Studies on DNA fingerprinting in Murrah buffaloes using microsatellite markers

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For the present study, blood samples from 35 Murrah dam-progeny pairs and semen samples of their sires were collected. All six primers successfully amplified the genomic DNA from all the animals. The mean observed heterozygosity \( (H_o) \) within the population was found to be 0.5438 and it ranged from 0.375 (CSSM43) to 0.775 (CSSM61). Average expected Levene’s and Nei’s heterozygosity \( (H_{ep}) \) were 0.6209 ±0.0894 and 0.6170 ±0.0889, ranging from 0.5155 to 0.7575 and 0.5123 to 0.7528, for loci CSSM43 and CSSM61, respectively. Both observed and expected heterozygosity was above 0.5, which shows that there is sufficient variability in the population and reflects presence of large number of polymorphic loci in the breed. The mean PIC value was 0.5661, ranging from 0.476 to 0.718, for the loci CSSM43 and CSSM61, respectively. Both observed and expected heterozygosity was above 0.5, which shows that there is sufficient variability in the population and reflects presence of large number of polymorphic loci in the breed. The mean PIC value was 0.5661, ranging from 0.476 to 0.718, for the loci CSSM43 and CSSM61, respectively. The combined PE \( (PEc) \) for all 6 loci was 0.845. Based on PE\( _c \), it was shown that DNA analysis in the examined herd of Murrah buffaloes allows incorrect parentage to be excluded with 84.5% probability.

**Keywords:** DNA fingerprinting, microsatellite markers, Murrah buffalo

**Introduction**

Improvement in domestic livestock is possible by successful selection of superior animals and their correct parentage records. The identification of proven sires has been of utmost importance in animal improvement programmes. Failure to record correct parentage can cause bias in sire evaluation by introducing errors in estimates of heritability and breeding values. Accuracy of genetic parameters and sire evaluation are dependent on correct recording of genetic relationships. Misidentification reduces genetic gain with sire models\(^1\) and may have an even greater effect with animal models that account for all assumed genetic relationships\(^2\). Misidentification rates vary from 1.3 to 30% in cattle\(^3\). Error in recording the sire of a progeny can occur at many stages, such as, handling of semen during freezing, transportation and artificial insemination (AI), in recording of progeny, due to misidentification of sire, mating with local bull after artificial insemination with recorded sire or changing of calf after parturition.

Conventional methods like blood groups, serum proteins and major histocompatibility complex (MHC) antigens can be used for solving parentage problems. However, these tests lack conclusion and need further confirmations. With recent development in area of molecular genetics, DNA marker technology has opened a new possibility for developing sophisticated and reliable methods for solving these problems. There are different approaches based on DNA polymorphism for paternity testing including restriction fragment length polymorphism (RFLPs)\(^4\), DNA fingerprinting using multilocus, minisatellite and oligosynthetic probes\(^5\), and PCR based amplification of minisatellites and microsatellites\(^6\). However, RFLPs generally have low level of polymorphism and low polymorphic information content (PIC), while the DNA fingerprints used in probes are difficult to interpret owing to complex nature of banding pattern. Out of various DNA markers, microsatellite are markers of choice as these are short tandemly repeated sequence of 1-6 bases, highly polymorphic, numerous, codominantly inherited, genetically conserved, distributed throughout the genome and amenable to automation. Microsatellites are also known as short tandem repeat (STR), simple sequence length polymorphism (SSLP). Microsatellite markers not only overcome many of the difficulties, but are also markers of choice for paternity verification and

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individual identification. STRs are used throughout world for DNA finger printing in human beings and also for identification and parentage verification of many farm animal species, mainly horses, cattle and sheep, but also wild animals\(^7\)\(^-\)\(^10\). For efficient breeding programmes, males used should have high genetic merit for traits of economic importance. Dairy breeding industry relies heavily on progeny testing for genetic improvement and, therefore, correct identification of sires is of immense importance. To date, no estimates are available on the reliability of sire identification in Indian buffalo dairy herds. Therefore, verification of parentage will serve as a valuable tool for success of progeny testing program.

The purpose of this study was to develop DNA fingerprinting technique for individual animal identification and to assess genetic divergence in Murrah buffaloes using microsatellite markers.

**Materials and Methods**

**Genomic DNA Isolation**

Blood samples from 35 dam-progeny pairs of Murrah breed and semen samples of their sires were collected. The high mol wt genomic DNA was isolated from blood samples\(^11\) and from semen samples\(^12\) using the earlier protocols with slight modification, like washing of DNA pellet with 70% ethanol was done at 12000 rpm instead of 11000 rpm.

**Evaluation of Quality and Quantity of Genomic DNA**

The quality of isolated genomic DNA was evaluated by agarose gel electrophoresis using 0.7% agarose gel and 1× TAE buffer. The concentration of DNA was also checked by UV spectrophotometer taking optical density (OD) at 260 and 280 nm. The 260/280 nm absorbance ratio for all the samples ranged from 1.7 to 1.9, indicating high quality DNA.

**Microsatellite-PCR Optimization**

The PCR reaction mixture consisted of 25 mM MgCl\(_2\), 10 mM dNTPs, 1 U Taq polymerase, 2 pmole primer with 30 ng of genomic DNA were finalized to be the components of the PCR mix. The amplification was carried out for 33 cycles with initial denaturation at 94°C for 5 min, second denaturation for 45 sec at 94°C, annealing at 58°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min. Six microsatellites used in the present study were CSSM61\(^13\), ILSTS017\(^14\), ILSTS28\(^14\), ETH152\(^14\), CSSM43\(^15\) and CSSM22\(^15\).

**Capillary Electrophoresis**

The fluorescently end-labeled microsatellite PCR products (with fluorescent dye: FAM, HEX, TAMRA) were run on ABI Prism 3130XL capillary based genetic analyzer and analyzed in the presence of GS LIZ 500 mol wt standards labeled with the fluorescent dye LIZ (PE- Applied Biosystems). The raw data were collected using ABI 3130XL Data Collection Software version 4.0. Microsatellite fragment sizing was performed by the Gene Mapper\textsuperscript{TM} version 4.0 (Applied Biosystems) as shown in Fig. 1.

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![Fig. 1—From top to bottom showing peaks of sire, dam and progeny, respectively w.r.t. locus ILSTS17 by GeneMapper\textsuperscript{TM} version 4.0 software](image-url)
Statistical Analysis
The genetic parameters like allele number, allele frequency, observed and expected heterozygosity, polymorphic information content and exclusion probability were obtained using POPGENE version 1.31\textsuperscript{10} and Cervus 3.0 softwares.

Results and Discussion
Microsatellite-PCR Analysis
All six primers successfully amplified the genomic DNA from all the animals. The amplifications were reproducible and distinct. The characteristics of the amplification profiles using 6 microsatellite primers have been presented in Table 1 and Fig. 2. The alleles varied in their size from 97-254 bp. All the 6 primers detected polymorphism among the population. From the 6 primers, a total of 38 alleles were scored. The number of alleles ranged from 3 for ETH152 to 11 for CSSM61 with an average of 6.33±2.8. The frequency of these alleles varied according to locus (Table 1).

Table 1—Amplification profile of different primers

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Approx. size of PCR product</th>
<th>Size on 2% agarose gel (bp)</th>
<th>Size range by Gene Mapper (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CSSM61</td>
<td>126</td>
<td>97-125</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ETH152</td>
<td>198</td>
<td>192-198</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ILSTS17</td>
<td>120</td>
<td>114-120</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ILSTS28</td>
<td>150</td>
<td>142-172</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CSSM22</td>
<td>212</td>
<td>205-217</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>CSSM43</td>
<td>254</td>
<td>240-256</td>
<td></td>
</tr>
</tbody>
</table>

Heterozygosity
The mean observed $H_o$ within the population found to be 0.5438 and it ranged from 0.375 (CSSM43) to 0.775 (CSSM61). Average Levene’s and Nei’s $H_{ep}$ were 0.6209±0.0894 and 0.6170±0.0889, ranging from 0.5155 to 0.7575 and 0.5123 to 0.7528, for loci CSSM43 and CSSM61, respectively. Both $H_o$ and $H_{ep}$ were above 0.5, which shows that there is sufficient variability in the population and reflects presence of large number of polymorphic loci in the breed. As there is no significant difference between $H_o$ and $H_{ep}$, random mating is expected in the population.

PIC
PIC value, which indicates the informativeness of a marker, was calculated for all the 6 loci. The mean PIC value was 0.5661, ranging from 0.476 to 0.718, for the loci CSSM43 and CSSM61, respectively, which revealed that the set of 6 polymorphic bovine microsatellite loci described in this study could be used to study parentage verification and genetic polymorphism in Murrah.

Ewens Watterson Test for Neutrality
To estimate the selection on each locus, the Ewens Watterson test for neutrality was conducted in the present study. The overall test for neutrality showed that microsatellite locus ILSTS17 was not neutral as the observed F values for these markers out laid the lower and upper limits of 95% confidence region of expected F values (Table 3). This shows that this locus may be associated with some coding region of the genome or linked to production trait.

Hardy-Weinberg Equilibrium (HWE)
In the present study, two loci (ILSTS28 & CSSM43) showed deviations from the HWE, indicating non-random mating. The results are further supported by the significant differences ($P \leq 0.05$) in the $H_o$ and $H_{ep}$. For the remaining loci, an excess of heterozygotes was observed, indicating the occurrence of random mating.

PE and Parentage Testing
PIC and degree of heterozygosity are indirect indicators of the usefulness of a genetic marker for parentage testing. A direct measure is the PE for incorrect parentage assignment. In the present study, the PE ranged from 0.0994 to 0.5535 for the loci CSSM43 and CSSM61, respectively. The combined PE ($P_{E_c}$) for all 6 loci was 0.845. Based on $P_{E_c}$, it was shown that DNA analysis in the examined herd of Murrah buffaloes allows incorrect parentage to be excluded with 84.5% probability.

In conclusion, the present study shows that microsatellites are highly polymorphic and proved...
very useful for parentage verification in Murrah buffalo population. The study can be extended to include more microsatellite loci with high PE and increased sample size to validate the results.

References

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### Table 2—Allele frequency analysis computed with Cervus 3.0

<table>
<thead>
<tr>
<th>Locus</th>
<th>Observed heterozygosity (H&lt;sub&gt;o&lt;/sub&gt;)</th>
<th>Expected heterozygosity (H&lt;sub&gt;ep&lt;/sub&gt;)</th>
<th>Polymorphic information content (PIC)</th>
<th>Exclusion probability (PE)</th>
<th>Frequency with null allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSSM61</td>
<td>0.775</td>
<td>0.758</td>
<td>0.718</td>
<td>0.553</td>
<td>+0.0241</td>
</tr>
<tr>
<td>ETH152</td>
<td>0.450</td>
<td>0.578</td>
<td>0.502</td>
<td>0.107</td>
<td>+0.1067</td>
</tr>
<tr>
<td>ILSTS17</td>
<td>0.575</td>
<td>0.697</td>
<td>0.630</td>
<td>0.261</td>
<td>-0.0135</td>
</tr>
<tr>
<td>ILSTS28</td>
<td>0.488</td>
<td>0.603</td>
<td>0.560</td>
<td>0.177</td>
<td>+0.0535</td>
</tr>
<tr>
<td>CSSM43</td>
<td>0.375</td>
<td>0.515</td>
<td>0.476</td>
<td>0.099</td>
<td>+0.4858</td>
</tr>
<tr>
<td>CSSM22</td>
<td>0.600</td>
<td>0.574</td>
<td>0.510</td>
<td>0.29</td>
<td>-0.0337</td>
</tr>
</tbody>
</table>

### Table 3—Ewens-Watterson test for neutrality for different loci

<table>
<thead>
<tr>
<th>Locus</th>
<th>Observed sum of squared allele frequencies (Observed F)</th>
<th>Expected sum of squared allele frequencies (Expected F)</th>
<th>Min</th>
<th>Max</th>
<th>Mean*</th>
<th>SE</th>
<th>Lower limit (L95)</th>
<th>Upper limit (U95)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSSM61</td>
<td>0.2472</td>
<td>0.0909</td>
<td>0.8828</td>
<td>0.2684</td>
<td>0.0089</td>
<td>0.1513</td>
<td>0.5149</td>
<td></td>
</tr>
<tr>
<td>ETH152</td>
<td>0.4253</td>
<td>0.3333</td>
<td>0.9753</td>
<td>0.6863</td>
<td>0.0366</td>
<td>0.3576</td>
<td>0.9752</td>
<td></td>
</tr>
<tr>
<td>ILSTS17</td>
<td><strong>0.3070</strong></td>
<td>0.2500</td>
<td>0.9632</td>
<td>0.6033</td>
<td>0.0352</td>
<td>0.3177</td>
<td>0.9388</td>
<td></td>
</tr>
<tr>
<td>ILSTS28</td>
<td>0.4007</td>
<td>0.1429</td>
<td>0.9278</td>
<td>0.4054</td>
<td>0.0197</td>
<td>0.2154</td>
<td>0.7505</td>
<td></td>
</tr>
<tr>
<td>CSSM43</td>
<td>0.4877</td>
<td>0.1429</td>
<td>0.9278</td>
<td>0.4008</td>
<td>0.0210</td>
<td>0.2131</td>
<td>0.7691</td>
<td></td>
</tr>
<tr>
<td>CSSM22</td>
<td>0.4299</td>
<td>0.1667</td>
<td>0.9395</td>
<td>0.4606</td>
<td>0.0257</td>
<td>0.2402</td>
<td>0.8458</td>
<td></td>
</tr>
</tbody>
</table>

*Values calculated using 1000 simulated samples
Observed F value in bold outlies the lower limit of Expected F values