Adv. Hort. Sci., 2014 28(2): 95-99

Characterization of chloroplast *matK* sequences of *Citrus tachibana* and *Citrus depressa*, two indigenous species in Japan

Y. Nagano[1], S. Inafuku-Teramoto[2,3], M. Hashimoto[4], T. Mimura[4], R. Matsumoto[4], M. Yamamoto[5(*)]

[1] *Analytical Research Center for Experimental Sciences, Saga University, Honjo-machi, Saga 840-8502, Japan.*

[2] *Faculty of Agriculture, University of The Ryukyus, Nishihara, Okinawa 903-0213, Japan.*

[3] *Botswana-JICA Jatropha Project, DAR, Sebele, Gaborone, Botswana.*

[4] *Faculty of Agriculture, Saga University, Honjo-machi, Saga 840-8502, Japan.*

[5] *Faculty of Agriculture, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan.*

Key words: cpDNA, genetic resources, Ryukyu islands, shiikuwasha, tachibana.

Abstract: *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* are indigenous mandarin species in Japan. We deduced their phylogenetic relationships from nucleotide sequences of the chloroplast *matK* gene. The results indicate that *C. tachibana*, *C. nippokoreana*, and *C. depressa* accessions can be classified into two types: type A, all sixteen *C. tachibana* and six *C. depressa*; type B, eleven *C. depressa* and one *C. nippokoreana*. Both type A and type B accessions of *C. depressa* were found on the Okinawa Islands, whereas only type B accessions of *C. depressa* were found on the Sakishima and Amami Islands. This cpDNA divergence seemed to indicate a polyphyletic origin of *C. depressa*. The *matK* genes of type A were found only in *C. tachibana* and some *C. depressa*. From these results, both species probably possess a characteristic chloroplast genome among various *Citrus* species.

1. Introduction

Citrus is one of the most important fruit crops in Japan and also worldwide. Various accessions of *Citrus* species are adapted to the southwest of Japan, and although they are cultivated in this region, almost all of them are non-native, that is, they were introduced from abroad, arose as chance seedlings, were selected from bud sports, and were bred by artificial pollination. Only two species, *Citrus tachibana* (Makino) Tanaka (Tachibana) and *Citrus depressa* Hayata (Shiikuwasha) were present in Japan before recorded history.

C. tachibana mainly grows indigenously on the Pacific side of the southwest of Japan's main islands (Kyushu, Shikoku, and Honshu). *C. tachibana* was recorded in "Kojiki", the oldest chronicle in Japan dating from the early 8th century. Its indigenous trees were also found on the Ryukyu Islands (islands including the Okinawa Islands, Sakishima Islands, and Amami Islands, which were ruled by Japan from the 17th to19th centuries) and Taiwan (Tanaka, 1931; Lin and Chen, 2006; Inafuku-Teramoto *et al*., 2010). *C. depressa* is indigenous to both the Ryukyu Islands and Taiwan (Tanaka, 1936; Lin and Chen, 2006). Compared to *C. tachibana*, *C. depressa* is considered to be adapted to a warmer climate; the former is usually used as an ornamental for gardens and its fruit is inedible. On the other hand, fruit of *C. depressa* is in much demand as an ingredient for food and drinks, to garnish dishes similar to a lemon or lime, to make juice and jam, and as an additive to soy sauce and distilled spirits. Recently, this fruit has attracted attention because it contains high levels of polymethoxyflavonoids, one of the most important health-promoting components of citrus (Inafuku-Teramoto *et al*., 2010).

We have investigated the phylogenetic relationships of *Citrus* and its relatives through the analysis of genes encoded in chloroplast DNA (cpDNA) (Tshering *et al*., 2010, 2013). In our recent study (Tshering *et al.*, 2013) in which various *Citrus* accessions were used as materials, we found that *C. tachibana* and *C. depressa* possess a characteristic cpDNA genome based on the sequences of the chloroplast *matK* genes, which encode a maturase involved in splicing type II introns from RNA transcripts (Hilu and Liang, 1997; Hilu *et al*., 2003; Olmstead and Palmer, 1994). There are many accessions in both species, and intraspecies diversity is found within each species (Hirai *et al*., 1990; Yamamoto *et al*., 1998; Kinjo, 2007; Inafuku-Teramoto *et al*., 2010; Yamamoto *et al*., 2011).

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

Received for publication 31 March 2014
Accepted for publication 1 July 2014

However, a limited number of accessions were used in our previous study (Tshering *et al*., 2013).

Therefore, for the present work, we analyzed the *matK* gene sequences of a number of *C. tachibana* and *C. depressa* plants grown in various regions in Japan to reveal their characteristic profiles of the cpDNA genome. *C. nippokoreana* (Korai Tachibana), a *C. tachibana* relative indigenous to Hagi City, Yamaguchi Prefecture, Japan, and Cheju Island, Korea (Kimura and Taninaka, 1995), was also investigated.

2. Materials and Methods

Plant materials

Sixteen *C. tachibana*, one *C. nippokoreana*, 17 *C. depressa*, and 13 control accessions were used in this study. The sources of the materials are shown in Table 1 and Figure 1.

PCR amplification and DNA sequencing

Genomic DNA was extracted from leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). By using this genomic DNA as a template, the *matK* gene was amplified by PCR using proofreading PrimeSTAR GXL DNA Polymerase (TAKARA BIO, Ohtsu, Shiga, Japan). The primers used for PCR amplification of the *matK* gene were matK1F (5′-ACCGTATCGCACTATGTATC-3′) and matK1R (5′-GAACTAGTCGGATGGAGTAG-3′). The amplified DNA fragments were purified using the NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Düren, Germany). The primers used for sequencing of the *matK* gene were matK1F, matK2F (5′-ACGGTTCTTTCTCCACGAGT-3′), matK3F (5′-GGTCCGATTTCTCTGATTCT-3′), matK1R, matK2R (5′-AGAATCAGAGAAATCGGACC-3′), and matK3R (5′-ACTCGTGGAGAAAGAACCGT-3′). The purified DNA fragments were sequenced in both directions in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) as described previously (Platt *et al*., 2007). Sequence data were submitted to DDBJ/GenBank/EBI and were assigned accession numbers ranging from AB839905 to AB839932. The sequences of the accessions from No. 29 to No. 34 were deposited in our previous study (Tshering *et al*., 2013).

Fig. 1 - Collection sites of *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* in the present study.

Phylogenetic analyses

The neighbor-joining (NJ) and maximum likelihood (ML) methods from the MEGA (version 5.2.1) program (Tamura *et al*., 2011) were used to create phylogenetic trees. The reliability of each branch was tested by bootstrap analysis with 1,000 replications.

3. Results and Discussion

We constructed multiple sequence alignments of 1,630-bp fragments containing the *matK* gene from different *Citrus* accessions. Each sequence contained a 1,530-bp protein-coding sequence and 100 bp of the 3′ UTR. One exception is the *matK* gene of trifoliate orange (*Poncirus trifoliata*), which has a 6 bp insertion at the 3′ UTR. Of these, 23 bases were variable and six bases were phylogenetically informative.

We created phylogenetic trees using the NJ and ML methods. The topologies of the different trees were identical (data not shown). Therefore, we present here only the ML tree (Fig. 2). *C. tachibana*, *C. depressa*, and *C. nippokoreana* accessions were classified into two types as follows:

Type A: all 16 *C. tachibana* and six *C. depressa* [Shiikuwasha-Okinawa#1 (No. 17), Shiikuwasha-Okinawa#3 (No. 19), Shiikuwasha-Okinawa#6 (No. 22), and Shiikuwasha-Oku (No. 26), Kabishi (No. 29), and Fusubuta (No. 31)].

Type B: Eleven *C. depressa* [Shiikuwasha-Taketomi (Nohara) (No. 11), Shiikuwasha-Taketomi (Takana) (No. 12), Shiikuwasha-Iriomote (No. 13), Shiikuwasha-Iriomote (Katoura) (No. 14), Shiikuwasha-Kohama (Ufudake) (No. 15), Shiikuwasha-Kohama (Omori) (No. 16), Ishikunibu (No. 28), Mikanguwa (No. 30), Kaachi (No. 32), Shiikunin (No. 33), and Shiikurubu (No. 34)] and one *C. nippokoreana*.

None of the control accessions belonged to type A, whereas all seven control mandarin accessions belonged to type B. The other control accessions were clearly distinguished from type A and type B. This finding is consistent with the results of our previous study (Tshering *et al*., 2013).

All 16 *C. tachibana* accessions carried an identical *matK* sequence. Previous studies (Hirai *et al.*, 1990; Yamamoto and Tominaga, 2003) reported that *C. tachibana* was genetically differentiated from *Citrus* species originating from all

Table 1 - *Citrus tachibana*, *C. nipponkoreana*, and *C. depressa* accessions used in the present study

No.	Accession	Latin name	Origin	Note
1	Tachibana-Dazaifu (uchi)	*Citrus tachibana* (Makino) Tanaka	Fukuoka, Kyushu	Planted tree
2	Tachibana-Dazaifu (soto)	*C. tachibana* (Makino) Tanaka	Fukuoka, Kyushu	Planted tree
3	Tachibana-Heian Jingu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
4	Tachibana-Iwashimizu Hachimangu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
5	Tachibana-Kitano Tenmangu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
6	Tachibana-Toshijima (Mie)	*C. tachibana* (Makino) Tanaka	Mie, Honshu	Native tree
7	Tachibana-Matsuoyama (Kochi)	*C. tachibana* (Makino) Tanaka	Kochi, Shikoku	Native tree
8	Tachibana-Nangoku (Kochi)	*C. tachibana* (Makino) Tanaka	Kochi, Shikoku	Planted tree
9	Korai Tachibana	*C. nippokoreana* Tanaka	Kochi, Shikoku	Planted tree
10	Tachibana-Ishigakijima	*C. tachibana* (Makino) Tanaka	Ishigaki-jima, Sakishima	Native tree
11	Shiikuwasha-Taketomi (Nohara)	*C. depressa* Hayata	Taketomi-jima, Sakishima	Native tree
12	Shiikuwasha-Taketomi (Takana)	*C. depressa* Hayata	Taketomi-jima, Sakishima	Native tree
13	Shiikuwasha-Iriomote	*C. depressa* Hayata	Iriomote-jima, Sakishima	Native tree
14	Shiikuwasha-Iriomote (Katoura)	*C. depressa* Hayata	Iriomote-jima, Sakishima	Native tree
15	Shiikuwasha-Kohama (Ufudake)	*C. depressa* Hayata	Kohama-jima, Sakishima	Native tree
16	Shiikuwasha-Kohama (Omori)	*C. depressa* Hayata	Kohama-jima, Sakishima	Native tree
17	Shiikuwasha-Okinawa#1	*C. depressa* Hayata	Okinawa-honto	Native tree
18	Tanibuta-Okinawa#2	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
19	Shiikuwasha-Okinawa#3	*C. depressa* Hayata	Okinawa-honto	Native tree
20	Tanibuta-Okinawa#4	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
21	Tanibuta-Okinawa#5	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
22	Shiikuwasha-Okinawa#6	*C. depressa* Hayata	Okinawa-honto	Native tree
23	Tanibuta-Okinawa#7	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
24	Tanibuta-Okinawa#8	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
25	Garagara	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
26	Shiikuwasha-Oku	*C. depressa* Hayata	Okinawa-honto	Native tree
27	Tanibuta	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
28	Ishikunibu	*C. depressa* Hayata	Okinawa-honto	Native tree
29	Kabishi	*C. depressa* Hayata	Okinawa-honto	Native tree
30	Mikanguwa	*C. depressa* Hayata	Okinawa-honto	Native tree
31	Fusubuta	*C. depressa* Hayata	Okinawa-honto	Native tree
32	Kaachi	*C. depressa* Hayata	Okinawa-honto	Native tree
33	Shiikunin	*C. depressa* Hayata	Tokuno-shima, Amami	Native tree
34	Shiikuribu	*C. depressa* Hayata	Okinoerabu-jima, Amami	Native tree
Control accessions				
	Satsuma mandarin ‘Aoshima’	*C. unshiu* Marcow.		
	Ponkan ‘Yoshida Ponkan’	*C. reticulata* Blanco		
	Mediterranean mandarin	*C. deliciosa* Ten.		
	Dancy	*C. tangerina* hort. ex Tanaka		
	Kinokuni ‘Hirakishu’	*C. kinokuni* hort. ex Tanaka		
	Sunki	*C. sunki* (Hayata) hort. ex Tanaka		
	Cleopatra	*C. reshni* hort. ex Tanaka		
	Yuzu ‘Yamane’	*C. junos* Siebold ex Tanaka		
	Sweet orange ‘Fukuhara’	*C. sinensis* (L.) Osbeck		
	Lemon ‘Eureka’	*C. limon* (L.) Burm. f.		
	Pummelo ‘Mato Buntan’	*C. maxima* (Burm.) Merr.		
	Citron ‘Maru Busshukan’	*C. medica* L.		
	Trifoliate orange ‘Standard’	*Poncirus trifoliata* (L.) Raf.		

other countries except Japan. The present study also confirmed that the *matK* sequence of *C. tachibana* was not identical to those of studied accessions originating from all other countries except Japan. However, we found that the *matK* sequence of *C. tachibana* was identical to those of some investigated *C. depressa* accessions that are indigenous to the Ryukyu Islands, Japan. This suggests that *C. tachibana* has been isolated from the mandarins elsewhere, and evolved in Japan in unique ways. We found no diversity within species. However, further study considering more accessions is needed since the materials used here did not cover the entire area where *C. tachibana* grows. The *matK* sequence of *C. nippokoreana* was not identical to that of *C. tachibana*, indicting genetic differentiation between the two species. Because it is considered that *C. nippokoreana* is related to *C. tachibana* (the Japanese name "Korai Tachibana" means "Tachibana from Korea"), this finding is interesting.

C. depressa accessions were divided into two types according to *matK* sequences. One was the same type as *C. tachibana* and the other was the same type as several mandarins such as *C. reticulata* and *C. sunki*. This result completely agrees with the results of our previous study (Tsheringet *al*., 2013). Differentiation of the cpDNA genome in *C. depressa* was also reported by Urasaki *et al*. (2005) and Yamamoto *et al*. (2013), who analyzed the *trnL-trnF* and *trnF-trnVr* regions, respectively. These results strongly suggest a polyphyletic origin of *C. depressa*. This divergence of *matK* genes was found in *C. depressa* accessions grown on Okinawa-honto (the main island of Okinawa Islands) but not in those grown on the Sakishima and Amami Islands. *C. depressa* possessing *C. tachibana*-type cpDNA (A type) was found only on Okinawa-honto. Similar results were reported by Yamamoto *et al*. (2013) who studied *C. depressa* on Okinawa-honto and the Amami Islands. However, Urasaki *et al.* (2005) found that *C. depressa* accessions possessed *C. tachibana*-type cpDNA (*trnL-trnF* sequence) on the Sakishima Islands. Thus, further study using many *C. depressa* accessions grown on various islands is necessary to resolve the distribution of each type.

There is a possibility that type A *C. depressa* is geneticallyclose to *C. tachibana* than type B. However, this hypothesis is not supported since the proportion of common bands from random amplified polymorphic DNA (RAPD) analysis between *C. depressa* of type A and *C. tachibana* was not so different from that of type B and *C. tachibana* (Yamamoto *et al*., 1998). Since the origin and/or relationship of *C. depressa* to *C. tachibana* cannot be elucidated only by cpDNA analysis, cpDNA analysis combined with nuclear genome analysis such as simple sequence repeat (SSR), sequence-related amplified polymorphism markers (SRAPs) (Barkley *et al*., 2006; Uzun *et al.*, 2009), and restriction site-associated DNA sequences (RAD-seq) (Baird *et al*., 2008) is considered to be necessary. For this purpose, structural analysis (Barkley *et al*., 2006) seems to be informative.

The present work demonstrates the characteristic profiles of the chloroplast genome of *Citrus tachibana* and *Citrus depressa*, two indigenous species in Japan, using a number of accessions grown in various regions based on the results of *matK* sequencing. Furthermore, the divergence of the cpDNA genome of *C. depressa* seems to indicate a polyphyletic origin of this species. These findings are a contribution to progress in the study of the genetic resources in *Citrus* and related genera.

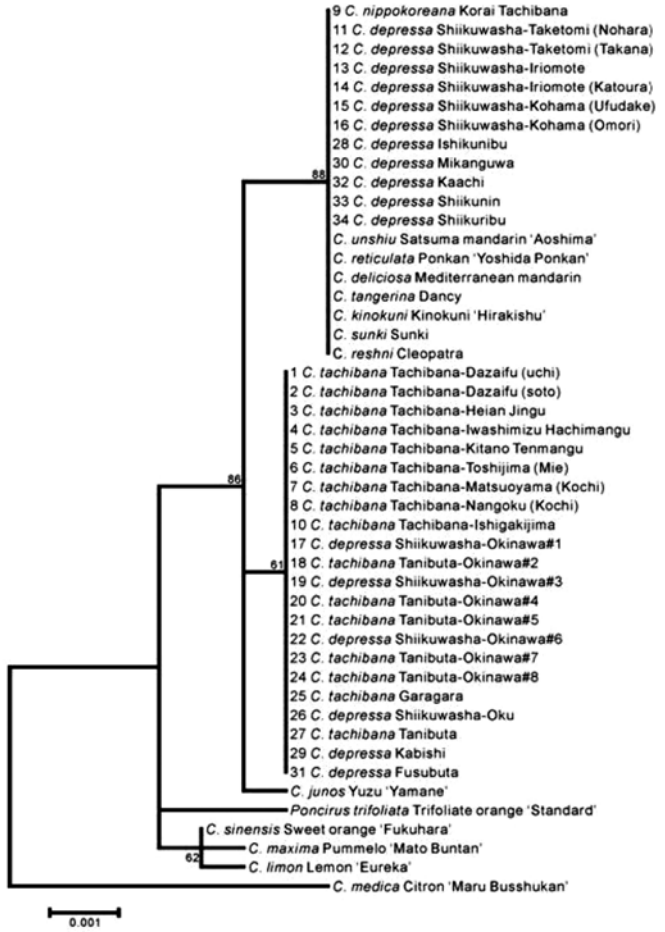

Fig. 2 - Maximum likelihood tree of the *matK* genes from *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* and their control accessions. Numbers at the nodes indicate bootstrap values (% over 1000 replicates). The scale bar shows the number of substitutions per site.

Acknowledgements

We would like to express our deep appreciation to Dr. Tetsuo Koyama, Kochi Prefectural Botanical Garden for help in obtaining tachibana samples. We also acknowledge the help of staff of the following prestigious shrines, Dazaifu Tenmangu, Kitano Tenmangu, Heian Jingu, and Iwashimizu Hachimangu, for supplying tachibana samples from their sacred trees.

References

BAIRD N.A., ETTER P.D., ATWOOD T.S., CURREY M.C., SHIVER A.L., LEWIS Z.A., SELKER E.U., CRESKO

W.A., JOHNSON E.A., 2008 - *Rapid SNP discovery and genetic mapping using sequenced RAD markers.* - PLOS ONE, 3: e3376.

BARKLEY N.A., ROOSE M.L., KRUEGER R.R., FEDERICI C.T., 2006 - *Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs).* - Theor. Appl. Genet., 112: 1519-1531.

HILU K.W., BORSCH T., MÜLLER K., SOLTIS D.E., SOLTIS P.S., SAVOLAINEN V., CHASE W., POWELL M.P., ALICE L.A., EVANS R., SAUQUET H., NEINHUIS C., SLOTTA T.A.B., ROHWER J.G., CAMPBELL C.S., CHATROU L.W., 2003 - *Angiosperm phylogeny based on* matK *sequence information.* - Am. J. Bot., 90: 1758-1776.

HILU K.W., LIANG H., 1997 - *The* matK *gene: sequence variation and application in plant systematics.* - Am. J. Bot., 84: 830-839.

HIRAI M., MITSUE S., KITA K., KAJIURA I., 1990 - *A survey and isozyme analysis of wild mandarin, tachibana (*Citrus tachibana *(Mak.) Tanaka. growing in Japan.* - J. Japan. Soc. Hort. Sci., 59: 1-7.

INAFUKU-TERAMOTO S., YAMAMOTO M., KINJO H., KITAJIMA A., WADA K., KAWAMITSU Y., 2010 - *Local citrus genetic resources and their polymethoxyflavones content in northern part of Okinawa island*. - Hort. Res., 9: 263-271.

KIMURA K., TANINAKA T., 1995 - *Acid citrus (In Japanese), Vol. 3. Acid citrus in Japan*. - Harada Co., Tokushima, Japan.

KINJO H., 2007 - *Acid citrus "Shiikuwasha" in Ryukyu Island*. - Studia Citorologica, 17: 137-148.

LIN S.Y., CHEN I.Z., 2006 - *Native citrus in Taiwan and current statues of research and utilization.* - Seed & Nursery, 8: 1-12.

OLMSTEAD R.G., PALMER J.D., 1994 - *Chloroplast DNA systematics: a review of methods and data analysis.* - Am. J. Bot., 81: 1205-1224.

PLATT A.R., WOODHALL R.W., GEORGE A.L., 2007 - *Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol*. - Biotechniques, 43: 58-62.

TAMURA K., PETERSON D., PETERSON N., STECHER G., NEI M., KUMAR S., 2011 - *MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.* - Mol. Biol. Evol., 28: 2731-2739.

TANAKA T., 1931 - *The discovery of* Citrus tachibana *in Formosa, and its scientific and industrial significance*. - Studia Citrologia, 5: 1-20.

TANAKA T., 1936 - *Citrus studies*. - Yokendo, Tokyo, Japan.

TSHERING P., ANAI T., NAGANO Y., MATSUMOTO R., YAMAMOTO M., 2010 - *Phylogenetic relationships of* Citrus *and its relatives based on rbcL gene sequences*. - Tree. Gen. Genomes, 6: 931-939.

TSHERING P., YAMAMOTO M., UEHARA M., IDE M., MATSUMOTO N., MATSUMOTO R., NAGANO Y., 2013 - *Phylogenetic relationships of* Citrus *and its relatives based on* matK *gene sequences*. - PLoS ONE, 8: e62574.

URASAKI N., YOSHIDA K., UEHARA T., INOUE H., ONDA S., KINJYO H., KAWANO S., 2005 - *Single nucleotide polymorphism in shiikuwasha (*Citrus depressa *Hayata) chloroplast DNA, trnL-trnF.* - Jpn. J. Trop. Agr., 49: 246-251.

UZUN A., YESILOGLU T., AKA-KACAR Y., TUZCU O., GULSEN O., 2009 - *Genetic diversity and relationships within* Citrus *and related genera based on sequence related amplified polymorphism markers (SRAPs)*. - Sci. Hortic., 121: 306-312.

YAMAMOTO M., KOUNO R., NAKAGAWA T., USUI T., KUBO T., TOMINAGA S., 2011 - *Isozyme and DNA analyses of local* Citrus *germplasm on Amami Islands, Japan*. - J. Japan. Soc. Hort. Sci., 80: 268-273.

YAMAMOTO M., MATSUO Y., KUNIGA T., MATSUMOTO R., YAMADA Y., 1998 - *Isozyme and RAPD analyses of shiikuwashas (*Citrus depressa *Hayata)*. - Bull. Natl. Inst. Fruit Tree Sci., 30/31: 39-51.

YAMAMOTO M., TOMINAGA S., 2003 - *High chromosomal variability of mandarin (*Citrus *spp.) revealed by CMA banding*. - Euphytica, 129: 267-274.

YAMAMOTO M., TSUCHIMOCHI Y., NINOMIYA T., KOGA T., KITAJIMA A., YAMASAKI A., INAFUKU-TERAMOTO S., YANG X., YANG X., ZHONG G., NASIR N., KUBO T., TOMINAGA S., 2013 - *Diversity of chloroplast DNA in various mandarins (*Citrus *spp.) and other citrus demonstrated by CAPS analysis.* - J. Japan. Soc. Hort. Sci., 82: 106-113.