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Identification of molecular markers to study the *Garcinia* spp. diversity

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The genus *Garcinia* shows a considerable variation in its morphological characters such as leaf, flower and fruit with taxonomic ambiguity. It is a potential under-exploited multipurpose crop that gained considerable attention for the presence of (–) hydroxycitric acid, an anti-obesity compound, in its fruit rind and leaves. Here, we evaluated the genetic relationship through molecular markers among the selected 9 species commonly available in the Western Ghats and the Northeastern Himalayan foot hills of India. The nucleotide sequence data obtained from two prominent monomorphic bands generated in ISSR profiling of the species was utilized for the study. The selected bands were found to be of ITS region (700 bp) and partial region of KNOX-1 gene (600 bp). The evolutionary cluster was formed using MEGA5 software. The study indicated 2 major clusters, influenced by floral morphology of the species and availability of (–) hydroxycitric acid in their fruit rinds. In the subclusters, one species from the Western Ghats were paired with another from Northeastern Himalayas with relatively similar morphological traits.

Keywords: ITS region, KNOX-1 gene, Northeastern Himalayas, Species diversity, Western Ghats

Garcinia, belonging to Clusiaceae family, are tropical evergreen plants of varying morphological features and show high rate of endemism. About 35 species are known to occur in India, of which 7 are endemic to the Western Ghats, 4 to Northeastern Himalayas and 6 to Andaman and Nicobar[1]. In Assam and Nagaland, *Garcinia* are all locally termed as "*Thekara*" though they have different fruit size, shape and color, where as in the Western Ghats, they are known differently *viz*. '*Kokum*' (*Garcinia indica*) and '*Kudampuli*' (*Garcinia gummi-gutta*).

Genus Garcinia suffers from the synonymity of several species. The Plant List reports that out of the total 572 reported occurrences of *Garcinia*, about 31% of the species names are synonyms, and 6.54% names (out of total 612 records) are unresolved[2]. The identification and grouping of the accessions become difficult when they are collected from their natural habitat and in the absence of a clear morphological attributes[3]. However, lack of awareness, coupled with habitat destruction, leads to genetic erosion of this forest resource and many such species have been pushed to the "threatened" state[4].

Utpala *et al*.[5] made extensive collections of *Garcinia* in the Western Ghats and in the Himalayan foot hills, studied their morphological, biochemical and molecular diversity and noted high variation for all the characters studied.

Molecular markers are the regions of DNA which can be used for genetic and evolutionary studies of an organism[6]. Our earlier comparative study of ISSR and RAPD markers for Indian *Garcinia* indicated that ISSR markers showed clear distinction among the species where as RAPD markers showed segregation based on geographical location as well as species based[7].

It has been reported that ISSR (Inter simple sequence Repeats) profiling is not much useful for distinguishing individuals, but for phylo-geographic analyses or may be delimiting species[8]. Though the polymorphic bands, in general, are important to understand the diversity among the different species of a genus[9], the monomorphic bands are potentially significant when the taxonomic analysis is done and the analysis could be extended to the samples collected from different geographic location[10]. Sompong *et al.* (2005) studied the phylogeny of 5 species of *Lansium* with DNA sequencing method of amplicons produced by RAPD primers[11]. Similarly, Allure *et al*.[12], who studied the diversity of *Drimia indica* (Roxb.) Jessop correlated molecular marker data with phenotypic traits analysis. For evolutionary studies, most researchers use sequence alignments that are based on nucleotide similarity. Comparative analysis of DNA sequences is becoming progressively more significant in plant systematic and taxonomy[6,13-15].

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In this study, the monomorphic bands of ISSR profiling, namely 700 and 600 bp, were sequenced for 9 species. The molecular diversity of the 9 species of *Garcina* collected from the Northeastern Himalayan foot hills and the Western Ghats were compared with the help of sequences of the two monomorphic bands of size 700 and 600 bp.

Materials and Methods

The DNA samples of 9 *Garcinia* species belonging to two distinct geographical locations were taken for the study. The species selected were *Garcinia indica*, *G. gummi-gutta*, *G. subelliptica*, and *G. mangostana* from the Western Ghats; and *G. lanceaefolia* (*Rupohi-thekara*), *G. kydia* (*kuji-thekara*) and *G. pedunculata* (*Bor-thekara*) from Northeastern Himalaya. *G. xanthochymus* was taken from both the ecosystems. Species selected were abundant in their ecosystem and are known for their high edible and economic utility.

The DNA from the leaves of selected plants was extracted using modified Doyle and Doyle method[16] and quantified in Agarose gel electrophoresis. 55 UBC primers available in the institute were first screened *in silico* (FastPCR). The selected 19 primers were used for wet-lab studies. The details of the primers are given in details in the Table 1.

Table 1—Showing the details of the primers with base pairs used in Wet Lab study

Primer Code	Sequence
809	AGAGAGAGAGAGAGAGG
810	GAGAGAGAGAGAGAGAT
812	GAGAGAGAGAGAGAGAA
815	CTCTCTCTCTCTCTCTGC
816	CACACACACACACACATC
835a	AGAGAGAGAGAGAGAGCC
835b	AGAGAGAGAGAGAGAGTC
840b	GAGAGAGAGAGAGAGATT
841a	GAGAGAGAGAGAGAGACC
841b	GAGAGAGAGAGAGAGATC
848a	CACACACACACACACAAG
848b	CACACACACACACACAGG
852a	TCTCTCTCTCTCTCTCAA
857a	ACACACACACACACACCG
857b	ACACACACACACACACTG
860a	TGTGTGTGTGTGTGTGAA
860b	TGTGTGTGTGTGTGTGGA
861	ACCACCACCACCACCACC
868	GAAGAAGAAGAAGAAGAA

The selected primers were 18 and 20-mers of various GT and CA repeats. The 700 bp band produced by primer 860b and 600 bp band by primer 810 were selected for sequencing. Eluted DNA was first ligated into pGEMT vector and then introduced into chemically competent DH5α cells. After selecting the transformed cells in a medium containing X-gal, the cells were cultured in LB broth. Plasmids were isolated from the cells using kit method (GeneJet plasmid mini prep kit – ThermoScientific). Purified plasmids were then sequenced. Sequences obtained were then compared using various bioinformatics tools.

Sequence analysis

Local alignment of sequence data of *Garcinia* species were conducted in BLAST (NCBI) and similarities with already available sequences of other plants were noted. The boundaries of the sequenced regions were determined by aligning the sequences of standard primers in FastPCR tool. Length and sequence features such as G-C content, conserved sites, variable sites, and singleton sites; nucleotide features such as transitions, transversions and substitutions for these regions were tabulated using software MEGA5[17].

Cluster analysis

An alignment-sequence data matrix for the sequences of both the region were first created, which was then used for evolutionary analysis in MEGA5. An evolutionary relationship tree (phylogenetic reconstruction) was constructed by neighbour-joining method utilizing genetic distance matrix calculated by Kimura-2 parameter model[18].

Results and Discussion

Suitable primers for ISSR (19) (Table 1) as selected by FastPCR based on their binding properties were used for wet-lab studies on the selected 9 *Garcinia* sp.. The 700 bp band was recorded monomorphic in 4 primers, (860a, 860b, 861, 857a), and 600 bp band was produced as monomorphic in two primers namely, 809 and 810. The 700 bp band produced by 860b {$(TG)_8GA$}primer and 600 bp band of primer 810 {$(CA)_8T$} were subsequently sequenced, as they were the most prominent among the 700 and 600 bp bands.

The study was focused on analysis of genomic regions of the crop, obtained through sequencing of the above UBC primer amplicons. The sequencing

and the BLAST analysis of the amplicons revealed that 700 bp band belonged to the ITS region, whereas 600 bp band belonged to KNOX-1. A similarity matrix was created using the BLAST tool (NCBI) by comparing with the sequences of ITS region already available in NCBI. The similarity ranges from 46-90%. For the KNOX-1 partial gene region, sequences were more than 95% similar. The boundaries of ITS regions were confirmed with the standard ITS region's primers as described by White *et al.*[19] using software FastPCR

The sequence data set for both the regions (ITS and KNOX-1) for the species namely, *G.indica*, *G. gummi-gutta*, *G. kydia*, *G. lanceaefolia*, *G. xanthochymus* (2), *G. pedunculata* and *G. mangostana* were deposited in the NCBI database. The accession numbers of ITS region sequences in NCBI are *G. gummi-gutta* clone GW860b (JX472233), *G. lanceaefolia* clone LH860b (JX472234), *G. kydia* clone KH860b (JX472235), *G. indica* clone IW860b (JX472236), *G. mangostana* clone MW860b (JX472237), *G. pedunculata* clone PH860b (JX472238), *G. subelliptica* clone SW860b (JX472239), *G. xanthochymus* clone XW860b (JX472240) and *G. xanthochymus* clone XH860b (JX472241). Accession numbers for the partial KNOX-1 region are KC848669.1 (*G. indica*), KC848670.1 (*G. lanceaefolia*), KC848671.1 (*G. gummi-gutta*), KC848672.1 (*G. pedunculata*), KC848673.1 (*G. subelliptica*), KC848674.1 (*G. xanthochymus* W.G), KC848675.1 (*G. xanthochymus* N.E.H), KC848676.1 (*G. mangostana*) and KC848677.1 (*G. kydia*).

The lengths of sequenced DNA regions are given in Table 2. Present study showed that ITS-1 region varied between 256 and 296 bp, while ITS-2 region varied from 283 to 321 bp. The 5.8S region was fixed length of 45 bp. ITS sequences are widely used in molecular phylogenetics at species level[11]. Partial KNOX region obtained was of 660 bp in length average.

Table 2—Length (bp) of ITS and KNOX-1 partial region of the selected (8) species of *Garcinia* & *Mahi-thekara*

Species	Total length	ITS region			KNOX-1 region
		ITS-1	5.8S	ITS-2	
G. gummi-gutta	701	289	45	300	667
G. indica	681	273	45	283	652
G. kydia	722	293	42	297	676
G. lanceaefolia	689	275	45	283	652
G. xanthochymus (NEH)	751	284	45	317	670
G. xanthochymus (WG)	753	285	45	318	670
G. mangostana	763	296	45	316	677
G. subelliptica	717	293	44	290	667
G. pedunculata	726	256	45	321	659

The phylogram obtained by N-J method of MEGA5 was given in (Fig. 1). Here, two distinct clusters with 5 subclusters were found with apparent variation in leaf morphology, flower shape and petal numbers; where as the subclusters were formed based on the leaf shape and fruit shape, size and colour. However, the important biochemical factor, HCA was absent in the 2nd cluster while it was present in the species of 1st cluster (Table 3).

In each subcluster, a pair of species, one each from the Western Ghats and the Northeastern Himalayas were observed (Fig. 1). The pairs with similar morphological traits are given in Table 3. The *G. gummi-gutta* and *G. pedunculata* were in subcluster one, belonging tothe Western Ghats and the Northeastern Himalayas, respectively. Both the species were having ridges on the fruit and were considerably big in size. *G. subelliptica* (Western Ghats) and *G. kydia* (Northeastern Himalaya), both were having elliptical leaf almost same sized fruit with 4 numbers of seeds. *G. indica* and *G. lanceaefolia* constituted the third sub cluster of the first cluster in which the former was from the Western Ghats and latter from Northeastern Himalaya. In both the cases, the leaves were lanceolate and the fruit size and colour of the ripened fruits were almost same. *G. mangostana* and *G. xanthochymus* had formed a separate cluster totally separated from other species and it seeks attention that both the species lack HCA (hydroxy citric acid) in their fruit rind or leaf, which is an important biochemical compound of *Garcinia*. It was also to be noted that both species were having 5-petaled flowers while all the species of the first cluster were having 4 petals in their flower.

The position and numbers of the bases of a molecular marker are important to find out the

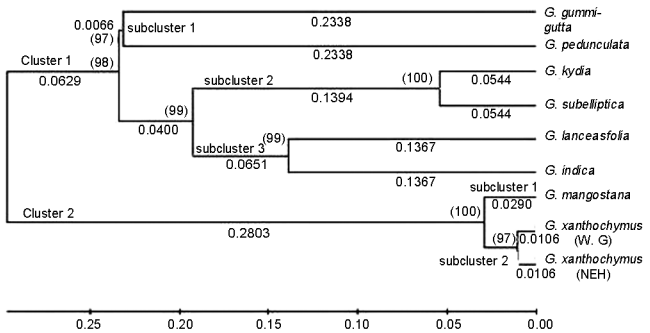

Fig. 1—Evolutionary relationships of taxa using ITS and KNOX-1 gene regions

Table 3—Showing the major morphological characters of the *Garcinia* species.

Cluster 1	Species	Canopy	Leaves	Flowers	Fruits	Biochemistry
Sub cluster 1	*G. gummi-gutta*	Spreading, erect	Obovate 8×6 cm	4 petals, red	Dm 4-7 cm. ovate with prominent groves (6-8). Yellow/orange coloured, Stigma crowned and mammilate at apex. Pale yellow arils.	Major acid-HCA (15%), Malic acid (4%)
	G. pedunculata	Spreading, erect	Oblong-lanceolate 12×8 cm	4 petals, white	Dm 6-10 cm. oblate with 5-8 mild groves-near to ends, yellow coloured, stigma attached to crown. Cream-yellow arils	Major acid-Malic acid (9%), HCA (1.5%)
Sub cluster 2	*G. subelliptica*	Spreading, erect	Ovate-elliptic 8×4 cm	4 petals, white	Dm 3-6 cm. ovoid, yellow coloured fruits with pale pink arils.	Major acid-Malic acid (4%), HCA (2%)
	G. kydia	Spreading, erect	oblong-lanceolate 8×4 cm	4 petals, pale yellow	Dm 4-5 cm, ovoid-oblique, yellow coloured fruits with pale yellow arils.	Major acid-Malic acid (13%), HCA (8%)
Sub cluster 3	*G. indica*	Oval, drooping	Lanceolate 6×3 cm	4 petals, pale yellow	Dm 3-5 cm. round, purple coloured fruits with pale pink arils.	Major acid-HCA (7%), malic acid (2%)
	G. lanceaefolia	Oval, drooping	Lanceolate 10×3 cm	4 petals, white	Dm 2-4 cm. ovoid, orange coloured fruits with pale pink arils.	Major acid-Malic acid (10%), HCA (1%)
Cluster 2	*G. xanthochymus*	Round, angular	Oblong-obovate 18×5 cm	5 petals, white	Dm 8-10 cm. round, yellow coloured fruits with yellow arils.	Major acid-Citric acid (8%), HCA (0.1%)
	G. mangostana	Round, angular	Oblong-obovate 18×5 cm	5 petals, red	Dm 6-8 cm. round, purple coloured fruits with pale pink arils and hard pericarp.	Major acid-Citric acid (8%), HCA (0.2%)

Leaf and fruit sizes are the average of 20 samples collected from various locations. Characters reported by Utpala and Nandakishore[14].

similarity or variation among the species. Results have shown that the sequences of all the species of both ITS and partial KNOX-1 regions had lower GC content indicating that the species of the same cluster also exhibit variation in AT/GC percentages. However, *G. xanthochymus*, though were collected from two different ecosystems, showed same percentages of AT/GC (Table 4).

Baldwin[20], stated that molecular characters that differentiate two species could represent the mutations which arise from a common ancestor. When DNA sequences of species are derived from a common ancestral sequence, descendent sequences gradually diverges by nucleotide substitution. For analyzing the evolutionary relationship among the sequence from the common ancestral sequence, the knowledge of nucleotide pairs are important[21]. Numbers of various nucleotide pairs are given in Table 5.

The R value is the ratio of the number of transitions to the number of transversions for a pair of sequences. R becomes 0.5 when there is no bias towards either transitional or transversion. When the R value is more than 0.5, it shows that the changes are significant and there are some variations, in comparison to the ancestral sequence. In this study R value for ITS region is 0.69, while in case of KNOX-1 region, R value is 0.87, which shows some evolution on the ancestral species has occurred. ITS (internal transcribed spacer) refers to a piece of non-functional DNA situated between structural ribosomal RNAs

Table 4—The GC and AT content of different *Garcinia* species

Species	ITS region			KNOX-1 region		
	G+C%	A+T%	AT/GC	G+C%	A+T%	AT/GC
G. gummi-gutta	43.08	56.92	1.32	43.48	56.52	1.30
G. pedunculata	40.63	59.37	1.46	43.55	56.45	1.30
G. indica	44.93	55.07	1.23	42.50	57.50	1.35
G. lanceaefolia	41.22	58.78	1.43	44.17	55.83	1.26
G. xanthochymus (NEH)	42.88	57.12	1.33	44.03	55.97	1.27
G. xanthochymus (WG)	42.90	57.10	1.33	43.88	56.12	1.28
G. mangostana	43.91	56.09	1.28	44.17	55.83	1.26
G. subelliptica	37.24	62.62	1.68	43.48	56.52	1.30
G. kydia	40.30	59.70	1.48	43.79	56.21	1.28

Table 5—Domain information of the base pairs of *Garcinia* ITS region

Domain information data	ITS region	KNOX-1 region
Identical pairs (ii)	414.00	526.00
Transitional Pairs (si)	111.00	61.00
Transversional Pairs (sv)	162.00	70.00
R (si/sv)	0.69	0.87

(rRNA) genes. Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny as it is easier to amplify even from small quantities of DNA owing to the high copy number of rRNA genes. It has a high degree of variation even between closely related species[22]. This region is also used for biogeographic investigations[23-25]. The ITS region is separated into ITS-1 and ITS-2, both immediately flanking the 5.8S gene sequence, with the former upstream and the latter downstream of that sequence. In this study, the entire ITS region was present within 700 bp, where ITS-1 256-297 and ITS-2 was ranging 283-323 bp and 5.8s region of 45bp in all the cases.

Chinawat *et al.*[26] worked on phylogenetic relationship of 17 species of *Garcinia* of Malaysia using standard ITS primers (used by White *et al*.)[19] and recorded uniform length of 5.8S region sized 43 bp and ITS-1 region sized 254-257 bp. In this study, the 700 bp band with UBC primer 860b yielded ITS region. It confirms that 700 bp band could be used as the marker for *Garcinia* as it contains ITS region within it.

KNOX genes comprise a small family of TALE homeobox genes that are found in all green plant lineages. Plant KNOX genes play an important role in regulating meristem function. Their loss or gain of function mutation can affect overall plant height, leaf shape, meristem development and floral development[27]. In this study, 600 bp band amplified by primer 810 was a monomorphic band and the sequence confirms KNOX-1 region through NCBI BLAST. Though there is a variation among the species, the percentage similarity of KNOX-1 region was very high (95%).

The domain information of the study reveals that in Garcinia ITS region 141 pairs were uniform out of 687 bp where as there are changes in 111 pairs which are called as transitional pairs formed by the transition mutation of ancestral sequence. Transversion pairs are the ones in which purines are replaced by pyramidines or *vise versa*. Here, the numbers of transversion pairs were 162. For the partial KNOX-1 region, out of 657 nucleotide pairs, 526 pairs were identical while 61 and 70 pairs are transitional and transversional, respectively.

The comparison of total sequences through cluster analysis showed an importance to the morphological characters of the flower and fruit. Geographical genotypes of species were not clear as in all the sub clusters out of two species one from the Western Ghats and another from the Himalayan group. Soltis *et al.*[28] reported that such comparison can significantly improve the understanding of the origin of species.

The evolutionary distances of two species present in the same subcluster were equal, indicating the sequence pattern of both the species to be equal in ITS-KNOX regions. So, hypothetically, it can be concluded that the origin of both the species of the same clusters were the same. The changes noticed in fruit shape, size and colour could be due to the modification by the difference in the two ecosystems, where the temperature, annual rainfall and rainfall periods are different.

It is interesting to note that the second cluster had *G. mangostana* and *G. xanthochymus*, which are not having HCA in their fruit or leaves. It was also recorded that the abundant organic acid for the Western Ghats species was HCA, whereas for the Northeastern Himalayan samples it was malic acid[29].

It may be concluded that each subcluster consists of two species and they are of from two different ecosystems. The reason could be that the same ancestral line subjected to different environmental conditions, slowly evolved differences. However, they share the same origin.

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References

1 Peter KV & Abraham Z, *Garcinia*. In: *Biodiversity in Horticultural Crops*, (Daya Books, New Delhi, India), 2007, 264.

2 The Plant List (2013) Version 1.1, http://wwwtheplantlistorg/. Accessed on 22 May 2016.

3 The ENCODE Project Consortium, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C & Snyder M, An integrated encyclopedia of DNA elements in the human genome, *Nature*, 489 (2012) (7414), 57.

4 Utpala Parthasarathy & OP Nandakishore, Morphological characterization of some important Indian *Garcinia* species. *Dataset Papers in Science*, 2014, Article ID 823705, http://dx.doi.org/10.1155/2014/823705.

5 Utpala P, Nirmal Babu K, Senthil Kumar R, Ashis GR, Mohan S & Parthasarathy VA, . Diversity of Indian Garcinia, a medicinally important spice crop in India. *Acta Hort* (ISHS), 979 (2013) 467.

6 Sharma BB, Mustafa M, Sharma T, Banerjee BD & Urfi AJ, Cross-species amplification of microsatellite markers in *Mycteria leucocephala* Pennant 1769: Molted feathers as successful DNA source. *Indian J Exp Biol*, 52 (2014) 1011.

7 Utpala Parthasarathy, OP Nandakishore, K Nirmal Babu & Senthil Kumar, Comparative effectiveness of inter-simple sequence repeat and randomly amplified polymorphic DNA markers to study genetic diversity of Indian Garcinia. *.Afr J Biotechnol*, 12 (2013) 443

8 Morgan L, Intersimple sequence repeats (ISSR) molecular markers to distinguish gametophytes of *Batrachospermum boryanum* (Batrachospermales, Rhodophyta). *Phycologia*, 38 (1999) 70.

9 Mei Z, Khan MA, Yang L, Yang M & Fu J, Genetic characterization and authentication of *Gardenia jasminoides* in different regions of China by using improved RAPD analysis. *Indian J Exp Biol*, 53 (2015) 164.

10 Mariela AK, Micheline SR, Caroline T, Alicia F & Ana CMA, Genetic similarity of Brazilian hull-less and malting barley varieties evaluated by RAPD markers. *Sci Agric* (Piracicaba, Braz), 62 (2005) 36.

11 Sompong T, Mongkol L & Mii M, Comparison of cultivar identification methods of longkong, langsat and duku: *Lansium* spp. *Songklanakarin J Sci Technol*, 27 (2005) 406.

12 Alluri N, Shivakameshwari MN, Manohar SH & Majumdar M, Diversity of *Drimia indica* (Roxb.) Jessop and its relationship to *Drimia nagarjunae* using phenotypic traits and molecular markers. *Indian J Exp Biol*, 53 (2015) 412.

13 Sethia B, Mustafa M, Manohar S, Patil SV, Jayamohan NS & Kumudini BS, Indole acetic acid production by fluorescent *Pseudomonas* spp. from the rhizosphere of *Plectranthus amboinicus* (Lour.) Spreng. and their variation in extragenic repetitive DNA sequences. *Indian J Exp Biol*, 53 (2015) 342.

14 Shashank PR, Ojha Rakshit, Venkatesan T, Jalali SK & Bhanu KRM, Molecular characterization of brinjal shoot and fruit borer, *Leucinodes orbonalis* (Guenée) (Lepidoptera: Crambidae) based on mitochondrial marker cytochrome oxidase I and their phylogenetic relationship. *Indian J Exp Biol*, 53 (2015) 51

15 Susana,SN & Laura,LF PLANT DNA sequencing for phylogenetic analysis.from plants to sequences. *Methods Moll Biol*,781 (2011) 183.

16 Asish G R, Utpala P & Nithya NG, Standardization of DNA isolation and PCR parameters in *Garcinia* spp. for RAPD analysis. *Indian J Biotechnol*, 9 (2010) 424.

17 Tamura K, Peterson D, Peterson N, StecherG, Nei M & Kumar S, MEGA5:Molecular evolutionary genetics analysis using maximum likelihoevolutionary distance andmaximum parsimony methods. *Mol Biol Evol*, 28 (2011) 2731.

18 Tamura K, Nei M & Kumar S,. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci* (USA) 101 (2004) 11030.

19 White TJ, Bruns T, Lee S & Taylor J, Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In: *PCR Protocols: a Guide to Methods & Applications*, (Eds. Innis MA, Gelfand DH, Sninsky JJ & White TJ; New York Academic press, New York), 1990, 315.

20 Baldwin BG, Molecular phylogenetics of *Calcydenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: Chromosomal and morphological evolution reexamined. *Am J Bot*, 80 (1993) 222.

21 Irina M, Kseniya G, Svetlana S & Alex&er B, Phylogenetic relationships of the Siberian iris species inferred from noncoding chloroplast DNA sequences. *Int J Plant Sci*, 164 (2003) 229.

22 Ghiurcuta CG, Models and Algorithms for Non-coding Genes. *Edic Research Proposal*, 2009, http://wwwepflch/ edicpublic, As accessed on 27 October 2013.

23 Suh Y, Thien LB, Reeve HE & Zimmer EA, Molecular evolution and phylogenetic implications of ribosomal DNA in Winteraceae. *Am J Bot*, 80 (1993) 1042.

24 Hsiao C, Chatterton NJ, Asay KH & Jensen KB, Phylogenetic relationships of 10 grass species: An assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome*, 37 (1994) 112.

25 Dubouzet JG & Shinoda K, Relationships among old and new world Alliums according to ITS DNA sequence analysis. *Theor Appl Genet*, 98 (1999) 422.

26 Chinawat Y, Sugranant S, Chitose H & Keizo Y, Phylogenetic Relationship of Mangosteen (*Garcinia mangostana*) & several wild relatives (*Garcinia* spp.) revealed by ITS sequence data. *J Am Soc Hort Sci*, 129 (2004) 368.

27 Kerstetter RA, Laudencia-Chingcuanco D, Smith LG & Hake S, Loss-of-function mutations in the maize homeobox gene, knotted1, are defective in shoot meristem maintenance. *Development*, 124 (1997) 3045.

28 Soltis DE, Pamela SS & John NT, Chloroplast DNA variation in *Lithophragma* (Saxifragaceae). *Syst Bot*, 17, (1992) 607.

29 Utpala P, Nandakishore OP, Senthil KR, Nirmal BK, Zachariah TJ & Parthasarathy VA, Chromatographic finger-printing and estimation of organic acids in selected *Garcinia* species. *Int J Innovative Hort*, 1 (2012) 68.