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MOLECULAR CHARACTERIZATION OF SOME PAKISTANI DATE PALM (*PHOENIX DACTYLIFERA* L.) CULTIVARS BY RAPD MARKERS

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Abstract

The present study was conducted with an aim to analyze the genetic diversity and phylogenetic relationship among twenty five date palm (*Phoenix dactylifera* L.) cultivars from Pakistan. The leaf samples for DNA isolation were collected from four provinces of Pakistan i.e., Sindh, Punjab, Khyber Pakhtoonkhwa and Balochistan. DNA was isolated by modified CTAB method. The study was carried out by using six universal RAPD primers. The RAPD primers showed polymorphism among all date palm cultivars. The bands obtained were successfully used to differentiate the genotypes. Based on the pair-wise comparison of amplification products, the genetic relationship was estimated. All date palm cultivars showed variation at the DNA level. The average of genetic diversity among the date palm cultivars ranged 79.4%. A dendrogram was constructed using NTSYSpc programme, on the basis of this analysis, the populations were clustered into two main clusters and sub-clusters. Therefore, the high level of polymorphism was detected and its reproducibility suggests that RAPD markers are powerful and reliable tool for genetic diversity analysis of date palm cultivars.

Introduction

Date palm (*Phoenix dactylifera* L.) is an important fruit crop mostly grown in the arid regions of Africa, the Middle East and South Asia (Khan *et al.,* 2012). In Pakistan, date palm is considered third major fruit crop (Khan & Bibi 2012; Ahmad & Mirani 2008) after citrus (Safdar *et al*., 2010) and mango (Ghafoor *et al*., 2010). Pakistan is the fifth largest dates producer in the world (Shafique *et al.,* 2011). Date palm is a long-lived, dioecious, monocotyledonous fruit tree having (2n=2x=36) chromosome number (Kumar *et al.,* 2010) and wind pollinated member of the Arecaceae family (Arabnezhad *et al.,* 2012; Akkak *et al.,* 2009). It represents a source of income to oases inhabitants, provides protection to under-crops from the harshness of the climate and reduces the damage from sand storms and wind erosion (Hussein *et al.,* 2005). It is one of the oldest known fruit trees cultivated for at least 5,000 years (Khan *et al.,* 2012; Al-Khalifah & Askari, 2003) and is believed to have originated in Mesopotamia (Hanachi *et al.,* 1998). The tolerance of date palm to high temperature, drought and salinity environments had favoured its introduction to other regions such as Eastern China, Northern India and California in recent history (Arabnezhad *et al.,* 2012). Date palm having high nutritional value, productivity and long yield-life (100 years) and referred as the "*tree of life*" in the Bible. It is a multi-purpose tree, being highly regarded as a national heritage in many countries (Mahmoudi *et al.,* 2008).

Date palms have always been clonally propagated to ensure the identity and uniformity of the cultivars. However, the existence of intra-cultivar variation could potentially cause confusion in cultivar nomenclature, preservation and utilization. Discrimination among closely related cultivars and clones is often extremely difficult (Akkak *et al.,* 2009). On the other hand introduction of new genotypes from neighboring countries together with traditional hand pollination systems may have lead to obtain recombinant genotypes and cause genetic variation of date palms in different geographical locations. Therefore, understanding genetic structure of date palm at the regional level is elementary for the efficient use of these valuable resources and better upkeep of date palm populations (Arabnezhad *et al.,* 2012) as has already been demonstrated in several other crops (Turi *et al.,* 2012; Shinwari *et al*., 2013).

Morphological markers of date palm are mainly based on fruit characteristics (shape, weight, color, skin aspect, consistency and texture) and the morphology of leaves and spines, have been used to describe many varieties. However, using morphometric characters alone for genetic diversity among closely related cultivars is often unreliable, especially because of the influence of environmental conditions (Khanam *et al.,* 2012; Zada *et al*., 2013). Future breeding programs depend on the availability of genetic variability to increase productivity. Traditionally, assessment of genetic diversity has been based on the differences in morphological and agronomic traits or pedigree information in different crops (Iqbal *et al.,* 2008). Biological macromolecules, such as DNA-based techniques have successfully been used for the analysis of genetic relationships and classifications of plants, which are difficult to be determined by the classical taxonomic methods due to environmental variations (Xuemei *et al.,* 2012; Akbar *et al*., 2012). Hence, DNA fingerprinting has proven to be the most suitable method for accurately identifying date palm cultivars and for analyzing their genetic diversity and phylogenetic relationships. DNA typing in plants is primarily used for identification of gene assortment, protection of biodiversity or germplasm conservation and identifying markers associated with specific traits. Genetic preservation is dependent on understanding the amount and distribution of the genetic diversity present in the existing germplasm (Khanam *et al.,* 2012; Jan *et al*., 2012).

Recently many researchers such as Hamza *et al.,* 2012; Khanam *et al.,* 2012; Hamza *et al.,* 2011; Khierallah *et al.,* 2011; Elshibli *et al.,* 2010; El-Rayes, 2009; Zehdi *et al.,* 2004, have successfully/efficiently attempted to study the genetic diversity among date palm cultivars by using different DNA based techniques. Molecular markers are used for the characterization and evaluation of genetic diversity among different plant species and population (Bakht *et al.,* 2012). According to Enjalbert *et al.,* 1999, molecular markers are more useful tool for accurate determination of genetic variation in plants. Various approaches are available for DNA fingerprinting such as; amplified fragment length polymorphism (AFLP), restriction fragments length polymorphism (RFLP), simple sequence repeats (SSRs) and randomly amplified polymorphic DNA (RAPD) (Paraksh & Staden, 2008).

Randomly amplified polymorphic DNA (RAPD) is a PCR based marker technique, requiring only tiny amount of genomic DNA and does not require expensive material as in molecular biology techniques like blotting and radioactive material (Malabadi, *et al*., 2006). DNA finger prints can be generated with RAPD using short nucleotide sequences of primers (usually 10bp) and does not need any prior knowledge of DNA sequence and also reveals a high level of polymorphism. RAPD-PCR is currently used as a common tool to assess different genetic markers for the assessment of genetic variation between genotypes which can be useful in different breeding programmes.

RAPD markers have been used for identification and DNA fingerprinting of the date palm varieties. Mostly this technique has been used for the genetic diversity and for analysis of phylogenetic relationships among the date palm cultivars (Khanam *et al.,* 2012). The RAPD markers in particular, have been successfully used to determine genetic diversity among species in tropical and semitropical forest plants (Elmeer & Almalki, 2011; Akbar *et al*., 2011). It contributed to early genetic diversity, research thanks to its technical simplicity and feasibility (Williams *et al.,* 1990). Successful RAPD applications assessing genetic diversity have been documented in many plants (Wangsomnuk *et al.,* 2011). Keeping in view the present study was conducted with an aim to investigate the level of polymorphism among twenty five date palm cultivars from Pakistan to assess genetic diversity as well as similarity and clear view of phylogenetic relationship among economically important and viable date palm cultivars.

Materials and Methods

The plant material consisted of twenty five important date palm cultivars from Pakistan. The date palm cultivars (Table 1) were selected on the basis of their fruit quality and revenue generation.

The young pale yellow (juvenile/unopened) leaves were collected randomly from different date palm growing areas of four provinces of Pakistan namely (Sindh, Khyber PakhtonKhawah, Punjab and Balochistan) from mature date palm trees and preserved at -20^{0}C until DNA extraction.

DNA extraction and estimation: Genomic DNA from the leaves of twenty five date palm cultivars was extracted by modified CTAB method of Doyle & Doyle (1990). Before PCR amplification DNA quantification as well as quality assessment was carried out by using UV Spectrophotometer (JENWAY, Model Genova, Serail No. 1489) at 260nm and 280nm and on 0.8% agarose gel according to Sambrok *et al*., (1989). The working DNA concentration was adjusted/diluted to 10 ng/µl in TE buffer for PCR amplification and other all coded DNA sample tubes were kept in the freezer at – 20^{0}C till use.

RAPD analysis: RAPD-PCR reactions were carried out with six RAPD primers, (Table 2) were purchased from Gene Link Technologies, USA; to amplify DNA of twenty five date palm cultivars (Table 1). The polymerase chain reactions (PCR) were conducted in a total reaction volume of 25 µl consisted of 10x PCR buffer, $MgCl_2$ 25 mM, 200 mM each of dATP, dCTP, dGTP and dTTP, primer 4 µM, *Taq* DNA polymerase 1 unit and 25 ng DNA. The PCR reaction mixture was assembled on ice box. PCR amplifications were carried out in a Hi-Temp 96 wells Thermal cycler (Master Cycler, Eppendorf®, Germany) with the following PCR program, initial denaturation at 94^{o}C for 5 min, 37 cycles comprising denaturation at 94^{o}C for 1 min, annealing at 36^{o}C for1 min, extension at 72^{o}C for 2 min and final extension was carried out at 72^{o}C for 10 min.

Electrophoresis: The amplified PCR products were separated on 1.2% agarose gel containing 0.5 µg/ml ethidium bromide and run in 1X TBE buffer at 60 Volts for 120 minutes and visualized under UV light Gel Documentation System (UV Tech TM, UK).

Table 1. Date palm cultivars used in the study were collected from four provinces of Pakistan.

1.	Aseel	2.	Asul khurmo	3.	Asul Kurh	4.	Dedhi	5.	Gajar
6.	Karbalaen	7.	Kashowari	8.	Khar	9.	Kupro	10.	Nar Aseel
11.	Noori	12.	Otaqin	13.	Dhakki	14.	Gulistan	15.	Halawee
16.	Seedless	17.	Sher Shahi Dokka	18	Begum Jangi	19.	Mazawati	20.	Shakkri
21.	Ab-e-dandan	22.	Shakkar	23.	Hussaini	24.	Basra	25.	Rabaee

Table 2. Polymorphism percentage in 25 date palm cultivars by using six RAPD primers.

Sequence of primer	Annealing temperature	MW (µg/µmole)	GC contents %	Total bands	Polymorphic bands	Polymorphism %
GLA-18-AGGTGACCGT	36^0C	3068.1	60	10	07	70
GLB-01-GTTTCGCTCC	36^0C	2970	60	10	08	80
GLG-16-AGCGTCCTCC	36^0C	2964	70	08	08	100
GLK-16-GAGCGTCGAA	36^0C	3077.1	60	05	04	80
GLJ-19-GGACACCACT	36^0C	2997	60	07	05	71.4
GLL-01-GGCATGACCT	36^0C	3028	60	08	06	75

Data analysis: For all 25 date palm cultivars six RAPD primers were used, two DNA samples from each individual were tested, only clear, unambiguous and reproducible bands amplified in both cases were considered for scoring the data. The clear visible band scored for the presence 1 and absence 0 for each primer. Data was subjected to genetic similarity matrix compiled using Dice's coefficient (Dice, 1945). By applying the unweighted pair-group arithmetic average (UPGMA) method (Sneath & Sokal 1973) using the SHAN subroutine by using the Numerical Taxonomy and Multivariate Analysis System NTSYS-pc version 2.10e (Rohlf, 1988), dendrograms representing the genetic relationship among twenty five date palm cultivars. Percentage of polymorphic bands was calculated as the percentage of polymorphic bands amplified by the primer to that of the total number of bands were produced by the same primer.

Results and Discussion

The present study is the first attempt/report to use RAPD markers for genetic diversity analysis in date palm cultivars from Pakistan. Our results confirmed that the RAPD markers are powerful tools for the detection of genetic analysis. Knowledge of genetic variation has important implications for the conservation of genetic resources and breeding programs. The relative genetic diversity can be estimated using various approaches including pedigree information, morphological and molecular markers. In case of present study the parentage (pedigree) of date palm cultivars in Pakistan is unknown. The cultivars have been developed by continuous selection by the farmers.

A total of 25 date palm cultivars from Pakistan were assessed for genetic diversity analysis using six decamer random primers. These six decamer primers generated reproducible polymorphic amplicons. The number of total amplicons generated by individual primers varied from 5-10, the maximum of 10 amplicons were generated with primer GLA-18 and GLB-01 while the minimum of 5 amplicons were observed with primer GLK-16. The monomorphic bands generated by the individual primers were 0-3 and the average number of bands is 1.6 with all primers. The range of polymorphism of individual primers was 70-100%, but the polymorphism of all six primers with 25 DNA templates were 79.4%. The size of amplified fragments ranged from 200 to 2,000bp, this is in agreement with the results of Xuemei *et al.,* (2012). One primer GLG-16 generated 100% polymorphic amplicons. RAPD profiles are shown in (Fig. 1). A total of 923 amplicons (number of cultivars analyzed x number of amplicons with all the primers) were amplified using six primers.

The pair-wise genetic distance were estimated by using Dice co-efficient for the RAPD data ranged from 0.636 to 0.950. This factor serves to ascertain the degree of genetic relatedness among the 25 commercial date palm cultivars from Pakistan. Rabae cultivar from Turbat Balochistan is most distinctly related from other date palm cultivars with similarity index (0.636) and Ab-e-dandan was most closely related with Mazawati and Halwaee having a value of 0.950 (Table 3).

The cluster analysis were estimated by unweighted paired group method of arithmetic mean (UPGMA) method and divided into two clusters. Cluster A comprises of two cultivars Seedless and Kashowari with 0.79 genetic similarity and cluster B comprises other 23 cultivars with 0.85-0.95 genetic similarity range, which were further divided into sub-clusters (Fig. 2). In cluster B the cultivars Aseel, Hussaini and Rabae fall into one group and show close relationship. On the other hand in the same cluster B cultivars Otaqin, Halawee, Dhakki and Mazawati showed closest relationship 95% similarity. In the light of above mentioned results of RAPD high level of polymorphism was observed in Pakistani date palm cultivars which indicates high level of genetic diversity among the cultivars of four provinces of Pakistan.

RAPD markers have been successively used for phylogenetic studies in many plant species (Xuemei *et al.,* 2012) and RAPD analysis could be used for an effective identification and DNA fingerprinting of date palm (Abdulla & Gamal, 2010; Jan *et al.,* 2011). The genetic similarity matrices were estimated for the date palm cultivars and used to develop dendrogram revealing the genetic relationships. Moreover, the polymorphism detected and its reproducibility suggests that RAPD markers are reliable for identification of date palm cultivars (Eissa *et al.,* 2009). Our results are supported by Sonboli *et al.,* 2011 and Xuemei *et al.,* 2012 that RAPD marker system reveal high levels of polymorphism among species indicating its effectiveness for evaluating intra- and inter-specific genetic diversity in the genus.

On the basis of above observations on DNA level, it can be concluded that there is high genetic diversity and stable difference between date palm cultivars. These results are supported by Markhand *et al*., 2010, with previous studies conducted on fruit characterization of Pakistani date palm that the fruits are significantly diverse from one cultivar to another.

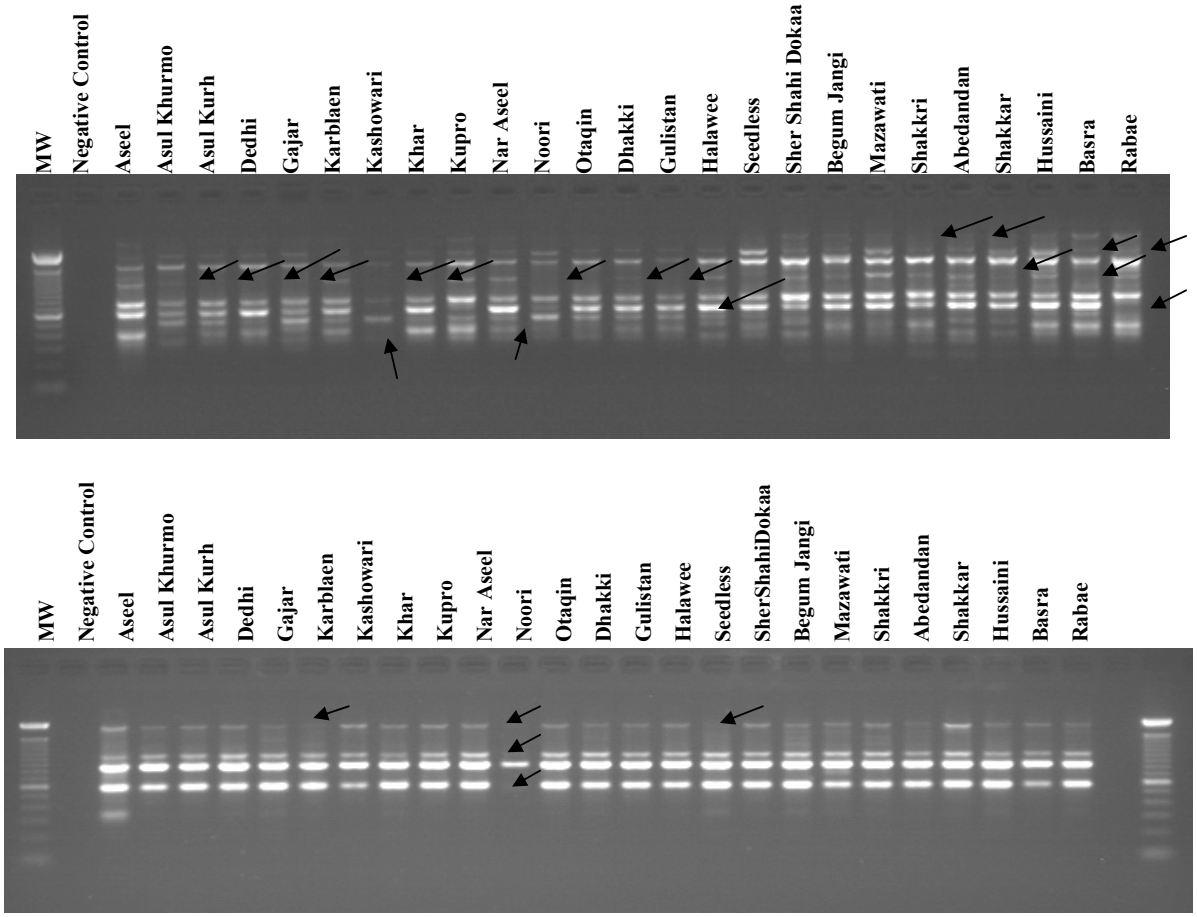

Fig. 1. RAPD-PCR gel images showing DNA polymorphism.

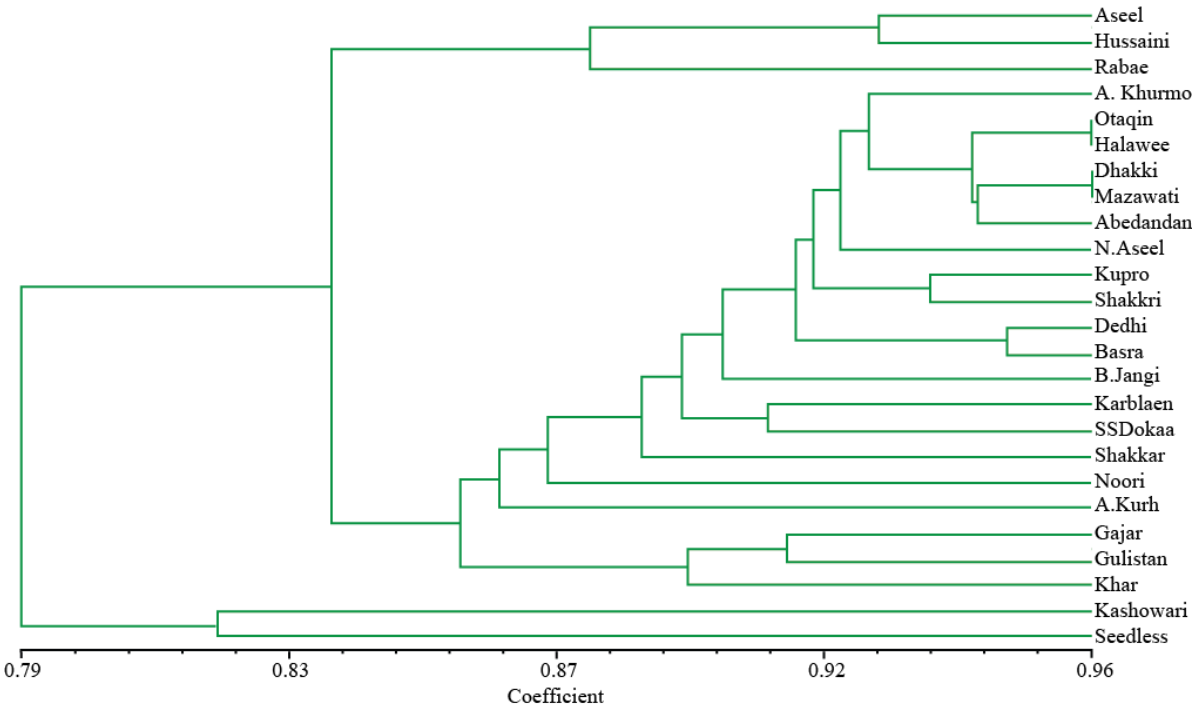

Fig. 2. Phylogenetic tree of 25 date palm cultivars derived from UPGMA cluser analysis of RAPD markers.

Table 3. Dice co-efficient similarity index.

		Aseel	AsulKhurmo	AsulKurh	Dedhi	Gajar	Karblaen	Kashowari	Khar	Kupro	Nar Aseel	Noori	Otaqin	Dhakki	Gulistan	Halawee	Seedless	SherShahiDokaa	Begum Jangi	Mazati	Shakkri	Abedandan	Shakkar	Hussaiani	Basra	Rabae
1.	**Aseel**	1.000																								
2.	**A.Khurmo**	0.853	1.000																							
3.	**A. Kurh**	0.835	0.880	1.000																						
4.	**Dedhi**	0.864	0.909	0.918	1.000																					
5.	**Gajar**	0.871	0.864	0.845	0.904	1.000																				
6.	**Karblaen**	0.864	0.909	0.864	0.894	0.849	1.000																			
7.	**Kashowari**	0.750	0.823	0.769	0.865	0.843	0.776	1.000																		
8.	**Khar**	0.815	0.861	0.811	0.873	0.882	0.873	0.773	1.000																	
9.	**Kupro**	0.878	0.897	0.880	0.935	0.864	0.883	0.823	0.861	1.000																
10.	**N. Aseel**	0.829	0.923	0.853	0.883	0.837	0.909	0.794	0.805	0.923	1.000															
11.	**Noori**	0.805	0.904	0.857	0.916	0.840	0.833	0.825	0.835	0.876	0.821	1.000														
12.	**Otaqin**	0.784	0.906	0.833	0.891	0.816	0.864	0.800	0.869	0.880	0.880	0.914	1.000													
13.	**Dhakki**	0.864	0.909	0.864	0.921	0.876	0.921	0.835	0.873	0.935	0.909	0.888	0.918	1.000												
14.	**Gulistan**	0.842	0.861	0.840	0.929	0.911	0.845	0.838	0.909	0.888	0.805	0.865	0.869	0.901	1.000											
15.	**Halawee**	0.843	0.936	0.868	0.897	0.853	0.923	0.782	0.876	0.911	0.936	0.864	0.947	0.948	0.876	1.000										
16.	**Seedless**	0.720	0.788	0.735	0.828	0.805	0.742	0.819	0.769	0.788	0.788	0.787	0.794	0.800	0.769	0.805	1.000									
17.	**SS Doka**	0.878	0.897	0.826	0.883	0.837	0.909	0.794	0.833	0.871	0.871	0.849	0.880	0.909	0.861	0.886	0.732	1.000								
18.	**B. Jangi**	0.850	0.894	0.894	0.906	0.833	0.853	0.787	0.828	0.921	0.894	0.845	0.849	0.906	0.828	0.883	0.782	0.842	1.000							
19.	**Mazawati**	0.843	0.936	0.868	0.923	0.826	0.923	0.811	0.849	0.936	0.936	0.891	0.947	0.948	0.849	0.950	0.805	0.936	0.909	1.000						
20.	**Shakkri**	0.888	0.909	0.837	0.894	0.876	0.894	0.805	0.901	0.935	0.883	0.861	0.891	0.947	0.901	0.923	0.800	0.883	0.906	0.923	1.000					
21.	**Abedandan**	0.843	0.936	0.842	0.897	0.853	0.897	0.782	0.849	0.911	0.936	0.864	0.921	0.948	0.849	0.950	0.805	0.911	0.909	0.950	0.923	1.000				
22.	**Shakkar**	0.878	0.897	0.880	0.909	0.891	0.883	0.794	0.833	0.871	0.871	0.876	0.880	0.909	0.861	0.911	0.788	0.871	0.842	0.886	0.857	0.911	1.000			
23.	**Hussaini**	0.926	0.871	0.853	0.883	0.918	0.883	0.764	0.861	0.897	0.846	0.821	0.800	0.883	0.861	0.860	0.760	0.897	0.868	0.860	0.883	0.860	0.897	1.000		
24.	**Basra**	0.853	0.897	0.880	0.935	0.864	0.909	0.823	0.861	0.897	0.897	0.876	0.906	0.909	0.861	0.911	0.788	0.923	0.894	0.936	0.883	0.936	0.923	0.871	1.000	
25.	**Rabae**	0.883	0.767	0.800	0.805	0.811	0.777	0.698	0.805	0.849	0.767	0.735	0.742	0.805	0.805	0.783	0.636	0.821	0.816	0.783	0.833	0.794	0.794	0.876	0.794	1.000

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