

Molecular Characterization in Different Cultivars of *Cicer arietinum* L.



**A DISSERTATION
SUBMITTED TO THE UNIVERSITY OF DHAKA
IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BOTANY**

**BY
KAZI NAHIDA BEGUM**

MAY 2014

**CYTOGENETICS LABORATORY
DEPARTMENT OF BOTANY
UNIVERSITY OF DHAKA
DHAKA 1000, BANGLADESH**

Dedicated
To
My Beloved Parents
Kazi Abdul Matin
&
Kazi Nujhat Afza Khatun

Sheikh Shamimul Alam

M. Sc. (Dhaka), Ph. D. (Japan), Post. Doc. (Germany)
(Humboldt Fellow)

Professor

**Department of Botany**

University of Dhaka
Dhaka-1000, Bangladesh
Phone: 880-2-9661920 extn. 7549
Res: 880-2-8144772
Fax: 880-2-8615583
E-mail: ssalam81@yahoo.com

Certificate

This is to certify that the thesis entitled "Molecular Characterization in Different Cultivars of Cicer arietinum L." submitted by Kazi Nahida Bagum has been carried out under my supervision in this laboratory. This is further to certify that it is an original work and suitable for submission for the award of doctor of philosophy in Botany.

Prof. Dr. Sheikh Shamimul Alam

Cytogenetics Laboratory

Department of Botany

University of Dhaka

Dhaka-1000, Bangladesh

Acknowledgement

All praises are due to Almighty Allah, the greatest, gracious and merciful, whose blessing enabled me to complete the research work successfully within due time.

I feel pleasure to express my immense respect and heartfelt gratitude to Professor Dr. Sheikh Shamimul Alam, Cytogenetics Laboratory, Department of Botany, University of Dhaka for his intellectual guidance, constructive criticism and untiring supervision during my Ph.D. research work and inspiration to work further in Plant Cytogenetics. I am always grateful to him for giving me a chance to work under his generous supervision.

Sincere thanks are due to the Ministry of Science and Technology for the “Bangabandhu Scholarship on Science and ICT”. I would like to thank Mr. Md. Saifullah, Project Director (Additional Secretary), Ministry of Science and Technology for his kind cooperation.

I am thankful to Professor Dr. Moniruzzaman Khondker, Chairman and Professor Dr. Abdul Aziz, former Chairman, Department of Botany, University of Dhaka for providing the necessary facilities of the Department.

I express my sincere and heartiest gratitude to Professor Dr. Imdadul Haque, Dean, Faculty of Biological Sciences, Department of Botany, University of Dhaka for his valuable suggestion, support and encouragement during my research work.

I would like to express my sincere and heartiest gratitude to Late Professor Abdul Matin, Professor Dr. Masuda Khatun, Professor Dr. Rokeya Begum and Professor Dr. Mihir Lal Saha, Department of Botany, University of Dhaka, for their generous support, help, invaluable suggestions and continuous encouragement during the course of my research work.

Sincere and heartiest gratitude to Professor Dr. Rakha Hari Sarkar, Professor Dr. M. Nurul Islam, Ms. Rita Sarah Borna, Lecturer and all the members of Plant Breeding and Biotechnology Laboratory specially Sonia Khan Sony, Department of Botany, University of Dhaka, for their valuable suggestion, support, encouragement as well as providing laboratory facilities along with their generous co-operation.

I also express my sincere and heartiest gratitude to Prof. Dr. M. Mozammel Haque, Prof. Dr. M. Abul Hasan, Prof. Dr. Md. Abul Bashar, Prof. Dr. Shamim Shamsi, Prof. Dr. Parvin Rashid, Prof. Dr. Nargis Jahan and Ms. Riffat Samad, Lecturer, Department of Botany, University of Dhaka for their encouragement and valuable suggestions.

I wish to express my deepest sense of gratitude to Prof. Dr. Hasna Hena Begum, Dean, Faculty of Life and Earth Science, Jagannath University, Prof. Dr. Md. Maniruzzman Khandaker, Chairman, Department of Botany, Jagannath University, Kazi Shakhawath Hossain, Associate Professor, GM Al-Amin, Assistant Professor, Ripa Akter Sharmin, Lecturer, Bivas Kumar Sarker, Lecturer, AMM Golam Adam, Lecturer and all other teachers and staffs of Department of Botany, Jagannath University for their opinion, recommendation, co-operation, encouragement and cordial behavior.

I thankfully acknowledge to Bangladesh Agricultural Research Institute (BARI) for providing sufficient research materials.

I express my heartiest gratitude to Syeda Sharmeen Sultana, Lecturer, Department of Botany, University of Dhaka, for her inspiration, immense assistance to my work and warm encouragement to complete the study.

I express my sincere and heartiest gratitude to Mr. Md. Ahashan Habib, Senior Scientific Officer, BCSIR, Dhaka and Mahin Afroz, Lecturer, Department of Botany, University of Barisal for their help, invaluable suggestions, inspiration and immense assistance to my work and the warm encouragement to complete the study.

Special thanks are due to the former and present students of the Cytogenetics Laboratory, Department of Botany, University of Dhaka for their generous co-operation namely - Dr. Kazi Kamrun Nahar, Senior Assistant Secretary, Ministry of Public Administration, Dr. A.N.M. Rubaiyath Bin Rahman, Assistant Professor, Kushtia Islamic University, Md. Alamgir Kabir, Lecturer, Department of Fisheries, University of Dhaka, Nusrat Sultana, Lecturer, Department of Botany, Jagannath University, Moontaha Mahboob, lecturer, Department of Botany, Jagannath University, Mousona Islam, Scientific Officer, BCSIR, Asma Ahmed Warasy, Assistant Professor, Department of Botany, Jahangirnagar University, Md. Shohidur Rahman, Lecturer, Department of Botany, Open University, Md. Yahia Zaman, Lecturer, Barisal Womens College, Rinat Fawzia, Lecturer, Sirajganj Govt. College, Mahfuza Binte Shukur, Officer, Sonali Bank Ltd., Shifat Shahla, Officer, Krishi Bank. Ltd., Sadia Munira, Officer, Madhumati Bank Ltd. and Nowreen Jahan, Israt Jahan Sonia, Riffat Ara Alam, Lutfun Nahar, Elham Ishrat, Ruhina Tasnim, Nasrin Sultana, Zannati Rahman, Sadia Islam Shima, Amika Ahmed Manzum Prithula, Laila Akhter, Kazi Imran, Uzzal Hossain and Riyad Hossen.

Sincere thanks are due to Mr. Mohammad Moniruzzaman (Porag Bhai), Senior Scientific Officer, BCSIR for his co-operation and encouragement throughout the work.

I specially thank to Dr. Rabeya Begum (Lipu) living in Sweden, for her help, inspiration and invaluable suggestions to prepare my thesis paper. I am really grateful to her from the core of my heart.

Special thanks to my uncle Professor Dr. Abdul Matin, Department of Geology University of Dhaka for giving inspiration and continuous co-operation to me.

I sincerely thanks to Shuvas Da, Jashim, Shuman, Tofazzal Bhai and Nuruzzaman, Gardeners, Department of Botany, University of Dhaka for providing all the garden facilities and co-operation.

I would like to express my heartfelt gratitude to my respected father Kazi Abdul Matin, mother Kazi Nujhat Afza Khatun, beloved three sisters Kazi Nazma Begum, Kazi Nasima Begum, Kazi Naima Begum, dearest three brothers Kazi Kawser Ahmed, Kazi Anisur Rahman, Kazi Arifur Rahman and all other members of my family.

I am indeed thankful to my respected parents-in-law Manirul Islam and Jaynob Begum for their continuous help and co-operation. Without their help it was not possible to complete the thesis work in time.

I express my heartiest gratitude one of my sweet apu Maisha Tabassum, HRO, Quantum Foundation for her continuous encouragement.

Special thanks to my beloved husband Adv. M. Ariful Islam, Legal Advisor, Quantum Foundation and my beautiful daughter Fairuz Islam.

Finally, this thesis is dedicated to my beloved parents Kazi Abdul Matin and Kazi Nujhat Afza Khatun.

– The Author

Contents

Titles	Page No.
List of Tables	i-vi
List of Figures	vii-xv
List of Abbreviations	xvi-xvii
Abstract	
Introduction	1-11
Materials and Methods	12-58
Results	59-167
Discussion	168-185
References	186-200

Sl. No.	Name of Tables	Page No.
1.	Composition of separating (10%) and stacking (5%) gel	27
2.	Composition of resolving (15%) and stacking (5%) gel	32
3.	Components and concentrations of extraction buffer	39
4.	Spectrophotometric absorbance readings at 260 nm wave length and concentration of DNA of nine cultivars of <i>Cicer arietinum</i> L.	50
5.	Preparation of working DNA solution (50 ng/μl) for PCR	52
6.	Twelve arbitrary RAPD primers used in the present study	53
7.	Ten SSR primers used in the present study	54
8.	Preparation of PCR cocktail (10 reactions) for RAPD primers	56
9.	Component of PCR cocktail (10 reactions) for SSR primers	57
10.	Types of interphase nuclei and prophase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. after staining with orcein	84
11.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-1 of <i>Cicer arietinum</i> L.	85
12.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-2 of <i>Cicer arietinum</i> L.	86
13.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-3 of <i>Cicer arietinum</i> L.	87
14.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-4 of <i>Cicer arietinum</i> L.	88

Sl. No.	Name of Tables	Page No.
15.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-5 of <i>Cicer arietinum</i> L.	89
16.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-6 of <i>Cicer arietinum</i> L.	90
17.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-7 of <i>Cicer arietinum</i> L.	91
18.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-8 of <i>Cicer arietinum</i> L.	92
19.	Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-9 of <i>Cicer arietinum</i> L.	93
20.	Comparative orcein stained karyotype analysis in nine cultivars of <i>Cicer arietinum</i> L.	94
21.	Comparative CMA-banding analysis in nine cultivars of <i>Cicer arietinum</i> L.	95
22.	Comparative DAPI-banding analysis in nine cultivars of <i>Cicer arietinum</i> L.	96
23.	Band formation rate and band color of different types of isozyme.	97
24.	Activity of acid phosphatase (APH) on Native-PAGE from leaf extract of nine cultivars of <i>Cicer arietinum</i> L.	97
25.	Summary of Nei's (1972) genetic distances on the basis of acid phosphatase activity in nine cultivars of <i>Cicer arietinum</i> L.	97
26.	Activity of esterase (EST) from leaf extract of nine	98

Sl. No.	Name of Tables	Page No.
	cultivars of <i>Cicer arietinum</i> L.	
27.	Summary of Nei's (1972) genetic distances on the basis of esterase in nine cultivars of <i>Cicer arietinum</i> L.	98
28.	Activity of peroxidase (PER) from leaf extract of nine cultivars of <i>Cicer arietinum</i> L.	99
29.	Summary of Nei's (1972) genetic distances on the basis of peroxidase activity in nine cultivars of <i>Cicer arietinum</i> L.	99
30.	Nei's (1972) genetic distances on the basis of combined analysis of acid phosphatase, esterase and peroxidase in nine cultivars of <i>Cicer arietinum</i> L.	100
31.	Molecular weight on the basis of SDS-PAGE of nine cultivars of <i>Cicer arietinum</i> L.	101
32.	Activity of SDS-PAGE from leaf extract of nine cultivars of <i>Cicer arietinum</i> L.	102
33.	Summary of Nei's (1972) genetic distances on the basis of SDS-PAGE activity of nine cultivars of <i>Cicer arietinum</i> L.	103
34.	RAPD analysis with primer OPA-1 (5'-CAG GCC CTT C-3') of nine cultivars of <i>Cicer arietinum</i> L.	104
35.	RAPD analysis with primer OPA-3 (5'-AGT CAG CCA C-3') of nine cultivars of <i>Cicer arietinum</i> L.	105
36.	RAPD analysis with primer OPA-4 (5'-AAT CGG GCT G-3') of nine cultivars of <i>Cicer arietinum</i> L.	106
37.	RAPD analysis with primer OPA-5 (5'-AGG GGT CTT G- 3') of nine cultivars of <i>Cicer arietinum</i> L.	107
38.	RAPD analysis with primer OPA-7 (5'-GAA ACG GGT G-3') of nine cultivars of <i>Cicer arietinum</i> L.	108
39.	RAPD analysis with primer OPA-8 (5'-GTG ACG TAG G-3') of nine cultivars of <i>Cicer arietinum</i> L.	109
40.	RAPD analysis with primer-2 (5'-GTT GCG ATC C-3') of	110

Sl. No.	Name of Tables	Page No.
	nine cultivars of <i>Cicer arietinum</i> L.	
41.	RAPD analysis with primer-12 (5'-GTA TGG GGC T-3') of nine cultivars of <i>Cicer arietinum</i> L.	111
42.	RAPD analysis with primer Primer-18 (5'-GTT TCG CTC C-3') of nine cultivars of <i>Cicer arietinum</i> L.	112
43.	RAPD analysis with primer-19 (5'-GAT GAC CGC C-3') of nine cultivars of <i>Cicer arietinum</i> L.	113
44.	RAPD analysis with Primer-23 (5'-GTC AGG GCA A-3') of nine cultivars of <i>Cicer arietinum</i> L.	114
45.	RAPD analysis with primer Primer-24 (5'-GGT CGG AGA A- 3') of nine cultivars of <i>Cicer arietinum</i> L.	115
46.	List of RAPD markers generated from twelve primers in nine cultivars of <i>Cicer arietinum</i> L.	116
47.	SSR analysis with primer GA26 CICER: Forward-5'-GTGC AGAGCATCATGCGATGCTCAAGACATCTGCCA-3'- Reverse-5'-TCATACTCAACAAATTCATTTCCC-3' of nine cultivars of <i>Cicer arietinum</i> L.	117
48.	SSR analysis with primer CASTMS2: Forward- 5'-ATTTTA CTTTACTACTTTTTTCCTTTC-3' – Reverse-5'-AATAAATGG A GTGTAAATTTTCATGTA-3' of nine cultivars of <i>Cicer arietinum</i> L.	118
49.	SSR analysis with primer CASTMS15: Forward-5'-CTTGT GAATTCATATTTACTTATAGAT-3'-Reverse-5'-ATCCGTAATT TAAGGTAGGTAAAATA-3' of nine cultivars of <i>Cicer arietinum</i> L.	119
50.	SSR analysis with primer CASTMS21:Forward-5'-CTACAG TCTTTTGTTCTTCTAGCTT-3'- Reverse-5'-ATATTTTTTAAGA GGCTTTTGGTAG-3' of nine cultivars of <i>Cicer arietinum</i> L.	120

Sl. No.	Name of Tables	Page No.
51.	SSR analysis with primer TA71: Forward-5'-CGATTTAAC ACAAAACACAAA-3'- Reverse-5'-CCTATCCATTGTCATCTC GT-3' of nine cultivars of <i>Cicer arietinum</i> L.	121
52.	SSR analysis with primer TA72:Forward-5'-GAAAGATTTA AAAGATTTTCCACGTTA-3'- Reverse-5'-TTAGAAGCATATTG TTGGGATAAGAGT-3' of nine cultivars of <i>Cicer arietinum</i> L.	122
53.	SSR analysis with primer TA130: Forward- 5'-TCTTTCTTT GCTTCCAATGT-3'-Reverse -5'-GTAAATCCCACGAGAAATC AA-3' of nine cultivars of <i>Cicer arietinum</i> L.	123
54.	SSR analysis with Primer TA135: Forward-5'-TGTTGGA AATTGATGTTTT-3' - Reverse-5'-GTGGTGTGAGCATAATT CAA-3' of nine cultivars of <i>Cicer arietinum</i> L.	124
55.	SSR analysis with primer TR29: Forward-5'-GCCCACTGAA AAATAAAAAG-3' - Reverse-5'-ATTTGAACCTCAAGTTCTC G-3' of nine cultivars of <i>Cicer arietinum</i> L.	125
56.	SSR analysis with primer TA118: Forward-5'-ACAAGTCAC ATGTGTTCTCAATA-3' - Reverse-5'-GGAAAGGTTAAGAAA TTT TACA ATAC-3' of nine cultivars of <i>Cicer arietinum</i> L.	126
57.	SSR markers generated from ten primers in nine cultivars of <i>Cicer arietinum</i> L.	127
58.	Summary of Nei's (1972) genetic distances of nine cultivars of <i>Cicer arietinum</i> L. by combined RAPD and SSR analysis.	128

Sl. No.	Name of Figures	Page No.
1-9.	Seed morphology of nine cultivars of <i>Cicer arietinum</i> L. 1. BC-1, 2. BC-2, 3. BC-3, 4. BC-4, 5. BC-5, 6. BC-6, 7. BC-7, 8. BC-8 and 9. BC-9.	129
10-18.	Plants morphology with flower of nine cultivars of <i>Cicer arietinum</i> L. 10. BC-1, 11. BC-2, 12. BC-3, 13. BC-4, 14. BC-5, 15. BC-6, 16. BC-7, 17. BC-8 and 18. BC-9.	130
19-27.	Orcein stained mitotic interphase nuclei of nine cultivars of <i>Cicer arietinum</i> L. 19. BC-1, 20. BC-2, 21. BC-3, 22. BC-4, 23. BC-5, 24. BC-6, 25. BC-7, 26. BC-8 and 27. BC-9. Bar = 5 μ m.	131
28-36.	CMA-stained mitotic interphase nuclei of nine cultivars of <i>Cicer arietinum</i> L. 28. BC-1, 29. BC-2, 30. BC-3, 31. BC-4, 32. BC-5, 33. BC-6, 34. BC-7, 35. BC-8 and 36. BC-9. Bar = 5 μ m.	132
37-45.	DAPI-stained mitotic interphase nuclei of nine cultivars of <i>Cicer arietinum</i> L. 37. BC-1, 38. BC-2, 39. BC-3, 40. BC-4, 41. BC-5, 42. BC-6, 43. BC-7, 44. BC-8 and 45. BC-9. Bar = 5 μ m.	133
46-54.	Orcein-stained mitotic prophase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 46. BC-1, 47. BC-2, 48. BC-3, 49. BC-4, 50. BC-5, 51. BC-6, 52. BC-7, 53. BC-8 and 54. BC-9. Bar = 5 μ m.	134
55-63.	CMA-stained mitotic prophase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 55. BC-1, 56. BC-2, 57. BC-3, 58. BC-4, 59. BC-5, 60. BC-6, 61. BC-7, 62. BC-8 and 63. BC-9. Bar = 5 μ m.	135
64-72.	DAPI-stained mitotic prophase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 64. BC-1, 65. BC-2, 66.	136

Sl. No.	Name of Figures	Page No.
	BC-3, 67. BC-4, 68. BC-5, 69. BC-6, 70. BC-7, 71. BC-8 and 72. BC-9. Bar = 5 μ m.	
73-81.	Orcein-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 73. BC-1, 74. BC-2, 75. BC-3, 76. BC-4, 77. BC-5, 78. BC-6, 79. BC-7, 80. BC-8 and 81. BC-9. Bar = 5 μ m.	137
82-90.	CMA-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 82. BC-1, 83. BC-2, 84. BC-3, 85. BC-4, 86. BC-5, 87. BC-6, 88. BC-7, 89. BC-8 and 90. BC-9. Bar = 5 μ m.	138
91-99.	DAPI-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 91. BC-1, 92. BC-2, 93. BC-3, 94. BC-4, 95. BC-5, 96. BC-6, 97. BC-7, 98. BC-8 and 99. BC-9. Bar = 5 μ m.	139
100-108.	Karyotype prepared from orcein-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 100. BC-1, 101. BC-2, 102. BC-3, 103. BC-4, 104. BC-5, 105. BC-6, 106. BC-7, 107. BC-8 and 108. BC-9. Bar = 5 μ m.	140
109-117.	Idiogram prepared from orcein-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 109. BC-1, 110. BC-2, 111. BC-3, 112. BC-4, 113. BC-5, 114. BC-6, 115. BC-7, 116. BC-8 and 117. BC-9. Bar = 5 μ m.	141
118-126.	Karyotype prepared from CMA-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 118. BC-1, 119. BC-2, 120. BC-3, 121. BC-4, 122. BC-5, 123. BC-6, 124. BC-7, 125. BC-8 and 126. BC-9. Bar = 5 μ m.	142

Sl. No.	Name of Figures	Page No.
127-135.	Idiogram prepared from CMA-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 127. BC-1, 128. BC-2, 129. BC-3, 130. BC-4, 131. BC-5, 132. BC-6, 133. BC-7, 134. BC-8 and 135. BC-9. Bar = 5 μ m.	143
136-144.	Karyotype prepared from DAPI-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 136. BC-1, 137. BC-2, 138. BC-3, 139. BC-4, 140. BC-5, 141. BC-6, 142. BC-7, 143. BC-8 and 144. BC-9. Bar = 5 μ m.	144
145-153.	Idiogram prepared from DAPI-stained mitotic metaphase chromosomes of nine cultivars of <i>Cicer arietinum</i> L. 145. BC-1, 146. BC-2, 147. BC-3, 148. BC-4, 149. BC-5, 150. BC-6, 151. BC-7, 152. BC-8 and 153. BC-9. Bar = 5 μ m.	145
154.	Acid phosphatase activity following polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3= BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6 =BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9.	146
155.	Zymogram of acid phosphatase banding profiles of gel electrophoresis, BC-1 to BC-9 same as Fig. 154.	146
156.	UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of <i>Cicer arietinum</i> L. by acid phosphatase isozyme analysis.	147
157.	Esterase activity following polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of	148

Sl. No.	Name of Figures	Page No.
	<i>Cicer arietinum</i> L. BC-1 =BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4 =BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7 =BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9.	
158.	Zymogram of esterase banding profiles of gel electrophoresis, BC-1 to BC-9 same as Fig. 157.	148
159.	UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of <i>Cicer arietinum</i> L. by esterase isozyme analysis.	149
160.	Peroxidase activity following polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of <i>Cicer arietinum</i> L. BC-1 =BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4 =BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7 =BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9.	150
161.	Zymogram of peroxidase banding profiles of gel electrophoresis, BC-1 to BC-9 same as Fig. 160.	150
162.	UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of <i>Cicer arietinum</i> L. by peroxidase isozyme analysis.	151
163.	UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of <i>Cicer arietinum</i> L. on the basis of acid phosphatase, esterase and peroxidase isozymes analysis.	152
164.	SDS polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of <i>Cicer arietinum</i> L.	153

Sl. No.	Name of Figures	Page No.
	following coomassie brilliant blue staining. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3 =BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6 =BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9.	
165.	Zymogram of SDS-PAGE banding profiles of BC-1 to BC-9 same as Fig. 164.	153
166.	UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of <i>Cicer arietinum</i> L. by SDS-PAGE analysis.	154
167.	Electrophoregram of ethidium bromide stained genomic DNA samples of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	155
168.	RAPD analysis with primer OPA-1 (5'-CAG GCC CTT C-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	156
169.	RAPD analysis with primer OPA-3 (5'-AGT CAG CCA C-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	156

Sl. No.	Name of Figures	Page No.
170.	RAPD analysis with primer OPA-4 (5'-AAT CGG GCT G-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	157
171.	RAPD analysis with primer OPA-5 (5'-AGG GGT CTT G-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	157
172.	RAPD analysis with primer OPA-7 (5'-GAA ACG GGT G-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	158
173.	RAPD analysis with primer OPA-8 (5'-GTG ACG TAG G-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	158
174.	RAPD analysis with primer-2 (5'-GTT GCG ATC C-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI eChola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	159

Sl. No.	Name of Figures	Page No.
175.	RAPD analysis with primer-12 (5'-GTA TGG GGC T-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	159
176.	RAPD analysis with primer-18 (5'- GTT TCG CTC C-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	160
177.	RAPD analysis with primer-19 (5'-GAT GAC CGC C-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	160
178.	RAPD analysis with primer-23 (5'-GTC AGG GCA A-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	161
179.	RAPD analysis with primer-24 (5'-GGT CGG AGA A-3') of nine cultivars of <i>Cicer arietinum</i> L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.	161

Sl. No.	Name of Figures	Page No.
180.	SSR analysis with primer pairs GA26 CICER: Forward-5'-GTGCAGAGCATCATGCGATGCTCAAGACATCTGCCA-3' Reverse- 5'-TCATACTCAACAAATTCATTTCCC-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	162
181.	SSR analysis with primer pairs CASTMS2: Forward-5'-AT TTTACTTTACTACTTTTTTCCTTTC-3'- Reverse -5'-AATAA ATGGAGTGTAATTTTCATGTA-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	162
182.	SSR analysis with primer pairs CASTMS15: Forward-5'-CTTGTGAATTCATATTTACTTATAGAT-3'- Reverse -5'-ATC CGTAATTTAAGGTAGGTAAAATA-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	163
183.	SSR analysis with primer pairs CASTMS21: Forward-5'-C TACAGTCTTTTGTCTTCTAGCTT-3'- Reverse -5'-ATATTT TTTAAGAGGCTTTTGGTAG-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	163
184.	SSR analysis with primer pairs TA71: Forward-5'-CGATT TAACACAAAACACAAA-3'- Reverse-5'-CCTATCCATTGTCA TCTCGT-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	164
185.	SSR analysis with primer pairs TA72: Forward-5'-GAAA GATTTAAAAGATTTTCCACGTTA-3'- Reverse-5'-TTAGAA GCATATTGTTGGGATAAGAGT-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	164
186.	SSR analysis with primer pairs TA130: Forward-5'-TCTT TCTTTGCTTCCAATGT-3'- Reverse -5'-GTAAATCCCACGA GAAATCAA-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	165

Sl. No.	Name of Figures	Page No.
187.	SSR analysis with primer pairs TA135: Forward-5'-TGG TTGGAATTGATGTTTT-3'- Reverse -5'-GTGGTGTGAGC ATAATTCAA-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	165
188.	SSR analysis with primer pairs TR29: Forward-5'-GCCC ACTGAAAAATAAAAAG-3'- Reverse-5'-ATTTGAACCTCAA GTTCTCG-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	166
189.	SSR analysis with primer pairs TA118: Forward-5'-ACAA GTCACATGTGTTCTCAATA-3'- Reverse-5'-GGAAAGGTTA AGAAATTTTACAATAC-3' of nine cultivars of <i>Cicer arietinum</i> L. M=100 bp DNA ladder.	166
190.	UPGMA dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of <i>Cicer arietinum</i> L. by RAPD and SSR analysis.	167

List of abbreviations

°C	: Degree centigrade
μ	: Micron
μg	: Microgram
μl	: Micro liter
μm	: Micrometer
μM	: Micro molar
1N	: 1 Normal
ac	: Acrocentric
AFLP	: Amplified Fragment Length Polymorphism
AT	: Adenine-Cytocine
BARI	: Bangladesh Agricultural Research Institute
bp	: Base pair
CI	: Centromeric Index
CMA	: Chromomycin A ₃
CTAB	: Cetyl trimethyl ammonium bromide
DAPI	: 4'-6-diamidino-2-phenylindole
ddH₂O	: Double distilled water
EDTA	: Ethylene diamine tetra acetic acid
et al.	: et alii (and others)
Fig(s).	: Figure(s)
FISH	Fluorescent <i>in situ</i> hybridization
g	: Gram
GC	: Guanine-Cytocine
h	: Hour
i.e.	: id est (that is)
ISSR	Inter-simple sequence repeat
Kb	: Kilo base
m	: Metacentric
m	: Minute
mg	: Milligram
MT	: Metric Ton
MW	: Molecular weight
ng	: Nanogram
No.	: Number
PCR	: Polymerase Chain Reaction

PVP	: Polyvinyl pyrrolidone
RAPD	: Random Amplified Polymorphic DNA
RFLP	: Restriction Fragment Length polymorphism
RL	: Relative length
rpm	: Revolution per minute
RTs	: Root tips
s	: Second
sm	: Sub-metacentric
SSR	: Simple Sequence Repeat
t	: Telocentric
t/ha	: Ton/hectare
TAE	: Tris-acetate-EDTA
TE	: Tris-EDTA
UPGMA	Unweighted Pair Group Method of Arithmetic Means
UV	: Ultra violet
viz.	: Videlicet, Namely
Vol.	: Volume
W/V	: Weight/Volume
α	: Alpha
β	: Beta
Φ	: Phi
θ	: Theta
λ	: Lambda
δ	: Delta
Ω	: Omega
%	: Percentage

ABSTRACT

Abstract

Nine cultivars of *Cicer arietinum* L. (chickpea) released by Bangladesh Agricultural Research Institute (BARI) viz BC1, BC-2, BC-3, BC-4, BC-5, BC-6, BC-7, BC-8 and BC-9 were investigated cytogenetically and at the molecular level using isozyme, SDS-PAGE, RAPD- and SSR-markers for authentic characterization. The nine chickpea cultivars have certain spectrum of variation for several phenotypic and agronomic traits. A prominent nucleolus was found in the interphase nuclei and prophase chromosomes after orcein staining in each cultivar. Although the nine cultivars were found to possess $2n=16$ metacentric chromosomes, differed in respect of other karyotypic features such as total length of $2n$ chromosome complements, range of relative chromosome length and orcein staining pattern of interphase nuclei and prophase chromosomes. The number of CMA positive bands was 2 to 6 in the metaphase chromosomes of nine cultivars with percentage of GC-rich repeats ranging from 2.66 (BC-8) to 14.52 (BC-2). Each cultivar had distinct CMA- banding karyotype formula. Heteromorphism in CMA banding revealed the occurrence of paracentric inversion in chromosome pair II of BC-2. The number of DAPI bands was ranging from 1 (BC-4) to 4 (BC-1, BC-5, BC-6, BC-7 and BC-9). DAPI bands were confined to centromeric and terminal regions of chromosomes among nine cultivars. Some DAPI bands were so unique that these chromosomes could easily be isolated from the rest and may use as marker chromosomes for the respective cultivars. Each cultivar has characteristic DAPI-banding formula.

Heteromorphicity in DAPI banding pattern between homologue members indicated the occurrence of deletion. Differential staining property of satellites revealed the stain specific nature of satellited portions. The nine cultivars showed different SDS-PAGE pattern. Cultivar BC-2, BC-6, BC-8 and BC-9 could be characterized by SDS-PAGE bands on the basis of their location, size and intensity. Three isozymes system namely acid phosphatase, esterase and peroxidase were investigated of which esterase found suitable for producing distinct polymorphic band among nine cultivars. DNA from the nine chickpea cultivars was studied with 12 oligonucleotide primers and 10 microsatellite primer pairs for RAPD and SSR assay, respectively. The ten RAPD primers generated 351 distinct bands with 91.61% polymorphisms indicated highly diversified nature. In addition to polymorphism, 19 unique RAPD sequences were identified in nine chickpea cultivars. The ten SSR primer pairs generated 143 distinct bands of which 62 were considered as polymorphic (43.36% polymorphisms). Moreover, one unique SSR sequence was identified in BC-5. The combined RAPD and SSR dendrogram made BC-8 and BC-9 distinct from the rest and placed in a separate sub cluster that correlated with their phenotypic, agronomic and cytogenetical features. Therefore, each germplasm could be characterized authentically by cytogenetical and molecular analysis. This information will be helpful for characterizing each cultivar of chickpea for improved breeding program.

1. INTRODUCTION

1. Introduction

The genus *Cicer* belongs to the family Fabaceae. There are 43 species in this genus of which nine annual and 34 perennial (Van der Maesen 1987). *Cicer arietinum* L. is one of the nine annual species and only the economically important species among *Cicer* genus (Heldreich 1862).

There are two distinct types of cultivated chickpea, "Kabuli" (also known as macrosperma) and "Desi" (also known as microsperma) differing in their geographic distribution and different plant type. The "Desi type" is found in the central Asia and in the Indian subcontinent while the "Kabuli type" mostly found in the Mediterranean region. "Kabuli type" is usually taller and has white flowers, lack anthocyanin pigmentation on stem, white or cream-colored seeds with a "ram's head" shape, thin seed coat and smooth seed surface. On the other hand, "Desi type" is generally shorter, possessing small leaflets, pods, seeds, predominantly pink-colored flowers and anthocyanin pigmentation on stems and almost brown colored thick seed coat. "Kabuli" chickpea seems to have evolved from the "Desi type" (Monero and Cubero 1978). The seed weight generally ranges from 0.1 to 0.3g and 0.2 to 0.6g in "Desi" and "Kabuli" types, respectively (Frimpong *et al.* 2009). The "Desi types" account for about 80-85% of the total chickpea area and are mostly grown in Asia and Africa. The "Kabuli type" is largely grown in West Asia, North Africa, North America and Europe (Pande *et al.* 2005).

Chickpea is the most important pulse crop. The whole dried seeds are eaten after soaking in water for a few hours or cooked, particularly by the Muslims after their fasting. Green pods and tender shoots are used as vegetables. The split dried seed (dal) is cooked as soup and whole seeds and husk are used as animal feed. The powder made from dried seeds (beson) is used in confectionary and in cosmetics. Aerial parts are used to treat fever, dysmenorrhoea, gonorrhoea, menstrual and urinary troubles. Seeds are astringent and used in dysentery. Boiled leaves are applied to sprains and dislocated limbs (van der Measen and Somaatmadja 1992)

Chickpea is a good and cheap source of protein for people in developing countries (especially in South Asia), who are largely vegetarian either by choice or because of economic reasons. Additionally, chickpea is rich in minerals (phosphorus, calcium, magnesium, iron and zinc), fiber, unsaturated fatty acids and β -carotene.

Chickpea is also a good source of carbohydrates together with protein, constituting about 80% of the total dry seed mass (Chibbar *et al.* 2010, Geervani 1991, Jukanti *et al.* 2012) in comparison to other pulses. Chickpea is cholesterol free and a good source of dietary fibre, vitamins and minerals (Agriculture and agri-Food Canada 2006, Wood and Grusak 2007). The total carbohydrate content in chickpea (60.7 g) is higher than other pulses such as Pigeonpea (23.8 g), Bean (7.0 g), Mung bean (52.6 g), Peas (14.5 g) (USDA 2010). Protein calorie malnutrition is observed in infants and young children in developing countries and includes a range of pathological conditions arising due

to lack of protein and calories in the diet (Haider and Haider 1984). Malnutrition affects about 170 million people especially pre-school children and nursing mothers of developing countries in Asia and Africa (Iqbal *et al.* 2005). Pulses provide a major share of protein and calories in Afro-Asian diet. Among the different pulses, chickpea is reported to have higher protein bioavailability (Sanchez-Vioque 1999).

Chickpea improves soil fertility by fixing atmospheric nitrogen, meeting up to 80% of its nitrogen (N) requirement from symbiotic nitrogen fixation (Saraf 1998, Sarker and Awal 1999). Chickpea returns a significant amount of residual nitrogen to the soil and adds organic matter, improving soil health and fertility. Growing interest in chickpea consumption, coupled with increased preference for vegetable-based protein has led to an increase in global demand for chickpea. Additionally, awareness of both economic (premium prices for large seeded Kabuli) and other benefits of chickpea including human health, crop diversification and sustainable agriculture have increased interest among farmers in cultivating chickpea.

Chickpea is grown in over 50 countries with 90% of its area in developing countries. Southern and South-Eastern Asia accounts for 79% of the global chickpea production. India is the largest chickpea producing country, with 68% of world chickpea production. The other major chickpea producing countries include Australia, Pakistan, Turkey, Myanmar, Ethiopia, Iran, Mexico, Canada and the USA. The number of chickpea importing countries has increased from 64 in 1990 to 142 in 2009 (FAOSTAT 2012) suggesting increasing global demand

of the chickpea. Globally, chickpea is the third most important pulse crop in production, next to dry beans and field pea (FAOSTAT 2011).

In Bangladesh, chickpea is the third major pulse crop after grass pea (Khesari) and lentil (Islam *et al.* 1981 and Anonymous 1998). Pulses covered about 225711.74 ha of cultivated area of Bangladesh of which chickpea covered 9352.63 ha in 2008 and produced 7168 metric tons with an average yield of 0.76 mt/ha (BBS 2008).

The major constraints limiting chickpea production globally include various abiotic and biotic stresses. Drought is the most important abiotic constraint to yield in chickpea, accounting for about 50% yield reduction in Bangladesh (Mandal *et al.* 2011). It generally occurs at the terminal stage as the crop is mostly raised on conserved soil moisture under rain-fed conditions. In addition to abiotic stress, there are some important biotic stresses, such as i) Fusarium wilt (FW), caused by *Fusarium oxysporum* f. sp. *ciceri*, ii) dry root rot caused by *Rhizoctonia bataticola* and iii) collar rot, caused by *Sclerotium rolfsi*. Moreover, there are some pest (pod borer, nematode) and viral disease (rust) of chickpea. These are the important diseases of chickpea in areas where the chickpea growing season is dry and warm. All these diseases cause plant mortality.

Being an important pulse crop, breeders of Bangladesh have been trying to develop a better variety in respect of both yield and disease resistant. As a consequence, Bangladesh Agriculture Research Institute (BARI) has released nine cultivars of this species, namely- BC-1, BC-2, BC-3, BC-4, BC-5, BC-6, BC-7, BC-8 and BC-9 (Mandal *et al.* 2011).

Successful breeding program depends on the complete knowledge and understanding of the genetic diversity within and among genetic resources of the available germplasms. This will enable plant breeders to choose parental sources that generate diverse populations for selection (Esmail *et al.* 2008). The extent of genetic diversity in species is important in determining the selection to be performed and for the ability to adapt to variable environmental conditions. At the onset of a breeding program, information concerning genetic relationships can be used to improve the breeding population by complementing phenotypic features. Though various breeding procedures like hybridization, mutation and polyploidy breeding etc. can be used for enlarging the available variability, it is always essential to quantify and qualify the genetic variability. Information about the relationships among cultivars or genotypes and the genetic diversity in available germplasms is important for the optimal design of chickpea breeding programs.

Moreover, a number of germplasms has been collected all over Bangladesh and stored in the gene bank of BARI. These germplasms with many lines are characterized on the basis of their morphological features and yield production. This kind of characterization sometimes creates problem since phenotypic features are not always reliable. There are many lines evolved from these germplasms but the screening of these lines is difficult due to the hybrid possibilities among them. For this purpose, an authentic characterization and streamlining of these germplasms are needed to avoid overlapping and misidentification for germplasms conservation (Akter and Alam 2005). Further the characterization is needed to make patent for each

cultivar of chickpea released by BARI. This characterization will prevent the trade piracy.

In order to characterize the cultivar on the basis of chromosomal information, many cytological works have been carried out on *Cicer* (Mercys *et al.* 1974, Lavania and Lavania 1982, Sharma and Gupta 1986, Venora *et al.* 1991, 1995, Galasso and Pignone 1992, Ocampo *et al.* 1992, Ahmad and Hymowitz 1993, Tayyar *et al.* 1994). The chromosomes of this species are difficult to stain and examine, due to their condensation and small size. As a result, comparative study of chromosome morphology and detail karyotypic features among genotypes in *Cicer arietinum* L. has not been well documented. Therefore, most of the earlier researches were confined to the counting of diploid chromosome number. As a consequence, information about detailed karyotypic features of chickpea is not available.

Karyotype analysis is one of the measures for proper characterization since it is a stable character and specific for each specimen. However, it is difficult in varietal level as well as cultivars, as varieties usually possessing the same chromosome numbers and even the same karyotypes (Sultana and Alam 2007, Shema *et al.* 2013, Afroz *et al.* 2013, Kondo and Hizume 1982, Hizume *et al.* 1988). In addition, conventional karyotype analysis is unable to express critically the differences among different germplasms of a species since the germplasms of a species possess similar 2n chromosomes numbers and even other karyotype parameters (Khatun and Alam 2010, Khatun *et al.* 2011). Moreover, the consideration of chromosome length, arm

ratio, position and number of secondary constrictions are not always sufficient to differentiate individual chromosome. Minute deletion, inversion, tandem duplication etc. could not be possible to detect by conventional karyotype analysis. In such a situation, modern cytogenetic approaches should be undertaken for comparative study among different cultivars of a species. This approach enables to find out minute differences in the genomes. This is usually done by differential staining with orcein, CMA and DAPI (Alam and Kondo 1995, Fawzia and Alam 2011, Alam *et al.* 2011, Shahla and Alam 2011).

Using fluorescent banding with chromomycin A3 (CMA) and 4'-6 diamidino-2-phenylindole (DAPI), it is now possible to determine the arrangement of GC-and AT-rich repeats in the genome, respectively (Akter and Alam 2005, Alam and Kondo 1995). Schweizer (1976) for the first time initiated this technique. CMA binds with GC (Guanine-Cytosine)-rich repetitive sequences of the genome and gives characteristics yellow color bands. On the other hand, DAPI binds to AT (Adenine-Thymine)-rich repeats giving characteristic blue color bands (Schweizer 1976, Alam and Kondo 1995, Kondo and Hizume 1982, Jessy *et al.* 2005, Akhter and Alam 2005, Islam and Alam 2011, Sultana *et al.* 2011). With the help of fluorescent banding it was possible to identify even a particular chromosome (Schweizer 1976, Kondo and Hizume 1982, Hizume *et al.* 1988, Alam and Kondo 1995). It is well known that each chromosome has one or more micro satellite composed of repetitive sequences. These are germplasm specific. If it is possible to mark those areas of chromosomes, karyotypes of a specimen could be authentically identified.

Moreover, the staining property of interphase nuclei and prophase chromosomes are another parameter for comparative karyological study. Tanaka (1971) classified the different types of interphase nuclei and prophase chromosomes on the basis of orciein staining property. The outcome of this study showed that various taxa including varieties of many plant species could be distinguished by their staining properties of interphase nuclei and prophase chromosomes.

Gel electrophoresis for protein analysis has become a standard and powerful research tool for application in a multitude of biological sciences. They provide valuable information with respect to hybridization and gene duplication, including polyploidy. Isozymes are originally defined by Markert and Moller (1959) as different variants of the same enzyme having identical functions. Isozymes are closely related to gene products (Soost and Torres 1981). Their electrophoretic mobilities are the results of different sizes and shapes of enzyme molecules and their variation is a good indicator of genetic variability. The three most widely used isozymes are acid phosphatase, esterase and peroxidase. These have been used in plants for various purposes, including taxonomic or genetic relationship studies (Hirai *et al.* 1986). Isozyme variations have been extensively used as genetic markers for characterization of species and cultivars (Bringham *et al.* 1981, Dewald *et al.* 1988, Mundges *et al.* 1990), establishment of phylogenetic relationships among taxa (Crawford 1983, Hirai *et al.* 1986, Perl-Trevers *et al.* 1986, Staub *et al.* 1988) and genetic studies in many plants (Arus and Orton 1983, Staub and Fredrick 1988, Wendel and Parks 1982). The enzyme variations have been also used in plant breeding successfully as

genetic markers for hybrid confirmation and for convenient screening of seedlings with target characters (Arus *et al.* 1985, 1982, Soost *et al.* 1981, Tanksley and Jones 1981). Compared with other conventional markers, isozymes have some advantages, such as environmental stability and co dominant expression (Gottlieb 1981). Therefore, the above mentioned three isozymes proved efficient for authentic characterization.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is widely used due to its validity and simplicity for describing genetic structure of crop germplasm (Javid *et al.* 2004). As storage proteins are not affected by environmental fluctuations, their profiling using SDS-PAGE technology is particularly considered as a reliable tool for distinguishing cultivars of particular crop species (Javid *et al.* 2004, Iqbal *et al.* 2005, Jha and Ohri 1996, Mennella *et al.* 1999). Leaf storage proteins have been used as genetic markers in four major areas: i) analysis of genetic diversity within and between species, ii) plant domestication in relation to genetic resources conservation and breeding, iii) genome relationship and iv) a tool in crop improvement (Gafoor *et al.* 2002). Therefore, SDS-PAGE would be another tool for molecular marker analysis.

DNA fingerprinting by Randomly Amplified Polymorphic DNA (RAPD) is one of the molecular methods for characterizing germplasms. The term DNA fingerprinting/profiling describes the combined use of several single locus detection systems. This method has been using as versatile tool for investigating various genomic aspects of organism. It includes characterization of genetic variability, genome fingerprinting,

genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy etc. The advantages of RAPD analysis over other methods are its low sample DNA requirement and detection of the high frequency of polymorphic bands (Williams *et al.* 1990). A diverse level of polymorphism in different crops have been reported such as Chickpea 98.14% (Rasool 2013), Chickpea 87% (Datta *et al.* 2010), Brassica 98.03% (Ghosh *et al.* 2009), Eggplant 57.89% (Biswas *et al.* 2009), Peanut 96% (Lang and Hang 2007), Peanut 42.7% (Raina *et al.* 2001) and Chilli 90% (Paran *et al.* 1998).

Simple sequence repeats (SSRs) are a class of molecular microsatellites markers based on tandem repeats of short (2-6 bp) DNA sequences (Litt and Luty 1989). These repeat sequences are often highly polymorphic, even among closely related cultivars, due to slippage mutations during DNA replication causing variation in the number of repeating units. Different alleles of a given locus can readily be detected using primers designed from the conserved DNA sequences flanking the SSR and the polymerase chain reaction (PCR). SSR markers are generally reported to detect higher levels of polymorphism than RFLPs, RAPDs and AFLPs (Powel *et al.* 1996, Milbourne *et al.* 1997, Russel *et al.* 1997, Crouch *et al.* 1999) and have been widely adopted for genetic analysis in plants (Panaud *et al.* 1996, Powell *et al.* 1996). Thus it is believed that SSR markers will provide the molecular differentiation to facilitate routine diversity analysis and molecular breeding applications.

Although some conventional karyotype, fluorescent banding and different molecular marker analysis of chickpea have been undertaken

earlier, these were scattered and not exactly used for characterization. No attempt has been taken earlier to combine cytogenetical and molecular data for characterizing chickpea cultivars. Moreover, no cytogenetical and molecular analysis has yet been initiated for the released chickpea cultivars of BARI. As a result, our cultivars have not yet been characterized.

In the present study, a combination of cytogenetical and molecular analysis was carried out for the first time to characterize nine chickpea cultivars released by BARI with a view to fulfill the following objectives:

- i. to compare the staining property of the interphase nuclei and prophase chromosomes after staining with orcein, CMA and DAPI.
- ii. to make the conventional orcein-stained karyotypes of each cultivar.
- iii. to compare the fluorescent banding pattern after staining with CMA- and DAPI- fluorochromes.
- iv. to compare RAPD and SSR patterns.
- v. to characterize each cultivar on the basis of cytogenetical and molecular data.

2. MATERIALS AND METHODS

2. Materials and Methods

2.1. Research materials

The following nine cultivars of *Cicer arietinum* L. were investigated in this study which collected from the Pulse Research Center (PRC) of Bangladesh Agricultural Research Institute (BARI). These nine cultivars were maintained in the Botanic garden, Department of Botany, University of Dhaka. The brief description of each cultivar of *Cicer arietinum* L. was given below-

i. BC-1 (BARI Chola-1)

Native chickpea known as "Nobin", herbaceous and perennial herb, stem erect type and light green color, plant height of about 65-70 cm and flowers are light pink in color, brick colored seed, 30-40% larger than local seed, weight of 100 seeds is 12 g, percentage of protein and carbohydrate is 25.50 and 47.30 respectively, yield 2200 kg/ha (Figs. 1, 10).

ii. BC-2 (BARI Chola-2)

'Deshi' chickpea known as "Boral", source ICRISAT ICCL-83228, stem semi spreading type and tip of the branch is slender with long internodes, pink color flower, plant height of about 65-70 cm and plant color is green, light brown colored seed, seed size is 40-50% larger than the local variety, both sides of the seed is flatter and light brown in color, 100 seed weight 15-16 g, percentage of protein and carbohydrate is 25.20 and 59.30, respectively, yield 2800 kg/ha (Figs. 2, 11).

iii. BC-3 (BARI Chola-3)

'Deshi' chickpea known as "Barendra", source ICRISAT ICCL-83105, stem semi spreading type and tip of the branch is slender with long internodes, leaflets are large, pink colour flower and plant colour is green, seed size is 50-60% larger than the local variety and 40-50% than BC-1, 100 seed weight 18-19 g, percentage of protein and carbohydrate is 25.20 and 52.8, respectively, yield 1900 kg/ha (Figs. 3, 12).

iv. BC-4 (BARI Chola-4)

'Deshi' chickpea known as "Pair Flower", source ICRISAT ICCL- 85222, two flowers and pod in a same peduncle, plant height of about 55 cm, semi erect stature and light green colour, grey colour pigmentation presentation is stem, hilum is very prominent, pink and white colour flower, plant colour is green, both sides of the seed is somewhat flattery, smooth and light brown in colour, 100 seed weight 13-14 g, protein and carbohydrate is 19.40% and 57.10%, respectively, yield 2800 kg/ha (Figs. 4, 13).

v. BC-5 (BARI Chola-5)

'Deshi' chickpea known as "Pabnai", source ICRISAT RBH-228, spread stature and light green colour, no pigmentation observed at seedling stage but light grey pigmentation observed in the matured stage stem, plant height of about 50 cm, pink colour flower, plant colour is green, small seed size and grey brown colour seed coat. Both sides of the seed is somewhat flattery, smooth, 100 seed weight 15.5 g, 21.07% protein and 54.37% carbohydrate, yield 2600 kg/ha (Figs. 5, 14).

vi. BC-6 (BARI Chola-6)

'Desi' chickpea known as "Navarun", source ICRISAT ICCL-83149, semi spreading type with medium and light green leaflets, no pigmentation observed in stem at seedling stage but light gray pigmentation observed in the matured stage, plant height of about 60 cm, pink colour flower, plant colour is green, small seed size, round shaped with deep brown colour seed coat, 100 seed weight 17-18 g, percentage of protein and carbohydrate is 19.42 and 60.21, respectively, yield 2600 kg/ha (Figs. 6, 15).

vii. BC-7 (BARI Chola-7)

'Desi' chickpea known as "Boral", source ICRISAT ICCL-3272, Erect type stature with large and green leaflets, plant height of about 55 cm, pink colour flower, plant colour is green, light brown coloured seed coat, seed size is larger than the local cultivar, 100 seed weight about 17-18 g, protein 20.42% and carbohydrate 58.21%, yield 2800 kg/ha (Figs. 7, 16).

viii. BC-8 (BARI Chola-8)

'Kabuli' chickpea, known as "Kabuli", source ICRISAT ICCL-8803, erect type stature with large and green leaflets, plant height of about 60-65 cm, white colour flower, plant colour is green, cream coloured seed and smooth, seed size is larger than the desi cultivar, 100 seed weight about 27 g, percentage of protein and carbohydrate is 19.42 and 60.21, respectively, yield 1500 kg/ha (Figs. 8, 17).

ix. BC-9 (BARI Chola-9)

'Deshi' chickpea, source ICRISAT ICCV-95138, this is erect type cultivar with plant height of about 60-65 cm. The leaves are dark green, with broad leaflets and fresh twigs. Flowers are pink and have dense trichomes on pods, plant colour is green, medium seeded high yielding cultivar, seeds are slight roundish and brown coloured, 100 seed weight 24-26 g, 21.20% protein and carbohydrate 59.50%, yield 1800 kg/ha (Figs. 9, 18).

2.2. Methods**2.2.1. Preparation of reagents****2.2.1.1. Pre-fixative- Paradichloro Benzene (PDB)**

One liter of distilled water was taken in a flask to make a saturated solution. Some amount of PDB was added to the flask and stirred until dissolved. After dissolving, some more PDB was added and stirred. This process was carried out until some PDB precipitation settled at the bottom of the flask. The bottle was kept at room (27-28 °C) temperature for future use.

2.2.1.2. Fixative (45% acetic acid)

45 ml of glacial acetic acid was mixed with 55 ml of distilled water.

2.2.1.3. Preservative (70% alcohol)

70 ml of absolute alcohol was mixed with 30 ml of distilled water.

2.2.1.4. Hydrolyzing agent (1N HCl)

36.5 ml of HCl were thoroughly dissolved in a liter of distilled water by constant stirring and kept at room (27-28 °C) temperature for future use.

2.2.1.5. Hydrolyzing solution

Two parts of 1N HCl was mixed with one part of 45% acetic acid.

2.2.2. Stains

2.2.2.1. Aceto-orcein (1%)

1 g of orcein dye (Sigma, USA) was added to a flask containing 45 ml (100%) acetic acid. These were heated gently (not boiling) for about 20 h and messed volume up to 100 ml by adding distilled water. It was then filtered quickly and stored at room temperature (27-28 °C) for future use.

2.2.2.2. Chromomycin A₃ (CMA) (0.1 mg/ml)

1 mg of CMA (Sigma, USA) was thoroughly dissolved in 10 ml of McIlvaine's buffer (McIlvaine 1921) supplemented with Mg⁺² by stirring and kept at -20 °C for future use.

2.2.2.3. 4', 6-Diamidino-2-Phenylindole (DAPI) (0.01 mg/ml)

1 mg of DAPI (Sigma, USA) was thoroughly dissolved in 100 ml McIlvaine's buffer (without Mg⁺²) by stirring and kept at -20 °C for future use.

2.2.3. Buffer

2.2.3.1. McIlvaine's buffer (pH 7.0)

About 59 (58.99) g of Na₂HPO₄.12H₂O (FW 358.14) and 3.71 g citric acid were measured and mixed in one liter distilled water. It was then autoclaved for 10 m and kept at 4 °C for future use.

2.2.3.2. McIlvaine's buffer with Mg^{+2} (pH 7.0)

About 59 (58.99) g of $Na_2HPO_4 \cdot 12H_2O$ (FW 358.14) and 3.71 g of citric acid were mixed in one liter of distilled water. After autoclaving, 2.46 g of $MgSO_4$ was added to it and kept at 4 °C for future use.

2.2.4. Antibiotics**2.2.4.1. Distamycin A (0.1 mg/ml)**

2.0 mg of distamycin A (Sigma, USA) thoroughly dissolved in 20 ml of McIlvaine's buffer by stirring and kept at -20 °C for future use.

2.2.4.2. Actinomycin D (0.25 mg/ml)

2.0 mg of Actinomycin D (Sigma, USA) thoroughly dissolved in 8 ml of McIlvaine's buffer by stirring and kept at -20 °C for future use.

2.2.5. Procedure for studying mitotic chromosomes**2.2.5.1. Collection of root-tips (RTs)**

The young healthy roots from seedling were cut 0.5 cm away from the tip by a clean blade.

2.2.5.2. Pre-treatment

The collected RTs were soaked on a filter paper to remove surface water and pretreated with PDB for 1.15 h at 20-25 °C.

2.2.5.3. Fixation

RTs were fixed in 45% acetic acid for 15 m at 4 °C.

2.2.5.4. Preparation of slide for Orcein-staining

The pretreated RTs were hydrolyzed for 10 s at 60 °C in a mixture of 1N HCl and 45% acetic-acid (2:1). Then the hydrolyzed RTs were soaked on a filter paper and taken on a clean slide. The meristematic region was cut with a fine blade. A drop of 1% aceto-orcein was added to the material and kept in an acetic acid chamber for 30-40 m. A clean cover glass was placed on the material. At first the materials were tapped gently by a tooth pick and then squashed by placing thumbs. During tapping and squashing care was taken so that the cover glass should not be moved because a minute displacement of it could damage the entire preparation. The slides were observed under Nikon microscope.

2.2.6. Preparation of slide for fluorescent staining

2.2.6.1. Preparation of air-dried slides

After hydrolyzing and dissecting, the materials were tapped and squashed with 45% acetic-acid and kept in -80 °C freeze for 3 m. The cover glass was removed quickly and dried in air for at least 24 h before study.

2.2.6.2. CMA-staining

Method proposed by Alam and Kondo (1995) was followed with slight modifications. After 24 h of air drying, the slide was first pre-incubated in McIlvaine's buffer (pH 7.0) for 30 m. At once one drop of 0.1 mg/ml distamycin-A was added to the materials of slides and a cover glass placed on it. The slide was kept in humid chamber for 10 m. Then the slide was washed with distilled water in such a way that the cover

glasses were removed. The slide was rinsed mildly in McIlvaine's buffer supplemented with MgSO_4 for 15 m. Then one drop of chromomycin A_3 (0.1 mg/ml) was added to the materials of slide and a clean cover glass was placed on it. The slide was kept in humid chamber for 12 m. The slide was washed with distilled water in such a way that the cover glasses were removed. The slides were treated again for 10 m in McIlvaine's buffer with Mg^{+2} and 10 m McIlvaine's buffer without Mg^{+2} . Slides were mounted in 50% glycerol and kept at 4 °C for overnight before observation. These were observed under Nikon (Eclipse 50i) fluorescent microscope with blue violet (BV) filter cassette.

2.2.6.3. DAPI-staining

For DAPI-staining method proposed by Alam and Kondo (1995) was followed after slight modifications. After 48 h of air drying, the slide was first pre-incubated in McIlvaine's buffer (pH 7.0) for twenty five m. The slide was treated in 0.25 mg/ml actinomycin D for 10 m in a humid chamber. After antibiotic treatment, the slide was washed with distilled water in such a way that the cover glass removed. The slide was immersed again in McIlvaine's buffer (pH 7.0) for 15 m followed by treating in DAPI solution (0.1 mg/ml) for 12 m. After rinsing in McIlvaine's buffer (pH 7.0) for 10 m, the slide was mounted with 50% glycerol and kept at 4 °C. These were observed under a Nikon (Eclipse 50i) fluorescent microscope with ultra violet (UV) filter cassette.

2.2.7. Determination of centromeric type, relative length and centromeric index

2.2.7.1. Centromeric type

Procedure proposed by Levan *et al.* (1964) for determining centromeric types of chromosomes was followed with slight modification. In this study, the l/s arm ratio (l = length of the longer arm and s = length of the shorter arm) was considered in the following manner:

Chromosome type (ct)	l/s ratio
(a) Metacentric (m)	1.00- 1.50
(b) Sub-metacentric (sm)	1.51- 3.00
(c) Acrocentric (ac)	3.01- 7.00
(d) Telocentric (t)	above 7.00

2.2.7.2. Relative length (RL) of chromosomes

The measurement of relative length was done by dividing the length of a particular chromosome with the total length of the diploid complements. It is represented as follows:

$$RL = \frac{\text{length of a particular chromosome}}{\text{total length of the diploid complements}}$$

2.2.7.3. Centromeric index (CI)

It was measured by the ratio of short arm to total length of that chromosome, expressed as per cent. It can be shown as below:

$$CI = \frac{\text{length of short arm}}{\text{total length of that chromosome}} \times 100$$

2.2.8. Classification of interphase nuclei and prophase chromosomes on the basis of Orcein-staining

The classification proposed by Tanaka (1971) was followed:

2.2.8.1. Interphase nuclei

- (1) Diffuse type
- (2) Simple chromocenter type
- (3) Complex chromocenter type
- (4) Rod pro-chromosome type
- (5) Round pro-chromosome type

2.2.8.2. Prophase chromosomes

- (1) Continuous type
- (2) Interstitial type
- (3) Gradient type
- (4) Proximal type
- (5) Tenuous type

2.2.9. Classification of fluorescent bands

According to the different fluorescent banding patterns observed in this study, bands were classified as follows:

α = Band in terminal region of short arm.

β = Band in terminal region of long arm.

λ = Band in terminal region of short arm and long arm.

Ω = Band in centromeric region.

Ψ = Dot like band.

θ = Fluoresced almost throughout the length.

δ = No band.

2.2.10. Idiogram

To get an accurate measurement of lengths, chromosomes from at least three metaphase plates were measured for each cultivar. Their average arm length was used to prepare the idiogram. The chromosomes were arranged gradually from bigger to smaller in length. The short arm placed on the upper side of the axis and long arm on the lower side.

2.2.11. Magnification

The photographs were taken by using 8 mega pixels canon power shoot A720 model with the magnification of 8.9x at auto mode. For measuring the magnification, at first the magnification was calculated by multiplying the magnification of objective (100X), tube length (1.25X) and camera lens (7X). From print the final magnification was calculated.

2.3. Isozyme analysis

2.3.1. Requirements

2.3.1.1. Required materials for protein isolation

1. Plant tissue (leaf tissue)
2. Digital weighting machine
3. Autoclave machine
4. Mortar and pestle (ice cold)
5. Eppendorf tubes (1.5 ml)
6. Centrifuge machine (providing 10,000 rpm – 14,000 rpm)
7. Ice machine
8. Refrigerator (-20 °C)

2.3.1.2. Required chemicals for protein isolation

1. Liquid Nitrogen
2. Phosphate buffer

2.3.1.3. Required chemicals for protein estimation

1. 0.1 M NaOH in 1% anhydrous Na₂CO₃
2. 1 % CuSO₄
3. 2 % Na-K-Tartrate
4. Folin-Ciocalteu's Phenol reagent
5. BSA (Bovine Serum Albumin)

2.3.1.4. Required equipment for isozyme analysis

For isozyme analysis Mini-PROTEAN Tetra Electrophoresis System from Bio-Rad was used. The Mini-PROTEAN Tetra system included a casting stand and glass plates with permanently bonded gel spacers (0.75 mm) that simplified hand casting and eliminated leaking during casting.

2.3.2. Extraction and estimation of proteins

For the extraction of proteins and isozyme study young leaf tissues of *ex vitro* grown plants were used. The leaves were collected from the field and homogenized in cold distilled water in an ice cold mortar pestel with liquid nitrogen. To estimate the protein leaf homogenates were centrifuged at 4 °C using 12000 rpm for 10 m in eppendorf centrifuge. Supernatant was discarded and pellet kept at -20 °C.

The quantities of proteins in various extract were estimated following the method of Lowry *et al.* (1951), for this purpose Bovine Serum Albumin (Sigma, UK) was used as a standard.

2.3.3. Preparing the solution

The Lowry solution is a mixture of 2 % sodium-potassium tartrate, 1 % copper sulphate and other chemicals such as 0.1 M NaOH, 1 % anhydrous Na_2CO_3 except the Folin Reagent. The Lowry solution was prepared freshly at the day of measurement. Generally the individual solutions for the Lowry solution were prepared in advance and then mixed at the day of measurement.

Solution A is a dilute alkaline solution. Folin and Ciocalteu's Phenol Reagent contain HCl and H_2PO_4 .

Solution A (alkaline solution) (250 ml)

NaOH 1.0000 gm

Na_2CO_3 0.2640 gm

Solution B (100 ml)

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.2500 gm

Solution C (100 ml)

Na-K-Tartrate 0.564 gm

Lowry Solution (freshly prepared)

Solution A + Solution B +Solution C with a ratio (vol:vol) of 100:1:1

Folin Reagent (instant fresh)

Folin and Ciocalteu's Phenol Reagent + double distilled water with a ratio of 1:1 is light sensitive. For this reason it was prepared at least 5 m of the first sample incubation and kept in an amber container.

The Lowry method relies on two different reactions

1. The first reaction is the formation of a copper ion complex with amide bonds, forming reduced copper in alkaline solutions. This is called a Biuret chromophore and is commonly stabilized by the addition of tartrate.

2. The second reaction is reduction of the Folin-Ciocalteu's reagent. The end product of this reaction had a blue color. The amount of protein in the sample was estimated via reading the absorbance (at 750 nm) of the end product of the folin reaction against a standard curve of a selected protein solution (in this case, BSA solution).

2.3.4. Polyacrylamide Gel Electrophoresis (Native PAGE) for Isozyme analysis

Polyacrylamide gel electrophoresis separates molecules in complex mixtures according to size and charge. During electrophoresis there is an intricate interaction of samples, gel matrix buffer and electric current resulting in separate bands of individual molecules. Polyacrylamide gel electrophoresis was performed in vertical mini slab gel (8 cm × 7 cm) separated by 0.75 mm thick spacer. The electrophoresis was done using the discontinuous buffer system.

2.3.5. Gel casting

2.3.5.1. Solutions and reagents

30% Acrylamide: 29.6 g of Acrylamide and 0.8 g of N, N' methelenebisacrylamide were dissolved in 100 ml of deionised doubled distilled water and stored in a dark bottle at 4°C.

Separating gel buffer (1.5 M Tris-Cl, pH 8.8): 18.16 g of Tris was dissolved in 80 ml of deionised doubled distilled water, pH was adjusted to 8.8 with concentrated HCl and volume was made up to 100 ml with deionised doubled distilled water.

Stacking gel buffer (1.0 M Tris-Cl, pH 6.8): 12.11 g of Tris was dissolved in 80 ml of deionised doubled distilled water, pH was adjusted to 6.8 with concentrated HCl and volume was made up to 100 ml with deionised doubled distilled water.

10% APS: 0.1 g of APS was dissolved in 1 ml of deionised doubled distilled water. APS provides the free radical which promotes the polymerization of Acrylamide and N, N' methelenebisacrylamide.

TEMED: TEMED was used directly. It accelerates the polymerization of Acrylamide and N, N' methelenebisacrylamide by catalizing the formation of free radicals from APS.

The above mentioned solutions and reagents were used in appropriate proportions to caste gel. Table 1 shows the different components of the gel and their amount needed for the casting of the two types of gel.

Table 1-Composition of separating (10%) and stacking (5%) gel

Solution components	Component volume for one gel mold (ml)	
	Separating gel (10%)	Stacking gel (5%)
Deionised double distilled water	4.4	3.0
1.5 M Tris-Cl (pH 8.8)	2.5	-----
1.0 M Tris-Cl (pH 6.8)	-----	0.63
Glycerol	-----	0.63
30% acrylamide solution	3.0	0.83
10% APS	0.1	0.05
TEMED	0.005	0.0025

After adding all the constituents of separating gel, the entire mixture was immediately poured between the glass plates leaving an appropriate gap for the stacking gel (about 3 cm from the top of the glass plate). After pouring, the solution of separating gel was carefully overlaid with deionised doubled distilled water and kept undisturbed for several minutes allowing the gel to polymerize. After removing the overlaying layer, a Teflon comb was inserted leaving a gap of 5 mm above the polymerized separating gel. All the constituents of 5% stacking gel was mixed and immediately poured on the polymerized separating gel. It was then kept undisturbed for half an hour for polymerization.

2.3.6. Gel Run

2.3.6.1. Solutions

Top electrode buffer (pH 8.3)

Tris	0.6 g/l
Glycine	2.7 g/l

Lower electrode buffer (pH 8.3)

Tris	3.08 g/l
------	----------

Sample buffer

Glycerol	75%
Bromophenol blue	25%

Before loading, the samples were diluted in a ratio of 1:1 with sample buffer. The gels were run as described of Arus and Orton (1983). Sample containing 30 µg of proteins was used for the running of gels.

After polymerization of the gel, combs were removed from the gel plates. The top and lower buffer tanks were filled up with top and lower electrode buffer respectively.

2.3.6.2. Power conditions

Power to the Mini-PROTEAN Tetra cell was supplied by an external DC voltage power supply and the electrophoresis was allowed to start. 120 V constant is recommended for native gel applications. 120 V was used for two gels. Run time was approximately 45 m.

2.3.7. Isozymes used in this study and their staining methods

Three isozyme systems, namely, acid phosphatase, esterase and peroxidase were used in the present investigation. These experiments were carried out following the method of Arus and Orton (1983). Three separate methods of staining were used to detect the enzyme activity on the gels.

2.3.7.1. Acid Phosphatase

Acid Phosphatase isozymes were visualized by the following principles

α -Naphthyl Phosphate $\longrightarrow$ α -Naphthol + Phosphate

α -Naphthol + Fast Garnet GBC $\longrightarrow$ Brown precipitate

2.3.7.2. Staining method

Solutions and reagents

1. 0.2 M Sodium acetate buffer, pH 5.0 (100 ml)

Sodium acetate	1.6408 g
----------------	----------

Deionised doubled distilled water was added to make upto 100 ml of final volume. pH was adjusted to acetic acid.

2. Staining solutions

α -Naphthyl acid phosphate mono sodium salt 100 mg
(dissolved in 500 μ l acetone and 500 μ l
deionised doubled distilled water)

Fast Garnet GBC	75 mg
-----------------	-------

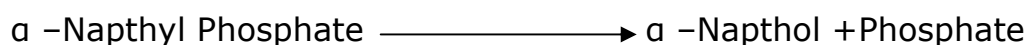
10% Magnesium chloride	0.5 ml
------------------------	--------

0.2 M Sodium acetate buffer, pH 5.0	100 ml
-------------------------------------	--------

Just after electrophoresis, the gel was immersed in staining solution and kept at room temperature in the dark for half an hour. Clear dark brown bands appeared.

2.3.7.3. Esterase

Esterase isozymes were visualized by the following principles



2.3.7.4. Staining method

Solutions and reagents

1. 0.2 M Sodium phosphate buffer, pH 7.0 (100 ml)

Solution A	0.2 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (100 ml)	2.76 gm
------------	---	---------

Solution B	0.2 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (100 ml)	5.365 gm
------------	--	----------

39.0 ml from Solution A and 61.0 ml from Solution B was mixed to make 100 ml Sodium phosphate buffer (pH 7.0).

2. Staining solutions –

α -Naphthyl acetate	30 mg
(dissolved in 1.0 ml absolute alcohol)	

Fast blue B salt	50 mg
------------------	-------

0.2 M Sodium phosphate buffer	100 ml (pH 7.0)
-------------------------------	-----------------

After electrophoresis, the gel was immersed in esterase staining solution for half an hour at room temperature in the dark until clear dark brown bands appear.

2.3.7.5. Peroxidase

Staining method

Solutions and reagents

Benzidine	0.3 g
Distilled water	300 ml
Acetic acid	1.8 ml
H_2O_2	150 μl

2.4. SDS-PAGE analysis

2.4.1. Required chemicals

15% Resolving gel

- a) Distilled water
- b) 1.5 M Tris-cl (pH 8.8)
- c) 30% Acrylamide solution
- d) 10% Ammonium persulfate (APS)
- e) 10% SDS
- f) TEMED

5% Stacking gel

- a) Distilled water
- b) 1.0 M Tris-cl (pH 6.8)
- c) 30% Acrylamide solution
- d) 10% Ammonium persulfate (APS)
- e) 10% SDS
- f) TEMED

2.4.2. Separation of seed proteins by SDS- PAGE

Protein extract (200 µg protein per sample) are separated by SDS-PAGE according to the method of Laemmli (1970) on a Biorad protean II system, with 15% resolving and 5% stacking gels, respectively, run at 200 V for 55 min.

5x Tris-glycine gel buffer (taken from Sambrook *et al.* 1989)

For 100 ml (final stock concentration):

Tris-Cl	1.51 g	[0.125 M]
Glycine	9.40 g	[0.125 M]
10% SDS	5.00 ml	[0.5%]

Deionized double distilled water was added to make 100 ml

2.4.3. Gel Preparation

1. The following gels were made in glass vials, without adding the 10% Ammonium persulfate and TEMED (Table 2).

Table 2: Composition of resolving (15%) and stacking gel (5%)

15% Resolving Gel		5% Stacking Gel	
Total Volume =10 ml	MI	Total Volume =2 ml	MI
H ₂ O	2.300	H ₂ O	1.400
30% Acrylamide mix	5.000	30% Acrylamide mix	0.330
1.5 M Tris-Cl, pH 8.8	2.500	1.0 M Tris, pH 6.8	0.250
10% SDS	0.100	10% SDS	0.020
10% Ammonium persulfate	0.100	10% Ammonium persulfate	0.020
TEMED	0.004	TEMED	0.002

2. The Biorad protean II system was assembled using 0.7 mm spacers.
3. Ammonium persulfate (10%) and TEMED was added to the resolving gel solution and quickly pipette into the assembled gel.
4. Poured butan-1-ol across the top of the resolving gel to produce a straight edge.
5. Allowed approximately 45 m for gel to polymerise.
6. Rinsed the butan-1-ol thoroughly with deionized double distilled water from the gel edge and dried prior to pouring the stacking gel.

7. Ammonium persulfate (10%) and TEMED was added to the stacking gel solution quickly pipette into the assembled gel.
8. Quickly placed the comb into the stacking gel.
9. Allowed 45 m for gel to polymerise.
10. Removed comb.
11. Rinsed wells with 1x Tris-glycine gel buffer to remove any un-polymerized gel.

2.4.4. Preparation of samples for SDS-PAGE

1. Protein samples was adjusted to a concentration of 3 μg and a volume of 10 μL using protein extraction Buffer (+DTT/+BB).
2. Vortexed briefly and pulsed spin.
3. Heated at 95 $^{\circ}\text{C}$ for 2 m.
4. Allowed to cool immediately on ice.

2.4.5. GEL loading and running

1. The upper chamber was filled with 1x Tris-glycine gel buffer.
2. Using a 10 μl pipette, loaded all samples into well carefully.
3. Loaded 10 μl Benchmark Prestained Ladder (Invitrogen 10748-010) in one lane.

Cat.No.		PR911654
Range		10-170 kDa
Number of bands		10
Concentration		0.2-0.4 mg of each protein
Recommended Load		3-5 μl /well
10 prestained coloured bands	10, 15, 20, 25, 35, 40, 55, 70, 100, 130,	170kDa

4. The lower chamber filled with 1x Tris-glycine gel buffer.
5. Runed at 200 V for 55 min.

2.4.6. Staining the Gel

The gel is fixed, stained and destained according to the manufacturer's instructions for (Sigma, B2025).

NOTE: Minigels required about 50 ml solutions to cover a square Petri dish (100 mm x 100 mm).

1. The protein was fixed for 30 m in fixing solution (7 % glacial acetic acid in 40 % (v/v) methanol) with rocking gently.
2. Decant the fixing solution into methanol waste.
3. Added staining suspension (40 ml Brilliant Blue G-colloidal concentrate, 10 ml methanol) to gel, rock for 1-2 h or overnight if destained.
4. The stain solution was decanted into methanol waste.
5. Decanted with Destain solution (10% acetic acid in 25%) (v/v) methanol with shaking for 10-30 s.
6. Decant the destain solution into methanol waste.
7. Rinsed the gel with 25 % methanol (destain solution II).
8. Decanted the 25 % methanol into methanol waste.
9. Destained the gel with 25 % methanol (destain solution II) for up to 24 h.
10. Decanted the 25% methanol into methanol waste.
11. If any precipitated dye remains on the surface of the gel, gently wiped with a clean lab wipe soaked in 25 % methanol.
12. Added deionized double distilled water to the gel.
13. Proceeded to analyzing Gel.

2.4.7. Data analysis for isozymes and SDS-PAGE

2.4.7.1. Band scoring

Based on polyacrylamide gel, different isozyme bands were scored as present (1) and absent (0) separately in data sheet to calculate Nei's (1972) genetic distance and dendrograms were generated with Unweighted Pair Group [1, 0] matrix. Then data were analyzed and similarity matrix and genetic distance was constructed from binary data with UPGMA (Unweighted Pair Group Method Arithmetic Average) using POPGENE32 software (Version 1.32) (Yeh *et al.* 1999).

2.4.7.2. Preparation of zymograms

The zymograms obtained from the gels were prepared by measuring the distance of each band from the point of gel loading. Then the retardation factor (*Rf*) of each band was calculated as a ratio of distance traveled by the band to the tracking dye. Bands were numbered based on increasing *Rf* values. The variation was determined based on the presence or absence of specific bands.

2.4.7.3. Determination of retention factor

$$Rf = \frac{\text{Migration distance of substance}}{\text{Migration distance of solvent front}}$$

2.4.7.4. Determination of polymorphic bands

$$\text{Percentage of polymorphic bands} = \frac{\text{No. of polymorphic bands}}{\text{No. of total bands}} \times 100$$

2.4.7.5. Cluster analysis and Dendrogram construction

Following electrophoresis, the size of protein bands were estimated by comparing the isozyme activity and protein profiling with molecular weight ranged from 10 to 170 kDa from photographic films. A few bands which were not reproducible were excluded. All distinct bands (protein bands) were thereby given the identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0) of bands, size of bands and overall polymorphism of bands for each variant. The scores obtained using all protein banding analysis were then combined to create a single data matrix. This was used for estimating polymorphic loci, Nei's (1972) gene diversity, genetic distance (D) and constructing a Unweighted Pair Group Method of Arithmetic Average (UPGMA) to generate a dendrogram among the cultivars using computer program "POPGENE 32" (Version 1.32) (Yeh *et al.* 1999).

2.5. RAPD and SSR marker analysis

2.5.1. Collection of leaf sample

To extract genomic DNA, young and actively growing fresh leaves were collected from field grown plants of the BC-1 to BC-9. These leaves were cut apart with sterilized scissors and washed well initially in distilled water and soaked on fresh tissue paper to remove spores of microorganisms and other sources of foreign DNA.

2.5.2. Preparation of stock solutions and working solutions used for DNA Isolation

For conducting the isolation procedures, the following stock solutions and working solutions were prepared.

2.5.2.1. Stock Solution of Tris - HCl 1 M, pH 8.0 (100 ml)

12.14 g of Trizma base (MW=121.14) was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0 by adding about 5 ml of concentrated HCl. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4 °C.

2.5.2.2. Stock Solution of EDTA (0.5 M) pH 8.0 (100 ml)

18.61 g of EDTA (EDTA. 2H₂O, MW = 372.24) was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 g of NaOH pellets was added to adjust the final pH to 8.0. It may mention that EDTA alone will not dissolve, unless NaOH is added. The final volume of the solution was adjusted to 100 ml by adding sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4 °C.

2.5.2.3. Stock Solution of NaCl (5 M) (100 ml)

29.22 g of sodium chloride (NaCl, MW = 58.44) was dissolved in 75 ml of distilled water. Then the final volume was made up to 100 ml with distilled water. Vigorous stirring along with heating was required to dissolve NaCl. After sterilization by autoclaving it was then stored at 4 °C. NaCl was added in small amount at once to be dissolved in solution.

2.5.2.4. β-Mercaptoethanol

β-Mercaptoethanol was available at a strength of 14.4 M solution and was stored in a dark bottle at room temperature.

2.5.2.5. Ribonuclease A

10 mg RNAase A was dissolved in 1 ml of deionized distilled water and was stored at -20 °C.

2.5.2.6. Tris-HCl saturated phenol

It was prepared by following procedure:

1. The crystal phenol was melted in a water bath at 65 °C for 30 m.
2. Then 100 ml of melted phenol was taken and same volume of Tris-HCl (pH 8.0) was added.
3. It was mixed with a magnetic stirrer for 10 minutes and then was left in rest for 5 m.
4. At this stage, two distinct phases were visible, colorless upper phase and colored lower phase.
5. The upper phase was removed with the help of a dropper.

This step was performed for six times which took about 3.5 h to obtain pH 7.75. After saturation, the phenol became the half of the initial volume. As phenol is very much corrosive and highly toxic, protective measures (Apron, Gloves, Mask etc.) were adopted.

2.5.2.7. Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) (100 ml)

50 ml of phenol, 48 ml of chloroform and 2 ml of isoamyl alcohol were added and mixed properly using vortex mixture. Mixing was done under fume hood for safety. The solution was shaken before every use.

2.5.2.8. 70% ethanol (100 ml)

30 ml double distilled water (ddH₂O) was added in 70 ml absolute ethanol.

2.5.2.9. Stock Solution of TE (Tris-HCl EDTA) buffer pH 8.0 (100 ml)

1 ml of 1 M Tris-HCl was added with 200 μ l of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4 °C.

2.5.2.10. Sodium acetate (3 M) pH 5.2 (100 ml)

40.824 g of sodium acetate was dissolved in 70 ml of ddH₂O and was adjusted to 100 ml in volume adding ddH₂O and was sterilized by autoclaving.

2.5.2.11. Extraction buffer (Homogenization buffer)

To prepare extraction buffer the following components with proper concentrations were used. For the economic use of chemicals, different volumes of solutions were prepared as in the tabular form given below (Table 3):

Table 3 –Components and concentrations of extraction buffer

Chemical Names	Molecular weight	Stock Con.	Working Con.	Working volume	
				100 ml	1000 ml
CTAB			2%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (pH 8.0)	372.24	0.5 M	20 mM	4 ml	40 ml
Tris-HCl (pH 8.0)	121.1	1 M	100 mM	10 ml	100 ml
β -Mercaptoethanol		14.4 M	100 mM	700 μ l	7 ml

Steps of extraction buffer preparation (100 ml)

Extraction buffer (100 ml) was prepared by the following steps

- i. 10 ml of 1 M Tris-HCl (pH 8.0) was taken in a 250 ml conical flask.
- ii. 28 ml of 5 M NaCl was added to it.
- iii. 4 ml of 0.5 M EDTA (pH 8.0) was added next.
- iv. The solution was then autoclaved.
- v. After autoclaving 1 g PVP and 2 g CTAB was added and stirred in the magnetic stirrer.
- vi. 700 μ l β -mercaptoethanol was added prior to use and was mixed by glass rod under fume hude.
- vii. pH of all solutions were adjusted to 5 with HCl and make up to 100 ml by adding sterile de-ionized distilled water.

Freshly prepared extraction buffer solution was used, β -mercaptoethanol was added to the solution prior to use. The whole task was performed in the fume hood.

2.5.3. Protocol used for genomic DNA isolation

DNA was isolated using the mini preparation CTAB method (Doyle and Doyle 1987) with minor modification. The protocol is described as follows:

2.5.3.1. Required equipment for plant genomic DNA isolation

1. Digital weighting machine
2. pH meter
3. Autoclave machine

4. Mortar and pestle
5. Centrifuge tubes (15 ml)
6. Eppendorf tubes (1.5 ml)
7. Micropipettes and nuclease free micropipette tips
8. Centrifuge machine (providing 10,000 rpm – 14,000 rpm)
9. Ice machine
10. Water bath (capable of maintaining 65°C)
11. Water de- ionizer
12. Water distillation plant
13. Fume hood
14. Refrigerator

2.5.3.2. Required chemicals for plant genomic DNA isolation

All the chemicals were prepared with de-ionized, sterile water and autoclaved.

1. Tris-HCl
2. EDTA
3. NaCl
4. CTAB
5. β -Mercaptoethanol
6. Phenol
7. Chloroform
8. Isoamyl alcohol
9. Isopropanol
10. Ethanol
11. Sodium acetate
12. Ribonuclease A

2.5.3.3. Required materials for agarose gel electrophoresis

The following equipments were used to carry out the agarose gel electrophoresis

1. A horizontal electrophoresis chamber and power supply
2. Gel casting tray and combs
3. Micropipettes and tips
4. Gel documentation system

The following chemicals were used to conduct the agarose gel electrophoresis

1. Electrophoresis buffer
2. Agarose
3. Ethidium bromide
4. 6x sample loading buffer

2.5.3.4. Procedure

1. Freshly harvested leaf tissue (1.5 g) was taken in liquid nitrogen in ice cold mortar and grinded to fine powder with the help of pestle. Five ml extraction buffer was added to it until it became homogenous paste.
2. The paste was then transferred to 15 ml centrifuge tube and allowed to incubate at 65 °C of preheated water bath with occasional mild shaking.
3. After incubation the homogenate was centrifuged at 13,000 rpm for 10 minutes at room temperature to remove non soluble debris.
4. The supernatants were then transferred to fresh tubes and an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1)

was added and mixed well by slow inversion followed by centrifuging at 13,000 rpm for 10 m. This step was repeated for three times.

5. The supernatants were then transferred to fresh tubes and an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed well by slow inversion followed by centrifuging at 13,000 rpm for 5 m. This step was repeated for three times.
6. The supernatants were then transferred to fresh tubes and an equal volume of chloroform was added and mixed well by slow inversion followed by centrifuging at 13,000 rpm for 5 minutes. This step was repeated for two times.
7. The supernatants were transferred to fresh tubes and 0.1 vol. of 3 M Sodium acetate (pH 5.2) was added followed by 0.6 vol. of 100% chilled Isopropanol was added and the solution shaken slowly. In this step, DNA became visible as whitish thread like substance in the solution.
8. These mixtures were then centrifuged at 13,000 rpm for 10 m at room temperature. The supernatants were discarded carefully by using adjustable micropipettes.
9. The pellets were washed with 70% ethanol. This washing step was repeated at least twice to thrice. The pellets were air dried on a towel paper for about one hour.
10. The dried DNAs were resuspended in 500 µl of TE buffer and treated with RNAase A for 30 m at 37 °C and stored at 4 °C.

2.5.4. Estimation of quality and quantity of isolated DNA samples

Before PCR amplification it is important to know the quality and quantity of genomic DNA because different DNA extraction methods results DNA of widely different purity. It is necessary to optimize the amount of DNA to achieve reproducibility and strong signal in PCR assay. Excessive genomic DNA may result smears lack of clearly defined bands in the gel. On the other hand, too little DNA will give non-reproducible patterns (Williams *et al.* 1993). Measurement of isolated DNA concentration can be done by comparing DNA with the standard DNA on agarose gel electrophoresis or by estimating the absorbance of DNA by spectrophotometer at 260 nm. Both the methods were carried out during this investigation.

2.5.5. Measurement of DNA concentration and quality by agarose gel electrophoresis

The following equipments and chemicals were used to conduct agarose gel electrophoresis:

1. A horizontal electrophoresis chamber and power supply
2. Gel casting tray and combs
3. Gel Documentation System (BioSciTec, Gelscan 6.0 Professional, Germany)
4. Gloves
5. Pipette and tips
6. DNA ladder (1 kb for RAPD and 100 bp for SSR)
7. Electrophoresis buffer (TAE)
8. 6× sample loading buffer
9. Agarose
10. DNA stain (Ethidium bromide)

2.5.6. Preparation of different solutions used for gel electrophoresis

For conducting the gel electrophoresis, following solutions were prepared

2.5.6.1. TAE buffer (50X, pH 8.3, 1 litre)

242 g Trizma base (MW=121.14) was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (pH 8.0) was added to it. They were mixed well. The pH of the solution was adjusted by mixing concentrated HCl at pH 8.3. The final volume of the solution was adjusted to 1000 ml.

2.5.6.2. Loading dye (10X)

This was required to load samples during gel electrophoresis for the visualization. For making 10 ml of 10X loading dye, 40 mg of Bromophenol blue (i.e. the final concentration was 0.4%), 40 mg of Xylene cyanol FF (i.e. the final concentration was 0.4%) and 5 ml of 98% glycerol (i.e. the final concentration was 50%) were added to 4 ml of sterile de-ionized distilled water and mixed properly. The final volume was adjusted to 10 ml with sterile de-ionized distilled water and stored at 4°C.

2.5.6.3. Ethidium bromide solution

For the preparation of 1 ml solution, 10 mg of Ethidium Bromide was dissolved in 1 ml of sterile de-ionized distilled water. It was then mixed by gentle shaking with hand. The solution was transferred to a dark

bottle and was stored at room temperature. Stock solution of 10 mg/ml can be purchased directly from companies.

2.5.6.4. Preparation of 1.2% Agarose gel electrophoresis

The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp *et al.* (1973).

- i. Agarose (1.2 g) was heated to melt into 100 ml of TAE buffer, ethidium bromide was added (10 µg/ml), poured into gel-tray and fixed with appropriate combs.
- ii. After solidification the gel was placed into gel-running tray containing 1 xTAE buffer.
- iii. Required plant DNA solutions were loaded with 6x gel loading dye and electrophoresis was continued until DNA fragments separated well.

2.5.6.5. Comb set-up

The ends of the gel casting tray was sealed or fitted with casting dams and the combs placed in the gel casting tray. The melted agarose solution was poured into the casting tray and let it to cool until solid. The comb was removed carefully from the solidified gel. The casting dams or gates were removed from the edges of the gel tray carefully so that the gel did not slide off the tray.

2.5.7. Preparation of DNA sample for electrophoresis

At first 2 µl 10X loading dye was placed on a piece of parafilm by a micropipette. 10 µl DNA sample (after thawing from frozen stock) was added to it and mixed 2-4 times with the adjustable micropipette.

2.5.7.1. Electrophoresis

The gel was placed in the electrophoresis chamber in such a way that the sample wells remained near to the cathode (negative end generally marked as black). DNA sample would migrate towards the anode (positive end generally marked as red) during electrophoresis.

The gel was covered by sufficient amount of 1X TAE buffer. The volume of the electrophoresis buffer should not above the maximum buffer mark on electrophoresis system. The prepared samples were loaded slowly and allowed to sink to the bottom of the wells. 10 μ l of 1 kb DNA ladder (marker) was also loaded at least in one well (generally the first one). Electrophoresis was carried out at 50 volts and 100 mA for 1.5 hours. The separation process was monitored by the migration of the dye on the gel. When the first dye (bromophenol blue) had reached about three-fourths of the gel length, the electrophoresis was completed and stopped.

2.5.7.2. Documentation of the DNA sample

- i. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed in gel documentation system (Biosens SC Series-645) for checking the DNA bands.
- ii. The DNA was observed as band and photographed using Gel Documentation system.
- iii. The electrophoregram of DNA samples of nine chickpea cultivars are shown in figure 167.

2.5.7.3. Quantification of DNA by measuring their OD in a spectrophotometer

For more confirmation, DNA was quantified by measuring their optical density in a spectrophotometer. The DNA concentration was obtained by multiplying the absorbance at 260 nm by a constant. The DNA purity was measured by dividing the absorbance at 260 nm wave length with the absorbance at 280 nm wave length.

High-quality DNA should give the ratio (A_{260}/A_{280}) in the range of 1.8–2.0. The A_{260}/A_{280} ratio higher than 2.0 generally indicates RNA contamination. If the ratio is lower than 1.8, it normally indicates protein contamination during extraction process. Following Sambrook *et al.* (1989), the DNA concentration was calculated (1 O. D.) at 260 nm corresponds to 50 $\mu\text{l}/\text{ml}$ of double standard DNA.

2.5.7.3.1. Measurement of OD in spectrophotometer

- i. The spectrophotometer (Jasco V-630 Spectrophotometer) was turned on and warmed up at 260 nm wave length for 30 m.
- ii. The absorbance reading was standardized by adjusting at zero with the help of a cuvette filled with 2 ml sterile distilled water.
- iii. The samples were prepared by taking 2 μl of each DNA sample in a cuvette containing 1,998 μl sterile distilled water. The samples were mixed well by using an adjustable micropipette.
- iv. The reading was taken by adjusting the Spectrophotometer at 260 nm and at 280 nm, respectively.
- v. After recording the absorbance readings, the cuvette was rinsed out with sterile distilled water. The values of spectrophotometric

absorption of different samples for determination of DNA quality are shown in the Table 4.

2.5.7.4. Calculation for determining the concentration of DNA

Before PCR for RAPD analysis, DNA concentrations were determined according to the following formula:

DNA concentration = A₂₆₀ × Dilution factor × Conversion factor

$$= A_{260} \times \frac{\text{Volume of distilled water } (\mu\text{l})}{\text{Amount of DNA sample } (\mu\text{l})} \times 50$$

$$= (\text{ng}/\mu\text{l})$$

$$= (\mu\text{g}/\text{ml}) \text{ [since } 1 \mu\text{g} = 10^{-3}\text{ng i.e. } \mu\text{g}/\text{ml} = \text{ng}/\mu\text{l}]$$

A₂₆₀= spectrophotometric absorbance reading at 260 nm of DNA sample.

Dilution factor= the ratio of distilled water (μl) to amount of DNA sample (μl).

Conversion factor 50 = the 50 μg/ml of DNA contained in a solution which gives the spectrophotometric absorbance reading at 260 nm equal to 1.

Note: The calculated DNA concentration expressed in μg/ml gave a fraction. To avoid fraction it was converted into ng/μl (1μg = 10⁻³ ng) and therefore, multiplied with 1000.

Table 4. Spectrophotometric absorbance readings at 260 nm wave length and concentration of DNA of nine cultivars of *Cicer arietinum* L.

Plants	Absorbance reading at 260 nm	Concentration of DNA (ng/μl)
BC-1	0.145	7250
BC-2	0.154	7700
BC-3	0.027	1350
BC-4	0.105	5250
BC-5	0.220	11000
BC-6	0.042	2100
BC-7	0.082	4100
BC-8	0.072	3600
BC-9	0.020	1000

2.5.8. Amplification of DNA by polymerase chain reaction (PCR) using RAPD and SSR primers

To perform the amplification of target DNA, RAPD and SSR primer sequences were mixed with genomic DNA in the presence of a thermostable DNA polymerase with suitable buffer and subjected to temperature cycling conditions typical PCR.

2.5.8.1. Preparation of working solution (50 ng/μl) of DNA samples for PCR

Original stock solution concentration of each DNA sample was adjusted to a unique concentration (50 ng/μl) using the following formula:

$$S_1 \times V_1 = S_2 \times V_2$$

$$V_1 = S_2 \times V_2 / S_1$$

Where, S_1 = stock DNA concentration (ng/ μ l)

V_1 = required volume (μ l)

S_2 = working DNA concentration (ng/ μ l)

V_2 = working volume of DNA solution (μ l)

Original stock DNA (2 μ l) was taken in a 1.5 ml eppendorf tube and required amount of TE buffer calculated from the above formula was added to it. Required volume of TE buffer for each sample was calculated and shown in the Table 5.

Table 5. Preparation of working DNA solution (50 ng/ μ l) for PCR

Plants	Working solution(50 ng/ μ l) for PCR	
	TE buffer/ de-ionized water (μ l) required	DNA (μ l) required
BC-1	288	2
BC-2	306	2
BC-3	52	2
BC-4	208	2
BC-5	42	2
BC-6	82	2
BC-7	162	2
BC-8	142	2
BC-9	38	2

2.5.8.2. Primers used in RAPD analysis

Primarily thirty five decamer primers were tested for RAPD amplification of which twelve primers exhibited good quality banding patterns and sufficient variability. These twelve primers were selected for further analysis. The details of the twelve primers were given in Table 6.

Table 6. Twelve arbitrary RAPD primers used in the present study

Primer code	Sequence (5'–3')	Annealing temp. (°C)	G+C content (%)
OPA-1	CAG GCC CTT C	36.4	70
OPA-3	AGT CAG CCA C	34.3	60
OPA-4	AAT CGG GCT G	35.1	60
OPA-5	AGG GGT CTT G	32.6	60
OPA-7	GAA ACG GGT G	33.2	60
OPA-8	GTG ACG TAG G	31.1	60
Primer-2	GTT GCG ATC C	34.2	60
Primer-12	GTA TGG GGC T	32.2	60
Primer- 18	GTT TCG CTC C	33.7	60
Primer-19	GAT GAC CGC C	38.8	70
Primer-23	GTC AGG GCA A	34.7	60
Primer-24	GGT CGG AGA A	33.2	60

2.5.8.3. Primers used in SSR analysis

Ten SSR primers were used to amplify simple sequence repeats of genomic DNA from nine cultivars of chickpea. Primers were evaluated on the basis of intensity or resolution of bands, repeatability of markers and consistency within individual and potential to differentiate cultivars (polymorphism). The details of the ten primers were given below:

Table 7. Ten SSR primers used in the present study

Pair No.	Primer names	Sequences (5'–3')	Annealing Temp. (°C)	G+C content (%)
I	GA26 CICER	F-GTGCAGAGCATCATGCGATGCTCAA GACATCTGCCA R- TCATACTCAACAAATTCATTTCCC	85.4 62.6	52.7 33.3
II	CASTMS2	F- ATTTTACTTTACTACTTTTTTCCTTTC R- AATAAATGGAGTGTAATTTTCATGTA	56.8 58.5	22.2 23.0
III	CASTMS15	F-CTTGTGAATTCATATTTACTTATAGAT R-ATCCGTAATTTAAGGTAGGTAAAATA	55.0 59.2	22.2 25.9
IV	CASTMS21	F- CTACAGTCTTTTGTCTTCTAGCTT R- ATATTTTTTAAGAGGCTTTTGGTAG	58.1 58.9	32.0 28.0
V	TA71	F- CGATTTAACACAAAACACAAA R- CCTATCCATTGTCATCTCGT	57.7 59.0	28.5 45.0
VI	TA72	F-GAAAGATTTAAAAGATTTTCCACGTTA R-TTAGAAGCATATTGTTGGGATAAGAGT	62.3 62.8	25.9 33.3
VII	TA130	F- TCTTTCTTTGCTTCCAATGT R- GTAAATCCCACGAGAAATCAA	58.9 60.4	35.0 38.0
VIII	TA135	F- TGGTTGGAAATTGATGTTTT R- GTGGTGTGAGCATAATTCAA	59.3 58.6	30.0 40.0
IX	TR29	F-GCCCACTGAAAAATAAAAAG R- ATTTGAACCTCAAGTTCTCG	57.8 58.4	35.0 40.0
X	TA118	F-ACAAGTCACATGTGTTCTCAATA R-GGAAAGGTTAAGAAATTTACAATAC	58.6 58.3	34.7 26.9

2.5.8.4. Preparation of primers

The supplied primers were diluted to 100 μM . To make 100 μM of each primer, the following mathematical deduction was followed:

We know,

$n = cv$ where n = number of mole

c = molarity *i.e.* concentration in molarity

v = volume.

Generally, 100 μM concentration of primer has to prepare as main stock solution.

In this case,

$C = 100 \mu\text{M}$ since 53.4 nM of primer was present in the vial used in the investigation, supplied from the company, $n = 53.4 \text{ nM}$.

v = required volume of TE buffer has to add in the supplied vial to make 100 μM main stocks, using the formula-

$$v = \frac{n}{c}$$

$$v = \frac{53.4 \text{ nmol}}{100 \mu\text{mol}}$$

$$v = \frac{53.4 \times 10^{-9} \text{ mol}}{100 \times 10^{-6} \text{ mol}}$$

$$v = \frac{53.4 \times 10^{-5} \text{ mol}}{\text{mol}}$$

$$v = \frac{53.4 \times 10^{-5} \text{ mol}}{\text{molL}^{-1}}$$

$$v = \frac{53.4 \times 10^{-5}}{\text{L}^{-1}}$$

$$v = 53.4 \times 10^{-5} \text{ L}$$

$$v = 53.4 \times 10^{-5} \times 100 \text{ ml}$$

$$v = 53.4 \times 10^{-2} \text{ ml}$$

$$v = 53.4 \times 10^{-2} \times 1000 \mu\text{l}$$

$$v = 534 \mu\text{l}$$

Therefore, 534 μl of TE buffer was added to the vial to make 100 μM main stocks. Using the above deduction method, all primer pairs were diluted to 100 μM main stock. All primers were diluted to 10 times i.e. 10 μM to make working solution for use.

2.5.8.5. PCR reaction for RAPD and SSR analysis

2.5.8.5.1. Preparation of PCR reaction mixture/PCR cocktail

The following components were used to prepare PCR cocktail (Table 8 and Table 9). The total volume of PCR reaction was 232 μl for 9 samples.

Table 8. Preparation of PCR cocktail (10 reactions) for RAPD primers

Sl. No.	Reagents	Amount per sample	Total
1	Sterile de-ionized distilled water	19.05 μl	190.5 μl
2	<i>Taq</i> buffer A 10X (Tris with 15 mM MgCl_2)	2.5 μl	25.0 μl
3	Primer (10 μM)	1.0 μl	10.0 μl
4	dNTPs (10 mM each)	0.25 μl	2.5 μl
5	<i>Taq</i> DNA Polymerase (5U/ μl)	0.2 μl	2.0 μl
6	Template DNA (25 ng/ μl)	2.0 μl	---
Total		25.0 μl	

Table 9. Component of PCR cocktail (10 reactions) for SSR primers

Sl. No.	Reagents	Amount per sample	Total
1	Sterile de-ionized distilled water	18.05 μ l	180.5 μ l
2	<i>Taq</i> buffer A 10X (Tris with 15 mM MgCl ₂)	2.5 μ l	25.0 μ l
3	Primer forward (10 μ M)	1.0 μ l	10.0 μ l
4	Primer reverse (10 μ M)	1.0 μ l	10.0 μ l
5	dNTPs (10 mM each)	0.25 μ l	2.5 μ l
6	<i>Taq</i> DNA polymerase (5U/ μ l)	0.2 μ l	2.0 μ l
7	Template DNA (25 ng/ μ l)	2.0 μ l	---
Total		25.0 μl	

During the experiment, PCR buffer, dNTPs, primers and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice. Template DNA (25 ng/ μ l) were pipetted (2.0 μ l) first into PCR tubes (0.5 ml) compatible with the thermocycler. For each DNA sample being tested, a pre-mix was prepared in the following order- buffer, dNTPs, DNA template and sterile distilled water. *Taq* DNA polymerase enzyme was added to the pre-mix. The pre-mix was then mixed well and aliquot into the tubes containing primers. The tubes were then sealed and placed in a thermocycler and the cycling started immediately.

2.5.8.5.2. PCR amplification for RAPD and SSR

PCR amplification was done in an oil-free thermal cycler (Biometra UNO II). The optimum amplification cycle was as follows-

Arbitrary primer-PCR profile

Denaturation/ Annealing/ Extension		Temperature	Time
35 cycles	Initial denaturation	95 °C	5 m
	Denaturation at	95 °C	1 m
	Annealing at	34-36 °C	30 s
	Extension at	72 °C	3 m
	Final extension at	72 °C	5 m

SSR marker-PCR profile

Denaturation/ Annealing/ Extension		Temperature	Time
35 cycles	Initial denaturation	95 °C	3 m
	Denaturation at	95 °C	30 s
	Annealing at	55-84.4 °C	30 s
	Extension at	72 °C	1 m
	Final extension at	72 °C	2 m

After completion of cycling program, the reactions were kept at 4°C.

2.5.8.5.3. Electrophoresis of the amplified products (RAPD and SSR) and documentation

The amplified products were separated electrophoretically on 1.2 % agarose gel. The gel was prepared using 1.2 g agarose powder

containing ethidium bromide and 100 ml 1× TAE buffer. Agarose gel electrophoresis was conducted in 1× TAE buffer at 50 volts and 100 mA for 1.5 h. As marker 1 kb and 100 bp DNA ladder was electrophoresed alongside the RAPD and SSR product, respectively. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system for both the markers.

2.5.9. RAPD and SSR marker data analysis

Cluster analysis and dendrogram construction

Following electrophoresis, the size of amplified PCR products were estimated by comparing the migration of each amplified fragment with that of a known size fragments of 1 kb (for RAPD) and 100 bp (for SSR) molecular weight marker from photographic films. A few bands which were not reproducible were excluded. All distinct bands were thereby given the identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0) of bands, size of bands and overall polymorphism of bands for each cultivars and each primer. The scores obtained using all primers in the RAPD and SSR analysis were then combined to create a single data matrix. This was used for estimating polymorphic loci, Nei's (1972) gene diversity, genetic distance (D) and constructing a Unweighted Pair Group Method of Arithmetic Average Analysis (UPGMA) to generate a dendrogram among the cultivars using computer program "POPGENE 32" (Version 1.32) (Yeh *et al.* 1999).

3. RESULTS

3. Results

3.1. Appropriate season for obtaining maximum number of dividing cells from the root-tips

Almost equal numbers of dividing cells were observed throughout the year in the root tip cells (RTCs) (about 70%). However, the number of dividing cells was very poor in extreme high or low temperature.

3.2. Appropriate time for obtaining maximum number of dividing cells from the root tips

Root tips of nine cultivars of chickpea (*Cicer arietinum* L.) were collected from 9.00, 9.30, 10.00, 10.30, 11.00, 11.30 a.m., 12:00 p.m., 12:30 p.m. and 1:00 p.m. It was found that 11.30 a.m. to 12.30 p.m. were the optimum time for obtaining maximum number of dividing cells (about 70 %).

3.3. Appropriate chemical for pretreatment

Different chemicals like 8-hydroxyquinoline, colchicine and para dichloro benzene (PDB) were tried for pretreatment to find out proper contraction and intact morphology of chromosomes. It was found that only PDB for 1.15 h gave the best results.

3.4. Karyotypes and Idiograms

On the basis of overall length and centromeric position, somatic chromosomes ($2n = 16$) of chickpea (*Cicer arietinum* L.) could be assembled in 8 pairs (Figs. 100-108, Tables 11-20). From the data, idiograms were made supplementing the karyotypes (Figs. 109-117).

3.5. Interphase nuclei

For every staining, at least 50 interphase nuclei were observed in each cultivar.

3.5.1. Orcein staining

The nine cultivars of chickpea have more or less similar staining type of interphase nuclei with orcein. Ten to sixteen darkly stained small heterochromatic regions were found in every nucleus of all cultivars except BC-2 and BC-3 (Figs. 19, 22-27). In the interphase nuclei of BC-2, small heterochromatins were aggregated forming large heteropycnotic bodies (Fig. 20). Few smaller heterochromatic bodies were present in the interphase nuclei of BC-3 (Fig. 21, Table 10).

3.5.2. CMA-staining

A numbers of brightly stained CMA-positive bands were found throughout the interphase nuclei of nine cultivars. Among the bands two were prominent and distinguishable from other especially in BC-1, BC-2, BC-4, BC-5, BC-6 and BC-9 (Figs. 28-36).

3.5.3. DAPI-staining

Many DAPI-positive bands were found throughout the interphase nuclei of nine chickpea cultivars (Figs. 37-45). These bands were brighter than those of CMA.

3.6. Prophase chromosomes

3.6.1. Orcein staining

Prophase chromosomes of nine cultivars stained with orcein in three different ways. Prophase chromosomes of BC-1 and BC-8 were stained

at the interstitial region. The other regions of these chromosomes were faintly stained (Figs. 46, 53). In BC-2, BC-6 and BC-9, the prophase chromosomes stained in one end and faint to the other end gradually (Figs. 47, 51, 54). Therefore, one end of these chromosomes was much darker than other end. Prophase chromosomes of BC-3, BC-4, BC-5 and BC-7 were stained uniformly along the entire length (Figs. 48, 49, 50, 52).

3.6.2. CMA-staining

The prophase chromosomes of nine chickpea cultivars were brightly and uniformly stained with CMA. Two bright and distinguishable CMA-positive dots were found in every prophase stage of these cultivars (Figs. 55-63, arrow).

3.6.3. DAPI-staining

The prophase chromosomes of nine chickpea cultivars were brightly stained. Several DAPI-positive bands were distributed within the prophase chromosomes of these cultivars (Figs. 64-72). The bands were different in respect of number, size and location.

3.7. Metaphase chromosomes

3.7.1. Orcein stained mitotic metaphase chromosomes

Total length, l/s ratio, centromeric index, relative length and centromeric type of each chromosome of nine chickpea cultivars were presented in Tables 6-14. All the cultivars of *Cicer arietinum* were found to possess $2n=16$ metacentric chromosomes (Figs. 73-81, 100-108, 109-117, Tables 11-20). The length of chromosomes was

gradually decreased in each cultivar (Figs. 100-108). Karyotype features of each cultivar were given below.

Total length of diploid chromosome complements in BC-1 was 39.19 μm . Relative length of individual chromosome ranged from 0.05-0.10 whereas individual chromosomal length ranging from 1.47-3.40 μm (Table 20). A pair of satellites was found on the short arm of both members of pair I and the length of each satellite was 0.64 μm (Figs. 73, 100, 109, arrows, Table 11).

The total length of 2n chromosome complements of BC-2 was 36.43 μm . The relative length of the individual chromosomes was ranging from 0.04-0.09. The individual chromosome length ranged from 1.29-3.31 μm (Table 20). A pair of satellites was found on the short arm of both members of pair I and the length of both satellites was 0.64 μm (Figs. 74, 101, 110, arrow, Table 12).

In BC-3, total length of diploid chromosome complements was 30.73 μm . Range of relative length of individual chromosome was 0.04-0.09. Individual chromosome length ranged was from 1.10-2.85 μm (Table 20). A pair of satellites was found on the short arm of both members of pair I. The length of two satellites were different i.e. 0.74 μm and 0.64 μm (Figs. 75, 102, 111, arrow, Table 13).

BC-4 possessed highest length of 2n chromosome complements. It was 37.21 μm . The relative length of chromosome was ranging from 0.04-0.08. On the other hand, individual chromosomal length was ranging from 1.61-3.04 μm (Table 20). A pair of satellites was present on the terminal end of short arm in each member of pair I and the

size of each satellite was 0.74 μm (Figs. 76, 103, 112, arrow, Table 14).

In BC-5, total length of diploid chromosome complements was 28.93 μm . This length was smallest among the nine chickpea cultivars. Relative length of individual chromosome was from 0.05-0.08 and the range of individual chromosomal length ranging from 1.38-2.30 μm (Table 20). A pair of satellites was found on the short arm of both members of pair I and the length of two satellites was 0.69 μm (Figs. 77, 104, 113, arrow, Table 15).

The total length of the 2n chromosome complements in BC-6 was 38.92 μm . The relative length of chromosome was ranging from 0.04-0.08. The individual chromosomal length ranged from 1.56-3.22 μm (Table 20). A pair of satellites was present on the terminal end of short arm in each member of pair I and the size of both satellites was 0.83 μm (Figs. 78, 105, 114, arrow, Table 16).

Length of diploid chromosome complements of BC-7 was 31.10 μm . Relative length of individual chromosome ranged from 0.04-0.07 and the range of individual chromosomal length from 1.29-2.30 μm (Table 20). A pair of satellites was found on the short arm of both members of pair I and the length of both satellites was 0.60 μm (Figs. 79, 106, 115, arrow, Table 17).

In BC-8, total length of diploid chromosome complements was 34.59 μm . Relative length of individual chromosome ranged from 0.04-0.09 whereas the range of individual chromosomal length was from 1.43-3.22 μm (Table 20). A pair of satellites was found on the short arm of

both members of pair I and the length of both satellites was 0.83 μm (Figs. 80, 107, 116, arrow, Table 18).

BC-9 had 36.52 μm of total length of diploid chromosome complements. Relative length of individual chromosome ranged from 0.04-0.07 and the range of individual chromosomal length was 1.47-2.71 μm (Table 20). A pair of satellites was found on the short arm of both members of pair I and the length of both satellites were 0.74 μm (Figs. 81, 108, 117, arrow, Table 19).

3.7.2. CMA-stained mitotic metaphase chromosomes

Although the nine cultivars of chickpea were found to possess $2n=16$ chromosomes, showed different types of CMA-banding pattern (Figs. 82-90, 118-126, 127-135, Table 21). The distinct characters of each cultivar after CMA-staining were described below:

In BC-1, two CMA-positive bright and thick bands were observed on the upper terminal region in pair I (Figs. 82, 118, 127). The total length of CMA-banded region was 1.84 μm which covered about 4.69% of the total chromatin length. The CMA banded karyotype formula of this cultivar was $2\alpha+14\delta$ (Table 21).

Six CMA-positive bands were found in BC-2. Both the members of pair I and V showed an upper terminal bright and thick CMA-positive band. In contrast, a member of pair II showed an upper terminal CMA-positive band while its homologue had a CMA-positive band at the centromeric region (Figs. 83, 119, 128). The size of the later two bands was almost same. The total length of CMA-banded region was 5.29 μm which was about 14.52% of the total chromatin length. The percentage of GC-rich repeats was the highest in this cultivar. The

CMA-positive karyotype formula of this cultivar was $5\alpha + 1\Omega + 10\delta$ (Table 21).

In BC-3, three CMA-positive bands were found. A pair of CMA-positive bands was found at the upper terminal region one in each member of pair I. Only a member of pair II had a prominent thick CMA-positive band that covered centromere and its both proximal sides. This band was the biggest among the nine cultivars. However, no such band was found in its homologue pair (Figs. 84, 120, 129). The total length of CMA-banded region was 2.76 μm , which covered about 8.98% of the total chromatin length. The CMA-positive karyotype formula of this cultivar was $2\alpha + 1\Omega + 13\delta$ (Table 21).

Two CMA-positive bands were found in two different chromosomes of BC-4. One member of pair I and V showed a centromeric and an upper terminal CMA-positive band, respectively (Figs. 85, 121, 130). However, their homologue members had no band. The total length of CMA-banded region was 1.84 μm which covered about 4.94% of the total chromatin length. The CMA-positive karyotype formula of this cultivar was $1\alpha + 1\Omega + 14\delta$ (Table 21).

In BC-5, a pair of CMA-positive bands was found at the upper terminal region on both the members of pair I (Figs. 86, 122, 131). The total length of CMA-banded region was 1.84 μm which was about 6.36% of the total chromatin length. The CMA-positive karyotype formula of this cultivar was $2\alpha + 14\delta$ (Table 21).

Three CMA-positive bands were found in BC-6. In pair I, both the members had upper terminal CMA-positive bands that occupied more than half of the length of short arms. On the other hand a dot like

CMA-positive banded region was found at the sub terminal region in a member of pair VIII. However, no band was observed in its homologue member (Figs. 87, 123, 132). The total length of CMA-banded region was 2.76 μm which covered about 7.09% of the total chromatin length. The CMA-positive karyotype formula of this cultivar was $2\alpha + 1\Psi + 13\delta$ (Table 21).

Only one member of pair VI and VII of BC-7 showed a CMA-positive band at the centromeric and upper terminal region, respectively (Figs. 88, 124, 133). Their homologue members had no such bands. The total length of CMA-banded region was 0.92 μm which was about 2.96% of the total chromatin length. The CMA-positive karyotype formula of this specimen was $1\alpha + 1\Omega + 14\delta$ (Table 21).

In BC-8, an upper terminal CMA-positive band was observed in a member of pair I. In addition, a member of pair VI showed a centromeric CMA-positive band. No such band was found in their homologue members (Figs. 89, 125, 134). The total length of CMA-banded region was 0.92 μm which covered about 2.66% of the total chromatin length. This percentage of GC-rich repeats was the lowest among nine cultivars. The CMA-positive karyotype formula of this specimen was $1\alpha + 1\Psi + 14\delta$ (Table 21).

Four CMA-positive bands were found in four different chromosomes of BC-9. One member of pair I and III showed upper terminal CMA-positive bands. On the other hand, a centromeric CMA-positive band was found in a member of pair IV and VIII. However, no such band was found in the respective homologue members (Figs. 90, 126, 135). The total length of CMA-banded region was 2.30 μm which was about

6.29% of the total chromatin length. The CMA-positive karyotype formula of this cultivar was $2\alpha + 2\Omega + 12\delta$ (Table 21).

3.7.3. DAPI-stained mitotic metaphase chromosomes

Nine cultivars of chickpea showed different types of DAPI-banding pattern and karyotype features (Figs. 91-99, 136-144, 145-153, Table 22). The distinct characteristic of each cultivar after DAPI-staining was described below:

In BC-1, five DAPI-positive bands were observed in four chromosomes. An upper terminal DAPI-positive band was found in a member of pair V and VIII. No band was found in their homologue members. One big and prominent DAPI-positive centromeric band was found in only a member of pair IV. A member of pair III had a band in both the ends. This chromosome was unique since DAPI bands in both terminal regions of a chromosomes was absent in other cultivars. No satellite was found after DAPI staining (Figs. 91, 136, 145). The total length of DAPI-banded region was 2.76 μm which covered about 7.04% of the total chromatin length. The DAPI-positive karyotype formula of this cultivar was $2\alpha + 1\Omega + 1\lambda + 12\delta$ (Table 22).

Two upper terminal DAPI-positive bands were found in each member of pair VII in BC-2. One satellite was found in each member of pair I (Figs. 92, 137, 146, arrow). The total length of DAPI-banded region was 0.92 μm which was about 2.53% of the total chromatin length. The DAPI-positive karyotype formula of this cultivar was $2\alpha + 14\delta$ (Table 22).

No DAPI-positive band was observed in BC-3 however, a pair of satellites was found in pair I (Figs. 93, 138, 147). DAPI-banded karyotype formula of this cultivar was 16δ (Table 22).

In BC-4, only a centromeric DAPI-positive band was found in a member of pair II however, no band present in its homologue member. Dully fluoresced dot like satellites were found in each member of pair I (Figs. 94, 139, 148). The total length of DAPI-banded region was $0.49\ \mu\text{m}$ which covered only about 1.32% (lowest among nine cultivars) of the total chromatin length. The DAPI-positive karyotype formula of this cultivar was $1\Omega + 15\delta$ (Table 22).

Four DAPI-positive bands were found in BC-5. A thick DAPI-band was found on the upper terminal region in a member of pair II. Dot like terminal band was found on the short arm in a member of pair V, whereas their homologue members had no such band. Both the members of pair VI were fluoresced almost throughout the length. No satellite was found in this cultivar with DAPI-staining (Figs. 95, 140, 149). The total length of DAPI-banded region was $3.22\ \mu\text{m}$ which covered about 11.13% of the total chromatin length. The DAPI-positive karyotype formula of this cultivar was $2\alpha + 2\theta + 12\delta$ (Table 22).

In BC-6, four chromosomes had DAPI-positive bands. The two members of pair I showed different locations of DAPI bands, *i.e.* one had an upper terminal whereas centromeric band was present in its homologue. This pair had satellites on the short arms. On the other hand, a member of pair II had two dots like DAPI-positive bands on both the ends, while band in only a terminal end was observed in its

homologue (Figs. 96, 141, 150, arrow). The total length of DAPI-banded region was 2.30 μm which covered about 5.91% of the total chromatin length. The DAPI-positive karyotype formula of this cultivar was $2\alpha + 1\lambda + 1\Omega + 12\delta$ (Table 22).

In BC-7, four centromeric DAPI-positive bands were observed in the members of pair I and Pair IV. No satellite was found in this cultivar with DAPI-stain (Figs. 97, 142, 151). The total length of DAPI-banded region was 3.68 μm which covered about 11.83% of the total chromatin length. The DAPI-positive karyotype formula of this specimen was $4\Omega + 12\delta$ (Table 22).

In BC-8, both the members of pair I had a centromeric DAPI-positive band. A pair of satellites was observed on the short arms of this pair (Figs. 98, 143, 152). The total length of DAPI-banded region was 0.92 μm which covered about 2.66% of the total chromatin length. The DAPI-positive karyotype formula of this specimen was $2\Omega + 14\delta$ (Table 22).

Four chromosomes showed DAPI-positive bands in BC-9. Two large fluorescent DAPI-positive regions were observed on the long arm in pair I. A pair of satellite was observed on the short arm one in each member of this pair. The satellite portions were brightly stained with DAPI. However, satellites in other cultivars were dully fluoresced. Both the members of pair V were almost entirely fluoresced with DAPI (Figs. 99, 144, 153). The total length of DAPI-banded region was 5.52 μm which was about 15.12% (highest among nine cultivars) of the total chromatin length. The DAPI-positive karyotype formula of this specimen was $2\beta + 2\theta + 12\delta$ (Table 22).

3.8. Isozymes analysis

3.8.1. Enzyme location on polyacrylamide gel following electrophoresis

Activity of three isozymes namely acid phosphatase, esterase and peroxidase from the leaf of the nine chickpea cultivars were examined in this study using vertical polyacrylamide gel electrophoresis. This isozyme study was conducted to characterize the variation among nine cultivars of chickpea plant which collected from BARI (Bangladesh Agriculture Research Institute). The gels were stained for different isozymes and the acid phosphatase, esterase and peroxidase isozyme were displayed as green, brown and blue bands respectively (Table 23). The enzyme activity interms of different intensities of the bands were termed as very dark, moderately dark and lightly dark. The activity of these enzymes as detected on polyacrylamide gels and their respective zymograms were described below:

3.8.1.1. Acid phosphatase polymorphism (ACP)

Acid phosphatase activity on polyacrylamide gel produced by the nine cultivars and the zymogram obtained from this observation was presented in Figs. 154 and 155. Electrophoresis patterns with *Rf* value of acid phosphatase were presented in Table 24.

A total of 4 bands of acid phosphatase isozymes was observed in the nine cultivars of chickpea of which band number 3 and band number 4 were common to all with similar *Rf* value i.e. 0.416 and 0.694 respectively. Band number 1 with *Rf* value 0.250 was present in all cultivars except in BC-7. In contrast, band number 2 (*Rf* value 0.333) was present only in BC-8 and BC-9. On the basis of intensities, there

were three categories of band such as i) dark (band no. 3), ii) moderately dark (band no. 1 and 2) and iii) light (band no. 4) (Figs. 154, 155).

3.8.1.1.1. Genetic distance on the basis of acid phosphatase markers

The genetic distance analysis was calculated based on the banding patterns followed by Nei's (1972). The distance was ranging from 0.00 to 0.6931. The highest genetic distance (0.6931) was found in BC-7 vs. BC-8 and BC-9, while the lowest (0.0000) found among BC-1 to BC-6 (Table 25).

3.8.1.1.2. Cluster analysis

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) segregated the nine cultivars of chickpea into two major clusters i.e. cluster 1 (C_1) and cluster (C_2). All cultivars formed cluster 1 (C_1) except BC-8 and BC-9, which formed cluster 2 (C_2). Cluster 1 divided into two sub-clusters such as SC_1 and SC_2 . Only BC-7 was comprised of sub-cluster 1 (SC_1) whereas, rest of the cultivars were included in sub-cluster 2 (SC_2) (Fig. 156).

3.8.1.2. Esterase polymorphism

Esterase activity on polyacrylamide gel produced by the nine cultivars and the zymogram obtained from this observation was presented in Figs. 157 and 158. Electrophoresis patterns with R_f value of esterase were presented in Table 26.

Esterase isozyme showed high level of variation (57%) with four polymorphic enzymatic bands out of total seven enzymatic bands. Band number 1 (*Rf* value 0.243) was present in all samples except BC-8. Band number 2 (*Rf* value 0.326) appeared in only BC-8 and BC-9 with high intensity and absent in others. Band number 3 and 6 (*Rf* value 0.444 and 0.833) were present in BC-2, BC-6, BC-7 and BC-8 of nine chickpea cultivars. Bands with high intensity were found in BC-9.

3.8.1.2.1. Genetic distance on the basis of esterase markers

The genetic distance analysis was calculated based on the banding patterns followed by Nei's (1972). The distance was ranging from 0.00 to 0.8473. The highest genetic distance (0.8473) was found between BC-8 vs. BC-1, BC-8 vs. BC-3, BC-8 vs. BC-4 and BC-8 vs. BC-5, while the lowest genetic distance (0.00) was found among BC-1 vs. BC-3, BC-4, BC-5 and BC-6 vs. BC-7 (Table 27).

3.8.1.2.2. Cluster analysis (Tree Diagram)

Dendrogram constructed based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) segregated the nine cultivars of chickpea into two major clusters i.e. cluster 1 (C_1) and cluster (C_2). Cluster 1 includes five cultivars such as BC-1, BC-3, BC-4, BC-5 and BC-2, while cluster 2 had the rest (BC-6, BC-7, BC-8 and BC-9) (Fig. 159).

3.8.1.3. Peroxidase polymorphism

Peroxidase activity on polyacrylamide gel produced by the nine cultivars and the zymogram obtained from this observation were presented in Figs. 160 and 161, respectively. Electrophoresis patterns with individual R_f value of peroxidase were presented in Table 28.

A total of three bands were observed of which, band number 1 and band number 3 present in all cultivars (R_f value 0.479 and 0.743). These bands could be considered as common band. Whereas band number 2 (R_f value 0.597) was absent in BC-2 and BC-6, thus considered as polymorphic enzymetic band. On the basis of intensities, there were three categories of bands such as i) dark (band no. 1), ii) moderately dark (band no. 3) and iii) light (band no. 2) (Figs. 160, 161)

3.8.1.3.1. Genetic distance and cluster analysis on the basis of peroxidase markers

The genetic distance analysis was calculated based on the banding patterns followed by Nei's (1972). The distance was ranging from 0.0000 to 0.4055. Dendrogram based on genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) segregated the nine cultivars of chickpea into two major clusters i.e. cluster 1 (C_1) and cluster (C_2). Cluster 2 included two cultivars BC-2 and BC-6 and rest of the cultivars formed cluster 1 (C_1) (Fig. 162, Table 29).

3.8.1.4. Combined cluster analysis with genetic distance based on different marker obtained from three isozyme systems

The genetic distance analysis was calculated based on the banding patterns followed by Nei's (1972). The distance was ranging from 0.0000 to 0.4418. Figure 163 showed a combined dendrogram based on markers of three different isozyme systems. The nine cultivars of chickpea were segregated into two major clusters i.e. cluster 1 (C_1) and cluster (C_2). All the cultivars were present in cluster 1 (C_1) except BC-8 and BC-9 which formed cluster 2 (C_2). Cluster 1 divided into two sub-clusters such as Sub-cluster 1 (SC_1) and Sub-cluster 2 (SC_2). SC_1 included BC-1, BC-3 BC-4 and BC-5 whereas, rest of the cultivars such as BC-2, BC-6 and BC-7 were in SC_2 (Fig. 163, Table 30).

3.9. SDS-PAGE analysis

3.9.1. Study of polymorphism by protein profile analysis

Present investigation revealed that protein profiling was one of the basic and reliable methods to detect genetic diversity and study of phylogenetic relationship among the nine cultivars of *Cicer arietinum* L.

3.9.1.1. SDS-PAGE of leaf protein/soluble protein profile analysis

Electrophoresis analysis of protein profiling was carried out on water soluble SDS-protein fraction for nine cultivars of chickpea. Densitometer analysis of SDS-PAGE representing protein bands with different molecular weight ranged from 10 kDa to 170 kDa (Table 31). All bands resolved on the gel are not prominently seen in the

photographs. An identical amount of protein (200 µg) was analyzed for all the samples. A total of twelve bands were recorded for all cultivars with *Rf* value through the SDS-PAGE photograph, as well as scoring (Table 32) and zymogram (Figs. 164, 165). Among twelve bands three were polymorphic (*Rf* value 0.099, 0.275 and 0.507) revealed 25% polymorphism.

3.9.2. Genetic distance and cluster analysis of SDS-PAGE

The genetic distance analysis was calculated based on the banding patterns followed by Nei's (1972). The distance was ranging from 0.0000 to 0.2877. Dendrogram was constructed based on unweighted pair group method using arithmetic averages (UPGMA). Cluster analysis of data placed the nine chickpea cultivars into two main clusters (Fig. 166) namely cluster 1 (C1) and cluster 2 (C2). Cluster 2 comprised in BC-8 and BC-9. On the other hand, cluster 1 further subdivided into sub cluster 1, where BC-1 and BC-5 were closely related with BC-2 and BC-6. In sub cluster 2, BC-3 and BC-4 were closely related with BC-7 (Table 33).

3.10. RAPD analysis

Twelve oligonucleotide primers combination were used for RAPD analysis of nine cultivars of *Cicer arietinum* L. Each primer combination showed different banding pattern in each cultivar. The primer wise RAPD banding pattern analysis of nine cultivars of chickpea was described below:

3.10.1. Primer OPA-1 (5'-CAG GCC CTT C-3')

No band was found in BC-4 and BC-5. Three similar bands (10000 bp, 1000 bp, 500 bp) were found in BC-1 and BC-3. Rest of the cultivars had four bands of different sizes. Fragment size 10000 bp was common in all cultivars. Cultivar BC-6 and BC-7 had similar pattern. On the other hand, cultivar BC-8 and BC-9 were different than other since a fragment of 3000 bp only present in these two cultivars (Fig. 168, Table 34).

3.10.2. Primer OPA-3 (5'-AGT CAG CCA C-3')

The nine cultivars showed different banding pattern. The highest numbers of bands (5 bands) were found in BC-2. Similar banding patterns (3000 bp, 2000 bp, 1500 bp and 1100 bp) were observed in BC-3, BC-4 and BC-5. Three bands were found in BC-1 and BC-7. The cultivar BC-8 was found to possess two bands (2000 bp and 1700 bp) whereas only one band was present in BC-6 and BC-9. Band size of 2500 bp and 5000 bp were unique in BC-7 and BC-2, respectively. On the other hand, a fragment of 2000 bp was common in all the nine cultivars (Fig. 169, Table 35).

3.10.3. Primer OPA-4 (5'-AAT CGG GCT G-3')

No band was found in BC-8 and BC-9. A band of 3000 bp was present in the rest cultivars. The highest band (7 bands) were found in BC-1 followed by BC-2 (5 bands) and BC-3 (3 bands). Two bands were found in BC-4 and BC-5 whereas BC-6 and BC-7 had only one band. Four unique bands (2500 bp, 2200 bp, 800 bp and 625 bp) were observed in BC-1. These four bands were absent in the rest cultivars (Fig. 170, Table 36).

3.10.4. Primer OPA-5 (5'-AGG GGT CTT G-3')

In BC-5 and BC-8, no band was found. Fragment size of 1500 bp and 1100 bp were present in BC-1, BC-2, BC-3, BC-4 and BC-9. A unique band of 2000 bp and 5000 bp was found in BC-1 and BC-4, respectively. On the other hand, BC-7 had two unique bands (1700 bp and 1300 bp). The cultivar BC-4 and BC-9 shared four fragments of 3200 bp, 2200 bp, 1500 bp and 1100 bp (Fig. 171, Table 37)

3.10.5. Primer OPA-7 (5'-GAA ACG GGT G-3')

The numbers of RAPD bands was ranging from one (BC-1) to six (BC-8 and BC-9) in the nine cultivars. The only band (1600 bp) in BC-1 was unique since absent in other cultivars. Among the six bands in BC-8 and BC-9, five were similar. A fragment of 2000 bp, 750 bp and 4500 bp was unique for BC-6, BC-8 and BC-9, respectively. Band size of 1800 bp and 1500 bp were common in all cultivars except BC-1. The band of 1000 bp was brightest and thickest in BC-3, BC-5 and BC-7 (Fig. 172, Table 38)

3.10.6. Primer OPA-8 (5'-GTG ACG TAG G-3')

The cultivar BC-5 did not have any band with this primer. The number of bands was ranging from two to nine. Cultivar BC-4, BC-8 and BC-9 had nine bands. Eight bands were found in BC-2, BC-3 and BC-7. Out of eight bands, a band of 1250 bp was unique in BC-7. Four and two bands were found in BC-1 and BC-6, respectively. All the cultivars shared four common bands (4000 bp, 2750 bp, 750 bp and 625 bp) except BC-6. The cultivar BC-6 showed polymorphism in respect of banding pattern. In this cultivar, three bands (4000 bp, 2750 bp and 750 bp) were absent which present in the rest cultivars. Band size 750

bp and 600 bp were the brightest and thickest from other cultivars (Fig. 173, Table 39).

3.10.7. Primer-2 (5'-GTT GCG ATC C-3')

The cultivar BC-7 and BC-9 had a unique band of 1500 bp and 700 bp, respectively. These two fragments were absent in other cultivars. Three bands (3000 bp, 2000 bp and 1200 bp) were common in all cultivars except BC-1. The fragment of 3000 bp was prominent but thickest and brightest in BC-3 and BC-9. Cultivar BC-1 showed polymorphism in respect of banding pattern because of lacking of 2000 bp and 1200 bp which were present in other cultivars. No band was found in BC-6 (Fig. 174, Table 40).

3.10.8. Primer-12 (5'-GTA TGG GGC T-3')

The nine cultivars shared four fragments (3000 bp, 2100 bp, 1700 bp and 1500 bp). Among the four fragments, 3000 bp was brightest. The band of 1250 bp and 700 bp were common to all except BC-5. Therefore, BC-5 showed distinct polymorphism in respect of banding pattern. A band of 1300 bp was only present in BC-4 and BC-9 (Fig. 175, Table 41).

3.10.9. Primer-18 (5'-GTT TCG CTC C-3')

Three cultivars (BC-1, BC-2 and BC-9) did not show any band with this primer. A band of 250 bp was common in other six cultivars. In contrast, a fragment of 1200 bp was present in BC-4, BC-5 and BC-7 (Fig. 176, Table 42).

3.10.10. Primer-19 (5'-GAT GAC CGC C-3')

Similar banding pattern was found in BC-3 and BC-5. On the other hand, cultivar BC-4 and BC-9 shared five common bands. A band of 1650 bp was only present in BC-2 and BC-7. Two bands (4200 bp and 2000 bp) were absent in BC-8. The cultivar BC-6 did not have any band with this primer (Fig. 177, Table 43).

3.10.11. Primer-23 (5'-GTC AGG GCA A-3')

No band was found in BC-6 with primer-23. Four bands (2000 bp, 1000 bp 750 bp and 500 bp) were common in all cultivars except BC-1. This cultivar showed polymorphism regarding banding pattern because two bands (2000 bp and 750 bp) were absent (Fig. 178, Table 44).

3.10.12. Primer-24 (5'-GGT CGG AGA A-3')

With this primer no band was found in BC-7. A band of 1800 bp was common in all cultivars. In addition, a band of 1000 bp in BC-8 and 250 bp in BC-9 were unique for these two cultivars since absent in others (Fig. 179, Table 45).

3.10.13. Level of polymorphism

A total of 351 products were amplified by the 12 polymorphic primers with average of 29.25 fragments per primer. The locus number ranged from 2 to 11 per primer while the band number ranged from 9 to 57 per primer. The minimum number of products was observed in primer-18 and the maximum in primer OPA-8 and Primer-12. Among the 351 amplified products, 342 were polymorphic with an average of 98.68%. The size of band was ranging from 250 bp to 10000.

Eighteen unique bands were generated in different cultivars with eight RAPD primers namely OPA-3, OPA-4, OPA-5, OPA-7, OPA-8, Primer-2 and Primer-24 (Table 46).

3.11. SSR analysis

Ten primer pairs were used for SSR analysis of nine chickpea cultivars. Each primer pair showed different banding patterns. The pair-wise SSR-banding pattern analysis of nine cultivars of chickpea was described:

3.11.1. Primer pair GA26 CICER

No band was found in BC-6. Two bands (80 bp and 300 bp) were present in six cultivars (BC-2, BC-3, BC-4, BC-7, BC-8 and BC-9). In contrast, BC-1 had only a band of 80 bp while a band of 300 bp was present in BC-5. Therefore BC-1 and BC-5 showed polymorphism in this respect (Fig. 180, Table 47).

3.11.2 Primer pair CASTMS2

Band was found in every cultivar. Although there was no common band, a band of 100 bp was present seven cultivars. On the other hand, a fragment of 50 bp was found in only four cultivars (BC-1, BC-2, BC-3 and BC-5). Cultivar BC-2 and BC-5 had both the bands (Fig. 181, Table 48).

3.11.3. Primer pair CASTMS15

Fragment size 70 bp was common in all cultivars whereas a band of 250 bp present in five cultivars (BC-5, BC-6, BC-7, BC-8 and BC-9). Both the band of BC-9 (250 bp and 70 bp) and a band of BC-7 (70 bp) were brightly stained (Fig. 182, Table 49).

3.11.4. Primer pair CASTMS21

Only one band of 80 bp was found in all cultivars of which band in BC-8 was the brightest (Fig. 183, Table 50).

3.11.5. Primer pair TA71

Every cultivar shared a common band of 50 bp whereas another band of 175 bp was present in all the cultivars except BC-6 and BC-7. Therefore, BC-6 and BC-7 showed polymorphism regarding this band (Fig. 184, Table 51).

3.11.6. Primer pair TA72

Maximum band was found with this primer. The number of band ranged from two to four. Four bands (380 bp, 300 bp, 220 bp and 20 bp) were observed in BC-3, BC-4, BC-5 and BC-6. Of these four bands, 380 bp was absent in BC-2 whereas band of 380 bp and 300 bp not found in BC-1. Fragment size 220 bp was common in all cultivars of which the band in BC-8 was the thickest and brightest. A unique band of 40 bp was found in BC-5 which absent in the rest cultivars (Fig. 185, Table 52).

3.11.7. Primer pair TA130

All the cultivars had only one band of 200 bp. The band in BC-8 was the brightest (Fig. 186, Table 53).

3.11.8. Primer pair TA135

Two bands viz. 200 bp and 80 bp were common in all cultivars. Band size 200 bp was brighter in BC-3, BC-5, BC-7 and BC-8. On the other hand, 80 bp fragments in BC-9 was the brightest (Fig. 187, Table 54).

3.11.9. Primer pair TR29

All the cultivars have shared a fragment of 60 bp. This band was more or less thick and bright, however brighter in BC-4, BC-5, BC-6, BC-7 and BC-8 (Fig. 188, Table 55).

3.11.10. Primer pair TA118

One common band (150 bp) was found in all cultivars with this primer pair of which BC-6 and BC-8 were brightly stained. In addition to this band, another band of 40 bp was present in BC-2, BC-3, BC-4, BC-7 and BC-9 (Fig. 189, Table 56).

3.11.11. Level of polymorphism

A total of 25 products ranging from 20 bp to 380 bp were amplified by the 10 polymorphic primers with average of 2.5 fragments per primer. The locus number ranged from 2 to 6 (20-380 bp) per primer. The maximum number of products was observed in primer pair TA72. Among the 25 amplified products, 19 were polymorphic with an average of 76% of the total fragments and obtained base pair size ranging from 20 bp to 380 bp (Table 57).

3.11.12. Genetic distance made by combined RAPD and SSR analysis

The genetic distance analysis was calculated based on the RAPD and SSR banding patterns followed by Nei's (1972). The distance was ranging from 0.1557 to 0.7975. Cultivar BC-1 vs. BC-7 showed the highest genetic distance (0.7975) whereas, the lowest (0.1557) found between BC-2 and BC-3 (Table 58).

3.11.13. Combined dendrogram from RAPD and SSR markers

Dendrogram was constructed based on unweighted pair group method using arithmetic averages (UPGMA). The nine chickpea cultivars were segregated into two major clusters namely cluster 1 (C1) and cluster 2 (C2) on the basis of combined RAPD and SSR data (Fig. 189). The first cluster comprised in two sub clusters. Cultivar BC-2, BC-3, BC-4 and BC-5 were placed in subcluster 1 (SC1) while BC-1 alone in SC2. The second cluster also formed two sub clusters where BC-7, BC-8 and BC-9 were in one subcluster 4 (SC4) and BC-6 in the other (SC3) (Fig. 190).

Table 10. Types of interphase nuclei and prophase chromosomes of nine cultivars of *Cicer arietinum* after staining with orcein

Name of chickpea cultivars	Orcein-stained interphase nuclei	Orcein-stained prophase chromosomes
BC-1	Simple chromocenter type	Interstitial type
BC-2	Complex chromocenter type	Gradient type
BC-3	Between simple and complex chromocenter type	Continuous type
BC-4	Simple chromocenter type	Continuous type
BC-5	Simple chromocenter type	Continuous type
BC-6	Simple chromocenter type	Gradient type
BC-7	Simple chromocenter type	Continuous type
BC-8	Simple chromocenter type	Interstitial type
BC-9	Simple chromocenter type	Gradient type

Table 11. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-1 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.70	1.70 *0.64	3.40	1.00	0.09	50.00	m
	1.70	1.70 *0.64	3.40	1.00	0.11	50.00	m
II	1.84	1.38	3.22	1.33	0.10	42.86	m
	1.84	1.38	3.22	1.33	0.10	42.86	m
III	1.38	1.38	2.76	1.00	0.09	50.00	m
	1.38	1.38	2.76	1.00	0.09	50.00	m
IV	1.29	1.01	2.30	1.27	0.07	44.00	m
	1.29	1.01	2.30	1.27	0.07	44.00	m
V	1.29	1.01	2.30	1.27	0.07	44.00	m
	1.29	1.01	2.30	1.27	0.07	44.00	m
VI	1.10	1.01	2.12	1.09	0.07	47.83	m
	1.10	1.01	2.12	1.09	0.07	47.83	m
VII	1.10	0.74	1.84	1.50	0.06	40.00	m
	1.10	0.74	1.84	1.50	0.06	40.00	m
VIII	0.92	0.92	1.84	1.00	0.06	50.00	m
	0.74	0.74	1.47	1.00	0.05	50.00	m
GT=39.19							

*Length of satellite not added

Table 12. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-2 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.93	1.38 *0.64	3.31	1.40	0.09	41.67	m
	1.93	1.38 *0.64	3.31	1.40	0.09	41.67	m
II	1.47	1.38	2.85	1.07	0.08	48.39	m
	1.47	1.38	2.85	1.07	0.08	48.39	m
III	1.38	1.29	2.67	1.07	0.07	48.28	m
	1.38	1.29	2.67	1.07	0.07	48.28	m
IV	1.20	1.10	2.30	1.08	0.06	48.00	m
	1.20	1.10	2.30	1.08	0.06	48.00	m
V	1.01	1.01	2.02	1.00	0.06	50.00	m
	1.01	1.01	2.02	1.00	0.06	50.00	m
VI	0.92	0.92	1.84	1.00	0.05	50.00	m
	0.92	0.92	1.84	1.00	0.05	50.00	m
VII	0.92	0.83	1.75	1.11	0.05	47.37	m
	0.92	0.83	1.75	1.11	0.05	47.37	m
VIII	0.83	0.83	1.66	1.00	0.05	50.00	m
	0.64	0.64	1.29	1.00	0.04	50.00	m
GT=36.43							

*Length of satellite not added

Table 13. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-3 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.66	1.20 *0.74	2.85	1.38	0.09	41.94	m
	1.66	1.20 *0.64	2.85	1.38	0.09	41.94	m
II	1.20	1.10	2.30	1.08	0.07	48.00	m
	1.20	1.10	2.30	1.08	0.07	48.00	m
III	1.10	1.01	2.12	1.09	0.07	47.83	m
	1.10	1.01	2.12	1.09	0.07	47.83	m
IV	1.01	0.92	1.93	1.10	0.06	47.62	m
	1.01	0.92	1.93	1.10	0.06	47.62	m
V	1.01	0.83	1.84	1.22	0.06	45.00	m
	1.01	0.83	1.84	1.22	0.06	45.00	m
VI	0.92	0.74	1.66	1.25	0.05	44.44	m
	0.92	0.74	1.66	1.25	0.05	44.44	m
VII	0.83	0.74	1.56	1.13	0.05	47.06	m
	0.83	0.74	1.56	1.13	0.05	47.06	m
VIII	0.55	0.55	1.10	1.00	0.04	50.00	m
	0.55	0.55	1.10	1.00	0.04	50.00	m
GT=37.73							

*Length of satellite not added

Table 14. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-4 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.56	1.47 *0.74	3.04	1.06	0.08	48.48	m
	1.56	1.47 *0.74	3.04	1.06	0.08	48.48	m
II	1.66	1.20	2.85	1.38	0.08	41.94	m
	1.66	1.20	2.85	1.38	0.08	41.94	m
III	1.61	1.15	2.76	1.40	0.07	41.67	m
	1.61	1.15	2.76	1.40	0.07	41.67	m
IV	1.38	1.10	2.48	1.25	0.07	44.44	m
	1.38	1.10	2.48	1.25	0.07	44.44	m
V	1.20	0.92	2.12	1.30	0.06	43.48	m
	1.20	0.92	2.12	1.30	0.06	43.48	m
VI	1.15	0.78	1.93	1.50	0.05	39.02	m
	1.15	0.78	1.93	1.50	0.05	39.02	m
VII	1.01	0.83	1.84	1.22	0.05	45.00	m
	1.01	0.83	1.84	1.22	0.05	45.00	m
VIII	0.92	0.69	1.61	1.33	0.04	42.86	m
	0.92	0.69	1.61	1.33	0.04	42.86	m
GT=37.21							

*Length of satellite not added

Table 15. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-5 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.38	0.92 *0.69	2.30	1.50	0.08	40.00	m
	1.38	0.92 *0.69	2.30	1.50	0.08	40.00	m
II	1.38	0.92	2.30	1.50	0.08	40.00	m
	1.15	0.92	2.07	1.25	0.07	44.44	m
III	1.06	0.87	1.93	1.21	0.07	45.24	m
	1.06	0.87	1.93	1.21	0.07	45.24	m
IV	1.01	0.87	1.89	1.16	0.07	46.34	m
	1.01	0.87	1.89	1.16	0.07	46.34	m
V	0.92	0.83	1.75	1.11	0.06	47.37	m
	0.92	0.83	1.75	1.11	0.06	47.37	m
VI	0.83	0.74	1.56	1.13	0.05	47.06	m
	0.83	0.74	1.56	1.13	0.05	47.06	m
VII	0.83	0.64	1.47	1.29	0.05	43.75	m
	0.83	0.64	1.47	1.29	0.05	43.75	m
VIII	0.69	0.69	1.38	1.00	0.05	50.00	m
	0.69	0.69	1.38	1.00	0.05	50.00	m
GT=28.93							

*Length of satellite not added

Table 16. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-6 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.84	1.38	3.22	1.33	0.08	42.86	m
	1.84	*0.83 1.38	3.22	1.33	0.08	42.86	m
II	1.84	1.38	3.22	1.33	0.08	42.86	m
	1.84	1.38	3.22	1.33	0.08	42.86	m
III	1.66	1.29	2.94	1.29	0.08	43.75	m
	1.66	1.29	2.94	1.29	0.08	43.75	m
IV	1.29	1.15	2.44	1.12	0.06	47.17	m
	1.29	1.15	2.44	1.12	0.06	47.17	m
V	1.29	0.92	2.21	1.40	0.06	41.67	m
	1.29	0.92	2.21	1.40	0.06	41.67	m
VI	1.10	0.92	2.02	1.20	0.05	45.45	m
	1.10	0.92	2.02	1.20	0.05	45.45	m
VII	0.92	0.92	1.84	1.00	0.05	50.00	m
	0.92	0.92	1.84	1.00	0.05	50.00	m
VIII	0.83	0.74	1.56	1.13	0.04	47.06	m
	0.83	0.74	1.56	1.13	0.04	47.06	m
GT=38.92							

*Length of satellite not added

Table 17. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-7 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.29	1.01	2.30	1.27	0.07	44.00	m
	1.29	*0.60 1.01	2.30	1.27	0.07	44.00	m
II	1.20	1.01	2.21	1.18	0.07	45.83	m
	1.20	1.01	2.21	1.18	0.07	45.83	m
III	1.10	1.01	2.12	1.09	0.07	47.83	m
	1.10	1.01	2.12	1.09	0.07	47.83	m
IV	1.10	1.01	2.12	1.09	0.07	47.83	m
	1.10	1.01	2.12	1.09	0.07	47.83	m
V	1.10	1.01	2.12	1.09	0.07	47.83	m
	1.10	1.01	2.12	1.09	0.07	47.83	m
VI	0.92	0.83	1.75	1.11	0.06	47.37	m
	0.92	0.83	1.75	1.11	0.06	47.37	m
VII	0.83	0.83	1.66	1.00	0.05	50.00	m
	0.83	0.83	1.66	1.00	0.05	50.00	m
VIII	0.64	0.64	1.29	1.00	0.04	50.00	m
	0.64	0.64	1.29	1.00	0.04	50.00	m
GT=31.10							

*Length of satellite not added

Table 18. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-8 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.75	1.47 *0.83	3.22	1.19	0.09	45.71	m
	1.75	1.47 *0.83	3.22	1.19	0.09	45.71	m
II	1.38	1.24	2.62	1.11	0.08	47.37	m
	1.38	1.24	2.62	1.11	0.08	47.37	m
III	1.20	1.10	2.30	1.08	0.07	48.00	m
	1.20	1.10	2.30	1.08	0.07	48.00	m
IV	1.10	0.92	2.02	1.20	0.06	45.45	m
	1.10	0.92	2.02	1.20	0.06	45.45	m
V	1.06	0.87	1.93	1.21	0.06	45.24	m
	1.06	0.87	1.93	1.21	0.06	45.24	m
VI	1.06	0.87	1.93	1.21	0.06	45.24	m
	1.06	0.87	1.93	1.21	0.06	45.24	m
VII	1.01	0.83	1.84	1.22	0.05	45.00	m
	1.01	0.83	1.84	1.22	0.05	45.00	m
VIII	0.78	0.64	1.43	1.21	0.04	45.16	m
	0.78	0.64	1.43	1.21	0.04	45.16	m
GT=36.43							

*Length of satellite not added

Table 19. Length, arm ratio, centromeric index, relative length and centromeric type of mitotic metaphase chromosomes of cultivar BC-9 of *Cicer arietinum* L.

Chromosome pair	Long arm (l) μm	Short arm (s) μm	Total length (T) μm	Arm ratio (l/s)	Relative length (RL)	Centromeric index (CI)	Centromeric type (CT)
I	1.61	1.10 *0.74	2.71	1.46	0.07	40.68	m
	1.61	1.10 *0.74	2.71	1.46	0.07	40.68	m
II	1.52	1.20	2.71	1.27	0.07	44.07	m
	1.52	1.20	2.71	1.27	0.07	44.07	m
III	1.38	1.20	2.58	1.15	0.07	46.43	m
	1.38	1.20	2.58	1.15	0.07	46.43	m
IV	1.38	1.06	2.44	1.30	0.07	43.40	m
	1.38	1.06	2.44	1.30	0.07	43.40	m
V	1.15	1.15	2.30	1.00	0.06	50.00	m
	1.15	1.15	2.30	1.00	0.06	50.00	m
VI	1.06	1.06	2.12	1.00	0.06	50.00	m
	1.06	1.06	2.12	1.00	0.06	50.00	m
VII	0.97	0.97	1.93	1.00	0.05	50.00	m
	0.97	0.97	1.93	1.00	0.05	50.00	m
VIII	0.74	0.74	1.47	1.00	0.04	50.00	m
	0.74	0.74	1.47	1.00	0.04	50.00	m
GT=36.52							

*Length of satellite not added

Table 20. Comparative orcein stained karyotype analysis in nine cultivars of *Cicer arietinum* L.

Name of cultivars	2n	No. of satellite	Total length of 2n chromosome complement (μm)	Range of chromosomal length (μm)	Centromeric formulae
BC-1	16	2	39.19	1.47-3.40	16m
BC-2	16	2	36.43	1.29-3.31	16m
BC-3	16	2	30.73	1.10-2.85	16m
BC-4	16	2	37.21	1.61-3.04	16m
BC-5	16	2	28.93	1.38-2.30	16m
BC-6	16	2	38.92	1.56-3.22	16m
BC-7	16	2	31.10	1.29-2.30	16m
BC-8	16	2	34.59	1.43-3.22	16m
BC-9	16	2	36.52	1.47-2.71	16m

m = metacentric chromosome

Table 21. Comparative CMA-banding analysis in nine cultivars of *Cicer arietinum* L.

Name of cultivars	No. of CMA-bands	Total length of CMA-positive banded region (μm)	% of CMA-positive banded region	CMA-banded karyotype formulae
BC-1	2	1.84	4.69	$2\alpha + 14\delta$
BC-2	6	5.29	14.52	$5\alpha + 1\Omega + 10\delta$
BC-3	3	2.76	8.98	$2\alpha + 1\Omega + 13\delta$
BC-4	2	1.84	4.94	$1\alpha + 1\Omega + 14\delta$
BC-5	2	1.84	6.36	$2\alpha + 14\delta$
BC-6	3	2.76	7.09	$2\alpha + 1\Psi + 13\delta$
BC-7	2	0.92	2.96	$1\alpha + 1\Omega + 14\delta$
BC-8	2	0.92	2.66	$1\alpha + 1\Psi + 14\delta$
BC-9	4	2.30	6.29	$2\alpha + 2\Omega + 12\delta$

Classification of CMA-positive bands:

α = Band in terminal region of short arm.

Ω = Band in centromeric region.

Ψ = Dot like band

δ = No band.

Table 22. Comparative DAPI-banding analysis in nine cultivars of *Cicer arietinum* L.

Name of cultivars	No. of satellites	No. of DAPI-bands	Total length of DAPI-positive banded region (μm)	% of DAPI-positive banded region	DAPI-banded karyotypic formulae
BC-1	-	4	2.76	7.04	$2\alpha + 1\Omega + 1\lambda + 12\delta$
BC-2	2	2	0.92	2.53	$2\alpha + 14\delta$
BC-3	2	-	-	-	16δ
BC-4	2	1	0.49	1.32	$1\Omega + 15\delta$
BC-5	-	4	3.22	11.13	$2\alpha + 2\theta + 12\delta$
BC-6	2	4	2.30	5.91	$2\alpha + 1\Omega + 1\lambda + 12\delta$
BC-7	-	4	3.68	11.83	$4\Omega + 12\delta$
BC-8	2	2	0.92	2.66	$2\Omega + 14\delta$
BC-9	2	4	5.52	15.12	$2\beta + 2\theta + 12\delta$

Classification of DAPI-positive bands:

α = Band in terminal region of short arm.

β = Band in terminal region of long arm.

λ = Band in terminal region of short arm and long arm.

Ω = Band in centromeric region.

Ψ = Dot like band.

θ = Fluoresced almost throughout the length.

δ = No band.

Table 23. Band formation rate and band color of different types of isozyme.

Sl. No.	Name of Isozymes	Band color	Band formation rate
1	Acid phosphatase (ACP)	Gray	Slow
2	Esterase (EST)	Deep Brown	Quick
3	Peroxidase (PRX)	Blue	Quick

Table 24. Activity of acid phosphatase (APH) on Native-PAGE from leaf extract of nine cultivars of *Cicer arietinum* L.

Band No.	R _f value	Cultivars (presence/absence of bands and their intensity)								
		BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
1	0.250	++	++	++	++	++	++	-	++	++
2	0.333	-	-	-	-	-	-	-	++	++
3	0.416	+++	+++	+++	+++	+++	+++	+++	+++	+++
4	0.694	+	+	++	+	+	+	+	+	+
Band in each		3	3	3	3	3	3	3	4	4

Table 25. Summary of Nei's (1972) genetic distances on the basis of acid phosphatase activity in nine cultivars of *Cicer arietinum* L.

Cultivars	BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
BC-1	****								
BC-2	0.0000	****							
BC-3	0.0000	0.0000	****						
BC-4	0.0000	0.0000	0.0000	****					
BC-5	0.0000	0.0000	0.0000	0.0000	****				
BC-6	0.0000	0.0000	0.0000	0.0000	0.0000	****			
BC-7	0.2877	0.2877	0.2877	0.2877	0.2877	0.2877	****		
BC-8	0.2877	0.2877	0.2877	0.2877	0.2877	0.2877	0.6931	****	
BC-9	0.2877	0.2877	0.2877	0.2877	0.2877	0.2877	0.6931	0.0000	****

Table 26. Activity of esterase (EST) from leaf extract of nine cultivars of *Cicer arietinum* L.

Band No.	Rf value	Cultivars (presence/absence of bands and their intensity)								
		BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
1	0.243	+++	+++	+++	+++	+++	+++	+++	-	+++
2	0.326	-	-	-	-	-	-	-	+++	+++
3	0.444	-	+	-	-	-	+	+	+++	+
4	0.674	+	++	+	+	+	+	+	+	+
5	0.750	+	+	+	+	+	+	+	+	+
6	0.833	-	++	-	-	-	++	++	++	++
7	0.882	+	++	+	+	+	++	++	++	++
Band in each		4	5	4	4	4	6	6	6	7

Table 27. Summary of Nei's (1972) genetic distances on the basis of esterase in nine cultivars of *Cicer arietinum* L.

Cultivars	BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
BC-1	****								
BC-2	0.1542	****							
BC-3	0.0000	0.1542	****						
BC-4	0.0000	0.1542	0.0000	****					
BC-5	0.0000	0.1542	0.0000	0.0000	****				
BC-6	0.3365	0.1542	0.3365	0.3365	0.3365	****			
BC-7	0.3365	0.1542	0.3365	0.3365	0.3365	0.0000	****		
BC-8	0.8473	0.5596	0.8473	0.8473	0.8473	0.3365	0.3365	****	
BC-9	0.5596	0.3365	0.5596	0.5596	0.5596	0.1542	0.1542	0.1542	****

Table 28. Activity of peroxidase (PER) from leaf extract of nine cultivars of *Cicer arietinum* L.

Band No.	Rf value	Cultivars (presence/absence of bands and their intensity)								
		BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
1	0.479	+++	+++	+++	+++	+++	+++	+++	+++	+++
2	0.597	+	-	+	+	+	-	+	+	+
3	0.743	++	++	++	++	++	++	++	++	++
Band in each		3	2	3	3	3	2	3	3	3

Table 29. Summary of Nei's (1972) genetic distances on the basis of peroxidase activity in nine cultivars of *Cicer arietinum* L.

Culti vars	BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
BC-1	****								
BC-2	0.4055	****							
BC-3	0.0000	0.4055	****						
BC-4	0.0000	0.4055	0.0000	****					
BC-5	0.0000	0.4055	0.0000	0.0000	****				
BC-6	0.4055	0.0000	0.4055	0.4055	0.4055	****			
BC-7	0.0000	0.4055	0.0000	0.0000	0.0000	0.4055	****		
BC-8	0.0000	0.4055	0.0000	0.0000	0.0000	0.4055	0.0000	****	
BC-9	0.0000	0.4055	0.0000	0.0000	0.0000	0.4055	0.0000	0.0000	****

Table 30. Nei's (1972) genetic distances on the basis of combined analysis of acid phosphatase, esterase and peroxidase in nine cultivars of *Cicer arietinum* L.

Cultivars	BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
BC-1	****								
BC-2	0.2412	****							
BC-3	0.0000	0.2412	****						
BC-4	0.0000	0.2412	0.0000	****					
BC-5	0.0000	0.2412	0.0000	0.0000	****				
BC-6	0.2412	0.0000	0.2412	0.2412	0.2412	****			
BC-7	0.2412	0.1542	0.2412	0.2412	0.2412	0.1542	****		
BC-8	0.4418	0.3365	0.4418	0.4418	0.4418	0.3365	0.3365	****	
BC-9	0.3365	0.2412	0.3365	0.3365	0.3365	0.2412	0.2412	0.0741	****

Table 31. Molecular weight on the basis of SDS-PAGE of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (kDa)	No. of bright band with molecular weight (kDa)	No. of polymorphic band with molecular weight (kDa)	No. of common band with molecular weight (kDa)
BC-1	10 (180, 130, 100, 85, 65, 57, 39, 23, 21, 15)	1 (39)	1 (57)	
BC-2	11 (180, 130, 100, 85, 65, 57, 39, 30, 23, 21, 15)	1 (39)	2 (30, 57)	
BC-3	11 (190, 180, 130, 100, 85, 65, 57, 39, 23, 21, 15)	1 (39)	2 (190, 57)	
BC-4	11 (190, 180, 130, 100, 85, 65, 57, 39, 23, 21, 15)	1 (39)	2 (190, 57)	9
BC-5	10 (180, 130, 100, 85, 65, 57, 39, 23, 21, 15)	1 (39)	1 (57)	(180, 130, 100, 85, 65, 39, 23, 21, 15)
BC-6	11 (180, 130, 100, 85, 65, 57, 39, 30, 23, 21, 15)	1 (39)	2 (30, 57)	
BC-7	11 (190, 180, 130, 100, 85, 65, 57, 39, 23, 21, 15)	1 (39)	2 (190, 57)	
BC-8	10 (180, 130, 100, 85, 65, 39, 30, 23, 21, 15)	1 (39)	1 (30)	
BC-9	10 (180, 130, 100, 85, 65, 39, 30, 23, 21, 15)	1 (39)	1 (30)	

Table 32. Activity of SDS-PAGE from leaf extract of nine cultivars of *Cicer arietinum* L.

Band No.	<i>Rf</i> value	Cultivars (presence/absence of bands and their intensity)								
		BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
1	0.099	-	-	++	++	-	-	++	-	-
2	0.123	++	++	++	++	++	++	++	++	++
3	0.158	+	++	+	+	+	++	++	++	++
4	0.197	++	++	++	++	++	++	++	++	++
5	0.222	++	++	+	+	+	++	++	++	++
6	0.275	+	++	+	+	+	++	+	-	-
7	0.317	+	++	+	+	+	+++	++	+++	++
8	0.426	+++	+++	+++	+++	+++	+++	+++	+++	+++
9	0.507	-	+++	-	-	-	+++	-	++	++
10	0.553	++	+++	++	++	++	+++	++	++	++
11	0.592	++	+	++	++	++	++	++	++	++
12	0.662	++	++	++	++	++	++	++	++	++
Band in each		10	11	11	11	10	11	11	10	10

Table 33. Summary of Nei's (1972) genetic distances on the basis of SDS-PAGE activity of nine cultivars of *Cicer arietinum* L.

Cultivars	BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
BC-1	****								
BC-2	0.0870	****							
BC-3	0.0870	0.1823	****						
BC-4	0.0870	0.1823	0.0000	****					
BC-5	0.0000	0.0870	0.0870	0.0870	****				
BC-6	0.0870	0.0000	0.1823	0.1823	0.0870	****			
BC-7	0.0870	0.1823	0.0000	0.0000	0.0870	0.1823	****		
BC-8	0.1823	0.0870	0.2877	0.2877	0.1823	0.0870	0.2877	****	
BC-9	0.1823	0.0870	0.2877	0.2877	0.1823	0.0870	0.2877	0.0000	****

Table 34. RAPD analysis with primer OPA-1 (5'-CAG GCC CTT C-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	3 (10000, 1000, 500)	2 (1000, 500)	1 (10000)	---	
BC-2	4 (10000, 4800, 1000, 500)	4 (10000, 4800, 1000, 500)	---	---	
BC-3	3 (10000, 1000, 500)	2 (1000, 500)	1 (10000)	---	---
BC-4	---	---	---	---	
BC-5	---	---	---	---	
BC-6	4 (10000, 4800, 1700, 600)	4 (10000, 4800, 1700, 600)	---	---	
BC-7	4 (10000, 4800, 1700, 600)	4 (10000, 4800, 1700, 600)	---	---	
BC-8	4 (10000, 3000, 1700, 600)	3 (3000, 1700, 600)	1 (10000)	---	
BC-9	4 (10000, 3000, 1700, 600)	3 (3000, 1700, 600)	1 (10000)	---	

Table 35. RAPD analysis with primer OPA-3 (5'-AGT CAG CCA C-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	3 (2500, 2000, 1500)	3 (2500, 2000, 1500)	---	1 (2500)	
BC-2	5 (5000, 3000, 2000, 1500, 1100)	4 (5000, 3000, 1500, 1100)	1 (2000)	---	
BC-3	4 (3000, 2000, 1500, 1100)	3 (3000, 1500, 1100)	1 (2000)	---	
BC-4	4 (2800, 2000, 1500, 1100)	3 (2800, 1500, 1100)	1 (2000)	---	
BC-5	4 (2800, 2000, 1500, 1100)	3 (2800, 1500, 1100)	1 (2000)	---	---
BC-6	1 (2000)	1 (2000)	---	---	
BC-7	2 (2800, 1700)	2 (2800, 1700)	---	---	
BC-8	1 (1700)	1 (1700)	---	---	
BC-9	---	---	---	---	

Table 36. RAPD analysis with primer OPA-4 (5'-AAT CGG GCT G-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	7 (3000, 2500, 2200, 1400, 1000, 800, 625)	7 (3000, 2500, 2200, 1400, 1000, 800, 625)	---	4 (2500, 2200, 800, 625)	
BC-2	5 (3000, 1400, 1000, 900, 500)	4 (1400, 1000, 900, 500)	1 (3000)	---	
BC-3	3 (3000, 1400, 900)	2 (1400, 900)	1 (3000)	---	
BC-4	2 (3000, 900)	1 (900)	1 (3000)	---	---
BC-5	2 (3000, 500)	2 (3000, 500)	---	---	
BC-6	1 (3000)	1 (3000)	---	---	
BC-7	1 (3000)	---	1 (3000)	---	
BC-8	---	---		---	
BC-9	---	---	---	---	

Table 37. RAPD analysis with primer OPA-5 (5'-AGG GGT CTT G- 3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	3 (2000, 1500, 1100)	2 (2000, 1500)	1 (1100)	1 (2000)	
BC-2	2 (1500, 1100)	1 (1500)	1 (1100)	---	
BC-3	2 (1500, 1200)	2 (1500, 1200)	---	---	
BC-4	5 (5000, 3200, 2200, 1500, 1200)	5 (5000, 3200, 2200, 1500, 1200)	---	1 (5000)	
BC-5	---	---	---	---	---
BC-6	2 (2200, 1500)	2 (2200, 1500)	---	---	
BC-7	2 (1700, 1300)	2 (1700, 1300)	---	2 (1700, 1300)	
BC-8	---	---	---	---	
BC-9	4 (3200, 2200, 1500, 1200)	4 (3200, 2200, 1500, 1200)	---	---	

Table 38. RAPD analysis with primer OPA-7 (5'-GAA ACG GGT G-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (1600)	1 (1600)	---	1 (1600)	
BC-2	3 (1800, 1500, 1000)	3 (1800, 1500, 1000)	---	---	
BC-3	3 (1800, 1500, 1000)	2 (1800, 1500)	1 (1000)	---	
BC-4	3 (1800, 1500, 1000)	3 (1800, 1500, 1000)	---	---	
BC-5	3 (1800, 1500, 1000)	2 (1800, 1500)	1 (1000)	---	
BC-6	4 (2000, 1800, 1500, 1300)	4 (2000, 1800, 1500, 1300)	---	1 (2000)	---
BC-7	3 (1800, 1500, 1000)	2 (1800, 1500)	1 (1000)	---	
BC-8	6 (3000, 1800, 1500, 1300, 1100, 750)	6 (3000, 1800, 1500, 1300, 1100, 750)	---	1 (750)	
BC-9	6 (4500, 3000, 1800, 1500, 1300, 1100)	6 (4500, 3000, 1800, 1500, 1300, 1100)	---	1 (4500)	

Table 39. RAPD analysis with primer OPA-8 (5'-GTG ACG TAG G-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	4 (4000, 2750, 750, 625)	2 (4000, 2750)	2 (750, 625)	---	
BC-2	8 (4000, 2750, 1500, 1200, 750, 625, 500, 400)	6 (4000, 2750, 1500, 1200, 500, 400)	2 (750, 625)	---	
BC-3	8 (4000, 2750, 1500, 1200, 750, 625, 500, 400)	6 (4000, 2750, 1500, 1200, 500, 400)	2 (750, 625)	---	
BC-4	9 (4000, 2750, 1500, 1200, 750, 625, 500, 400, 250)	7 (4000, 2750, 1500, 1200, 500, 400, 250)	2 (750, 625)	---	---
BC-5	---	---	---	---	
BC-6	2 (900, 625)	1 (900)	1 (625)	---	
BC-7	8 (4000, 2750, 1500, 1250, 750, 625, 500, 400)	6 (4000, 2750, 1500, 1250, 500, 400)	2 (750, 625)	1 (1250)	
BC-8	9 (4000, 2750, 1500, 900, 750, 625, 500, 400, 250)	7 (4000, 2750, 1500, 900, 500, 400, 250)	2 (750, 625)	---	
BC-9	9 (4000, 2750, 1500, 900, 750, 625, 500, 400, 250)	7 (4000, 2750, 1500, 900, 500, 400, 250)	2 (750, 625)	---	

Table 40. RAPD analysis with primer-2 (5'-GTT GCG ATC C-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (3000)	1 (3000)	---	---	
BC-2	4 (3000, 2000, 1200, 600)	4 (3000, 2000, 1200, 600)	---	---	
BC-3	4 (3000, 2000, 1200, 600)	1 (600)	3 (3000, 2000, 1200)	---	
BC-4	4 (3000, 2000, 1200, 600)	4 (3000, 2000, 1200, 600)	---	---	
BC-5	4 (3000, 2000, 1200, 600)	4 (3000, 2000, 1200, 600)	---	---	---
BC-6	---	---	---	---	
BC-7	5 (3000, 2000, 1500, 1200, 600)	5 (3000, 2000, 1500, 1200, 600)	---	1 (1500)	
BC-8	4 (3000, 2000, 1200, 600)	3 (2000, 1200, 600)	1 (3000)	---	
BC-9	5 (3000, 2000, 1200, 700, 600)	2 (700, 600)	3 (3000, 2000, 1200)	1 (700)	

Table 41. RAPD analysis with primer-12 (5'-GTA TGG GGC T-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	6 (3000, 2100, 1700, 1500, 1100, 500)	5 (2100, 1700, 1500, 1100, 500)	1 (3000)	---	
BC-2	6 (3000, 2100, 1700, 1500, 1100, 500)	5 (2100, 1700, 1500, 1100, 500)	1 (3000)	---	
BC-3	7 (3000, 2100, 1700, 1500, 1100, 900, 500)	6 (2100, 1700, 1500, 1100, 900, 500)	1 (3000)	---	
BC-4	6 (3000, 2100, 1700, 1500, 1250, 500)	5 (2100, 1700, 1500, 1250, 500)	1 (3000)	1 (1250)	
BC-5	4 (3000, 2250, 2000, 1600)	3 (2250, 2000, 1600)	1 (3000)	---	1 (3000)
BC-6	7 (3000, 2250, 2000, 1600, 1250, 900, 500)	6 (2250, 2000, 1600, 1250, 900, 500)	1 (3000)	---	
BC-7	6 (3000, 2250, 2000, 1600, 1250, 500)	5 (2250, 2000, 1600, 1250, 500)	1 (3000)	---	
BC-8	7 (3000, 2250, 2000, 1600, 1250, 900, 500)	5 (2250, 2000, 1600, 1250, 900)	1 (3000, 500)	---	
BC-9	8 (3000, 2250, 2000, 1600, 1250, 1100, 900, 500)	7 (2250, 2000, 1600, 1250, 1100, 900, 500)	1 (3000)	---	

Table 42. RAPD analysis with primer Primer-18 (5'-GTT TCG CTC C-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	---	---	---	---	
BC-2	---	---	---	---	
BC-3	1 (250)	1 (250)	---	---	
BC-4	2 (1200, 250)	2 (1200, 250)	---	---	---
BC-5	2 (1200, 250)	2 (1200, 250)	---	---	
BC-6	1 (250)	1 (250)	---	---	
BC-7	2 (1200, 250)	2 (1200, 250)	---	---	
BC-8	1 (250)	1 (250)	---	---	
BC-9	---	---	---	---	

Table 43. RAPD analysis with primer-19 (5'-GAT GAC CGC C-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	---	---	---	---	
BC-2	5 (4000, 3300, 2000, 1800, 1500)	4 (4000, 3300, 2000, 1800)	1 (1500)	1 (4000)	
BC-3	5 (4200, 3300, 2500, 2250, 1500)	4 (4200, 3300, 2500, 2250)	1 (1500)	---	
BC-4	5 (4200, 3300, 2500, 2000, 1500)	4 (4200, 3300, 2500, 2000)	1 (1500)	---	
BC-5	5 (4200, 3300, 2500, 2100, 1500)	4 (4200, 3300, 2500, 2100)	1 (1500)	---	---
BC-6	---	---	---	---	
BC-7	6 (4200, 3300, 2500, 2000, 1800, 1500)	5 (4200, 3300, 2500, 2000, 1800)	1 (1500)	---	
BC-8	3 (3300, 2500, 1500)	2 (3300, 2500)	1 (1500)	---	
BC-9	5 (4200, 3300, 2250, 1800, 1500)	4 (4200, 3300, 2250, 1800)	1 (1500)	---	

Table 44. RAPD analysis with Primer-23 (5'-GTC AGG GCA A-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	2 (1000, 500)	1 (500)	1 (1000)	---	
BC-2	4 (2000, 1000, 750, 500)	3 (2000, 750, 500)	1 (1000)	---	
BC-3	4 (2000, 1000, 750, 500)	3 (2000, 750, 500)	1 (1000)	---	
BC-4	4 (2000, 1000, 750, 500)	3 (2000, 750, 500)	1 (1000)	---	
BC-5	4 (2000, 1000, 750, 500)	3 (2000, 750, 500)	1 (1000)	---	---
BC-6	---	---	---	---	
BC-7	4 (2000, 1000, 750, 500)	3 (2000, 750, 500)	1 (1000)	---	
BC-8	4 (2000, 1000, 750, 500)	3 (2000, 750, 500)	1 (1000)	---	
BC-9	4 (2000, 1000, 750, 500)	3 (2000, 750, 500)	1 (1000)	---	

Table 45. RAPD analysis with primer Primer-24 (5'-GGT CGG AGA A-3') of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (1800)	---	1 (1800)	---	
BC-2	1 (1800)	---	1 (1800)	---	
BC-3	1 (1800)	---	1 (1800)	---	
BC-4	1 (1800)	---	1 (1800)	---	
BC-5	1 (1800)	---	1 (1800)	---	---
BC-6	1 (2000)	1 (2000)	---	---	
BC-7	---	---	---	---	
BC-8	2 (2000, 1000)	2 (2000, 1000)	---	1 (1000)	
BC-9	2 (1800, 250)	1 (250)	1 (1800)	1 (250)	

Table 46. List of RAPD markers generated from twelve primers in nine cultivars of *Cicer arietinum* L.

Primer codes	Sequences (5'—3')	No. of total bands	Size ranges (bp)	No. of polymorphic bands	Number and size unique bands (bp)	Polymorphism (%)
OPA-1	CAG GCC CTT C	26	500-10000	26	---	100
OPA-3	AGT CAG CCA C	24	1100-5000	15	BC-1 (2500) BC-2 (5000)	62.5
OPA-4	AAT CGG GCT G	21	500-3000	21	BC-1 (2500, 2200, 800, 625)	100
OPA-5	AGG GGT CTT G	20	1100-5000	20	BC-1 (2000), BC-4 (5000), BC-7 (1700, 1300)	100
OPA-7	GAA ACG GGT G	32	750-4500	32	BC-1 (1600), BC-6 (2000), BC-8 (750), BC-9 (4500)	100
OPA-8	GTG ACG TAG G	57	250-4000	57	BC-7 (1250)	100
Primer-2	GTT GCG ATC C	31	580-3000	31	BC-7 (1500), BC-9 (700)	100
Primer-12	GTA TGG GGC T	57	600-3000	21	---	36.84
Primer-18	GTT TCG CTC C	9	250-1200	9	---	100
Primer-19	GAT GAC CGC C	34	1500-4000	34	---	100
Primer-23	GTC AGG GCA A	30	500-2000	30	---	100
Primer-24	GGT CGG AGA A	10	250-2000	10	BC-8 (1000), BC-9 (250)	100
Total	Twelve primers	351	250-10000	306	19	91.61

Table 47. SSR analysis with primer GA26 CICR: Forward-5'-GTGCAG AGCATCATGCGATGCTCAAGACATCTGCCA-3' - Reverse-5'-TCATACTCAACAAATCATTTC-3' of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (80)	1 (80)	---	---	
BC-2	2 (300, 80)	2 (300, 80)	---	---	
BC-3	2 (300, 80)	1 (80)	1 (300)	---	
BC-4	2 (300, 80)	2 (300, 80)	---	---	
BC-5	1 (300)	---	1 (300)	---	---
BC-6	---	---	---	---	
BC-7	2 (300, 80)	2 (300, 80)	---	---	
BC-8	2 (300, 80)	2 (300, 80)	---	---	
BC-9	2 (300, 80)	2 (300, 80)	---	---	

Table 48. SSR analysis with primer CASTMS2: Forward- 5'-ATTTTACTTTACTACTTTTTTCCTTTC-3' – Reverse-5'-AATAAATGGAGTGTAAATTTTCATGTA-3' of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (50)	1 (50)	---	---	
BC-2	2 (200, 50)	2 (200, 50)	---	---	
BC-3	1 (50)	1 (50)	---	---	
BC-4	1 (200)	1 (200)	---	---	
BC-5	2 (200, 50)	2 (200, 50)	---	---	---
BC-6	1 (200)	1 (200)	---	---	
BC-7	1 (200)	1 (200)	---	---	
BC-8	1 (200)	---	1 (200)	---	
BC-9	1 (200)	---	1 (200)	---	

Table 49. SSR analysis with primer CASTMS15: Forward-5'-CTTGTGA ATTCATATTTACTTATAGAT-3' – Reverse –5'-ATCCGTAATTTAA GG TAGGT TAAATA-3' of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (70)	1 (70)	---	---	
BC-2	1 (70)	1 (70)	---	---	1 (70)
BC-3	1 (70)	1 (70)	---	---	
BC-4	1 (70)	1 (70)	---	---	
BC-5	2 (250, 70)	2 (250, 70)	---	---	
BC-6	2 (250, 70)	2 (250, 70)	---	---	
BC-7	2 (250, 70)	1 (250)	1 (70)	---	
BC-8	2 (250, 70)	2 (250, 70)	---	---	
BC-9	2 (250, 70)	---	2 (250, 70)	---	

Table 50. SSR analysis with primer CASTMS21: Forward-5'-CTACAGT CTTTGTCTCTTAGCTT-3' – Reverse-5'-ATATTTTAAAGAGGC TTTTGGTAG-3' of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (80)	1 (80)	---	---	
BC-2	1 (80)	1 (80)	---	---	
BC-3	1 (80)	1 (80)	---	---	
BC-4	1 (80)	1 (80)	---	---	
BC-5	1 (80)	1 (80)	---	--	1 (80)
BC-6	1 (80)	1 (80)	---	---	
BC-7	1 (80)	1 (80)	---	---	
BC-8	1 (80)	---	1 (80)	---	
BC-9	1 (80)	1 (80)	---	---	

Table 51. SSR analysis with primer TA71: Forward -5'-CGATTTAACAC AAAACACAAA-3' – Reverse-5'-CCTATCCATTGTCATCTCGT-3' of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total No. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	2 (175, 50)	2 (175, 50)	---	---	
BC-2	2 (175, 50)	2 (175, 50)	---	---	
BC-3	2 (175, 50)	2 (175, 50)	---	---	
BC-4	2 (175, 50)	2 (175, 50)	---	---	
BC-5	2 (175, 50)	1 (50)	1 (175)	---	1 (50)
BC-6	1 (50)	1 (50)	---	---	
BC-7	1 (50)	1 (50)	---	---	
BC-8	2 (175, 50)	1 (50)	1 (175)	---	
BC-9	2 (175, 50)	2 (175, 50)	---	---	

Table 52. SSR analysis with primer TA72: Forward-5'-GAAAGATT TAAAAGATTTTCCACGTTA-3' – Reverse-5'-TTAGAAGCATATTG TTGGGATAAGAGT-3' of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total No. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	2 (220, 20)	2 (220, 20)	---	---	
BC-2	3 (300, 220, 20)	3 (300, 220, 20)	---	---	
BC-3	4 (380, 300, 220, 20)	4 (380, 300, 220, 20)	---	---	
BC-4	4 (380, 300, 220, 20)	4 (380, 300, 220, 20)	---	---	
BC-5	4 (380, 300, 220, 40)	4 (380, 300, 220, 40)	---	1 (40)	1 (220)
BC-6	4 (380, 300, 220, 20)	2 (380, 300, 220, 20)	---	---	
BC-7	2 (220, 100)	2 (220, 100)	---	---	
BC-8	3 (380, 220, 100)	2 (380, 100)	1 (220)	---	
BC-9	3 (380, 220, 20)	3 (380, 220, 20)	---	---	

**Table 53. SSR analysis with primer TA130: Forward- 5'-TCTTTCT
TTGCTTCCAATGT-3' - Reverse-5'-GTAAATCCCACGAGAAATC
AA -3' of nine cultivars of *Cicer arietinum* L.**

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (200)	1 (200)	---	---	
BC-2	1 (200)	1 (200)	---	---	
BC-3	1 (200)	1 (200)	---	---	
BC-4	1 (200)	1 (200)	---	---	
BC-5	1 (200)	1 (200)	---	---	1 (200)
BC-6	1 (200)	1 (200)	---	---	
BC-7	1 (200)	1 (200)	---	---	
BC-8	1 (200)	---	1 (200)	---	
BC-9	1 (200)	1 (200)	---	---	

Table 54. SSR analysis with Primer TA135: Forward-5'-TGGTTG GAAATTGATGTTTT-3' - Reverse – 5'-GTGGTGTGAGCATAATTC AA-3' of nine cultivars of *Cicer arietinum* L.

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	2 (200, 80)	2 (200, 80)	---	---	
BC-2	2 (200, 80)	2 (200, 80)	---	---	
BC-3	2 (200, 80)	1 (80)	1 (200)	---	
BC-4	2 (200, 80)	2 (200, 80)	---	---	
BC-5	2 (200, 80)	1 (80)	1 (200)	---	
BC-6	2 (200, 80)	2 (200, 80)	---	---	
BC-7	2 (200, 80)	1 (80)	1 (200)	---	2 (200, 80)
BC-8	2 (200, 80)	1 (80)	1 (200)	---	
BC-9	2 (200, 80)	1 (200)	1 (80)	---	

**Table 55. SSR analysis with primer TR29: Forward-5'-GCCCACTG
AAAAATAAAAAG-3' – Reverse-5'-ATTTGAACCTCAAGTTCTC
G-3' of nine cultivars of *Cicer arietinum* L.**

Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (60)	1 (60)	---	---	
BC-2	1 (60)	1 (60)	---	---	
BC-3	1 (60)	1 (60)	---	---	
BC-4	1 (60)	---	1 (60)	---	
BC-5	1 (60)	1 (60)	---	---	1 (60)
BC-6	1 (60)	---	1 (60)	---	
BC-7	1 (60)	---	1 (60)	---	
BC-8	1 (60)	---	1 (60)	---	
BC-9	1 (60)	1 (60)	---	---	

Table 56. SSR analysis with primer TA118: Forward-5'-ACAAGTC ACATGTGTTCTCAATA-3' – Reverse-5'-GGAAAGGTTAAGAAAT TTTACAATAC-3' of nine cultivars of *Cicer arietinum* L.

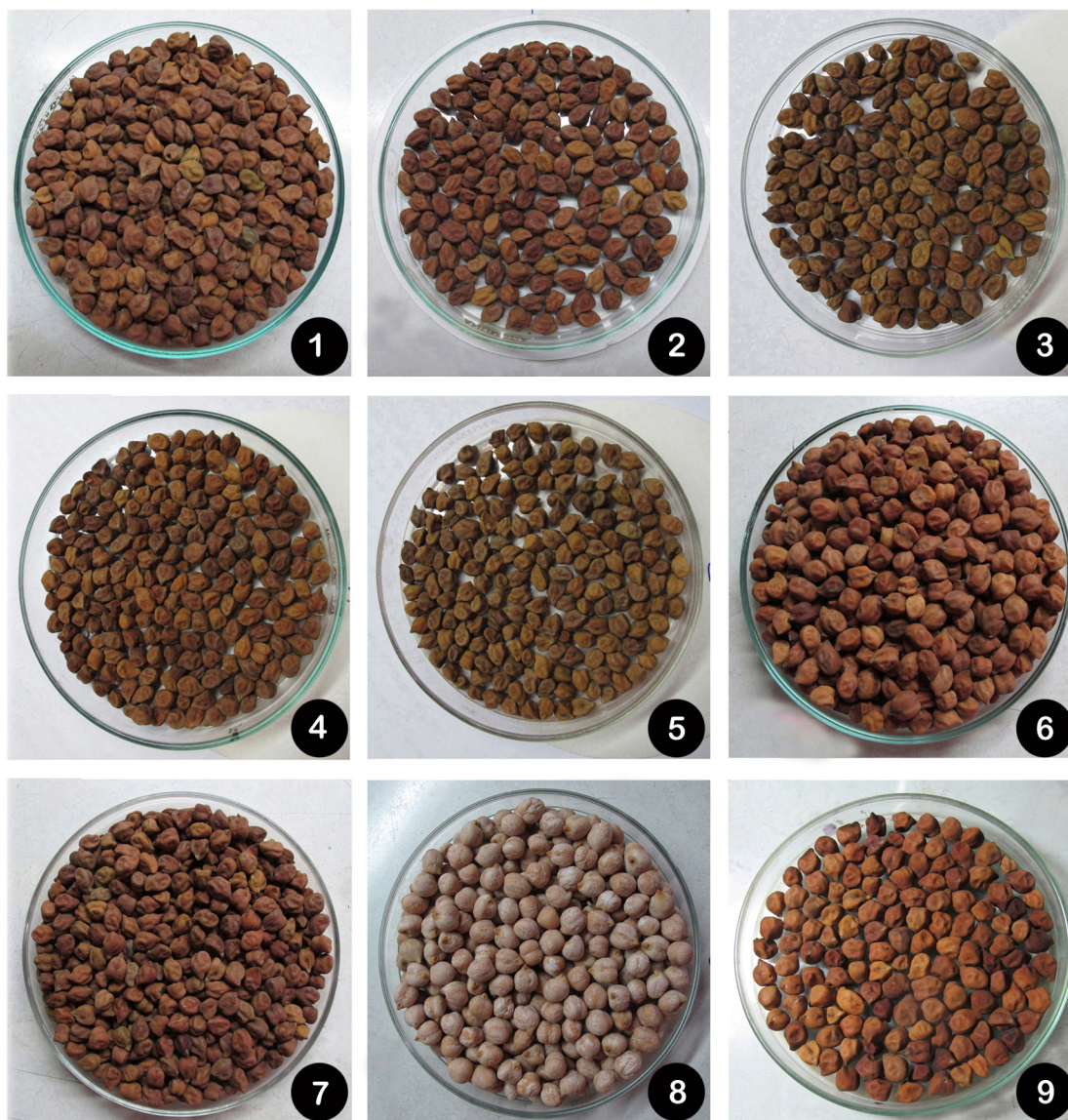
Name of cultivars	Total no. of bands with molecular weight (bp)	No. of light band with molecular weight (bp)	No. of bright band with molecular weight (bp)	No. of unique polymorphic band with molecular weight (bp)	No. of common band with molecular weight (bp)
BC-1	1 (140)	1 (140)	---	---	
BC-2	2 (140, 40)	2 (140, 40)	---	---	
BC-3	2 (140, 40)	2 (140, 40)	---	---	
BC-4	2 (140, 40)	2 (140, 40)	---	---	
BC-5	1 (140)	1 (140)	---	---	
BC-6	1 (140)	---	1 (140)	---	
BC-7	2 (140, 40)	2 (140, 40)		---	1 (140)
BC-8	1 (140)	---	1 (140)	---	
BC-9	2 (140, 40)	2 (140, 40)	---	---	

Table 57. SSR markers generated from ten primers in nine cultivars of *Cicer arietinum* L.

Sl. No.	Primer codes	Sequences (5'—3')	Total bands	Size ranges (bp)	Common bands	Poly morphic bands	No. of unique band (bp)	Polymorphism (%)
1	GA26 CICER	F-GTGCAGAGCATCATGCGATGCTCAAG ACATCTGCCA R-TCATACTCAACAAATTCATTTCCC	14	80-300	--	14	--	100
2	CASTM S2	F-ATTTTACTTTACTACTTTTTTCCTTTC R-AATAAATGGAGTGTAATTTTCATGTA	11	50-200	--	11	--	100
3	CASTM S15	F-CTTGTGAATTCATATTTACTTATAGAT R-ATCCGTAATTTAAGGTAGGTTAAAATA	14	70-250	9	5	--	35.71
4	CASTM S21	F-CTACAGTCTTTTGTTCTTCTAGCTT R-ATATTTTTTAAGAGGCTTTTGGTAG	9	80-100	9	--	--	00
5	TA71	F-CGATTTAACACAAAACACAAA R-CCTATCCATTGTCATCTCGT	16	50-175	9	7	--	43.75
6	TA72	F-GAAAGATTTAAAAGATTTTCCACGTTA R-TTAGAAGCATATTGTTGGGATAAGAGT	29	20-380	9	20	BC-5 (40)	68.97
7	TA130	F-TCTTTCTTTGCTTCCAATGT R-GTAAATCCCACGAGAAATCAA	9	170-200	9	--	--	00
8	TA135	F-TGGTTGGAAATTGATGTTTT R-GTGGTGTGAGCATAATTCAA	18	80-200	18	--	--	00
9	TR29	F-GCCCACTGAAAAATAAAAAAG R-ATTTGAACCTCAAGTTCTCG	9	50-60	9	--	--	00
10	TA118	F-ACAAGTCACATGTGTTCTCAATA R-GGAAAGGTTAAGAAATTTACAATAC	14	40-140	9	5	--	35.71
Total			143	20-380	81	62	1	43.36

Table 58. Summary of Nei's (1972) genetic distances of nine cultivars of *Cicer arietinum* L. by combined RAPD and SSR analysis.

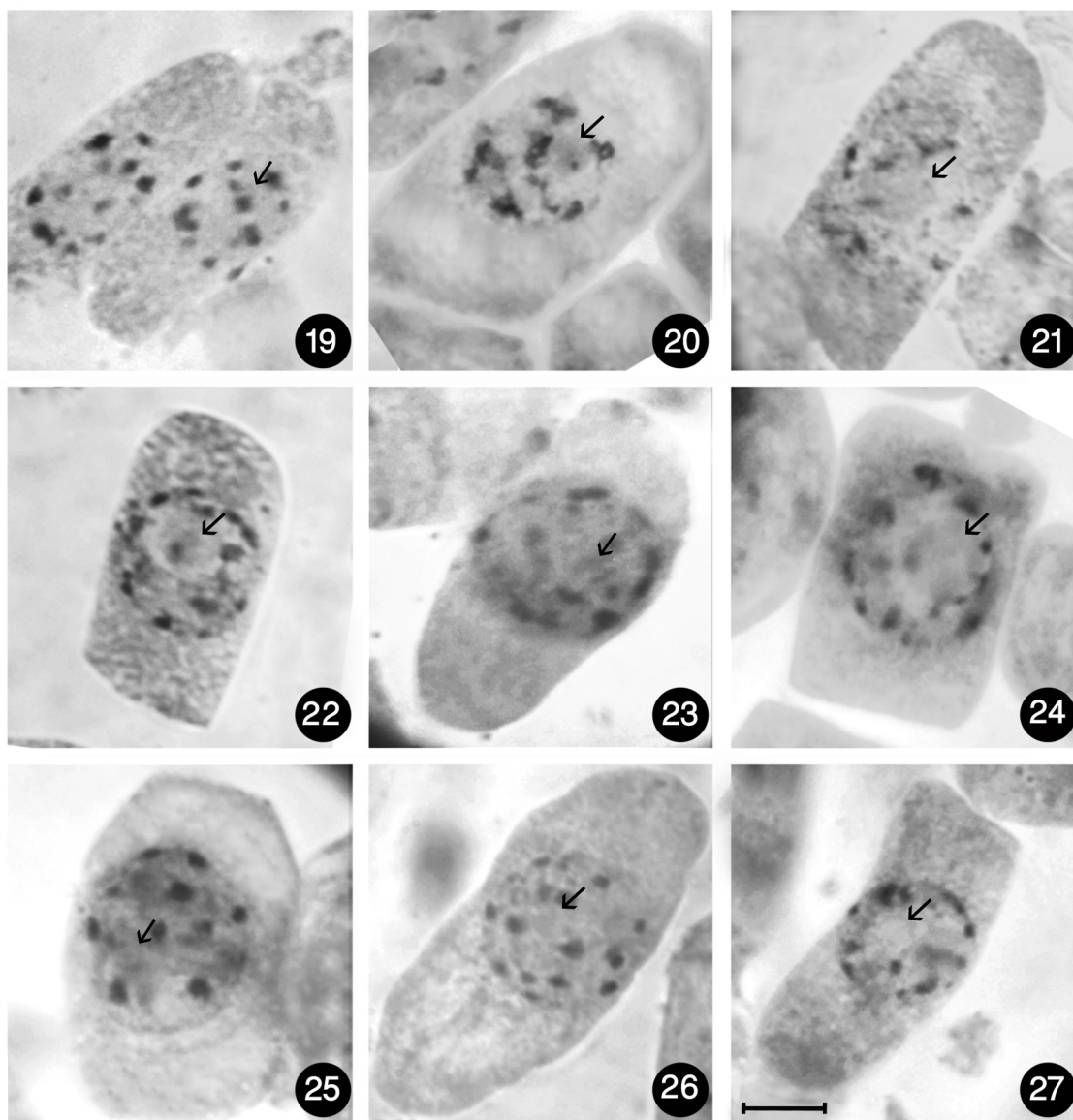
Cultivars	BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9
BC-1	****								
BC-2	0.3151	****							
BC-3	0.3401	0.1557	****						
BC-4	0.5506	0.3275	0.1877	****					
BC-5	0.5664	0.3920	0.3401	0.3528	****				
BC-6	0.5351	0.7022	0.6320	0.7205	0.4754	****			
BC-7	0.7975	0.5199	0.4610	0.4754	0.4055	0.4900	****		
BC-8	0.7583	0.6842	0.4900	0.5664	0.5199	0.4055	0.2907	****	
BC-9	0.7777	0.6320	0.5048	0.4055	0.6320	0.5048	0.4055	0.2097	****



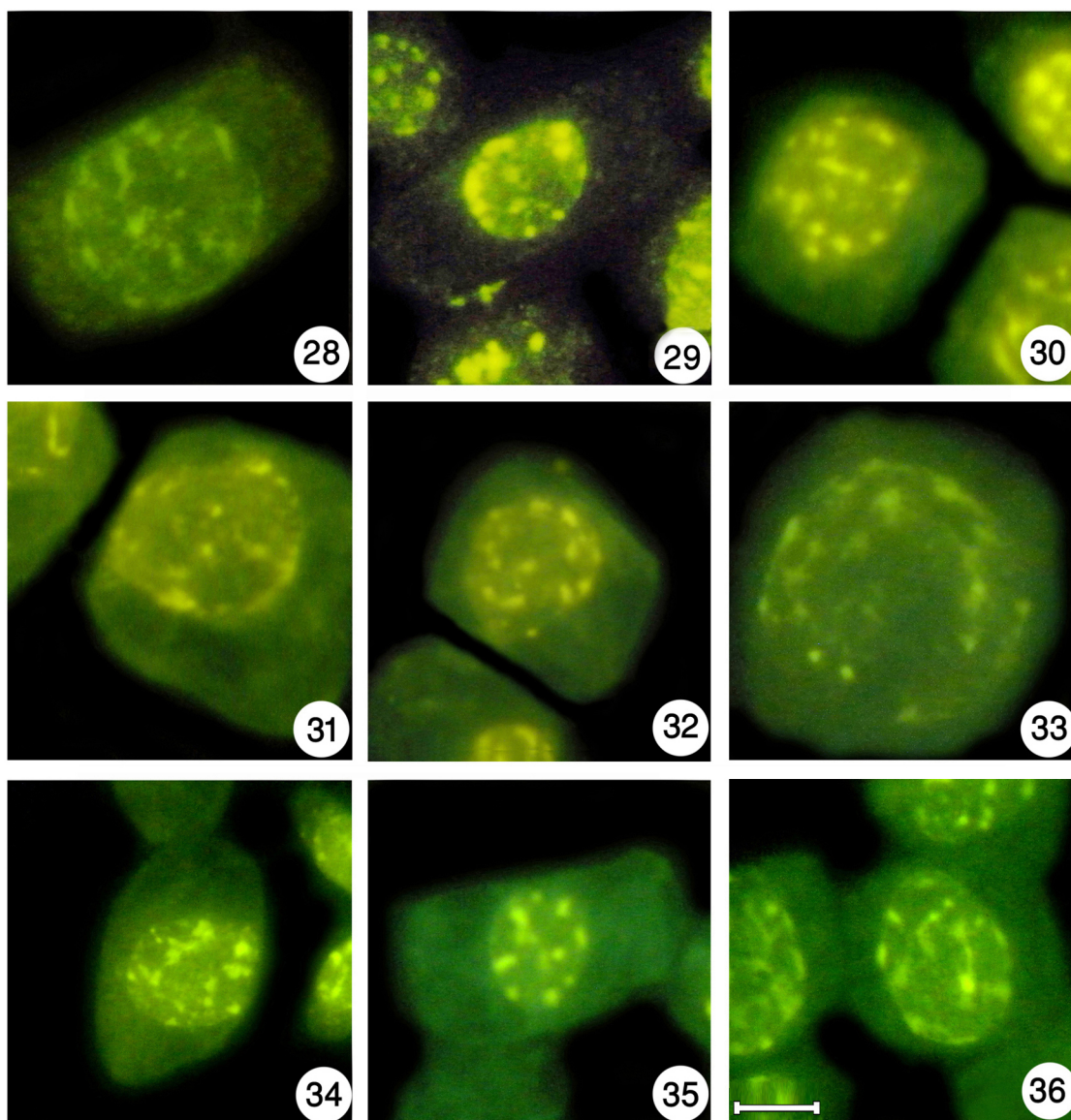
Figs. 1-9. Seed morphology of nine cultivars of *Cicer arietinum* L. 1. BC-1, 2. BC-2, 3. BC-3, 4. BC-4, 5. BC-5, 6. BC-6, 7. BC-7, 8. BC-8 and 9. BC-9.



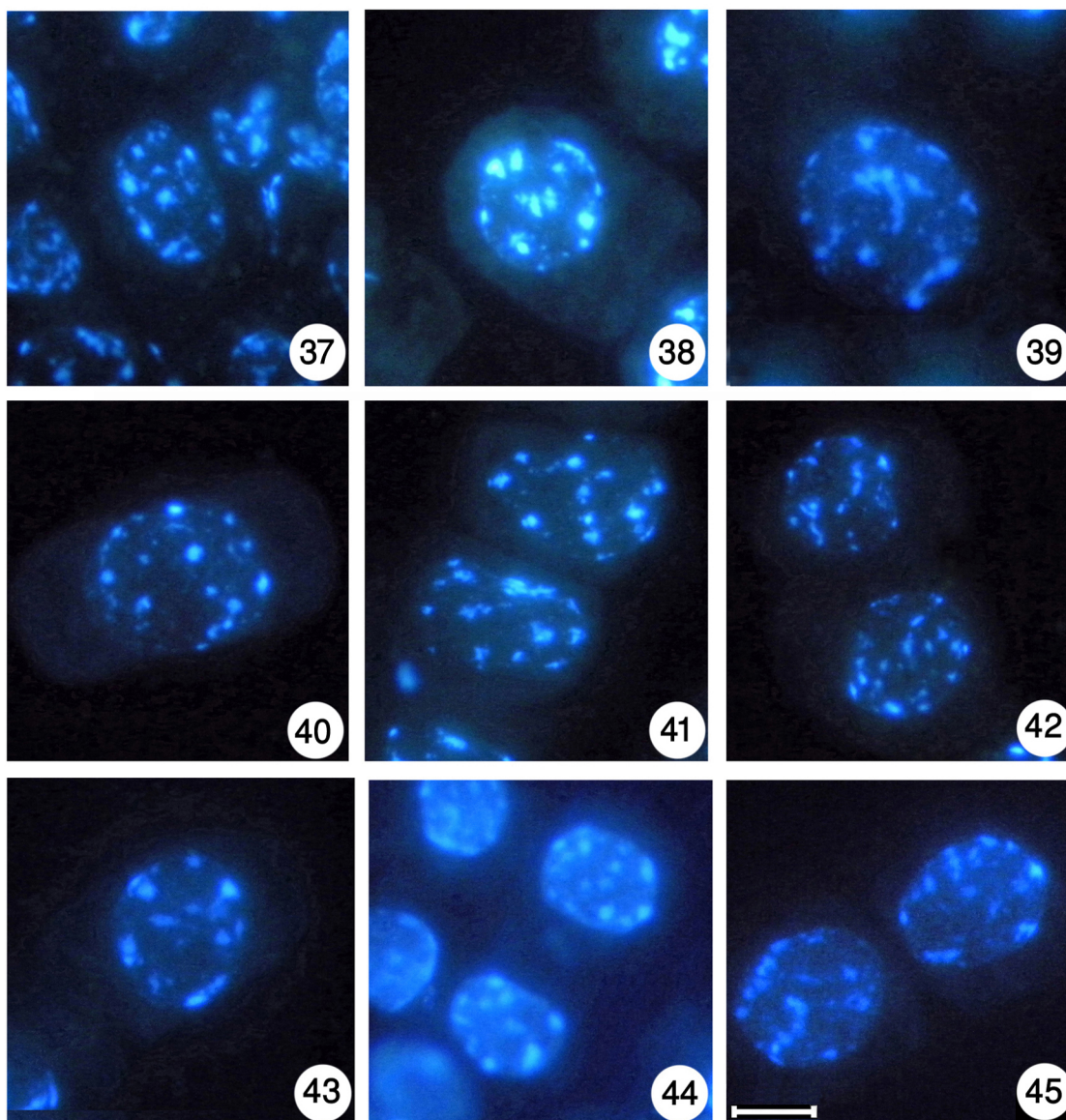
Figs. 10-18. Plants morphology with flower of nine cultivars of *Cicer arietinum* L.
 10. BC-1, 11. BC-2, 12. BC-3, 13. BC-4, 14. BC-5, 15. BC-6, 16. BC-7, 17.
 BC-8 and 18. BC-9.



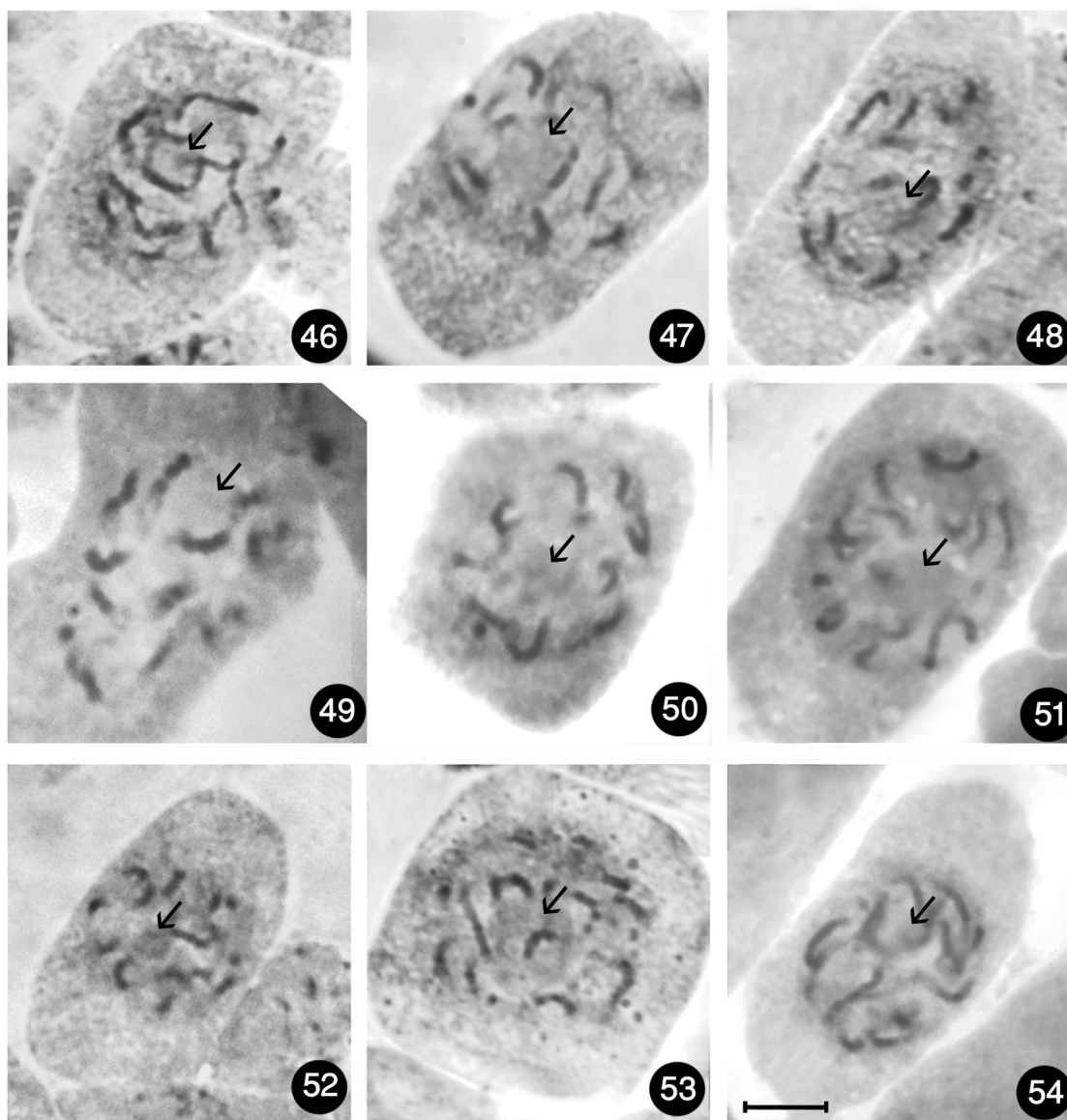
Figs. 19-27. Orcein stained mitotic interphase nuclei of nine cultivars of *Cicer arietinum* L. 19. BC-1, 20. BC-2, 21. BC-3, 22. BC-4, 23. BC-5, 24. BC-6, 25. BC-7, 26. BC-8 and 27. BC-9. Bar = 5 μ m, arrow indicates nucleolus.



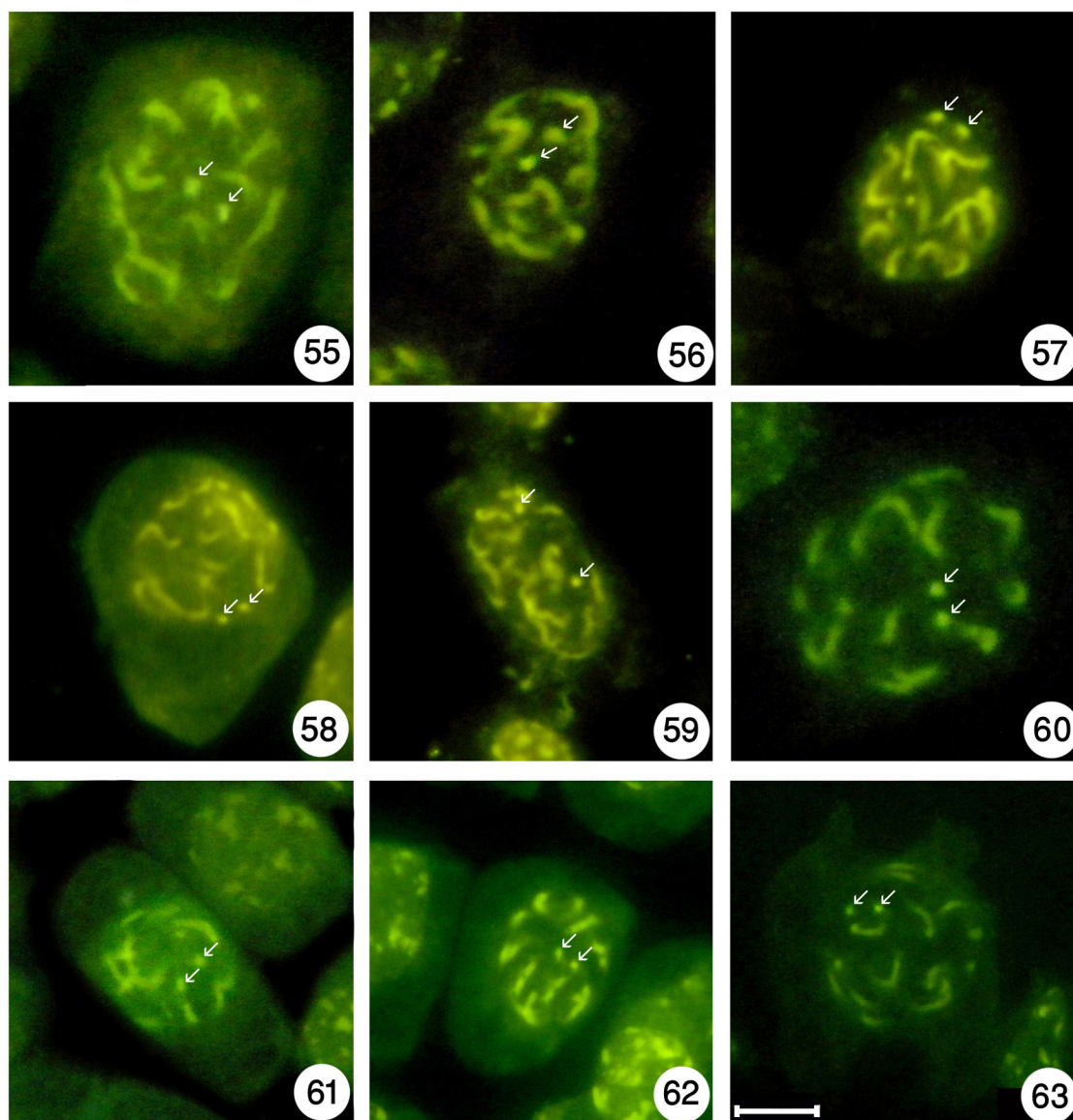
Figs. 28-36. CMA-stained mitotic interphase nuclei of nine cultivars of *Cicer arietinum* L. 28. BC-1, 29. BC-2, 30. BC-3, 31. BC-4, 32. BC-5, 33. BC-6, 34. BC-7, 35. BC-8 and 36. BC-9. Bar = 5 μ m.



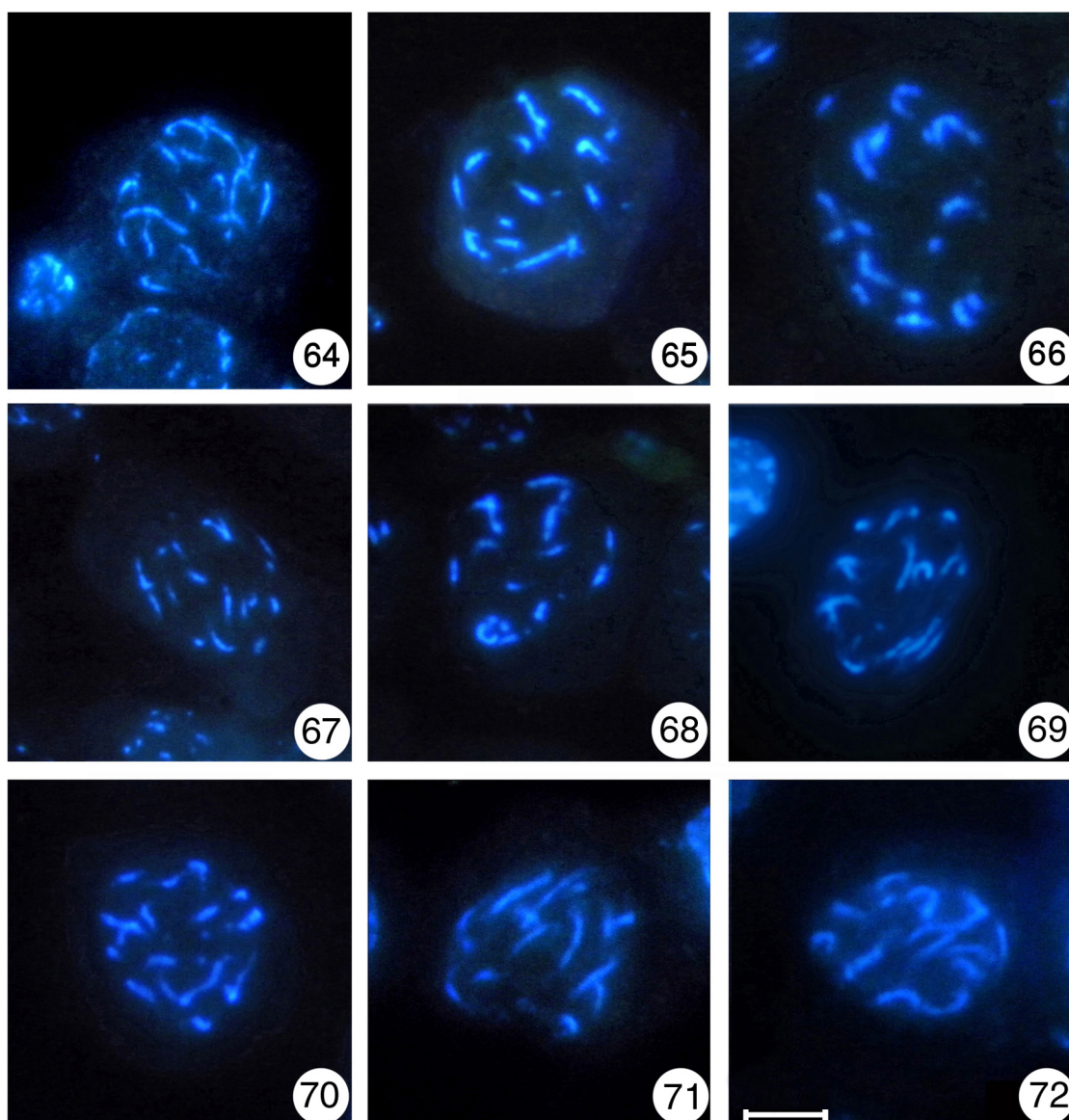
Figs. 37-45. DAPI-stained mitotic interphase nuclei of nine cultivars of *Cicer arietinum* L. 37. BC-1, 38. BC-2, 39. BC-3, 40. BC-4, 41. BC-5, 42. BC-6, 43. BC-7, 44. BC-8 and 45. BC-9. Bar = 5 μ m.



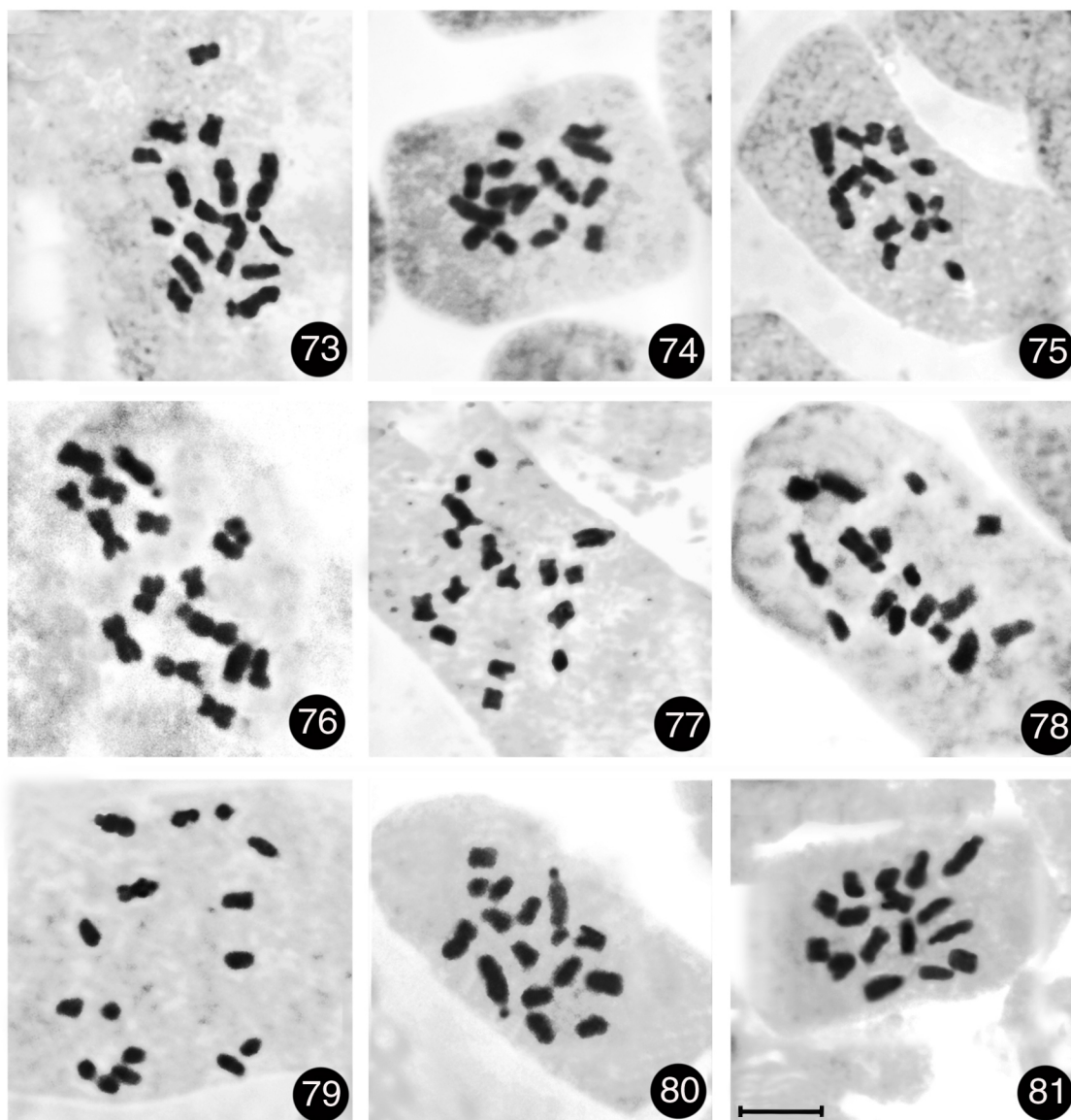
Figs. 46-54. Orcein-stained mitotic prophase chromosomes of nine cultivars of *Cicer arietinum* L. 46. BC-1, 47. BC-2, 48. BC-3, 49. BC-4, 50. BC-5, 51. BC-6, 52. BC-7, 53. BC-8 and 54. BC-9. Bar = 5 μ m, arrow indicates nucleolus.



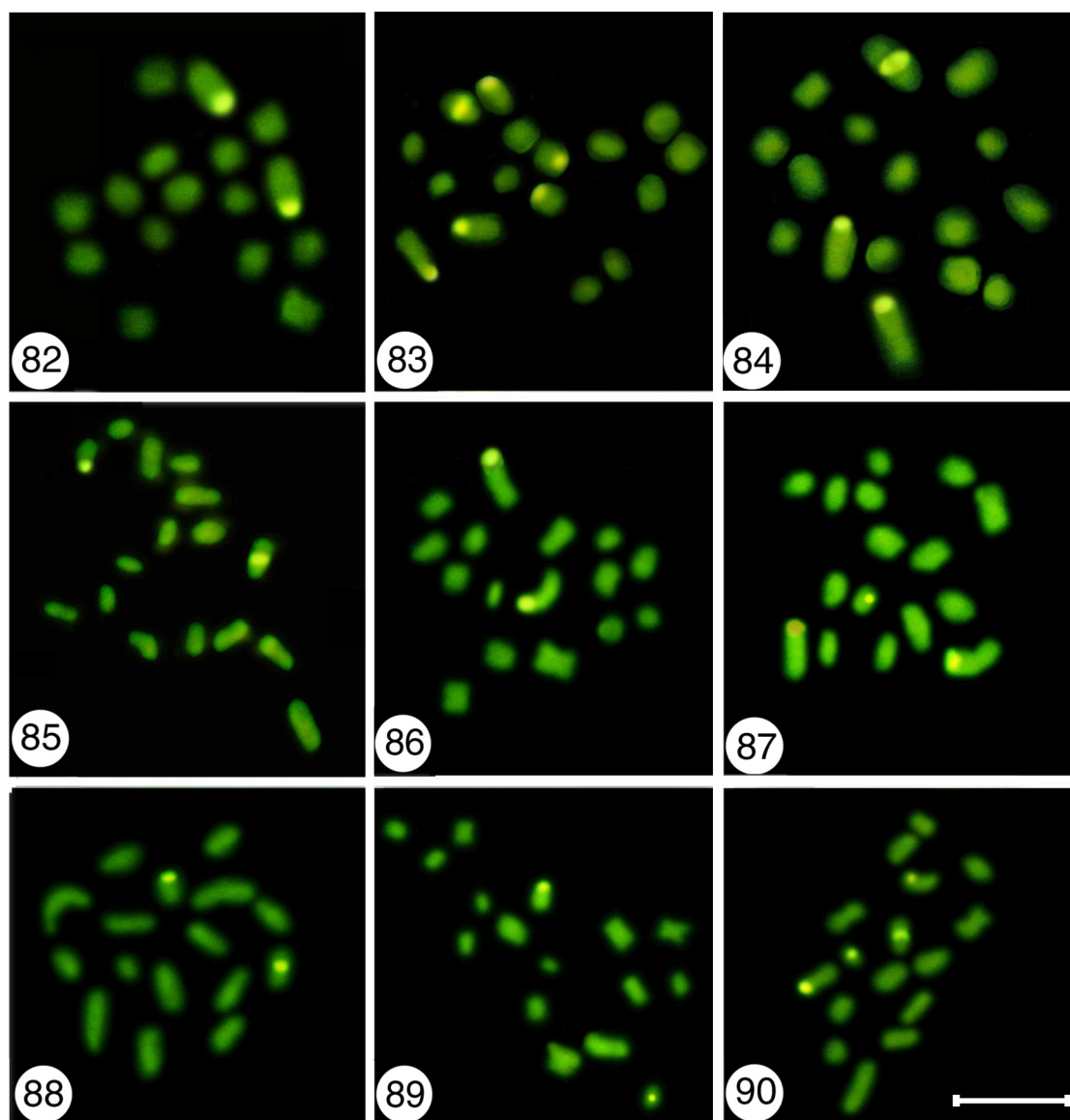
Figs. 55-63. CMA-stained mitotic prophase chromosomes of nine cultivars of *Cicer arietinum* L. 55. BC-1, 56. BC-2, 57. BC-3, 58. BC-4, 59. BC-5, 60. BC-6, 61. BC-7, 62. BC-8 and 63. BC-9. Bar = 5 μ m.



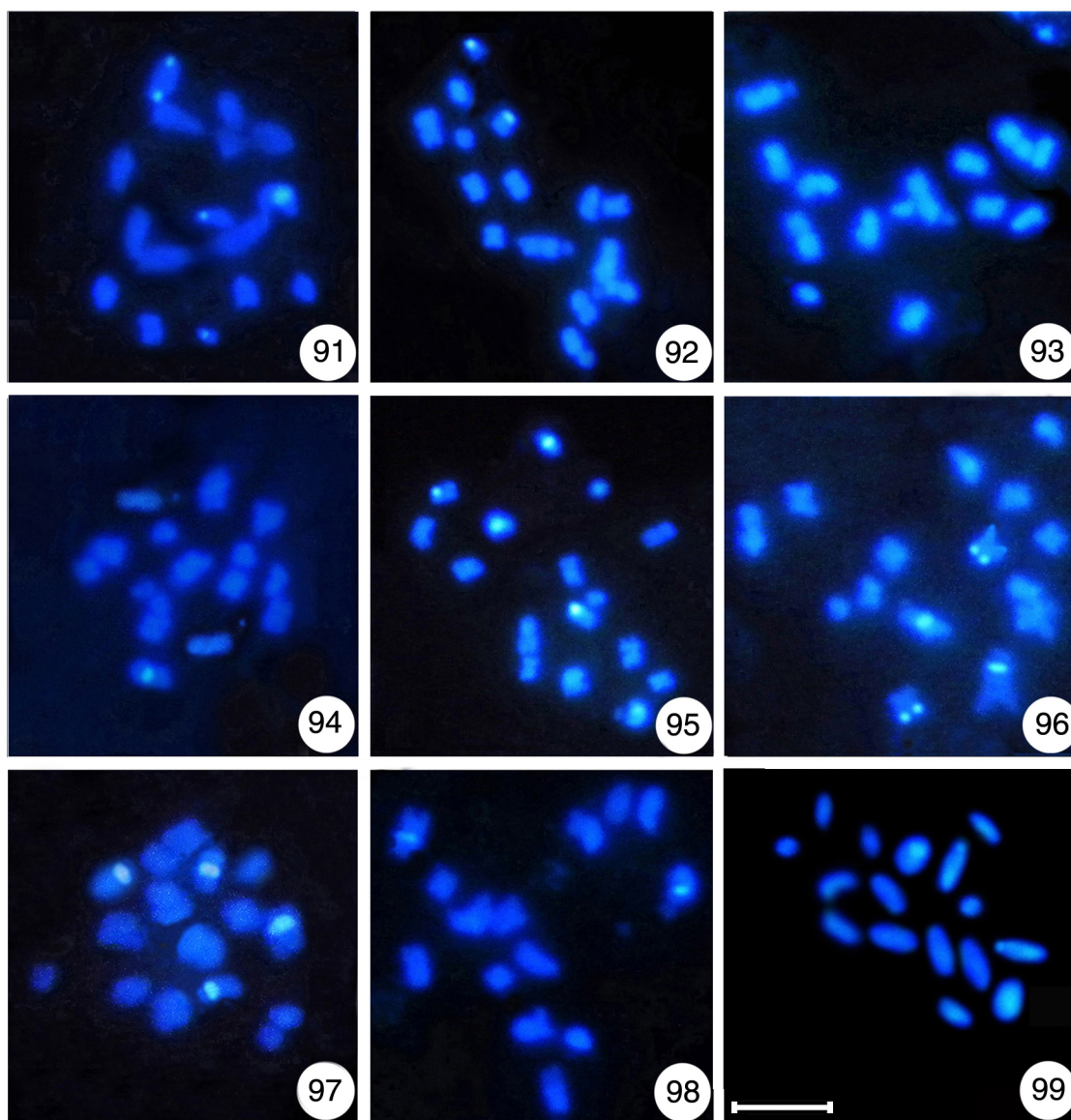
Figs. 64-72. DAPI-stained mitotic prophase chromosomes of nine cultivars of *Cicer arietinum* L. 64. BC-1, 65. BC-2, 66. BC-3, 67. BC-4, 68. BC-5, 69. BC-6, 70. BC-7, 71. BC-8 and 72. BC-9. Bar = 5 μ m.



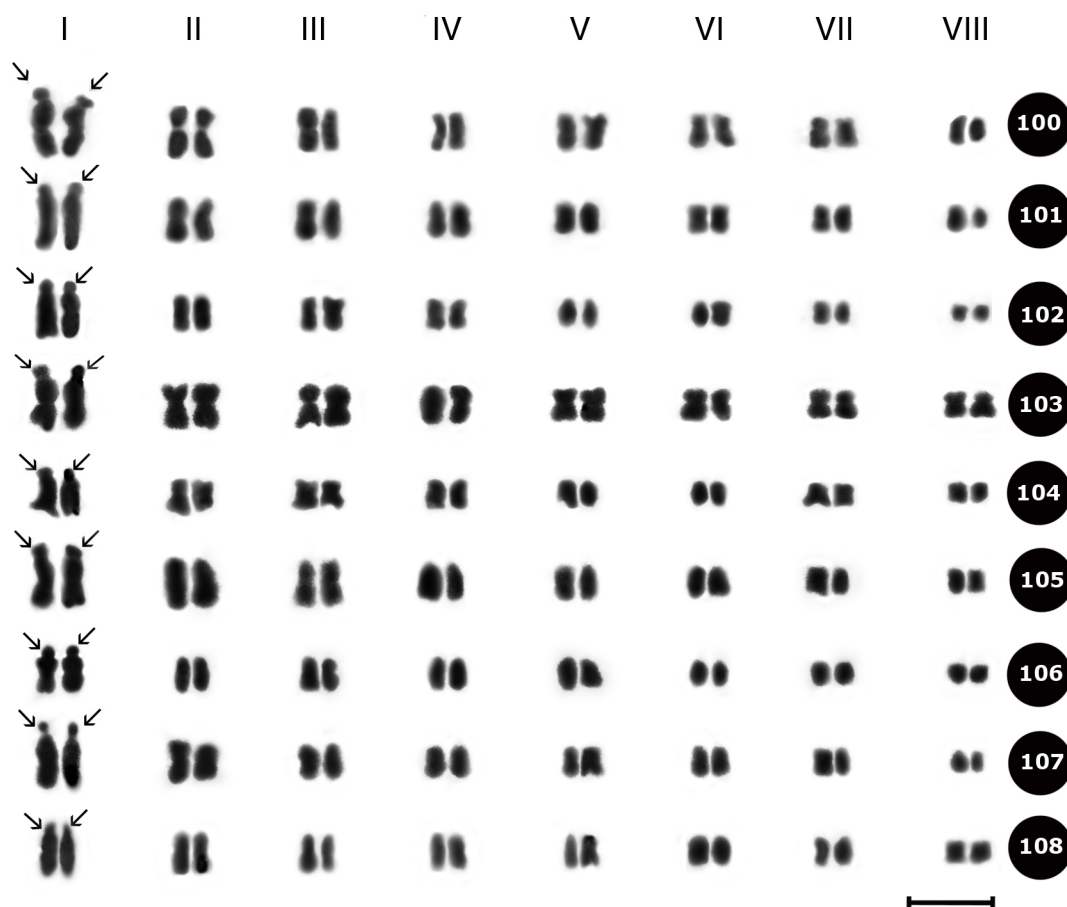
Figs. 73-81. Orcein-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 73. BC-1, 74. BC-2, 75. BC-3, 76. BC-4, 77. BC-5, 78. BC-6, 79. BC-7, 80. BC-8 and 81. BC-9. Bar = 5 μ m.



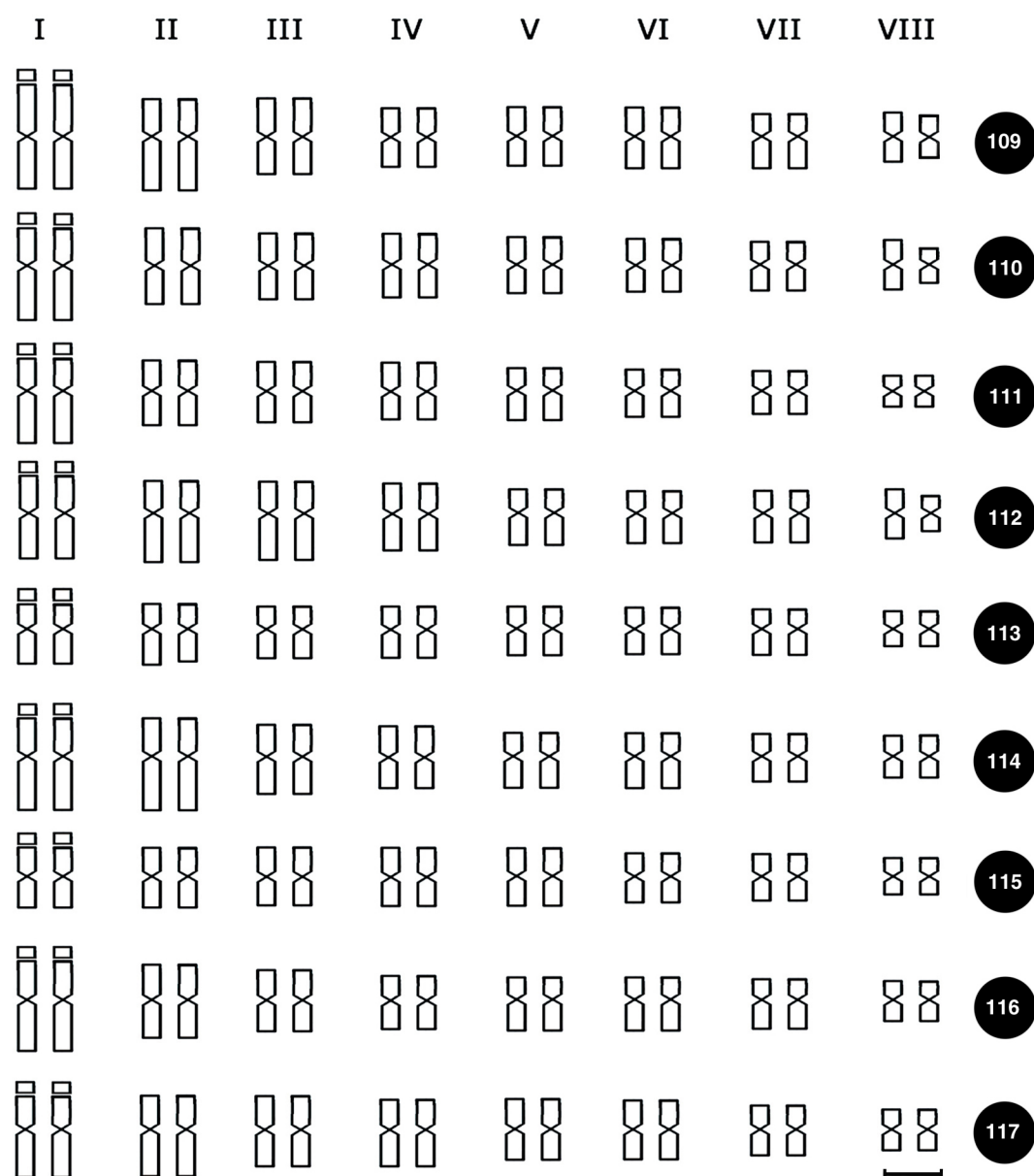
Figs. 82-90. CMA-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 82. BC-1, 83. BC-2, 84. BC-3, 85. BC-4, 86. BC-5, 87. BC-6, 88. BC-7, 89. BC-8 and 90. BC-9. Bar = 5 μ m.



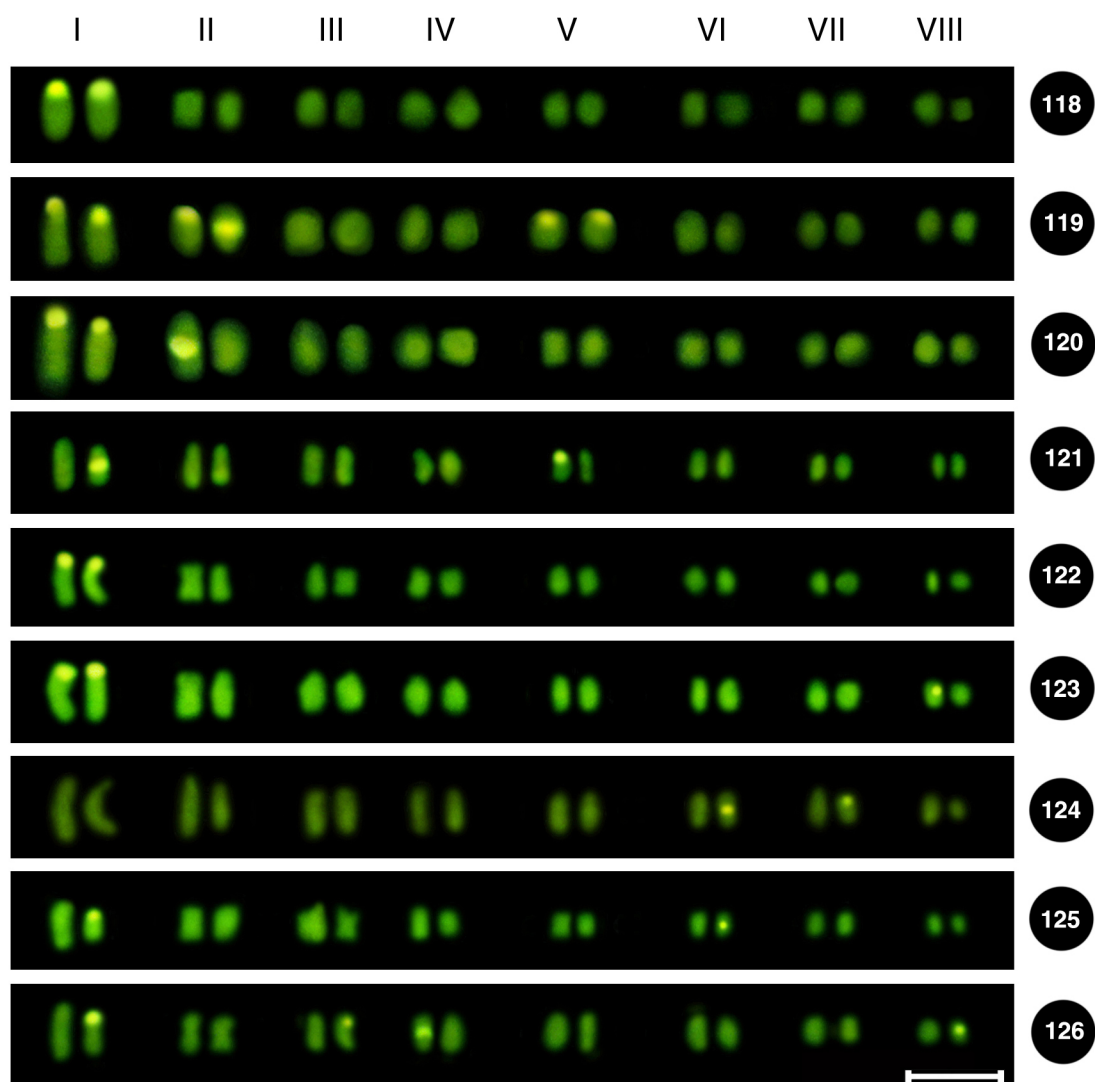
Figs. 91-99. DAPI-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 91. BC-1, 92. BC-2, 93. BC-3, 94. BC-4, 95. BC-5, 96. BC-6, 97. BC-7, 98. BC-8 and 99. BC-9. Bar = 5 μ m.



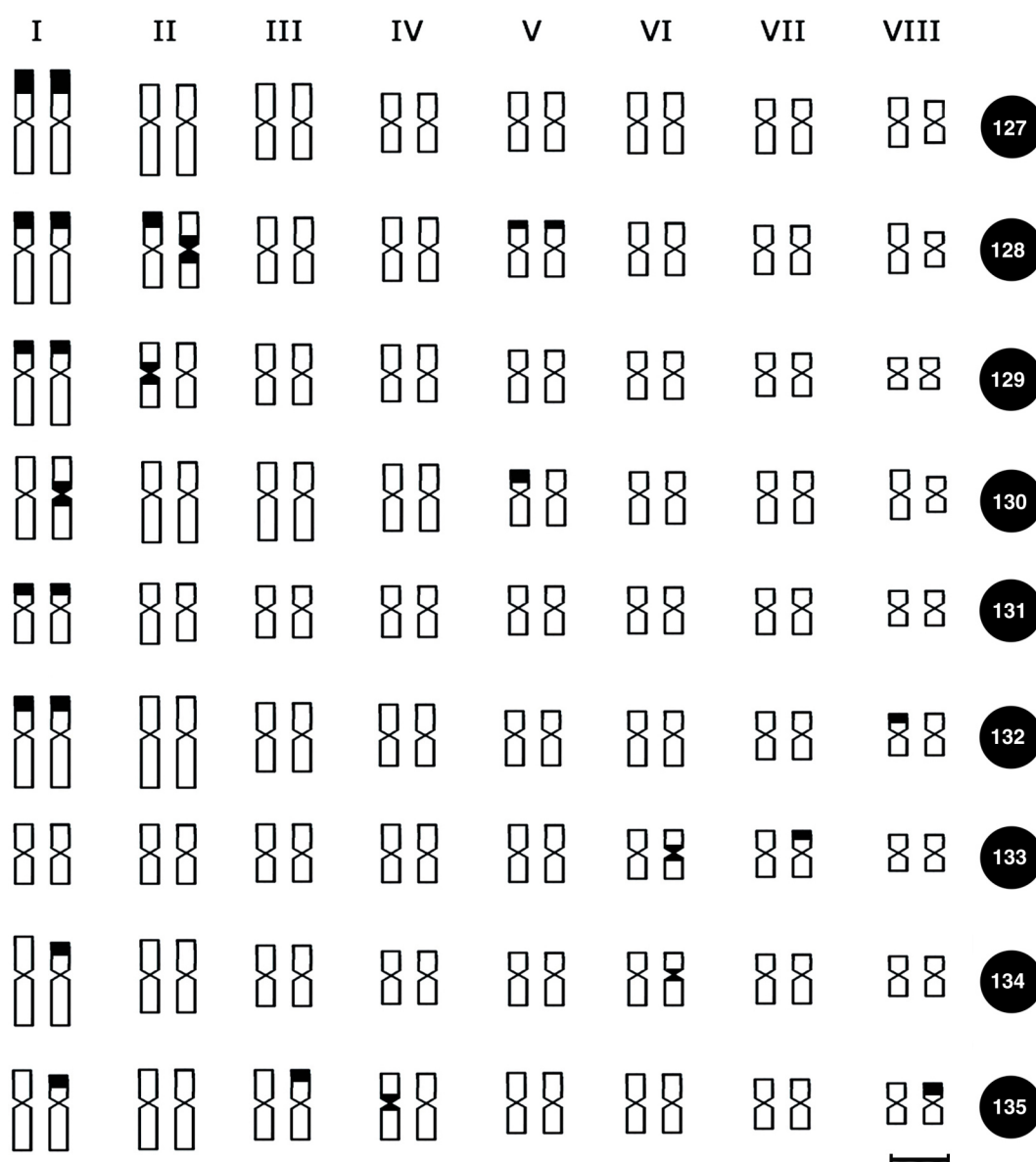
Figs. 100-108. Karyotype prepared from orcein-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 100. BC-1, 101. BC-2, 102. BC-3, 103. BC-4, 104. BC-5, 105. BC-6, 106 BC-7, 107. BC-8 and 108. BC-9. Bar = 5 μ m, arrow indicates satellites.



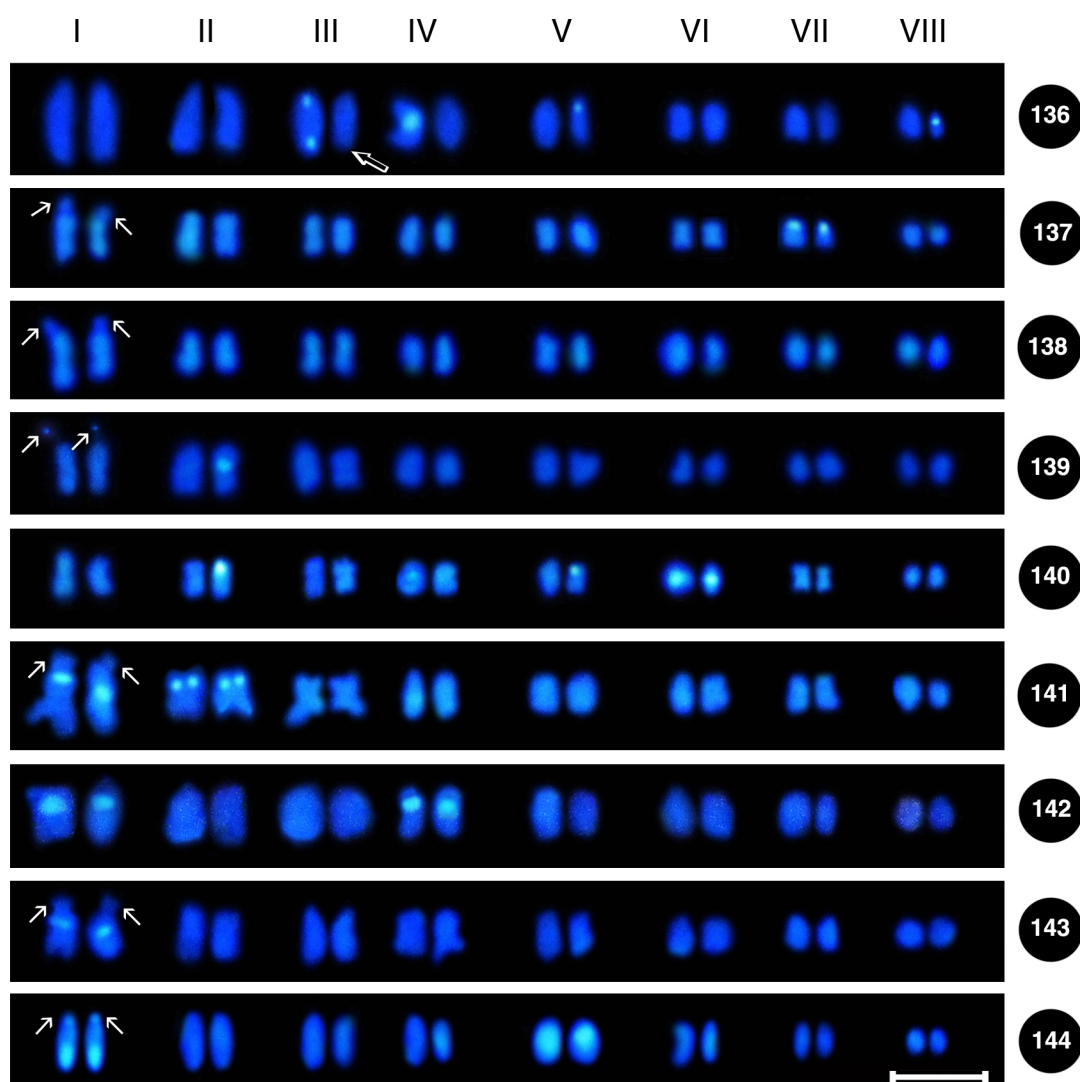
Figs. 109-117. Idiogram prepared from orcein-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 109. BC-1, 110. BC-2, 111. BC-3, 112. BC-4, 113. BC-5, 114. BC-6, 115. BC-7, 116. BC-8 and 117. BC-9. Bar = 2 μ m.



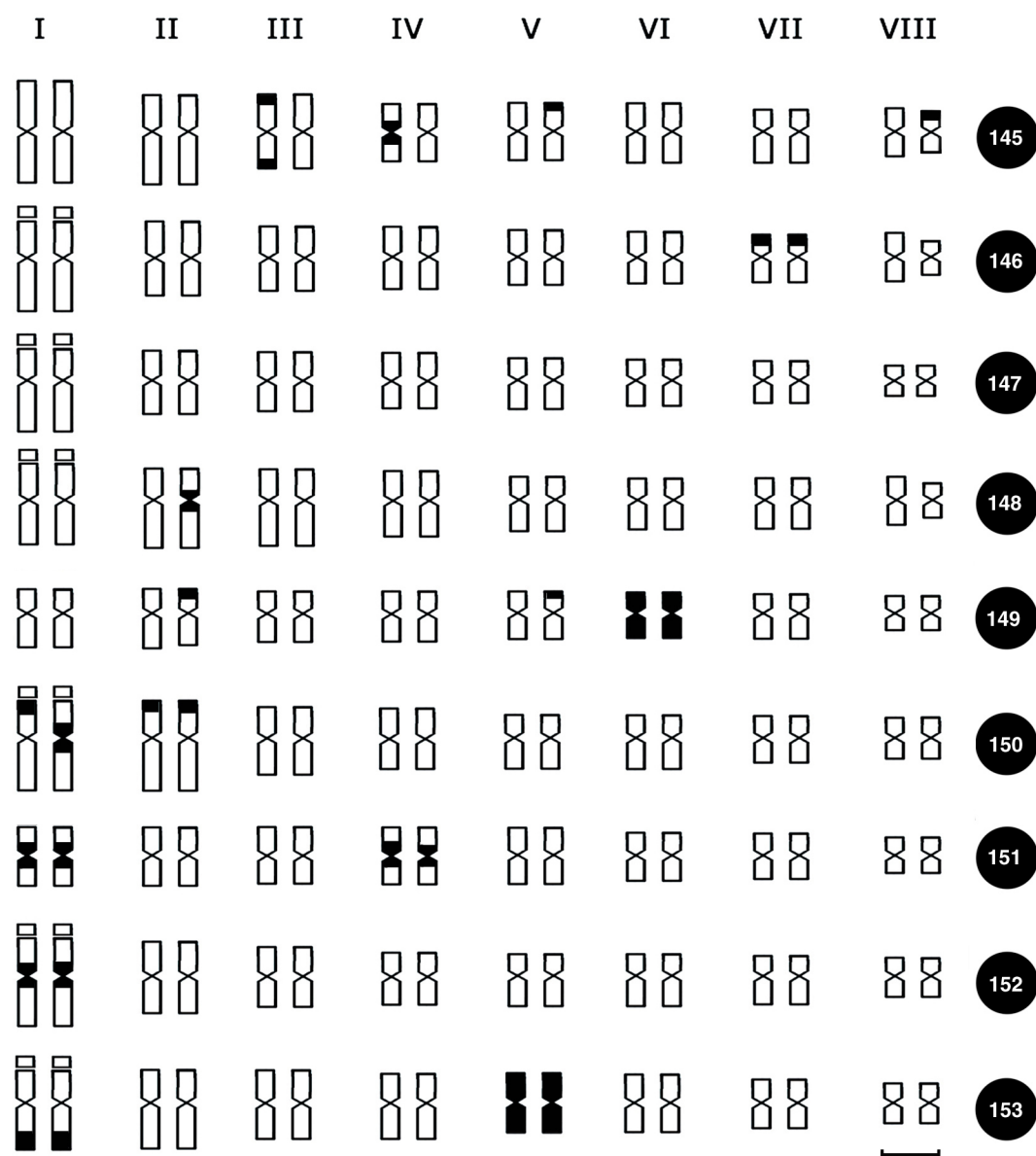
Figs. 118-126. Karyotype prepared from CMA-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 118. BC-1, 119. BC-2, 120. BC-3, 121. BC-4, 122. BC-5, 123. BC-6, 124. BC-7, 125. BC-8 and 126. BC-9. Bar = 5 μ m.



Figs. 127-135. Idiogram prepared from CMA-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 127. BC-1, 128. BC-2, 129. BC-3, 130. BC-4, 131. BC-5, 132. BC-6, 133. BC-7, 134. BC-8 and 135. BC-9. Bar = 2 μm. ■ = CMA-positive banded region



Figs. 136-144. Karyotype prepared from DAPI-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 136. BC-1, 137. BC-2, 138. BC-3, 139. BC-4, 140. BC-5, 141. BC-6, 142. BC-7, 143. BC-8 and 144. BC-9. Bar = 5 μ m, $\Rightarrow$ = indicates satellites.



Figs. 145-153. Idiogram prepared from DAPI-stained mitotic metaphase chromosomes of nine cultivars of *Cicer arietinum* L. 145. BC-1, 146. BC-2, 147. BC-3, 148. BC-4, 149. BC-5, 150. BC-6, 151. BC-7, 152. BC-8 and 153. BC-9. Bar = 2 μ m. ■ = DAPI-positive banded region.

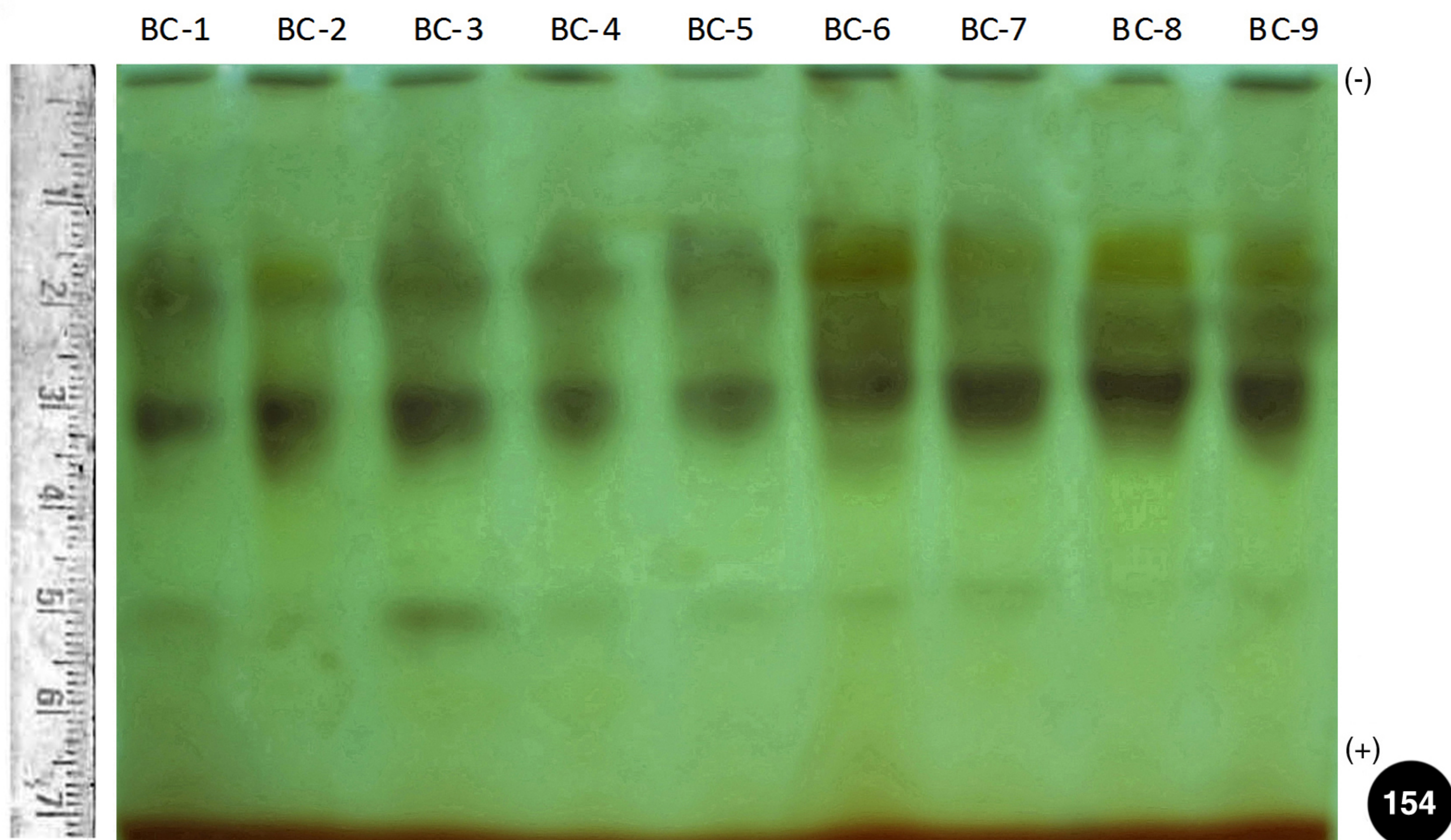


Fig. 154. Acid phosphatase activity following polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9.

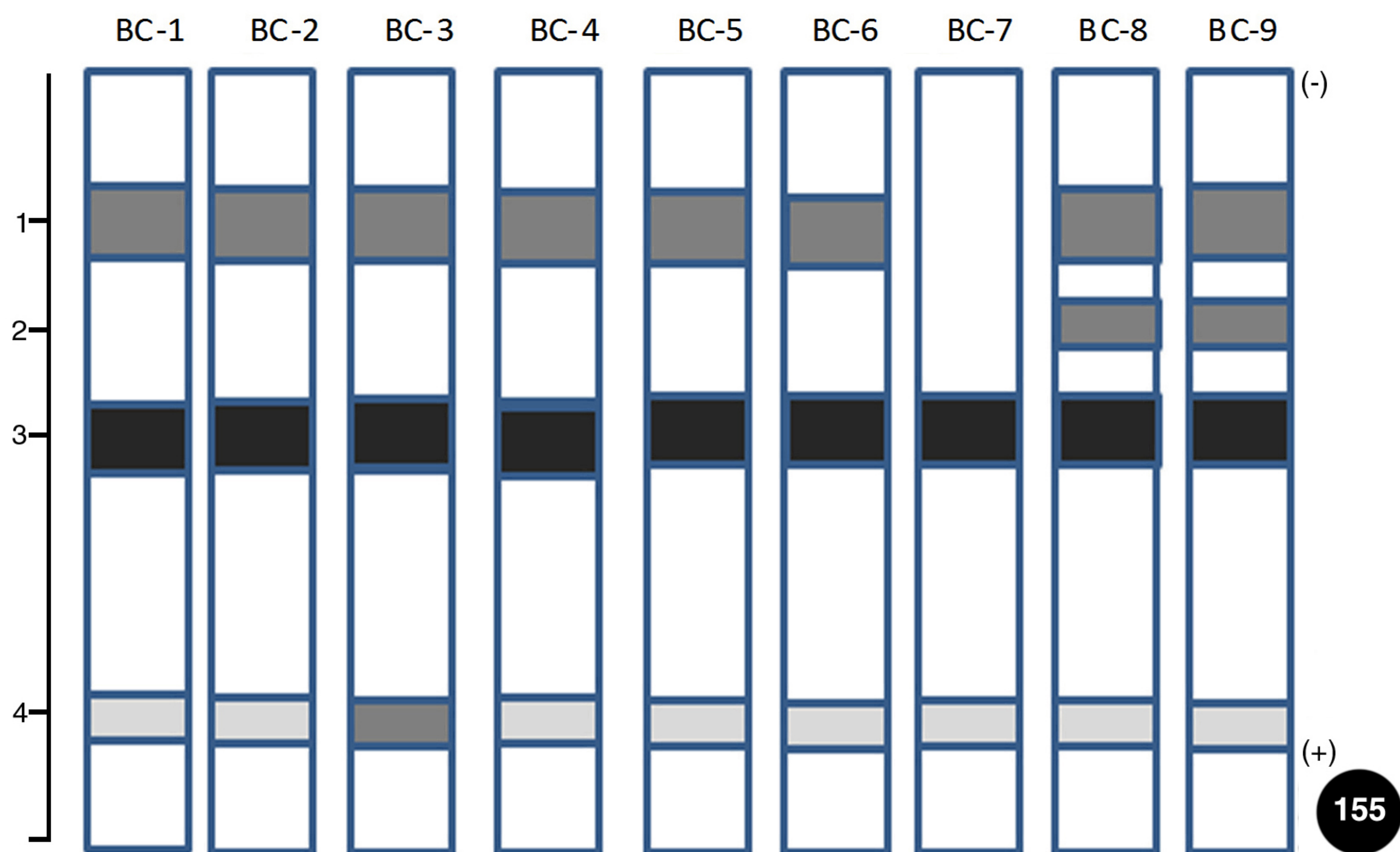
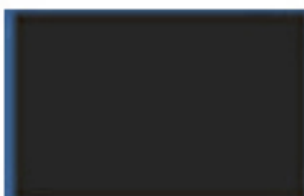
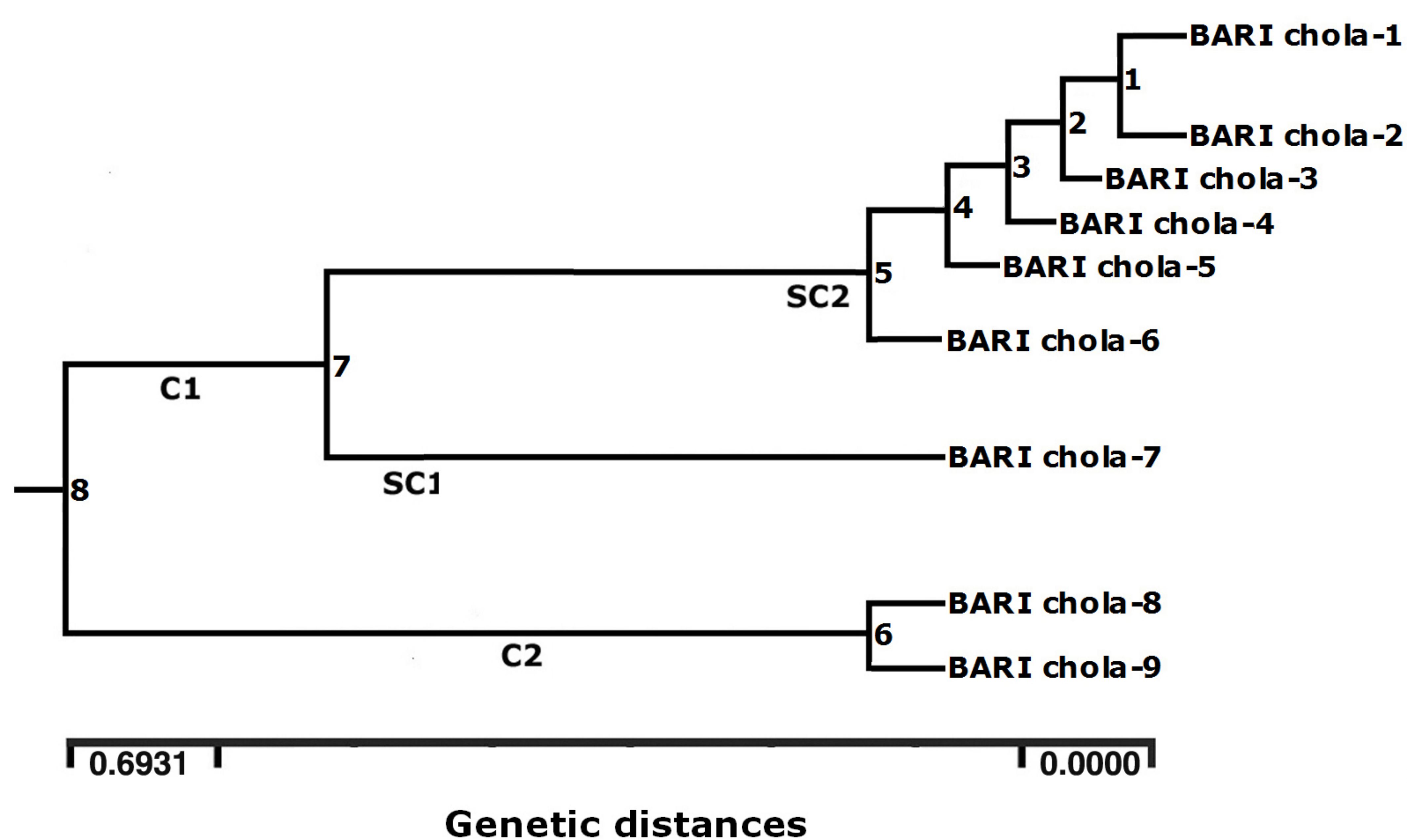


Fig. 155. Zymogram of acid phosphatase banding profile of gel electrophoresis, BC-1 to BC-9 same as Fig. 154.

 =Dark Band
 =Moderately Dark Band
 =Light Band



156

Fig. 156. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of *Cicer arietinum* L. by acid phosphatase isozyme analysis.

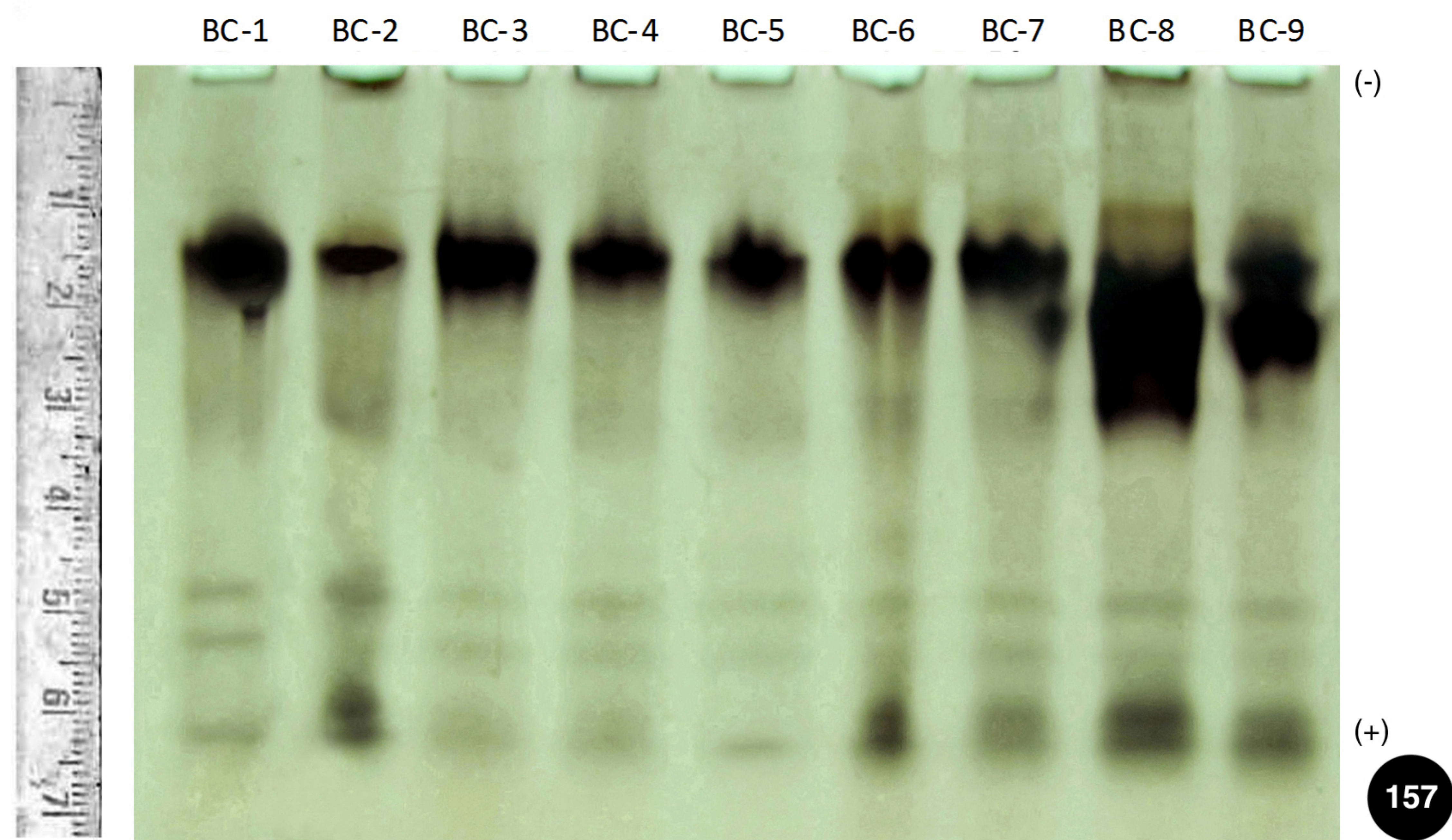


Fig. 157. Esterase activity following polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of *Cicer arietinum* L. BC-1 =BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4 =BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7 =BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9.

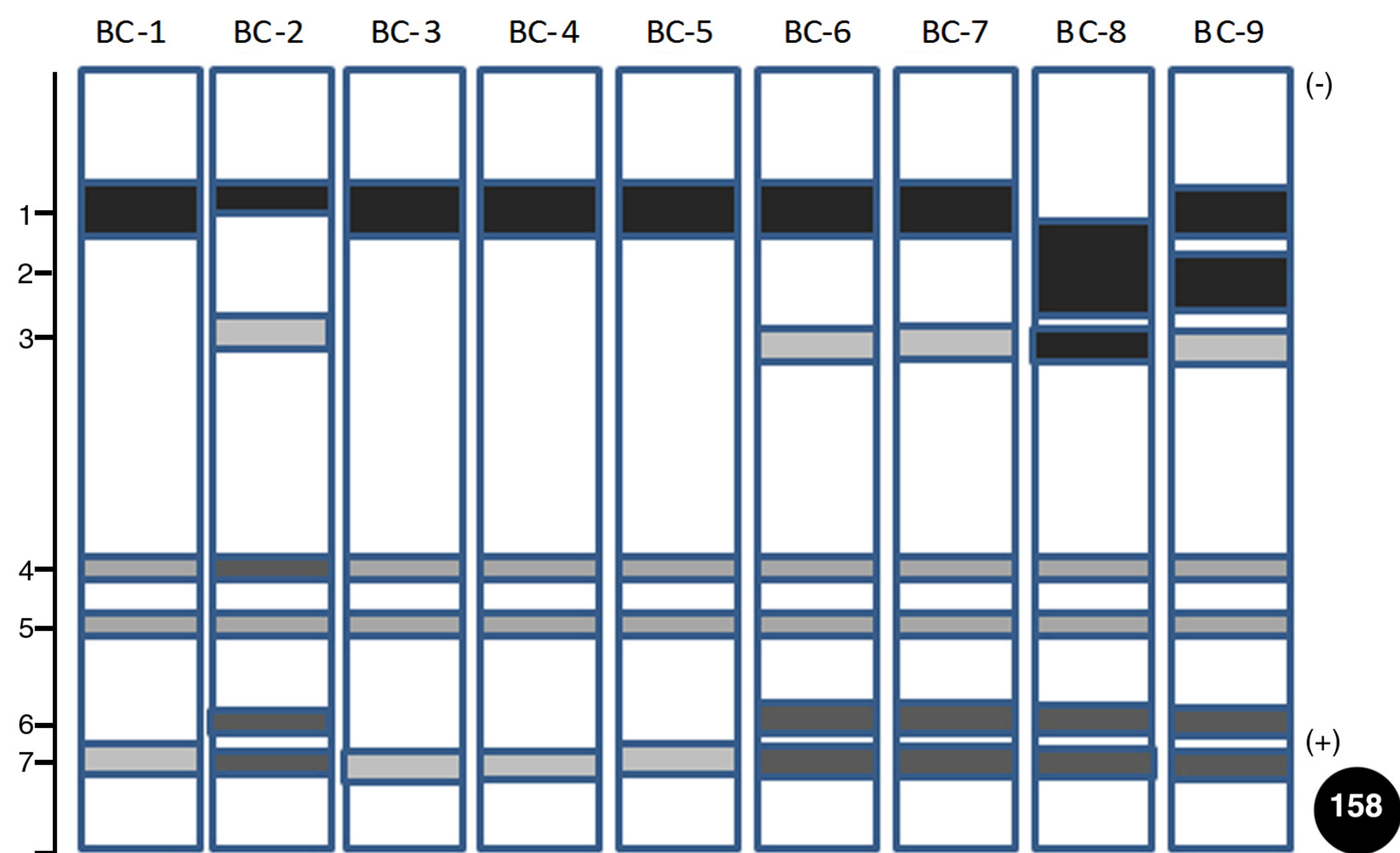


Fig. 158. Zymogram of esterase banding profile of gel electrophoresis, BC-1 to BC-9 same as Fig. 157.

■ =Dark Band
■ =Moderately Dark Band
■ =Light Band

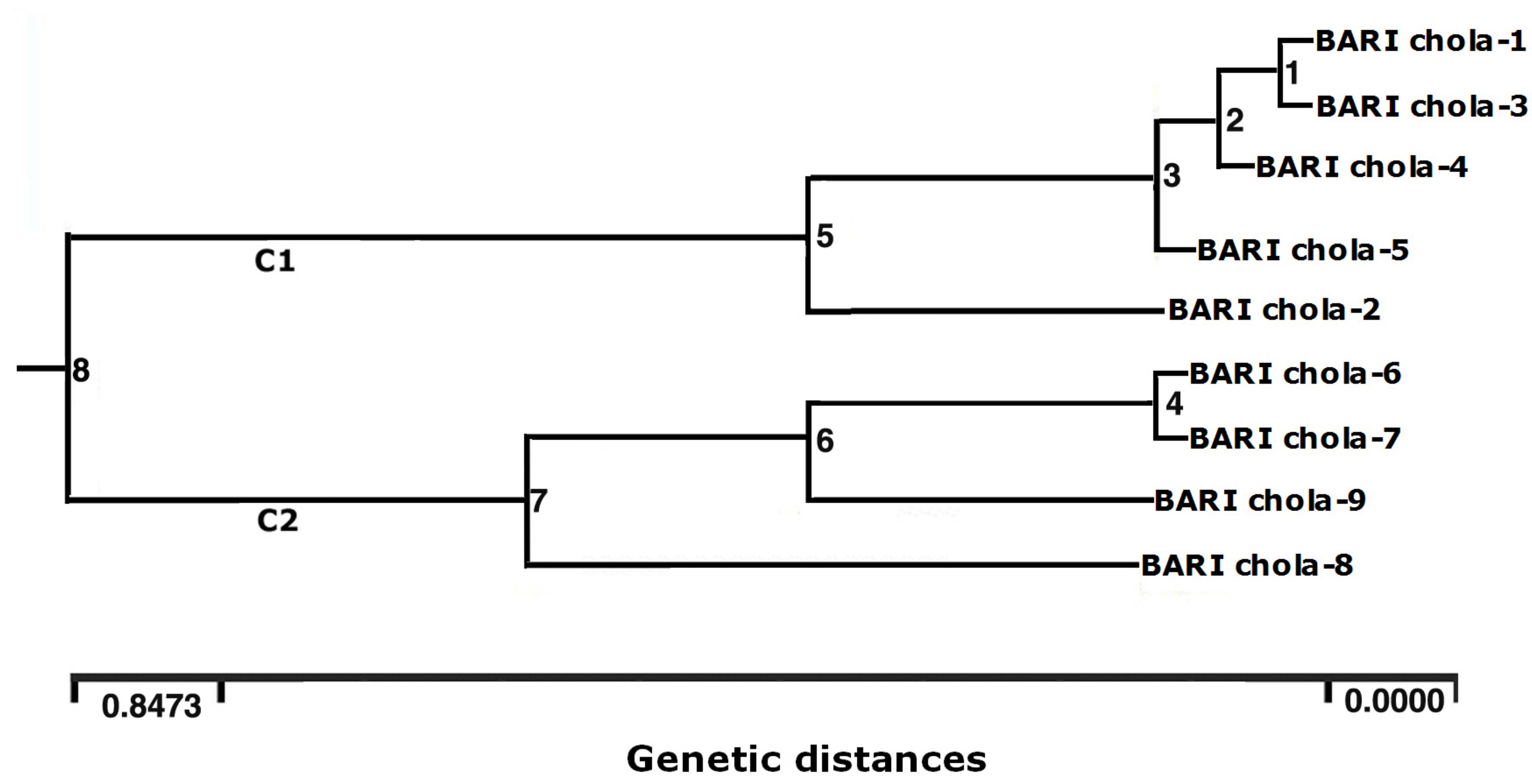


Fig. 159. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of *Cicer arietinum* L. by esterase isozyme analysis.

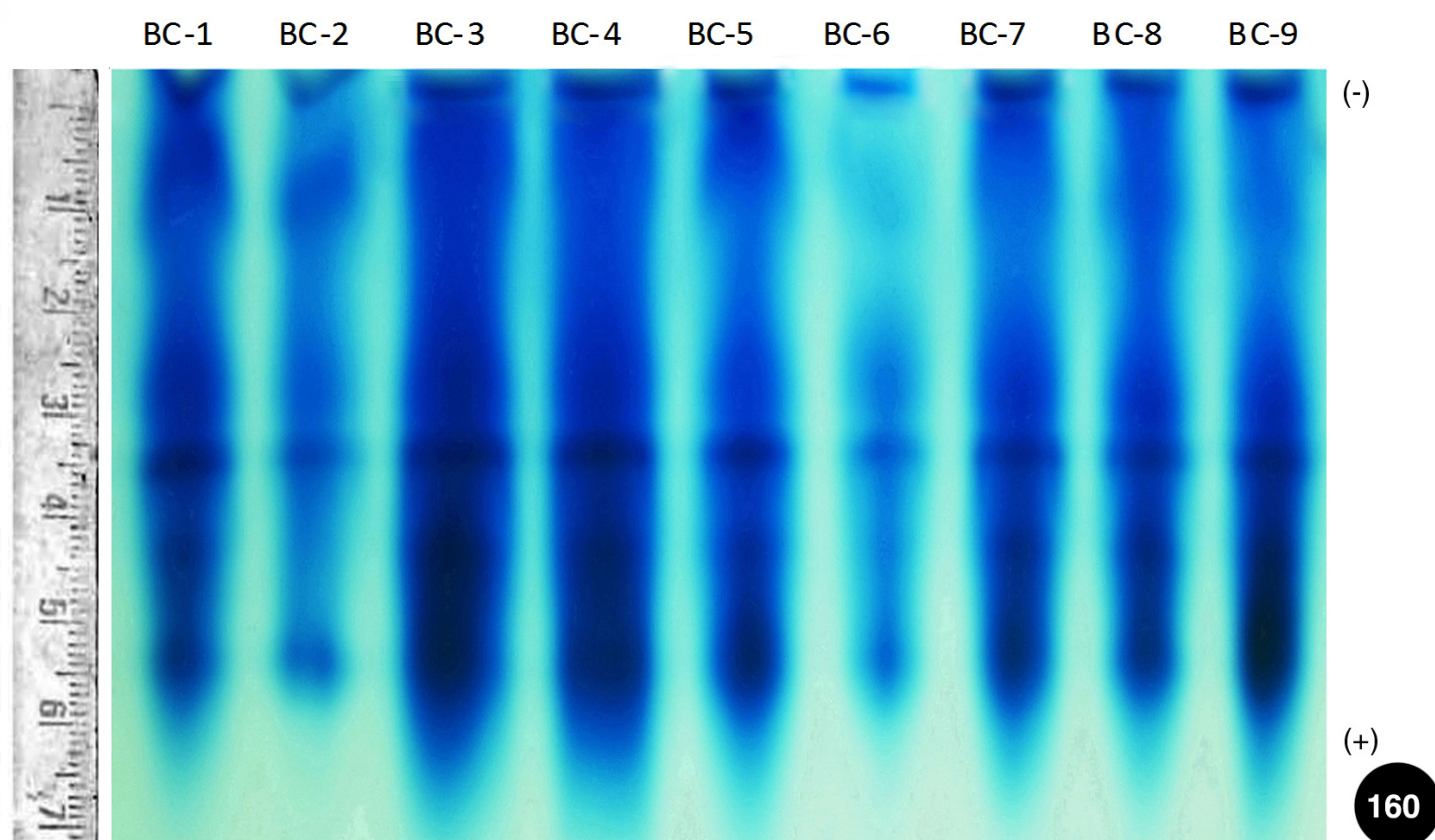


Fig. 160. Peroxidase activity following polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of *Cicer arietinum* L. BC-1 =BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4 =BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7 =BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9.

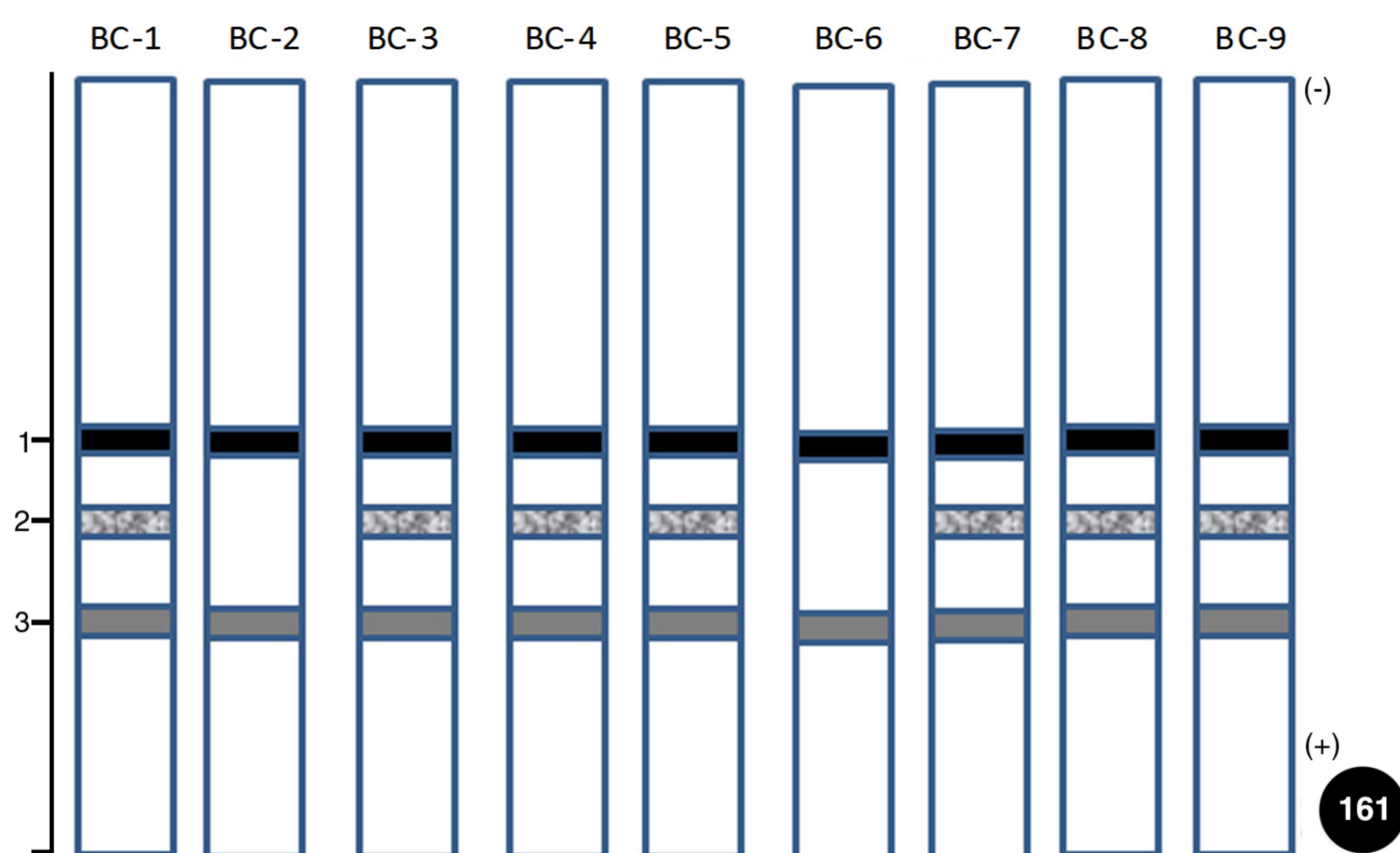


Fig. 161. Zymogram of peroxidase banding profile of gel electrophoresis, BC-1 to BC-9 same as Fig. 160.

■ =Dark Band
 ■ =Moderately Dark Band
 ■ =Light Band

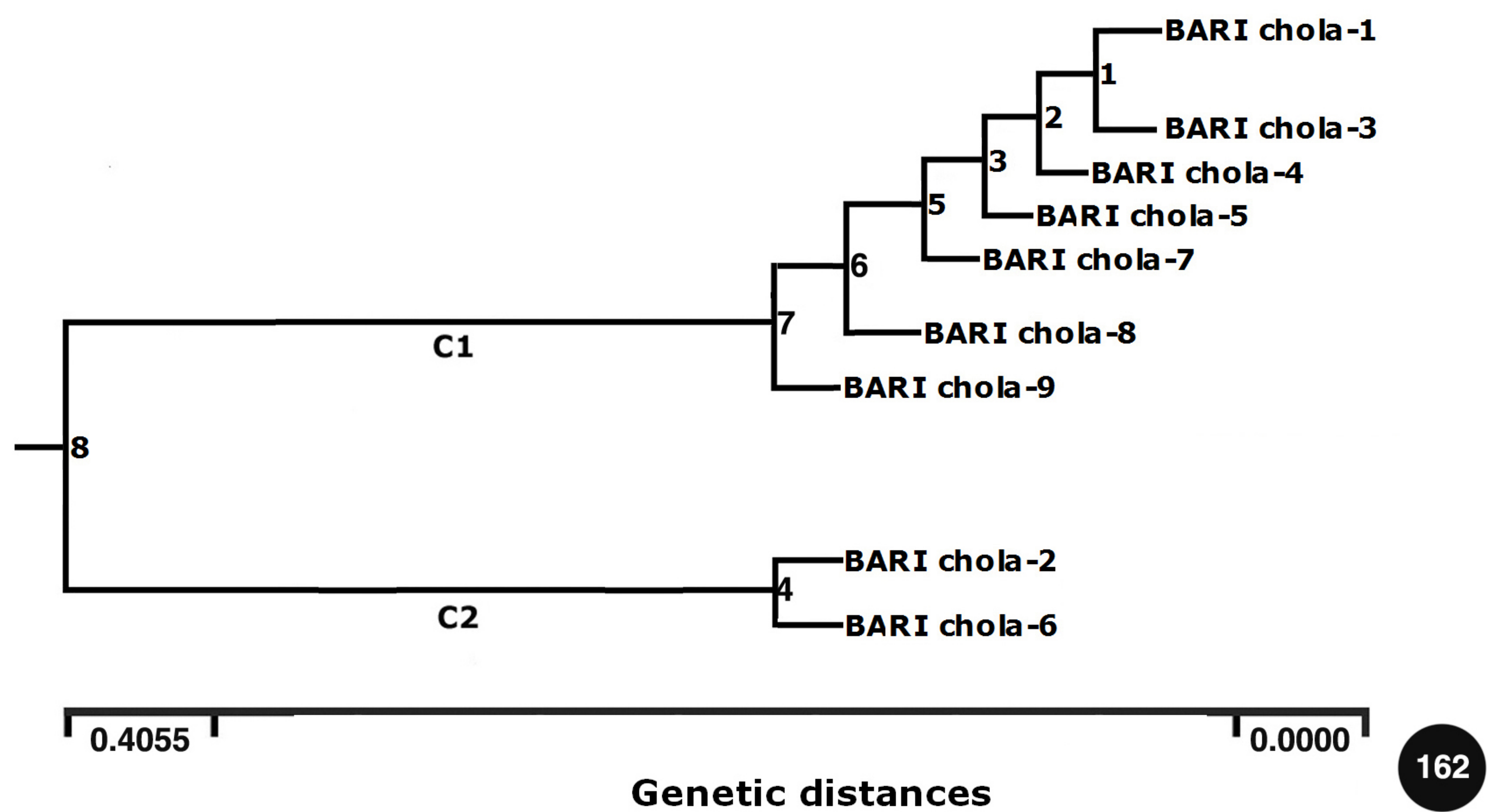
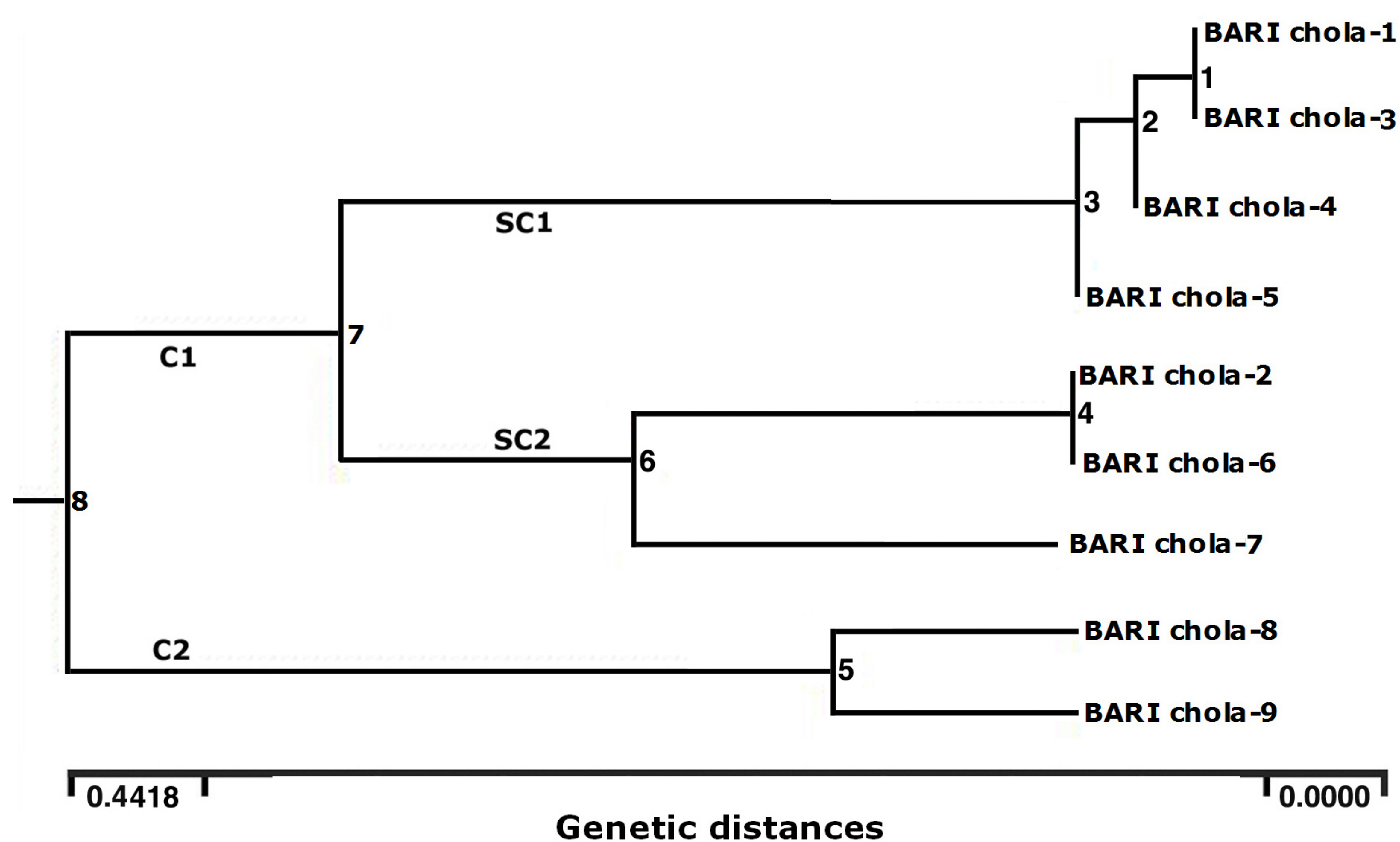


Fig. 162. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of *Cicer arietinum* L. by peroxidase isozyme analysis.



163

Fig. 163. UPGMA combined dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of *Cicer arietinum* L. on the basis of acid phosphatase, esterase and peroxidase isozymes analysis.

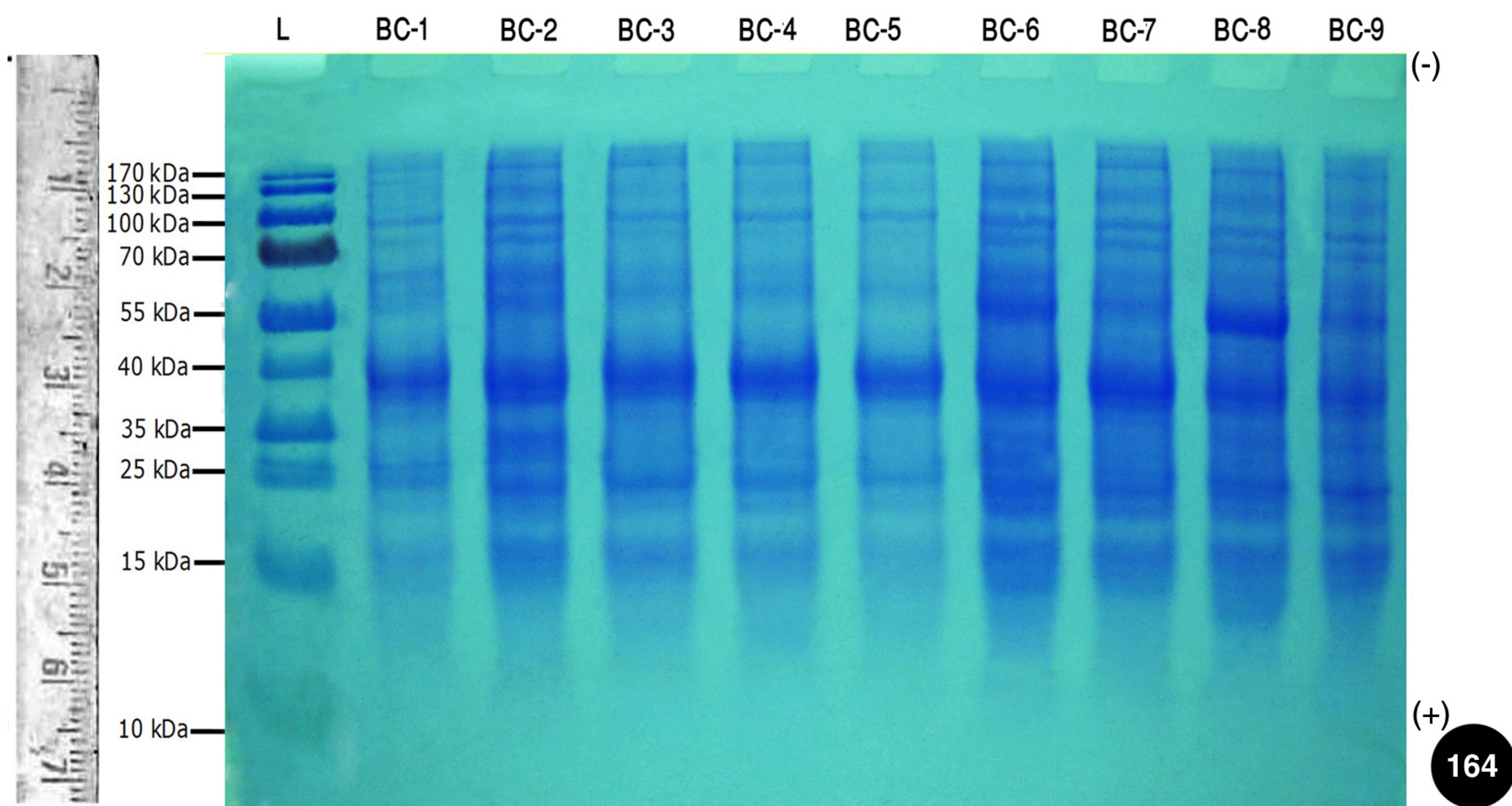


Fig. 164. SDS polyacrylamide gel electrophoresis of the leaf extract from nine cultivars of *Cicer arietinum* L. following coomassie brilliant blue staining. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, L= Protein ladder.

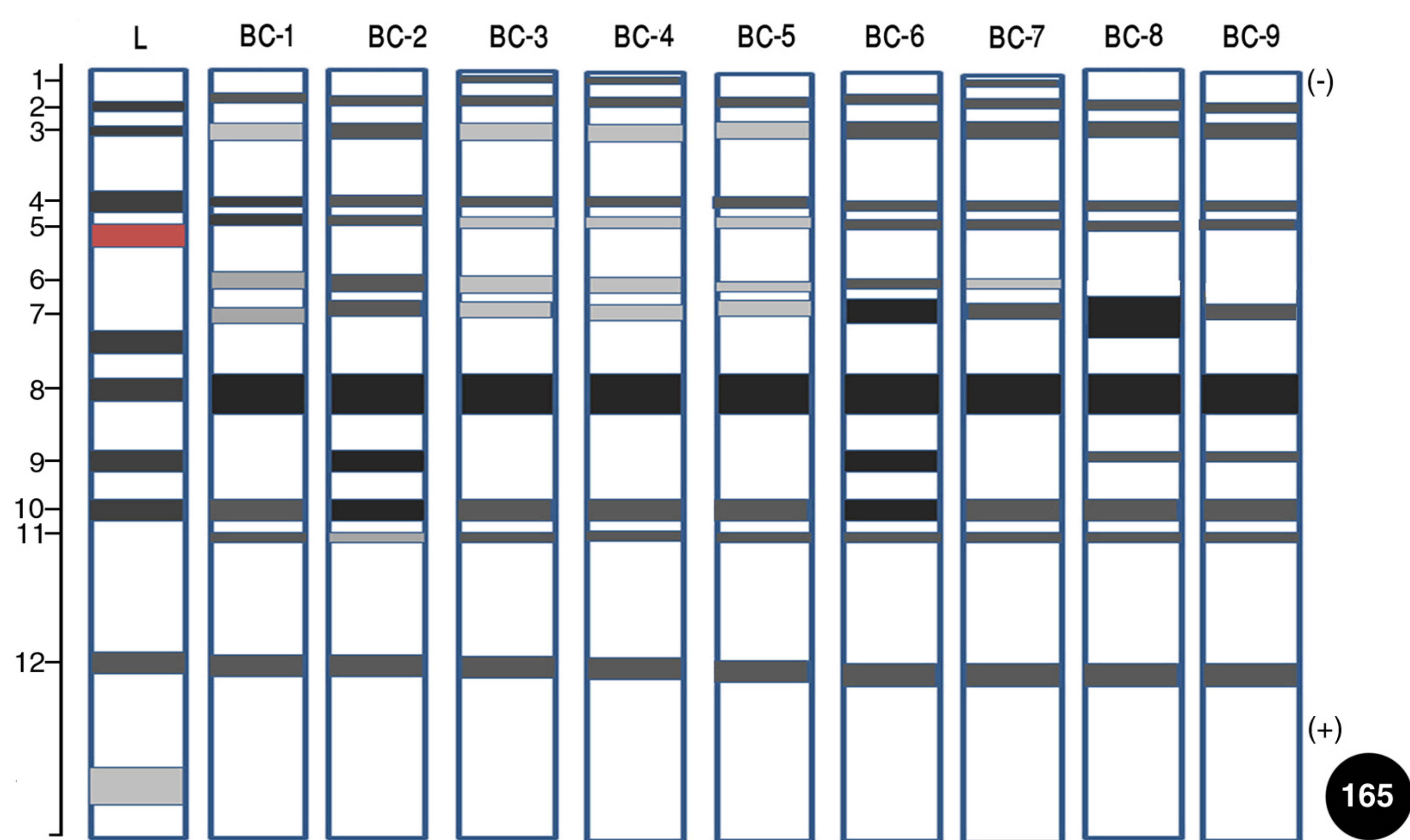
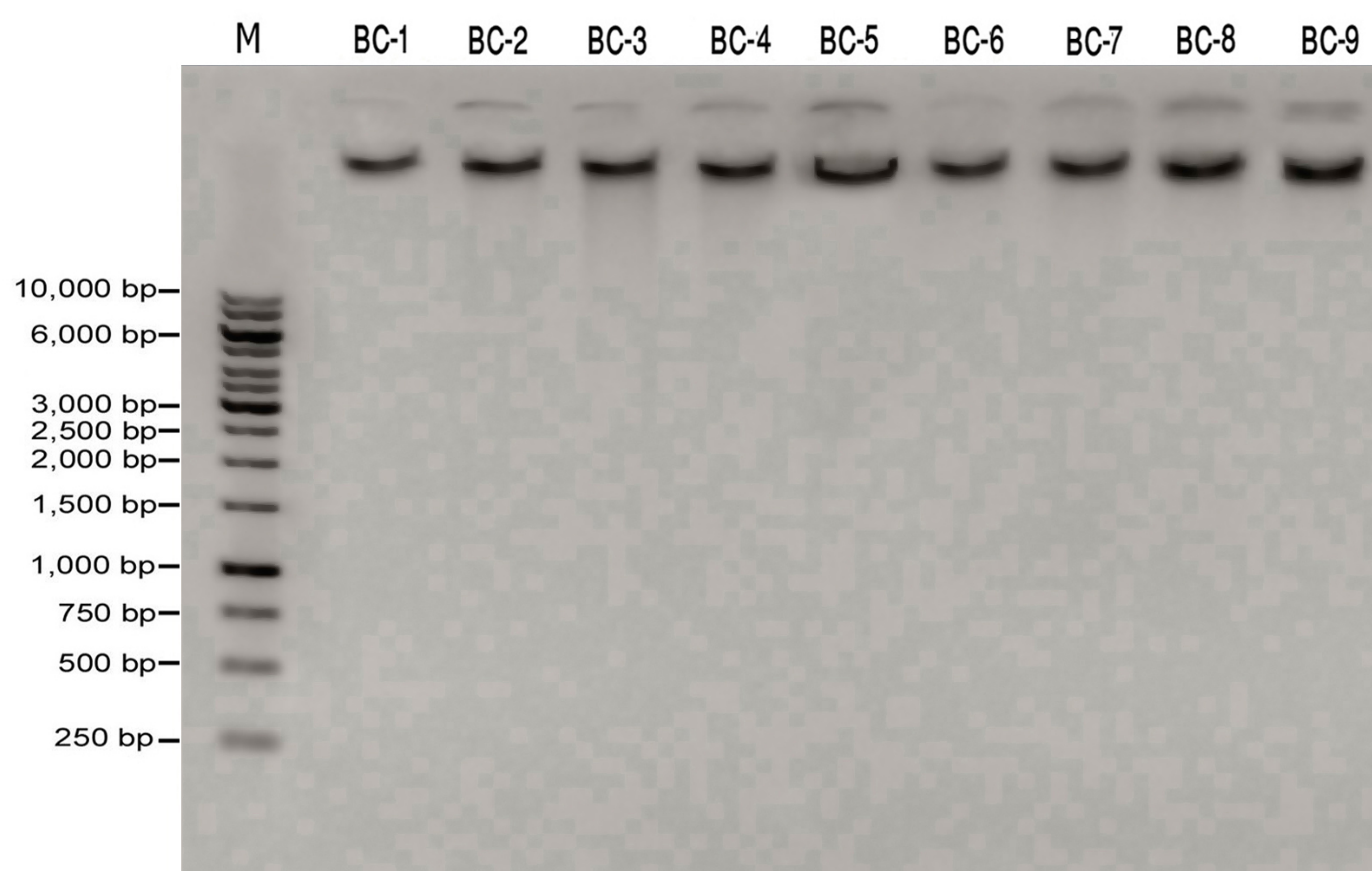


Fig. 165. Zymogram of SDS-PAGE banding profile of gel electrophoresis, BC-1 to BC-9 same as Fig. 164.

■ =Dark Band
■ =Moderately Dark Band
■ =Light Band

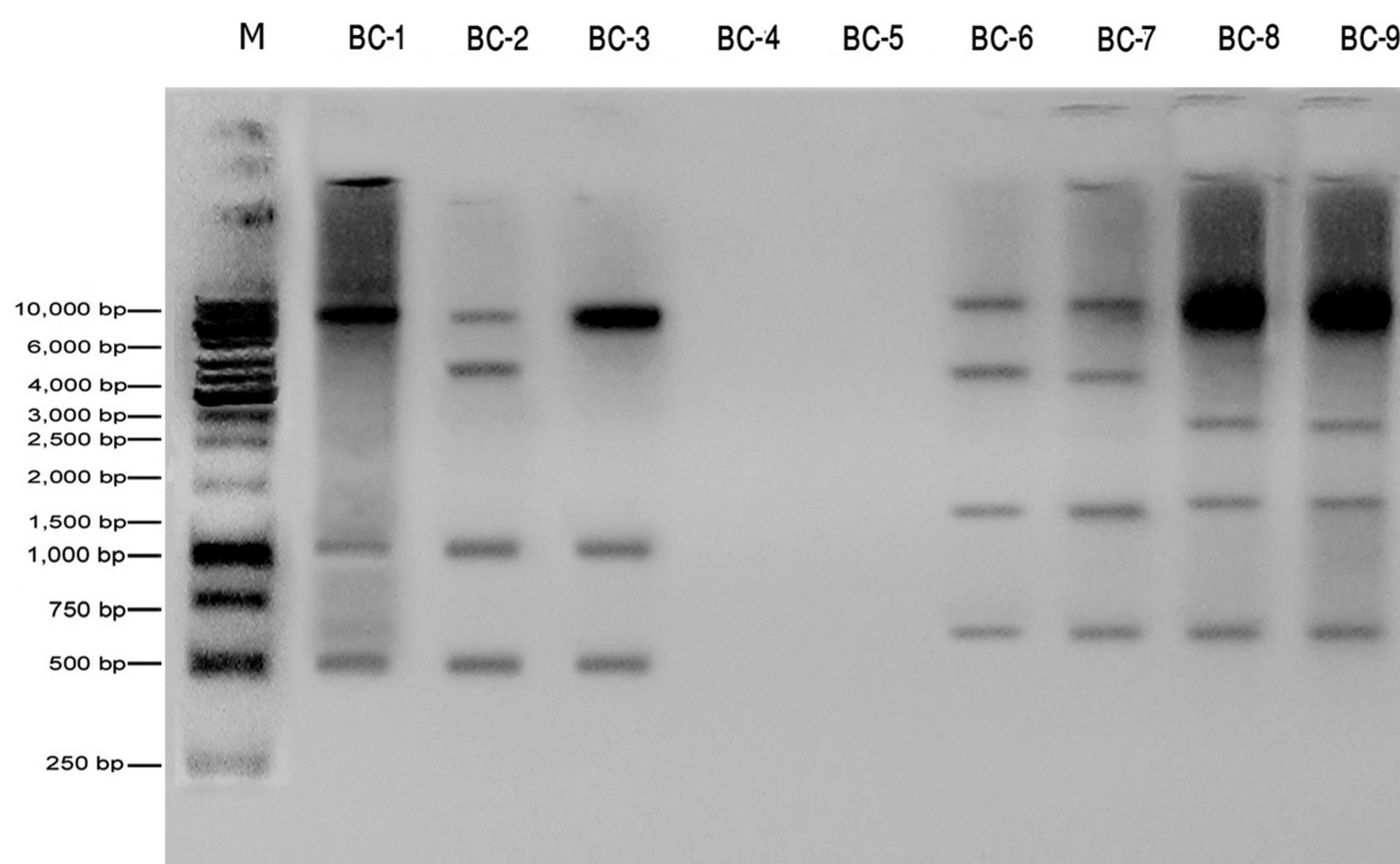


154



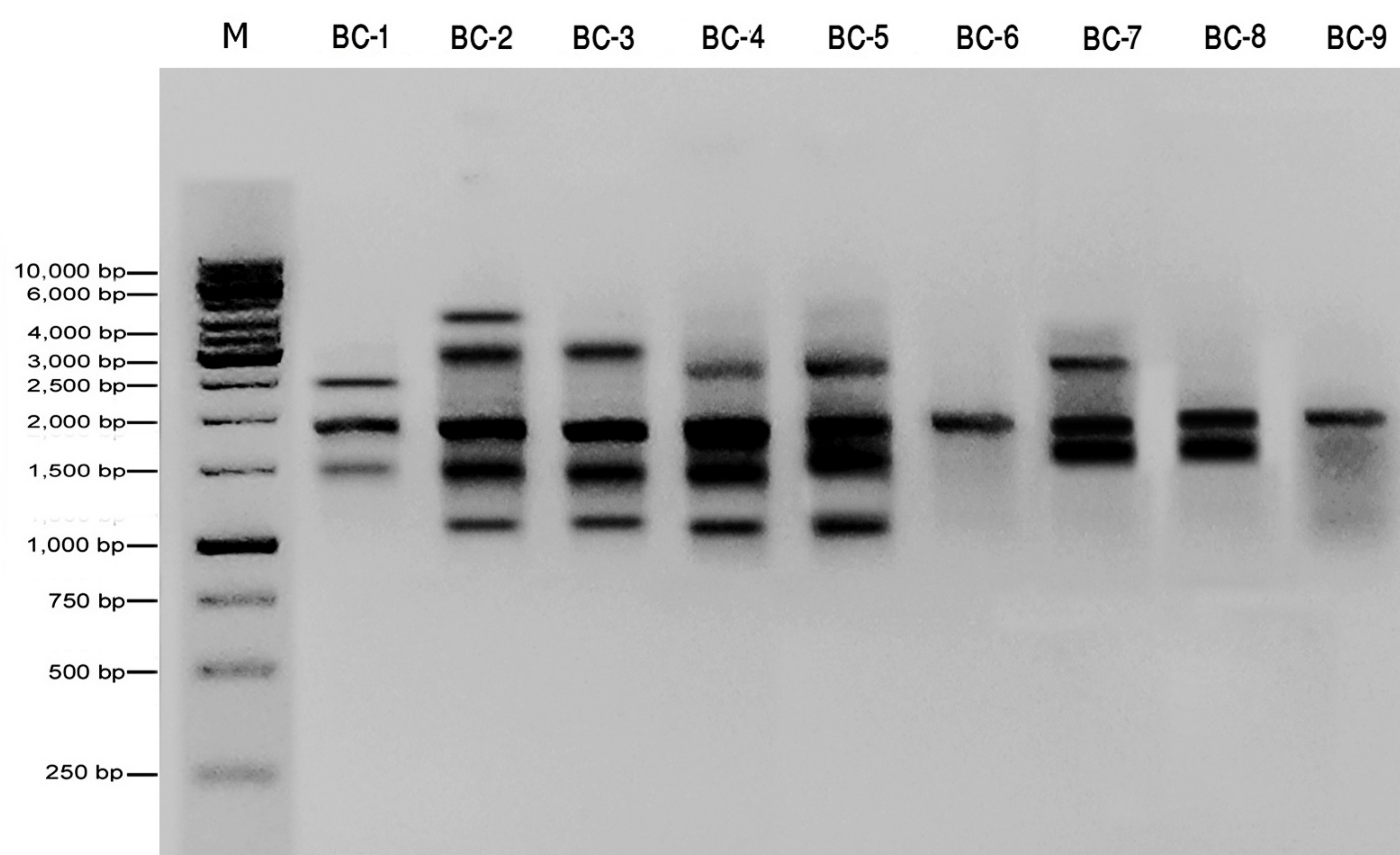
167

Fig. 167. Electrophoregram of ethidium bromide stained genomic DNA samples of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



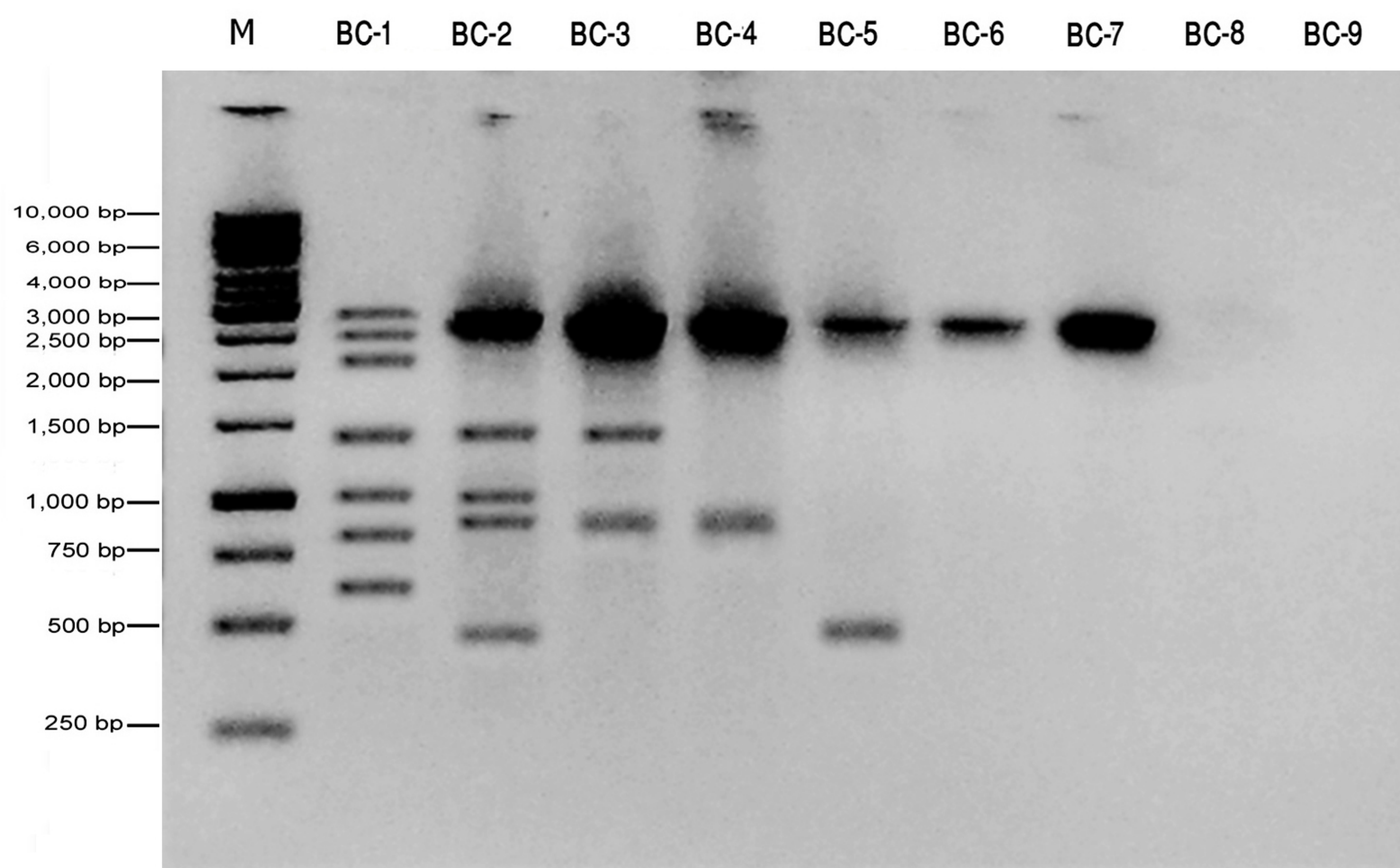
168

Fig. 168. RAPD analysis with primer OPA-1 (5'-CAG GCC CTT C-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



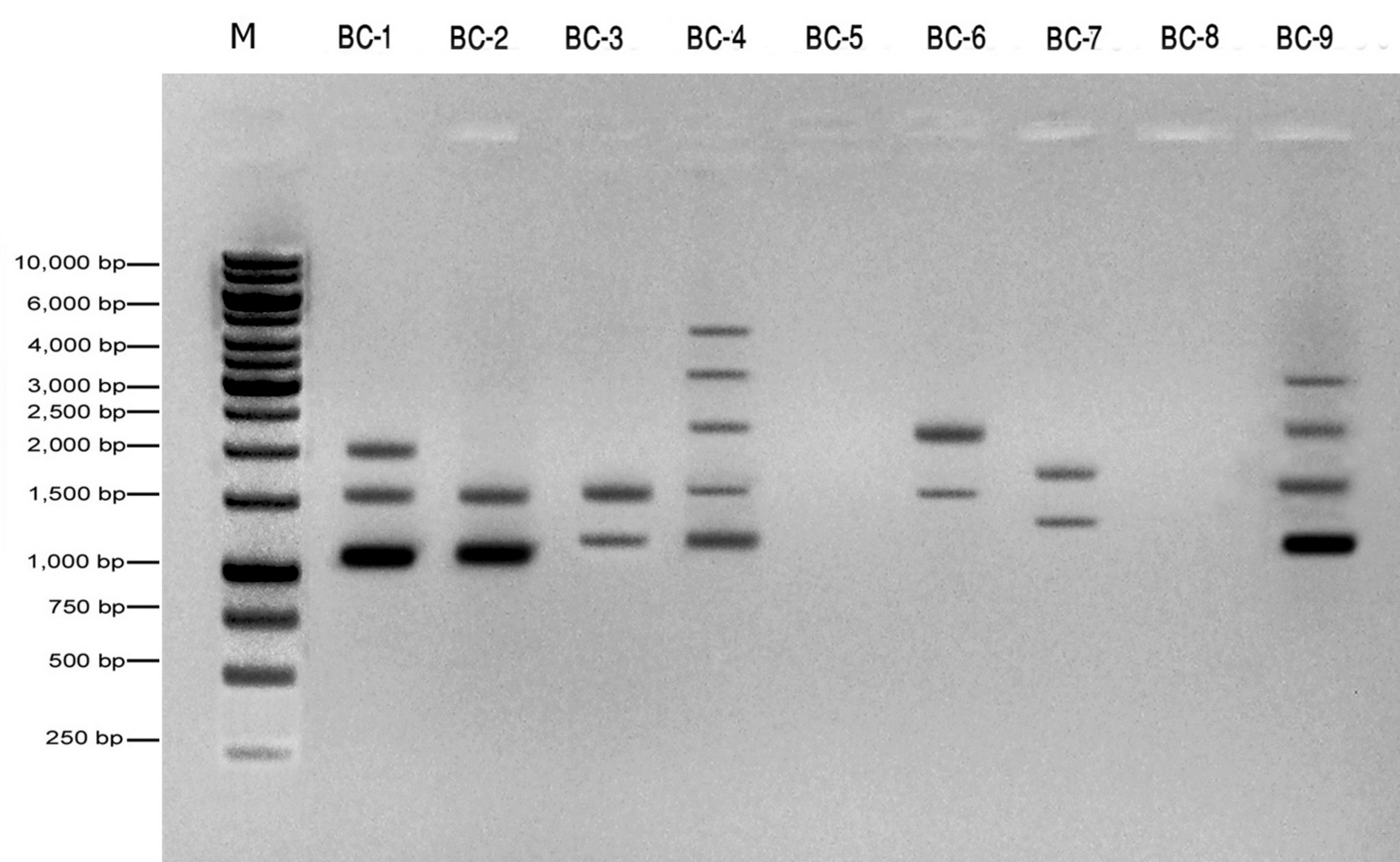
169

Fig. 169. RAPD analysis with primer OPA-3 (5'-AGT CAG CCA C-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



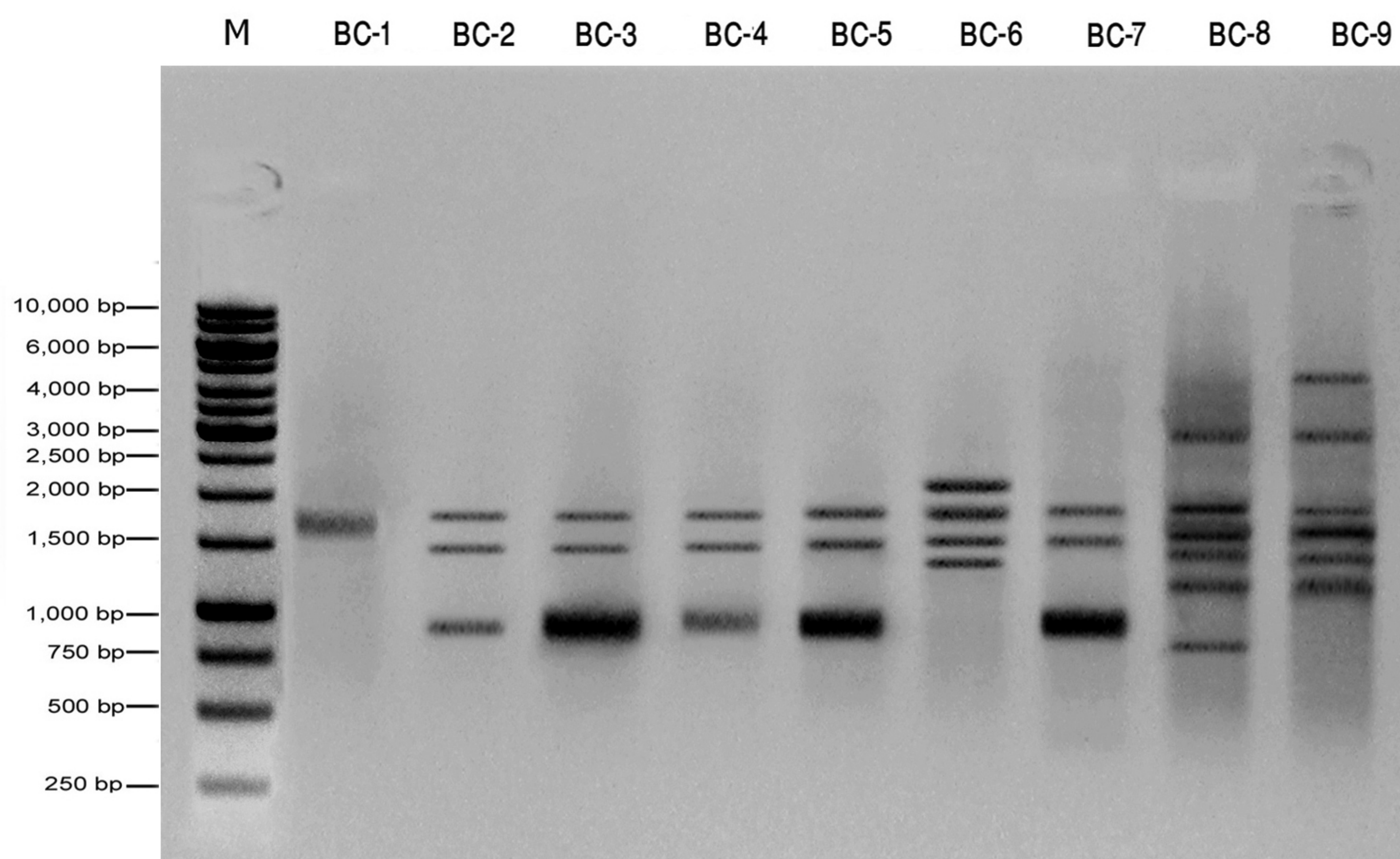
170

Fig. 170. RAPD analysis with primer OPA-4 (5'-AAT CGG GCT G-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



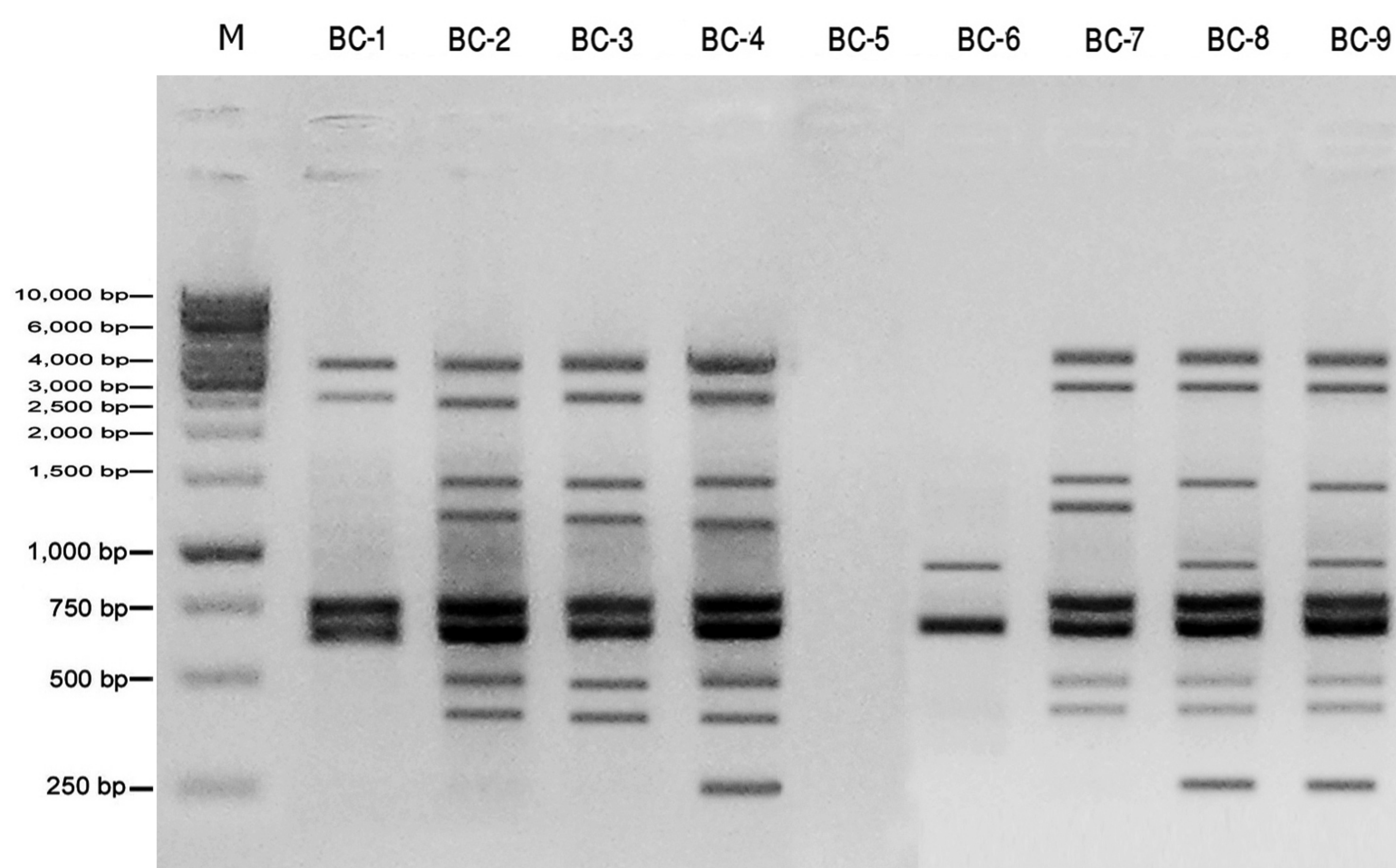
171

Fig. 171. RAPD analysis with primer OPA-5 (5'-AGG GGT CTT G-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



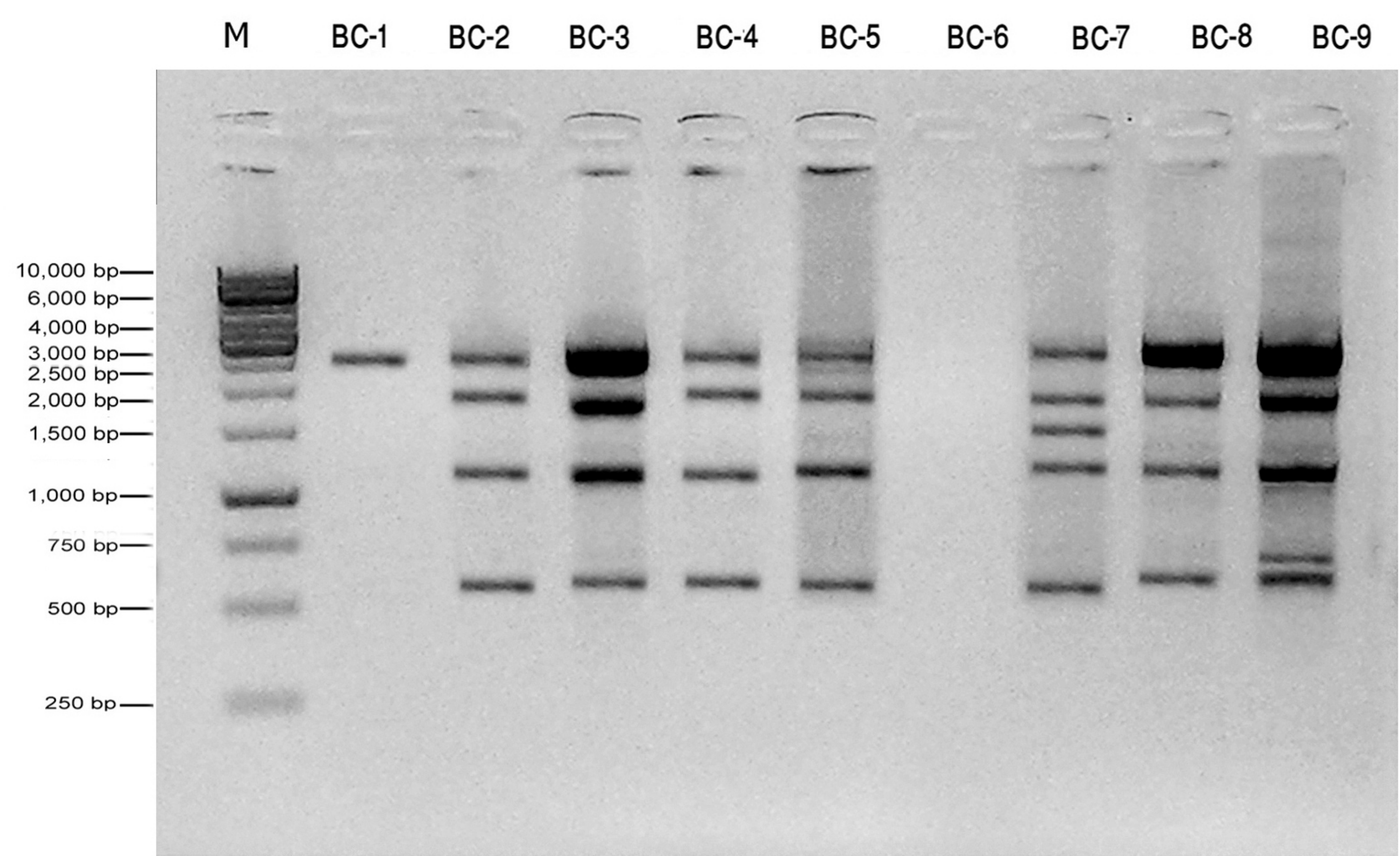
172

Fig. 172. RAPD analysis with primer OPA-7 (5'-GAA ACG GGT G-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



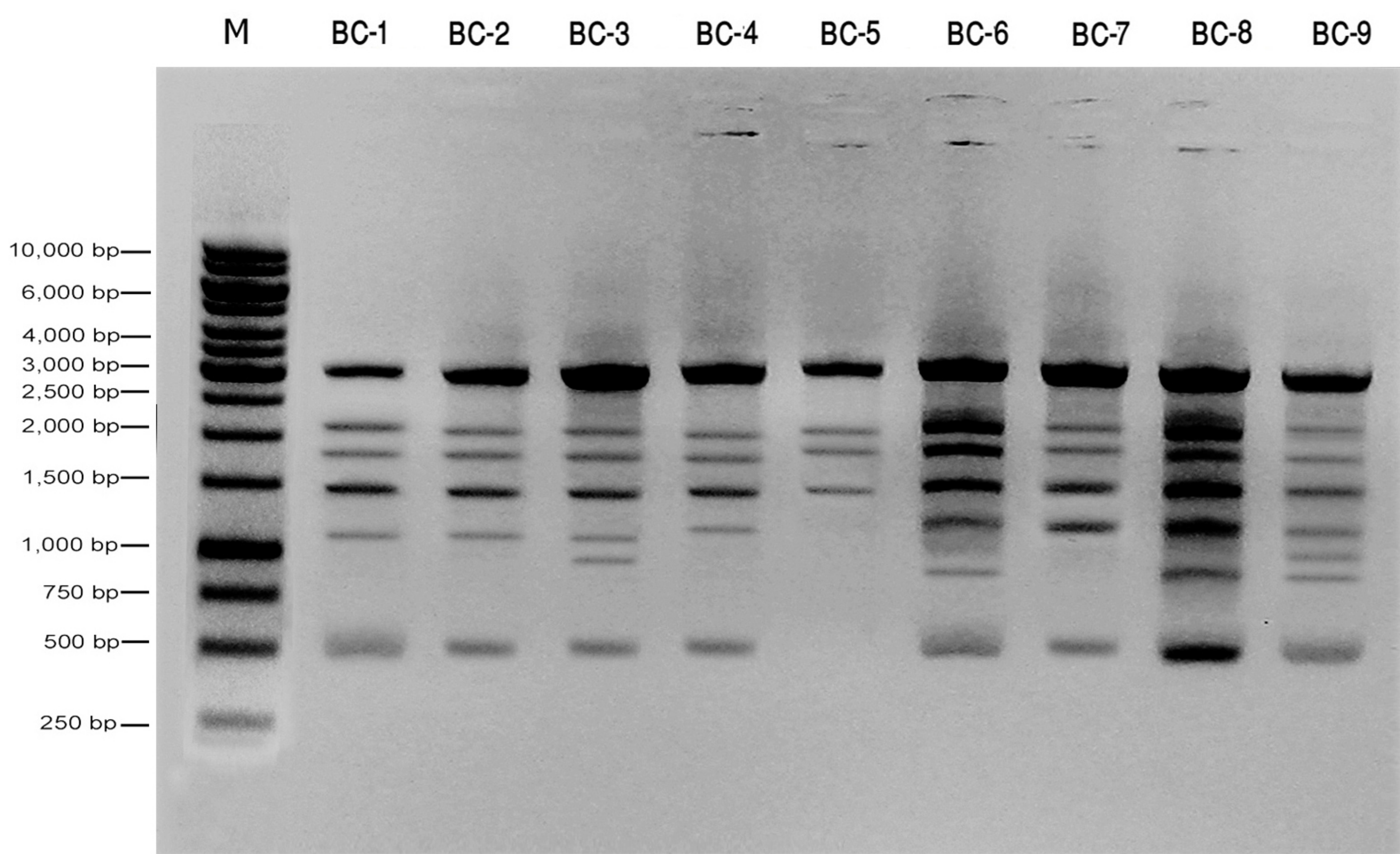
173

Fig. 173. RAPD analysis with primer OPA-8 (5'-GTG ACG TAG G-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



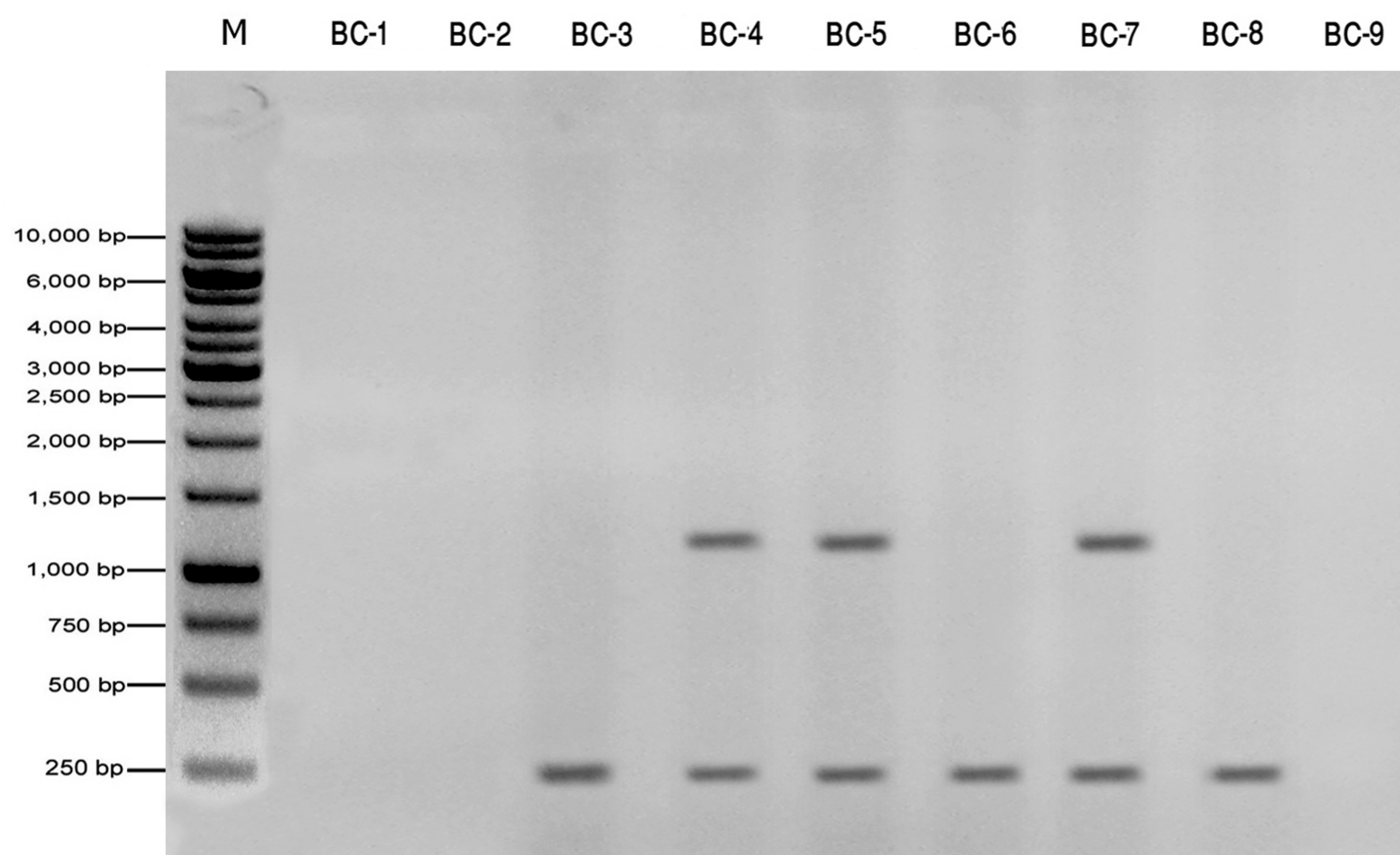
174

Fig. 174. RAPD analysis with primer-2 (5'-GTT GCG ATC C-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



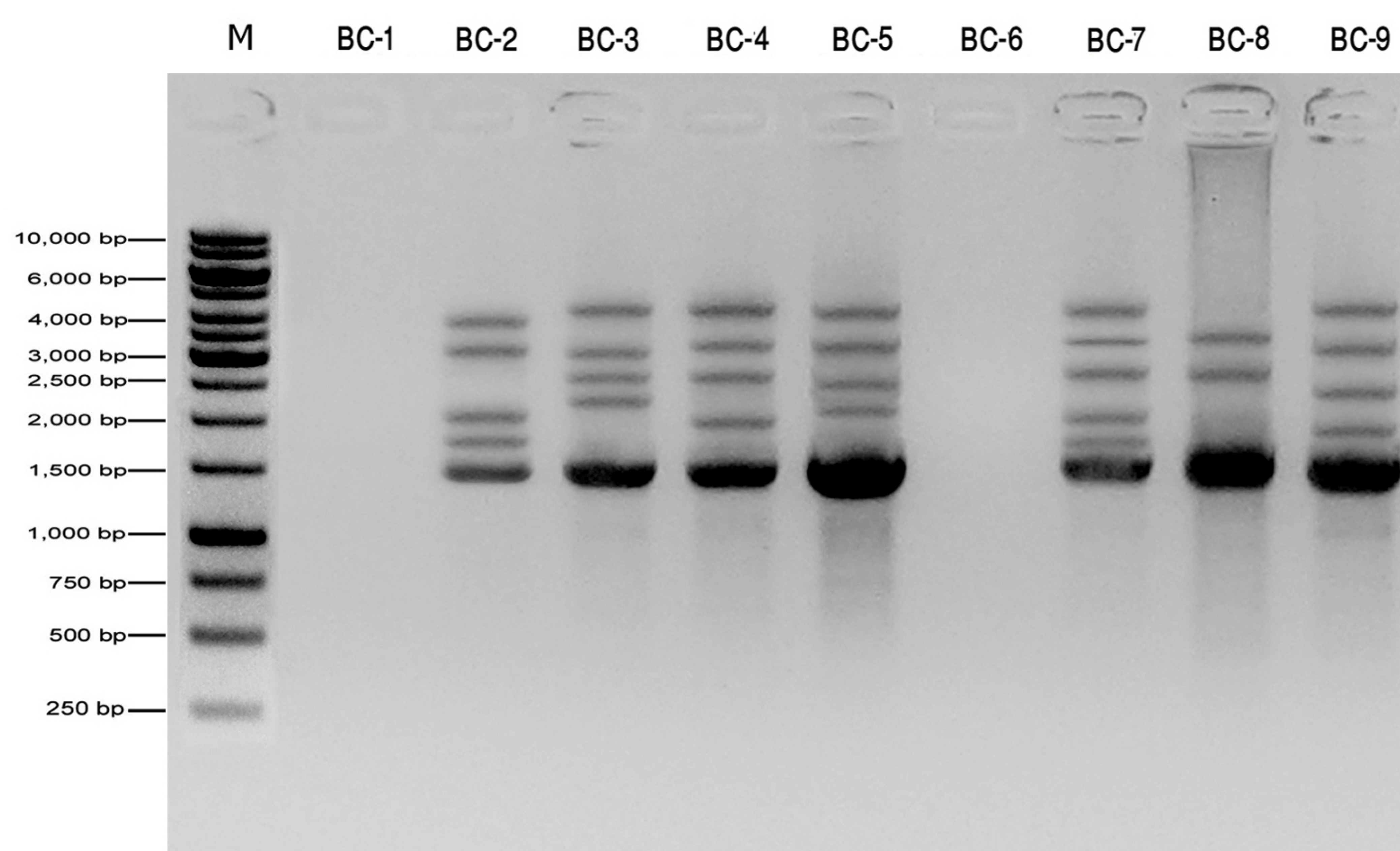
175

Fig. 175. RAPD analysis with primer-12 (5'-GTA TGG GGC T-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



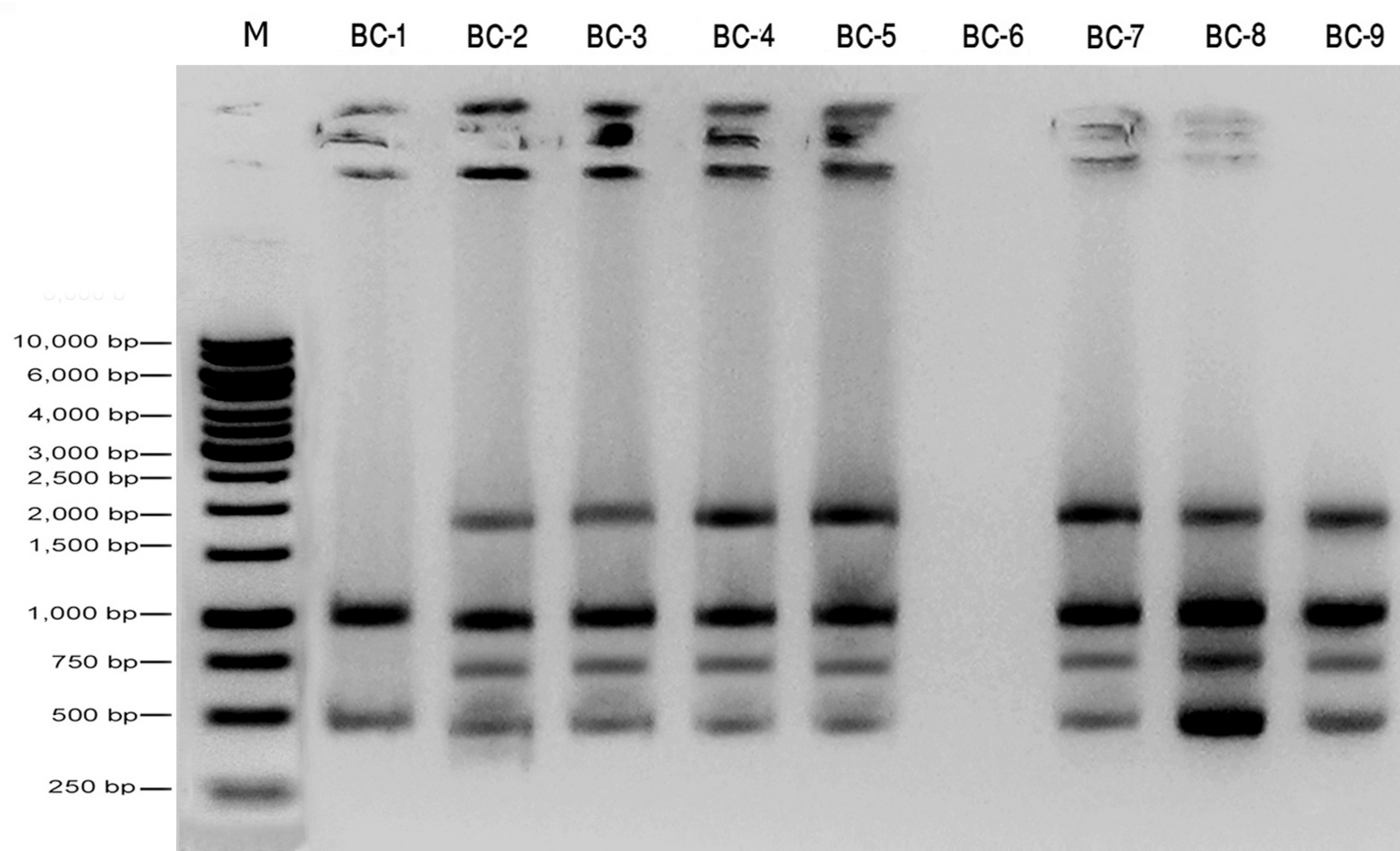
176

Fig. 176. RAPD analysis with primer-18 (5'- GTT TCG CTC C-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



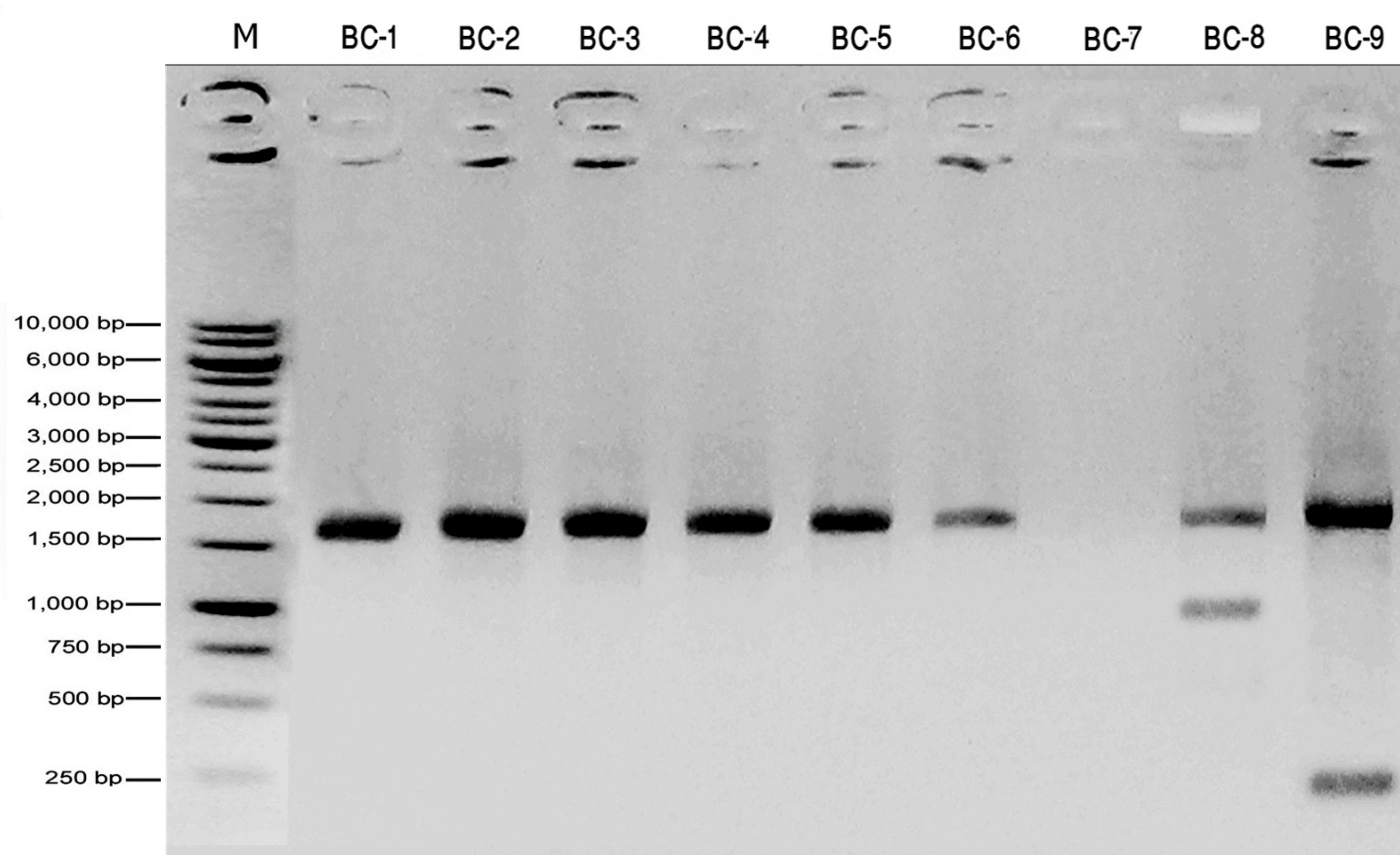
177

Fig. 177. RAPD analysis with primer-19 (5'-GAT GAC CGC C-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



178

Fig. 178. RAPD analysis with primer-23 (5'-GTC AGG GCA A-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.



179

Fig. 179. RAPD analysis with primer-24 (5'-GGT CGG AGA A-3') of nine cultivars of *Cicer arietinum* L. BC-1=BARI Chola-1, BC-2=BARI Chola-2, BC-3=BARI Chola-3, BC-4=BARI Chola-4, BC-5=BARI Chola-5, BC-6=BARI Chola-6, BC-7=BARI Chola-7, BC-8=BARI Chola-8 and BC-9=BARI Chola-9, M=1 Kb DNA ladder.

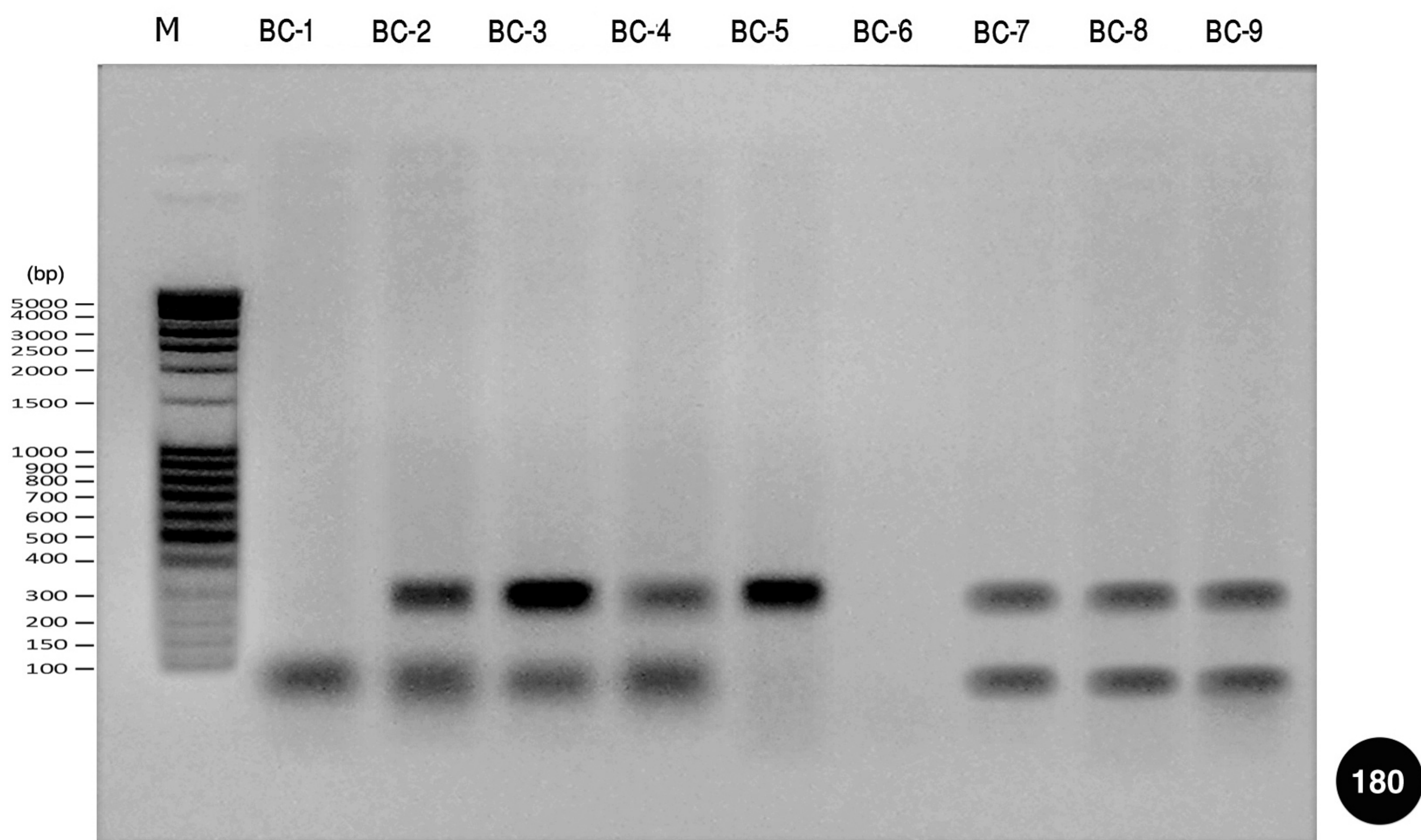


Fig. 180. SSR analysis with primer pairs GA26 CICER: Forward-5'-GTGCAG AGCATCATGCGATGCTCAAGACATCTGCCA-3'- Reverse-5'-TCATACTCAACA AATTCATTTCCC-3' of nine cultivars of *Cicer arietinum* L. M=100 bp DNA ladder.

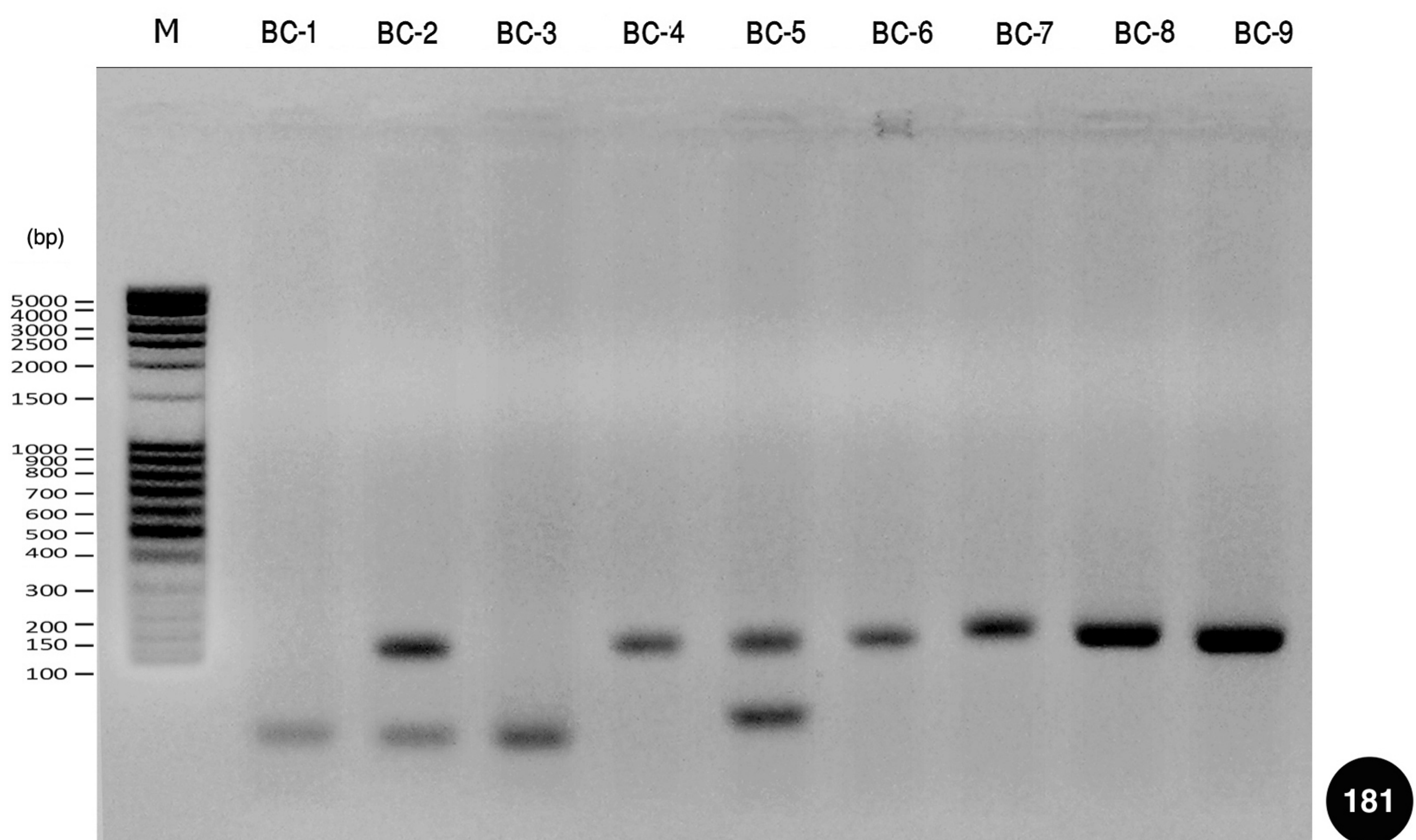
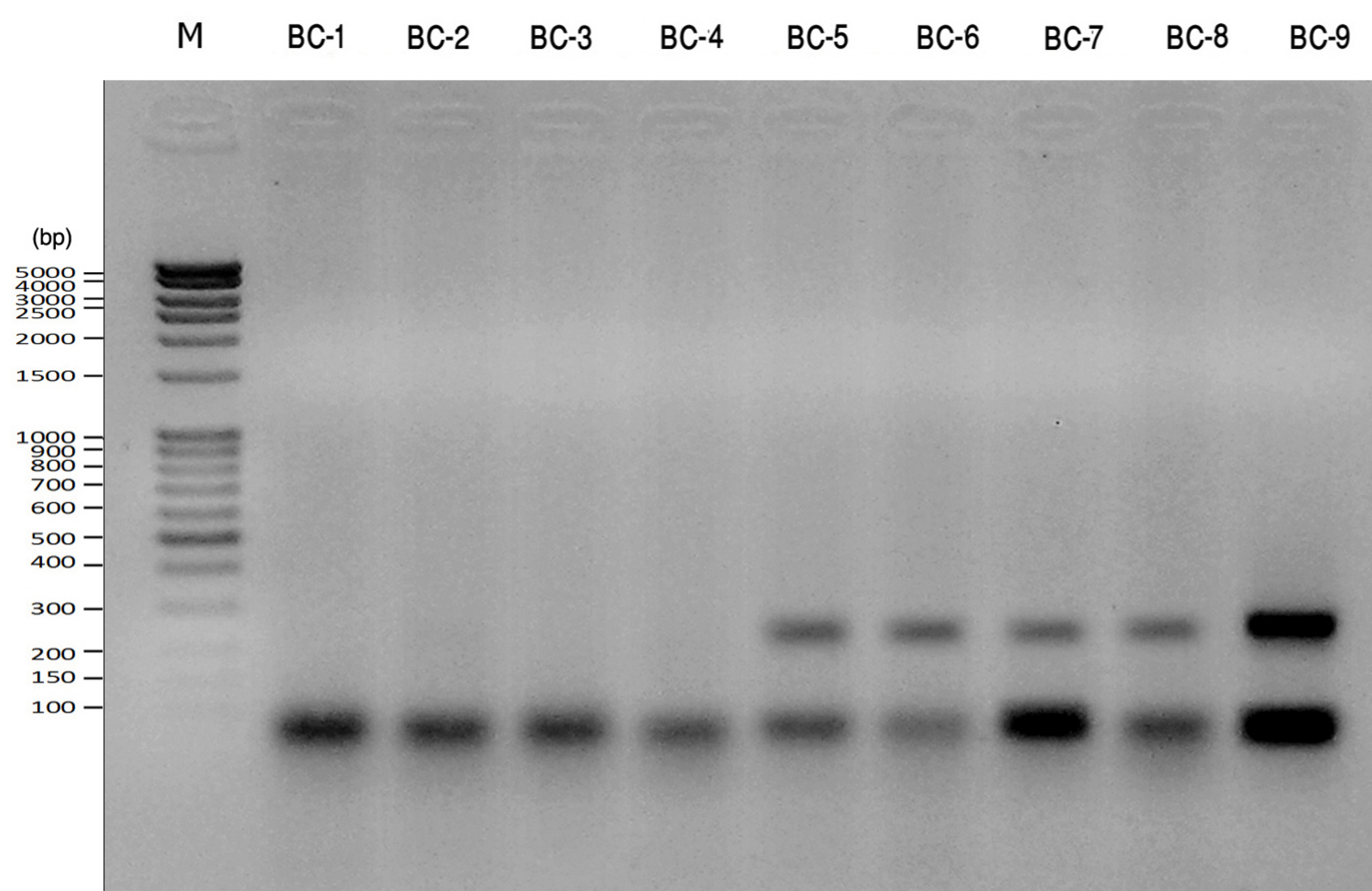
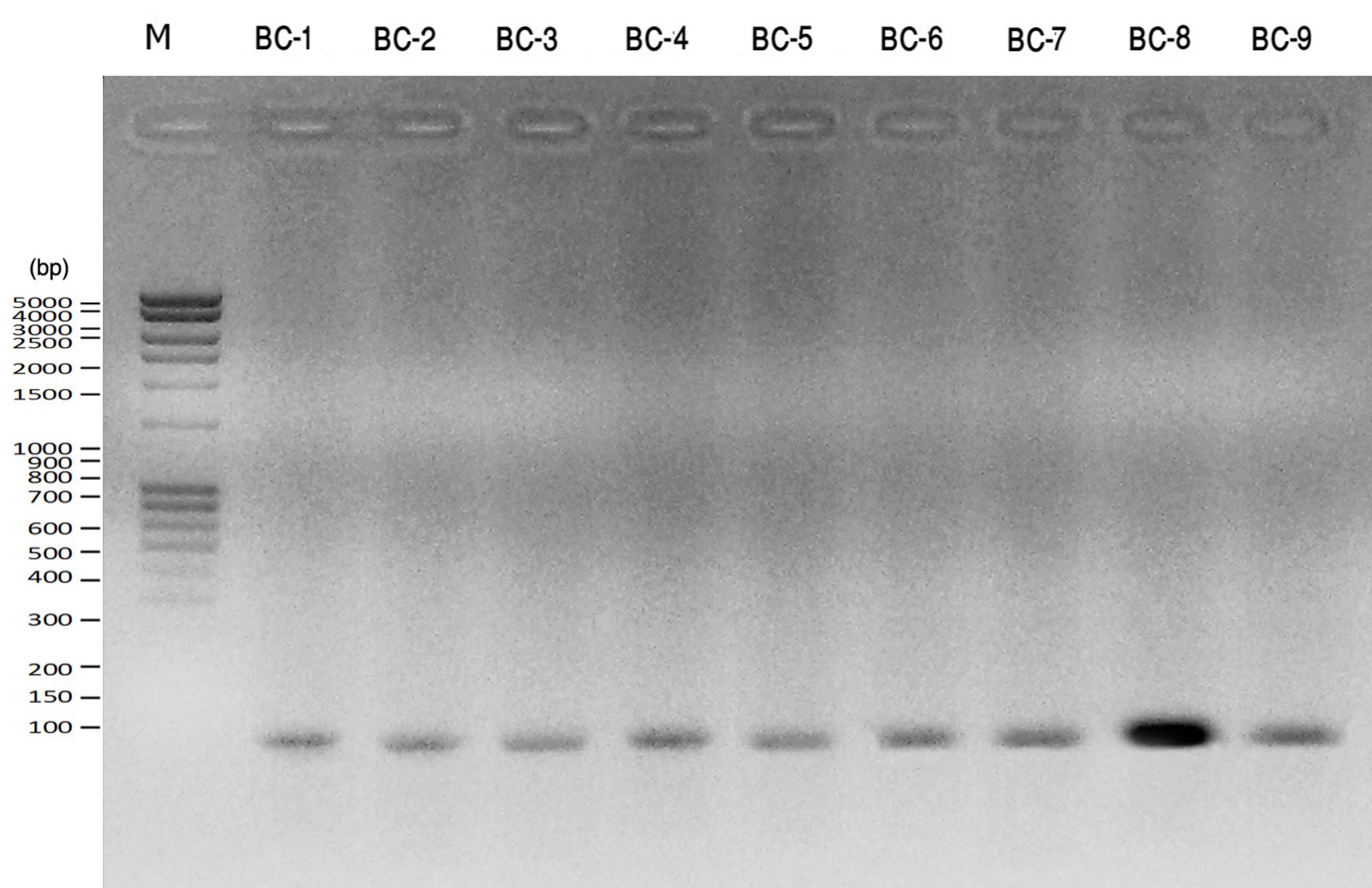


Fig. 181. SSR analysis with primer pairs CASTMS2: Forward-5'-ATTTTACTT TACTACTTTTTTCCTTTC-3'- Reverse-5'-AATAAATGGAGTGTAATTTCATGT A-3' of nine cultivars of *Cicer arietinum* L. M=100 bp DNA ladder.



182

Fig. 182. SSR analysis with primer pairs CASTMS15: Forward-5'-CTTGTGA ATTCATATTTACTTATAGAT-3'- Reverse-5'-ATCCGTAATTTAAGGTAGGTTAA AATA-3' of nine cultivars of *Cicer arietinum* L. M=100 bp DNA ladder.



183

Fig. 183. SSR analysis with primer pairs CASTMS21: Forward-5'-CTACAGT CTTTGTTCTTCTAGCTT-3'- Reverse-5'-ATATTTTAAAGAGGCTTTTGGTA G-3' of nine cultivars of *Cicer arietinum* L. M=100 bp DNA ladder.

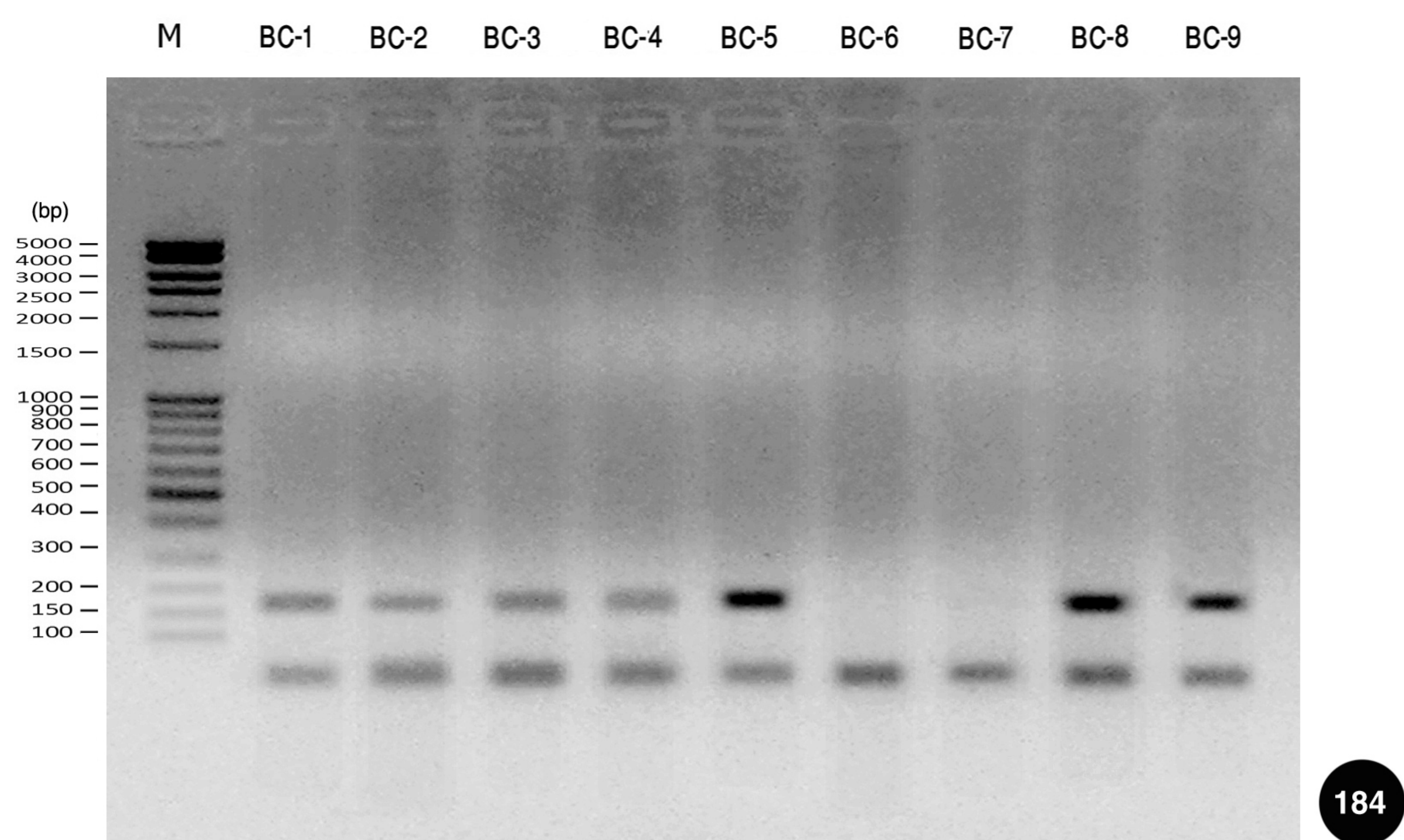


Fig. 184. SSR analysis with primer pairs TA71: Forward-5'-CGATTTAACAC AAAACACAAA-3'- Reverse-5'-CCTATCCATTGTCATCTCGT-3' of nine cultivars of *Cicer arietinum* L. M= 100 bp DNA ladder.

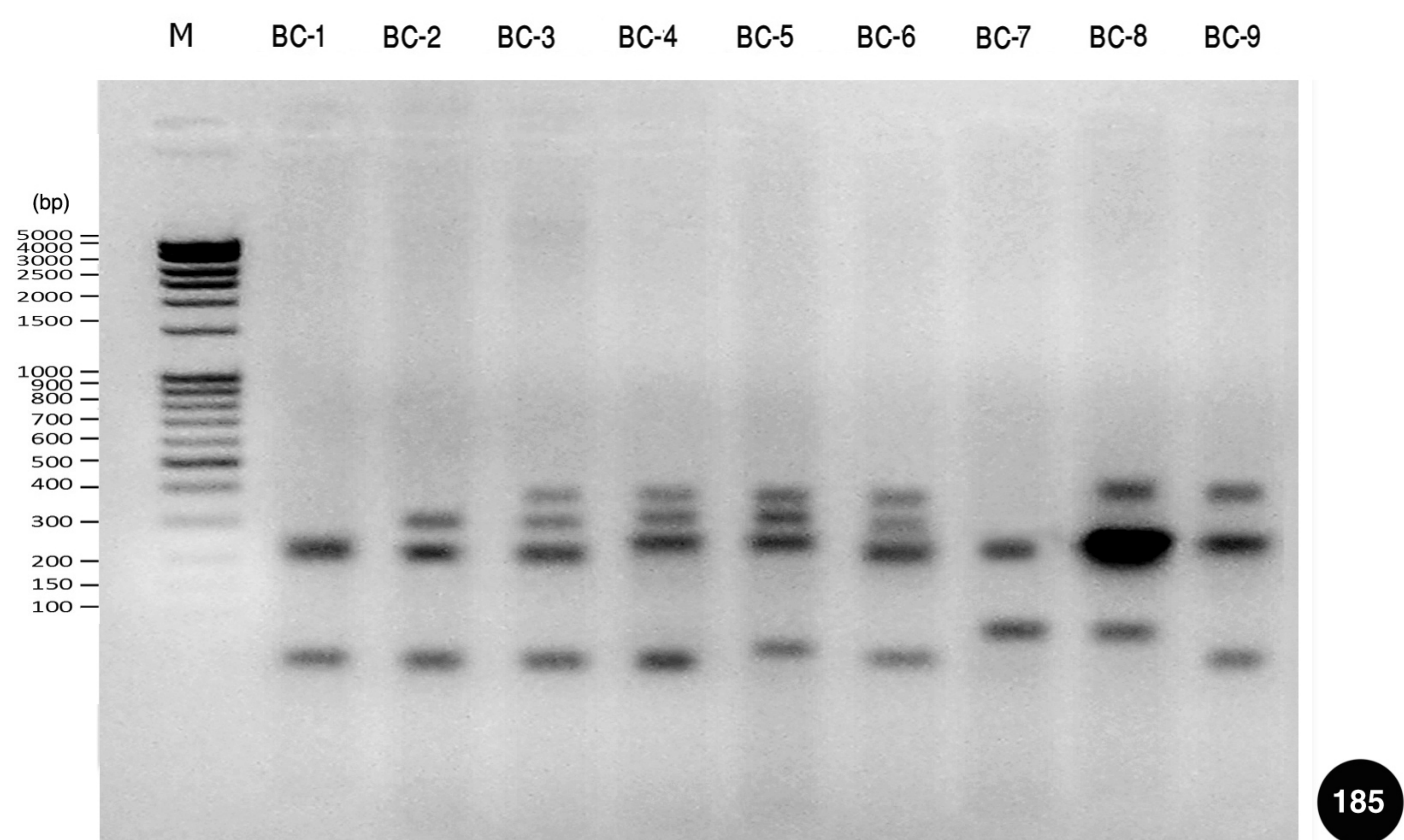
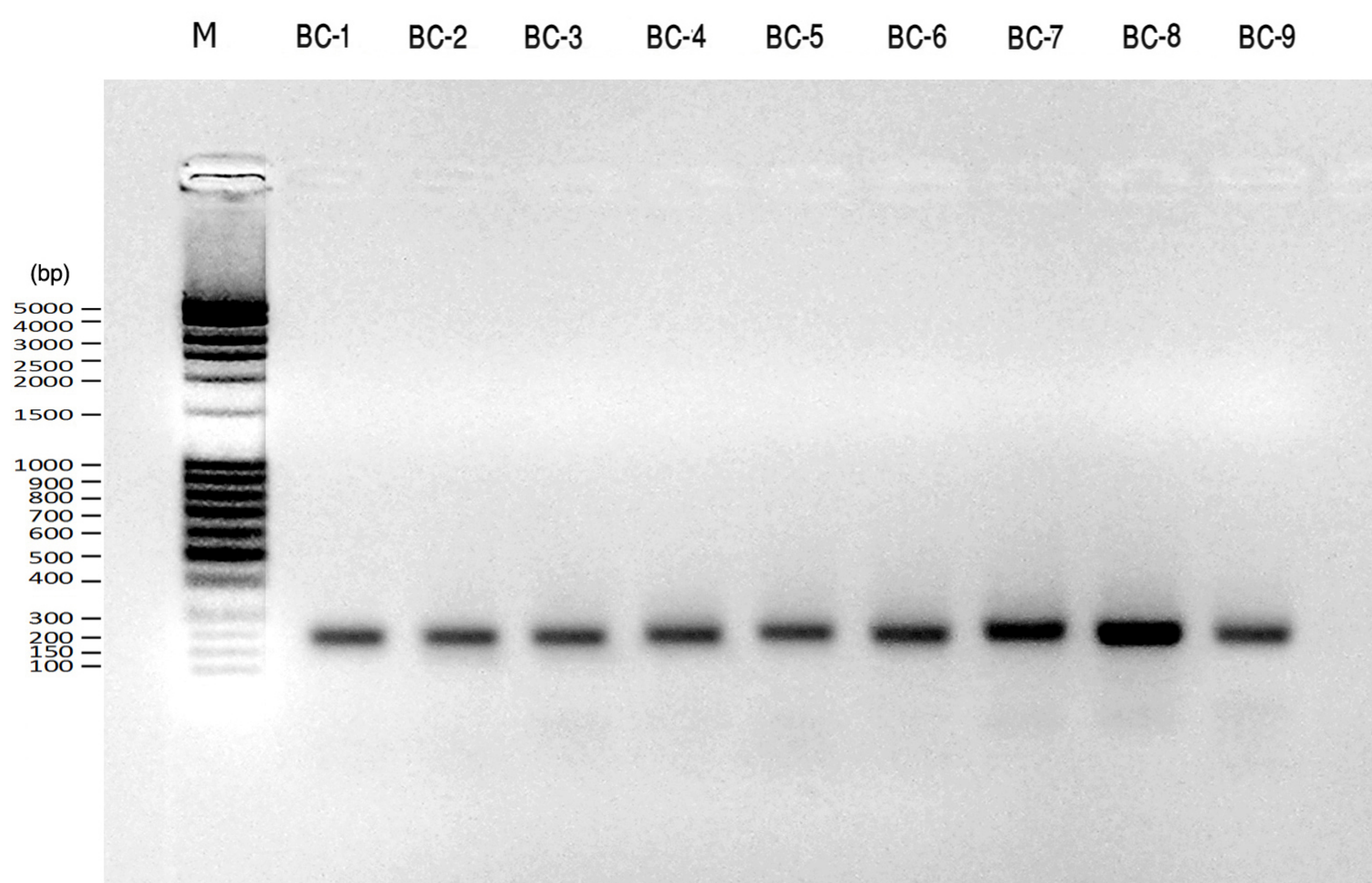
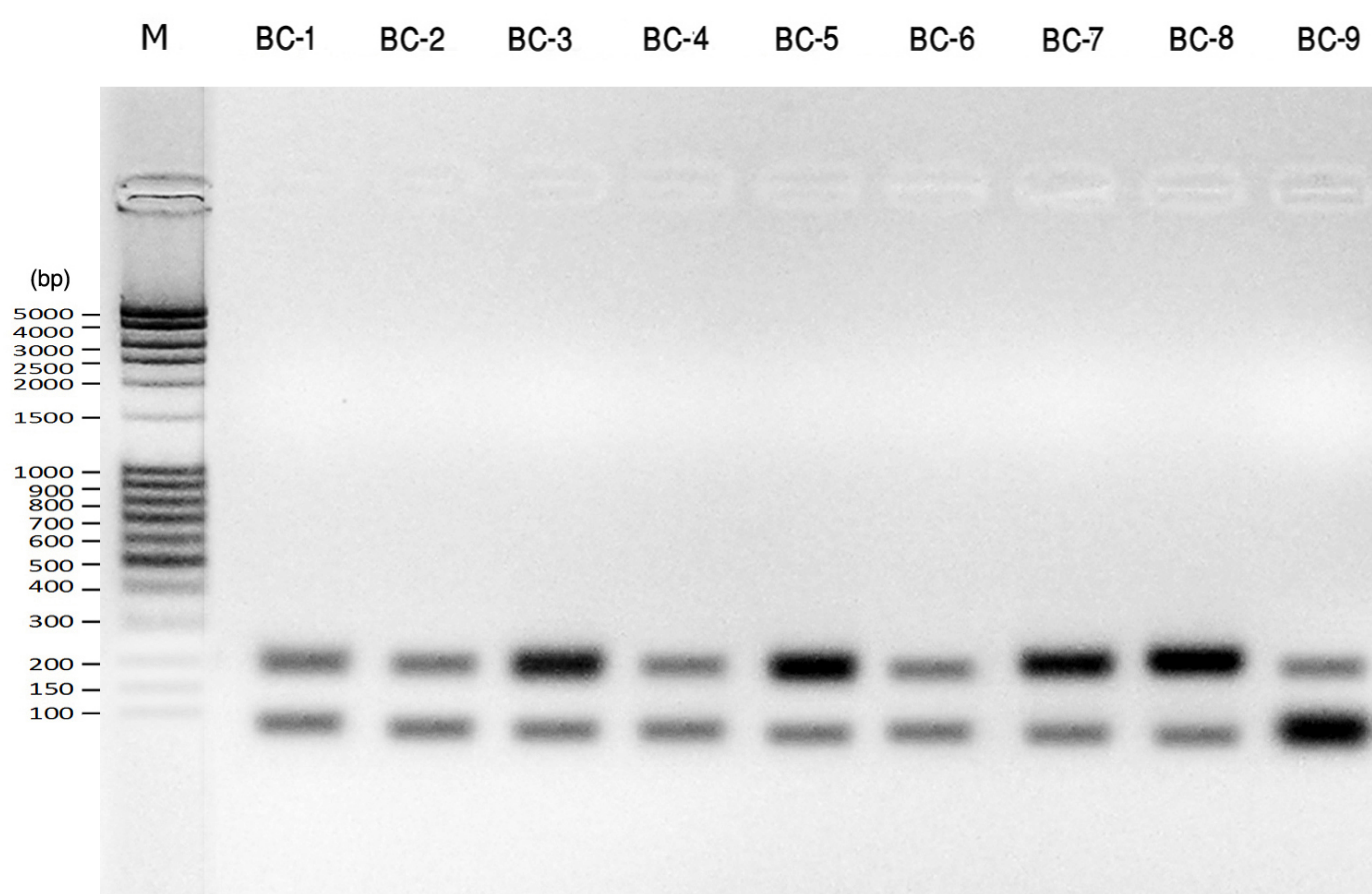


Fig. 185. SSR analysis with primer pairs TA72: Forward-5'-GAAAGATTAA AAGATTTCCACGTTA-3'- Reverse-5'-TTAGAAGCATATTGTTGGGATAAGAG T-3' of nine cultivars of *Cicer arietinum* L. M= 100 bp DNA ladder.



186

Fig. 186. SSR analysis with primer pairs TA130: Forward-5'-TCTTTCTTTGC TTCCAATGT-3'- Reverse-5'-GTAAATCCCACGAGAAATCAA-3' of nine cultivars of *Cicer arietinum* L. M= 100 bp DNA ladder.



187

Fig. 187. SSR analysis with primer pairs TA135: Forward-5'-TGGTTGGAAA TTGATGTTTT-3'- Reverse-5'-GTGGTGTGAGCATAATTCAA-3' of nine cultivars of *Cicer arietinum* L. M= 100 bp DNA ladder.

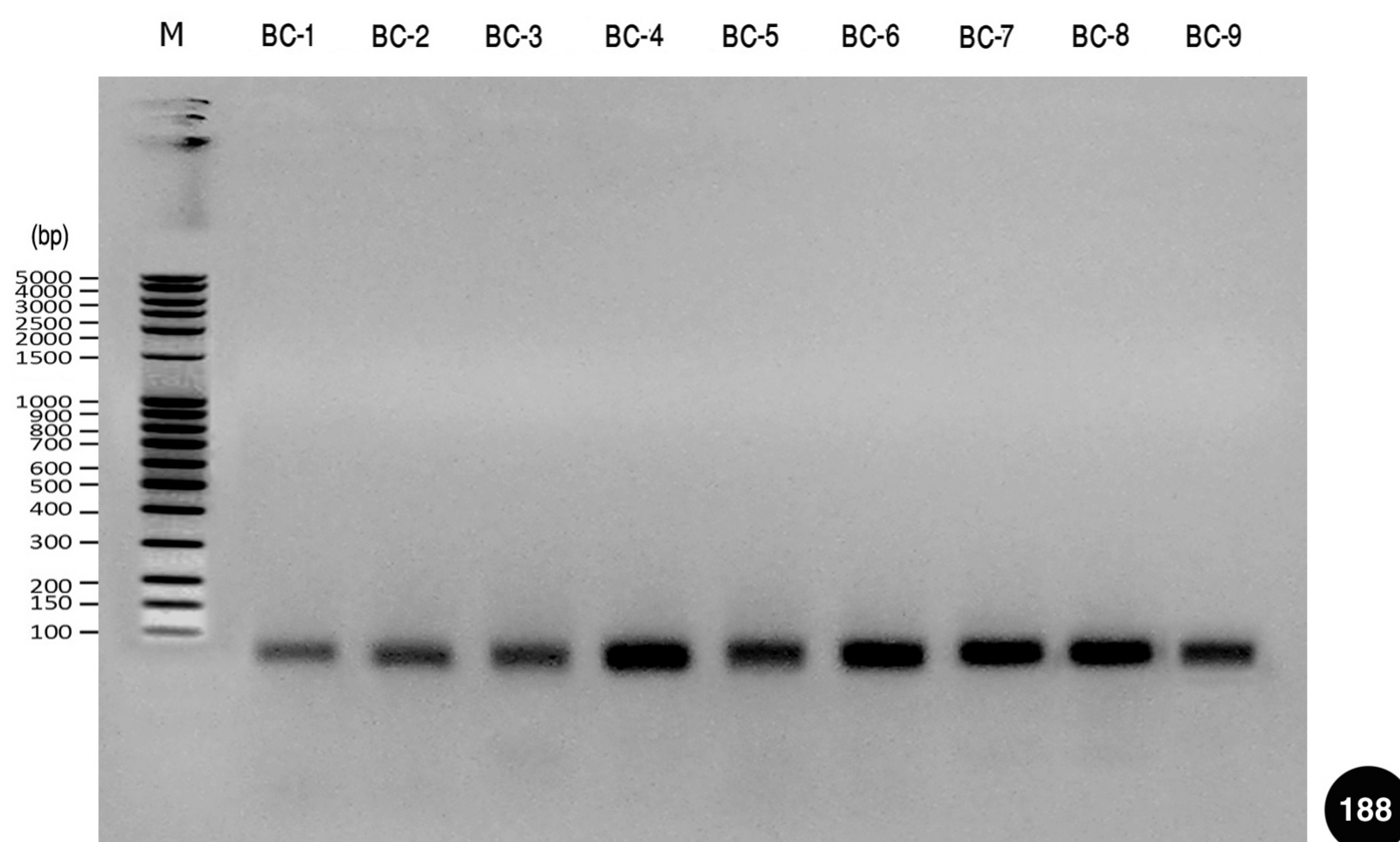


Fig. 188. SSR analysis with primer pairs TR29: Forward-5'-GCCCACTGAAA AATAAAAAG-3'- Reverse-5'-ATTGAACTCAAGTTCTCG-3' of nine cultivars of *Cicer arietinum* L. M= 100 bp DNA ladder.

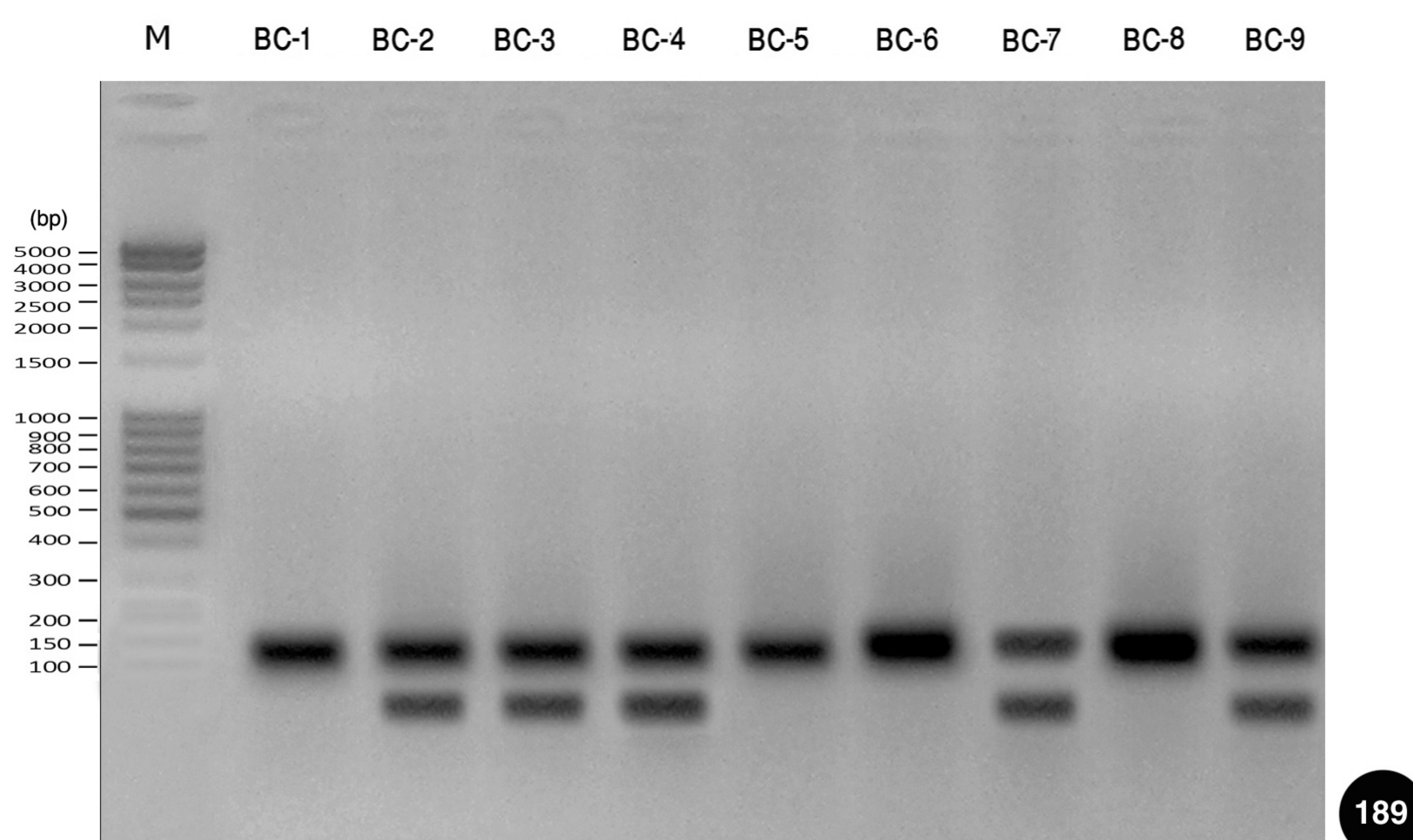


Fig. 189. SSR analysis with primer pairs TA118: Forward-5'-ACAAGTCACA TGTGTTCTCAATA-3'-Reverse-5'-GGAAAGGTTAAGAAATTTACAATAC-3' of nine cultivars of *Cicer arietinum* L. M=100 bp DNA ladder.

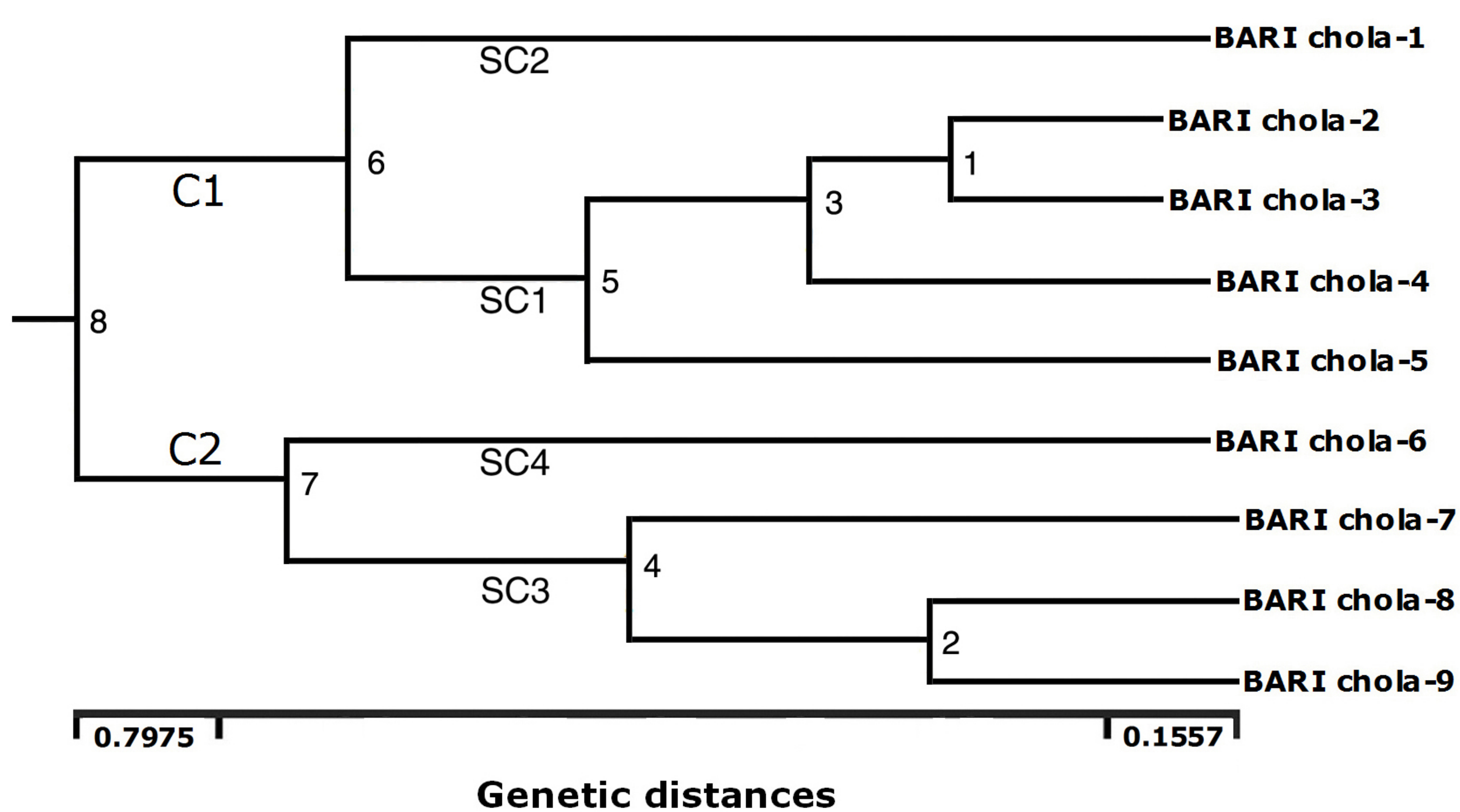


Fig. 190. UPGMA combined dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation among nine cultivars of *Cicer arietinum* L. by RAPD and SSR analysis.

4. DISCUSSION

4. Discussion

Nine cultivars of *Cicer arietinum* L. viz. BC-1 (BARI Chola-1), BC-2, BC-3, BC-4, BC-5, BC-6, BC-7, BC-8 and BC-9 were cytogenetically studied after staining with orcein, CMA and DAPI. The nature of differential staining property of interphase nuclei and prophase chromosomes were accomplished to get additional information. Besides, isozymes, SDS-PAGE analysis and DNA fingerprinting using RAPD and SSR markers were also carried out for molecular characterization.

4.1. The properties of orcein-stained interphase nuclei and prophase chromosomes

The orcein staining properties of interphase nuclei and prophase chromosomes show some characteristic karyomorphological features often helpful in distinguishing different germplasms. This idea was first proposed by Tanaka (1971). The nature of staining of heterochromatins present in the interphase nuclei and prophase chromosomes were different in different species. On the basis of the staining property Tanaka (1971) classified interphase nuclei and prophase chromosomes in five different categories in each case. Later different workers applied these criteria in characterizing different plant materials (Alam *et al.* 1993, Begum and Alam 2004).

In this study, different types of staining properties in the interphase nuclei and the prophase chromosomes were observed in nine chickpea cultivars. These cultivars showed three different types of staining properties of interphase nuclei such as- i) darkly stained small

heterochromatic regions were found in every nucleus of all cultivars except BC-2 and BC-3 (Figs. 19, 22-27). This type of staining was named as "Simple Chromocenter Type" by Tanaka (1971), ii) small heterochromatins were aggregated forming large heteropycnotic bodies in BC-2 (Fig. 20). This character was not found in the other cultivars. Tanaka (1971) considered this feature as "Complex Chromocenter Type" and iii) few smaller heterochromatic bodies were present in the interphase nuclei of BC-3 (Fig. 21). This type of staining was between "Simple and Complex Chromocenter Type".

Prophase chromosomes of BC-1 and BC-8 were stained at the interstitial region. The other regions of these chromosomes were faintly stained (Figs. 46 and 53). This type of staining was named "Interstitial Type" by Tanaka (1971). In BC-2, BC-6 and BC-9, the prophase chromosomes stained in one end and faint to the other end gradually (Figs. 47, 51, 54). Therefore, one end of these chromosomes were much darker than other end. This type of staining was named "Gradient Type" by Tanaka (1971). Rest of the cultivars (BC-3, BC-4, BC-5 and BC-7) were found to possess "Continuous Type" of staining since the prophase chromosomes stained uniformly along the entire length (Figs. 48, 49, 50, 52).

Tanaka (1971) proposed that i) the species possessing "Diffused Type" of interphase nuclei usually show "Continuous Type" of prophase chromosomes, ii) species having "Complex Chromocenter Type" of interphase nuclei possess "Interstitial Type" of prophase chromosomes and iii) the "Simple Chromocenter Type" of interphase nuclei show "Gradient Type" of prophase chromosomes. "Simple

chromocenter type” of staining of interphase nuclei in BC-6 and BC-9 showed “Gradient Type” of staining of prophase chromosomes as per expectation. Except BC-6 and BC-9, the other cultivars did not follow this usual features correlated with the staining property of interphase nuclei and prophase chromosomes.

The probable reasons for the disagreement of Tanaka’s (1971) proposal regarding the staining property of the interphase nuclei and prophase chromosomes might be due to the presence of facultative heterochromatins. The heterochromatin might be condensed in the interphase nuclei and then diffused along the prophase chromosomes instead of localized in a particular region (Afroz *et al.* 2013).

The above findings indicated that although the nine cultivars of chickpea possessed symmetrical karyotype differed sharply in respect of staining property of interphase nuclei and prophase chromosomes. It further indicated that Tanaka’s (1971) above mentioned characterization of the interphase nuclei and the prophase chromosomes may not be applicable in these chickpea cultivars.

4.1.1. Nucleolus

A conspicuous nucleolus was found in interphase, prophase and even late prophase of each cultivar in this study (Figs. 19-27, 46-54). This was a feature of persistent nucleolus. Persistent nucleolus was found in other species *viz* *Spartocera fusca* (Cattani and Papeschi 2004), *Zea mays* (Zirkle 1928), telophase stage in *Oryza sativa* (Ramanujam 1938), *Ceiba pentandra* (Tijo 1948), 16 species of *Cassia* (D’Amato Avanzi 1953), 10 species of *Reseda* (Gori 1956) and 45 species of the family Gramineae (Walter and Emery 1957). This nature might be due

to the late transcription of rDNA to rRNA and late transportation of rRNA from the nucleus to the cytoplasm. Therefore, persistent nucleolus was a karyomorphological characteristic feature of chickpea.

4.2. Orcein-karyotype

4.2.1. Diploid (2n) Chromosome number

The nine cultivars of *Cicer arietinum* studied in the present investigation were found to possess $2n=16$ chromosomes (Figs. 100-108). The present findings agreed with the previous reports (Mercy *et al.* 1974, Ladizinsky and Adler 1976, Ahmad and Godward 1980, Lavania and Lavania 1982, Sharma and Gupta 1983, 1986, Kutarekar and Wanjari 1983, van der Maesen 1987, Mukherjee and Sharma 1987, Ahmad *et al.* 1987, Venora, 1991, 1995, Ohri and Pal 1991, Galasso and Pignone 1992, Ocampo *et al.* 1992, Ahmad and Hymowitz 1993, Tayyar *et al.* 1994, Galasso *et al.* 1996, Ahmad 2000). However, there were some reports on different $2n$ chromosome numbers of this species *viz.* $2n=14$ (Fedorov 1969, Dixit 1932), $2n=24$ (Meenakshi and Subramaniam 1966), $2n=32$ (Phadnis and Narkhede 1972, Oke 1955, Sen and Jana 1956, Meenakshi and Subramaniam 1967) and $2n=33$ (Sen and Jana 1956, Meenakshi and Subramaniam 1967). The diploid chromosome number $2n=24$ and 32 might be considered as triploid and tetraploid, respectively. There are two probable reasons for obtaining $2n=33$ chromosomes such as i) either this was due to miscounting the chromosome number of $2n=32$ since chromosomes were very small in size or ii) a special kind of numerical aberration. The reason for obtaining $2n=14$ chromosomes was not clear, however there also two probable explanations – i)

either the specimen was a different cytotype of *Cicer arietinum* or ii) might be a double monosomic. Therefore, ignoring the few exceptions, the diploid chromosome number of *Cicer arietinum* was almost stable and conserved.

The 16 chromosomes of nine cultivars of chickpea were metacentric with the centromeric formula of 16m (Figs, 100-108, 109-117, Table 20). Hejazi (2011) reported 16 metacentric chromosomes in *Cicer arietinum*. Presence of all metacentric chromosomes is a feature of symmetric karyotype (Stebbins 1971). Symmetric karyotype is generally primitive in nature. Therefore, *Cicer arietinum* may be considered as primitive.

Besides, reports on different centromeric formulae of this species were available such as 8m+ 8sm (Kordi *et al.* 2006) and 10m+ 6sm (Ahmad 2000, Venora *et al.* 1995). The different centromeric formula indicated the occurrence of structural aberration within the genome of *Cicer arietinum*.

4.2.2. Satellite

A pair of satellites was observed one in each member of pair I in nine cultivars (Figs. 100-108, arrow). Different numbers of satellites were also reported such as i) two pairs (Kutarekar and Wanjari 1983, Mercy *et al.* 1974, Ahmad and Godward 1980, Sharma and Gupta 1983, Ahmad *et al.* 1987) and ii) three pairs (Mukherjee and Sharma 1987). In contrast, no satellite was reported for this species (Sharma and Gupta 1986, Ocampo *et al.* 1992). This observation clearly indicated the gradual loss of satellited portion from the genome of *Cicer arietinum* L.

4.3. Fluorescent banding analysis

Chromomycin A₃ (CMA) and 4'-6-Diamidino-2-Phenylindole (DAPI) are two fluorochromes specific to GC-and AT-rich base specific segments, respectively (Schweizer 1976). Fluorescent banding gives decisive analysis of karyotype, even chromosome having similar morphology and other conventional karyotypic features. In this study, these two fluorochromes were used for critical analysis of karyotypes of nine cultivars of chickpea.

4.3.1. CMA-banding pattern

CMA-banding patterns of nine chickpea cultivars were discussed under different heads-

4.3.1.1. Nature of CMA-bands

Most of the CMA-bands were thick, prominent and present at the terminal regions of respective chromosomes in nine chickpea cultivars (Figs. 82-90, 118-126, 127-135, Table 21). The presence of terminal CMA bands indicated a tendency of accumulating GC-rich repetitive sequences at the chromosomal ends (Zaman and Alam 2009). In addition to terminal bands, few CMA bands were found at centromeric regions of the respective chromosomes in BC-2, BC-3, BC-4, BC-7 and BC-9 cultivars revealing the presence of GC repeats in those regions (Figs. 119, 120, 121 and 126). The centromeric bands were smaller than the terminal bands except a band in BC-3 (Fig. 120, arrow). Few dots like terminal bands were also observed in BC-6 and BC-8 (Figs. 125, 123). Terminal CMA positive bands were found in *Solanum nigrum* (Sultana and Alam 2007), *Capsicum frutescens* L. (Shema et

al. 2013) and 3 species of *Solanum* (Alam *et al.* 2013). These finding indicated that GC-rich repeats were preferentially distributed at the terminal regions of chromosomes.

4.3.1.2. CMA Karyotype

Prominant CMA-bands were present at the terminal regions of first pair in five cultivars (Figs. 118, 119, 120, 122 and 123). Besides this similarity the nine cultivars of chickpea have distinct CMA banding pattern. The number, location, distribution and intensities of CMA band varied in different cultivars (Figs. 82-90, 118-126). On the basis of number and location of CMA-bands, karyotypes formulae were prepared for each germplasms. It has seen that each cultivar has distinct CMA-banded karyotype formula (Table 21). Moreover, the percentage of GC-rich region was ranging from 2.66 (BC-8) to 14.52 (BC-2) (Table 21). This finding was used as another parameter for karyotype analysis.

4.3.1.3. Heteromorphicity

After CMA-staining, heteromorphicity was found in chromosome pair II of BC-2. In this pair, one chromosome had upper terminal band and an interstitial band was present in its homologue, indicated a paracentric inversion either from terminal to interstitial or vice versa (Fig. 119).

Pair II of BC-3, pair I and V of BC-4, pair VIII of BC-6, pair VI and VII of BC-7, pair I and VI of BC-8 and pair I, III, IV and VIII of BC-9 showed heteromorphicity in respect of CMA banding pattern. In these pairs, a member had a CMA band while no such band was observed in

its homologue suggesting deletion of the banded region from the respective chromosomes (Figs. 120, 121, 123, 124, 125 and 126).

Why deletion of this portion did not affect the cultivar might be a question. The probable reason was that those portions composed of heterochromatin and thus did not have any impact on the plant (Khatun *et al.* 2011).

4.3.2. DAPI- banding pattern

DAPI-banding patterns of nine chickpea cultivars were discussed under different heads-

4.3.2.1. DAPI-karyotypes

The number of DAPI bands less than those of CMA and ranging from one to four among nine cultivars. The cultivar BC-3 had no band. Only one band was found in BC-4 and maximum (4) bands in BC-1, BC-5, BC-6, BC-7 and BC-9 (Figs. 136, 140, 141, 142 and 144). The DAPI bands were more or less equally located at the terminal and centromeric regions of respective chromosomes of different cultivars.

Schweizer (1976) first reported that DAPI bands revealed the presence of AT-rich repeats. Later many workers supported this idea (Alam and Khanam 2005, Alam and Zerin 1998, Hizume *et al.* 1988, Alam and Kondo 1995). Silva *et al.* (2010) reported that centromeric DAPI band was AT-rich. They also mentioned that the DAPI banded areas were heterochromatic in nature. Unlike SAT and telomeric sequence, centromeric sequences were not conserved (Farr 2004). King (1980) mentioned that the centromeric sequence may be euchromatic or heterochromatic in nature. In the present

investigation, the centromeric DAPI bands revealed the existence of heterochromatic regions while the non-banded centromeres as euchromatic parts of the respective chromosomes.

Although the DAPI bands of nine cultivars were present either at the centromeric or terminal regions, each cultivar differed regarding the size, number, position and intensity of DAPI bands (Figs. 91-99, 136-144). The percentage of AT-rich region was also variable among the cultivars (Table 22). Few cultivars could be identified by unique DAPI banded chromosomes. For example, one member of pair III in BC-1 had dot like terminal band on upper and lower arms (Fig. 136). In BC-5 and BC-9, two chromosomes were fluoresced almost throughout the length (Figs. 140, 144). These chromosomes could easily be identified from the rest and used as marker for the respective cultivars. On the basis of DAPI banding patterns it was possible to construct karyotype formula. Each cultivar has characteristic DAPI banded karyotype formula (Table 22).

4.3.2.2. Heteromorphicity

Heteromorphicity in respect of DAPI band was found in three cultivars viz. BC-1, BC-4 and BC-5. In these cultivars, DAPI bands were found only in a member of respective pairs whereas no such band present in their homologue member. There may be two possible reasons for this heteromorphicity – i) either the chromosomes are naturally made up in such a way or ii) the banded area may be deleted from the respective chromosomes.

Heteromorphicity in DAPI band was reported earlier by several workers in different specimen (Afroz *et al.* 2013, Zaman and Alam

2007, Khatun and Alam 2011). In these reports the authors mentioned that deletion was the result for heteromorphicity.

In cultivar BC-1, a member of pair III has similar size DAPI bands on both the terminal ends. No band was found in any terminal end of its homologue member. Moreover, one terminal end of the non-banded chromosome looked smaller when compared to the banded one (Fig. 136, arrow). The probable reason of this heteromorphicity might be unequal crossing over of that portion.

4.3.2.3. Satellite

A pair of satellites was found one in each member of pair I in all cultivars except BC-1, BC-5 and BC-7 after DAPI staining (Figs. 137, 138, 139, 141, 143, 144, Table 22 and arrow). In contrast, satellites in these pairs were found in all cultivars after orcein staining. On the other hand, no satellite was found in any cultivar after CMA staining. Since prominent satellites were found with orcein staining, in principle satellite should be observed after CMA and DAPI staining. In this investigation, no satellite could be detected with CMA staining and only six cultivars showed satellite after DAPI staining.

This preferential staining revealed that these satellites were stain specific. Alam and Kondo (1995) also reported on the nature of a satellite and a small chromosome in *Drosera ericksonea*. They carried out sequential staining of the same metaphase plate with Giemsa, CMA and DAPI. They found a satellite and a small chromosome in Giemsa and DAPI, whereas it was not observed in CMA. From these observations, they suggested that there were stain specific satellites and chromosomes. In the present study, the pair of satellites those

were not observed after staining with CMA might possess some kind of DNA sequences which made it stain-specific. However, no report about stain specific satellite was available for *Cicer arietinum* L.

4.4. SDS-PAGE and Isozyme analysis

In the present investigation, a total number of 12 SDS-PAGE bands was observed among nine cultivars of chickpea (Figs. 164, 165, Table 32). Although most of the bands were common in these cultivars, some polymorphism regarding banding pattern could be recorded. Band number 6 and 7 were much darker in BC-2 and BC-6. Band number 7 of BC-8 was so thick that could be easily isolated from the same band of different cultivars (Figs. 164, 165). Band number 9 was only present in BC-2, BC-6, BC-8 and BC-9. Parker *et al.* (1998) mentioned that SDS-PAGE was a powerful tool in studying population genetics. Since storage proteins (seed proteins) are not affected by environmental fluctuation, many workers used SDS-PAGE profiling technology as a reliable tool for authentic characterization of germplasm. Moreover, Jha and Ohri (1996) reported SDS-PAGE patterns as promising tool for distinguishing cultivars of particular species. In contrast, other workers reported that cultivar identification was not possible with the SDS-PAGE method (Ahmad and Slinkard 1992, De Vries 1996). In this investigation, it was possible to characterize some chickpea cultivars with SDS-PAGE protein markers and thus became a reliable tool for chickpea diversity study.

Three isozyme systems *viz.* acid phosphatase, esterase and peroxidase were carried out in this study with a view to identify genetic diversity in nine chickpea cultivars. The isozyme patterns were

genetically controlled and remained constant for cultivar identification at different conditions (Substain *et al.* 1996, Maquet *et al.* 1997). Isozyme characterization is becoming a supplement to morphological characterization because of its speed, repeatability, stability and relative independence of environmental conditions (Tanksley and Orton 1983, Hamrick and Godt 1997).

In this study, the highest seven bands were found in esterase system. Besides common bands, few specific bands were found in these cultivars. Band number 1 of BC-8 was much bigger than the other band number 1 of different cultivars (Figs. 157, 158). In BC-2, the same band (band number 1) was the thinnest among these bands. Band number 2 was only present in BC-9 (Figs. 157, 158). These bands could be used as markers of these three chickpea cultivars. Band number 3 and 6 were absent in BC-1, BC-3, BC-4 and BC-5. Therefore, esterase banding profile showed distinct polymorphism among nine cultivars. On the other hand, no significant polymorphism was found in acid phosphatase and peroxidase banding profile (Figs. 154, 155, 160 and 161). Sarker and Haque (1996) observed similar pattern in esterase and peroxidase systems. Therefore, activities of esterase would be a useful tool for characterizing chickpea cultivars.

An attempt was undertaken to determine the extent of genetic affinities and distance among nine chickpea cultivars. For this reason, a combined dendrogram was prepared on the basis of protein and isozyme markers. The dendrogram clearly indicated close affinity between BC-8 and BC-9 (Figs. 156, 159, 162, 163 and 166). These two cultivars are genetically far away from the rest and thus made

separate cluster. This information will be helpful for future breeding of chickpea.

4.5. DNA fingerprinting

The nine chickpea cultivars selected for the present study represented a broad spectrum of variation for several phenotypic traits. DNA from the nine cultivars of chickpea was studied with twelve oligonucleotide primers and ten microsatellite primer pairs for RAPD and SSR assay, respectively.

4.5.1. DNA Fingerprinting by Random Amplification of Polymorphic DNA (RAPD)

RAPD is a PCR based marker technique that has been used for estimation of genetic diversity of populations and for studying the genetic relationships among different genotypes (Esmail *et al.* 2008).

4.5.1.1. Primer selection

The main aim of this study was to investigate the potential of RAPD markers in assessing the genetic variation in nine cultivars of *Cicer arietinum* L. A total of thirty five primers was used initially in this study those differed greatly in their efficiency for revealing polymorphism. Twenty three of them failed to amplify DNA. Since all of the reaction parameters were identical for all primers, differences in the clarity of the banding patterns are likely due to the specific requirements (such as PCR reagents or temperature profile) of a primer. Weeden *et al.* (1992) also reported variation in the efficiency of primer amplification. Fritsch *et al.* (1993) demonstrated the importance of the G+C content of primers on the PCR yield of

detectable on amplified products. However, no correlation between G+C content and the clarity of the banding pattern was noted in the present study.

On the other hand, the remaining 12 primers generated reproducible RAPD patterns (Table 46). This suggested that 12 primers could be able to bind with suitable priming sites of the genomic DNA. Moreover, this revealed the proper adjustment of PCR parameters (reagents and thermocycler parameters) and thus optimized the amplification (Devos and Gale 1992). Therefore, these twelve primers have been selected for subsequent analysis.

4.5.1.2. Polymorphism on the basis of RAPD pattern

In the present study, the variation among the chickpea cultivars was assessed with RAPD markers. Twelve RAPD primers amplified a total of 351 DNA fragments with an average of 29.25 fragments per primer. Band size ranging from 250 to 10000 bp of PCR amplification products scored for all primers. Among the twelve primers OPA-8 and primer-12 produced the highest number (57) of polymorphic bands. In contrast, the primer-18 generated the least number (9) of polymorphic bands (Table 46). Light and bright bands were produced in the RAPD reactions. Light bands produced from low homology between the primer and the pairing site on the DNA strand (Thormann *et al.* 1994) (Tables 34-45).

Out of the total amplified fragments, only 45 were common and the remaining 306 (91.61%) were polymorphic indicating high level of diversification (Table 46).

Several workers carried out RAPD analysis on chickpea. Rasool (2013) and Datta *et al.* (2010) found 98.14% and 87.00% polymorphism in chickpea genotypes, respectively. In contrast, only 14.56% polymorphic products with 10 polymorphic primers were observed among 29 elite chickpea cultivars by Sant *et al.* (1999). Moreover, Sonnante *et al.* (1997) observed 25.5% polymorphic products among six chickpea accessions using 16 polymorphic primers. These results revealed that chickpea genotypes showed both high and low level of polymorphism. High level and low level RAPD polymorphisms suggested a wide range of diversification existing among different genotypes of chickpea. This information would be useful for improved breeding program of chickpea.

4.5.1.3. Unique RAPD markers

In addition to polymorphism, 19 unique RAPD sequences were identified in nine chickpea cultivars using twelve different primer combinations. The term unique sequence means that the sequence found in a cultivar with a certain primer was absent in other cultivars (Figs. 169, 170, 171, 172, 173, 174, 179, Table 46). The unique bands were stable and specific for the respective cultivars and thus could be used as a tool for characterization. In the earlier literature, there was no information about unique band (Rasool 2013, Datta *et al.* 2010). The earlier authors considered all bands as polymorphic band. The unique band has a number of potential applications including the determination of cultivar purity, efficient use and management of a genetic resource collection, particularly the identification of mis-labelled accessions, and the establishment of property rights (plant variety protection and patenting).

4.6. DNA fingerprinting by Simple Sequence Repeats (SSRs)

Ten SSR primer pairs were used in this study of which all generated well-defined and reproducible polymorphic bands. The primer sequence, band size and banding pattern of nine chickpea cultivars were given in Tables 47-57 and in Figs. 180-189.

4.6.1. Polymorphism as detected by SSR analysis

The ten primer pairs generated 143 distinct bands of which 62 were considered as polymorphic and thus showed 43.36% polymorphisms which indicated the moderate level of polymorphisms (Table 57). Datta *et al.* (2010) studied 93% average polymorphism among chickpea and pigeonpea genotypes. Like RAPD, a wide range of SSR polymorphism indicated broad diversity within chickpea cultivars. The size of band was ranging from 20 to 380 bp (Table 57). Out of ten, the primer pair TA72 produced highest number (20) of polymorphic bands followed by 14 with primer pair GA26 CICER (Figs. 180, 185, Tables 47, 52). In contrast, the primer pair TA135 did not generate any polymorphic band (Fig. 187, Table 54).

Besides polymorphic bands, fifty four common bands were observed among nine cultivars. The common band indicated the sharing of similar DNA fragments among nine chickpea cultivars.

The present study supported the observations of several workers on the potential utility of SSR markers in characterization of different plant species (Satyavathi *et al.* 2006, Sethy *et al.* 2006, Bharadwaj *et al.* 2010). The high rate of polymorphism points towards the scope for further utilization of these markers for chickpea improvement.

4.6.2. Unique SSR primers

In BC-5, one unique SSR sequence of 40 bp was identified with primer TA-72 (Table 52). The unique band was stable and specific for this cultivar and thus could be used as marker. The occurrence of unique alleles or rare STMS alleles provides an immense opportunity for generation of comprehensive fingerprint database. The resources of many unique STMS alleles may be an indication of addition or deletion of small number of repeats (Goldstein and Pollock 1997) and most rational explanation for high mutation rate is polymerase slippage (Levinson and Gutman 1987). The sequences analysis at two microsatellite loci by Sethy *et al.* (2006), revealed copy number changes at repeats motifs as the major reason for microsatellite variability in chickpea.

4.7. Genetic relationships among nine chickpea cultivars

The values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among nine cultivars of chickpea were computed from combined data obtained from twelve RAPD primers and ten SSR primer pairs. The value was ranging from 0.1557 to 0.7975 (Fig. 190, Table 58). The highest genetic distance (0.7975) was found between BC-1 with BC-7. On the other hand, the lowest (0.1557) genetic distance was observed between BC-2 and BC-3. Moreover, the cultivar BC-8 and BC-9 showed close affinities forming sub-cluster and thus placed far away from the rest cultivars. This results correlates with SDS-PAGE, isozymes and cytogenetical data where these two cultivars showed almost similar banding patterns and placed into a sub-cluster far away from the rest. The difference between the highest and the lowest value of genetic

distance revealed the wide range of variability persisting among the nine cultivars. High genetic distance values between cultivar pairs were found due to difference in genetic constituent. The cultivars of lowest genetic distance can be used as parental source for breeding line to improve chickpea cultivars.

4.8. Conclusions

In the available literatures and internet sources, report on the nature of mitotic interphase nuclei and prophase chromosomes, CMA and DAPI banding pattern, unique RAPD and SSR bands and combined cytogenetical and molecular analysis of *Cicer arietinum* L. was not found. Therefore, in this study, characterization of nine chickpea cultivars by the above parameters was the pioneer attempt.

Alterations of chromosomal segments were found in nine chickpea cultivars. In spite of similar $2n$ chromosome number, diversification and reshuffling of CMA- and DAPI-positive banded regions were observed in these nine chickpea cultivars. The number, location and distributions of GC- and AT-rich repeats were specific for each cultivar revealing characteristic CMA- and DAPI-banding pattern. The nine cultivars showed unique RAPD and SSR DNA fingerprinting useful for authentic characterization.

Therefore, the nine cultivars of *Cicer arietinum* L. released from BARI could be characterized authentically by combined cytogenetical and molecular analysis.

5. REFERENCES

5. References

- Afroz M, Sultana SS and Alam SkS 2013. Karyotype and RAPD analysis of three morphological forms of *Alocasia fornicata* (Roxb.) Schott. Cytologia **78**(3): (in press).
- Agriculture and Agri-Food Canada 2006. Chickpea: Situation and outlook. Bi-weekly Bulletin **19**. <http://www.agr.gc.ca>.
- Ahmad F 2000. A comparative study of chromosome morphology among the nine annual species of *Cicer* L. Cytobios **101**: 37-53.
- Ahmad F and Hymowitz T 1993. The fine structure of chickpea (*Cicer arietinum* L.) chromosomes as revealed by pachytene analysis. Theor. Appl. Genet. **86**: 637-641.
- Ahmad F and Slinkard AE 1992. Genetic relationships in the genus *Cicer* L. as revealed by polyacrylamide gel electrophoresis of seed storage proteins. Theor. Appl. Genet. **84**: 688-692.
- Ahmad F, Slinkard AE and Scoles GJ 1987. Karyotype analysis of annual *Cicer* L. species. Genet. Soc. Canada Bull. **18**: 130 pp.
- Ahmad S and Godward MBE 1980. Cytological studies on the cultivars of *Cicer arietinum* L. from Pakistan. Caryologia **33**: 55-67.
- Akhter S and Alam SkS 2005. Differential fluorescent banding pattern in three varieties of *Cicer arietinum* L. (Fabaceae). Cytologia **70**(4): 441-445.
- Alam SkS and Kondo K 1995. Differential staining with Orcein, Giemsa, CMA and DAPI for comparative chromosome study of 12 species of Australian *Drosera* (Droseraceae). American J. Bot. **82**(10): 1278-1286.

- Alam SkS, Kondo K and Tanaka R 1993. A chromosome study of eight orchid species in Bangladesh. *La Kromsomo* **11**-71-72: 2456-2464.
- Alam SkS, Nahar L, Habib MA and Sultana SS 2013. Cytogenetical and molecular characterization of *S. violaceum*, *S. sisymbirifolium* and a Putative Hybrid between ♀ *S. violaceum* and ♂ *S. melongena*. *Cytologia* **78**(1): 35-41.
- Alam SkS, Sukur MB and Zaman MY 2011. Karyotype analysis in two morphological forms of *Xanthium strumarium* L. *Cytologia* **76**(4): 493-498.
- Alam SkS and Khanam N 2005. Fluorescent karyotype analysis of four *Aloe* species. *Bangladesh J. Bot.* **34**(1): 17-20.
- Alam SkS and Zerín N 1998. Differential fluorescent banding and isozyme assay to distinguish three varieties in *Zinnia elegans* L. (Compositae). *Cytologia* **63**: 387-393.
- Arus P and Orton TJ 1983. Inheritance and linkage relationships of isozyme loci in *Brassica oleracea*. *J. Hered.* **74**: 405-412.
- Arus P, Shields CR and Orton TJ 1985. Application of isozyme electrophoresis for purity testing and cultivar identification of F1 hybrids *Brassica oleracea*. *Euphytica* **34**: 651-657.
- Arus P, Tanksley SD, Orton JJ and Jones RA 1982. Electrophoretic variation as tool for determining seed purity and for breeding hybrid variety of *Brassica oleracea*. *Euphytica* **31**: 417-428.
- BBS 2008. A pocket book of agricultural statistics. Bangladesh Bureau of statistics, Ministry of Planning, People's Republic of Bangladesh.
- Begum R and Alam SkS 2004. Karyomorphological study in two orchid species. *Dhaka Univ. J. Biol. Sci.* **13**(1): 99-101.

- Bharadwaj C, Chauhan SK, Rajguru G, Srivastava R, Satyavathi TC and Yadav S. 2010 Diversity analysis of chickpea (*Cicer arietinum* L.) using STSM markers. Indian J. Agric. Sci. **80**: 947-951.
- Biswas MS, Akhond AAY, Alamin M, Khatun M and Kabir MR 2009. Genetic relationship among ten promising eggplant varieties using RAPD markers. Plant Tiss. Cul. Biotech. **19**(2): 119-126.
- Bringhurst RS, Arulsekhar S, Hancock JJF and Volt V 1981. Electrophoretic characterization of strawberry cultivars. J. American Hort. Sci. **106**: 684-687.
- Cattani MV and Papeschi AG 2004. Nucleolus organizing regions and semi-persistent nucleolus during meiosis in *Spartocera fusca* (Thunberg) (Coreidae, Heteroptera). Hereditas **140**: 105-111.
- Chibbar RN, Ambigaipalan P and Hoover R 2010. Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. Cereal Chem. **87**: 342-352.
- Crawford DJ 1983. Phylogenetic and systematic inferences from electrophoretic studies. In : Tanksley SD and Orton TJ (eds.) Isozymes in plant genetics and breeding. Part A. Elsevier, Amsterdam. pp. 257-287.
- Crouch JH, Crouch HK, Constandt H, Cysel VA, Breyne P, Montagu VM, Jarret RI and Ortiz R 1999. Comparison of PCR-based molecular marker analyses of *Musa* breeding populations. Mol. Breed. **5**: 233-244.
- *D'amanto-Avanzi MG 1953. Nuove osservazioni sulla persistenza del nucleolo durante la mitosi del genere *Cassia*. Caryologia **5**: 133-135.

- Datta J, Lal N, Kaashyap M and Gupta PP 2010. Efficiency of three PCR based marker systems for detecting DNA polymorphism in *Cicer arietinum* L. and *Cajanus cajan* L. Millspaugh. Genet. Engin. Biotech. **5**: 1-15.
- De-Vries IM 1996. Characterisation and identification of *Lactuca sativa* cultivars and wild relatives with SDS-electrophoresis (*Lactuca* sect. *Lactuca*, Compositae). Grace. **43**: 193-202.
- Devos KM and Gale MD 1992. The use of random amplified polymorphic DNA markers in wheat. Theor. Appl. Genet. **84**: 567-572.
- Deward MG, Moore GA and Shermin WB 1988. Identification of pineapple cultivars by isozyme genotypes. J. American Hort. Sci. **113**: 935-938.
- Dixit PD 1932. Studies in Indian pulses. A note on the cytology of 'Kabuli and Desi' gram types. Indian j. Agric. Sci. **2**: 385-390.
- Doyle JJ and Doyle JL 1987. A rapid DNA isolation procedure from small quantities of fresh leaf tissues. Phytochem. Bull. **19**: 11-15.
- Esmail RM, Zhang JF and Abdel-Hamid AM 2008. Genetic diversity in elite cotton germplasm lines using field performance and RAPD markers. World J. Agri. Sci. **4**(3): 369-375.
- FAOSTAT 2011. <http://faostat.fao.org/site/567/Desktop, Default.aspx>. (Accessed 12th December 2011).
- FAOSTAT 2012. Available online: <http://faostat.fao.org/site/567/Desktop, Default.aspx> (accessed on 10 January 2012).
- Fawzia R and Alam SkS 2011. Fluorescent karyotype analysis in four varieties of *Solanum melongena* L. Cytologia **76**(3): 345-351.
- Fedorov 1969. Chromosomes number of flowering plants, ed. Fedorov. ANA Academy of science of the USSR. V.L. Komarov Botanical Institute.

- Farr C.J. 2004. Centromeres, kinetochores and the segregation of chromosomes. *Chromosome Research* **12**: 517-520.
- Frimpong A, Sinha A and Tar'an B 2009. Genotype and growing environment influence chickpea (*Cicer arietinum* L.) seed composition. *J. Sci. Food Agric.* **89**: 2052-2063.
- Fritsch P, Hanson MA, Spore CD, Pack PE, Riseberg LH 1993. Constancy of RAPD primers amplification strength among distantly related taxon of flowering plants. *Plant Mol. Bio. Rep.* **11**: 10-20.
- Galasso I and Pignone D 1992. Characterization of chickpea chromosomes by banding techniques. *Genet. Res. Crop Evol.* **39**: 115-119.
- Galasso I, Ferediani M, Cremonini R and Pignone D 1996. Chromatin characterization by banding techniques, *in situ* hybridization and nuclear DNA content of six species of *Astragalus* (Leguminosae). *Cytologia* **60**: 329-335.
- Geervani P 1991. Utilization of chickpea in India and scope for novel and alternative uses. In *Proceedings of a Consultants Meeting, AP, India: ICRISAT*. 47-54 pp.
- Ghafoor A, Ahmad Z, Qureshi AS and Bashir M 2002. Genetic relationship in *Vigna mungo* L. Hepper and *V. radiata* L. R. Wileczek based on morphological traits and SDS-PAGE. *Euphytica* **123**: 378-2002.
- Ghosh KK, Huque ME, Parvin MS, Akhter F and Rahim MM 2009. Genetic diversity analysis in *Brassica* varieties through RAPD marker. *Bangladesh J. Agri. Res.* **34**(3): 493-503.
- Goldstein DB and Pollack DD 1997. Launching microsatellites: a review of mutation process and methods of phylogenetic inheritance. *J. Hered.* **88**: 335-342.

- *Gori C 1956. Persistenza nucleolare durante la mitosi nel genere *Reseda*. *Caryologia* **9**: 45-55.
- Gottlieb LD 1981. Electrophoretic variation in plant populations. *Prog phytochem.* **7**: 1-46.
- Haider M and Haider S 1984. Assessment of protein-calorie malnutrition. *Clin. Chem.* **30**: 1286-1299.
- Hamrick JL and Godt MJW 1997. Allozyme diversity in cultivated crops. *Crop Sci.* **37**: 26-30.
- Hejazi SMH 2011. Karyological study on three *Cicer* L. species (Fabaceae) in Iran. *Asian J. cell Biol.* **6**(3): 97-104.
- *Heldreich Th V 1862. Die Nutzpflanzen Griechenlands. *Botanical Gazette.* **701**.
- Hirai M, Kozaki I and Kajiura I 1986. Isozyme analysis and phylogenetic relationships of *Citrus*. *Japan. J. Breed.* **36**: 377-389.
- Hizume M, Abe KK and Tanaka A 1988. Fluorescent chromosome bandings in the Taxodiaceae. *La Kromosomo* **11-50**: 1609-1619.
- Iqbal SH, Ghafoor A and Ayub N 2005. Relationship between SDS-PAGE markers and Ascochyta blight in chickpea. *Pakistan J. Bot.* **37**: 87-96.
- Islam M and Alam SkS 2011. Karyotype characterization with fluorescent banding in one released and two wild germplasms of *Hibiscus cannabinus* L. *Cytologia* **76**(2): 223-227.
- Islam MQ, Sarker AT, Quader M and Begum L 1981. Status of chickpea improvement at BARI. In *Proc. national workshop on pluses at Joydebpur*. Kaul AK (Ed.) 56-64 pp.

- Javid A, Ghafoor A and Anwar R 2004. Seed storage protein electrophoresis in ground nut for evaluating genetic diversity. *Pakistan J. Bot.* **36**: 87-96.
- Jha SS and Ohri D 1996. Phylogenetic relationships of *Cajanus cajan* L. Millsp. (pigeonpea) and its wild relatives based on seed protein profiles. *GRACE* **43**: 275-281.
- Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): A review. *Brazil J. Nutr.* **108**: 512-526.
- Khatun M and Alam SkS 2010. Confirmation of species status of *Corchorus trilocularis* by differential chromosome banding and isozyme assay. *Cytologia* **75** (1): 83-88.
- Khatun M, Sultana SS, Ara H, Islam MN and Alam SkS 2011. Differential chromosome banding and isozyme assay of three *Corchorus* spp. *Cytologia* **76** (1): 27-32.
- King M 1980. C-banding studies of Australian hybrid frogs: secondary constriction structure and the concept of euchromatin transformation. *Chromosoma* **80**: 191-217.
- Kondo T and Hizume M 1982. Banding for the chromosomes of *Cryptomeria japonica* D. Don. *Japan J. Forest. Soc.* **64**: 356-358.
- Kordi FM, Majd A, Valizadeh M, Sheidai M and Sabaghpour H 2006. A comparative study of chromosome morphology among some genotypes of *Cicer arietinum* L. *Pakistan J. Biol. Sci.* **9**(7): 1225-1230.
- Kutarekar DR and Wanjeri KB 1983. Karyomorphological studies in some of the varieties of Bengal gram (*Cicer arietinum* L.). *Cytologia* **48**: 699-705.

- Ladizinsky G and Adler A 1975. The origin of chickpea as indicated by seed protein electrophoresis. *Israel J. Bot.* **24**: 183-189.
- Laemmli UK 1970. Cleavage of structural proteins during the assembly of head of Bacteriophage T4. *Nature* **227**: 680-685.
- Lang NT and Hang PTC 2007. Genetic divergence analysis on peanut by RAPDs. *Omonrice* **15**: 174-178.
- Lavania UC and Lavania S 1982. Chromosome banding pattern in some Indian pulses. *Ann. Bot.* **49**: 235-239.
- Lowery OH, Rosebrough NJ, Farr AL and Randall RJ 1951. Protein measurement with the Follin-Phenol reagent. *J. Biol. Chem.* **193**: 256-261.
- Levan A, Fredga K and Sandberg AA 1964. Nomenclature for centromeric position on chromosomes. *Hereditas* **52**: 201-220.
- Levinson G and Gutman GA 1987. Slipped strand mispairing, a major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **4**: 203-221.
- Litt M and Luty JA 1989. A hypervariable microsatellite revealed by *in vitro* amplification of dinucleotide repeat within the cardiac muscle actin gene. *Amer. J. Human Genet.* **44**: 397-401.
- Mandal MRI, Islam MS, Bhuiya MAZ, Rahman MM, Alam MS and Rahman MHH 2011. *Krishi prajukti handboi*, BARI udbhabito krishi prajuktir bibarani, BARI **1**: 1-488.
- Maquet A, Zoro Bi I, Delvaux M, Wathélet B and Baudion JP 1997. Genetic structure of lima bean base collection using allozyme markers. *Theor. Appl. Genet.* **95**: 980-991.
- Markert CL and Moller F 1959. Multiple forms of enzyme: tissue, ontogenetic and species specific patterns. *Proc. Natl. Acad. Sci.* **45**: 753-763.

- Meenakshi G and Subramaniam MK 1967. Tetrasomy in roots of *Cicer arietinum* L. Proc. Indian Acad. Sci. Sect. **66**:192-199.
- Mennella G, Onofaro SV, Tonini A and Magnifico V 1999. Seed storage protein characterization of *Solanum* species and of cultivars and androgenetic lines of *S. melongena* L. by SDS-PAGE and AE-HPLC. Seed Sci. Tech. **27**: 23-35.
- Mercys T, Kakar SK and Chowdhry JB 1974. Cytological studies in three species of the genus *Cicer*. Cytologia **39**:383-390.
- Milbourne D, Meyer R, Bradshaw JE, Baird E and Bonar N 1997. Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivated potato. Mol. Breed. **3**: 127-136.
- Moreno M, Cubero JI 1978. Variation in *Cicer arietinum* L. Euphytica **27**: 465-485.
- Mukherjee S and Sharma AK 1987. Structure and behaviour of chromosomes in the strains of *Cicer arietinum* L. Cytologia **52**: 707-713.
- Mundges H, Kohler W and Friedt W 1990. Identification of rape seed cultivars (*Brassica napus*) by starch gel electrophoresis of enzymes. Euphytica **45**: 179-187.
- Nei M 1972. Genetic distance between populations. American Nat. **106**: 283-292.
- Ocampo B, Venora G, Ericco A, Singh KB and Saccardo F 1992. Karyotype analysis in the genus *Cicer*. J. Genet. Breed. **46**: 229-240.
- Ohri D and Pal M 1991. The origin of chickpea (*Cicer arietinum* L.), karyotype and nuclear DNA content. Heredity **66**: 367-372.

- Oke JG 1955. Natural tetraploidy in *Cicer arietinum* L. Proc. Indian Acad. Sci. Sect. B. **41**:220-226.
- Panaud O, Chen X and McCouch SR 1996. Development of SSR markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). Mol. Gen. Genet. **252**: 597-607.
- Pande S, Siddique KHM and Kishore GK 2005. *Ascochyta blight* of chickpea: biology, pathogenicity and disease management. Australian J. Agric. Res. **56**: 317-332.
- Paran I, Aftergoot E and Shifriss C 1998. Variation in *Capsicum annuum* revealed by RAPD and AFLP markers. Euphytica **99**: 167-173.
- Parker PG, Snow AA, Schug MD, Booton GC and Fuerst PA 1998. What molecules can tell us about populations: choosing and using a molecular markers. Ecology **79**: 361-382.
- Perl-Treves R, Zamir D, Navot N and Galun E 1986. Phylogeny of Cucumis based on isozyme variability and its comparison with plastome phylogeny. Theor. Appl. Genet. **71**: 430-436.
- Phadnis BA and Narkhede MN 1972. Chromosome behavior in colchicines induced autotetraploids of *Cicer arietinum* L. Cytologia **37**: 415-421.
- Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S and Rafalski A 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. **2**: 225-238.
- Raina SN, Rani V, Kijima T, Ogihara Y, Singh KP and Devarumath RM 2001. RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivar and wild species. Genome **44**(5): 763-72.

- Ramanujam S 1938. Cytogenetical studies in the Oryzeae. I. Chromosome studies in the Oryzeae. *Ann. Bot.* **2**: 107-125.
- Rasool S 2013. Genetic diversity as revealed by RAPD analysis among chickpea genotypes. *Pakistan J. Bot.* **45**(3): 829-834.
- Russell JR, Fuller JD, Macaulay M, Hatz BG, Jahoor A, Powell W and Waugh R 1997. Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theor. Appl. Genet.* **95**: 714-722.
- Sabstain A, Sujata VS, Nybe EN, Nair GS and Augustine A 1996. Isozyme variation and species relationship in the genus *Piper*. *Tropical agricul.* **34**: 85-92.
- Sambrook J, Fritsch EF and Maniatis T 1989. *Molecular Cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, USA. 2001-2344 pp.
- Sánchez-Vioque R, Clemente A, Vioque J, Bautista J, and Millán F 1999). Protein isolate from chickpea (*Cicer arietinum* L.): chemical composition, functional properties and protein characterization. *Food Chem.* **64**: 237-243.
- Sant VJ, Patankar AG, Sarode ND, Mhase LB, Sainani MN, Deshmukh RB, Ranjekar PK and Gupta VS 1999. Potential of DNA markers in detecting divergence and in analysing heterosis in Indian elite chickpea cultivars. *Theor. App. Genet.* **98**: 1217-25.
- Saraf CS, Rupela OP, Hegde DM, Yadav RL, Shivkumar BG, Bhattarai S, Razzaque MA, Sattar MA 1998. Biological nitrogen fixation and residual effects of winter grain legumes in rice and wheat cropping systems of the Indo-Gangetic plain. In residual effects of legumes in rice and wheat cropping systems of the Indo-Gangetic plain, Rao

- KJVDK, Johansen C and Rego TJ. Eds. Oxford and IBH Publishing Co. Pvt. Ltd: New Delhi, India, 14–30 pp.
- Sarker RH and Awal T 1999. *In-vitro* morphogenesis in chickpea (*Cicer arietinum*). Plant Tiss. Cul. **9**: 141-150.
- Sarker RH and Haque MK 1996. Study of isozyme markers in characterizing jute germplasm. Bangladesh J. Bot. **25**: 139-145.
- Satyavathi TC, Bhat KV, Bharadwaj C, Tiwari SP and Chaudhary V 2006. AFLP based DNA profiling and genetic diversity assessment of Indian soybean (*Glycine max* L.) varieties. Genet. Resour. Crop Evol. **53**: 1069–1079.
- Schweizer D 1976. Reverse fluorescent chromosome banding with Chromomycin and DAPI. Chromosoma **58** : 307-324.
- Sen NK and Jana MK 1956. A spontaneous autotetraploid gram (*Cicer arietinum* L.) Curr. Sci. **25**:231-232.
- Sethy NK, Shokeen B, Edwards KJ and Bhatia S 2006. Development of microsatellite markers and analysis of intra specific genetic variability in chickpea (*Cicer arietinum* L.). Theor. Appl. Genet. **112**: 1416–1428.
- Shahla S and Alam SkS 2011. Comparative fluorescent banding in two forms of *Leonurus sibiricus* L. Cytologia **76**(3): 361–366.
- Sharma PC and Gupta PK 1986. Cytogenetics of legume genera *Cicer* L. and *Lens* L. In: genetics and crop improvement, Gupta PK and Bahl JR (Eds.). Rastogi and Co. Meerut, India, 321-340 pp.
- Shema SI, Habib MA, Begum R and Alam SkS 2013. Differential chromosome banding and RAPD analysis of four varieties of *Capsicum frutescens* L. Cytologia **78** (3): (in press).

- Silva SC, Martins MIG, Santos RC, Penaloza APS, Filho PAM, Iseppon AMB, Vallis JFM and Carvalho R 2010. Karyological features and banding patterns in *Arachis* species belonging to the Heteranthae section. *Plant Syst. Evol.* **285**: 201-207.
- Sonnante G, Marangi A, Venora G and Pignone D 1997. Using RAPD markers to investigate genetic variation in chickpea. *Journal of Genetics and Breeding* **51**: 303-307.
- Soost RK and Ores 1981. Leaf isozymes as genetic markers in *Citrus*. *Proc. Intl. Soc. Citricult.* **1**: 7-10.
- Soost RK, Williams TE and Torres AM 1980. Identification of nucellar and zygotic seedlings of *Citrus* with leaf isozymes. *Hort. Sci.* **15**: 728-729.
- Staub JE and Fredrick I 1988. Inheritance of three enzyme loci in cucumber. *Hort. Sci.* **23**: 757-758.
- Stebbins GL 1971. Chromosomal evolution in higher plants (1st Eds.) Edward Arnold Ltd. London 219 pp.
- Sultana SS and Alam SkS 2007. Differential fluorescent chromosome banding of *Solanum nigrum* and *Solanum villosum* from Bangladesh. *Cytologia* **72**: 213-219.
- Sultana SS, Ara H and Alam SkS 2011. Karyotype analysis with orcein and CMA in two species of *Alocasia* (Schott) G. Don. *Bangladesh J. Bot.* **40**(1): 53-56.
- Sumner AT 1990. Chromosome banding. Unwin Hyman, London. 434 pp.
- Tanaka R 1971. Types of resting nuclei in Orchidaceae. *Bot. Mag. Tokyo* **84**: 118-122.

- Tanksley SD and Jones RA 1981. Application of alcohol dehydrogenase allozymes in testing the purity of F1 hybrids of tomato. Hort. Sci. **16**: 179-181.
- Tanksley SD and Orton TJ 1983. Isozymes in plant breeding and genetics. Part A, Elsevier Science Publishers BV, Amsterdam, The Netherlands.
- Tayyar RI, Lukaszewski AJ and Waines JG 1994. Chromosome banding patterns in the annual species of *Cicer*. Genome **37**: 656-663.
- Thormann CE, Ferreira ME, Camargo LEA, Tivang JG and Osborn TC 1994. Comparison of RFLP and RAPD markers for estimating genetic relationships within and among Cruciferous species. Theor. Appl. Genet. **88**: 973-980.
- Tijo JH 1948. Notes on nucleolar conditions in *Ceiba petandra*. Hereditas **34**: 204-208.
- United States Department of Agriculture (USDA) 2010. National nutrient database for standard reference, release 22 (2009). <http://www.nal.usda.gov/fnic/foodcomp/search/> (accessed 01/07/2010, 12/07/2010, 02/08/2010).
- van der Maesen LJG 1987. Origin, history and taxonomy of chickpea. In: The Chickpea, Saxena, M.C. and K.B. Singh (Eds.). International Publications, UK. 11-34 pp.
- Van-Der-Measen LJG and Somaatmadja S 1992. Plant resources of south-east Asia. Pulse. Prosea Foundation, Bogor, Indonesia **1**: 106 pp.
- Venora G, Conicella C, Ericco A and Saccardo F 1991. Karyotyping in plants by in image analysis system. J. Genet. Breed. **45**: 133-240.
- Venora G, Galasso I and Pignon D 1995. Quantitative heterochromatin determination by means of image analysis. J. Genet. Breed. **49**: 187-190.

- Walter VB and Emery WHP 1957. Persistent nucleoli and grass systematics. American J. Bot. **44**(7): 585-590.
- Weeden NF, Tirnmerman GM, Hernmant M, Kneen BE, and Lodhi MA 1992. Inheritance and reliability of RAPD markers and applications of RAPD technology to plant breeding. Crop Sci. Soc. Arner, Madison, WI. 12-17 pp.
- Wendel JF and Parks CR 1982. Genetic control of isozyme variation in *Camellia japonica* L. J. Hered. **73**: 197-204.
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. **18**: 6531-6535.
- Wood JA and Grusak MA 2007. Nutritional value of chickpea. In Chickpea breeding and management. [SS Yadav, R Redden, W Chen and B Sharma, editors]. Wallingford, UK: CAB International. 101-142 pp.
- Yeh FC, Yang RC and Boyle T 1999. POPGENE Version 1.31: Microsoft Window-based free software for population genetic analysis. <http://www.ualberta.ca/fy.ch>.
- Zaman MY and Alam SkS 2009. Karyotype diversity in three varieties of *Momordica charantia* L. Cytologia **74**(4): 473-478.
- Zirkle C 1928. Nucleus in root tip mitosis in *Zea mays*. Bot. Gaz. **86**: 402-418.

*Original not seen