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Taxonomic distinction of *Calamus nambariensis* Becc., in Northeast India based on morphological and molecular markers

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ABSTRACT: *Calamus nambariensis* Becc. is a high quality of rattan, threatened to the Northeastern region of India. The species was first described by O. Beccari based on a fragmentary specimen collected by G. Mann from Nambor Wildlife Sanctuary of Assam, India. In this study, an extensive survey was carried out to locate the occurrence of *C. nambariensis* in the Northeastern part of India and to determine their diversity and discrimination through morphological and molecular markers. Specimens collected from six localities; Nambor Wildlife Sanctuary (WLS), Hoollongapar Gibbon Sanctuary, Dehing Patkai WLS of Assam and Kimin, Khonsa and Namdapha National Park of Arunachal Pradesh showed considerable morphological variations among themselves. These differences are accommodated by assigning the status of subspecies as *Calamus nambariensis* subsp. *nambariensis* and *Calamus nambariensis* subsp. *arunachalensis*, and varieties such as *Calamus nambariensis* subsp. *nambariensis* var. *nambariensis* and *Calamus nambariensis* subsp. *nambariensis* var. *hoollongapariensis.* The taxonomic delimitation is also endorsed by *mat*K, *trn*L-CD, and *trn*L-EF sequence analysis which strengthened our findings. The variations observed among the *C. nambariensis* occurring in northeastern India based on morphological and genetic diversity might be due to their adaptation in different biogeoclimatic conditions over the time.

KEY WORDS: Arecaceae, morphological diversity, genetic diversity, phylogenetic analysis, *mat*K, *trn*L-CD, *trn*L-EF.

INTRODUCTION

Rattans are spiny climbing palms belonging to the family Arecaceae that comprise around 600 species belonging to 14 genera in the world and mostly confined to South East Asia (Dransfield, 1981). Of the 14 genera of rattans, *Calamus* is the largest genus with about 370 species. These are naturally distributed in the South East Asia from Fiji Island to Africa and from southern China to Queensland (Australia) with the greatest concentration in the Dipterocarp Rain Forests of the Malay Archipelago (Weidelt, 1990).

India is one of the countries harboring high repositories of rattans with over 60 species under five genera namely *Calamus*, *Daemonorops*, *Salacca, Korthalsia* and *Plectocomia* (Renuka, 1999). They are mostly confined in the wet evergreen forests in three areas; Western Ghats of Peninsular India, sub-Himalayan tracts of the North-Eastern Himalaya and the Andaman and Nicobar Islands (Uma Shaanker *et al*., 2004).

Of the genera of Arecaceae, *Calamus* Linnaeus belonging to the sub-family Calamoideae is by far the largest genus with 374 species widely distributed from India eastwards to Fiji and a few in tropical Africa (Asmussen *et al.*, 2006; Dransfield *et al*., 2008; Sreekumar and Henderson, 2014). The highest concentration of the species under the genus is located in tropical Asia, especially the Malay Peninsula, Borneo and Sumatra. In India, 46 species of *Calamus* are distributed in three major regions *i.e.,* Northeastern India, the Western Ghats and the Andaman and Nicobar Islands (Rama Bhat *et al*., 2010; Renuka, 1999; Renuka and Sreekumar, 2012).

Calamus nambariensis was first described by O. Beccari based on material collected by G. Mann from Nambar forest in Assam (India) in 1888 (Beccari, 1908; Deka *et al*., 2018a,b). The species was originally known to be occurring only in Nambar forest of Assam but subsequently, the species has also been reported from Lohit, Changlang, Tirap and Upper Subansiri districts of Arunachal Pradesh; India (Thomas and Haridasan, 1997; Haridasan *et al*., 2002; Baruah *et al*., 2017; Deka *et al*., 2017).

In this study, details morphological analyses were carried out for taxonomic distinction among the *C. nambariensis* occurred in Northeastern part of India. Moreover, the taxonomic delimitation was also endorsed by few evolutionary based molecular markers for strengthening our findings.

MATERIALS AND METHODS

Collection of the plant materials

Field survey was carried out in different parts of North East India during 2014-2016 covering different seasons of the year to find out the species in its natural habitats and collection of specimens. The species initially identified as *Calamus nambariensis* could be found in six different localities were collected, studied in their natural habitats, documented and brought to the laboratory for further analyses. In Assam, it was collected from Nambor WLS, Hoollongapar Gibbon Sanctuary and Dehing Patkai WLS and in Arunachal Pradesh, from Kimin forest, Khonsa forest and Namdapha National Park (Fig.1).

Fig. 1. Distribution of ***C. nambariensis*** in Northeastern India (red stars indicate the areas of occurrences)

Morphological analysis

The specimens of *C. nambariensis* were collected with their reproductive parts for morphological analysis. Morphological features viz., the diameter of the stem with or without leaf sheath, length of internodes, the colour of leaf sheath in juvenile as well as mature stage, the shape of ocrea, presence or absence of indumentum and its color, type and arrangement of the spine, presence of flagellum or cirrus, arrangements of leaflet i.e. regular/irregular and grouped, type of knee, color, and length of inflorescence, type, and length of inflorescence bract, length, shape, and arrangement of rachillae, size, shape, and color of fruits, etc. were recorded during the fieldwork. Although descriptions of the specimens were drawn mainly from fresh and live specimens during fieldwork, herbarium specimens were also used to record certain features. Relevant literature was consulted along with voucher specimens of different herbaria viz., ASSAM, CAL, NEHU, APF, ARUN, GUBH (Beccari, 1908; 1913; 1914; Basu, 1985; 1992; Thomas and Haridasan, 1997; Renuka, 1999; Evans *et al.,* 2001; Haridasan *et al.,* 2002; Rama Bhat *et al.,* 2010; Renuka and Sreekumar, 2012). All the specimens of *C. nambariensis* were preserved as herbarium specimens (Jain and Rao, 1977) and deposited in the Botanical Survey of India, Eastern Regional Centre (ASSAM) and the Gauhati University Herbarium (GUBH).

DNA extraction, amplification and sequencing

Fresh leaf material was grounded with liquid nitrogen and total DNA was extracted using the CTAB protocol of Doyle and Doyle (1987) and purified with the HiPurA^{TM} Plant Genomic DNA Miniprep Purification Kit (HiMedia; MB507-50PR). DNA amplification and sequencing of the *mat*K, *trn*L-CD and *trn*L-EF regions were performed using the primers (*mat*K390f: 5′-CGATCTATTCATTCAATATTTC-3′ and *mat*K1326r: 5′-TCTAGCACACGAAAGTCGAAGT-3′; *trn*LC-f: 5′-CGAAATCGGTAGACGCTACG-3′and *trn*LD-r: 5′-GGGGATAGAGGGACTTGAAC-3′; *trn*LE-f: 5′-GGTTCAAGTCCCTCTTATCCC-3′and *trn*LF-r: 5′-ATTTGAACTGGTGACACGAG-3′). The PCR amplification was performed in a total volume of 25µl with 50 ng DNA template, 1 µl 10× PCR buffer, 0.2 Mm dNTPs, 5 pmol each primer and 1 unit *Taq* DNA polymerase (Qiagen, Germantown, Maryland, USA) following 5 min pre-heating at 95ºC, 1 min denaturation at 95°C, 1 min annealing at 50°C (*mat*K), 53.8°C (*trn*L-CD) and 57°C (*trn*L-EF) and 1 min extension at 72°C. The reaction was repeated for 34 cycles before the final extension step at 72°C for 5 min and the reaction was stopped at 4°C. The PCR amplification was performed using the SimpliAmp Thermal Cycler PCR System (Applied Biosystems). The PCR products were checked by 1.5 % agarose gel, stained with ethidium bromide for the quality and quantity prior to cycle sequencing. Fragment sizes were estimated comparing with the 100 bp DNA ladder (Chowdhury *et al.,* 2014; Rahman *et al.,* 2012).

The PCR products were purified using the HiPurA^{TM} PCR Product Purification Kit (HiMedia; MB512-50PR) according to the manufacturer's instruction. The purified PCR products were sequenced in both directions with the primers used for DNA amplification. Sequencing was

Table 1. Morphological attributes of ***C. nambariensis*** subsp. ***nambariensis*** and ***C. nambariensis*** subsp. ***arunachalensis***.

Attributes	***C. nambariensis*** subsp. ***nambariensis***	***C. nambariensis*** subsp. ***arunachalensis***
Colour of leaf sheath	Grey or yellowish grey in juvenile and yellowish green in mature stage, sometimes having straw coloured patches.	Grey with dark blackish transverse stripes in juvenile and greenish yellow with grayish tint in mature stage.
Large spine		
Shape and size	Deflexed with broad and concavo- convex swell base, margin of spine smooth	Deflexed with flat base, margin of spine undulates or rarely smooth.
Colour	Base of the spine is shinny dark brown and middle part is yellowish gray.	Base of the spine is dull grey and middle part is greenish gray.
Small spine		
Shape	Facing outward, base swollen, spine triangular, compactly arranged, stout, sharply pointed.	Facing upward, often slightly bent outward, base flat, triangular, bluntly pointed.
Colour	Shiny blackish brown in colour.	Dull blackish brown in colour.
Knee		
Shape and colour	Nose sharp; shinning yellowish green with transverse brownish strips.	Nose flattened often with a basal constriction; dull grayish green with brownish transverse strips.
Spine on knee	Spine present or absent on the lateral parts of knee, facing upwards; Dorsal surface of knee often have spine, facing upwards, 0.1–0.2 cm.	Both lateral and dorsal surface having spine; facing downward, 0.1–0.6 cm.
Indumentum	Juvenile leaf may be or may not be contain hair like indumentum	Juvenile leaf contains hair like indumentum.
Shape of petiole	Slightly triangular in cross section	Elliptical in shape in cross section
Spine on petiole	Dorsal surface have larger spine with several small scattered spines, 0.1–0.5 cm; ventral surface often have scattered unequal spines, facing outwards, base sought.	Dorsal surface have larger spine with occasional few small scattered spines, 0.1–0.3 cm; Ventral surface have scattered, unequal spines, facing upwards, base flattened.
Shape of rachis	Basal part triangular and distal part quadrangular in cross section	Basal part elliptical and distal part triangular in cross section
Spine on rachis	Spines on the dorsal surface shiny, yellowish green in colour, facing downwards; marginal spines flat, basal part swollen, shining yellowish and distal part shiny blackish-brown in colour	Spines on the dorsal surface dull blackish-brown in colour; facing upwards; marginal spines, flat, basal part dull straw coloured and distal part dull blackish brown in colour.
Cirrus	Yellowish green	Dull green
Spine of Cirrus	Base of the spine shiny straw colour and distal part of spine shiny black; 4–11 number of spine in a cluster.	Base of the spine dull straw coloured and distal part of spine dull black; 2–8 number of spine in a cluster.
Ocrea	Tip blunt; 0.8–1.3 cm long and 4–4.7cm in diameter.	Tip pointed; 0.5–0.7 cm in length and 4–4.5 cm in diameter.
Fruit	Ovoid to ellipsoid with abruptly short-beaked, Shiny brown, 1.6–2 cm.	Ovoid, globose to ellipsoid, with a distinct long beak, rusty brown, 1.8–2 cm

performed using custom sequencing services with the use of the BigDye® Terminator v3.1 cycle sequencing kit with Applied Biosystems highest capacity-based genetic analyzer platforms.

Data analysis and construction of phylogenetic tree

The sequence data obtained by amplification using *mat*K, *trn*L-CD and *trn*L-EF primers were searched for homology using BLAST (Altschul*et al*., 1990) against the NCBI non-redundant database. However, no homologous sequences against the queries of *trn*L-CD and *trn*L-EF sequences were found and homologous sequences with more than 95% identity were retrieved for *mat*K sequence. Here, the alignment followed by phylogeny was constructed among the experimental sequences for *trn*L-CD and *trn*L-EF. The analyzed dataset for *mat*K consisted of 19 nucleotide sequences: 6 experimental and 13 downloaded sequences respectively. The sequences were aligned using CLUSTAL X2 (Thompson *et al*., 1994). Pairwise distance among the individuals in and out-groups was calculated using MEGA6.2, maximum likelihod (ML) tree was generated using default parameters (Kimura, 1980; Kumar *et al.,* 2016; Nei and Kumar, 2000; Rzhetsky and Nei, 1992; Saitou and Nei, 1987). The phylogenetic tree was constructed using the bootstrap value at 500 replicates (Felsenstein, 1983; 1985).

RESULTS

Morphologicla features

Morphological features of all the specimens of *C. nambariensis* collected from six localities; three each from Assam and Arunachal Pradesh (India) clearly separated into two distinct groups. Although the specimens from Nambor WLS and Hoollongapar Gibbon Sanctuary of Assam showed certain differences, yet they could be considered in one clade. Similarly, specimens collected from Dehing Patkai of Assam, and three localities of Arunachal Pradesh *viz*., Khunsa, Kimin and Namdapha could be considered as another group. The most distinguishing features of both the samples of *C. nambariensis* of Assam and Arunachal Pradesh were mentioned in Table 1. Further, the specimens from Nambor WLS and Hoollongapar Gibbon Sanctuary also exhibited certain differences mentioned in Table 2. On the other hand, the specimens from Dehing Patkai, Namdapha, Kimin, and Khunsa did not exhibit marked differences except in their color of the leaf sheath. However, these color variations of the leaf sheath were not assigned to any

Table 2. Morphological attributes of ***C. nambariensis*** ssp. ***nambariensis*** var. ***nambariensis*** and ***C. nambariensis*** ssp. ***nambariensis*** var. ***hoollongapariensis***

Attributes	***C. nambariensis*** ssp. ***nambariensis*** var. ***nambariensis***	***C. nambariensis*** ssp. ***nambariensis*** var. ***hoollongapariensis***
Colour of leaf sheath	Grey in juvenile and yellowish green in mature stage.	Yellowish grey in juvenile and yellowish green with straw coloured patches in mature stage.
Large spine		
Shape and Size	Large spine 4–4.5 cm long and 0.6–0.8 cm in width	Large spine 3.5–4.2 cm long and 0.6–1cm in width.
Colour	Base of the spine is straw- or copper coloured, shiny and middle part is yellowish gray.	Base of the spine is dark chocolate in colour, shiny and middle part is yellowish gray.
Small spine		
Shape	Compactly arranged, arranged linearly with scattered scars of undeveloped spines.	Loosely arranged, arranged scattered.
Colour	Dark brown in colour, shiny.	Shiny blackish brown in colour.
Shape and colour of Knee	Nose sharp; shinning yellowish green with transverse brownish strips.	Nose sharply pointed; shinning yellowish green, transverse strips absent.
pine on Knee	Spine present or absent on the lateral parts of knee, facing upwards; dorsal surface of knee often have spine, facing upwards, 0.1–0.2 cm.	Spine absent on the lateral as well as dorsal surface of knee.
Indumentum	Juvenile leaf does not contain hair like indumentum	Juvenile leaf contains hair like indumentum.
Spine on petiole	Dorsal surface has larger spine with several small scattered spines, 0.1–0.5 cm; ventral surface often has scattered unequal spines,	Dorsal surface have larger spine with several small scattered spines, 0.1–0.4; ventral surface often have scattered unequal spines
Spine on rachis	Distal part of spine shiny blackish- brown in colour	Distal part of spine shiny dark in colour.
Cirrus	Yellowish green	Shiny yellowish green
Ocrea	1–1.3 cm long and 4–4.5 cm in diameter.	0.8–1 cm in length and 4–4.7 cm in diameter.
Fruit	Ovoid	Ovoid to ellipsoid

Table 3. Downloaded sequences from GenBank showing high similarity with *mat*K sequence of ***C. nambariensis***.

Species	Location	voucher ID	E- value	identity	Query coverage	Accession No.
C. brandisii	Trichur, Kerala, India	C.bra1	0.0	100%	100%	JX502796.1
C. erectus	Kunming, Yunnan, China	KUN:Yanghq0026	0.0	--	--	JQ041985.1
C. tenuis	Trichur, Kerala, India	--	0.0	99%	90%	JX390640.1
C. guruba	Kunming, Yunnan, China	KUN:Yanghq0058	0.0	99%	94%	JQ042013.1
C. gracilis	Kunming, Yunnan, China	KUN:Yanghq0023	0.0	98%	94%	JQ041982.1
C. viminalis I	Trichur, Kerala, India	--	0.0	99%	98%	JX502789.1
C. viminalis II	Richmond, Surrey, UK	MWC812835	0.0	98%	100%	JQ435566.1
C. khasianus	Trichur, Kerala, India	C.gaz1	0.0	100%	99%	JX502794.1
C. rotang	Trichur, Kerala, India	--	0.0	100%	100%	JX185544.1
C. metzianus	Trichur, Kerala, India	--	0.0	100%	100%	JX185543.1
C. longisetus	Trichur, Kerala, India	--	0.0	100%	100%	JX185542.1
C. delessertianus	Trichur, Kerala, India	--	0.0	99%	100%	JX502795.1
C. hookerianus	Trichur, Kerala, India	--	0.0	100%	99%	JX502793.1

taxonomic status considering the fact that the variations among them might be related to age of the plant, altitude and climate of the localities where they occurred.

Phylogenetic analyses

PCR amplified products of *mat*K, *trn*L-CD and *trn*L-EF genes of *C. nambariensis* were sequenced that yielded ~ 950 bp, 520 bp and 500 bp amplicon sizes respectively. Multiple sequence alignment was done among the DNA sequences of *mat*K gene of our six experimental samples (Detail in supplmetary table) of *C. nambariensis* along with 13 downloaded accessions of different species of *Calamus* (Baker *et al*., 1999). The accession numbers, query coverage, E-value and identity percentage were mentioned in Table 3. However, not a single homologous sequence of *Calamus* sp. could be retrieved from DNA databank against *trn*L sequences of *C. nambariensis* and therefore, alignment was conducted among the *trn*L-CD and *trn*L-EF genes sequences of our experimental samples only. Phylogenetic trees for *mat*K, *trn*L-CD and *trn*L-EF sequences were created using the maximum likelihood (ML) method.

The present investigation of ML analysis based on the *mat*K sequences produced very consistent phylogeny and all the major clades were well supported. To compare the *mat*K sequences obtained from the present study with other 13 related *Calamus* sp. retrieved from DNA database, phylogenetic tree was constructed from partial *mat*K sequences (Nt. 950). Here, most of the species formed distinct clades as shown in Fig. 2A. The phylogenetic tree produced distinctly into two major clusters with five different clades in each cluster. *C. nambariensis* collected from three location of Arunachal Pradesh and Dehing Patkai Wildlife Sanctuaryof Assamwere included in the same clades. In contrast, two other samples of *C. nambariensis* from Nambor and Hoollongapar constituted a separate clade although both exhibited considerable amount of variations with regard to *mat*K sequences. Rest of the three clades in cluster I included the other species of *Calamus,* commonly found

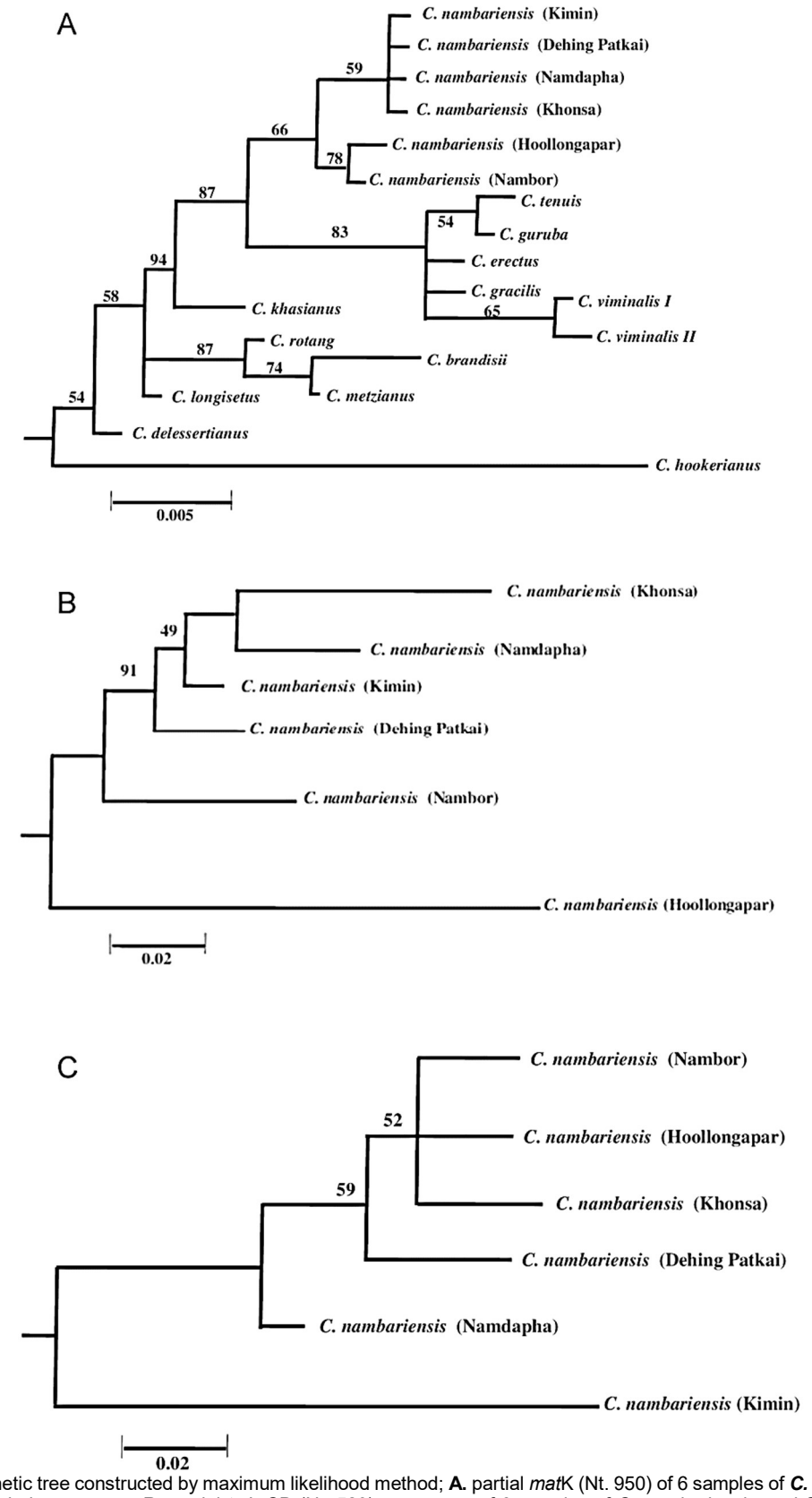

Fig. 2. Phylogenetic tree constructed by maximum likelihood method; **A.** partial *mat*K (Nt. 950) of 6 samples of ***C. nambariensis*** along with 13 downloaded sequences; **B.** partial *trn*L-CD (Nt. 520) sequences of 6 samples of *C. nambariensis,* and **C.** partial *trn*L-EF (Nt. 500) sequences of 6 samples of ***C. nambariensis***

in Northeastern region of India. However, the cluster II comprising five different clades included the *Calamus* spp. commonly occurred in Western Ghats of Peninsular India and Andaman - Nicobar Island.

We also conducted phylogenetic analysis of our experimental samples of *C. nambariensis* using partial-length *trn*L-CD (Nt. 520) and *trn*L-EF (Nt. 500) sequences and the results pointed to the high likelihood of their being in the same clades (Fig. 2B-C). Based on morphological distinction, we regarded them broadly as two different subspecies, while plants from Nambor and Hoollongapar into two different varieties. Our phylogenetic analysis using *mat*K, *trn*L-CD and *trn*L-EF data showed similar results and clustered into two distinct clades, plants of Nambor and Hoollongapar in one clade and rest four were positioned within a common clade. Our morphological observations showed identical and did not contradict with molecular analysis. Further 13 downloaded sequence of some related *Calamus* sp. of *mat*K sequence could finally confirm their common clustering with respect to their occurrence and biogeographical distinctions i.e., Western Ghats of Peninsular India and Andaman - Nicobar Island. As a whole, *C. nambariensis* occurring in Northeastern regions of India by molecular phylogenetic analysis resolved the ambiguity and strengthened our morphological data for proper discrimination and to establish into subspecies and varieties

TAXONOMIC TREATMENTS

Calamus nambariensis Becc. Ann. Roy. Bot. Gard. Calcutta. 11:433. 1908 & Appendix pl. 193. 194. Basu, Rattans (Canes) Monogr. Revi. India 56, 1992; Thomas *et al*., Arunachal Forest News. 15 (1 & 2): 29-30, 1997. ***Type***: Assam, Nambar Forest, 1988, *G. Mann s. n.* (Holotype?: FI. B; Isotype: K000522016!)

Fig. 3

Scandent, rather robust, clump forming, climbing up to 40–67 m. 1–4 mother plants with 1–3 saplings. Leaf cirrate, with cirrus 389–465 cm, without cirrus 180–265 cm; leaf sheath 25–30 cm long; stem with leaf sheath 13–15 cm in diam., without sheath 5–7 cm in diam., the diameter of the stem is continuously increased from base to apex; colour of leaf sheath in juvenile stage grey with scattered light indumentum, which turn to yellowish green in mature plant. Leaf sheath having two types of spines. Among the large spines, the smaller spines are found to be scattered. Large spine deflexed with broad and concavo-convex swollen base, while scares of immature spines are prominent on maturity. Base of spine shiny straw or copper coloured, middle part of spine yellowish gray whereas tip part is dark brown in colour; margin of spine smooth; large spine 4–4.5 cm long and 0.6–0.8 cm in width; small spine arranged linearly with scattered scars of undeveloped spines, facing outward, base swollen, triangular, compactly arranged, stout, sharply pointed, shiny dark brown in colour, 0.1–0.5 cm in long. Knee prominent, nose sharp, 4.5–5 cm long, 7–7.8 cm width; shiny yellowish green with transverse brownish strips, lateral parts of knee may or may not have spine, dorsal surface often have spine, facing upwards, 0.1–0.2 cm long. Ocrea blunt, marcescent, dry, dark brown in colour, 1–1.3 cm long, 4–4.5 cm in diam. Petiole slightly triangular in cross section, 8.5–9 cm long, 6–6.5 cm in diam; adaxially flat with prickles of 0.1–0.5 cm long, arranged linearly at a distance of 2–2.5 cm with several small scattered spines; abaxially rounded and armed with scattered unequal spines with swollen base, singly or basally fused forming groups of 2, 3, 4 or 5, facing outwards, 1–2 cm long, base sought, shiny dark brown in colour, marginal spines 1.5–1.8 cm long, 0.4–0.6 cm in width thick with swelling base, deflexed, shiny dark brown in colour, addressed scares present; rachis slightly triangular at the basal part and quadrangular at the distal part in cross section, spines of rachis on the dorsal surface linearly arranged and progressively increased from 1 to 6 from base which are basally fused forming claws; small spines randomly distributedbetween dorsal and lateral rows of spines up to the 1/3 of the length of the rachis, basal part of spine shiny yellowish green and apical part shiny blackish-brown in colour, 0.3–0.6 cm long, half of the have usually fused 3–7 spines in variable orientation, often individual or two fused spines are there in between fused groups of spines, flat, facing downwards, 0.1–0.5 cm long, distal ventral side devoid of spines; marginal spines 0.8–1.5 cm long, flat, basal part swollen, shiny yellowish and distal part shiny blackish-brown in colour. Cirrus 150–167 cm long, shiny yellowish green, spine cluster forming claws, 4–11 in number; base of spine shiny straw coloured, distal part shiny black, 0.1–0.5 cm long, 0.1–0.2 cm in width. Pinnae lanceolate, regularly alternate, 46 - 58 in number, middle pinnae 40–54 cm long, 3.2–4.2 cm wide at mid-point, 3–5 nerve, bristiles on dorsal surface of leaf only, margins bristly, strongly divaricate on juvenile leaves. Male inflorescence slender, simply decompound, flagellate, 88–120 cm long excluding terminal flagellum, pendulous, 5–9 partial inflorescences on each side, deeply inserted in the mouth of the primary bract, 30–45 cm long; young stems that produce flower for the first time usually bear short partial inflorescences; whereas the mature stems produce much larger inflorescences. Male inflorescences with primary bracts tightly sheathing, armed with scattered claws, primary bract tubular, 6.5–17 cm long, 3–3.5 cm diameter, shiny yellowish green; rachillae slightly curved, having 6–10 rachillae on each side, up to 4 cm long, inserted in or arising just outside the mouths of the tertiary bracts, rachillae with very small bracts at 1.5 mm intervals having 10–22 flower bud very closely set on each side; Male flower small, 0.5 cm long, rather apiculate,

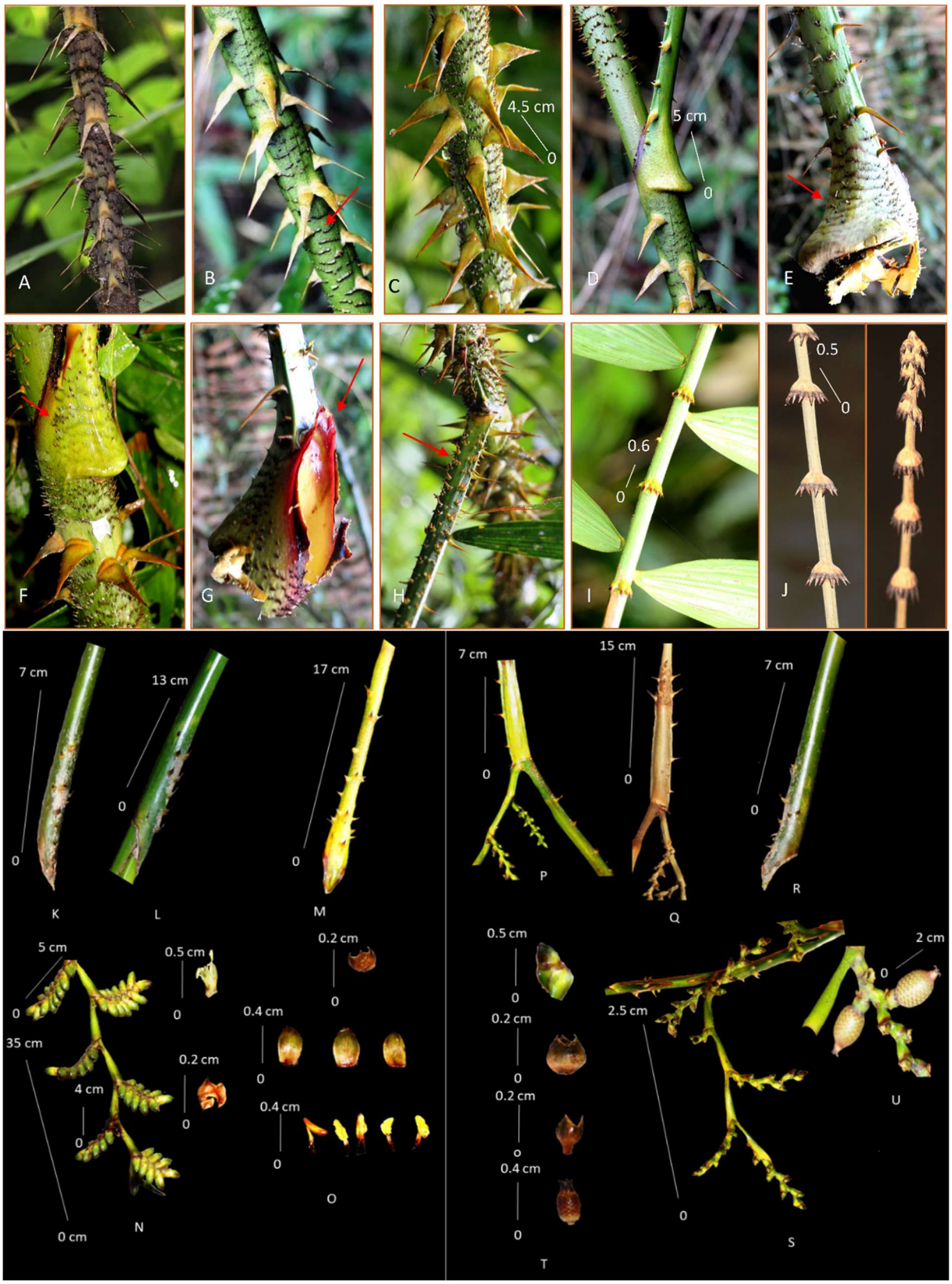

Fig. 3. ***Calamus nambariensis***; **A**-**C**. leaf sheath with spine (juvenile to mature stage), **D**-**F**. knee, **G**. ocrea, **H**. petiole (ventral view), **I**. rachis (dorsal view of distal part), **J**. cirrus. **K**-**O**. male inflorescence: **K**-**M**. bract, **N**. partial inflorescence with rachillae, **O**-complete flower with floral parts; **P**-**S**. female inflorescence: **P**-**R**. bract, **S**. partial inflorescence with rachillae, **T**. complete flower with floral pats, **U**. fruit.

corolla 0.3 cm long; stamens five, 0.4 cm long. Female inflorescence simply decompound, flagellate, 75–165 cm long, 7–10 partial inflorescence arise on each side, 28–43 cm long, exerted from the primary bracts.Primary bract tubular, 7–15 cm long, 3–3.2 cm diam., shiny yellowish green, having spine; lower partial inflorescence longer than that of upper one, having 5–11 rachillae on each side, rachillae up to 5 cm, zigzag, rachillae bracts tightly sheathing, with 12–18 flower bud very closely set on each side; flower small, 0.5 cm long, gynoecium 0.4 cm long. Fruit ovoid with a distinct long beak, stalked, deeply channeled yellowish green with dark brown scale margin, length with beak 1.6–2 cm, 2.5–3 cm in diam; epicarp scales 21 vertical rows, seed homogenous (Fig. 3).

***Phenology*:** Flowering from April to June; Fruiting from September to October.

***Habitat*:** The plant grows in hilly slopes near streams and associated species are *Elaeocarpus serratus* L., *Terminalia chebula* Retz., *Mallotus ferrugineus* (Roxb.) Müll. Arg., *Mangifera sylvatica* Roxb., *Antidesma bunius* (L.) Spreng., *Beilschmiedia fagifolia*Nees, *Cinnamomum bejolghota* (Buch.-Ham.) Sweet, *Castanopsis armata* (Roxb.) Spach, *Aesculus assamica* Griff., *Actinodaphne obovata* (Nees) Blume, *Garcinia kydia* Roxb., *Calamus flagellum* Griff. ex Mart., *Calamus erectus* Roxb., *Salacca secunda* Griff., *Toona ciliata* M. Roem., *Vatica lanceifolia* (Roxburgh) Blume etc.

***Distribution*:** Nambor Wildlife Sanctuary, Karbi Anglong, Assam (India)

Key to the sub-species of *Calamus nambariensis*

1a. leaf sheath without transverse stripes, nose sharp, ocrea blunt …… ……..…… ***C. nambariensis*** subsp. ***nambariensis***
1b. leaf sheath with dark blackish transverse stripes, nose flattened, ocrea pointed ……..…… ***C. nambariensis*** subsp. ***arunachalensis***

Calamus nambariensis subsp. ***arunachalensis*** Deka, Borthakur & Tanti, ***subsp. nov.***

Fig. 4

***Types*:** India, Arunachal Pradesh, Changlang district, Namdapha National Park, 27°23′30″N, 96°15′12″ E; 24 Feb 2016,3500 m a.s.l., K. *Deka, S. K. Borthakur & B. Tanti 634* (holotype: ASSAM, isotypes: GUBH).

Leaf sheath grey with dark blackish transverse stripes in juvenile, which turn to greenish yellow with grayish tint in mature stage. Large spine deflexed with flat base, base of the spine is dull grey in colour, greenish gray at middle merging into dull blackish tip; margin of spine undulates or rarely smooth, 3.7–4.2 cm long and 0.5–0.8 cm in width. The space between the larger spines having loosely arranged linearly as well as scattered smaller spines; facing upward, often slightly bent outward, base flat, bluntly pointed, dull blackish brown in colour. Nose flattened often with a basal constriction; dull grayish green with brownish transverse strips, 6.5–7.6 cm width, both lateral and dorsal surface having spine, facing downward. Ocrea pointed dry, 0.5–0.7 cm in length. Petiole elliptical in cross section, dorsal surface of petiole having larger spine with occasional few small scattered spines, ventral surface has scattered, unequal spines, marginal spines deflexed, base flat, pale dark brown in colour. Basal part of rachis slightly elliptical and distal part triangular in cross section, spines on the dorsal surface dull blackish-brown in colour; half of the have usually fused 3–5 spines in variable orientation, basal part dull straw coloured and distal part dull blackish brown in colour. Cirrus dull green in colour, spine cluster forming claws, 2-8 in number; base of spine dull straw coloured, distal part dull black; Juvenile leaf contain hair like indumentum. Fruit ovoid, globose to ellipsoid, abruptly short beaked. Length with beak 1.8–2 cm, 2.5–3 cm in diam; epicarp scales in 18 vertical rows, scale rusty brown with dark brown margin (Fig. 4).

***Phenology*:** Flowering from April to June; Fruiting from September to October.

Etymology: The sub-specific epithet derived from the state of occurrence i.e., Arunachal Pradesh.

Habitat: Grows near hilly streams and occasionally in between rocks. The common associated species are *Areca triandra* Roxb. ex Buch.-Ham., *Ardisia japonica* (Thunb.) Blume, *Cinnamomum bejolghota* (Buch.-Ham.) Sweet, *Hedychium coccineum* Buch.-Ham. ex Sm., *Hedychium thyrsiforme* Sm., *Larsenianthus assamensis* S. Dey, Mood & S. Choudhury, *Myrioneuron nutans* Wall. ex Hook.f., *Salacca secunda* Griff., *Helicia robusta* (Roxb.) R.Br. ex Blume, *Mycetia longifolia* (Wall.) Kuntze, *Oxyspora paniculata* (D. Don) DC., *Sarcopyramis nepalensis* Wall., *Elatostema heterolobum* (Wedd) Hall. f. etc.

***Distribution*:** *C. nambariensis* subsp. *arunachalensis* is present in Namdapha National Park, Changlang district; Kimin, Papumpare district; Khonsa, Tirap district (Arunachal Pradesh) and Dehing Patkai Wildlife Sanctuary, Dibrugarh district (Assam), India. Population of *C. nambariensis* is relatively poor in Kimin, Khonsa and Dehing Patkai Wildlife Sanctuary as compared with Namdapha National Park, where it is abundantly found.

Other specimen examied: India, Assam, Dibrugarh district, Dehing Patkai Wildlife Sanctuary, 27°17′53″N, 95°30′49″E, 25 Nov 2016, 187 m a.s.l., *K. Deka, S. K. Borthakur & B. Tanti 753A* (GUBH); Arunachal Pradesh, Papumpare district, Kimin, 27°18′24″N, 93°58′21″E, 12 Dec 2016, 1120 m a.s.l., *K. Deka, S. K. Borthakur & B. Tanti 753B*, *753C* (ASSAM); Tirap district, Khonsa, 26°99′41″N, 95°54′07″E, 27 Dec 2016, 370 m a.s.l., *K. Deka, S. K. Borthakur & B. Tanti 753D* (GUBH).

Key to the varities of *Calamus nambariensis* subsp. *nambariensis*

1a. small spine compactly arranged, knee with transverse brownish strips, fruit ovoid …… …… ***C. nambariensis*** subsp. ***nambariensis*** var. ***nambariensis***
1b. small spine loosely arranged, knee without transverse strips, fruit ovoid to ellipsoid ……..…… …… ***C. nambariensis*** subsp. ***nambariensis*** var. ***hoollongapariensis***

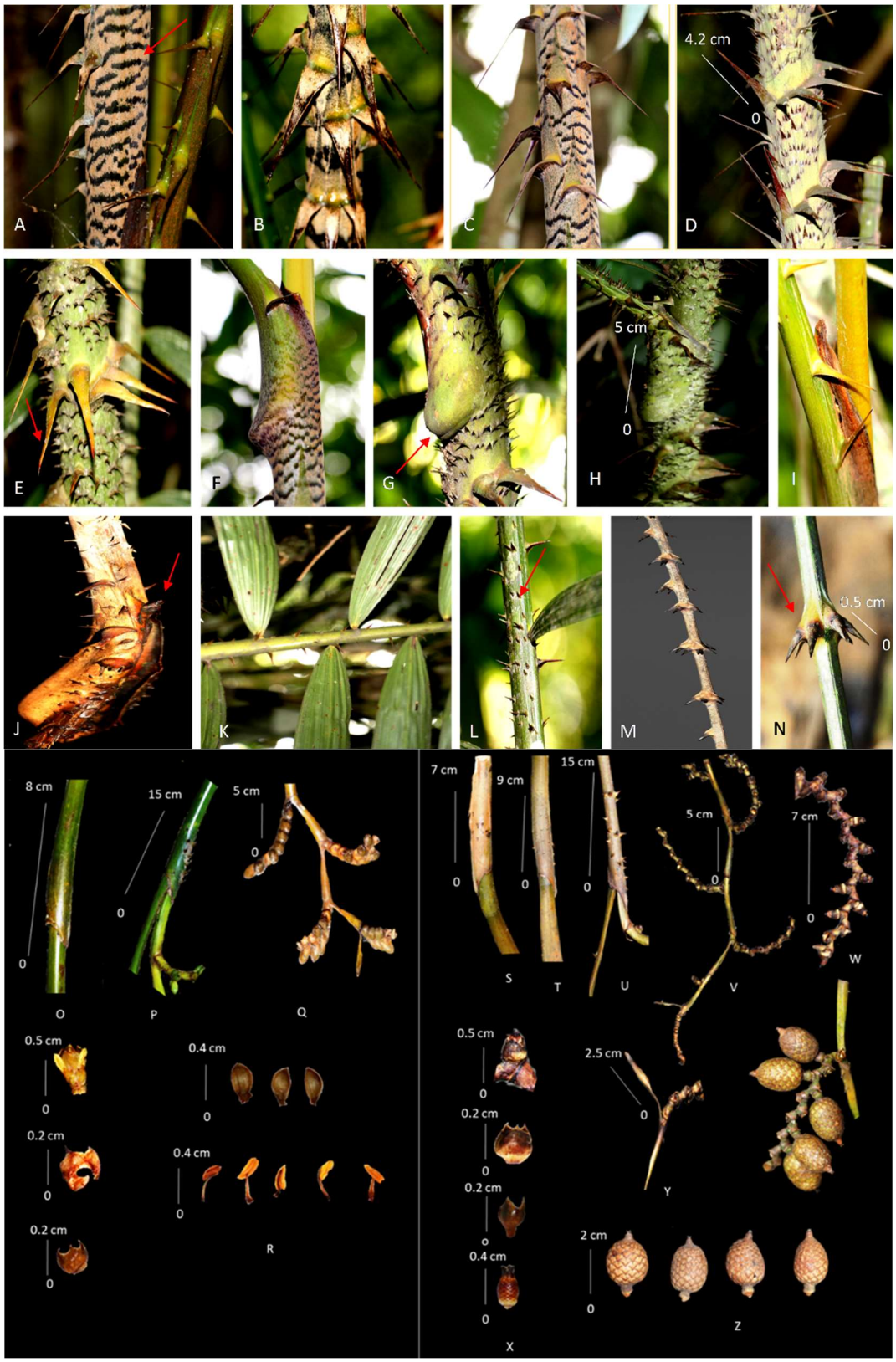

Fig. 4. ***Calamus nambariensis*** subsp. ***arunachalensis***; **A**-**E**. Leaf sheath with spine (juvenile to mature stage); **F**-**H**. Knee. **I**-**J**. Ocrea **K**. Rachis (dorsal view of apical part) **L**. Rachis (ventral view of apical part) **M**. Cirrus **N**. Claws. Male inflorescence (**O**-**R**): **O**-**P**. Bract, **Q**. Partial inflorescence with rachillae, **R**. Complete flower with floral parts; Female inflorescence (**S**-**Z**): **S**-**U**. Bract, **V**. Partial inflorescence with rachillae, **W**. Rachillae, **X**. Complete flower with floral parts, **Y**. Flagella, **Z**. Fruit

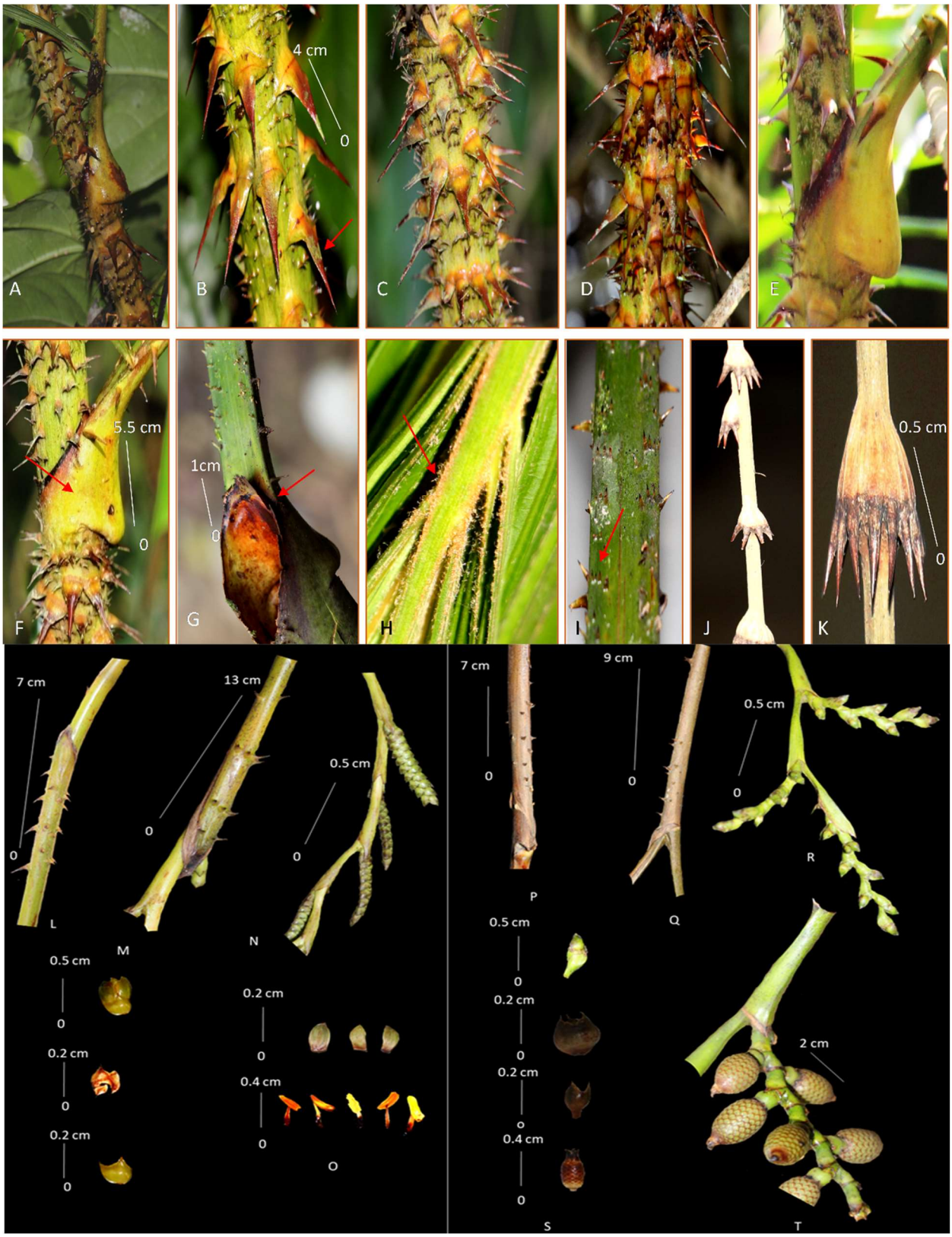

Fig. 5. ***Calamus nambariensis*** subsp. ***nambariensis*** var. ***hoollongapariensis***; **A**-**D**. Leaf sheath with spine (juvenile to mature stage); **E**-**F**. Knee **G**. Ocrea **H**.Indumentum in juvenile leaf **I**. Petiole (ventral view) **J**. Cirrus **K**. Claws. Male inflorescence (**L**-**O**): **L**-**M**. Bract, **N**. Partial inflorescence with rachillae; **O**. Complete flower with floral parts; Female inflorescence (**P**-**T**), **P**-**Q**. Bract; **R**. Partial inflorescence with rachillae; **S** Complete flower with floral pats; **T**. Fruit.

Calamus nambariensis subsp. ***nambariensis*** var. ***hoollongapariensis***, Deka, Borthakur & Tanti, ***var. nov.*** **Fig. 5**

Type: India, Assam, Jorhat district, Hoollongapar Gibbon Sanctuary, Bhelaguri site, 26˚40′30″N, 94˚21′41″E, 18 Dec 2015, 110 m a.s.l., *K. Deka, S.K. Borthakur & B. Tanti 932* (holotype: ASSAM, isotypes: GUBH).

Colour of leaf sheath in juvenile stage yellowish grey, which turn to yellowish green with straw-coloured patches in mature stage. The space between the large spines having loosely arranged scattered smaller spines. Large spine shiny dark brown or chocolate in coloured at base, yellowish gray at middle merging into dark brown tip. Small spine arranged scattered, shiny blackish brown in colour. Knee nose sharply pointed, transverse strips absent; spine absent on the lateral as well as dorsal surface of knee. Ocrea forming a brittle margin to the sheath mouth. Hair like indumentum abaxially in juvenile leaves. Fruit ovoid to ellipsoid, epicarp scales 19 vertical rows (Fig. 5).

***Phenology*:** Flowering from April to June; Fruiting from September to October.

***Etymology*:** The varietal epithet derived from the places of occurrence i.e., Hoollongapar Gibbon Sanctuary.

***Habitat*:** Grows near the streams and as forest undergrowth. The species is found growing along with species like *Dipterocarpus macrocarpus* Vesque, *Mangifera sylvatica* Roxb., *Shorea assamica* Dyer, *Mesua ferrea* L., *Gynocardia odorata* R.Br., *Garcinia acuminata* A. Chev., *Garcinia kydia* Roxb., *Magnolia griffithii* Hook. f. & Thomson, *Phoebe goalparensis* Hutch., *Litsea assamica* Hook. f., *Terminalia myriocarpa* Van Heurck & Müll. Arg., *Ixora acuminate* Roxb., *Dillenia indica* L., *Areca triandra* Roxb. ex Buch.-Ham. etc.

***Distribution*:** *C. nambariensis* subs. *nambariensis* var. *hoollongapariensis* occurs in Assam, India. We found many juvenile as well as fruiting plants from Bhelaguri site and Boideha site of Hoollongapar Gibbon Sanctuary, Jorhat district of Assam.

Other specimen examied: India, Assam, Jorhat district, Hoollongapar Gibbon Sanctuary, Boideha site, 26˚43′35″N, 94˚24′64″E, 12 Jan 2016, 100 m a.s.l., *K. Deka, S. K. Borthakur & B. Tanti 968A, 968B* (GUBH).

DISCUSSION

The present investigation of ML analysis based on the *mat*K sequences produced very consistent phylogeny and all the major clades were well supported. To compare the *mat*K sequences obtained from the present study with other 13 related *Calamus* sp. retrieved from DNA database, phylogenetic tree was constructed from partial *mat*K sequences (Nt. 950). Here, most of the species formed distinct clades as shown in Fig. 2A. The phylogenetic tree produced distinctly into two major clusters with five different clades in each cluster. *C. nambariensis* collected from three location of Arunachal Pradesh and Dehing Patkai Wildlife Sanctuary of Assam were included in the same clades. In contrast, two other samples of *C. nambariensis* from Nambor and Hoollongapar constituted a separate clade although both exhibited considerable amount of variations with regard to *mat*K sequences. Rest of the three clades in cluster I included the other species of *Calamus,* commonly found in Northeastern region of India. However, the cluster II comprising five different clades included the *Calamus* spp. commonly occurred in Western Ghats of Peninsular India and Andaman - Nicobar Island.

We also conducted phylogenetic analysis of our experimental samples of *C. nambariensis* using partial-length *trn*L-CD (Nt. 520) and *trn*L-EF (Nt. 500) sequences and the results pointed to the high likelihood of their being in the same clades (Fig. 2B-C). Based on morphological distinction, we regarded them broadly as two different subspecies, while plants from Nambor and Hoollongapar into two different varieties. Our phylogenetic analysis using *mat*K, *trn*L-CD and *trn*L-EF data showed similar results and clustered into two distinct clades, plants of Nambor and Hoollongapar in one clade and rest four were positioned within a common clade. Our morphological observations showed identical and did not contradict with molecular analysis. Further 13 downloaded sequence of some related *Calamus* sp. of *mat*K sequence could finally confirm their common clustering with respect to their occurrence and biogeographical distinctions i.e., Western Ghats of Peninsular India and Andaman - Nicobar Island. As a whole, *C. nambariensis* occurring in Northeastern regions of India by molecular phylogenetic analysis resolved the ambiguity and strengthened our morphological data for proper discrimination and to establish into subspecies and varieties.

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