperus iria* Linn.
32	Niwas-4-MP	*Cyperus iria* Linn.
33	Amarkantak-3-MP	*Cyperus iria* Linn.
34	Dindori-2-MP	*Cyperus iria* Linn.
35	Jagdalpur-9-CG	*Cyperus iria* Linn.
36	Mandla-4-MP	*Cyperus iria* Linn.
37	Mandla-2-MP	*Cyperus iria* Linn.
38	Amarkantak-1-MP	*Cyperus kyllingia* Endl.
39	Jabalpur-16-MP	*Cyperus kyllingia* Endl.
40	Kapildhara-2-MP	*Cyperus kyllingia* Endl.
41	Pantnagar-1-UK	*Cyperus kyllingia* Endl.
42	Chabi-1-MP	*Cyperus microiria* Steud.
43	Jabalpur-4-MP	*Cyperus microiria* Steud.
44	Jabalpur-7-MP	*Cyperus microiria* Steud.
45	Jabalpur-8-MP	*Cyperus microiria* Steud.
46	Jabalpur-9-MP	*Cyperus microiria* Steud.
47	Jagdalpur-2-CG	*Cyperus microiria* Steud.
48	Jagdalpur-4-CG	*Cyperus microiria* Steud.
49	Jagdalpur-5-CG	*Cyperus microiria* Steud.
50	Kundam-1-MP	*Cyperus microiria* Steud.
51	Niwas-1-MP	*Cyperus microiria* Steud.
52	Niwas-3-MP	*Cyperus microiria* Steud.
53	Shahpura-1-MP	*Cyperus microiria* Steud.

54	Lucknow-1-UP	*Cyperus microiria* Steud.
55	Kapildhara-3-MP	*Cyperus nutans* Vahl.
56	Kundam-3-MP	*Cyperus nutans* Vahl.
57	Chennai-3-TN	*Cyperus odoratus* Linn.
58	Kausani-1-UK	*Cyperus odoratus* Linn.
59	Rewa-1-MP	*Cyperus retrorsus* Chapman.
60	Amarkantak-5-MP	*Cyperus rotundus* Linn.
61	Anand-1-GJ	*Cyperus rotundus* Linn.
62	Dindori-3-MP	*Cyperus rotundus* Linn.
63	Jabalpur-10-MP	*Cyperus rotundus* Linn.
64	Jabalpur-19-MP	*Cyperus rotundus* Linn.
65	Jabalpur -20-MP	*Cyperus rotundus* Linn.
66	Jagdalpur-1-CG	*Cyperus rotundus* Linn.
67	Jagdalpur-6-CG	*Cyperus rotundus* Linn.
68	Corbett National Park-1-UK	*Cyperus rotundus* Linn.
69	Corbett National Park-2-UK	*Cyperus rotundus* Linn.
70	Pantnagar-3-UK	*Cyperus rotundus* Linn.
71	Anand-3-GJ	*Cyperus rotundus* Linn.
72	Chennai-1-TN	*Cyperus rotundus* Linn.
73	Jaipur-1-RJ	*Cyperus rotundus* Linn.
74	Jabalpur-18-MP	*Cyperus rotundus* Linn.
75	Jabalpur-22-MP	*Cyperus tenuispica* Linn.
76	Pantnagar-2-UK	*Fimbristylis autumnalis* (L.) Vahl.
77	Jagdalpur-7-CG	*Fimbristylis autumnalis* (L.) Y. Vahl.
78	Jabalpur-17-MP	*Fimbristylis littoralis* Gaud.
79	Jabalpur-15-MP	*Fimbristylis milliacea* (L.) Vahl.
80	Karanjia-1-MP	*Kyllinga odorata* Vahl.
81	Karanjia-2-MP	*Kyllinga odorata* Vahl.
82	Jagdalpur-3-CG	*Kyllinga brevifolia* Rottb.

4.1.4 Assessment of the morphological traits to identify a sedge plant species

In order to find out which morphological traits were actually responsible for grouping of individual plant samples in to separate clusters, the species specific data set was divided out in to three parts; vegetative traits (including observations on plant colour, root, rhizome, nut, stem/culm and leaves), inflorescence (including observations on inflorescence pattern, spike and spikelets) and floral traits (including observations on glume colour, glume shape, keel, bracts and rachilla); and each data set was analyzed separately by constructing dendrogram using J or SM coefficients. The vegetative traits grouped all the 17 species individually in case of J coefficient used (Fig 4.3). Similar results were observed in case of inflorescence (Fig 4.4). Different species have shown the different pattern of inflorescence, but the floral traits failed to differentiate between the species *C. compressus* and *F. autumnalis* (Fig 4.5).

4.1.5 Best morphological trait for the identification of a sedge plant species

It was also attempted to identify a single morphological trait that could be observed for the identification and clustering of the sedge plants under a particular species. The dendrograms were constructed based on most variable morphological traits among the plants of 17 identified species which were glume (glume colour, glume shape) and spikelet (colour and shape). The clustering based on glume did not identify the species *C. odoratus*, *C. retrorsus* and *F. autumnalis* separately but into a single cluster only (Fig 4.6). Spikelet was found to be the best for identifying a sedge plant to its species level, since, the dendrogram constructed based on the observations on spikelet color and shape grouped all the 17 species individually in case of both the J and SM coefficients (Fig 4.7).

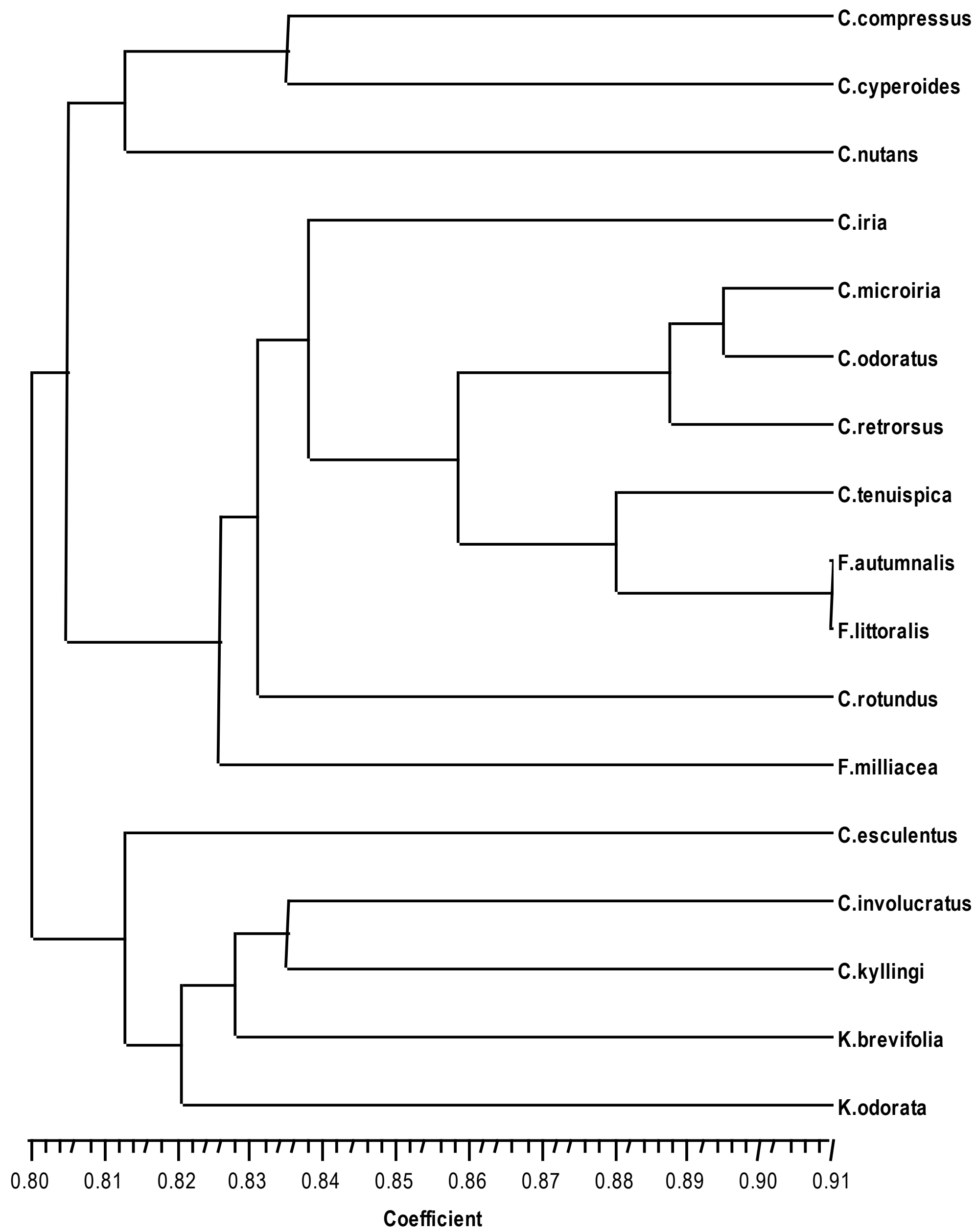


Fig 4.3 Dendrogram showing clustering of different sedge species based on vegetative traits

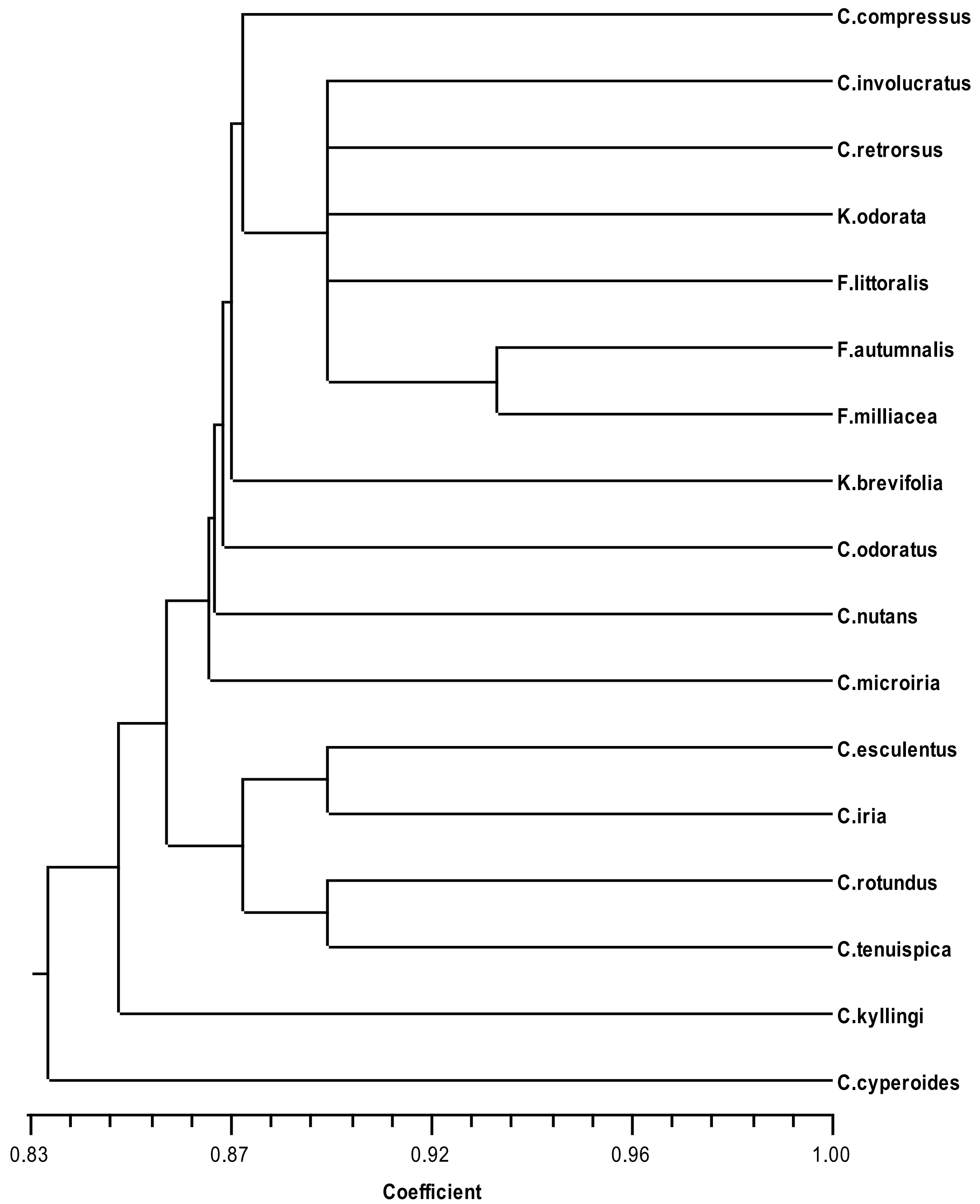


Fig 4.4 Dendrogram showing clustering of different sedge species based on inflorescence

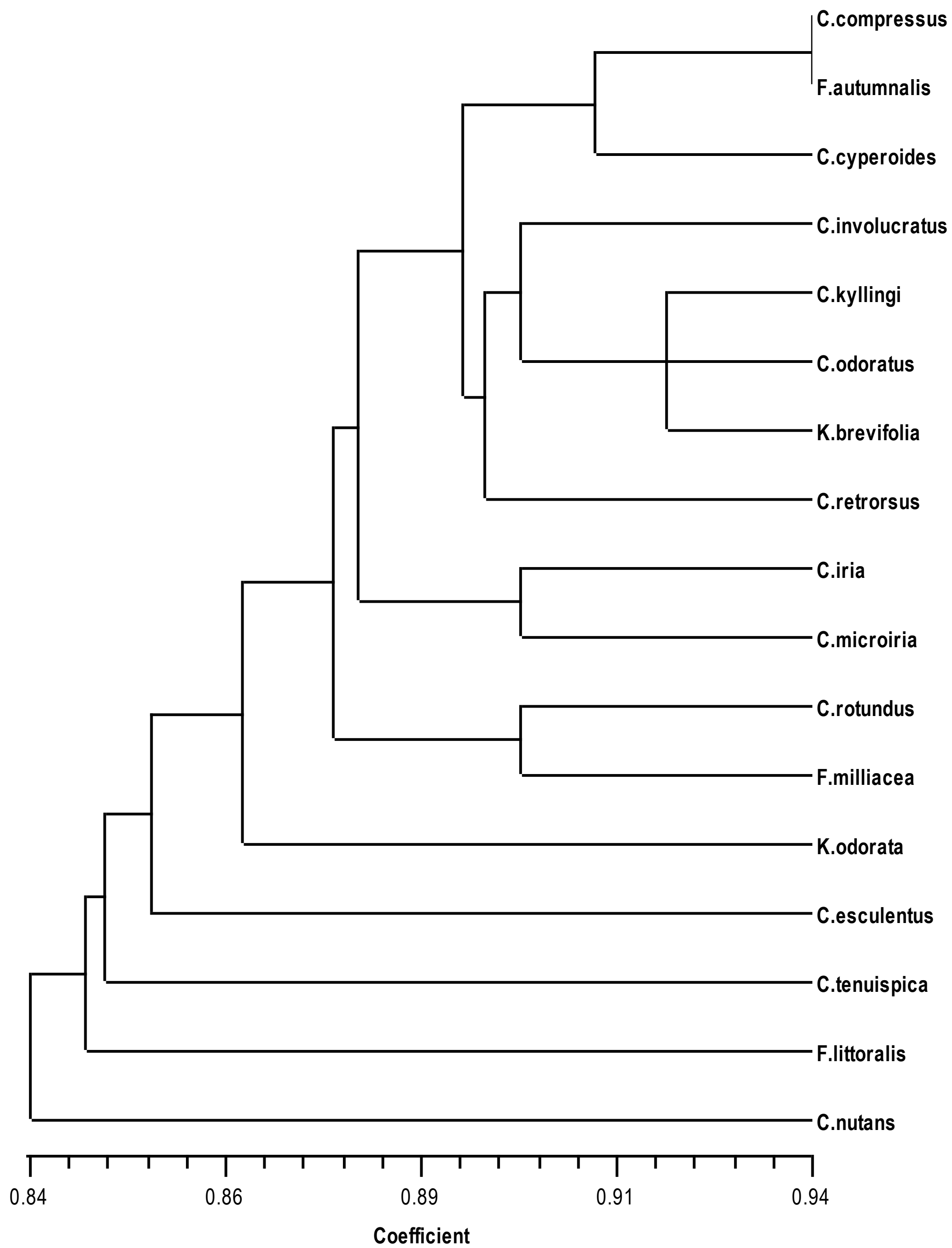


Fig 4.5 Dendrogram showing clustering of different sedge species based on floral traits

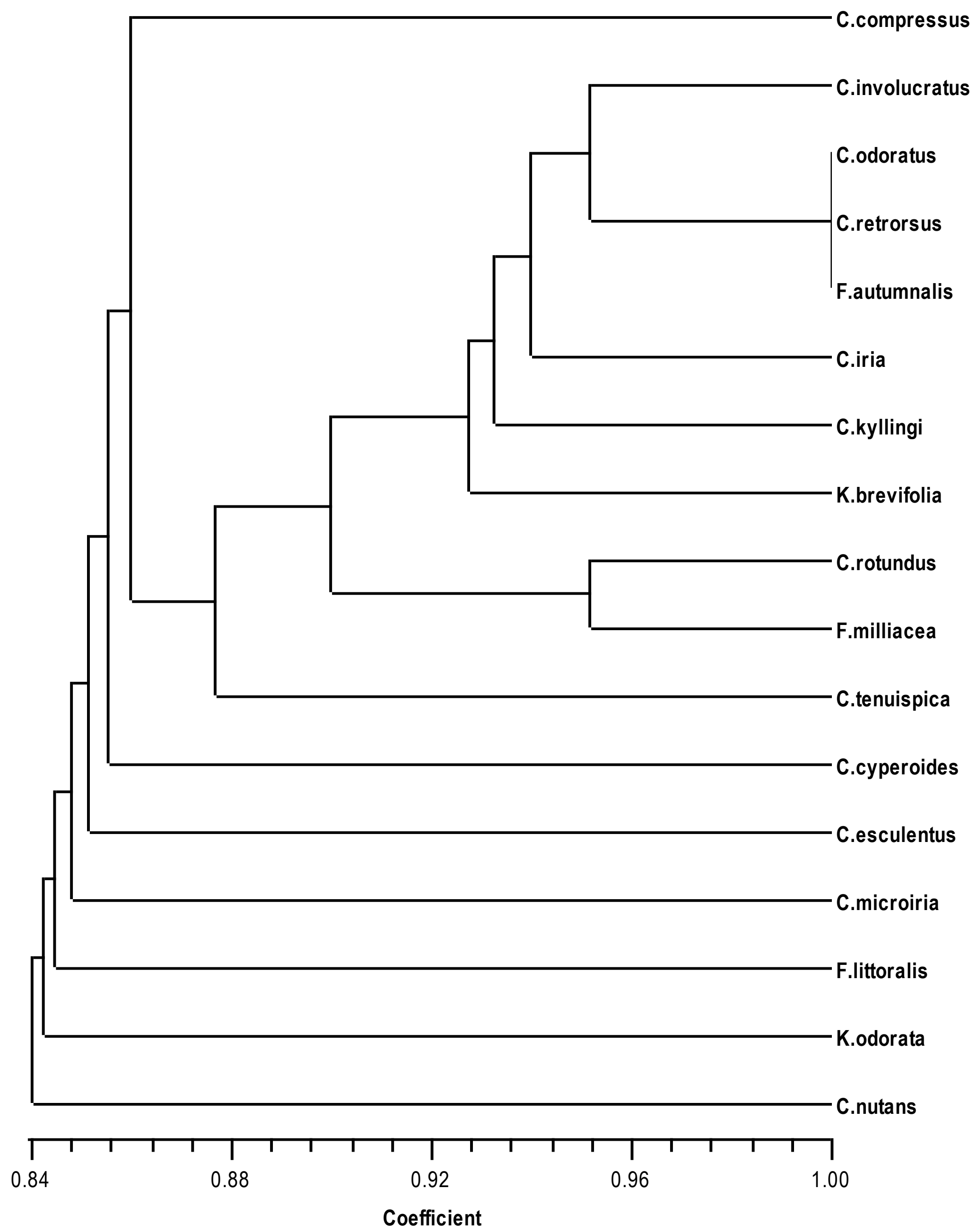


Fig 4.6 Dendrogram showing clustering of different sedge plant species based on Glume

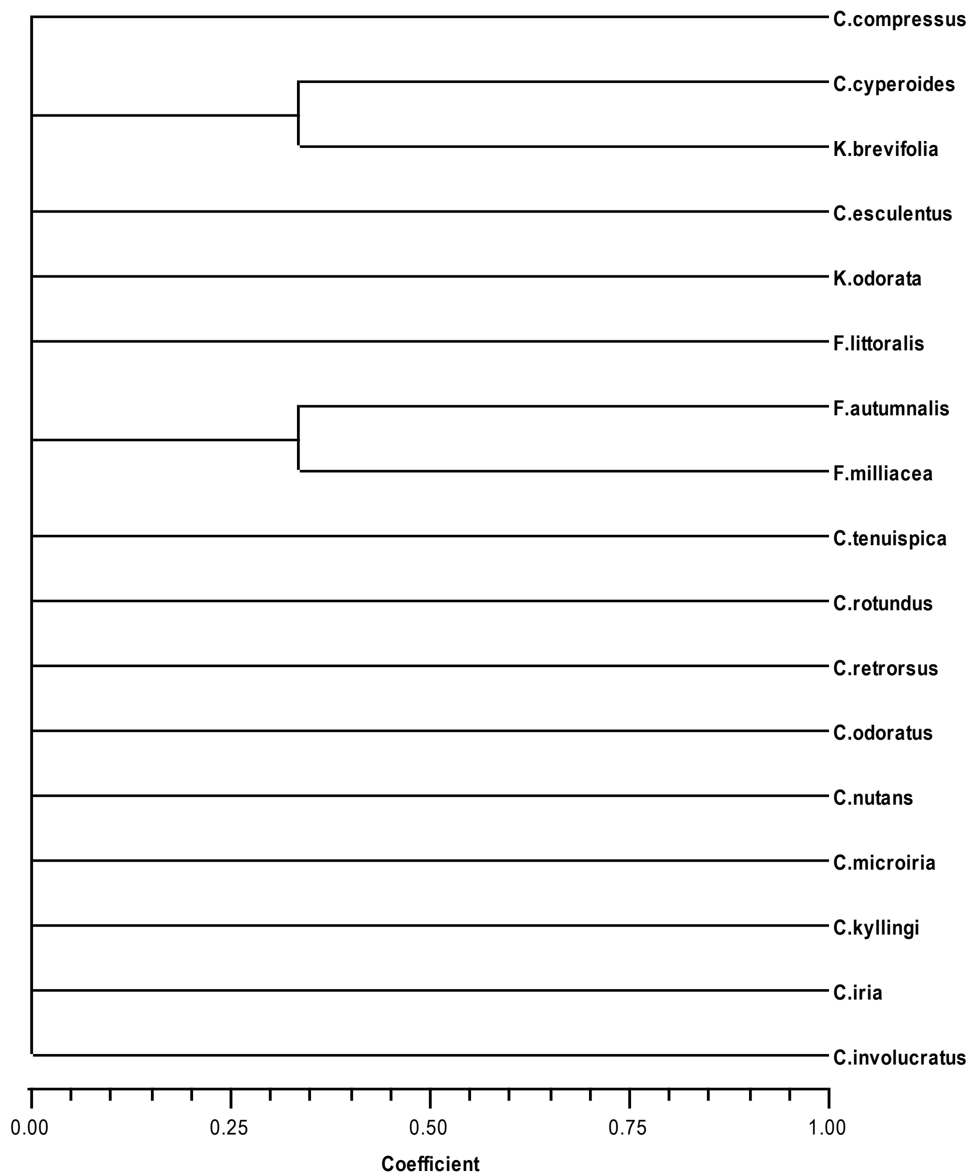


Fig 4.7 Dendrogram showing clustering of different sedge plant species based on Spikelet

4.2 Species identification based on DNA barcodes

4.2.1 Barcode amplification and sequencing

During the present course of investigation, a total of 40 samples representing all the identified species based on morphological analysis were finally selected for generation of DNA barcode through PCR amplification using four barcode gene loci specific primers from chloroplast genome namely *rbcL*, *matK, trnH-psbA* and *ycf1(b)*. High quality and good yield of DNA was

obtained during extraction from all the 40 plant samples (Plate 5). Barcode region *rbcL* could be amplified in 36 plant DNA samples, *matK* in 17 samples, *trnH-psbA* in 14 samples while *ycf1(b)* in only three samples (Plate 6).The *rbcL* region was successfully sequenced for 36 samples, *matK* for 17 samples, *trnH-psbA* for 14 samples while *ycf1(b)* was only sequenced for three samples. Sequences were edited and assembled in BioEdit program. The final length of sequences varied in the ranges from 314-347 bp for *rbcL*, 540-826 bp for *matK,* 856-883 bp for *trnH-psbA* and 688-793 bp for *ycf1(b)*. All sequences were deposited in GenBank and given accession numbers (Table 4.3).

4.2.2 Similarity based species identification through BLAST (NCBI) search

Each and every sequence was subjected to online BLASTn search analysis to identify the species of the plant sample. The searches resolved the plant sequences up to genus level. However, due to several hits at the same parameters of max score, total score, query cover, E value and identity %, most of the plant species were not identified unambiguously. The *ycf1(b)* barcode sequences showed similarity with reference barcodes of plants from other genera as no reference was found in *Cyperus* spp. Based on BLAST searches, some of the barcodes generated in present study were found to be unique barcodes for the sedge plant species (Table 4.4).

4.2.3 Similarity based species identification through BOLD database search

Barcode of Life Datasystems (BOLD) has a collection of plant barcodes for *rbcL* and *matK*. The *rbcL* and *matK* barcode sequences of present study were subjected to similarity search in the BOLD database. The searches resolved the plant sequences up to genus level. However, due to several hits at the same parameters of max score, total score, query cover, E value and identity %, most of the plant species were not identified unambiguously.

Table 4.3 Sequence details for all the four DNA barcode loci

Sequence code	Collection site	Species	Accession Number	Barcode	Nucleotide
KR1	Lucknow	*Cyperus microiria*	KX219762	*rbcL*	329
KR2	Lucknow	*Cyperus iria*	KX219763	*rbcL*	331
KR3	Jabalpur	*Cyperus rotundus*	KX219764	*rbcL*	319
KR4	Jabalpur	*Cyperus iria*	KX219765	*rbcL*	318
KR5	Rewa	*Cyperus retrorsus*	KX219766	*rbcL*	314
KR6	Jagdalpur	*Cyperusr otundus*	KX219767	*rbcL*	343
KR7	Anand	*Cyperus esculentus*	KX219768	*rbcL*	318
KR8	Jabalpur	*Cyperus rotundus*	KX219769	*rbcL*	315
KR9	Shahpura	*Cyperus microiria*	KX219770	*rbcL*	321
KR10	Dindori	*Cyperus compressus*	KX219771	*rbcL*	331
KR11	Amarkantak	*Kyllinga monocephala*	KX219772	*rbcL*	301
KR12	Amarkantak	*Cyperus iria*	KX219773	*rbcL*	343
KR13	Kapildhara	*Kyllinga monocephala*	KX219774	*rbcL*	328
KR14	Kapildhara	*Cyperus esculentus*	KX219775	*rbcL*	321
KR15	Amarkantak	*Cyperus rotundus*	KX219776	*rbcL*	302
KR16	Mandla	*Cyperus compressus*	KX219777	*rbcL*	309
KR17	Mandla	*Cyperus microiria*	KX219778	*rbcL*	325
KR18	Niwas	*Cyperus microiria*	KX219779	*rbcL*	320
KR19	Chabi	*Cyperus microiria*	KX219780	*rbcL*	323

KR20	Jabalpur	*Cyperus rotundus*	KX219781	*rbcL*	325
KR21	Jabalpur	*Cyperus iria*	KX219782	*rbcL*	308
KR22	Jabalpur	*Cyperus tenuispica*	KX219788	*rbcL*	329
KR23	Jaipur	*Cyperus rotundus*	KX219783	*rbcL*	344
KR24	Pantnagar	*Cyperus rotundus*	KX219784	*rbcL*	309
KR25	Kosani	*Cyperus odoratus*	KX219785	*rbcL*	340
KR26	Corbett Park	*Cyperus rotundus*	KX219786	*rbcL*	319
KR27	Chennai	*Cyperus rotundus*	KX219787	*rbcL*	347
KR28	Jabalpur	*Fimbristylis miliacea*	KX499535	*rbcL*	327
KR29	Jabalpur	*Fimbristylis littoralis*	KX499527	*rbcL*	329
KR30	Jagdalpur	*Kyllinga nemoralis*	KX499528	*rbcL*	325
KR31	Kundam	*Cyperus nutans*	KX499529	*rbcL*	324
KR32	Karangia	*Kyllinga odoratus*	KX499530	*rbcL*	320
KR33	Karangia	*Kyllinga odoratus*	KX499531	*rbcL*	326
KR34	Pantnagar	*Fimbristylis autumnalis*	KX499532	*rbcL*	330
KR35	Garjiya	*Cyperus cyperoides*	KX499533	*rbcL*	327
KR36	Jabalpur	*Cyperus involucratus*	KX499534	*rbcL*	331
M1	Lucknow	*Cyperus iria*	KY386827	*matK*	744
M2	Rewa	*Cyperus retrorsus*	KY386828	*matK*	651
M3	Jagdalpur	*Fimbristylis autumnalis*	KY386829	*matK*	826
M4	Dindori	*Cyperus iria*	KY386830	*matK*	648
M5	Kapildhara	*Cyperus kyllingia*	KY386831	*matK*	775

M6	Pantnagar	*Fimbristylis autumnalis*	KY386832	*matK*	816
M7	Garjiya	*Cyperus cyperoides*	KY386833	*matK*	540
M8	Garjiya	*Cyperus cyperoides*	KY386834	*matK*	545
M9	Chennai	*Cyperus rotundus*	KY386835	*matK*	769
M10	Jabalpur	*Cyperus involucratus*	KY386836	*matK*	771
M11	Jabalpur	*Fimbristylis miliacea*	KY386837	*matK*	815
M12	Jabalpur	*Fimbristylis littoralis*	KY386838	*matK*	820
M13	Shahpura	*Cyperus microiria*	KY386839	*matK*	648
M14	Kundam	*Cyperus nutans*	KY386840	*matK*	762
M15	Karangia	*Kyllinga odorata*	KY386841	*matK*	793
M16	Corbett Park	*Cyperus rotundus*	KY386842	*matK*	823
M17	Corbett Park	*Cyperus rotundus*	KY386843	*matK*	645
TP1	Lucknow	*Cyperus iria*	KY386813	*trnH-psbA*	864
TP2	Jabalpur	*Cyperus kyllingia*	KY386814	*trnH-psbA*	859
TP3	Rewa	*Cyperus retrorsus*	KY386815	*trnH-psbA*	861
TP4	Jagdalpur	*Cyperus microiria*	KY386816	*trnH-psbA*	865
TP5	Dindori	*Cyperus iria*	KY386817	*trnH-psbA*	865
TP6	Amarkantak	*Cyperus compressus*	KY386818	*trnH-psbA*	882
TP7	Mandla	*Cyperus iria*	KY386819	*trnH-psbA*	858
TP8	Jabalpur	*Cyperus rotundus*	KY386820	*trnH-psbA*	856
TP9	Pantnagar	*Fimbristylis autumnalis*	KY386821	*trnH-psbA*	883
TP10	Garjiya	*Cyperus cyperoides*	KY386822	*trnH-psbA*	859

TP11	Corbett Park	*Cyperus cyperoides*	KY386823	*trnH-psbA*	880
TP12	Chennai	*Cyperus rotundus*	KY386824	*trnH-psbA*	827
TP13	Jabalpur	*Cyperus involucratus*	KY386825	*trnH-psbA*	870
TP14	Jabalpur	*Cyperus rotundus*	KY386826	*trnH-psbA*	673
Y1	Jabalpur	*Cyperus esculentus*	KY386844	*ycf1(b)*	793
Y2	Jabalpur	*Fimbristylis miliacea*	KY386845	*ycf1(b)*	688
Y3	Jabalpur	*Fimbristylis littoralis*	KY386846	*ycf1(b)*	742

Table 4.4 Unique DNA barcodes generated in present study

DNA barcode Loci	Species specific unique barcodes
rbcL	*Cyperus nutans* (KR31), *Kyllinga nemoralis* (KR30), *Kyllinga odoratus* (KR32, KR33)
matK	*Cyperus microiria* (M13), *Cyperus nutans* (M14), *Cyperus kyllingia* (M5), *Fimbristylis miliacea* (M11), *Fimbristylislittoralis* (M12)
trnH-psbA	*Cyperus microiria* (TP4), *Cyperus cyperoides* (TP10, TP11), *Cyperus kyllingia* (TP2), *Fimbristylis autumnalis* (TP9)
ycf1(b)	*Cyperus esculentus* (Y1), *Fimbristylis miliacea* (Y2), *Fimbristylis littoralis* (Y3)

4.2.4 Species identification through species identifier program of TaxonDNA

DNA barcode sequence data for *rbcL*, *matK*, *trnH-psbA* and the concatenated dataset for *rbcL+matK*, *rbcL+trn*H-*psb*A, *matK+trnH-psbA* and *rbcL+matK+trnH-psbA* obtained from the Mesquite program were analyzed in TaxonDNA/ SpeciesIdentifier-1.8 program for evaluating the specimen identification success for the seven barcode datasets using the ‘best match’ and ‘best close match’ function of the program. The results are presented in Table 4.5.

Table 4.5 Results from similarity based analysis using SpeciesIdentifier-1.8 program of TaxonDNA (% value shown in parentheses)

Barcode regions							
	rbcL	*matK*	*trn*H-*psb*A	*rbcL*+*matK*	*rbcL*+*trn*H-*psb*A	*matK*+*trn*H-*psb*A	*rbcL*+*matK*+*trn*H-*psb*A
Best Match							
Correct	**22 (61.11)**	9 (52.94)	8 (57.14)	**26 (66.66)**	22 (57.89)	10 (52.63)	**29 (74.35)**
Ambiguous	0	0	0	0	1 (2.63)	0	1 (2.56)
Incorrect	14 (38.88)	8 (47.05)	6 (42.85)	13 (33.33)	15 (39.47)	9 (47.36)	9 (23.07)
Best Close Match							
Correct	**22 (61.11)**	9 (52.94)	5 (35.71)	**26 (66.66)**	22 (57.89)	7 (36.84)	**25 (64.1)**
Ambiguous	0	0	0	0	0	0	0
Incorrect	7 (19.44)	4 (23.52)	0	4 (10.25)	5 (13.15)	2 (10.52)	0
Sequence without any match closer than threshold (3 %)	7 (19.44)	4 (23.52)	9 (64.28)	9 (23.07)	11 (28.94)	10 (52.63)	14 (35.89)

The results of similarity based analysis through SpeciesIdentifier program indicated *rbcL* as best barcode region followed by *matK* among individual barcode locus, while *rbcL+matK* as best barcode loci combination followed by *rbcL+matK+trnH-psbA* on the basis of their correct identification percentage by 'best close match' method. The threshold value of 3% was system calculated.

4.3 Phylogenetic analysis for the clustering based identification of sedge plant species by UPGMA method

In order to identify the sedge plant species based on evolutionary relationship, the barcode sequences of *rbcL*, *matK*, *trnH-psbA* and *ycf1(b)* were subjected to UPGMA based phylogenetic analysis along with all the available reference species-specific sequences from GenBank (shown with GenBank accession numbers). The analysis led to correct identification of monophyletic sample sequences with respect to the reference sequences.

Each species was grouped in to a single species specific cluster. The results are presented in Fig 4.8 – Fig 4.11. The optimal tree with the sum of branch length is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA7 program.

The UPGMA analysis showed species specific clusters with respect to the reference sequences for *rbcL*, *matK* and *trn*H-*psb*A. Since, there is no reported barcode for *ycf1(b)* in *Cyperus* spp., it showed similarity with plants of other genera.

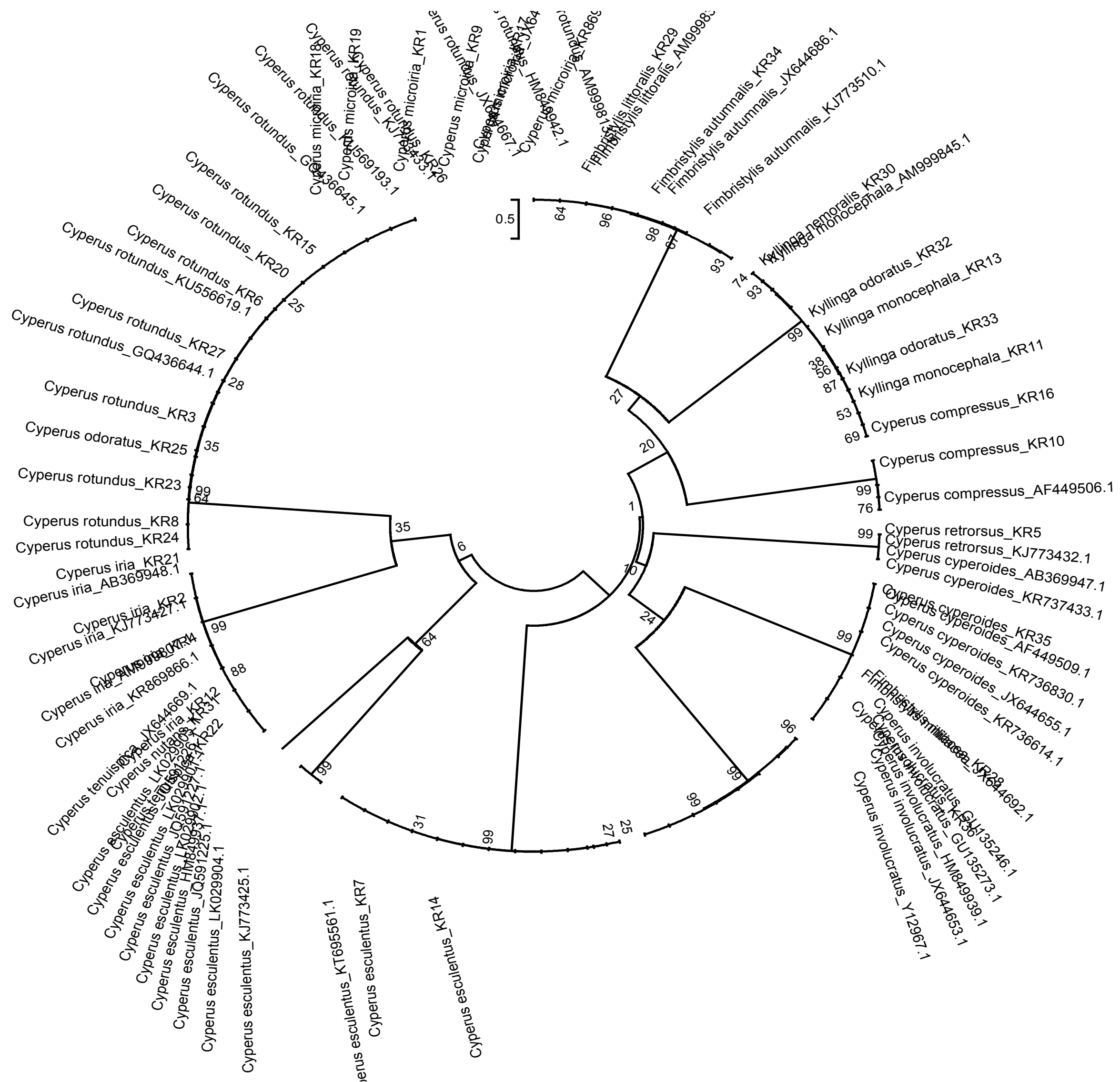


Fig 4.8 UPGMA based phylogenetic analysis of *rbcL* barcode sequences (species specific reference sequences are shown with GenBank accession numbers)

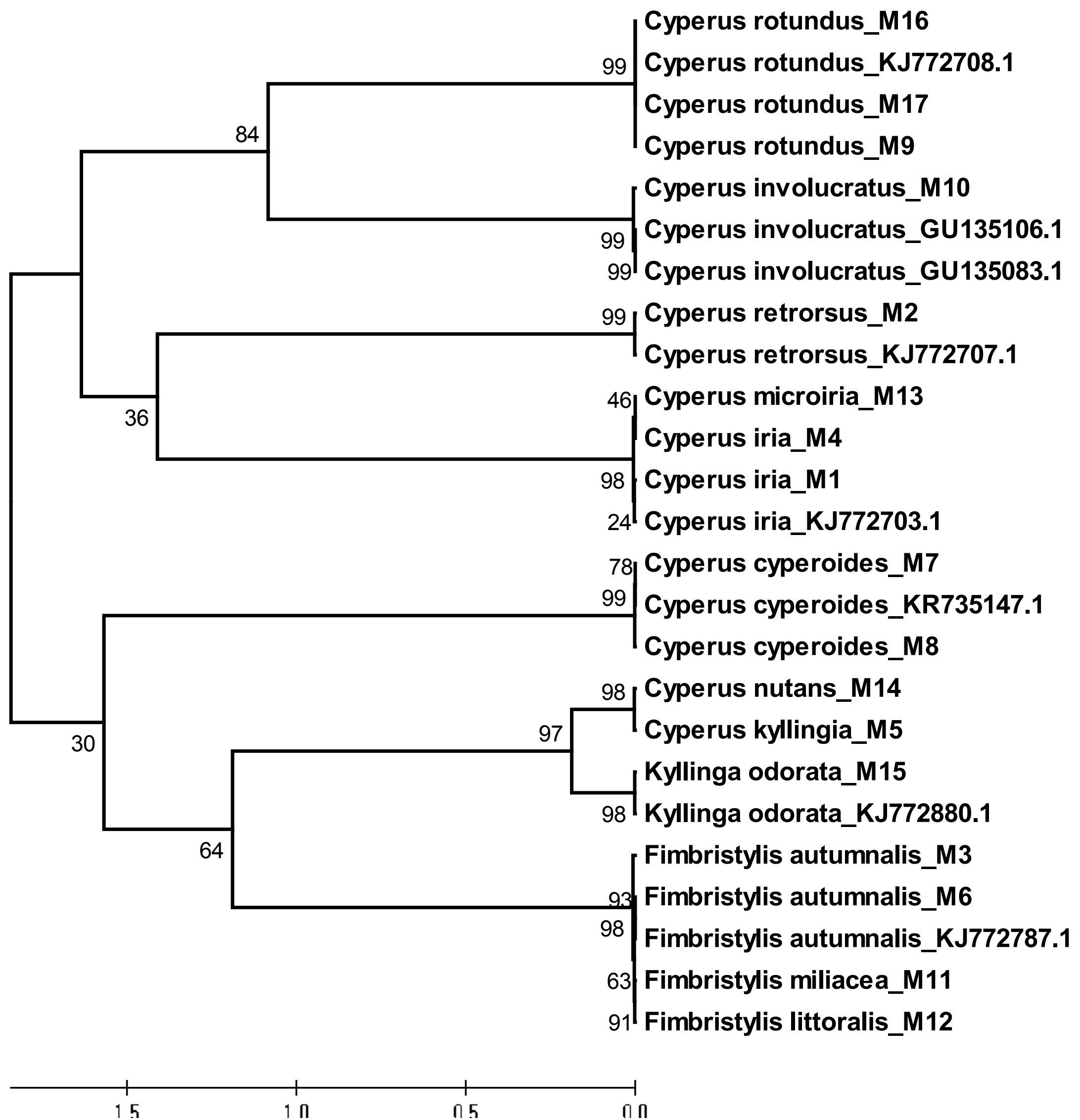


Fig 4.9 UPGMA based phylogenetic analysis of *matK* barcode sequences (species specific reference sequences are shown with GenBank accession numbers)

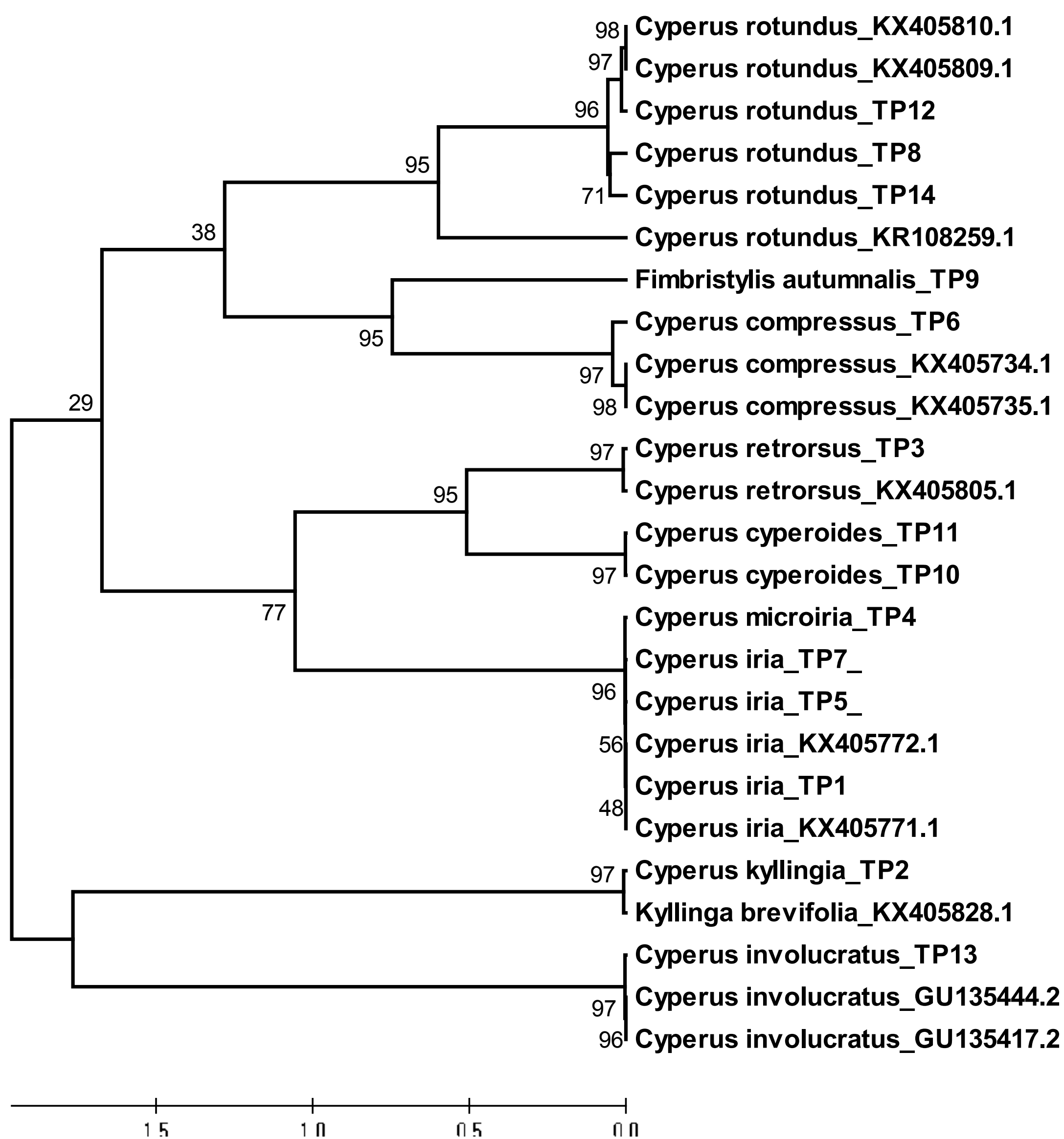


Fig 4.10 UPGMA based phylogenetic analysis of *trnH-psbA* barcode sequences (reference shown with GenBank accession numbers)

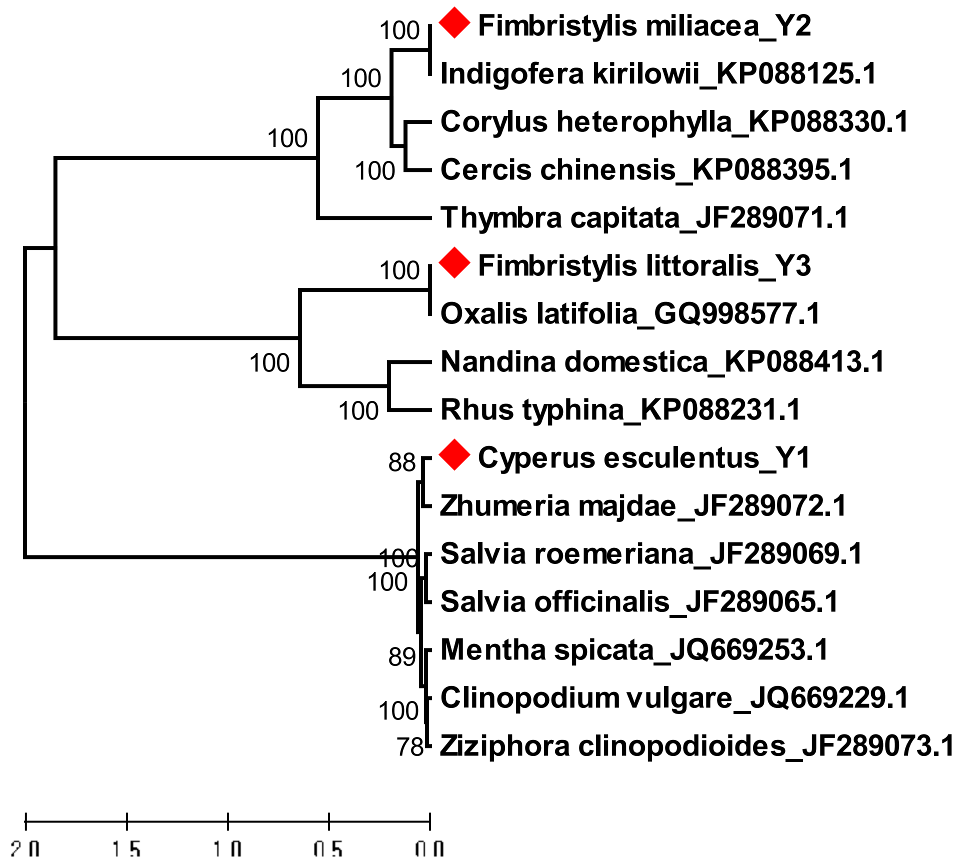


Fig 4.11 UPGMA based phylogenetic analysis of *ycf1(b)* barcode sequences(reference shown with GenBank accession numbers)

4.4Tree based analyses (Maximum parsimony and bootstrap joining)

In order to assess the most efficient barcoding loci for the identification of sedge plant species and to infer on the taxonomy of the sampled sedge plants, phylogenetic analysis based on maximum parsimony (MP) method was conducted on all the seven barcode datasets in MEGA7 program. The tree was constructed by the bootstrap method in 1000 replications using the Tree-Bisection-Regrafting (TBR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences in10 replicates. The tree was drawn to scale, with branch lengths calculated using the average pathway method and in the units of the number of changes over the whole sequence. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated except for *rbcL+matK+trnH-psbA* for which site coverage cut off was kept at 50%. Only

individual barcodes were analysed with reference sequences and outgroup species. The results of the analysis are presented in Table 4.6 and the phylogenic trees are shown in Fig4.12 – Fig 4.18.

Table 4.6 Maximum Parsimony (MP) analysis results for seven barcode datasets

MP parameters	*rbcL*	*matK*	*trn*H-*psb*A	*rbcL*+*matK*	*rbcL*+*trn*H-*psb*A	*matK*+*trn*H-*psb*A	*rbcL*+*matK*+*trn*H-*psb*A
No. of taxa/ sequences	80*	26*	26*	39	38	19	39
No. of Pi Sites	280	399	774	331	276	28	698
No. of most parsimonious tree	2	5	4	2	1	3	1
Tree length	1672	1427	3296	2134	1626	135	4097
CI	0.466	0.662	0.555	0.444	0.478	0.562	0.467
RI	0.919	0.881	0.798	0.780	0.814	0.677	0.696
RCI	0.429	0.584	0.444	0.346	0.389	0.381	0.325

*includes reference sequences and outgroup species, **Pi** (Parsimony informative), **CI** (Consistency index), **RI** (Retention index), **RCI** (Rescaled Index for all sites)

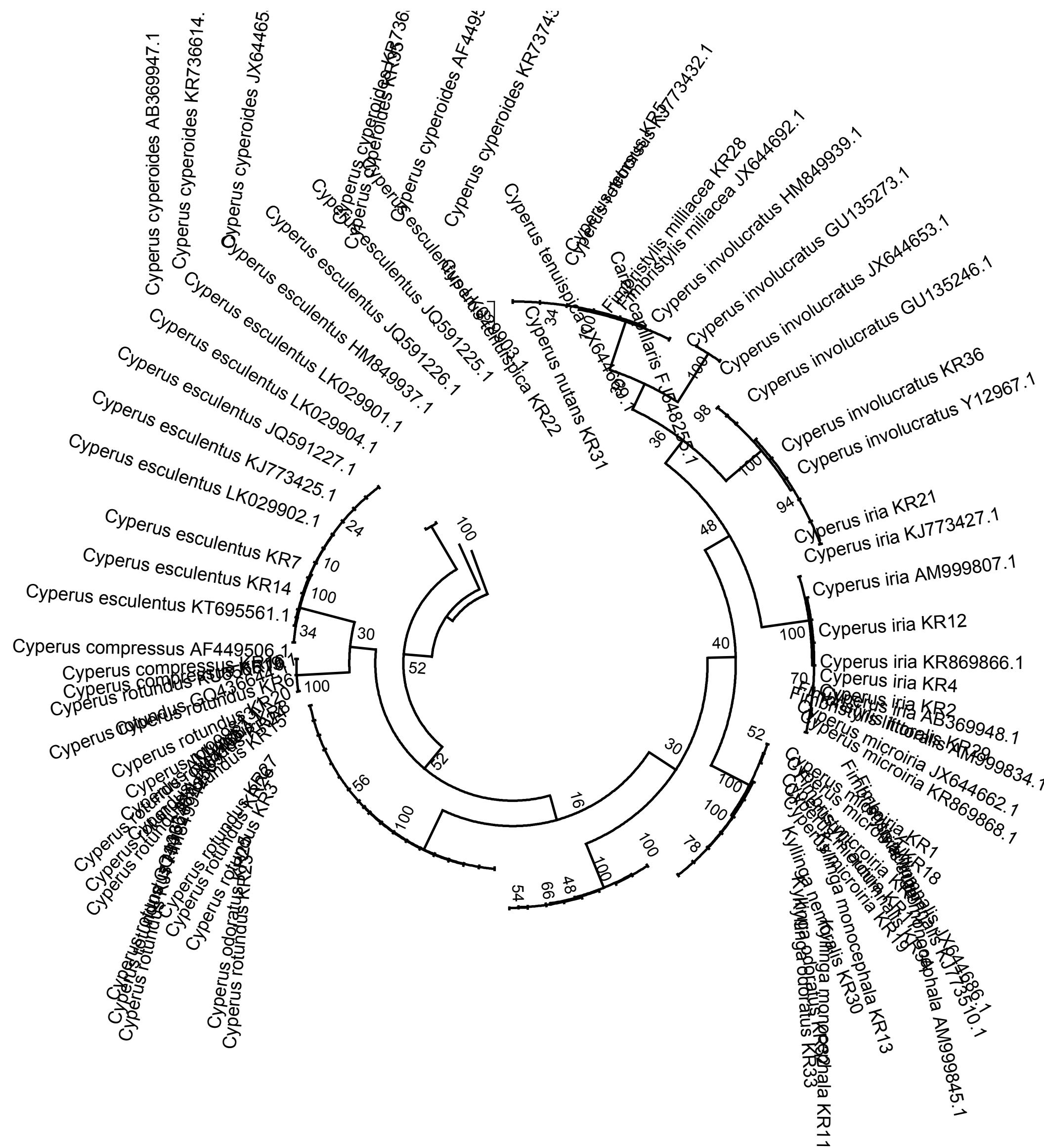


Fig 4.12 Maximum Parsimony analysis of taxa based on *rbcL* barcode sequences (reference sequences shown with GenBank accession numbers; *Carex capillaris* as outgroup species)

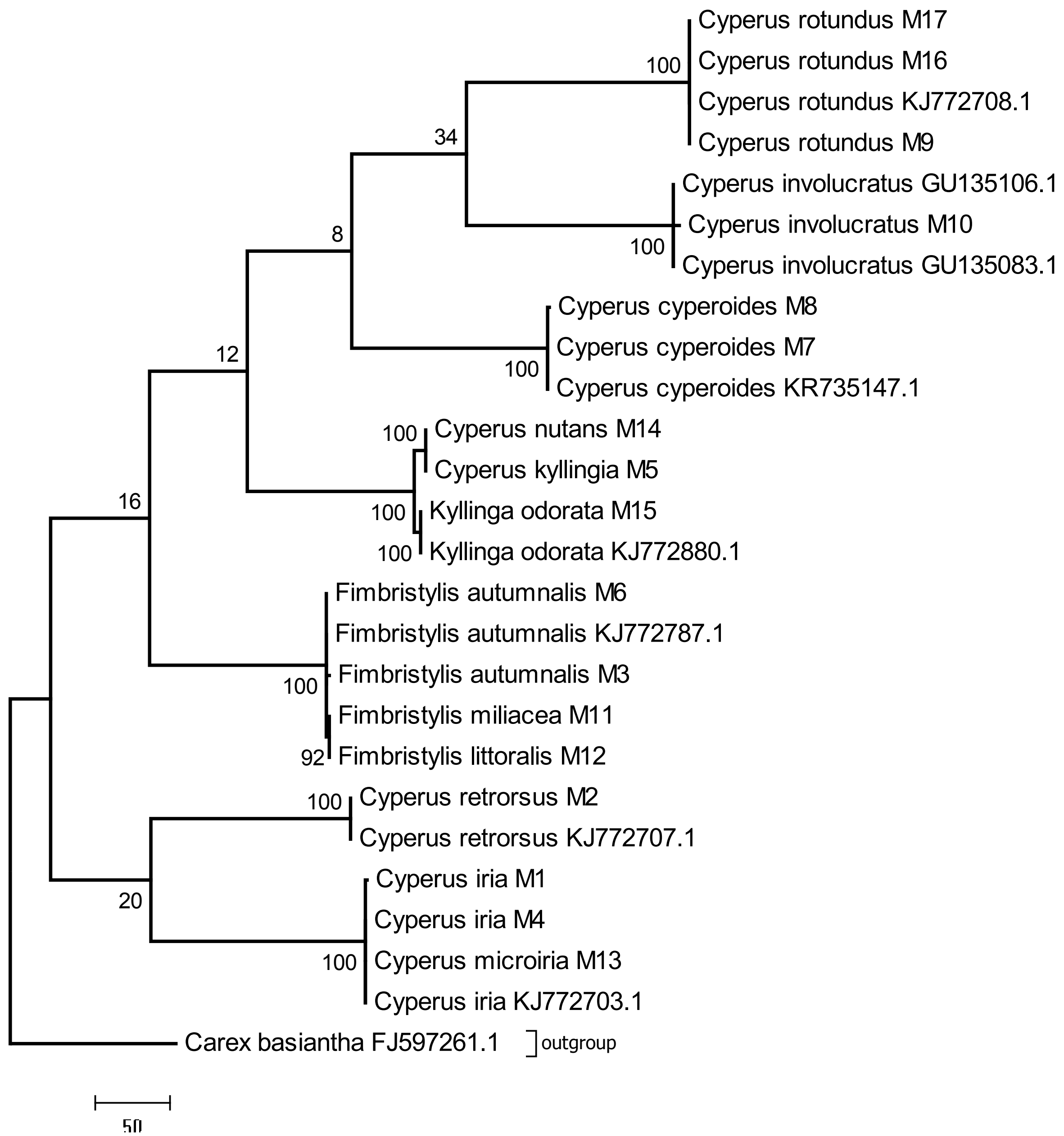


Fig 4.13 Maximum Parsimony analysis of taxa based on *matK* barcode sequences (reference sequences shown with GenBank accession numbers; *Carex basiantha* as outgroup species)

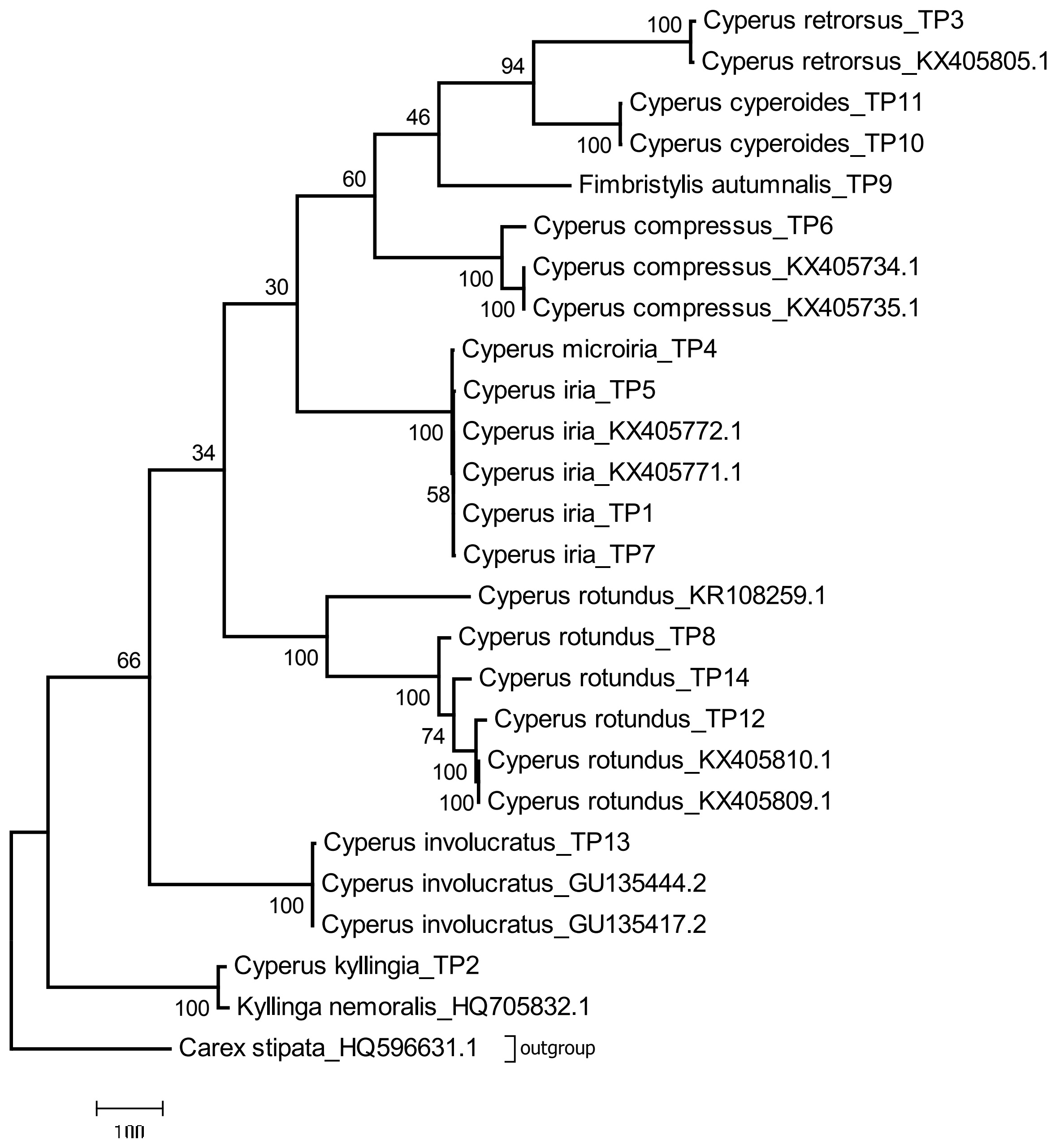


Fig 4.14 Maximum Parsimony analysis of taxa based on *trnL-psbA* barcode sequences (reference sequences shown with GenBank accession numbers; *Carex stipata* as outgroup species)

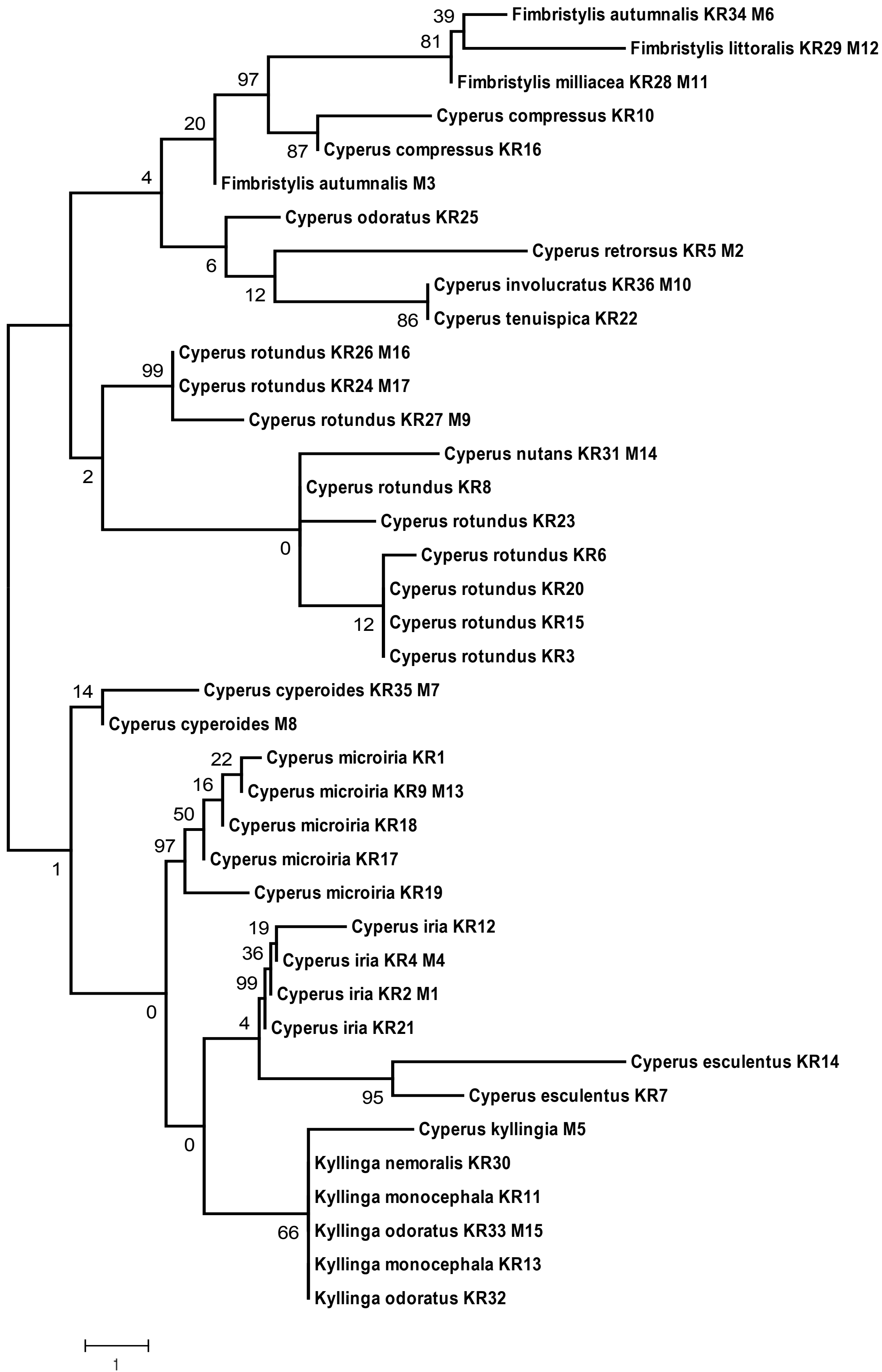


Fig 4.15 Maximum Parsimony analysis of taxa based on *rbcL+matK* barcode sequences dataset

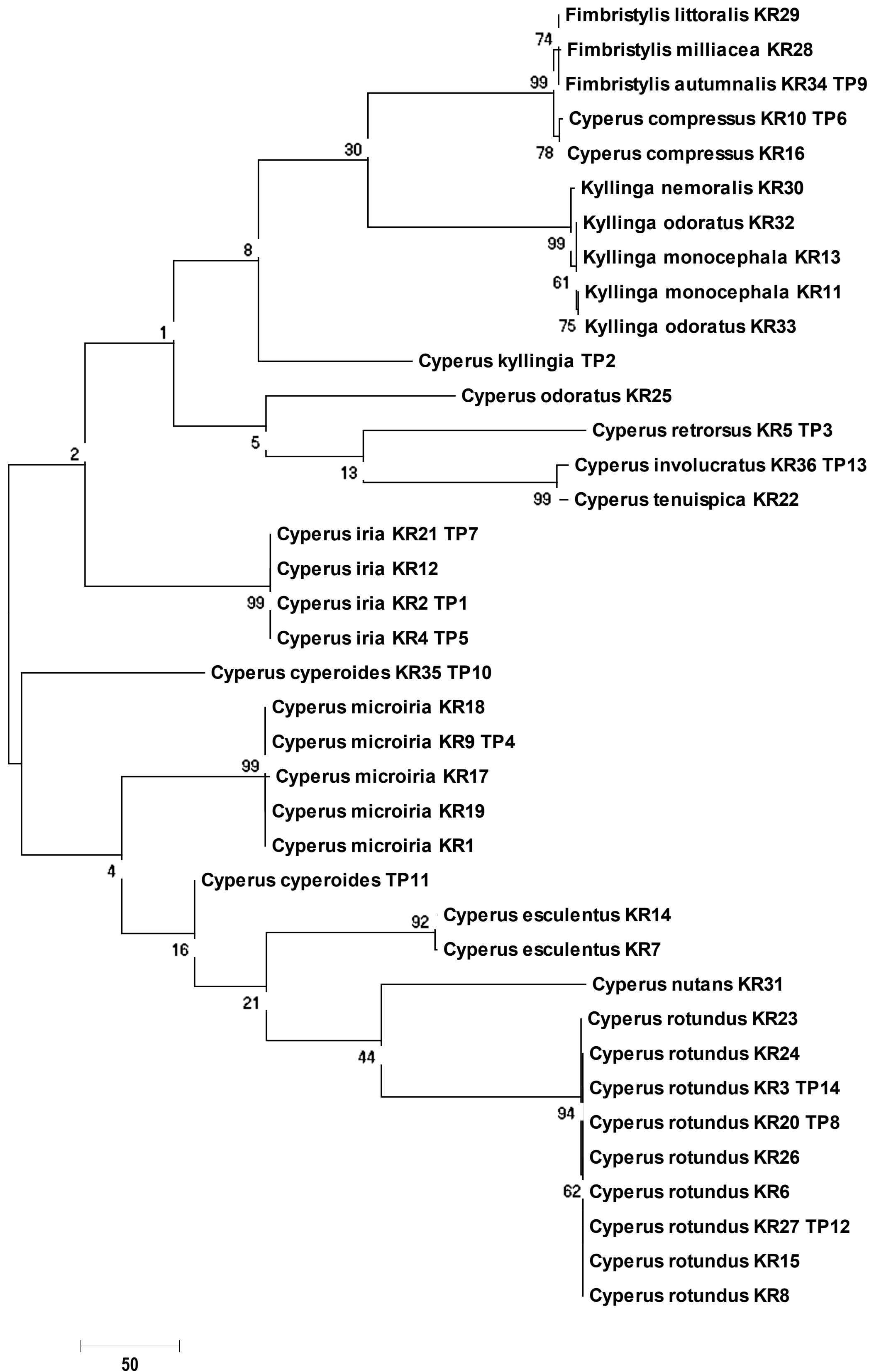


Fig 4.16 Maximum Parsimony analysis of taxa based on *rbcL+trnH-psbA* barcode sequences dataset

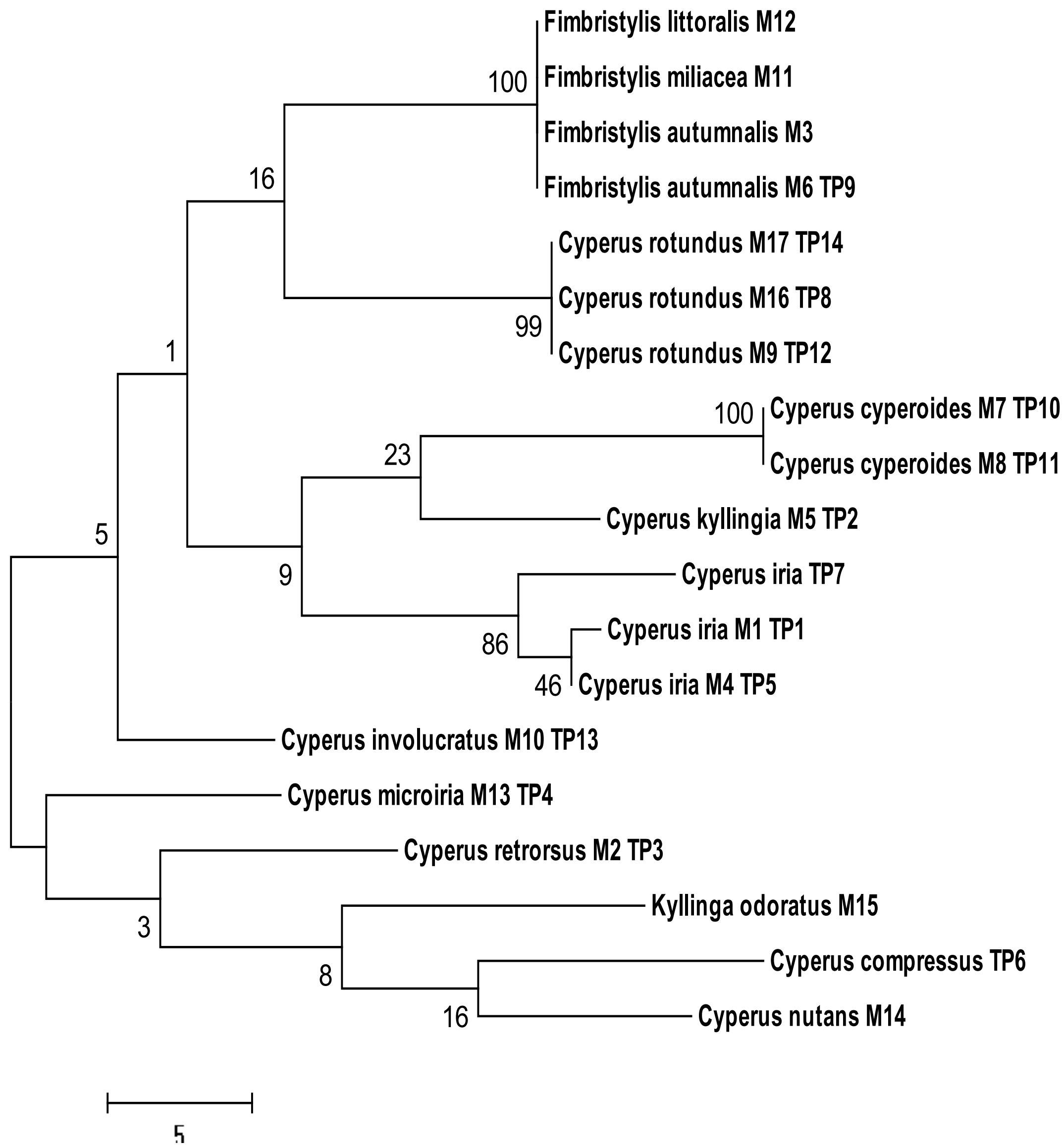


Fig 4.17 Maximum Parsimony analysis of taxa based on *matK+trnH-psbA* barcode sequences dataset

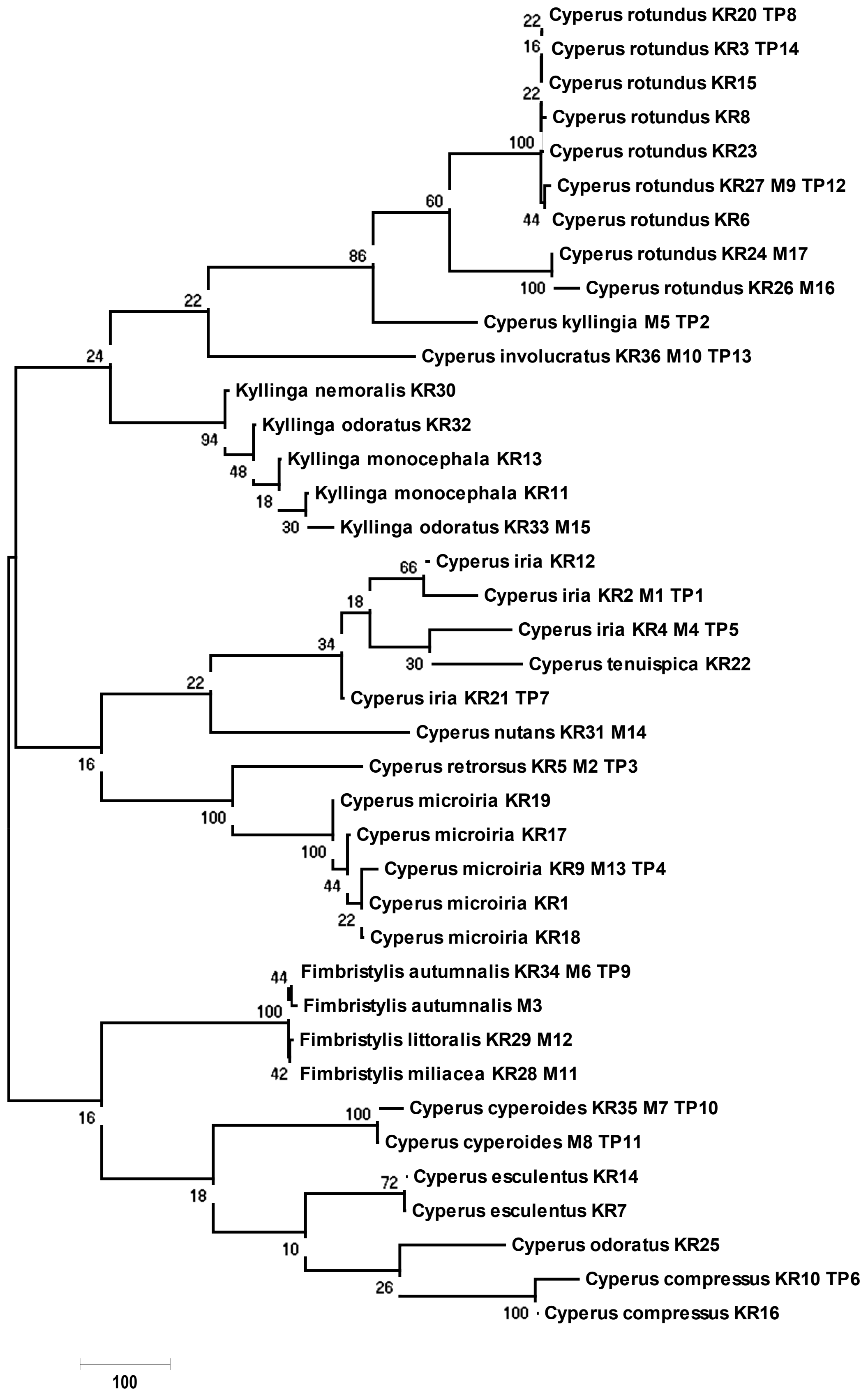


Fig 4.18 Maximum Parsimony analysis of taxa based on *rbcL+matK+trnH-psbA* barcode sequences dataset

Maximum parsimony analysis for *rbcL* barcode (Fig 4.12) although shown monophyletic clusters for most of the species along with reference sequences but failed to group the species belonging to genus *Fimbristylis* in to a single cluster or to sister clusters. *Fimbristylis miliacea* was grouped with *Cyperus involucratus* while *Fimbristylis littoralis* was grouped with *Cyperus microiria*, both with a bootstrap value of 100%. Phylogenetic tree constructed by *matK* (Fig 4.13) and *trnH-psbA* (Fig 4.14) resolved all the included species as monophyletic. The combination barcode *rbcL+matK* (Fig 4.15) resolved all the species better than *rbcL* alone and grouped all the species of genus *Fimbristylis* in to a single major cluster, however nested by *Cyperus compressus*. The combination barcode *rbcL+trnH-psbA* (Fig 4.16) resolved all the species better than *rbcL* alone and grouped all the species of genus *Fimbristylis* in to a single cluster with a sister cluster of *Cyperus compressus* with a bootstrap value of 99%. The combination barcode *matK+trnH-psbA* (Fig 4.17) resolved all the species of *Cyperus*, *Kyllinga* and *Fimbristylis* as monophyletic group with low bootstrap values. The combination barcode *rbcL+matK+trnH-psbA* (Fig 4.18) resolved all the species included in this study as monophyletic species/genus cluster with bootstrap values above 60 for most of the clusters.

4.5 Distance analysis and barcoding gap for *rbcL*, *matK*, *trnH-psbA*and combined barcodes

DNA barcode sequence data for *rbcL*, *matK*, *trnH-psbA* and the concatenated datasets of *rbcL+matK*, *rbcL+trn*H-*psb*A, *matK+trnH-psbA* and *rbcL+matK+trnH-psbA* were analyzed in TaxonDNA/ SpeciesIdentifier-1.8 program for calculating uncorrected *p*-distances between all sequence pairs and the distance data was used to calculate mean and range of intraspecific and interspecific distances for all the seven barcode datasets (Table 4.7).Barcode *trnH-psbA* had the minimum interspecific distance value 0.4408 which was larger than the maximum value of intraspecific distance 0.1144 indicating a significant barcode gap. The barcode gap was absent for all other loci indicating overlaps between inter and intraspecific divergence. However, the mean interspecific divergence was significantly higher than that of the corresponding intraspecific divergence for each of the loci studied indicating

very low overlap between inter and intraspecific divergences (Fig 4.19) and hence suitability of the same as DNA barcode locus for the identification of plant species.

Table 4.7 Characteristics of the seven barcode datasets evaluated in TaxonDNA

Barcode	No. of specimens/No. ofspecies	Alignment length(bp)	Mean intraspecific distance (range)	Mean interspecific distance (range)
rbcL	36/17	347	0.0112 (0.0-0.0290)	0.6362 (0.0029-0.7738)
matK	17/12	826	0.0027 (0.0-0.0098)	0.6727 (0.0012-0.7333)
*trn*H-*psb*A	14/09	883	0.0476 (0.0011-0.1144)	0.6446 (0.4408-0.6980)
rbcL+*matK*	39/17	1149	0.0160 (0.0-0.1222)	0.7256 (0.0066-0.7794)
rbcL+*trn*H-*psb*A	38/17	1213	0.1084 (0.0-0.7645)	0.7205 (0.0066-0.7890)
matK+*trn*H-*psb*A	19/13	1699	0.0330 (0.0059-0.0799)	0.6803 (0.0012-0.7244)
rbcL+*matK*+*trn*H-*psb*A	39/17	2029	0.1556 (0.0-0.7174)	0.7232 (0.2197-0.9266)

4.6 Most efficient barcoding loci

Based on similarity searches, maximum parsimony and DNA barcode gap analyses, *rbcL+matK* followed by *matK* and *trnH-psbA* were found best barcode region to unambiguously identify a sedge plant species as they could group each species in a monophylectic cluster group. The DNA barcodes generated during present investigation are presented in the form of illustrated barcode graphic Figures in Table 4.8.

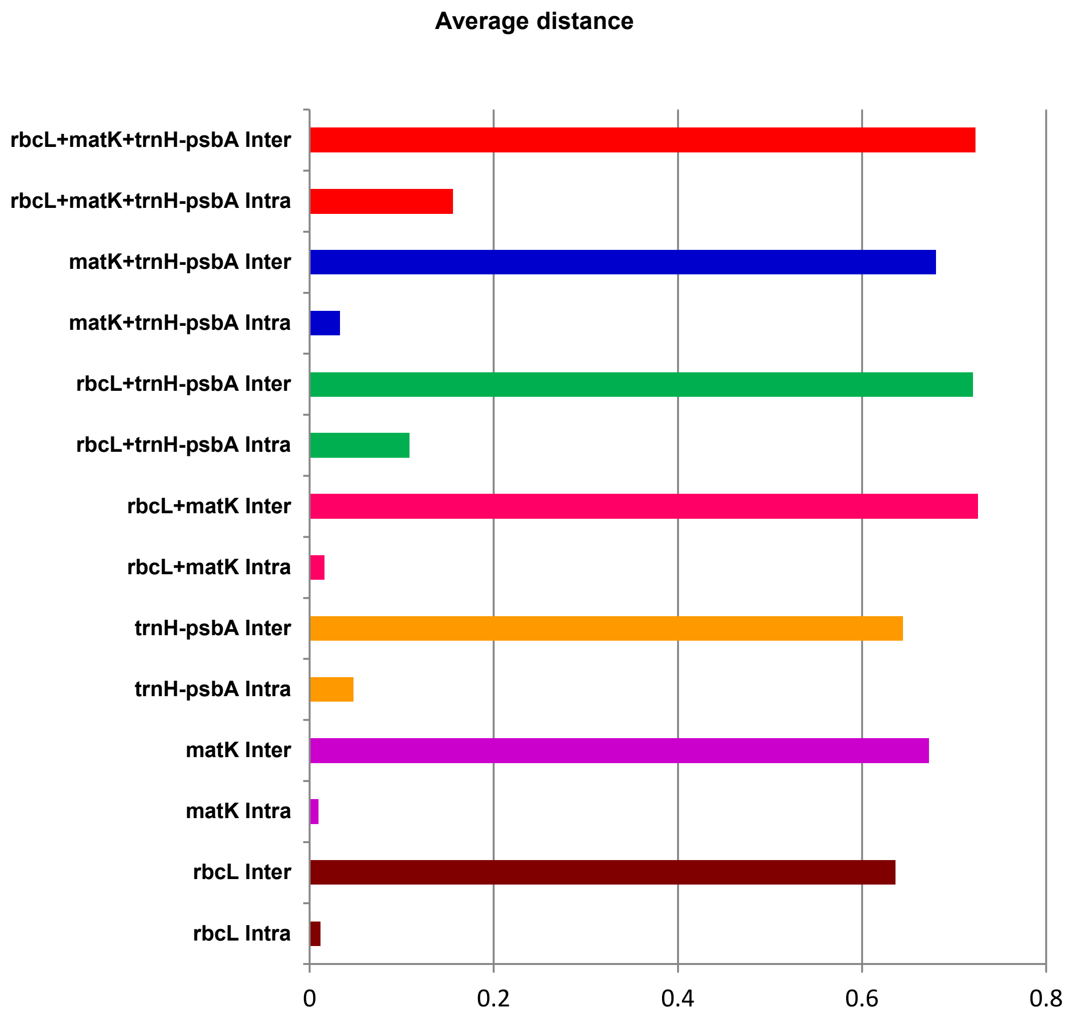


Fig 4.19 The barcoding gap: Graph between average interspecific and intraspecific distances

A. *Cyperus rotundus*

B. *Cyperus esculentus*

C. *Cyperus iria*

D. *Cyperus microiria*

Plate 1 Morphological identification of *Cyperus* species of A. *Cyperus rotundus*, B. *Cyperus esculentus*, C. *Cyperus iria* and D. *Cyperus microiria*

E. *Cyperus cyperoides*

F. *Cyperus compressus*

G. *Cyperus involucratus*

H. *Cypeus nutans*

Plate 2 Morphological identification of *Cyperus* species of E. *Cyperus cyperoides,* F. *Cyperus compressus,* G. *Cyperus involucratus* and H. *Cyperus nutans*

I. *Cyperus retrorsus*

J. *Cyperus odoratus*

K. *Cyperus tenuispica*

L. *Cyperus kyllingi*

Plate 3 Morphological identification of *Cyperus* species of I. *Cyperus retrorsus*, J. *Cyperus odoratus*, K. *Cyperus tenuispica* and L. *Cyperus Kylligi*

M. *Fimbristylis miliacea*

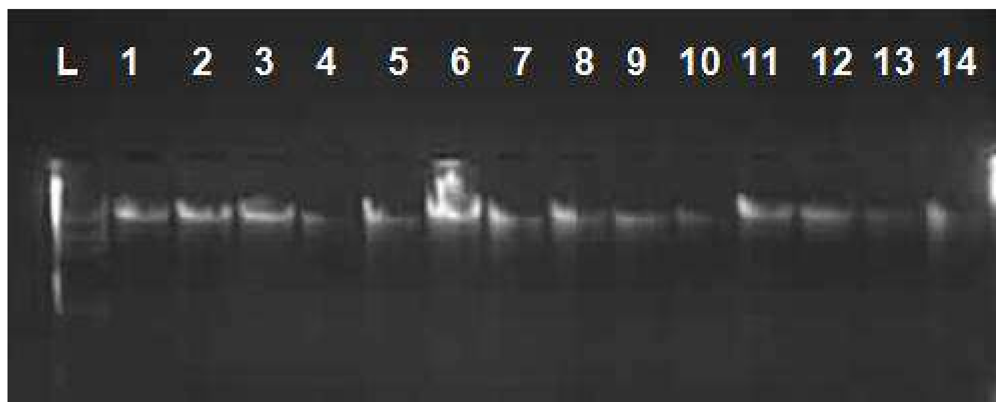
N. *Fimbristylis littoralis*

O. *Fimbristylis autumnalis*

P. *Kyllinga brevifolia*

Q. *Kyllinga odorata*

Plate 4 Morphological identification of *Fimbristylis* and *Kallinga* species



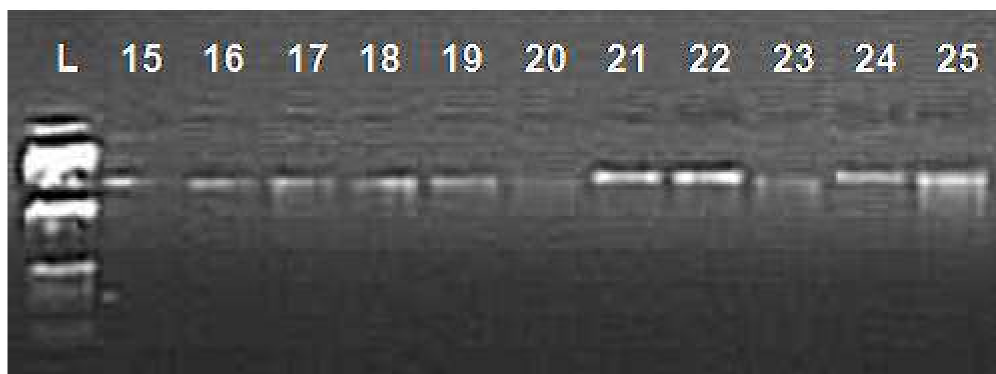


Plate 5 DNA Isolation and Quantification from Plant Samples
L – Uncut λ DNA ladder

Plate 6a Lane 1 to 36 - PCR Product (400bp) using *rbcL* Primer
L - GeneRuler 100bp DNA ladder

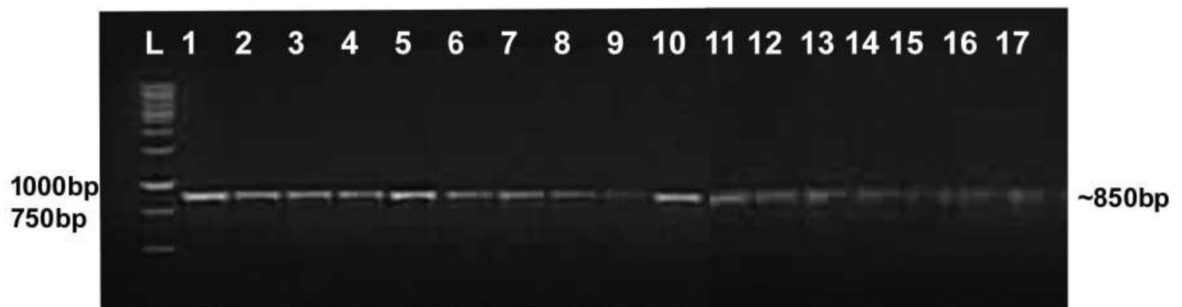


Plate 6b Lane 1 to 17 - PCR Product (~850bp) using *matK* Primer
L - GeneRuler 1kb DNA ladder

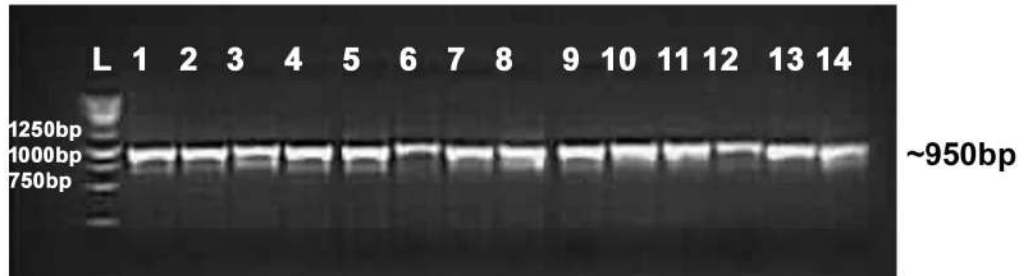


Plate 7a Lane 1 to 14 - PCR Product (~950bp) using *trnH-psbA* Primer
L - GeneRuler 1kb DNA ladder

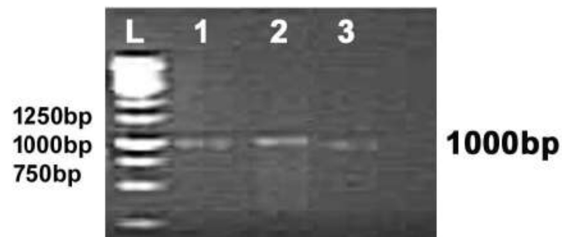
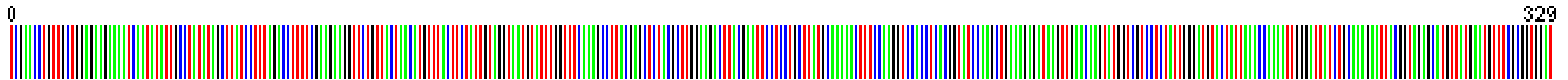
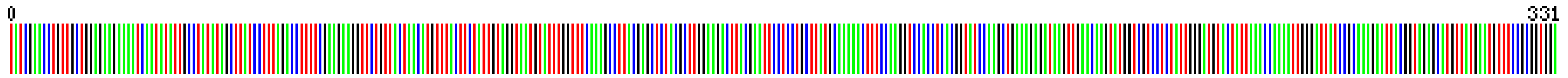
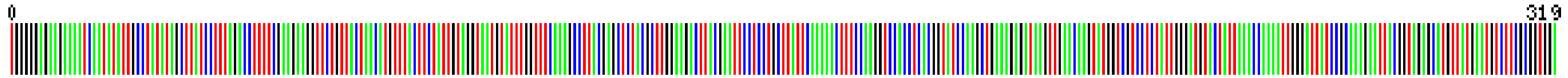
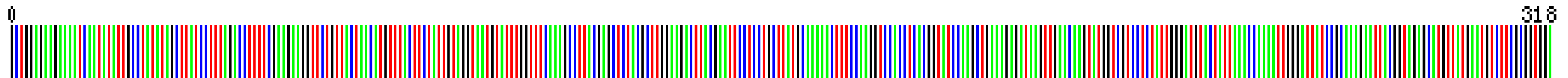
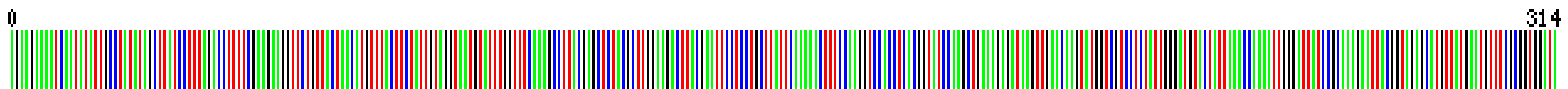
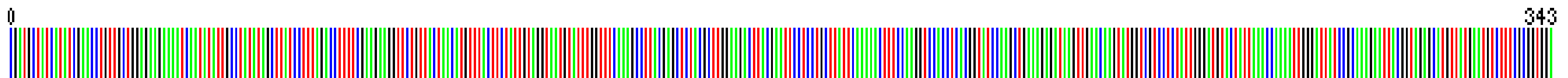
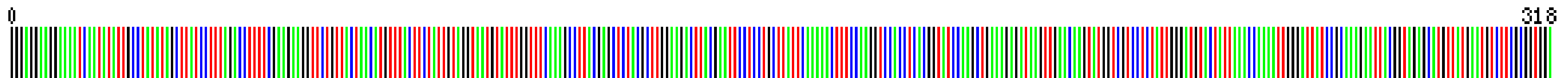
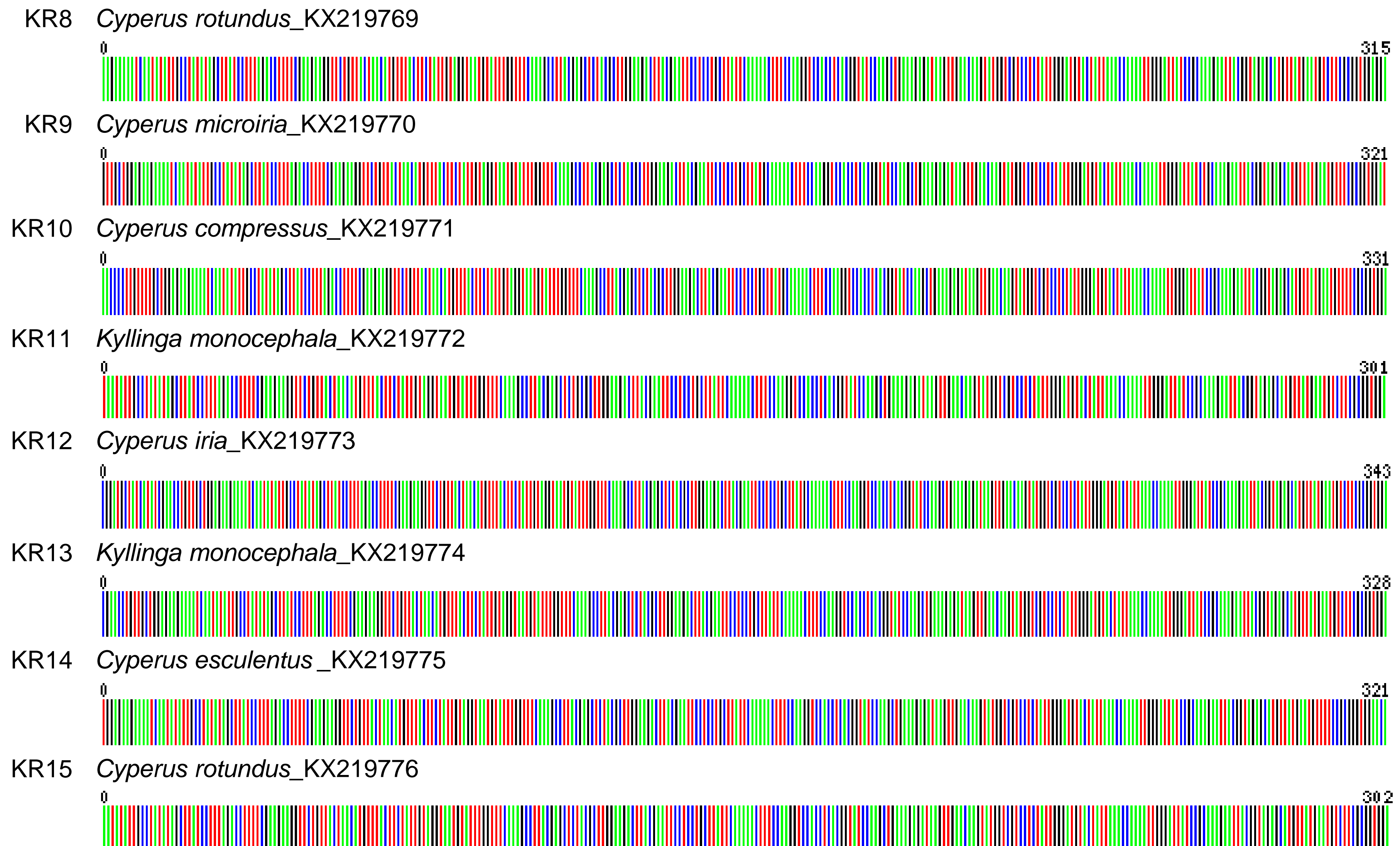


Plate 7b Lane 1 to 3 - PCR Product (1000bp) using *ycf1(b)* Primer
L - GeneRuler 1kb DNA ladder

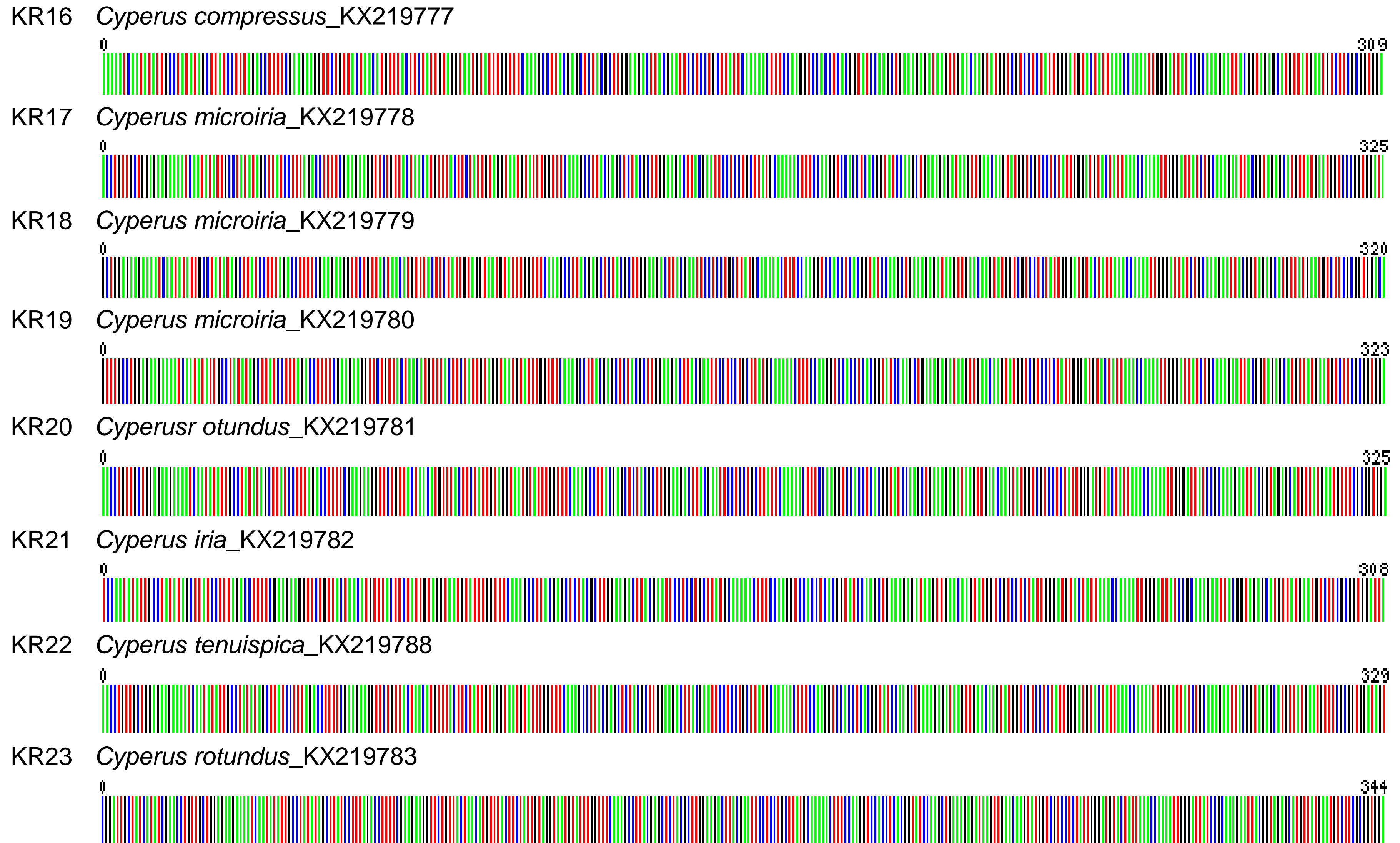
Table 4.8 Species specific DNA Barcodes (Nucleotide bar colours: G-Black, C-Blue, A-Green and T-Red)

SN	Species specific illustrative barcode with GenBank submitted accession numbers
KR1	*Cyperus microiria*_KX219762 
KR2	*Cyperus iria*_KX219763 
KR3	*Cyperus rotundus*_KX219764 
KR4	*Cyperus iria*_KX219765 
KR5	*Cyperus retrorsus*_KX219766 
KR6	*Cyperus rotundus*_KX219767 
KR7	*Cyperus esculentus* _KX219768 

Barcode of species in bold letters are reported first time in public domain

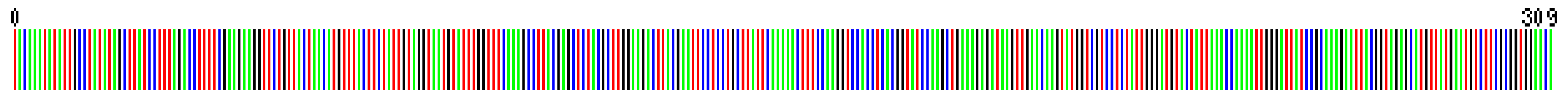


Barcode of species in bold letters are reported first time in public domain

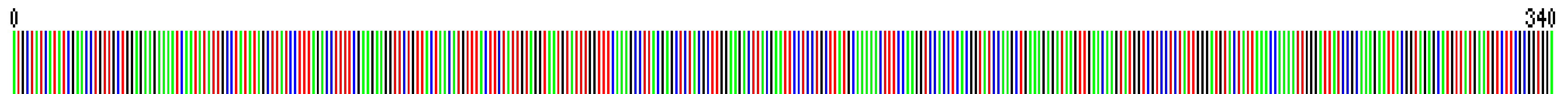


Barcode of species in bold letters are reported first time in public domain

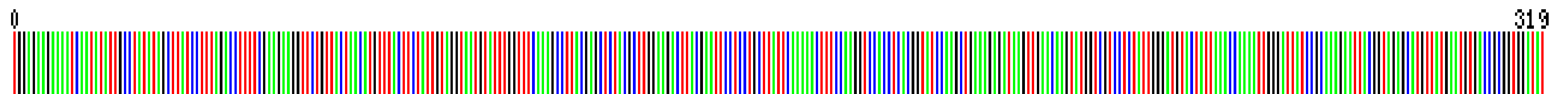
KR24 *Cyperus rotundus*_KX219784



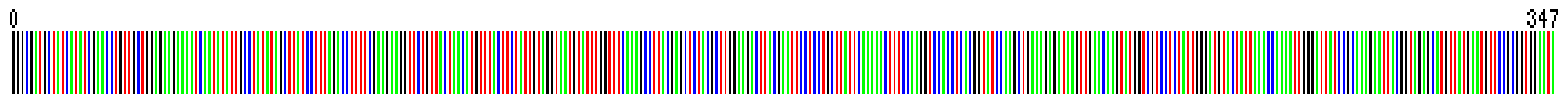
KR25 *Cyperus odoratus*_KX219785



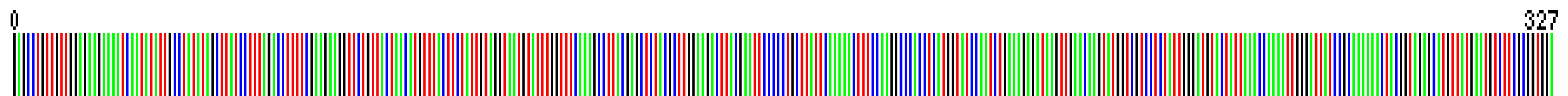
KR26 *Cyperus rotundus*_KX219786



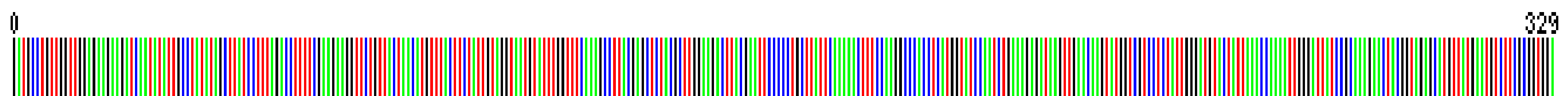
KR27 *Cyperus rotundus*_KX219787



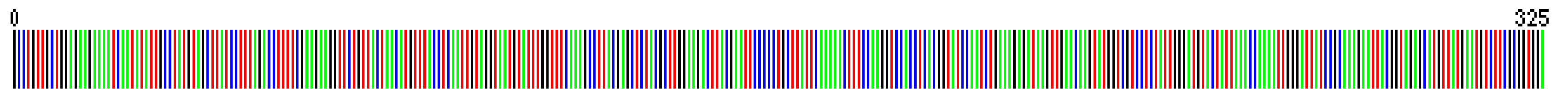
KR28 *Fimbristylis miliacea*_KX499535



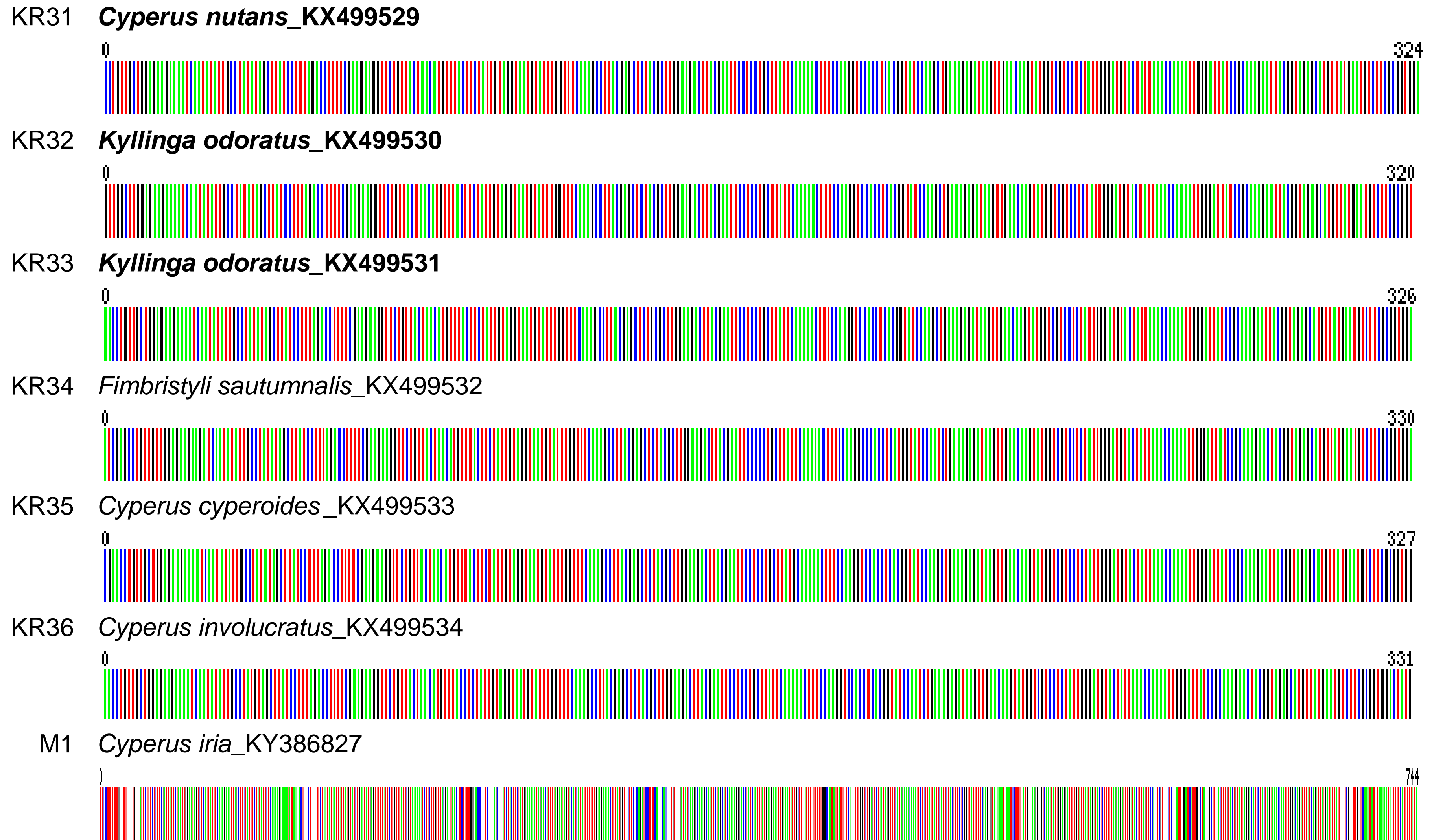
KR29 *Fimbristylis littoralis*_KX499527



KR30 ***Kyllinga nemoralis*_KX499528**

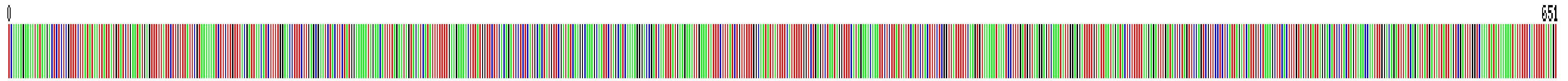


Barcode of species in bold letters are reported first time in public domain

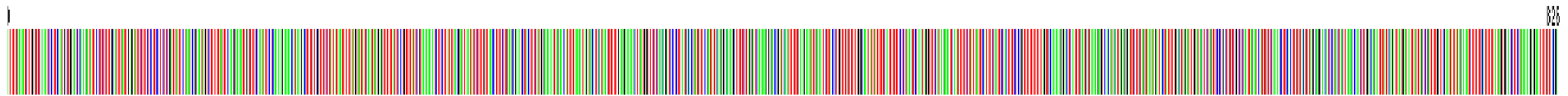


Barcode of species in bold letters are reported first time in public domain

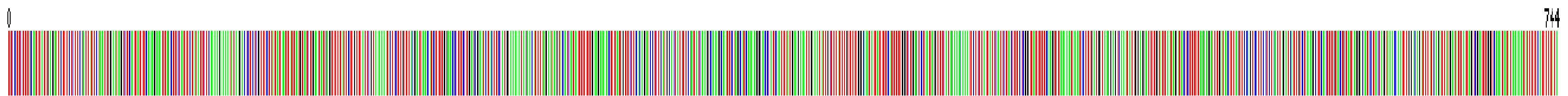
M2 *Cyperus retrorsus*_KY386828



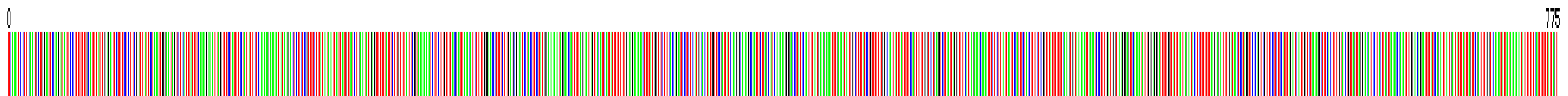
M3 *Fimbristylis autumnalis*_KY386829



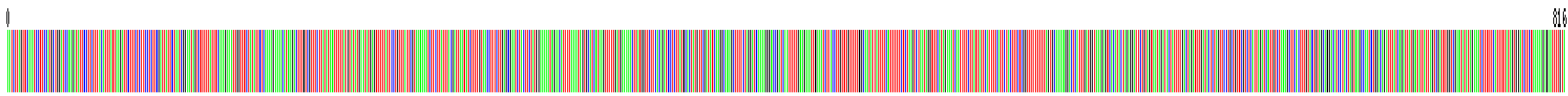
M4 *Cyperus iria*_KY386830



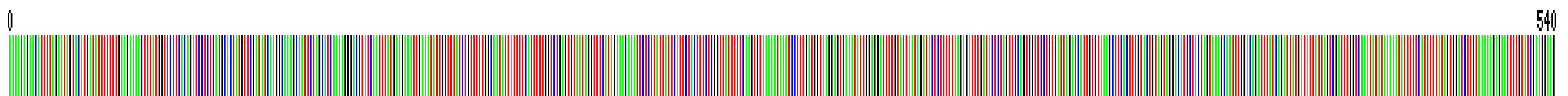
M5 ***Cyperus kyllingia*_KY386831**



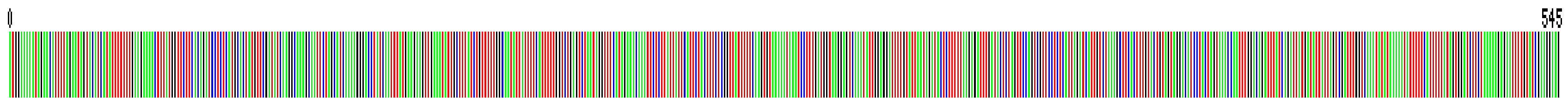
M6 *Fimbristylis autumnalis*_KY386832



M7 *Cyperus cyperoides*_KY386833



M8 *Cyperus cyperoides*_KY386834



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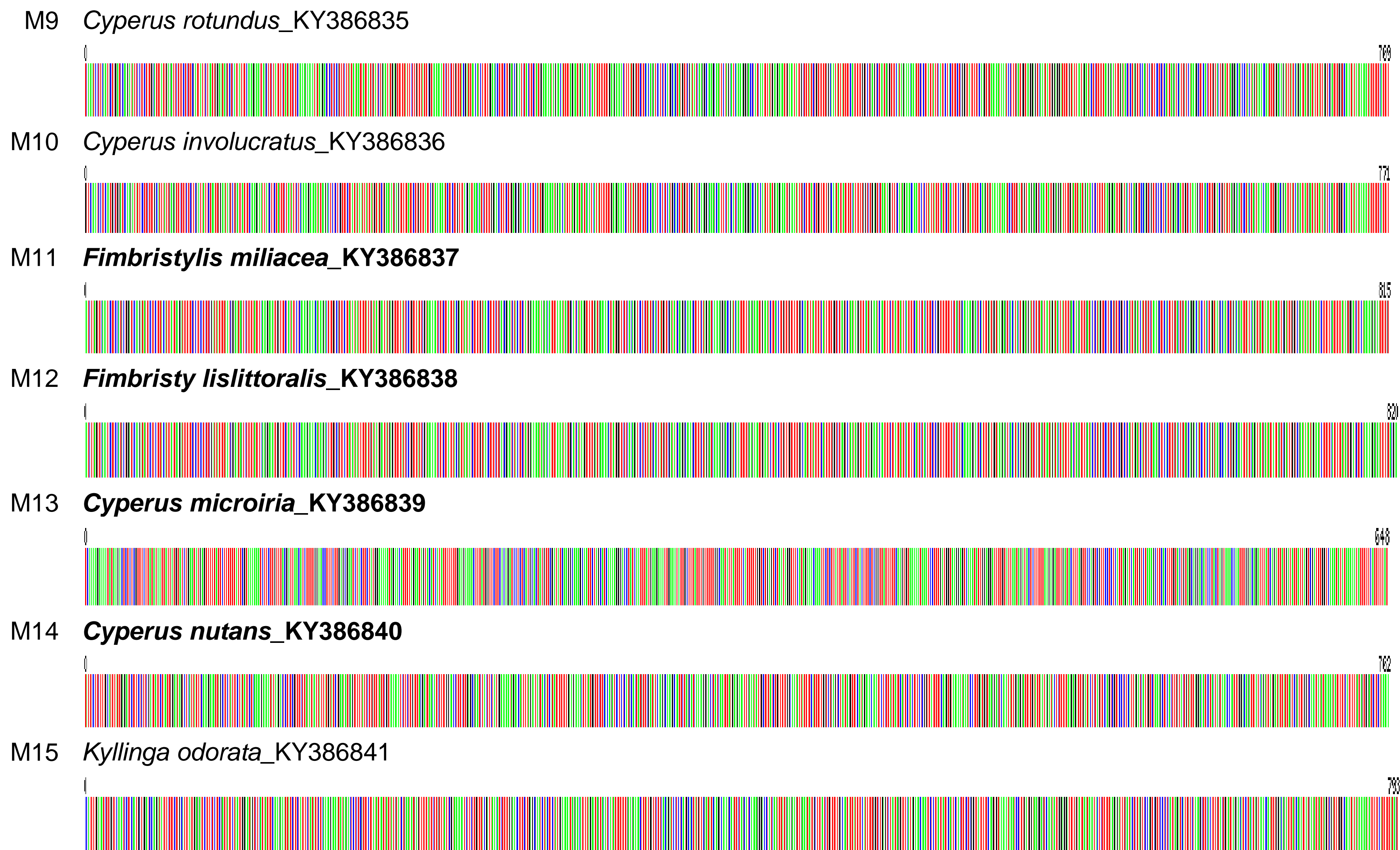
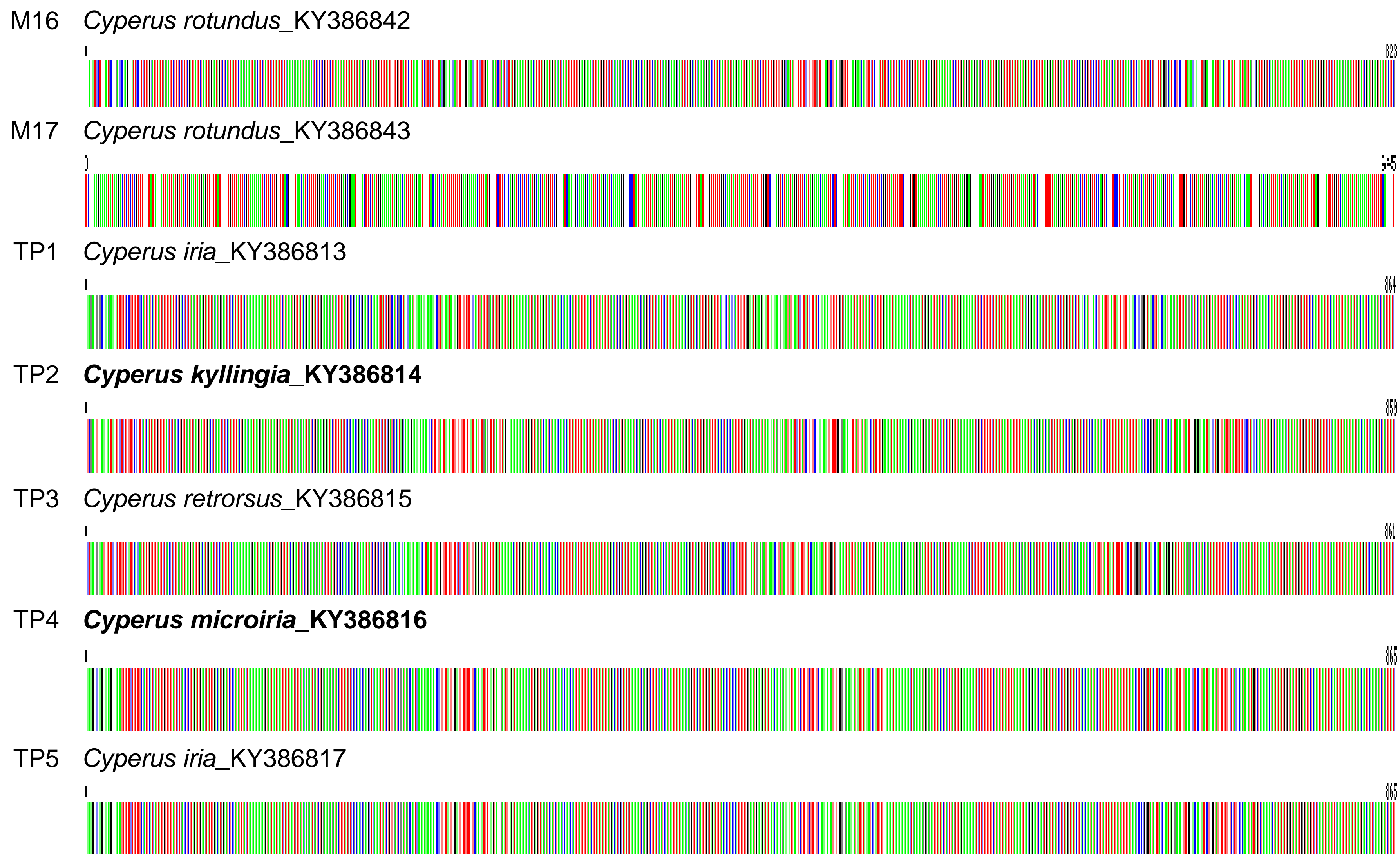
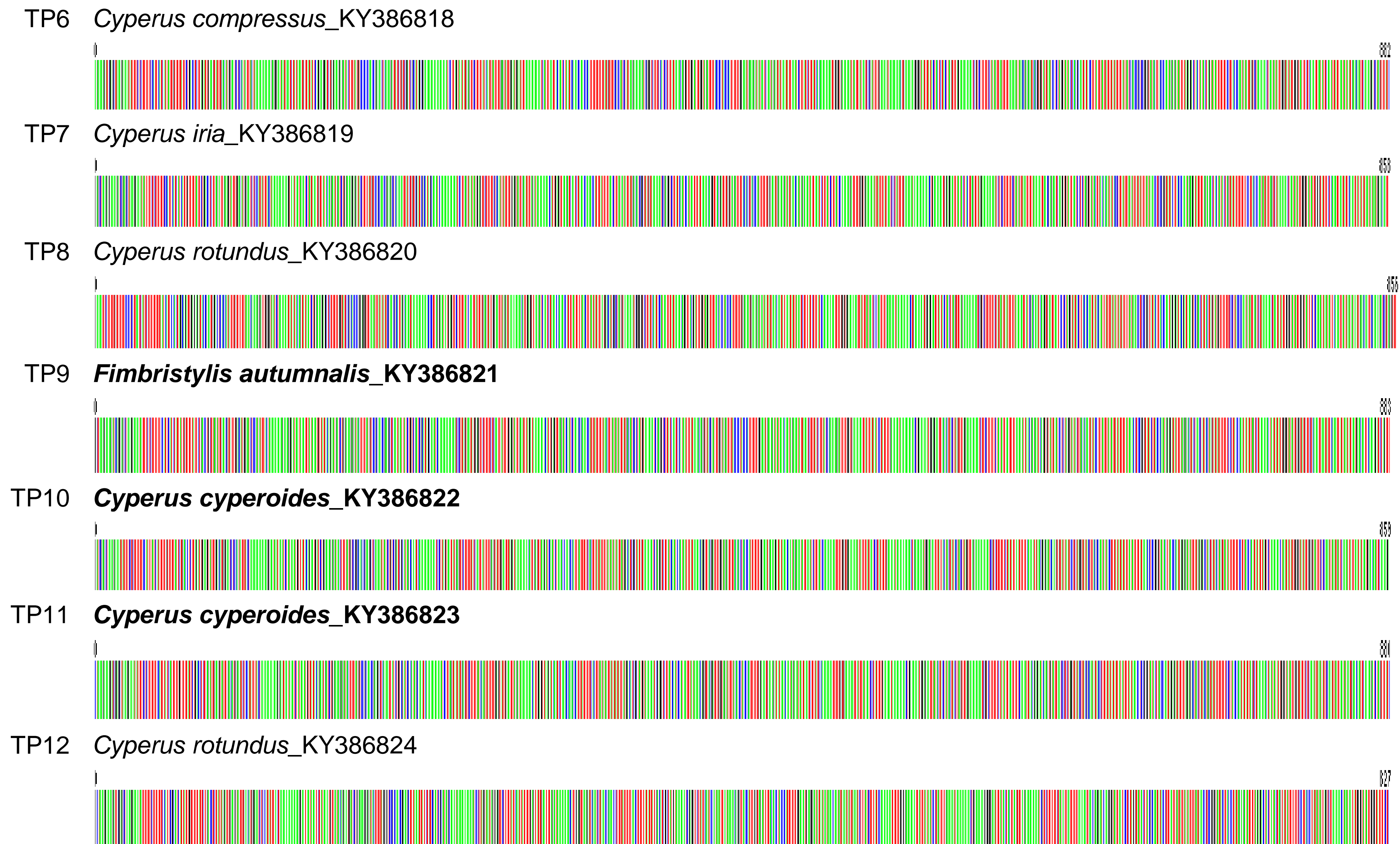


Barcode of species in bold letters are reported first time in public domain

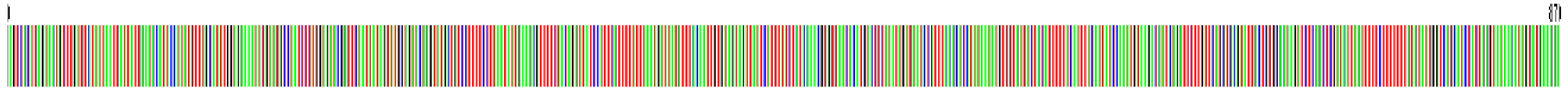


Barcode of species in bold letters are reported first time in public domain

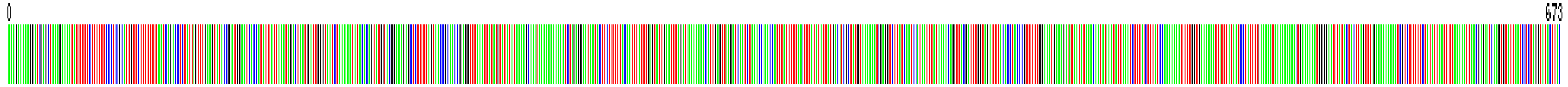


Barcode of species in bold letters are reported first time in public domain

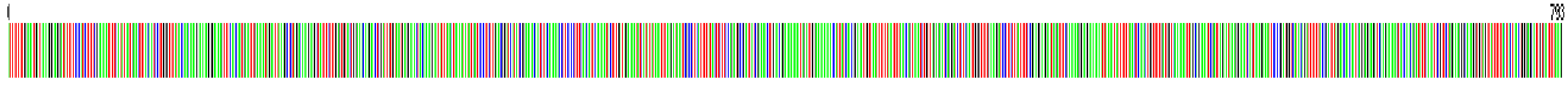
TP13 *Cyperus involucratus*_KY386825



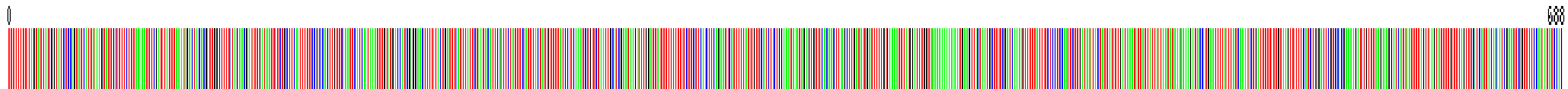
TP14 *Cyperus rotundus*_KY386826



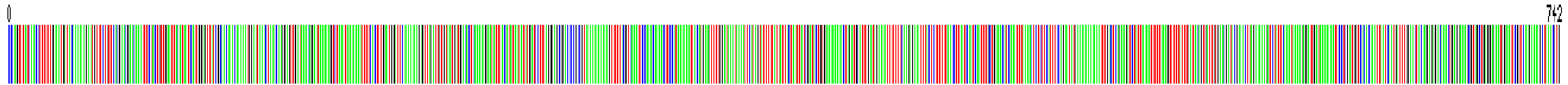
Y1 ***Cyperus esculentus*_KY386844**



Y2 ***Fimbristylis miliacea*_KY386845**



Y3 ***Fimbristylis littoralis*_KY386846**



Barcode of species in bold letters are reported first time in public domain

DISCUSSION

Biodiversity in weedy populations results from taxonomic diversity, as well as diversity in those weedy traits that affect the survival, mortality, and reproduction of individual weeds. The ecological diversity of sedges is tremendous; with species occurring in almost all habitats. Cyperaceae is sedge family of monocotyledonous flowering plants, a division of the order poales. Plants of the family Cyperaceae are grass-like herbaceous plants found in wet regions throughout the world. *Cyperus* having about 650 species, is the second largest genus after *Carex* in the Cyperaceae family. Conservation of plant species is an essential part of reaching the target of the Convention on Biological Diversity 2020 on improving the status of global biodiversity.

The first crucial step in conserving and managing plant species is correct identification of the target species that traditionally relies on morphological characters of especially reproductive parts, such as flowers and fruits, which is not accessible throughout the year. Accurate identification in species-rich or taxonomically complex groups also typically requires expert knowledge, which is not always available. DNA barcoding is a potential method to meet these identification challenges. DNA barcoding is the identification of species by a short universal DNA sequence, that exhibits a sufficient level of variation to discriminate among species. The key advantage of DNA barcoding is that once a solid reference database has been established, the method does not require expert taxonomic knowledge in order to identify specific plant samples. Further, identification can be done with small tissue samples from virtually any part of the plant, does not always require reproductive material, and the identification process is in general fast and reproducible. A limitation of the method is that no single universal DNA region has been identified that can be used across all taxonomic groups.

The present work was undertaken to check the applicability of the concept of DNA barcoding to weedy plants of *Cyperus* spp. and to evaluate the regions of chloroplast already being used for other plants, as possible barcodes for different species in family Cyperaceae. Based on previous reported research on plants, four loci chosen to be tested as DNA barcodes were *rbcL*, *matK*, *trnH-psbA* and *ycf1(b)*. These four selected loci were evaluated for three major criteria laid down by CBOL, Plant Working Group (2009). These were (i) Universality and robust amplification: ability to retrieve sequence of the targeted locus (ii) using single primer pair across the species belonging to different genera of family Cyperaceae (iii) Sequence quality and coverage: which loci are most amenable to the production of bidirectional sequences with few or no ambiguous base calls and require less manual editing of trace sequences and (iv) Discrimination: which loci enable most species to be distinguished using genetic distances, phylogenetic trees and BLAST analysis. In the present investigation, the species specific barcodes were developed for *Cyperus* spp. and other sedge plants of Cyperaceae family based on the above mentioned criteria. The details of the methodology followed and the results obtained are discussed further.

5.1 Collection and morphological characterization of *Cyperus* species

A total of eighty two sedge plant samples were collected from 21 different locations in seven states of India (Fig 3.1). Plant samples were named according to their places of collection (Table 3.1). All the variable morphological traits (Table 3.4) were observed in each sample and tabulated for generating a qualitative data. This data was subjected to Unweighted pair group method with arithmetic averages (UPGMA) based cluster analysis and dendrogram was generated using NTSYSpc version 2.02e software program (Rohlf, 1997). The data analysis was performed adopting two different similarity coefficients i.e. Jaccard (Jaccard, 1901) and Simple Matching (Sokal and Michener, 1958) similarity coefficients and was tested by applying SIMQUAL, SAHN, TREE procedures in NTSYSpc program.

Cluster analysis based on recorded morphological traits in all the eighty two sedge plant samples resulted in to grouping of plant samples in to a total of 17 clusters in case of both the clustering methods based on Jaccard (J) and Simple Matching (SM) similarity coefficients (Fig 4.1 A & B). The plant samples in the 17 clusters were identical in the groups made by both the clustering methods. Both the methods had shown the plant samples Jagdalpur-3-CG, Jabalpur-15-MP, Jabalpur-17-MP, Jabalpur-22-MP, Jabalpur-23-MP and Rewa-1-MP as groups with single individuals. However, the differences were observed in the major clusters containing various groups of plants. As first major cluster from top of the dendrogram (Fig 4.1 A & B) made from J coefficient showed only two individual sample groups of Jagdalpur-3-CG and Jabalpur-23-MP while the first major cluster made from SM coefficient showed individual sample groups of Jabalpur-15-MP, Jabalpur-17-MP, Rewa-1-MP and Jagdalpur-3-CG. However, some difference in both the clustering methods is not unexpected as the Jaccard coefficient provides different results compared to the simple matching coefficients, because these do not consider the negative co-occurrences (Sesli and Yegenoglu, 2010a).

The Mantel test correlation coefficient value 0.97169 between the two similarity coefficients J and SM was found to be significant at $p < 0.05$, shown in graph (Fig 4.2) which suggests that the dendrograms constructed from J and SM coefficients are highly correlated. The correlation coefficients calculated with the Mantel Test enables the finding of correlation between the similarity matrix and the phenetic trees obtained as a result of cluster analysis. The correlation matrices calculated show the goodness of fit of cluster analysis in accordance with the similarity matrix (Sesli and Yegenoglu, 2010b).

5.2 Identification of the species of the collected plant samples

The sedge plant samples of each group in different clusters were identified to the species level under family Cyperaceae on the basis of comparing and matching the recorded morphological trait observations with

the documented flora texts (Oomachand and Shrivastava 1996; Hooker 1894; Verma et al 1984; Shrivastava 1976; Naidu 2012; eFloras). Each cluster was found to represent a group of plants of an individual species based on the characteristic morphological traits (Table 4.1). The collected samples were identified as belonging to 17 different species (Table 4.2) among which twelve species were from genus *Cyperus* namely *C. compressus, C. cyperoides, C. esculentus, C. involucratus, C. iria, C. kyllingia, C. microiria, C. nutans, C. odoratus, C. retrorsus, C. rotundus and C. tenuispica.* Three species were belonging to genus *Fimbristylis* namely *F. autumnalis, F. littoralis and F. milliacea* and two were from genus *Kyllinga* of species *K. brevifolia and K. odoratus* (Plates 1-4). While the collected sedge plant samples varied from 83-100% (Fig 4.1), the visible appearances of plant samples of a particular species did not vary in different locations throughout India, indicating that all possessed several traits common within the species. This result is in agreement with the observations of Wills (1998) wherein variations occurred in the flower parts including the spike lets in purple nutsedge collections from 13 states within the continental United States and from 21 other locations around the world. Cluster analysis using morphological characteristics among purple nutsedge collected from 21 countries and 14 states in the United States showed variations providing evidence for divergent groups of purple nutsedge (Molin et al, 2009). However, our results show no location specific morphological variations within an individual species in India. Wills (1998) also found greater variation among plants from different locations around the world than from within the continental United States. Morpho-phenological evaluations conducted among three populations of Redroot Pigweed (*Amaranthus retroflexus* L.) from Jordon also indicated that populations had similar phenological and morphological traits (Ghosheh, 2014). This is due to the fact that more variations is found in quantitative traits than qualitative traits in plants like yellow nutsedge, where vegetative propagation is the major means of reproduction and maintenance of populations (Holt, 1994).

5.3 Most efficient morphological trait for identification of *Cyperus* spp.

In order to find out which morphological traits were actually responsible for grouping of individual plant samples in to separate clusters, the species specific data set was divided out in to three parts; vegetative traits (including observations on plant colour, root, rhizome, nut, stem/culm and leaves), inflorescence (including observations on inflorescence, spike and spikelets) and floral traits (including observations on glume colour, glume shape, keel, bracts and rachilla); and constructed dendrogram using J and SM coefficients. The vegetative traits grouped all the 17 species individually in case of both the coefficients used (Fig 4.3). Similar results were observed in case of inflorescence (Fig 4.4). Different species have shown the different pattern of inflorescence, but the floral traits failed to differentiate between the species *C. compressus* and *F. autumnalis* (Fig 4.5).

In order to find out a single morphological trait that could be observed for the identification and clustering of the *Cyperus* plants, dendrograms were constructed based on most variable morphological traits among the plants of 17 identified species which were Glume (glume colour, glume shape) and spikelet (colour and shape). The clustering based on glume did not identify the species *C. odoratus*, *C. retrorsus* and *F. autumnalis* separately but into a single cluster only (Fig 4.6). Spikelet was found to be the best for identifying a sedge plant to its species level, since, the dendrogram constructed based on the observations on spikelet color and shape grouped all the 17 species individually in case of both the J and SM coefficients (Fig 4.7). This result is in agreement with earlier report that the type of inflorescence, rachilla disarticulation and spikelet prophylls are most functional characters in Cyperaceae family (Desai and Raole, 2014).

5.4 DNA barcoding technique

DNA barcoding, on the basis of its initial success in lepidopteron insects, fishes and birds (Hebert et al, 2003) was projected as a powerful

technique for proper identification of all eukaryotes. The short DNA sequence proposed as the universal barcode was a 658 bp long region of *CO1* gene, commonly known as ‘Folmer’ region. Subsequently, various DNA barcode programmes were initiated to generate species specific molecular signatures for identifying animals and plants. Shortly, it was realized that this region of the *CO1* gene, suggested as universal barcode for all organism, might not work in plants, except in some macro algae, as *CO1* sequences in land plants are highly invariant (Chase et al 2005; Kress et al 2005). Moreover, in plants the events of hybridization, introgression and allopolyploids are more pronounced than in animals and the species identification based on one locus was considered to be insufficient (Chase et al, 2005). Initially taxonomists had viewed the technique and applicability of DNA barcoding to plants with a great deal of suspicion and skepticism (Chase et al, 2005). Thus, investigations in various laboratories using both *In silico* and experimental approaches, focused on the identification of a corresponding locus or multi-locus combination, which could become a barcode for plants (Fazekas et al 2008; Lahaye et al 2008; CBOL Plant Working Group 2009; Yao et al 2010; Chen et al 2010; Hollingsworth et al 2011).

5.5 DNA barcoding of the *Cyperus* spp.

5.5.1 Selection of barcoding loci

The loci to be verified or developed as DNA barcode for plants could be from the nuclear or chloroplast genome. These, may be from either coding or non-coding regions. However, to prove to be suitable as a DNA barcode this locus should possess certain pre-defined characteristics (Kress et al, 2005). An ideal barcode sequence needs to have conserved flanking regions and should be short, so that it can be amplified by universal primers and sequenced routinely in single pass sequencing (Kress et al, 2005). Moreover, the primers used should not be prone to non-specific annealing that result in production of double bands or amplification of loci other than the targeted one (Kress et al 2005; Ford et

al 2009). Most importantly, they should be capable of generating comparable data which enable species to be distinguished from one another. It should have an adequate sequence variations among the species with no or low divergence values within the species to provide a distinct barcode gap (Hebert et al 2003; Lahaye et al 2008). It should be easy to align and could be recovered from dry herbarium samples and parts/ fragments (http://www.kew.org/barcoding/ rationale.html).

The initial *in silico* analysis and laboratory assessment of various barcode loci suggested regions should be mainly from the chloroplast genome and one from the nuclear genome are suitable for plant barcoding (Kress et al 2005; Chase et al 2005; Newmaster et al 2006; Chase et al 2007; Kress and Erickson 2007). Kress et al (2005) recommended the use of nuclear ribosomal *ITS* and *trnH-psbA* spacer from the plastid genome for discriminating plant species. Chase et al (2005) using *in silico* approach tested the utility of *rbcL* and *ITS* sequences available in GenBank for species identification using BLAST method and suggested that multi-locus combination with regions from plastid genome and one from nuclear genome would suffice as universal barcode for plants. Newmaster et al (2006) advocated the use of *rbcL* as a DNA barcode for land plants. Chase et al. (2007) again proposed multi locus barcode and recommended that either of the two 3-locus combinations, *matK+rpoB+rpoC1* or *matK+rpoC1+trnH-psbA*, may be used as universal barcodes for plants. Kress and Erickson (2007), on the other hand, proposed the use of coding *rbcL* in combination with a non-coding intergenic spacer, *trnH-psbA* as a two-locus global DNA barcode for land plants. Taberlet et al (2007) proposed that the *trnL* (UUU) intron and its shorter P6 loop (10-143bp) could be a suitable plant barcode. Though this region provided low species resolution (67.3%), because of highly conserved primers and very robust amplification it was considered the most suitable barcode to be used in applications other than taxonomy (forensic science, biotechnology and food industry, animal diet). Mattia et al (2010) evaluated four candidate barcode regions (*rpoB, rbcL, matK and trnH-psbA*) in different genus of Lamiaceae

family. Recently, a chloroplast region *ycf1(b)* has been suggested as the most promising plastid DNA barcode of land plants (Dong et al, 2015). Based on the above mentioned reports, four plastid regions *rbcL, matK*, *trnH-psbA* and *ycf1(b)* were selected for DNA barcoding of *Cyperus* spp. in the present study.

5.5.2 Barcode amplification and sequencing

The individuals, branches or tissues of the collected species were brought to the laboratory in their native state. These were preserved under the conditions ensured for the DNA minimal damage. The plants or their parts were wrapped in aluminum foil and were sealed in plastic bags. Once in the laboratory, these were stored at -80°C in a deep freezer to minimize the degradation of DNA in the samples and to preserve them till DNA was extracted. The methodology followed in the present investigations was similar to the one used by other groups for the safe transfer and preservation of the plants or their collected parts (Sass et al 2007; Newmaster and Ragupathy 2009).

During the present course of investigation, a total of 40 samples representing all the identified species based on morphological analysis were finally selected for generation of DNA barcode through PCR amplification using four selected barcode gene loci specific primers from chloroplast genome namely *rbcL*, *matK, trnH-psbA* and *ycf1(b)*. Genomic DNA was extracted by widely used CTAB method for plant DNA extraction (Doyle and Doyle 1987; Simpson et al 2003). High quality and good yield of DNA was obtained during extraction from all the 40 plant samples (**Plate 5**). Barcode region *rbcL* could be amplified in 36 plant DNA samples, *matK* in 17 samples, *trnH-psbA* in 14 samples while *ycf1(b)* in only three samples (**Plate 6**).

The higher amplification rate of *rbcL* (RUBISCO large sub-unit) locus, obtained in the present study, is in agreement with most of the investigations reporting the similar amplification rates of 90-100% (Kress et al 2005; Kress and Erickson 2007; Fazekas et al 2008; Hollingsworth et al

2009; Kress et al 2009; CBOL Plant Working Group 2009; Ebihara et al 2010; Bhagwat et al 2015). The locus *rbcL* has been reported as easily retrievable and well suited for recovery of high quality bidirectional sequences across 93 land plant species from North America (Fazekas et al, 2008). However, lower success (97%) was obtained from the samples of 16 species of *Berberis* (Roy et al, 2010). This locus has been varyingly amplified in Araucaria, Inga and liverworts with 93%, 100% and 98% success, respectively (Hollingsworth et al, 2009). China Plant BOL Group (2011) reported 94.5% amplification success of *rbcL* among 6,286 individuals belonging to 1,757 species from 141 genera across 75 families of seed plants. The PCR success for *rbcL* was observed to be 96.91% in 36 species of *Dendrobium* (Singh et al, 2012). Beside high universality, *rbcL* is also the most suitable gene for studying phylogeny of various plant groups; therefore, a huge number of *rbcL* sequences are already available in the public databases. Newmaster et al. (2006) retrieved 10,000 *rbcL* sequences belonging to diverse group of land plants and proposed it as core barcode region for identification of lands plants. Due to its high recoverability and ease of sequencing, *rbcL* frequently appears as one of the core loci in different suggested barcode locus combinations (Kress and Erickson 2007; Fazekas et al 2008; CBOL Plant Working Group 2009; China Plant BOL Group 2011).

The amplification success rate of *matK* loci in present study was low. Poor PCR amplification efficiency of *matK* loci has also been reported in several studies. Very poor or no amplification has been reported in *Acacia* and *Albizia* species (Shinwari et al, 2014). Mattia et al (2010) reported failure of PCR amplification in some sample of Lamiaceae family while using *matK* primers. In 34 species of *Carex*, the primers for *matK* could amplify 98% of the analyzed species, but with weak bands in 21% of the samples (Starr et al, 2009). The Plant Working Group, CBOL (2009) reported *matK* amplification as more problematic with only 50% success in gymnosperms and cryptogams. Similarly, this locus could be amplified in 76% and 85% samples of *Berberis* and *Ficus* species, respectively (Roy et

al, 2010). The low amplification success of *matK* and the difficulties encountered in sequencing could be attributed to higher variability of the *matK* region especially in monocots (Chase et al 2007; Fazekas et al 2008). Therefore, it can be inferred that successful *matK* amplification and sequencing might require not only family or genera specific primers but also species specific primers in some cases. Rather, it was suggested by Hollingsworth et al (2011) that to increase the *matK* recovery rates either taxonomic group specific primers or modified universal or a cocktail of primers should be used for cost effective and efficient plant barcoding. Lower PCR amplification rate (74.0%) for *trnH-psbA* in tropical tree species of India has been reported by Tripathi et al. (2013). The low success rate in PCR amplification of *trnH-psbA* and *ycf1(b)* may be due to the absence of the common primer binding sites in different plant species in particular monocots like *Cyperus* spp.

Following amplification, the amplicons are generally sequenced directly using cycle sequencing method or can be cloned in a vector before being sequenced. For barcoding, direct sequencing of the amplicons is advocated (http://www.barcoding.si.edu/protocols.html) as cloning requires additional time and cost. In the present study, PCR products were directly used for sequencing. Sanger's di-deoxy chain termination (Sanger et al, 1977) and pryosequencing (Ronaghi et al, 1998) are the two major techniques that can be used to sequence DNA. The pyrosequencing technique is based on detection of pyrophosphate (PPi) molecules released on incorporation of every nucleotide in a growing chain on a template DNA molecule (Ronaghi et al, 1998). It uses a series of enzymes which results in generation of visible light on incorporation of each nucleotide. It is rapid but has an inherent limitation that it can produce sequence reads of only 300-500bp in one reaction (Ronaghi et al, 1998). This renders the pyrosequencing technique unsuitable for the sequencing of DNA barcodes that are expected to have a length of 600-800bp. In contrast, the modern automated ABI 3730XL capillary sequencer based on

Sanger's di-deoxy chain termination method can be used to sequence ~1000bp long DNA molecule in a single reaction (Chan, 2005).

In the present investigation, the DNA sequencer based on Sanger's di-deoxy chain termination method (Sanger et al, 1977) was used to sequence the amplicons obtained from PCR. The *rbcL* region was successfully sequenced for 36 samples, *matK* for 17 samples, *trnH-psbA* for 14 samples while *ycf1(b)* was only sequenced for three samples. Sequences were edited and assembled in BioEdit program. The final length of sequences varied in the ranges from 314-347 bp for *rbcL*, 540-826 bp for *matK,* 856-883 bp for *trnH-psbA* and 688-793 bp for *ycf1(b)*. All sequences were deposited in GenBank and given accession numbers (Table 4.3).

Among the plastid genes, *rbcL* is the best characterized gene sequence. Therefore, most of the investigating groups tested its suitability in barcoding. It encodes the large subunit of rubilose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO). As RUBISCO is a critical photosynthetic enzyme, *rbcL* was the first gene that was sequenced from the plants (Zurawski et al, 1981). *RbcL* has been a marker of choice for the phylogenetic studies in family Cyperaceae (Muasya et al 1998, 2001, 2002) Primers for PCR amplification and sequencing for such short sequence within the *rbcL* gene have been developed accordingly for most of the taxa. In present study, the sequence length in range of 314-347 bp obtained from *rbcL* primers proved very short in comparison to other published reports (Wang et al 2010; Tripathi et al 2013). The sequence length of *rbcL* was 522bp in Lemnaceae family as reported by Wang et al. (2010) showing higher sequence length in comparison to sequence obtained during present investigation. The sequence length of *rbcL* sequence obtained from tropical tree of India was 618bp reported by Tripathi et al (2013) also contradicts the present results. in order to obtain enough species discrimination, the entire ~1430bp needs to be sequenced, which acts as a limiting factor for its use as a barcoding sequence because an ideal DNA barcoding region should be short enough to be amplified from

degraded DNA and analyzed via single-pass sequencing. One solution for this phenomenon is to amplify short sequences with enough variability. Primers for PCR amplification and sequencing for such short sequence within the *rbcL* gene have been developed accordingly for most of the taxa.

5.6 Species identification ability of all the barcode loci

For species identification, three approaches were followed namely, similarity-based approach (BLASTn, BOLD), distance based analyses (SpeciesIdentifier / TaxonDNA) and Phylogenetic analyses (UPGMA and Maximum parsimony). At first each barcode sequence was subjected to similarity analysis using BLASTn tool of NCBI to look for its similarity with sequences available in GenBank repository. The searches resolved the plant sequences up to genus level. However, due to several hits at the same parameters of max score, total score, query cover, E value and identity %, most of the plant species were not identified unambiguously. Because at the same parameters more than one identical sequences were recovered with different species names under genus *Cyperus*. This may be due to conserved nature of the barcode region and in particular short barcode sequence of *rbcL*. The *ycf1(b)* barcode sequences showed similarity with reference barcodes of plants from other genera as no reference was found in *Cyperus* spp. Based on BLAST searches, some of the barcodes generated in present study were found to be unique barcodes for the sedge plant species (Table 4.4) because no barcode sequences of the same locus for the same species could be found in the sequence data repository. Barcode of Life Data systems (BOLD) has a collection of plant barcodes for *rbcL* and *matK*. The searches in the BOLD plant identification tool with the *rbcL* and *matK* barcode sequences of present study also could resolve the plant sequences up to genus level. However, due to several hits at the same parameters of score, query cover, E value and identity %, most of the plant species were not identified unambiguously. a possible reason could be the paucity of reference sequences in *Cyperus* genus as well as conserved nature of the barcode region. However, from different studies, it appears that finding a universal barcode or even a barcode at

family level is difficult and it may be possible to establish a discriminating barcode only at genus level (Riaz et al 2011). Moreover, the slow evolving coding regions of plastid genomes might not possess enough variation to discriminate closely related plant species and this could lower their potential as effective barcodes (Pettengill and Neel, 2010). This can be overcome by analyzing the selected loci either individually or in combination (Chase et al 2005; Kress and Erickson 2007).

A combined barcode sequence dataset for each of the combinations *rbcL*+*matK*, *rbcL*+*trnH-psbA*, *matK*+*trnH-psbA* and *rbcL*+*matK*+*trnH-psbA* was obtained by concatenating the sequences generated as barcodes for *rbcL*, *matK* and *trnH-psbA* for testing the effects of mixing the information for identifying the species. The data set for all the seven barcode (four combined) was analyzed in TaxonDNA / SpeciesIdentifier-1.8 program for evaluating the specimen identification success using the 'best match' and 'best close match' function of the program. The results are presented in Table 4.5. The analysis led to the correct identification rate of 64% by best close match method for the combined barcode *rbcL*+*matK*+*trnH-psbA* and correct identification rate of 67% for the combined barcode *rbcL*+*matK*. Barcodes *rbcL* and *matK* individually showed correct identification rates of 61% and 53%, respectively. However, higher correct match percentage up to 100% has been observed for *matK*, *matK*+*rbcL* and *matK*+*trnH-psbA* loci in *Dalbergia* Species (Bhagwat et al, 2015). This difference in results may be due to the fact that Bhagwat et al (2015) have used multiple accessions of each species while in present study only the sequences of test species were used i.e. without reference sequences.

The generated barcode sequences were further analyzed for their species identification success through statistical methods of UPGMA analysis and Maximum parsimony analysis, along with all the species specific reference sequences from GenBank. The UPGMA analysis showed species specific clusters with respect to the reference sequences for *rbcL*, *matK* and *trnH-psbA* (Fig 4.8 – Fig 4.10). Since, there is no reported barcode for *ycf1(b)* in *Cyperus* spp., it showed similarity with

plants of other genera (Fig 4.11). The analysis led to correct identification of monophyletic sample sequences with respect to the reference sequences. Each species was grouped in to a single species specific cluster. Further, in order to assess the most efficient barcoding loci for the identification of sedge plant species and to infer on the taxonomy of the sampled sedge plants, phylogenetic analysis based on Maximum Parsimony (MP) method was conducted on all the seven barcode datasets. The number of parsimony informative sites (Table 4.6) varied across the dataset in the range of 28 (*matK+trnH-psbA*) to 774 (*trnH-psbA*). The *matK* produced five most parsimonious tree followed by four from *trnH-psbA*.

Maximum parsimony analysis for *rbcL* barcode (Fig 4.12) although shown monophyletic clusters for most of the species along with reference sequences but failed to group the species belonging to genus *Fimbristylis* in to a single cluster or to sister clusters. *Fimbristylis miliacea* was grouped with *Cyperus involucratus* while *Fimbristylis littoralis* was grouped with *Cyperus microiria*, both with a bootstrap value of 100%. Phylogenetic tree constructed by *matK* and *trnH-psbA* (Fig 4.13, Fig 4.14) resolved all the included species as monophyletic. The barcode combination *rbcL+matK* (Fig. 4.15) resolved all the species better than *rbcL* alone and grouped all the species of genus *Fimbristylis* in to a single major cluster, however nested by *Cyperus compressus*. The barcode combination *rbcL+trnH-psbA* (Fig. 4.16) resolved all the species better than *rbcL* alone and grouped all the species of genus *Fimbristylis* in to a single cluster with a sister cluster of *Cyperus compressus* with a bootstrap value of 99%. The barcode combination *matK+trnH-psbA* (Fig. 4.17) resolved all the species under genera *Cyperus*, *Kyllinga* and *Fimbristylis* as monophyletic group with low bootstrap values. The combination barcode *rbcL+matK+trnH-psbA* (Fig 4.18) resolved all the species included in this study as monophyletic species/genus cluster with bootstrap values above 60 for most of the clusters. Maximum parsimony analyses have been widely reported for phylogenetic inferences in order to identify a species, genus or subfamily in family Cyperaceae (Simpson et al 2003, 2007; Muasya et al 2009). In

phylogenetic tree based method, a species is considered to be resolved if the accessions under the species form a monophyletic group (Tripathi et al, 2013). Similar to present results, Newmaster and Ragupathy (2009) reported that all the three barcoding regions (*rbcL, matK, trnH-psbA*) could discriminate sister species within the *Acacia.*

It has been reported that the coding plastic regions *rbcL* and *matK* not always yield high discrimination rates, other barcode locus seems to be a valid supplement to the core barcode (Hartvig et al., 2015).

5.7 Intraspecific, interspecific variations and barcode gap analysis

The assessment of intra-specific and inter-specific variations is important for the correct identification of species by generation of DNA barcodes (Hebert et al 2003a/b; Lahaye et al 2008). The minimum inter-specific variation has to be higher than the maximum intra-specific variation. The difference between the two is referred to as barcode gap (Meyer and Paulay, 2005). An ideal barcode must exhibit a barcode gap, so that, the distribution of intraspecific and interspecific divergences does not overlap (Lahaye et al, 2008). The accuracy of barcoding for species identification depended to a large extent on the barcoding gap between intra-specific and inter-specific sequence variations.

Effective barcoding weakens when inter-specific and intra-specific distances overlap. To evaluate significant barcoding gap, average inter-specific and intra-specific divergences were calculated for each barcode dataset. DNA barcode sequence data for *rbcL*, *matK*, *trnH-psbA* and the concatenated datasets of *rbcL*+*matK*, *rbcL*+*trnH-psbA*, *matK*+*trnH-psbA* and *rbcL*+*matK*+*trnH-psbA* were analyzed in TaxonDNA / SpeciesIdentifier-1.8 program for calculating uncorrected *p*-distances between all sequence pairs and the distance data was used to calculate mean and range of intraspecific and interspecific distances for all the seven barcode datasets (Table 4.7). Barcode *trnH-psbA* had the minimum interspecific distance value 0.4408 which was larger than the maximum value of intraspecific distance 0.1144 indicating a significant barcode gap.

The barcode gap was absent for all the other marker loci used in the present study, indicating overlaps between inter and intraspecific distances (Fig 4.19). However, the mean interspecific divergence was significantly higher than that of the corresponding intraspecific divergence for each of the loci. Barcode regions *matK* and *rbcL* showed low level of overlapping between mean intraspecific and interspecific distances. Among combined barcodes, *rbcL+matK* and *matK+trnH-psbA* showed lower values for mean intra- and interspecific distances (*p*-distances) indicating low overlapping (Fig 4.19). Lower level of overlapping between intra- and inter-specific distances nevertheless indicates the barcode gap for most of the sequences and their suitability as a DNA barcode for the identification of plant species. In similar studies, though barcode gap has not been observed but lower overlapping has been observed in *Dalbergia* species (Bhagwat et al, 2015).

Mattia et al (2011) showed inter-specific (2.31%) and intra-specific (0.2%) variation in Lamiaceae family revealed by *trnH-psbA* loci. Whereas, inter-specific and intra-specific variations in family Schisandraceae were 6.96% and 0.09 as revealed by *trnH-psbA* loci. The large variation in present results and previous results is due to the fact that *trnH-psbA* loci which is known as most variable genome segments in the chloroplasts of angiosperms. It has an average length of approximately 450bp varying from 296 to 1120bp (CBOL–Plant Working Group, 2009). *trnH-psbA* has been reported as the most divergent locus at the inter-specific level (Tripathi et al, 2013).

5.8 Most efficient barcoding loci for the identification of *Cyperus* spp.

The approach of maximum parsimony analysis, distance-based analyses in TaxonDNA and barcoding gap analysis has been found optimum for establishing an efficient barcode for discrimination and identification of plants (Hartvig et al, 2015). In present investigation, based on similarity searches, TaxonDNA best close match method, maximum parsimony and DNA barcode gap analyses, Loci *rbcL+matK* showed

highest species resolution ability among all the barcode loci followed by *matK* and *trnH-psbA* while using these three species resolution methods and was found best barcode region to unambiguously identify a sedge plant species as they could group each species in a monophylectic cluster group. The result of this study is in close agreement with the recommendation of core 2-locus combination of *rbcL+matK* as the plant barcode in order to provide a universal framework for the routine use (CBOL-Plant Working Group, 2009). DNA barcode *rbcL+matK* has also been reported as most efficient loci for discriminating plant species in local temperate flora (Burgess et al, 2011) and in tree species *Dalbergia* (Bhagwat et al, 2015; Hartvig et al, 2015). Among the chloroplast genes, *matK* is one of the most rapidly evolving gene and has been used as a marker to construct plant phylogenies because of its rapid evolution and the ubiquitous presence in plants. Phylogenetically, the rate of evolution of *matK* was found to be suitable for resolving intergeneric as well as interspecies relationships in many angiosperms (Soltis et al, 1998). CBOL Plant Working Group (2009) identified this gene as one of the universal DNA barcode for flowering plants. Another barcode locus which gave promising results during present study was *trnH-psbA*. The species resolution ability of *trnH-psbA* barcode loci is supported by the earlier work of Mattia et al (2011) in different barcode loci for family Lamiaceae.

Overall, during the present course of investigation, for *Cyperus* species resolution ability, *rbcL+matK* showed 100% efficiency in all samples studied using all the three species resolution methods namely similarity based method, phylogenetic tree based method and barcode gap method. Apart from identifying target *Cyperus* species, DNA barcoding can also be applied in floristics. Constructing a DNA barcoding reference library of entire local floras can allow for fast and effective floristic analyses without expert knowledge (Burgess et a, 2011), or even be a method of estimating species richness in a taxonomically poorly known flora. There is also a huge potential for application of DNA barcoding to the vast collections at herbaria and Natural History Museums, which could serve as

excellent reference databases and help identify the many un-identified specimens present in most collections, as well as identify new collections. This would contribute significantly to improved knowledge about distribution patterns of each species. DNA barcoding thus has the potential of becoming an important supporting tool for conservation and biodiversity assessments in areas with a high number of plant species, a shortage of expert taxonomists, and limited descriptions of the flora.

The results of present study clearly indicated the significance of species identification through DNA barcoding technique and adding emphasis to the existing methods of morphological identification. The DNA barcodes developed in this study could be directly used for the identification and/or authentication of plants of *Cyperus* spp. in taxonomic, phylogenetic as well as in herbal formulations and other industrial applications.

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary

The present work entitled “Studies for identification and conservation of *Cyperus* spp. using DNA barcoding” was carried out with the objectives of collection and morphological characterization of *Cyperus* species, DNA barcoding of the *Cyperus* spp. and to assess the most efficient barcoding loci for the identification of *Cyperus* spp.

Present study was conducted on 82 sedge plant samples collected from 21 places covering seven states of the country. The species of the plant samples were identified by characterizing and analyzing several morphological descriptors characteristic to a particular sedge plant species. Cluster analysis based on recorded morphological traits in all the 82 sedge plant samples resulted in to grouping of plant samples in to a total of 17 clusters representing 17 different species among which twelve species were from genus *Cyperus* namely *C. compressus, C. cyperoides, C. esculentus, C. involucratus, C. iria, C. kyllingia, C. microiria, C. nutans, C. odoratus, C. retrorsus, C. rotundus and C. tenuispica.* Three species were belonging to genus *Fimbristylis* namely *F. autumnalis, F. littoralis and F. miliacea* and two were from genus *Kyllinga* of species *K. brevifolia and K. odoratus*. The collected sedge plant samples varied from 83-100%, the visible appearances of plant samples of a particular species did not vary in different locations throughout India. Spikelet was found to be the best morphological trait for identifying a sedge plant to its species level.

During present study, four previously described barcode loci for land plants viz., *rbcL*, *matK, trnH-psbA* and *ycf1(b)* was used for DNA barcoding of the collected sedge plant samples. Barcode region *rbcL* generated 36 barcodes of length 314-347 bp, *matK* in 17 samples of length 540-826 bp, *trnH-psbA* in 14 samples of length 856-883 bp, while *ycf1(b)* in only three samples of length 688-793 bp. All the barcode sequences were deposited in GenBank and given accession numbers. Similarity based searches by BLASTn and BOLD plant identification tools were helpful for correctly

assigning a particular barcode sequence to genus level and giving several options at species level with identical parameters. However, the BOLD plant identification tool is limited to the identification by *rbcL* and *matK* barcode sequences only. Seventeen unique species specific barcodes were generated in present study which has not been reported in the GenBank for the same species. For the first time, barcode of *ycf1(b)* has been generated for *Cyperus spp.* in present study. Similarity search in SpeciesIdentifier program using DNA barcode sequence data for *rbcL*, *matK*, *trnH-psbA* and the concatenated dataset for *rbcL+matK*, *rbcL+trnH-psbA*, *matK+trnH-psbA* and *rbcL+matK+trnH-psbA* led to the correct identification rate of 64% by best close match method for the combined barcode *rbcL+matK+trnH-psbA* and correct identification rate of 67% for the combined barcode *rbcL+matK*. *matK* alone showed correct identification rate of 53%.

The generated barcode sequences were further analyzed for their species identification success through statistical methods of UPGMA analysis and Maximum parsimony analysis, along with all the species specific reference sequences from GenBank. The barcode loci *rbcL+matK*, *matK* and *trnH-psbA* showed clear monophyletic groups of sequences nested with reference sequences. Since, there is no reference sequence available in case of *ycf1(b)*, hence, the *ycf1(b)* sequences shown similarity with sequences from other genera. For assessment of best DNA barcode loci, the intra- and inter-specific distances were calculated by uncorrected *p*-distance measure, *trnH-psbA* showed a significant barcode gap as the minimum interspecific distance value 0.4408 was larger than the maximum value 0.1144 for intraspecific distance. The barcode gap was absent for all other loci indicating overlaps between inter and intraspecific divergence. However, the mean interspecific divergence was significantly higher than that of the corresponding intraspecific divergence for each of the loci studied indicating lower overlaps between inter and intraspecific divergences and hence suitability of the same as barcode locus. Based on similarity searches, UPGMA, maximum parsimony and DNA barcode gap analyses, *rbcL+matK* was found the best barcode locus followed by *matK* and *trnH-psbA* to unambiguously identify a sedge plant of *Cyperus* spp.

6.2 Conclusion

The present study revealed spikelet as the most variable morphological descriptor for the identification of *Cyperus* spp. Among DNA barcode loci *rbcL+matK* was found as the most efficient barcode region for the correct identification of plants of *Cyperus* spp. depicting highest species resolution using all the three species identification methods. Barcode region *trnH-psbA* was found to satisfy the requirements of an ideal barcode locus showing significant barcode gap and also revealing considerably good species resolving capacity. Although good result obtained but the *rbcL* alone failed to resolve all the species of the plants identified in present study. The results presented herein, adequately address to the concerns about the applicability of DNA barcoding to sedge plants. However, quest for a perfect universal barcode for plants providing 100% species resolution across the plant kingdom appears to be unrealistic, as DNA barcoding, like any other technology, is not expected to be 100% perfect. However, within a taxonomic group 100% species resolution could possibly be obtained by taxa specific barcodes. Thus, the projection that DNA barcodes, once available for all the described species, would be able to provide a correct identity up to species level to any unknown sample, whether available in vegetative, fragmented or DNA form. Perfect species resolution by some barcode loci emphatically demonstrates the efficacy of the DNA barcode technique.

6.3 Suggestion for further works

- The PCR amplification conditions for *ycf1(b)* needs further standardization in *Cyperus* spp. and bigger length of *rbcL* barcode should be obtained by using different set of primer pairs.
- The species resolution ability of the barcode loci of this study should be tested on large number of samples both at genus and species level.
- The multiplex PCR protocol should be developed for the combined barcode region.
- Owing to the medicinal importance of *Cyperus* spp., study is needed to authenticate herbal formulations containing them by DNA barcoding.

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APPENDIX

List of Publications

(1) One Patent (Published on 20.01.2017)

Tantwai K, Tiwari S and Tripathi N. 2017. DNA Barcode for species identification of sedge plants and methods thereof. The Patent Office, Mumbai. Application number 201721001636. Patent Journal page № 1452. Issue number 03/2017 date: 20.1.2017

(2) One Research Paper (Published on 12.11.2016)

Tantwai K, Tiwari S and Anshari T. 2016. Morphological variability in the common sedge plants in India. International Journal of Agriculture Sciences, Volume 8, Issue 55, pp.-3000-3007. ISSN: 0975-3710 & EISSN: 0975-9107 (**NAAS 4.20**).

(3) One Abstract Published in International Conference

Tantwai K, Tripathi N and Tiwari S. 2016. Identification of medicinal sedges of Cyperus species using rbcL genetic marker. 1st International Agrobiodiversity Congress organized by Indian society of Plant Genetic Resources at New Delhi during **6-9 November, 2016**. Abstract № 342 (P-321) Page 205.

(4) Seventy Sequences in GenBank Database (1-27 published on 01.02.2017)

1. Tantwai K, Tripathi N, Thakur VV, Kumar S and Tiwari S. 2016. *Cyperus microiria* KR1 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219762.
2. Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus iria* KR2 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219763.
3. Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR3 Ribulose-bisphosphate carboxylase L-2 gene. partial sequence. GenBank Accession № KX219764.
4. Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus iria* KR4 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219765.
5. Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus retrorsus* KR5 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219766.
6. Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR6 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219767.
7. Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus esculentus* KR7 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219768.

8 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR8 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219769.
9 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus microiria* KR9 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219770.
10 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus compressus* KR10 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219771.
11 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Kyllinga monocephala* KR11 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219772.
12 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus iria* KR12 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219773.
13 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Kyllinga monocephala* KR13 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219774.
14 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus esculentus* KR14 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219775.
15 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR15 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219776.
16 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus compressus* KR16 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219777.
17 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus microiria* KR17 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219778.
18 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus microiria* KR18 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219779.
19 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus microiria* KR19 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219780.
20 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR20 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219781.
21 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus iria* KR21 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219782.
22 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus tenuispica* KR22 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219788.
23 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR23 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219783.

24 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR24 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219784.
25 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus odoratus* KR25 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219785.
26 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR26 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219786.
27 Tantwai K, Tripathi N, Kumar S and Tiwari S. 2016. *Cyperus rotundus* KR27 Ribulose-bisphosphate carboxylase L gene. partial sequence. GenBank Accession № KX219787.
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(57) Abstract :
Present invention discloses a method of generating a DNA barcode for identification of sedge plant species comprising the steps of Collection of a sedge plant sample, DNA isolation from plant or a part thereof, Amplification of a plastid based barcode region selected from ycfl(b), matK, trnH-psbA and rbcL, with the help of barcode loci specific primer pair consisting of forward and reverse primers, Obtaining a sequence of the amplified product, Identifying the plant by matching the obtained sequence with a reference library and/or by constructing a phylogenetic tree, and finally Obtaining a species specific DNA barcode from the analyzed sequence. The invention also discloses a method of identification or authentication of a herbal formulation containing sedge plant or part thereof e.g. Cyperus rotundus or Cyperus esculentus, by application of the disclosed method.

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FORM 2
THE PATENTS ACT, 1970
(39 of 1970)
&
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COMPLETE SPECIFICATION
(*See* section 10 and rule 13)

1. TITLE OF THE INVENTION

DNA Barcode for species identification of sedge plants and methods thereof

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3. PREAMBLE TO THE DESCRIPTION

COMPLETE
The following specification particularly describes the invention and the manner in which it is to be performed.

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DNA BARCODE FOR SPECIES IDENTIFICATION OF SEDGE PLANTS AND METHODS THEREOF

FIELD OF INVENTION

The present invention relates to DNA barcodes for species identification of sedge plants belonging to several species under family Cyperaceae, genera *Cyperus*, *Kyllinga* and *Fimbristylis*. It also relates to the methods of generating species specific DNA barcodes and the methods of identification of the sedge plant species using the DNA barcodes.

BACKGROUND OF THE INVENTION AND PRIOR ART

Sedges are monocotyledonous flowering plants belonging to family Cyperaceae which is commonly known as sedge family. Sedges belonging to the genus *Cyperus* and family Cyperaceae are the most invasive agricultural weeds known, having spread out to a worldwide distribution in tropical and temperate regions. Weed populations exhibit a vast range of biodiversity resulting from taxonomic diversity among them including diversity in particular weedy traits that influences their survival, mortality, and reproduction.

The ecological diversity of sedges is tremendous; with species occurring in almost all habitats. Commonly, sedge plants in India are belonging to three main genera *Cyperus*, *Kyllinga* and *Fimbristylis*. *Cyperus* having about 650 species is the second largest genus after *Carex* in the Cyperaceae family. Members of the Cyperaceae (sedge family) resemble the Gramineae (grass family) but are distinguished by three-ranked leaves with one-third phyllotaxy and leaves that have closed leaf sheaths, usually solid stems, absence of a ligule and each flower subtended by a single glume or scale. The morphology of plants belonging to the genus *Cyperus* reported so far is relatively consistent with uniform embryo type. Morphologically all the *Cyperus* species have common characteristics of leaves that are shiny, light to dark green, three-ranked and corrugated in cross section. Leaf initiation terminates with the formation of seed bearing culm. The culm that grows through the center of the leaf fascicle is erect, simple, smooth and

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triangular in cross section. The culm supports a terminal inflorescence, which is a simple, or slightly compound loose umbel subtended by two or four leaf-like bracts.

The *Cyperus* species are herbaceous plants and mostly used as fodder. In particular, *C. rotundus* and *C. esculentus* are used as medicinal herb. They are also called as purple nut sedge and yellow nut sedge, respectively. They are commonly found as a perennial weed with slender, scaly creeping rhizomes, bulbous at the base which is about 1-3 cm long. They possess pharmacologically active substances such as α-cyperone, β-selinene, cyperene, cyperotundone, kobusone, isokobusone etc. They are useful for bowel disorder and inflammatory diseases as well as uterine relaxation in both pregnant and non-pregnant women and relieving pain. *C. rotundus* is a traditional herbal medicine used widely as antimalarial, analgesic, sedative, and treating stomach disorder etc. Anti-bacterial effect is also found in rhizome extract of *C. rotundus*. Antimalarial, anticancer, antimicrobial activities in essential oil from the aerial parts of *Cyperus kyllingia* have also been reported. *Cyperus involucratus* is planted as an ornamental plant. *Cyperus iria* is used as fodder and its stem is woven into mats. Leaf and tuber parts of *C. iria* are used as Tonic, stimulant, stomachic and astringent. *Kyllinga odorata* is known for diaphoretic and diuretic properties. *Kyllinga brevifolia* is a Paraguayan folk medicine and used as sedative and tonic for nervous system. *Fimbristylis miliacea* has been found of an important role in phytoremediation to absorb heavy metals and zinc in waste water treatment.

In view of the aforementioned uses and importance of the sedge plants, the identification of an exact species of the plant is highly needed inter alia in areas such as effective control of agricultural weed, identification of species suitable as fodder, herbaria and biodiversity conservation, plant quarantine, authenticating or diagnosing adulteration in herbal medicinal formulations etc. Biological species are authenticated traditionally according to their morphological features which usually require the expertise of an experienced professional taxonomist. In the case if the diagnostic morphological trait of a specimen is lacking, or variations due to environmental factors, it becomes difficult even for the specialists to

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recognize it correctly. Authentication using DNA barcodes overcomes these problems. The short DNA sequence is taken from standard region of genome to generate DNA barcode. DNA barcode is short DNA sequence made of four nucleotide bases A (Adenine), T (Thymine), C (Cytosine) and G (Guanine). Even non experts can identify species even from small, damaged or industrially processed material.

Since the advent of DNA barcoding technology, it has been widely used in animal studies wherein the standard used is a fragment of mitochondrial COI gene sequence approximately 650bp. However, in plants, research progress in DNA barcoding is relatively slow, it is because the plant genome is difficult to find a standard like animal's COI-kind of common single gene fragment. Due to slower rate of plant mitochondrial genome evolution, barcoding regions mainly in the chloroplast genome is selected. The genes identified as suitable barcode standard in plants are four coding genes in plastid namely *matK*, *rbcL*, *rpoB* and *rpoC1* and three non-coding genes *atpF-atpH*, *trnH-psbA* and *psbK-psbl* (CBOL plant working group, 2009). In addition to this nuclear ITS gene fragments have also been proposed. Recently, *ycf1* gene has been proposed as a promising plastid DNA barcode of land plants. Currently, the plant DNA barcoding is still in comparison and evaluation stage for each gene segment proposed as barcode. studies suggest that the ideal DNA barcoding should meet the following criteria: (1) a single site will be able to effectively identify different species; (2) in a clear interspecific genetic variation and differentiation, while intraspecific variation is small enough; (3) short enough for easy DNA extraction, PCR amplification and to facilitate DNA sequencing reaction and; (4) the presence of conserved regions, designed to facilitate universal primers. From the point of view of biodiversity, relying on several conserved gene fragments to identify and discriminate a wide variety of plant species is comparatively challenging task. But for a limited number of species of a family or genus, DNA bar codes are accurately distinguishing between different plant species. Nevertheless DNA barcodes have the huge potential application in the identification and protection of species.

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CN102332064 discloses biological species identification method in prokaryotes based on genetic barcode, the method comprises the steps of making gene bar code image and gene bar code image database of prokaryote DNA, and determining similarity of the sample with the database.

CN105177151 discloses DNA bar code primer pair and method for identifying *Taxus chinensis* species using primer pair upstream primer Taxus Indel-F and a downstream primer Taxus Indel-R. The barcode generated consists of a sequence length of 212-215bp.

CN104450892 discloses universal detection primer and DNA barcode molecular identification method for pine wood nematode *Bursaphelenchus xylophilus*.

CN104404131 discloses DNA bar code technology for identifying rosewood and rosewood product by constructing a high capacity database through a strategy by merging database of rosewood samples experiment data and a target plant; sequencing a DNA bar code sequence of the rosewood samples; merging the sequencing sequences with homologous sequences published in the Genbank database to construct a rosewood DNA bar code gene database with wide range of data sources; and establishing an algorithm and decision rules for the identification.

CN103695536 discloses a *Curcuma kwangsiensis* DNA barcoding standard sequence of 625bp, wherein said bar code standard testing gene is ITS gene.

US/2015/0080266 disclose DNA barcodes for multiplexed sequencing and provides methods for optimizing barcode design for multiplex DNA sequencing and optimized DNA barcodes.

WO/2008/000090 discloses DNA barcode sequence classification and method for indexing DNA barcode sequences for a barcode database, the method comprising the steps of: receiving a DNA barcode sequence; cleaning the received DNA barcode sequence by removing all characters except those characters uniquely identifying one of the four nucleotide subunits of a DNA strand; segmenting the

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cleaned DNA barcode sequence into a number of words, each word having a predefined number of nucleotides, the segmented DNA barcode representing a barcode index; and associating the barcode index with its species name for storage in the barcode database.

WO/2014/190111 discloses system and method for producing and reading dna barcodes by using DNA oligomers to encode for specific characteristics of biological materials leading to a readable barcode for biological material identification.

As discussed above, there is paucity of information on identification and/or authentication of sedge plants belonging to Cyperaceae family. Accordingly, there is a need for a highly selective and specific method for identification and/or authentication of sedge plants belonging to Cyperaceae family.

OBJECTS OF THE INVENTION

An object of the present invention is to provide a highly specific DNA barcode for correct and authentic species identification of sedge plants.

Another object of the present invention is to provide a method of generating highly specific DNA barcode for correct and authentic species identification of sedge plants.

Further object of the present invention is to provide a method of identification or authentication of sedge plants or any part thereof, up to species level in family Cyperaceae.

SUMMARY OF THE INVENTION

In one aspect present invention relates to a method of generating a DNA barcode for identification of sedge plant species comprising the steps of:

(a) Collection of or providing a sedge plant,
(b) DNA isolation from plant or a part thereof,
(c) Amplification of a plastid based barcode region selected from *ycf1(b)*, *matK*, *trnH-psbA* and *rbcL*,

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(d) Amplification of the step (c) by any one of primer pair consisting of forward and reverse primers of SEQ ID NOs: 1 and 2 for *ycf1 (b)*, SEQ ID NOs: 3 and 4 for *matK*, SEQ ID NOs: 5 and 6 for *trnH-psbA*, or SEQ ID NOs: 7 and 8 for *rbcL*;

(e) Obtaining a sequence of the amplified product,

(f) Identifying the plant by matching the obtained sequence with a reference library and/or by constructing a phylogenetic tree,

(g) Obtaining a species specific DNA barcode from the analyzed sequence in step (f).

In another aspect the invention relates to a method of identification or authentication of a herbal formulation containing sedge plant or part thereof comprising the steps of:

i. providing a herbal formulation containing sedge plant or its part,
ii. DNA isolation from herbal formulation,
iii. Amplification of a plastid based barcode region selected from *ycf1 (b)*, *matK*, *trnH-psbA* and *rbcL*,
iv. Amplification of the step (iii) by any one of primer pair consisting of forward and reverse primers of SEQ ID NOs: 1 and 2 for *ycf1 (b)*, SEQ ID NOs: 3 and 4 for *matK*, SEQ ID NOs: 5 and 6 for *trnH-psbA*, or SEQ ID NOs: 7 and 8 for *rbcL*;
v. Obtaining a sequence of the amplified product,
vi. Identifying the plant by matching the obtained sequence with a reference library and/or by constructing a phylogenetic tree.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

The above and other aspects, features, and advantages of certain exemplary embodiments of the present invention will be more apparent from the following description taken in conjunction with the accompanying drawings.

Fig. 1 represents the Phylogenetic tree constructed based on *matK* barcode sequences and reference sequences from GenBank repository.

Fig. 2 represents an illustration of a barcode sequence.

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DETAILED DESCRIPTION OF THE PRESENT INVENTION

The following description with reference to the accompanying drawings is provided to assist in a comprehensive understanding of exemplary embodiments of the invention. It includes various specific details to assist in that understanding but these are to be regarded as merely exemplary.

Accordingly, those of ordinary skill in the art will recognize that various changes and modifications of the embodiments described herein can be made without departing from the scope of the invention. In addition, descriptions of well-known functions and constructions are omitted for clarity and conciseness.

The terms and words used in the following description and claims are not limited to the bibliographical meanings, but, are merely used by the inventor to enable a clear and consistent understanding of the invention. Accordingly, it should be apparent to those skilled in the art that the following description of exemplary embodiments of the present invention are provided for illustration purpose only and not for the purpose of limiting the invention as defined by the appended claims and their equivalents.

It is to be understood that the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

By the term "substantially" it is meant that the recited characteristic, parameter, or value need not be achieved exactly, but that deviations or variations, including for example, tolerances, measurement error, measurement accuracy limitations and other factors known to those of skill in the art, may occur in amounts that do not preclude the effect the characteristic was intended to provide.

Features that are described and/or illustrated with respect to one embodiment may be used in the same way or in a similar way in one or more other embodiments and/or in combination with or instead of the features of the other embodiments.

It should be emphasized that the term "comprises/comprising" when used in this specification is taken to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

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"DNA barcoding" is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species.

"DNA Barcode" refers to a short DNA sequence from a standard region of genome to be used for accurate identification of a species.

"Sedge plants" are herbaceous plants belonging to family Cyperaceae.

For the purpose of generating a plant DNA barcode it is utmost needed to identify a standard region of genome to be used as a genetic marker for the accurate identification of a plant species. The inventors of present application have selected four different regions of chloroplast/plastid as genetic marker in order to generate DNA barcode for identification sedge plant species; *rbcL* (RuBisCo large subunit), *matK* (maturase K), *trnH-psbA* and *ycf1(b)*.

Collection of sedge plant samples:

Plant samples were collected from several places located in seven states of India namely Chhattisgarh, Gujarat, Madhya Pradesh, Rajasthan, Tamil Nadu, Uttarakhand and Uttar Pradesh. The samples were coded as per the location of collection.

DNA isolation from plant samples

Genomic DNA was isolated from young leaflets using a modified cetyl-tri-methyl ammonium bromide (CTAB) method with some modifications as required. Isolated DNA was quantified using Nanodrop spectrophotometer and diluted as per the requirements.

Polymerase Chain Reaction (PCR) DNA amplification

Amplification of plastid genome regions *rbcL*, *matK*, *trnH-psbA* and *ycf1(b)* was carried out in a thermal cycler using gene specific primers as given in Table 1 individually as well as in combination by multiplexing of primers.

Table 1. DNA barcoding gene specific Primers and their sequences

Primer	SEQ ID NO:	Forward 5'-3'	SEQ ID NO:	Reverse 5'-3'
ycf1(b)	1	TCTCGACGAAA ATCAGATTGTTG TGAAT	2	ATACATGTCAAA GTGATGGAAAA

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matK	3	CGTACAGTACTT TTGTGTTTACGA G	4	ACCCAGTCCATCT GGAAATCTTGGT TC
trnH-psbA	5	CGCGCATGGTG GATTCACAATC C	6	GTTATGCATGAA CGTAATGCTC
rbcL	7	GACAACTGTGT GGACCGATG	8	CCACCGCGAAGA CATTCATA

Gel Electrophoresis

PCR amplified products were analyzed on denaturing 4% agarose gel for determining the desired base pair size of the products by comparison with appropriate DNA ladder.

DNA sequencing

Amplified fragments were sequenced through the dideoxynucleotide chain-termination method with a DNA-sequencer.

Sequence analysis

All the sequences obtained from the sequencing reaction were subjected to sequence alignments using Clustal W program and alignments were subsequently adjusted manually using BioEdit analysis plateform.

Genetic distance analysis

For each barcode primers, pair wise distances were calculated with the simplest K2P model using MEGA 7.0. Intra and interspecific variation were calculated in an attempt to define the levels of molecular variability within each group of species.

Phylogenetic analysis

To evaluate whether species were recovered as monophyletic with each barcode, the inventors used standard phylogenetic techniques. Neighbour-joining (NJ) analysis, implemented in MEGA 7 was employed to both examine relationships among taxa in the profiles and for the subsequent classification of 'test' taxa. Bootstrap analyses were based on 500-1000 replicates in all cases. To estimate whether a species is resolved, for a given genomic region or combination, the

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inventors scored how well supported the monophyly of individual species was in bootstrap analysis.

Sedge plant Species Identification from all barcode loci

For the identification of sedge plant species the inventors performed three methods of species identification, including BLASTn, barcoding gap methods and neighbor-joining (NJ) tree construction. In the BLASTn method, correct identification was based on the best BLAST hit of the query sequence from the expected species. However, due to lack of sequence information in GenBank repository some of the species were identified with other methods. The inventors generated some barcodes which are entirely new, and identification thus relied on placing the unknown species in a phylogenetic tree with near relatives. In the barcoding gap, approach, a species was considered to be resolved if the minimum inter-specific divergence was higher than the maximum intra-specific divergence of the species. In NJ tree method, a species was considered to be resolved if the accessions under the species were forming a monophyletic group.

Barcode generation

For visual depiction to identify similarities, differences, and nucleotide compositions of each sequence, the inventors generated barcode for each barcode loci region using software FINGERPRINT or PAK.

Identification/authentication of herbal formulations containing sedge plant species

Herbal formulations containing sedge plant or part thereof can be identified/authenticated using the method steps detailed above.

The means and method for sedge plant species identification using DNA barcode can be performed and analyzed and can be readily understood in view of the disclosure made herein.

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EXAMPLES

Example 1

A total of eighty two sedge plant samples were collected from several places located in seven states of India namely Chhattisgarh, Gujarat, Madhya Pradesh, Rajasthan, Tamil Nadu, Uttarakhand and Uttar Pradesh. Plant samples were named according to their places of collection. Plant samples were tentatively identified on the basis of morphological observations.

Genomic DNA was isolated by CTAB method and checked for integrity on 4% agarose gel. Isolated DNA was quantified using Nanodrop and diluted to the concentration of 25 nanogram per microliter. DNA was amplified in a thermal cycler with *rbcL, matK, trnH-psbA* and *ycf1(b)* gene specific forward and reverse primers of SEQ ID NOs: 1-8 as given in Table 1. The PCR reaction was performed for 35 cycles with the reactants as given in Table 2 at annealing temperature of 55 degree C for all except *ycf1(b)* for which the annealing temperature was 58 degree C with a denaturation temperature of 94 degree C and elongation temperature of 72 degree C.

Table 2. List of components with their concentrations used for PCR

S. No.	Components	Concentrations
1.	10X PCR buffer	1 x
2.	25mM $MgCl_2$	2.5 mM
3.	10mM dNTPs	100μM
4.	Forward Primer	5pM
5.	Reverse Primer	5pM
6.	*Taq* Polymerase (5 Unit/μl)	1 unit
7.	Nuclease free H_2O	For volume making
8.	DNA	25ng

The amplified product was sequenced in a DNA sequencer and the sequences were obtained. DNA sequences were analysed by Clustal W and BioEdit software program. The final sequences were aligned by BlastN and the plant species were

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identified as detailed above by Phylogenetic analysis (Fig. 1). Based on the analysis, the collected 82 samples were identified as 17 different species (Table 3) belonging to three genera of Cyperaceae i.e. Cyperus, Kyllinga and Fimbristylis. The barcode sequences are given in Table 3. An exemplary visual depiction of a Barcode is shown in Fig 2.

Example 2

The method detailed in Example 1 was also performed by multiplexing of Barcode gene specific primers as *rbcL+matK*, *rbcL+trnH-psbA*, or *matK+trnH-psbA*. The annealing temperature was kept at 55 degree C. The other steps of the method were same as detailed for Example 1 and above.

Example 3

Herbal formulation sample containing powdered nuts of *Cyperus rotundus* was nixed with DNA extraction buffer, the mixed DNA was isolated containing DNA from other plants also. The DNA was amplified with selected Barcode gene specific primers and the further steps of identification were same as for Example 1. The barcode fragment specific to *Cyperus rotundus* was observed.

Table 3. Species specific Barcode sequences generated in present invention

SEQ ID NO.	DNA BARCODE SEQUENCES WITH DESCRIPTION
9	***rbcL* barcode of 347 nucleotides for species *Cyperus rotundus*** GGGCGATGCTATCATATCGAACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTAT ATAGCTTATCCTTTAGACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTAT TGTAGGTAATGTATTTGGTTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGA ATTCCTCCTGCTTATTCAAAAACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAA GAGATAAGTTGAACAAGTATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATT GGGATTATCCGCAAAGAATTACGGTAGAGCATGTTATGAATGTTCCGCGGTGGAATA
10	***rbcL* barcode of 329 nucleotides for species *Cyperus microiria*** TCGAACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGA CCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTG GTTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATGC AAAAACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAA GTATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCAAAG AATTACGGTAGAGCATGTTATGAATGTTTCGCGGTGGAT
11	***rbcL* barcode of 343 nucleotides for species *Cyperus iria*** CGGATGCTATCATATCGAACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATA GCTTATCCTTTAGACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGT

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	AGGTAATGTATTTGGTTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATT CCTCCTGCTTATGCAAAAACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAG ATAAGTTGAACAAGTATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGG ATTATCCGCAAAGAATTACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
12	***rbcL*** **barcode of 314 nucleotides for species** ***Cyperus retrorsus*** AGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGACCTTTTCGAAGAAGGT TCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGGTTTCAAAGCCTTACG AGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATTCAAAAACTTTCCAAGGT CCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAAGTATGGTCGTCCTCTA TTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGAATTACGGTAGAGCA TGTTATGAATGTTTCGCGGTGGATA
13	***rbcL*** **barcode of 327 nucleotides for species** ***Cyperus cyperoides*** CGAACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGAC CTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGG TTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATGCA AAAACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAAG TATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGA ATTACGGTAGAGCATGTTATGAATGTCTTCGCGGTGG
14	***rbcL*** **barcode of 331 nucleotides for species** ***Cyperus involucratus*** AACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATGTAGCTTATCCTTTAGACCT TTTCGAAGAAGGTTCTGTTACTAACATGTTTACCTCTATTGTAGGTAATGTATTTGGTT TCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATTCAAA AACTTTCCAAGGTCCACCTCACGGTATCCAATCTGAAAGAGATAAGTTGAACAAGTA TGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGAAC TACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGACTATG
15	***rbcL*** **barcode of 324 nucleotides for species** ***Cyperus nutans*** CCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGACCTTT TCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGGTTTC AAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATTCAAAAA CTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAAGTATG GTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGAATTA CGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
16	***rbcL*** **barcode of 331 nucleotides for species** ***Cyperus compressus*** AACCCCTTGTTTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTA GACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATT TGGTTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTAT GCAAAAACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAAC AAGTATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAA AGAATTACGGTAGAGCATGTTATGAATGTTTTCGCGGTGGA
17	***rbcL*** **barcode of 321 nucleotides for species** ***Cyperus esculentus*** TGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGACCTTTTCGAAGAA GGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGGTTTCAAAGCCTT ACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATTCAAAAACTTTCCAA GGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAAGTATGGTCGTCCT CTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGAATTACGGTAGA GCATGTTATGAATGTTTTCCGCGGGTGGAACA
18	***rbcL*** **barcode of 329 nucleotides for species** ***Cyperus tenuispica*** AACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGACCT TTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGGTT TCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATGCAAA AACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAAGTA TGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCTGCAAAGAAT TACGGTAGAGCATGTTATGAATGTTTCGCGGTGGATAGT
19	***rbcL*** **barcode of 340 nucleotides for species** ***Cyperus odoratus*** ATGCTATCATATCGAACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCT TATCCTTTAGACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGG TAATGTATTTGGTTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCT CCTGCTTATGCAAAAACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGAT

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	AAGTTGAACAAGTATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGAT TATCCGCAAAGAATTACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
20	***rbcL* barcode of 328 nucleotides for species *Kyllinga monocephala*** CGAACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGAC CTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGG TTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCTCCTGCTTATTCA AAAACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAAG TATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGA ATTACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
21	***rbcL* barcode of 326 nucleotides for species *Kyllinga odoratus*** AACCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGACCT TTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGGTT TCAAAGCCTTACGAGCTCTGCGCTTGGAAGACTTACGAATTCCTCCTGCTTATTCAAA AACTTTCCAAGGTCCACCTCACGGTATCCAAGCTGAAAGAGATAAGTTGAACAAGTA TGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGAAT TACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
22	***rbcL* barcode of 325 nucleotides for species *Kyllinga nemoralis*** GCCTGTTGCTGGAGAAGAAAATCAATATATTGCCTATGTAGCTTATCCTTTAGACCTT TTCGAAGAAGGTTCTGTTACTAACATGTTTACCTCAATTGTAGGTAATGTATTTGGTTT CAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCCCCTGCTTATTCAAAA ACTTTCCAAGGTCCACCTCACGGTATCCAATCTGAAAGAGATAAGTTGAACAAGTAT GGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAGAATT ACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
23	***rbcL* barcode of 330 nucleotides for species *Fimbristylis autumnalis*** ATCGAGCCTGTTGTTGGAGAAGAAGATCAATATATTGCCTATATAGCTTATCCTTTAG ACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTT GGTTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCCCCTGCTTATT CAAAAACTTTCCAAGGCCCACCTCATGGTATCCAATCTGAAAGAGATAAGTTGAACA AGTATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAA GAACTACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
24	***rbcL* barcode of 327 nucleotides for species *Fimbristylis miliacea*** GAGCCTGTTGTTGGAGAAGAAAATCAATATATTGCCTATATAGCTTATCCTTTAGACC TTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTGGT TTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCCCCTGCTTATTCAA AAACTTTCCAAGGCCCACCTCATGGTATCCAATCTGAAAGAGATAAGTTGAACAAGT ATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAAAA CTACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
25	***rbcL* barcode of 329 nucleotides for species *Fimbristylis littoralis*** GATGCCTGTTGGTTGGAGAAGAAGATCAATATATTGCCTATATAGCTTATCCTTTAGA CCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCTATTGTAGGTAATGTATTTG GTTTCAAAGCCTTACGAGCTCTACGCTTGGAAGACTTACGAATTCCCCCTGCTTATTC AAAAACTTTCCAAGGCCCACCTCATGGTATCCAATCTGAAAGAGATAAGTTGAACAA GTATGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAG AACTACGGTAGAGCATGTTATGAATGTCTTCGCGGTGGA
26	***matK* barcode of 823 nucleotides for species *Cyperus rotundus*** TTCAAATCCTTCAATGCTGGATCCAAGATATTCCTTCTTTGCATTTATTGAGATTCTTT CTCTTCGATTATTCTAATTGGAATAGTCTCATTATTTCAAAGAAATTAGCTTCTATTTC TATATTCTCAAAAGAAAATATAAGACTCTCTCGTTTCTTATATAATTATTATGTATCTG AATATGAGTTTTTATTCTTGTTTATTCGTAAAAAATCTTCTTGTTTACGATTAACATCT TTTGGAACCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGAACATTTTAGAATAG TACATCATATTTTTTTGAAGAAAACTTTATGGTTCTTCACAGATCCTCTCATGCACTAT GTTCGATATCAAGGCAAAGCAATTCTAGCATCAAAAGGGACCTATCAATTTATGAAG AAATTGAAATATTGCTTTATCTGTTTTTGGCAATATTATTTTCATTTTTGGTCTGAGTC TAATAGGTTTCATAGAAACAAATTCTCTTATTATTCATTCTACTTTCTCGGTTATTTTT CAAGTGTAAAAATAAATCCTTTGATGGTAAGGAGTCAAATATTGGAGGATTTTGTATT AATAGATACTCTTTTTAAGAGATTTGATACTCTAGTTCCAGTCGTTCCTCTCATTAGAT CATTGTCTAAAGCTTCACTTTGTACTGTATTAGGACATCCTACTAGTAAACCAATTTG GACAGATTTATCAGATTATGATATTATTAGTCGATTTGGTCAAATATATAAAAATATT TTTCATTTTTATAGTGGATCTTCTAAAAAGAGAATTTTGTATCGAATGAAGTATATAC

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	TTCG
27	***matK* barcode of 648 nucleotides for species *Cyperus microiria*** TCAAAAGAAAATATAAGACTCTCTCGTTTCTTATATAATTATTATGTATCTGAATATG AGTTTTTATTCTTTTTTATTCGTAAAAAATCTTCTTGTTTACGATTAACATCTTTTGGAA CCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGAACATTTTAGAATAGTACATCA TATTTTTTTGAAGAAAACTTTATGGTTCTTCACAGATCCTCTCATGCACTATGTTCGAT ATCAAGGCAAAGCAATTCTAGCATCAAAAGGGACCTATCAATTTATGAAGAAATTGA AATATTGCTTTATTTGTTTTTGGCAATATTATTTTCATTTTTGGTCTGAGTCTAATAGGT TTCATAGAAACAAATTCTCTTATTATTCATTCTACTTTCTCGGTTATTTTTCAAGTGTA AAAATAAATCCTTTGATGGTAAGGAGTCAAATATTGGAGGATTTTGTATTAATAGATA CTCTTTTTAAGAGATTTGATACTTTAGTTCCAGTCGTTCCTCTCATTAGATCATTGTCT AAAGCTTCACTTTGTACTGTAGCAGGACATCCTACTAGTAAACCAATTTGGACAGATT TATCAGATTATGATATTATTAGTCGATTTGGTCAAATATATAAAAATATTTTTCATTTT TAT
28	***matK* barcode of 744 nucleotides for species *Cyperus iria*** TTCCTTCTTTGCATTTATTGAGATTCTTTCTCTTCGATTATTCTAATTGGAATAGTCTCA TTATTTCAAAGAAATTAGCTTCTATTTCTATATTCTCAAAAGAAAATATAAGACTCTC TCGTTTCTTATATAATTATTATGTATCTGAATATGAGTTTTTATTCTTGTTTATTCGTAA AAAATCTTCTTGTTTACGATTAACATCTTTTGGAACCTTTCTTGAGCGAATCTACTTCT ATGGAAAAATAGAACATTTTAGAATAGTACATCATATTTTTTTGAAGAAAACTTTATG GTTCTTCACAGATCCTCTCATGCACTATGTTCGATATCAAGGCAAAGCAATTCTAGCA TCAAAAGGGACCTATCAATTTATGAAGAAATTGAAATATTGCTTTATTTGTTTTTGGC AATATTATTTTCATTTTTGGTCTGAGTCTAATAGGTTTCATAGAAACAAATTCTCTTAT TATTCATTCTACTTTCTCGGTTATTTTTCAAGTGTAAAAATAAATCCTTTGATGGTAAG GAGTCAAATATTGGAGGATTTTGTATTAATAGATACTCTTTTTAAGAGATTTGATACT TTAGTTCCAGTCGTTCCTCTCATTAGATCATTGTCTAAAGCTTCACTTTGTACTGTATT AGGACATCCTACTAGTAAACCAATTTGGACAGATTTATCAGATTATGATATTATTAGT CGATTTGGTCAAATATATAAAAATATTTTTCATTTTTATA
29	***matK* barcode of 651 nucleotides for species *Cyperus retrorsus*** TCAAAAGAAAATATAAGACTCTCTCGTTTCTTATATAATTATTATGTATCTGAATATG AGTTTTTATTCTTGTTTATTCGTAAAAAATCTTCTTGTTTACGATTAACATCTTTTGGA ACCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGAACATTTTAGAATAGTACATC ATATTTTTTTGAAGAAAACTTTATGGTTCTTCACAGATCCTCTCATGCACTATGTTCGA TATCAAGGCAAAGCAATTCTAGCATCAAAAGGGACCGATCAATTTATGAAGAAATTG AAATATTTCTTTATCTGTTTTTGGCAATATTATTTTCATTTTTGGTCTGAGTCTAATAG GTTTCATAGAAACAAATTCTCTTATTATTCATTCTACTTTCTCGGTTATTTTTCAAGTG TAAAAATAAATCCTTTGATGGTAAGGAGTCAAATATTGGAGGATTTTGTATTAATAGA TACTCTTTTTAAGAGATTTGATACTCTAGTTCCAGTCGTTCCTCTCATTAGATCATTGT CTAAAGCTTCACTTTGTACTGTATTAGGACATCCTACTAGTAAACCAATTTGGACAGA TTTATCAGATTATGATATTATTAGTCGATTTGGTCAAATATATAAAAATATTTTTCATT TTTATAGT
30	***matK* barcode of 545 nucleotides for species *Cyperus cyperoides*** ATGGAAAAATAGAACATTTTAGAATAGTACATCATATTTTTTTGAAGAAAACTTTATG GTTCTTCACAGATCCTCTCATGCACTATGTTCGATATCAAGGCAAAGCAATTCTAGCA TCAAAAGGGACCTATCAATTTATGAAGAAGTTGAAATATTGCTTTATCTGTTTTTGGC AATATTATTTTCATTTTTGGTCTGAGTCTAATAGGTTTCATAGAAACAAATTCTCTTAT TATTCATTCTACTTTCTCGGTTATTTTTCAAGTGTAAAAATAAATCCTTTGATGGTAAG GAGTCAAATATTGGAGGATTTTGTATTAATAGATACTCTTTTTAAGAGATTTGATACT CTAGTTCCAGTCGTTCCTCTCATTAGATCATTGTCTAAAGCTTCACTTTGTACTGTAGT AGGACATCCTACTAGTAAACCAATTTGGACAGATTTATCAGATTATGATATTATTAGT CGATTTGGTCAAATATATAAAAATATTTTTCATTTTTATAGTGGATCTTCTAAAAAGA GAATTTTGTATCGAAGGAAG
31	***matK* barcode of 771 nucleotides for species *Cyperus involucratus*** GTTCAAATCCTTCAATGCTGGATCCAAGATATTCCTTCTTTGCATTTATTGAGATTCTT TCTCTTCGATTATTCTAATTGGAATAGTCTGATTATTTCAAAGAAATTAGCTTCTATTT CTATATTCTCAAAAGAAAATATAAGACTCTCTCGTTTCTTATATAATTATTATGTATCT GAATATGAGTTTTTATTCTTGTTTATTCGTAAAAAATCTTCTTGTTTACGATTAACATC TTTTGGAACCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGAACATTTTAGAATA GTACATCATATTTTTTTGAAGAAAACTTTATGGTTCTTCACAGATCCTCTCATGCACTA TGTTCGATATCAAGGCAAAGCAATTCTAGCATCAAAAGGGACCGATCAATTTATGAA

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32	***matK* barcode of 762 nucleotides for species *Cyperus nutans*** TATTCCTTCTTTGCATTTATTGAGATTCTTTCTCTTCGATTATTCTAATTGGAATAGTCT CATTATTTCAAAGAAATTAGCTTCTATTTCTATATTCTCAAAAGAAAATATAAGACTC TCTCGTTTCTTATATAATTATTATGTATCTGAATATGAGTTTTTATTCTTGTTTATTCGT AAAAAATCTTCTTGTTTACGATTAACATCTTTTGGAACCTTTCTTGAGCGAATCTACTT CTATGGAAAAATAGAACATTTTAGAATAGTACATCATATTTTTTTGAAGAAAACTTTA TGGTTCTTCACAGATCCTCTCATGCACTATGTTCGATATCAAGGCAAAGCAATTCTAG CATCAAAAGGGACCTATCAATTTATGAAGAAATTGAAATATTGCTTTATCTGTTTTTG GCAATATTATTTTCATTTTTGGTCTGAGTCTAATAGGTTTCATAGAAACAAATTCTCTT ATTATTCATTCTACTTTCTCGGTTATTTTTCAAGTGTAAAAATAAATCCTTTGATGGTA AGGAGTCAAATATTGGAGGATTTTGTATTAATAGATACTCTTTTTAAGAGATTTGATA CTCTAGTTCCAGTCGTTCCTCTCATTAGATCATTGTCTAAAGCTTCACTTTGTACTGTA TTAGGACATCCTACTAGTAAACCAATTTGGACAGATTTATCAGATTATGATATTATTA GTCGATTTGGTCAAATATATAAAAATATTTTTCATTTTTATAGTGGATCTTCTAAAAA
33	***matK* barcode of 775 nucleotides for species *Cyperus kyllingia*** TCAAATCCTTCAATGCTGGATTCAAGATATTCCTTTTTTGCATTTATTGAGATTCTTTC TCTTCGATTATTCTAATTGGAATAGTCTCATTATTTCAAAGAAATTAGTTTCTATTTCT ATATTCTCAAAAGAAAATATAAGACTCTCTCGTTTCTTATATAATTATTATGTATCTGA ATATGAGTTTTTATTCTTGTTTATTCGTAAAAAATCTTCTTGTTTACGATTAACATCTTT TGGAACCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGAACATTTTAGAATAGTA CATCATATTTTTTTGAAGAAAACTTTATGGTTCTTCACAGATCCTCTCATGCACTATGT TCGATATCAAGGCAAAGCAATTCTAGCATCAAAAGGGACCTATCAATTTATGAAGAA ATTGAAATATTGCTTTATCTGTTTTTGGCAATATTATTTTCATTTTTGGTCTGAGTCTA ATAGGTTTCATAGAAACAAATTCTCTTATTATTCATTCTACTTTCTCGGTTATTTTTCA AGTGTAAAAATAAATCCTTTGATGGTAAGGAGTCAAATATTGGAGGATTTTGTATTAA TAGATACTCTTTTTAAGAGATTTGATACTCTAGTTCCAGTCGTTCCTCTCATTAGATCA TTGTCTAAAGCTTCACTTTGTACTGTAGTAGGACATCCTACTAGTAAACCAATTTGGA CAGATTTATCAGATTATGATATTATTAGTCGATTTGGTCAAATATATAAAAATATTTTT CATTTTTATAGT
34	***matK* barcode of 793 nucleotides for species *Kyllinga odoratus*** CCATTTTGAAATCTTGGTTCAAATCCTTCAATGCTGGATCCAAGATATTTCTTTCTTGC ATTTATTGAGATTCTTTCTCTTCGATTATTCTAATTGGAATAGTCTCATTATTTCAAAG AAATTATCTTCTATTTCTATATTCTCAAAAGAAAATATAAGACTCTCTCGTTTCTTATA TAATTATTATGTATCTGAATATGAGTTTTTATTCTTGTTTATTCGTAAAAAATCTTCTT GTTTACGATTAACATCTTTTGGAACCTTTCTTGAGCGAATCTACTTCTATGGAAAAAT AGAACATTTTAGAATAGTACATCATATTTTTTTGAAGAAAACTTTATGGTTCTTCACA GATCCTCTCATGCACTATGTTCGATATCAAGGCAAAGCAATTCTAGCATCAAAAGGG ACCTATCAATTTATGAAGAAATTGAAATATTGCTTTATCTGTTTTTGGCAATATTATTT TCATTTTTGGTCTGAGTCTAATAGGTTTCATAGAAACAAATTCTCTTATTATTCATTCT ACTTTCTCGGTTATTTTTCAAGTGTAAAAATAAATCCTTTGATGGTAAGGAGTCAAAT ATTGGAGGATTTTGTATTAATAGATACTCTTTTTAAGAGATTTGATACTCTAGTTCCAG TCGTTCCTCTCATTAGATCATTGTCTAAAGCTTCACTTTGTACTGTATTAGGACATCCT ACTAGTAAACCAATTTGGACAGATTTATCAGATTATGATATTATTAGTCGATTTGGTC AAATATATAAAAATATTTTTCATTTTTATAGT
35	***matK* barcode of 826 nucleotides for species *Fimbristylis autumnalis*** ATTTTGAAATCTTGGTTCAAATCCTTCAATGCTGGATCCAAGATATTCCTTCTTTGCAT TTATTGAGATTCTTTCTCTTCCTCTTCGATTATTCTAATCGGAATAGTCTTTTTATTTCA AGGAAATTGGTTTCTATATTCTCAAAAGAAAACATAAGACTTTGTCGTTTCTTATATA ATTTTTATGTATATGAATATGAGTTTTTATTCCTGTTTATTCGTAAAAAATCTTCTTATT TACGATTAATATCTTTTGTAACCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGA ACATTTTAAAATAGTACATCATAAGTTTTTGAAGAAAAACTTATGGTTCTTCACAGAT CCTCTTATGCACTATGTTCGATATCAAGGCAAAGCTTTTCTAGGATCAAAAGGGACCG ATCAATTTTTGAAGAAATTGAAATCTTACCTTGTTTGTTTTTGGCAATATTATTTTCAT

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	TTTTTCTCTAAGTCTAATAGGTTTCATAGAAATCAATTTTCTTATTATTCATTTTCATTC TACTTTCTCGGTTTTTTTTTTAGTGCAAAAAGGACTCATTTGATGGTAAAGAGTCACAT ACTAGAGGATTCTGTAGTAATAGATACTATTTTGACTAGATTTGATACTCTAGTTCCT GCTGTTCGTCTCATTAGATCATTGTCTAAAGCTTCACTTTGTACTGTAGCAGGACATCC TACTAGTAAACCAATCTGGACAGATTTATCAGATTATGATATTATTAGTCGATTTGGT CGAATATATAGAAATCTTTTTCATTTTTATAGTGGATCTTCTAAAAAGAGAATTTTGT ACGA
36	***matK*** **barcode of 815 nucleotides for species *Fimbristylis miliacea*** AATCTTAGTTCAAATCCTTCCATGCTGGATCCAAGATATTCCTTCTTTACATTTATTAA GATTTTTTCTCTTCCTCTTCGATTATTTTAATCGGAATAGTCTTTTTATTTCAAAGAAAT TGGTTTCTATATTCTCAAAAGAAAACATAAGACTTTGTCGTTTCTTATATAATTTTTAT GTATATGAATATGAGTTTTTATTCCTGTTTATTCGTAAAAAATCTTCTTATTTACGATT AATATCTTTTGTAACCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGAACATTTT AAAATAGTGCATCATAAGTTTTTGATGAAAAACTTATGGTTCTTCACAGATCCTCTTA TGCACTATGTTCGATATCAAGGCAAAGCTTTTCTAGGATCAAAAGGGACCGATCAATT TTTGAAGAAATTGAAATCTTACCTTGTTTGTTTTTGGCAATATTATTTTCATTTTTGGT CTAAGTCTAATAGGTTTCATAGAAATCAATTTTCTTATTATTCATTTTCATTCTACTTT CTCGGTTTTTTTTCTAGTGCAAAAAGGACTCATTTGATGGTAAAGAGTCACATACTAG AGGATTCTGTAGTAATAGATACTATTTTGACTAGATTTGATACTCTAGTTCCTGCTGTT CGTCTCATTAGATCATTGTCTAAAGCTTCACTTTGTACTGTAGCAGGACATCCTACTA GTAAGCCAATCTGGACAGATTTATCAGATTATGATATTATTAGTCGATTTGGTCGAAT ATATAGAAATCTTTTTCATTTTTATAGTGGATCTTCTAAAAAGAGAATTTTGT
37	***matK*** **barcode of 820 nucleotides for species *Fimbristylis littoralis*** AATCTTAGTTCAAATCCTTCCATGCTGGATCCAAGATATTCCTTCTTTACATTTATTAA GATTTTTTCTCTTCCTCTTCGATTATTTTAATCGGAATAGTCTTTTTATTTCAAGGAAAT TGGTTTCTATATTCTCAAAAGAAAACATAAGACTTTGTCGTTTCTTATATAATTTTTAT GTATATGAATATGAGTTTTTATTCCTGTTTATTCGTAAAAAATCTTCTTATTTACGATT AATATCTTTTGTAACCTTTCTTGAGCGAATCTACTTCTATGGAAAAATAGAACATTTT AAAATAGTGCATCATAAGTTTTTGATGAAAAACTTATGGTTCTTCACAGATCCTCTTA TGCACTATGTTCGATATCAAGGCAAAGCTTTTCTAGGATCAAAAGGGACCGATCAATT TTTGAAGAAATTGAAATCTTACCTTGTTTGTTTTTGGCAATATTATTTTCATTTTTGGT CTAAGTCTAATAGGTTTCATAGAAATCAATTTTCTTATTATTCATTTTCATTCTACTTT CTCGGTTTTTTTTCTAGTGCAAAAAGGACTCATTTGATGGTAAAGAGTCACATACTAG AGGATTCTGTAGTAATAGATACTATTTTGACTAGATTTGATACTCTAGTTCCTGCTGTT CGTCTCATTAGATCATTGTCTAAAGCTTCACTTTGTACTGTAGCAGGACATCCTACTA GTAAGCCAATCTGGACAGATTTATCAGATTATGATATTATTAGTCGATTTGGTCGAAT ATATAGAAATCTTTTTCATTTTTATAGTGGATCTTCTAAAAAGAGAATTTTGTACGAA
38	***trnH-psbA*** **barcode of 856 nucleotides for species *Cyperus rotundus*** GAAAATTCTTTTTGTTTTTTCCCTCTTAATCATTCTTTTTTTTAATCATTCCACTAGCAG ATTGAGAGATACCGAATGGACTCACCATATTTGTTTTTTAAACAAAAGGATTGGGTAA TCTAAAAAATATATACAGTATAATCTGGAAAGAGCTCTTTTTGGTATCCGCACCCGGA GGTTTAAATTATATATCGAACGCATAGCAAATAAAAAAAAAAAACCCTTAGGAATATG AGTTCTTTTTATCAATTATTTTGAGTATGGAATTTAGATAAAAAAAAACATTGAGTTAA GATCAATCTAAACCAAACCATTTATTTTTAACTTTATATATTATGACATCGCTATGTAA AAAATCGGGGTCTTATCAATCCATAATTTATAAAACAGGTTACGATTTGGTAGATTTT ACACCTACCCATTTTTTGGAAATGAAAGATATAATATAAACAATATAAATATTAAATC TTTATCTTTATCCAAAAAAATTTTTGGTGAAAAAATTATTTATAATCCTATTTTGAAAA ATAAAAAAAAAACTGAAAAAATGGAGAATTTATACTACTTAGTTTAGAAAAAAAAATC GTCTTTTTTCTATATTAATTTTTAAAAATTATCGACAATCAAGTGAATTAATCCTCTAG GTATACTTACACAATATACAATTATTTATATTATATTTTATAAACCTCCGGGTATACTT ACCCAATAGAGATACGATAAAGTCGACTATACTAGTATGTTTTTTTCCTTACCCAATA TATTAGTCTAAAAAACTAATATATTGGGTATATTGGGTATTTCTCAAATTAATATTCA AATTATTAATATAATGAACAATTCAAAAGATCGTAGGTTT
39	***trnH-psbA*** **barcode of 865 nucleotides for species *Cyperus microiria*** GAAAAGATCGAGCTAAAGAAAATATTTTCTCTTTTTTCCATTCATCATTATTGTTTTTA TTCTGACCTCTTAGATTGAAGTATTTGACATGGAATCTCCATATTTACTAAAAAAAAA AGGTAATAATAAATTTATAAATGTTATACAGAATGATCTGGAAAGAGATACTTTTATG TCCGCAACCGAAGCTCAAAATTATCTGTCGATCGCATAGCAAATCAAAAAAAAAAAT CCTTAGTAATATGAGTTCTTTTTCTCAATTATTTTGAGTATTGAATTTAGATAAAAAAA ACATTGAGTTAAGATCAATCTAAACCAAACCATTTATTTTTAACTTTATATATTATGA

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	CATCGCTATGTAAAAAATCGAGGTCTTATCAATCCATAATTTATAAAACAGGTTACGA TTTGGTAGATTTTACACCTACCCATTTTTTGGAAATGAAAGATATAAACAATAGAAAT ATTAAATCTTTATCTTTATCCAAAAAGATTTTTGGTGAAAAAATTATTTATAATCCTAT TTTGAAAAATAAAAAAAAAAACTGAAAAAATGGAGAATTTACACTACTTAGTTTAGAA AAAAAAATCTTCTTTTTCTATATTAATTTTTAAAAATTAACGAGAATCAAGTGTATT AATCCTCTAGGTATACTTACACAATATACCTTTATTTATATTATATTTTATAAACCTCC GGGTATACTTACCCAATAGAGATATTTTAGAGACTACTATACTAGTATATTTTTTTCCT TACCCAATATATTAGTCTAAAAAACTAATATATTGGGTATATTGGGTATTTCTCAAAT TAATATTCAAATTATTAATATAATGAACAATTCAAAAGATCTAGTTTATC
40	***trnH-psbA* barcode of 865 nucleotides for species *Cyperus iria*** GAAAAGATCGAGCTAAAGAAAATATTTTCTCTTTTTTCCATTCATCATTATTGTTTTTA TTCTGACCTCTTAGATTGAAGTATTTGACATGGAATCTCCATATTTACTAAAAAAAAA AAGTAATAATAAATCTATAAATGTTATACAGAATGATCTGGAAAGAGATACTTTTAT GTCCGCAACCGAAGCTCAAAATTATCTGTCGATCGCATAGCAAATCAAAAAAAAAAA TCCTTAGTAATATGAGTTCTTTTTCTCAATTATTTTGAGTATTGAATTTAGATAAAAAA AACATTGAGTTAAGATCAATCTAAACCAAACCATTTATTTTTAACTTTATATATTATG ACATCGCTATGTAAAAAATCGAGGTCTTATCAATCCATAATTTATAAAACAGGTTACG ATTTGGTAGATTTTACACCTACCCATTTTTTGGAAATGAAAGATATAAACAATAGAAA TATTAAATCTTTATCTTTATCCAAAAAGATTTTTGGTGAAAAAATTATTTATAATCCTA TTTTGAAAAATAAAAAAAAAAACTGAAAAAAGGGAGAATTTATACTACTTAGTTTAGA AAAAAAAATCTTCTTTTTCTATATTAATTTTTAAAAATTAACGAGAATCAAGTGTAT TAATCCTCTAGGTATACTTACACAATATACCTTTATTTATATTATATTTTTATAAACCT CCGGGTATACTTACCCAATAGAGATATTTTAGAGACTACTATACTAAGTATATTTTTT TCCTTACCCAATATATTAGTCTAAAAAACTAATATATTGGGTATATTGGGTATGTTCT CAAATTAATATTCAAATTATTAATATAATGAACAATTCAAAAGAATCTAGTT
41	***trnH-psbA* barcode of 861 nucleotides for species *Cyperus retrorsus*** AGCTAAGAGAAATATTTTCTCTTTTTTCCATTCATCATTATTGTTTTTATTCTGACCTCT TAGATTAAAGTATTTGACATGGAATCTCCATATTTACTAAAAAAAAAAAAGGAATAAA AAATCTGTAAATGTTATACAGAATGATCTGGAAAGAGATACTTTTATGTCCGCAACCG AAGCTCAAAATTATCTGTCGATCGCATAGCAAATCAAAAAAAAAAATCCTTAGTAATA TGAGTTCTTTTTCTCAATTATTTTGAGTATTGAATTTAGATAAAAAAAACATTGAGTTA AGATCAATCTAAACCAAACCATTTATTTTTAACTTTATATATTATATATATTATGACAT CGCTATGTAAAAAATCGAGGTCTTATCAATCCATAATTTATAAAACAGGTTACGATTT GGTAGATTTTACACCTACCCATTTTTTGGAAATGAAAGATATAAACAATATAAATATT AAATCTTTATCCAAAAAAATTTTTGGTGAAAAAATTATTTATAATCCTATTTTGAAAA ATAAAAAAAAAACTGAAAAAATGGAGAATTTATACTACTTAGTTTAGAAAAAAAAAT CGTCTTTTTTCTATATTAATTTTTAAAAATTAACGAGAATCAAGTGTATTAATCCTCTA GGTATACTTACGCAATATACCTTTATTTATATTATATTTTATAAACCTCCGGGTATACT TACCCAATAGAGAGAGATATTTTAGAGACTACTATACTAGTATATTTTTTTCCTTACC CAATATATTAGTTTAAAAAACTAATATATTGGGTATATTGGGTATTTCTCAAATTAAT ATTCAAATTTTTAATATAATTAATAGATCCAAAGATCTAGTTTATC
42	***trnH-psbA* barcode of 880 nucleotides for species *Cyperus cyperoides*** CTAAAGAAAAGATCGAGCTAAAGAAAATATTTTCTCTTTTTTCCATTTATCATTTTTGT TTTTATTCTGACCTCTTAGATTGAAGTATTTGACATGGAATCTCCATATTTACTAAAAA AAAAAAGGAATAATAAATCTATAAATGTTATACAGAATGATCTGGAAAGAGATACTT TTATGTCCGCAACCGAAGCTCAAAATTATCTGTCGATCGCATAGCAAATCAAAAAAA AAAATCCTTAGTAATATGAGTTCTTTTTATCAATTATTTTGAGTATTGAATTTAGATAA AAAAAACATTGAGTTAAGATCAATCTAAACCAAACCATTTATTTTTCACTTTATATAT TATATATATTATGACATCGCTATGTAAAAAATCGAGGTCTTATCAATCCATAATTTAT AAAACAGGTTACGATTTGGTAGATTTTACACCTACCCATTTTTTGGAAATGAAAGATA TAAACAATAGAAATATTAAATCTTTATCCAAAAAAATTTTTGGTGAAAAAATTATTTA TAATCCTATTTTGAAAAATAAAAAAAAAACTGAAAAAATGGAGAATTTATACTACTAT ACTACTTAGTTTAGAAAAAAAAAATCGTCTTTTTTCTATATTAATTTTTCAAAATTAACG AGAATCAAGTGTATTAATCCTCTAGGTATACTTACGCAATATACCTTTATATATATTA TATTTTATAAACCTCCGGGTATACTTACCCAATAGAGATATTTTAGAGACTACTATAC TAGTATATTTTTTTCCTTACCCAATATATTAGTCTAAAAAACTAATATATTGGGTATAT TGGGTATTTCTCAAATTAATATTCAAATTATTAATATAATGAACAATTCAAAAGATCT AGTTTATC
43	***trnH-psbA* barcode of 870 nucleotides for species *Cyperus involucratus***

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	GAAGTTCCTAGCTATTGAAGGATAAACTAGATCTTTTGAATTGTTCATTATATTAAAA ATTTGAATATTAATTTGAGAAATACCCAATATACCCAATATATTAGTTTTTAGACTA ATATATTGGGTAAGGAAAAAAATATACTAGTATAGTAGTCTCTAAAATCTCTCTATTG GGTAAGTATACCCGGAGTTTTCTAAAATATAAATAAAGGTATATTGCGTAAGTATACC TAGAGGATTAATAGACTTGATTCTCGTTTTTTTTCTCTTTTAAAAATTAATATAGAAAA AAGACGATTTTTTTTTCTAAACTAAGTAGTATAAATTCTCCATTTTTTCAGTTTTTTTTT ATTTTTAAAAATAGGATTATAAATGATTTTTTCAACAAAAATTTTGTTGGATAAAGAT AAAGATTTAATATTCCTATTGTTTATATCTTTCATTACCAAAAAACGGGTAGGTGTAA AATCTACCAAATCGTAACCTGTCTTATAAATTATGGATTGATAATACATCGATTTTTT ACACAGCGATCTCATAATATAAAAATAAATGGTTTGGTTTAGATTGATCTTAACTCAA TGTTTTTTTTATCTAAATTTTTATCTAAATTAAATACTCAAAATAATTGATAAAAAGAA CTCATATTACTAAGGATTTTCTTTTTTGATTTGCTATGCGATCGACAGATAATTTTGAG CTTCGGTTGCGGACATAAAAGTATTTCTTTCCAGATCCTTCTGTATAACATTTATAGAT TTTTTTTTCCTTTTTTTTTTTTTTTAGTAAGTAAATATGGGGATTCCATGTCAAATACCTC AATCTAAGAGGTTAAAATAAAAACAATAATGATGAATGGAAAAAAGAGAA
44	***trnH-psbA* barcode of 882 nucleotides for species *Cyperus compressus*** AGAAAAGATCGAGCTAAAGAAAATATTTTCTCTTTTTTCCATTCATCATTATTGTTTTT ATTCTGACCTCTTAGATTGAAGTATTTGACATGGAATCTCCATATTTACTAAAAAAAA AAAGAGGAATAATAAATCTATAAATGTTATATTCAGAAGGATCTGGAAAGAGATACT TTTATGTCCCCCCAACCGAAACTCAAAGATTATCTGTCGATCGCATAGCGAAAAAAA AAAAAAAAATCCTTAGTAATATGAGTTCTTTTTATCAATTATTTTGAGTATTGAATGA ATTTAAAAAAAAAAAATCATTTAATTACAATCTAATCCAAACCAACCCATTTTTTTTTT ATGTTTTCGCCATCTATAAGAAAAAAATCGGGTCTTTCCATCCTTTATAAAACAGGTT TGGATTTGGTAGATTTTACCCCTACCCATTTTTTGGAAATGAAAGATATAATCAATAG AATATAAATATTAAATCTTTATCTTTATCACAAAAAAAAATTGGGTGAAAAAATTATTT ATAATCCGATTTTGAAAAATAAAAAAAACTGAAAAAAGGGGTAATTTATACTACTCT ACTTAGTTTAGAAAAAAAAATCGTCTTTTTTCTATATTAATTTTTAAAAATTAACGAG AATCACGTGTATTAATCCTCTAGGTATACTCACGCAATATACCTTTATTTATTATTTAT ATTTTATTATCCACCGCTCCGGGTATACTTACCCAATAGAGATATTTTAGAGACTACT ATACTAGTATATTTTTTTCCTTACCCAATATATTAGTCTAAAAAACTAATATATTGGGT ATATTGGGTATTTCTCAAATTAATATTCAAATTATTAATATAATGAACAATTCAAAAG ATCTAGTTTATC
45	***trnH-psbA* barcode of 859 nucleotides for species *Cyperus kyllingia*** GATCGAGCTAAAAAAAATATTTTCTCTTTTTTCCATTCATCATTATTGTTTTTACTCTG ACCTGTTAGATTGAAGTATTTGACCTGAAATCTCCATATTTACTGAACAAATAAAGGA ATAATAAATGTATAAATGTGATACAGAATGATCTGGAAAGAGATACTTTTATGTCCG CAACCGAAGCTCAAAATTATCTGTCGATCGCATAGCAAATGAAAAAAAAAAATCCTTA GTAATATGAGTTCTTTTTATCAATTATTTTGAGTATTGAATTTAGATAAAAAAAACAT TGAGTTAAGATCAATCTAAACCAAACCATTTATTTTTAACTTTATATATTATGACATC GCTATGTAAAAAATCGAGGTCTTATCAATCCATAATTTATAAAATAGGTTACGATTTG GTAGATTTTACACCTACCCATTTTTTGGAAATGAAAGATATAAACAATAGAAATATTA AATCTTTATCTTTATCCAAAAAAATTTTTTGGTGAAAAAATTATTTATAATCCTATTTT GAAAAATAAATAAAAAACTGAAAAAATGGAGAATTTATACTACTTAGTTTAGAAAAA AAAATCGTCTTTTTTCTATATTAAATTAATTTTTAAAAATTAACGAGAATCAAGTGTA TTAATCCTCTAGGTATACTTACGCAATATACCTTTATTTATATTATATTTTATAAACCT CCGGGTATACTTACCCAATAGAGATATTTTAGAGACTACTATACTAGTATATTTATAT TTTTTTCCTTACCCAATATATTAGTCTAAAAAACTAATATATTGGGTATATTGGGTATT TCTCAAATTATTAATATAATGAACAATTCAAAAGATCTAGTTTATC
46	***trnH-psbA* barcode of 883 nucleotides for species *Fimbristylis autumnalis*** GCTAAAGAAAAGATCAGGAGCTAAAGAAAATATTTTCTCTTTTTTCCATTCATCTATT ATTGTTTTTATTCTGACCTGCTTAGATTGAACGTATTTGACATGGAATCTCCATATTTA CTAAAAAAAAAGAAAGGAATAATAAATTTATAAATGTTATACAGAATGATCTGGAAA GAGATACTTTTATGTCCGCAACCGAAGCTCAATAATTATCTGTCGATCGCATAGCAAA TCAAAAAAAAAAAAATCCTTAGTAATATGAGTTCTTTTTCTCAATTATTTTGAGTATTG AATTTAGATAAAAAAAAACATTGAGTTAAGATCAATCTAAACCAAACCATTTATTTTTA ACTTTATATATTATGACATCGCTATGTAAAAAATCGAGGTCTTATCAATCCATAATTT ATAAAACAGGTTACGATTTGGTAGATTTTACCCCTACCCATTTTTTGGAAATGAAAGA TATAAACAATAGAAATATTAAATCTTTATCTTTATCCAAAAAGATTTTTGGTGAAAAA ATTATTTATAATCCTATTTTGAAAAATAAAAAAAAAAACTGAAAAAATGGAGAATTTA

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	CACTACTTAGTTTAGAAAAAAAAATCTTCTTTTTTCTATATTAATTTTTAAAAATTAAC GAGAATCAAGTGTATTAATCCTCTAGGTATACTTACACAATATACCTTTATTTATATT ATATTTTATAAACCTCCGGGTATACTTACCCAATAGAGATATTTTAGAGACTACTAAC TATACTAGTATATTTTTTTCCTTACCCAATATATTAGTCTAAAAAACTAATATATTGGG TATATTGGGTATGTTCTCAAATTAAATATTCAAATTATTAATATAATGAACAATTCAA AAGATCTAGTTT
47	***ycf1(b)* barcode of 793 nucleotides for species *Cyperus esculentus*** ATTTTTTTGGAAATGATAAAAGGAAGGATATTTTCCCATCTTTTCGAAAAATTTTCAT ATAATGAACTTTACAATCCTTGGGTTTATACCAACAAAGAAAAAGGGAAAAGTTTCA ACAATGAATTTGAAAATAGAATTAAAGCTCTAGACAAAGAAGCTATTTCTTTGAGTG TACTGGAAACAAGAACTCGATTGTGTAATGAGGAAACTACAAAAGAATATTTAACTA AAATATATGATCCCTTCTTGAACGGATCATATCGGAAAACAATCTACAAAAACCTTCC CCTTTCAACATTCAAAAATACTTTGATAGAAAATATCATATATAAATTTAAGATAAAT CGCCTTCATTTTATACTTCTTCCAGATGCCGATTATCGAAAACTTGAACAGAAAAAAA ATAGATTGGAAAAAAAACCATTATCAACGGAAATTGTTAATTTTTTAATTTTAACCAG TAAATCTGTTAGAGAATCAGGATCTCTCAATTGGGTTTGAAAGATCCTTCTTTATTTT CCGAAGGAAGAATAGATTTCGAAAAAGGAAGAAAAAAATTAAAATATTTTTTAACTC AAACTGGTTTTGATGGTCAAAAAATTAGCAGAAAATCGATTAGAATAAAGGAAATCA GTAAAGAAATTCCCCGATGGTCATACAGATTTTTTACCGATTTAGAACAACAATCAGG AGAAGATCAAGAAAAAGTACCAACAGATATTCAAATTCGTTCTAGAAAGGGCAAAG GTGTAGTTATTTTTACTACTAACTGGGAGCATTGTGATTTTAAAG
48	***ycf1(b)* barcode of 688 nucleotides for species *Fimbristylis miliacea*** TTTTTTTTTTAGTATTATCGGTATCTCCAGTATCATTATAAGTATTGTCTTTCTTTAAAA ATTTCGATTTATTTAAAATGACTACACGTTTGGCTTTTCTAGAACGAATTTGATAATTC TCTGCTTCAATTTTTCCGTCCAGTTGTTCTAATTCATCAATAAATTTGTATGACCATCG AGGAACTTTTTTACTGATTTCATTTATTCTAATACATTCAGTTCTATTTCGATTTACTAT TGTTTTATCCTTCAAATCTGTTCTAATTGCATCGAATAAGATTTGGATTATTTTTTTTT TTCTTCTTCATCTTCAGAATTCATTTTTATTTGTTCATCTTCTGGAAATAAATAGAGTG TTTCACAACTTAAGCTTGATAGTGATTTTTGTGAAAATTTATGGATTTGGTTAAAAAA AAAAAATGAAGTTACTAATGCTTTTCGATCAAATGTTTTTATTTTCTCCTCCAATTCTT GATAATTACTATTATTTTGATAAATATTATTATTATTAATATAAAGAAAGATACCATG AATTTTATTTATCAAAATCTGATTTTTTTTGTAAGTTTTTTCATCTTGGATTCGTCCACG AAAAGATCTGTTTAAGAAAGGATCATATATTTTAGTTAAGTATTTTGTTTTAGTTTCAT CATTACACAATCGAATTCGGTTTTCCAATATATCCAG
49	***ycf1(b)* barcode of 742 nucleotides for species *Fimbristylis littoralis*** CCCAGTTTATCAACCTTTTTTGAAATGATACAAAAAAGAATGTTTCTGTTCACGAACG AACAAATTTCTACTGTTGAATTACATAATCATTGGAGTTATCGCAACAAACAAAAATG GAATAAACTAAACAAGGAGTTGATAAACAGAATAAAAAGTCTAAATAAAAAAATTTT CACTTTGAATGGATTCAAAAAAAGAGTTAGATTGTGTAATGATGACACTCAAAAAGA ATATTTACATATAATATATGATCCTTTAGGGAGCGGACCCCGCCGTAAAAAAAAAAA GTTTTTACGTCAAATTCCAAACAAAACTTCCATAAAAAAATTACATGAAGTTTTGGATA AATAAAATTCATAGTATTTTTATTATTACTAATTATCGATACTGTGAAAAAAAAACTAG ATGCATTTGATAGAAAATTTTTTTCAAGAGAAATTTACTCTTTTTTAACCTTAATCAAT CAATTTGCTGAAACATCAAATTTAAAACTCTCTTCATTTCCAGAATATGAAAAAAAAA AATTTGACTCAGAAGATCTATTAAAATTTTTAAAGTTTTTATTTGATACAGTGATTGTG AACGATACGAAACTTAGAAAAAGATCTATTCAAATAAAAGAGATTGATAAAAAAATT CCTCGATGGTCACACAAATTAATCAATGATTTGGAACTACAAGAGAGACAAACTGAG GAAAGCGTAGCTGAGGAGAATGGAATTCGTTCAAGAAAAGCTAAACGTG

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CLAIMS

I claim:

1. A method of generating a DNA barcode for identification of sedge plant species comprising the steps of:
   (a) Collection of or providing a sedge plant,
   (b) DNA isolation from plant or a part thereof,
   (c) Amplification of a plastid based barcode region selected from *ycf1(b)*, *matK*, *trnH-psbA* and *rbcL*,
   (d) Amplification of the step (c) by any one of primer pair consisting of forward and reverse primers of SEQ ID NOs: 1 and 2 for *ycf1(b)*, SEQ ID NOs: 3 and 4 for *matK*, SEQ ID NOs: 5 and 6 for *trnH-psbA*, or SEQ ID NOs: 7 and 8 for *rbcL*;
   (e) Obtaining a sequence of the amplified product,
   (f) Identifying the plant by matching the obtained sequence with a reference library and/or by constructing a phylogenetic tree,
   (g) Obtaining a species specific DNA barcode from the analyzed sequence in step (f).
2. The method as claimed in claim 1 wherein the amplified product of step (e) varies from 688-793 bp for *ycf1(b)*, 545-826 bp for *matK*, 856-883 bp for *trnH-psbA* and 314-347 bp for *rbcL*.
3. The method as claimed in claim 1, wherein the species specific DNA barcodes from *ycf1(b)* are SEQ ID NOs: 47-49.
4. The method as claimed in claim 1, wherein the species specific DNA barcodes from *matK* are SEQ ID NOs: 26-37.
5. The method as claimed in claim 1, wherein the species specific DNA barcodes from *trnH-psbA* are SEQ ID NOs: 38-46.
6. The method as claimed in claim 1, wherein the species specific DNA barcodes from *rbcL* are SEQ ID NOs: 1-25.
7. The method as claimed in any one of claims 1-6, wherein the plant is at least identified as a species of the genus *Cyperus*.

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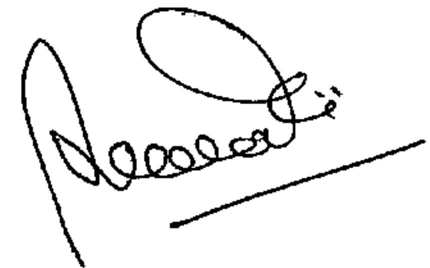
8. The method as claimed in claim 1, wherein step (d) further comprises multiplexing of barcode region primers.
9. A method of identification or authentication of a herbal formulation containing sedge plant or part thereof comprising the steps of:
   a) providing a herbal formulation containing sedge plant or its part,
   b) DNA isolation from herbal formulation,
   c) Amplification of a plastid based barcode region selected from *ycf1(b)*, *matK*, *trnH-psbA* and *rbcL*,
   d) Amplification of the step (c) by any one of primer pair consisting of forward and reverse primers of SEQ ID NOs: 1 and 2 for *ycf1(b)*, SEQ ID NOs: 3 and 4 for *matK*, SEQ ID NOs: 5 and 6 for *trnH-psbA*, or SEQ ID NOs: 7 and 8 for *rbcL*;
   e) Obtaining a sequence of the amplified product,
   f) Identifying the plant by matching the obtained sequence with a reference library and/or by constructing a phylogenetic tree.
10. The method as claimed in claim 9, wherein the sedge plant is *Cyperus rotundus* or *Cyperus esculentus*.

Dated: 10 January 2017



(Tiwari, Sharad)

Applicant

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DRAWING

Applicant: Tiwari, Sharad page 1/1

Application No.:

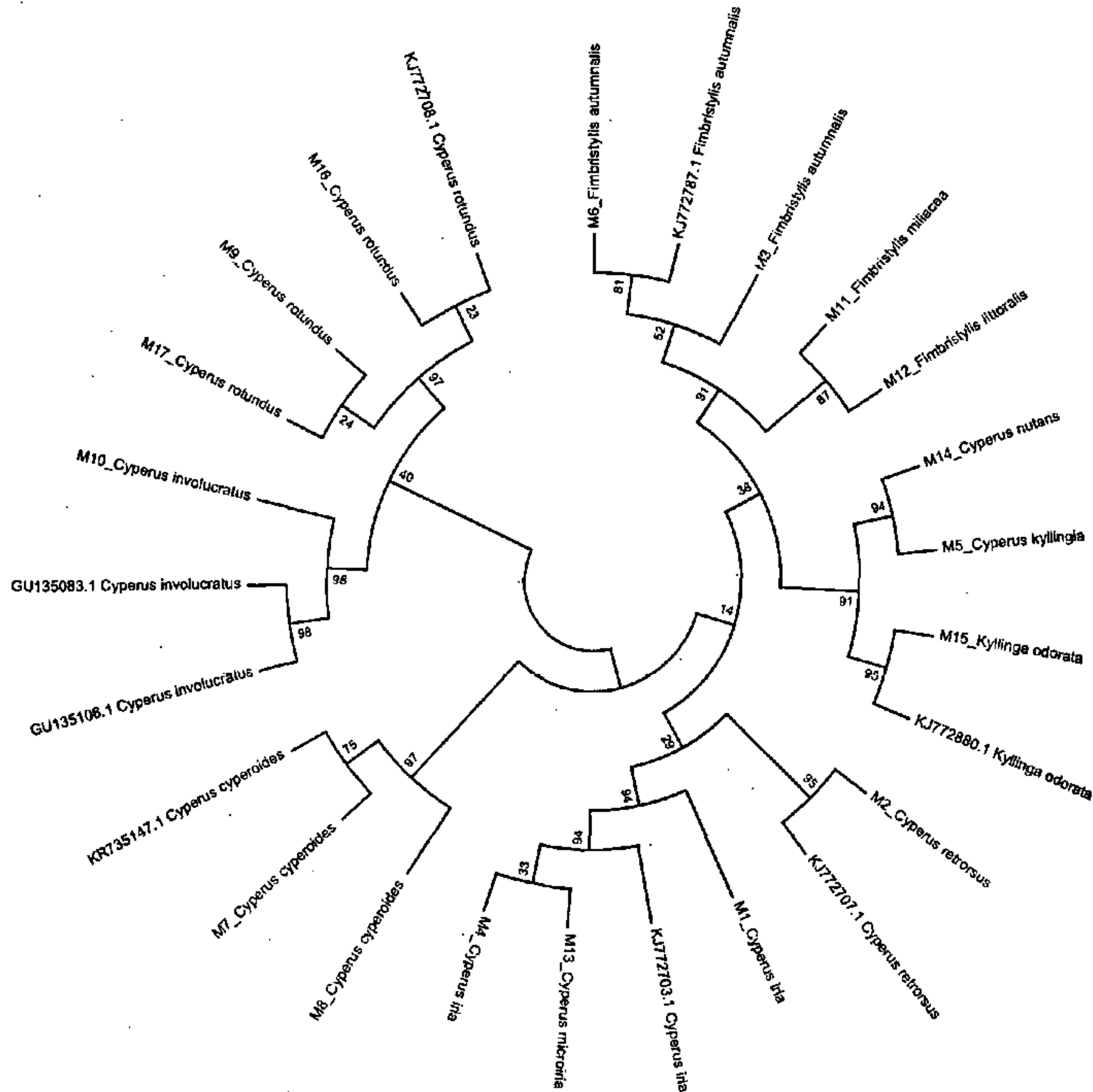

Fig.1: Phylogenetic tree based on *matK* barcode sequences of sedge plant species.

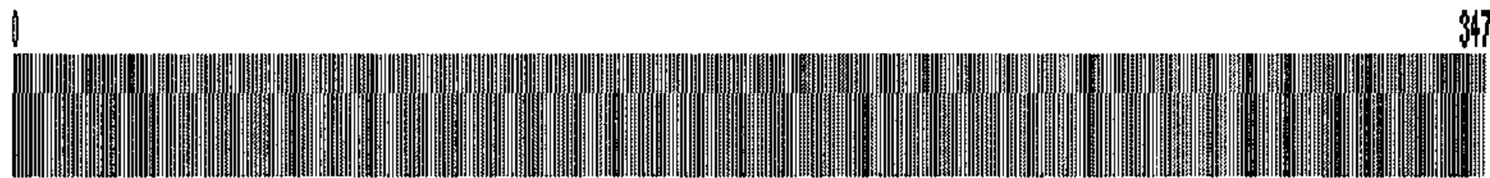

Fig. 2: An illustrative *rbcL* barcode of 347 nucleotides for species *Cyperus rotundus*

Applicant: **Tiwari, Sharad**

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ABSTRACT

"DNA BARCODE FOR SPECIES IDENTIFICATION OF SEDGE PLANTS AND METHODS THEREOF"

Present invention discloses a method of generating a DNA barcode for identification of sedge plant species comprising the steps of Collection of a sedge plant sample, DNA isolation from plant or a part thereof, Amplification of a plastid based barcode region selected from *ycf1(b)*, *matK*, *trnH-psbA* and *rbcL*, with the help of barcode loci specific primer pair consisting of forward and reverse primers, Obtaining a sequence of the amplified product, Identifying the plant by matching the obtained sequence with a reference library and/or by constructing a phylogenetic tree, and finally Obtaining a species specific DNA barcode from the analyzed sequence. The invention also discloses a method of identification or authentication of a herbal formulation containing sedge plant or part thereof e.g. *Cyperus rotundus* or *Cyperus esculentus*, by application of the disclosed method.

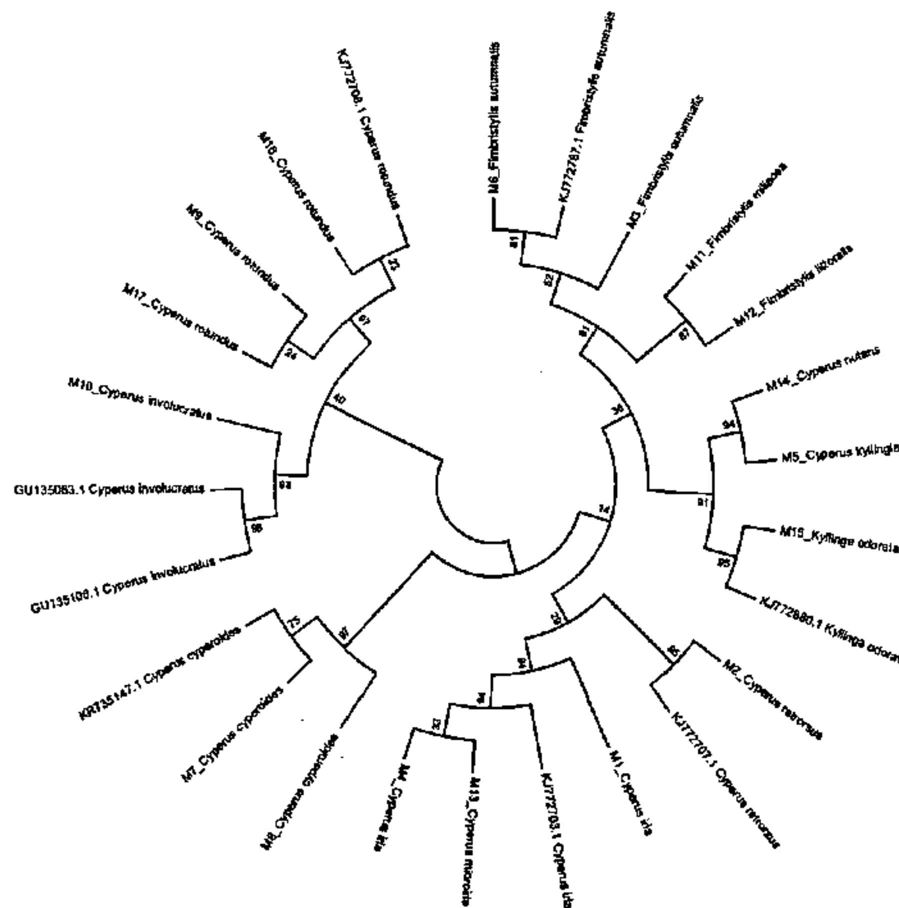

Fig.1: Phylogenetic tree based on *matK* barcode sequences of sedge plant species.

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Research Article

MORPHOLOGICAL VARIABILITY IN THE COMMON SEDGE PLANTS IN INDIA

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Abstract- *Cyperus* is the second largest genus of the sedges or Cyperaceae family, the plants of which are identified as one of the most common agricultural weeds. A total of eighty two sedge plants were collected from twenty one different places covering seven states of India. The plant species and morphological variations among different species were determined based on overall plant growth characteristics. The samples were identified as belonging to seventeen different species of *Cyperus* and related genera on the basis of UPGMA cluster analysis using Jaccard and Simple Matching coefficients. The mantel test coefficient between these two similarity coefficients was 0.97169. Based on morphological variations, plant samples were identified to be belonging to twelve different species of *Cyperus*, three species from genus *Fimbristylis* and two species from genus *Kyllinga*. Several morphological traits were assessed for identification of plants up to species level, among those spikelet was found the best to be used for the identification of sedge species.

Keywords- Sedge, Cyperaceae, Morphological variation, Cluster analysis

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Introduction

Weed populations exhibit a vast range of biodiversity resulting from taxonomic diversity among them including diversity in particular weedy traits that influences their survival, mortality, and reproduction [1]. The ecological diversity of sedges is tremendous; with species occurring in almost all habitats. Sedges mostly prefer moist and sunny sites. Sedges are monocotyledonous flowering plants belonging to family Cyperaceae which is commonly known as sedge family. Sedge plants are mostly grass-like herbs, which are abundantly found in moist and damp areas throughout the world. *Cyperus* having about 650 species, is the second largest genus after *Carex* in the Cyperaceae family. Members of the Cyperaceae (sedge family) resemble the Gramineae (grass family) but are distinguished by three-ranked leaves with one-third phyllotaxy and leaves that have closed leaf sheaths, usually solid stems, absence of a ligule and each flower subtended by a single glume or scale [2]. The morphology of plants belonging to the genus *Cyperus* reported so far is relatively consistent with uniform embryo type. Morphologically all the *Cyperus* species have common characteristics of leaves that are shiny, light to dark green, three-ranked and corrugated in cross section. Leaf initiation terminates with the formation of seed bearing culm. The culm that grows through the center of the leaf fascicle is erect, simple, smooth and triangular in cross section. The culm supports a terminal inflorescence, which is a simple, or slightly compound loose umbel subtended by two or four leaf-like bracts [2].

The *Cyperus* species are herbaceous plants and mostly used as fodder. In particular, *C. rotundus* and *C. esculentus* are used as medicinal herb. They are also called as purple nut sedge and yellow nut sedge, respectively. They are commonly found as a perennial weed with slender, scaly creeping rhizomes, bulbous at the base which is about 1-3 cm. long. They are useful for bowel disorder and inflammatory diseases as well as uterine relaxation in both pregnant and non-pregnant women and relieving pain. *C. rotundus* is a traditional herbal medicine used widely as antimalarial, analgesic, sedative, and treating stomach disorder etc. Anti-bacterial effect is also found in rhizome extract of *C. rotundus* [3]. Antimalarial, anticancer, antimicrobial activities in essential oil from the aerial parts of *Cyperus kyllingia* has also been reported [4]. *Cyperus involucratus* is planted as an ornamental plant. *Cyperus iria* is used as fodder and its stem is woven into mats. Leaf and tuber parts of *C. iria* are used as Tonic, stimulant, stomachic and astringent [5]. *Kyllinga odorata* is known for diaphoretic and diuretic properties [6]. *Kyllinga brevifolia* is a Paraguayan folk medicine and used as sedative and tonic for nervous system [7]. *Fimbristylis miliacea* has been found of an important role in phytoremediation to absorb heavy metals and zinc in waste water treatment [8].

Identification of the plant species of a potential use is not always easy in the absence of knowledge of the most preferred morphological trait to look upon on site of collection. It is also not feasible to apply costly and sophisticated molecular techniques to identify plants, which are otherwise regarded as weeds only. Their correct identification not only helps to formulate effective control strategy but to explore their potential uses too. Morphological parameters are commonly used as tools for investigating genetic relatedness and diversity among plant populations. It is pertinent to study variations in morphological traits among species to determine how plant genotype and diverse environmental conditions could influence the plant morphology of sedges under uniform conditions. We are reporting in the present study, the qualitative genetic--variations in morphological and phonological traits in sedges and the identification of sedge plants of Cyperaceae family up to the species level in plant samples collected at several locations from seven states of India. This study was conducted to document morphological characteristics of most common sedge plant species of *Cyperus* and related genera from different localities in India.

Materials and Methods

A total of eighty two sedge plant samples were collected from several places

located in seven states of India namely Chhattisgarh (CG), Gujarat (GJ), Madhya Pradesh (MP), Rajasthan (RJ), Tamil Nadu (TN), Uttarakhand (UK) and Uttar Pradesh (UP) in the year 2015 [Table-1]. Plant samples were named according to their places of collection. The morphological observations were recorded at various stages of growth at the site of collection as well as from some of the collected plants grown in pots till flowering that occurs in rainy season in India (July-October). Observations were recorded for each plant sample on various morphological traits including root, rhizome, nut, stem or culm, leaf, inflorescence, spike, spikelet, floret, glume etc. For each trait, different observations were tabulated in Microsoft excel sheet and a qualitative data was generated by recording 0 and 1 for the absence and presence of the trait, respectively, in a particular plant sample. This data was subjected to Unweighted Pair Group Method with Arithmetic Averages (UPGMA) based cluster analysis and dendrogram was generated using NTSYSpc version 2.02e software program [9]. The data analysis was done using two different similarity coefficients, Jaccard [10] and Simple Matching [11]. Routine procedures of NTSYSpc program (like SIMQUAL, SAHN, TREE) in appropriate modules were followed to test the clustering analysis based on these two similarity coefficients. For the comparison of original matrices generated by implementing Jaccard (J) and Simple Matching (SM) similarity coefficients, Mantel test [12] was applied in the option of MXCOMP in NTSYSpc program. The plant samples clustered in a group were identified up to species level by comparing the recorded morphological traits with various documented texts such as 'Flora of Jabalpur' [13], 'Flora of British India' [14], 'Flora of India' [15], 'Flora of Gorakhpurensis' [16], 'Hand Book on Weed Identification' [17] including 'eFloras' [18].

Table-1 *Eighty two plant samples collected from different locations and their identification (All are annual to perennial herb, Status: Common, Least Concern in IUCN Red List)*

S.N.	Place and Sample ID	Latitude_Longitude	Collection date	Species Identified	Vernacular Name
1	Chabi-2-MP	"22.825720 N 80.700650 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
2	Chennai-2-TN	"12.990738 N 80.185958 E"	20-Nov-2015	*Cyperus compressus* Linn.	Mothi
3	Jabalpur-1-MP	"23.2203 N 79.9638 E"	15-July-2015	*Cyperus compressus* Linn.	Mothi
4	Jabalpur-2-MP	"23.2095 N 79.9533 E"	15-July-2015	*Cyperus compressus* Linn.	Mothi
5	Kundam-2-MP	"22.845746 N 81.075466 E"	26-Aug-2015	*Cyperus compressus* Linn.	Mothi
6	Mandla-1-MP	"22.611542 N 80.372824 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
7	Mandla-3-MP	"22.601269 N 80.378423 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
8	Niwas-2-MP	"23.035570 N 80.437791 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
9	Amarkantak-2-MP	"22.677726 N 81.758913 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
10	Amarkantak-4-MP	"22.682933 N 81.748302 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
11	Dindori-1-MP	"22.938539 N 81.080024 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
12	Kapildhara-1-MP	"22.700877 N 81.705569 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
13	Sagartola-1-MP	"22.941793 N 81.076845 E"	27-Aug-2015	*Cyperus compressus* Linn.	Mothi
14	Garjiya-1-UK	"29.462150 N 79.153404 E"	30-Sep-2015	*Cyperus cyperoides* Linn.	Flatsedge
15	Garjiya-3-UK	"29.468026 N 79.146967 E"	30-Sep-2015	*Cyperus cyperoides* Linn.	Flatsedge
16	Jabalpur-6-MP	"23.2095 N 79.9533 E"	15-July-2015	*Cyperus cyperoides* Linn.	Flatsedge
17	Jabalpur-13-MP	"23.2203 N 79.9638 E"	15-July-2015	*Cyperus cyperoides* Linn.	Flatsedge
18	Corbett National Park -3-UK	"29.5486 N 78.9353 E"	30-Sep-2015	*Cyperus cyperoides* Linn.	Flatsedge
19	Garjiya-2-UK	"29.466382 N 79.145765 E"	30-Sep-2015	*Cyperus cyperoides* Linn.	Flatsedge
20	Anand-2-GJ	"22.569384 N 72.931226 E"	15-Oct-2015	*Cyperus esculentus* Linn.	Yellow nut sedge, Chufa flatsedge
21	Anand-4-GJ	" 22.56451 N 72.92887 E"	15-Oct-2015	*Cyperus esculentus* Linn.	Yellow nut sedge, Chufa flatsedge
22	Jabalpur-3-MP	"23.2203 N 79.9638 E"	15-July-2015	*Cyperus esculentus* Linn.	Yellow nut sedge, Chufa flatsedge
23	Jabalpur-12-MP	"23.2095 N, 79.9533 E"	15-July-2015	*Cyperus esculentus* Linn.	Yellow nut sedge, Chufa flatsedge
24	Kapildhara-4-MP	"22.700877 N 81.705569 E"	27-Aug-2015	*Cyperus esculentus* Linn.	Yellow nut sedge, Chufa flatsedge
25	Jabalpur-23-MP	"23.2203 N 79.9638 E"	20-Nov-2015	*Cyperus involucratus* Roxb.	Umbrella plant
26	Jabalpur-5-MP	"23.2203 N 79.9638 E"	18-July-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
27	Jabalpur-11-MP	"23.2095 N 79.9533 E"	15-July-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
28	Jabalpur-14-MP	"23.2203 N 79.9638 E"	15-July-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
29	Jabalpur -21-MP	"23.233164 N 79.967455 E"	30-Aug-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
30	Jagdalpur-8-CG	"19.075409 N 82.012864 E"	10-Sep-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
31	Lucknow-2-UP	"26.8429 N 80.9544 E"	01-Jan-2014	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
32	Niwas-4-MP	"23.035570 N 80.437791 E"	27-Aug-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
33	Amarkantak-3-MP	"22.677726 N 81.758913 E"	27-Aug-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
34	Dindori-2-MP	"22.938539 N 81.080024 E"	27-Aug-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
35	Jagdalpur-9-CG	"19.075409 N 82.012864 E"	10-Sep-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
36	Amarkantak-1-MP	"22.677726 N 81.758913 E"	27-Aug-2015	*Cyperus kyllingia* Endl.	Java grass, Red nut sedge
37	Jabalpur-16-MP	"23.2203 N 79.9638 E"	15-July-2015	*Cyperus kyllingia* Endl.	Java grass, Red nut sedge
38	Kapildhara-2-MP	"22.700877 N 81.705569 E"	27-Aug-2015	*Cyperus kyllingia* Endl.	Java grass, Red nut sedge
39	Pantnagar-1-UK	"29.025347 N 79.477147 E"	20-Sep-2015	*Cyperus kyllingia* Endl.	Java grass, Red nut sedge
40	Chabi-1-MP	"22.825720 N 80.700650 E"	27-Aug-2015	*Cyperus microiria* Steud.	Asian flat sedge
41	Jabalpur-4-MP	"23.2095 N 79.9533 E"	15-July-2015	*Cyperus microiria* Steud.	Asian flat sedge
42	Jabalpur-7-MP	"23.2203 N 79.9638 E"	15-July-2015	*Cyperus microiria* Steud.	Asian flat sedge
43	Jabalpur-8-MP	"23.2203 N 79.9638 E"	18-July-2015	*Cyperus microiria* Steud.	Asian flat sedge
44	Jabalpur-9-MP	"23.2095 N 79.9533 E"	18-July-2015	*Cyperus microiria* Steud.	Asian flat sedge
45	Jagdalpur-2-CG	"19.075409 N 82.012864 E"	10-Sep-2015	*Cyperus microiria* Steud.	Asian flat sedge
46	Jagdalpur-4-CG	"19.078032 N 82.004904 E"	10-Sep-2015	*Cyperus microiria* Steud.	Asian flat sedge
47	Jagdalpur-5-CG	"19.078722 N 82.006148 E"	10-Sep-2015	*Cyperus microiria* Steud.	Asian flat sedge
48	Kundam-1-MP	"22.845746 N 81.075466 E"	26-Aug-2015	*Cyperus microiria* Steud.	Asian flat sedge
49	Niwas-1-MP	"23.035570 N 80.437791 E"	27-Aug-2015	*Cyperus microiria* Steud.	Asian flat sedge
50	Niwas-3-MP	" 23.04752 N 80.44701 E"	27-Aug-2015	*Cyperus microiria* Steud.	Asian flat sedge

51	Shahpura-1-MP	"23.185804 N 80.703249 E"	26-Aug-2015	*Cyperus microiria* Steud.	Asian flat sedge
52	Lucknow-1-UP	"26.8429 N 80.9544 E"	01-Jan-2015	*Cyperus microiria* Steud.	Asian flat sedge
53	Kapildhara-3-MP	"22.700877 N 81.705569 E"	27-Aug-2015	*Cyperus nutans* Vahl.	-
54	Kundam-3-MP	"22.845746 N 81.075466 E"	26-Aug-2015	*Cyperus nutans* Vahl.	-
55	Chennai-3-TN	"12.990738 N 80.185958 E"	20-Sep-2015	*Cyperus odoratus* Linn.	Fragrant flatsedge, Rusty flatsedge
56	Kausani-1-UK	"29.846996 N 79.604959 E"	30-Sep-2015	*Cyperus odoratus* Linn.	Fragrant flatsedge, Rusty flatsedge
57	Rewa-1-MP	"24.536571 N 81.272274 E"	20-July-2015	*Cyperus retrorsus* Chapman.	Pine barren flatsedge
58	Amarkantak-5-MP	"22.681982 N 81.753827 E"	27-Aug-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
59	Anand-1-GJ	"22.569384 N 72.931226 E"	15-Oct-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
60	Dindori-3-MP	"22.938539 N 81.080024 E"	27-Aug-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
61	Jabalpur-10-MP	"23.2203 N 79.9638 E"	15-July-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
62	Jabalpur-19-MP	"23.208857 N 79.955460 E"	18-Aug-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
63	Jabalpur -20-MP	"23.233164 N 79.967455 E"	30-Aug-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
64	Jagdalpur-1-CG	"19.075409 N 82.012864 E"	10-Sep-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
65	Jagdalpur-6-CG	"19.078032 N 82.004904 E"	10-Sep-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
66	Corbett National Park-1-UK	"29.5486 N 78.9353 E"	30-Sep-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
67	Corbett National Park-2-UK	" 29.52744 N 78.77467 E"	30-Sep-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
68	Pantnagar-3-UK	"29.025347 N 79.477147 E"	20-Sep-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
69	Anand-3-GJ	"22.569384 N 72.931226 E"	15-Oct-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
70	Chennai-1-TN	"12.990738 N 80.185958 E"	20-Nov-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
71	Jaipur-1-RJ	"26.9000 N 75.8000 E"	30-Oct-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
72	Jabalpur-18-MP	"23.208857 N 79.955460 E"	18-Aug-2015	*Cyperus rotundus* Linn.	Motha, Nagramotha, Purple nut sedge
73	Jabalpur-22-MP	"23.233164 N 79.967455 E"	30-Aug-2015	*Cyperus tenuispica* Linn.	Slender spiked sedge
74	Mandla-4-MP	"22.601269 N 80.378423 E"	27-Aug-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
75	Mandla-2-MP	"22.611542 N 80.372824 E"	27-Aug-2015	*Cyperus iria* Linn.	Morphula, Umbrella sedge, Rice flat sedge
76	Pantnagar-2-UK	"29.025347 N 79.477147 E"	20-Sep-2015	*Fimbristylis autumnalis* (L.) Vahl.	Slender fimbry
77	Jagdalpur-7-CG	"19.075409 N 82.012864 E"	10-Sep-2015	*Fimbristylis autumnalis* (L.) Y. Vahl.	Slender fimbry
78	Jabalpur-17-MP	"23.2203 N 79.9638 E"	15-July-2015	*Fimbristylis littoralis* Gaud.	Lesser frimbristylis
79	Jabalpur-15-MP	"23.2203 N 79.9638 E"	15-July-2015	*Fimbristylis milliacea* (L.) Vahl.	Grass like fimbry Slender fimbry
80	Karanjia-1-MP	"22.710416 N 81.639093 E"	27-Aug-2015	*Kyllinga odorata* Vahl.	Fragrant Spikesedge; Whitehead Sedge
81	Karanjia-2-MP	"22.710550 N 81.638962 E"	27-Aug-2015	*Kyllinga odorata* Vahl.	Fragrant Spikesedge; Whitehead Sedge
82	Jagdalpur-3-CG	"19.075409 N 82.012864 E"	10-Sep-2015	*Kyllinga brevifolia* Rottb.	Green Kyllinga

Results and Discussion

Cluster analysis based on recorded morphological traits in all the eighty two sedge plant samples resulted in to grouping of plant samples in to a total of 17 groups in case of both the clustering methods based on Jaccard and Simple Matching similarity coefficients. The dendrogram generated for both the similarity coefficients has been presented in [Fig-1A & B]. The plant samples in the 17 groups were identical in the groups made by both the methods. Both the methods had shown the plant samples Jagdalpur-3-CG, Jabalpur-15-MP, Jabalpur-17-MP, Jabalpur-22-MP, Jabalpur-23-MP and Rewa-1-MP as groups with single individuals. However, the differences were observed in the major clusters containing various groups of plants. As first major cluster (from top of the dendrogram [Fig-1A & B] made from J coefficient showed only two individual sample groups of Jagdalpur-3-CG and Jabalpur-23-MP while the first major cluster made from SM coefficient showed individual sample groups of Jabalpur-15-MP, Jabalpur-17-MP, Rewa-1-MP and Jagdalpur-3-CG. However, some difference in both the clustering methods is not unexpected as the Jaccard coefficient provides different results compared to the simple matching coefficients, because these do not consider the negative co-occurrences [19].

The Mantel test correlation coefficient value 0.97169 between the two similarity coefficients J and SM was found to be significant at $p < 0.05$, shown in graph [Fig-2] which suggests that the dendrograms constructed from J and SM coefficients are highly correlated. The observed correlation between similarity matrix and phenetic trees indicates the goodness of fit of cluster analysis in accordance with the similarity matrix [20].

The sedge plant samples of each group in different clusters were identified to the species level under family Cyperaceae on the basis of comparing and matching the recorded morphological trait observations with the documented flora texts [13-18]. They were identified as belonging to 17 different species [Table-1] among which twelve species were from genus *Cyperus* namely *C. compressus, C. cyperoides, C. esculentus, C. involucratus, C. iria, C. kyllingia, C. microiria, C. nutans, C. odoratus, C. retrorsus, C. rotundus and C. tenuispica.* Three species were belonging to genus *Fimbristylis* namely *F. autumnalis, F. littoralis and F. milliacea* and two were from genus *Kyllinga* of species *K. brevifolia and K. odoratus* [Plate-1]. Comparisons of different morphological structures of the sedge plant species from the genus *Cyperus, kyllinga* and *Fimbristylis* species from different locations around India are presented in [Table-2]. While the collected sedge plant samples varied from 83-100% [Fig-1], the visible appearances of plant samples of a particular species did not vary in different locations throughout India, indicating that all possessed several traits common within the species. This result is in agreement with the observations of Wills [2] wherein variations occurred in the flower parts including the spike lets in purple nuts edge plant samples collected from 13 states in United States of America and 21 other locations around the world. Cluster analysis using morphological characteristics among purple nutsedge collected from 21 countries and 14 states in the United States showed variations providing evidence for divergent groups of purple nuts edge [21]. However, our results show no location specific morphological variations within a species in India. Wills [2] also found greater variation among plants from different locations around the world than from within the continental United States. Morpho-phenological evaluations conducted among three populations of Redroot Pigweed (*Amaranthus retroflexus* L.) from Jordon also indicated that populations had similar phenological and morphological traits [22]. This is due to the fact that more variations is found in quantitative traits than qualitative traits in plants like yellow nuts edge, where vegetative propagation is the major means of reproduction and maintenance of populations [23].

In order to find out which morphological traits were actually responsible for grouping of individual plant samples in to separate clusters, we divided out the species specific data set in to three parts; vegetative traits (including observations on plant colour, root, rhizome, nut, stem/culm and leaves), inflorescence (including observations on inflorescence, spike and spikelets) and floral traits (including observations on- -glume colour, glume shape, keel, bracts and rachilla); and constructed dendrogram using J and SM coefficients. The vegetative traits grouped all the 17 species individually in case of both the coefficients used [Fig-3]. Similar results were observed in case of inflorescence [Fig-4]. Different species have shown the different pattern of inflorescence, but the floral traits failed to differentiate between the species *C. compressus* and *F. autumnalis* [Fig-5].

Chabi-2-MP
Chennai-2-TN
Jabalpur-1-MP
Jabalpur-2-MP
Kundam-2-MP
Mandla-1-MP
Mandla-3-MP
Niwas-2-MP
Amarkantak-2-MP
Amarkantak-4-MP
Dindori-1-MP
Kapildhara-1-MP
Sagartola-1-MP
Garjiya-1-UK
Garjiya-3-UK
Jabalpur-6-MP
Jabalpur-13-MP
Corbett-3-UK
Garjiya-2-UK
Pantnagar-2-UK
Jagdalpur-7-CG
Jabalpur-15-MP
Jabalpur-17-MP
Kapildhara-3-MP
Kundam-3-MP
Rewa-1-MP
Jagdalpur-3-CG
Anand-2-GJ
Anand-4-GJ
Jabalpur-3-MP
Jabalpur-12-MP
Kapildhara-4-MP
Amarkantak-1-MP
Kapildhara-2-MP
Pantnagar-1-UK
Jabalpur-16-MP
Jabalpur-5-MP
Mandla-4-MP
Jagdalpur-9-CG
Amarkantak-3-MP
Lucknow-2-UP
Jabalpur-21-MP
Jabalpur-11-MP
Mandla-2-MP
Dindori-2-MP
Niwas-4-MP
Jabalpur-14-MP
Jagdalpur-8-CG
Jabalpur-22-MP
Amarkantak-5-MP
Jabalpur-18-MP
Anand-3-GJ
Corbett-2-UK
Jagdalpur-6-CG
Jabalpur-20-MP
Jabalpur-10-MP
Anand-1-GJ
Chennai-1-TN
Pantnagar-3-UK
Corbett-1-UK
Jagdalpur-1-CG
Jabalpur-19-MP
Dindori-3-MP
Jaipur-1-RJ
Jabalpur-23-MP
Chennai-3-TN
Kausani-1-UK
Karanjia-1-MP
Karanjia-2-MP
Chabi-1-MP
Lucknow-1-UP
Niwas-3-MP
Kundam-1-MP
Jagdalpur-4-CG
Jabalpur-9-MP
Jabalpur-7-MP
Niwas-1-MP
Jagdalpur-5-CG
Jagdalpur-2-CG
Jabalpur-8-MP
Jabalpur-4-MP
Shahpura-1-MP

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

0.09 0.32 0.54 0.77 1.00
J Coefficient

Chabi-2-MP
Chennai-2-TN
Jabalpur-1-MP
Jabalpur-2-MP
Kundam-2-MP
Mandla-1-MP
Mandla-3-MP
Niwas-2-MP
Amarkantak-2-MP
Amarkantak-4-MP
Dindori-1-MP
Kapildhara-1-MP
Sagartola-1-MP
Garjiya-1-UK
Garjiya-3-UK
Jabalpur-6-MP
Jabalpur-13-MP
Corbett-3-UK
Garjiya-2-UK
Anand-2-GJ
Anand-4-GJ
Jabalpur-3-MP
Jabalpur-12-MP
Kapildhara-4-MP
Amarkantak-1-MP
Kapildhara-2-MP
Pantnagar-1-UK
Jabalpur-16-MP
Karanjia-1-MP
Karanjia-2-MP
Jagdalpur-3-CG
Jabalpur-23-MP
Jabalpur-5-MP
Mandla-4-MP
Jagdalpur-9-CG
Amarkantak-3-MP
Lucknow-2-UP
Jabalpur-21-MP
Jabalpur-11-MP
Mandla-2-MP
Dindori-2-MP
Niwas-4-MP
Jabalpur-14-MP
Jagdalpur-8-CG
Jabalpur-22-MP
Chabi-1-MP
Lucknow-1-UP
Niwas-3-MP
Kundam-1-MP
Jagdalpur-4-CG
Jabalpur-9-MP
Jabalpur-7-MP
Niwas-1-MP
Jagdalpur-5-CG
Jagdalpur-2-CG
Jabalpur-8-MP
Jabalpur-4-MP
Shahpura-1-MP
Chennai-3-TN
Kausani-1-UK
Rewa-1-MP
Pantnagar-2-UK
Jagdalpur-7-CG
Jabalpur-17-MP
Amarkantak-5-MP
Jabalpur-18-MP
Anand-3-GJ
Corbett-2-UK
Jagdalpur-6-CG
Jabalpur-20-MP
Jabalpur-10-MP
Anand-1-GJ
Chennai-1-TN
Pantnagar-3-UK
Corbett-1-UK
Jagdalpur-1-CG
Jabalpur-19-MP
Dindori-3-MP
Jaipur-1-RJ
Jabalpur-15-MP
Kapildhara-3-MP
Kundam-3-MP

1 2 9 10 16 8 14 11 12 17 15 7 3 5 13 4 6

0.83 0.87 0.91 0.96 1.00
SM Coefficient

Fig-1 UPGMA clustering based on Jaccard (A) and Simple Matching (B) similarity coefficients of morphological data profiles between the 82 individuals of Cyperaceae family. Individual sedge plants are specified by their places of collection. Each cluster group represents a species: 1. *Cyperus compressus*, 2. *Cyperus cyperoides*, 3. *Fimbristylis autumnalis* , 4. *Fimbristylis milliacea*, 5. *Fimbristylis littoralis*, 6. *Cyperus nutans*, 7. *Cyperus retrorsus*, 8. *Kyllinga brevifolia*, 9. *Cyperus esculentus*, 10. *Cyperus kyllingia*, 11. *Cyperus iria*, 12. *Cyperus tenuispica*, 13. *Cyperus rotundus*, 14. *Cyperus involucratus*, 15. *Cyperus odoratus*, 16. *Kyllinga odorata*, 17. *Cyperus microiria*

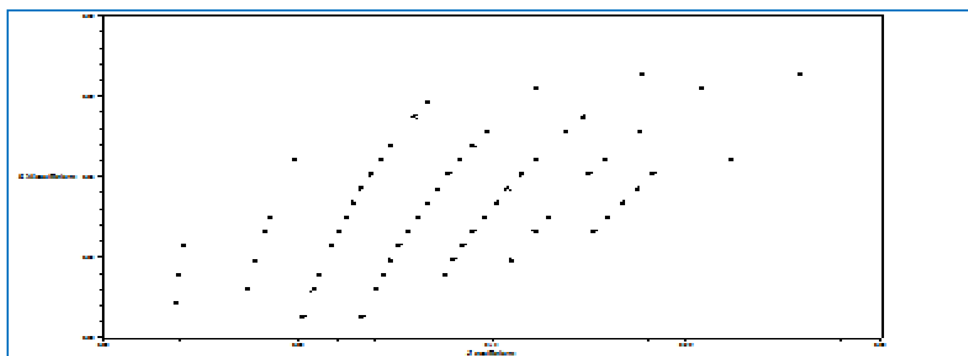

Fig-2 Mantel test graph showing correlation between the two similarity coefficients J & SM

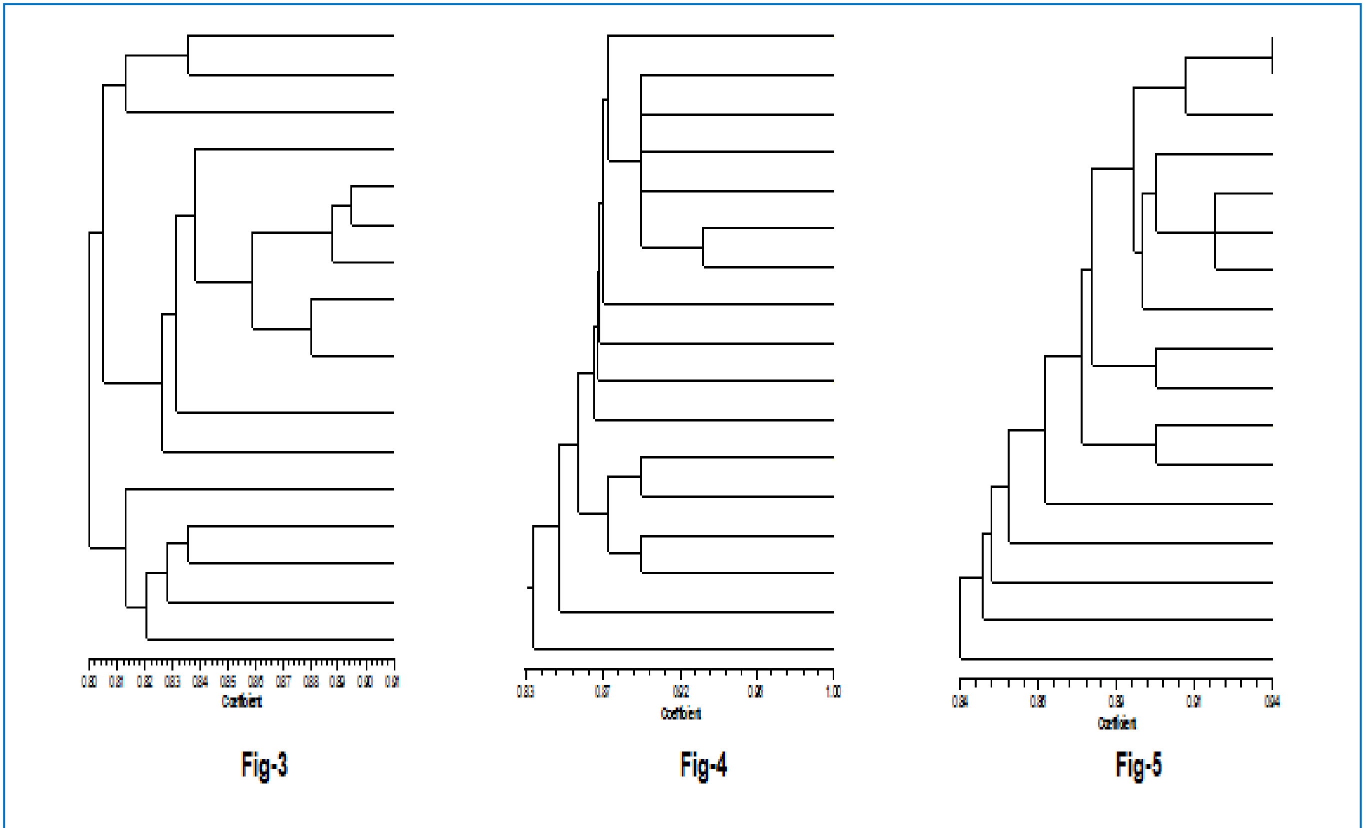

Fig-3-5 Dendrogram showing clustering of different species based on vegetative traits [Fig-3], inflorescence [Fig-4] and floral traits [Fig-5].

Further, we tried to find out a single morphological trait that could be observed for the identification and clustering of the sedge plants. We constructed dendrograms based on most variable morphological traits among the plants of 17 identified species which are Glume (glume colour, glume shape) and spikelet (colour and shape). The clustering based on glume did not identify the species *C. odoratus*, *C. retrorsus* and *F. autumnalis* separately but into a single cluster only [Fig-6]. Spikelet was found to be the best for identifying a sedge plant to its species since the dendrogram constructed [Fig-7] based on the observations on spikelet color and shape grouped all the 17 species individually in case of both the J and SM coefficients

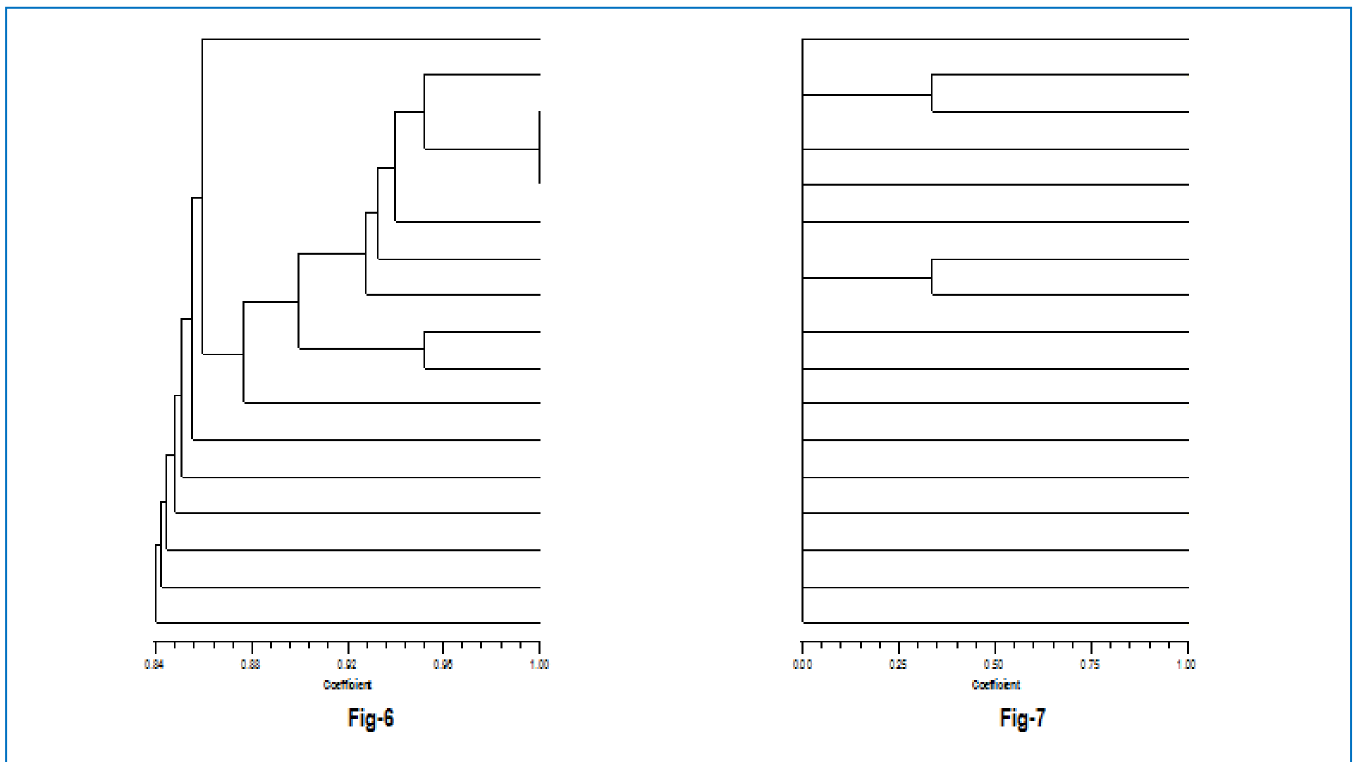

Fig-6-7 Dendrogram showing clustering of different species based on Glume [Fig-6] and Spikelet [Fig-7].

Table-2 *Morphological characters observed in sedge plant samples of different species*

S.N.	Species and its Morphological Characters
1	***Cyperus compressus* Linn.** Annual, Erect herb with numerous, fine tufted root, plant greenish or grayish-green, 5-10 cm high, Stem 3-gonous, Leaves basal, as long as or longer than the stem, Inflorescence umbellate, Spikelets condensed linear or oblong, grey-green or straw-color, Anthela simple, lax, glumes ovate, mucronate, strongly keeled, Glumes green on the sides winged, Stamens 3, shortly apiculate, dark brown to blackish, Nuts broadly triquetrous, dark brown [13, 15].
2	***Cyperus cyperoides* Linn.** A slender grass like sedge, 30-75 cm high. Stems several on a short creeping rhizome covered with the remains of old leaf-sheath. Leaves often as long as or exceeding stem. Spikletes closely spirally arranged in cylindric pedunculate spikes in simple terminal umbel. Fruiting spikelet after falling consists of 2 sub-equal glumes, outer lanceolate oblong, muticous many nerved, inner cymbiform with curved keel. Nuts brown, curved oblong, triquetrous [16].
3	***Cyperus esculentus* Linn.** Stem at base erect, stolons lateral long very slender bearing tubers, leaves and bracts long, spikletets yellow or yellow-brown, glumes over nearly their whole breadth plicate striate (otherwise as *C. rotundus*) [14].
4	***Cyperus involucratus* Roxb.** Perennial plant of height about 2 m with fibrous root, hard rhizomes, triangular stem with leaf sheaths at stem base. Large umbrella-like Inflorescence with 12-25 leaf-like bracts beneath 15 to 27stalked spikelet clusters (rays). A ray has 8-20 shiny brown clusters of tiny flowers (spikelets). Fruit small dark brown triangular achene [24].
5	***Cyperus iria* Linn.** Erect, glabrous annual variable in size and form. Stems striate, 3-quetrous. Leaves basal, blade linear, with papery sheaths and blades scabrid towards apex, sheath brown. Spikelets brown-yellow. Rachilla glabrous, wingless. Glumes obovate. Nuts obovate-elliptic, exceeding the glumes when mature [13].
6	***Cyperus kyllingia* Endl.** An erect, glabrous sedge upto 30cm tall, with well-developed rhizomes. Stem slender, covered with leaves linear, acuminate, rough on midrib and margin bracts 3 to 4, unequal. Spikes splitary, sub-globose white; spikelets obliquely lanceolate, elliptic, containing on flower. The lowest glume hyaline, second glume narrower; third glume boat-shaped with 3 well marked nerves on each side of the prominent winged keel; fourth glume slightly narrower; stamen 2-3. Nut elliptic-ovoid flattened [16].
7	***Cyperus microiria* Stud.** It resembles the rice-field flatsedge (*C. iria*), but has smaller achenes and spikelets. The inflorescence is at the tip of the plant and branched. Inflorescence a compound or decompound anthela; rays 5-9, mostly to 13 cm, unequal in length. Spikes ovoid, broadly ovoid, with many spikelets. Spreading spikelets linear to ovoid shape bearing 8-24 flowers; straight or hyaline white winged rachilla, straw-colored to pale glumes on rachilla, Glumes broadly obovate, rounded apex, keel abaxially extended into a mucro beyond apex [18].
8	***Cyperus nutans* Vahl.** Large size, spikletes recemose (i.e. loosely spicate) ripe suberect, glumes somewhat remote often minutely mucronate. Usually 2-3.5 feet. Umbel primary rays often 8-12 inch spikes bowing on the ultimate rays. Spikletes in ripe fruit collapsing in a tassel [14].
9	***Cyperus odoratus* Linn.** Annual, herb, culms trigonous, spikelets cylindric to subcylindric, corky rachilla disarticulated at base of scale. Segmented mature spikelet consisting of a scale and an internode of the rachilla, achene clasped into corky wings of rachilla [18].
10	***Cyperus retrorsus* Chapm.** Herbs, perennial, shortly rhizomatous. Culms trigonous, glabrous. Inflorescence: spike 1, densely oblong-ovoid often with small basal branches, rachilla deciduous, wings persistent, Spikelets 40–120, oblong-lanceoloid, subterete, distal spikelet spreading or ascending; floral scales persistent [18].
11	***Cyperus rotundus* Linn.** Erect, perennial herb with a woody, stoloniferous rhizome, which is clothed with fibrous. Stems nodose at base leaves radical, shorter than the stem, linear. Inflorescence a compound umbel of short spikes. Spikelets pale yellow or brown often with reddish tinge, glume ovate, straw-colored. Nuts broadly obovoid, greyish-black [13].
12	***Cyperus tenuispica* Steud.** Erect, annual, glabrous sedge. Stems tufted, 15-20 cm tall. Leaves shorter or longer than the stem, linear acute. Umbels compound or decompounds. Spikelets 3-6 mm linear lanceolate. Glumes 0.8 mm long, brown, oblong, rounded. Stamen one. Nuts globosely obovoid [16].
13	***Fimbristylis autumnalis* (L.) Y. Vahl.** Tiny sedge grows in clusters, 5-8 cm tall plants with thread-like leaves, larger leaves are wider than 1.0 mm. Small egg-shaped spikes (3-7 mm long) borne in clusters on top of the flat stems. anthelae compound and mostly diffuse, filiform to linear scapes, usually single bract, blade exceeded by anthela. Mostly narrowly lanceoloid to narrowly ellipsoid brown or red-brown spikelets, fertile keeled lanceolate scales [18].
14	***Fimbristylis littoralis* Gandich.** A glabrous, leafy annual. Stems 15-60 cm long, slender, obtusely angled below and triquetrous above. Leaves shorter than the stem, tapering to a firm point and with nearly smooth margins. Umbels decompounds rays unequal, suberect or spreading, filiform bearing may scattered very small pedicellate spikeletes. Spikletes sub-globose or sub-cylindric, obtuse, brown; rachilla stout, pitted, not winged. Glumes closely imbricate, ovate, stamens 1-3. Nuts obovoid, obtusely trigonous [16].
15	***Fimbristylis miliacea* (Linn.) Vahl.** An erect, tufted, annual sedge. Stems angular. Leaves basal, longer or shorter than the stems scabrid on the margins and midrib beneath. Spikletes very numerous, small, ellipsoid or oblong lanceolate, Spikletes brown, Inflorescence decompound umbels. Glumes ovate; keel 3-nerved, slightly excurrent. Nuts globosely obovoid, pale brown or whitish, minutely tuberculate [13, 16].
16	***Kyllinga brevifolia* Rottb.** A glabrous, 8-20 cm high, slender sedge with horizontally running rhizomes. Stems covered towards the base with usually brown leaf sheath. Leaves few erect, 2-8 cm long, sometimes exceeding the stems, bracts very similar to the leaves. Spikes green oblong cylindrical; Spikelets lanceolate or ovate-lanceolate, containing one flower. The lowest glumes empty and sub-equal; third glume boat-shaped, acuminate, 2 nerves on either side of the keel; fourth glume slightly larger; stamen 2. Nuts obovate ellipsoid, strongly compressed laterally; styles with two filiform arms [16].
17	***Kyllinga odorata*Vahl.** Erect, tufted, rhizomatous perennial; culms 12-29 cm tall, triquetrous. Leaves few, linear, acute, scabrid on upper margins; sheaths, pale brown. Inflorescence a head with 1-3 spikes, whitish-green; central spike cylindrical; lateral spikes globose, much shorter than the central spike; leafy bracts 3-5. Spikelets elliptic, flattened. Glumes 4, braodly ovate, folded with green, smooth keel. Nuts brown to black oblong, apiculate, biconvex.

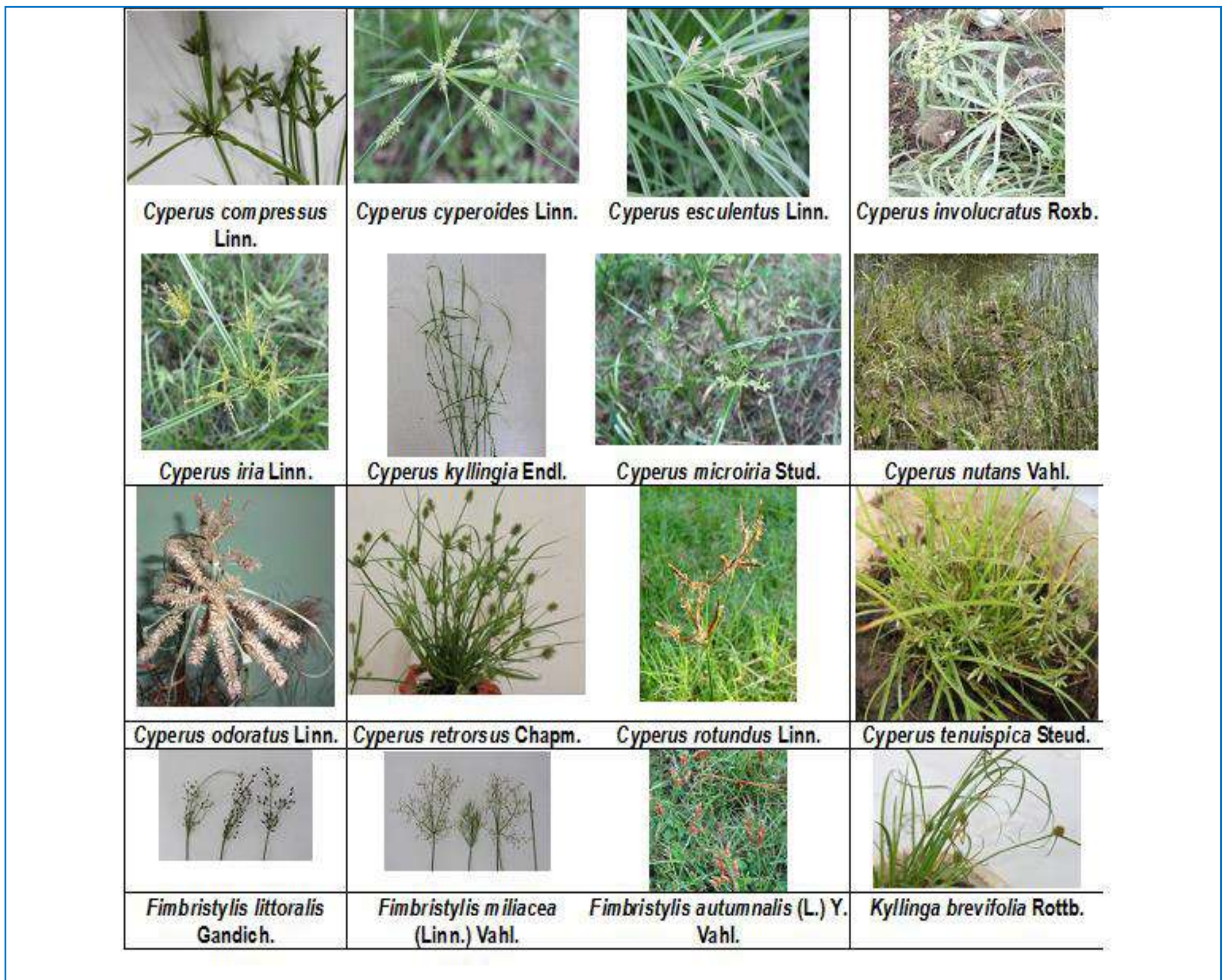

Plate-1 Photo of sedge plant samples from different species

Conclusion

Morphological variations among weed populations play an important role in their competitiveness in actual field situation where they have to compete with agricultural crops for their survival. These variations may also influence their response to chemical or other control strategies. *Cyperus* spp. have shown drastic change in their growth according to climatic conditions for their better adoption and growth. Although morphological descriptions of Cyperaceous have been widely reported [13-16], results of this study shows that to identify a sedge plant spikelet or inflorescence is the best morphological trait to be observed at first. Morphological observations analyzed for cluster analysis can serve as easy and cheapest method of identification in sedge plants. We conclude that sedge plants commonly prevalent in India are mostly belonging to three genera *Cyperus, Fimbristylis,* and *Kyllinga* with significantly distinctive morphological characters. Morphological variations analyzed among seventeen different sedge species of India also indicates that several different ecotypes of sedge species can be passively identified in many different geographical locations not only in India but in around the world

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