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RAPD-PCR: A POTENT TOOL FOR BREED CHARACTERIZATION IN DAIRY BREEDS

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The Rapid Amplification of Polymorphic DNA (RAPD) technique is highly informative even without prior knowledge of sequence information. It has achieved a great deal of acceptance due to its simplicity, low cost investment, and requirement of little amount of DNA. The technique has been successfully applied for genome mapping, differentiating geographically isolated population or breeds, species identification, establishing genetic relationship, estimating genetic diversity in different livestock species and characterization of different livestock breeds. This technique is comparable to that of RFLP and micro satellite techniques and possibility of converting a RAPD marker of interest into a single locus PCR marker-SCAR (Sequence Characterized Amplified Region) have made RAPD a promising technique. Further it is helpful in multilocus fingerprinting in contrast to protein polymorphisms examining a single locus. Therefore this technique may be a potent tool for the species and breed characterization of cattle and buffalo.

Key words: RAPD-PCR, Characterization, Polymorphism, Genetic distance, dairy breeds

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The sequences revealing variations at the DNA level are referred to as the molecular or DNA markers. Such DNA markers which are inherited in Mendelian fashion facilitate the study of inheritance of a trait or a linked gene. It must be polymorphic to be used for mapping. Molecular marker technology offers a wide range of novel approaches for improving the efficiency of selection strategies in livestock improvement. These markers are numerous and represent a milestone in genetics by providing the capacity for complete coverage of nuclear and mitochondrial genomes. Several kinds of molecular markers are available, but commonly used are, amplified fragment length polymorphism (AFLP), allele specific PCR (AS-PCR), DNA amplification fingerprinting (DAF), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), sequence characterized amplified region (SCAR), single strand conformation polymorphism (SSCP), simple sequence length polymorphism (SSLP), simple sequence repeat (SSR), PCR-RFLP and single nucleotide polymorphism (SNP) etc. These DNA markers (Table-1) can be broadly classified into hybridization-based DNA markers such as RFLP and DNA fingerprinting, PCR-based DNA markers such as RAPDs, SSRs, AFLP and ISSRs and DNA chip and sequencing-based DNA markers such as SNPs.

DNA polymorphisms can be used in marker assisted selection programmes, paternity testing (Scott *et al.*, 1992), species identification (Min *et al.*, 1996 and Koh *et al.*, 1998), breed characterization (Gwakisa *et al.*, 1994) and population genetic studies (Crowhurst *et al.*, 1991). RAPD analysis was developed independently by Welsh and McClelland (1990) and Williams *et al.*

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(1990). RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotides primer (usually decamer), and thus do not require prior knowledge of the DNA sequence of the genome.

Table 1. Different DNA markers and their characteristics

Marker type	**Acronym**	**Requires molecular information**	**Mode of Inheritance**	**Type**	**Locus under study**	**Likely allele number**	**Polymorphism**	**Major application**
Allozyme	-	Yes	Mendelian ,codominance	1	Single	2-6	Low	Linkage mapping, population study
Mitochondrial DNA	mt-DNA	No	Maternal inheritance	-	-	Multiple haplotypes	-	Maternal lineage
Restriction fragment length polymorphism	RFLP	Yes	Mendelian , Codominance	1 or 2	Single	2	Low	Linkage mapping,
Random amplified polymorphic DNA	RAPD/ AP-PCR	No	Mendelian , dominant	2	Multiple	2	intermediate	Finger printing for population studies ,hybrid identification
Amplified fragment length polymorphism	AFLP	No	Mendelian , dominant	2	Multiple	2	High	population studies Linkage mapping,
Microsatelites	SSR	Yes	Mendelian, Codominance	2	Single	Multiple	High	population studies Linkage mapping, paternity analysis
Expressed sequence tags	EST	Yes	Mendelian, codominance	1	single	2	Low	Linkage mapping, physical mapping ,comparative mapping
Single nucleotide polymorphism	SNP	Yes	Mendelian, codominance	1 or 2	Single	2-4	High	population studies Linkage mapping,
Insertions/deletion	Indels	Yes	Mendelian, codominance	1 or 2	Single	2	Low	Linkage mapping,

RAPD data can contain artifacts and are not fully reproducible (Paterson 1996). However, RAPDs have been ~~be~~ used to generate large numbers of genetic

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markers useful for linkage mapping quickly and cheaply. RAPD polymorphisms can occur due to base substitutions at the primer binding sites or indels (insertions and deletions) region between the sites. The potential power is relatively higher for detection of polymorphism as 5-20 bands may be produced using a given primer pair and multiple sets of random primers can be used to scan the entire genome for differential RAPD bands. Because each band is considered a bi-allelic locus (presence or absence of amplified products), polymorphism information content (PIC) values for RAPD may not be as informative as AFLP because few loci are generated simultaneously. RAPD marker are inherited as Mendelian marker in a dominant fashion (Bachmann, 1994) and scored as present or absent.

In breeding studies, the number of RAPD bands seen in the F1 generation should equal the sum of bands seen in the parents, assuming parental homozygosity at each locus, polymorphic RAPD then segregates in a 3:1 ratio in the F2 population (Liu and Cordes, 2004) and thus do not require prior knowledge of a DNA sequence. Generation of RAPD markers is based on the probability that DNA sequence, homologous to that of a single, short oligonucleotide primer will occur at different sites on opposite strands of a DNA template within a distance that is amplifiable by PCR. When these criteria are met, the single oligonucleotide will prime exponential DNA amplification in a PCR reaction. Polymorphisms result from mutations or indel (insertions and deletions) either at or between the priming sites and are most frequently detected as presence or absence of amplification product. RAPD primers with at least 60 percent G+C content are used to ensure efficient annealing and to avoid internal pairing that can produce PCR artifacts.

Advantages of using RAPD technology

It is relatively simple, cheap, quick, and requires no prior information of target genome. It needs less amount of template and require no radioactive elements and detect good number of polymorphism. And the degree of polymorphism observed is also comparable to RFLP and microsatellite techniques and it may be also converted into a single locus PCR marker-SCAR (Sequence Characterized Amplified Region) have made RAPD. In contrast to protein polymorphisms examining a single locus, RAPD markers are helpful in multilocus fingerprinting (Gwakisa *et al*., 1994). Rothuizen and Wolferen (1994) suggested that the standardization and interpretation of the complex fingerprint pattern can be overcome by careful observation. This technique detects abundant polymorphism resulting from either nucleotide changes in the DNA sequence at the primer binding site or the structural alterations.

Applications of RAPD-PCR

The advantage of using RAPD analysis over other methods of examining polymorphism is its ease and simplicity for identification of very informative markers without prior knowledge of sequence information. It has become a powerful tool in following areas:

- Fingerprinting analysis of various genomes (Welsh and McClelland, 1990; Williams *et al*., 1990).
- Estimation of genetic differences in human beings (Caetano-Anolles *et al*., 1991),
- Identification of intra and inter species differences (Cushwa and Medrano, 1996),
- Gene mapping studies (Reiter et al., 1992 and Levin et al., 1993),
- Population analysis (Bishop et al., 1993; Bostock et al., 1993 and Majiwa et al., 1993),
- Linkage analysis (Martin et al., 1991),
- Determining taxonomic identity (Hadrys et al., 1992; Yeo et al., 2002),
- Assessing kinship relationships (Hadrys et al., 1992; Yeo et al., 2002),
- Analyzing mixed genome samples (Hadrys et al., 1992; Yeo et al., 2002),
- Characterization of livestock breeds (Nagaraja et al., 2003; Bhattacharya et al., 2004; Saifi et al., 2004)

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- Differentiating geographically isolated populations or breeds (Bowditch et al., 1993).
- Determination of paternity and maternity (Scott et al., 1992).
- To detect Y-chromosome specific marker (Gutierrez et al., 1997; Horng et al., 2000; Horng et al., 2004).
- Species-specific markers (Stepniak et al., 2002; Huang et al., 2003)
- Markers specific to genetically diverged populations within species (Anbarasan et al., 2001).
- Detect breed specific markers (Ramesha et al., 2002; Appannavar et al., 2003 and Sharma et al., 2004).

Limitations of RAPD-PCR

Despite of diverse advantages, there are some limitations too, which restrict practical application of RAPD analysis (e.g. dominance, reproducibility, homology inferences and artifact fragments). Dominance is a major limitation of the RAPD approach. RAPD markers are thought to be dominant, with polymorphisms detected as either band presence or absence. Dominant markers are not as efficient as co-dominant markers for population genetics studies (Lewis and Snow, 1992; Lynch and Milligan, 1994). Lynch and Milligan (1994) estimated that 2–10 times more individuals need to be sampled per locus for dominant markers compared to co-dominant markers. Krauss and Peakall (1998) suggested that this disadvantage may be overcome because of the large number of available polymorphisms; typically over 100 polymorphisms per gel-lane are possible. Concerns about reproducibility of RAPDs have limited their wider use in environmental biology. Several studies have reported poor reproducibility for RAPD markers (Weeden et al., 1992; Penner et al., 1993; Skroch and Nienhuis, 1995). Bagley et al. (2001) assessed polymorphism and reproducibility of the two common fingerprinting techniques, RAPD and AFLP in pedigreed populations of rainbow trout (*Oncorhynchus mykiss*) to derive general rules for selective removal of problematic fingerprint bands. They found that by excluding bands that comprised less than1% of total intensity, and by excluding the largest and smallest 10% of the bands, they could achieve nearly 100% reproducibility of AFLP fingerprints. Similar application of band exclusion criteria to RAPD fingerprints did not significantly enhance their reproducibility, and on average at least 15% of RAPD bands were not fully repeatable, heritable, or transmittable. The character homology inferences for RAPDs might be corrected only at the population or variety level (Hseu et al., 1996). More generally, the use of RAPDs as systematic characters has several limitations, and the relevance and taxonomic meaning of RAPD groupings requires careful comparison with results of other sources of data (Hseu et al., 1996). Utility of RAPDs as systematic characters is limited because of difficulties in assessing character homologies.

There are some more limitations such as the nature of the genomic change that is scored is not known and it may not screen the genome as randomly as expected. Given that most RAPD primers have a high GC content, necessary for successful annealing at low temperatures, they may tend to screen GC-rich regions which are not evenly distributed across the genome (Harris, 1999). Finally, due to the random nature of amplification, both nuclear and organelle DNA may be amplified during PCR. For example, it was showed that up to 19% of the bands from the RAPD fingerprints of the Douglas fir (*Pseudotsuga menziesii*) could be attributed to organelle DNA (Aagaard *et al.,* 1998). Unlike RAPD bands generated by nuclear DNA; amplified organelle fragments which cannot be regarded as Mendelian markers, since they are haploid and clonally inherited. Hence, organelle DNA amplification may complicate the interpretation of RAPD fingerprints.

RAPD-PCR of Cattle and Buffalo

There are following several studies pertaining to RAPD-PCR of cattle and buffalo. In one RAPD-PCR analysis a battery of 11 random primers was used for characterization of Bhadawari and Murrah breeds of buffalo (Saifi *et al.*, 2004). The primers OPA-04 and BG-15 resolved Bhadawari specific amplicon whereas primers OPA-14, BG-27 and BG-28

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produced Murrah specific amplicon. The genetic identity index pooled over the primers was 0.59±0.03 between these two breeds. The highest mean average percentage difference (MAPD) estimate (53.9) between the two breeds was obtained with the primer BG-27 and the lowest (14.3) with the primer OPA-01. Singru (1998) also characterized Surti, Jaffarabadi, Murrah and Nagpuri breeds of buffalo using RAPD-PCR. Four out of twelve primers revealed some specific patterns. P2 and A2 primers amplified specific fragments of 1.6 kb and 1.5 kb in Surti and 1.75kb and 1.45 kb in Nagpuri breeds, respectively, while primer ILO-14 gave reproducible bands with molecular weight 4.5 kb in Jaffarabadi and 0.95 kb in Murrah breed. The potential use of RAPD was evaluated as a source of development of alternative genetic markers for studying variation in buffalo (*Bubalus bubalis*) and other related species of the Artiodactyla family Bovidae, in order to ascertain genetic relationships and diversities. Fourteen arbitrary primers were used to amplify DNA fragments in four species such as Indian Zebu cattle (*Bos indicus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*) and goat (*Capra hircus*). A clear and distinct RAPD pattern with a higher level of polymorphism was detected between species, while fewer polymorphisms were found within the species (Appa Rao *et al.,* 1996).

Genetic variation was also studied in Jersey x Red Sindhi cattle, Ongole cattle, Murrah and Surti buffaloes using random primers (Aravindakshan and Nainar 1998). The percentage of polymorphism as revealed by four primers ranged from 26.7 to 85.0 percent. Clustering analysis showed two distinct clusters in cattle whereas no distinct clustering was observed in buffalo breeds. Joshi et al., (2007) studied RAPD-PCR technique for the genetic identity and diversity in two identical cattle breeds Kankrej and Gir with a battery of 16 decamer primers. The phylogenetic tree based on Nei's formula was able to show the genetic similarity and diversity within and between breeds.

Genome analysis of five cattle breeds, viz. Korean, Aberdeen-Angus, Charolais, Hereford and Holstein was conducted using 150 arbitrary primers (Cho and Han, 1994). Ten primers identified polymorphic sequences between and within breeds. Bands specific for Korean cattle were produced with four primers and one primer differentiated Charolais from Holstein. One primer generated multiple bands that were suitable for paternity testing. Genetic similarity coefficients among the breeds were generated from the distribution of 66 polymorphic RAPD bands. The greatest similarity of 0.737 was found between the Korean and Holstein breeds. Breed specific RAPD markers in Zebu cattle of Tanzania were performed (Gwakisa *et al*. 1994). Lee *et al*. (1994) also reported uniform RAPD patterns in Hanwoo, Holstein, Hereford, Angus, Brown Swiss, Limousin and Simmental cattle breeds. The size of PCR products ranged from 0.5 to 2.0 kb. These breeds were clustered in two distinct groups i.e. Hanwoo and other cattle breeds on the basis of RAPD patterns. A product of 1.2 kb was found to be a marker for Hanwoo cattle.

Parentage and pedigree analysis in Holstein dairy cattle (Chung *et al*., 1995), Charolais (Jo *et al*. 1994), Finnish cattle (Kantanen *et al*., 1995) were studied using RAPD-PCR. The results revealed that all the RAPD products in offspring were present in either one or both parents indicating that the RAPD markers were inherited in a Mendelian fashion (Chung *et al*., 1998). RAPD-PCR technology was used for authentication and differentiation of Kangayam cattle by deducing breed specific fragments (Thiagarajan and Thangaraju, 2011). DNA samples of Holstein (Horng *et al.* 2000), Kangayam and Khillari breed (Shivakumar 1997), Deoni breed (Appannavar *et al*. (2003) of cattle were amplified using different random primers. All the primers produced many amplification patterns in random amplified microsatellite polymorphism (RAMPO) fingerprints. A comparative analysis using the RAPD technique was performed in pooled DNA of three cattle breeds (Holstein Friesian, Creole and Hereford) in order to evaluate their amplification patterns(Rincon *et al*., 2000). A total of 215 loci ranging between 300 bp and 2500 bp were amplified

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using 40 primers (Rincon *et al.*, 2000). Specific RAPD bands were identified in the three DNA pools and they were tested in every individual sample of each breed.

Korean cattle breed, Hanwoo was identified (Ahn *et al*. (1999) and sequenced using a specific marker of 519 bp of RAPD-PCR for identification from six other breeds (Yeo *et al*. (2002). Two different repetitive sequences, $(AAC)_5$ and $(GAAGA)_2$, were selected and designed to use specific probe to develop a DNA marker for Hanwoo. When the $(AAC)_5$ probe was applied, the 10 kb specific DNA marker showed in the DNA fingerprinting from 237 of 281 Hanwoo individuals. This novel Hanwoo specific DNA probe is useful to perform the marker–assisted selection for screening Hanwoo purity as a unique genetic source.

RAPD polymorphism and genetic relatedness

Genetic similarity coefficient from the RAPD data can be computed by Jaccard's coefficient and Nei's and Li's coefficient (Lamboy, 1994). Nei's and Li's coefficients had the lowest percentage bias with specific biological meaning that should be preferred for the routine use of RAPD data. RAPD technique was employed to analyse the genetic variation in cattle breeds (Parmar *et al.,* 2004; Guneren et al, 2010), buffalo breeds (Aravindakshan and Nainar 1998), sheep (Kantanen *et al*., 1995; Hentati et al. 2012), goat (Gaali and Satti, 2009 and Parmar *et al.,* 2004), camel (Mahrous et al. 2011), chicken (Jallad et al. 2012) as well as in East-European breeds of swift hounds, Russian Psovyi and Hortyi Borzois (Semenova *et al.,* 2002). A high level of intra- and interbreed variation was found in Russian Psovyi and Hortyi Borzois (Semenova *et al.,* 2002).

Genetic analysis of Hanwoo (Korean cattle) using 150 arbitrary primers was performed (Cho and Han, 1994). Polymorphic sequences between and within breeds were identified using ten primers. Genetic similarity coefficients were highest (0.73) between the Korean and Holstein breeds. Three Zebu cattle breeds with 141 arbitrary primers showed many polymorphic loci (Gwakisa *et al*. 1994). And higher degree of homogeneity within breeds was found than between breeds. Similarly, RAPD-PCR analysis of Korean Native, Holstein, Charolais, Aberdeen-Angus and Hereford cattle revealed band sharing between above said breed and they yielded sharing of 0.82, 0.82, 0.86 and 0.84, respectively; Similarity indices based on the band sharing method varied from 0.76 to 1.0 among animals (Carpio *et al*., 1996). The band sharing and probability of two individuals to exhibit identical fingerprints in the buffalo, as compared to cattle, were considerably low (Ganai *et al.,* 2000).

RAPD was used to characterize Crioulo Lageano cattle breed and to compare it to the Holstein and Nellore breeds. Forty-three primers generated 77 polymorphic bands. Holstein breed presented lowest genetic diversity (0.12) while Crioulo Lageano herd presented the highest (0.31). The observed genetic difference was highest between Nellore and Holstein breeds (0.37) (Spritze *et al*. 2003).

Genetic diversity of Holsten Friesian crossbred cattle from different countries viz. Australia, Denmark, France, Israel and USA were estimated using RAPD assay. Progeny of the same sire within the group were found to have higher similarity coefficient (Kaur *et al.* 2005). Rathi and Tharparkar breeds of cattle using RAPD revealed 86 and 87 percent polymorphic bands respectively. Averages of between breeds genetic similarities pooled over primers were 0.97 and 0.92 according to band frequency and band sharing, respectively, which reflect higher degree of genetic similarity between the two breeds.

Genetic diversity between and within Murrah and Bhadawari breeds using RAPD-PCR revealed high genetic diversity between breeds as compared to within breeds. Ahlawat *et al.*, (2006) studied Murrah and Nili-Ravi breeds for breed characterization and found that out of the battery of 40 decamer primers screened; only 11 amplified the genomic DNA, generating 3000 bp to 150 bp bands. From the 11 random primers, a total of 110 bands were amplified and 78 of these (about 71%) were found to be polymorphic among the population of Murrah breed. The number of polymorphic loci ranged from 2 to 13 within

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Murrah breed with an average of 7.10. From the 8 polymorphic random primers, a total of 57 bands were amplified and 34 of these (about 60%) were found to be polymorphic among the population of Nili-Ravi breed. The number of polymorphic loci ranged from 1 to 10 within Nili-Ravi breed with an average of 4.86. From the 11 polymorphic random primers, a total of 114 bands were amplified and 56 of these (about 49%) were found to be polymorphic between breed. The number of polymorphic loci ranged from 2 to 11 between the breeds with an average of 5.82. Four primers viz. OPB-06, OPI-01, OPI-04 and OPI-07 resolved Murrah breed specific amplicons. Barwar et al., (2008) also used RAPD technique to characterize murrah and bhadawari breeds of buffalo using 9 primers. Murrah and Bhadawari showed 60.72% and 56.36% polymorphism with genetic diversity of 0.81 and 0.80 respectively. Atta et al., (2009) used RAPD markers to detect genetic variations among the six locations using 10 random primers against bulked DNAs of the 100 individuals of each location. Mufti et al., (2009) performed RAPD analysis of Red Chittagong and found that genetic diversity was relatively higher for a prescribed breed and suggested selective breeding for improvement of red Chittagong cattle.

Genetic distance by RAPD-PCR

Genetic divergence was narrower between Murrah and Surti breeds of buffalo than Jersey crossbred and Ongole breeds of cattle by using RAPD-PCR technique (Aravindakshan and Nainar, 1998). In Zebu cattle breeds in Tanzania it was observed that within breed band sharing frequency was greater than between breeds and measurable genetic divergence existed among the three cattle breeds analyzed (Gwakisa *et al*., 1994). Genetic structure and diversity were also investigated by RAPD markers in sheep breeds (Khaldi et al. 2010), indigenous sheep breeds of Balochistan (Tariq et al. 2012) and Turkish sheep breed (Yildiran and Arica, 2009). And it was suggested that RAPD-PCR can effectively be used to determine the genetic distances among the sheep breeds (Tariq et al. 2012).

Ramesha *et al.*, (2002) conducted RAPD assay to identify polymorphic markers in Amritmahal, Krishna Valley, Hallikar, Deoni, Khillari, Ongole and Malnad Gidda breeds of South Indian cattle using 26 primers. Of the 93 RAPD markers obtained, 53 were present in all breeds, 22 were individual specific and 18 were polymorphic for different breeds. Dual-purpose breeds viz., Krishna Valley and Ongole showed less genetic divergence between them as compared to their genetic divergence from draft breeds viz., Amritmahal, Hallikar and Khillari. Malnad Gidda was found to be distantly different from others studied.

Genetic distance analysis was also performed among Korean Native, Holstein, Charolais, Angus and Hereford cattle by RAPD-PCR technique (Jeon *et al.* 1998). The lowest genetic distance was found between Korean Native and Holstein cattle (0.52). The distances among the other breeds ranged from 0.60 between Hereford and Korean Native to 0.75 between Hereford and Charolais. In another index of genetic distance between Rathi and Tharparkar breeds of cattle based on band frequency and band sharing frequency was found to be 0.03±0.01 and 0.09±0.03, respectively (Sharma *et al.* 2004). These results confirm high degree of genetic similarity between two breeds of cattle (Rathi and Tharparkar) was present. Similarly Yunnan DeHong cattle breed is closely related to the Brahman (*Bos indicus*), and the Yunnan DiQing cattle breed is closely related to the Simmental, Shorthorn and MurryGrey (*Bos taurus*) breeds (Yu *et al.* 2004).

In buffalo there was higher genetic similarity of 0.79 and 0.85 within Murrah and Nili-Ravi breeds, respectively as compared to the genetic similarity between (0.62) groups (Ahlawat *et al.*, 2006). Overall low genetic distance was observed within Murrah and Nili-Ravi breeds respectively as comparison to between breeds genetic distance of. The MAPD between breed was 36.64 % and within Murrah and Nili-Ravi were 21.29 % and 14.57 % respectively.

CONCLUSION

RAPD techniques unravels polymorphic pattern in DNA of individuals. This approach is cheaper and easier as compared to other methods of DNA fingerprinting.

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These polymorphisms are further utilized in characterization of different breeds or strains. Breeds of Buffalo and cattle belonging to different geographical region show more genetic diversity or polymorphism than breeds belonging same geographical region. Inter and intra breed variation at DNA sequence level can be useful in planning strategies for breeding and conservation of different breeds of buffalo, cattle and endangered species of other animals. It may be an important tool in species identification, meat speciation, breed and strain identification as well as phylogeny analysis. Moreover it may be also helpful in cataloging and germplasm characterization of livestock.

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