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Authentication and DNA Bar-Coding of *Curcuma caesia* Roxb. Genotypes and other *Zingiberaceae* Species Using ITS-2 Gene

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Abstract

Curcuma caesia Roxb. is widely distributed in Indian subcontinent, presently the species has been categorized as critically endangered. As a conservation strategy DNA bar-coding with ITS-2 marker (ITS2-F: ATGCGATACTTGGTGTGAAT, ITS2-R: ACGCTTCTCCAGACTACAAT) was done. The present study aims to identify species with at most precision and to avoid mismatch. All 20 samples taken for study yielded PCR product of ITS-2 gene of around 700 base pairs. The MOLE-BLAST results clearly showed that the percent identity of the all the species ranges from 79.14 to 99.9 per cent, where 75 to 98 per cent BLAST sequence identity is suggested. The percentage of individual sequence of the genotypes aligned to a sequence in gene bank is ranged from 62 to 92 per cent. Highest query coverage was observed for GKJ-5 (92 %) and GMV-6 (92 %) and least was for GNF-27 (62 %). The E value of the sequence blast ranges from $1e^{-83}$ to $9e^{-115}$, this clearly shows high quality match of the sequence with database. Genotype GKK-30 had least E value (e^{1-83}) followed by GGR-10 (e^{1-100}), GMF-21 (e^{1-114}), GAP-20 (e^{1-130}) and GMR-31 (e^{1-132}). BG-1 and BG-2 species were identified as *Kaempferia parviflora* (Black ginger), The unknown species (UNK-7) was identified as *Curcuma kwangsiensis* with an accession number KF694813.1 in the NCBI library record. These results helped us to identify the species in precision, the mole-blast sequences of each genotype of black turmeric and other species can be used as an identity marker.

Keywords : *Curcuma caesia* Roxb., DNA bar-coding, Nuclear marker, ITS-2, Mole-blast, Query coverage, E value, Phylogeny

Curcuma caesia Roxb. is commonly known as black turmeric, is an important, lesser known, non-conventional medicinal plant of Zingiberaceae family. Species is widely distributed in India, Bangladesh, China, Nepal, Malaysia and Thailand also reported from Java and Myanmar as cultivated species (Liu *et al.,* 2013). In India black turmeric is found in West Bengal, Madhya Pradesh, Orissa, Chhattisgarh and Uttar Pradesh States. The species is native to northeast and central India also sparsely found in papi hills of Godavari, foot hills of the Himalaya and Northern hill forests of Sikkim (Anonymous, 2001).

Presently black turmeric has been categorized as critically endangered by the Central forest department of India. National medicinal plant board (NMPB) of India has listed this plant as vulnerable species. Ministry of Environment imposes restrictions on export without permission of the legal competent authorities. Research work so far carried out to exploit the medicinal value of the herb is limited.

By looking in to the present status of the herb and broad spectrum activity on several ailments, conservation measures need to be taken for this economically important plant. Thus efforts should be made to conserve and work for the betterment of this plant species.

There are 40 curcuma species present in the Indian subcontinent. Some times because of collection of false taxonomic identification of the specimen, confusion may arise with the morphological characters like, emergence of the flower, colour and position of the coma bract, rhizome characters, including essential oil biochemical constituents. To avoid this and precise

identification of the species and accessions of *C. caesia* DNA barcoding with ITS-2 marker has taken.

DNA barcoding is a route for taxonomic identification using a short, standard DNA region that is universally present in the target lineages and has enough sequence variation to identify species and assign unidentified individuals to their correct species (Hebert *et al.,* 2003 and Kress & Erickson, 2007).

Initially *rbcL* and *matK* genes were proposed as plant core barcodes but recently other regions such as ITS-2 (Chen *et al.,* 2010) and ITS (Anonymous, 2011) were added. DNA barcoding has been proved and has provided a potential effectiveness in the identification and evaluation of quality for medicinal plants; stands advantageous over phylogenetic analysis. (Newmaster *et al.,* 2006; Chen *et al.,* 2007; Taberlet *et al.,* 2007; Valentini *et al.,* 2009 and Chen *et al.,* 2010). Earlier studies had tested the ability of DNA barcoding to identify *Curcuma* species through sequence data stored in gene bank so that others can use it as reference library (Shi *et al.,* 2011; Záveská *et al.,* 2012; Vinitha *et al.,*2014 and Chen *et al.,* 2015).

The species selected for the study were rare medicinal plants and having high medicinal values. In this direction 17 promising black turmeric genotypes, two black ginger and one unknown species of *Zingiberaceae* family were subjected for DNA barcoding using ITS2 marker (Table 1), mainly to confirm the black turmeric genotypes precisely, and to identify the unknown species accurately.

TABLE 1

Details of black turmeric genotypes and other Zingiberaceae sps. used in the study

Species	Code	Place of collection Latitude	Longitude	Longitude	Altitude (m)	State
Black turmeric	GKM-2	Mangalore	12°55'2.03"N	74°51'21.71"E	22	Karnataka
Black turmeric	GKJ-5	Joida	15.1688° N	74.4848° E	532	Karnataka
Black turmeric	GMV-6	Vidarbha-Gadchirolli	21.1286° N	79.0964° E	1000	Maharashtra
Unknown species	UNK-7	Nagpur	21.1458^{0}N	79.0882^{0}E	310	Maharashtra
Black turmeric	GBH-9	Hajipur	25.6858392N	85.2145907E	56	Bihar
Black turmeric	GGR-10	Rajkote	22° 17' 30N	70° 47' 36E	252	Gujarat
Black turmeric	GAB-13	Bokoliya	26.0564°N	93.1955°E	600	Assam
Black turmeric	GMA-17	Aizwal	23.727106°N	92.717636°E	1132	Mizoram
Black turmeric	GOK-19	Koraput	18.82°N	82.72°E	870	Odisha
Black turmeric	GAP-20	Pasighat Area	28.0619° N	95.3260° E	153	Arunachal Pradesh
Black turmeric	GMI-21	Manipur – Forest	24° 48' 50.2812" N	93° 57' 1.0044" E	900	Manipur
Black turmeric	GMT-22	Imphal	24.8170° N	93.9368° E	786	Manipur
Black ginger	BG-1	Thoubal	24.63^{0}N	94.02^{0}E	765	Manipur
Black turmeric	GMS-24	Sagar	23.8388° N	78.7378° E	427	Madhya Pradesh
Black turmeric	GNF-27	Nepal – Forest	27° 42' 2.7684" N	85° 18' 0.5040" E	330	Nepal
Black ginger	BG-2	Mandalay	21.98^{0}N	96.08^{0}E	80	Burma
Black turmeric	GKK-30	IISR Kozhikode	11.2588° N	75.7804° E	1	Kerala
Black turmeric	GMR-31	Ri-Bhoi	25.8432° N	91.9856° E	485	Meghalaya
Black turmeric	GJG-35	Godda	24.8255° N	87.2135° E	87	Jharkhand
Black turmeric	GNP-36	Phek	25.6634° N	94.4703° E	1524	Nagaland

Material and Methods

Isolation of DNA

Isolation of total genomic DNA of black turmeric genotypes was carried out according to Porebski *et al.* (l997) using CTAB with some modifications, the standardized protocol is as follows;

- Genomic DNA was extracted from fresh young leaves using a modified CTAB method (Saiki *et al.,* 1988).
- Two hundred mg of young leaves were ground into fine powder in liquid nitrogen with the help of pestleand mortar, while crushing extraction buffer (consisting of 100 mM Tris HCl of 8 pH, 2M NaCl, 25 mM EDTA, 2% C-TAB, 2% PVP and 0.2% b- Mercapto ethanol) is used. Homogenate was transferred to 2 ml eppendorf tube
- The tubes were incubated at 60 °C for one hour with occasional shaking. After incubation, the tubes were cooled to the room temperature
- Equal volume of (100 ml) chloroform: Iso-amyl alcohol (24:1) was added and the tubes were inverted gently for minimum twenty times for mixing two phases and centrifuged @ 11000 rpm for 10 minutes at 10 ºC for separation of DNA from rest of the materials (proteins). The upper aqueous phase (supernatant) was taken without disturbing the lower solid portion to another tube then this step is repeated once again.
- The supernatant was transferred without disturbing the lower solid portion to fresh labeled 1.5 ml tubes then 750 ml isopropanol (IPA) was added and the tubes were inverted gently for mixing two phases and then kept in deep freezer for 30 min. and later centrifuged @ 10000 rpm for 10 min at 4 °C.
- The pellet formed after centrifugation was washed with 70 per cent (v/v) ethanol for 30 min. Then alcohol was decanted and pellets were dried at least for 30 min till there was no alcohol smell. After drying the pellet was later dissolved in (150 ml) T10E1 buffer and stored at -20 °C until use.
- RNase treatment: Required quantity (3 ml) of RNAse was mixed to the DNA sample and tubes were incubated on water bath at 37 °C for one hour and 50 °C for five minutes to remove the RNA present in the DNA.

The quantity and purity of DNA was confirmed by electrophoresis, using agarose (0.8 %) gel. DNA concentration was calculated based on standard ladder which was 1Kb. Column purification was done using spin column-based tube (cat. No. 69702) with a solid phase of silica layer (0.45 µm pore size) to get rid of salt contamination (Table 2 and Plate 1).

Table 2

DNA quantification of promising black turmeric genotypes and other Zingiberaceae species for sequencing studies

Species	Code	Nucleic acidconcen tration (ng/µl)	A260/A280 ratio
Black turmeric	GKM-2	35.4	1.61
Black turmeric	GKJ-5	123.0	1.86
Black turmeric	GMV-6	125.5	1.62
Un Identified species	UNK-7	68.3	1.80
Black turmeric	GBH-9	73.3	1.74
Black turmeric	GGR-10	49.0	1.81
Black turmeric	GAB-13	53.5	1.55
Black turmeric	GMA-17	47.6	1.75
Black turmeric	GOK-19	101.3	1.82
Black turmeric	GAP-20	61.7	1.76
Black turmeric	GMF-21	134.3	1.80
Black turmeric	GMI-22	224.8	1.82
Black Ginger	BG-1	124.4	2.40
Black turmeric	GMS-24	38.8	1.80
Black turmeric	GNF-27	215.4	1.92
Black Ginger	BG-2	122.3	1.81
Black turmeric	GKK-30	397.2	1.83
Black turmeric	GMR-31	82.2	1.64
Black turmeric	GJG-35	98.7	1.80
Black turmeric	GNP-36	84.9	1.84

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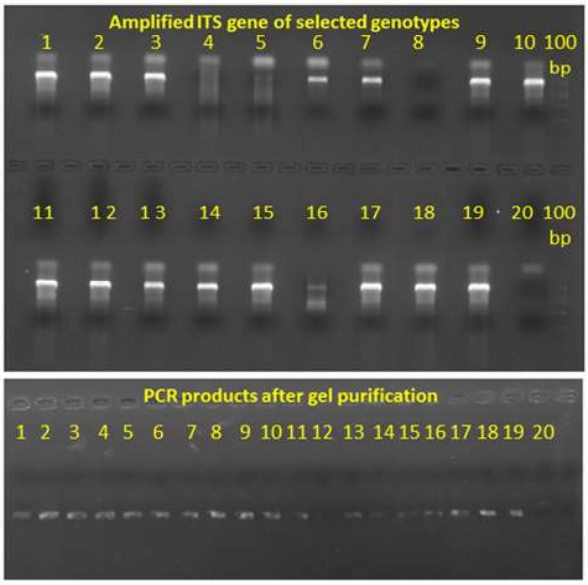

Plate 1 : A: Amplification of ITS2 gene of selected genotypes and species; B: Purified PCR products ready for Sanger sequencing.

Amplification of Nuclear Gene

The target genes were amplified using ITS-2 forward and reverse primers (ITS2-F: ATGCGATACTTGGTGTGAAT, ITS2-R: ACGCTTCTCCAGACTACAAT). The reaction mixture for PCR amplification of the isolated DNA was set after gel electrophoresis. Reaction mixture contained 25ng of genomic DNA, 10pM of forward and reverse primers, 10pM dNTP's, 0.5U of Taq polymerase and 10x reaction buffer was prepared and PCR plate was kept inside the thermo cycler which was programmed to target the respective gene; initial denaturation 95 °C for 2 minutes, final denaturation 95 °C for 30 seconds, annealing 55 °C for 30 seconds, elongation 72 °C for 1 minute for 30 cycles followed by final elongation 72 °C for 10 minutes. After PCR amplification, entire reaction volume was run on gel to observe the amplified product. The PCR bands were cut precisely, dissolving in buffer and was eluted to obtain purified DNA for Sanger sequencing.

Sanger Sequencing

After gel purification, the samples were sequenced bidirectional by performing sequencing PCR using ITS2 primers which is followed by post sequencing PCR purification. The program used is as follows: initial denaturation 95 °C for 2 minutes, final denaturation 95 °C for 30 seconds, annealing 55 °C for 30 seconds followed by termination 60 °C for 4 minutes for 30 cycles. The purified plate was linked in a 16 capillary genetic analyser (3130XL, Applied Biosystems). The obtained sequences were queried against NCBI's sequence databases using on-line BLAST search (Madden *et al.,* 1996). The hit having maximum coverage and more similarity percentage will be considered as relevant species.

Phylogeny

The DNA sequences obtained were minimally edited and manually aligned and partition homogeneity of sequences were implemented to get a total molecular evidence analysis. Further, all the sequences obtained from BLAST were aligned using the clustal omega computer program to understand the relationship between the species (Compson *et al*., 1997).

Results and Discussion

The nuclear ITS-2 is regarded as an appropriate DNA barcode region because of its high variability and has capacity to distinguish even closely related species. All the sequences obtained from Sanger sequencing were extracted to fasta sequences, and were subjected for multiple sequence alignment using Clustal Ω.

All 20 samples taken for study yielded PCR product of ITS2 gene of around 700 base pairs (Plate 1A, 1B). The sequences obtained were subjected to NCBI BLAST data base to check its similarity with nearest hits (Plate 2).

MOLE-BLAST is an experimental tool that helps taxonomists to find the closest database neighbors of submitted query sequences. It computes a multiple sequence alignment (MSA) between the query sequences along with their top BLAST database hits, and generates a phylogenetic tree. Query sequences in the tree are denoted with highlighted node yellow labels.

The present study aims to identify species with atmost precision and to avoid mismatch. The MOLE-BLAST

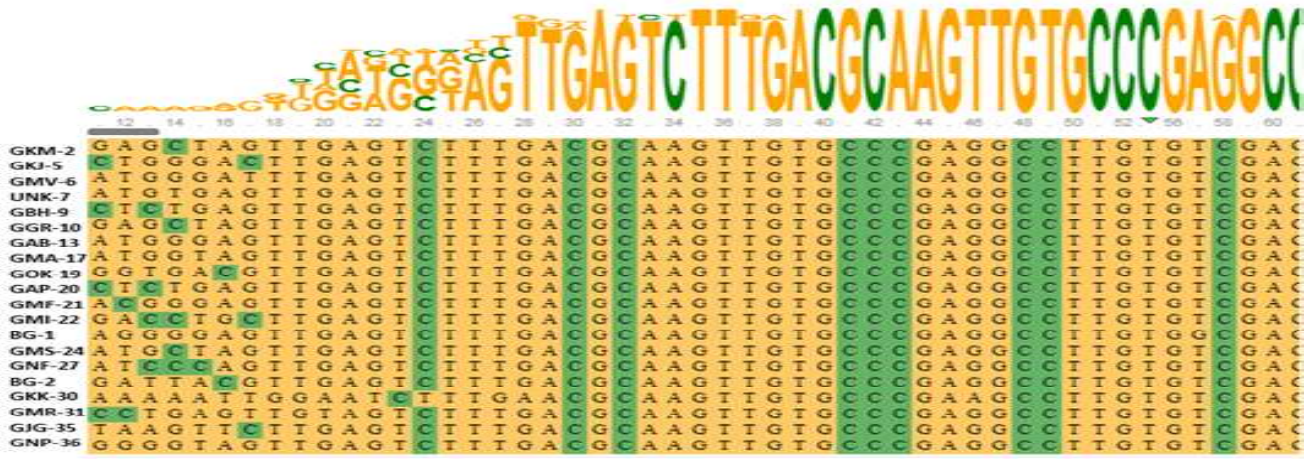

Plate 2: All the sequences of *Curcuma caesia* and other *Zingiberaceae* species aligned using Clustal Ω

results clearly shows that the percent identity of the all the species ranges from 79.14 to 99.9 per cent, where 75 to 98 per cent BLAST sequence identity is suggested. The genotypes GMS-24 and GJG-35 had exhibited highest percentage of sequence identity with the database. Followed by GAB-13 (98.50 %), GNF-27 (95.86 %), GMA-17 (92.88 %), GOK-19 (92.88 %), GMF-21 (92.13 %) and GKM-2 (90.03 %), clearly denotes that all the species are identified precisely and accurately (Table 3).

MOL-BLAST results of 17 promising *Curcuma caesia* Roxb genotypes are; genotype GKM-2, GKJ-5, GBH-9, GAB-13, GMA-17, GOK-19, GAP-20, GMF-21, GMI-22, GNF-27, GKK-30, GMR-31, GJG-35 and GNP-36 had equal Max score and Total score, which denotes highest alignment of the sequence for the matched nucleotide.

The percentage of individual sequence of the genotypes aligned to a sequence in gene bank is ranged

TABLE 3

NCBI BLAST results of *Curcuma caesia* and other Zingiberaceae species

Code	Description	Max score	Total score	Query coverage	E value	Identity(%)	Accession number
GKM-2	*Curcuma caesia* isolate Zn_58.6 clone 2 18S ribosomal RNA gene	379	379	69%	$8e^{-101}$	90.03	KF304492.1
GKJ-5	*Curcuma caesia* isolate cgbottu36 5.8S ribosomal RNA gene and internal transcribed spacer 2,	536	536	92%	$4e^{-148}$	88.81	MF076980.1
GMV-6	*Curcuma caesia* isolate cgbottu36 5.8S ribosomal RNA gene and internal transcribed spacer 2	436	672	92%	$5e^{-118}$	85.16	MF076980.1
UNK-7	*Curcuma wenyujin* voucher DQY32 5.8S ribosomal RNA gene, partial sequence	588	588	96%	$1e^{-163}$	89.70	KF694813.1
GBH-9	*Curcuma caesia* isolate Zn_58.6 clone 7 18S ribosomal RNA gene,	488	488	68%	$2e^{-133}$	96.01	KF304497.1
GGR-10	*Curcuma caesia* isolate Zn_58.6 clone 2 18S ribosomal RNA gene,	379	578	78%	$1e^{-100}$	90.03	KF304492.1

Code	Description	Max score	Total score	Query coverage	E value	Identity(%)	Accession number
GAB-13	*Curcuma caesia* isolate Zn_58.6 clone 9 18S ribosomal RNA gene	468	468	65%	$2e^{-127}$	98.5	KF304499.1
GMA-17	*Curcuma caesia* isolate Zn_58.6 clone 7 18S ribosomal RNA gene	425	425	66%	$9e^{-115}$	92.88	KF304497.1
GOK-19	*Curcuma caesia* isolate Zn_58.3 clone 10 18S ribosomal RNA gene	425	425	66%	$9e^{-115}$	92.88	KF304479.1
GAP-20	*Curcuma caesia* isolate cgbottu 36 5.8S ribosomal RNA gene and internal transcribed spacer 2,	479	479	71	$1e^{-130}$	85.71	MF076980.1
GMF-21	*Curcuma caesia* isolate Zn_58.6 clone 1 9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	425	425	64%	$1e^{-114}$	92.13	KF304499.1
GMI-22	*Curcuma caesia* isolate cgbottu 36 5.8S ribosomal RNA gene and internal transcribed spacer 2,	307	307	68%	$5e^{-79}$	79.44	MF076980.1
BG-1	*Kaempferia parviflora* isolate K96_5667 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	496	799	86%	$1e^{-135}$	99.9%	1 KY701332.1
GMS-24	*Curcuma caesia* voucher BSI/WRC/IDEN.CER./2017/H3-28 5.8S ribosomal RNA gene	333	647	68%	$6e^{-87}$	99.9	MG725946.1
GNF-27	*Curcuma caesia* isolate Zn_58.6 clone 7 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	507	507	62%	$4e^{-139}$	95.86	KF304497.1
BG-2	*Kaempferia parviflora* voucher BKF:J. Mood 3087 18S ribosomal RNA gene, partial sequence	435	435	68%	$2e^{-117}$	98.78%	KU159396.1
GKK-30	Curcuma caesia 18S ribosomal 1 RNA gene, partial sequence; internal transcribed spacer	322	322	80%	1e-83	82.01	KX148596.1
GMR-31	Curcuma caesia 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	484	484	81%	1e-132	89.56	KX148596.1
GJG-35	Curcuma caesia voucher BSI/WRC / IDEN.CER./2017/H3-28 5.8S ribosomal RNA gene and internal transcribed spacer 2	366	366	60%	8e-97	99.9	MG725946.1
GNP-36	Curcuma caesia isolate cgbottu36 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence	448	448	70%	3e-121	85.14	MF076980.1

from 62 to 92 per cent. Higest query coverage was observed for GKJ-5 (92 %) and GMV-6 (92 %) and least was for GNF-27 (62 %).

The E value of the sequence blast ranges from $1e^{-83}$ to $9e^{-115}$, E value (expect value) is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size. Blast results are sorted by E-value by default (best hit in 1st line), the smaller the E value better the match. Genotype GKK-30 had least E value e^{1-83}, followed by GGR-10 (e^{1-100}), GMF-21 (e^{1-114}), GAP-20 (e^{1-130}) and GMR-31 (e^{1-132}). This clearly shows high quality match of the sequence with database. All the genotypes were precisely identified and confirmed that the genus belongs to *Curcuma caesia* Roxb.

BG-1 and BG-2 species were identified as *Kaempferia parviflora* (Black ginger) with an accession number KY701332.1 and KU159396.1 in the NCBI library records, respectively. NCBI blast resulted 86 and 68 per cent query coverage, 99.9 and 98.78 per cent identity along with 496 and 435 max score out of 799 and 435 Total score and $1e^{-135}$ and $2e^{-117}$ E value for BG-1 and BG-2 species respectively. This result clearly shows the precision of the MOL-BLAST and both the species belongs to *Kaempferia parviflora* (black ginger) species.

The unknown species (UNK-7) was identified as *Curcuma kwangsiensis* with an accession number KF694813.1 in the NCBI library record. The per cent identity of the specimen is 89.70 per cent, having 96 per cent query coverage. The blast had scored 588 Max score out of 588 Total score with $1e^{-163}$ E values. These results are clearly helps to identify the unknown species (Table 3).

These results are helpful in identifying the species in precision (Fig.1-5), the sequence obtained from the

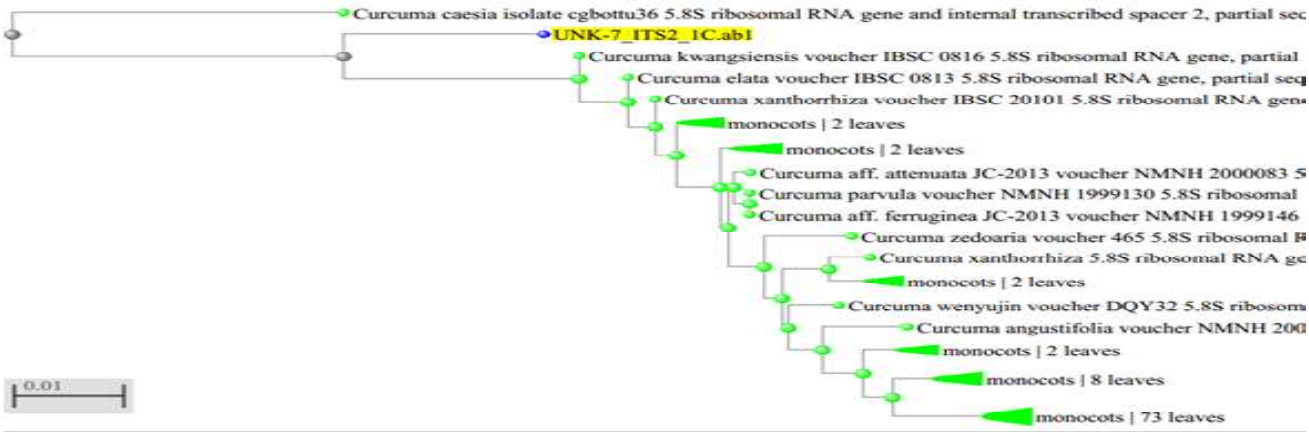

Fig. 1: Phylogenetic tree created through MOLE-BLAST for UNK-7

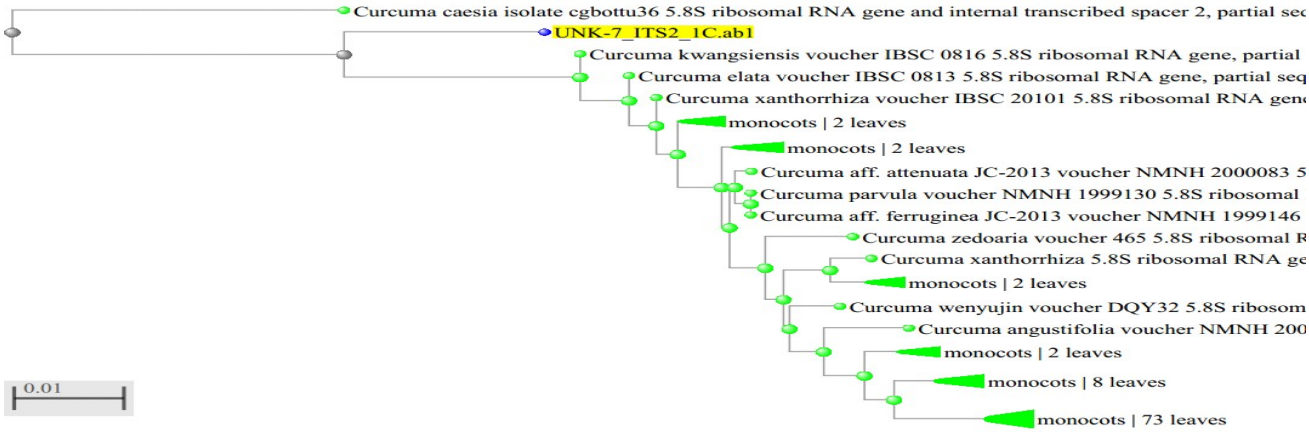

Fig. 2 : Phylogenetic tree created through MOLE-BLAST for BG-1

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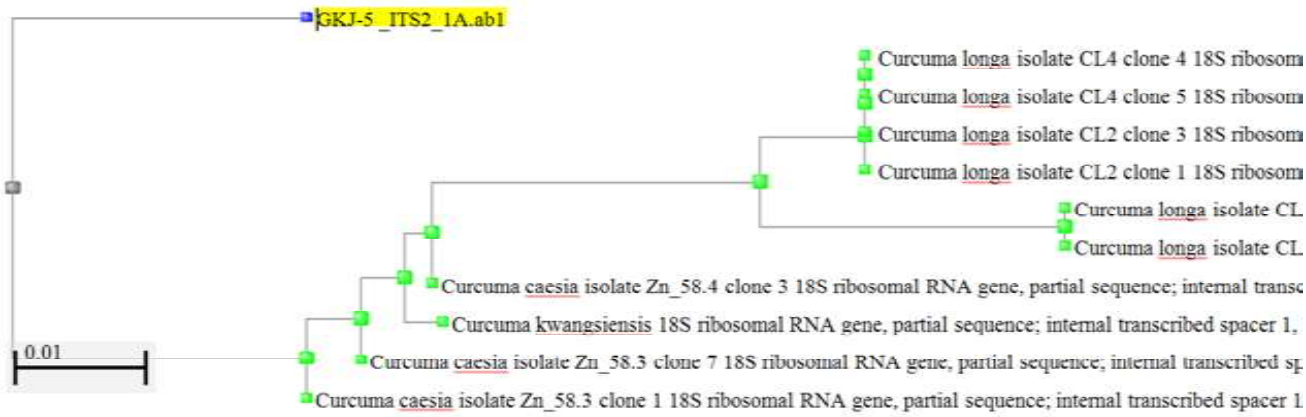

Fig. 3: Phylogenetic tree created through MOLE-BLAST for GKJ-05

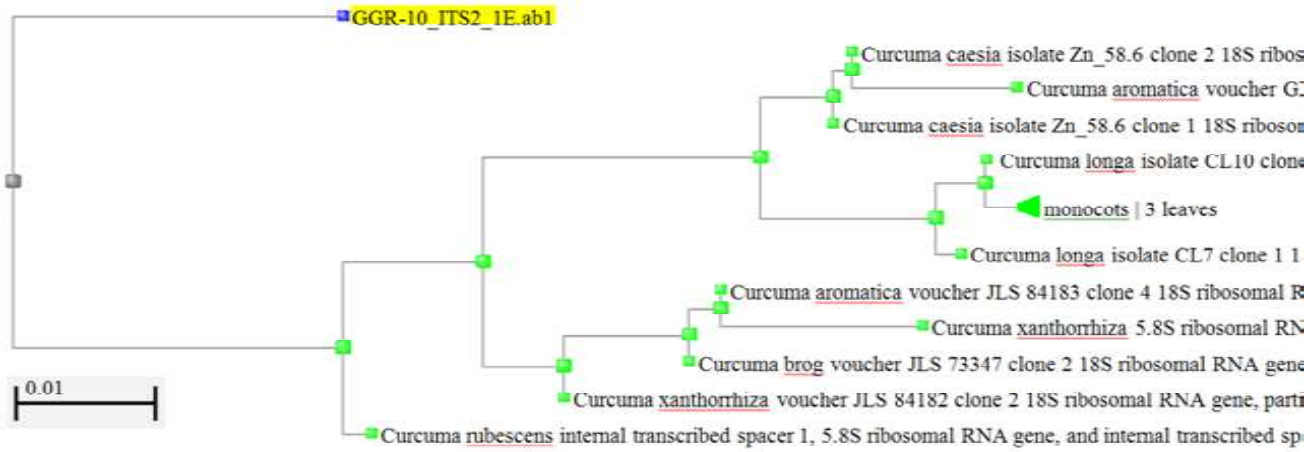

Fig.e 4: Phylogenetic tree created through MOLE-BLAST for GGR-10.

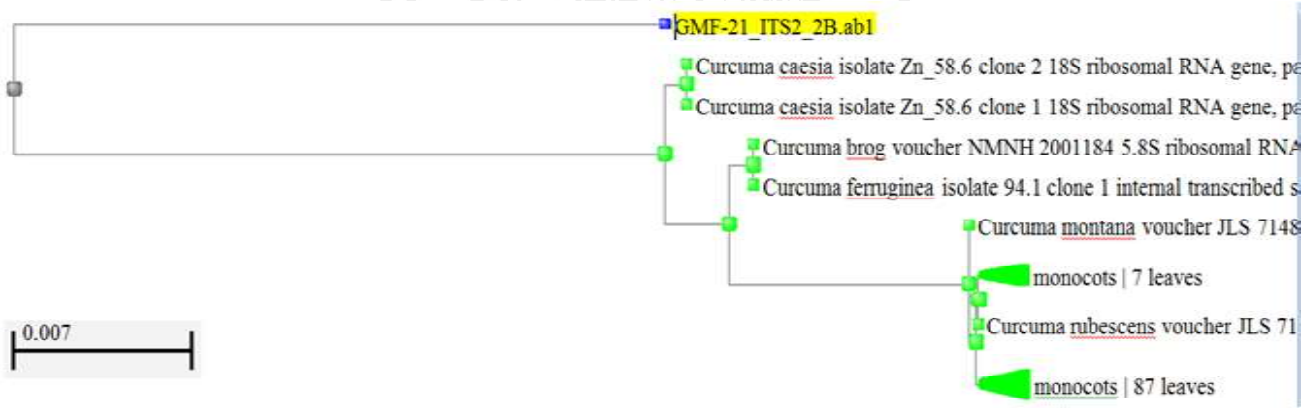

Fig. 5: Phylogenetic tree created through MOLE-BLAST for GMF-21

mole-blast sequencing of each genotypes of black turmeric and other species can be used as an identity marker for the respective genotypes or species (Plate 2). Hence, there is an urgent need to submit the sequences to NCBI and get accession numbers for *Curcuma caesia* genotypes, *Kaempferia parviflora* (BG-1 and BG-2*)* and *Curcuma kwangsiensis* (UNK-7) species.

The results are confirmatory with the findings of Sharma and Lamichhane (2020) who reported 100

per cent sequence similarity with NCBI blast by using ITS barcode candidate gene for black turmeric accessions collected from different locations of Nepal.

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