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RESEARCH ARTICLE

GENETIC DIVERSITY ANALYSES USING ISSR MARKERS ON *BACOPA MONNIERI* OCCURRING IN KERALA

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SUMMARY This study is aimed at the assessment of present status of genetic diversity of *Bacopa monnieri* (L.) Wettst. in its gene pool in Kerala. Sixty accessions of the species collected from localities throughout Kerala were subjected to molecular analysis using 16 ISSR markers. The following parameters were estimated for the assessment of genetic diversity of the species in the germplasm: Number of alleles per locus (Na), Nei's genetic diversity measure (H), Shannon's information index (I) and percentage of polymorphism (P). Cluster analysis of the accessions was also undertaken using the software POPGENE based on UPGMA, and the details were depicted in a dendrogram. The results revealed that the accessions subjected to the study are genetically different, but most importantly, genetic diversity of the species in the region is only moderate. The details of the results are useful for formulating strategies on conservation of genetic resource of the species as well as its utilization for the development of elite genotypes/cultivars and their genetic improvement.

Keywords: *Bacopa monnieri,* ISSR markers, germplasm, genetic diversity, improvement.

INTRODUCTION

Bacopa monnieri (L.) Wettst. (Plantaginaceae) is a perennial herbaceous species, having immense therapeutic potential. It is widely used in the Indian traditional systems of medicine such as Ayurveda, Siddha, Unani and Homeopathy (Singh & Singh 1980), and also for industrial scale production of herbal formulations. The Task Force of the erstwhile Planning Commission of India on Conservation and sustainable use of medicinal plants has identified it as one of the core species in great demand in pharmaceutical industry of the Indian traditional systems of medicine (Planning Commission 2000). *B. monnieri* occupied top position in the priority list of species significant to the Export Import Bank

of India, based on evaluation of its medicinal importance, commercial value and potential for further research and development (Bammidi et al. 2011).

Being a highly valued medicinal species with great demand in pharmaceutical industry, this species is being collected indiscriminately from the wild leading to depletion of the resource and consequent gene erosion of the species. Bansal et al. (2014) reported that *B. monnieri* is a locally endangered species. Loss of genotypes and shrinkage of its germplasm is detrimental to the prospects of its cultivation, genetic improvement of cultivars and conservation of the species.

ISSR markers are versatile high-resolution tools for molecular characterization of genotypes of a species and the data generated are useful for the assessment of genetic variability and grouping them based on their genetic interrelationship. This study is concerned with ISSR based genetic analyses of 60 accessions of *B. monnieri* collected from diverse localities all over Kerala, using 16 ISSR markers and estimation of the parameters for assessing its genetic diversity such as number of alleles per locus (Na), Nei's genetic diversity index (H) and Shannon's information index (I). The objectives of this investigation were, molecular characterisation of 60 accessions of the species, assess the genetic variability in the gene pool of the species in Kerala and to understand genetic interrelationship of its genotypes, and the data generated will be useful for conservation as well as utilization of genetic resource of the species.

MATERIALS AND METHODS

Sixty accessions of *B. monnieri*, were collected from different parts of Kerala, representing multiple populations from all the districts of the State (Table 1). Taxonomic identity of the accessions was confirmed by matching their herbarium specimens with authenticated ones in the Herbarium of the Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram (TBGT). The voucher specimens of all the accessions were deposited in the Herbarium. Planting materials of the accessions collected during the field surveys were planted in the nursery of the Institute. On establishment, the plants were transplanted to the Field Gene Bank (FGB) of medicinal, aromatic and spice plants at JNTBGRI. DNA was isolated by using the Origin Plant Genomic DNA Purification Kit. The concentration and purity of the isolated DNA was analysed by BioPhotometer (Eppendorf, Germany), 0.8 % agarose gel electrophoresis and was diluted to a final concentration of 50 ng/µl. Polymerase chain reaction (PCR) was carried out on the plant samples each in 25 µl reaction consisting of 50 ng template DNA, 1× PCR buffer (Origin, Kerala), 200 µM of each of the 4 dNTPs, 15 pM primer and one unit of Taq DNA polymerase (Origin, Kerala). Forty-eight ISSR primers (IDT, New Delhi) were checked and evaluated for clarity, consistency and polymorphism. Sixteen primers which

TABLE 1: Details of collection localities of the 60 accessions of *B. monnieri.*

Sl. No.	Acc. No.	Place of collection	District	Date of collection	Latitude	Longitude	Altitude (m)
1	Bm 1	Viraly	Thiruvananthapuram	25-12-2011	8°18'N	77°05'E	14
2	Bm 2	Kadakkavoor	Thiruvananthapuram	27-12-2011	8°40'N	76°45'E	14
3	Bm 3	Karikkakom	Thiruvananthapuram	30-12-2011	8°30'N	76°54'E	15
4	Bm 4	Aakkulam	Thiruvananthapuram	30-12-2011	8°31'N	76°54'E	16
5	Bm 5	Vettamukku	Kollam	28-12-2011	9°01'N	76°33'E	17
6	Bm 6	Panayam	Kollam	07-04-2012	8°57'N	76°37'E	18
7	Bm 7	Kochuplamoodu	Kollam	07-04-2012	9°00'N	76°39'E	9
8	Bm 8	Dalavapuram	Kollam	28-12-2011	8°56'N	76°33'E	12
9	Bm 9	Padukkottukal	Pathanamthitta	02-06-2012	9°11'N	76°43'E	34
10	Bm 10	Thakazhi	Alappuzha	28-12-2011	9°22'N	76°24'E	7
11	Bm 11	Pattaniyidukku	Alappuzha	28-12-2011	9°30'N	76°19'E	10
12	Bm 12	Pathirapally	Alappuzha	28-12-2011	9°32'N	76°19'E	9
13	Bm 13	Vattayil	Alappuzha	28-12-2011	9°28'N	76°20'E	10
14	Bm 14	Nerekadavu	Kottayam	30-05-2012	9°38'N	76°30'E	48
15	Bm 15	Koduppadom	Kottayam	30-05-2012	9°47'N	76°23'E	8
16	Bm 16	Kumarakom	Kottayam	30-05-2012	9°36'N	76°25'E	9
17	Bm 17	Vazhikkadavu	Kottayam	30-05-2012	9°40'N	76°53'E	972
18	Bm 18	Kumbalam	Ernakulam	14-01-2012	9°54'N	76°18'E	10
19	Bm 19	Thripunithura	Ernakulam	13-01-2012	9°57'N	76°20'E	11
20	Bm 20	Charthedam	Ernakulam	13-01-2012	10°11'N	76°13'E	12
21	Bm 21	Near Aarch dam	Idukki	31-05-2012	9°50'N	76°58'E	562
22	Bm 22	Vellayamkudi	Idukki	31-05-2012	9°46'N	77°05'E	907
23	Bm 23	Munnar	Idukki	27-10-2012	10°05'N	77°03'E	1462
24	Bm 24	Chellarkovil	Idukki	31-05-2012	9°40'N	77°10'E	1095
25	Bm 25	Kumily	Idukki	31-05-2012	9°37'N	77°09'E	892
26	Bm 26	Chelakkara	Thrissur	20-05-2011	10°40'N	76°21'E	54
27	Bm 27	Karuppadannapalam	Thrissur	29-12-2011	10°15'N	76°12'E	12
28	Bm 28	SNpuram beach	Thrissur	29-12-2011	10°16'N	76°10'E	9
29	Bm 29	Undaikadavu	Thrissur	14-01-2012	10°13'N	76°10'E	13
30	Bm 30	Kodakara	Thrissur	29-12-2011	10°22'N	76°18'E	17
31	Bm 31	Pattambi	Palakkad	27-04-2012	10°48'N	76°11'E	35
32	Bm 32	Kambram	Palakkad	28-04-2012	10°53'N	76°11'E	97
33	Bm 33	Pulakad	Palakkad	28-04-2012	10°47'N	76°39'E	78
34	Bm 34	Varakulam	Malappuram	28-04-2012	10°20'N	76°17'E	36
35	Bm 35	Paravanna	Malappuram	28-04-2012	10°54'N	75°53'E	11
36	Bm 36	Kannoorkettu	Kozhikode	12-04-2012	11°27'N	75°46'E	15

(Continued...)

TABLE 1: (Concluded)

Sl. No.	Acc. No.	Place of collection	District	Date of collection	Latitude	Longitude	Altitude (m)
37	Bm 37	Musankandi	Kozhikode	12-04-2012	11°21'N	75°43'E	10
38	Bm 38	Kakkoor	Kozhikode	12-04-2012	11°22'N	75°49'E	20
39	Bm 39	Pathinonnammile	Kozhikode	12-04-2012	11°22'N	75°50'E	17
40	Bm 40	Chelapram	Kozhikode	12-04-2012	11°20'N	75°47'E	10
41	Bm 41	Manicherry hills	Kozhikode	12-04-2012	11°30'N	75°51'E	478
42	Bm 42	Payyannur	Kannur	13-05-2012	12°05'N	75°11'E	10
43	Bm 43	Muttukatti	Kannur	13-05-2012	12°00'N	75°15'E	12
44	Bm 44	Kakkad	Kannur	13-05-2012	11°53'N	75°23'E	20
45	Bm 45	Andalloorkavu	Kannur	13-05-2012	11°47'N	75°28'E	11
46	Bm 46	Sasimala	Wayanad	13-04-2012	11°48'N	76°11'E	761
47	Bm 47	Channothukolli	Wayanad	13-04-2012	11°49'N	76°12'E	728
48	Bm 48	Seethamount	Wayanad	13-04-2012	11°50'N	76°12'E	752
49	Bm 49	Nadavayal	Wayanad	13-04-2012	11°44'N	76°07'E	797
50	Bm 50	Meppadi	Wayanad	13-04-2012	11°55'N	76°13'E	870
51	Bm 51	Padannakkadu	Kasaragode	12-05-2012	12°15'N	75°06'E	13
52	Bm 52	Kumbla	Kasaragode	12-05-2012	12°35'N	74°56'E	9
53	Bm 53	Edayilekkadu	Kasaragode	12-05-2012	12°08'N	75°09'E	10
54	Bm 54	Madakkara	Kasaragode	12-05-2012	12°13'N	75°07'E	14
55	Bm 55	Manakkakadavu	Kottayam	30-05-2012	9°48'N	76°24'E	8
56	Bm 56	Channikkadavu	Alappuzha	28-12-2011	9°51'N	76°18'E	6
57	Bm 57	Pedikkattuthuruthu	Ernakulam	13-01-2012	10°58'N	76°20'E	8
58	Bm 58	Cheriyathuruthu	Ernakulam	13-01-2012	10°03'N	76°14'E	10
59	Bm 59	Chungam	Alappuzha	28-12-2011	9°29'N	76°20'E	8
60	Bm 60	Kinanoor	Kasaragode	12-05-2012	12°16'N	75°10'E	15

created clear bands and amplification profile were selected for the study (Table 2). Amplification was carried out with the following conditions: 2 min at 94° C, followed by 35 cycles of denaturation for 30 s at 94° C, annealing for 1 min, extension for 2 min at 72° C, and for 7 min at 72° C for the final extension using Agilent Sure Cycler (Agilent Technologies, Malaysia). The amplified products were resolved with the aid of 1.4 % agarose gel electrophoresis in 1 × TBE buffer and visualized using Safe View™ Classic (Applied Biological Materials, Canada) in gel documentation system (UVP, UK). 1 kb or 100 bp DNA ladder (Origin, Kerala) was loaded in the gel as the size marker. The bands obtained with each primer were scored as diallelic characters: 1 denotes present and 0 denotes absent (Lamyai et al. 2014, Mark et al. 2015). A binary qualitative

TABLE 2: Details of ISSR primers used to analyze genetic diversity in 60 accessions of *B. monnieri.*

Sl. No.	ISSR primer	Sequence	Tm (˚C)
1	811	5'-GAGAGAGAGAGAGAGAT-3'	48
2	812	5'-GAGAGAGAGAGAGAGA-3'	52.8
3	815	5'-CTCTCTCTCTCTCTCTT-3'	48
4	825	5'-ACACACACACACACT-3'	52
5	807	5'-TATATATATATATATAC-3'	51
6	891	5'-HVHTGTGTGTGTGTGTG-3'	46
7	860	5'-ACACACACACACACACTG-3'	58
8	873	5'-GACAGACAGACAGACA-3'	54.5
9	889	5'-DBDACACACACACACAC-3'	40.2
10	844	5'-CTCTCTCTCTCTCTCTRA-3'	43.6
11	842	5'-GAGAGAGAGAGAGAGAYC-3'	55
12	814	5'-CTCTCTCTCTCTCTCTA-3'	39.6
13	810	5'-AGAGAGAGAGAGAGAGG-3'	43.8
14	880	5'-CTTCACTTCACTTCA-3'	54.5
15	S44	5'-GAGAGAGAGAGAGAGAC-3'	52.6
16	S45	5'-CTCTCTCTCTCTCTCTG-3'	44

TABLE 3: Estimates of the 3 genetic parameters (Na, I and H) in 60 accessions of *B. monnieri* (Multiple populations).

Sl. No.	Allele/ Primer	Na	I	H
1	811	2.00	0.68	0.49
2	812	2.00	0.53	0.35
3	815	2.00	0.50	0.32
4	825	2.00	0.69	0.50
5	807	2.00	0.50	0.32
6	891	2.00	0.68	0.49
7	860	2.00	0.44	0.27
8	873	2.00	0.54	0.36
9	889	1.00	0.00	0.00
10	844	2.00	0.63	0.44
11	842	1.00	0.00	0.00
12	814	2.00	0.59	0.40
13	810	2.00	0.50	0.32
14	880	2.00	0.69	0.50
15	S44	2.00	0.52	0.34
16	S45	1.00	0.00	0.00
	Mean	1.81	0.47	0.32

data matrix was formed and genetic parameters such as number of alleles per locus (Na) (Kimura & Crow 1964), Nei's (1973) genetic diversity (H) and Shannon's Information Index (I) (Lewontin 1972) were estimated statistically using the software POPGENE ver. 1.31 (Amit et al. 2014). A dendrogram was constructed from the data using TREEVIEW ver. 1.6.6.

OBSERVATIONS

DNA extracted from the accessions of *B. monnieri* used for ISSR analyses showed high purity. Out of the 48 primers tested for amplifying the DNA of the species, 16 primers provided reproducible polymorphic patterns. The parameters of genetic diversity such as Na, I, H and P in the 60 accessions were estimated based on 13 loci, the bands of which were clearly distinguishable. The number of amplified segments of the 16 primers ranged from 2 to 6, having the size, ranging from 100 bp to 1000 bp. The estimates of the genetic diversity parameters Na, H, I and P among each population of the 60 accessions (single populations) are given in Table 4, and its graphical representation is given in Fig.1. The results of the analysis can be summed up as given below. Number of alleles per locus (Na) ranged from 1.13 (Acc. No. Bm 23) to 1.69 (Acc. No. Bm 30 and 57) with mean 1.43, and 35 accessions showed higher values than the mean. Shannon's information index (I) ranged from 0.08 (Acc. Bm 23) to 0.45 (Acc. No. Bm 30) and with mean value

TABLE 4: Estimates of the genetic diversity parameters (Na, I, H and P) in 60 accessions of *Bacopa monnieri* (Single populations).

Acc. No.	Na	I	H	P%	Acc. No.	Na	I	H	P%
Bm 1	1.25	0.17	0.12	25.00	Bm 31	1.25	0.17	0.12	25.00
Bm 2	1.50	0.34	0.24	50.00	Bm 32	1.31	0.21	0.15	31.25
Bm 3	1.31	0.21	0.15	31.25	Bm 33	1.38	0.25	0.17	37.50
Bm 4	1.38	0.25	0.18	37.50	Bm 34	1.44	0.28	0.20	43.75
Bm 5	1.44	0.30	0.21	43.75	Bm 35	1.31	0.20	0.14	31.25
Bm 6	1.31	0.21	0.15	31.25	Bm 36	1.50	0.32	0.23	50.00
Bm 7	1.31	0.21	0.15	31.25	Bm 37	1.44	0.29	0.21	43.75
Bm 8	1.19	0.12	0.09	18.75	Bm 38	1.38	0.23	0.16	37.50
Bm 9	1.50	0.33	0.24	50.00	Bm 39	1.31	0.20	0.14	31.25
Bm 10	1.44	0.29	0.21	43.75	Bm 40	1.56	0.37	0.26	56.25
Bm 11	1.50	0.34	0.24	50.00	Bm 41	1.56	0.35	0.24	56.25
Bm 12	1.50	0.32	0.23	50.00	Bm 42	1.44	0.28	0.20	43.75
Bm 13	1.50	0.33	0.23	50.00	Bm 43	1.38	0.25	0.18	37.50
Bm 14	1.63	0.41	0.29	62.50	Bm 44	1.38	0.16	0.23	37.50
Bm 15	1.50	0.33	0.23	50.00	Bm 45	1.56	0.38	0.27	56.25
Bm 16	1.25	0.17	0.12	25.00	Bm 46	1.50	0.22	0.32	50.00
Bm 17	1.31	0.21	0.15	31.25	Bm 47	1.44	0.27	0.19	43.75
Bm 18	1.63	0.41	0.29	62.50	Bm 48	1.56	0.38	0.27	56.25
Bm 19	1.38	0.25	0.18	37.50	Bm 49	1.56	0.38	0.27	56.25
Bm 20	1.31	0.21	0.15	31.25	Bm 50	1.31	0.21	0.15	31.25
Bm 21	1.44	0.28	0.20	43.75	Bm 51	1.50	0.33	0.23	50.00
Bm 22	1.50	0.31	0.22	50.00	Bm 52	1.38	0.25	0.18	37.50
Bm 23	1.13	0.08	0.06	12.50	Bm 53	1.50	0.34	0.25	50.00
Bm 24	1.19	0.12	0.09	18.75	Bm 54	1.56	0.37	0.27	56.25
Bm 25	1.38	0.24	0.17	37.50	Bm 55	1.63	0.39	0.27	62.50
Bm 26	1.19	0.13	0.09	18.75	Bm 56	1.50	0.34	0.25	50.00
Bm 27	1.50	0.34	0.24	50.00	Bm 57	1.69	0.43	0.30	68.75
Bm 28	1.25	0.16	0.11	25.00	Bm 58	1.63	0.41	0.29	62.50
Bm 29	1.44	0.28	0.20	43.75	Bm 59	1.63	0.41	0.29	62.50
Bm 30	1.69	0.45	0.32	68.75	Bm 60	1.56	0.36	0.26	56.25

0.28 and 32 accessions showed higher values than the mean. Nei's genetic diversity index (H) ranged from 0.06 (Acc. No. Bm 23) to 0.32 (Acc. No. Bm 30) with mean 0.20 percentage of polymorphism (P) in the 60 accessions ranged from 12.50 (Acc. No. Bm 23) to 68.75 (Acc. Nos Bm 30 and 57) with mean 43.23.

The estimates of overall genetic variability in

TABLE 5: Similarity matrix of the 60 accessions of *B. monnieri* based on ISSR data.

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	****	0.91	0.91	0.93	0.93	0.92	0.86	0.92	0.90	0.79	0.93	0.88	0.88	0.88	0.82	0.84	0.91	0.80	0.83	0.90	0.89	0.84	0.93	0.91	0.86	0.91	0.90	0.93	0.79	0.84
2	0.10	****	0.99	0.98	0.97	0.92	0.94	0.92	1.00	0.88	0.94	0.96	1.00	0.90	0.96	0.88	0.92	0.90	0.96	0.95	0.98	0.92	0.91	0.88	0.95	0.92	0.92	0.91	0.92	0.91
3	0.09	0.01	****	0.98	0.94	0.94	0.95	0.93	0.93	0.81	0.87	0.94	0.91	0.84	0.92	0.89	0.97	0.85	0.91	0.96	0.94	0.85	0.91	0.89	0.95	0.92	0.90	0.91	0.88	0.83
4	0.08	0.02	0.02	****	0.97	0.91	0.96	0.91	0.94	0.79	0.94	1.00	0.95	0.90	0.95	0.88	0.90	0.92	0.94	0.97	0.98	0.89	0.93	0.91	0.96	0.90	0.88	0.92	0.91	0.92
5	0.07	0.04	0.06	0.03	****	0.95	0.89	0.95	0.89	0.78	0.98	0.96	0.91	0.85	0.91	0.81	0.95	0.89	0.90	0.90	0.90	0.84	0.94	0.91	0.89	0.94	0.92	0.93	0.87	0.87
6	0.08	0.08	0.06	0.09	0.05	****	0.93	0.97	0.94	0.80	0.97	0.95	0.93	0.86	0.93	0.87	0.93	0.88	0.88	0.94	0.87	0.82	0.92	0.90	0.88	0.89	0.94	0.97	0.89	0.79
7	0.15	0.06	0.05	0.04	0.12	0.07	****	0.93	0.96	0.87	0.92	0.95	0.97	0.85	0.97	0.97	0.87	0.91	0.97	0.99	0.94	0.85	0.94	0.92	0.93	0.92	0.91	0.92	0.98	0.83
8	0.09	0.08	0.07	0.09	0.05	0.03	0.08	****	0.89	0.80	0.91	0.88	0.88	0.79	0.88	0.85	0.93	0.83	0.93	0.89	0.88	0.82	0.96	0.94	0.88	0.93	0.88	0.89	0.90	0.81
9	0.11	0.00	0.08	0.06	0.11	0.06	0.04	0.12	****	0.90	0.98	0.98	1.05	0.95	0.98	0.90	0.84	0.92	0.92	0.97	0.96	0.90	0.87	0.85	0.91	0.84	0.93	0.92	0.93	0.90
10	0.23	0.13	0.21	0.23	0.25	0.23	0.14	0.23	0.11	****	0.82	0.81	0.89	0.91	0.92	0.93	0.83	0.86	0.92	0.86	0.91	0.89	0.82	0.88	0.89	0.84	0.81	0.80	0.84	0.85
11	0.07	0.07	0.14	0.06	0.02	0.03	0.08	0.10	0.02	0.20	****	0.98	0.98	0.92	0.94	0.86	0.86	0.93	0.89	0.93	0.91	0.85	0.91	0.89	0.87	0.88	0.95	0.96	0.90	0.88
12	0.12	0.04	0.07	0.00	0.05	0.05	0.05	0.12	0.02	0.22	0.02	****	0.99	0.95	1.00	0.87	0.85	0.98	0.91	0.98	0.93	0.87	0.87	0.84	0.92	0.83	0.89	0.94	0.93	0.90
13	0.13	0.00	0.09	0.05	0.10	0.07	0.03	0.13	-0.04	0.12	0.02	0.01	****	0.95	1.00	0.89	0.83	0.93	0.93	0.98	0.98	0.92	0.89	0.86	0.91	0.85	0.92	0.93	0.95	0.91
14	0.12	0.11	0.18	0.10	0.16	0.15	0.16	0.24	0.05	0.09	0.08	0.05	0.05	****	0.95	0.87	0.81	0.93	0.87	0.93	0.93	1.00	0.82	0.84	0.90	0.78	0.82	0.89	0.83	0.97
15	0.19	0.04	0.09	0.05	0.10	0.07	0.03	0.12	0.02	0.08	0.06	0.00	0.00	0.05	****	0.94	0.89	1.00	0.99	0.98	0.99	0.93	0.89	0.91	0.99	0.85	0.89	0.93	0.96	0.91
16	0.18	0.13	0.12	0.12	0.21	0.14	0.03	0.16	0.11	0.07	0.15	0.14	0.12	0.14	0.06	****	0.86	0.89	0.95	0.96	0.91	0.87	0.90	0.93	0.94	0.89	0.84	0.90	0.90	0.81
17	0.10	0.08	0.04	0.11	0.05	0.07	0.14	0.08	0.17	0.18	0.15	0.16	0.19	0.22	0.12	0.15	****	0.82	0.88	0.88	0.91	0.82	0.91	0.93	0.94	0.94	0.91	0.91	0.79	0.80
18	0.23	0.11	0.16	0.08	0.12	0.13	0.10	0.19	0.08	0.15	0.07	0.02	0.07	0.07	0.00	0.11	0.20	****	0.94	0.91	0.92	0.87	0.82	0.84	0.93	0.78	0.90	0.87	0.98	0.98
19	0.19	0.04	0.09	0.06	0.10	0.13	0.03	0.08	0.08	0.08	0.12	0.09	0.07	0.14	0.01	0.05	0.12	0.06	****	0.92	0.98	0.93	0.93	0.95	0.99	0.90	0.83	0.86	0.95	0.91
20	0.10	0.05	0.05	0.03	0.11	0.06	0.01	0.12	0.03	0.15	0.07	0.02	0.02	0.07	0.02	0.04	0.13	0.09	0.08	****	0.95	0.90	0.94	0.91	0.94	0.91	0.92	0.99	0.94	0.84
21	0.12	0.02	0.06	0.02	0.10	0.13	0.06	0.13	0.04	0.10	0.10	0.07	0.03	0.07	0.01	0.09	0.09	0.09	0.02	0.05	****	0.96	0.91	0.94	1.02	0.88	0.89	0.90	0.90	0.95
22	0.17	0.08	0.16	0.12	0.17	0.20	0.16	0.19	0.10	0.12	0.17	0.13	0.09	0.00	0.07	0.14	0.19	0.14	0.08	0.10	0.04	****	0.87	0.89	0.93	0.83	0.79	0.86	0.84	0.97
23	0.07	0.10	0.09	0.07	0.07	0.08	0.06	0.04	0.14	0.19	0.09	0.14	0.12	0.20	0.12	0.10	0.10	0.20	0.08	0.06	0.09	0.14	****	0.99	0.90	0.98	0.90	0.94	0.90	0.81
24	0.10	0.13	0.12	0.10	0.09	0.11	0.08	0.06	0.17	0.12	0.12	0.17	0.15	0.17	0.09	0.07	0.07	0.17	0.05	0.09	0.07	0.12	0.01	****	0.94	0.96	0.87	0.92	0.87	0.84
25	0.15	0.06	0.05	0.04	0.12	0.12	0.07	0.13	0.10	0.11	0.14	0.08	0.09	0.10	0.01	0.06	0.06	0.07	0.01	0.06	-0.02	0.07	0.11	0.06	****	0.87	0.85	0.90	0.88	0.92
26	0.10	0.08	0.08	0.11	0.06	0.11	0.09	0.07	0.17	0.18	0.13	0.18	0.16	0.25	0.16	0.11	0.06	0.24	0.11	0.09	0.13	0.19	0.02	0.04	0.14	****	0.91	0.92	0.86	0.77
27	0.11	0.08	0.10	0.12	0.08	0.06	0.10	0.13	0.08	0.21	0.05	0.12	0.08	0.20	0.12	0.17	0.09	0.11	0.18	0.09	0.12	0.23	0.11	0.14	0.16	0.09	****	0.95	0.93	0.86
28	0.07	0.10	0.09	0.08	0.07	0.03	0.08	0.11	0.08	0.23	0.04	0.06	0.07	0.12	0.07	0.11	0.10	0.14	0.16	0.01	0.10	0.16	0.06	0.08	0.11	0.09	0.05	****	0.87	0.80
29	0.24	0.09	0.13	0.09	0.14	0.11	0.02	0.11	0.07	0.18	0.11	0.07	0.05	0.19	0.04	0.11	0.23	0.02	0.05	0.07	0.11	0.18	0.11	0.14	0.13	0.15	0.07	0.14	****	0.90
30	0.18	0.09	0.18	0.09	0.15	0.23	0.18	0.22	0.10	0.17	0.13	0.11	0.09	0.03	0.09	0.22	0.22	0.02	0.09	0.17	0.05	0.03	0.21	0.18	0.09	0.26	0.15	0.23	0.10	****
31	0.11	0.03	0.02	0.03	0.07	0.05	0.07	0.05	0.06	0.23	0.13	0.04	0.06	0.14	0.06	0.18	0.10	0.13	0.08	0.07	0.06	0.12	0.10	0.13	0.07	0.15	0.16	0.12	0.10	0.14
32	0.05	0.12	0.07	0.05	0.04	0.06	0.13	0.06	0.16	0.35	0.09	0.10	0.17	0.14	0.17	0.22	0.08	0.21	0.17	0.12	0.13	0.15	0.08	0.11	0.13	0.12	0.13	0.09	0.21	0.15
33	0.14	0.07	0.08	0.09	0.03	0.11	0.13	0.11	0.16	0.17	0.10	0.14	0.14	0.19	0.07	0.16	0.01	0.16	0.08	0.12	0.05	0.14	0.08	0.05	0.05	0.05	0.08	0.08	0.18	0.17
34	0.04	0.09	0.13	0.06	0.05	0.11	0.17	0.13	0.10	0.19	0.04	0.10	0.10	0.09	0.10	0.20	0.07	0.14	0.13	0.14	0.03	0.13	0.11	0.09	0.06	0.15	0.10	0.09	0.24	0.08
35	0.10	0.05	0.01	0.03	0.10	0.02	0.01	0.08	0.03	0.20	0.09	0.02	0.04	0.12	0.04	0.10	0.09	0.11	0.10	0.01	0.07	0.16	0.10	0.13	0.06	0.14	0.09	0.05	0.08	0.19
36	0.09	0.14	0.11	0.15	0.09	0.15	0.26	0.18	0.22	0.25	0.16	0.20	0.24	0.21	0.22	0.27	0.04	0.15	0.24	0.22	0.15	0.27	0.20	0.17	0.14	0.16	0.06	0.17	0.26	0.12
37	0.10	0.17	0.17	0.15	0.11	0.13	0.18	0.07	0.19	0.31	0.13	0.20	0.20	0.21	0.25	0.28	0.18	0.18	0.19	0.23	0.22	0.20	0.12	0.15	0.25	0.16	0.12	0.22	0.13	0.09
38	0.11	0.13	0.14	0.06	0.05	0.13	0.14	0.13	0.18	0.36	0.08	0.11	0.15	0.13	0.15	0.24	0.15	0.19	0.16	0.13	0.12	0.10	0.09	0.12	0.14	0.13	0.15	0.10	0.20	0.11
39	0.17	0.11	0.12	0.11	0.07	0.14	0.10	0.08	0.19	0.26	0.13	0.19	0.16	0.32	0.16	0.18	0.11	0.15	0.11	0.14	0.13	0.23	0.05	0.08	0.15	0.04	0.03	0.13	0.06	0.16
40	0.18	0.08	0.12	0.03	0.08	0.16	0.12	0.16	0.12	0.31	0.11	0.05	0.10	0.10	0.09	0.22	0.22	0.02	0.10	0.11	0.10	0.08	0.15	0.18	0.12	0.20	0.13	0.16	0.05	-0.02
41	0.15	0.18	0.23	0.13	0.12	0.21	0.24	0.24	0.20	0.30	0.11	0.18	0.19	0.12	0.16	0.25	0.17	0.10	0.19	0.20	0.10	0.11	0.18	0.15	0.12	0.22	0.09	0.15	0.21	0.00
42	0.03	0.05	0.08	0.05	0.01	0.10	0.16	0.12	0.09	0.26	0.03	0.09	0.09	0.14	0.15	0.26	0.07	0.20	0.18	0.13	0.08	0.18	0.11	0.13	0.13	0.10	0.06	0.08	0.23	0.14
43	0.12	0.12	0.07	0.13	0.09	0.09	0.17	0.10	0.20	0.23	0.17	0.19	0.22	0.25	0.15	0.18	0.01	0.13	0.15	0.16	0.12	0.22	0.12	0.09	0.08	0.10	0.04	0.12	0.16	0.15
44	0.13	0.13	0.08	0.13	0.09	0.10	0.20	0.12	0.21	0.26	0.18	0.18	0.22	0.24	0.15	0.23	0.02	0.14	0.18	0.17	0.12	0.22	0.14	0.12	0.09	0.14	0.05	0.12	0.19	0.15
45	0.10	0.11	0.16	0.12	0.08	0.19	0.20	0.19	0.16	0.19	0.09	0.18	0.16	0.17	0.16	0.21	0.09	0.09	0.15	0.19	0.08	0.19	0.14	0.12	0.11	0.13	0.03	0.15	0.17	0.04
46	0.17	0.16	0.17	0.12	0.07	0.19	0.21	0.18	0.25	0.41	0.14	0.17	0.22	0.21	0.22	0.31	0.16	0.17	0.22	0.20	0.18	0.16	0.14	0.18	0.21	0.14	0.08	0.16	0.17	0.08
47	0.09	0.06	0.10	0.07	0.01	0.15	0.18	0.17	0.14	0.28	0.06	0.11	0.13	0.19	0.17	0.28	0.08	0.22	0.20	0.15	0.11	0.21	0.13	0.16	0.16	0.09	0.07	0.10	0.25	0.18
48	0.15	0.11	0.15	0.11	0.03	0.07	0.12	0.14	0.12	0.27	0.02	0.06	0.11	0.19	0.10	0.20	0.14	0.02	0.17	0.11	0.18	0.25	0.13	0.16	0.19	0.12	0.00	0.08	0.06	0.13
49	0.15	0.01	0.06	0.02	0.03	0.13	0.09	0.14	0.09	0.23	0.08	0.03	0.08	0.08	0.07	0.17	0.14	0.09	0.07	0.08	0.08	0.06	0.13	0.16	0.09	0.12	0.16	0.14	0.12	0.06
50	0.09	0.03	0.05	0.05	0.01	0.07	0.08	0.07	0.11	0.24	0.05	0.09	0.09	0.21	0.09	0.15	0.04	0.17	0.09	0.07	0.06	0.16	0.04	0.07	0.08	0.03	0.04	0.04	0.14	0.18
51	0.20	0.04	0.10	0.12	0.13	0.18	0.18	0.20	0.10	0.07	0.17	0.13	0.10	0.09	0.03	0.15	0.07	0.10	0.06	0.14	0.01	0.07	0.22	0.14	0.01	0.20	0.19	0.19	0.22	0.08
52	0.11	-0.01	0.02	0.00	0.04	0.05	0.03	0.05	0.02	0.19	0.07	0.01	0.01	0.11	0.01	0.12	0.10	0.07	0.02	0.03	0.02	0.08	0.07	0.10	0.03	0.11	0.12	0.08	0.05	0.09
53	0.18	0.16	0.18	0.15	0.07	0.10	0.17	0.16	0.17	0.11	0.07	0.11	0.17	0.11	0.05	0.14	0.06	0.07	0.11	0.16	0.12	0.19	0.18	0.11	0.08	0.16	0.14	0.12	0.21	0.17
54	0.16	0.02	0.05	0.07	0.11	0.18	0.11	0.17	0.10	0.10	0.17	0.13	0.09	0.07	0.07	0.15	0.06	0.17	0.08	0.11	0.01	0.02	0.15	0.12	0.03	0.12	0.13	0.16	0.18	0.06
55	0.17	0.06	0.11	0.05	0.10	0.24	0.16	0.25	0.12	0.17	0.13	0.09	0.10	0.04	0.08	0.20	0.14	0.12	0.11	0.13	0.02	0.03	0.19	0.16	0.05	0.19	0.17	0.17	0.22	0.02
56	0.09	0.06	0.08	0.06	-0.01	0.07	0.14	0.08	0.14	0.29	0.04	0.08	0.13	0.13	0.13	0.22	0.07	0.15	0.13	0.13	0.13	0.11	0.09	0.12	0.14	0.08	0.11	0.09	0.18	0.12
57	0.28	0.19	0.24	0.21	0.28	0.31	0.24	0.43	0.17	0.23	0.20	0.20	0.19	0.09	0.19	0.20	0.26	0.10	0.26	0.20	0.17	0.15	0.37	0.34	0.18	0.33	0.13	0.24	0.24	0.04
58	0.19	0.21	0.28	0.23	0.17	0.14	0.26	0.25	0.16	0.26	0.08	0.16	0.18	0.10	0.16	0.22	0.21	0.05	0.23	0.22	0.22	0.18	0.28	0.25	0.20	0.31	0.10	0.16	0.20	0.06
59	0.11	0.08	0.15	0.13	0.03	0.06	0.17	0.15	0.07	0.12	0.00	0.06	0.08	0.06	0.06	0.19	0.08	0.07	0.14	0.14	0.11	0.17	0.18	0.15	0.12	0.16	0.08	0.09	0.20	0.13
60	0.08	0.07	0.12	0.09	0.01	0.11	0.21	0.13	0.12	0.30	0.05	0.10	0.12	0.18	0.16	0.31	0.11	0.09	0.20	0.17	0.13	0.21	0.14	0.18	0.18	0.14	0.02	0.12	0.14	0.07

ID	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	0.89	0.95	0.87	0.96	0.90	0.91	0.90	0.89	0.85	0.83	0.86	0.97	0.89	0.88	0.90	0.84	0.91	0.86	0.86	0.91	0.82	0.89	0.83	0.85	0.84	0.91	0.76	0.83	0.90	0.92
2	0.97	0.89	0.94	0.91	0.95	0.87	0.85	0.87	0.90	0.93	0.84	0.95	0.89	0.88	0.89	0.85	0.94	0.90	0.99	0.97	0.96	1.01	0.86	0.98	0.94	0.94	0.83	0.81	0.92	0.94
3	0.98	0.93	0.93	0.88	0.99	0.90	0.84	0.87	0.89	0.89	0.80	0.92	0.93	0.93	0.85	0.85	0.91	0.86	0.95	0.95	0.91	0.98	0.84	0.96	0.89	0.92	0.79	0.76	0.86	0.89
4	0.97	0.95	0.92	0.94	0.97	0.86	0.86	0.94	0.89	0.97	0.88	0.95	0.88	0.88	0.89	0.89	0.93	0.90	0.99	0.96	0.89	1.00	0.86	0.93	0.95	0.94	0.81	0.79	0.88	0.91
5	0.94	0.96	0.97	0.95	0.90	0.91	0.90	0.95	0.93	0.92	0.88	0.99	0.91	0.91	0.93	0.93	0.99	0.97	0.97	0.99	0.88	0.96	0.93	0.90	0.90	1.01	0.75	0.84	0.97	0.99
6	0.95	0.94	0.89	0.89	0.98	0.86	0.88	0.88	0.87	0.85	0.81	0.90	0.91	0.91	0.83	0.83	0.86	0.93	0.87	0.93	0.84	0.95	0.90	0.84	0.79	0.93	0.74	0.87	0.94	0.90
7	0.93	0.88	0.88	0.84	0.99	0.77	0.84	0.87	0.91	0.89	0.79	0.85	0.85	0.82	0.82	0.81	0.83	0.88	0.91	0.92	0.84	0.97	0.84	0.89	0.85	0.87	0.79	0.77	0.84	0.81
8	0.95	0.94	0.89	0.88	0.93	0.84	0.94	0.88	0.92	0.86	0.79	0.88	0.91	0.89	0.83	0.83	0.85	0.87	0.87	0.93	0.82	0.95	0.85	0.85	0.78	0.93	0.65	0.78	0.86	0.88
9	0.94	0.85	0.85	0.90	0.97	0.80	0.83	0.84	0.83	0.89	0.82	0.91	0.82	0.81	0.85	0.78	0.87	0.88	0.91	0.90	0.91	0.98	0.84	0.91	0.89	0.87	0.84	0.85	0.93	0.88
10	0.80	0.71	0.85	0.83	0.82	0.78	0.74	0.69	0.77	0.73	0.74	0.77	0.80	0.77	0.83	0.66	0.76	0.76	0.79	0.79	0.93	0.83	0.89	0.90	0.84	0.75	0.80	0.77	0.88	0.74
11	0.88	0.91	0.90	0.96	0.91	0.85	0.87	0.93	0.88	0.89	0.90	0.97	0.84	0.83	0.92	0.87	0.94	0.98	0.92	0.95	0.84	0.93	0.93	0.84	0.88	0.96	0.82	0.92	1.00	0.95
12	0.97	0.91	0.87	0.90	0.98	0.82	0.82	0.90	0.83	0.95	0.84	0.91	0.83	0.84	0.84	0.84	0.89	0.94	0.97	0.91	0.88	0.99	0.90	0.88	0.91	0.93	0.82	0.85	0.95	0.91
13	0.94	0.85	0.87	0.90	0.96	0.78	0.82	0.86	0.85	0.91	0.83	0.91	0.81	0.81	0.85	0.80	0.88	0.90	0.93	0.91	0.91	0.99	0.85	0.91	0.90	0.88	0.82	0.83	0.93	0.89
14	0.87	0.87	0.83	0.92	0.89	0.81	0.81	0.88	0.73	0.90	0.89	0.87	0.78	0.79	0.84	0.81	0.83	0.83	0.92	0.81	0.92	0.90	0.89	0.93	0.96	0.88	0.91	0.90	0.94	0.84
15	0.95	0.85	0.93	0.91	0.96	0.80	0.78	0.86	0.85	0.91	0.85	0.86	0.86	0.86	0.85	0.80	0.84	0.90	0.93	0.91	0.97	0.99	0.95	0.93	0.92	0.88	0.83	0.85	0.95	0.85
16	0.83	0.80	0.85	0.82	0.91	0.76	0.76	0.79	0.83	0.80	0.78	0.77	0.84	0.79	0.81	0.74	0.75	0.82	0.85	0.86	0.86	0.89	0.87	0.86	0.82	0.81	0.82	0.80	0.83	0.73
17	0.90	0.93	0.99	0.93	0.91	0.96	0.84	0.86	0.90	0.80	0.85	0.93	0.99	0.98	0.92	0.86	0.92	0.87	0.87	0.96	0.93	0.90	0.94	0.94	0.87	0.93	0.77	0.81	0.93	0.89
18	0.88	0.81	0.85	0.87	0.89	0.86	0.84	0.82	0.86	0.98	0.91	0.82	0.88	0.87	0.91	0.85	0.81	0.98	0.91	0.85	0.91	0.93	0.94	0.85	0.88	0.86	0.90	0.95	0.94	0.91
19	0.93	0.84	0.92	0.88	0.90	0.79	0.83	0.85	0.90	0.91	0.83	0.83	0.86	0.83	0.86	0.80	0.82	0.85	0.93	0.91	0.94	0.98	0.89	0.93	0.89	0.88	0.77	0.79	0.87	0.82
20	0.94	0.88	0.89	0.87	0.99	0.80	0.79	0.87	0.87	0.89	0.82	0.88	0.86	0.85	0.82	0.82	0.86	0.89	0.92	0.93	0.87	0.97	0.85	0.90	0.88	0.88	0.82	0.80	0.87	0.84
21	0.94	0.87	0.96	0.97	0.94	0.86	0.80	0.89	0.88	0.91	0.91	0.93	0.89	0.89	0.93	0.83	0.90	0.84	0.93	0.94	0.99	0.98	0.89	0.99	0.98	0.87	0.84	0.81	0.90	0.87
22	0.88	0.86	0.87	0.88	0.85	0.77	0.82	0.91	0.79	0.93	0.89	0.83	0.80	0.80	0.83	0.85	0.81	0.78	0.95	0.85	0.93	0.93	0.83	0.98	0.97	0.89	0.86	0.84	0.85	0.81
23	0.91	0.92	0.93	0.89	0.90	0.82	0.89	0.91	0.95	0.86	0.84	0.90	0.89	0.87	0.87	0.87	0.88	0.88	0.88	0.96	0.81	0.93	0.84	0.86	0.82	0.91	0.69	0.76	0.84	0.87
24	0.88	0.90	0.95	0.92	0.88	0.84	0.86	0.89	0.93	0.83	0.86	0.88	0.91	0.89	0.89	0.84	0.85	0.85	0.85	0.93	0.87	0.91	0.90	0.89	0.85	0.89	0.71	0.78	0.86	0.84
25	0.93	0.88	0.96	0.94	0.94	0.87	0.78	0.87	0.86	0.89	0.89	0.87	0.92	0.91	0.90	0.81	0.85	0.83	0.91	0.93	0.99	0.97	0.92	0.97	0.95	0.87	0.83	0.82	0.89	0.83
26	0.86	0.89	0.95	0.86	0.87	0.85	0.85	0.88	0.96	0.82	0.80	0.91	0.90	0.87	0.88	0.87	0.92	0.89	0.89	0.97	0.82	0.90	0.85	0.89	0.83	0.93	0.72	0.74	0.85	0.87
27	0.85	0.88	0.93	0.90	0.91	0.95	0.89	0.87	0.97	0.88	0.91	0.94	0.96	0.95	0.97	0.93	0.93	1.00	0.85	0.96	0.83	0.89	0.87	0.88	0.84	0.90	0.88	0.90	0.92	0.98
28	0.89	0.91	0.92	0.91	0.95	0.84	0.80	0.91	0.88	0.85	0.86	0.92	0.89	0.89	0.86	0.86	0.90	0.93	0.87	0.96	0.83	0.93	0.89	0.85	0.84	0.91	0.78	0.85	0.92	0.89
29	0.90	0.81	0.83	0.79	0.92	0.77	0.88	0.82	0.94	0.95	0.81	0.80	0.85	0.83	0.84	0.85	0.78	0.94	0.89	0.87	0.80	0.95	0.81	0.83	0.80	0.84	0.79	0.82	0.82	0.87
30	0.87	0.86	0.85	0.92	0.83	0.88	0.92	0.90	0.85	1.02	1.00	0.87	0.86	0.86	0.96	0.93	0.84	0.88	0.95	0.83	0.92	0.91	0.84	0.94	0.98	0.89	0.96	0.94	0.88	0.93
31	****	0.93	0.88	0.88	0.98	0.83	0.87	0.86	0.86	0.92	0.78	0.89	0.88	0.89	0.80	0.81	0.85	0.84	0.93	0.90	0.89	1.01	0.82	0.91	0.86	0.90	0.70	0.75	0.86	0.88
32	0.08	****	0.89	0.93	0.93	0.89	0.94	0.99	0.87	0.91	0.90	0.94	0.91	0.91	0.86	0.94	0.90	0.88	0.93	0.93	0.80	0.92	0.85	0.89	0.88	0.99	0.78	0.84	0.87	0.91
33	0.13	0.11	****	0.95	0.88	0.92	0.80	0.91	0.94	0.84	0.90	0.95	0.95	0.95	0.95	0.90	0.97	0.91	0.91	1.00	0.94	0.92	0.95	0.96	0.94	0.94	0.79	0.80	0.94	0.91
34	0.13	0.07	0.05	****	0.88	0.94	0.85	0.92	0.85	0.86	0.96	0.99	0.91	0.92	0.98	0.87	0.95	0.88	0.88	0.93	0.93	0.91	0.93	0.92	0.95	0.92	0.82	0.88	0.97	0.94
35	0.02	0.08	0.13	0.13	****	0.84	0.83	0.86	0.85	0.88	0.78	0.89	0.89	0.89	0.80	0.81	0.85	0.87	0.90	0.91	0.86	0.98	0.85	0.90	0.85	0.87	0.78	0.78	0.87	0.85
36	0.18	0.11	0.08	0.06	0.18	****	0.88	0.83	0.88	0.84	0.93	0.94	1.01	1.01	1.00	0.90	0.93	0.92	0.83	0.89	0.89	0.82	0.90	0.89	0.87	0.89	0.86	0.89	0.92	0.98
37	0.14	0.06	0.23	0.16	0.18	0.12	****	0.90	0.91	0.93	0.88	0.86	0.89	0.87	0.88	0.93	0.81	0.89	0.86	0.84	0.73	0.85	0.78	0.82	0.78	0.92	0.77	0.87	0.82	0.93
38	0.15	0.01	0.10	0.08	0.15	0.19	0.11	****	0.88	0.95	0.95	0.93	0.84	0.84	0.88	0.99	0.92	0.89	0.97	0.94	0.79	0.91	0.84	0.90	0.94	1.00	0.82	0.85	0.86	0.89
39	0.15	0.14	0.06	0.16	0.16	0.13	0.10	0.13	****	0.90	0.88	0.89	0.94	0.92	0.94	0.94	0.90	0.95	0.87	0.96	0.79	0.90	0.82	0.87	0.82	0.90	0.76	0.79	0.82	0.94
40	0.09	0.09	0.17	0.15	0.13	0.18	0.07	0.05	0.10	****	0.95	0.87	0.86	0.86	0.89	0.98	0.85	0.94	1.00	0.88	0.83	0.96	0.80	0.90	0.93	0.95	0.89	0.89	0.83	0.95
41	0.25	0.11	0.10	0.04	0.24	0.08	0.12	0.05	0.12	0.05	****	0.92	0.91	0.91	1.00	0.98	0.89	0.92	0.89	0.89	0.86	0.84	0.87	0.90	0.95	0.92	0.95	0.97	0.89	0.95
42	0.12	0.06	0.05	0.01	0.12	0.06	0.15	0.07	0.11	0.14	0.09	****	0.90	0.90	0.97	0.91	1.02	0.92	0.92	0.97	0.87	0.91	0.88	0.92	0.94	0.96	0.81	0.83	0.95	0.98
43	0.12	0.10	0.05	0.10	0.12	-0.01	0.11	0.17	0.06	0.15	0.10	0.11	****	1.03	0.96	0.90	0.88	0.92	0.83	0.93	0.89	0.88	0.91	0.90	0.83	0.89	0.81	0.87	0.89	0.94
44	0.12	0.10	0.05	0.09	0.12	-0.01	0.14	0.17	0.08	0.15	0.10	0.10	-0.03	****	0.95	0.90	0.89	0.91	0.83	0.92	0.89	0.88	0.90	0.90	0.84	0.88	0.80	0.85	0.89	0.95
45	0.22	0.15	0.05	0.02	0.22	0.00	0.12	0.13	0.06	0.11	0.00	0.03	0.04	0.05	****	0.94	0.96	0.96	0.86	0.93	0.90	0.85	0.92	0.91	0.93	0.90	0.91	0.92	0.94	1.00
46	0.21	0.06	0.11	0.14	0.21	0.11	0.08	0.01	0.06	0.02	0.02	0.09	0.10	0.11	0.06	****	0.92	0.95	0.95	0.92	0.76	0.85	0.81	0.89	0.92	0.98	0.88	0.88	0.83	0.96
47	0.16	0.10	0.03	0.05	0.17	0.08	0.21	0.09	0.10	0.16	0.11	-0.02	0.13	0.12	0.04	0.08	****	0.93	0.93	0.98	0.86	0.89	0.88	0.92	0.94	0.96	0.80	0.79	0.94	0.97
48	0.17	0.13	0.10	0.13	0.14	0.09	0.12	0.12	0.05	0.06	0.09	0.08	0.09	0.10	0.05	0.05	0.07	****	0.91	0.94	0.81	0.90	0.92	0.82	0.84	0.95	0.86	0.94	0.96	1.00
49	0.08	0.07	0.10	0.13	0.11	0.19	0.15	0.03	0.14	0.00	0.12	0.08	0.18	0.19	0.15	0.06	0.07	0.09	****	0.94	0.89	0.98	0.86	0.97	0.99	1.01	0.88	0.85	0.90	0.91
50	0.10	0.07	0.00	0.07	0.09	0.11	0.18	0.06	0.04	0.12	0.11	0.03	0.07	0.08	0.07	0.08	0.02	0.06	0.06	****	0.88	0.96	0.89	0.93	0.91	0.97	0.79	0.81	0.92	0.94
51	0.12	0.22	0.06	0.08	0.15	0.12	0.31	0.24	0.23	0.18	0.16	0.13	0.11	0.11	0.10	0.27	0.15	0.22	0.11	0.13	****	0.92	0.94	0.98	0.96	0.85	0.85	0.83	0.94	0.85
52	-0.01	0.09	0.08	0.10	0.02	0.19	0.16	0.10	0.11	0.04	0.17	0.09	0.13	0.13	0.16	0.16	0.11	0.11	0.02	0.04	0.08	****	0.85	0.93	0.91	0.94	0.77	0.80	0.89	0.91
53	0.20	0.16	0.05	0.07	0.17	0.10	0.25	0.18	0.19	0.22	0.14	0.13	0.10	0.11	0.09	0.21	0.13	0.08	0.15	0.11	0.06	0.16	****	0.88	0.89	0.91	0.82	0.91	1.02	0.87
54	0.10	0.11	0.04	0.08	0.10	0.12	0.20	0.10	0.14	0.11	0.11	0.08	0.10	0.11	0.10	0.11	0.08	0.20	0.03	0.07	0.02	0.07	0.13	****	1.02	0.92	0.91	0.80	0.89	0.86
55	0.15	0.12	0.07	0.05	0.16	0.14	0.24	0.06	0.20	0.07	0.05	0.06	0.18	0.17	0.07	0.09	0.06	0.18	0.01	0.10	0.04	0.09	0.12	-0.02	****	0.93	0.94	0.83	0.90	0.88
56	0.11	0.01	0.06	0.08	0.13	0.12	0.08	0.00	0.10	0.05	0.09	0.04	0.11	0.12	0.10	0.02	0.04	0.05	-0.01	0.03	0.16	0.07	0.10	0.08	0.07	****	0.83	0.89	0.94	0.96
57	0.36	0.25	0.24	0.20	0.25	0.15	0.26	0.19	0.28	0.12	0.06	0.21	0.21	0.22	0.10	0.12	0.22	0.16	0.13	0.24	0.17	0.26	0.20	0.10	0.06	0.19	****	0.96	0.85	0.83
58	0.29	0.17	0.22	0.13	0.25	0.12	0.13	0.16	0.24	0.11	0.03	0.19	0.14	0.16	0.09	0.13	0.23	0.06	0.17	0.21	0.18	0.22	0.10	0.22	0.18	0.11	0.04	****	0.95	0.91
59	0.16	0.14	0.06	0.03	0.14	0.08	0.20	0.15	0.20	0.18	0.12	0.05	0.12	0.12	0.06	0.18	0.06	0.04	0.11	0.09	0.06	0.12	-0.02	0.12	0.10	0.06	0.17	0.05	****	0.95
60	0.12	0.10	0.09	0.06	0.17	0.02	0.08	0.11	0.07	0.05	0.05	0.02	0.06	0.05	0.00	0.05	0.03	0.00	0.09	0.06	0.16	0.10	0.14	0.15	0.13	0.04	0.19	0.10	0.05	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

TABLE 6: Composition of the three clusters of the accessions of *B. monnieri* based on POPGENE analysis.

Clusters	No.of Accs	Accessions
I	2	Bm 57, Bm 58
II	24	Bm 2, Bm 52, Bm 9, Bm 13, Bm 4, Bm 12, Bm 7, Bm 20, Bm 35, Bm 3, Bm 31, Bm 19, Bm 29, Bm 15, Bm 18, Bm 14, Bm 22, Bm 51, Bm 21, Bm 25, Bm 54, Bm 55, Bm 10, Bm 16
III	34	Bm 1, Bm 6, Bm 8, Bm 11, Bm 28, Bm 26, Bm 23, Bm 24, Bm 49, Bm 5, Bm 56, Bm 32, Bm 38, Bm 33, Bm 50, Bm 34, Bm 42, Bm 47, Bm 17, Bm 44, Bm 36, Bm 43, Bm 53, Bm 59, Bm 37, Bm 39, Bm 60, Bm 27, Bm 48, Bm 30, Bm 40, Bm 46, Bm 41, Bm 45

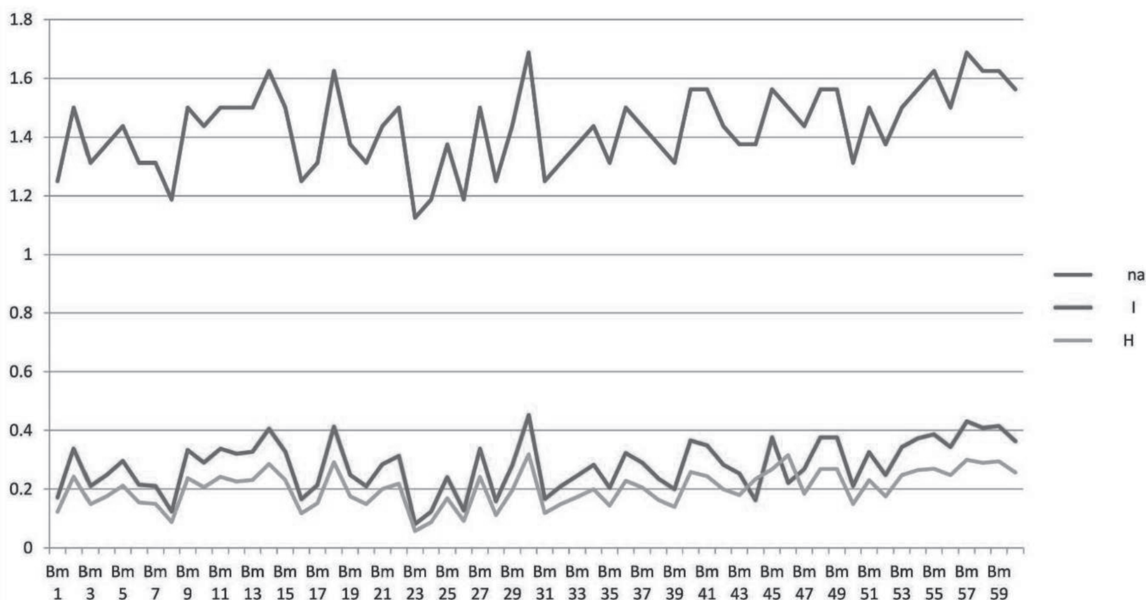

Fig. 1: Profiles of Na, I and H in the 60 accessions of *B. monnieri.*

the 60 accessions based on Na, H and I are given in Table 4, and the results of the analysis are summarised below. Number of alleles per locus (Na) ranged from 1 to 2 (mean 1.81), Shannon's information index (I) ranged from 0 to 0.69 (mean 0.47) and Nie's genetic diversity index (H) ranged from 0 to 0.50 (mean 0.32). The pairwise genetic similarity and distance matrix among the accessions (Table 5) were prepared on the basis of ISSR data given in Table 3. The genetic similarity

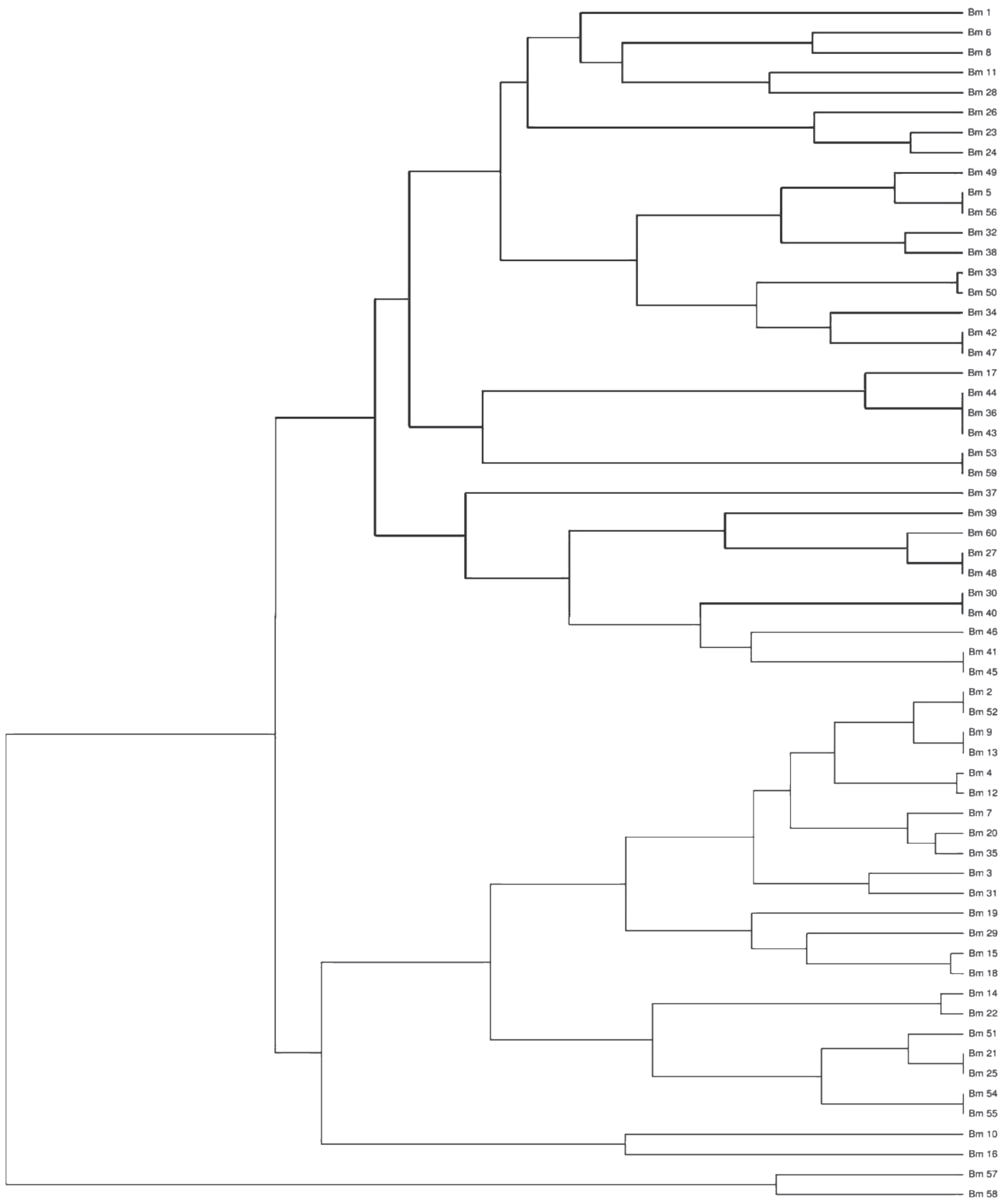

Fig. 2: Dendrogram showing the clusters of the 60 accessions of *B. monnieri* based on POPGENE analysis.

values given above the diagonal in Table 4 ranged from 0.65 to 1.05 (the observed maximum value, 1.05 has to be treated as 1, as the value is expected to range from 0 to 1, where 0 refers to no similarity and 1 maximum similarity). The mean genetic identity among the accessions was 0.89. The highest genetic identity (1.00) was found among 19 pairs of accessions and lowest genetic identity was between 2 accessions (Acc. Nos 8 and 57). The genetic diversity studies on the 60 accessions of the species using 16 ISSR primers generated 2890 bands, among which 921 were only polymorphic (31.81%).

The cluster analyses using POPGENE grouped the 60 accessions into 3 main groups, Clusters I, II and III. Composition of the clusters is given in Table 6. Cluster I includes 2 accessions, Cluster II, 24 accessions in 12 subgroups and Cluster III, 34 accessions in 21 subgroups. The dendrogram revealed the details of the groups of the 3 clusters and the interrelationship of the accessions (Fig. 2).

DISCUSSION

Molecular markers are the best tools for genetic diversity analysis in plants, since they detect variation at the nucleotide level and this can provide reliable means for determining genetic variability among the genotypes of a species. The present day advancements in molecular biology have increased the chances of using data on polymorphism of genes in breeding programmes and remarkable achievements have been made in the field of crop improvement. This became possible since molecular markers are powerful tools for assessing intraspecific genetic variation and for understanding genetic relationship within and among related species (Chakravarthi & Naravaneni 2006). Unlike morphological characters, the molecular parameters used are not affected by environment (Staub et al. 1997). The advent of DNA marker technology has revolutionised the field of genetics by enhancing the pace and precision of genetic analysis (Collins 2002).

Although there are some preliminary attempts for assessing the variability in the germplasm of *B. monnieri* (Darokar et al. 2001, Mathur et al. 2003, Ramesh et al. 2011, Tripathi et al. 2012, Bansal et al. 2014, Roshini et al. 2014, Srivastava et al. 2016), a comprehensive study on the species in its gene pool in Kerala by including a large number of accessions from all parts of the State has not been attempted so far.

Taking into consideration the estimates (Table 4) of the 4 parameters, Na, H, I and P for assessing genetic diversity among the accessions showed that they are diverse recognizably, as regard to the 13 loci and the genetic diversity profiles of the 60 accessions based on Na, I and H were almost similar (Fig. 1). The estimated values of Na (1.13–1.69), I (0.08–0.45), H (0.06–0.32) and P (12.50–68.75) substantiate the presence of genetic variation between the accessions.

The cluster analysis using POPGENE and the dendrogram generated based on the analysis revealed that the 60 accessions differ from each other genetically (Fig. 2). This observation indicates that the accessions represent 60 different genotypes of the species. The clustering of the 60 accessions into 3 major groups and many subgroups reveal genetic interrelationship among the accessions as well as genetic variability among the accessions of the species. The accessions, Bm 57 and Bm 58 collected from Pedikattuthuruthu and Cheriyathuruthu respectively belong to the same district (Ernakulam) showed very high genetic similarities between them and coming under the same cluster (Cluster I). These 2 accessions are much diverse from other accessions. On the contrary, this study also revealed that the accessions within the same cluster showed no closeness in their geographical locations. The present findings support those of the previous study that the accessions from diverse geographical regions can be genetically similar (Jain et al. 2004).

The average level of genetic polymorphism among the 60 populations is 31. 81%, revealing the presence of only moderate genetic diversity in the germplasm of *B. monnieri* in Kerala. Christopher et al (2017) based on HPTLC densitometric analysis reported significant variation percentage composition of the marker compounds of the species, Bacoside A and Bacopaside I present in the same 60 accessions. This study revealed the influence of genetic diversity of the species on the percentage composition of the bioactive compounds in its genotypes. The morphological analysis on these 60 accessions also showed significant variation on qualitative and quantitative traits of the species (Christopher 2016).

The analysis of overall genetic similarity in the 60 accessions based on Na, H and I corroborate this finding. Similar results were reported by Kumar et al. (2013) and Bansal et al. (2014) in the accessions of the species from different parts of India. This could be due to the factors such as predominant vegetative mode of reproduction of the species and gene erosion in the species as a result of habitat destruction taking place in the region. This points to the need for conservation of genetic resources of this high value medicinal species, both in situ and ex situ methods for attaining the purpose before the loss of the remaining genetic diversity of *B. monnieri*. Since genetic variability of many crop species is rapidly declining by direct or indirect consequence of human intervention, human influences such as habitat destruction and over exploitation from the wild cause limited gene flow and limiting genetic variation in plant species (Lee et al. 2018). High level of genetic diversity of plant species results in greater ability of the plants to adapt to adverse environments (Li & Chen 2004). Low genetic variation leads to decreased adaptability and it even elevates the occurrence of less beneficial genes leading to extinction of the genotypes and further lowering the level of genetic diversity

within populations (Dasgupta et al. 2015).

In addition to conservation of *B. monnieri*, this study also assumes importance for the utilisation of genetic resources of the species. Since it provides information for selecting genotypes of the species having divergent characters, which may be considered as candidates as parents of hybridisation programmes for exploiting hybrid vigour. The results of the study are also beneficial for undertaking bioactive compound-based expression markers, which is useful for crop improvement programmes of the species.

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Declaration

The authors declare no conflict of interest.

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RESEARCH ARTICLE

CHROMATOGRAPHIC AND BIOCHEMICAL CHARACTERIZATION OF PHYTOCHEMICALS IN LEAF EXTRACTS OF *PSEUDERANTHEMUM BICOLOR* (SCHRANK) RADLK. EX LINDAU

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SUMMARY *Pseuderanthemum bicolor* leaves were extracted with methanol, chloroform and ethyl acetate solvents. The extracts were subjected to phytochemical analysis through HPLC and HPTLC methods to separate different phytoconstituents and by fingerprinting formulations. Methanolic leaf extracts showed the presence of carbohydrates, glycosides, saponins, terpenoids, steroids, gums and mucilage. Chloroform leaf extract revealed the presence of carbohydrates, proteins, glycosides, saponins, alkaloids, flavonoids, gums and mucilage. On the other hand, ethyl acetate leaf extract showed, glycosides, saponins, phenolics, flavonoids, steroids, gums and mucilage. The HPLC and HPTLC analyses confirmed that stigmasterol was present in methanol leaf extract of *P. bicolor*. The results suggested that *P. bicolor* is a source of various bioactive phytochemical and pharmacological compounds.

Keywords: *Pseuderanthemum bicolor*, leaf extract, phytochemicals, HPLC, HPTLC.

INTRODUCTION

Phytochemicals are the chemicals produced by plants through metabolism and can be characterized into primary and secondary metabolites. Primary metabolites such as proteins, carbohydrates, lipids etc., are essential for metabolic processes in plants, but secondary metabolites like phenols, alkaloids, flavonoids, terpenoids etc., are not involved in the growth and development of the plants and are having medicinal and biological importance like antimicrobial, antioxidant, anticancerous, antiinflammatory, antidiabetic etc., (Velu et al. 2018). Plants are the rich source of phytochemicals and provide useful drugs. A crude drug may be obtained from seeds, fruits, flowers, leaves, roots and bark of stem or wood. Several studies have shown that many ornamental plants also have medicinal properties. Phytochemical constituents of ornamental plants are pharmaceutically important. *Pseuderanthemum bicolor,* an important ornamental undershrub commonly known as “limang–sugat" belongs to the family Acanthaceae, Traditionally, the decoction of aerial parts of this plant

were used for aphthae, cicatrizant and ulcers. The Gujjar tribe of sub-Himalayan tract, Uttarakhand, India, used the plant paste to treat boils. Leaves are fried in mustard oil and applied externally on cracked feet. The present study was carried out to screen the phytochemical compounds by qualitative analysis, HPLC and HPTLC methods in the methanol, chloroform and ethyl acetate leaf extracts of *P. bicolor*.

MATERIAL AND METHODS

Material was collected from Gudemaranahalli village, Magadi taluk, Ramanagara district (13. 051951˚N, 77.263177˚E) and authenticated by late Dr. K. Gopalakrishna Bhat. Stem cuttings were potted and maintained in the garden of Department of Botany, Bangalore University, Jnanabharathi, Bengaluru. Voucher specimen was deposited in the Herbarium, Department of Botany, Bangalore University, Bengaluru.

Fresh and healthy leaves of *P. bicolor* were collected and washed under the running tap water to remove dust and unwanted debris, shade dried for 8–10 d. The dried leaves were ground into fine powder using an electric mixer grinder and stored in air-tight containers for further use. 10 g of powder was extracted individually using 150 ml each of 3 different solvents viz., methanol, chloroform and ethyl acetate for 4 h in a water bath at 50˚ C by decoction method. The contents were then filtered through the Whatman No.1 filter paper. The extracts thus obtained were allowed to evaporate the solvents in a hot air oven. The condensed extracts were stored in microcentrifuge vials at 4˚ C for further use. 1 g each of leaf extract was redistributed in 50 ml of respective solvents and obtained the samples.

Qualitative phytochemical screening tests of the leaf extracts were performed according to the standard protocols for detecting the presence of various phytochemicals (Harborne 1998, Khandelwal 2008).

High performance liquid chromatography (HPLC)

Mobile phase composition used was the mixture of acetonitrile and water in the ratio of 75:25. 10 mg of stigmasterol, used as standard, was dissolved in 25 ml of methanol and heated in a water bath for 15–20 min at 60°–70° C and made up the volume to 25 ml with methanol. This standard solution was filtered before injecting it in the column.

100 mg of plant sample was extracted in 25 ml of methanol, heated in a water bath for 15–20 min at 60°–70° C and the volume was concentrated to 25 ml with methanol. This sample solution was filtered before injecting it in the column.

10 ml mixture of acetonitrile and water in the ratio of 75:25 was transferred to the chromatographic tank containing HPLC column. 20 µl of sample and standard solutions were injected separately on HPLC column. The area of stigmasterol in the standard was calculated and the sample was compared with standard by employing the following formula:

$$\frac{\text{Standard weight}}{\text{Sample weight}} \times \frac{\text{Sample area}}{\text{Standard area}} \times \text{\% of Standard assay}$$
$$= \text{\% of sample assay}$$

High performance thin layer chromatography (HPTLC)

The purified compound was obtained by column chromatography and was compared with standard stigmasterol by performing HPLC.

It was carried out in Model Waters–510 isocratic system using acetonitrile:methanol: water mobile phase in the ratio of 25:25:50 (v/v/v). It is equipped with C18 column (4.6 mm dia, 250 mm length and 5 µ particle size), flow rate of 1 ml/min, pressure 1350 psi, with injection volume 20 µL was used and the eluted fractions were detected at 254 nm.

10 mg of stigmasterol (reference standard) was extracted with 25 ml methanol for 10 min on a water bath at 40°–60° C, filtered and concentrated to 10 ml and proceeded for spotting. 1 g of extract with 50 ml methanol was heated on a water bath at 40º–60º C for 10 min, filtered and concentrated to 10 ml and proceeded for spotting.

Mobile phase composition used was the mixture of toluene and ethyl acetate in the ratio of 93:7. 10 ml of this mixture was transferred to the chroma-tographic tank. Whatman filter paper disc was placed and closed with the lid (for faster saturation of the tank with the solvent system) and the tank was allowed to saturate for 30 min. 10 µL of sample(s) and 10 µL standard (as 10 mm bands separated by a distance of 15 mm; at 10 mm from the base) were loaded on a HPTLC silica plate using a Linomat HPTLC applicator. The plate was then left in the fume hood to facilitate the solvent to evaporate. The plate was placed in the tank as near vertical as possible ensuring that the line of application was well above the solvent level and the lid was placed tightly and allowed the solvent to ascend to 1.5 cm below the top of the plate. The plate was removed and allowed to air dry in the fume hood. For the visualization of bands anisaldehyde sulphuric acid reagent was sprayed and observed under 366 nm UV light.

OBSERVATIONS

The qualitative phytochemical analysis of methanolic leaf extract showed the presence of carbohydrates, glycosides, saponins, terpenoids, steroids, gums and mucilage. Chloroform leaf extract revealed the presence of carbohydrates, proteins, glycosides, saponins, alkaloids, flavonoids, gums and mucilage. Ethyl acetate leaf extract showed the presence of glycosides, saponins, phenolics, flavonoids, steroids, oils, gums and mucilage.

During the HPLC analysis, the concentration of stigmasterol showed a peak at retention time (RT) 5.27 min, with an area of 100% (Table 1, Fig. 1).

In the plant sample analyzed, a peak appeared at RT 5.29 min, with an area of 1.11% confirming the presence of stigmasterol in the methanolic leaf extract at concentration of 1.11%. The standard lane also showed the presence of 5 other compounds. Among them, the first compound

TABLE 1: HPLC analysis of standard stigmasterol and leaf extract of *P. bicolor.*

Peak	RT	Area	Height	Area (%)	Height (%)
Stigmasterol	5.27	18813170	429887	100.00	100.00
1	2.64	6525753	502012	92.51	93.53
2	3.23	53810	6197	0.76	1.15
3	3.84	306486	19980	4.34	3.72
4	5.29	78781	4544	1.11	0.84
5	6.68	65901	2680	0.93	0.49
6	7.45	22912	1293	0.32	0.24
TOTAL		7053642	536706	100.00	100.00

Sl. No 1-6 in column 1 represent the peaks in the chromatogram.

band of stigmasterol in the standard lane and at the same position in the sample lane of methanolic leaf extract (Fig. 3).

DISCUSSION

Qualitative phytochemical analysis revealed the presence of various bioactive compounds viz., carbohydrates, proteins, glycosides, phenolics, gums and mucilage. Saponins, alkaloids were majorly found in chloroform followed by methanol extract. Flavonoids were recorded in chloroform and ethyl acetate extracts. Terpenoids

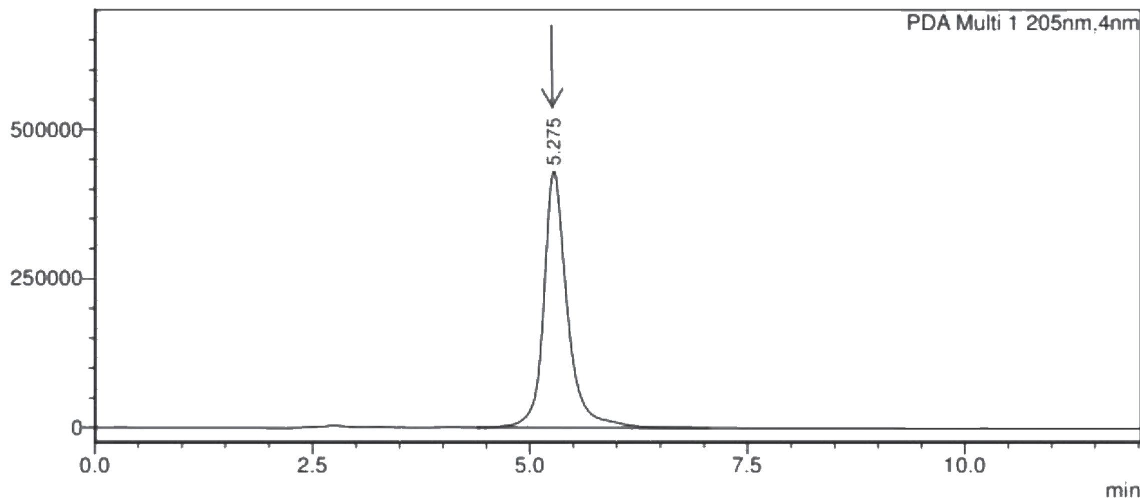

Fig. 1: *P. bicolor.* HPLC chromatogram of standard stigmasterol.

appeared at RT of 2.64 min, had a highest concetration of peak area of 92.51%. The second, third, fourth and fifth compounds appeared at a RT of 3.23 min, 3.84 min, 6.68 min and 7.45 min with a peak area of 0.76%, 4.34%, 0.93% and 0.32% respectively (Table 1, Fig. 2).

HPTLC studies revealed the presence of

were found in methanol extracts. Steroids were found in methanol and ethyl acetate extracts. Similar results were reported from the ethyl acetate and methanol leaf extracts of *Crossandra infundibuliformis* by Madhumitha & Saral (2011). They showed the presence of alkaloids, saponins, steroids, phenolics, flavonoids, tannins,

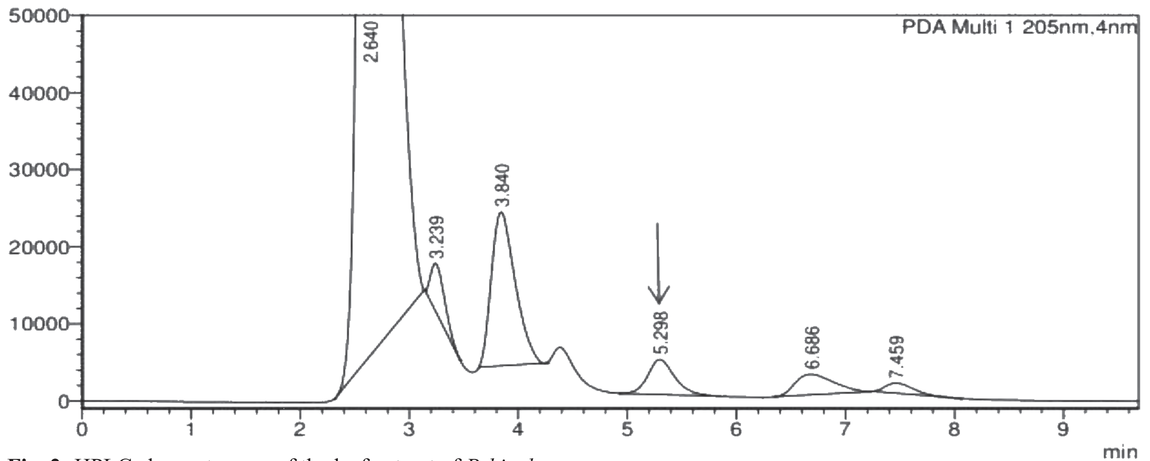

Fig. 2: HPLC chromatogram of the leaf extract of *P. bicolor.*

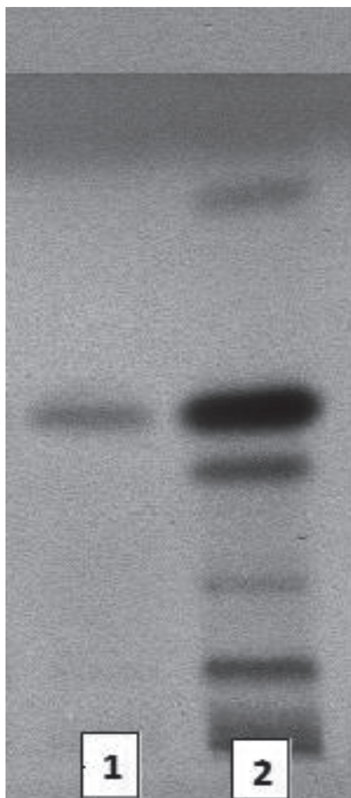

Fig. 3: Spectrogram of *P. bicolor* leaf extract and the standard stigmasterol analyzed by HPTLC. 1. Standard stigmasterol. 2. *P. bicolor.*

carbohydrates and terpenoids. Petsangkrit & Kittipongpatana (2015) screened phytochemicals in ethanolic leaf and callus extracts of *P. palatiferum* and confirmed the presence of flavonoids, saponins, terpenoids and alkaloids.

Sikri & Dalal (2018) observed the occurrence of phenols, flavonoids, saponins, alkaloids and tannins in the distilled water and methanol leaf extracts of *Andrographis paniculata* and *Justicia adhatoda.* Hanoon et al. (2019) recorded alkaloids and flavonoids in the leaf extracts of chloroform, methanol and aqueous, except for hexane extract. But tannins, saponins, steroids and reducing sugars were detected in all 4 different leaf extracts of *P. malabaricum.* The study conducted by Rajanna et al. (2017) on 3 crude extracts of tubers of *Ruellia tuberosa* prepared by using the solvents viz., methanol, chloroform and petroleum ether have shown positive response to carbohydrates, alkaloids, tannins, terpenoids and flavonoids. Saponins were found to be absent in

methanolic and petroleum ether extracts but were present in chloroform extracts. The aqueous root extract of *R. tuberosa* contained the presence of flavonoids, tannins, ascorbic acids and phenolic compounds, whereas the negative response was noticed for saponins, steroids, alkaloids and terpenoids as reported by Safitri et al. (2020).

By HPLC analysis it was confirmed that stigmasterol was present in methanol leaf extract of *P. bicolor*. Dandin & Murthy (2012) quantitatively analysed the presence of andrographolide content in micropropagated plants of *A. paniculata.* HPLC analysis showed the presence of the 0.01% of andrographolide compound in *A. alata* investigated by Bhavani & Bangajavalli (2020). Venkatachalapathi & Subban Ravi (2012) quantified lupeol in the petroleum ether extract by HPTLC method and found it to be 0.16% w/w in *Strobilanthes ciliatus*. Jana et al. (2023) conducted RP–HPLC and HPTLC study in *A. paniculata* and confirmed the presence of andrographolide.

The study conducted by Lahre & Kumar (2019) using HPTLC analysis determined the presence of sitosterol in methanol extract followed by petroleum ether and chloroform extracts of leaf, stem and root of *A. paniculata*. Kavitha et al. (2014) quantitatively analyzed and isolated sitosterol in the methanolic leaf extract of *J. gendarussa*. Vasicine is a quinazoline alkaloid which was isolated, identified and confirmed in the leaf extract of *Adhatoda vasica* by HPTLC technique (Sakthi Priya et al. 2021).

Das et al. (2022) estimated quercetin, catechol and tannic acid in the ethanol leaf extract of *Barleria prionitis* by RP–HPLC technique. The chromatographic condition was developed and optimized, where 3 distinct standard peaks of quercetin, catechol and tannic acid with a RT of 3.31 min, 4.70 min and 8.55 min respectively in the total runtime of 12 min. The HPTLC study revealed the presence of echioidin, eluted at RF value 0.61, as one of the major compounds in the methanolic extracts of *A. echioides* in the aerial parts and roots (Ghule et al. 2021).

Stigmasterol is an unsaturated phytosterol belonging to the class of tetracyclic triterpenes which shows anticancer, antiosteoarthritis, anti-inflammatory, immunomodulatory, neuroprotective, antidiabetic, antibacterial, antifungal, antioxidant, antiparasitic and antiviral properties (Bakrim et al. 2022). During the present investigation also stigmasterol was identified and quantified through HPTLC and HPLC techniques. Further isolation and quantification of stigmasterol is very essential for the formulation of novel drugs.

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Declarations

Consent to participate

We hereby certify that we have participated sufficiently in the analysis and interpretation of the data as well as the writing of the manuscript, to take public responsibility for it and have agreed to have our names listed as contributors.

Authors' contribution

The study was initiated by LR. BSR was involved in the collection of material and experimentation. The data collection was carried out by both BSR and NSS. Supervision of the study as well as drafting and correction of the manuscript was done by LR.

Conflict of interest

The authors declare no conflict of interest.

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RESEARCH ARTICLE

AMPELOCALAMUS PATELLARIS: A UNIQUE BAMBOO WITH CHROMOSOMAL MOSAICISM

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SUMMARY *Ampelocalamus patellaris* (Poaceae) was subjected to mitotic and meiotic studies by following standard procedures. The studies revealed the occurrence of chromosomal numeric mosaicism, a rather infrequent abnormal cytological phenomenon. The results of the study also point to the allotetraploid origin of *A. patellaris*. The chromosomal numeric mosaicism observed in seed-raised progenies of the plants indicates genetic determination of this phenomenon in the species. Previous workers observed that chromosomal numeric mosaicism can be due to the effect of genes and/or environment. Whether, this abnormal cytogenetic phenomenon exhibited by *A. patellaris* has some beneficial effects enabling easy adaptation of the species to new ecological conditions as evident from its wide distribution, is an enigma to be investigated.

Keywords: *Ampelocalamus patellaris*, bamboo, chromosomal mosaicism, aneusomaty.

INTRODUCTION

Ampelocalamus patellaris (Gamble) Stapleton (Poaceae) is one of the bamboo species having wide range of distribution (India: Assam, Sikkim, China: South-Central, East Himalaya, Laos, Myanmar, Nepal, Tibet, Vietnam) (POWO 2023). It occurs at an altitude of 1400–1800 m (De-Zhu Li et al. 1996). The members of the subfamily Bambusoideae of the family Poaceae, coming under the group commonly known as bamboos, include approximately 1680 species under 123 genera (BTSG-KFRI 2024), of which ca. 430 species are known cytologically. Out of the 430 species, which are known cytologically, about 100 of them belong to a single genus *Bambusa*, indicating that most of the genera of the subfamily have not been studied cytologically so far, including *Ampelocalamus*.

The term, chromosome numerical mosaicism or aneusomaty is used to describe the occurrence of different chromosome numbers in the cells of the same tissue or organ of an organism (Venkateswarlu & Krishna Rao 1969). According to them, this rather infrequent phenomenon has been recorded both in naturally occurring species and in artificially produced hybrids and their

derivatives. Aneusomaty is reported occasionally in anther and root tip materials (Gildenhuys & Brix 1958, Fukumoto 1962). The present investigation deals with mitotic and meiotic analyses and occurrence of aneusomaty in *A. patellaris*.

MATERIAL AND METHODS

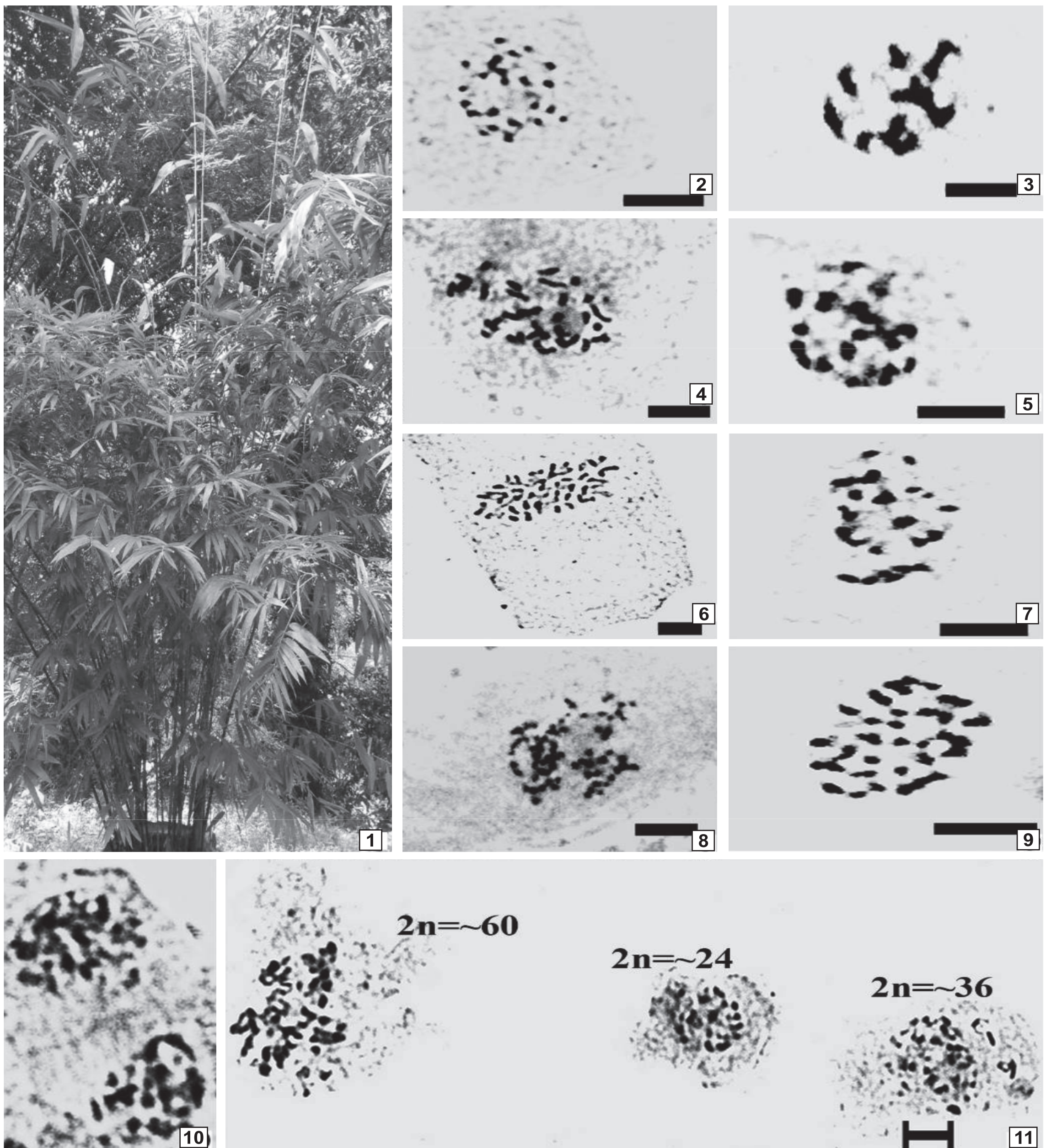
One of us (KCK) collected plants of this species from Sikkim in 2011 (Offset; India: Sikkim, Gangtok, Tadong, Wayside Gardens & Nurseries, N 27° 18. 530′ E 088° 35. 316′, 1097 m, 23.02.2011, *K C Koshy 66698*) and introduced to the bambusetum of JNTBGRI (Accession No. 956). After their establishment in the bambusetum they were propagated vegetatively and produced 25 plants. Out of these, 12 plants were selected using simple random sampling method. The selected plants were repotted and maintained in the nursery. New roots started emerging from the rhizomes of the plants after about a month, and 10 root tips of about 1 cm long were collected randomly from each of the plants for mitotic studies. In addition, mitotic studies were also made in seed-raised progenies of the accession.

For mitotic analysis, the root tips were pretreated with 0.002 Mol 8-hydroxyquinoline at 10.15 a.m. for 3 h at 4° C, washed thoroughly in water and fixed in Carnoy's fluid at 1.15 p.m. Squash preparations of the root tips were made using 2% acetocarmine (Beeks 1955). About 100 cells per root tip were observed under Leica DM 2500 trinocular microscope, lengths of chromosomes were measured using Leica Application Suite (version 4.12) and computed the range of lengths of chromosomes of the cells.

For meiotic studies, young flower buds were fixed in Carnoy's fluid at 9.15 a.m. Smear preparations of the anthers dissected from these flower buds were made using 2% acetocarmine (Beeks 1955) and pollen mother cells (PMCs) at different stages of meiotic divisions were analysed. Appropriate chromosome preparations were photomicrographed using Leica DM 100 digital camera attached with Leica DM 2500 trinocular microscope. Voucher specimens were deposited in the Herbarium of JNTBGRI (TBGT).

OBSERVATIONS

The results of the present study on chromosomal numeric mosaicism in *A. patellaris* are summarized in Table 1. Mitotic studies in root tip cells of the species showed 6 ploidy levels based on x = 12, viz. 2n = 2x = 24, 3x = 36, 4x = 48, 5x = 60, 6x = 72, and 8x = 96 and a few aneuploids, of which cells with tetraploid (4x) chromosome constitution were having the highest frequency (31.94 %) (Figs 2, 4, 6, 8). Mitotic studies also showed unequal separation of chromosomes during anaphase and early telophase (Fig. 10). The occurrence of adjacent cells of root meristematic tissues, which were in metaphase stage of mitotic division, and observed in the same field under same magnification (Fig. 11) were having varying number of chromosomes (ca. 2n = 60, 24 and 36). Range in length of chromosomes of



Figs 1–11: *A. patellaris*. 1. Whole plant. 2. Mitotic metaphase showing 24 chromosomes. 3. Meiotic metaphase showing 12 bivalents. 4. Mitotic metaphase showing 36 chromosomes. 5. Meiotic metaphase showing 18 bivalents. 6. Mitotic metaphase showing 48 chromosomes. 7. Meiotic metaphase showing 24 bivalents. 8. Mitotic metaphase showing 72 chromosomes. 9. Meiotic metaphase showing 36 bivalents. 10. Root tip cells showing unequal separation of chromosomes during early telophase. 11. 3 adjacent root tip cells showing unequal chromosome constitution. Scale bar = 5 µm.

TABLE 1 : Details of mitotic and meiotic analysis in *A. patellaris*.

Ploidy	*2n*	Cells having different ploidy levels (%)	Chromosome length range (µm)	*n*	Chromosomal association at diakinesis and metaphase I
2x	24	18.05	1.18–0.58	12	$4_{II} + 2_{IV} + 1_{VIII}$
3x	36	27.77	2.48–0.91	18	$2_{I} + 9_{II} + 2_{IV} + 1_{VIII}$
4x	48	31.94	1.67–0.61	24	$2_{I} + 6_{II} + 4_{IV} + 1_{VI} + 1_{XII}$
6x	72	13.19	0.72–0.49	36	$1_{I} + 12_{II} + 1_{III} + 5_{IV} + 4_{VI}$
Others*	-	9.02	-	-	-

*8 x and aneuploids – data not given except frequency distribution.

the complements belonging to the cells having different ploidy levels was also observed (Table 1). Meiotic studies showed PMCs having n = 12, 18, 24 and 36 (Figs 3, 5, 7, 9).

DISCUSSION

Cytogeneticaly, bamboo species are considered to be of alloploid origin. Previous workers have postulated that origin of temperate woody bamboos is through allotetraploidization and that of tropical woody bamboos either by allotetraploidization or allohexaploidization (Triplett et al. 2014, Guo et al. 2019). Allopolyploidy emerged independently in the woody bamboos of temperate and tropical regions (Triplett et al. 2014, Guo et al. 2019, Basak et al. 2021). Jero Mathu et al. (2023) reported alloploid genome constitution in 8 *Ochlandra* species endemic to the Western Ghats. In the present species, chromosomal numeric mosaicism has been found in cells of root tips and anthers. This phenomenon is the result of endoreduplication of chromosomes in somatic cells followed by unequal separation of chromosomes during mitosis. The high frequency of tetraploid cells in its somatic tissues and the occurrence of higher number of tetravalents in PMCs at diakinesis and metaphase I, irrespective of their ploidy levels are also suggestive of allotetraploid origin of the species. Variation in the range of chromosome length exhibited by the cells having different ploidy levels revealed that chromosome numerical variation in the somatic cells was also associated with structural variations of the chromosomes. Meiotic studies showed that in PMCs, the gametic number varied from n = 12 to 48, corresponding to the ploidy levels observed in the somatic cells. Hence, it may be inferred that the gametic number in a pollen grain is mainly determined in accordance with the ploidy level of the premeiotic cell from which it has been formed. This study also revealed that meiosis in this species was strikingly abnormal with the

occurrence of clumped chromosomes comprising up to 6 secondary associated bivalents (Fig. 7), univalents and trivalents, resulting in irregular anaphase separation of chromosomes and high pollen sterility. This in turn might have led to poor seed set in the species as evidenced from the development of only 29 seeds in the plants grown in the bambusetum, in spite of production of innumerable flowers during its long period of flowering and fruiting extending to about 20 months.

Chromosome numerical mosaicism was reported in many plant species which include natural polyploids (Thompson 1962, Pantulu & Narasimha Rao 1977, Lathakumari & Jayalakshmi 1984, Rao & Nirmala 1986), artificial hybrids (Shahare & Shastry 1963, Yang 1965, Venkateswarlu & Raja Rao 1979), in induced auto- and amphiploids (Sachs 1952, Yang 1964, Venkateswarlu & Krishna Rao 1969, Lydia Prasad 1982), in chemically induced mutants (Vaarama 1949, Sharma & Bhattacharjee 1953, Mitra & Steward 1961, Ross 1962, Rajhathy 1963, Siddiq 1967, Gottschalk 1971, Kasha 1974, Sadasivaiah & Lesins 1974, Rao & Rao 1977). In spite of the many reports of its occurrence, the mechanism involved and exact causes are not yet clearly understood (Rao et al. 1987). This phenomenon of intraplant chromosome numerical variation can be transmitted from generation to generation, hence it is considered to be genotypically controlled (Pantulu & Narasimha Rao 1977). Sachs (1952) attributed the phenomenon of chromosome numerical mosaicism to certain gene combinations and it is not dependant upon the magnitude of chromosome number of the amphiploid. According to him, this phenomenon is the result of gene-controlled spindle abnormalities in the premeiotic mitoses and this feature is passed onto the progeny. He postulated that disharmonious gene combinations are responsible for premeiotic somatic instability which leads to chromosome mosaicism observed during meiosis in PMCs. The accumulation of genes with minor effects or certain environmental conditions will be required to trigger chromosome numerical mosaicism (Venkateswarlu & Krishna Rao 1969). Gene combinations are responsible for chromosomal instability and it is not always associated with higher gene dosage and hence higher polyploidy (Pantulu & Narasimha Rao 1977). Occurrence of aneusomaty in seed-raised progenies of the species points to genetic determination of this anomaly in the species. But, at the same time the report of good seed set in *A. patellaris* by the earlier workers in its areas of distribution also indicates the possibility of occurrence of aneusomaty in the species due to the influence of the entirely different environmental conditions in ex situ at the bambusetum, compared to its habitat (Naithani et al. 2014, Lepcha & Naithani 2018).

Rao et al. (1987) reported colchicine induced chromosome mosaicism in chili pepper (*Capsicum annuum* L.), and they noted stunted

growth in the chromosome mosaic plants. But, *A. patellaris*, a naturally occurring species with aneusomaty is growing luxuriantly in the wild as well as in the bambusetum at JNTBGRI and revealed that this abnormal phenomenon has not at all affected adversely the growth and development of this species. On the other hand, whether chromosomal numeric mosaicism has some beneficial effects enabling easy adaptation of this species to new ecological conditions, as evident from its wide distribution across bio-geographic regions, is an enigma to be investigated.

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Declaration

The authors have no conflict of interest.

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OBITUARY

PROFESSOR (DR.) P. M. MATHEW (1930–2024)

Professor P. M. Mathew was born on 16 March 1930 at Pallom in Kottayam district, Kerala, India. His primary and secondary school education was in and around Kottayam. His brilliance and hardworking ability were evident even in the early years of his education. He excelled in Intermediate course in Biology at C. M. S. College, Kottayam. He studied B. Sc. Botany (University of Kerala) at S. B. College, Changanacherry and passed with First Class and First Rank. He did M.Sc. Botany (by research) at University College, Thiruvananthapuram with UGC Fellowship and passed outstandingly.

Beginning his career as a lecturer in the Department of Botany, University of Kerala, in 1959, he rose to the position of Professor in 1979 and appointed as Head, Department of Botany in 1989. Prof. Mathew's commitment to research extended far beyond his retirement in 1990. While working in the post of lecturer, as part of post-doctoral programme, during 1967–68, he underwent training in Population Genetics and

Human Genetics at North Carolina State University and Wane State University in USA with Fulbright Scholarship.

Prof. Mathew's research expertise spanned a wide range of disciplines including plant and human cytogenetics, population genetics, human inbreeding, cytotaxonomy, palynology and bio-systematics. The prestigious degree of Doctor of Science of the University of Kerala, has been awarded to him in the year 2022, at the age of 92, for his contributions in various fields, inspiring young researchers working in the disciplines and reaffirming the significance of basic research. During his post-doctoral programme in USA, he met Professor G. L. Stebbins – a stalwart in cytogenetics – at the University of California, Berkeley, who had been working in the University even in his late seventies. During the meeting, Prof. Stebbins told him 'Prof. Mathew, a scientist has no retirement'. He imbibed Prof. Stebbins' advice in his heart and put it in practise in his life. He was so passionate to research work and very particular in publishing the findings, until his last days.

His pioneering studies on human inbreeding among tribal populations of Kerala and Tamil Nadu revealed the highest percentage (97%) of consanguineous marriages in the world. As part of the studies, he pointed out that the series of infant deaths reported in the tribal hamlets of Attappadi region in Kerala is due to accumulation of deleterious alleles in the foetus, an aftermath of marriages between close relatives. He challenged the prevailing notion that the infant mortality is due to malnutrition of mothers, brought public attention to this drastic problem and suggested preventive measures to be undertaken by the government.

Prof. Mathew's expertise in cytogenetics and talent in making unequivocal chromosome preparations, karyotype analysis etc. were exemplary. He corrected the earlier findings of pioneers in cytological studies on Black pepper (*Piper nigrum* L.) and reported the correct mitotic and meiotic chromosome numbers (2n = 52, n = 26) of the species. Moreover, he proposed the cytogenetics, phylogeny and evolution of the taxon and revealed that *P. nigrum* is an allotetraploid. This fundamental, basic knowledge about the species is advantageous for progress of all the studies, aimed at genetic improvement of this spice crop. In honour of Prof. Mathew, a newly discovered wild genotype of the species with unique characteristics has been named, *P. nigrum* L. 'PMM'. The Field Gene Bank of Black Pepper developed at the Department of Botany, University of Kerala has also been named after him.

He led cytotaxonomic and cytogenetic studies on about 20 angiosperm families, about 50 species of pteridophytes and many species of gymnosperms. The paper on cytological studies

of about 100 species of Ferns, published in 1962, jointly with Professor C. A. Ninan and Professor A. Abraham, fetched ‘Sree Chitra’ award for the best paper published in the year from India. The paper on cytology of coconut endosperm published in ‘Annals of Botany’ with Professor Abraham, received wide acclaim and as a result of these achievements ‘Abraham’s School of Cytogenetics’ in the University of Kerala became famous internationally. His close association with Dr. P. K. K. Nair, Father of Indian Palynology, enabled him to recognize the excellent possibilities of palynology in biosystematic studies and conservation of biodiversity, and he initiated palynological studies on species occurring in the Western Ghats.

Prof. Mathew, a gifted teacher and mentor, nurtured the academic and personal growth of a number of students. He supervised 17 Ph. D. students, 15 M. Phil. students and generously assisted many others in their doctoral pursuits. He published about 220 research papers in national and international journals and authored eight books, many of which after his retirement from service. Among the books, the book titled ‘Fundamentals of Population Genetics with emphasis on Human inbreeding’ is unique. In population genetics, the books available are written by foreign authors and students of biology find it difficult to follow, since they are obsessed with high level mathematics. But, in his book he explained the subject with the aid of simple mathematics. This publication also satisfied the long felt need for an Indian book on population genetics. Prof. C. A. Ninan, former Head, Department of Botany and Dean, Faculty of Science, University of Kerala, and a long-term associate of Prof. Mathew at the Department of Botany, wrote in his foreword to the book ‘Professor Mathew is a botanist with a mathematical brain. It is seldom that we come across such persons among classical biologists’.

Professor Mathew has been associated with several scientific bodies in India. He was an active member of the Society of Cytologists and Geneticists and served as the Associate Editor of the Journal of Cytology and Genetics, the chief organ of the Society, and made significant contribution to improve the journal through his research articles. In addition, he was the source of inspiration to a large number of his students to contribute papers to this journal.

He was a man of deep faith in God, and humility was the hallmark of his personality. In his autobiography, he stated that his life was profoundly influenced by his mentor, Professor A. Abraham, founder Professor and Head, Department of Botany, University of Kerala, a visionary and an institution builder in plant science in the country. Prof. Mathew’s wife,

Mrs. Aleyamma Mathew, was also a source of motivation in his life, who passed away in 2012 at the age of 78.

Following a brief period of illness, Prof. Mathew left for his heavenly abode on 17 August 2024, at the age of 94. He is survived by his son Prof. Babu Mathew and daughter Mrs. Molly Mathew. His journey from humble beginnings to becoming a leading figure in science, through dedication, hard work and perseverance will be an inspiration to the younger generations, since he proved in his life 'Bumblebees can fly'.

P. J. MATHEW
Former KSCSTE Emeritus Scientist
Department of Botany
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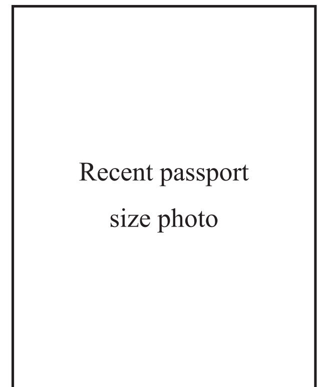

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