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GENETIC RELATIONSHIP AMONG *Citrus Species* ON THE BASIS OF PROTEIN PROFILING OBTAINED BY COMPARATIVE ELECTROPHORESIS

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ABSTRACT

Soluble seed protein of different *Citrus species* was compared on discontinuous electrophoretic system. Five polyembryonic and three monoembryonic species with elite ecotypes formed 31 distinct, unambiguous bands in the range of 4 to 71 kD with four conserved bands in all the ecotypes irrespective of species with average molecular weight of 4, 10, 20, 35 KD, respectively. The major storage protein globulin showed a conserved nature without having any differential expression relevant to species or ecotype marking. A 46 kilo Dalton band was unique to monoembryonic types. Protein profile reveals close association of *C. reticulata* and *C. sinensis* indicating a probable co-evolution. Hierarchical Cluster analysis showed the monoembryonic types in a single cluster while in polyembryonic types *C. reticulata* paired with *C. sinensis* while *C. limon* and *C. aurantifolia* came together. The closeness of two polyembryonic species *C. reticulata* and *C. sinensis* with the monoembryonates suggested evolution of the previous types from the later or by hybridization. Study revealed the uniqueness of *Citrus grandis* among monoembryonates. *C. papedia* showed altogether different protein profile with some homology with *C. aurantifolia*. The study confirms the role of cross breeding and mutation followed by natural selection in co evolution of different ecotypes.

Key Words : *Citrus, discontinuous electrophoretic system, protein profile, polyembryonic, monoembryonic, dendogram.*

A minute seed harbours the diverse genetic combination for generating a new progeny with desirable combination of genes. Species with different ecotypes and landraces are important source of greater variation. This genetic variation is presented by different morphological traits and exploited by the breeders for a long time. The dependence on morphological character alone may not be effective to assess the genetic variability of a species. The environmental effect on different traits renders these measurements relatively insensitive. More sensitive markers are thus required to study minute differences. Protein markers are widely used to reveal seed protein and isozyme variation. They operate at the gene product level where the environment has very little influence (1). Protein markers have been regarded as important taxonomic tool since these proteins are highly conserved in evolution (2) and these proteins are not susceptible to damage or mutation caused by environmental factors (3). The genetic comparison across species as well as within species is important for germplasm collection and conservation and that may lead to a stable yield from genetically heterogeneous populations than homogeneous counterparts (4).

Seed protein electrophoresis is increasingly being used as an approach for species identification and as a useful tool for tracking back the profile for taxonomic and evolutionary purposes by several investigators, (6). The SDS-PAGE is also utilized by several scientists for broad spectrum of studies. (7) used it for separation of citrus seed storage protein, was used for assessing the sensitivity of Citrus jambhiri to Alternaria alternata toxin (8), for identifying a specific morphological type in Chenopodium helped in taxonomic grouping and comparison with flavonoid compound distribution (9). Though SDS-PAGE analysis of soluble seed protein was done for major cereals like rice (1). This kind of attempt is very sparse in citrus. The genetic association study with protein based marker was meagre in citrus (10).

Citrus is a polyembryonic species and the extent of this specific trait is very broad considering the number of embryos, zygotic or nucellar types, or abnormalities etc. An inter and intraspecific study of

citrus germplasms is very important before indulging into intricate and sophisticated embryological or pathogenic molecular analysis. Therefore the purpose of the present study is to characterize the genetic diversity of citrus germplasms collected from different parts of India. In order to achieve this objective the inter and intra-specific variation was ascertained and ecotypic variability was measured by protein banding pattern using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Collection of fruits/seeds : Fruits from different species were collected mostly from orchards of state of West Bengal, Manipur, Delhi and Punjab. The elite clones with good bearing habits were selected according to growers' interpretation. Minimum 5 matured fruits from each species were collected and brought to the laboratory and seeds were extracted from the fruits and used for study of SDS-PAGE on a discontinuous buffer system.

Preparation of seed sample : For extraction of protein individual seeds were ground with mortar and pestle with Phosphate buffer (pH 6.8). The solution was taken into centrifuge tube and centrifuged at 14,000 rpm for 10 minutes. The extracted crude protein was recovered as clear supernatant. The total protein was estimated by Lowry method using Bovine Serum Albumin (BSA) standard curve. The crude protein was purified with trichloroacetic acid and after repeated washing the protein is air-dried and dissolved in sample buffer (0.05 M Tris, 0.192M Glycine, 0.1% SDS).

Preparation of gel: Seed protein was analyzed through slab type SDS-PAGE followed by Laemmli using 15% polyacrylamide gel (11). Electrophoresis was carried out at 60 Volt for 4 hrs. In order to check the reproducibility of the method two separate gels were run under similar electrophoreic conditions. In each gel SDS PAGE Track Protein MW Marker (Merck-Genei, 3.5- 43.5 kDa) was loaded for tracking and size reference. After electrophoresis gels were stained with 0.2% (w/v) Coomassie brilliant blue R250 for about 2 hour. Then the gel was destained with Ezee Fast Blue by placing the gel immersed in the solution for 2 hour. With each set of experiment a negative control is included by running one lane with sample buffer for controlling any contamination from water or buffer.

Analysis of Electrophoregrams: Finally the gel is photographed and analyzed using Alphaease ver. 4.0 Gel Imaging software. The cluster analysis is performed using SPSS (var. 16) with Hierarchical cluster with Ward's minimum variance algorithm for understanding the relationship of different ecotypes and species. The presence of a particular molecular weight protein band was indicated by 1, while the absence by 0 to form a binary matrix.

RESULTS AND DISCUSSION

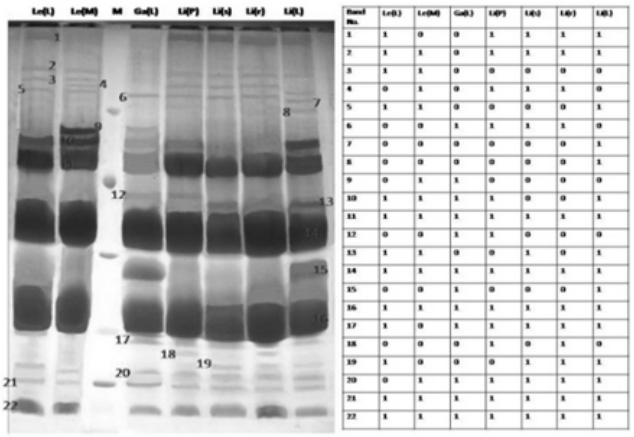
Thirteen elite germplasms including two ecotypes of *Citrus reticulata,* one of *Citrus sinensis*, two ecotypes of *Citrus limon*, four ecotypes of *Citrus aurantifolia* and one of *Citrus papedia* from polyembryonic species and three monoembryonic types *Citrus medica, Citrus latipes* and *Citrus grandis* were analyzed using SDS-PAGE. Electrophoregrams are analyzed carefully. Two trials were done for each protein types. SDS treated protein shows presence of 31 distinct bands covering all the species. The faint and unambiguous bands are rejected and not included in analysis. The resulting bands from SDS-PAGE are documented with Alphaease imaging software (var.4.0). The molecular weight and relative mobility (Rm) value of the bands are determined and used for construction of a dendogram. Band ranges from approximately 4 kDa to 70 kDa on 15 percent gel. The gel percentage was selected for detecting

Table-1 : Different *Citrus* species and ecotypes collected by peer survey.

Genotypes	State	Embryonic state
Citrus reticulata	West Bengal	poly
Citrus reticulata	Delhi	poly
Citrus sinensis	West Bengal	poly
Citrus limon	West Bengal	poly
Citrus limon	Manipur	poly
Citrus aurantifolia	West Bengal	poly
Citrus aurantifolia, smooth skinned	Manipur	poly
Citrus aurantifolia, rough skinned	Manipur	poly
Citrus aurantifolia	Punjab	poly
Citrus papedia	West Bengal	poly
Citrus medica	Manipur	mono
Citrus latipes	Manipur	mono
Citrus grandis	West Bengal	mono

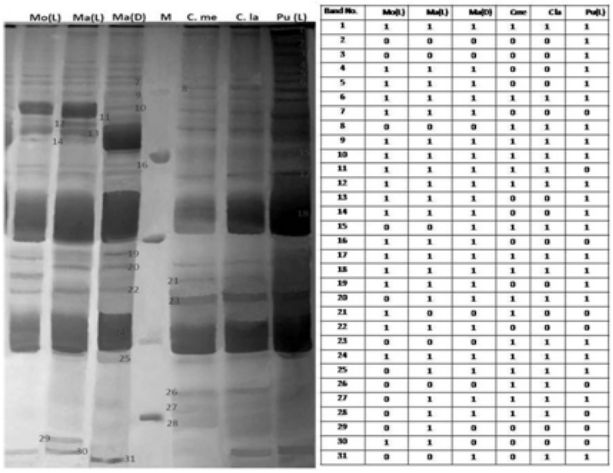
Table-1 : Similarity Index of water soluble proteins of Thirteen Ecotypes of Citrus.

Band	Citrus limon (West Bengal)	Citrus limon (Manipur)	Citrus papedia (West Bengal)	Citrus aurantifolia (Punjab)	Citrus aurantifolia Smooth skin (Manipur)	Citrus aurantifolia Rough skin (Manipur)	Citrus aurantifolia (West Bengal)	Citrus sinensis (West Bengal)	Citrus reticulata (West Bengal)	Citrus reticulata (Delhi)	Citrus medica (Manipur)	Citrus latipes (Manipur)	Citrus grandis (West Bengal)
1	1	0	0	1	1	1	1	1	1	1	0	1	1
2	1	1	0	1	1	1	1	0	0	0	1	1	1
3	1	1	0	0	0	0	0	0	0	0	0	0	1
4	0	1	0	1	1	1	0	1	1	1	0	0	1
5	1	1	0	0	0	0	1	1	1	1	0	0	1
6	1	1	1	1	1	1	1	1	1	1	1	1	1
7	0	0	0	0	0	0	1	1	1	1	0	0	0
8	0	0	0	0	0	0	1	0	0	0	1	1	1
9	0	1	1	0	0	0	0	1	1	1	1	1	1
10	1	1	1	1	0	0	1	1	1	1	1	1	1
11	0	0	0	0	0	0	0	1	1	1	1	1	0
12	0	0	0	0	0	0	0	1	1	1	1	1	1
13	0	0	0	0	0	0	0	1	1	1	0	0	1
35	1	1	1	1	1	1	1	1	1	1	0	0	1
15	0	0	1	1	0	0	0	0	0	1	1	1	1
16	1	1	0	0	1	0	1	1	1	1	0	0	0
17	0	0	0	0	0	0	0	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1	1	1	1	1
19	0	0	1	0	0	0	1	1	1	1	0	0	1
20	0	0	0	0	0	0	0	0	1	1	1	1	1
21	0	0	0	0	0	0	0	1	0	0	1	0	0
22	0	0	0	0	0	0	0	1	1	1	0	0	0
23	0	0	0	0	0	0	0	0	0	0	1	1	1
24	1	1	1	1	1	1	1	1	1	1	1	1	1
25	1	0	1	1	1	1	1	0	1	1	1	1	1
26	0	0	0	1	0	1	0	0	0	0	1	1	0
27	1	0	0	0	1	1	1	0	1	1	1	1	1
28	0	1	1	1	1	1	1	0	1	1	1	1	0
29	1	1	1	1	1	1	1	0	1	0	0	0	0
30	1	1	1	1	1	1	1	1	1	0	0	1	1
31	0	0	0	0	0	0	0	0	0	1	0	1	1



Band No.	Le(L)	Le(M)	M	Ga(L)	Li(P)	Li(s)	Li(v)	Li(L)
1	1	0	0	1	1	1	1	
2	1	1	0	1	1	1	1	
3	1	1	0	0	0	0	0	
4	0	1	0	1	1	1	0	
5	1	1	0	0	0	0	1	
6	0	0	1	1	1	1	0	
7	0	0	0	0	0	0	1	
8	0	0	0	0	0	0	1	
9	0	1	1	0	0	0	0	
10	1	1	1	1	0	0	1	
11	1	1	1	1	1	1	1	
12	0	0	1	1	0	0	0	
13	1	1	0	0	1	0	1	
14	1	1	1	1	1	1	1	
15	0	0	1	0	0	0	1	
16	1	1	1	1	1	1	1	
17	1	0	1	1	1	1	1	
18	0	0	0	1	0	1	0	
19	1	0	0	0	1	1	1	
20	0	1	1	1	1	1	1	
21	1	1	1	1	1	1	1	
22	1	1	1	1	1	1	1	

Fig.-1: SDS-PAGE of Water Soluble Proteins from Seeds of *Citrus* Species; Lane 1. *Citrus limon* of West Bengal, Lane 2. *Citrus limon* of Manipur, Lane 3. Protein Marker (Merck Genei), Lane 4. *Citrus papedia* of West Bengal, Lane, 5. *Citrus aurantifolia* of Punjab, Lane 6. *Citrus aurantifolia* (smooth skinned) of Manipur, Lane 7. *Citrus aurantifolia* (rough skinned) of Manipur, Lane 8. *Citrus aurantifolia* of West Bengal



Band No.	Mo(L)	Ma(L)	Ma(D)	M	C. me	C. la	Pu (L)
1	1	1	1	1	1	1	
2	0	0	0	0	0	1	
3	0	0	0	0	0	1	
4	1	1	1	0	0	1	
5	1	1	1	0	0	1	
6	1	1	1	1	1	1	
7	1	1	1	0	0	0	
8	0	0	0	1	1	1	
9	1	1	1	1	1	1	
10	1	1	1	1	1	1	
11	1	1	1	1	1	0	
12	1	1	1	1	1	1	
13	1	1	1	0	0	1	
14	1	1	1	0	0	1	
15	0	0	1	1	1	1	
16	1	1	1	0	0	0	
17	1	1	1	1	1	1	
18	1	1	1	1	1	1	
19	1	1	1	0	0	1	
20	0	1	1	1	1	1	
21	1	0	0	1	0	0	
22	1	1	1	0	0	0	
23	0	0	0	1	1	1	
24	1	1	1	1	1	1	
25	0	1	1	1	1	1	
26	0	0	0	1	1	0	
27	0	1	1	1	1	1	
28	0	1	1	1	1	0	
29	0	1	0	0	0	0	
30	1	1	0	0	0	0	
31	0	0	1	0	1	1	

Fig 2: SDS-PAGE of Water Soluble Proteins from Seeds of *Citrus* Species Lane1. *Citrus sinensis* of West Bengal; Lane 2. *Citrus reticulata* of West Bengal, Lane3. *Citrus reticulata* of Manipur, Lane 4. Protein Marker (Merck Genei), Lane 5. *Citrus medica* of Manipur, Lane 6. *Citrus latipes* of Manipur, Lane 7. *Citrus grandis* of West Bengal

comparatively low molecular weight proteins mostly the globulins and albumins. Seed storage protein genes shows tissue specific expression and used as useful marker for studying seed developmental processes (12). In this study it is revealed that the major seed storage protein citrin (7), which is a globulin is highly conserved in nature with high level of quantitative expression without significant differential expression as in avocado (13). The water soluble protein part is composed of predominantly polar amino acids like threonine, serine, glycine and are more diverse than the salt soluble fraction. The extracted water soluble proteins came from cell and are mostly metabolic proteins and are more diverse than salt soluble proteins.

Most wide range of SDS treated protein bands ar exhibited by *Citrus limon* ecotype of West Bengal. The band ranges from 4 to 70 kDa. Highest number of band was exhibited by one ecotype of *Citrus reticulata* collected from West Bengal. *Citrus medica* differs from others in having lowest number of bands with a narrow range of 6-50 kDa. Four bands in the range of 35 kDa, 20kDa, 10 kDa and 4kDa were conserved in all the species. The 4kDa band is only absent in *Citrus medica*. The 35 kDa and 20 kDa bands with consistent appearance showed similarity with the finding of salt soluble globulin seed storage protein fractions in Citrus sinensis [14]. Some species specific unique bands were also present. *Citrus papedia* and only one ecotype of *Citrus aurantifolia* collected from Punjab only showed the presence of a 26 kDa band. Four bands of 18 kDa, 29kDa, 48 kDa and 54kDa are present in *Citrus sinensis* and two ecotypes of *Citrus reticulata* which are absent in other species. A 37 kDa band in ecotype of *Citrus aurantifolia* is unique and absent in all other species and ecotypes of the same species. Two unique bands of 54 kDa, 60 kDa were present only in two ecotypes of *Citrus reticulata*. A band of 46 kDa is found in only monoembryonates and was absent in other polyembryonic collections. Presence of three unique bands of 67 kDa, 55kDa and 50kDa made *Citrus grandis* a distinct species. A previous study with *Citrus species* identified 12 distinct bands of high molecular weight. The previous finding by (7) along with our study may suggest the necessity of running a gradient gel for proper understanding of all the soluble seed proteins. 2D gel electrophoresis along with isoelectric focusing may ease the complication (5).

In order to study the relatedness of the species, a dendogram was constructed by inputting the values of distance matrix with SPSS software (var.16.0) using Ward's minimum variance method. All the monoembryonic species shared the same cluster showing strong association between *C. latipes* and *C. medica* than *C. grandis* under the same sub-cluster. Polyembryonic *Citrus reticulata* and *Citrus sinensis*

clustered together in a single sub-cluster confirming the finding made by (7). *C. limon* paired with *C. aurantifolia* of West Begal showing interspecific similarity of seed protein. All other ecotypes of *C. aurantifolia* clustered on a subgroup with some homology in banding pattern with *C. Papedia* but *C. papedia* with its unique banding pattern maintained a separate entity. This association of *C. papedia* with *C. aurantifolia* reveal their relatedness. Association of *C. limon* and *C. aurantifolia* are in accordance with the RAPD analysis of citrus cultivars and rootstocks of North Eastern Himalayan region [16]. This cluster analysis also shows similarity of three monoembryonic species with *C. sinensis* and *C. reticulata.* The two subcluster one with *C. limon* types and the other with *C. aurantifolia* merged showing close association of lemon and lime genotypes. The analogy of *C. papedia* with other species and ecotypes contradicts the previous analysis made by RAPD primers using different citrus species and rootstocks and need repetition as *C. papedia* is neglected in most of the previous molecular work with sparse data on its evolutionary origin. This experiment very clearly identified the variation of different polyembryonates and the closeness of monoembryonic species. Comparative electrophoresis is very useful in biosystematics because the homology or analogy exhibited by species have theoretical significance in studying relatedness and diversity of different taxa. Citrus shows diverse embryological states. Some of the species are polyembryonic others monoembryonic. The closeness of different species and difference in ecotype of a same species may have generated due to cross breeding and mutation with advantageous natural selection (5). The protein profiling of soluble seed protein can certainly be utilized as an important tool to identify association or diversification of different species. This kind of study may be utilized for management and improvement of Citrus germplasms and create the preliminary platform for sophisticated embryological and pathological analysis.

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