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Optimization of Autosomal STR Markers for Equine Genotyping Using Multiplex PCR

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ABSTRACT

The investigation of horse lineage was of paramount importance in the registration of different breeds, trade, and formulation of studbooks. The pioneering technique of DNA fingerprinting emerged as the first highly responsive method reliant on DNA for individual identification and the examination of genetic affiliations. Microsatellites were a valuable tool for analyzing the genetic variations present among different horse breeds. The International Society for Animal Genetics (ISAG) has endorsed a set of 17 specific Short Tandem Repeats (STRs) for the equine identification, although these can be quite expensive to obtain through commercially available multiplex kits. **Objective:** To determine five autosomal STR markers (HMS6, HMS7, ASB23, VHL20, and LEX14) were optimized using multiplex PCR for equine genotyping. **Methods:** DNA was extracted from a Thoroughbred horse blood sample via an organic extraction method. Sensitivity analysis determined the optimal PCR concentration. Genotyping was performed on the ABI PRISM® 3100XL, and data were processed with Gene Mapper ID 3.2v software. **Results:** The optimal conditions for multiplex PCR of HMS6, HMS7, ASB23, VHL20, and LEX14 primers were 60°C annealing temperature, 3ng DNA concentration and 6µM primer concentration. A 12.5µL PCR reaction volume was recommended for cost efficiency. **Conclusions:** The results of this research have the potential to create a cost-effective, regionally produced multiplex PCR kit. This kit would be designed for analyzing parentage lineage within the Equine family in Pakistan, incorporating ISAG-recommended markers: VHL20, HMS6, HMS7, ASB23, and additionally LEX14. It could significantly streamline the import and export of horses in Pakistan.

INTRODUCTION

Horses are truly remarkable beings, acclaimed for their unparalleled fusion of speed and potency. Horses *(Equus caballus)* hold the distinction of being the most ancient animals intertwined with human civilization, exerting a profound influence on human culture. The earliest evidence of the pivotal role of horses in human culture within the Eurasian context dates back around 15,000 years ago and originates from the southern region of France and adjacent areas. This evidence is manifested through archaeological findings, including faunal remains and cave paintings [1]. The classification of horses was established based on criteria encompassing their size, body morphology, physiology, and anatomical features. Among horse breeds the Thoroughbred is the valuable breed of light horses. The equestrian industry supports this breed because of its great physiological qualities i.e. in racing, jumping, fox hunting and steeplechase [2]. The process of breeding involved the introduction of Arabian, Barb, or Turk stallions to mate with indigenous. English horse mares, resulting in the creation of the Thoroughbred horse breed [3]. Thoroughbred was originally a crossbred breed so it is necessary to record back to its ancestry. For around 200 years, a stud book has been used to keep track of the family history of this breed and to make sure the breed stays pure and healthy [4]. The inaugural publication of the Thoroughbred Stud Book took place in England in the year 1791 [5]. When horses are imported and exported, a parentage analysis is necessary. At the outset in 1960, the verification of equine parentage relied on blood typing antigens like EAC, EAA, EAD, AIB, and PGD [6]. Nevertheless, with the progression of molecular technology, a shift occurred towards utilizing STR markers

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instead of blood typing antigens. Presently, the examination of genetic variance in horse parentage is conducted through STR genotyping, offering both precision and efficiency in performing parentage analysis as well as assessing genetic diversity. Short Tandem Repeats (STRs) are sequences of DNA consisting of 2 to 6 base pairs, which are dispersed across the genome and varies from person to person [7]. This advancement has led to improved precision in confirming parentage. Microsatellite genotyping is widely employed in forensic applications and parentage investigations, as well as in clinical settings, agriculture, animal breeding, and the food industry [8]. From 2001, Genetic testing facilities were required to conduct testing on TB horses using autosomal DNA STR loci by following the guidelines established by the International Society for Animal Genetics and (ISBC). The PCR molecular technique is employed to amplify STR markers, facilitating parentage verification [9]. Subsequently, the amplified PCR product undergoes genotyping, resulting in data that is presented in the form of peaks [10]. In Korea, the parentage verification of Thoroughbred (TB) horses was carried out utilizing a set of 14 microsatellites, which are part of the ISAG panel [11]. To evaluate phylogenetic relationships and genomic variability in horses, ten microsatellite markers (HTG10, ASB2, ASB17, HMS3, AHT4, HMS7, VHL20, AHT05, HTG4, and HMS6) were examined in Pakistan [12]. In Colombia certification and filiation tests were accurately performed by utilizing 17 STRs markers recommended by ISAG [13]. Meanwhile in Syria, an assessment of the genetic diversity and lineage of horses was conducted using 16 STR markers as suggested by ISAG [14]. This study has been done to develop multiplex PCR of five Thoroughbred horses STR markers (VHL20, HMS6, HMS7, ASB23 and LEX 14). Because commercially available kits are expensive, it's really important to create a cheaper local kit for accurately testing horse's parentage. To address, this study has meticulously adjusted several critical factors in a multiplex PCR setup for the recommended ISAG STR markers. These factors include the annealing temperature, minimum template DNA and primer concentrations, as well as the optimal PCR volume. This study will provide a platform to establish a mega multiplex system that will lead to establish the equine stud book in the country. Creating a stud-book through DNA profiling would facilitate the seamless import and export of horses in Pakistan. Which would ultimately contribute to the economic advancement of the country.

METHODS

Sample collection: Blood samples of horses ranging in age approximately from 6 to 7 years and weight from 400 to 500 kg, were taken from healthy and active individuals from Horse shed UVAS Lahore Pakistan. Intravenously drawn blood samples of 3 ml were taken from the jugular vein and placed in pre- labeled EDTA vacutainer vials with the horse's data like name, age and the date of collection. Following sample collection, the samples were properly delivered to the forensic laboratory CAMB for further proceedings. To prevent blood sample degradation, transport them immediately to the lab. To prevent blood sample degradation, samples were transported immediately to the lab. A cargo box with perforated sponge layers was used on the bottom and top of the samples, which were placed in securely closed zip-top bags. An unperforated sponge layer was added before sealing the box. The box temperature was maintained at 10°C during transport. Then samples were stored at 4°C. Organic Method of DNA Extraction: By following ethical guideline all the experiments like wet lab or Insilco labs were performed at Forensic DNA lab CAMB Lahore. The DNA was extracted using the organic method and purified with a microcon centrifugal device. The yield was estimated by 1% agarose gel electrophoresis and the gel was visualized using the BIO-RAD GelDoc system. The DNA was extracted by organic method and purified by microcon centrifugal device and estimation was done by 1% yield gel by using BIO-RAD Gel Doc system [15]. The primers were synthesized by ABI which were labeled with fluorescent dye as mentioned (Table1).

Table 1: Characteristic of Primers

S. No.	Gene/ Marker	Primer	Dye	Size Range (bp)	Repeats
1	VHL20	F:CAAGTCCTCTTACT TGAAGACTAG R:AACTCAGGGAGAAT CTTCCTCAG	FAM- 6	87-105	30
2	HMS7	F:TGTTGTTGAAACAT ACCTTGACTGT R:CAGGAAACTCATGT TGATACCATC	FAM- 6	165-185	1
3	HMS6	F:GAAGCTGCCAGTAT TCAACCATTG R:CTCCATCTTGTGAA GTGTAACTCA	VIC	151-169	4
4	ASB23	F:GAGGGCAGCAGGT TGGGAAGG R:ACATCCTGGTCAA ATCACAGTCC	VIC	176-212	3
5	LEX14	F: CCTTACTCACTG GGGAAT R: AGACTGAACACC TAACTATGA	VIC	204-206	5

After completing the gel electrophoresis, the stained gel was placed in a BIO-RAD GelDoc system connected to a computer. Using the Image Lab™ Software, the system was controlled to capture an image of the gel under UV light, making the stained bands visible. The software facilitated the analysis, such as measuring the intensity of the bands and comparing them with the 100 bp ladder. The optimal annealing temperature was determined using gradient PCR on BIO-RAD TIOOTM Thermal Cycler. The setup included 10ng DNA, 10uM primers, and a temperature range of 54°C to

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64˚C. The PCR protocol consisted of initial denaturation at 95˚C (5min), denaturation at 95˚C (30sec), annealing at 54˚C to 64˚C (75s), and final extension at 72˚C (10min) in a 25uL reaction volume. Three sensitivity analyses were conducted to optimize PCR conditions using the Conventional PCR GeneAMP PCR System 9700. The analyses focused on determining the optimal lowest DNA concentration(ranging from 0.25ng to 10ng)(Table 2).

Table 2: Plan for DNA Sensitivity Analysis(8 Reactions)

S. No.	Chemical Reagents	Quantities of Volume
1	Primer Mix	80uL (10ul for Each Reaction)
2	Template DNA	24ul (24ng)(3ng for Each)
3	Taq DNA Polymerase	4uL (0.5uL for Each)
4	Master Mix	79.5uL
5	T.E Buffer	19.75uL

Primer sensitivity with concentrations from 2uM to 10uM, and volume quantities (25uL, 12.5uL, and 7.5uL) while maintaining consistent conditions and conserving reagents (Table 3). PCR products were genotyped using Capillary electrophoresis. A 96-well microtiter plate was prepared with the first well as a negative control containing 0.4 uL Liz (GS500) and 13 uL Hi-Di formamide. The other wells contained 2 uL of PCR product in addition to Liz and formamide. After loading and spinning, the plate was incubated at 95^{0}C for 5 minutes, followed by a 3-4-minute heat shock. After another spin, the plate was wrapped and sent for capillary electrophoresis on an ABI prism 3130XL Genetic analyzer. Genotyping data was then analyzed using Gene Mapper ID 3.2v software.

Table 3: Reaction mix for Sensitivity Analysis of Primer (5 Reactions)

S. No.	Chemical Reagents	Quantities of Volume
1	Master Mix	50uL (10uL Each Reaction)
2	Taq DNA Polymerase	2.5uL (0.5 for Each Reaction)
3	Primer Mix	30uL (2ul to 10uL) for 5 Reactions
4	T.E Buffer	27.5uL
5	Template DNA	3ng (3uL) for Each Reaction

RESULTS

Below these concentrations the amplification was not significant. The genotyping data analysis also revealed that the best RFU (Relative Fluorescence Units) values were observed within the DNA concentration range of 3ng to 10ng(Table 4).

Table 4: Genotyping Data(DNA Sensitivity Analysis)

Microsatellite Markers	Dye-Labelled	Peak Heights at different Concentration of DNA in Electropherogram								
		Amplicon Size	10ng	5ng	4ng	3ng	2ng	1ng	0.5ng	0.25ng
VHL20	FAM-6	91-104	2878-1752	2023-1246	1737-1029	2706-1612	1382-1895	1632-930	136-76	557-385
HMS7	FAM-6	172-174	783-509	958-651	473-344	926-946	205-159	391-308	97-80	-
HMS6	VIC	167	2185	1360	891	834	643	892	263	-
ASB23	VIC	192	2234	1141	536	1109	445	405	186	-
LEX14	VIC	206	2926	1631	763	1512	457	678	291	-

Similarly, the RFU values for primer sensitivity showed that there was a good amplification at 6uM to 10uM(Table 5).

Table 5: Genotyping Data of Primer Sensitivity Analysis

Microsatellite Markers	Dye-Labelled	Peak Heights at different Primer Concentrations in Electropherogram					
		Amplicon Size	10µM	8µM	6µM	4µM	2µM
VHL20	FAM-6	91-104	1456-970	1664-1083	867-685	69-<50	66-53
HMS7	FAM-6	172-174	787-489	1200-731	490-265	62-<50	<50
HMS6	VIC	167	2067	2267	1154	95	85
ASB23	VIC	192	969	1034	441	<50	227
LEX14	VIC	206	1083	1457	604	250	380

The genotyping data for primer sensitivity analysis was also represented graphically(Figure 8). Subsequently the peak height (RFU) suggested that there was good amplification at 25uL and 12.5uL. But at 7.5uL there was a decline in RFU values which may result in allelic drop out(Table 6).

Table 6: Genotyping Data of Primer Sensitivity Analysis

Microsatellite Markers	Dye-Labelled	Amplicon Size	Peak Heights of different Volumes of PCR Mix in Electropherogram		
			25µl	12.5µl	7.5µl
VHL20	FAM-6	91-104	1664-1083	1212-708	930-640
HMS7	FAM-6	172-174	1200-731	1271-742	897-552
HMS6	VIC	167	2267	2723	1766
ASB23	VIC	192	1034	469	202
LEX14	VIC	206	1457	569	296

In the examination of the 4% product gel, amplification was detected at temperatures of 56°C, 58°C and 60°C. Among these, the most robust amplification was consistently observed at 60°C(Figure 1).

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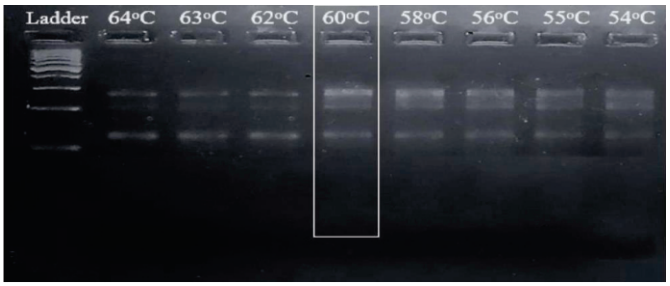

Figure 1: Product Gel of Gradient PCR

In figure 2 gel electrophoresis illustrating DNA sensitivity analysis, displaying bands corresponding to different DNA template concentrations, indicating successful amplification across a range of sensitivities."

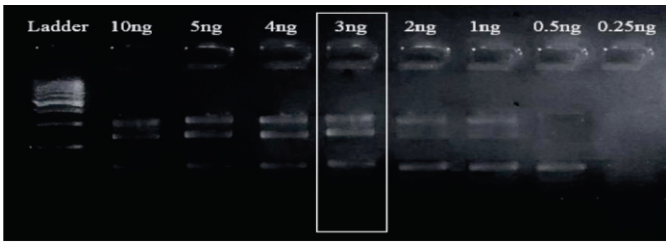

Figure 2: Product Gel of DNA Sensitivity Analysis

In figure 3 gel electrophoresis showing the results of primer sensitivity analysis, with distinct bands indicating successful amplification at varying primer concentrations (Figure 3).

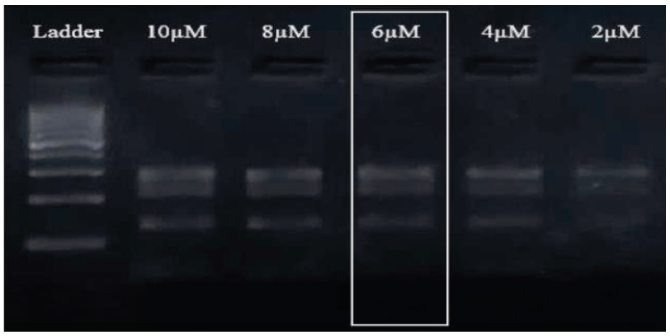

Figure 3: Product Gel of Primer Sensitivity Analysis

In figure 4 gel electrophoresis showing the results of optimal volume analysis, with bands representing varying reaction volumes to determine the ideal conditions for efficient amplification.

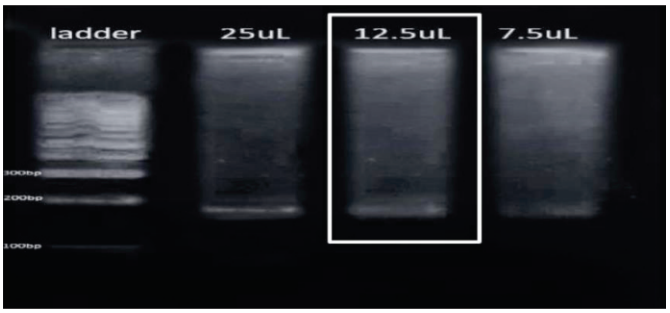

Figure 4: Product Gel of Optimal Volume Analysis

Notably, there was a decline in RFU values at the 2ng and 1ng DNA concentrations, heightening the risk of allelic dropout particularly in cases of heterozygosity. Moreover, the RFU values for the VHL20 and HMS7 loci resulted in allele dropout at 0.5ng. Additionally, at the 0.25ng DNA concentration, only the VHL20 locus exhibited amplification. In light of these findings, the DNA concentration of 3ng was identified as the optimal DNA concentration(Figure 5).

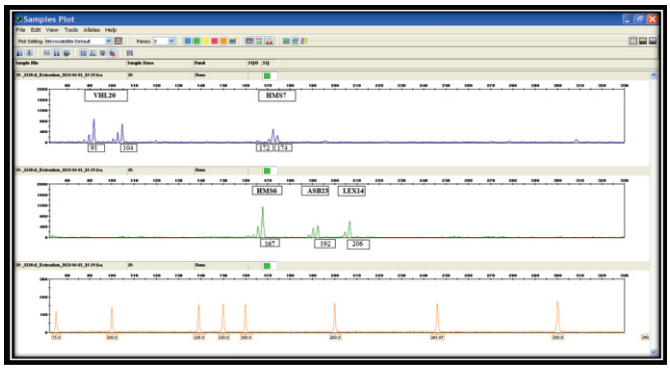

Figure 5: DNA Sensitivity Analysis(3ng)

The genotyping data for DNA sensitivity analysis was also represented graphically(Figure 6).

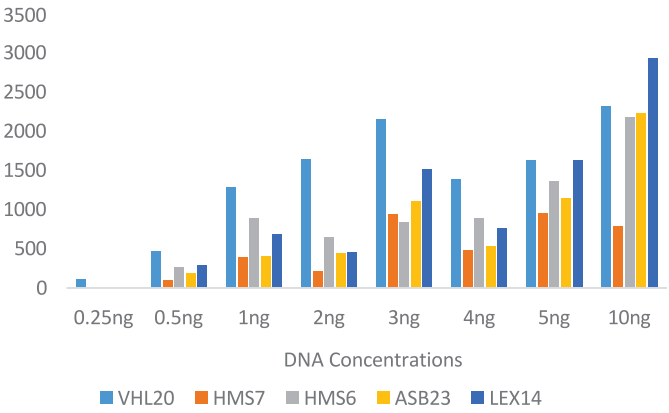

Figure 6: Graphical Representation of DNA Sensitivity Analysis (0.2 ng to 10ng)

While primer concentrations 4uM and 2 uM result in allelic drop out. So 6uM was reported as least primer concentration that give best amplification(Figure 7).

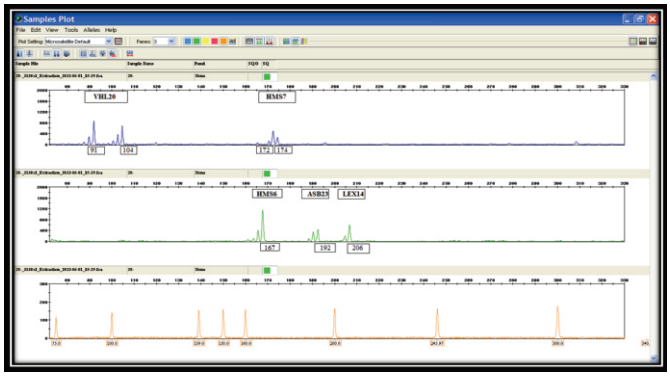

Figure 7: Primer Sensitivity Analysis(6uM)

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A graph showing primer sensitivity analysis from 2 µM to 10 µM typically plots primer concentration on the x-axis and PCR efficiency or product yield on the y-axis. The curve helps determine the optimal primer concentration for maximum amplification success while minimizing nonspecific products(Figure 8).

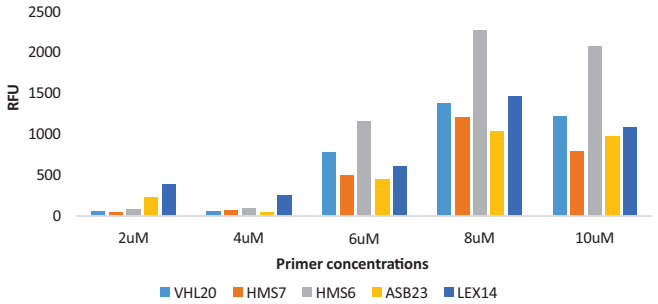

Figure 8: Graphical Representation of Primer Sensitivity Analysis (2uM to 10uM)

To prevent the wastage of costly reagents, it was recommended that a final PCR reaction volume of 12.5 would be optimal(Figure 9).

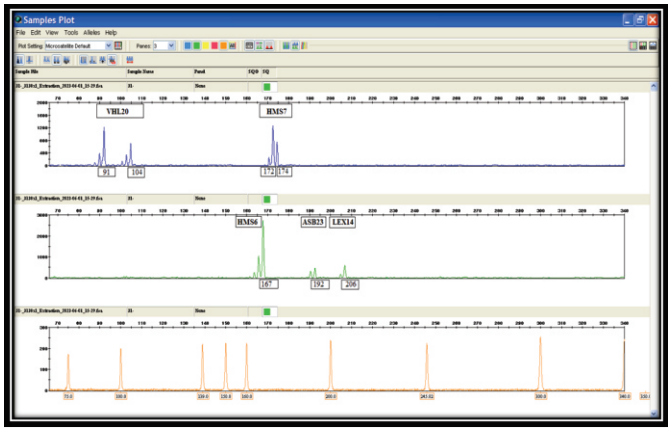

Figure 9: Volume sensitivity Analysis(12.5uL)

The genotyping data for primer sensitivity analysis was also represented graphically(Figure 10)

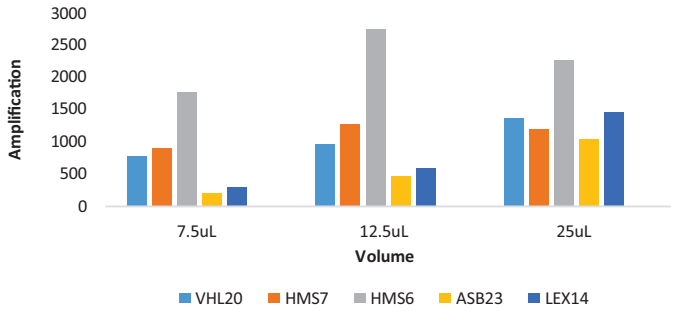

Figure 10: Graphical Representation of Volume Sensitivity Analysis

DISCUSSION

Besides other methods, which have been previously used, this study has been done to develop multiplex PCR of five Thoroughbred horses STR markers (VHL20, HMS6, HMS7, ASB23 and LEX 14). This study puts forward a novel idea to create a cheaper local kit for accurately testing horse's parentage. IT considered several critical factors in a multiplex PCR setup for the recommended ISAG STR markers. These factors include the annealing temperature, minimum template DNA and primer concentrations, as well as the optimal PCR volume. The research conducted on Arabian horses and Caspian horses which coincided with the creation of multiplex PCR assays for 17 STR loci and 7 STR loci respectively, revealed a consistent identical annealing temperature with this study finding. In Korea, a multiplex analysis was conducted utilizing 14 STR markers, employing an annealing temperature of 60°C [16-18]. The DNA sensitivity analysis was found to be interconnected with related research, such as the advancement of a 19-plex PCR system. This study indicated that DNA concentrations ranging from 2ng to 20ng yielded successful amplification, while using 1ng of template DNA led to allelic dropouts. Consequently, utilizing 1ng or less of template DNA was not recommended based on these finding [19]. In the development of 13-plex PCR a primer concentration range of 5uM was used which was closely in line with this findings [19]. The finding of this research suggested 12.5uL as the optimum reaction volume. Research in Portugal however, employed 10uL of final PCR reaction volume to test the paternity of Portuguese autochthonous horse breeds [20]. To validate the paternity of the Syrian Arabian horse breed, a microsatellite analysis was conducted using a 12uL sample from the final volume, demonstrating alignment with this research outcomes [21]. This study will provide a platform to establish a mega multiplex system that will lead to establish the equine stud book in the country.

CONCLUSIONS

In this study, five autosomal STR markers HMS6, HMS7, ASB23, VHL20 and LEX14 have been optimized using multiplex PCR for equine genotyping analysis. The best annealing temperature for the multiplex PCR of HMS6, HMS7, ASB23, VHL20 and LEX14 primers was found as 60°C.The least DNA concentration that gave the amplification of all five primers in multiplex PCR was found as 3ng. The minimum primer concentration needed for successful amplification of this penta-plex was found to be 6uM. This study recommends the use of 12.5uL PCR reaction volume.To make the assay more economical. This study will provide a platform to establish a mega multiplex system for Equine genotyping which will lead to establish the equine stud book in the country. The establishment of stud-book on the basis of DNA profiling would ultimately aid in the import and export of horses into Pakistan.

Authors Contribution

Conceptualization: Z, MH
Methodology: Z, SS
Formal analysis: UM, J
Writing, review and editing: MH

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All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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REFERENCES

[1] Rook L, Bernor RL, Avilla LS, Cirilli O, Flynn L, Jukar A *et al.* Mammal biochronology (Land Mammal Ages) around the world from Late Miocene to Middle Pleistocene and major events in horse evolutionary history. Frontiers in Ecology and Evolution. 2019 Jul; 7: 278. doi: 10.3389/fevo.2019.00278.

[2] Huntington PJ, Brown-Douglas CG, Pagan JD. Growth and development of Thoroughbred horses. Animal Production Science. 2020 Nov; 60(18): 2093-102. doi: 10.1071/AN19629.

[3] Lebedeva LF, Atroshchenko MM, Naumenkova VA, Solodova EV. Comparative analysis of various technologies of breedings of horses. InIOP Conference Series: Earth and Environmental Science 2021 Jul; 624(1): 012035. doi: 10.1088/1755-1315/624/1/012035.

[4] Cosenza M, La Rosa V, Rosati R, Chiofalo V. Genetic diversity of the Italian thoroughbred horse population. Italian Journal of Animal Science. 2019 Jan 2;18(1):538-45.

[5] Bergmann IM. Towards Interspecies Sustainability: The Future for Thoroughbreds and Thoroughbred Racing [Doctoral dissertation]; 2020.

[6] Petersen JL. Horse Breeding. InAnimal Breeding and Genetics 2022 Nov: 279-295. doi: 10.1007/978-10716-2460-9_1120.

[7] Flynn P, Morrin-O'Donnell R, Weld R, Gargan LM, Carlsson J, Daly S *et al.* Comparative analysis of single nucleotide polymorphisms and microsatellite markers for parentage verification and discovery within the equine Thoroughbred breed. bioRxiv. 2021 Jul: 2021-07. doi: 10.1101/2021.07.28.453868.

[8] Zhu J, Lv M, Zhou N, Chen D, Jiang Y, Wang L *et al.* Genotyping polymorphic microhaplotype markers through the Illumina® MiSeq platform for forensics. Forensic Science International: Genetics. 2019 Mar; 39: 1-7. doi: 10.1016/j.fsigen.2018.11.005.

[9] Khrabrova LA, Blohina NV, Suleymanov OI, Rozhdestvenskaya GA, Pustovoy VF. Assessment of line differentiation in the Thoroughbred horse breed using DNA microsatellite loci. Вавиловский журнал генетики и селекции. 2019; 23(5): 569-74. doi: 10.18699/VJ19.526.

[10] Al-Shuhaib MB and Hashim HO. Mastering DNA chromatogram analysis in Sanger sequencing for reliable clinical analysis. Journal of Genetic Engineering and Biotechnology. 2023 Nov; 21(1): 115. doi: 10.1186/s43141-023-00587-6.

[11] Park CS, Lee SY, Cho GJ. Evaluation of recent changes in genetic variability in Thoroughbred horses based on microsatellite markers parentage panel in Korea. Animal Bioscience. 2022 Apr; 35(4): 527. doi: 10.5713/ab.21.0272.

[12] Atiq I, Zahoor I, Basheer A, Khan W. Genetic Diversity, Population Structure And Phylogenetic Relationship Of Race, Sports, Draft And Wild Type Horses. Pakistan Journal of Agricultural Sciences. 2018 Mar; 55(1). doi: 10.21162/PAKJAS/17.5087.

[13] Pérez JR, Palacios DM, Garro JM. Genetic characterization of the Colombian Creole Horse population via STR markers used in filiation tests and equine certification. Forensic Science International: Animals and Environments. 2023 Dec; 3: 100065. doi: 10.1016/j.fsiae.2023.100065.

[14] Machmoum M, Boujenane I, Azelhak R, Badaoui B, Petit D, Piro M. Genetic diversity and population structure of Arabian horse populations using microsatellite markers. Journal of Equine Veterinary Science. 2020 Oct; 93: 103200.doi:10.1016/j.jevs.2020.103200.

[15] Paul R, Ostermann E, Wei Q. Advances in point-of-care nucleic acid extraction technologies for rapid diagnosis of human and plant diseases. Biosensors and Bioelectronics. 2020 Dec; 169: 112592. doi: 10.1016/j.bios.2020.112592.

[16] Ropka-Molik K, Stefaniuk-Szmukier M, Musiał AD, Velie BD. The genetics of racing performance in Arabian horses. International Journal of Genomics. 2019 Sep; 2019(1): 9013239. doi: 10.1155/2019/9013239.

[17] Sadeghi R, Moradi-Shahrbabak M, Miraei Ashtiani SR, Schlamp F, Cosgrove EJ, Antczak DF. Genetic diversity of Persian Arabian horses and their relationship to other native Iranian horse breeds. Journal of Heredity. 2019 Mar; 110(2): 173-82. doi: 10.1093/jhered/esy061.

[18] Yaralı C, Köseman A, Özşensoy Y, Şeker İ, Toprak B, Zengin K. Parentage verification and genetic diversity of the Arabian and Thoroughbred horse populations in Türkiye using microsatellite analysis. Überprüfung der Abstammung und genetischen Vielfalt der Araber-und Vollblutpferde in der Türkei mittels Mikrosatellitenanalyse. Arch. Tierheilkd. 2023 Nov; 165: 716-25. doi: 10.52973/rcfcv-e33262.

[19] Shang S, Zhang M, Zhao Y, Dang W, Hua P, Zhang S *et al.* Development and validation of a novel 13-plex PCR system for commonly used short tandem repeats in

DOI: https://doi.org/10.54393/fbt.v4i03.128

horses (Equus caballus). Equine Veterinary Journal. 2019 Sep; 51(5): 688-95. doi: 10.1111/evj.13047.

[20] Cozzi MC, Valiati P, Longeri M, Ferreira C, Abreu Ferreira S. Genetic variability trend of lusitano horse breed reared in Italy. Animals. 2022 Jan; 12(1): 98. doi: 10.3390/ani12010098.

[21] Sargious MA, Ahmed HA, El-Shawarby RM, Bakery HH, Ramadan SI, Cothran EG *et al*. Genetic diversity and population assignment of Arabian horses. 2021 Nov: 1-9. doi: 10.17582/journal.pjz/20201013221026.