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Isozyme and DNA Analyses of Local *Citrus* Germplasm on Amami Islands, Japan

Masashi Yamamoto*, Rumiko Kouno, Tsuyoshi Nakagawa, Takuma Usui, Tatsuya Kubo and Shigeto Tominaga

Faculty of Agriculture, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan

Genetic relationships among local *Citrus* accessions on the Amami islands, Japan, were evaluated using isozyme, random amplified polymorphic DNA (RAPD) and cleaved amplified polymorphic sequence (CAPS) of chloroplast DNA (cpDNA) analyses. Four loci were detected for three enzymes examined in isozyme analysis. Four and three kinds of genotypes were detected in glutamate oxaloacetate transminase (GOT)-1 and GOT-2, respectively. At least six genotypes were observed in peroxidase (PX). All accessions showed the same genotype except for one accession of superoxide dismutase (SOD)-3. In general, accessions that belong to the same species or type showed identical genotypes; however, some diversity of genotypes was observed within the same species and type. On the basis of the RAPD data, genetic relationships were assessed using NJ cluster analysis. From this result, accessions could be classified into three major clusters, A, B, and C. Cluster A included *C. nobilis* (Kunenbo), *C. keraji* ('Keraji-Kikaijima' and Kabuchii), *C. oto* (Oto), 'Keraji-Kakeromajima', and 'Oto-Okinoerabujima'. Cluster B included only control accessions. Cluster C could be divided into four subclusters as follows: subcluster *C. depressa* (Shiikuwasha), some Sour orange relatives such as 'Fusuu' and 'Kusa', 'Kikaimikan-Okinoerabujima', and 'Shimamikan'. Accessions were classified into three types in cpDNA analysis. Type I included *C. nobilis* (Kunenbo), *C. keraji* ('Keraji-Kikaijima' and Kabuchii), *C. oto* (Oto), 'Keraji-Kakeromajima', 'Oto-Okinoerabujima', and *C. rokugatsu*. Type II was composed of *C. depressa* (Shiikuwasha), 'Kikaimikan-Okinoerabujima', 'Kusa', and 'Shiikuu'. Type III consisted of only 'Shimamikan'. All mandarin accessions that were determined to belong to cluster A in RAPD analysis were included in type I of cpDNA analysis. Meanwhile, mandarins in types II and III in cpDNA analysis consisted of accessions in cluster C of RAPD analysis.

Key Words: CAPS, cpDNA, genetic resources, RAPD, Ryukyu (Nansei) islands.

Introduction

The Amami islands are located between a latitude of approximately 27 and 29 degrees north and are in the northern part of the Ryukyu (Nansei) islands, Japan. Various local *Citrus* are grown in this subtropical region. Among them, *Citrus depressa* Hayata (Shiikuwasha) is the only indigenous species. Others have been introduced from China or Southeast Asia or as new seedlings that have arisen by chance from indigenous or introduced species (Tanaka, 1946). Since there are large geographical and climatic differences between the Amami islands and the main islands (Honshu, Kyushu, and Shikoku) of Japan, there are unique local *Citrus* genetic resources on the Amami islands.

Recently, the number of local *Citrus* in this region has been decreasing. Economically important fruit crops such as *C. tankan* Hayata (Tankan), *Mangifera indica* L. (mango), and *Passiflora edulis* Siems (passionfruit) have been replanted. Many *Citrus* trees have been in danger of lose owing to serious pests (white-spotted longicorn beetle) and disease (Huanglongbing). Thus, exploration and preservation of these local *Citrus* accessions are very important and urgent. In the 1990s and 2000s, Ishihata et al. (1997), Nakano et al. (2001), and Yamamoto et al. (2006) investigated local *Citrus* accessions on the Amami islands. Although many accessions were recorded and classified in these studies, the genetic relationships among them were not clarified.

Studies of genetic relationships among citrus have developed rapidly on the basis of the results of isozyme (Ashari et al., 1989; Hirai and Kajiura, 1987; Hirai et al., 1986b; Torres et al., 1978) and DNA analyses (Barkley et al., 2006; Federici et al., 1998; Nicolosi et al., 2000; Yamamoto et al., 1993). It is necessary to conduct this kind of study on local *Citrus* on the Amami islands; therefore, in this study, we analyzed isozymes

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* Corresponding author (E-mail: yamasa@agri.kagoshima-u.ac.jp).

and DNA of local *Citrus* accessions collected during our exploration of this region (Yamamoto et al., 2006). For isozymes, glutamate oxaloacetate transminase (GOT), peroxidase (PX), and superoxide dismutase (SOD), which are known to provide valuable data for *Citrus* classification, were analyzed (Hirai and Kajiura, 1987; Hirai et al., 1986b). Random amplified polymorphic DNA (RAPD) and cleaved amplified polymorphic sequence (CAPS) of chloroplast DNA (cpDNA), both of which are useful in the analysis of genetic relationships among *Citrus* (Asadi Abkenar et al., 2004; Coletta Filho et al., 1998; Nicolosi et al., 2000), were performed for DNA analysis.

Materials and Methods

Plant material

In our exploration of eight of the Amami islands (Yamamoto et al., 2006), leaves of local *Citrus* accessions were collected, brought back to Kagoshima University, and stored at −75°C until used. The control accessions in this study were preserved at the Faculty of Agriculture, Kagoshima University except for Fassarai lime. We analyzed 67 local and 23 control accessions for isozymes. For DNA analysis, 23 local and 8 control accessions were used (Table 1). Types and species of local *Citrus* accessions were determined according to morphological traits. *Citrus* accessions were basically classified according to Tanaka's system (Tanaka, 1969, 1977).

Isozyme analysis

Leaves were homogenized with extraction buffer (95 mM Tris, 9.5 mM dithiothreithol, 4.3 mM EDTA, 1% Triton X-100, pH 7.5). After the centrifugation of the homogenate, the supernatant was used as an extract for polyacrylamide gel electrophoresis (7.5%). The gel was stained for GOT (E.C.2.6.1.1), PX (E.C.1.11.1.7) and SOD (E.C.1.15.1.1) according to the procedures of Hirai et al. (1986a), Hirano (1980), and Vallejos (1983), respectively. Locus and allele nomenclature procedures adopted were those of Hirai et al. (1986b) and Hirai and Kajiura (1987).

RAPD analysis

Total DNA was extracted from leaves according to the SDS method of Honda and Hirai (1990). Primers were purchased from Operon (Alameda, USA) and Bex (Tokyo, Japan). OPA04, OPA09, OPA12, OPA17, OPB09, OPB11, and OPB17 (Operon primers) and D02, D03, D04, D21, D67, and D87 (Bex Common primers) were used. These primers showed highly reproducible results in preliminary experiments. The PCR reaction mixture of 12.5 μL consisted of 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 10 ng template DNA, 0.2 mM dNTPs, 10 pmol primer and 1.25 unit Taq polymerase (Bioneer, Daejeon, Korea). PCR reactions were performed in a PC320 (Astec, Fukuoka, Japan) thermal cycler programmed as follows: initial heating at 95°C for 3 min, 45 cycles of denaturing at 94°C for 1 min, annealing at 37°C for 2 min, extension at 72°C for 3 min, and final extension of 15 min at 72°C. Amplified products were electrophoresed on 1.5% agarose gels (Seakem GTG Agarose, Takara Bio, Otsu, Japan), and detected by staining with Mupid-Blue (Advance, Tokyo, Japan). Reproducibility of the patterns was tested by running some of the reactions in duplicate. The bands were recorded as 1 for present and as 0 for absent. Genetic distance was calculated between each pair of accessions (Dice, 1945). For phenetic analysis, cluster analysis was conducted with Molecular Evolutionary Genetic Analysis (MEGA, ver. 3.1) software (Kumar et al., 2004) by applying the neighbor-joining (NJ) method.

Chloroplast (cp) DNA analysis

The template DNA used for cpDNA analysis was the same as that in RAPD analysis. Regions of cpDNA, *rbc*L (5'-ATGTCACCACAAACAGAAACTAAAGCAAGT-3')- ORF106 (5'-ACTACAGATCTCATACTACCCC-3'), were amplified using universal primers (Arnold et al., 1991). The composition of PCR reaction mixture was as described above except for the primers. Ten picomoles of each primer were used in cpDNA analysis. PCR reactions were performed in a PC320 (Astec) thermal cycler programmed as follows: initial heating at 94°C for 1 min, 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and final extension of 10 min at 72°C. Aliquots of amplified products were digested with 5 units of *Hha*I and *Hin*fI (Nippon Gene, Tokyo, Japan) for 4 h at 37°C. Digested amplified products were electrophoresed on 1.5% agarose gels (Seakem GTG Agarose, Takara Bio), and detected by staining with Mupid-Blue (Advance).

Results

Of the three enzyme systems tested, GOT had two zones of activity resulting in four loci, all showing variability among local *Citrus* accessions of the Amami islands. All alleles observed in this study have already been reported (Hirai and Kajiura, 1987; Hirai et al., 1986b). Since clear zymograms were not obtained for four accessions ('Masakunin' (No. 39), 'Kubutu' (No. 48), 'Tunge' (No. 50), and 'Bontan' (No. 58) in PX, their genotypes could not be determined.

The genotypes of four loci in local *Citrus* accessions are shown in Table 1. Although four *GOT-1* genotypes in local accessions were used in this study, that of many accessions was *SS*. The genotypes of a few accessions were *SA*, *FS*, and *FF*. Three genotypes of *GOT-2* were observed: *AA*, *MA*, and *MM*. *AA* was only detected in 'Masakunin' (No. 39). All *C. depressa* (Shiikuwasha) (No. 1–8) except for No. 9, *C. keraji* hort. ex Tanaka (Kabuchii) (No. 18–25) except for No. 26, and *C. oto* hort. ex Tanaka (Oto) (No. 29, 30) showed *MA* in *GOT-2*,

Table 1. Isozyme genotypes of local *Citrus* accessions grown on Amami islands and accessions used for DNA analysis.

No.	Type	Accession[z]	Latin name	Island	Genotype[y]				DNA analysis[x]	Note[w]
					GOT-1	*GOT-2*	*PX*	*SOD-3*		
	Mandarin									
	Shiikuwasha and its relatives									
1		Tachibana	*Citrus depressa* Hayata	Amami Oshima	SS	MA	CD	AA		A: 7001
2		Yamakunin	*C. depressa* Hayata	Tokunoshima	SS	MA	AD	AA		A: 6004
3		Shiikunin-Ama	*C. depressa* Hayata	Tokunoshima	SS	MA	CD	AA	X	A: 6009
4		Shiikunin-Kara	*C. depressa* Hayata	Tokunoshima	SS	MA	AD	AA	X	A: 6010
5		Shiikuribu-Kamishiro2	*C. depressa* Hayata	Okinoerabujima	SS	MA	AD	AA	X	A: 11002
6		Shiikuribu-Kamishiro6	*C. depressa* Hayata	Okinoerabujima	SS	MA	AD	AA		A: 11006
7		Shiikuribu-Tamina	*C. depressa* Hayata	Okinoerabujima	SS	MA	AD	AA		A: 11009
8		Kinkan	*C. depressa* Hayata	Yoronjima	SS	MA	AD	AA	X	A: 12001
9		(Yamato-8)	*C. sp.*	Amami Oshima	SS	MM	DD	AA		A: 7008
	Kunenbo and its relatives									
10		Tokunebu	*C. nobilis* Lour.	Amami Oshima	SS	MM	DD	AA	X	A: 7005
11		Kunenbo	*C. nobilis* Lour.	Amami Oshima	SS	MM	DD	AA		A: 7021
12		(Kuji-22)	*C. nobilis* Lour.	Amami Oshima	SS	MM	DD	AA		A: 7022
13		Tokunebu	*C. nobilis* Lour.	Kakeromajima	SS	MM	DD	AA		A: 8009
14		Toku	*C. nobilis* Lour.	Kikaijima	SS	MM	DD	AA		A: 1003
15		Tokunin	*C. nobilis* Lour.	Tokunoshima	SS	MM	DD	AA	X	A: 6003
16		Tokuribu	*C. nobilis* Lour.	Okinoerabujima	SS	MM	DD	AA	X	A: 11019
17		Unjoki	*C. nobilis* Lour.	Yoronjima	SS	MM	CD	AA	X	A: 12004
	Kabuchii and its relatives									
18		(Tokuhama-18)	*C. keraji* hort. ex Tanaka	Kakeromajima	SS	MA	DD	AA		A: 8018
19		(Ikechi-6)	*C. keraji* hort. ex Tanaka	Yoroshima	SS	MA	DD	AA		A: 9006
20		(Ikechi-7)	*C. keraji* hort. ex Tanaka	Yoroshima	SS	MA	DD	AA		A: 9007
21		Erabumikan	*C. keraji* hort. ex Tanaka	Ukeshima	SS	MA	DD	AA		A: 10012
22		Kikaimikan	*C. keraji* hort. ex Tanaka	Kikaijima	SS	MA	DD	AA	X	A: 1002
23		Natsukunin	*C. keraji* hort. ex Tanaka	Tokunoshima	SS	MA	DD	AA		A: 6002
24		Kabocha	*C. keraji* hort. ex Tanaka	Okinoerabujima	SS	MA	DD	AA	X	A: 11003
25		Kikaimikan	*C. keraji* hort. ex Tanaka	Yoronjima	SS	MA	DD	AA		A: 12006
26		Irabuoto	*C. keraji* hort. ex Tanaka	Yoronjima	SS	MM	CD	AA	X	A: 12007
27		(Kasari-3)	*C. sp.*	Amami Oshima	SS	MM	CD	AA		A: 7003
	Keraji									
28		Keraji	*C. keraji* hort. ex Tanaka	Kikaijima	SS	MM	DD	AA	X	A: 1001
	Oto									
29		Kurushima	*C. oto* hort. ex Yu. Tanaka	Okinoerabujima	SS	MA	CD	AA	X	A: 11015
30		Yunnuoto	*C. oto* hort. ex Yu. Tanaka	Yoronjima	SS	MA	CD	AA	X	A: 12011
	Dancy tangerin									
31		Akamikan	*C. tangerina* hort. ex Tanaka	Amami Oshima	SS	MM	DD	AA		A: 7007
	Shimamikan									
32		Shimamikan	*C. sp.*	Amami Oshima	SS	MM	CD	AA	X	A: 7004
33		Shimamikan	*C. sp.*	Kakeromajima	SS	MM	CD	AA		A: 8010
34		(Kanyu-11)	*C. sp.*	Kakeromajima	SS	MA	CD	AA		A: 8011
35		Sumimikan	*C. sp.*	Kakeromajima	SS	MM	CD	AA		A: 8020
36		Chinazekunin	*C. sp.*	Tokunoshima	SS	MM	CD	AA		A: 6007
	Other mandarin									
37		(Saneku-12)	*C. sp.*	Kakeromajima	SS	MM	CD	AA		A: 8012
38		Keraji	*C. sp.*	Kakeromajima	FS	MA	DD	AA	X	A: 8015
39		Masakunin	*C. sp.*	Tokunoshima	SS	AA	—[v]	AA		A: 6005
40		(Serikatsu-1)	*C. sp.*	Okinoerabujima	SS	MA	CD	AA		A: 11001
41		Kikaimikan	*C. sp.*	Okinoerabujima	SS	MA	AD	AA	X	A: 11004
42		(Kamishiro-5)	*C. sp.*	Okinoerabujima	SS	MA	CD	AA		A: 11005
43		(Tamina-11)	*C. sp*	Okinoerabujima	SS	MA	CD	AA		A: 11011
44		Oto	*C. sp*	Okinoerabujima	SS	MM	CD	AA	X	A: 11010
45		Shiioto	*C. sp*	Okinoerabujima	SS	MM	CD	AA		A: 11021
	Sour orange and its relatives									
46		(Yoro-1)	*C. aurantium* L.	Yoroshima	SA	MM	BD	AA		A: 9001

Table 1. Continued.

No.	Type	Accession[z]	Latin name	Island	Genotype[y]				DNA analysis[x]	Note[w]
					GOT-1	*GOT-2*	*PX*	*SOD-3*		
47		(Yoro-2)	*C. aurantium* L.	Yoroshima	SA	MM	BD	AA		A: 9002
48		Kubutu	*C. rokugatsu* hort. ex Yu. Tanaka	Yoroshima	SS	MA	—[v]	AA		A: 9008
49		Fusuu	*C. rokugatsu* hort. ex Yu. Tanaka	Kikaijima	SS	MA	DD	AA	X	A: 1010
50		Tunge	*C. rokugatsu* hort. ex Yu. Tanaka	Okinoerabujima	SS	MA	—[v]	AA	X	A: 11016
51		Ishikata	*C. rokugatsu* hort. ex Yu. Tanaka	Yoronjima	SS	MA	DD	AA	X	A: 12009
52		(Sachiyuki-14)	*C. rokugatsu* hort. ex Yu. Tanaka	Kakeromajima	SS	MA	DD	AA		A: 8014
53		Kusa	*C. sp.*	Amami Oshima	SS	MA	CD	AA	X	A: 7002
54		Kusa	*C. sp.*	Yoroshima	SS	MA	CD	AA		A: 9004
55		Shiikuu	*C. sp.*	Kikaijima	SS	MA	CD	AA	X	A: 1008
56		Tunugekunin	*C. sp*	Tokunoshima	SS	MM	CD	AA		A: 6006
57		(Naze-6)	*C. sp.*	Amami Oshima	SS	MM	BD	AA		A: 7006
	Pummelo									
58		Bontan	*C. maxima* (Burm.) Merr.	Kakeromajima	FF	MM	—[v]	AA		A: 8016
	Tangelo									
59		(Ikechi-5)	*C. sp.*	Yoroshima	SS	MM	AC	AA		A: 9005
60		(Ukeamuro-9)	*C. sp.*	Ukeshima	FS	MM	BD	AA		A: 10009
61		(Ukeamuro-10)	*C. sp.*	Ukeshima	FS	MM	BD	AA		A: 10010
62		(Tamina-8)	*C. sp.*	Okinoerabujima	SS	MA	AD	AA		A: 11008
	Miscellaneous									
63		(Uken-23)	*C. sp.*	Amami Oshima	SA	MM	CC	AE		A: 7023
64		(Yoro-3)	*C. sp.*	Yoroshima	SS	MM	CD	AA		A: 9003
65		(Ukeamuro-11)	*C. sp.*	Ukeshima	SS	MA	CD	AA		A: 10011
66		(Tamina-7)	*C. sp.*	Okinoerabujima	SS	MM	CD	AA		A: 11007
67		Noborumikan	*C. sp.*	Yoronjima	FS	MM	AC	AA		A: 12005
	Control									
68		Hamlin	*C. sinensis* (L.) Osbeck		SS	MM	DD	AA	X	C: 117307
69		Otsu 4 gou	*C. unshiu* Marcow.		SS	MM	DD	AA		C: 117319
70		Kinokuni	*C. kinokuni* hort. ex Tanaka		SS	MM	DD	AA		D: p20
71		Sunki	*C. sunki* (Hayata) hort. ex Tanaka		SS	MM	DD	AA		B: 117403
72		Clementine	*C. clementina* hort. ex Tanaka		SS	MM	DD	AA		D: p. 19
73		Cleopatra	*C. reshni* hort. ex Tanaka		SS	MM	DD	AA	X	B: 117402
74		Kunenbo	*C. nobilis* Lour.		SS	MM	DD	AA	X	C: 117387
75		Koji	*C. leiocarpa* hort. ex Tanaka		SS	MM	CD	AA		D: p. 20
76		Yoshida ponkan	*C. reticulata* Blanco		SS	MM	CC	AA		C: 113178
77		Shiikuwasha-Okinawa	*C. depressa* Hayata		SS	MA	AD	AA	X	C: 117406
78		Oto	*C. oto* hort. ex Yu. Tanaka		SS	MA	CD	AA	X	D: p: 19
79		Tachibana	*C. tachibana* (Makino) Tanaka		SS	AA	CC	AA	X	D: p. 20
80		Kabosu	*C. sphaerocarpa* hort. ex Tanaka		SS	MB	CC	AA		C: 117381
81		Yuzu	*C. junos* Siebold ex Tanaka		SS	MB	AC	AA		D: p. 19
82		Kabusu	*C. aurantium* L.		SA	MM	BD	AA	X	C: 117365
83		Allen eureka	*C. limon* (L.) Burm. f.		FS	SM	CD	AE		D: p. 17
84		Hassaku	*C. hassaku* hort. ex Tanaka		FS	MM	BD	AA		C: 117286
85		Madohongyou	*C. maxima* (Burm.) Merr.		FF	MM	—[v]	AA		D: p. 17
86		Genshokan	*C. genshokan* hort. ex Tanaka		—[u]	—[u]	—[u]	—[u]	X	B: 113159
87		Busshukan	*C. medica* L.		FF	MM	CC	EE		D: p. 17
88		Purrut	*C. hystrix* DC.		SM	MM	CC	—[u]		D: p. 17
89		Fassarai lime	*C. sp* (Sect. *Limonellus*)		FF	SM	CC	—[u]		E
90		Calamondin	*C. madurensis* Lour.		SM	MM	DF	—[u]		D: p. 20

[z] Accessions in parentheses have tentative names.
[y] Locus and allele nomenclature procedures adopted are those of Hirai et al. (1986b) and Hirai and Kajiura (1987).
[x] Accessions used in DNA analysis are indicated by 'X'.
[w] A: Accession number of a previous report (Yamamoto et al., 2006). B: An accession identical to that of NIAS Genebank and its JP number. C: An accesion probably identical to that of NIAS Genebank and its JP number. D: An accession without accession number listed in a previous list (Yamamoto et al., 2005) and its page number appeared. E: Its isozyme genotype was reported in Tominaga et al. (2003).
[v] The genotype could not be determined because clear zymogram was not obtained.
[u] The genotype was not analyzed.

whereas that of all *C. nobilis* Lour. (Kunenbo) (No. 10–17) and Shimamikan (No. 32, 33, 35, 36) except for No. 34 was *MM*. Both *MA* and *MM* were found in type Other mandarin (No. 37, 38, 40–45), Sour orange and its relatives (No. 46–57), Tangelo (No. 59–62), and Miscellaneous (No. 63–67). At least six genotypes of *PX* were designated as *AC*, *AD*, *BD*, *CC*, *CD*, and *DD*. Allele *D* existed in almost all accessions analyzed. *CD* and *DD* were the predominant genotypes. Some *C. depressa* (Shiikuwasha) (No. 2, 4–8) and the other two accessions (No. 41, 62) showed *AD*. *C. aurantium* L. (Sour orange) (No. 46, 47), one of its relatives (No. 57), and two Tangelo (No. 60, 61) had *BD*. *AC* and *CC* were found in a few accessions (No. 59, 63, 67). The *SOD-3* genotype of all accessions was *AA* except for 'Uken-23' (No. 63) of type Miscellaneous.

In RAPD analysis, the sizes of polymorphic fragments ranged from 500 to 2000 base pairs. There were 28 polymorphic fragments in total and 2.2 polymorphic fragments per primer on average. The number of polymorphic fragments produced by each primer ranged from one by OPA17, OPB09, OPB17, D21, and D87 to four by OPB11, D03, and D04.

On the basis of the RAPD data, genetic relationships among local *Citrus* accessions were assessed using NJ cluster analysis (Fig. 1). From this result, the 31 accessions including controls could be classified into three major clusters, A, B, and C. Cluster A included *C. nobilis* (Kunenbo) (No. 10, 15–17), *C. keraji* ('Keraji-Kikaijima' (No. 28) and Kabuchii (No. 22, 24, 26)), *C. oto* (Oto) (No. 29, 30), 'Keraji-Kakeromajima' (No. 38), and 'Oto-Okinoerabujima' (No. 44). *C. nobilis* and *C. keraji* were closely related. Cluster B included only control accessions. Cluster C could be divided into four subclusters as follows: subcluster *C. depressa* (Shiikuwasha) (No. 3–5, 8), some Sour orange relatives (No. 49–51, 53, 55) such as 'Fusuu' (No. 49) and 'Kusa' (No. 53), 'Kikaimikan-Okinoerabujima' (No. 41), and 'Shimamikan' (No. 32).

In a two primer/enzyme combination, *rbcL*-ORF106/*Hin*fI and *Hha*I, each showed two types of banding pattern among the accessions used in this study (Table 2). From the two combined results, accessions were classified into three types. Type I included *C. nobilis* (Kunenbo) (No. 10, 15–17), *C. keraji* ('Keraji-Kikaijima' (No. 28) and Kabuchii (No. 22, 24, 26)), *C. oto* (Oto) (No. 29, 30), 'Keraji-Kakeromajima' (No. 38), 'Oto-Okinoerabujima' (No. 44), and *C. rokugatsu* hort. ex Yu. Tanaka (No. 49–51). Type II was composed of *C. depressa* (Shiikuwasha) (No. 3–5, 8), 'Kikaimikan-Okinoerabujima' (No. 41), 'Kusa' (No. 53), and 'Shiikuu' (No. 55). Type III consisted of only 'Shimamikan' (No. 32). All local mandarin accessions determined to belong to cluster A in RAPD analysis were included in type I of cpDNA analysis. Meanwhile, local mandarins in types II and III in cpDNA analysis consisted of accessions in cluster C of RAPD analysis.

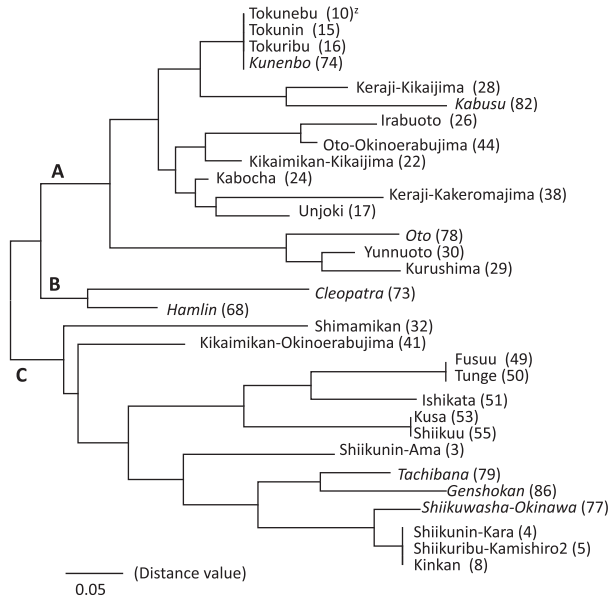

Fig. 1. Genetic relationships of local *Citrus* accessions of Amami islands assessed by neighbor-joining method cluster analysis of RAPD data. Accession names in italics are controls. [z] Number in parentheses indicates No. in Table 1.

Table 2. Types of cpDNA of *Citrus* accessions of Amami islands used in this study.

Type	Accession	*rbc*L-ORF106[z]	
		*Hin*fI[y]	*Hha*I
I	Tokunebu (10), Tokunin (15), Tokuribu (16), Unjoki (17), Kikaimikan (22)[w], Kabocha (24), Irabuoto (26), Keraji-Kikaijima (28)[w], Kurushima (29), Yunnuoto (30), Keraji-Kakeromajima (38), Oto-Okinoerabijima (44), Fusuu (49), Tunge (50), Ishikata (51), *Hamlin*[v] (68), *Kunenbo* (74)[w], *Oto* (78), *Kabusu* (82)	a[x]	a
II	Shiikunin-Ama (3), Shiikunin-Kara (4)[w], Shiikuribu-Kamishiro2 (5)[w], Kinkan (8), Kikaimikan-Okinoerabujima (41), Kusa (53), Shiikuu (55), *Cleopatra* (73), *Shiikuwasha-Okitnawa* (77), *Tachibana* (79)[w]	b	a
III	Shimamikan (32), *Genshokan* (86)	b	b

[z] Primers.
[y] Restriction enzyme.
[x] Same letter indicates the same banding pattern.
[w] Their cpDNA types were reported in Yamamoto et al. (2010a).
[v] Accession names written in italics are controls.

Discussion

In the analytical methods used in this study, isozyme analysis detected differences in the size and shape of enzyme molecules based on electrophoretic mobilities. Among the various DNA analyses, RAPD is a fast and easy technique which amplified template DNA using an arbitrary oligonucleotide primer. CAPS is a method which detects polymorphisms by digestion of the amplified products with endonuclease. Among them, occasional spurious products can be detected in RAPD (Waugh and Powell, 1992); thus, we conducted RAPD in duplicate and confirmed reliable bands. On the other hand, three enzymes used in isozyme and CAPS of cpDNA analyses are well known for their ability to produce highly reliable results (Asadi Abkenar et al., 2004; Hirai and Kajiura, 1987; Hirai et al., 1986b; Nicolosi et al., 2000).

Recent DNA analyses (Barkley et al., 2006; Federici et al., 1998; Nicolosi et al., 2000; Yamamoto et al., 1993) revealed only three basic true species of *Citrus*; *C. medica* L. (citron), *C. maxima* (Burm.) Merr. (pummelo), and *C. reticulata* Blanco (mandarin). Others are of hybrid origin and some have genes from papeda, *Poncirus*, or *Fortunella*. In our exploration of the Amami islands (Yamamoto et al., 2006), no local citron, papeda, *Poncirus* or *Fortunella* was found and they were not considered as ancestor of local accessions investigated in accordance with morphological traits. The main objective of the present study was to clarify genetic relationships among local citrus accessions in the Amami islands. Thus, citron, papeda, *Poncirus*, and *Fortunella* were not included as control accessions.

C. depressa (Shiikuwasha) was the only indigenous species used in this study. This species is distributed over almost all of the Ryukyu (Nansei) islands. On the other hand, *C. tachibana* (Makino) Tanaka, another indigenous species in Japan, is distributed in the main islands (Honshu, Kyushu, and Shikoku) of Japan to the Ryukyu (Nansei) islands. *C. depressa* and *C. tachibana* can be clearly distinguished, although there is a genetic relationship between them (Yamamoto et al., 1998, 2010a). Although phylogenic analysis of *C. depressa* in the Okinawa islands has been performed (Urasaki et al., 2005; Yamamoto et al., 1998), its analysis in the Amami islands has never been conducted. This is the first report on isozyme and DNA analyses of *C. depressa* in the Amami islands. There are two types of isozyme genotype in *C. depressa* in the Amami islands. Both types have already been reported in *C. depressa* from Okinawa (Yamamoto et al., 1998). A small difference in genotype was observed between *C. depressa* on Amami and Okinawa; *AD* for *PX* is predominant in *C. depressa* from Amami whereas *CD* for *PX* is predominant in that from Okinawa. In RAPD analysis, three out of four *C. depressa* accessions from the Amami islands always showed identical banding patterns. Close relationships between *C. depressa* from the Amami and Okinawa islands were observed. Similar results were obtained in inter simple sequence repeat (ISSR) analysis (Yamamoto et al., 2010a). These results suggested high genetic homology among the *C. depressa* in the Amami islands and relatedness between those from Amami and Okinawa, although a large number of accessions require further analysis.

C. nobilis (Kunenbo), which originated in Vietnam, is considered to be a natural tangor (a hybrid of mandarin and sweet orange) and was introduced into Japan more than 400 years ago (Tanaka, 1948). The type of cpDNA of *C. nobilis* is distinguished from those of most mandarins, but is identical to that of *C. maxima* (pummelo) and is probably derived from *C. sinensis* (sweet orange) (Yamamoto and Kobayashi, 1996). Thus, the female ancestor of *C. nobilis* is considered to be *C. sinensis*, although its male parent has not been elucidated. All *C. nobilis* accessions showed identical isozyme genotypes, and RAPD and cpDNA profiles in this study owing to clonal propagation, with the exception of 'Unjoki'. The fruit trait of 'Unjoki' is slightly different from that of Kunenbo (Yamamoto et al., 2006) and this is considered to be the result of seedlings that arose by chance from *C. nobilis*.

The type of cpDNA of *C. keraji* and *C. oto* is the same as that of *C. nobilis*. Moreover, these three species were determined to belong to the same cluster in RAPD analysis. In particular, 'Keraji-Kikaijima' was closely related to *C. nobilis*, suggesting that they arose from *C. nobilis* as a female ancestor. These results agree with a previous report (Yamamoto et al., 2010a). On the other hand, involvement of Japanese mandarins such as *C. depressa* in the development of Kabuchii and *C. oto* was observed. Their *GOT-2* genotype was *MA* and the *A* is a characteristic allele of Japanese mandarin (Hirai et al., 1986b). It could be considered that *C. nobilis* and *C. depressa* played a part in the origin of various mandarin accessions that arose as chance seedlings on the Amami islands. Limited genetic variability within Kabuchii was detected in isozyme and RAPD analyses; 'Irabuoto' was slightly different from other accessions. 'Irabuoto' is grown in Yoronjima, which is very close to Okinawa island. Since Kabuchii is distributed on Okinawa island (Inafuku-Teramoto et al., 2010), it is necessary to conduct analysis of genetic relationships of Kabuchii grown on both Amami and Okinawa islands.

'Shimamikan' was distinct from both *C. depressa* and *C. nobilis* in RAPD analysis. Moreover, its cpDNA was distinguished from those of all other local *Citrus* on the Amami islands. Although in this study this type of cpDNA was detected only in 'Shimamikan' and 'Genshokan', it was observed in several mandarins, such as *C. unshiu* Marcow., *C. reticulta* Blanco, and *C. kinokuni* hort. ex Tanaka (Yamamoto, et al., 2010b). It has not been possible to confirm whether the origin of 'Shimamikan' is as a chance seedling that arose on

the Amami islands or whether it was introduced.

C. rokugatsu, 'Kusa', and 'Shiikuu' have been considered to be *C. aurantium* relatives on the basis of morphological traits (Tanaka, 1969); however, their genetic relationship with *C. aurantium* is unclear. By contrast, the involvement of *C. depressa* in the development of *C. rokugatsu*, 'Kusa', and 'Shiikuu' was proposed; all possessed *A* in *GOT-2*, a characteristic allele of Japanese mandarin, and they were determined to belong to a *C. depressa* cluster in RAPD analysis. Although a close relationship between *C. rokugatsu* and 'Kusa'/'Shiikuu' was observed in RAPD analysis, their cpDNA profiles were different. These results suggest that *C. rokugatsu* and 'Kusa'/'Shiikuu' arose independently.

Morphologically different 'Keraji' were found in both Kikaijima and Kakeromajima. The results of isozyme and RAPD analyses proved that these two accessions are completely different. The same result was observed for 'Kikaimikan' grown in Kikaijima and Okinoerabujima. These two accessions are genetically different. 'Oto' of Okinoerabujima is different from 'Oto' (*C. oto*) on Okinawa.

For the results mentioned above, studies of genetic relationships among local *Citrus* accessions in the Amami islands has developed. In particular, we could demonstrate that *C. depressa* and *C. nobilis* played a part in the origin of many local *Citrus* in this region; however, the origin of both species is still unresolved. Barkley et al. (2006) reported genes from *Fortunella*/papeda and *Poncirus* to *C. depressa* and *C. nobilis*, respectively; therefore, it is necessary to conduct DNA analysis including the above mentioned species and basic species. In addition, the Ryukyu (Nansei) islands consist mainly of the Amami and Okinawa islands. There are close geographical and historical relationships between these two regions; thus, it is necessary to conduct studies of the genetic relationships among local *Citrus* accessions grown all over the Ryukyu (Nansei) islands.

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Literature Cited

Arnold, M. L., C. M. Bucker and J. J. Robinson. 1991. Pollen-mediated introgression and hybrid speciation in Louisiana irises. Proc. Natl. Acad. Sci. USA 88: 1398–1402.

Asadi Abkenar, A., S. Isshiki and Y. Tashiro. 2004. Phylogenic relationships in the "true citrus fruit trees" revealed by PCR-RFLP analysis of cpDNA. Sci. Hortic. 102: 233–242.

Ashari, S., D. Aspinall and M. Sedgley. 1989. Identification and investigation of relationships of mandarin types using isozyme analysis. Sci. Hortic. 40: 305–318.

Barkley, N. A., M. L. Roose, R. R. Krueger and C. T. Federici. 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple seaquence repeat markers (SSRs). Theor. Appl. Genet. 112: 1519–1531.

Coletta Filho, H. D., M. A. Machado, M. L. P. N. Targon, M. C. P. Q. D. G. Moreira and J. Pompeu Jr. 1998. Analysis of the genetic diversity among mandarins (*Citrus* spp.) using RAPD markers. Euphytica 102: 133–139.

Dice, L. R. 1945. Measures of the amount of ecologic association between species. Ecology 26: 297–302.

Federici, C. T., D. Q. Fang, R. W. Scora and M. L. Roose. 1998. Phylogenic relationships within the genus *Citrus* (*Rutaceae*) and related genera as revealed by RFLP and RAPD analysis. Theor. Appl. Genet. 96: 812–822.

Hirai, M. and I. Kajiura. 1987. Genetic analysis of leaf isozymes in citrus. Japan. J. Breed. 37: 377–388.

Hirai, M., I. Kozaki and I. Kajiura. 1986a. The rate of spontaneous inbreeding of trifoliate orange and some characteristics of the inbred seedlings. Japan. J. Breed. 36: 138–146.

Hirai, M., I. Kozaki and I. Kajiura. 1986b. Isozyme analysis and phylogenic relationship of citrus. Japan. J. Breed. 36: 377–389.

Hirano, H. 1980. Thremmatological studies of protein variation in mulberry. Bull. Sericul. Exp. Sta. 28: 67–186 (In Japanese with English abstract).

Honda, H. and A. Hirai. 1990. A simple and efficient method for identification of hybrids using nonradioactive rDNA as probe. Japan. J. Breed. 40: 339–348.

Inafuku-Teramoto, S., M. Yamamoto, H. Kinjo, A. Kitajima, K. Wada and Y. Kawamitsu. 2010. Local citrus genetic resources and their polymethoxyflavones content in northern part of Okinawa island. Hort. Res. (Japan) 9: 263–271 (In Japanese with English abstract).

Ishihata, K., H. Shiota and M. Onjo. 1997. Some kinds of tropical and subtropical fruit trees and characteristics of some native citrus fruits in Amami islands. Bull. Exp. Farm. Fac. Agr. Kagoshima Univ. 22: 1–13 (In Japanese with English abstract).

Kumar, S., K. Tamura and M. Nei. 2004. MEGA 3: Integrated software for molecular evotionary genetic analysis and sequence alignment. Brief Bioinformatics 5: 150–163.

Nakano, M., H. Nesumi and T. Yoshida. 2001. Exploration for citrus genetic resources on the Nansei archipelago. Ann. Rep. Explore. Intro. Plant Genet. Resour. 17: 39–48 (In Japanese with English abstract).

Nicolosi, E., Z. N. Deng, A. Gentile, S. La Malfa, G. Continella and E. Tribulato. 2000. Citrus phylogeney and genetic origin of important species as investigated by molecular markers. Theor. Appl. Genet. 100: 1155–1166.

Tanaka, T. 1969. Misunderstanding with regards citrus classification and nomenclature. Bull. Univ. Osaka Pref., Ser. B. 21: 139–145.

Tanaka, T. 1977. Fundamental discussion of citrus classification. Studia Citrologia 14: 1–5.

Tanaka, Y. 1946. An iconograph of Japanese citrus fruits. Vol. 1 (In Japanese). Yokendo, Tokyo.

Tanaka, Y. 1948. An iconograph of Japanese citrus fruits. Vol. 2 (In Japanese). Yokendo, Tokyo.

Tominaga, S., M. Yamamoto, R. Kohno, B-J. Park, K. Tsuda, M. Onjo and K. Ichitani. 2003. Classification of citrus species on Yap Island and Ulthi Atoll of the FSM. Kagoshima Univ. Res. Center S. Pac. Occasional Papers 39: 93–98.

Torres, A. M., R. K. Soost and U. Deidenhofen. 1978. Leaf isozyme as genetic markers in citrus. Amer. J. Bot. 65: 869–881.

Urasaki, N., K. Yoshida, T. Uehara, H. Inoue, S. Onda, H. Kinjyo and S. Kawano. 2005. Single nucleotide polymorphism in shiikuwasha (*Citrus depressa* Hayata) chloroplast DNA, trnL-trnF. Jpn. J. Trop. Agr. 49: 246–251.

Vallejos, C. E. 1983. Enzyme activity staining. p. 469–516. In: S. D. Tanksley and T. J. Orton (eds.). Isozymes in plant genetics and breeding Part A. Elsevier, Amsterdom.

Waugh, R. and W. Powell. 1992. Using RAPD markers for crop improvement. Tib. Tech. 10: 186–191.

Yamamoto, M. and S. Kobayashi. 1996. Polymorphism of chloroplast DNA in citrus. J. Japan. Soc. Hort. Sci. 65: 291–296.

Yamamoto, M., M. Fukuda, T. Koga, T. Kubo and S. Tominaga. 2010a. Examination of the origin of Keraji (*Citrus keraji*), local citrus of Kikaijima island, Kagoshima prefecture. Hort. Res. (Japan) 9: 7–12 (In Japanese with English abstract).

Yamamoto, M., S. Kawaguchi, O. Tanimura and H. Nakano. 2005. List of fruit tree genetic resources in the Toso Orchard of the Experimental Farm, Faculty of Agriculture, Kagoshima University. 1. Citrus. Bull. Exp. Farm. Fac. Agr. Kagoshima Univ. 28: 15–21 (In Japanese).

Yamamoto, M., S. Kobayashi, Y. Nakamura and Y. Yamada. 1993. Phylogenic relationships of citrus revealed by RFLP analysis of mitochondrial and chloroplast DNA. Japan. J. Breed. 43: 355–365.

Yamamoto, M., Y. Matsuo, T. Kuniga, R. Matsumoto and Y. Yamada. 1998. Isozyme and RAPD analyses of shiikuwashas (*Citrus depressa* Hayata). Bull. Natl. Inst. Fruit Tree Sci. 30/31: 39–51 (In Japanese with English abstract).

Yamamoto, M., K. Matsushima, T. Ijichi, Y. Uechi, S. Kawaguchi, H. Nakano, T. Nomura, O. Tanimura, T. Kubo and S. Tominaga. 2006. Exploration and preservation of local citrus germplasms in the Amami archipelago. Bull. Exp. Farm. Fac. Agr. Kagoshima Univ. 29: 5–11 (In Japanese with English abstract).

Yamamoto, M., Y. Tsuchimochi, A. Kitajima, S. Inafuku-Teramoto, X. Yang, J. Yang, G. Zhong, T. Kubo and S. Tominaga. 2010b. Diversity of chloroplast DNA in *Citrus*. Hort. Res. (Japan) 9 (Suppl. 1): 47 (In Japanese).