

Genetic differentiation of Indian Zebu Cow (*Bos taurus*)  
breeds using random oligonucleotide primers (RAPD-PCR) in  
Amravati region, Maharashtra, India.

**Abstract:**

In India, insufficient characterization skills and extensive crossbreeding of cattle have led to the loss of several breeds. The purpose of this work is to find polymorphic primers for identifying Zebu cattle breeds. Random crossbreeding has resulted in genetic losses among the Gaolao, Krishna Valley, and Hallikar breeds. Genetic characterization and cryopreserving semen are critical components of conservation and breeding initiatives. The study highlights the urgent need for effective genetic characterization of Zebu cattle breeds in India, identifying 8 polymorphic and 4 monomorphic primers through RAPD-PCR. These findings are crucial for accurate breed identification, which is essential for conservation efforts and informed breeding strategies to preserve genetic diversity and prevent the decline of indigenous breeds like Gaolao, Krishna Valley, and Hallikar. Genomic DNA was isolated from whole blood using a DNA extraction kit and amplified with RAPD-PCR. A total of 12 primers were evaluated, and agarose gel electrophoresis revealed that eight (OPA-09, OPA-15, OPB-13, OPB-07, OPB-05, OPA-13, OPA-18, and OPA-01) were polymorphic and four (OPB-06, OPA-04, OPB-03, and OPA-02) were monomorphic. Polymorphic primers demonstrated relatedness between some of the available breeds. Primers OPA-09 and OPA-15 showed a higher degree of polymorphism than primers OPB-13, OPB-07, OPB-05, OPA-13, OPA-18, and OPA-01, indicating that primers OPA-09 and OPA-15 may successfully identify various cattle breeds.

**Keywords:** Indian Zebu, polymorphic, characterization, RAPD-PCR, Gaolao, Krishna Valley, Hallikar.

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1. **Introduction:**

India has many different species of indigenous cattle. The primary indigenous breeds include Gaolao, Krishna Valley and Hallikar, in addition to various alien breeds. Since 2000, India has experienced indiscriminate cow breeding as a result of random livestock movements across farms. As a result, it is critical to screen Randomly Amplified Polymorphic Deoxyribonucleic Acid (RAPD) primers for polymorphisms that can be utilized to determine cattle genotypes.

Indiscriminate crossbreeding has resulted in the loss of breeds (Gwakisa *et al.*, 1994), such as

the Gaolao cattle breed (More *et al.*, 2022), Krishna Valley (Karthickeyan *et al.*, 2006), and Hallikar (Kumar *et al.*, 2006).

Molecular markers based on DNA sequence differences can successfully discriminate between closely related genotypes and can accurately identify closely related genotypes due to their resistance to selection (Hassen *et al.*, 2007; Matthew, 2010). For effective breeding and conservation programs to be carried out, it is critical to screen RAPD primers to determine the genotypes in cattle. The polymerase chain reaction (PCR) approach uses amplified DNA sequences as molecular markers with minimal template DNA (Munthali *et al.*, 1992; Weber *et al.*, 1989).

“Random Amplified Polymorphic Deoxyribonucleic Acid-Polymerase Chain Reaction (RAPD-PCR) annealing occurs when a random primer of any sequence binds to specific related priming sites of the template genomic DNA, producing amplicons if the priming sites are within an amplifiable distance of each other” (Gwakisa *et al.*, 1994; Hardys *et al.*, 1992). Williams *et al.* (1990) developed “the RAPD analysis method, which employs short oligonucleotide primers to amplify a wide range of fragments from a template DNA in PCR processes with a lower annealing temperature. The RAPD PCR detects polymorphisms used in genetic mapping and strain identification. It is the superior method because it is (a) less expensive than previous methods for finding genetic variants, such as protein markers; (b) faster; and (c) without the need for previous sequencing details. The ability of RAPDs to survey many loci has been used to compute genetic distance and reconstruct phylogenies”.

“Awareness of genetic variation is required for breed characterization in order to undertake effective breeding efforts across and within individuals. The RAPD technique has been successfully utilized to analyze bovine species” (Gwakisa *et al.*, 1994; Kemp *et al.*, 2009; Kemp *et al.*, 1994). “Other studies that have used RAPD markers include Japanese black cattle (Wagyu), Zebu cattle, German native cattle, and Korean native cattle” (Yeo *et al.*, 2000). Hwanget *al.* (2001) states that RAPD markers are an important tool for correlation studies in cattle.

RAPD and microsatellite markers are both used for genetic characterization, but each has unique advantages and limits. The advantages of RAPD over microsatellite markers include its simplicity and cost-effectiveness. It requires no prior knowledge of the DNA sequence, making it suitable for studies with few resources. RAPD is a rapid procedure that uses a single PCR

reaction with arbitrary primers. RAPD may discover polymorphisms across the genome without requiring precise sequence information.

Reasons why microsatellite markers might not be used: Microsatellite markers necessitate the use of unique primers for each locus, which necessitates more complicated and costly procedures, such as sequence information and primer design. The use of microsatellite markers necessitates greater technical skill and more advanced equipment, which may not be available in all research locations. Microsatellite marker development and analysis take longer than RAPD markers do.

In this Zebu cattle breed study, RAPD was most likely chosen because of its low cost, simplicity, and ability to generate polymorphic markers fast without prior sequence information. This makes it an appropriate choice for initial genetic characterization and conservation efforts, particularly when resources and time are limited.

This study verified RAPD primers to find polymorphic primers for Indian Zebu cow breeds. This study is relevant to India's conservation and breeding programs. As a result, identifying RAPD primers capable of determining genotypes in cattle is significant.

## **2. Materials and methods:**

### **2.1. Blood collection:**

A syringe and 18G needle were used to collect blood from three cows after the region was sterilized with 70% ethanol. To prevent clotting, the blood was promptly transferred into ethylene diamine tetra acetic acid (EDTA) containing blood tubes and gently inverted ten times. Blood tubes were transported to the laboratory in ice bags and stored in a freezer at -20°C.

### **2.2. DNA extraction:**

High-molecular-weight genomic DNA was extracted from whole blood by using the Qiagen DNeasy® Blood & Tissue Kit (50), Cat. No. 69504. By taking a 2-ml microcentrifuge tube, add 20 µl of proteinase K and added 100 µl of anticoagulant blood sample, adjusted the volume to 220 µl with PBS (Potassium Buffer Saline, pH 7.2). Added 200 µl of lysis buffer AL (without ethanol addition), mixed thoroughly by vortexing and incubated at 56 °C for 10 minutes. Added 200 µl of ethanol (96–100%) to the sample and mixed thoroughly by vortexing to yield a homogeneous solution. Pipetted the mixture from step 3 into the DNeasy Minispincolumn placed in a 2 ml collection tube. Centrifuged at  $\geq 6000$  g (8000 rpm) for 1

minute. Discard the supernatant through the collection tube. Placed the DNeasy Mini spin column in a new 2 ml collection tube, added in it 500  $\mu$ l Buffer AW1 and centrifuged for 1 minute at  $\geq 6000$  g (8000 rpm). Discard the flow through the collection tube. Place the DNeasy Mini spin column in a new 2 ml collection tube, added 500  $\mu$ l Buffer AW2 and centrifuged for 3 minutes at 20,000g (14,000 rpm) to dry the DNeasy membrane. Placed the DNeasy Mini spin column in a clean 2ml microcentrifuge tube and pipetted 200 $\mu$ l buffer AE directly onto the DNeasy membrane. Incubated at room temperature for 1 minute and then centrifuged for 1 minute at  $\geq 6000$  g (8000 rpm) to elute. The eluted genomic DNA from Zebu cattle blood was stored at -20 °C.

### **2.3. RAPD-PCR reaction:**

RAPD-PCR was performed in a 25  $\mu$ l reaction using 1.5  $\mu$ l of Taq polymerase 7.5 units (Thermo, HSN). 1.5  $\mu$ l taq buffer (Thermo, HSN), 2  $\mu$ l dNTP's 0.4 mM (Thermo, HSN) 3  $\mu$ l Tris and HCl buffer (pH 8.0) 3  $\mu$ l primer (10 mM. Operon technology) and 1  $\mu$ l (50 to 100 ng) diluted genomic DNA mix with 12  $\mu$ l nuclease-free water in a 0.2 ml PCR tube. The BioEra's thermocycler was used to perform the polymerase chain reaction (PCR). The cycling conditions were as follows: pre-denaturation: 95°C for 3 min; denaturation: 95°C for 1 min. annealing: 36°C for 1 min. extension: 72°C for 1 min. final extension: 72°C for 8 min. a total of 41 cycles. Electrophoresis of amplification products on 1.5% agarose gels in 1X Tris base, acetic acid, and EDTA (TAE) buffer with ethidium bromide at 150V was performed for 45 minutes.

### **2.4. Gel electrophoresis:**

To prepare a 1.5% agarose gel, dissolved 3.0 g of agarose powder in 200 ml of TAE buffer (1X). A hot magnetic stirrer was used to dissolve the gel in a conical flask covered with aluminium foil. When the gel was still hot, added ethidium bromide (10mg/ml) and stirred into the agarose solution to create a homogenous solution. After cooling until the conical flask could be grasped; the solution was placed on a gel box (Galileo Biosystems). After solidification, the gel was suspended in 1X TAE buffer. The RAPD-PCR products (10  $\mu$ l PCR amplification products along with 2  $\mu$ l of DNA loading dye) were separated on 1 percent Agarose gel containing Ethidium bromide (0.5  $\mu$ l 1/10 ml of gel) at 100 volts for three hours using 1 x TBE buffer. A UV transilluminator was used to visualize the bands, which were then photographed with a Canon T5 EOS Rebel DSLR digital camera.

### **3. Results:**

Out of 25 random primers tested on DNA samples, 13 were eliminated due to the absence of distinct amplified bands. The remaining 12 primers were used based on the number

and strength of bands to assess genetic variability. We scored fingerprints based on the assumption

that each band represented a single locus. Based on the quantity and strength of the bands, they were utilized to assess the variability of the genome. To obtain fingerprint scores, one band was thought to correspond to one locus. RAPD-PCR was carried out with 12 primers: OPA-01, OPA-02, OPA-04, OPA-09, OPA-13, OPA-15, OPA-18, OPB-03, OPB-05, OPB-06, OPB-07 and OPB-13. There are 2 primers that showed 100% polymorphism (OPA-9 and OPA-15), and 10 primers were found to have different polymorphisms in nature (OPA-1, OPA-02, OPA-13, OPA-18, OPB-03, OPB-05, OPB-06, OPB-07, and OPB-13).

These primers amplified 2–36 bands with sizes ranging from 250 to 1500 bp. Among the 248 loci amplified, 131 (52.82%) showed polymorphisms. Table 1 shows the average number and size of bands obtained from the different random primers. Primers OPB-07 and OPB-13 showed a higher degree of polymorphism. Figure 1 displays the RAPD fingerprints of primer-amplified bands. Data from Table I was used to create a dendrogram indicating genetic relationships among the selected breeds (Figure 2). The genetic links among the breeds showed two separate groups: Gaolao is a separate group, and the other one includes Krishna Valley and Hallikar.

Table 1 Average number and size of bands obtained from different random primers

Primers	Sequence (5'-----3')	GC%	Average No. of Bands	Polymorphic Bands & %	Range (bp)
OPA-01	CAGGCCCTTC	70	19	10(52.63)	400-1200
OPA-02	TGCCGAGCTG	70	25	10(40)	300-1500
OPA-04	AATCGGGCTG	60	21	06(28.51)	300-700
OPA-09	GGGTAACGCC	70	15	15(100)	250-500
OPA-13	CAGCACCCAC	70	27	15(55.56)	300-1000
OPA-15	TTCCGAACCC	60	06	06(100)	250-300
OPA-18	AGGTGACCGT	60	27	15(55.56)	300-1000
OPB-03	CATCCCCCTG	70	18	03(16.67)	250-700
OPB-05	TGCGCCCTTC	70	36	21(58.34)	300-1000
OPB-06	TGCTCTGCCC	70	12	00(0)	250-600
OPB-07	GGTGACGCAG	70	30	21(70)	250-1200
OPB-13	TTCCCCCGCT	70	12	09(75)	300-1200

#### 4. Discussion:

Traditional cattle **molecular** characterization approaches, such as protein markers, are time consuming and difficult to understand when compared to molecular **genetic** analysis like RAPD-PCR (Sarkar et al., 2012). Molecular DNA polymorphisms are recommended for determining genetic variety in animal breeds (Zulu, 2008). The results demonstrate that

polymorphic primers (Fig.1), OPA-09, OPA-15, OPB-13, OPB-07, OPB-05, OPA-13, OPA-18, and OPA-01, are appropriate primers for distinguishing Gaolao, Krishna Valley, and Hallikar cow breeds. RAPD-PCR provides several features that make it the preferred molecular approach in Asia. During the screening of the 12 primers, primers OPA-09, OPA-15, OPB-13, OPB-07, OPB-05, OPA-13, OPA-18, and OPA-01 displayed polymorphisms, indicating potential differences among the three (Gaolao, Krishna Valley, and Hallikar) cow genotypes. This means that when these primers are used on control samples, genotypic variations in purebred animals become clear. The monomorphic primers OPB-06, OPA-04, OPB-03, and OPA-02 revealed no polymorphisms and hence cannot be utilized to distinguish genotypic differences amongst the breeds used in this investigation. Ramesha *et al.*, (2002) investigated the genetic distance between South Indian Zebu cattle breeds Krishna Valley and Hallikar by using different random amplified DNA markers.

Strucken *et al.*, (2021) observed genetic diversity and effective population sizes of thirteen Indian cattle breeds including Krishna Valley and Hallikar cattle. This is the first time to study the genetic diversity and population structure of Gaolao cow breeds which is presented in this paper.

In a study of Rathi and Tharparkar cattle breeds, Sharma *et al.*, (2004) discovered that primers OPA-01, OPA-02, OPA-04, and OPB-07 yield polymorphic bands. Primers OPA-02 and OPA-04 produced monomorphic bands while primers OPA-01 and OPB-07 produced polymorphic bands. This could have been the result of genotypic variances among the breeds included in the study.

According to Strucken *et al.*, (2021), “the phenotypic traits of the breeds employed in their study suggested that the animals were crossbreeds between Krishna Valley and Hallikar”. Ramesha *et al.*, (2002) discovered “a high degree of similarity in DNA bands between Ongole and Krishna Valley cattle breeds. They indicated that the Krishna Valley breed is a blend of four unique breeds i.e., the Gir, Ongole, Kankrej, and Hallikar. The Hallikar cattle breed was found to have a lower genetic distance from Amritmahal due to breeding tract overlap. They also found that dual-purpose breeds Krishna Valley and Ongole have less genetic divergence than draft breeds Amritmahal, Hallikar and Khillari. The polymorphism revealed by the primers OPA-09 and OPA-15 showed that all three breeds were differentiated by 100% polymorphism. All breeds shared a band size of 250 to 300 bp. This indicated that the breeds are connected or that the band size is peculiar to these cattle”.

In the present work, polymorphisms of OPA-18 and OPB-13 are shown by three distinct breeds Gaolao, Krishna Valley, Hallikar. Primer OPB-05 had four different banding patterns. This

demonstrates the existence of three distinct breeds or genotypes. A present study found that primer OPB-05 produced 21 polymorphic bands ranging in size from 300 to 1000 bp. Sharma *et al.*, (2004) studied in Rathian and Tharparkar indigenous (*Bos indicus*) cattle breeds that primer OPG-07 yielded a total of 8 polymorphic bands ranging in size from 400 to 1475 bp. Similarly, primer OPB-05 can be efficiently utilized to define various cow breeds based on genotypic differences.

According to Mhuka *et al.*, (2016) RAPD-PCR is successful in finding polymorphisms in bovine species. Polymorphic primers can identify genetic variants in crossbred animals across multiple breeds using RAPD-PCR to identify breed genotype. The OPB-03 polymorphic pattern identified three distinct breeds based on their banding patterns. Lanes 2 and 3 had similar band sizes; however, lane 1 representing Gaolao only had one 400-bp band, which was not shared by the remaining, indicating genetic variation or species specificity in a Gaolao cow. The common band size indicated that, despite being crosses of two different breeds, the two animals shared genetic material from a common ancestral lineage. OPB-13 revealed amazing polymorphisms, yet certain lanes did not show a single band. This could be due to nonspecific binding during RAPD-PCR or if the template DNA did not match the existing breeds have the priming locations for OPB-15. This study provides baseline information on the primers suitable for genetic variability analysis of Gaolao, Krishna Valley and Hallikar cows in India. “Likewise, with the polymorphisms acquired with OPX-15, some lanes failed to exhibit even one band. This could have been caused by non-specific binding during RAPD-PCR or the template DNA of the breeds present did not include the OPX-15 priming sites” (Mhuka *et al.*, 2016).

## **5. Conclusion:**

RAPD-PCR is found to be successful in detecting polymorphisms within the bovine species. Polymorphic primers (OPA-09, OPA-15, OPB-13, OPB-07, OPB-05, OPA-13, OPA-18, and OPA-01) may detect genotype differences in animals, while monomorphic primers (OPB-06, OPA-04, OPB-03, and OPA-02) cannot do so. Primer OPA-04, which was used by Sharma *et al.*, (2004) shown polymorphism in Rathian and Tharparkar breeds, and monomorphic in this study. Polymorphic primers demonstrated relatedness between some of the available breeds. Primers OPA-09 and OPA-15 showed a higher degree of polymorphism than primers OPB-13, OPB-07, OPB-05, OPA-13, OPA-18, and OPA-01, indicating that primers OPA-09 and OPA-15 may successfully identify various cattle breeds.

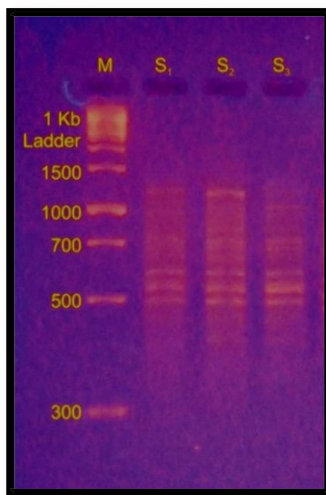
## **Conflict of Interest:**



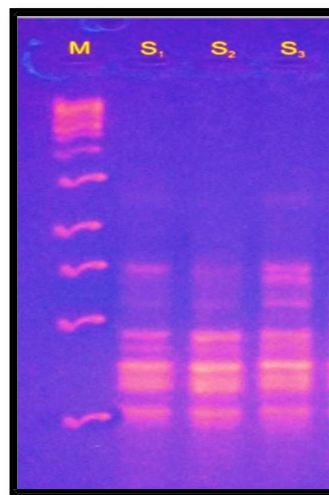
The authors declare that there is no conflict of interest regarding the publication of this paper.

**Acknowledgements:**

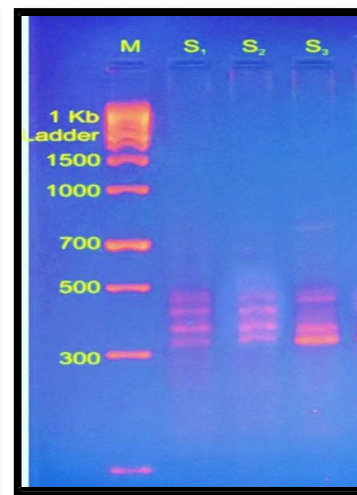
The authors would like to thank the Chhatrapati Shahu Maharaj Research Training and Human Development Institute, SARTHI, Pune, M.S., for giving the research grant for this research work, as well as HOD department of Zoology, Sant Gadge Baba Amravati University, Amravati and Supervisor, Dr. H. P. Nandurkar madam and Asstt. Professor Shri V. K. Nagale sir. We would also like to thank department of Zoology, Sant Gadge Baba Amravati, University Amravati for providing equipments and personnel to assist with the protocols, with special thanks to Dr. N. Warghat sir. The authors also want to thank the Gaushala for providing us with the cattle blood sample and laboratory staff members.



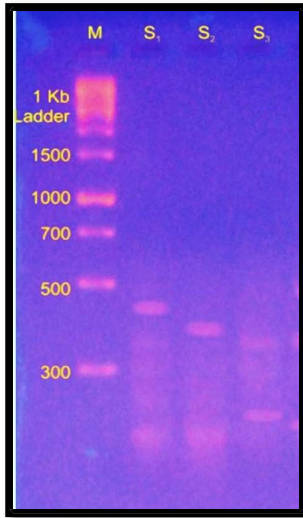
OPA-01



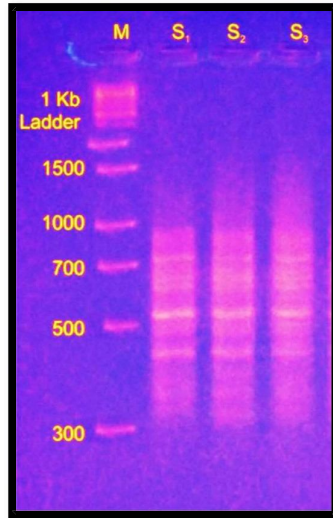
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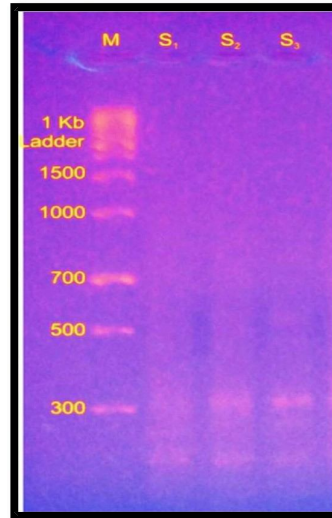
OPA-04



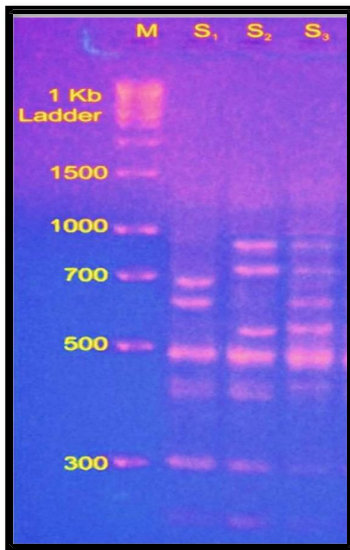
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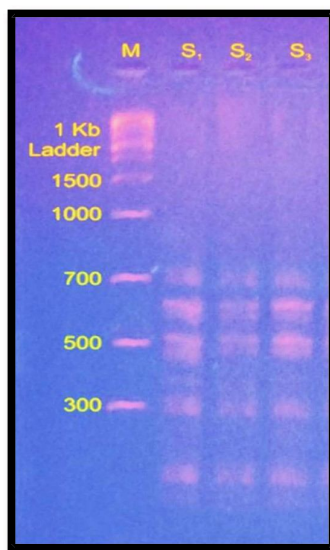
OPA-13



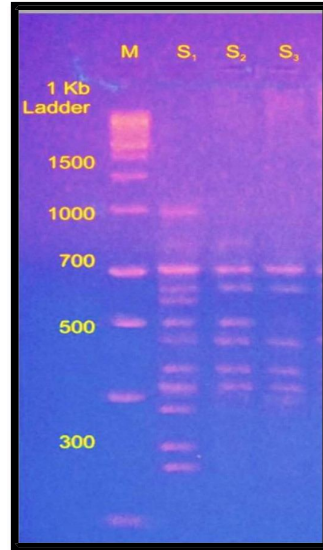
OPA-15



OPA-18



OPB-03



OPB-05

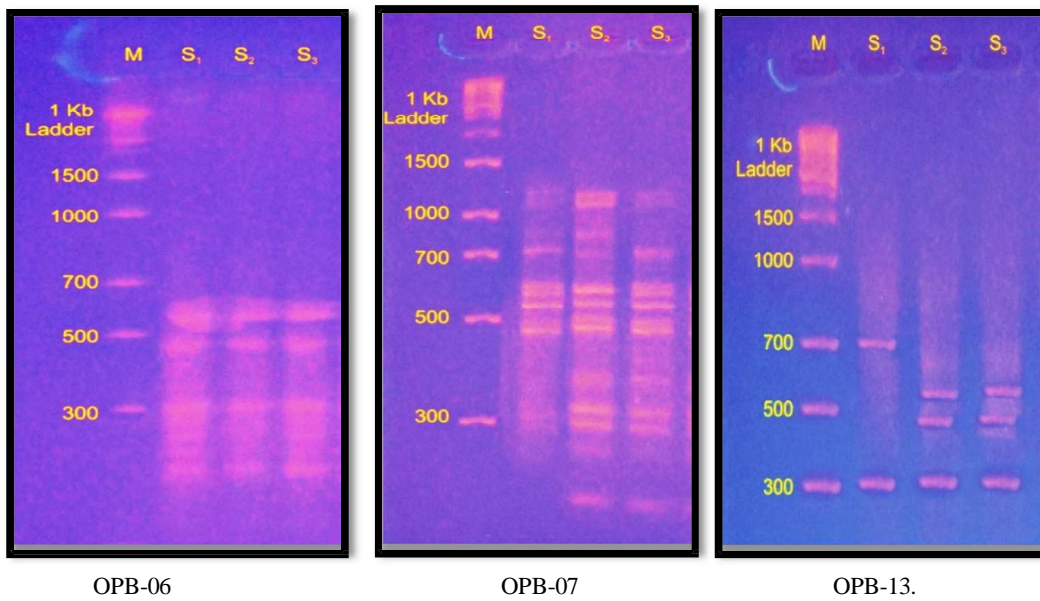


Figure 1: Agarose gels showing polymorphic and monomorphic primers (OPA-01, OPA-02, OPA-04, OPA-09, OPA-13, OPA-15, OPA-18, OPB-03, OPB-05, OPB-06, OPB-07, OPA-13).

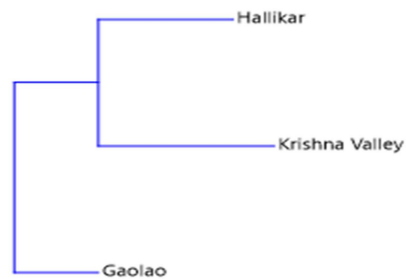


Figure 2: Dendrogram constructed using Nei coefficient of similarity between the breeds.

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