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Full Length Article

Phylogenetic Studies of Selected *Citrus* Species Based on Chloroplast Gene, *rps14*

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Abstract

Citrus, one of the major genus of Rutaceae family, has a tropical to semi tropical origin and is well known for its medicinal, nutritional and commercial importance. In order to assess the phylogenetic relationships among eight samples of six *Citrus* species (C. *aurantium*, *C. sinensis* var. mousami, *C. medica*, *C. limon*, *C. maxima*, *C. sinensis* var. malta, *C. reticulata*, *C. sinensis* var. feutrell) a chloroplast gene *rps*14 was successfully amplified and sequenced. The overview of phylogram illustrated an overall genetic distance of 0.02 indicating close genetic relationship among *Citrus* species. Pairwise distance was calculated for *rps*14 gene and low genetic diversity values were observed that ranges from 0.10-0.41. On the basis of results obtained it was concluded that lowest genetic diversity value 0.003 was observed from *C. sinensis* var. mousami and *C. medica* indicating that both are closely related species. However, most distant members are *C. limon* and *C. sinensis* var. feutrell with highest genetic diversity value 0.404. © 2013 Friends Science Publishers

Keywords: Rutaceae; Phylogeny; *rps* 14; Chloroplast; *Citrus*

Introduction

Citrus is a genus of family Rutaceae that comprise some 158 genera and 1900 species (Mabberley, 2008). It is mainly tropical to semi tropical in origin and is assumed to have originated from the region within Northeast India, South China, Indonesia and Peninsular Malaysia (Swingle and Reece, 1967). *Citrus* grows particularly well in areas where there is enough rainfall or irrigation to maintain growth and freezing conditions are not severe enough to kill the tree (Whiteside *et al.*, 1998). *Citrus* is also one of the most important fruit crops in the world and its international production has reached 122 million tons (FAO, 2008). The increased interest in their consumption is not only due to their sweet refreshing properties but also as a result of increased knowledge of their nutritional and medicinal values. Such as orange fruit and its juice have a number of beneficial, nutritive and health properties (Okwu and Emenike, 2006). For medical point of view, based on epidemiological studies it has been reported that dietary *Citrus* flavonoids can enhance a reduction in the risk of coronary heart disease and chronic asthma (Hertog *et al.*, 1993; Di-Majo *et al.*, 2005) and is attracting more and more attention not only due to their antioxidant properties, but as anti-carcinogenic, anti-inflammatory, anti-fungal agents and blood clot inhibition activities (Kaur and Kapoor, 2001; Martın *et al.*, 2002; Abeysinghe *et al.*, 2007).

Citrus fruits and juices are a great source of bioactive compounds including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins that are important to human nutrition (Ebrahimzadeh *et al.*, 2004; Fernandez-Lopez *et al.*, 2005; Jayaprakasha and Patil, 2007). The peel which represents almost one half of the fruit mass has the highest concentrations of flavonoids in the *Citrus* fruit (Manthley and Grohmann, 1996, 2001; Anagnostopoulou *et al.*, 2006). A wide range of DNA markers is available and has been used to study the classification of *Citrus* genus, and phylogenetic relationships within *Citrus* and with related genera. These molecular studies have provided some insight to *Citrus* phylogeny. In this context, microsatellite or simple sequence repeats (SSR) have been employed in *Citrus* for the assessment of genetic variability, phylogenetic studies and for the construction of genetic maps (Kijas *et al.*, 1995; Corazza-Nunes *et al.*, 2002; Zane *et al.*, 2002; Cristofani *et al.*, 2003; Pang *et al.*, 2003; Golein *et al.*, 2005; Barkley *et al.*, 2006). The application of DNA sequence data to address phylogenetic problems is now a routine (Small *et al.*, 2004). Araújo *et al.* (2003) first attempted it in *Citrus* and its relatives using two segments of chloroplast DNA (cpDNA) (Jena *et al.*, 2008). In order to analyze the phylogenetic relationship among selected *Citrus* species, a chloroplast gene encoding ribosomal protein for smaller subunit (*rps*) was amplified and sequenced and finally the sequence data was analyzed with the help of computational tools.

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Materials and Methods

Plant Material

Eight samples from different *Citrus* species (*C. aurantium, C. sinensis* var. mousami, *C. medica, C. limon, C. maxima, C. sinensis* var. malta, *C. reticulata, C. sinensis* var. feutrell) were selected and the botanical names were given according to Muhammad *et al.* (2006) and Naeem *et al.* (2011). Young leaves were removed from the plant and stored at 4ºC.

DNA Extraction and Primer Design

For DNA extraction CTAB (Cetyl Trimethyl Amonium Bromide) protocol of DNA isolation was adopted (Richards, 1997). A pair of primer that can amplify ribosomal protein S14 (*rps*14) were designed from tobacco chloroplast genome (Accession No. Z00044.2) available in NIH (National Institutes of Health, United States) GenBank. Primers were designed by using online available Primer 3 (version 0.4.0) software (http//primer3.source-forge.net/). The sequence of the used primers is given below:

*rps*14 F 5′ ATGGCAAGGAAAAGTTTGATTC 3′
*rps*14 R 5′ TTACCAACTTGATCTTGTTGCTCCT 3′

PCR Optimization

PCR amplification was performed in a volume of 25 µL containing 12.5 µL of 2× PCR Master Mix (Fermentas), 9.5 µL of nuclease free water, 1 µL (25 pmol) of forward and reverse primer each and 1 µL (25–50 ng/µL) of DNA template. The reaction was carried out in Multi Gene thermal cycler (Labnet). The PCR conditions used after optimization were pre PCR denaturation at 95ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing for 1 min at 60ºC and extension at 72ºC for 1 min. Final cycle was same except extension at 72ºC for 10 min. The amplified PCR products along with Gene Ruler™ 50 bp and 100 bp DNA Ladders (Fermentas) were resolved on 1.5% agarose gel prepared in 0.5X TAE buffer and then gel staining (by using ethidium bromide) was performed and finally gel was visualized in gel documentation system (Wealtech, Dolphin Doc^{Plus}).

Purification of Amplified Products and Sequencing

PCR products were purified with the help of JETquick (Genomed) PCR Product Purification Spin Kit by following the manufacturer's instructions. The purified products were analyzed on 2% agarose gel prepared in 0.5X TAE buffer. The purified DNA samples were stored at -20°C to be used for sequencing. Sequencing reaction was performed in 200 µL centrifuge tubes by using Dye Terminator Cycle Sequencing (DTCS) Quick start kit by Beckman and Coulter as per instruction provided in the manufacturer's manual. The sequencing PCR cycling conditions were as follows; pre PCR denaturation at 96°C for 1 min then 30 cycles of denaturation at 96°C for 25 sec, annealing at 55°C for 25 sec, extension at 60°C for 4 min. At the end a final extension step at 60°C for 10 min was also performed. The samples were loaded to the available wells of CEQ sample plate and a proper sequencing program was run by using Beckman Coulter CEQ 8000 sequencer.

Gene Sequence Analysis

Blastn was performed for all the sequences by using NCBI website http://blast.ncbi./nlm.nih./gov/Blast.cgi/ to verify the results of sequenced samples. The sequence of *rps*14 gene of eight samples of six *Citrus* species including *C. aurantium, C. sinensis* var. mousami, *C. medica, C. limon, C. maxima, C. sinensi* var. malta, *C. reticulata* and *C. sinensis* var. feutrell was given in the form of query one by one and blastn was performed to compare them with already reported sequences in GenBank. After performing BLAST, the sequence data of eight samples of six *Citrus* species was submitted to GenBank in order to get the accession numbers. The *rps*14 sequences from all samples were then aligned by using online multiple alignment software ClustalW and aligned sequences were further used for phylogenetic tree construction using the software Molecular Evolutionary Genetics Analysis (MEGA 5). Phylogenetic tree of sequenced samples was constructed to find out the evolutionary relationships among the *rps*14 sequences.

Results

Sequence Analysis

The sequences of purified amplified products of *rps*14 gene from *C. aurantium, C. sinensis* var. mausami, *C. medica, C. limon, C. maxima, C. sinensi* var. malta, *C. reticulata* and *C. sinensis* var. feutrell showed 90, 94, 91, 90, 85, 88, 85 and 82% similarity respectively with *C. sinensis* chloroplast complete genome (Accession no. DQ864733). The similarity scores were obtained using "nblast" (http//www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The sequences were submitted in GenBank and following accession numbers were obtained JQ250613, JQ250614, JQ250615, JQ250616, JQ250617, JQ250618, JQ250619 and JQ250620 for *C. aurantium, C. sinensis* var. mausami, *C. medica, C. limon, C. maxima, C. sinensi* var. malta, *C. reticulata* and *C. sinensis* var. feutrell, respectively.

Phylogenetic Analysis

The phylogram constructed by using MEGA 5 software revealed two clusters denoted by cluster I and cluster II (Fig. 1). The tree has shown that speciation occurred after gene duplication event making two clusters (I and II). All the members of cluster I and II are orthologs with their cluster members. The overview of this phylogram illustrated that *Citrus* species showed very little overall genetic distance of 0.02 indicating close genetic affinity among studied citrus varieties.

Table 1: Data analysis for eight samples of six *Citrus* species on the basis of pair wise distance calculation

Citrus species	1	2	3	4	5	6	7	8
C. aurantium	0.000							
C. sinensis var. mausami	0.102							
C. medica	0.099	0.003						
C. limon	0.266	0.258	0.253					
C. maxima	0.096	0.187	0.183	0.294				
C. sinensis var. malta	0.030	0.114	0.110	0.276	0.107			
C. reticulate	0.150	0.235	0.240	0.327	0.167	0.167		
C. sinensis var. feutrell	0.221	0.316	0.322	0.404	0.258	0.240	0.234	0.000

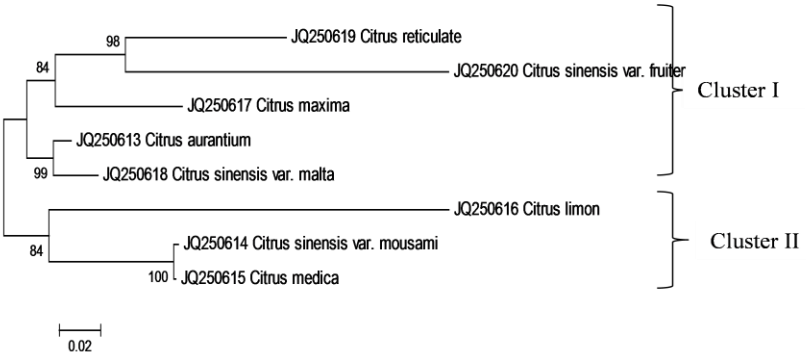

Fig. 1: Phylogenetic tree produced by Neighbor Joining method of Bootstrap Test of Phylogeny based on rps14 gene sequences from eight samples of six *Citrus* species

Cluster I includes five samples namely *C. reticulata*, *C. sinensis* var. feutrell, *C. maxima*, *C. aurantium* and *C. sinensis* var. malta (Fig. 1). Although several methods for tree estimation (or inferring trees) are currently available, very little inferential theory is available for quantifying uncertainty for these trees. The most widely used tool for inference is a version of the bootstrap introduced by Felsenstein (1983). The closely related *Citrus* species in cluster I (*C. aurantium* and *C. sinensis* var. malta) have shown bootstrap value of 99, similarly *C. reticulata* and *C. sinensis* have shown close genetic affinity with a bootstrap value of 98. The phylogram (Fig. 1) indicates that *C. maxima* is genetically associated with *C. reticulata* and *C. sinensis* var. feutrell with a bootstrap value 84. In cluster I branch length indicates that *C. sinensis* var. feutrell is recently evolved member with a large branch length of 0.15637, while *C. aurantium* is the earliest evolved member with a smallest branch length of 0.00879.

Cluster II includes three varieties *C. limon, C. sinensis* var. mausami and *C. medica* (Fig. 1). It was observed that *C. sinensis* var. mausami and *C. medica* are closely related and have shown less genetic diversity with bootstrap value of 100. According to Berry and Gascuel (1996) if the bootstrap value for a certain clade is close to 100, nearly all of the characters informative for this group agree that it is a group. Moreover, *C. limon* is genetically allied with *C. sinensis* var. mausami and *C.* medica with a bootstrap value 84. Among these three citrus cultivars, *C. limon* has a recent origin having significantly large branch length that is 0.19336, while *C. medica* is distantly evolved member in cluster II with small branch length of 0.00108.

The phylogram based on *rps*14 gene sequence constructed by using NJ method in MEGA 5, an overall genetic distance of 0.02 was observed indicating close genetic distance among them. Pairwise distance was calculated and the values for genetic diversity were in the range of 0.10-0.41. It was revealed that *C. sinensis* var. mausami and *C. medica* were closely related with bootstrap value of 100, they have shown low genetic divergence value that is 0.003. Similarly *C. aurantium* and *C. sinensis* var. malta with bootstrap value of 99 were sibilings having very close genetic affinity. It is depicted from the phylogram that there is very little overall genetic divergence (0.02) among the eight varieties of six citrus species indicating close genetic correlation.

Pairwise Distance Calculation

Pairwise distance was calculated on the basis of *rps*14 gene sequences (Table 1). The values for genetic diversity range from 0.10-0.41 with an average of 0.202. These values indicate that genes were genetically associated with each

other and there was little genetic diversity among them. Lowest genetic diversity value (0.003) was observed between *C. sinensis* var. mausami and *C. medica*, while highest genetic diversity value (0.404) was observed in *C. limon* and *C. sinensis* var. feutrell.

Discussion

The cpDNA sequences are the primary source of characters for phylogenetic studies in plants (Bayer *et al.*, 2000; Small *et al.*, 2005). Protein-coding gene sequences such as *rbc*L have been used to elucidate phylogenetic relationships among higher-level taxa (Chase *et al.*, 1993). Subsequently, the potential utility of non-coding regions of the chloroplast genome was recognized for lower-level studies (Taberlet *et al.*, 1991). Recently, Lu *et al.* (2011) investigated the molecular phylogeny of 30 genotypes from six genra of the true citrus fruit trees by conducting research on three cpDNA regions. Earlier, with the help of cpSSR Cheng *et al.* (2005) and Deng *et al.* (2007) have reported the molecular phylogeny of *Citrus*.

In a recent article, ten *Fritillaria* taxa were phylogenetically analyzed using cpDNA sequences with the help of NJ method for phylogram construction. The phylogeny analysis revealed two major clades dividing ten *Fritillaria* taxa based on the DNA sequences of the chloroplast trnL-trnF region (Turktaş *et al.*, 2012). In our study, the phylogeny of *rps*14 gene from eight different *citrus* members has shown two major clusters. The cluster I having five members *C. reticulata*, *C. sinensis* var. fruiter, *C. maxima*, *C. aurantium* and *C. sinensis* var. malta, while the cluster II including *C. sinensis* var. mausami, *C. medica* and *C. limon*. In another report, Morton *et al.* (2003) carried out molecular phylogenetic studies on Aurantioidea subfamily based on plastid DNA sequences of citrus and its close relatives, in this report, within the subfamily Aurantioidea two tribes *Citreae* and *Clauseneae* were claded phylogenetically based on rps16 and trnL-trnF sequences.

In the present study, *rps*14 gene, that encodes ribosomal protein S14 was used to assess phylogenetic relationship with *Citrus* Spp. investigated. After analyzing the sequence data it was observed that within the genus, the level of polymorphism was very low, which might be due to the self-pollinated nature of plant or could be due to restricted distribution, non-effective gene flow, low fecundity, local selection pressure, low pollen flow, inbreeding systems or less possibility of introgressions during evolution (Loveless and Hamrick, 1984; Loveless, 1992). Research on *Citrus* genetics has faced many serious impediments due to genetic heterozygosity, longer juvenility, nucellar embryo interference, self-sterility or incompatibility of partial species. Moreover, most *Citrus* physiological and morphological traits are controlled by quantitative trait loci (QTLs) (Liu and Deng, 2007).

Overall, it has been observed here that genus *Citrus* showed higher level of similarity and low genetic diversification among eight samples of six selected species. So, it can be concluded that the *rps*14 gene sequence is highly conserved in genus *Citrus* and it does not provide much information for establishing phylogeny of genus *Citrus*. It is evident that all the species are monophyletic with very little genetic diversity.

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