



## RAPD Analysis of Three Deer Species in Malaysia

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**ABSTRACT** : The genetic variability within and among three deer species in Malaysia, namely *Cervus nippon* (sika), *Cervus timorensis* (rusa) and *Cervus unicolor* (sambar), were evaluated using the RAPD technique. The DNA extracted from the buffy coat of 34 sika, 38 rusa and 9 sambar were analysed using ten primers that gave bands which showed good resolution. The primers generated 164 RAPD markers in total, and these ranged in size from 150 to 900 bp. The percent of polymorphism of the bands generated per primer ranged from 66.66-93.33% for rusa, 36.84-61.14% for sambar and 52.38-100% for sika. The overall percent polymorphism observed for the 164 RAPD markers was 99.39%. The results revealed five exclusive, monomorphic markers for sambar and one exclusive, monomorphic marker for sika; none was observed for rusa. However, these cannot be declared as markers for the identification of the species without analysis of more samples, populations and species. The means of within population genetic distances, based on Dice's and Jaccard's similarity indices, were similar for the rusa (0.383 and 0.542, respectively) and sika (0.397 and 0.558, respectively) populations with the sambar population being the least variable (0.194 and 0.323, respectively). The Dice based genetic distances within the species ranged from 0.194 to 0.397 and the genetic distances among the species were 0.791-0.911. The genetic distances based on Dice's and Jaccard's similarity indices between the rusa and sambar were 0.556 and 0.713, between the rusa and sika populations were 0.552 and 0.710, and between sambar and sika were 0.622 and 0.766, respectively. (**Key Words** : RAPD, Deer, Rusa, Sambar, Sika)

### INTRODUCTION

Deer as wildlife are often exposed to extensive loss of genetic variability as a result of increasing isolation and subdivision of wild populations by alteration of the landscape and constant slaughtering by humans for venison, hides and antlers, and for sports. In recent decades, there has been much interest in the domestications and farming of deer under varying degrees of intensification. The establishment of artificial populations in enclosures with consequent limited population sizes, closed breeding practices and selection of breeding animals are also contributory factors to losses in genetic variations. In Malaysia, for many years, deer have been kept in captivity in zoos and parks, where they do not serve as an economic enterprise. However, in the last two decades deer farming has become a popular livestock industry which is likely to grow rapidly in the future since there is both high local demand and good export potential for venison (Vidyadaran

et al., 1993). Deer farms have been established for the production of venison, velvet and as part of local agrotourism. Deer of various species and subspecies have been imported from different countries, such as Australia, Thailand, Mauritius and New Zealand, for this purpose. This has resulted in the introduction of new germplasm into the country and the risk of mixtures among these, making it necessary to evaluate and monitor the genetic variability among the deer species in Malaysia. Limited genetic studies to this purpose are available. It is, therefore, vital that a detailed study to evaluate and document the genetic makeup of the deer species and populations in Malaysia be conducted before the gene pool is indiscriminately diluted or altered.

Variability in animal populations, especially among species, may be studied at the morphological, chromosomal, and molecular levels. Over the past four decades, polymorphism at the molecular level has become a popular indicator of inherited genetic variations within and between populations and species. DNA markers can serve as useful tools to evaluate and compare the available genetic resources, and to monitor changes in populations over time as a result of human activities. The randomly amplified polymorphic DNA (RAPD) fingerprinting technique, which

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**Table 1.** Primers and optimised parameters for the RAPD-PCR analysis

| Primer name | Primer sequence (5' → 3') | Mg <sup>+2</sup> concentration (mM) | Annealing temperature (°C) | GC content (%) |
|-------------|---------------------------|-------------------------------------|----------------------------|----------------|
| 52A         | AGAGACATAGTT              | 3                                   | 30                         | 33             |
| 57A         | ATCATTGGCGAA              | 3.25                                | 34                         | 41             |
| 60B         | AGCCCTTATTTA              | 3.25                                | 28                         | 33             |
| 62B         | ACTCCAAATGTG              | 3                                   | 34                         | 41             |
| 64B         | GAGACTATGAAA              | 3.25                                | 28                         | 33             |
| 67B         | GCCCTTTTGGAC              | 3                                   | 37                         | 58             |
| 95A         | TACGTGGTAACA              | 3                                   | 32                         | 41             |
| 105R        | GAGGGTGGT                 | 3                                   | 39                         | 66             |
| 108R        | TTCGAGCCAG                | 2.5                                 | 38                         | 60             |
| BRL06B      | CTCAAGCGTACA              | 3                                   | 36                         | 50             |

Source: Tamate et al., 1995.

**Table 2.** Number of RAPD markers (and marker size range, bp) generated by the ten primers for the three deer species

| Primer | Parameter              | Number of RAPD Markers |         |         |
|--------|------------------------|------------------------|---------|---------|
|        |                        | Rusa                   | Sambar  | Sika    |
| 52A    | No. of markers         | 11                     | 11      | 10      |
|        | Marker size range (bp) | 180-850                | 150-900 | 180-850 |
|        | Percent polymorphism   | 100.00                 | 72.73   | 100.00  |
| 57A    | No. of markers         | 12                     | 10      | 12      |
|        | Marker size range (bp) | 200-800                | 200-750 | 200-800 |
|        | Percent polymorphism   | 100.00                 | 80.00   | 100.00  |
| 95A    | No. of markers         | 11                     | 11      | 11      |
|        | Marker size range (bp) | 180-750                | 220-750 | 180-750 |
|        | Percent polymorphism   | 100.00                 | 72.73   | 100.00  |
| 06B    | No. of markers         | 15                     | 9       | 13      |
|        | Marker size range (bp) | 150-800                | 150-580 | 150-800 |
|        | Percent polymorphism   | 100.00                 | 77.78   | 92.31   |
| 60B    | No. of markers         | 16                     | 7       | 12      |
|        | Marker size range (bp) | 150-750                | 150-750 | 150-750 |
|        | Percent polymorphism   | 100.00                 | 0.00    | 100.00  |
| 62B    | No. of markers         | 13                     | 9       | 13      |
|        | Marker size range (bp) | 280-800                | 280-800 | 280-800 |
|        | Percent polymorphism   | 84.62                  | 0.00    | 92.31   |
| 64B    | No. of markers         | 14                     | 8       | 13      |
|        | Marker size range (bp) | 200-820                | 200-800 | 200-820 |
|        | Percent polymorphism   | 100.00                 | 75.00   | 100.00  |
| 67B    | No. of markers         | 13                     | 11      | 12      |
|        | Marker size range (bp) | 200-750                | 200-700 | 200-700 |
|        | Percent polymorphism   | 92.31                  | 63.64   | 91.67   |
| 105R   | No. of markers         | 15                     | 14      | 14      |
|        | Marker size range (bp) | 200-850                | 150-850 | 250-850 |
|        | Percent polymorphism   | 100.00                 | 100.00  | 78.57   |
| 108R   | No. of markers         | 12                     | 7       | 13      |
|        | Marker size range (bp) | 150-830                | 250-750 | 150-750 |
|        | Percent polymorphism   | 100.00                 | 100.00  | 100.00  |

involves the polymerase chain reaction (PCR) amplification of genomic DNA using a single primer of arbitrary nucleotide sequence, has been proven to be a reliable technique for population studies (Chen et al., 2001; Su et al., 2006), and it can easily and precisely distinguished species according to the band distribution of RAPD patterns (Huang et al., 2003). In the present study, this technique was adopted to investigate the genetic variations within and

between three popular farmed deer species in Malaysia.

## MATERIALS AND METHODS

### Experimental materials

The three deer species evaluated in the present study were *Cervus timorensis* (rusa), *Cervus nippon* (sika) and *Cervus unicolor* (sambar). The rusa herd was located at the Deer Breeding Unit of the University Agricultural Park, Universiti Putra Malaysia (UPM), Serdang. The sika herd was located at the Deer Unit of Pusat Ternakan Haiwan, Batu Arang, Selangor. The sambar was a very small herd purchased recently by UPM from Pusat Pembiakan Ternakan Sabray, Kenungau, Sabah. The sample sizes used were 38 for rusa, 34 for sika and only 9 for sambar.

### RAPD analysis

Blood samples were collected by means of jugular vein puncture, and DNA was extracted from the buffy coat using the Qiagen DNA extraction kit. The RAPD technique was used to detect the variations at the molecular level. Sixteen 12-mer oligonucleotide primers of arbitrary sequences with 33-66% G+C content were selected to screen the samples. These 16 primers were selected from the 70 primers that were reported to have given clear RAPD patterns for three sika populations in Japan (Tamate, 1995).

PCR was performed in a thermal cycler (MJ Research Mini cycler) using 20 µl mixtures containing 25 ng genomic DNA, 60 pmole of primer (Introgen), 0.5 mM of dNTP, 5U of Taq DNA polymerase (Promega), 2 µl of PCR buffer and 3-3.5 mM MgCl<sub>2</sub>. The PCR protocol included an initial denaturation at 94°C for 2 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 28-42°C for 20 s and extension at 72°C for 1 min. At the end of the 38 cycles, a final extension at 72°C for 5 min was performed. The amplified products were electrophoresed on 2% agarose gel. The gel was stained with ethidium bromide, and visualised and photographed under UV. The BIOID++ software of the gel documentation system (Vilber Lourmat, France) was

**Table 3.** Percentage of shared RAPD markers detected using ten primers

| Bands                                 | Number of polymorphic markers | Number of monomorphic markers | Total number of markers <sup>1</sup> |
|---------------------------------------|-------------------------------|-------------------------------|--------------------------------------|
| Exclusive for rusa                    | 10                            | -                             | 10 (6.09)                            |
| Exclusive for sambar                  | 10                            | 5                             | 15 (9.15)                            |
| Exclusive for sika                    | 10                            | 1                             | 11 (6.71)                            |
| Shared exclusively by rusa and sambar | 16                            | -                             | 16 (9.75)                            |
| Shared exclusively by rusa and sika   | 46                            | -                             | 46 (28.05)                           |
| Shared exclusively by sambar and sika | 6                             | -                             | 6 (3.66)                             |
| Shared by rusa, sambar and sika       | 59                            | 1                             | 60 (36.58)                           |
| Total                                 | 157                           | 7                             | 164 (100)                            |

<sup>1</sup> Values in parentheses indicate percentages.

used to estimate the size of the bands representing the RAPD markers with reference to the 100 bp DNA step ladder. A conservative approach was taken for band scoring, with only bright, distinct bands and reproducible bands being recorded. The data were analysed using the RAPDistance software (Armstrong et al., 1994).

## RESULTS

Of the 16 arbitrary RAPD primers screened, only ten primers produced bands which showed good resolution (Table 1). These primers produced a range of 14-21 bands per primer from the three deer species, with band sizes ranging from 150 to 900 bp (Table 2). Eight primers revealed polymorphism in all the three deer species. Two primers (60B and 62B) produced monomorphic banding patterns for sambar, although the markers produced for rusa and sika were generally polymorphic.

The ten primers generated a total of 164 RAPD markers from the three species, with an overall percent polymorphism of 99.39%. Of these, 131 markers were amplified in rusa, 97 in sambar and 124 in sika. Sixty RAPD markers were shared by all three species, of which one marker, 62B-1 (800 bp), was monomorphic for all three species.

When the three species were considered individually, 128 RAPD markers (97.71%) were polymorphic for rusa, 66 (68.04%) were polymorphic for sambar and 118 (92.18%) were polymorphic for sika. Sambar had five exclusive monomorphic markers: 52A-14 (150 bp), 95A-14 (220 bp), 06B-14 (350 bp), 60B-11 (320 bp) and 67B-7 (550 bp). The marker 105R-9 (520 bp) was monomorphic and exclusive for sika. Rusa had no exclusive monomorphic marker. The marker 67B-13 (350 bp) was monomorphic for both the rusa and sambar populations, but was polymorphic for sika. The markers 105R-5 (620 bp) and 105R-6 (600 bp)

**Table 4.** Genetic distance (mean±SE) within and between the rusa, sambar and sika populations based on Dice's and Jaccard's similarity index

| Species         | Genetic distance |                 |
|-----------------|------------------|-----------------|
|                 | Dice's index     | Jaccard's index |
| Within species  |                  |                 |
| Rusa            | 0.383±0.120      | 0.542±0.023     |
| Sambar          | 0.194±0.007      | 0.323±0.010     |
| Sika            | 0.397±0.020      | 0.558±0.021     |
| Between species |                  |                 |
| Rusa and Sambar | 0.556±0.002      | 0.713±0.002     |
| Rusa and Sika   | 0.552±0.002      | 0.710±0.001     |
| Sambar and Sika | 0.622±0.003      | 0.766±0.003     |

were monomorphic for sika but were polymorphic for rusa and sambar. Table 3 shows the percentage of RAPD markers that were exclusive or shared among the three species.

The mean genetic distances among individuals within populations and between populations, based on the similarity indices of Dice (1945) and Jaccard (1912), are shown in Table 4. The genetic distance values based on Jaccard's similarity indices were higher than that based on Dice's similarity indices. The mean within population genetic distances for the rusa and sika populations were similar for both the indices. The Dice based genetic distance values among the rusa deer ranged between 0.08-0.60 while the Jaccard based ones were 0.15-0.77. Among the sika deer the Dice based genetic distance values ranged between 0.12-0.60 while the Jaccard based ones were 0.22-0.75. The sambar was the least variable population, with the Dice based genetic distances ranging 0.11-0.27 while the Jaccard based ones were 0.20-0.43. When the genetic distances among the three deer species were compared, the genetic distances between the rusa and sambar and between rusa and sika populations were similar. The genetic distance values between sambar and sika were slightly higher.

## DISCUSSION

The 16 primers used in the present study were reported to have given clear RAPD patterns for three sika populations in Japan (Tamate et al., 1995). However, only 10 primers managed to amplify the genomic DNA of the three deer species investigated. One reason for the failure of the six primers to amplify the DNA could be that these primers may have special requirements for amplification in terms of PCR reagents or temperature profile or time which were not utilised during the optimization in the present study. The PCR conditions reported by Tamate and coworkers (1995) were initially followed before further attempts at optimization. In the present study, only the Mg<sup>2+</sup> concentrations and the primer annealing temperature were optimised for each primer and both were relatively low

when compared to those used in other reported studies. The optimal  $Mg^{+2}$  concentrations were moderate (3-3.5 mM), and the annealing temperatures ranged from 28 to 39°C. Increase in annealing temperature reduces the events of non-specific priming and thus results in a less complex and reproducible banding pattern (Bowditch et al., 1993). However, in the present study higher annealing temperatures did not produce any detectable bands.

The concentration and source of Taq DNA polymerase,  $Mg^{+2}$  concentration, concentration and GC content of the primers, and the thermal cycler's efficiency have also been shown to influence amplification (Zhang et al., 1996). Meunier and Grimont (1993) and Schiewater and Ender (1993) reported that variability in the commercial preparation of Taq DNA polymerase was a major source of variation in RAPD reactions. In this study, Taq DNA polymerase from Promega was used in all reactions, therefore, eliminating the possible inconsistency of RAPD banding patterns due to different sources of the enzyme.

Despite the various attempts at optimization, the six primers failed to amplify the DNA of the three populations. Therefore, it may be correctly assumed that the six primers did not show amplification probably due to lack of annealing sites in the genomic DNA of the deer species/populations studied. Frameshift mutations, resulting from insertion or deletion of one or more bases, and point mutations (substitution of a base) may abolish primer binding sites (Gwakisa et al., 1994). For the rusa and sambar this could have occurred during speciation, however, for the sika population in Malaysia it probably occurred recently in the population from which the founder animals of the herd studied originated.

The percentage of the primers screened that were amplification in the present study was much higher than that reported by Sodhi et al. (2006). Of the 50 primers they used to screen two buffalo breeds, only 26 generated reproducible banding patterns. However, Sharma et al. (2004) reported a similar percent amplification of the primers used as the present study. Fifteen of the 23 primers used for molecular characterization of two indigenous cattle breeds yielded satisfactory amplification.

The results from the analysis of the RAPD markers generated by the 10 primers are acceptable for rusa and sika, based on the sample sizes representing these populations. However, the results for sambar may not reflect the real situation due to the small sample size of only nine animals used in this study. Primack (2002) reported a correlation between population size and genetic variability; larger populations had greater variability. A minimum sample size of 15-20 is recommended for any population genetic study using molecular markers (Mathur et al., 1994).

Collecting blood samples from deer proved to be a difficult task. Although deer are currently being farmed in

Malaysia, they are kept under an extensive management system. The animals are allowed to roam free in large paddock areas and are often quite wild. Catching as well as restraining these animals for blood sampling were major problems, especially when many farms had no dark house and body crush. The farmers were also not receptive to the idea of anaesthetising the animals. That was the reason why the sambar deer used were from a small sample of animals brought over to UPM from a population in the state of Sabah, East Malaysia. At UPM a dark house was available, although there was no body crush.

The large number of markers generated by the 10 primers for the three species and the high percent polymorphism observed among these markers are characteristics of the RAPD technique that are responsible for it being popularly used in population genetic studies. Sharma et al. (2004) detected 86-87% polymorphism among the bands generated for two indigenous cattle breeds using the RAPD technique. The primers used in the RAPD analysis are non specific. Therefore, they scan the entire genome and detect high variation among animals of a population and between populations/species.

The sambar exhibited five exclusive, monomorphic RAPD markers, sika had one, and rusa none. For a DNA marker to be considered as one to identify a species and to distinguish it from other species, it must be exclusive for the species as well as being monomorphic. However, since only nine sambar deer were analysed in this study the five markers cannot be confirmed as being monomorphic. As for the sika, marker 105R-9 can be considered as a potential marker for identification of this species. However, before it could be declared as the marker for the identification of the sika deer, its presence in other populations of sika, particularly in Malaysia, and its absence in other animal species, especially deer species, must be tested. It is difficult to find monomorphic, unique RAPD markers for species, breed or population distinction and thus identification due to its highly polymorphic nature. Sodhi et al. (2006) found two RAPD markers that were present in the NiliRavi buffalo breed but absent in the Murrah buffalo, however, these were polymorphic. Similarly, in the study comparing catla (*Catla catla*) from three major river systems, Islam et al. (2005) found two RAPD markers unique to the Halda river population and one RAPD marker unique to the Padma river. These three markers were all polymorphic.

The genetic distance values, based on Dice's and Jaccard's similarity indices, for among individuals within populations indicated that the genetic variabilities in sika and rusa were moderate. The farmed deer populations kept in enclosures and descendants of introduced founder populations showed moderate genetic variability, with mean within population genetic distance values higher than that

reported for some other livestock populations (Kumar et al., 1997; Chen et al., 2001). This may be attributed to the lack of selection and controlled breeding in the management practices. Sharma et al. (2004), however, reported similar genetic distance values, on the basis of band sharing based on RAPD analysis using 15 primers, for the Rathi and Tharparkar indigenous cattle populations. The genetic distances among the three species found in this study were high, much higher than the values for similar parameters reported for between breed comparisons by Panandam et al. (2003) and Ali (2003). The results reveal the species as being genetically different and justifies them being classified as different species. The results also suggest that the divergence among the rusa, sambar and sika either from the common ancestral origin or from each other during evolution (Todd, 1975, 1985; Fontana and Rubini, 1990; Habiba, 2005) must have taken place a very long time ago.

Based on the polymorphisms observed in the present study and the genetic distance values for within species and between species, it may be concluded that the rusa, sambar and sika deer species in Malaysia are genetically diverse. The industrialisation of deer farming and their establishment from limited founder populations have not affected the genetic variability within the deer species in Malaysia.

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