Phylogenetic Relationships Among the "True Citrus Fruit Trees" by Glutamate Oxaloacetate Transaminase Isozymes Analysis

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Summary

Leaf isozymes of glutamate oxaloacetate transaminase (EC 2.6.1.1; GOT) in the "true citrus fruit trees" group of Citrinae were analyzed using polyacrylamide gel electrophoresis to elucidate phylogenetic relationships among them. Segregation at each of three loci, *Got-1*, *Got-2* and *Got-3* of GOT isozymes in the progenies obtained from intergeneric crosses between *Citrus* and *Microcitrus* species followed a simple Mendelian inheritance, suggesting that *Microcitrus* and *Citrus* species have similar genetic systems and the species of these two genera are closely related to each other. Some common alleles at the three loci controlling GOT isozymes were recognized between the genera of "true citrus fruit trees". These findings suggest that a genetic identity is conserved among the "true citrus fruit trees" group; even some of them climatically, geographically and reproductively isolated.

Introduction

The "true citrus fruit trees" group of the subtribe Citrinae (Rutaceae) is comprised of six genera. Among them, *Fortunella*, *Poncirus*, and *Citrus* are native to southeast Asia, *Microcitrus* and *Eremocitrus* to Australia, and *Clymenia* to New Ireland (New Guinea) (Swingle and Reece, 1967).

Cross compatibility has been reported among the members of "true citrus fruit trees" (Barrett, 1977, 1981, 1985; Iwamasa et al., 1988), thus indicating their close relationships. Graft compatibility was also reported among them (Bitters et al., 1977; Sykes, 1988). To date, all phylogenetic studies which have been carried out are mostly concentrated on *Citrus* (Potvin et al., 1983; Niedz et al., 1991; Gogorcena and Oritz, 1993), but comprehensive phylogenetic relationships among the "true citrus fruit trees" at the genetic level have not yet been clarified.

The present investigation was aimed at elucidating the phylogenetic relationships among the genera of "true citrus fruit trees" by isozyme analysis.

Materials and Methods

Plant materials

Parent trees and their adult progeny (about 5 years old), which had been obtained from the previous work (Iwamasa et al., 1988), were used to assess genetic determinism (Table 1). All plant materials were maintained in a greenhouse under natural light. The genotypes of *Fortunella*, *Eremocitrus*, *Microcitrus*, and *Citrus* were identified on the basis of progeny segregation (Rahman and Nito, 1994a).

Protein extraction

The leaves were selected for protein extraction from the previous spring cycle of growth (approximately 6 months old). Fresh leaf tissue (0.2 g) was homogenized with 0.5 ml of 100 mM K-acetate buffer, pH 5.4 (Esen and Soost, 1976). The crude homogenate was centrifuged at $14,000 \times g$ for 20 min. The resulting supernatant was desalted with Sephadex G-25 (Hirai et al., 1986b). All operations were carried out at 4 °C.

Electrophoresis

Vertical polyacrylamide slab gel electrophoresis (PAGE) was conducted at 4 °C. The separations

were performed on 2.5 % stacking gel and 7 % separation gel consisting of 1.49 M Tris-HCl, pH 8.9. The electrode buffer contained 0.04 M Tris and 0.38 M glycine, pH 8.3. Protein content was estimated by Lowry's procedure (Lowry et al., 1951) and 50 μ g protein equivalent of each sample was loaded. The electrophoresis was carried out at a constant current of 12 mA per gel until the bromophenol blue dye began to run off the gel.

Staining of gels

At the end of electrophoresis, gels were stained for glutamate oxaloacetate transaminase (EC 2.6.1.1; GOT) in the dark at 30 °C until a clear blue band appeared (Torres et al., 1978a). GOT is synonymous with aspartate aminotransferase (AAT). Stained gels were washed and fixed in 7 % acetic acid.

Genetic interpretation

The principles followed for interpretation of banding patterns and for designation of gene loci and alleles were the same as outlined by Hirai et al. (1986a) and Hirai and Kajiura (1987).

The genetic control of isozyme variants was determined by chi-square analysis of progeny for goodness-of-fit to expected ratios in available crosses.

For making comparisons among the "true citrus fruit trees", the reported alleles of GOT isozymes of *Fortunella*, *Eremocitrus*, *Poncirus*, *Microcitrus*,

and *Citrus* (Torres et al., 1978b; Hirai et al., 1986a; Hirai and Kajiura, 1987) and *Clymenia* (Soost and Torres, 1981) were adopted from the previous works.

Results

Three zones of activity, GOT-1, GOT-2, and GOT-3 (sharing the same zone with GOT-2) were detected on gels stained for GOT. The mobilities of GOT-2 homodimers overlapped those of the GOT-3, making interpretation of some phenotypes difficult. However, it was successfully resolved, based on the progeny segregation patterns. A sample of the different banding patterns observed in this isozymes is shown in Fig. 1.

Crosses between parents with three bands and single-banded GOT-1 phenotypes gave two classes of progenies with single-band and three-bands and were produced in approximately equal frequency (Table 1). The results can be explained on the basis of progeny segregation for a single gene, (*Got-1*). The presence of the 'hybrid' band denotes the dimeric structure of this enzyme.

In the GOT-2 zone, progenies obtained from 2 crosses (cross no. 1 and 3) among the 4 intergeneric crosses were segregated. In the cross no. 2 and 4 no segregations were obtained because a common homozygous allele is present in both parents (Table 1). The results of segregation at GOT-2 zone are in agreement with the hypothesis that enzymes observed in this zone are coded by a

Fig. 1. Glutamate oxaloacetate transaminase zymograms of progeny from *C. iyo* (cv. Miyauchi Iyo) (B) $\times$ *M. inodora* (A). Segregation was observed for GOT-1 (FS $\times$ SS) and GOT-3 (En $\times$ Mn). The origin of migration is at the bottom of the photograph and the band migration is towards the anode. Genotypes within brackets are not distinguishable.

Table 1. Goodness-of-fit to tests for single locus segregation of genotypes in progenies of controlled crosses between *Citrus* and *Microcitrus*.

Locus	Cross ^z	Parental genotypes	Progeny no. and their genotypes	Expected ratio	Chi- ^x square	P
<i>Got -1</i>	1	FS × SS	8 FS : 4 SS	1:1	1.34	0.10-0.25
	2	FS × SS	41 FS : 38 SS	1:1	0.12	0.90-0.95
	3	MM × MS	14 MM : 4 MS	1:1	5.56	0.01-0.05
	4	FS × SS	25 FS : 20 SS	1:1	0.56	0.25-0.50
<i>Got -2</i>	1	MM × MR	7 MM : 5 MR	1:1	0.34	0.50-0.75
	2	MM × MM	79 MM (100%)	—	—	—
	3	MM × MR	12 MM : 6 MR	1:1	2.00	0.10-0.25
	4	MM × MM	45 MM : (100%)	—	—	—
<i>Got -3</i>	1	Mn × AM	4 An : 4 AM : 4 (MM+Mn) ^y	1:2:1	2.00	0.25-0.50
	2	Mn × Mn	79 (Mn+MM+nn)	—	—	—
	3	AM × AA	14 AM : 4 AA	1:1	5.56	0.01-0.05
	4	En × Mn	16 En : 25 (Mn+nn) : 4 EM	1:2:1	6.95	0.01-0.05

^z Cross designation : 1=*C. grandis* (cv. Tosa buntan) × 'Sydney hybrid', 2=*C. grandis* (cv. Tosa buntan) × *M. inodora*, 3=*C. macroptera* × *M. australasica*, 4=*C. iyo* (cv. Miyouchi Iyo) × *M. inodora*.

^y Genotypes within brackets are not distinguishable.

^x Degrees of freedom were, 1 and 2 for 1:1 and 1:2:1 ratios respectively.

Table 2. Comparison of common alleles among the genera of "true citrus fruit trees."

Locus	Allele ^z	<i>Microcitrus</i>	<i>Citrus</i>	<i>Fortunella</i>	<i>Poncirus</i>	<i>Eremocitrus</i>	<i>Clymenia</i>
<i>Got -1</i>	P	—	—	+	+	—	—
	F	+	+	—	—	—	—
	M	+	+	+	+	+	—
	W ^y	+	—	—	—	—	—
	A	—	+	—	—	—	—
	S	+	+	+	—	+	+
	E ^y	—	—	—	—	+	—
<i>Got -2</i>	A	—	+	—	—	—	—
	M	+	+	+	+	+	—
	B	—	+	—	—	—	—
	S	—	+	—	+	+	—
	R ^y	+	—	—	—	—	—
	W ^y	+	—	—	—	—	—
	G ^y	+	—	—	—	—	—
	C ^x	—	—	—	—	—	+
<i>Got -3</i>	n ^y	+	—	—	—	—	—
	A	+	+	—	—	—	—
	C	—	+	—	—	—	—
	E	—	+	+	—	—	—
	D	—	+	—	—	—	—
	B	—	—	+	—	—	—
	M ^y	+	—	—	—	?	—
<i>Got -3</i>	n	+	+	—	+	?	—

+ : Present, — : Absent, ? : Genotype not determined.

^z Data adopted from Hirai et al. (1986 a) and Hirai and Kajiura (1987). Allele 'A' at *Got-2* correspond to 'F' allele of Torres et al. (1978 b) and Soost and Torres (1981).

^y Alleles were newly discovered by the present authors (Rahman and Nito, 1994 a, b).

^x Data adopted from Torres et al. (1978 b) and Soost and Torres (1981).

single gene (*Got-2*) (Table 1). The presence of an intermediate intensively staining band in the heterozygotes indicates that the enzymes in GOT-2 enzyme are dimeric.

The segregation of GOT-3 zone from 4 intergeneric crosses (Table 1) suggests that a gene, *Got-3*, codes for the enzymes in that zone. The results from intergeneric crosses (cross no. 1, 2, and 4) suggest that a null allele 'n' is present in one or both of the parents (Table 1).

Goodness-of-fit tests for single locus segregation of GOT isozymes suggested that each of three loci controlling GOT isozymes in *Citrus* and *Microcitrus* have similar genetic systems (Table 1).

Alleles recognised at three GOT loci in "true citrus fruit trees" are presented in Table 2. A total of 23 alleles of which 7 at *Got-1*, 9 at *Got-2* and 7 at *Got-3* have been identified. Common 'M' alleles were observed in *Fortunella*, *Eremocitrus*, *Poncirus*, *Microcitrus*, and *Citrus* at *Got-1* and *Got-2*; whereas 'S' allele at *Got-1* was common to *Fortunella*, *Eremocitrus*, *Clymenia*, *Microcitrus*, and *Citrus* (Table 2).

Discussion

The disjunctive distribution of the "true citrus fruit trees" group is considered to be the result of geological and climatic events. It is assumed that *Microcitrus* and *Eremocitrus* migrated into Australia from Asia via New Guinea during the mid-Miocene about 15 MYBP (Raven and Axelrod, 1974; Scora, 1988). According to continental drift hypothesis, Australia and New Guinea were attached to southern Asia before the separation took place during the late Oligocene and Miocene (Benson, 1923; Brough, 1933; Scora, 1988). It is predicted that before separation all the "true citrus fruit trees" had distributed sequentially in Asia and originated from the same ancestor (Scora, 1988; Iwamasa and Nito, 1988).

Some common isozyme bands of amylase and peroxidase were observed among "true citrus fruit trees" to indicate their close relationships (Esen and Soost, 1976; Esen and Scora, 1977). Handa et al., (1986) on the basis of Fraction I protein analysis suggested that *Fortunella*, *Poncirus*, and *Citrus* are closely related to one another. The same results were obtained in numerical taxonomic studies based on morphological characters (Barrett and Rhodes, 1976; Handa and Oogaki,

1985).

The chromosomal numbers of the six genera of "true citrus fruit trees" reported to be identical ($2n=18$) (Krug, 1943; Marcelo and Guerra, 1984, 1985). On the basis of chromosome pairing in meiosis in the intergeneric hybrids, it was concluded that the four genera (*Fortunella*, *Poncirus*, *Microcitrus*, and *Citrus*) of "true citrus fruit trees" represent the same genome (Nakamura, 1934, 1942; Naithani and Raghuvanshi, 1962; Iwamasa and Nito, 1988).

In this study, some common alleles between "true citrus fruit trees" at the three loci controlling GOT isozymes were recognized (Table 2), confirming the close affinity among the genera. As both our finding and all the above mentioned literatures show evidence of close affinity relationships among "true citrus fruit trees", it would be conceivable to consider that six genera of the "true citrus fruit trees" originated from the same ancestor as reported previously (Scora, 1988; Iwamasa and Nito, 1988).

Cross compatibility has been reported between some members of "true citrus fruit trees", and no isolating barrier of intersterility was observed between *Citrus* and *Microcitrus* species (Barrett, 1977, 1981, 1985; Iwamasa et al., 1988). Somatic hybridizations were also reported between the genera of "true citrus fruit trees" (Ohgawara et al., 1985; Vardi et al., 1989; Deng et al., 1992). Though the species of these two genera have been isolated from each other for tens of million of years, genetic identity between the two genera has been clarified to be conserved. Isozyme analysis in this study indicated that *Citrus* and *Microcitrus* had similar genetic systems. These findings support that a close affinity exists between the genome of *Citrus* and *Microcitrus*. This genome affinity of the species in the two genera is supported by the cytogenetic evidence from an intergeneric hybrid (Iwamasa and Nito, 1988).

The genus *Clymenia* has no sexual compatibility with *Citrus*; whereas the other four genera of "true citrus fruit trees" have the compatibility (Sykes, 1988). The *Clymenia* is considered to be a somewhat distant relative of the other five genera of "true citrus fruit trees" (Swingle and Reece, 1967). In this isozyme study, alleles 'M' at *Got-1* and 'M' at *Got-2* were commonly observed in *Fortunella*, *Eremocitrus*, *Poncirus*, *Microcitrus*, and

Citrus, but they were not found in *Clymenia* (Table 2), suggesting divergence of *Clymenia* from the other five genera of "true citrus fruit trees".

The 'S' allele at *Got-1* is commonly observed in the five genera of "true citrus fruit trees" including *Clymenia*. The existence of this common allele suggests that the *Clymenia* still conserves a genetic identity with *Fortunella*, *Eremocitrus*, *Microcitrus*, and *Citrus*; although, now *Clymenia* has been highly differentiated from the other "true citrus fruit trees".

In this research, we recognized some common alleles at three loci of GOT isozymes between the six genera of "true citrus fruit trees", suggesting that a certain degree of genetic identity is maintained among the genera.

The electrophoretic results of this study support the previous phylogenetic assessment (Barrett and Rhodes, 1976; Handa and Oogaki, 1985; Handa et al., 1986; Iwamasa and Nito, 1988) regarding "true citrus fruit trees" that all six genera viz. *Fortunella*, *Eremocitrus*, *Poncirus*, *Clymenia*, *Microcitrus* and *Citrus* represent the same genome and originated from the same ancestor. The data were consistent with the cytogenetic evidence in this regard. This discovery contributes to understanding the evolution of species in subtribe Citrinae (Rutaceae).

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グルタミン酸オキザロ酢酸アミノ基転移酵素 (GOT) アイソザイム分析による 真正カンキツ類の系統分化の解明

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摘 要

カンキツ亜連, 真正カンキツ類の系統分化および類縁関係を検討するため, 葉のグルタミン酸オキザロ酢酸アミノ基転移酵素 (EC 2.6.1.1; GOT) アイソザイムについてポリアクリルアミドゲル電気泳動法を用いて分析を行った。

カンキツ属とミクロシトラス属の属間交雑実生のアイソザイム分析の結果, GOT を支配する *Got-1*, *Got-2* および *Got-3* の3遺伝子座における遺伝子型の分離が

それぞれ単純なメンデル遺伝に従うことを確認した。このことから, これら2属が類似の遺伝系を持ち, 近縁であることが推察できた。

真正カンキツ類6属の *Got-1* *Got-2* および *Got-3* において互いに共通するいくつかの対立遺伝子が認められた。真正カンキツ類6属は地理的分布や交雑親和性などで隔離されているが, ある程度の遺伝子的相似度を互いに維持していることが示唆された。