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MARKER BASED ASSESSMENT OF GENETIC DIVERSITY IN NUTMEG (*Myristica fragrans* Houtt.) USING ISSR PRIMERS

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ABSTRACT

Inter Simple Sequence Repeats (ISSR) constitute a reliable molecular marker system for genetic diversity analysis among species. The present study was conducted to evaluate genetic diversity / relatedness among superior accessions of *Myristica fragrans* (Nutmeg) maintained at the germplasm of Regional Agricultural Research Station, Kumarakom, Kerala, India using ISSR markers. Genomic DNA was extracted from 19 superior genotypes of nutmeg. Among the 34 ISSR primers used, only 16 primers developed reproducible banding pattern. The selected primers generated 145 amplified bands, of which 97 were found to be polymorphic. The amplicon size varied between 300 to 1500 bp. The polymorphism percentage for the tested primers ranged between 33% (UBC 860) to 100% (UBC 852a) with an average value of 65.73%. Among the different primers studied the primer UBC 815 showed the highest PIC value (0.48) and the primer UBC860 provided the lowest value (0.10). The mean value for marker index (MI) observed in this study was 1.62. The Jaccard's similarity coefficient for the genotypes studied varied from 0.565 to 0.939. The dendrogram derived from UPGMA analysis separated the nineteen nutmeg accessions into two major clusters. The information obtained from this study could be used for further breeding programmes in nutmeg.

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1 Introduction

Myristica fragrans Houtt. (Myristicaceae), commonly known as nutmeg, is an evergreen aromatic tree which produces two economically important spices, namely nutmeg seed and mace. Nutmeg is originated from spice islands of Indonesia (Leela, 2008) It is a tropical tree and commonly available in Malaysia, India, Indonesia and South East of Asia (Al-Rawi et al., 2011). The dried kernel of broadly ovoid seed of the nutmeg is known for its aromatic properties (Tajuddin et al., 2003). The nutmeg as a spice is used mainly in food processing industry and the mace is used in savory dishes (Leela, 2008). The seed and mace are widely used in pharmaceutical industries. Various researchers have narrated the anti oxidant properties of nutmeg (Madsen & Berteldsen, 1995; Lagouri & Boskou, 1995). *M. fragrans* is considered as a source of medicinally active compounds with various pharmacological effects like anti-oxidant, anti-cancerous, anti-diabetic, anti-depressant and hypocholesterolemic (Asgarpanah & Kazemivash, 2012). Myristicin extracted from nutmeg is reported to have hepatoprotective effects (Morita et al., 2003). The nutmeg is traditionally used for treating digestive disorders and kidney ailments. South East Asians use this for treating fever, headaches and bronchial problems. The Chinese consider this as an aphrodisiac (Leela, 2008). The nutmeg oil also has many therapeutic properties and is used in Ayurveda and Homeopathic treatments. Fruit powder is used (3-6gms) in Ayurveda for Atisara, Grahini, Chardi, Mukhroga, Pinasa, Rasa, Svasa and Sukrameha (Tripathi & Dwivedi, 2015). The fruit processes several pharmacological properties viz. stimulant, narcotic, carminative, astringent, aphrodisiac, acrid, astringent, sweet, bitter, thermogenic, diuretic, hypolipidaemic, antithrombotic, anti-platelet aggregation, antifungal, anti-inflammatory, anodyne, vulnerary, alternate, stomachic, laxative, carminative, digestive, anthelmintic, cordiotonic, aphrodisiac, antiseptic, febrifuge, depurative and tonic (Muchtaridi et al.,2010; Bamidele et al.,2011; Shafiei et al., 2012) According to Krishnamoorthy & Rema (2001) the nutmeg oil is used externally as a stimulant.

In India the area under nutmeg cultivation is showing an increasing trend, especially in the southern region owing to the high price of nutmeg and mace prevailing in international markets. In India, it is grown throughout Kerala, parts of TamilNadu, Karnataka, Goa, Assam and Andaman and Nicobar Islands (Thangaselvabai et al.,2011).

According to Khan et al. (2015) genetic diversity analysis involving molecular markers are useful for understanding the genomic constitution, genes responsible for important traits and germplasm conservation. Ni et al (2002) opined that genetic diversity analysis is helpful for developing genetically unique germplasm which compliments existing cultivars. ISSR markers are simple, reproducible and inexpensive. They are widely used for analysing genetic diversity between closely related cultivars (Gonzalez et al., 2002), molecular characterisation of germplasm (Charters & Wilkinson, 2000), phylogenetic studies (Ajibade et al., 2000), marker assisted selection in plant breeding and genome mapping (Reddy et al., 2002). It also provides information about markers linked to agronomic traits, and disease resistance (Ratnaparkhe et al., 1998).

Variability is observed in the growth rate, productivity, size and shape of leaf, flower, fruit and seed in nutmeg (Krishnamoorthi, 1996; Haldankar et al., 2004). Sheeja et al. (2006) reported molecular variation between clonal and seedling progenies of nutmeg using RAPD markers. Authors have already reported (Krishnan et al., 2017) the diversity analysis using RAPD primers in nutmeg The aim of the present study was to assess the genetic variability and to characterise the germplasm of *M. fragrans* using ISSR markers.

2 Materials and methods

2.1 *Plant sample collection*

The ISSR analysis was conducted on nineteen promising nutmeg accessions conserved at the Regional Agricultural Research Station, Kumarakom, Kerala, India (Table 1). Among these accessions 18 genotypes were collected from different locations

Table 1 Selected nutmeg accessions and their site of collection for genetic diversity analysis using ISSR markers

Sl No.	Genotypes/Accession	Place of Collection
1	ACC1	IISR Viswasree (a released variety)
2	ACC2	Kadapoor
3	ACC3	Vaikom
4	ACC4	Vaikom
5	ACC5	Piravom
6	ACC6	Piravom
7	ACC17	Piravom
8	ACC7	Chengalam
9	ACC8	Chengalam
10	ACC9	Arunootimangalam
11	ACC21	Arunootimangalam
12	ACC22	Arunootimangalam
13	ACC12	Poovarani
14	ACC19	Poovarani
15	ACC13	Elikulam
16	ACC14	Chengalam
17	ACC20	Pinakanad
18	ACC15	Kanjirappally
19	ACC11	Kanjirapally

of Kottayam district in Kerala and one was a released variety IISR Viswasree,. The local genotypes were selected based on their fruit characters and yield. Emerging pale green leaves collected from the selected genotypes were wrapped in aluminium foil and kept in ice boxes until reaching the laboratory. Then these were plunged in liquid nitrogen and stored at -80 °C until DNA extraction.

2.2 *DNA extraction*

The genomic DNA extraction was performed using the modified CTAB protocol described by Divyasree et al. (2014). For this protocol 0.2 g of fresh leaf tissue was ground in a preheated (60 °C) mortar and pestle using 1 ml extraction buffer, 50µl β-mercaptoethanol and a nip of PVP. Further, the ground material was subjected to incubation in a water bath at 60°C for 30 minutes, with mixing at intervals. This was centrifuged at 7200 rpm for 10 minutes at 25 °C after adding equal volume of Chloroform: Isoamyl alcohol (24:1). After that, the aqueous phase on the top was pipetted in to a sterile 1.5 ml tube. This was mixed with 2/3rd volume of ice cold isopropanol. The tube was inverted gently to facilitate DNA precipitation. Then centrifugation carried out at 7200 rpm at 25 °C for 3 minutes. 1ml of wash buffer (76% ethanol, 10 mM ammonium acetate) was added to this after discarding supernatant. Then the samples were kept for incubation at room temperature for 20 minutes. Again this was centrifuged at 7200 rpm for 10 minutes at 25 °C. The pellet obtained after discarding the supernatant was dissolved in 30 µl of sterile distilled water. The quantity and quality of DNA were checked with spectrophotometer (Shimadzu) and agarose gel (0.8%) electrophoresis.

2.3 *ISSR analysis*

PCR amplification was performed in an Agilent Sure Cycler 8800 (Agilent Technologies, USA) using 34 ISSR primers (The University of British Columbia, Canada) as suggested by Sheeja et al. (2008) (Table 2). The reaction mixture contained deionised water 17 µL, *Taq* buffer (10x) 2.5 µL, $MgCl_2$ (25 mM) 1 µL, ISSR Primer (10 pmol/ µL) 2 µL, dNTP mix (2.5mM each) 1 µL, *Taq* DNA Polymerase (2 U/µL) 0.5 µL, Template DNA (25 ng/µL) 1 µL with a final volume of 25 µL. The PCR programme was designed as follows: initial denaturation of 4 min at 94°C; followed by 35 cycles of denaturation for 35 s at 94°C, primers annealing for 1 min at 50-55 °C , and extension for 10 min at 72°C, followed by a final extension for 10 min at 72°C. Amplified products were size-separated on 2% agarose gel and documented using a Bio-Rad Gel Documentation System (Bio-Rad Laboratories, Hercules, CA).

Table 2 List of ISSR primers used for the study

Sl. No.	Primer	Nucleotide sequence
1	UBC 807	5'-AGAGAGAGAGAGAGAGT-3'
2	UBC 809	5'-AGAGAGAGAGAGAGAGG-3'
3	UBC 810	5'-GAGAGAGAGAGAGAGAT-3'
4	UBC 812	5'-GAGAGAGAGAGAGAGAA-3'
5	UBC 815	5'-CTCTCTCTCTCTCTCTG-3'
6	UBC 816	5'-CACACACACACACACAT-3'
7	UBC 834a	5'-AGAGAGAGAGAGAGAGCT-3'
8	UBC 834b	5'-AGAGAGAGAGAGAGAGTT-3'
9	UBC 835	5'-AGAGAGAGAGAGAGAGCC-3'
10	UBC 836a	5'-AGAGAGAGAGAGAGAGCA-3'
11	UBC 838	5'-TATATATATATATATAAC-3'
12	UBC 840a	5'-GAGAGAGAGAGAGAGACT-3'
13	UBC 840b	5'-GAGAGAGAGAGAGAGATT-3'
14	UBC 841a	5'-GAGAGAGAGAGAGAGACC-3'
15	UBC 841b	5'-GAGAGAGAGAGAGAGATC-3'
16	UBC 842b	5'-GAGAGAGAGAGAGAGATG-3'
17	UBC 843a	5'-CTCTCTCTCTCTCTCTAA-3'
18	UBC 844a	5'-CTCTCTCTCTCTCTCTAC-3'
19	UBC 848a	5'-CACACACACACACACAAG-3'
20	UBC 850a	5'-GTGTGTGTGTGTGTGTCC-3'
21	UBC 852a	5'-TCTCTCTCTCTCTCTCAA-3'
22	UBC 855	5'-ACACACACACACACACYT-3'
23	UBC 857a	5'-ACACACACACACACACCG-3'
24	UBC 857b	5'-ACACACACACACACACTG-3'
25	UBC 858	5'-TGTGTGTGTGTGTGTGRT-3'
26	UBC 860	5'-TGTGTGTGTGTGTGTGAA-3'
27	UBC 862	5'-AGCAGCAGCAGCAGCAGC-3'
28	UBC 865	5'-CCGCCGCCGCCGCCGCCG-3'
29	UBC 866	5'-CTCCTCCTCCTCCTCCTC-3'
30	UBC 868	5'-GAAGAAGAAGAAGAAGAA-3'
31	UBC 872	5'-GATAGATAGATAGATA-3'
32	UBC 873	5'-GACAGACAGACAGACA-3'
33	UBC 874	5'-CCCTCCCTCCCTCCCT-3'
34	TC 10G	5'-TCTCTCTCTCTCTCTCTCTCG-3'

2.4 *Data analysis*

The molecular size of the amplicons was estimated based on the relative position of bands with the 100 bp ladder marker which was run along with the amplified product. The amplified bands were scored as present (1) or absent (2). Only those bands which were clear and reproducible were considered for scoring. The percentage of polymorphism was calculated using the binary data generated. The Polymorphism Information Content (PIC) was calculated as PIC = 2fi (1 - *fi*), where fi is frequency of amplified allele (band present) and (1 - *fi*) is frequency of null allele (band absent) for the allele *i* (Roldan-Ruize et al., 2000). The marker index (MI) was calculated as a product of PIC and number of polymorphic bands per assay unit as explained by Powel et al. (1996). The MI value assesses the overall utility of a marker system. The genetic relationship between selected accessions was analysed using the software programme Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc, Version 2.02e, Exeter Software, NY, USA) (Rohlf, 1998). Jaccard's coefficient of similarity (Jaccard, 1908) was calculated with the SIMQUAL program. These similarity coefficients were used to generate UPGMA (Unweighted Pair Group with Arithmetic Averages) dendrogram using SAHN module of NTSYS-pc (Sneath & Sokal 1973).

3 Result and discussion

Different types of molecular markers have been used in nutmeg for assessing genetic diversity such as RAPD (Sheeja et al., 2006; Krishnan et al., 2017) and ISSR (Sheeja et al., 2013). Inter simple sequence repeat markers are one of the robust tool used to evaluate genetic diversity in agronomically important crops (Brantestam et al., 2004). In this study, 34 ISSR primers were screened for their amplification efficiency in 19 superior accessions of nutmeg. Among them 16 primers generated clear and reproducible banding profile. These ISSR primers yielded 145 amplified bands of which 97 were polymorphic. The number of amplified fragments for each primer ranged from 3 (UBC 860) to 13 (UBC 840A and UBC 810) with a mean of 8.5 bands per primer which varied in size from 300 to 1500 bp. The percentage of polymorphism among the primers studied showed an average value of 65.73% and it varied between 33% (UBC 860) to 100% (UBC 852a). The highest number of polymorphic bands was yielded by the primers UBC 857b, UBC 807 and UBC 852a (Figure 1). Sheeja et al. (2013) reported that 16 ISSR primers produced 262 bands with a polymorphism of 97.3% in seven *Myristica* sp., two of its related genera and one unidentified species. In a related study with eight accessions of *Cinnamomum tamala*, Gawari et al. (2016) obtained an average polymorphism of 86.3% for 3 ISSR markers. The rate of polymorphism obtained in this study was higher when compared to ISSR analysis in *Garcinia xanthochymas* where polymorphism was only 21.25% (Anerao et al., 2016)

The discriminatory power of ISSR primers for genetic relationship analysis was evaluated using polymorphic information content (PIC), and marker index. In the present study the PIC value of 16 ISSR primers ranged from 0.10 to 0.48 with an average of 0.28, which indicate that these ISSR markers are highly informative (Table 3). The most informative marker in this study was UBC 815 since it recorded highest PIC value (0.48). Thimmappaiah et al. (2009)

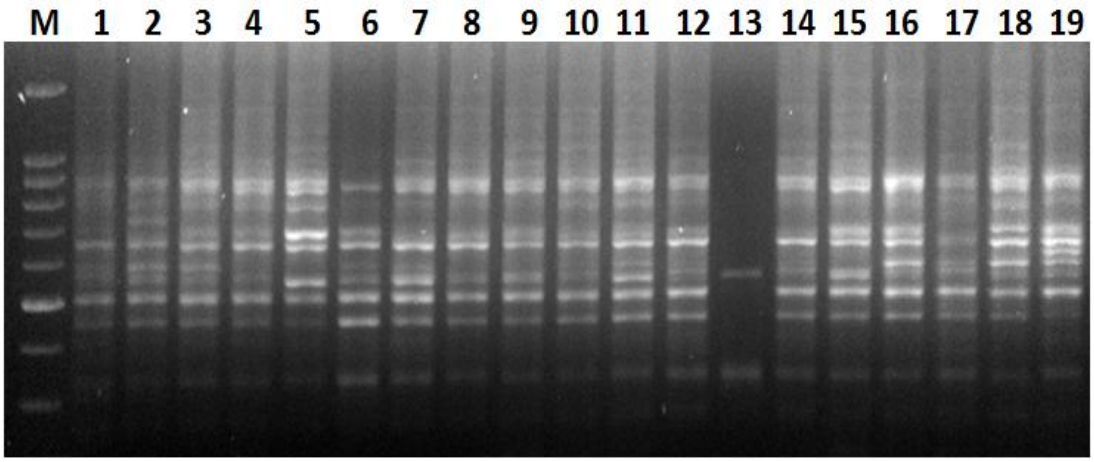

Figure 1 ISSR profiling of selected Nutmeg genotypes using UBC807. Lane M: 100bp DNA Ladder; Lane 1: Acc. 1; Lane2: Acc. 2; Lane 3:Acc.3; Lane 4 Acc. 4; Lane 5 : Acc.5; Lane 6 : Acc.6; Lane 7 : Acc.17; Lane 8: Acc.7; Lane 9: Acc.8; Lane 10: Acc.9; Lane 11: Acc.21; Lane 12: Acc.22; Lane 13: Acc.12; Lane 14: Acc.19; Lane 15: Acc.13; Lane 16: Acc.14; Lane 17: Acc.20; Lane 18: Acc.15; Lane 19: Acc.11.

Table 3 ISSR data obtained with 16 primers analysed on 19 accessions of *M. fragrans*.

Primer	Total number of bands	Percentage of polymorphic bands	Percentage of monomorphic bands	PIC	MI
UBC 840A	13	53.85	46.15	0.34	2.35
UBC 860	3	33.00	66.00	0.10	0.10
UBC 844A	9	55.00	45.00	0.26	1.27
UBC 807	11	81.81	18.18	0.24	2.20
UBC 858	7	85.71	14.28	0.22	1.29
UBC 840B	10	70.00	30.00	0.29	2.02
UBC 852A	9	100.00	0.00	0.20	1.79
UBC 842B	5	80.00	20.00	0.30	1.20
UBC 857B	10	90.00	10.00	0.24	2.13
UBC 850A	6	50.00	50.00	0.22	0.66
UBC 855	9	55.00	44.00	0.34	1.69
UBC 812	10	50.00	50.00	0.40	2.01
TC-10	6	83.33	16.66	0.26	1.29
UBC 815	6	33.33	66.66	0.48	1.92
UBC 848	10	60.00	40.00	0.27	1.59
UBC 834A	8	75.00	25.00	0.30	1.78
UBC 810	13	61.50	38.50	0.29	2.29
Total	145				
Average	8.5	65.73%	36.27%	0.28	1.62

obtained similar results in Cashew (*Anacardium occidentale* L.) where the PIC value varied from 0.180 to 0.419 with an average of 0.295 using 10 ISSR primers. Sheeja et al. (2013) observed an average PIC value of 0.35 for 16 ISSR primers in *Myristica* sp. and related genera. The marker index (MI) calculated for the 16 primers employed in the present investigation ranged from 0.10 to 2.35 with a mean of 1.62 (Table 3). MI reflects the efficiency of marker to analyse a large number of bands, rather than level of polymorphism detected (Powell et al., 1996).

The Jaccard's similarity co-efficient ranged between 0.565 and 0.939 (Table 4) revealing a moderate level of genetic diversity among the accessions studied. The accessions ACC7 and ACC11 showed most distinct genetic relationship with the lowest similarity index (0.565) whereas the accessions ACC22 and ACC19 were closer in their genetic makeup with highest similarity index (0.939). Jaccard's similarity coefficient ranged from 0.55 to 1.00 in 22 Castor (*Ricinus communis* L.) genotypes using 21 ISSR primers (Gajera et al., 2010). The genetic similarity coefficient of 48 okras ranged from 0.6558 to 0.9935 using 22 ISSR markers (Yuan et al., 2015).

The data generated was used to estimate genetic similarity for pair wise accessions based on Jaccard's similarity coefficient. The UPGMA dendrogram constructed based on Jaccard's similarity coefficient clustered the 19 genotypes into two major clusters at a coefficient value of 0.62 (Figure 2). In the

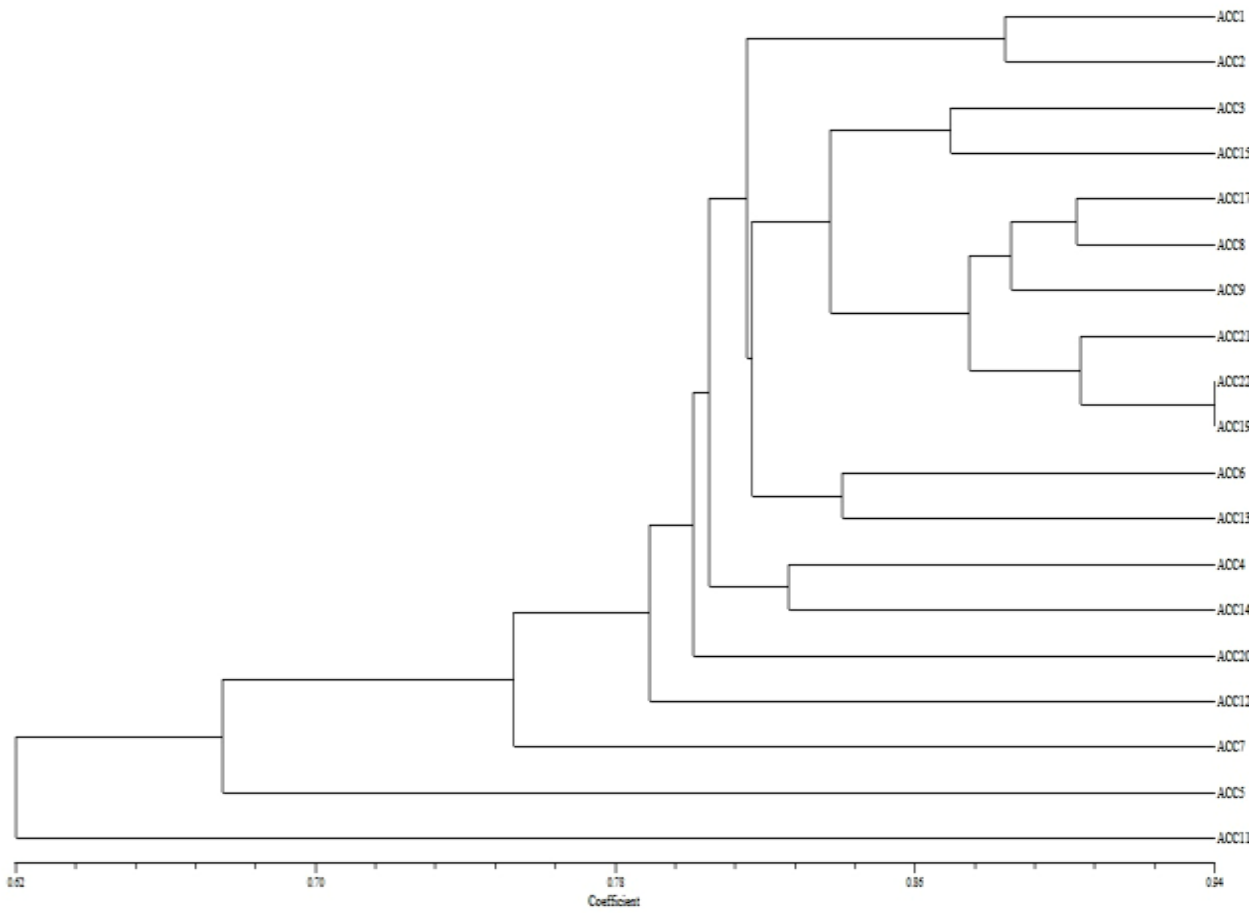

Figure 2 Dendrogram obtained from 19 accessions of Nutmeg with UPGMA based on Jaccard's coefficient

dendrogram ACC 11 was most genetically dissimilar and was separated from all other accessions. The remaining accessions were again divided into 2 with accession ACC 5 alone forming a separate category. The accessions ACC19 & ACC22 showed maximum amount of similarity.

Assessment of genetic variation is essential for developing high yielding varieties (Kumar et al., 2015). Hence the information generated under this study about the variability of nutmeg trees in Kerala will be useful for further crop improvement programme of nutmeg.

In conclusion, the current study demonstrated the genetic diversity existed between the 19 superior accessions of nutmeg maintained in farm of RARS, using ISSR markers. Predominance of duplicates is common in germplasm banks. They can be eliminated by molecular characterization of the accessions and thereby reducing the cost of maintenance of redundant in clonal repositories.

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Conflict of Interest

Authors hereby declare no conflict of interest.

Table 4 Jaccard's co-efficient of similarity between 19 nutmeg accessions based on ISSR data.

	ACC1	ACC2	ACC3	ACC4	ACC5	ACC6	ACC17	ACC7	ACC8	ACC9	ACC21	ACC22	ACC12	ACC19	ACC13	ACC14	ACC20	ACC15	ACC11
ACC1	1.000																		
ACC2	0.883	1.000																	
ACC3	0.827	0.848	1.000																
ACC4	0.785	0.801	0.858	1.000															
ACC5	0.611	0.635	0.693	0.680	1.000														
ACC6	0.835	0.836	0.807	0.750	0.650	1.000													
ACC17	0.793	0.835	0.848	0.822	0.679	0.852	1.000												
ACC7	0.762	0.770	0.725	0.725	0.689	0.763	0.788	1.000											
ACC8	0.824	0.867	0.840	0.813	0.644	0.826	0.902	0.779	1.000										
ACC9	0.818	0.860	0.876	0.821	0.677	0.798	0.868	0.772	0.902	1.000									
ACC21	0.779	0.821	0.848	0.796	0.705	0.815	0.882	0.735	0.874	0.881	1.000								
ACC22	0.774	0.816	0.850	0.803	0.674	0.823	0.876	0.747	0.854	0.861	0.904	1.000							
ACC12	0.739	0.768	0.770	0.705	0.662	0.789	0.816	0.707	0.807	0.815	0.830	0.824	1.000						
ACC19	0.785	0.841	0.841	0.801	0.672	0.792	0.861	0.754	0.881	0.888	0.903	**0.939**	0.850	1.000					
ACC13	0.744	0.815	0.800	0.748	0.654	0.8391	0.836	0.738	0.884	0.835	0.822	0.801	0.795	0.828	1.000				
ACC14	0.794	0.837	0.817	0.824	0.730	0.833	0.858	0.748	0.796	0.803	0.816	0.812	0.791	0.797	0.770	1.000			
ACC20	0.792	0.822	0.802	0.743	0.662	0.793	0.816	0.746	0.781	0.828	0.816	0.851	0.776	0.822	0.743	0.818	1.000		
ACC15	0.745	0.800	0.868	0.787	0.708	0.740	0.820	0.752	0.838	0.819	0.807	0.827	0.730	0.813	0.774	0.757	0.755	1.000	
ACC11	0.578	0.625	0.650	0.598	0.604	0.603	0.647	**0.565**	0.647	0.622	0.653	0.628	0.583	0.617	0.626	0.632	0.580	0.632	1.000

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