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Assessing Genetic Diversity of Shishi Date Palm Cultivars in Saudi Arabia and Qatar Using Microsatellite Markers

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Abstract
In this study fourteen microsatellite primer pairs were used to study the genetic diversity of Shishi Date palm in Qatar. A total of 32 date palm (15 Shishi cultivar, 10 Khalas and seven male date palms) were collected from Qatar and 5 Shihi cultivars were collected from Saudi Arabia for comparison. The Shishi set collected from Qatar was selected from different regions to represent the genetic diversity of this cultivar. The results indicated 98 alleles produced from the 14 microsatellite markers, and the cluster analysis showed four major clusters corresponding o the geographical areas. Similarly, the structure analysis indicated four populations according to statistic K value. PCoA analysis showed three groups (A, B and C) separating Shishi (from Qatar) in group A, Khalas in group B and Shishi (from Saudi Arabia) in group C and no clear group separated the male genotypes. This indicates that the sexual propagation by seeds is the main source of variation in the date palm. This is the first study focusing on Shishi cultivar in Qatar and Saudi Arabia by using molecular markers.

Keywords: Date Palm, *Phoenix dactylifera*, Microsatellite, Shihi, Khalas.

Introduction

Date palm (*Phoenix dactylifera* L.) of the family Arecaceae is a dioecious tree and one of the most important members of this family. It is also one of the oldest cultivated crops grown in the desert and semi-desert areas. Individual varieties are valued primarily for fruit-related traits including moisture and sugar content, and as many as 3,000 varieties are recognized worldwide (Johnson et al., 2013). The tremendous advantages of the tree its resilience, requirement for limited inputs, long-term productivity and multiple purposes attributes (Bekheet, 2013).

Conventionally, date palm is propagated sexually through seeds and vegetative by offshoot (Shah, 2014). Clonal propagation of elite cultivars with known high performance is highly desired, but the availability of the date palm offshoots is limited because their number produced by each palm tree is low (Popenoe, 1973) and some of which will die when separated from the mother plants. Some date palm

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cultivars are a mixture of plants derived from seeds with significant progeny variation. Propagation by seeds is much manageable; seeds germinate easily and are available in large numbers, and sometimes seeds germinate just under their mother trees (Elmeer and Mattat, 2015) .

Over years many varieties have been transplanted to the areas other than original regions, and they may have been adapted and cultivated with different names. As a result, a variety may have a different name in different plantation areas or even two genetically different varieties may have the same name (Torres and Tisserat, 1980). Most of the description of date cultivars are based on farmer's selection which is why there is no evidence of the majority of cultivars being breeder's clones (Elshibli and Korpelainen, 2009). Also, their vulnerability to biotic and abiotic stresses suggests an urgent need for their genetic conservation.

Many studies have pointed out how farmers recognize and name the population of crops that they grow according to their agro-morphological, ecological-adaptive, quality and use characteristics. Additionally, information on distinctiveness of farmers varieties bearing similar names would help in preserving and using this diversity because it might be lost if such varieties are discarded because they bear the same name (Jarvis et al., 2007). Farmers characterization of the crop diversity they manage may range from a simple application of generic crop name, to all varieties of the crop, even if the different population is managed differently, to a location-specific name modified by an accompanying set of traits. This recognition that the name may or may not represent the level of diversity of farmer management has helped to refine methods to understand how farmer manage diversity on farm (Jarvis et al., 2007).

The development of molecular tools has changed the way in which individual cultivars can be identified and useful agronomic characteristics can be analyzed.

The current study aimed to assess the genetic diversity of date palm cultivars by microsatellite markers, and to investigate the correlation between geographical areas and the genetic variation of date palm cultivars.

Materials and Methods

Plant materials

A total of 32 date palm samples consisted of 15 represent Shishi cultivar (5 from Saudi Arabia and 10 from Qatar) and 10 represent Khalas in addition to seven male date palms were collected from Qatar (Table 1). The Shishi and Khalas considered as a well-known and noble cultivar in Gulf region.

Table 1. Thirty-two samples representing 5 Saudi Arabia Shishi, 10 Qatari Shishi, 10 Qatari Khalas and 7 male date palms from Qatar

No	Country	Cultivar	Location	Code	No.	Country	Cultivar	Location	Code
1	KSA	Shishi	Al-Hasa	SK1	17	Qatar	Khalas	East	KQ2
2	KSA	Shishi	Al-Hasa	SK2	18	Qatar	Khalas	East	KQ3
3	KSA	Shishi	Al-Hasa	SK3	19	Qatar	Khalas	East	KQ4
4	KSA	Shishi	Al-Hasa	SK4	20	Qatar	Khalas	East	KQ5
5	KSA	Shishi	Al-Hasa	SK5	21	Qatar	Khalas	East	KQ6
6	Qatar	Shishi	North	SQ1	22	Qatar	Khalas	West	KQ7
7	Qatar	Shishi	North	SQ2	23	Qatar	Khalas	South	KQ8
8	Qatar	Shishi	East	SQ3	24	Qatar	Khalas	South	KQ9
9	Qatar	Shishi	East	SQ4	25	Qatar	Khalas	South	KQ10
10	Qatar	Shishi	East	SQ5	26	Qatar	Male	East	MQ1
11	Qatar	Shishi	West	SQ6	27	Qatar	Male	East	MQ2
12	Qatar	Shishi	South	SQ7	28	Qatar	Male	East	MQ3
13	Qatar	Shishi	South	SQ8	29	Qatar	Male	West	MQ4
14	Qatar	Shishi	South	SQ9	30	Qatar	Male	West	MQ5
15	Qatar	Shishi	TC*	SQ10	31	Qatar	Male	West	MQ6
16	Qatar	Khalas	North	KQ1	32	Qatar	Male	West	MQ7

*Tissue culture derived

DNA extraction

Young leaf tissues of the 32 mature adulate trees which producing date fruits have been collected for DNA extraction. The tissue samples were stored at -80°C, until DNA extraction. The frozen young leaf tissues were first cleaned carefully with distilled water. The DNA of this collection were extracted using the cetyl trimethyl ammonium bromide (CTAB) method, as described by Rogers and Bendich (1985). In brief, young green leaves were collected from well grown palm trees was frozen in liquid nitrogen and ground into a fine powder, which was subsequently added to a 2 mL Eppendorf tube with 1 mL pre-warmed 2 X CTAB buffer (2 % CTAB, 0.1 M Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM ethylene diamine tetra acetic acid (EDTA)). The suspension was mixed and incubated at 65 °C for 30 min. The suspension was cooled at room temperature (RT) for 5 min, and then 1 mL chloroform-isoamyl alcohol (24:1) was added to the tube and the suspension gently mixed by shaking for 10 min. The suspension was centrifuged at 4,500 rpm for 20 min at RT and the supernatant transferred to a new tube. The DNA was precipitated with 700 µL of cold isopropanol. The DNA was transferred into a micro-centrifuge tube and washed twice with a washing buffer (75 % ethanol and 200 mM sodium acetate) for 20 min. After air-drying for about 10–20 min, the DNA was dissolved in 100 µL of 1X TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The obtained DNA was quantified and qualified using a Nanodrop Spectrophotometer.

Microsatellite amplification

Fourteen labeled microsatellite primer pairs published by Billotte et al. (2004) and synthesized by Applied Biosystems (Life Technologies Europe BV, Belgium) have been used in this study to study the genetic diversity (Table 2). A polymerase chain reaction (PCR) was performed using a 25-µL reaction mixture containing 2 µL (5 ng) total genomic DNA, 12.5 µL AmpliTaq Gold® 360 Mastermix (Applied Biosystems), 1 µL 5 µM forward primer (labeled), 1 µL reverse primer, and 8.5 µL nuclease free water. Amplification was carried out in a Veriti 96 Well Fast Thermal Cycler (Applied Biosystems) under the following profile: initial denaturation at 95 °C for 10 min; 35 cycles of denaturation at 95 °C for 30 s, annealing temperature depending on primer for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min.

The SSR fragments were electrophoresed by using 3130 Genetic Analyzer (Applied Biosystems). The running mixture was consisted of 1 µL PCR product, 10 µL Hi-Di Formamide and 0.3 µL GS500LIZ. An initial denaturation at 95 °C for 3 min was conducted for samples loading and then samples were kept on ice until loading. Automatic genotyping and allele scoring was performed by the GeneMapper® Software v4.0 (Applied Biosystems).

Data analysis

Data were analyzed with the PowerMarker software v3.0 (Liu and Muse, 2005) to determine the percentage of heterozygosis, major allele frequency, number of alleles, gene diversity, and polymorphic information content. The phylogenetic relationships among the genotypes and phylogenetic diagrams were drawn using the Past software version 1.91 (Hammer et al., 2001) on the basis of the Hamming similarity index with 100 bootstraps. The principal coordinate analysis (PCoA) was analyzed using PAST software v1.91 (Hammer et al., 2001) using Hamming distance measures with 95% elipses. The analysis of molecular variance (AMOVA), and the genetic variation within and among population were analyzed by using GenAlex 6.3 software (Peakall and Smouse, 2006).

The software program Structure 2.0 (Pritchard et al., 2000) was utilized to infer population structure and assign individuals to populations based on the 14

SSR genotype data. Structure uses model-based clustering in which a Bayesian approach is used to identify clusters based on fit to Hardy–Weinberg equilibrium and linkage equilibrium. Multiple runs of structure were performed by setting K (the number of populations) from 1 to 11. The burn-in time and replication number was set to 10000 for each run and each run was replicated five times. A calculation of gene flow was carried out with Popgene software version 1.21 (Yeh et al., 1997).

Table 2. Forward and reverse microsatellite primers and the melting temperature (Tm) based on Billotte et al. (2004)

No.	Primer code	Repeat motif	Primer sequences (5'-3')	Optimal Tm (°C)
1	mPdCIR010	$(GA)_{22}$	F: ACCCCGGACGTGAGGTG R: GTCGATCTCCTCCTTTGTCTC	55.9
2	mPdCIR015	(GA)15	F: AGCTGGCTCCTCCCTTCTTA R: GCTCGGTTGGACTTGTTCT	51.6
3	mPdCIR016	(GA)14	F: AGCGGGAAATGAAAAGGTAT R: ATGAAAACGTGCCAAATGTC	51.7
4	mPdCIR025	(GA)22	F: GCACGAGAAGGCTTATAGT R: CCCCTCATTAGGATTCTAC	49.3
5	mPdCIR032	(GA)19	F: CAAATCTTTGCCGTGAG R: GGTGTGGAGTAATCATGTAGTAG	51.5
6	mPdCIR035	(GA)15	F: ACAAACGGCGATGGGATTAC R: CCGCAGCTCACCTCTTCTAT	53.9
7	mPdCIR044	(GA)19	F: ATGCGGACTACACTATTCTAC R: GGTGATTGACTTTCTTTGAG	51.7
8	mPdCIR048	(GA)32	F: CGAGACCTACCTTCAACAAA R: CCACCAACCAAATCAAACAC	51.4
9	mPdCIR057	(GA)20	F: AAGCAGCAGCCCTTCCGTAG R: GTTCTCACTCGCCCAAAAATAC	55.4
10	mPdCIR070	(GA)17	F: CAAGACCCAAGGCTAAC R: GGAGGTGGCTTTGTAGTAT	48.7
11	mPdCIR078	(GA)13	F: TGGATTTCCATTGTGAG R: CCCGAAGAGACGCTATT	49.6
12	mPdCIR085	(GA)29	F: GAGAGAGGGTGGTGTTATT R: TTCATCCAGAACCACAGTA	50.4
13	mPdCIR090	(GA)26	F: GCAGTCAGTCCCTCATA R: GCAGTCAGTCCCTCATA	48.6
14	mPdCIR093	(GA)16	F: CCATTTATCATTCCCTCTCTTG R: CTTGGTAGCTGCGTTTCTTG	51.8

Results and Discussion

The 14 primers that used in this study successfully produced clear amplified SSR peaks with sizes ranging from 120 bp with primer mPdCIR010 and mPdCIR015 to 302 bp with primers mPdCIR032 and mPdCIR044 (Table 3), similar to the results of Elmeer and Mattat (2015) where the band sizes ranged from 104 to 330 bp.

A total of 98 alleles were observed using 14 microsatellite markers, and there was an average of 7 alleles per locus. This high number of polymorphism at these microsatellite loci reveals a high level of genetic diversity in the existing date palm germplasm. However, the number of alleles varied from 4 (mPd-CIR057) to 10 (mPdCIR015 and mPdCIR078) (Table 3).

Table 3. Major allele frequency, allele number, gene diversity and polymorphic information continent of 14 SSR

Primer code	Allele size (bp)	Major allele frequency	Genotype No.	Allele No.	Gene diversity	Heterozygosity	PIC
mPdCIR010	120-160	0.20	14	9	0.83	0.94	0.81
mPdCIR015	120-140	0.28	12	10	0.83	1.00	0.81
mPdCIR016	130-138	0.38	8	5	0.71	0.72	0.65
mPdCIR025	201-229	0.70	7	8	0.49	0.43	0.47
mPdCIR032	290-302	0.39	7	5	0.71	0.75	0.65
mPdCIR035	185-197	0.38	6	5	0.73	0.66	0.69
mPdCIR044	282-302	0.54	8	8	0.67	0.00	0.64
mPdCIR048	160-192	0.32	10	8	0.73	0.60	0.68
mPdCIR057	254-266	0.50	5	4	0.58	0.81	0.49
mPdCIR070	182-204	0.45	9	7	0.71	0.50	0.67
mPdCIR078	122-148	0.34	12	10	0.81	0.47	0.79
mPdCIR085	158-182	0.22	13	9	0.84	0.97	0.82
mPdCIR090	144-156	0.69	6	5	0.47	0.16	0.41
mPdCIR093	155-175	0.70	7	5	0.47	0.31	0.43
Mean		**0.43**	**8.86**	**7**	**0.68**	**0.59**	**0.64**

Yusuf et al. (2015) obtained 7 alleles per locus using fourteen cultivars of date palm from Nigeria and Saudi Arabia, while Hamza et al. (2012) identified 7.2 alleles per locus when they evaluated the genetic diversity of twenty-six Tunisian cultivars using five SSR loci, also Zehdi et al. (2004), was detected 7.14 alleles per locus when examining 46 Tunisian date palm accessions using 14 microsatellite loci. Elshibli and Korpelainen (2008) reported 21.4 alleles per locus, which is more than the number of alleles per locus detected in this study. This may be a result of using different genotypes (45 female and 23 male of date palm accessions from Sudan and Morocco).

The genetic diversity was ranged from 0.47 to 0.84 with an average of 0.68 (Table 3). This high genetic diversity in these palm trees might be due to geographical isolation and gene flow. The gene flow identified in this study is relatively high (0.56), it may be due to cross pollination nature in date palm in addition to the Qatari farmer's are dealing and bringing the date palm offshoots from Saudi Arabia.

The average PIC value in the 14 polymorphic microsatellites was 0.64 (Table 3), where 10 loci (71.5%) showed a high number of polymorphisms (PIC $\geq$ 0.64) and 4 loci (28.5%) were low polymorphic loci (PIC $\leq$ 0.49). Markers with high PIC values could be effectively used in genetic diversity studies on date palms.

Genetic diversity using microsatellites in date palm

A total of 32 date palm cultivars according to neighbor joining method were divided into four major clusters corresponding to the geographical areas from which they were collected (Fig. 1). Date palm trees in cluster "A", "B" and "D" were mainly collected from Qatar. The cluster "A" included the male tress, cluster "B" consisted of the Khalas genotypes. Cluster "D" included Shishi cultivar collected form Qatar. Shishi cultivars collected from Saudi Arabia were clustered separately in cluster C.

The value of K was fixed to 4 according to the statistic of Evanno et al. (2005) as shown in (Fig. 2-A). Structure analysis doesn't identified clearly separation of the populations. The clearest population was Khalas followed by Shishi collected from Saudi Arabia (Fig. 2-B). The population of male trees showed mixture of bars indicating high level of allele shared with other female populations used in this study. This is expected as male is always used to fertilize the

female trees of different varieties. First two axis of the Principal coordinate analysis (PCoA) explained a total of 65 % of the variations (Gower, 1966). PCoA showed three groups (A, B and C) separating Shishi (from Qatar) in group A, Khalas in group B and Shishi (from Saudi Arabia) in group C.

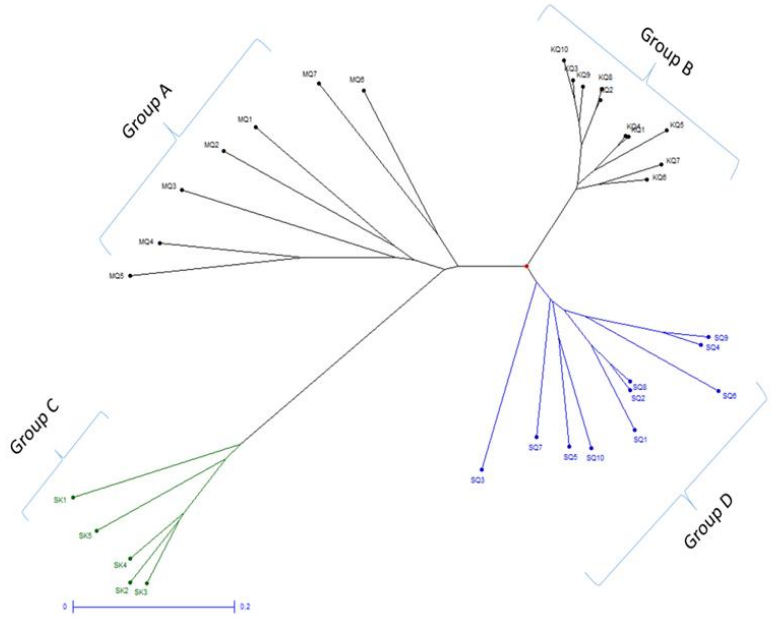

Fig. 1. Phylogenetic relationship between the individual date palm cultivars in Saudi Arabia and Qatar based on Hamming coefficient analysis.

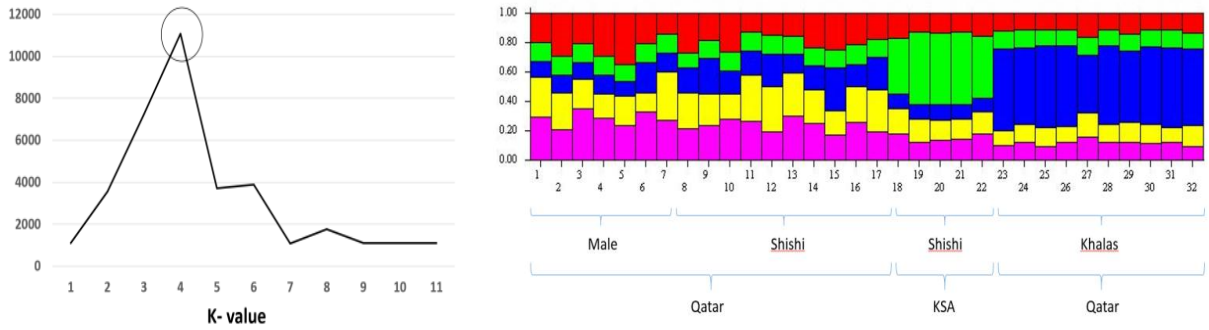

Fig. 2. Estimated population structure. A) K-value indicating the optimal number of populations based on the genotype data developed from 14 SSR. B) Each individual bar represents a genotype of 32 Date palm genotypes of Shishi, Khalas and male genotypes.

Interestingly, no clear group separated the male genotypes (Fig. 3) but they were distributed among the three groups (MQ2 and MQ7), or with Qatari date palm whichever Shishi (Group A) or Khalas (Group B). The male date palm from east location in Qatar (MQ3) grouped with the Khalas from the same location (KQ2, KQ3, KQ4, KQ5, KQ6), and the male date palm from west location in Qatar (MQ4) grouped with the Shishis from the same location (SQ6), indicating that quantity of date palm in Qatar were propagated by seeds resulting from Qatari males pollination, furthermore link between Saudi Shishi (Group C) and males because there was no Saudi male

in this study (Fig. 3). According to Zaid and de Wet (2002), the mixture of cultivars during sexual propagation by seeds is the main source of variation in the date palm. Seed propagation of date palm has been reported to occur also in other countries. Elshibli and Korpelainen (2008) while analyzing Sudan date palm resource, because of high genetic diversity observed within the groups and the weak clustering of the cultivars suggested that they are not a result of a full cloning process. The ease and rapidity of seed reproduction coupled with their large availability support the maintenance of this practice among farmers; consequently, date palm plantations are a mixture of plants both clonally or seed propagated with a high genetic variability within cultivars. Clonal propagation, beyond to guarantee genetic uniformity of the cultivars, also limits the negative effect of inbreeding.

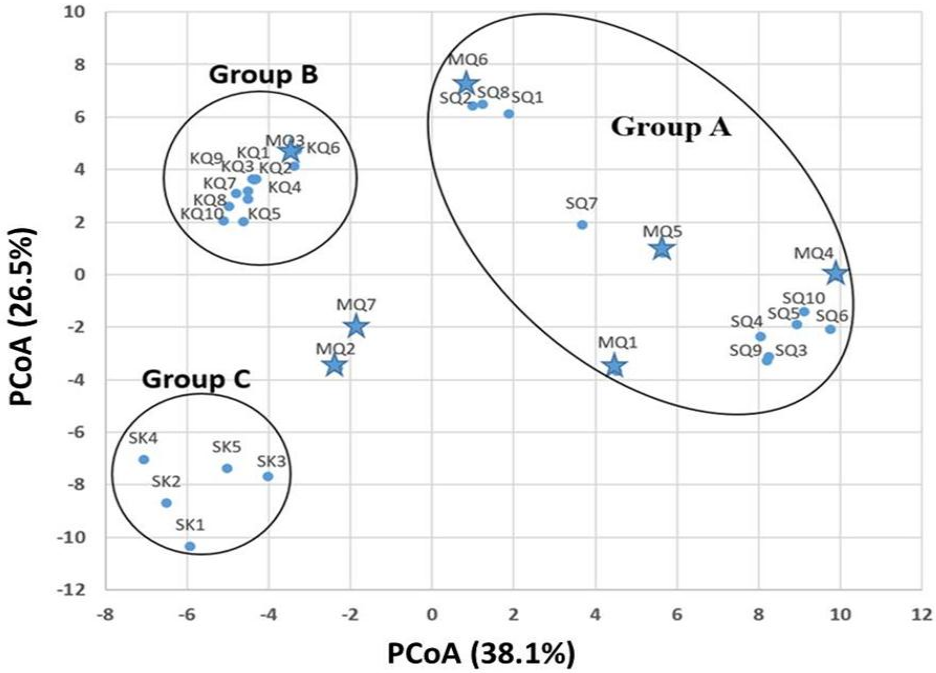

Fig. 3. Scatter plot of Principle Coordinate analysis (PCoA analysis) of 14 microsatellites loci in 32 date palm cultivars.

Analysis of molecular variance (AMOVA) showed 57% of variability among population, emphasizing the geographical effect on the genetic diversity, while 43% within population (Fig. 4). This percentage of variability within and among the studied populations explains the high level of genetic diversity within each population and the high genetic diversity between the genotypes and the geographic regions even if the varieties were given the same name.

Date palm domestication and the nature of date palm culture may have played a vital role in the composition of date palm genomes. Continuous selection was carried following asexual reproduction implemented by farmers may resulted in new cultivars. Emergence exchange of propagules, which are a mixture of vegetative and seed-propagated materials, has been conducted among farmers. All these processes together may result in a mixed genome within the same country (Elshibli and Korpelainen, 2008).

Name and traits to describe named varieties have the potential to differ not only over spatial scales but also over time.

A name may stay the same, but the traits used to describe the named variety may change. Likewise, the traits that farmers used to describe a variety may stay the same, but the name associated with these sets of traits may change over time as new farmer's adopt and grow the materials. It could also be that the most important traits for the farmer to distinguish a variety are not the genetically distinct ones that researcher used to distinguish varieties (Jarvis et al., 2007).

Conclusion

The results obtained from current study revealed that the date palm in Saudi Arabia and Qatar are very diverse. In addition, cluster analysis and PCoA clustering had divided the Shishi cultivar into two groups which are consistent with two regions. This study demonstrates that the genetic diversity and population structure of date palm in Saudi Arabia and Qatar had close relationships with the indigenous migration and cultural interflow.

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Conflict of interest: The authors declare that there is no conflict of interest.

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