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

Abstract.

In India, lack of characterization ~~resources~~ and excessive crossbreeding of cattle have resulted in the extinction of some 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. 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.

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, indiscriminate cattle breeding has occurred in India as a result of random cattle transfers

between farms. Indiscriminate breeding have an impact on bull reproductive success by resulting in variation in semen quality. 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).

~~Kary Mullis invented PCR in 1980 based on denaturation, annealing, and extension (Karp., 2009).~~ When Random Amplified Polymorphic Deoxyribonucleic Acid-Polymerase Chain Reaction (RAPD-PCR) annealing occurs, a random primer of an arbitrary sequence will bind 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) established the RAPD analysis approach, which uses short oligonucleotide primers to amplify a variety of fragments from a template DNA in PCR reactions with a lower annealing temperature ~~(Ramesha *et al.,* 2001)~~. The RAPD PCR detects polymorphisms for genetic mapping and strain identification. It is the preferable method since it is (a) less expensive than previous methods for determining genetic variants, such as protein markers; (b) faster; and (c) does not require prior sequence

information. The ability of RAPDs to survey numerous loci has been utilized to calculate genetic distance and reconstruct phylogenies.

Awareness of genetic variation is essential for the characterization of breeds to conduct efficient breeding campaigns across and within individuals (Hetzel *et al.,* 1992). The RAPD approach has been used effectively to study bovine species (Gwakisa *et al.,* 1994; Kemp *et al.,* 2009; Kemp *et al.,* 1994). Other investigations that used RAPD markers include Japanese black cattle (Wagyu), Zebu cattle, German native cattle and Korean native cattle (Yeo *et al.,* 2000). According to Hwang *et al.,* (2001), RAPD markers are a valuable tool for linkage studies in cows.

This study verified RAPD primers to find polymorphic primers for Indian Zebu cow breeds. This material 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 tetraacetic 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^{O}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 Mini spin column placed in a 2 ml collection tube. Centrifuged at ≥ 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 µl Buffer AW1 and centrifuged for 1 minute at ≥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 µl 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 200µl buffer AE directly onto the DNeasy membrane. Incubated at room temperature for 1 minute and then centrifuged for 1 minute at ≥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 µl reaction using 1.5 µl of *Taq* polymerase 7.5 units (Thermo, HSN). 1.5 µl *taq* buffer (Thermo, HSN), 2 µl dNTP's 0.4 mM (Thermo, HSN) 3 µl Tris and HCl buffer (pH 8.0) 3 µl primer (10 mM. Operon technology) and 1 µl (50 to 100 ng) diluted genomic DNA mix with 12 µl 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 (10 mg/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 µl PCR amplification products along with 2 µl of DNA loading dye) were separated on 1 percent Agarose gel containing Ethidium bromide (0.5 µl 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 genomic 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 genotypic characterization approaches, such as protein markers, are time-consuming and difficult to understand when compared to molecular genomic 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.

~~The procedure is affordable, trustworthy and rapid. This method requires no prior understanding of the DNA under investigation and only employs small quantities of genomic DNA. To optimize RAPD analysis, primers were chosen with ten nucleotides and having at least 60% G-C content as high A-T content can cause melting of the DNA-primer complex during polymerization at 72 °C (Yeo *et al.,* 2000).~~

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 pure-breed 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 crossbreds between Krishna Valley and Halliakr. 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 250–300 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 study found that primer OPB-05 produced 21 polymorphic bands ranging in size from 300 to 1000 bp. Sharma *et al.,* (2004) studied in Rathi 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.

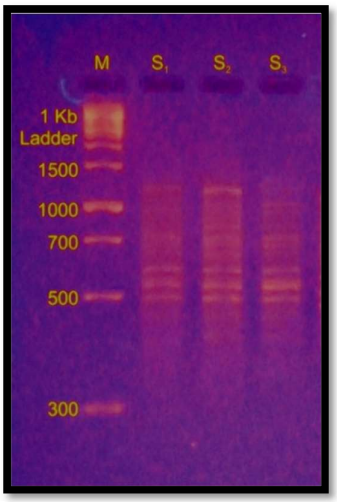
The OPB-03 polymorphic pattern identified three distinct breeds based on their banding patterns. Lanes 2 and 3 had similar band sizes; however, lane 1 representing Gaolao only had one 400-bp band, which was not shared by the remaining, indicating genetic variation or species specificity in a Gaolao cow.

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 non-specific 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).

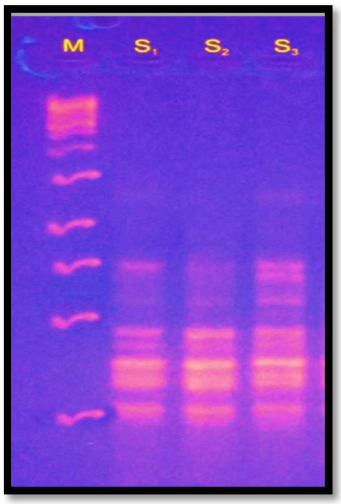
5.Conclusion

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 Shrama *et al*., (2004) shown polymorphism in Rathi 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.

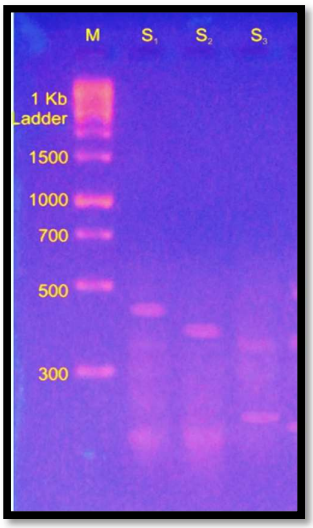


OPA-01

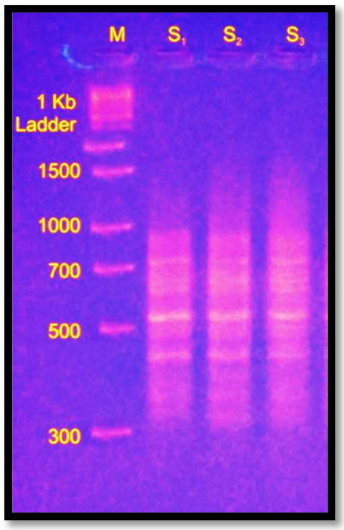


OPA-02

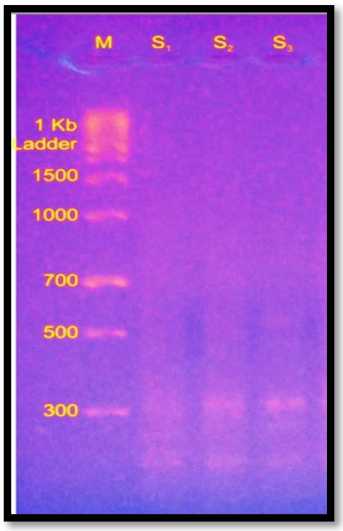
OPA-04



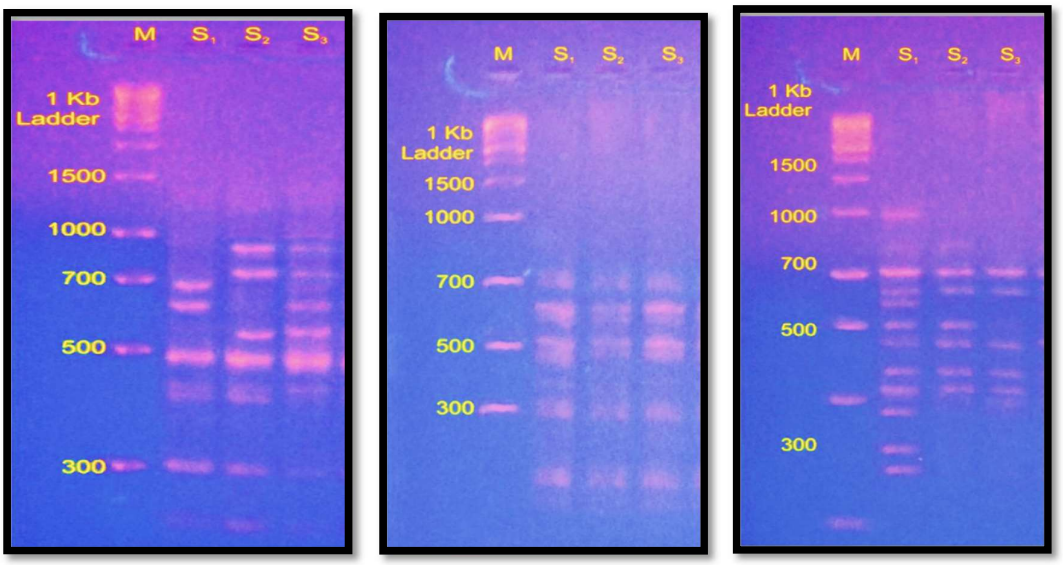
OPA-09



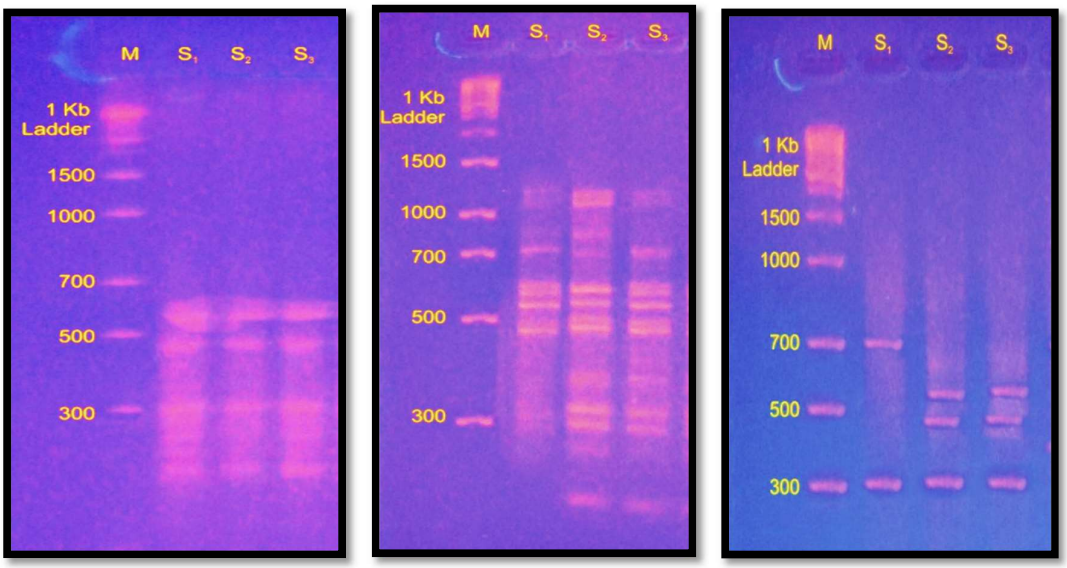
OPA-13



OPA -15



OPA-18 OPB-03 OPB-05



OPB-06 OPB-07 OPB-13.

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, 0OPB-13).

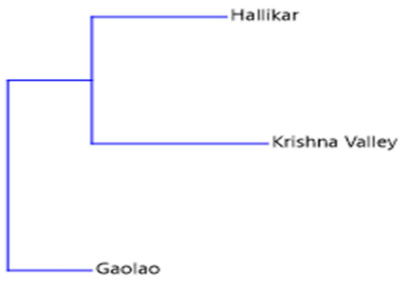


Figure 2: Dendrogram constructed using Nei coefficient of similarity between the breeds.

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