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Development of RAPD-Derived STS Markers for Genetic Diversity Assessment in Melon (*Cucumis melo* L.)

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Abstract: Random amplified polymorphic DNA (RAPD) has been used widely in diversity studies, including population structure and phylogenetics at all taxonomic levels. However, there is a problem in stability and repeatability of RAPD in some cases. Therefore, conversion of RAPD markers into new type of PCR-based marker to overcome low levels of repeatability of RAPD marker is needed. The aim of this study was to develop sequence-tagged site (STS) markers by designing specific primers based on RAPD marker sequences to provide the potential markers for analyzing genetic diversity of melon germplasm. Eight RAPD-STS markers were successfully converted from RAPD markers and have two polymorphism types: A20 and B99 showed different sizes of fragment; A22, A31, A57, B15, B71 and C00 showed presence/absence polymorphism in melon germsplasm. The applicability of new RAPD-STS markers has been demonstrated by comparing genotype analysis of 41 melon accessions using RAPD and RAPD-STS markers. Both of RAPD markers and RAPD-STS markers divided them into two major clusters. However, the RAPD-STS markers were more polymorphic than RAPD markers (polymorphic index content (PIC) values were 0.346 and 0.274, respectively). Mantel's test showed significant correlation (r = 0.896, P < 0.01) between RAPD-STS dendrogram and RAPD dendrogram. Furthermore, RAPD-STS markers could give more information in population structure and identify admixture individuals by using STRUCTURE software. Eight RAPD-STS markers developed in this study are useful for genetic diversity analysis and population studies in melon.

Key words: RAPD, STS, *Cucumis melo* L., genetic diversity, marker.

1. Introduction

Melon (*Cucumis melo* L.) is considered as one of the most morphologically diverse species among the major cucurbit crops, even among vegetables. Pitrat [1] has classified 15 botanical groups of melon, including five botanical groups in *Cucumis melo* ssp. *agrestis* (*acidulous*, *conomon*, *momordica*, *makuwa*, *chinensis*) and 10 botanical groups in *Cucumis melo* ssp. *melo* (*chate*, *flexuosus*, *tibish*, *adana*, *ameri*, *cantalupensis*, *chandalak*, *reticulatus*, *inodorus*, *dudaim*). However, recent findings of the African and Asian melons not included in this taxonomy caused confusion [2, 3]. Application of molecular markers to assess genetic diversity is essential to refine morphological-based classification.

Abundant of molecular markers are applicable to reveal the variation within melon accessions, including restriction fragment length polymorphisms (RFLP) [4], random amplified polymorphic DNA (RAPD) [5, 6], simple sequence repeats (SSR) [7] and single nucleotide polymorphisms (SNP) [8]. PCR-based markers, especially RAPD and SSR, have received much attention; however, RAPD markers are dominant markers and incapable to detect heterozygote, and also difficult to reproduce RAPD profile between different laboratories due to their lack

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of specificity. In contrast, SSR often require high resolution and laboratory intensive techniques. Therefore, genetic diversity analysis requires a new type of marker targeted to specific sites and needs no manipulation of amplified products for polymorphism detection.

In broad sense, sequence-tagged site (STS) markers are generated by a pair of primers (18-21 nucleotides), which are designed based on known DNA sequences. In narrow sense, STS markers are produced based on the conversion from RAPD/AFLP/RFLP markers which are referred as another name sequence characterized amplified region (SCAR). STS marker was firstly introduced by Olson et al. [9] as a DNA landmark in physical map of human, which occurred only once in a genome or chromosome. STS marker is now used as a valuable genetic marker to distinguish individuals with many advantages, including high specificity and reproducibility, low time-consuming and easiness to conduct [10-14].

The goal of this study was to develop RAPD-STS markers by designing specific primer sets from RAPD sequences to provide potential markers for genetic diversity analysis in melon.

2. Materials and Methods

2.1 DNA Materials

A total of 24 melon accessions were selected based on RAPD profile from previous studies for screening polymorphism of RAPD-STS markers [5, 6]. The polymorphic RAPD-STS markers were used to genotype of 41 melon accessions [6].

Total DNA from melon leaves was extracted by cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson [15] with minor modifications.

2.2 Cloning and Sequencing of RAPD-Generated DNA Fragments

RAPD reactions were performed and analyzed following the method described by Nhi et al. [6]. The bright and highly reproducible products of 16 RAPD markers were excised from agarose gel and purified by QIAquick PCR purification kit (QIAGEN, USA). The RAPD fragments were cloned into pCR2.1-TOPO of the TOPO TA cloning kit (Invitrogen, USA) followed by the manufacturer's instructions and sequenced by using an ABI PRISMR 3730 DNA analyzer (Applied Biosystems, USA).

2.3 RAPD-STS Primer Design

To design the primer set specific to each RAPD marker sequence, the additional 5' and 3' flanking sequences were obtained from the melon genome database by BLAST using the marker sequence as a query[1]. Based on the genomic sequence, including RAPD marker sequence and 5' and 3' flanking sequences obtained, primers consisting of 18-21 nucleotides were designed by Primer3[2]. Whole or part of the original RAPD primer sequence was included in the new primers.

2.4 RAPD-STS Analysis

The 10 μL PCR mixture for RAPD-STS analysis comprised of 50 ng of genomic DNA, 1 μL PCR buffer (Sigma, USA: 10 mM Tris-HCl, pH 8.3, 50 mM KCl), $MgCl_2$, 0.1 mM dNTP, 0.25 μM of each primer and 0.25 U Taq polymerase (Sigma, USA). Amplification reactions were performed using an iCycler (Bio-Rad, USA). The PCR cycle started with an initial denaturing step at 95 °C for 3 min, followed by 35 cycles of 1 min at 95 °C, 1 min for annealing and 2 min at 72 °C. The final step was at 72 °C for 5 min. The annealing temperature and $MgCl_2$ concentration were adjusted depending on RAPD-STS primer sets.

The PCR products were separated on 1.5% agarose gels in TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

[1] http://melonomics.net/.
[2] http://bioinfo.ut.ee/primer3-0.4.0/primer3/.

2.5 Data Analysis

The RAPD marker band and RAPD-STS marker band were scored as 1 for present and 0 for absent. From these data, the polymorphic index content (PIC) was calculated according to Anderson et al. [16]. Genetic similarity (GS) among accessions was calculated as described by Apostol et al. [17] and their genetic distance (GD) was calculated by Eq. (1):

$$GD = 1 - GS \quad (1)$$

A dendrogram was constructed by the PHYLIP program using the unweighted pair group method with arithmetic mean (UPGMA) method. Mantel's test of XLSTAT software was used to find out the correlation between dendrograms constructed by RAPD-STS markers and RAPD markers, respectively. Population structure and identification of admixed individuals was performed using STRUTURE 2.2 software.

3. Results and Discussion

3.1 Development of RAPD-STS Markers

A total of 18 polymorphic bands of RAPD markers were cloned and successfully sequenced. Basically, based on the internal sequence within the cloned RAPD markers, RAPD-STS markers were designed either by adding 10 to 14 bases to 3' end of the original primer sequence [11, 14, 18] or by settling inner side of the cloned RAPD fragments without regard to the sequence of the RAPD primers [19, 20]. Upstream and downstream of sequences flanking to the RAPD marker sequence were obtained using BLAST tool. RAPD-STS primers were then designed (Table 1).

3.2 Detection Efficiency and Sensitivity of RAPD-STS Markers

Three out of 18 RADP-STS markers (A23, B32 and B68) failed to amplify. Fifteen primers successfully produced a single band of expected sizes from melon genome, except for A22. Marker A22-RAPD-STS amplified a 350-bp fragment, which was much smaller than those amplified by A22-RAPD (1,520 bp-fragment). The sequence of 350 bp-fragment was 99.99% identical to the downstream sequence of 1,520 bp-fragment.

Reference accessions, including "plus accessions" (can be amplified according to RAPD profile) and "minus accessions" (can not be amplified according to RAPD profile), were used for selecting the polymorphic marker among 15 RAPD-STS markers. Two different types of polymorphism were observed. A20 and B99 showed different sizes of fragment, while A22, A31, A57, B15, B71 and C00 showed presence or absence of band (Fig. 1). For the latter six markers, positive control primer sets shown in Table 2 were combined in the PCR reaction and multiplex PCR was performed, in order to confirm the absence of marker band. The other seven markers showed no polymorphism.

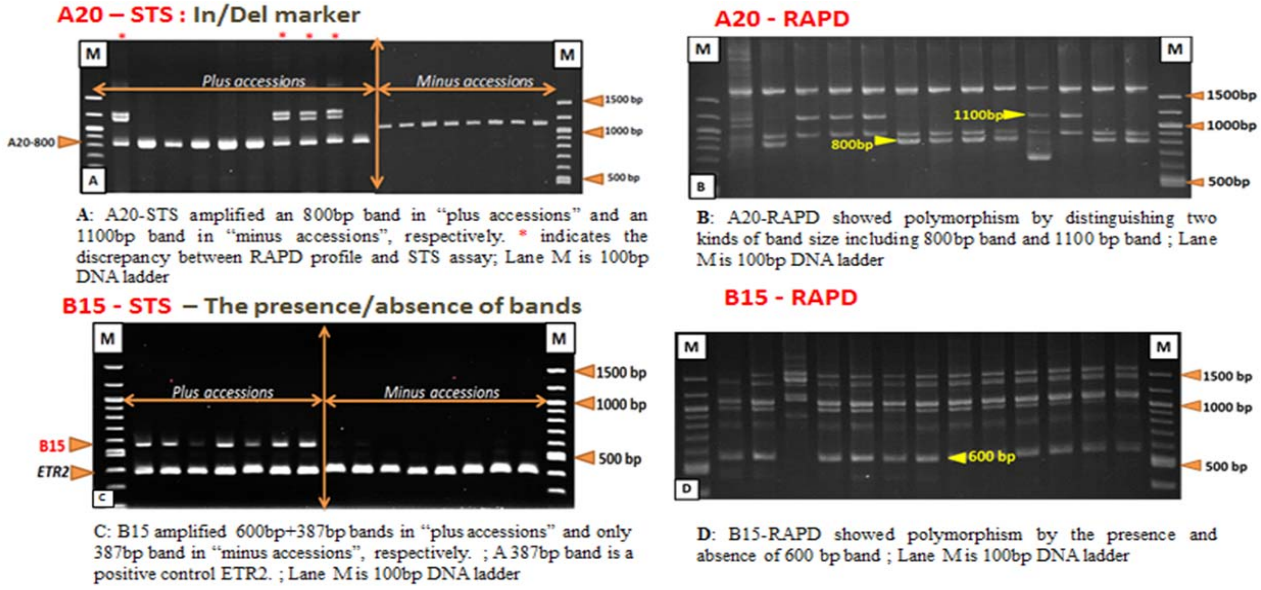
In this study, the loss of polymorphism was observed in 10 out of 18 RAPD-STS markers. The difficulty in polymorphism reproduction was also observed by Horejsi et al. [11], Mammadov et al. [13] and Paran et al. [14]. Paran et al. [14] explained that the lack of specificity in RAPD primer can lead to mismatch at the primer sites and produce the artifactual bands (false positives) corresponding to rearranged fragments. Therefore, the sequence data of RAPD marker fragment did not provide information on these mismatches, which explains why RAPD-STS markers lost the polymorphism when converted from RAPD markers.

3.3 Validation of RAPD-STS Markers

To confirm the accuracy and stability of new RAPD-STS markers (A20, A22, A31, A57, B15, B71, B99 and C00), 41 melon accessions were genotyped and compared with RAPD profile [6]. All markers produced clear and reproducible polymorphic fragments in the 41 accessions, except for C00. C00 did not show polymorphism among 41 melon accessions so that it was eliminated during establishment of phylogenetic tree.

Table 1 Sequence of 18 RAPD-STS primer sets and their expected size.

RAPD-STS marker	Primer sequence	Expected size (bp)
A20	ATAGATCACCTAGCGGGACCA ACGAACTCAAACCGGGACCA	800
A22	TTTTCCAAGAACGGGAAGG GGTGAAGAAGCCAAACTACCA	350
A23	AGTGGTGGTATACCTGT CAGGAGTAAGCCGAATC	1,200
A26	GTTAATGGAGCTGCGTATTCA TGAGGATTCATTAGGCAAAGC	1,400
A31	GGCAGCAGTGGTGGCATC GGAACAAATAAGTGTGTGGTATC	800
A41	AATAGACCCTTGTACGGTAT GATGAGGAGTTGGTACTGTAT	930
A57	GCTAATAAGCAATTGGCGAAC GCCAAAGATCGATTGTCGAA	800
B15	CCTTGGCATCGGTATGTA CTTGGCATCGGCACCTTT	600
B32	TTTTTACGTACGTGGATACCAA TCATCTAAAATTAATGGATCGT	700
B68	CACACTCGTCATATACA CACACTCGTCATGTTT	1,078
B71	GGACCTCCATCGATAA GGACCTCCATCGTATCC	1,220
B84-600	CTTATGGATCCGCTTGGTATG CTTATGGATCCGACGAAGAG	600
B84-700	CTTATGGATCCGTTTGGTAA CTTATGGATCCGACGAAGAG	700
B86	TCGAGCGAACGTAATGAAGA TACTGTTATCCCGAGCGAATG	1,370
B96-750	AAAGACTGCTACCAAAACGAA TGGTCGTGTGAATACTATGGA	750
B96-850	GGCTACAAAGGTGAAAACTATGG TGAGATTATGAATCCTATGTATG	850
B99	TTCTGCTCGAAACTCTAGG TTCTGCTCGAAATACAAGAAA	1,550
C00	GAGTTGTATGCGGAGTTGGA GTTGTATGCGATAAAGTCAACA	1,350



A: A20-STS amplified an 800bp band in "plus accessions" and an 1100bp band in "minus accessions", respectively. * indicates the discrepancy between RAPD profile and STS assay; Lane M is 100bp DNA ladder

B: A20-RAPD showed polymorphism by distinguishing two kinds of band size including 800bp band and 1100 bp band ; Lane M is 100bp DNA ladder

C: B15 amplified 600bp+387bp bands in "plus accessions" and only 387bp band in "minus accessions", respectively. ; A 387bp band is a positive control ETR2. ; Lane M is 100bp DNA ladder

D: B15-RAPD showed polymorphism by the presence and absence of 600 bp band ; Lane M is 100bp DNA ladder

Fig. 1 The comparison of two RAPD-STS markers with the corresponding RAPD markers.

Table 2 PCR conditions of eight sets of RAPD-STS primers and the PIC values of RAPD-STS and RAPD polymorphism in 41 melon accessions.

RAPD-STS markers	Annealing temperature (°C)	Concentration of $MgCl_2$ (mM)	Expected size (bp)	Type of polymorphism	Positive control primers[a]	Amount of primers[b]	PIC	
							RAPD-STS	RAPD
A20	62	2.5	800	InDel	Not used		0.450	0.485
			1,100				0.433	0.433
A22	62	2.5	350	Presence/absence	ACO1-MS3	2:1	0.485	0.476
A31	64	2.5	800	Presence/absence	ACO1-MS3	2:1	0.414	0.314
A57	62	2.5	800	Presence/absence	ETR-MS2	1:1	0.450	0.000
B15	62	2.5	600	Presence/absence	ETR-MS2	2:1	0.136	0.136
B71	60	2.5	1,220	Presence/absence	B86-1370	2:1	0.214	0.214
B99	64	2.5	1,400	InDel	Not used		0.393	0.136
			1,550				0.136	-
C00	62.5	2.0	1,350	Presence/absence	ETR-MS2	2:1	0.000	0.000

[a] Sequence of positive control primers:

ACO1-MSF3CCACCTCCTCTCTTTCCCACATA ACO1-MSR3 CTCCTTAAACCTCTCTTCCATAC

ETR-MSF2ATCGATTGTTGAAGCAACTTT ETR-MSR2GAGACCCAGAAAGGCGTTTAG

B86-1370-F2 TCGAGCGAACGTAATGAAGA B86-1370-RTACTGTTATCCCGAGCGAATG

[b] Amount of RAPD-STS primers (amount of positive control primers).

-: means the marker band was not scored by Nhi et al. [6].

The average PIC value of all the RAPD-STS markers was 0.346, with a maximum of 0.485 for A22 and a minimum of 0.136 for B99 and B15 (Table 2). Most of PIC values obtained from RAPD-STS markers were higher than those obtained from RAPD markers. Thus, RAPD-STS markers were more polymorphic than RAPD markers.

3.4 Genetic Diversity Analysis of Melon Germplasm

A total of 41 melon accessions were used to assess their genetic diversity. Of these, 27 accessions were collected from Vietnam. These accessions mainly belong to var. *conomon* and var. *makuwa*, and have a large phenotypic diversity and a low genetic diversity [6]. Despite of the low genetic diversity of 41 melon accessions, both of RAPD markers and RAPD-STS markers still divided them into two major clusters (Fig. 2). Cluster I consisted of 28 accessions, which were dominated by andromonoecious type, except for VN139, whereas the remaining accessions were included in cluster II, which was further divided into two subclusters. Subcluster IIa included 10 monoecious accessions, while subcluster IIb was composed of three andromonoecious accessions of European and American origin. Furthermore, the correlation between RAPD-STS based dendrogram and RAPD based dendrogram was 0.896 by Mantel's test.

Based on the membership coefficients, the accessions which had the coefficients over 70% were assigned to the corresponding subgroups, while others categorized as the admixture (Fig. 3). With RAPD-STS markers, a monoecious accessions VN139 is considered as admixture form with inferred value from cluster I and cluster II 69% and 31%, respectively. The admixture is likely the result of hybridization between two groups. As one more remarkable case, VN13 was regarded as admixture with inferred value from cluster II quite high (34.8%) by RAPD analysis, while this value was only 3.4% by RAPD-STS analysis. In other words, the genetic architecture of divergent accessions can be estimated by assessing the STRUTURE of the population using RAPD-STS markers.

In short, the results derived from analysis of genetic diversity and population structure strongly supported that eight RAPD-STS markers can be utilized as efficient molecular markers to analyze diversity of melon.

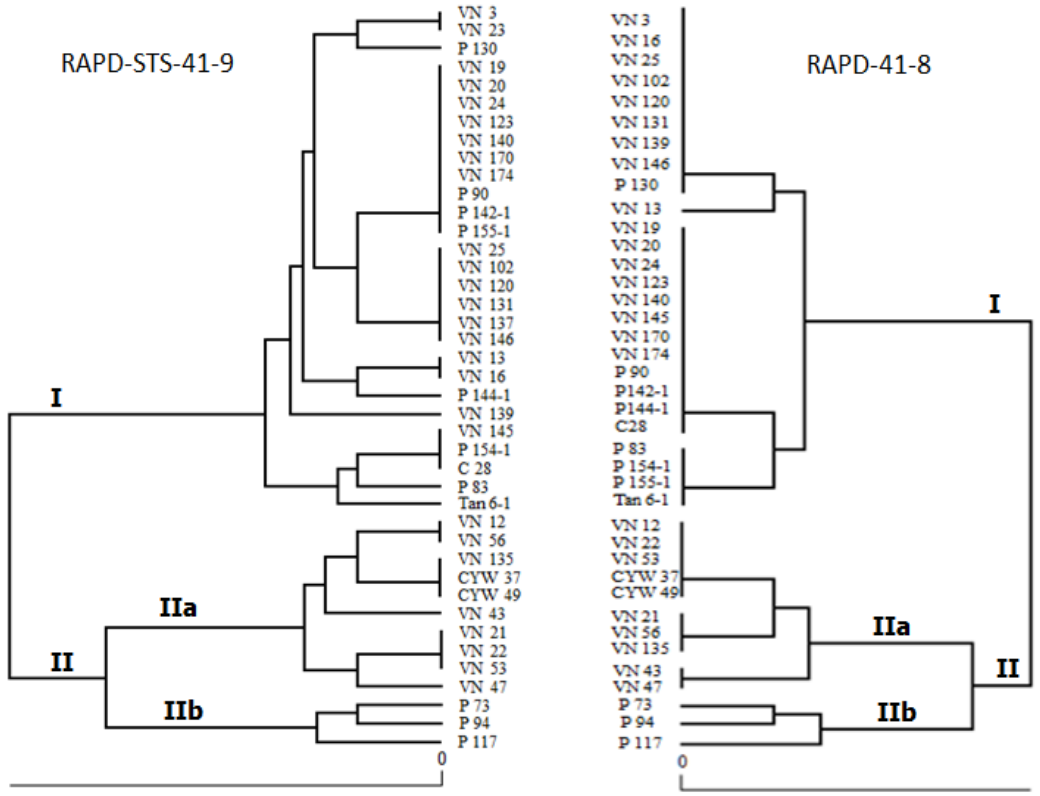


Fig. 2 Comparison of phylogenetic trees constructed by RAPD-STS analysis (RAPD-STS-41-9) and RAPD analysis (RAPD-41-8).

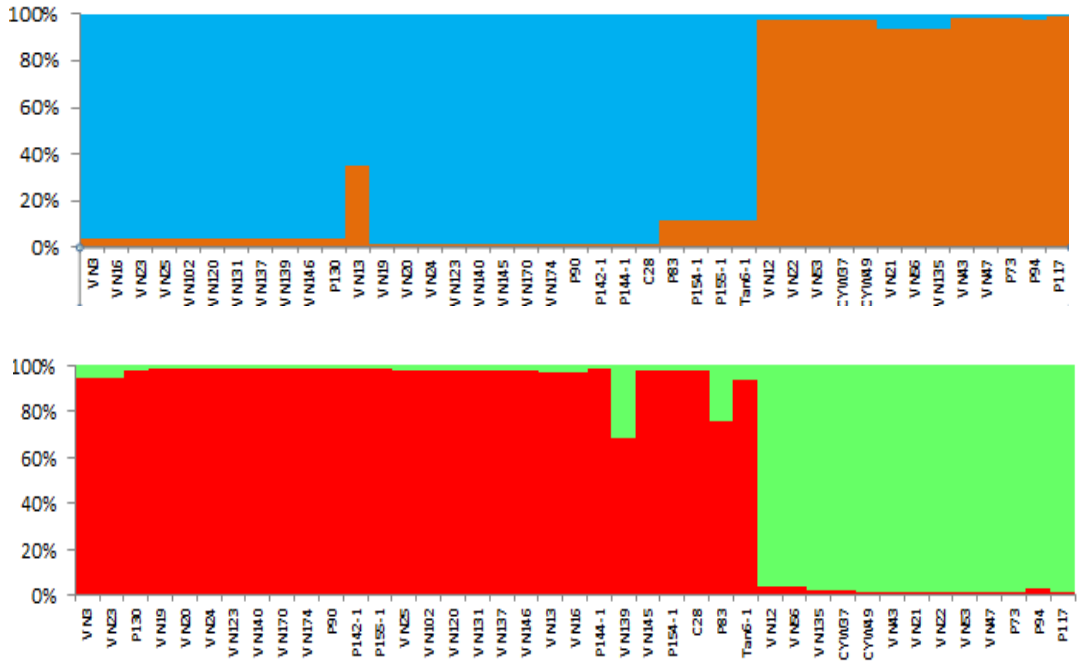


Fig. 3 Bar plots for individual melon accessions by Structure 2.2 using the admixture model based on eight RAPD markers (upper) and nine RAPD-STS markers (lower).

4. Conclusions

In general, this study contributed advantages of STS markers compared with RAPD markers. With the use of primer sets designed using the external sequences of the cloned RAPD fragments, it can be identified the position of RAPD-STS markers in the melon genome, which is meaningful not only for diversity analysis but also for mapping projects. Conversion of RAPD markers into RAPD-STS markers was not easy task. Especially, the polymorphism loss tended to occur at high frequency. However, the success of conversion of eight RAPD-STS markers (A20, A22, A31, A57, B15, B71, B99 and C00) will be useful for genetic diversity analysis as well as population mapping. Two RAPD-STS markers A20 and B99 showed polymorphism with different sizes of fragment; while the remaining markers (A22, A31, A57, B15, B71 and C00) showed presence/absence polymorphism. These eight markers could amplify various sizes of marker fragment ranging from 350 bp to 1,350 bp, indicating that they are valuable for diversity analysis. Indeed, validation of these eight markers using 41 melon accessions was initial evidence for the applicability of them. In order to widely apply the set of these RAPD-STS markers, it is suggested that these eight markers need to continue being validated on various melon populations. If they are stable and reliable, eight markers should be used as efficient molecular markers to analyze diversity of melon and also *Cucumis* sp..

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