A STUDY ON DNA FINGERPRINTING OF AREOLATED GROUPER (*Epinephelus areolatus*) USING RAPD ANALYSIS

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ABSTRACT

This study reports the application of Random Amplified Polymorphic DNA (RAPD) technique in establishment of DNA fingerprinting of areolated grouper, *Epinephelus areolatus,* an economically important fish species of the subfamily Epinephelinae (popularly known as grouper). The RAPD technique was applied to determine the genetic variability within the species for the purpose of implementing and managing its breeding program. Among 20 screened arbitrary primers, six RAPD primers namely OPA-06, OPA-10, OPA-15, OPA 16, OPA-17, and OPA-19 were selected to be used in this study to analyze the RAPD profile, polymorphism, similarity index and genetic distance of *E. areolatus* in the DNA level marker. The results showed that the six primers generated a total of 46 scorable loci (fragments) with 52% polymorphic loci (24 fragments). The RAPD fragment ranged from two to eight fragments with a size range of 350-3000 bp and the number of genotypes of each primer varied from three to five. Average similarity index among individuals was 0.60156 ± 0.1045 and genetic distance level ranged from 0.119 to 0.462.

KEYWORDS: ***Epinephelus areolatus*, DNA fingerprinting, PCR, RAPD**

INTRODUCTION

Areolated grouper, *Epinephelus areolatus* is one of the important commercial groupers (*Epinephelus* spp.) caught in the coral reef areas of Southeast Asian countries. It belongs to the subfamily Epinephelinae, and the family Serranidae. Sixty-three species of the genus *Epinephelus* have been identified by Randall (1987) and at least 21 of them have been reported to have potential for commercial culture as well as a good source of protein (Shamsudin, 1992). *E. areolatus* is commonly named as aerolated grouper, yellow-spotted rockcod or squaretail rockcod. The body of this species is covered with brownish yellow spots with usually hexagonal in shape on head, body, and fins (sometimes close-set forming a pale meshwork), bigger in size and lighter in shade and the caudal fin is usually emarginate (Kohno *et al.*, 1990). There is little information available on the molecular genetics of this species. Determination of genetic variation of this species on the DNA level marker is necessary to design future management and conservation for its breeding program.

Detection of genetic variability is essential to a wide range of comparative genetic endeavors. These include studies as diverse as gene mapping, individual identification, parentage determination, population genetic, molecular phylogenetic, conservation and DNA fingerprinting. Williams *et al.* (1990) described a novel Polymerase Chain Reaction (PCR) based method called Random Amplified Polymorphic DNA (RAPD) fingerprinting. This technique allows detection of DNA polymorphisms by randomly amplifying multiple regions of the genome by PCR using a single arbitrary primer designed independent of target DNA sequence. DNA fingerprinting is a relatively new technique by which a set of polymorphic markers can be simultaneously detected, resulting in a pattern unique to individual, species, or strain. The RAPD fingerprinting technique has been reported to be extremely efficient for detection of molecular genetic markers in assessment of genetic variation in fish (Dinesh *et al.*, 1993). This technique also has several advantages over other published methods available: (i) a universal set of primers can be used for genomic analyses in a wide variety of species; (ii) no preliminary work, such as isolation of cloned DNA probes, preparation of filter for hybridization, or nucleotide sequencing, is required; and (iii) each RAPD marker is the equivalent of a sequence target site, which can greatly simplify information transfer in collaborative research programs (Williams *et al.*, 1990). The aim of this present study is to establish DNA fingerprinting of areolated grouper, *E. areolatus* using RAPD analysis.

MATERIALS AND METHODS

Fish Samples

Seventeen areolated grouper, *E. areolatus,* with 11.0-20.4 cm length and 50-186 g body weight, were

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collected from South Sulawesi waters. Approximately 50 mg of the muscle tissue was taken out from each individual and placed in a 1.5 mL microcentrifuge tube containing 250 mL TNES-Urea preservation buffer. This buffer consists of 6 M urea; 10mM Tris-HCl; 125 mM NaCl; 10 mM EDTA; and 1% SDS, at pH 7.5 (Asahida *et al*., 1996). The samples were transported at ambient temperature from the field and kept at room temperature (25°to 28°C) in the laboratory prior to DNA extraction.

DNA Extraction

Genomic DNA from the muscle tissue was extracted using the Phenol-Chloroform technique. Five hundred microlitres of lysis buffer (0.5 M NaCl, 0.001 M EDTA, 1% SDS, 0.8% Triton X-100, and 0.1 M Tris-HCl, at pH 9.0) was added to the sample and followed by adding 40 mL SDS 10% and 40 mL Proteinase K (20 mg/mL solution). The sample was incubated at 55°C for 1-3 hours until completely lysed. Samples were then treated with 25 mL *RNAase* (20 mg/mL solution) and left at room temperature (25-28°C) for 15-30 minutes.

The sample was treated with 500 mL solvent mixture phenol: chloroform:isoamyl alcohol (25:24:1) and then gently vortexed to homogenize it. The sample was left at room temperature (25-28°C) for 10 minutes before centrifugation at 13,000 rpm (11,000 x g) for four minutes. The top aqueous layer was removed to a new 1.5-mL microcentrifuge tube. The step of adding the solvent mixture was repeated twice. The sample was treated with one volume of chloroform : isoamyl alcohol (24:1) and then centrifuged at 13,000 rpm (11,000 x g) for four minutes. Two volumes of cold absolute ethanol were mixed to the top aqueous layer by inversion of the tubes several times. Precipitated DNA was collected at the bottom of the tubes as a white pellet after centrifugation at 6,000 rpm (4,000 x g) for 30 minutes. The pellet was washed with one milliliter ethanol 70% and then centrifuged at 6,000 rpm (4,000 x g) for 15 minutes. The genomic DNA was allowed to dry at room temperature (25-28°C) and then resuspended with TE (Tris-EDTA) buffer.

The genomic DNA was electrophoresed in a 0.8% horizontal agarose gel at 55 volts for 1-2 hours in 1 x TBE (Tris-Borate-EDTA) buffer. The gel was stained with 0.5 µg/mL ethidium bromide for 20-30 minutes and then washed with distilled water for 5-10 minutes prior to photographing.

DNA Purity and Quantity

Ten microliters of genomic DNA were diluted in 490 mL distilled water (dilution factor of 50) in a 0.5-mL cuvette tube prior to measuring the DNA purity and quantity. Genomic DNA and purity of DNA were estimated using a UVIKON Spectrophotometer (Kontron Instrument) at wavelengths of 260 nm and 280 nm. The reading of absorbency at 260 nm (OD_{260}) allows calculation of DNA concentration of the sample. The quantity of DNA was quantitatively determined by following the formula of Linacero *et al.* (1998). Purity of DNA was quantitatively estimated from the ratio between the reading of absorbency at 260 nm and 280 nm (OD_{260}/OD_{280}) and also qualitatively observed through the appearance of the single band formed on the agarose gel.

DNA Amplification

A total of 20 arbitrary primers (OPA01-20) were screened for a randomly single individual in order to find the suitable primer for DNA amplification of grouper. Among them, six primers (based on the clarity and sharpness of the fragments) were selected to use in further analysis (Table 1). DNA amplification reactions consisted of 1 x reaction buffer; 3.5 mM $MgCl_2$;

Table 1. The sequence, nucleotide length, and G+C content of primers used in this study

Primer*	Sequences (5' to 3')**	Nucleotide length	G+C content (%)
OPA-06	GGTCCCTGAC	10-mer	70.0
OPA-10	GTGATCGCAG	10-mer	60.0
OPA-15	TTCCGAACCC	10-mer	60.0
OPA-16	AGCCAGCGAA	10-mer	60.0
OPA-17	GACCGCTTGT	10-mer	60.0
OPA-19	CAAACGTCGG	10-mer	60.0

* : OPA=Operon Technologies Primer Set A

** : A=Adenine, C=Cytosine, G=Guanine, T=Thymine

0.4 mM dNTPs mixture; 0.4 mM primer; 2.0 units taq polymerase; 50 ng template DNA and distilled water in a final volume of 25 mL. The amplification was performed using a programmable temperature cycler of GeneAmp PCR system 2400 from Perkin Elmer. The amplification was programmed at 45 cycles for 30 seconds of denaturation at 94°C, 30 seconds of annealing at 36°C, 1 minute of extension at 72°C, and 2 minutes of final extension at 72°C.

A mixture of 10 mL PCR product and 2.5 mL loading dye was electrophoresed on a 2.0% horizontal agarose gel at 55 volts for 2-3 hours and then stained with 0.5 mg/mL of ethidium bromide for 20-30 minutes. The gel was washed with distilled water for 5-10 minutes prior to documentation with Image Master VDS (Pharmacia-Technology).

Data Analysis

The molecular weight of fragments was estimated based on the standard DNA fragment pattern from GeneRuler DNA ladder marker. The fragments were valued as polymorphic when they are absent in some samples but changes in banding intensity are not considered as polymorphic. Clear fragments were manually scored as present (1) or absent (0) at a particular position or distance migrated on the gel. A data matrix of 1's and 0's was entered into the data analysis package. Data analysis was performed by using the program RAPDistance Package Software Version 1.04 (Amstrong *et al.*, 1994) and Numerical Taxonomy and Multivariate Analysis System (NTSYS) Version 1.80 (Rohlf, 1994). The similarity index of grouper was calculated across all possible pairwise comparisons of individuals using the formula: $S_{xy} = 2n_{xy}/n_x + n_y$ (Nei and Li, 1979). Where n_{xy} is the number of fragments shared by individual x and y; n_x and n_y are the number of fragments scored for each individual. The index of similarity was used to calculate the genetic distance values and to construct the dendrogram. The dendrogram was constructed using the Unweighted Pair-Group Method of Aritmethic (UPGMA) employing Sequential, Agglomerative, Hierarchical and Nested Clustering (SAHN) from NTSYS-pc program (Rohlf, 1994).

RESULTS AND DISCUSSION

DNA Extraction

Genomic DNA of *E. areolatus* was successfully extracted by the Phenol-Chloroform technique. The DNA purity and quantity of *E. areolatus* obtained from this technique was 2.0049 ± 0.1436 and 67.03 ± 15.52 ng/mL respectively (Table 2). Linacero *et al.* (1998) noted that the determination of DNA purity and quantity is an important step in performing a genetic analysis such as RAPD. The purity of genomic DNA should be in the range of 1.8-2.0 for PCR requirement of DNA

Table 2. The purity and quantity of genomic DNA of *E. areolatus*

Ind. Code	OD_{260}	OD_{280}	Purity	Quantity (ng/µL)
1	0.0367	0.0186	1.9731	91.75
2	0.0176	0.0080	2.2000	44.00
3	0.0221	0.0110	2.0091	55.25
4	0.0194	0.0092	2.1087	48.50
5	0.0271	0.0132	2.0530	67.75
6	0.0399	0.0196	2.0357	99.75
7	0.0365	0.0180	2.0278	91.25
8	0.0287	0.0138	2.0797	71.75
9	0.0225	0.0103	2.1845	56.25
10	0.0253	0.0125	2.0240	63.25
11	0.0282	0.0143	1.9720	70.50
12	0.0516	0.0318	1.6226	129.00
13	0.0618	0.0363	1.7025	154.50
14	0.0448	0.0235	1.9064	112.00
15	0.0325	0.0156	2.0833	81.25
16	0.0327	0.0160	2.0438	81.75
17	0.0461	0.0224	2.0580	115.25
	Average*		**2.0049±0.1436**	**67.03±15.52**

* = mean±SD

amplification. However the result of this study indicated that the DNA purity of slightly lower than 1.8 and higher than 2.0 showed good RAPD banding patterns. It is necessary to determine the quantity of genomic DNA in order to find out the DNA concentration for optimization of PCR-RAPD analysis.

The high purity level and the clear bands formed on agarose gel of genomic DNA obtained in this study indicated that Phenol-Chloroform is a very powerful technique for DNA extraction in fish especially *E. areolatus*. Using the Phenol-Chloroform technique for routine total DNA isolation, Taggart *et al.* (1992) obtained a consistently good quality of high molecular weight DNA extracted from 12 species of the genus *Salmo, Salvelinus, Oncorhynchus, Coregonus,* and *Thymalus*. Shima (1999) also reported that DNA extraction using the Phenol-Chloroform technique on the mudskipper, *Periophthalmus schosseri* was more reliable than the DNA extraction of wizard genomic DNA purification kit. The Phenol-Chloroform technique for DNA level extraction was also employed in studies on DNA marker of river catfish, *Mystus numerus* (Kim, 1998) and tiger barb, *Puntius tetrazona* (Asma, 1999).

RAPD Profiles

The RAPD banding patterns were examined for the presence or absence of bands associated with a certain primer used. A set of each 10-mer arbitrary primers of different oligonucleotide sequences was found to generate different RAPD profiles within individuals. Using the six primers, 46 random markers (loci) were generated from DNA amplification of E. areolatuis. The RAPD banding patterns from representative samples of *E. areolatus* generated by each primer are shown in Fig. 1.

The number and size of fragments amplified by different primers varied from two to eight and 350-3000 bp, respectively (Table 3). The OPA-10 produced the highest number and size of fragments. Asma (1999) also reported the different number and size of fragments revealed by different primers in a study on genetic differences of tiger barb, *P. tetrazona*. The number and size of amplification products ranged from one to nine fragments and 210-255 bp respectively. A range of the molecular weight fragments amplified by the four different primers (OPA-14, OPA-17, OPA-18, and OPA-19) on genomic DNA of Malaysian river catfish, *Mystus numerus* was 200-2000 bp (Lim, 1998).

Different RAPD profiles between two species of groupers have been reported by Bakar & Azizah (1999). They found that a fragment of 700bp produced from a primer OPA-05 was present in *E. tauvina* but not in *E. bleekeri*. Similar findings were also reported by Williams *et al.* (1990) and Dinesh *et al.* (1993). Their results showed that DNA amplification in the fingerprinting technique can be tailored to produce patterns of varying complexity by changing the primer sequence length.

Polymorphism

Levels of variability were estimated according to the proportion of polymorphic bands within primers

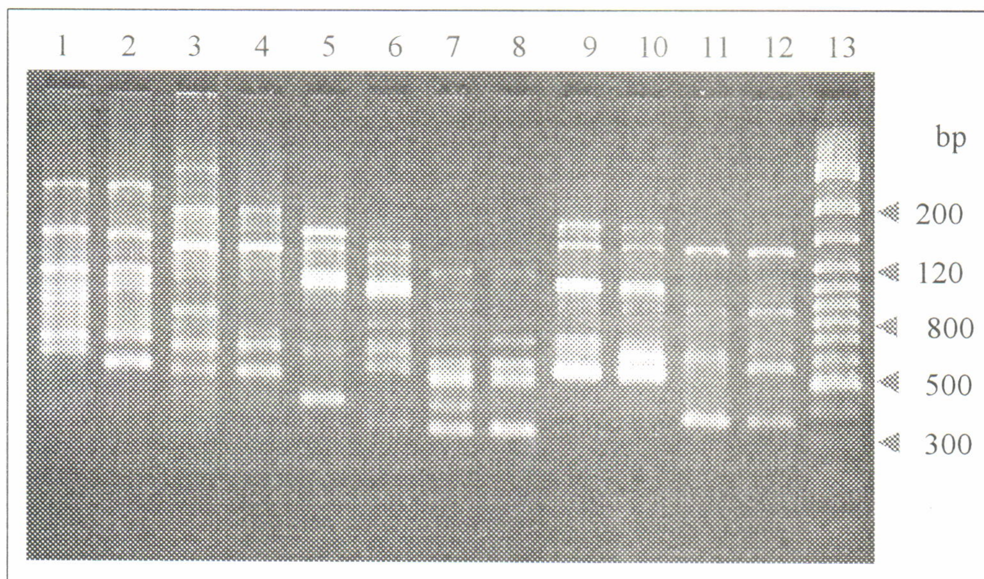

Fig. 1. RAPD banding patterns of the two representative individuals of *E. areolatus* generated by various primers formed on a 2.0% agarose gel. DNA amplification generated by primers OPA-06 (lanes 1-2), OPA-10 (lanes 3-4), OPA-15 (lanes 5-6), OPA-16 (lanes 7-8), OPA-17 (lanes 9-10), OPA-19 (lanes11-12) and 100bp DNA Ladder Plus from Fermentas (lane 13)

Table 3. The number and size of fragments amplified by different RAPD primers

Primer	Number of fragment	Size range of fragment (bp)
OPA-06	5-8	400-2500
OPA-10	2-8	550-3000
OPA-15	5-7	350-1600
OPA-16	3-7	350-1200
OPA-17	3-6	550-1600
OPA-19	4-7	350-1600

(Table 4) and index similarity among individuals (Table 5). Among the primers, the OPA-10 was most variable, having an average of polymorphic bands of 63%. There was considerable variation in the level of polymorphism seen in different primers, ranging from as high as 63% in OPA-10 to 44% in OPA-06. The high polymorphism scored with RAPD markers is probably due to preferential amplification of non-coding repetitive regions of the genome. Since primers are constructed at random, both coding and non-coding regions may be targets of PCR amplification. Lynch and Milligan (1994) noted that the RAPD technique was expected to scan the genomic more randomly than conventional methods. Genotypes obtained in each primer varied from three to five in percentage ranging from 5.9% to 58.8%. The highest genotype number was revealed by primer OPA-10 and the lowest by primer OPA-16. The high genotype observed indicated the great genetic variability of this species.

A high level of diversity ranging from 33.33% to 50.00% was detected in the freshwater shrimp, *Macrobrachium borellii* with the RAPD marker (D'Amato and Corach, 1996). Different species of tilapine fishes showed different polymorphic bands (Mwanja *et al*., 1996). The results of their investigation on five species of tilapia showed that the number of polymorphic bands was 49% to 81% (*Oreochromis niloticus*), 33% to 78% (*O. esculentus*), 64% to 78% (*O. leocostictus*), 70% (*Sarotherodon galilaeus*), and 55% to 75% (*Tilapia zilli*).

Similarity Index and Genetic Distance Level

The similarity index among individuals of *E. areolatus* ranged from 0.4137-1.0000 with an average of 0.60156 ± 0.1045 (Table 5). The similarity index of 1.000 indicated that individuals showed the genetic identical such as individual between 1 and 4; and 2 and 7. The similarity index was found to be lowest (0.4137) between individual 10 and 12. The similarity index of tiger barb, *P. tetrazona* varied from 0.62778-0.73131 in Perak population and 0.33333-0.83222 in Johor population (Asma, 1999).

Table 4. Total number of fragments (TF), number of polymorphic fragments (PF), and proportion of polymorphic fragments (PPF), number and percentage of genotypes of *E. areolatus*

	OPA-06	OPA-10	OPA-15	OPA-16	OPA-17	OPA-19
TF	9	8	8	7	6	8
PF	4	5	4	4	3	4
PPF	0.44	0.63	0.5	0.57	0.5	0.5
No.Genotype	4	5	4	3	4	4
Percentage of Genotype*	47.1 (8)	35.3 (6)	47.1 (8)	58.8 (10)	47.1 (8)	58.8 (10)
	23.5 (4)	29.4 (5)	23.5 (4)	35.3 (6)	29.4 (5)	17.6 (3)
	17.6 (3)	17.6 (3)	17.6 (3)	5.9 (1)	17.6 (3)	11.8 (2)
	11.8 (2)	11.8 (2)	11.8 (2)		5.9 (1)	11.8 (2)
		5.9 (1)				

* ( ) = number of individuals

Figure 2 shows the dendrogram of seventeen individuals of *E. areolatus* constructed according to the genetic distance from Nei and Li's indices. The genetic distance level obtained ranged from 0.119 to 0.462. A high level of genetic distance of this species indicated a low level of inbreeding among individuals. It is also supported by the fact that the samples were collected from a wild population. On the other hand, the low genetic distance, indicating the high inbreeding activity level, has been reported for several cultivable fishes such as tiger barb, tilapia, discus and hilsa shad. Asma (1999) found a low genetic distance level on domesticated tiger barb, *P. tetrazona* among individuals of different varieties and populations. The genetic distance values ranged from 0.048 to 0.294 in normal variety from Perak population, 0.032 to 0.833 in green variety from Perak population, 0.030-0.286 in yellow variety from Perak population, 0.043 to 0.529 in normal variety from Johor population, 0.033 to 0.304 in green variety from Johor population, and 0.034 to 0.429 in yellow variety from Johor population. The different genetic distance on different fish species has been shown on tilapia (Bardakci and Skibinski, 1999), discus (Koh *et al.*, 1999) and hilsa shad (Dahle *et al.*, 1997) where the genetic distance was 0.04-0.34, 0.07-0.18 and 0.08-0.16, respectively.

CONCLUSION

A total of 46 loci (fragments) with 52% polymorphic fragments in size length of 350-3000 bp and three to five genotypes of *E. aerolatus* were generated by the six primers. The high genetic distance level observed in this species indicates a low inbreeding activity among individuals. The RAPD demonstrated a useful technique in detection of polymorphism and generating a potential analysis for DNA fingerprinting of fish species.

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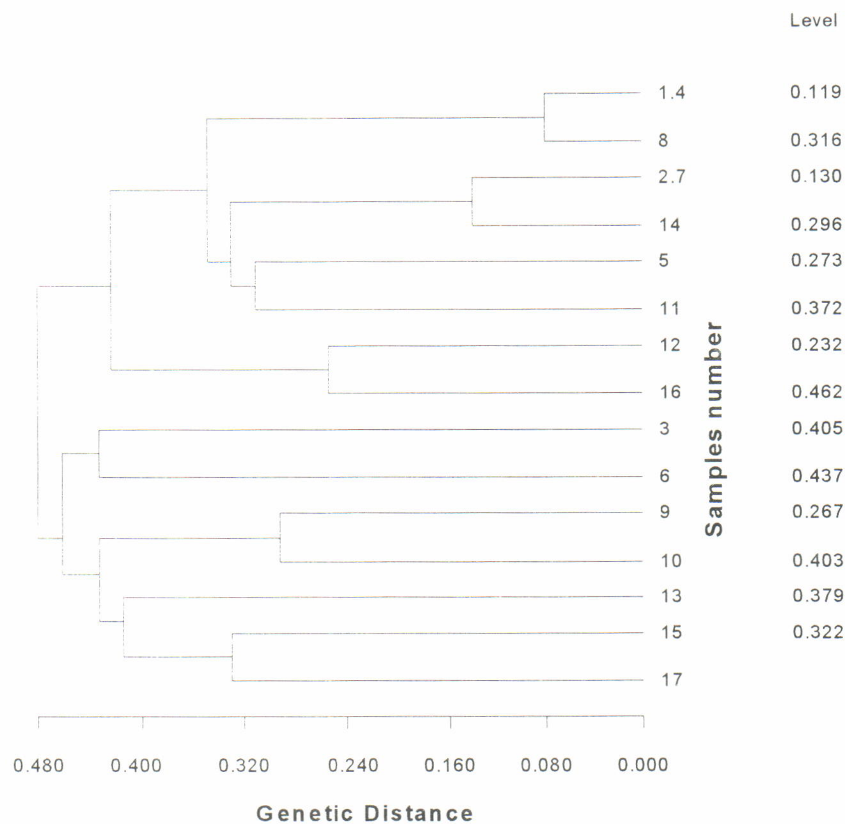

Fig. 2. UPGMA cluster analysis based on the genetic distance generated from Nei and Li's indices of *E. areolatus*

Table 5. The matrix of similarity index among individuals of *E. areolatus*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1																	
2	0.6718																
3	0.5000	0.5607															
4	1.0000	0.6718	0.5000														
5	0.7072	0.7183	0.5984	0.7072													
6	0.5457	0.5000	0.5955	0.5457	0.4419												
7	0.6718	1.0000	0.5607	0.6718	0.7183	0.5000											
8	0.8813	0.6938	0.5120	0.8813	0.7308	0.5248	0.6938										
9	0.4593	0.5451	0.5607	0.4593	0.4805	0.5774	0.5451	0.4697									
10	0.4226	0.5000	0.5528	0.4226	0.4419	0.5697	0.5000	0.4320	0.7327								
11	0.6815	0.6889	0.5753	0.6815	0.7268	0.4836	0.6889	0.7029	0.5600	0.5169							
12	0.6903	0.6518	0.5328	0.6903	0.6440	0.5670	0.6518	0.6667	0.4778	0.4137	0.5528						
13	0.5375	0.5248	0.6159	0.5337	0.6335	0.5170	0.5249	0.5463	0.5601	0.5528	0.6108	0.5219					
14	0.6518	0.8698	0.5451	0.6518	0.6938	0.4870	0.8698	0.6718	0.5682	0.5224	0.7183	0.6335	0.5120				
15	0.5394	0.6556	0.5451	0.5394	0.5670	0.5607	0.6556	0.5528	0.7089	0.6026	0.6220	0.5595	0.6667	0.6349			
16	0.6489	0.5736	0.5886	0.6489	0.6064	0.5670	0.5736	0.6273	0.7076	0.4410	0.5860	0.7675	0.5219	0.5948	0.5268		
17	0.5670	0.6496	0.5774	0.5670	0.5984	0.5528	0.6496	0.5821	0.6203	0.5528	0.6159	0.5528	0.5754	0.6286	0.6784	0.5886	

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