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DNA BARCODING IN THE ENDEMIC BAMBOO GENUS *OCHLANDRA* OF THE WESTERN GHATS

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ABSTRACT OF THE PROJECT PROPOSAL

Project Number	:	KFRI RP 629/2011
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Objectives	:	Development of species specific DNA barcodes for the genus *Ochlandra*
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CONTENTS

ABSTRACT

The genus *Ochlandra* Thw. (Poaceae: Bambusoideae), commonly known as reed bamboos is a dominant genus of the Western Ghats, India comprising many taxonomically challenging species. They are one of the economically as well as ecologically important groups of bamboo genus. The similarities between the species in the vegetative characteristics, phenotypic plasticity as well as relative rarity of flowering lead to identification issues in the genus. DNA barcoding has been reported as an effective approach and a supplementary tool to resolve these taxonomic complexities. This study envisages demonstrating the usefulness of DNA barcoding technique to tackle the species identification problems in the endemic reed bamboo genus *Ochlandra* of the Western Ghats. Multiple accessions along with the type specimens of the reported ten *Ochlandra* species were collected from their natural distribution zones. PCR amplification and sequencing in all the species have been done using CBOL recommended four barcode regions (*rbcL, matK, rpoC1* and *trnH-psbA).* The intra and interspecific divergence parameters were calculated using Kimura 2 parameter (K2P) in *MEGA v.6.0*. The results showed that *trnH-psbA+rpoC* combination can identify the species with 100 % species discrimination accuracy with a significant barcode gap. The results suggest synonymisation of *O. spirostylis* and *O. keralensis* under *O. travancorica* as the barcode derived phylogram clustered these three species together with high confidence level in addition to the existing morphological similarities. DNA barcoding has demonstrated as an efficient supplementary tool for the identification of taxonomically challenging species of the endemic bamboo genus *Ochlandra*.

1. INTRODUCTION

Bamboos which belong to family Poaceae and subfamily Bambusoideae, are one of the most successful and diverse group of monocots. For over centuries, bamboos held an important position among the non-timber forest products, supporting livelihood of people living in rural areas. Given the multifarious purposes in both socio-economical as well as ecological aspects, bamboos are often known as 'Poor man's timber' and 'Green Gold of India' (Orhnberger and Georrings, 1986; Tewari, 1992). Worldwide there are 1400 species of bamboo belonging to 115 genera (BPG, 2012). In India, 130 bamboo species under 18 genera are distributed in the Western Ghats, North Eastern India and Andaman and Nicobar Islands, constitute the second largest reserve of bamboo resources in the world (Orhnberger and Georring, 1986; Kumar, 2011). In India, 22 species under 7 genera occur in the Western Ghats and forms a major component of bamboo diversity with a high degree of endemism next to Northeastern India (Seethalakshmi and Kumar, 1998; Kumar, 2011). *Ochlandra* (reed bamboos), an endemic dominant genus in the Western Ghats is considered to be one of the ecologically and economically important groups among the bamboo species. Even though they are heavily exploited for paper and pulp industry with corresponding depletion of natural genetic resources over years, no stringent scientific management practices have been followed in their extraction. Hence this endemic genus requires proper attention from conservation point of view.

1.1. Genus *Ochlandra* - Distribution

The Western Ghats are one among the 34 biodiversity hotspots of the world (Mittermeier, 2008), particularly the Western Ghats of India bordering the States of Kerala and Karnataka. The central Western Ghats, considered as a microspot of endemic

bamboo diversity harbours the endemic genus *Ochlandra* (Gamble, 1896; Kumar, 1990; Uma Shaanker *et al.,* 2004). The region occupies an area of 1600 km and supports about eight major forest types, of which moist evergreen and moist deciduous forests are the dominant ones. Genus *Ochlandra* are confined to the tropical moist deciduous and evergreen forests of the Western Ghats (Fig. 1) as large reed brakes, which extend considerably along the stream sides (Champion and Seth, 1978). *Ochlandra* species are distributed in Kerala part of the Western Ghats except *O. talboti*, which is confined to Karnataka state (Table 1). Most of the *Ochlandra* species prefer moist soil (Thomas and Sujatha, 1992) and require an annual rainfall of more than 1500 mm (Sujatha *et al.,* 2003). All the species are endemic to the Western Ghats, at the same time *O. ebracteata, O. beddomeii, O. setigera* and *O. talboti* are reported to be endangered (Kumar, 1990) and are distributed from an elevation of 200 m upto 1000 m (Gamble, 1896; Raizada and Chatterjee, 1963; Seethalakshmi and Kumar, 1998).

1.2. Flowering pattern

Bamboos have characteristic flowering and fruiting cycle, ranging from a few to 120 years. On the basis of flowering cycles they are classified as annually (*O. scriptoria*) or sporadically (*O. travancorica*) or gregariously flowering bamboos (Gaur, 1987). *O. travancorica* and *O. wightii* are monocarpic in nature and regenerated naturally through seeds (Janzen, 1976). Some species (*O. wightii, O. scriptoria, O. travancorica, O. setigera*) within the genus exhibit both gregarious as well as sporadic flowering behaviour (Basha and Kumar, 1994; Koshy and Harikumar, 2001; Seethalakshmi *et al.,* 2008). *O. scriptoria* flowers annually without subsequent death of the clumps (Gamble, 1896). Later reports revealed that species did not flower annually instead the clump died off after flowering and the flowering period varies from 1-5 years (Koshy and Harikumar, 2001; Koshy and

Mathew, 2009). Seethalakshmi *et al.* (2009) reported the flowering of *O. travancorica, O. sodestromiana* and *O. spirostylis* during 1997-1998. The anthropogenic activities had a greater influence on their regeneration status and both *ex situ* as well as *in situ* methods are recommended for their conservation.

1.3. Propagation

The gregarious flowering of some *Ochlandra* species is found to be a major constraint for propagation from seeds. The unpredictable flowering and the consequent death of the clump along with short seed viability (Seethalakshmi and Kumar, 1998) became major hurdles for raising large scale plantations. Macro and micro propagation methods have also been standardised for the planting stock production in *O. travancorica, O. scriptoria* and *O. travancorica* var. *hirsuta* for raising large scale plantations (Somen *et al.,* 2011). Several reports are available on the vegetative propagation of *O. travancorica, O. scriptoria* and *O. beddomeii* by rooting the culm cuttings (Surendran and Seethalakshmi 1985; Seethalakshmi *et al.,* 1990). Micropropagation through callus induction from isolated embryos in *O. travancorica* and *O. travancorica* var. *hirsuta* (Philip, 1997) and nodal segments in *O. wightii* has been reported (Bejoy *et al.,* 2012). The main limitation reported in vegetative mode of propagation is the unavailability of plant material as well as difficulty in handling the large propagules.

1.4. Ecosystem services and economic importance

Genus *Ochlandra* is one of the economically as well as ecologically important group of bamboos which offers numerous ecosystem services. *Ochlandra* species are usually found as a top cover along the streams with their fibrous roots serving to prevent soil erosion and thereby found to be the potential species in soil conservation (Thomas

and Sujatha, 1992), *O. spirostylis* acts as a potential species in river bank stabilization (Seethalakshmi *et al.,* 2009). *Ochlandra* species provides food and shelter for forest fauna (Basha, 1991), the fruiting of *O. wightii* supported the population of an endemic rodent *Platacanthomys lasyrus* (Gopakumar and Motwani, 2013). *O. travancorica* is the host plant of butterfly larvae of *Parantirrhoea marshalli* (Churi *et al.,* 2014) and *Raorchestes ochlandrae, a* small-sized frog, inhabits the tubular internodes of *O. setigera* brakes (Gururaja *et al.,* 2007). The major *Ochlandra* growing areas are elephant corridors and the high dung encounter rate indicates the heavy inhabitation of elephants in the reed dominant areas (Varma, 2001).

The presence of long fibre in *Ochlandra* species makes it a suitable raw material for paper and pulp industry. They play a significant role for the support of livelihood of the rural communities by providing source materials for construction, fencing, handicraft industries, food and fodder for cattle and for medicinal purposes (Van Rheede, 1685; Mauria and Arora, 1988; Khader *et al.,* 2001; Jayaraman *et al.,* 2008; Prasad and Raveendran, 2010; Gopakumar and Motwani, 2013).

1.5. Taxonomy

The genus *Ochlandra* Thwaites was first mentioned in Van Rheedes' Hortus Malabaricus (1685) but scientifically explained by Thwaites (1864). At first, Beddome (1873) treated the genus *Ochlandra* as 'Beesha'. Gamble (1896) in 'The Bambuseae of British India' described seven species and one variety within the genus *Ochlandra*. Orhnberger and Georring (1986) described 11 species and three varieties within the genus *Ochlandra* and later on various taxonomic revisions have happened and many of the already described species have been merged within the genus. Basha and Kumar (1994) re-described three *Ochlandra* species, *viz*. *O. setigera, O. beddomeii* and

O. travancorica var*. hirsuta* based on the morphology explained by Gamble. After several revisions, nine species and one variety have been described in the genus (Kumar, 1995; Seethalakshmi and Kumar, 1998).

Morphological characteristics employed in the traditional taxonomic classification of the genus showed close affinities among some species and all the revisions have been reported accordingly over the last few years. *Ochlandra* (reed bamboos) attain a maximum height of 10 m and their identification is mainly based on vegetative characters such as culm, culm sheath, leaf, nodes, rhizomes etc. Floral characters are also used for identification if available. The taxonomic keys used for their identification are summarised in Table 1. Culms are usually small, thin walled, erect with comparatively longer internodes and are caespitose in nature. Usually culm size varies from species to species. Culm sheaths are the modified leaves, possess thin, small persistent auricles which are found to be one of the important keys for identification. Leaves are small to large sized, linear or oblong with numerous short petiolated veins and leaf sheaths have striated cartilaginous margin. The presence of ligule at the leaf base is found to be a characteristic feature for identification (Tewari, 1992). The inflorescence is a large compound, spicate panicle with semi-verticillate cluster of spikelets with numerous stamens. *Ochlandra* seeds which are large in size possess thick fleshy pericarp separated from the seed coat. With regards to the rhizomes, the genus *Ochlandra* possess prominent sympodial rhizome with short, thick and curved branching pattern with longer internodes and solitary lateral buds (Seethalakshmi and Kumar, 1998) .

Table 1. The taxonomical key used for the identification of *Ochlandra* species (Kumar 2001a; 2011)

Sl No	Characters	Species
1a	Leaves broad, 8-10 cm across, spikelet ovate-oblong, lodicules broad, 3-4, fruit subglobose to ovate-oblong	**2**
1b	Leaves narrow, 1.5-3.5 cm across, spikelets ovate-lanceolate, lodicules narrow, 6-7, fruit oblong- lanceolate	**7**
2a	Internodes rough, ventral side of the leaf rough, stamens around 40	***O. kadambaranii***
2b	Internodes smooth, ventral side of the leaf smooth, stamens 55-130	**3**
3a	Ligule conspicuous	**4**
3b	Ligule inconspicuous	**5**
4a	Ligule stiff, short, lacerate, 0.3-0.5 cm long	***O. ebracteata***
4b	Ligule membranous, long, fimbriate, 1.5-2.5 cm long	***O. wightii***
5a	Auricle conspicuous; leaf sheath hirsute	***O. keralensis***
5b	Auricle inconspicuous; leaf sheath smooth	**6**
6a	Style coiled or having a bend	***O. spirostylis***
6b	Style straight	***O. travancorica***
7a	Branches few, unequal	**8**
7b	Branches numerous, subequal	***O. talboti***
8a	Sheath tip thin; spikelets glabrous, inner sides of the blade glabrous	**9**
8b	Culm sheath tip thick; spikelets hirsute, inner side of the blade hirsute	***O. beddomei***
9a	Sheath papery, persistent, blade needle like	***O. setigera***
9b	Sheath coriaceous, deciduous, blade narrow	***O. scriptoria***

Later on, *O. spirostylis* and *O. sodestromiana* (Kumar *et al.,* 1999) and *O. keralensis* (Kumar *et al.,* 2001a) were identified as new reed species in the genus *Ochlandra* from

southern Western Ghats. Unnikrishnan (2003) on his revision in 'Bamboos of South India' reported a new species, *O. kadambaranii* from Kollam. Later, Kumar (2011) synonymised *O. travancorica* var. *hirsuta* Gamble, *O. sivagiriana* (Gamble) Camus and *O. soderstromiana* Muktesh & Stephen under *O. travancorica* (Bedd.) Benth. The morphological evaluation of the genus *Ochlandra* revealed that the species belonging to the genus are categorised into two groups *viz. O. scriptoria* group *and O. travancorica* group based on clear morphological differences. As per the latest revision, the genus comprises of ten species distributed in the Western Ghats except *O. stridula* which is confined to Sri Lanka (Kumar, 2011).

Taxonomic identification of the species is mainly relied on morphological characters particularly vegetative characters. This has resulted in poor representation of specimen in the herbaria which leads to taxonomic complexities. The available taxonomic information of the genus indicates a disparity in the number of species reported from various locations and the need for a taxonomic review and field re-survey have also been emphasized (Kumar and Sequira, 1999). *O. scriptoria* in the Western Ghats exhibits morphological similarity with *O. stridula* of Sri Lanka which reflects the affinities of both species (Koshy and Harikumar, 2001; Kumar, 2011). The field identification guide of bamboos provides the vegetative as well as floral features of bamboo species including the genus *Ochlandra* for taxonomic identification (Kumar *et al.,* 2001b). Among the *Ochlandra* species, *O. ebracteata* represents the sixth position having largest leaf among the old world bamboo followed by *O. wightii* and *O. travancorica* (Koshy *et al.,* 2010). *O. ebracteata*, differs from *O. travancorica* mainly on culm sheaths with long and reflexed blades having lacerate ligules (Raizada and Chatterji, 1963). Since most of the vegetative characters are not stable, anatomy of vascular bundle

was also used as a diagnostic character for taxonomic identification in *O. scriptoria* and *O. travancorica* (Appasamy, 1989).

1.6. DNA barcoding for species identification

DNA barcoding is now a well-established technique for species identification in animals. *CO1* region is widely recommended and accepted with high confidence for this purpose because of its low variation within species and high variation among species (Hebert *et al.* 2004). However, *CO1* cannot be adopted for barcoding purposes in plants as the mitochondrial genome is having lower mutation rate than the plastid or the nuclear genome. Chloroplast genome is analogous to the mitochondrial genome of animals due to the conserved gene order, amenability to PCR amplification and availability of universal primers. But the chloroplast genes have a slow rate of evolution and hence species discriminatory power is comparatively lower than that of mitochondrial genes in animals (Ledford, 2008). Chloroplast (*matK*, *rbcL trnH-psbA*) as well as nuclear (ITS1 & ITS2) gene regions have been studied in great detail in taxonomic contexts as the barcode loci for plants (Kress *et al.* 2005, Chase *et al.* 2007; Erickson *et al.* 2008; Kane and Cronk, 2008; Lahaye *et al.* 2008; Chase and Fay, 2009). Beyond the candidate barcodes described above, there are many other widely used plastid barcoding regions, such as *rpoB*, *rpoC*, *atpF-atpH*, *psbK-psbI* and *trnL* (Taberlet *et al.* 2007*).* Thus, there is no single plastid gene region that alone can stand as a universal barcode for all groups of plants. The alternate solution for this problem is to use a core barcode system or by using combinations of barcodes (Chase *et al.* 2007). Different research groups have tested different combinations using various taxa, however universal agreement is yet to be reached.

2. OBJECTIVES

Since morphological characteristics often failed to delimit the species boundaries in the genus *Ochlandra*, a precise molecular tool for species identification is indispensable to tackle the taxonomic complexities. In this context, DNA barcoding, the process of species identification using short standard DNA sequences, has been experimented as a supplementary tool to arrive at a suitable DNA barcode for ensuring taxonomic identity of species within the genus. In our study, the suitability of widely accepted chloroplast gene regions viz., *rbcL, matk, rpoC,* and an intergenic spacer region, *psbA-trnH* were tested to discriminate the species of the endemic bamboo genus *Ochlandra* in the Western Ghats.

This study therefore has been undertaken with the objective of:

- Development of species specific DNA barcodes in the genus *Ochlandra*

3. MATERIALS AND METHODS

3.1. Study area

The Western Ghats bordering the States of Kerala and Karnataka, is considered as a microspot of endemic bamboo diversity, harbours almost all the endemic species (Gamble, 1896; Kumar, 1990; Tewari, 1992; Uma Shaanker *et al.,* 2004). 22 species under 7 genera of bamboo are distributed in the moist deciduous and evergreen forests of the Western Ghats (Kumar, 2011). *Ochlandra*, an endemic genus is mainly confined to the Western Ghats as well as to Sri Lanka and their distribution is shown in Fig.1.

Figure 1. Distribution of *Ochlandra* species

3.2. Sample collections

Leaf samples of all the species of *Ochlandra* were collected from distribution areas especially from their type localities as well as from the available type specimens in the herbarium. Multiple accessions collected were stored in silica gel for further use. The voucher specimens were deposited in KFRI herbarium. The species collected and their distribution zones are shown in Table 2.

Table 2. Species distribution and type locations

Species	**Locations**
Ochlandra beddomei	Thariyode, Kombian
O. travancorica	Agasthyamala, Achankovil, Thenmala, Mananthavady, Ponmudi, Nelliyampathy, Silent Valley, PTR, Malyattur, Pooyamkutty, Sholayar, Sringeri, Nedumgayam
O. spirostylis	Chaatuparakudy, Idukki
O. keralensis	Pachakanam, Pathanamthitta
O. wightii	Kallar, Palode, Bonacaud, Ponmudi
O. kadambaranii	Nilamel, Mukkada, Villumala, Choondipara
O. scriptoria	Peruvannamoozhi, Manimala, Vazhachal
O. setigera	Gudallur, Nadugani, Vazhikadavu
O. ebracteata	Paruthipally range, Kottur Reserve, Ambanad Waterfalls
O. talboti	Karike, Jog Falls, Mookambika WLS

3.3. DNA Extraction

Genomic DNA extraction was carried out using modified Cetyl trimethyl ammonium bromide (CTAB) method as well as DNeasy Plant Mini Kit (Qiagen, USA) according to manufacturer's protocol. Total genomic DNA was extracted from all the collected samples.

In the CTAB method, 20 mg of silica dried leaf tissues was ground in liquid nitrogen using a chilled mortar and pestle. The powdered tissue sample was transferred to pre-warmed (65^{o}C) CTAB extraction buffer. RNase (10 mg/ 100 ml) was added to this slurry and the samples were incubated at 65^{o}C on a water bath for two hours with gentle inversion for every 5 minutes. After incubation, the lysate was allowed to cool to room temperature. An equal volume of chloroform: isoamylalcohol (24:1) was added to the lysate and mixed properly by inversion and centrifuged at 8,000 rpm for 5 minutes. The supernatant was pipetted out carefully and transferred to a 1.5 ml microcentrifuge tube. A half volume of 5M NaCl and double volume of ice cold ethanol were added to precipitate the DNA. The solution was centrifuged at 5000 rpm for 10 minutes. The DNA pellet was air dried to remove the residual ethanol. The pellet was dissolved in sterile water and stored in -20˚C deep freezer for further use. DNA extractions were carried out using this commercial kit as per manufacturer's protocol (Qiagen, USA) with slight modifications. The DNA sample was stored in the deep freezer at 20^{0}C until further use.

The isolated genomic DNA was subjected to electrophoresis to visualise the DNA. The samples were separated on 1.5 per cent agarose gel and then stained in ethidium bromide and visualised under UV transilluminator (Fig. 2). DNA was quantified using a spectrophotometer (Nanodrop Fisher Thermo., USA).

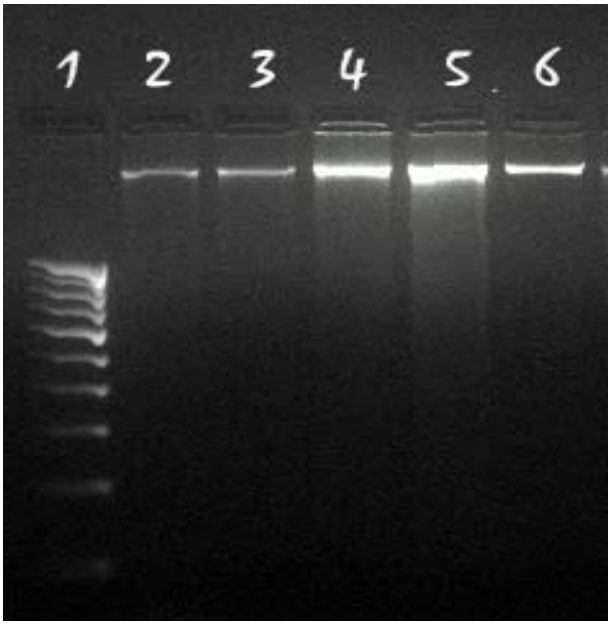


Figure 2. Total genomic DNA

3.3. Polymerase Chain Reaction

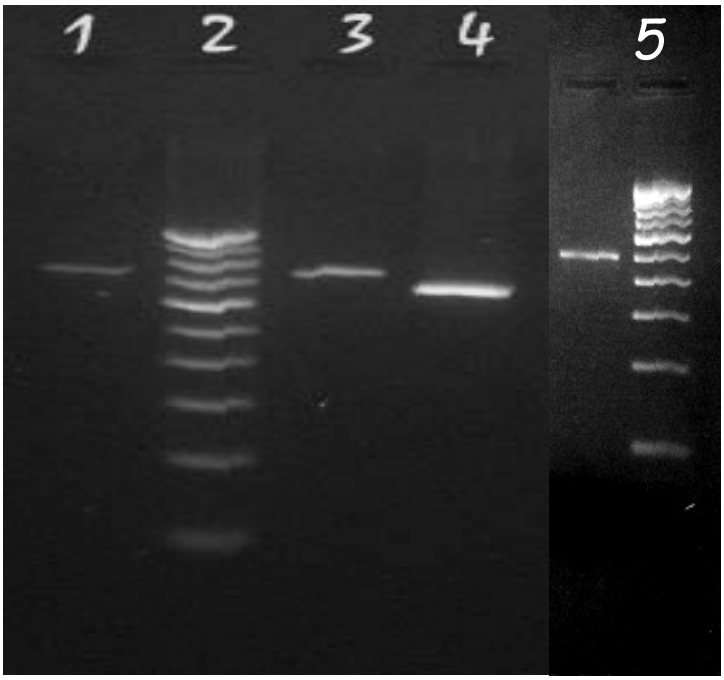
Four candidate barcode regions including three coding genes (*matk, rbcL, rpoC*) and an intergenic spacer, *trnH-psbA* from the chloroplast genome have been selected for DNA barcoding of ten *Ochlandra* species. The barcode regions, the primer sequences and the reaction conditions used are provided in Table 3.

PCR was performed to amplify the gene sequences in 20 µl reaction volume, containing 1 µl genomic DNA, 2 µl primer (20 picomoles), 200 µM dNTPs, 2 µL of 10x Taq buffer, 2U of Taq DNA polymerase. DNA was amplified by a programmable thermal cycler PTC 200 (MJ Research Inc., USA). PCR reaction was performed with the following conditions, initial denaturation of 5 minutes at 94 ^{0}C, cycle denaturation of 1 minute at 94 ^{0}C, cycle annealing of 1 min at 60 ^{0}C and cycle extension of 1 min at 72 ^{0}C for 35 cycles and final extension at 72 ^{0}C for 10 minutes. Annealing temperature varies from primer to primer as well as species to species.

Table 3. Primer sequences of four candidate DNA barcodes and their reaction conditions

Barcode region	**Primer**	**Primer sequence 5'-3'**	**Reaction condition**
rbcL	1F 724R	ATGTCACCACAAACAGAAAC TCGCATGTACCTGCAGTAGC	94°C 5 min. 94°C 1 min. 60°C 1min. 72°C 1min. 34 cycles 72 °C 10 min.
matk	472F 1248R	CCCRTYCATCTGGAAATCTTGGTT GCTRTRATAATGAGAAAGATTTCTGC	94°C 10 min. 94°C 1 min. 57°C 1min. 72°C 1.5 min. 34 cycles 72 °C 12 min.
psbA- *trnH*	*psbA* *trnH*	GTWATGCAYG AACGTAATGCTC CGCGCATGGTGGATTCACAATCC	94°C 5 min. 94°C 1 min. 60.5°C 1min. 72°C 45 min. 34 cycles 72 °C 10 min.
rpoC	*rpoC* Forward *rpoC* Reverse	GGCAAAGAGGGAAGATTTCG CCATAAGCATATCTTGAGTTGG	94°C 5 min. 94°C 1 min. 57°C 1min. 72°C 1min. 34 cycles 72 °C 10 min.

PCR products were resolved by 2% agarose with the same protocol as above. Electrophoresis was performed in agarose gel by applying constant voltage to resolve the products and documented with Alpha Imager (Alpha Innotech, USA) (Fig. 3).



Lane1: *matK*
Lane 2: 100bp DNA ladder
Lane 3: *rbcL*
Lane 4: *trnH-psbA*
Lane 5: *rpoC*

Figure 3. PCR amplification of *matK, rbcL* and *trnH-psbA* barcode regions

3.4. Elution of PCR products

PCR reaction has been scaled up to 50 µL volume for the purpose of elution. Elution of the PCR product was done by Nucleospin gel and PCR clean up kit as per the manufacturer's protocol (Machery-Nagel, U.S.A.). DNA sequencing was performed for the eluted PCR products in both forward and reverse directions employing Sanger's dideoxy chemistry.

3.5. Sequence data analysis

The raw sequence chromatograms obtained after sequencing were edited using *BioEdit* Software v.7.0. The edited sequences were used for multiple sequence alignment in *CLUSTAL X* (Thompson *et al.,* 1997). The sequences after alignment were then subjected to BLAST sequence similarity search in NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The Kimura 2-parameter (K2P) nucleotide substitution model was used for calculating pairwise distances with *MEGA v.6.0* (Tamura *et al.,* 2013). Average pairwise intraspecific divergence (K2P distance), mean theta (ϑ), and average coalescent depth were used to characterize intraspecific divergence, as described in Meyer and Paulay (2005). An ideal barcode should have high interspecific but low intraspecific divergence. Intraspecific as well as interspecific divergence parameters were compared for all the analysed barcode regions along with their possible combinations. DNA barcoding gaps were calculated by comparing intra and interspecific genetic distances (Kress and Erickson, 2008). Wilcoxon signed rank tests for testing the significance of inter-intraspecific divergence were also performed (Lahaye *et al.* 2008). Positive species identification by a candidate barcode was counted only if multiple individuals formed a monophyletic cluster in the Neighbor-Joining trees (*MEGA v.6.0*), according to the method of Starr *et al.* (2009). Clade support was estimated with 1,000 heuristic bootstrap replicates (100 random addition cycles per replicate, with tree bisection-reconnection and branch-swapping) to test the reliability of the inferred phylograms.

4. RESULTS AND DISCUSSION

4.1. PCR Amplification, sequencing and alignment

All the analysed four barcode regions (*matK*, *rbcL*, *rpoC* and *trnH-psbA*) were successfully amplified with 100 % PCR efficiency with the primers recommended by CBOL Plant Working Group (2009). Multiple Sequence Alignment (MSA) of *rbcL* and *matK* gene didn't show any nucleotide differences where as *trnH-psbA* and *rpoC* sequences exhibits variations within and among the species in the *CLUSTAL X* program for multiple sequence alignment (Fig. 4). All the sequences were subjected to *BLAST* similarity searches and were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 4.).

Table 4. Genbank accession numbers for the generated barcode sequences

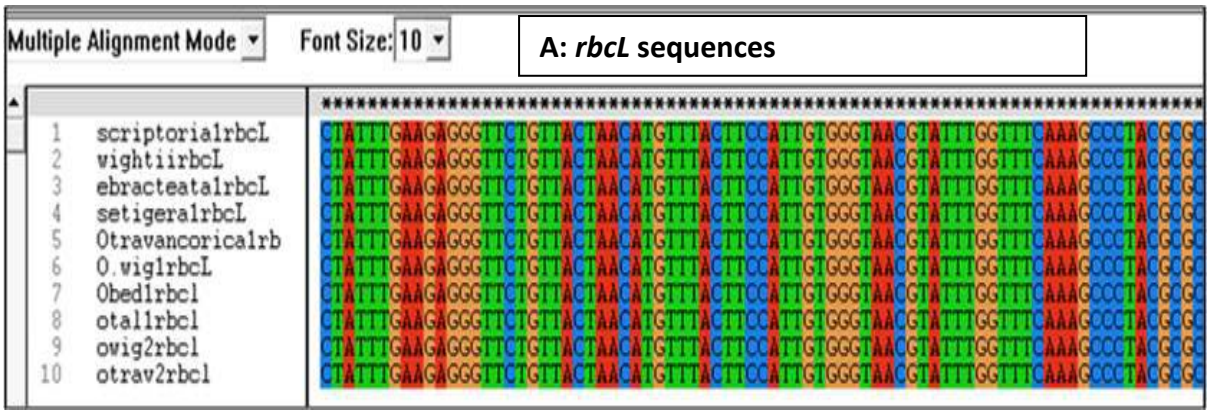
Sl No	Species	Gene region	Accession No.	BankIt ID
1	*Ochlandra travancorica*	*rbcL*	JQ 710845	Banklt 1516995
2	*O. setigera*	*rbcL*	JQ 710844	Banklt 1516988
3	*O. ebracteata*	*rbcL*	JQ710842	Banklt 1516950
4	*O. wightii*	*rbcL*	JQ 710846	Banklt 1516996
5	*O. talboti*	*rbcL*	JX185540	Banklt 1545339
6	*O. scriptoria*	*rbcL*	JQ 710843	Banklt 1516981
7	*O. travancorica*	*matk*	JX 390634	Banklt 1551866
8	*O. setigera*	*matk*	JX 390635	Banklt 1551871
9	*O. ebracteata*	*matk*	JX 390636	Banklt 1551875
10	*O. talboti*	*matk*	JX 185551	Banklt 1545337
11	*O. setigera*	*trnH-psbA*	JX 502807	Banklt 1557800
12	*O. wightii*	*trnH-psbA*	JX 502809	Banklt 1557804
13	*O. talboti*	*trnH-psbA*	JX 502808	Banklt 1557801
14	*O. scriptoria*	*trnH-psbA*	JX 502805	Banklt 1557794

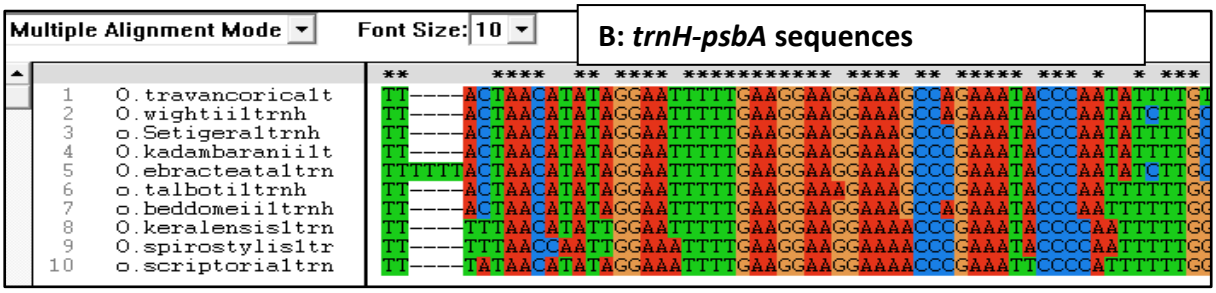
Based on the number of conserved sites, among the barcode regions, *rbcL* was the most conserved locus (680/ 690 nucleotides) where as *trnH-psbA* (43/ 621) had the greatest nucleotide variation followed by *rpoC* (56/ 482). The parsimony informative sites were highest for *rpoC* (33/ 482), followed by *trnH-psbA* (27/ 621). Sequence length and basic sequence statistics like conserved sites, variable sites and singletons based on the results of *CLUSTAL X* alignment as well as with alignment explorer in *MEGA v.6.0,* are provided in Table 5.

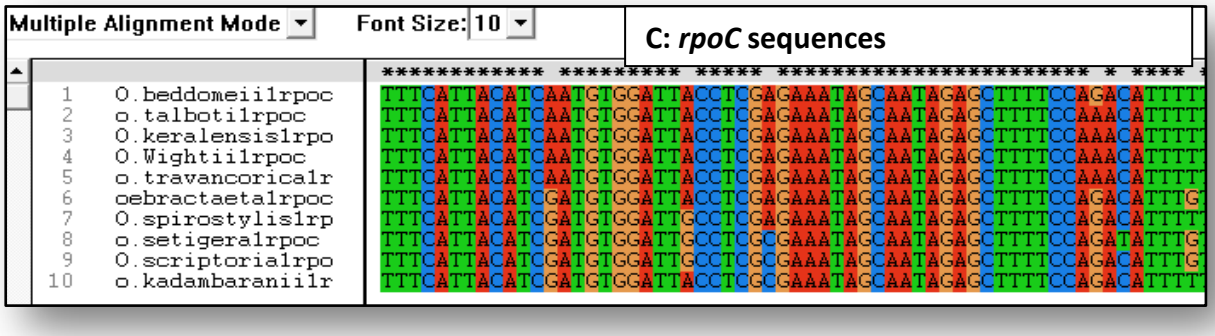
Table 5. Basic sequence statistics

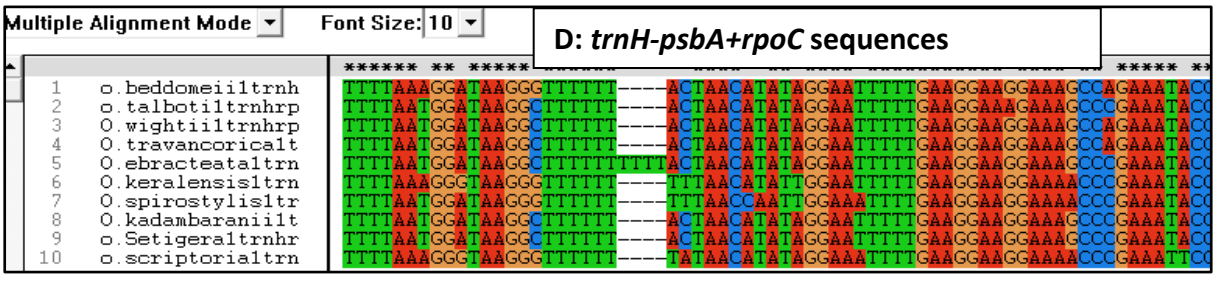
Comparison	***rbcL***	***matk***	***trnH-psbA***	***rpoC***	***trnH-psbA+rpoC***
Sequence length (bp)	690	724	621	482	1095
Conserved sites	680	717	559	424	970
Variable sites	1	4	43	56	105
Informative sites	1	2	27	33	66
Singleton site	0	2	15	21	38

Figure 4. Multiple sequence alignment of gene sequences: A-*rbcL*; B-*trnH-psbA*; C-*rpoC*, D-*trnH-psbA +rpoC* combined sequence data









4.2. Species divergence and barcode gap analysis

Three parameters (*viz.* average interspecific distance, theta prime, and the minimum inter-specific distance) were employed to characterize interspecific divergence, and the average intraspecific distance, mean theta, and coalescent depth were employed to calculate intraspecific variation (Table 6.). By comparing the interspecific divergences of four candidate DNA regions (i.e., *rbcL, matK, rpoC* and *trnH-psbA*), *rpoC* had the highest inter-specific divergence, average interspecific distance and theta prime followed by *trnH-psbA*. Six parameters for the intraspecific and interspecific genetic divergence were calculated for the two-locus combinations of *rpoC+trnH-psbA* as well. Using Wilcoxon signed rank test, a significant difference between the inter and intraspecific divergences could be observed only for the *rpoC* barcode and the two locus combination of *trnH-psbA*+r*poC*, with their interspecific divergences significantly higher than the intraspecific variations. Thus a well defined DNA barcoding gap was exhibited by *trnH-psbA*+*rpoC* combination, while other regions *viz*. *matK* and *rbcL* did not show any significant barcoding gap (Fig. 5).

Figure 5. DNA barcoding gap for the *rpoC* barcode and the *trnH-psbA+rpoC* combination

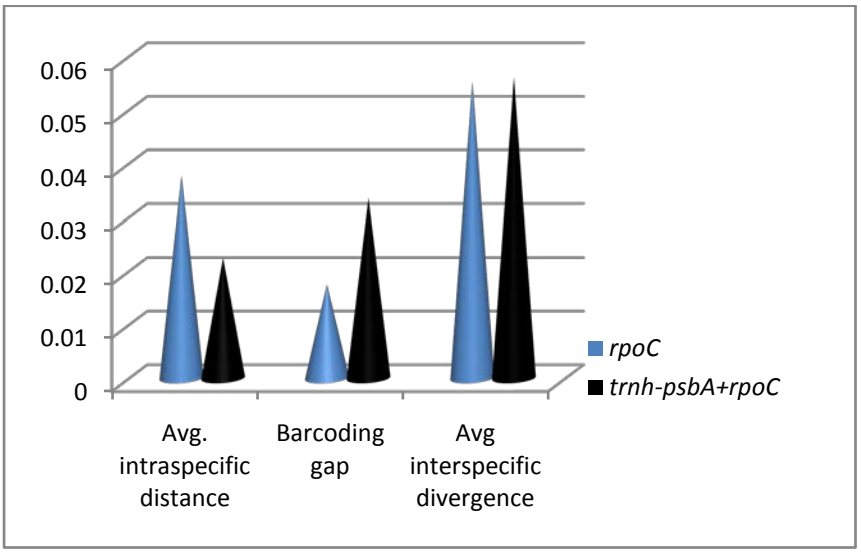


Table 6. Genetic divergence parameters

Parameters	***rbcL***	***matk***	***trnH-psbA***	***rpoC***	***trnH-psbA +rpoC***
Average intraspecific distance	0	0.0003± 0.0008	0.0122 ± 0.0034	0.0378 ± 0.0077	0.0225 ± 0.0038
Average theta	0	0.0082±0. 0050	0.0059 ± 0.0017	0.0119 ± 0.0025	0.0043 ± 0.0044
Average coalescent depth	0	0.0027± 0.0019	0.0027 ± 0.0067	0.0031 ± 0.0050	0.0102 ± 0.0017
Average interspecific divergence	0	0.0073± 0.0053	0.0177± 0.0036	0.0553 ± 0.0111	0.0561 ± 0.0046
Minimum interspecific distance	0	0	0	0	0
Average theta prime	0	0.0097± 0.0033	0.0176 ± 0.0046	0.0455 ± 0.0114	0.0217± 0.0049

Thus DNA barcoding technique has been reported to be a valid tool to confirm species identity in case of taxonomic complexities (Pang *et al.* 2010, Hou *et al.* 2013). An ideal DNA barcode region should be evolutionarily conserved so as to have lower variability within a species and greater variability among congeneric species of a genus (Taberlet *et al.* 2007). The CBOL-Plant Working Group recognizes *rbcL* as one of the gene sequences with potential for DNA barcoding in plants. However, due to its low ability for species discrimination, most of the working groups suggested the use of *rbcL* in conjunction with other gene regions (Chase *et al.,* 2007; Hollingsworth *et al.,* 2009). Similarly, *matK* has proved its utility as a potential barcode in several closely related groups, such as *Compsoneura* (Newmaster *et al.,* 2007), orchids (Lahaye *et al.,* 2008), sedges (Starr *et al.,* 2009) and *Acacia* (Newmaster and Ragupathy, 2009), but universality of this barcode remains uncertain. In the present study, we tested the feasibility of four gene regions (*matK*, *rbcL, rpoC,* and *psbA-trnH*) as potential barcodes in *Ochlandra* species. Out of the four barcode loci (*rbcL, matK, rpoC* and *psbA-trnH*), *viz. rbcL* and *matK* failed to discriminate *Ochlandra* species.

Chloroplast intergenic *trnH-psbA* spacer has been a popular barcode locus for species discrimination in plants (Monkheang et al., 2011; Newmaster et al., 2008). The presence of higher number of variable sites in this region could offer high level of species discrimination in plants (Kress *et al.,* 2005; Kress and Erickson, 2007). Eventhough, the presence of mononucleotide repeats, inversion and duplication events in *trnH-psbA* spacer region pose a problem in sequencing, these nucleotide changes are stable within species and hence exploited for plant barcoding studies (Fazekas *et al.,* 2008). From the pooled sequence data of angiosperms, gymnosperms and cryptogams, *trnH-psbA* region showed a success rate of 93 % amplification, good discrimination and their sequence length varies from 300 to 1000

bp from species to species (CBOL, 2009). The *trnH-psbA* region has been reported for the species level identification in *Ocimum* (Christiana and Annamalai 2014), *Dendrobium* (Yao *et al*., 2009), *Rhododendron* species (Liu *et al.,* 2012), to identify the Amazonian trees (Gonzalez *et al.,* 2009), Italian trees (Piredda *et al.,* 2010) and also as a potential barcode in the large family Umbelliferae (Degtjareva *et al.,* 2012). In the genus *Ochlandra*, the sequence length of *trnH-psbA* ranges from 550-600 bp, which is found to be constant in all the species, even though mononucleotide repeats were present. *trnH-psbA* displayed a wide variation but the amount of intraspecific variation was slightly higher than interspecific variation and in the absence of a well defined DNA barcoding gap, cannot be used alone for discriminating *Ochlandra* species.

Combinations of barcode loci often result in a more efficient species discriminant core barcode and have been reported for species discrimination in various plant genera. *trnH-psbA* exhibited more efficiency as a barcode when combined with other gene regions in certain taxa such as in pteridophytes (Ebihara *et al.,* 2010) and in Myristicaeae using *trnH-psbA+matK* (Newmaster *et al.,* 2007). Out of the eight chloroplast regions tested, the core combination *rbcL+matK* showed only 50 % resolution in Proliferae where as *trnH-psbA + ITS* showed 100 % resolution (Yan *et al.,* 2011). Zuo *et al.* (2011) reported that *trnH-psbA+*ITS combination was sufficient for identifying all the species of Ginsengs (*Panax*, Araliaceae). In woody angiosperms, a combination of *trnH-psbA+ITS* showed higher levels of variation and potential discriminatory power (Clement and Donoghue, 2012). Armenise *et al.* (2012) suggested *rbcL+trnH-psbA* combination, in terms of universality and efficacy, for identifying the taxonomically challenging conifers where as in the genus *Lamium*, the best-performing barcode were found to be *matK+trnH-psbA* (Krawczk *et al.,* 2014). For the authentication of

Cassia for medicinal purpose, Purushothaman *et al.* (2014) found that *rbcL+trnH-psbA* showed 100 % species discrimination *where as matK+rbcL* showed only 90 % discrimination.

Core barcode has greatly improved identification ability in Roscoea (Zingiberaceae), where *ITS+trnH-psbA* could effectively discriminate 90 % species when compared to *rbcL* and *matK* gene regions (Zhang *et al.,* 2014). The identification efficiency of Apiaceae was increased to 82.2 % using *ITS+trnH-psbA* marker combination which was significantly higher than that of *rbcL+matK* (40 %) (Liu *et al.,* 2014). Similarly, *rpoC* gene region alone may not generate a good barcode in some taxa, but proposed to be used in combination with other gene regions for efficient species discrimination (Chase *et al.,* 2007; Fazekas *et al.,* 2008; Hollingsworth *et al.,* 2009; Shneyer, 2009). The highest discriminatory power in *Populus* species was reported using the combination of two intergenic spacers and a coding region, *trnG-psbK+psbK-psbl+rpoC* (Schroeder *et al.,* 2012).

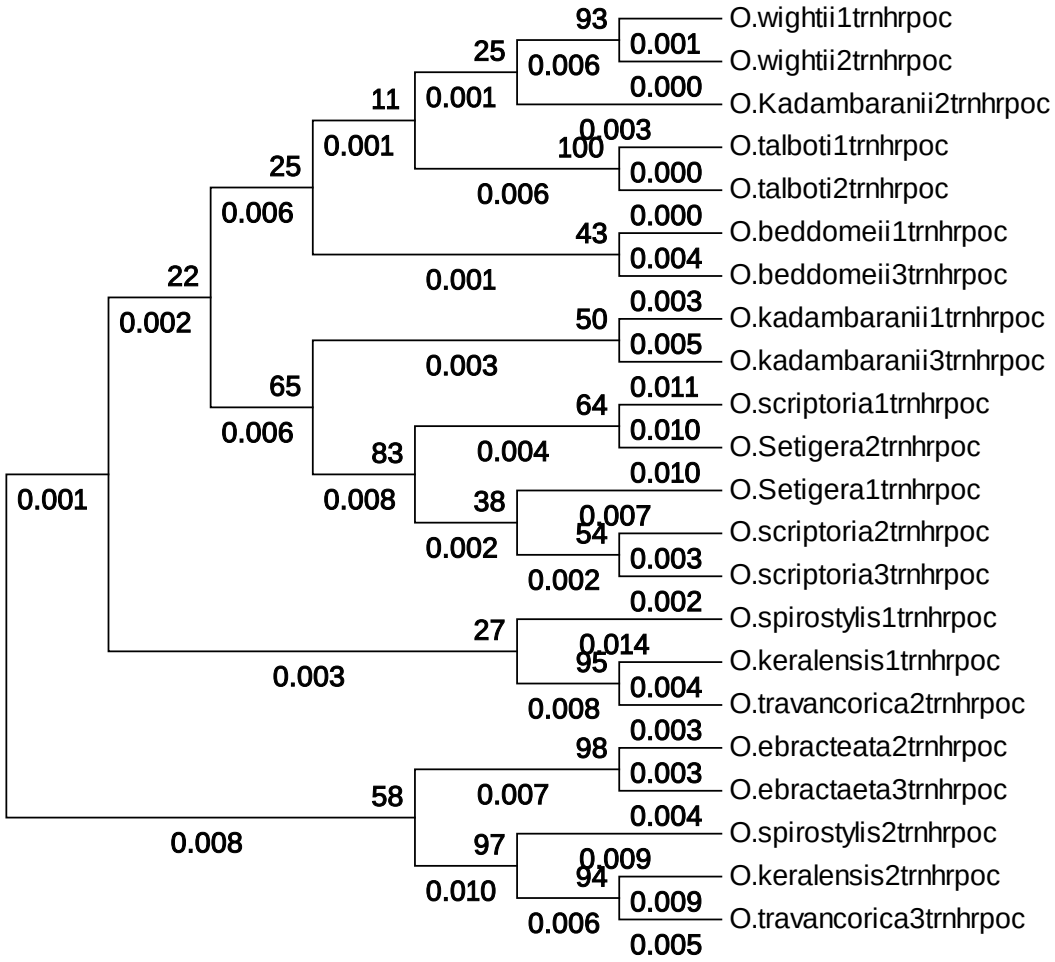
Eventhough, all the analysed barcode regions have high efficiency in PCR amplification and sequencing, only combination of *trnH-psbA* and *rpoC* barcode regions showed species discrimination in the genus *Ochlandra*. *trnH-psbA+rpoC* combination discriminated all the analysed species of *Ochlandra* with a significant barcode gap. Monophyletic clustering was displayed by most of the species in the phylogram generated using K2P parameter in *MEGA v.6.0*. *Ochlandra spirostylis*, *O. travancorica* and *O. keralensis* clustered together in the phylogram without any discrimination among the species. The morphological similarities displayed by these three *Ochlandra* species also point out that the species boundaries of *O. keralensis* and *O. spirostylis* are not so distinct from *O. travancorica*. This suggests the need for synonymising *O. keralensis* and *O. spirostylis* under *O. travancorica.* Based on the analysis of all the four recommended barcodes, *trnH-psbA+rpoC* combination is appeared to be the best barcode for discriminating the *Ochlandra*

species in the present study. DNA barcoding thus provides a supplementary tool for the species identification of morphologically similar species of *Ochlandra* as well as to tackle the taxonomical issues prevailing in the genus.

4.3. Taxonomic implications

O. spirostylis is a species published by Kumar et al. (1999) from Chattuparakkudy settlement in Adimali, Idukki district. The authors mentioned that the species is closely allied to *O. setigera*, but Unnikrishnan (2002) suggested that this species is closely related to *O. travancorica*. Major difference of this species from *O. travancorica* is in its coiled or bend nature of style. Kumar (2011) pointed that two types of flowers were found within this species and in majority of flowers the style is spirally coiled and in some cases with an 'S' shaped bend. The molecular analysis has confirmed that this species is a synonym of *O. travancorica.* Similarly, *O. keralensis* is another species reported from Pachakkanam forest area in Pathanamthitta district (Kumar et al., 2001). In the protologue, the authors mentioned that this species is closely related to *Ochlandra wightii* and differs in having hairs on the leaf sheath of the flowering twigs and hairs on the basal part of the mucronate tip of the sterile glumes. The number of lodicules present in this species is reported to be four (Kumar et al., 2001). However, Kumar (2011) stated that this species is closely allied to *O. travancorica* and a majority of flowers have three lodicules and when four lodicules are present one is very small. The present barcode analysis on the samples representing the type locality has proven that this species is a morphological variant of *O. travancorica*.

Figure 6. Phylogram based on *rpoC+trnH-psbA* sequences



5. SUMMARY AND CONCLUSIONS

Species identification within the genus *Ochlandra*, using morphological features alone, is a challenging task due to intraspecific variability of vegetative parts and the unavailability of floral features in most instances. This study evaluated the role of DNA barcoding as an alternative or as a supplementary tool to address the taxonomic complexities prevailing in the ten reported species of the endemic bamboo genus *Ochlandra*. The CBOL recommended four barcode regions *viz. rbcL, matK, rpoC and trnH-psbA* were examined for their usefulness as a DNA barcode in discriminating the *Ochlandra* species. Of the candidate barcode regions analyzed, *trnH-psbA+rpoC* combinations demonstrated lowest intra-specific variation and highest inter-specific divergence with significant barcoding gap and species identification accuracy. Our findings showed that *the trnH-psbA+rpoC* region can be used as a core barcode to identify *Ochlandra* species and to discriminate the species more effectively than other barcode regions. In the generated phylogram of the above combined barcode sequence data, monophyletic clustering was displayed by most of the *Ochlandra* species except for *O. keralensis* and *O. spirostylis* which clustered along with *O. travancorica* at high confidence level. The morphological similarities displayed by these three *Ochlandra* species also pointed out that the species boundaries of *O. keralensis* and *O. spirostylis* are not so distinct from *O. travancorica*. This suggests the need for synonymising *O. keralensis* and *O. spirostylis* under *O. travancorica.*

DNA barcoding technique was able to discriminate the species boundaries in the endemic bamboo genus *Ochlandra* of the Western Ghats. The reported core barcode, *trnH-psbA+rpoC* can be adopted for addressing the biosystematics in other bamboo genus as well. This core barcode can serve as a rapid species identification tool for the certification of planting materials in priority bamboo species at the nursery stage before the establishment

of plantations. As such, this method provides a robust technique that complements conventional methods for the identification of taxonomically complex and morphologically indistinguishable species.

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