Genetic Diversity of Kedah Kelantan Cattle Breed and Its Crossbred types in Malaysia Based on Microsatellite Markers

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Haytham Hago Abdelwahid  hhago2000@yahoo.com
University of Bahri
Corresponding Author
ORCiD: 0000-0002-5636-5207

Jothi M Panandam
Universiti Putra Malaysia

Reuben S K Sharma
Universiti Putra Malaysia

Halimatun Yaakub
Universiti Putra Malaysia

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Abstract

Background

The Kedah Kelantan (KK) is the indigenous cattle breed of Malaysia and is mainly kept by small farmers for meat production because of its small and compact body, and low maintenance requirement. This breed faces risk of germplasm dilution due to extensive crossbreeding and breeds replacement practices in the country. The population size of purebred KK is fast decreasing and most of the commercial populations are actually crossbreds. There is a lack of information on the genetic characteristics of KK. The genetic relationships between the KK, the synthetic breeds developed using the KK as the maternal line, as well as the non-descriptive KK crossbred types are also unknown. It is with these in mind that the present study was conducted. The objective of the study was to evaluate the genetic variability within and among the indigenous KK cattle and its crossbred types in Malaysia using 30 microsatellites loci.

Results

All the 30 microsatellites loci used were polymorphic in all populations. Heterozygosity values observed were moderate and lower than the expected values. The inbreeding was present in all populations and could lead to loss of genetic diversity if not addressed. In general, the genetic differentiation measures were moderate, with a mean FST of 0.054. The structure analysis grouped the populations into three clusters. Analysis of zebu and taurine diagnostic alleles showed that all population had high proportion of Indian zebu alleles and very low proportions of African taurine and European taurine diagnostic alleles.

Conclusions

It may be concluded that there is still some genetic variation present in the KK. However, this genetic diversity is at risk of being lost if no appropriate breeding practices are implemented.
Background

Indigenous breeds of livestock represent valuable resource to their owners and their countries [1]. However, these breeds are often not fully evaluated and are neglected in terms of genetic improvement. Many indigenous breeds, particularly those adapted to harsh environments of developing countries, have not yet been sufficiently characterized [2]. Moreover, many of these breeds, especially in the Asian countries, have been subjected to crossbreeding with improved breeds in order to improve their productivity [3]. The adoption of controlled crossbreeding strategy does not always produce the expected results [4], because of adaptation problems faced by the crossbred animals. Indiscriminate crossbreeding is the main threat for indigenous breeds especially under smallholder farming conditions [5], and this has led to severe reduction of indigenous breeds in many countries [6]. Industrial livestock farming is also a great threat to indigenous breeds; a few improved commercial breeds have already replaced or are fast replacing the latter. Indigenous breeds may perish before their potentials are fully recognized and exploited [2]. Therefore, there is an urgent need to conserve indigenous breeds [7]. Understanding the origin and subsequent history of indigenous breeds as well as their genetic diversity is essential to design strategies for their sustainable use and conservation [8].

In Malaysia the Kedah Kelantan (KK) is the only indigenous cattle breed and is mainly kept for meat production. It is popular among the small farm owners because of its small compact body size and low maintenance requirement [9]. However, the owners of bigger herds prefer the larger KK crossbreds or the imported exotic breeds. Genetic improvement of the KK has been by controlled crossbreeding strategies adopted by the government of Malaysia [10] through the Department of Veterinary Services Malaysia (DVS) and the Malaysian Agriculture Research and Development Institute (MARDI) to improve its
productivity and increase the local beef production. However, uncontrolled crossbreeding of the KK with improved beef breeds has also been popular, especially among farmers and smallholders [11], resulting in non-descript KK crossbred types. As a result of these activities, the population size of the purebred KK population is fast decreasing, and most of the current commercial beef cattle populations are actually crossbreds of KK. The KK breed faces risk of germplasm dilution due extensive crossbreeding and breed replacement practices. There is a lack of information on the genetic characteristics of the KK. The proportions of KK genes in the KK crossbred populations are also unknown. These make it necessary to evaluate the genetic makeup of the KK and KK crossbred breed types and evaluate the genetic variation among these populations. The objective of this study was to evaluate the genetic variability within and among the indigenous KK cattle and its crossbred breed types in Malaysia.

Methods

A total of 312 animals were used in this study. These animals could be classified into three groups: indigenous KK, KK crossbreds and exotic breed. The indigenous KK used in this study were from the nucleus herd at the DVS Livestock Centre in Tanah Merah, Kelantan. The crossbreds were of two types: the crossbreds developed through planned crossbreeding programmes, which included the Brakmas and Charoke, and the non-descriptive KK crosses. The Brakmas animals used in the present study were from the nucleus herd maintained at the MARDI Station in Muadzam Shah, Pahang, while the Charoke animals were from the MARDI Station in Kluang, Johor. The two non-descriptive crosses used are referred to as KK cross 1 (KXX1) and KK cross 2 (KXX2). The KXX1 herd initially began as a nucleus herd of pure KK. However, it was not maintained as such and the animals were mixed with other breeds of both Zebu and Taurine types. KXX2 was a herd belonging to a commercial meat production farm in Kluang, Johor (Kulim Livestock
This herd was the result of crossing KK with the Brahman breed. Since no breeding design was followed and mating was random these animals were considered as a non-descriptive KK cross. These latter two KK crossbred types were included in the present study as they represent many of the cattle herds in the country. The exotic breed used in the study was the Brahman breed. The animals were from the nucleus herd maintained at the DVS Livestock Centre in Kuala Berang, Terengganu. The Brahman has contributed to the beef industry development in Malaysia, and many owners of big farms have Brahman herds and smaller farm owners cross their cattle with the Brahman; therefore, this breed was included as an out-group, a non KK breed type. Figure 1 shows the locations of the sampled populations for each breed.

**Samples**

Blood samples from 56 random animals from each herd were used, except for the Brahman where samples from only 32 animals were used.

**Microsatellite markers**

Thirty microsatellite markers were investigated in the present study. These markers were those recommended by FAO/ISAG advisory group for genetic diversity studies in cattle [12] (Table1).

Table 1. Microsatellite loci investigated with chromosomal location, primer sequences, annealing temperatures and reported allele size ranges
| No. | Marker | Ch. | Primer | Sequence | Annealing Temperature (°C) |
|-----|--------|-----|--------|----------|---------------------------|
| 1   | BM181  | 8   | F: AGCTGGGAATATAACCAAAGG | R: AGTGCTTTCAAGGTCCATGC | 56-60 |
|     |        |     | F: GAGCAAGGTGTTTTCCCAATC | R: CATTCTCCACGTTTCCCTTG | 55-60 |
| 3   | BM211  | 3   | F: GCTGCCTTCTACAAATACCC | R: CTTCCGAGAGGAAGACCACCC | 55-60 |
|     | CSRM6  | 0   | F: AAGATGTTGATCAAAGAGGCA | R: AGGACCACTGCTAAGGGCATAAG | 55-56 |
|     | CSSM6  | 6   | F: ACACAAATCCTTCTGCCAGCTGA | R: AGGACCACTGCTAAGGGCATAAG | 55-65 |
|     | ETH3   | 19  | R: GAACTGCTTCTCCTCATTAGG | F: GATCACCTTGCCACTTTCCT | 55-65 |
| 7   | ETH10  | 5   | R: CCTCCAGCCCACCTTCTCTTCTC | F: TACTCGTACGGCCAGCTGCTG | 55-60 |
|     | ETH15  | 5   | R: GCACCTCCAAGAAGTCCCATCAG | F: TGCATGGACAGAGCAGCTGGC | 58-67 |
|     | ETH18  | 5   | R: GCACCCCAACGAAAGCTCCCAG | F: GACACCTCCAAGAAGTCCCATCAG | 55-56 |
|     | ETH22  | 9   | R: GATCACCTTGGCCACTATTTCTC | F: GCAGGATCACTTGTTAGGGA | 55-56 |
| 12  | HAUT2  | 4   | R: ACATGACAGGCAGCTGCTACT | F: CTCTGTGCCTCTGTCCCTGT | 52-55 |
|     | HAUT2  | 7   | R: AATACACTTTAGGAGAAAAATA | F: TTTTATGTCTATTTTTGACTGG | 57 |
|     | HEL1   | 15  | R: AAATCTGAAATCTCCATCTGA | F: CAACAGCTTATTTAAACAGA | 54-57 |
|     | HEL5   | 21  | R: AGGGCTACGATCCATGTTAGGA | F: GCACGTTAGTGTCACATATAC | 52-57 |
| 15  | HEL9   | 8   | R: CATCACTGCTTCCAGAGGT | F: CCCATTCACTCCATCACCAC | 52-57 |
|     | HEL13  | 11  | F: TAAAGACTTGGGATTAAGGAG | R: CCATCTACCTCCCATCTTAAC | 52-57 |
| 1   | ILSTS005 | 10 | F: GGAAGCAATGAAAATCTTATAGCC | R: CCATCTACCTCCCATCTTAAC | 54-58 |
R: TGTTCTGTGAGTTTGTAAGC

1  ILSTS006  7  F:TGTCTGTATTTCTGCTGTGG  55°C

R: ACACGGAAGCGATCTAAACG

1  INRA005  12  F: CAATCTGCATGAAGTATAAATAT  55

R: CTTCAGGCATACCCCTACACC

2  INRA023  3  F: GAGTAGAGCTACAAGATAAACTTC  55

R: TAACCTACAGGGTGGTAGATGAACTC

2  INRA032  11  F: AACTGTATTTCTCTAATAGCTAC  55-58

R: GCAAGACATATCTCCATTCCTTT

2  INRA035  16  F: ATCCTTGCAGCCTCCACCATTG  55-60

R: TTGTGCTTTATGACACTATCCG

2  INRA037  11  F: GATCCTGCTTATATTTAACCAC  57-58

R: AAAATTCCATGGAGAGAAAC
| No. | Accession | Length | Forward | Reverse |
|-----|-----------|--------|---------|---------|
| 2   | INRA063   | 18     | F: ATTTGCACAAGCTAAATCTAACC 55-5 | R: AAACCACAGAAATGCTTGGAAG |
| 2   | MM12      | 9      | F: CAAGACAGGTGTTTCAATCT       50-55 | R: ATCGACTCTGGGATGATGT |
| 2   | SPS115    | 15     | F: AAAGTGACACACAGCTTCCAG      55-60 | R: AACGAGTGCCTAGTTTGGCTGT |
| 2   | TGLA53    | 16     | F: GCTTTCAAGAAATAGTTTGCATTCA  55 | R: ATCTTCACATGATATTACAGCAG |
| 2   | TGLA122   | 21     | F: CCCTCCTCCAGGTAATACGC       55-58 | R: AATCACATGGCAAATAAGTACATA |
| 2   | TGLA126   | 20     | F: CTAATTAGAATGAGAGGCTTCT     55-58 | R: TGGTCTCTATTCTCTGAATATTCC |
| 3   | TGLA227   | 18     | F: CGAATTCCAAATCTGTAATTTGCT   55-56 |
PCR amplification of Microsatellites

DNA was extracted from the blood samples using the QIAamp DNA blood kit (Qiagen, Germany) according to the manufacturer’s instructions. PCR was carried out in a total volume of 15 μl containing 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs (Promega), 0.4 μM each of forward and reverse primers, 1U Taq DNA polymerase (Promega) and 50 ng/μl of genomic DNA. PCR was accomplished by using a touchdown programme. The PCR cycling conditions were as follows: initial denaturation for 8 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 45 s, annealing at temperatures ranging from 64 – 54 °C for 45 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR products were separated using an automated capillary sequencer (CEQ 8000; Beckman Coulter). The CEQ 6.0 software was used to automate allele sizing by comparisons with size standard 400 (Beckman Coulter, USA), which includes DNA fragments from 60 to 420 bp. Data analysis was performed using the AE2 subroutine (dye mobility correction). This analysis included analysis of raw data to estimate the fragment sizes, and filtration of low quality and unwanted samples. Binning was also performed to estimate the allele sizes.

Data analyses

The mean of observed and effective number of alleles (MNA and MNE) and Shannon information index per locus per population were estimated using the Popgene software [13]. The informativeness of the marker and polymorphism information content (PIC) were
calculated according to Botstein [14] using the Power Marker software [15]. The Popgene software was used to estimate the observed heterozygosity ($H_0$) and expected heterozygosity ($H_E$) and the deviation from Hardy-Weinberg equilibrium expectation for each locus in the six populations, as well as the inbreeding coefficient ($F_{IS}$) for the populations. The genetic variation between KK and its crossbred breed types were evaluated by estimation of F-statistics, genetic admixture and genetic distance, as well as by analysis phylogenetic analysis and principal component analysis (PCA).

F-statistics ($F_{IS}$, $F_{ST}$, and $F_{IT}$) for each locus and overall, including breed pairwise $F_{ST}$ and $F_{IT}$ values were calculated using the Popgene software. The genetic admixture in the KK and the non-descript KK breeds were estimated using two methods: Structure analysis and analysis for Zebu and Taurine diagnostic alleles. The Structure software version 2.3 [16] implements a model-based clustering for inferring population structure using genotype data. To choose the appropriate number of inferred clusters (K), two to seven inferred clusters were performed with three independent runs each. All analyses used a burn-in period of 10000 and 10000 iterations for data collection. The phenomenon that once the real K is reached the likelihood for larger K's ($\text{LnP(D)}$) plateaus and the variance among run increases were not observed. When K was increased, the $\text{LnP(D)}$ increased continuously. Therefore, the Evanno method was used. The values of $\text{LnP(D)}$ for each K were plotted and the delta K (DK) statistics were estimated, which is based on the rate of change in $\text{LnP(D)}$ between successive K values [17]. One to ten inferred clusters were performed with 20 independent runs each. Based on references [18-20] that suggest groups of alleles which may be used as diagnostic markers of Indian zebu, African taurine and European taurine breeds, the frequencies of these alleles at the loci were averaged to estimate of proportion of introgression from the individual cattle groups.
Pairwise genetic distances between the six breed-types were estimated using Nei’s standard genetic distances (DS) [21]. Phylogenetic trees were constructed based on four genetic distance measures: Cavalli-Sforza [22], Nei’s DA genetic distances [23], Goldstein [24] and Shriver [25] using neighbour joining (NJ) method [26]. The robustness of tree topologies was evaluated with a bootstrap test of 1000 resampling across loci; the PowerMarker program [15] was used for this purpose. The phylogenetic trees were edited using MEGA 5 program [27]. The principal components analysis (PCA) was performed using the allele frequencies according to Cavalli-Sforza [28] with the aid of the Unscrambler X 10.1 software.

Results

**Microsatellite Loci Analysis**

All 30 loci were amplified successfully and were polymorphic. As shown in Table 2 the total number of alleles (TNA) detected across the breed types was 360, and the mean number of alleles (MNA) per locus was 12. The most polymorphic loci were INRA035 and MM12 with 18 alleles each, and the least polymorphic were ETH10, INRA005 and TGLA126 with 8 alleles each. All microsatellite markers showed high polymorphism content in all the breed types. The observed allele size for all 30 loci were within the allele size ranges reported in the literature.

| No. | Locus  | Na | Ne | PIC | I   | Allele Size Ranges (bp) | Observed  | Reported in Literature |
|-----|--------|----|----|-----|-----|-------------------------|-----------|------------------------|
| 1   | BM1818 | 10 | 3.9| 0.72| 1.74| 252-270                 | 248-278   |                        |
| 2   | BM1824 | 11 | 3.5| 0.66| 1.49| 167-191                 | 176-197   |                        |
| 3   | BM2113 | 11 | 6.6| 0.83| 2.04| 120-140                 | 122-156   |                        |
| 4   | CSRM60 | 15 | 7  | 0.84| 2.22| 81-111                  | 79-115    |                        |
|     | Code     | Breed | Genotype | Virginity | Birth Weight | Death Weight | Mean  | Std Dev |
|-----|----------|-------|----------|-----------|--------------|--------------|--------|---------|
| 5   | CSSM66   |       | 12       | 4.9       | 0.78         | 1.97         | 175-197| 171-209 |
| 6   | ETH3     |       | 12       | 3.2       | 0.65         | 1.54         | 95-133 | 103-133 |
| 7   | ETH10    |       | 8        | 4         | 0.72         | 1.64         | 205-221| 207-231 |
| 8   | ETH152   |       | 11       | 4.9       | 0.77         | 1.84         | 185-205| 181-211 |
| 9   | ETH185   |       | 15       | 3.9       | 0.73         | 1.9          | 204-240| 214-246 |
| 10  | ETH225   |       | 14       | 4.2       | 0.74         | 1.83         | 133-159| 131-159 |
| 11  | HAUT24   |       | 12       | 5.5       | 0.8          | 1.95         | 102-126| 104-158 |
| 12  | HAUT27   |       | 13       | 4.4       | 0.74         | 1.8          | 124-152| 120-158 |
| 13  | HEL1     |       | 12       | 4.2       | 0.74         | 1.85         | 95-117 | 99-119  |
| 14  | HEL5     |       | 11       | 4.7       | 0.76         | 1.79         | 141-175| 145-171 |
| 15  | HEL9     |       | 12       | 6.3       | 0.82         | 2.06         | 141-165| 141-173 |
| 16  | HEL13    |       | 9        | 2.7       | 0.6          | 1.43         | 172-190| 178-200 |
| 17  | ILSTS005 |       | 11       | 5.2       | 0.79         | 1.92         | 174-196| 176-194 |
| 18  | ILSTS006 |       | 11       | 3.2       | 0.66         | 1.56         | 275-301| 277-309 |
| 19  | INRA005  |       | 8        | 5         | 0.77         | 1.69         | 131-145| 135-149 |
| 20  | INRA023  |       | 13       | 3         | 0.63         | 1.54         | 185-213| 195-225 |
| 21  | INRA032  |       | 12       | 6.3       | 0.82         | 2.02         | 154-202| 160-204 |
| 22  | INRA035  |       | 18       | 6.6       | 0.83         | 2.21         | 94-134 | 100-124 |
| 23  | INRA037  |       | 13       | 5.8       | 0.81         | 2.01         | 112-148| 112-148 |
| 24  | INRA063  |       | 8        | 5.4       | 0.79         | 1.78         | 171-187| 167-189 |
| 25  | MM12     |       | 18       | 6         | 0.82         | 2.15         | 95-133 | 101-145 |
| 26  | SPS115   |       | 9        | 5         | 0.77         | 1.8          | 240-256| 234-258 |
| 27  | TGLA53   |       | 13       | 2.4       | 0.56         | 1.46         | 149-181| 143-191 |
| 28  | TGLA122  |       | 17       | 7.9       | 0.86         | 2.29         | 132-164| 136-184 |
| 29  | TGLA126  |       | 8        | 4.1       | 0.72         | 1.64         | 113-127| 115-131 |
| 30  | TGLA227  |       | 13       | 2.6       | 0.58         | 1.44         | 72-98  | 75-105  |

|     | Mean     |       |         | Std Dev  |             |             |        |         |
|-----|----------|-------|---------|----------|--------------|--------------|--------|---------|
| 5   | 12       | 4.7   | 0.74    | 1.82     |              |              |        |         |

**Genetic diversity within breeds**
Table 3 shows the genetic variability at the 30 microsatellite loci typed in the KK cattle breed, its crossbred types and the Brahman. The results showed that the Brahman had the lowest TNA (232) and MNA (7.7), while KXX1 had the highest TNA (280) and MNA (9.3). The TNA and MNA of KK (245 and 8.2, respectively) were lower than the TNA and MNA of the crossbred types (TNA: 256 – 280, MNA: 8.5 – 9.3). The MNE values were less than the MNA values for all the breed types. As for heterozygosity, KK had lower mean observed (Ho) and expected (He) heterozygosity (0.54 and 0.70, respectively) compared to the KK crossbreds (Ho: 0.57 – 0.65, He: 0.73 – 0.78) and the Brahman (Ho: 0.58, He: 0.75). The mean values of Ho were lower than the mean values of He for all breed types. All breed types had positive inbreeding coefficient ($F_{IS}$) estimates, ranging from 0.149 in Charoke to 0.232 in KXX2. Of the 180 Hardy - Weinberg equilibrium (HWE) tests (30 loci in six breed types), 144 tests gave significant ($p < 0.05$) deviation from HWE. The deviations from HWE were for 27 loci in KXX2, 24 loci in KXX1, 23 loci in Brakmas, 22 loci in KK, 19 loci in Charoke and 18 loci in Brahman. No breed exhibited deviation from HWE for all the loci.

Table 3: Genetic variability at the microsatellites typed in the KK cattle breed and KK breed types

| Breed           | N  | TNA | MNA | MNE | Ho  | He  | FIS  | HWED |
|-----------------|----|-----|-----|-----|-----|-----|------|------|
| Kedah Kelantan | 56 | 245 | 8.2 | 3.6 | 0.54| 0.7 | 0.212| 22   |
| Brakmas         | 56 | 259 | 8.6 | 4.0 | 0.58| 0.73| 0.205| 23   |
| Charoke         | 56 | 271 | 9   | 4.7 | 0.65| 0.78| 0.149| 19   |
| KK crossbred 1  | 56 | 280 | 9.3 | 4.3 | 0.59| 0.74| 0.169| 24   |
| KK crossbred 2  | 56 | 256 | 8.5 | 4.1 | 0.57| 0.74| 0.232| 27   |
| Brahman         | 32 | 232 | 7.7 | 4.1 | 0.58| 0.75| 0.215| 18   |

$N = \text{number of individuals; } TNA = \text{total number of allele; } MNA = \text{mean number of allele; } MNE = \text{mean number of effective alleles; } Ho = \text{observed heterozygosity; } He = \text{expected heterozygosity; } HWED = \text{number of Hardy-Weinberg equilibrium deviated loci.}$

**Genetic Variation and Relationship between KK and KK Crossbred Types**

Table 4 shows the values of the gene differentiation ($F_{ST}$) and gene flow ($N_M$) among the
KK breed types and the Brahman. High $F_{ST}$ value was found between KXX2 and Brahman (0.049), while the lowest value was found between KK and KXX1 (0.015). The latter $F_{ST}$ and that between KK and KXX2 (0.018) was less than the $F_{ST}$ between KK and Brakmas (0.03) and between KK and CK (0.041). The $F_{ST}$ between Brahman and Brakmas (0.031) was less than the $F_{ST}$ between KK and Brahman (0.045). As for the gene flow ($N_M$), high value was observed between KK and KXX1 (16.84), while the lowest value was found between KXX2 and Brahman (4.88). The gene flow between KK and KXX1 (16.84) and KK and KXX2 (13.39) was higher than the gene flow between KK and Brakmas (8.067) and KK and Charoke (5.862). The gene flow between Brahman and Brakmas (7.873) was higher than the gene flow between KK and Brahman (5.321).

Table 4. $F_{ST}$ and gene flow among the Kedah Kelantan and KK breed types.

| Breed 1 | Breed 2 | $F_{ST}$ | $N_M$ |
|---------|---------|----------|-------|
| KK      | Brakmas | 0.030    | 8.067 |
| KK      | Charoke | 0.041    | 5.862 |
| Brakmas | Charoke | 0.047    | 5.039 |
| KK      | KXX1    | 0.015    | 16.843|
| Brakmas | KXX1    | 0.029    | 8.466 |
| Charoke | KXX1    | 0.027    | 9.037 |
| KK      | KXX2    | 0.018    | 13.385|
| Brakmas | KXX2    | 0.025    | 9.713 |
| Charoke | KXX2    | 0.038    | 6.348 |
| KXX1    | KXX2    | 0.018    | 13.646|
| KK      | Brahman | 0.045    | 5.321 |
| Brakmas | Brahman | 0.031    | 7.873 |
| Charoke | Brahman | 0.044    | 5.415 |
| KXX1    | Brahman | 0.035    | 6.804 |
| KXX2    | Brahman | 0.049    | 4.880 |

$KK = $ Kedah Kelantan, $KXX1 = $ Kedah Kelantan crossbred 1, $KXX2 = $ Kedah Kelantan crossbred 2, $F_{ST} =$ the degree of gene differentiation among populations in terms of allele frequencies, $N_M =$ gene flow.
Table 5 shows F-statistics for each locus based on pool data from all six breed types. FIS was positive (0.198), and FIT (0.242) exceeded FST (0.054) indicating inbreeding in all breeds. The level of genetic differentiation among KK and the KK crosses measured in terms of FST was moderate; 5.4 % of the total genetic variation corresponded to between breed differences, and 94.6% corresponded to within-breed differences.

Table 5. F-statistics and gene flow at the 30 microsatellite loci across the Kedah Kelantan and KK breed types.
| Locus        | $F_{IS}$ | $F_{IT}$ | $F_{ST}$ | $N_M$ |
|--------------|----------|----------|----------|-------|
| BM1818       | 0.272    | 0.295    | 0.031    | 7.87  |
| BM1824       | 0.106    | 0.164    | 0.065    | 3.59  |
| BM2113       | 0.084    | 0.119    | 0.037    | 6.43  |
| CSRM60       | 0.203    | 0.250    | 0.058    | 4.03  |
| CSSM66       | 0.011    | 0.057    | 0.046    | 5.15  |
| ETH3         | 0.289    | 0.311    | 0.030    | 8.08  |
| ETH10        | 0.191    | 0.252    | 0.075    | 3.07  |
| ETH152       | 0.079    | 0.138    | 0.064    | 3.66  |
| ETH185       | 0.217    | 0.243    | 0.034    | 7.10  |
| ETH225       | 0.208    | 0.300    | 0.116    | 1.90  |
| HAUT24       | 0.378    | 0.407    | 0.047    | 5.12  |
| HAUT27       | 0.543    | 0.558    | 0.034    | 7.06  |
| HEL1         | 0.154    | 0.221    | 0.079    | 2.92  |
| HEL5         | 0.647    | 0.678    | 0.088    | 2.60  |
| HEL9         | 0.106    | 0.135    | 0.033    | 7.37  |
| HEL13        | 0.105    | 0.143    | 0.042    | 5.67  |
| ILSTS005     | 0.182    | 0.225    | 0.052    | 4.53  |
| ILSTS006     | 0.412    | 0.458    | 0.077    | 2.99  |
| INRA005      | 0.129    | 0.165    | 0.041    | 5.88  |
| INRA023      | 0.069    | 0.116    | 0.050    | 4.75  |
| INRA032      | 0.047    | 0.089    | 0.045    | 5.33  |
| INRA035      | 0.185    | 0.225    | 0.049    | 4.86  |
| INRA037      | 0.158    | 0.196    | 0.045    | 5.29  |
| INRA063      | 0.269    | 0.328    | 0.082    | 2.81  |
| MM12         | 0.150    | 0.179    | 0.034    | 7.07  |
| SPS115       | 0.074    | 0.133    | 0.064    | 3.65  |
| TGLA53       | 0.314    | 0.373    | 0.086    | 2.66  |
| TGLA122      | 0.117    | 0.154    | 0.042    | 5.76  |
| TGLA126      | 0.215    | 0.254    | 0.050    | 4.75  |
| TGLA227      | 0.105    | 0.133    | 0.031    | 7.82  |
| **Mean**     | **0.198**| **0.242**| **0.054**| **4.38**|

$F_{IS} = \text{the deficiency or excess of average heterozygotes}$, $F_{IT} = \text{the deficiency or excess of}$
average heterozygotes in a group of populations, $F_{ST} = \text{the degree of gene differentiation among populations in terms of allele frequencies.}$

The genetic structure of the study populations is shown by the clustering assignment of the 312 animals representing the six breed types (Figure 3). The value of $K = 3$ was chosen as this showed the highest delta $K$ (Figure 3) as suggested by Evanno [17]. Table 6 shows the average proportions of memberships ($q$) to the three clusters. KK, Charoke and Brahman were grouped in cluster 1, 2 and 3, respectively, with $q \geq 0.80$; while KXX2 and Brakmas were grouped in cluster 1 and 3, respectively, with $q \leq 0.80$. The KXX1 animals were split among the three clusters. The genetic compositions of the 56 KK individuals ($q$-values) are shown in Figure 4.

Table 6: Membership of each breed type in each of the three clusters inferred

| Given population | Proportion in inferred Clusters | Number of individuals |
|------------------|---------------------------------|-----------------------|
|                  | 1 | 2 | 3 |                     |                       |
| KK               | 0.85 | 0.05 | 0.11 | 56                   |
| Brakmas          | 0.17 | 0.05 | 0.78 | 56                   |
| Charoke          | 0.08 | 0.85 | 0.07 | 56                   |
| KXX1             | 0.49 | 0.35 | 0.16 | 56                   |
| KXX2             | 0.73 | 0.09 | 0.18 | 56                   |
| Brahman          | 0.05 | 0.13 | 0.82 | 32                   |

Table 7 shows the zebu and taurine diagnostic alleles and the frequencies of these alleles in the KK and its crossbred types. As shown in Figure 5, all breeds had higher proportions of the zebu alleles compared to the taurine alleles. The mean percentage of zebu
diagnostic alleles in the breed types ranged from 18.4% (Brakmas) to 25.8% (KXX2), while the mean percentage of African taurine diagnostic alleles ranged from 2.5% (KK) to 7.4% (Charoke). The mean percentage of European taurine diagnostic alleles ranged from 1.6% (KK) to 4.7% (Charoke).

Table 7. Zebu and Taurine diagnostic alleles and their frequencies in KK and its crossbred breed types.

| Group            | Locus | Allele | Allele Frequency |
|------------------|-------|--------|------------------|
|                  |       |        | KK   | BK   | CK   | KXX1 | KXX2 | BR   |
| Indian zebu      | BM2113| 130    | 0.036 | 0.039 | 0.010 | 0.010 | 0.020 | 0.033 |
|                  |       | 140    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
|                  | CSSM66| 181    | 0.027 | 0.088 | 0.157 | 0.052 | 0.163 | 0.050 |
|                  | ETH10 | 207    | 0.100 | 0.060 | 0.091 | 0.155 | 0.239 | 0.121 |
|                  |       | 209    | 0.582 | 0.420 | 0.318 | 0.409 | 0.424 | 0.293 |
|                  |       | 211    | 0.046 | 0.000 | 0.000 | 0.046 | 0.076 | 0.017 |
|                  | ETH152| 191    | 0.191 | 0.052 | 0.315 | 0.255 | 0.092 | 0.111 |
|                  | ETH225| 153    | 0.536 | 0.524 | 0.000 | 0.524 | 0.509 | 0.481 |
|                  |       | 155    | 0.036 | 0.000 | 0.363 | 0.024 | 0.155 | 0.000 |
|                  | HEL1  | 101    | 0.009 | 0.063 | 0.125 | 0.057 | 0.066 | 0.071 |
|                  |       | 107    | 0.028 | 0.115 | 0.298 | 0.047 | 0.132 | 0.119 |
|                  |       | 117    | 0.000 | 0.000 | 0.039 | 0.000 | 0.000 | 0.024 |
|                  | HEL13 | 182    | 0.000 | 0.090 | 0.046 | 0.031 | 0.123 | 0.096 |
|                  |       | 186    | 0.028 | 0.000 | 0.011 | 0.021 | 0.038 | 0.058 |
|                  | TGLA122| 144   | 0.000 | 0.020 | 0.075 | 0.051 | 0.028 | 0.091 |
| Mean             |       | 0.202  | 0.184 | 0.231 | 0.210 | 0.258 | 0.196 |
| African taurine  | BM1824| 181    | 0.000 | 0.050 | 0.010 | 0.048 | 0.052 | 0.000 |
|                  | BM2113| 122    | 0.127 | 0.058 | 0.167 | 0.133 | 0.143 | 0.017 |
|                  | ETH10 | 219    | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
|                  | ETH152| 195    | 0.000 | 0.010 | 0.019 | 0.018 | 0.000 | 0.056 |
|                  | HEL1  | 109    | 0.037 | 0.031 | 0.000 | 0.000 | 0.019 | 0.048 |
|                  | HEL13 | 190    | 0.009 | 0.020 | 0.307 | 0.146 | 0.009 | 0.115 |
|                  | INRA23| 199    | 0.000 | 0.096 | 0.018 | 0.009 | 0.018 | 0.000 |
| Mean             |       | 0.025  | 0.038 | 0.074 | 0.051 | 0.035 | 0.034 |
Table 8 shows the genetic distance among the breed types. The KK breed was found to be very similar to the KXX1 (GD = 0.074) and KXX2 (GD = 0.092), and was most distant from the Brakmas (GD = 0.159) and Charoke (GD = 0.254). The Brakmas and Charoke breeds had the highest genetic distance (GD = 0.335).

Table 8. Genetic distance and genetic identity among KK and KK breed types

| Population | KK    | BK    | CK    | KXX1  | KXX2  | BR    |
|------------|-------|-------|-------|-------|-------|-------|
| KK         | ****  | 0.853 | 0.776 | 0.929 | 0.912 | 0.769 |
| BK         | 0.159 | ****  | 0.715 | 0.843 | 0.866 | 0.831 |
| CK         | 0.254 | 0.335 | ****  | 0.833 | 0.768 | 0.717 |
| KXX1       | 0.074 | 0.171 | 0.183 | ****  | 0.902 | 0.795 |
| KXX2       | 0.092 | 0.143 | 0.264 | 0.103 | ****  | 0.723 |
| BR         | 0.263 | 0.185 | 0.333 | 0.230 | 0.324 | ****  |

Nei's genetic identity (above diagonal), genetic distance (below diagonal). KK = Kedah Kelantan, BK = Brakmas, CK = Charoke, KXX1 = Kedah Kelantan crossbred 1, KXX2 = Kedah Kelantan crossbred 2, BR = Brahman.

Figure 6 shows Phylogenetic trees of the study populations. All the trees were in agreement and revealed three clusters. The first cluster consisted of KK and KXX2, and the second cluster consisted of Brahman and Brakmas, while the third cluster contained
Charoke and KXX1. Bootstrap values for the trees ranged from 56 to 89 indicating reliable topology of the phylogeny constructed from these distances.

Figure 7 shows an individual-animal-based NJ tree for the 312 individuals. This was built from the Nei’s DA genetic distance. The tree displayed three clusters; KK and KXX2 were clustered into one group, and Charoke and KXX1 into another cluster, while Brahman and Brakmas were in a third cluster.

The results of the PCA on the allele frequencies of the 30 microsatellite loci in the six breed types are shown in Figure 8. The first axis, which accounted for 36% of the variation, separated KK, KXX2, KXX1 and Charoke from Brahman and Brakmas. The second axis accounted for 32% of the variation, and separated the KXX1 and Charoke from KK and KXX2, each in a different cluster.

Discussion

**Genetic Diversity at the Microsatellite Loci**

A TNA of 360 was observed for the six breed types in the present study. This was higher than that reported for five Cuban cattle breeds (n = 317; TNA = 299) screened for the same 30 microsatellite loci [29]. This may be due to the fact that the Cuban cattle breeds were purebreds, while the present study included crossbred animals with a number of contributing breeds. The TNA was, however, lower than that reported for 27 indigenous Chinese cattle breeds (n = 1638; TNA = 480) for the same loci [30]. This most likely is due to the big sample size and larger number of breeds from different parts of China used in the Chinese study. The range of the PIC values, however, was similar to that reported for seven of the native Chinese cattle breeds (PIC = 0.74 – 0.75) [30] and for the five Cuban breeds [29]. In general, the high number of alleles and PIC values for the individual loci showed that the 30 microsatellite loci recommended by ISAG/FAO for genetic diversity evaluation of cattle are highly informative and suitable for the purpose.
Genetic Diversity within KK Cattle and KK Crossbred Types

The genetic diversity within the breed types was evaluated using the allelic variation. The MNA is consider as a good indicator of genetic variation. The Brahman breed exhibited the lowest MNA (7.7) among the six breed types. This may be due to the smaller sample size used (n = 32), but most likely due to founder effects and population bottlenecks as a result of breeding practices. The Brahman were introduced into Malaysia to be used for crossbreeding as well as to increase the local cattle population. The KK breed had lower MNA than the KK crossbred types. This may be attributed to crossbreeding incorporating the alleles of the parental breeds into the crossbred types [31]; the KK crossbred types would have the KK alleles as well as those of the other parental breeds, increasing the number of allele.

The MNA of KK and KK breed types were lower than that reported for four Chinese native cattle breeds (10.1-10.5) [30], but was higher than those reported for six Spanish native cattle breeds (4.9 - 6.7) [32]. This may be attribute to the differences in population sizes and the sampling technique used. The sample size for the Spanish breeds were between 29 to 50 individuals. Moreover, the breeds occurred as isolated populations and four of them were considered as endanger. Isolated small populations lose genetic variability over time but they become distinctively different.

The average heterozygosity is the best general measure of genetic variation [33]. The KK breed had the lowest Ho (0.54) and He (0.70) among the breed types studied, while the Cheroke had the highest values (0.65 and 0.78, respectively). Low heterozygosity could be attributed to isolation and inbreeding, which if not addressed could eventually result in loss of unexploited genetic potential [31]. High heterozygosity in Cheroke could be attributed to the mixed nature of this breed type. The rest of the breed types generally showed similar Ho values, ranging from 0.57 (KKX2) to 0.59 (KKX1). The mean values of
Ho were lower than the mean values of He for all the breed types, indicating heterozygous deficiency.

The Ho estimation for KK and Brahman (0.58) were lower than that reported for 27 Chinese native breeds (0.61-0.76) [30], six Indian breeds (0.60-0.72) [34] and ten Ethiopian breeds (0.64-0.70) [35]. This may be due to the fact that China, India and Ethiopia are considered as cattle domestication centres, where it was believed there was contact between immigrant Asiatic indicine and taurine cattle [36]. Therefore, the high heterozygosity values observed in the cattle breeds in these countries most probably are the consequence of the initial admixture of *B. indicus* and *B. taurus* cattle that formed the foundation stocks in the past.

F$_{IS}$ value indicates excess or deficit of homozygotes. In the present study, the mean values of F$_{IS}$ were positive for all six breed types, which indicates excess of homozygotes due to inbreeding in all the breed types. This may be due to non-random mating which may be expected in livestock herds. Selection and controlled mating were practised to a certain extent in the farms concerned, especially the nucleus farms. However, proper record keeping was generally lacking; records were often available for growth traits but pedigree records were often not available. The observed F$_{IS}$ values were higher than that reported for Chinese and Ugandan native cattle breeds [30, 37] screened using the same 30 microsatellite loci.

A population is said to be in Hardy-Weinberg equilibrium (HWE) when the gene and genotype frequencies remain constant from generation to generation, and the latter is of a definite proportion [38]. In the present study the deviations from HWE was observed in all six breed types. Deviation from HWE could be attributed to many causes, among which are selection, assortative mating, migration and small population size, all of which could
have influenced these populations. The overall numbers of loci that deviated from HWE were high compared to that reported for 10 Ethiopian and 10 Portuguese native cattle for the 30 microsatellite loci [35, 39].

In general, the low genetic diversity observed in the present study in terms of low mean number of the alleles, heterozygote deficiency and deviation from HWE could be attributed to many reasons, but the most probable reasons are inbreeding, small population sizes and assortative mating. The KK, Brakmas, Cherokee and Brahman animals used in the present study were from single nucleus herds. According to Phillips [40] there are many factors associated with establishing and managing nucleus cattle herd which lead to inbreeding. These include the nucleus herd size, whether the nucleus is open or close, the desired age structure of the nucleus, selection criteria and selection accuracy for the bulls and replacement cows, and completeness of the performance and pedigree records. When these factors were investigated, it was noticed that the records in these nucleus farms were limited and often incomplete. Vital pedigree information was often missing. Consequently, this would have affected the selection accuracy for the bulls and replacement cows. The record keeping at these farms has to be improved and the system reviewed regularly if genetic variability is to be maintained and herd performance is to be improved.

**Genetic Variation and Relationship between the KK and KK Crossbred Breed Types**

The genetic variation between KK and KK crossbred types were evaluated by estimation of F-statistics, gene flow, genetic admixture and genetic distance, as well as by phylogenetic analysis and principal component analysis (PCA). F-statistics ($F_{IS}$, $F_{ST}$, and $F_{IT}$) are measures of the deficit of heterozygotes relative to expected HWE proportions in the specified population [33] For large, random mating populations, it is expected that the
observed heterozygosity would be equal to the expected heterozygosity, and \( F_{IS} \) would be equal or close to zero. In this case, \( F_{IT} \) would be approximately equal to \( F_{ST} \). However, when \( F_{IS} \) is negative which implies no inbreeding, \( F_{ST} \) would generally exceed \( F_{IT} \). On the other hand, when \( F_{IS} \) is positive, implying inbreeding in the population, \( F_{IT} \) would exceed \( F_{ST} \).

In the present study \( F_{IS} \) was positive (0.198), and \( F_{IT} \) (0.242) exceeded \( F_{ST} \) (0.054) indicating inbreeding in all breed types. The level of genetic differentiation among KK and its crossbred types measured in terms of \( F_{ST} \) (5.4%) was moderate. This means that 5.4% of the total genetic variation corresponded to between breed type differences, and 94.6% of the total genetic variation corresponded to within-breed type differences. This could be attributed to the fact that the most of the studied breed types were developed or originated from crosses with KK as the maternal line, and the Brahman breed too was involved in many of the crosses; thus the breeds types sharing some common alleles. The value of \( F_{ST} \) observed in the present study was lower than the \( F_{ST} \) values for the three Indian cattle breeds, Sahiwal, Hariana and Deoni (\( F_{ST} = 11.3\% \)), reported by [41] and 27 Chinese indigenous cattle breeds (\( F_{ST} = 8\% \)) [30]. This was probably due to the fact that the breeds used in these earlier studies originated from different parts of the respective countries. For example, in the Indian study the Sahiwal breed was native to Pakistan and found along the India-Pakistan border in the North, while the Hariana and Deoni were found in northern and western India respectively. In the study by Zhang [30] the 27 breeds were representatives from all parts of the vast land area of China, from north, south, east and west. The \( F_{ST} \) value in the present study was, however, higher than that reported by [35] for 10 Ethiopian cattle breeds (\( F_{ST} = 1.3\% \)). The most probable cause of
this low level of genetic differentiation in these Ethiopian breeds is the fact that Ethiopian cattle breeds have common historical origins, and shared common grazing lands and watering points. Moreover, an uncontrolled mating practice, which is predominant in Ethiopia, increases the gene flow among the breeds.

It is very clear that the $F_{ST}$ between KK and KKX1 ($F_{ST} = 1.5\%$) and between KK and KKX2 ($F_{ST} = 1.8\%$), were lower than the $F_{ST}$ between KK and Brakmas ($F_{ST} = 3\%$) and between KK and Charoke ($F_{ST} = 4\%$). KKX1 and KKX2 represent unplanned breed types and the KK was probably the most common breed used in the mating, thus being a major gene contributor. The Brakmas and Charoke are synthetic breeds developed using planned breeding design, and, therefore they are more different from the KK than the other KK crosses. The genetic makeup of a synthetic breed is not easy to manage and monitor; it is influenced by inbreeding and selection for fitness and desired traits (fertility, fleshing ability, mature weight and coat colour, etc.) which may be bias to one of the parental breeds. In this study, it is clear that all breed types were bias towards the KK breed genes.

The degree between breed differentiations indicated a relatively moderate to high gene flow between the six cattle breeds ($Nm = 4.38$). The highest gene flow (16.84 \%) was between the KK and KKKX1 population. The gene flow between the KK and KKKX1 populations (16.84\%), between KKKX1 and KKKX2 populations (13.65 \%), and between KK and KKKX2 populations (13.39\%) reflect the genetic similarity between these three breeds, supporting the findings based on the F statistics. High gene flow was also observed between the Brakmas and KKKX2 (9.71\%) and the Charoke and KKKX1 (9.04\%) also indicating their genetic closeness between these two pairs. The earlier association may be due to the fact that both Brakmas and KKKX2 are crossbreds of KK and Brahman. As for the second pair,
both Charoke and KXX1 shared the same ancestral KK population which was then kept as a nucleus herd at MARDI Station in Kluang, Johor. However, due to funding shortage this KK herd was not maintained and the animals were crossed with the different available breeds. Moreover, since both these breed types were from the same farm, there is a possibility that at times there was interbreeding between the two herds. Although the Brakmas and Charoke are crossbreds of KK they showed low inter breed gene flow (5.04%) compared to the others pairs. This could be attributed to the physical separation of the two breeds, and the breeding and selection programs practised in the respective farms; the latter may have been bias towards the genes of the exotic breeds.

The genetic admixture in the KK cattle and the KK breed types was estimated using structure analysis and frequency analysis of the zebu and taurine diagnostic alleles. The results of the structure analysis showed that the studied populations were split into three clusters: KK and KXX2 in the first cluster, Brakmas and Brahman in the second cluster and Charoke in the third cluster. KXX1 was distributed in all three inferred clusters. KK, Charoke and Brahman had more than 80 % membership coefficients in their respective inferred clusters. The genetically defined clusters agreed with the breeds’ histories.

Although the KK and Brahman breeds are assumed to be pure breeds, the results showed that both these breeds had admixed (hybrid) individuals. There were genetic contributions from the Brahman (11%) and Charoke (5%) breeds to the KK cattle. The Charoke contribution may be due to a possible use of Charolais or Charoke semen for artificial insemination in this herd. It could also be attributed to the introduction of KK crosses from other farms (government and non government) which may have had Charoke as one of its ancestors into the KK herd. The results of the present study are in agreement with Payne [9] who stated that the majority of indigenous cattle breeds of Southeast and East Asia are subjected to crossbreeding, and so have genes from the *Bos taurus* and *Bos indicus*
species.

The existence of admixed individuals in the Brahman breed may be attributed to the fact that the Brahman cattle in the present study were imported from Australia (Australian Brahman), which in turn originates from founder population imported from United States of America (USA). According to the American Brahman Breeders Association (ABBA) the Brahman breed in the USA was developed in the early 1900s from progeny of four Indian cattle breeds with some infusion of British-bred cattle [42].

The structure analysis also showed that there were contributions from KK to Brakmas (17%) and the Charoke (8%), though these were less compared to the contributions of KK to the composite crosses, 49% to KXX1 and 73% to KXX2. These results are concordance with the F-statistics and gene flow results. Once again this could be attributed to the effects of the breeding designs and selection programs for both Brakmas and Charoke that ensured that high proportion of the genes from the exotic breeds maintained in the synthetic breeds. The KXX1 was identified as having a complicated genetic background.

The animals of this breed type displayed membership in all the three clusters. This finding was consistent with the KXX1's history, which revealed that its ancestors were crossed with different breeds, which included both zebu and Taurine breeds. This explained the high TNA, MNA and Ho. These genetic characteristics of the KXX1 may also be the result of a lack of breeding goals and controlled mating for this herd. Although the KXX2 is outcome of unplanned crossing of KK and Brahman animals, the results revealed higher genetic similarity between the KK and the KXX2 than the Brahman. This result corroborated the gene flow results between KXX2 and KK (13.4%) and between KXX2 and Brahman (4.9%).

This is as expected in outcome of most crossbreeding activities at non-research farms. The mating beyond the initial crosses producing the F1 are not controlled to ensure the desirable proportion of the parental breeds. Often the crosses are backcrossed with the
indigenous breed as these animals are more readily available and in larger numbers, thereby, the eventual population losing a large proportion of the exotic genes incorporated into the crosses.

Concerning Zebu and Taurine diagnostic alleles, the results show that Charoke had the highest proportion of African and European Taurine diagnostic alleles among the six breed types (7.4% and 4.7%, respectively). This was as expected as Charoke was a Charolais (B. taurus) cross, whereas the other crosses were B. indicus types. The introgression of Indian Zebu genes into the KK and the KK breed types (18.4 – 25.8%) was higher than African zebu genes (2.5 - 7.4%) and the European Taurine genes (1.6 – 5.2 %). The high frequency of the Indian Zebu diagnostic alleles is supported by the history of introduction of Zebu animals into Southeast Asia, where it was believed that the Indian Zebu cattle was spread from India through the human migrations and ancient sea trading routes [9]. Similar levels of introgression of Indian zebu genes (17 – 26.3%) in seven indigenous cattle breeds in central and southern China have been reported by Zhang [30]. Higher level of introgression of Indian zebu genes into indigenous cattle breeds from North Ethiopia (55.16 - 63.78%) was reported by Zerabruk [43] and among west-central African cattle breeds (58.1–74.0%) by Ibeagha-Awemu [44]. This may be due to the fact that Ethiopia has been a gateway for cattle immigrations into Africa. It was believed that a major wave of B. indicus introgression may have started with the Arab settlements along the east coast of Africa [36]. In general, the analysis of the diagnostic alleles produced results suggesting that the KK and the Brahman breeds in the present study was not genetically pure Zebu; they exhibited a proportion of Taurine backgrounds. This result was in agreement with that of the structure analysis. As stated earlier the taurine alleles are possibly the result of historical crossbreeding activities in the country using taurine breeds to improve production of the local cattle.
All phylogenetic trees reconstructed from the NJ method, based on the four genetic distance methods yielded trees, which were consistent with the historical information. Generally, the accuracy of the phylogenetic tree is confirmed by bootstrap values; nodes with high bootstrap values (above 0.70) are considered significant, whereas nodes with low values (below 0.50) were considered not significant. The tree topologies generated in the present study were confirmed by relatively high bootstrap values, ranging from 56 to 89. Concerning the four genetic measures, all trees showed similar results. However, the trees obtained using Cavalli-Sforza [22] and Nei’s DA genetic distances had high bootstrap values (80 – 89) compared to the Goldstein’s and Shriver’s trees (56 – 73).

The PCA revealed the relationship between KK and the other breed types. The central position of Brakmas between KK and Brahman, and of KKX1 between KK and Chaoke as revealed by PCA have been clarified by their admixed nature. The distant positioning of Brakmas and Charoke is evidence of high genetic divergence between these two breed types. The results indicate high frequency of KK genes in KKX1 and KKX2 higher than those of the exotic breeds that have been used in the initial crossing. In contrary, the Brakmas and Charoke have high frequencies of the genes of the exotic breeds. This may be attributed to these breed types being developed through planned crossing and selection for high performance traits of the Brahman and Charolais, respectively. These results are in agreement with those of the structure analysis and the phylogenetic analysis.

Conclusions

In conclusion, the following salient findings are highlighted. The results showed that the microsatellite loci recommended by ISAG/FAO and used in the present study are highly informative and suitable for genetic diversity evaluation in the cattle breeds. There are some genetic variations present in the KK and the non-descript KK crossbreds. However,
this genetic diversity risks being lost if no appropriate breeding practices are implemented. The KK is a zebu breed with a very low percentage of taurine alleles. The KK herd used in the present study is to be maintained as a nucleus herd and the purity of the KK has to be conserved. The animals identified as free from admixture should be strategically used for breeding. The non-descript KK crossbreds are very similar to the KK although the two populations exhibited different percentages of genes of exotic breeds. The Charoke and Brakmas are genetically distinct breeds. Proper mating designs should be adopted to maintain these, and the genetic structure of the herds should be monitored so that the time, effort and money invested in their development are not in vain.

Abbreviations

- **bp**: base pair
- **Ch**: chromosome
- **DNA**: deoxyribonucleic acid
- **dNTP**: deoxyribonucleotide triphosphates
- **DVS**: Department of Veterinary Services Malaysia
- **EDTA**: ethylenediaminetetraacetic acid
- **FAO**: Food and Agriculture Organization of the United Nations
- **FIS**: inbreeding coefficient
- **FIT**: deficiency or excess of average heterozygotes in a group of populations
- **FST**: degree of gene differentiation among populations
- **HE**: expected heterozygosity
- **HO**: observed heterozygosity
- **HWE**: Hardy-Weinberg equilibrium
- **ISAG**: International Society for Animal Genetics
- **KK**: Kedah Kelantan cattle
- **KKX1**: Kedah Kelantan cross 1
- **KKX2**: Kedah Kelantan cross 2
- **MARDI**: Malaysian Agriculture Research and Development Institute
- **MgCl2**: magnesium chloride
- **ml**: milliliter
- **mM**: millimole
- **MNA**: mean number of allele
- **MNE**: mean number of effective allele
- **μl**: microliter
- **μM**: micromole
- **Na**: observed number of alleles
- **Ne**: effective number of alleles
- **ng**: nanogram
- **NJ**: Neighbor-joining
- **0°C**: degree centigrade / celsius
- **PCR**: polymerase chain reaction
- **PIC**: polymorphic information content
- **STR**: short tandem repeats
- **TBE**: Tris/Borate/EDTA
- **Tm**: melting temperature
- **UPGMA**: Unweighted Pair Group Method with Arithmetic Mean
- **UV**: ultraviolet
Declarations

**Ethics approval and consent to participate**

Blood samples were collected by veterinarians from the Department of Veterinary Services Malaysia (DVS) as part of their routine screening of animal herds in the country. Random samples were obtained by the researchers from DVS for the present study. We obtained written informed consent to use these animals in this study from the owner(s) of the animals.

**Consent for publication**

Not applicable

**Availability of data and material**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

H A, J P, R S designed the research. H A conducted the research. H A and J P analysed the data. H A, H Y and J P prepared the manuscript. H A is responsible for the final content. All authors read and approved the final manuscript.

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Figures

![Fig. 1](image.png)

**Figure 1**

Location of the breeds used in this study KXX1: Kedah Kelantan cross 1, KXX2: Kedah Kelantan cross 2.
Clustering assignments of 312 animals representing the six cattle breed types. K = number of clusters. When K (the inferred number of cluster) was two, KK and KXX2 were grouped into one cluster, and Charoke and Brahman were separated in the other cluster, while Brakmas and KXX1 showed admixture from both clusters.

For K=3, KK and KXX2 were grouped again into one cluster, Brakmas and
Brahman were grouped into another cluster, while Charoke was clearly separated into a different cluster; KKKI showed admixture from the three clusters. With $K=6$, the six populations were inferred to six cluster.

Figure 3

Evanno method to detect the real $K$ (A) $L(K)$; Mean likelihood for each $K$ value over 20 runs. (B) Rate of change of the likelihood distribution (mean) calculated as $L'(K) = L(K) - L(K - 1)$. (C) Absolute values of the second order rate of change of the likelihood distribution (mean) calculated according to the formula: $|L''(K)| = |L'(K + 1) - L'(K)|$. (D) $\Delta K$; delta $K$ which calculated as $\Delta K = \text{mean of } |L''(K)| / \text{standard deviation of } L(K)$. 
Figure 4

Genetic compositions of the 56 KK individuals based on q-values. Each vertical bar represents a single individual. The colours represent the three clusters. The colours in each vertical bar represent proportion of each individual’s loci that are drawn from each of the three predefined clusters. For example, the genetic makeup of individual No. 25 shows that about half of its alleles come from cluster 2.
Figure 5

Frequency distribution of diagnostic alleles for Indian zebu, African and European taurine breeds in KK, KXX1, KXX2, Brakmas, Charoke and Brahman.
Figure 6

Consensus tree generated from 1000 bootstrap value using Neighbor-joining based on four genetic distance measures. KK = Kedah Kelantan, BK = Brakmas, CK = Charoke, KXX1 = Kedah Kelantan crossbred 1, KXX2 = Kedah Kelantan crossbred 2, BR = Brahman.
Figure 7

Dendrogram of genetic relationship among the 312 animals based on Nei’s DA genetic distances. Each tip represents a single animal. Breeds are distinguished by different colours according to the legend. KK = Kedah Kelantan, BK = Brakmas, CK = Charoke, KX1 = Kedah Kelantan crossbred 1, KX2 = Kedah Kelantan crossbred 2, BR = Brahman.
Figure 8

Principal component analysis of microsatellite diversity among KK cattle and KK breed types. KK = Kedah Kelantan, BK = Brakmas, CK = Charoke, KXX1 = Kedah Kelantan crossbred 1, KXX2 = Kedah Kelantan crossbred 2, BR = Brahman.