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Genetic diversity of orange fruit (*Citrus sinensis* L.) cultivars in Tunisia using AFLP markers

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Abstract

In Tunisia, *Citrus sinensis* culture is spread especially in Cap Bon region in the North East. It is represented by a large number of varieties. AFLP (Amplified Fragment Length Polymorphism) markers were used in order to study genetic diversity. Thirty accessions representing the majority of orange germplasm were collected from Cap Bon region. AFLP products were analyzed by capillary electrophoresis on an automated ABI Prism 3130 DNA sequencer. Using GeneMapper, AFLP bands were scored, across all genotypes, for presence (1) or absence (0) and transformed into 0/1 binary matrix. A total of 330 of polymorphic markers were revealed using 3 AFLP primer combinations. These markers expressed a high level of polymorphism allowing the distinction of all accessions. Resolving power (Rp) showed a high rate of collective Rp (97.75) with an average of 32.58. The Polymorphism Information Content (PIC) ranged from 0.16 to 0.22 with an average of 0.18 per primer pair. Genetic similarities were estimated basing on Nei and Li's (1972) formula. The similarity coefficient between cultivars ranged from 0.15 to 0.96 with an average of 0.76. Most of the accessions showed a high degree of genetic similarity. Additionally, the relationship of the cultivars was also estimated using principal coordinate analysis (PCoA); the first three principal axes explained 94.56 of the total variation. Bioinformatic tools were very useful for investigating the genetic diversity of orange genotypes. Results of this study showed that AFLP markers can be useful tool for investigating the genetic diversity of orange genotypes.

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Introduction

Sweet orange, the most widely grown and consumed citrus type, presents something of a mystery. Four kinds of sweet oranges are recognized: the common, or blond, orange, which is the most important and of which there are many varieties; the acidless orange, of minor importance; the blood orange, which has a red pigmentation in the flesh due to the accumulation of anthocyanins; and the navel orange, grown for fresh consumption (Swingle and Reece, 1967). The sweet orange originated from Asia and its hybrid characteristic seems to come from a cross between mandarin (*Citrus reticulate Blanco*) and pummel (*Citrus grandis* L. Osbeck) (Davies and Albrigo, 1992; Nicolosi *et al.*, 2000). Citrus fruit is produced throughout the tropical and subtropical regions of the world, where the winter temperatures are adequate for tree survival and avoidance of freeze devastation, and where there is sufficient water and suitable soils to support tree growth and fruit production (Talon and Gmitter, 2008). Tunisia has a long tradition in citrus culture. Its introduction probably dates from the Xth century. Industrial culture of citrus was established after the French occupation in the early XXth century. Since 1934, the export trade has expanded greatly and the producers were referred to the culture of maltaise orange ½ sanguine (Mzali and Lasram, 2007). Citrus germplasm is very various and diversified regarding number of varieties, adaptation and fruit qualities. Tunisian varieties as Maltaise demi-sanguine, Chami, Sakasli, L'sén asfour are very appreciated locally and internationally regarding their gustative qualities. The main cultivated variety is "Maltaise". At the moment, *citrus* germplasm is represented by nearly 20.400 ha and 6.3 millions of trees. Production varied from 210.000 to 243.000 tons in these last ten years. Citrus Exportation represents 14% of total agricultural exportation (DGPA, 2008). Economic pressure has slanting agricultors to substitute local varieties by new ones more productive. This fact may anticipate the genetic erosion of very well adapted varieties to local environment. Taking in account these considerations, many prospects were done and permitted the establishment of citrus collections. Recently, Tunisian National Gene Bank (BNG) was established in order to conserve and evaluate genetic resources. In this scope, this work is one of the research programs of fruit trees genetic conservation. Furthermore, it is the first one interested on molecular characterization of Tunisian *Citrus* germplasm based on Amplified Fragment Length Polymorphism markers (AFLP). AFLP is an efficient PCR based assay for plant DNA fingerprinting that reveals significant levels of polymorphism (Vos *et al.*, 1995) The advantages of this technique are reproducibility, high level of polymorphism detection, genome-wide distribution of markers and no pre-requisite of knowledge of the genome being studied (Mueller and Wolfenbarger, 1999). Meudt and Clarke (Meudt and Clarke, 2007) evidenced that AFLP technique is robust and reveals high number of polymorphic and reproducible bands with few primer combinations. AFLP has been widely used for several applications including the study of fig genetic diversity (Baraket *et al.*, 2009), the identification of DNA markers linked to traits in Ponkan mandarin (*Citrus reticulate Blanco*) (JinPing *et al.*, 2009) and identification of linked markers to a major gene essential for nucellar embryony (apomixis) in *Citrus maxima* × *Poncirus trifoliata* (Kepiro and Roose, 2010). Principally in this study, we were interested on molecular characterization of citrus varieties. Thus, several bioinformatic tools were used in order to assess genetic diversity, elucidate its structure and to establish relationships between the considered ecotypes. The information engendered will be of great interest for the management of *in situ* and *ex situ* orange genetic resources in Tunisia.

Materials and Methods

Plant material

Accessions were collected from citrus collection in Kobba and from two citrus farms in Kobba and Menzel Bouzelfa. These regions are situated in Cap Bon in the North Est of Tunisia. This region is well known and specialized in citrus agriculture. A total of 30 accessions were collected in order to fingerprint citrus varieties (Table 1). Each accession is well defined and has its accession number recognized at Tunisian National Gene Bank (BNG).

Table 1. Accessions of citrus varieties studied.

Accession name	***Label***	***Accession number (BNG)***	***Origin***		
		Group	**Sub group**	**BNG code**	**origin**
Malti abiadh	C 1	Sweet orange	blonde orange	BNG3 000600001	Collection Koba
Boukhbza	C 2	Sweet orange	deep blood orange	BNG3 000600002	(11)
Nucelaire F	C 3	Sweet orange	semi blood orange	BNG3 000600003	
Sanguineli	C 4	Sweet orange	deep blood orange	BNG3 000600004	
Chami	C 5	Sweet orange	common blood orange	BNG3 000600005	
Moro	C 6	Sweet orange	deep blood orange	BNG3 000600006	
Nucelaire I	C 9	Sweet orange	semi blood orange	BNG3 000600011	
Nucelaire G	C 10	Sweet orange	semi blood orange	BNG3 000600010	
Nucelaire H	C 11	Sweet orange	semi blood orange	BNG3 000600012	
Malti sanguine	C 12	Sweet orange	common blood orange	BNG3 000600013	
Malti demi sanguine	C 13	Sweet orange	semi blood orange	BNG3 000600009	
Beldi	M 1	Sweet orange	blonde orange	BNG3 000600019	Menzel Bouzelfa
Beldi ahmar	M 2	Sweet orange	semi blood orange	BNG3 000600028	(13)
Malti abiadh boujneb	M 3	Sweet orange	blonde orange	BNG3 000600017	
Sanguine	M 4	Sweet orange	deep blood orange	BNG3 000600020	
Beldi abiadh acide	M 5	Sweet orange	blonde orange	BNG3 000600052	
Sakasli	M 7	Sweet orange	deep blood orange	BNG3 000600022	
Bourouhine 2	M 8	Sweet orange	Navel orange	BNG3 000600016	
Chami	M 10	Sweet orange	semi blood orange	BNG3 000600025	
Malti turcki	M 11	Sweet orange	semi blood orange	BNG3 000600026	

Bourouhine 1	M 12	Sweet orange	Navel orange	BNG3 000600027	
Malti ahmar	M 13	Sweet orange	common blood orange	BNG3 000600018	
Meski ansli	M 14	Non acid orange	blonde orange	BNG3 000600029	
Bourouhine 3	M 16	Sweet orange	blonde navel orange	BNG3 000600023	
Malti abiadh	K 3	Sweet orange	blonde orange	BNG3 000600042	Koba (6)
Malti turcki	K 7	Sweet orange	semi blood orange	BNG3 000600051	
L'sen asfour	K 8	Sweet orange	deep blood orange	BNG3 000600038	
Double fine	K 11	Sweet orange	semi blood orange	BNG3 000600035	
Meski sifi	K 15	Non acid orange	blonde late orange	BNG3 000600031	
Sifi (Valencia Late)	K 17	Sweet orange	blonde late Valencia orange	BNG3 000600049	

DNA isolation

Total genomic DNA was extracted from frozen young leaves according to the procedure slightly modified of Saghai-Maroof *et al.* (1984). The DNA concentration was estimated by spectrophotometer and by analytical [1% (w/v)] agarose gel electrophoresis (Sambrook *et al.*, 1989).

AFLP analysis

Template DNA (500 ng) was double digested with EcoRI and MseI restriction enzymes in a final volume of 40 µL. Ligation products were diluted 5 times. Five microliter of the ligation product were pre-amplified with EcoRI + A and MseI + C primers in a total volume of 25 µl in a thermocycler for 2 min at 94°C, 30 cycles at 94°C denaturation (30s), 56°C annealing (30 s) and 72°C extension (1 min) and a final hold at 72°C for 10 min. Pre-amplified DNA was analyzed by 1% agarose gel electrophoresis. The pre selective amplification product was diluted 25X in TBE buffer 1X and stored at 4°C for amplification, or stored at –20°C for later use. Five microliter of this solution was used as a template for selective amplification using 5'end. Amplifications were carried out using a touch-down PCR program: 1 cycle of 94 C for 30 s, 65 C for 30 s, and 72°C for 60 s, then 13 cycles with the annealing temperature lowered by 0.7°C per cycle, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. one microliter of the amplified product were mixed with 13.5 µl of deionized formamide and 0.5 µl of GeneScan - 500 Liz internal size standard, denaturized at 95°C for 5 min and analyzed by capillary electrophoresis on an automated ABI Prism 3130 DNA sequencer.

Data analysis

Clear and unambiguous bands in length ranging from 50 to 500 pb were considered as usable. AFLP bands were scored, across all genotypes, for presence (1) or absent (0) and transformed into 0/1 binary matrix. Total number of bands was calculated for all primers. Polymorphic bands were only taken into account to estimate the percentage of polymorphic bands (%PB). The ability of the most informative primers to discriminate among cultivars was assessed by calculating the resolving power (Rp) (Prevost and Wilkinson, 1999) which has been reported to correlate between accessions. Evaluation of the Rp

was performed according to the formula of Gilbert *et al.* (1999):

$Rp = \sum Ib$, $IB = 1 - \lceil 2X \mid 0.5 - p \mid \rfloor$

P is the proportion of the accessions containing the I band. In addition, the discriminating power of derived markers was made by the assessment of the polymorphism information content (PIC) using the following formula:

$$\mathbf{PIC = 1 - \sum_{i=1}^{k} \bar{p}_i^2}$$

Where k is the total number of alleles detected for a given marker locus and pi is the frequency of the i^{th} allele in the set of genotypes investigated (Lynch and Walsh, 1998). The binary matrix was processed using NTSYS pc software package, version 2.02 (Rohlf, 1998). Estimates of genetic similarity among all genotypes were also calculated using Nei and Li (1979) coefficient of similarity between two individuals (i and j), according to the formula: Nei and Lei's coefficient = 2a/(b+c), where a is the number of shared bands present in both samples i and j; b the total number of bands of individual i and c the total number of bands of individual j. The similarity matrix was used to construct a dendrogram by the unweighted pair group method arithmetic averages (UPGMA) procedure (Sokal and Michener, 1958). The goodness of fit of the clustering was tested using the MxCOMP program, which directly compares the original similarity matrix and the cophenetic value matrix, as suggested by Rholf (1998). Principal coordinate analysis (PCoA) was also performed via distance matrix to describe the relationship between accessions using Past software.

Results and Discussion

DNA polymorphism

The three AFLP primer combinations produced a total of 510 amplification products with 330 polymorphic AFLP bands for the 30 individuals. Figure 1 shows an example of AFLP profil for the combination E-ACA/M-CTA.

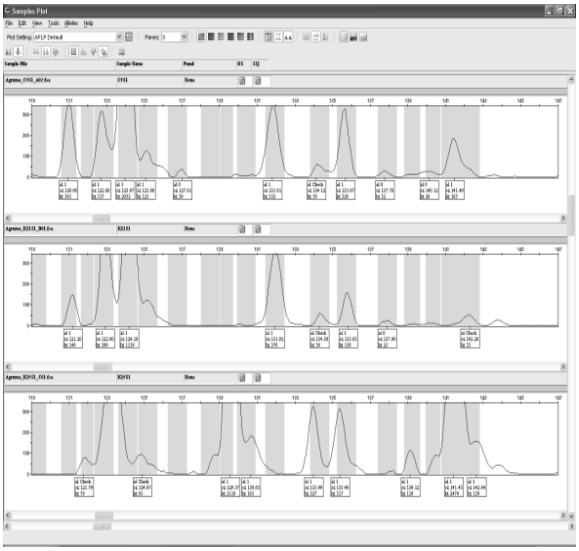

Fig. 1. AFLP of orange varieties (E-ACA/M-CTA).

The average number of polymorphic bands scored per primer pair was 110 (Table 2). The largest number of polymorphic bands 144 was produced with primer combination E-ACA/M-CTA and the least number of polymorphic bands 88 was detected using primer combination E-ACG/M-CTG. Thus, we assume that all the tested primers are powerful to detect DNA polymorphisms in orange fruit trees. Moreover, estimates of the resolving power (Rp) showed a high rate of collective Rp (97.75) with an average of 32.58. The most informative primer combination for distinguishing the genotypes was E-ACG/M-CTA with the highest Rp value (48.28). The Polymorphism Information Content (PIC) ranged from 0.16 to 0.22 with an average of 0.18 per primer pair (Table 2).

Assessment of relationship between cultivars

The similarity coefficient between cultivars ranged from 0.15 to 0.96 with an average of 0.76 using Nei and Li's method. Most of the accessions showed a high degree of genetic similarity. The lowest genetic distance value of 0.15 has been scored between both 'Meski sifi' and 'Malti ahmar' and 'Meski sifi' and 'Meski ansli'. 'L'sen asfour' and 'Moro' were the most similar (0.96). The UPGMA dendrogram among 30 cultivars was generated (Figure 2).

There were no significant clusters corresponding to sampling localities. Accessions collected from different provinces were mixed among the clusters,

and no relation was found between the clusters and the agro-ecological zones of distribution. Since no large differences exist in environmental conditions, the localities considered may share the same orange cultivars introduction origin or intensive germplasm exchange may happened between farmers. Based on similarity indexes, Meski sifi (Kobba) and Sanguinelli (Collection) diverge from all the other orange cultivars. This indicates a diverse genetic base. It is worth to note the significant distinction of Meski Sifi from all the other cultivars (0.42). Many AFLP markers were exclusively detected on this cultivar. The cophenetic coefficient was r= 0.98, indicating that there is a good fit between dendrogram clusters and the similarity matrix. Discarding Sanguinelli and Meski Sifi cultivars, all remind orange accessions have showed a narrow genetic base, referring to genetic similarity index. This result has been finding in Iranian sweet orange using SSR markers. The majority of sweet orange accession showed a narrow genetic base suggesting that the observed morphological polymorphism within the group must be associated with somatic mutations, which were not exactly detected by these molecular markers (Golein *et al.*, 2005). Barrett and Rhodes (1976) have described that the members of this species are thought to have undergone only minor somatic mutational variants from the original biotype resulting in such variants as seedlessness, pigmentation and time of maturity.

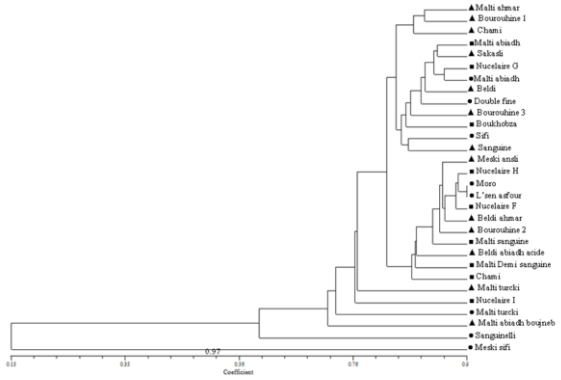

Fig. 2. Dendrogram based on 330 markers from 30 orange cultivars constructed by UPGMA.
(▲: Menzel Bouzelfa, ●: Kobba, ■: Collection)

Table 2. AFLP primer combination characteristics.

Primer combination	***TNB***	***NPB***	***% PB***	***Rp***	***PIC***
E-ACA/M-CTA	222	144	64.86%	31.11	0.17
E-ACG/M-CTG	136	88	64.70%	18.36	0.16
E-ACG/M-CTA	152	98	64.47%	48.28	0.22
Total	510	330	-	97.75	-
Average	170	110	64.67%	32.58	0.18

TNB, Total number of bands; NPB, number of polymorphic bands; % PB percentage of polymorphic bands; Rp, resolving power; PIC, polymorphic information content.

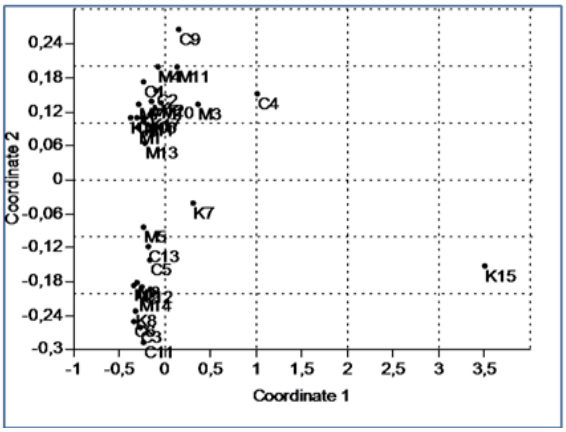

Fig. 3. Distribution of orange accessions revealed by principal coordinate analysis (PCoA) based on AFLP data.

Additionally the relationship of the cultivars was also estimated using principal coordinate analysis (PCoA); the first three principal axes explained 94.56 of the total variation (Figure 3). The first two axes allowed the distinction of the cultivar Meski sifi (Kobba) from all the other accessions. Moreover, the second axe permitted the distinction of two main groups.

Identification of SCAR markers

The two primers E-ACG/M-CTG and E-ACG/M-CTA used in this study were selected regarded to a candidate markers linked to the seedless trait. JinPing *et al.* (2009) employed the AFLP technique to find molecular markers linked to seedless trait in Ponkan mandarin (*Citrus reticulate Blanco*). The AFLP marker selected were converted to a SCAR marker of 195pb and 229 respectively for E-ACG/M-CTG and E-ACG/M-CTA. In this study, it was possible to detect only the 195pb marker corresponding to SCAR marker generated from the E-ACG/M-CTG primer combination. As SCAR markers are dominant markers it was possible to detect the 195 pb marker for Malti abiadh cultivars, Malti abiadh boujeneb, Beldi, Sanguine, Saksli, Boukhobza, Nucelaire I, Nucelaire G, Meski sifi and Sifi. The SCAR marker detected has presented a high homology (73%) with TTN8 gene encoding for structural maintenance of chromosome 1 cohesion, and is known to interact with condensins in some eukaryotes in the regulation of chromosomes dynamics (Liu *et al.*, 2005). Thus, this study confirms the result found by JinPing *et al.* (2009).

Conclusions

AFLP markers have shown their efficiency in orange cultivars molecular characterization. It was possible to study the relationship between cultivars. These markers were suitable for genetic characterization of many fruit species as pomegranate (Jbir *et al.*, 2008; Moslemi *et al.*, 2010) fig (Baraket *et al.*, 2009) and apricot (Krichen *et al.*, 2010). AFLP markers were useful in *Olea europea* for characterizing intraspecific variation among cultivated accessions. The cluster distribution emphasizes the existence of recognizable genetic similarity within varieties and genetic heterogeneity between them (Sensi *et al.*, 2003). The seedless is a desirable trait in Citrus and has been an important breeding objective. Using AFLP markers it was possible to identify a SCAR marker relying on seedlessness on *Citrus sinenesis* L. The marker selected could be useful for accelerating Citrus breeding programs by enabling early screening for seedlessness mutations using marker assisted selection. Finally, the conservation of genetic diversity is important for the long-term interest of any species (Falk and Holsinger, 1991). Molecular markers are effective methods for delineate genetic diversity and structure of populations and can provide effective conservation and management strategies for species (Song *et al.*, 2010).

References

Baraket G, Chatti K, Saddoud O, Mars M, Marrakchi M, Trifi M, Salhi Hannachi A. 2009. Genetic analysis of Tunisian fig (*Ficus carica* L.) cultivars using amplified fragment length polymorphism (AFLP) markers. Scientia Horticulturae **120,** 487-492.

Barrett H C, Rhodes A M. 1976**.** A numerical taxonomy study affinity relationships in cultivated citrus and its close relatives. Systematic Botany **1,** 105-136.

Davies F S, Albrigo L G. 1992. History, distribution and uses of citrus fruits. In: Citrus. CAB International, University Press, 1-11 Cambridge, UK.
DGPA. 2008. General Direction of Agricultural Production. Ministry of Agriculture and Hydraulic resources, Tunisia.

Falk DA, Holsinger KE. 1991. Genetics and conservation of rare plants. *New York: Oxford University Press*.

Gilbert JE, Lewis RV, Wilkinson MJ, Galigari PDS. 1999. Developing and appropriate strategy to assess genetic variability in plant germplasm collections. Theorical and Applied Gentics **98**, 1125-1131.

Golein B, Talaie A, Zamani Z, Ebadi A, and Behjatina A. 2005. Assessment of genetic variability in some Iranian sweet oranges (*Citrus sinensis* [L.] Osbeck) and mandarins (*Citrus reticulate* Blanco) using SSR markers. International Journal of Agriculture and Biology **7**, 167-170.

Jbir R, Hasnaoui N, Mars M, Marrakchi M, Trifi M. 2008. Characterization of Tunisian pomegranate (*Punica granatum* L.) cultivars using amplified fragment length polymorphism analysis. Scientia Horticulturae **115**, 231-237.

JinPing X, LiGeng C, Ming X, HaiLin L, and WeiQi Y. 2009. Identification of AFLP fragments linked to seedlness in Ponkan mandarin (*Citrus reticulate* Blanco) and conversion to SCAR markers. Scientia Horticulturae **121**, 505-510.

Kepiro JL, Roose ML. 2010. AFLP markers closely linked to a major gene essential for nucellar embryony (apomixis) in *Citrus maxima* × *Poncirus trifoliate*. Tree Genetics and Genomes **6**, 1–11.

Krichen L, Bourguiba H, Audergon J M, Trifi-Farah N. 2010. Comparative analysis of genetic diversity in Tunisian apricot germplasm using AFLP and SSR markers. Scientia Horticulturae **127**, 54-63.

Liu CM, McElever J, Tzafrir I, Joosen R, Wittich P, Patton DV, An Lammeren AAM, Meinke D W. 2002. Condensin and cohesion knockouts in Arabidopsis exhibit a titan seed phenotype. The Plant Journal **4**, 405-415.

Lynch M., Walsh J.B. 1998. Genetics and analysis of quantitative traits. Sinauer Assocs: Inc. Sunderland, MA.

Meudt HM, Clarke AC. 2007. Almost forgotten or latest practice? AFLP applications, analyses, advances. Trends in Ecololgy and Evolution **12**, 106-117.

Moslemi M, Zahravi M, Bakhshi Khaniki G. 2010. Genetic diversity and population genetic structure of pomegranate (*Punica granatum* L.) in Iran using AFLP markers. Scientia Horticulturae **126**, 441-447.

Mueller UG, Wolfenbarger LL. 1999. AFLP genotyping and fingerprinting. Trends in Ecololgy and Evolution **14**, 389-394.

Mzali MT, Lasram M. 2007. L'arboriculture fruitière en Tunisie, Vol3: Les arbres à pépins, les agrumes et la vigne de table.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences **76,** 5269-5273.

Nicolosi E, Deng Z N, Gentile A, La Malfa S, Continella G, Tribulato E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theorical and Applied Gentics **100**, 1155-1166.

Prevost A, Wilkinson M J. 1999. A new system of comparing PVR primers applied to ISSR fingerprinting of potato cultivars. Theorical and Applied Gentics **98**, 107-112.

Rohlf FJ. 1989. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System version 2.02, Exeter software. New York: Setauket

Saghai-Maroof NA, Soliman KM, Jorgensen RA, Allard R. 1984. Ribososmal RNA spacer-length polymorphism in barley: mendelian inheritance, chromosomal location and population dynamics. Proceedings of the National Academy of Sciences **81**, 8014-8018.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed. NewYork: San Francisco, Cold Spring Harbor laboratory, Cold Spring Harbor.

Sensi E, Vignani R, Scali M, Masi E, Cresti M. 2003**.** DNA fingerprinting and genetic relatedness among cultivated varieties of *Olea europea* L. estimated by AFLP analysis. Scientia Horticulturae **97**, 379-388.

Sokal RR, Michener CD. 1958. A statistical method for evaluating systematic relationships. The University of Kansas science bulletin **38**, 1409-1438.

Song N, Zhang X M, Gao TX. 2010. Genetic diversity and population structure of spotted tail goby (*Synechogobius ommaturus*) based on AFLP analysis. Biochemical Systematics and Ecology **38**, 1089-1095.

Swingle WT, Reece PC. 1967. The botany of citrus and its wild relatives; In: The Citrus industry, Vol 1, ed. W Reuther H J Webber L D Batchelor, 389-390 USA, University of California Press, Berkley, CA.

Talon M., Gmitter FGJ. 2008. Citrus genomics. International Journal of Plant Genomics 1-17.

Vos P, Hoger R, Bleeker M, Rejans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research **23**, 4407-4417.