APPLICATION OF MICROSATELLITE MULTIPLEX PANELS FOR PARENTAGE VERIFICATION IN WATER BUFFALOES AND CATTLE

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ABSTRACT

The Philippine Carabao Center has made available the country's first paternity testing service for buffaloes and cattle that aimed to address pedigree errors that could lower the accuracy of breeding value prediction in genetic evaluations. The testing was upgraded from a singleplex into a multiplex panel to make it more efficient. Thus, this study aimed to utilize 16 microsatellite markers developed into four multiplex panels to conduct parentage verification in different buffaloes and cattle breeds. Also, the study conducted throughput and cost analysis to measure the time-efficiency and cost-effectiveness of the test. DNA fragments were amplified using polymerase chain reaction (PCR) and then genotyped through fragment analysis using the ABI 3500xL Genetic Analyzer. Allele sizing and parentage assignment were done using GeneMapper and Cervus software. At 99% strict confidence, of the 20 offspring examined, 3 had matched parent-pairs, 8 had matched dams, 4 had matched sires, and 1 had reassigned parent-pair. However, the findings have revealed that certain of the offspring's assigned parents do not conform to their pedigree records. These findings emphasize the sensitivity and reliability of multiplex panels in detecting mismatches and correctly assigning true parentage. Hence, applying multiplex panels in genetic testing could achieve high throughput with minimal resource input. This initiative significantly enhances genetic evaluation systems by ensuring accurate pedigree information and promoting efficient genotyping schemes.

Keywords: fragment analysis, genetic evaluation, microsatellite markers, multiplex panel, pedigree

INTRODUCTION

Parentage verification is a critical component of livestock breeding programs, providing accurate genetic information that supports selective breeding, genetic improvement, and overall herd management. Misassigned parentage can lead to incorrect pedigree records, which may in turn affect genetic evaluations, skew breeding value estimates, and hinder the effectiveness of breeding programs. Microsatellite (MS) markers are widely recognized as powerful tools for parentage verification due to their high polymorphism, co-dominant inheritance, and ease of scoring (Van Eenennaam *et al.*, 2007; Ashton *et al.*, 2016).

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Traditionally, parentage testing has relied on singleplex PCR methods, which analyze one marker at a time, making it a time-consuming and costly approach when multiple markers are needed. However, the development of multiplex panels, which allow for the simultaneous amplification of multiple markers in a single PCR reaction, has significantly improved both efficiency and throughput in genetic testing (Clarke *et al.*, 2014). These multiplex panels enable faster and more cost-effective parentage analysis without compromising accuracy. Furthermore, studies have shown that microsatellite (MS) markers used in multiplex panels can achieve comparable accuracy to singleplex methods while reducing the resources required for large-scale testing (Izadpanah *et al.*, 2018; Vallecillos *et al.*, 2022).

This process is followed by fragment analysis to determine allele sizes, enabling efficient genotyping through automated systems. Fragment analysis is widely recognized as a reliable method for sizing alleles in parentage verification, as it minimizes human error and ensures accuracy in allele assignment (Rico *et al.*, 1996; Jones *et al.*, 2010).

In cattle and buffalo breeding, accurate parentage verification is essential for ensuring the integrity of genetic evaluations, which inform breeding decisions and drive genetic gains in traits of interest (Van Eenennaam *et al.*, 2007). By verifying parentage with multiplex MS markers, breeding programs can improve the reliability of pedigree information, reduce the risk of inbreeding, and promote the genetic improvement of herds (Junqueira *et al.*, 2017). As such, the application of MS markers in multiplex panels is an invaluable advancement in genetic testing, with broad implications for sustainable livestock breeding and productivity (Husein *et al.*, 2024).

These advancements highlight the importance of maintaining accurate pedigree data in breeding programs, as errors or gaps in genealogical information can undermine genetic progress and the effectiveness of selection programs aimed at improving desirable traits. Thus, the study aimed to utilize 16 microsatellite markers developed into multiplex panels to conduct parentage verification in Philippine buffaloes and cattle breeds. Also, the study compares the time efficiency and cost-effectiveness of these multiplex panels to traditional singleplex methods.

MATERIALS AND METHODS

DNA extraction

A Promega Extraction Kit was used to isolate genomic deoxyribonucleic acid (gDNA). One thousand microliters of 0.14 M Ammonium Chloride were used to lyse and wash 500 µL blood samples following the 1:2 ratios. Cell lysis, nuclei lysis, and protein precipitation were utilized to isolate the gDNA from blood debris and other molecules that could cause impurities. Further, isopropanol and ethanol were used to wash the isolated gDNA. After washing, the solution was removed, and the pellet was air-dried in the fume hood. DNA rehydration solution was used to reconstitute the DNA and stored at 4°C.

Polymerase chain reaction (PCR) amplification

gDNA was amplified using an ESCO thermal cycler through the process of polymerase chain reaction. The PCR component includes sdH_2O, 4 µL of 10x PCR buffer with 15mm $MgCl_2$, 1.6 µL of 2.5mM dNTPs, 10ng Forward and Reverse Primers, 0.4 µL of *Taq* Polymerase, and 2 µL of ≥50ng DNA. In this study, sixteen labeled primers (Table

1) were used and categorized into panels based on their dye type, size, GC content, and co-amplification compatibility. Specific details of the markers are documented in the VeriSire utility model, registered under No. 2/2020/050497. After several trials, the following PCR profile was the optimum setting for co-amplification to all panels which subsequently follows initial denaturation at 95°C for 10 minutes, 30 cycles of denaturation at 95°C for 15 seconds, annealing temperature at 58°C for 30 seconds, extension at 72°C for another 30 seconds, and then with a final extension of 72°C for 10 minutes. After having PCR products, it was then stored at 4°C.

Table 1. Loci location, allele size range and fluorescent dye used

LOCI	PANEL	CHROMOSOME NO.	ALLELE SIZE RANGE	FLUORESCENT DYE
1	A	9	102-130	6-FAM
2	A	4q	203-213	6-FAM
3	A		<100	NED
4	A		138-152	VIC
5	B	3	118-142	6-FAM
6	B	16	232-254	6-FAM
7	B	3q	127-162	NED
8	B		170-200	VIC
9	C	1q	131-161	6-FAM
10	C		129-145	VIC
11	C	11	163-187	PET
12	C		119-139	NED
13	D	10	90-130	VIC
14	D	17	130-180	6-FAM
15	D	22	93-115	VIC
16	D	3p	217-239	VIC

Gel documentation

PCR products were loaded on an agarose gel to check and quantify the amplicons. The gel was prepared using 1x Tris-Acetate-EDTA (TAE) buffer, 2% agarose gel, 1% Low Melting Sieve Agarose, and 2% dye. Fifty base-pair ladders were used to determine the sizes of amplicons. Mupid-Ex gel electrophoresis was used to run the PCR products, while Enduro GST Gel documentation was used to view gel products.

Fragment analysis

PCR reaction will be diluted subjectively from 1-100 µL. Ten microliter mixture of highly deionized (Hi-Di™) formamide and GeneScan 600 LIZ size standard was used to resuspend the sample before electrokinetic injection on Capillary Electrophoresis (CE) using Applied Biosystems 3500xl Genetic Analyzer by Thermo Fisher Scientific.

Gene mapping and parentage analysis

Loci sizing was analyzed using GeneMapper® software. The likelihood ratio for parentage assignment was done using Cervus version 3.0.7 (Marshall, 2014).

RESULTS AND DISCUSSION

The genetic testing of 100 reference samples for buffalo and cattle, reveals significant insights into the accuracy of parentage records across different breeds. Table 2 provides detailed information on various offspring of buffaloes and cattle, highlighting their breed, recorded parentage, and assigned parentage. Each offspring is identified by a unique ID, with species including Riverine Buffalo, Swamp Buffalo, Beef Cattle, and Dairy Cattle. For each animal, the recorded column lists the dam (mother) and sire (father) IDs as per registration records, while the assigned column displays the results of genetic testing. The comparison reveals instances where recorded and assigned parents matched, affirming the accuracy of pedigree records for those animals.

Based on Table 2, among the 20 offspring tested at 99% strict confidence, 3 offspring matched with their parent-pair, 8 matched with their dam, and 4 matched with their sire. However, at the same confidence level, 9 offspring had unmatched sires, 1 had an unmatched parent-pair, and 3 had unassigned parentage for either parent. To provide further context, Table 3 outlines three types of parentage conditions.

First, in cases of confirmed parentage, the reported parents matched the most likely genetic parents, verifying the correct pedigree (e.g., AnBuff_O2, AnBuff_O8, and AnCatt_O4). Second, in instances where the reported parents did not match the most likely genetic parents, the reassignment of the correct parents was supported by strong evidence. This included a positive LOD score and no more than two mismatched alleles between the offspring and candidate parents at 99% strict confidence (e.g., AnBuff_O1, AnBuff_O3, and AnCatt_O5). Finally, unresolved parentage occurred when the reported parents were not the true genetic parents. This was characterized by more than two mismatched alleles and a negative LOD score of 99% strict confidence. In such cases, true parents could not be assigned due to the absence of their genetic profiles in the database or because they had not been sampled (e.g., AnCatt_O5, AnCatt_O6, and AnCatt_O7).

The findings indicate potential gaps in genealogical records, possibly resulting from incomplete data entry during the registration process or inaccuracies in earlier generational records. Such discrepancies underscore the necessity of continuous validation and periodic updates to pedigree data to maintain its accuracy in breeding programs (Srikanth *et al*., 2024). Incomplete or erroneous genealogical information can pose significant risks to genetic progress and herd management, as unverified parentage may compromise the effectiveness of selection programs designed to enhance desirable traits such as meat quality, growth rate, and disease resistance (Moreno *et al.*, 2024).

Generally, Cervus evaluates the likelihood ratio (LR) for each candidate parent regardless of the recorded identity of the dam and sire (Marshall *et al.*, 1998). Therefore, for each offspring, Cervus considers all potential pairings of candidate individuals from the given set of probable parents, called parent pairs. It compares the offspring's genetic data (such as microsatellite genotypes) with that of each candidate parent pair.

The LR is calculated after Cervus identifies mismatches between the offspring genotypes and the candidate parent pair. A mismatch occurs when the alleles at a particular

locus in the offspring do not match those from the corresponding locus in the candidate parent pair. Mismatched loci decrease the LR because they suggest a lower probability of parentage. Based on the computed LR, each candidate pair is ranked, and then the parent pair with the highest LR is typically considered the most likely parent (Kalinowski *et al.*, 2007).

Table 2. Parentage of buffalo and cattle breeds based on recorded pedigree as compared to assigned parents.

NO.	OFF-SPRING ID	BREED	RECORDED PARENTS		ASSIGNED PARENTS	
			DAM	SIRE	CANDIDATE MOTHER ID	CANDIDATE FATHER ID
1	AnBuff_O1	Swamp Buffalo	AnBuff_D5	AnBuff_S3	AnBuff_D5	AnBuff_S1
2	AnBuff_O2	Swamp Buffalo	AnBuff_D7	AnBuff_S2	AnBuff_D7	AnBuff_S2
3	AnBuff_O3	Swamp Buffalo	AnBuff_D3	AnBuff_S1	AnBuff_D7	AnBuff_S3
4	AnBuff_O4	Swamp Buffalo	AnBuff_D2	AnBuff_S2	AnBuff_D1	AnBuff_S2
5	AnBuff_O5	Swamp Buffalo	AnBuff_D10	AnBuff_S5	AnBuff_D11	AnBuff_S13
6	AnBuff_O6	Swamp Buffalo	AnBuff_D11	AnBuff_S7	AnBuff_D11	AnBuff_S12
7	AnBuff_O7	Swamp Buffalo	AnBuff_D15	AnBuff_S6	AnBuff_D15	AnBuff_S12
8	AnBuff_O8	Swamp Buffalo	AnBuff_D16	AnBuff_S4	AnBuff_D16	AnBuff_S4
9	AnBuff_O9	Riverine Buffalo	AnBuff_D12	AnBuff_S8	AnBuff_D12	AnBuff_S12
10	AnBuff_O10	Riverine Buffalo	ND	AnBuff_S10	ND	AnBuff_S11
11	AnBuff_O11	Riverine Buffalo	ND	AnBuff_S10	ND	AnBuff_S11
12	AnBuff_O12	Riverine Buffalo	ND	AnBuff_S9	ND	AnBuff_S11
13	AnBuff_O13	Riverine Buffalo	ND	AnBuff_S9	ND	AnBuff_S11
14	AnCatt_O1	Beef Cattle	ND	AnCatt_S5	ND	AnCatt_S5
15	AnCatt_O2	Beef Cattle	ND	AnCatt_S1	ND	AnCatt_S1
16	AnCatt_O3	Beef Cattle	ND	AnCatt_S8	ND	AnCatt_S8
17	AnCatt_O4	Dairy Cattle	AnCatt_D1	AnCatt_S12	AnCatt_D1	AnCatt_S12
18	AnCatt_O5	Dairy Cattle	AnCatt_D2	AnCatt_S13	AnCatt_D2	No Assigned Parents
19	AnCatt_O6	Dairy Cattle	AnCatt_D3	AnCatt_S12	AnCatt_D3	No Assigned Parents
20	AnCatt_O7	Dairy Cattle	AnCatt_D5	AnCatt_S14	No Assigned Parent	AnCatt_S14

Cervus presents the likelihood ratio as a LOD score, which is the natural logarithm of the likelihood ratio. A negative LOD score suggests that the candidate parent is unlikely to be the true parent, often due to multiple mismatches at genetic loci with the offspring. Candidate parents who are unrelated to the offspring receive negative LOD scores. Conversely, a positive LOD score indicates a higher likelihood that the candidate parent is indeed the true parent and establishes parentage assignment. True parents consistently show a positive LOD score with mostly no mismatches or at most two. In contrast, a LOD score of zero indicates an equal likelihood that the candidate parent is or is not the true parent (Marshall *et al.*, 1998). Additionally, the software performs simulations to establish confidence levels, providing probabilistic thresholds (99% strict confidence or 80% relaxed confidence) that enhance the reliability of assignments.

Table 3. Parentage analysis of buffalo and cattle breeds based on paired loci mismatch, strict confidence, and logarithm of the odds.

Offspring ID	Loci typed	Strict Confidence	Candidate mother ID	Pair loci mismatching	Pair LOD score	Remarks	Candidate father ID	Pair loci mismatching	Pair LOD score	Remarks
AnBuff_O1	16	99%	AnBuff_D5	2	1.73E+01	True Parent	AnBuff_S1	4	4.50E+00	
AnBuff_O2	16	99%	AnBuff_D7	2	2.00E+01	True Parent	AnBuff_S2	6	-8.50E+00	
AnBuff_O3	16	99%	AnBuff_D7	2	1.66E+01	True Parent	AnBuff_S3	2	1.44E+01	True Parent
AnBuff_O4	16	99%	AnBuff_D1	3	1.02E+01		AnBuff_S2	4	8.29E+00	True Parent
AnBuff_O5	16	99%	AnBuff_D11	0	1.90E+01	True Parent	AnBuff_S13	5	-1.11E+01	
AnBuff_O6	16	99%	AnBuff_D11	2	7.45E+00	True Parent	AnBuff_S12	5	-1.44E+01	
AnBuff_O7	16	99%	AnBuff_D15	0	1.15E+01	True Parent	AnBuff_S12	5	-1.68E+01	
AnBuff_O8	16	99%	AnBuff_D16	0	1.62E+01		AnBuff_S4	1	4.63E+00	True Parent
AnBuff_O9	16	99%	AnBuff_D12	0	1.66E+01	True Parent	AnBuff_S12	3	-7.91E+00	
AnBuff_O10	16	99%	ND	ND	ND		AnBuff_S11	6	-2.12E+01	
AnBuff_O11	16	99%	ND	ND	ND		AnBuff_S11	6	-2.19E+01	
AnBuff_O12	16	99%	ND	ND	ND		AnBuff_S11	6	-2.22E+01	
AnBuff_O13	16	99%	ND	ND	ND		AnBuff_S11	6	-2.22E+01	
AnCatt_O1	16	99%	ND	ND	ND		AnCatt_S5	1	4.23E+00	True Parent
AnCatt_O2	16	99%	ND	ND	ND		AnCatt_S1	0	7.57E+00	True Parent
AnCatt_O3	16	99%	ND	ND	ND		AnCatt_S8	0	1.10E+01	True Parent
AnCatt_O4	16	99%	AnCatt_D1	0	8.86E+00		AnCatt_S12	1	3.54E+00	True Parent
AnCatt_O5	16	99%	AnCatt_D2	1	8.48E+00		No Assigned Parent	No Assigned Parent	No Assigned Parent	
AnCatt_O6	16	99%	AnCatt_D3	0	1.17E+00		No Assigned Parent	No Assigned Parent	No Assigned Parent	
AnCatt_O7	16	99%	No Assigned Parent	1			AnCatt_S14		5.78E+00	

Results were generated using Cervus software version 3.0.7

Table 4. Parentage Analysis workflow per sample.

PROCESS	TIME
DNA Extraction	30 mins
PCR Amplification	150 mins
Gel Electrophoresis	45 mins
Amplification Scoring	1 min
Fragment Analysis	40 mins
Allele Scoring	5 mins
Parentage Analysis	5 mins
TOTAL	276 mins / 4hrs 36 mins

Table 5. Comparison of resource input per mple using singleplex and multiplex.

16 MARKERS PER SAMPLE	SINGLEPLEX	MULTIPLEX
Reaction	16	4
Gel	16	4
Gel Grades	16	4
Dilution	16	4
Capillary Electrophoresis	16	4
Gene Mapping	1:1	1:4

The efficiency and cost-effectiveness of genetic analysis methods are critical for high-throughput environments, such as parentage testing in livestock. Table 4 demonstrates that the process workflow for parentage analysis per sample takes approximately 4 hours and 36 minutes. This includes various stages such as DNA Extraction, PCR amplification, gel electrophoresis, and allele scoring. When considering singleplex analysis, the resource input ratio is 1:16, making it significantly more expensive than multiplex analysis, which operates at a 1:4 ratio of preparation and input (refer to Table 5). The high resource input for singleplex analysis is primarily due to the necessity of conducting multiple reactions, gel preparations, and dilutions for each marker individually, whereas multiplex analysis consolidates these processes, significantly reducing the resource and time requirements.

The cost efficiency of multiplex analysis is further highlighted in Table 6, which presents the estimated cost of parentage analysis resources per sample. The total cost per sample using singleplex analysis is PhP 2617.33, whereas multiplex analysis reduces this to PhP 1209.33. This significant cost reduction, amounting to 53.80% savings, is attributable to the consolidation of processes in multiplex analysis, which decreases the number of reactions, gel preparations, and other resource-intensive steps.

The findings align with existing literature that underscores the advantages of multiplex analysis over singleplex analysis in terms of cost and efficiency. Studies have shown that multiplexing can significantly enhance throughput and reduce operational costs without compromising data quality (Sint *et al.*, 2012; Hawkins and Guest 2017). The reduced preparation time and resource input in multiplex analysis, as detailed in Tables 4

and 5, make it a preferable choice for laboratories aiming to optimize their workflows and budget allocations.

However, it is important to note that developing multiplex assays can be more complex and time-consuming due to issues like cross-reactivity and the need for a wide dynamic range (Hayden *et al.*, 2008). Despite these challenges, the benefits of multiplexing generally outweigh the drawbacks, making it a preferable choice for many laboratories aiming to enhance their genotyping schemes and ensure accurate genetic evaluations.

Table 6. Estimated cost of parentage analysis resources per sample.

COSTING	SINGLEPLEX	MULTIPLEX
DNA Extraction	300	300
PCR Amplification	597.33	149.33
Fragment Analysis	1,280	320
Utilities, Maintenance, and Labor	247.2	247.2
Other MOOEs	192.8	192.8
Total	**PhP 2,617.33**	**PhP 1,209.33**

CONCLUSION

The study confirmed that there is no difference in the parentage results between singleplex and multiplex methods regarding allele size calling and parentage assignment. Adopting the multiplex protocol for parentage verification significantly improves cost-effectiveness and efficiency, benefiting various clients, including regional centers and farmers, and the operating unit itself. Multiplex analysis, verified by Cervus software, provided accurate parentage assignments while reducing costs and resource use, making it the preferred method for high-throughput genetic testing.

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