SHORT COMMUNICATION

Isoenzyme Polymorphism in *Citrus* spp. and *Poncirus trifoliata* (L.) Raf.

T Dhurjati*, GM Poornima and Awtar Singh
National Research Centre for Citrus, Nagpur 440 010 (Maharashtra)

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The genus *Citrus*, belonging to the family Rutaceae, subfamily Aurantioideae, tribe Citreae and subtribe Citrinae having its origin in North-eastern India and Southeast Asia is one of the most widely produced fruit species in the world. Several species used as scion cultivars, such as sweet oranges, mandarins, lemons and grapefruits are cultivated commercially. Many other *Citrus* species and hybrids with related genera are used as rootstocks on which the commercially important scion is grafted. Basically the "true *Citrus*" group of fruit trees consists of six closely related genera namely *Citrus, Fortunella, Poncirus, Microcitrus, Eremocitrus* and *Clymenia,* which are highly inter-fertile, with the possible exception of *Clymenia.*

The taxonomic relationships among *Citrus* species have been a source of debate for a long time with respect to the species status and relationships. The well-known taxonomies, of Swingle (1943) and Tanaka (1969) differ widely in the number of species, the former recognizing 16 and the latter 159 in the citrus group. Another classification by Barrett and Rhodes (1976) recognized three basic species for subgenus *Citrus,* namely pummelo (*Citrus grandis* (L.) Osbeck), citron (*Citrus medica* L.) and mandarin (*Citrus reticulata* Blanco) (Torres *et al.*, 1978). *Citrus* species have been identified for taxonomy and systematic purposes using both morphological and non-morphological descriptors (biochemical and molecular markers). Recently a more comprehensive study of the genetic diversity of orange subfamily Aurantioideae using a diverse set of descriptors including molecular markers revealed the existence of two basic affinity groups namely the Orange-mandarin group and Lime-lemon-citron-pummelo group (Herrero *et al.*, 1996).

Biochemical marker studies involving isozyme polymorphism have been very useful in cultivar identification (Barret and Rhodes, 1976), classification of *Citrus* species (Herrero *et al.*, 1996), differentiation of nucellar and zygotic seedlings (Torres *et al.*, 1982) and genetic mapping (Jarrell *et al.*, 1992). Isoenzymatic polymorphism is expected in citrus due to the fact that most of the present day cultivars arose from natural hybridization. The present study was undertaken as a precursor to the ongoing molecular marker based analysis of citrus genetic diversity to specifically gain a preliminary insight into the allelic diversity of four isozyme systems in nine modal citrus species as defined by Torres *et al.* (1978). Therefore, the aims of this current survey were: 1) to conduct a preliminary investigation of levels of genetic diversity within and between species and to assess the suitability of the technique for further studies within the genus using four isozyme systems, and 2) to use the data to construct a dendrogram describing similarities between taxa and to compare the results with the accepted taxonomy for the genus *Citrus*. Owing to limited intraspecific sampling and the low number of isozyme loci used, values for genotype and allele frequencies and observed heterozygosity need to be treated with caution. Further sampling and analysis is required, before accurate estimates of these indicators of genetic diversity are obtained.

Leaf samples for this study were sampled from the National Citrus Germplasm Repository of National Research Centre for Citrus, Nagpur (Table 1). Young leaves (minimum of 5 samples) from nine different citrus species [*Citrus aurantifolia* (Christm) Swingle, *C. aurantium* (L); *C. grandis,* (L) Osbeck; *C. jambhiri* (Lush); *C. medica,* L; *C. paradisi,* Macf; *C. reticulata* Blanco; *C. sinensis* (L) Osbeck; *C. limon* (L) Burm. f] and one citrus relative [*Poncirus trifoliata* (L).Raf] were collected in polyethylene bags and subjected to a survey of isozyme variation. In case of mandarin, rough lemon and trifoliate orange, 16, 24 and 15 cultivars, respectively, were assayed (Table 1). Fresh leaf tissue from each sample was extracted by grinding in cold (4°C) 0.1 M Tris buffer containing 10mM KCl, 0.10 mM $MgCl_2$, 1mM EDTA, 14 mM beta-mercaptoethanol, 0.10 M ascorbic acid and 0.20 g/ml PVP for AAT, PGM

Corresponding author E-mail: tarakdhurjati@yahoo.com

Table 1. Citrus species and cultivars

Species	Cultivar/Local name/ Cultivar name/No. of samples
Citrus aurantifolia (Christm.) Swingle	Acid lime (Kagzi lime)
Citrus aurantium L.	Sour Orange (Tirupati)
Citrus grandis (L.) Osbeck	Pummelo (Local)
Citrus jambheri Lush	Rough lemon (Jamberi)
	(14-9-13, Limonaria, Assam, Sohsarkar, Mithitulia, Telankhedi, Sohmyndong, 58-8-III 4,8780, katajamir, Sohjhalia, S. Africa, Jullandahari khatti, Chethali, Tharsa, MP, Jattikhatti, Citrunelle, Tirupati, Rahuri, Chase, Florida, Local, Schaub
Citrus medica L.	Citron (Etrog Citron)
Citrus paradisi Macf.	Grapefruit (Flame, Ray Ruby)
Citrus reticulata Blanco	Mandarins (Corsica no. 2(21), Page Mandarin, Citrus Clementine, Caffin Clementine, Corsica No.1, Clementine Montreal, Dhakula Local, Corsica No. 1 (4CN11), Marisol, Nagpur Seedless, Nagpur Mandarin, Mudkhed Seedless, Yonezawa, Frost Owari, Dobashibeni, Sunchusha)
Citrus sinensis (L.) Osbeck	Sweet Orange (Mosambi, Washington Navel, Shamouti)
Citrus limon (L.) Burm.f.	Lemon (NRCC repository)
Poncirus trifoliata (L.) Raf.	Trifoliate Orange (Yamaguchi Roubidoux, Argentina, Williams, Florida, Srirampur, Gotharoad, Large-flowered, USA, English Large, Flying Dragon, Rich 16-6, Local, Gonicoppal, Chethali).

and PGI and 0.1M cold (4°C) potassium phosphate buffer, pH7.2 for the isozyme Peroxidase. The ground tissue was centrifuged at 27000Xg for 20 min. (2 to 4°C). The samples were assayed for four isozyme systems namely Aspartate amino transferase (EC2.6.1.1; AAT), Phospho gluco isomerase (EC5.3.1.9; PGI), Phospho gluco mutase (EC2.7.5.1; PGM) and Peroxidase (EC 1.11.1.7; PRX) which were chosen based on their robustness and stability.

Polyacrylamide gel electrophoresis was performed in a vertical slab gel apparatus. The gel slab consisted of a stacking portion of 5% acrylamide in 1M, tris-HCl, pH 6.8 and a resolving part of 12% acrylamide in 1.5M, tris-HCl, pH 8.8. The electrode buffer was 25mM Tris, 250mM glycine pH 8.3 for all the isozymes studied. Electrophoresis was carried out at 4°C for 3-4 hours. Gels were stained according to Shields *et al.* (1983). Allele scoring and notation was according to Torres *et al.* 1978. Allele designation by alphabet letters in increasing order of speed of migration is as follows, S specifying a slowly migrating enzyme or subunit, M for medium, I for intermediate; F for fast and T for faster. Alleles were scored by numbering from '1' to 'n' from the fastest to slowest to migrate from the origin. To exemplify a specific allele (FS) with fast and slow migrating bands, three bands would be considered as a heterozygote and a single band (FF/SS) as a homozygote for dimeric enzymes and two bands (FS) would be considered as a heterozygote and a single band (FF/SS) is considered a homozygote for monomeric enzyme. Allele frequencies for seven isozyme loci were calculated and analyzed to develop a matrix of genetic distances calculated as per the Nei's (1972) genetic distance measure, to describe the variation between different citrus species.

The standard Nei's genetic distance is $D_{xy} = -\ln (I_{xy})$, where I_{xy} (Genetic Identity) is the average interpopulation heterozygosity (J_{xy}) divided by the square root of the product of J_x and J_y, where J_x & J_y are average homozygosity values of population x and population y, respectively. The values of D vary from zero (identical allele frequencies) to infinity (do not share alleles). Genetic distances were calculated based on allele frequencies, according to Nei (1972), and then used to generate a UPGMA dendrogram with the software package TFPGA (Miller Mark, 1997).

The results showed distinct isozymic polymorphism between different citrus species. All the cultivars within a species showed monomorphic isozyme profiles with similar alleles in all the four isozyme systems used in the study. The number of alleles and loci varied according to the isozyme system used. Two loci each were scored for AAT (Aat-1 and 2), PGM (Pgm-1 and Pgm-2), PGI (Pgi-1and 2) and one locus was scored for PRX (Prx-1) profile. PGI and AAT isozymes having a dimeric quartenary structure gave three bands in a heterozygote, while PRX and PGM which are monomers, resolved two bands in a heterozygote. The number of alleles varied with a minimum of two (F or T and S) for Pgm-1and Prx locus to a maximum of five alleles (F or T, S, M and I) in Pgi-1 locus (Fig 1).

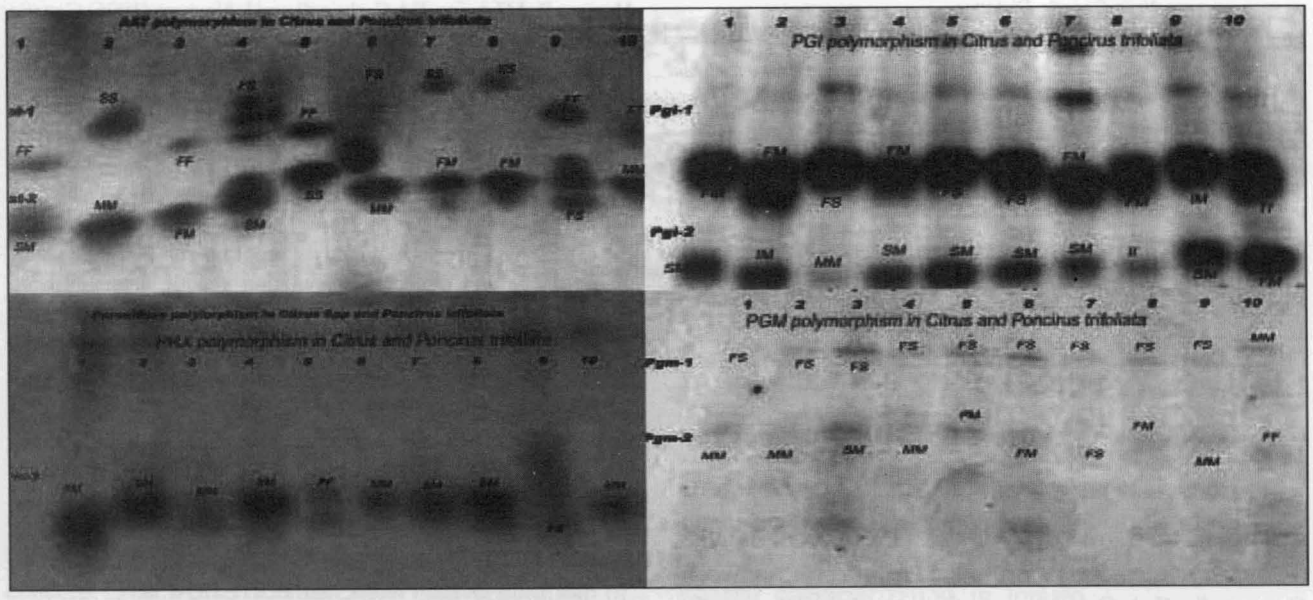


Fig 1. Representative Zymograms (Clockwise, AAT, PGI, PGM and PRX) of different Citrus species and Poncirus trifoliata Lanes 1-10: 1) Acid lime, 2) Sour orange, 3) Pummelo, 4) Rough lemon, 5) Citron, 6) Grapefruit, 7) Nagpur mandarin, 8) Sweet orange, 9) Lemon and 10) Trifoliate orange

A total of seven polymorphic loci were resolved and 24 alleles were scored with an average of 3.4 alleles per locus. All the samples analyzed within the species showed similar banding patterns. The average expected heterozygosity of or Nei's genetic diversity of 0.59 was obtained between species considered. The results of Nei's original measures of genetic identities/distances revealed highest genetic identity (GI) of 0.8839 between rough lemon and acid lime and the least GI of 0.15 between lemon and trifoliate orange (Table 2). The genetic relationships between different *Citrus* species were expressed based on UPGMA (Unweighted pair group method using arithmetic averages) cluster constructed from the genetic distance matrix with resampling of 1000 replicates bootstrapping for validation.

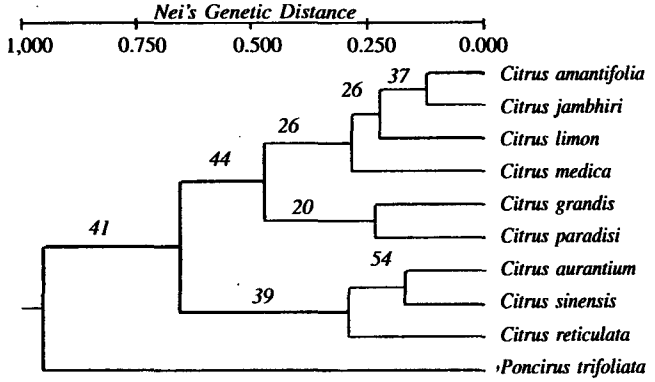
Mandarin clustered with sweet orange and sour orange, which belong to the mandarin-orange group, while pummelo clustered with grapefruit (Fig. 2). This conforms with the fact that grapefruit has originated in the new world as a natural hybrid of pummelo with sweet orange. Further, it is known that limes have evolved from citrons and the clustering of limes, lemons in the citron affinity group confirms the results according to the established citrus taxonomy (Swingle, 1943; Tanaka, 1967; Herrero *et al.*, 1996) although rough lemon and lemon are known to have a more complicated origin.

Table 2. Distance matrix based on Nei's (1972) distance measure

Pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.63	0.7	0.88	0.79	0.72	0.47	0.44	0.83	0.40
2	0.45	****	0.55	0.83	0.30	0.73	0.72	0.84	0.47	0.38
3	0.30	0.59	****	0.67	0.55	0.79	0.55	0.42	0.57	0.52
4	0.12	0.17	0.39	****	0.61	0.76	0.68	0.64	0.76	0.37
5	0.23	1.20	0.59	0.48	****	0.52	0.33	0.26	0.73	0.28
6	0.32	0.30	0.23	0.26	0.64	****	0.70	0.61	0.44	0.65
7	0.75	0.31	0.58	0.37	1.09	0.34	****	0.76	0.41	0.42
8	0.81	0.17	0.86	0.43	1.33	0.49	0.26	****	0.38	0.35
9	0.18	0.74	0.54	0.26	0.30	0.81	0.88	0.94	****	0.15
10	0.91	0.96	0.64	0.98	1.25	0.42	0.85	1.04	1.89	****

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

1) *Citrus aurantifolia* 2) *Citrus aurantium* 3) *Citrus grandis* 4) *Citrus jambheri* 5) *Citrus medica* 6) *Citrus paradisi* 7) *Citrus reticulata* 8) *Citrus sinensis* 9) *Citrus limon* 10) *Poncirus trifoliata*



Numbers at nodes indicate bootstrap values

Fig 2. UPGMA dendrogram depicting relationships among Citrus spp. and Poncirus trifoliata

According to Barret and Rhodes (1976), sour orange (*C. aurantium*), probably originated from a cross between *C. grandis* (L.) Osbeck and *C. reticulata* Blanco. In conclusion, *Citrus* species clustered largely into two basic affinity groups of lime-lemon-citron-pummelo and orange-mandarin clusters and the isoenzyme phenotypes could differentiate the inter-specific relationships between nine modal citrus species and *Poncirus trifoliata.*

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