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Identification of genetic diversity and relationships of some Iranian tea genotypes using SRAP markers

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ABSTRACT

Purpose: The tea plant is one of the most important products in the northern region of Iran, and plays an essential role in the region's economy. Since today many tea plants in the region are being destroyed for various reasons, so having information about the genetics of those trees helps design breeding programs to reach appropriate plants for specific purposes. **Research Method:** SRAP markers, using eight primer combinations, were used to study the genetic relationships of 27 tea plant samples. **Findings:** In total, these eight combinations produced 41 scorable bands, 70.63% of which were polymorphic. The calculated PIC for all combinations was from 0.23 to 0.43 at an average of 0.36. Data analysis was performed by NTSYS software using Jaccard's similarity coefficient to determine the amount of similarity and the dendrogram was drawn based on UPGMA. Based on molecular data, the range of similarity between samples varied from 0.393 to 0.933. Samples were divided into five groups at a similarity level of 0.65. The fifth group (E) was divided into four subgroups at a similarity level of 0.75. **Research limitations:** Application of another marker system such as SSR and AFLP can help to understand the relationships of samples better. **Originality/Value:** In general, the study of genetic diversity showed that the SRAP marker could be useful in identifying polymorphic regions and estimating genetic distances and germplasm management in tea plants.

INTRODUCTION

Tea (*Camellia sinensis* L., O. Kuntze) is a woody, evergreen, permanent and cross-pollinated plant (Mondal et al., 2003). The young leaves of this plant are processed for the production of the popular non-alcoholic beverage in the world called herbal tea. These plants are grown and consumed in China for over 2000 years (Li, 1983). Tea originates from Southeast Asia, at the intersection point between 29 degrees north longitude and 98 degrees east latitude near the source of the Irrawaddy River at the intersection of Northeastern India, Northern Burma, Southwestern China and the Tibet Province (Wight, 1959).

Due to the method of cultivating and maintaining the tea plant, it is essential that vegetative properties must be used to identify the differences between taxa. However, since the tea is cross-pollinated and very heterogeneous, these types of characteristics may not reflect the genetic diversity within the product (Purseglove, 1968; Wickremaratne, 1981). Also, most of the vegetative properties used as descriptors can only be evaluated at the time of maturity and highly influenced by the environment. Therefore, using mechanisms that do not have these problems is more desirable. Environmental and systematic studies often depend on the use of molecular tools to answer questions about genetic relationships among individuals, population structure and phylogenetic relationships. The dominant PCR-based marker systems, such as RAPD, ISSR and AFLP, have shown that they can be used as tools for understanding genetic relationships (Pang et al., 2007; Simmons et al., 2007). Since these types of marker systems produce multiple bands and do not require genome sequencing information for primer design, they are used to evaluate genetic diversity in plants (Wolfe & Elisens, 1993; Fang et al., 1997; Aggarwal et al., 1999; Culley & Wolfe, 2001; Koopman et al., 2008; Khadivi-Khub et al., 2014; Khiavi et al., 2015; Khiavi et al., 2016; Khaivi & Ashourpour, 2017).

Li and Quiros (2001) introduced, for the first time, a new molecular marker called SRAP. SRAP is a relatively new marker technique that targets the genome coding sequence by 17-19 nucleotide primers (Lu & Foo, 2002), which is particularly used to reproduce the open reading frame (ORF) sequence and is based on two replication primers (Seanz-Romero et al., 2006).

SRAP markers have all the features of a suitable molecular marker, such as co-dominance (Liu et al., 2008). The forward primer has 17 nucleotide pairs with 14 constant nucleotides and rich in G and C, and its three variable nucleotides are in 3'-terminal region. This primer preferably reinforces the exon area. The reverse primer has 19 nucleotide pairs with 16 constant nucleotides and rich in A and T, and its 3 variable nucleotides are in 3'-terminal region. This primer preferably reinforces the region of introns and promoters (Liu et al., 2008). Polymorphism is essentially caused by the diversity in the length of these introns and promoters and distances. Three SRAP terminal variable nucleotides can be modified according to the design of the various primer combinations. Primer pairs can create new combinations by combining forward and reverse primers to reduce the synthesis of primers and increase the use of useful primers. Ferriol et al. (2003) reported that SRAP markers are most consistent with morphological changes compared to other markers (Ferriol et al., 2003). The SRAP marker is a new marker that so far there has not been much research on this marker and the genus *Camellia*.

Yong (2013) used 11 SRAP primer combinations to study the genetic diversity of the 19 *Camellia meiocarpa* populations. The results showed that the range of genetic differences was between 0.021 and 0.164 among the studied populations, which indicates the close relationship between these plants. The polymorphism percentage, Nei's genetic diversity (H),

Shannon index (I) among the studied populations were 84.96-95.58, 0.321-0.388, and 0.473-0.567, respectively.

Peng et al. (2012) examined the genetic diversity of *Camellia oleifera* using two types of ISSR and SRAP markers. The bands produced by the ISSR marker showed 88.14% polymorphism. Nei's genetic similarity index (H) was also found in the range of 0.52-1.00. Of the 12 pairs of SRAP markers, 284 bands were detectable, which 244 bands were polymorphic. Nei's genetic similarity index (H) was also found in the range of 0.65-0.95. The results of the two markers, alone and in total, showed a close relationship, indicating the high power of these two markers for the genetic study of this plant.

Lin et al. (2010) used the 15 SRAP primers for identifying and analyzing the genetics of *Camellia oleifera*, which these primers were able to reproduce 423 sites, with 394 polymorphic sites (93% polymorph). After calculating the genetic distance and cluster analysis, the genetic distance between 0.502 and 0.816 was obtained. In cluster analysis, the 12 studied clones were differentiated into three groups at a different level of 0.35.

Li et al. (2015) used 24 pairs of SRAP markers to study the genetic diversity of 30 *Toona ciliate* populations and reported that these numbers of primer pairs reproduced 656 bands ranging from 100bp to 1500bp, which 77% of these reproduction bands were polymorphic. The polymorphism information content (PIC) value for these primers was in the range of 0.32-0.45 at an average of 0.41. The AMOVA analysis also showed that the maximum diversity identified is within populations, and the difference between populations is low.

In this study, we first tried to use SRAP markers to study the genetic diversity of tea plants cultivated in Iran and some of the imported clones in order to increase the knowledge and recognition of the tea germplasm in the country and, given the specific features that are considered in breeding programs, there could be a more precise plan for selecting parents to cross among samples.

MATERIALS AND METHODS

Plant material and DNA extraction

In this study, 27 tea plants, including 17 imported clones and Ten selective tea genotypes were tested (Table 1). The DNA extraction was performed using the Dellaporta method (Dellaporta et al., 1983) with minor modifications. The quantity and quality of DNA were investigated by the spectrophotometric method and agarose gel electrophoresis.

SRAP molecular analysis

PCR reaction mixture for samples at a volume of 20 µl was prepared from a mixture of 2 mM magnesium chloride, 0.2 mM of each dNTP, one unit enzyme of *Taq* DNA polymerase, 2 µl of polymerase chain reaction buffer (10x concentration) without magnesium chloride, 0.3 µl of each primer (forward and reverse) and 50 ng of genomic DNA from each sample. The PCR conditions were adjusted as follows: initially, initial denaturation at 94°C for 4 min, 5 cycles of denaturation at 94°C for one min, annealing primers at 32°C for one min, and extension at 72°C for 2 min, 35 cycles, including denaturation at 94°C for one min, annealing primers at 55°C for one min, and extension at 72°C for 2 min and finally, 10 min of final extension at 72°C. Table 2 shows the combinations of SRAP primers used.

Table 1. Tea samples used in the analysis, the code recorded in the Tea Institute, the site of their sampling and coding

Plant code	TRC No.	Location	Scientific name	Plant code	TRC No.	Location	Scientific name
G1	Assam	Lahijan	*Camellia* sp.	G15	Japan 2	Ezbaram	*Camellia* sp.
G2	Sri Lanka 1	Ezbaram	*Camellia* sp.	G16	Japan 3	Ezbaram	*Camellia* sp.
G3	Sri Lanka 2	Ezbaram	*Camellia* sp.	G17	468	Fajr Lahijan	*Camellia* sp.
G4	Sri Lanka 3	Ezbaram	*Camellia* sp.	G18	440	Fajr Lahijan	*Camellia* sp.
G5	Sri Lanka 4	Ezbaram	*Camellia* sp.	G19	455	Fajr Lahijan	*Camellia* sp.
G6	Sri Lanka 5	Ezbaram	*Camellia* sp.	G20	548	Fajr Lahijan	*Camellia* sp.
G7	Sri Lanka 6	Ezbaram	*Camellia* sp.	G21	Seedling 2	Fajr Lahijan	*Camellia* sp.
G8	Sri Lanka 7	Ezbaram	*Camellia* sp.	G22	591	Fajr Lahijan	*Camellia* sp.
G9	Sri Lanka 8	Ezbaram	*Camellia* sp.	G23	578	Fajr Lahijan	*Camellia* sp.
G10	Sri Lanka 9	Ezbaram	*Camellia* sp.	G24	791	Fajr Lahijan	*Camellia* sp.
G11	Sri Lanka 10	Ezbaram	*Camellia* sp.	G25	703	Fajr Lahijan	*Camellia* sp.
G12	Sri Lanka 11	Ezbaram	*Camellia* sp.	G26	100	Fajr Lahijan	*Camellia* sp.
G13	Sri Lanka 12	Ezbaram	*Camellia* sp.	G27	444	Fajr Lahijan	*Camellia* sp.
G14	Japan 1	Ezbaram	*Camellia* sp.				

Table 2. Characteristics of SRAP primers used in PCR reaction

Combination name	Forward and Reverse Primers	Primers sequences (5'—3')
S1	Me1 (forward)	TGAGTCCAAACCGG ATA
	Em2 (reverse)	GACTGCGTACGAATTTGC
S2	Me1 (forward)	TGAGTCCAAACCGG ATA
	Em4 (reverse)	GACTGCGTACGAATTTGA
S3	Me1 (forward)	TGAGTCCAAACCGG ATA
	Em6 (reverse)	GACTGCGTACGAATTGCA
S4	Me2 (forward)	TGAGTCCAAACCGGAGC
	Em3 (reverse)	GACTGCGTACGAATTGAC
S5	Me2 (forward)	TGAGTCCAAACCGGAGC
	Em4 (reverse)	GACTGCGTACGAATTTGA
S6	Me2 (forward)	TGAGTCCAAACCGGAGC
	Em5 (reverse)	GACTGCGTACGAATTAAC
S7	Me2 (forward)	TGAGTCCAAACCGGAGC
	Em6 (reverse)	GACTGCGTACGAATTGCA
S8	Me3 (forward)	TGAGTCCAAACCGGACAT
	Em1 (reverse)	GACTGCGTACGAATTAAT

Table 3. Total No. of fragments, number of polymorphic bands, polysemy percentage and Polymorphic Information Content (PIC)

Combination name	Total No. of fragments	No. of polymorphic fragment	No. of monomorphic fragment	Polymorphic percent (%)	Polymorphic Information Content (PIC)
S1	4	2	2	50.00	0.28
S2	3	3	0	100.00	0.25
S3	5	3	2	60.00	0.32
S4	7	5	2	71.43	0.38
S5	7	6	1	85.71	0.43
S6	4	4	0	100.00	0.31
S7	5	2	3	40.00	0.23
S8	6	6	0	100.00	0.41
Total	41	31	10	75.61	0.36
Average	5.12	3.87	1.15	-	-

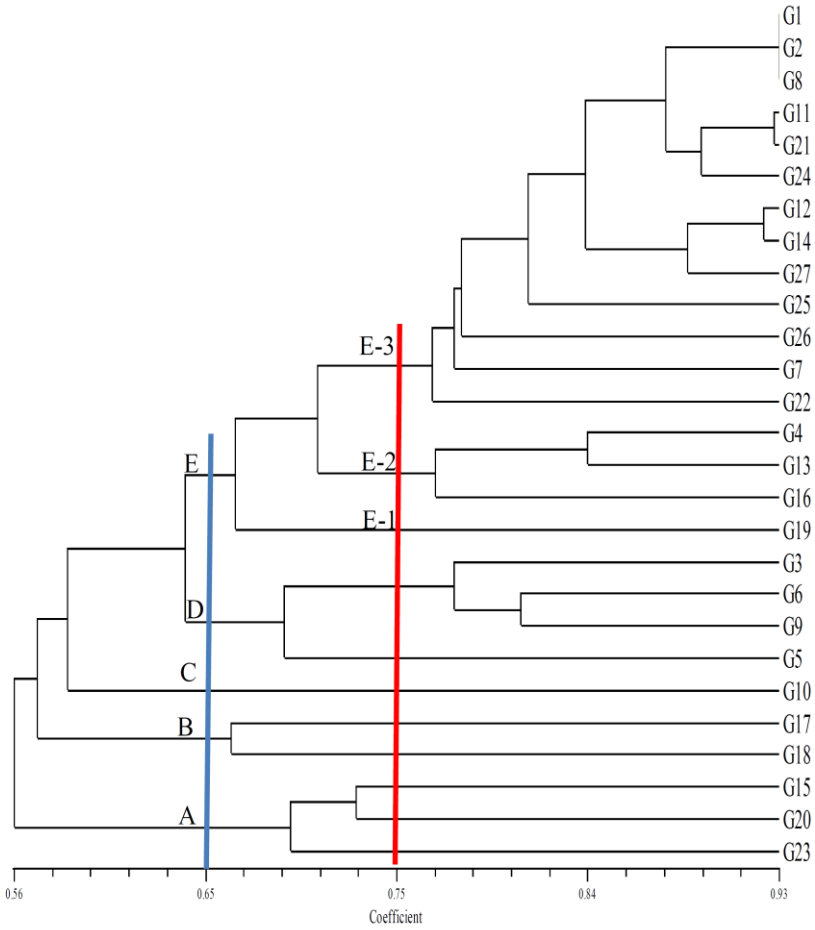

Fig. 1. Cluster analysis of Tea genotypes based on SRAP data

Table 4. Calculated cophenetic coefficient based on SRAP data

	Dice coefficient	Jaccard coefficient	SM coefficient
UPGMA algorithm	0.78	0.81	0.78

Data analysis

The amplified products were separated on 2.5% agarose gels cast in a Tris-borate-EDTA buffer (1×), by running at 90 V for 120 minutes. Each mixture loaded in agarose gel contained 8µl of PCR product, 3µl of loading buffer and 2µl double distilled water. The gel was stained with ethidium bromide and the fragments were observed by UV light and they were photographed. The bands with the desired resolution were given 1 or zero based on the presence or the absence of the band. NTSYS-pc (ver. 2.02) software was used to estimate genetic similarity based on Jaccard's coefficient and genotypes were grouped according to the UPGMA method. Genetic statistics such as the average of effective alleles, Shannon index, minimum total diversity, diversity within the population, diversity index between populations, Nei's genetic distance were carried out by POGGENE software, and the degree of polymorphism of markers was performed by Excel software. The polymorphic information content (PIC) of the combinations was calculated according to the method described by Roldain-Ruiz et al. (2000).

RESULTS AND DISCUSSION

The usefulness of used SRAP primers

Of the 18 SRAP markers tested on four random tea samples that were not included in this study, eight compounds with the best reproductive power were selected. Table 3 shows the number of amplified bands, the number of polymorphic bands, the polymorphism percentage, and the polymorphic information content. The total number of amplified bands for primers used was 41, of which 31 bands were polymorphic. The range of the number of bands was changed from three bands for S2 to seven bands for the S4 and S5 combinations. The average number of bands for each compound was 5.12. According to the number of total bands and polymorphic bands, the polymorphism percentage was calculated in the range of 40.00% to 100.00% at an average of 75.61%. Because the SRAP marker was not used in tea plants, it was not possible to compare with other studies. Yong et al. (2013), however, using the SRAP marker to study the genetic diversity of the *Camellia meiocarpa*, reported the polymorphism percentage of 84.96% to 95.50%. Lin et al. (2010) also reported the polymorphism percentage of 93% on average using SRAP marker in *Camellia oleifera*. In another study on *Camellia oleifera*, Peng et al. (2012), reported the polymorphism percentage of 85.91% with the use of SRAP marker. Given that the polymorphism percentage calculated in the present study is within the range of these percentages, it can be concluded that the selected combinations are suitable. In order to accurately determine the precision of the combinations used, the polymorphic information content (PIC) of the combinations was obtained from maximum 0.43 to minimum 0.23 with a mean value of 0.36 by using the formula presented by Roldain-Ruiz et al. (2000) to determine the ability of primers to detect diversity and polymorphism. According to the total value of polymorphic information content, it was found that the primers used in this study had good power identifying the genetic diversity of tea samples. The amplified fragment of some samples in the combination S5 is shown in Figure 1.

Similarity matrix analysis of SRAP marker data

In order to determine the best method for analyzing and calculating the similarity between the studied samples, the cophenetic coefficient was calculated for the three coefficients of Jaccard similarity, simple matching (SM) and Dice and UPGMA algorithm (Table 4) and it was determined that if Jaccard's similarity coefficient and the UPGMA algorithm was used, 81% of the similarity matrix information was transmitted to the chart. Therefore, based on this coefficient, it was determined that the best similarity coefficient to calculate the similarity between the samples studied and the cluster analysis using UPGMA method was the use of Jaccard's coefficient.

The similarity range of the calculated similarity matrix was different from 0.393 to 0.933. The minimum level of similarity was obtained between G3 (Sri Lankan cultivar, Siahkal Ezbaram Station) and G15 (Japanese cultivar, Siahkal Ezbaram Station) and G15 (Japanese cultivar, Siahkal Ezbaram Station) and G5 (Sri Lankan cultivar, Siahkal Ezbaram Station) and G15 (Japanese cultivar, Siahkal Ezbaram Station). The maximum similarity was observed between G1 (Asami cultivar, Shahid Slami Station, Central tea growing region) and G2 (Sri Lankan cultivar, Siahkal Ezbaram Station), and G2 and G8 (Sri Lankan cultivars, Siahkal Ezbaram Station). The important point of the results is that Sri Lanka, as one of the main regions of the tea plant has the highest variations, which is also evident in this matrix. The average similarity for all samples was equal to 0.715. In different studies carried out on tea using other markers, similar results have been reported regarding similarity value (Ben-Ying et al., 2010; Jahangirzadeh Khiavi et al., 2016; Jahangirzadeh Khiavi & Falkro, 2017). The

matrix of similarity obtained by Jaccard's similarity coefficient for the 27 tea samples is shown in Table 5.

Cluster analysis of samples based on SRAP marker data

The high cophenetic coefficient in cluster analysis showed the efficiency of the dendrogram in expressing the calculated similarities. The cophenetic coefficient can range from zero to one. If the coefficient is more than 0.9 ($r > 0.9$), the expression of data has been very good, if the range of the coefficient is between 0.7 and 0.9 ($0.7 < r < 0.9$), the expression of data has been good, and if the coefficient is less than 0.7 ($r > 0.7$), the calculated attributes in the similarity matrix have poorly been shown in the clustering. Rincon et al. (1996), based on clustering the samples by SRAP data (Fig. 1), separated the samples in five main clusters at a similarity level of 65%.

The first group had three members, which the samples of G15 (Japanese cultivar), G20 and G23 (collected from the central tea growing region) were placed in this group. The second group had two members, which the samples of G17 and G18 (collected from the central tea growing region) were in this group. The third group had only one member, and the G10 sample (Sri Lankan cultivar) was in this group. The fourth group at a similarity level of 0.65 had four members. The G3, G5, G6 and G9 samples, all of which were imported from Sri Lanka, were included in this group. The fifth group had 17 members. Indeed, this group was the largest group of molecular analyzes with the SRAP marker, which accounted for about 63% of all samples (more than half the samples). The important point in this group is the placing of samples of the central region entered in the study along with samples from other countries, which indicates the close relationship between the genotypes of tea with each other. This group can form three sub-groups at a similarity level of 0.75. At this similarity level, the samples were divided into three sub-groups of 5-1, 5-2 and 5-3, which the 5-1 sub-group had only one G19 member from the central tea growing area. The second subgroup (5-2) also had three members: G4 and G13 (Sri Lankan cultivars) and G16 (Japanese cultivar). The third sub-group at a similarity level of 75% had the highest number of members (13 members). There were almost all samples from different countries in the study of samples in this sub-group, indicating the close relationship of tea bushes.

CONCLUSION

SRAP markers in plants such as buckwheat (Li et al., 2009), the hybrid of *Populus adenopoda* Maxim × *P. alba* L. (Wang et al., 2010), *Toona ciliata* Roem (Li et al., 2015) and *Salvia* species (Saebnazar & Rahmani, 2013) have been introduced as an appropriate marker for studying genetic diversity. On the other hand, the efficiency of the technique of a molecular marker depends on the amount of polymorphism and its ability to distinguish the difference between germplasms, especially indicators such as polysemy percentage and PIC. Furthermore, given that SRAP examines the ORF regions, it can be concluded that the individuals who are in the same group are more similar and more genetically related. For this reason, it can be argued that individuals of the same group have a primary origin and have been scattered for different reasons in different regions, and if there are the differences between them, it is due to cross-pollination with other genotypes. Overall, due to the high polymorphism of SRAP markers, this technique is a useful method for evaluating the genetic diversity between these genotypes of tea.

Table 5. Jaccard similarity coefficients matrix of tea sample based on SRAP markers

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14
G1	1.000													
G2	0.933	1.000												
G3	0.633	0.581	1.000											
G4	0.759	0.759	0.556	1.000										
G5	0.690	0.633	0.667	0.556	1.000									
G6	0.800	0.742	0.800	0.621	0.731	1.000								
G7	0.800	0.800	0.667	0.679	0.667	0.786	1.000							
G8	0.933	0.933	0.581	0.700	0.690	0.742	0.742	1.000						
G9	0.759	0.700	0.750	0.630	0.680	0.808	0.621	0.759	1.000					
G10	0.667	0.724	0.367	0.593	0.367	0.484	0.586	0.667	0.483	1.000				
G11	0.900	0.839	0.655	0.667	0.655	0.828	0.767	0.900	0.786	0.581	1.000			
G12	0.897	0.897	0.533	0.714	0.643	0.700	0.821	0.897	0.714	0.679	0.862	1.000		
G13	0.828	0.767	0.571	0.840	0.517	0.633	0.690	0.767	0.704	0.667	0.733	0.786	1.000	
G14	0.897	0.897	0.586	0.714	0.643	0.759	0.889	0.833	0.714	0.679	0.800	0.926	0.786	1.000
G15	0.600	0.655	0.393	0.464	0.393	0.517	0.630	0.600	0.464	0.600	0.567	0.607	0.536	0.667
G16	0.759	0.700	0.500	0.692	0.448	0.567	0.621	0.700	0.630	0.654	0.667	0.714	0.840	0.714
G17	0.586	0.586	0.370	0.444	0.423	0.500	0.680	0.533	0.393	0.520	0.500	0.593	0.464	0.654
G18	0.677	0.677	0.536	0.500	0.483	0.714	0.714	0.677	0.552	0.630	0.700	0.633	0.567	0.690
G19	0.690	0.690	0.481	0.556	0.481	0.552	0.667	0.690	0.615	0.519	0.655	0.769	0.630	0.769
G20	0.655	0.655	0.444	0.519	0.444	0.517	0.692	0.600	0.519	0.538	0.567	0.667	0.593	0.731
G21	0.900	0.839	0.600	0.667	0.655	0.767	0.767	0.900	0.724	0.581	0.931	0.862	0.733	0.800
G22	0.800	0.742	0.552	0.567	0.607	0.724	0.724	0.800	0.679	0.586	0.828	0.821	0.690	0.759
G23	0.633	0.633	0.429	0.556	0.481	0.552	0.731	0.581	0.448	0.577	0.600	0.643	0.571	0.704
G24	0.897	0.833	0.586	0.655	0.643	0.759	0.700	0.897	0.778	0.567	0.929	0.857	0.724	0.793
G25	0.867	0.867	0.516	0.690	0.621	0.677	0.677	0.867	0.633	0.600	0.774	0.767	0.759	0.767
G26	0.793	0.733	0.536	0.607	0.720	0.655	0.778	0.793	0.667	0.571	0.759	0.815	0.741	0.815
G27	0.862	0.862	0.500	0.741	0.607	0.667	0.786	0.800	0.621	0.704	0.767	0.889	0.815	0.889

Table 5. Continued

	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25	G26	G27
G15	1.000												
G16	0.414	1.000											
G17	0.500	0.500	1.000										
G18	0.500	0.607	0.667	1.000									
G19	0.560	0.615	0.609	0.536	1.000								
G20	0.727	0.519	0.565	0.448	0.625	1.000							
G21	0.567	0.667	0.552	0.645	0.714	0.567	1.000						
G22	0.467	0.741	0.500	0.655	0.607	0.517	0.828	1.000					
G23	0.696	0.448	0.423	0.483	0.481	0.696	0.600	0.552	1.000				
G24	0.607	0.655	0.536	0.633	0.704	0.607	0.862	0.759	0.533	1.000			
G25	0.586	0.690	0.517	0.613	0.621	0.533	0.833	0.677	0.516	0.767	1.000		
G26	0.615	0.607	0.538	0.586	0.654	0.615	0.759	0.714	0.654	0.750	0.724	1.000	
G27	0.630	0.679	0.556	0.600	0.667	0.630	0.767	0.724	0.667	0.759	0.793	0.846	1.000

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