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Whispers of the Genome: Unmasking SNP through Simple Modified PCR

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Abstract: Single nucleotide polymorphisms (SNPs) are essential in research, diagnostics, agriculture, medicine, animal husbandry, anthropological research, and many more, as they shed light on genetic diversity, population dynamics, and evolutionary connections. The present study focused on the identification of the Duffy intronic variant (a SNP) (C5411T; C>T) (rs863002), which has been shown to be strongly associated with malaria. A total of 242 samples were analysed by a modified allele-specific PCR and TaqMan assay. About 5% of the sample's genotypes were confirmed on Sanger Sequencing.

In the present study, an efficient allele-specific PCR (AS-PCR) methodology was developed and validated to successfully identify the Duffy intronic variant by end-point PCR. The results obtained by AS-PCR and TaqMan SNP genotyping assay technique were compared for concordance, and the percentage of identity between the two techniques was found to be 100%. Single nucleotide polymorphisms (SNPs) are frequently used as genetic markers in studying population structure and genetics. The present study focused on the identification of a specific genetic variant associated with malaria. The study collected blood samples from different geographical regions and developed an efficient PCR methodology to identify the variant. The modified PCR technique used in this study does not require additional treatments, making it cost-effective and time-efficient. The simple AS-PCR methodology developed can be used in anthropological studies to understand human genetic diversity and migration patterns.

Keywords: Allele-specific PCR (AS-PCR), DNA Sequencing, single nucleotide polymorphism (SNP), markers, TaqMan assay.

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Introduction

Single nucleotide polymorphisms (SNPs) are essential in research diagnostics. (Schrijver *et al*. 2012), agriculture (Huang *et al*., 2010; Huang *et al*., 2015), medicine (Manolio *et al*., 2009), animal husbandry (Rubin *et al*., 2012; Qanbari *et al*., 2014; Hayes & Daetwyler, 2018), anthropological research (Dash *et al*., 2022). They are frequently employed as genetic markers in studying population structure and genetics (Pickrell & Pritchard, 2012). Researchers can evaluate genetic diversity, identify community boundaries, and identify migration patterns, admixture, and isolation by examining the distribution of SNPs among various groups. SNP analysis sheds light on population dynamics and humans' evolutionary history by demonstrating the genetic differences and relatedness between different groups.

Researchers can find genetic markers linked to specific geographic areas or ancestral populations by looking at the frequency of a particular SNP or SNP haplotype, making it possible to recreate migratory paths, population shifts, and the ancestry of various groupings. Studies examining population mixing, such as the genetic contributions of extinct hominin groups to contemporary human populations, frequently employ SNP-based ancestry analysis (Sankararaman *et al*., 2016).

Studying genetic adaptations and illness risk in various groups also heavily relies on SNPs (Visscher *et al*., 2017; Hindorff *et al*., 2009). Due to genetic drift or natural selection, the frequencies of some SNPs might differ between populations and be linked to particular traits or illnesses. Through the examination of SNPs associated with characteristics like skin pigmentation, lactase persistence, or vulnerability to diseases like malaria, scientists can acquire a deeper understanding of the evolutionary processes that have moulded human populations and their ability to adapt to diverse surroundings.

SNP analysis is vital in anthropological studies to yield important insights into human genetic diversity, population structure, migration patterns, genetic adaptations, and illness susceptibility (Bamshad *et al*., 2004; Reich *et al*., 2009). Researchers can learn more about the complex patterns of human populations, the genetic foundations of human evolution, and the forces that have moulded species by examining SNPs that have shaped various species.

SNP analysis is widely used to study genetic diversity within specific populations. SNP study provides insight into particular groups' genetic diversity, demographic makeup, and unique genetic characteristics. This knowledge is necessary to understand the pharmaceutical responses, population-specific health disparities, and the genetic foundations of diseases.

The present study focused on the identification of the Duffy intronic variant (a SNP) (C5411T; C>T) (rs863002), which has been shown to be strongly associated with malaria in Sudan's inhabitants. (Kempińska-Podhorodecka *et al*., 2012). This SNP has been studied so far by real-time TaqMan-based assay. In the present study, an efficient allele-specific PCR (AS-PCR) methodology was developed and validated to identify this variant successfully by end-point PCR. The AS-PCR principle is based on a mismatch at the 3' end of the primers. However, it requires an endogenous control gene to address the presence of PCR inhibitors. In the present modified technique, we used two pairs of primers, averting the need for any other separate endogenous control primers. One set of primers flanked the region of the SNP, while two allele-specific primers were designed at the polymorphic site in reverse polarity to accurately identify the SNP at the locus (Figure 1).

Materials and Methods

Study Site and Subject

The present study collected 162 unrelated blood samples from blood banks of different geographical regions (Mumbai (Mu, n=23) – Maharashtra; Surat (Su. N=26) – Gujarat; Manipur North-East (NE, n=88) and Mangalore (Ma, n=25) – Karnataka, which were positive by rapid identification test.

Peripheral blood was collected (3 cc. in EDTA) and stored at 4°C till samples reached the testing centre. Males and females aged over 18 years participated in the study. Information on the patient's ethnic background and other disease conditions, including diabetes and infectious disease (including malaria), were obtained by interviewing the individual. Blood from healthy controls without any history of fever in the last year was also collected from each geographical location (n=20 each). All the control samples were negative for malaria infection on peripheral blood slide examination and immunochromatography test.

Inclusion criteria

Participants of the age group (15- 65 years old) were included in the present study. Only participants having either a confirmed malaria infection (by microscopy or rapid diagnostic test) at the time of recruitment or having a documented history of malaria infection within the past year were included in the study as cases. The study included the participants or their legal guardians (for minors), who provided informed consent to participate in the study.

Exclusion criteria

Participants with severe or complicated forms of malaria were excluded to avoid potential confounding factors or ethical concerns. Individuals with chronic or severe conditions, such as HIV/AIDS, sickle cell disease, or other significant illnesses, were excluded to reduce potential confounding factors. Similarly, pregnant women were also excluded.

Participants who had a history of malaria or were on an antimalaria treatment regime were excluded from the control group. Individuals who had received a blood transfusion within 6 months before the study were not included to ensure accurate genetic analysis. Individuals who did not consent to participate in the study were excluded. Participants who have a history of travel with a year and malaria incidence were not included in the study.

DNA extraction

DNA was extracted and purified from 200 µL of blood by the organic extraction method described by Green and Sambrook 2012. After the purification step, the extracted DNA was eluted with 50 µL of elution buffer and quantified by Nanodrop-1000 (Thermo Fisher Scientific, Massachusetts, United States).

AS-PCR Genotyping

PCR was performed using 150ng of extracted DNA, 8 pmol of flanking primers, 5 pmol of allele-specific primers (table 1), 3.5 nmol of each dNTP, 1.5 U Taq polymerase, and buffer (Genei Labs, Bangalore, India), in a total volume of 25µL. Touch-down (55°C - 48°C) PCR was performed in a thermal cycler (S-96 Gradient Thermal Cycler, Quanta Biotech, USA) with the cycling conditions as 95 °C for 5 min; 13 cycles of denaturation at 95 °C for 45 sec; annealing at 63 °C for 45 sec (-0.5°C decrement/cycle); elongation at 72 °C for 45 sec; followed

Table 1: Primers Sequences for Genotyping rs863002 [C5411T] Polymorphism

Primer identity	Sequence (5′ → 3′)	PCR Product Size (bp)	Tm (◦C)
FP_5411C>T	GCTTCCTGATGCCCCCTGTCC	540	TD 63-55
RP_5411C>T	AGGTCGGGTGGGAGAACAAGGTC		
AS_RP_5411C>T	CTAGGAGGCTAGCATAGGAAGGAGAG	300	
AS_FP_5411C>T	CCTCTTCCTTCAAAGTCTTTTTCCTTT	240	

by 25 cycles of denaturation at 95 °C for 45 sec; annealing at 55 °C for 45 sec; elongation at 72 °C for 45 sec; final extension of 5-min incubation at 72 °C; and 4 °C 5-min. The PCR products were loaded on 1% agarose in Tris-acetate-EDTA (TAE) buffer to check for amplification efficiency (Figure 1).

Figure 1: Primer designing a scheme to identify rs863002 polymorphism. A. Schematic representation of primers location with respect to the polymorphic site of SNP rs863002. B. Sequence spanning polymorphism rs863002 (C5411T) and allele-specific primers are marked on the sequence. The allele-specific forward and reverse primer sequences are underlined.

DNA Sequencing

About 5% of the samples were randomly selected to verify the authenticity of allele-specific PCR. FP_5411C>T + FP_5411C>T primers generated products of size 539-bp. The amplified PCR product was cleaned with ExoSAP-IT (USB Corporation, Cleveland, Ohio) and then sequenced using a 3700XL automated DNA sequencer (Applied Biosystems, USA). Sequencing was performed using the fluorescent Big-Dye Terminator v.1.1 cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's protocol. The BigDye Terminator reaction was cycled at 96°C for one minute, then 25 cycles of 96°C for ten seconds, 50°C for five seconds, and 60°C for four minutes The NCBI reference genomic sequence used for DARC was NM_002036.2.

TaqMan assay

TaqMan single tube assay was procured from ABI for real-time TaqMan-based allele calling. The single tube format assay mix (part number: C___7480790_10; Cat no.: 4351379) contained primers (forward and reverse primer), probes that perfectly matched to the sequence variants [wild type sequence C labelled with VIC; polymorphic variant labelled with 6-carboxyfluorescein (FAM)] TaqMan-MGB genotyping master mix was available at 40X concentration. The assay included 1.25 µL genomic DNA (50 ng), along with 0.25 µL of TaqMan-MGB genotyping assay mix (20X), 2.5 µL Taq Man Genotyping Master Mix (Applied Biosystems, Carlsbad CA, USA). 1.25 µL of nuclease-free water was added to no template control (NTC). Each test was run in duplicate in the plate, and the assay was run with the default setting on ABI real-time StepOne v 2.0.5 software. The AB standard PCR method was used, which included initial denaturation and activation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute.

The first step in the TaqMan assay is to pre-read the plate that contains the background fluorescence data. The instrument captured the fluorescence in real time during each cycle. The software calculated the fluorescence levels before and after and normalised the dye fluorescence (ΔRn) as a function of cycle number for either Allele2 (mutant) or Allele1 (wild type). The software used this value, which indicated the proportional number of possible outcomes, to identify an individual as heterozygous automatically (C/T: VIC/FAM), homozygous (C/C: VIC/VIC), or variant allele (T/T: FAM/FAM).

Results

AS-PCR

The polymorphic site of the intronic variant for rs863002 (C5411T) lacks recognition by available restriction enzymes, rendering genotyping by PCR-RFLP technique a remote possibility. Therefore, the allele-specific duplex PCR technique was modified to genotype this intronic variant using end-point PCR without the need for RFLP or TaqMan assay.

The Flanking primers (FP_5411C>T + RP_5411C>T) amplify a 540-bp product. This amplicon contained the polymorphic site and served as the amplification control. The allele-specific forward primer (AS_FP_5411C>T) along with reverse primer (RP_5411C>T) amplify a product of 240-bp. Similarly, allele-specific reverse primer (AS_RP_5411C>T) along with forward primer (FP_5411C>T) amplify a 300-bp product (fig. 1). This single-tube PCR was independent of the use of endogenous PCR control.

The PCR products were resolved over 1% agarose gel in 1x TAE buffer (80 volts for 25 mins) for separation of allele bands. A PCR product of 539-bp was severed as the PCR control (reagent control) to identify any inhibitor that could substantially affect PCR amplification efficiency (fig. 2). The allele products (300-bp and 240-bp) were amplified with the presence of the corresponding allele in the template. The presence of a 300-bp fragment predicted the occurrence of TT homozygous at the 5411th position at the locus. Similarly, the 240-bp fragment is the product produced by allele-specific reverse primer and reverse primer combination predicted the occurrence of CC at the 5411th locus. Amplification of both the products (viz 300-bp and 240-bp) (fig. 2) predicted the locus is heterozygous with C and T at 5411th.

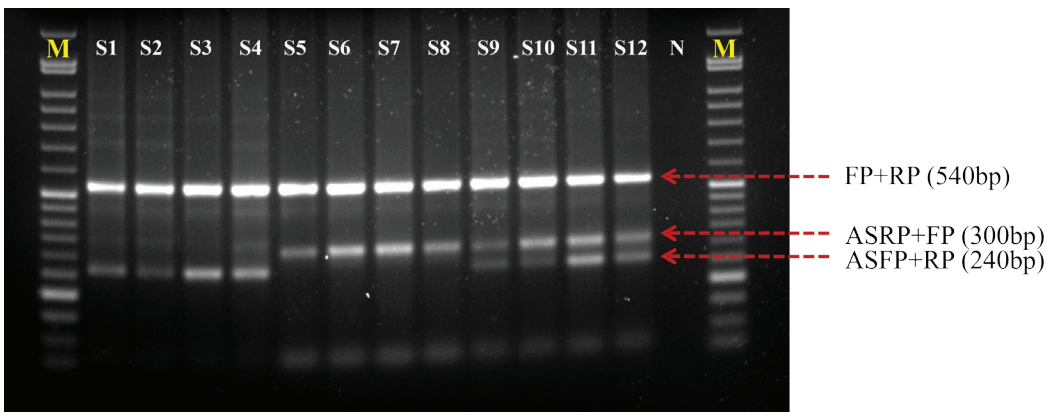

Figure 2: Agarose Gel Electrophoresis to study rs863002 (C5411T) polymorphism. S1-S4: homozygous C/C, S5-S8: homozygous T/T, S9-S12: heterozygous C/T. M: 50bp molecular marker, N (Cat. No.: DM012-R500, GeneDirex), No template control (NTC).

Sanger Sequencing

The results obtained were validated by sequencing the polymorphic locus using Sanger sequencing to confirm the genotype studied by newly modified allele-specific PCR. The AS-PCR results were confirmed initially by Sanger sequencing before analysing the bulk samples. Fig. 3 shows the presence of three genotypes of rs863002 SNP. The AS-PCR genotyping was validated by Sanger sequencing, and it was found that there is a 100% concordance between AS-PCR and Sanger sequencing.

TaqMan Probe-based Assay

The polymorphic locus rs863002 was genotyped in 162 cases and 80 controls by TaqMan SNP genotyping assay technique. The results obtained by AS-PCR

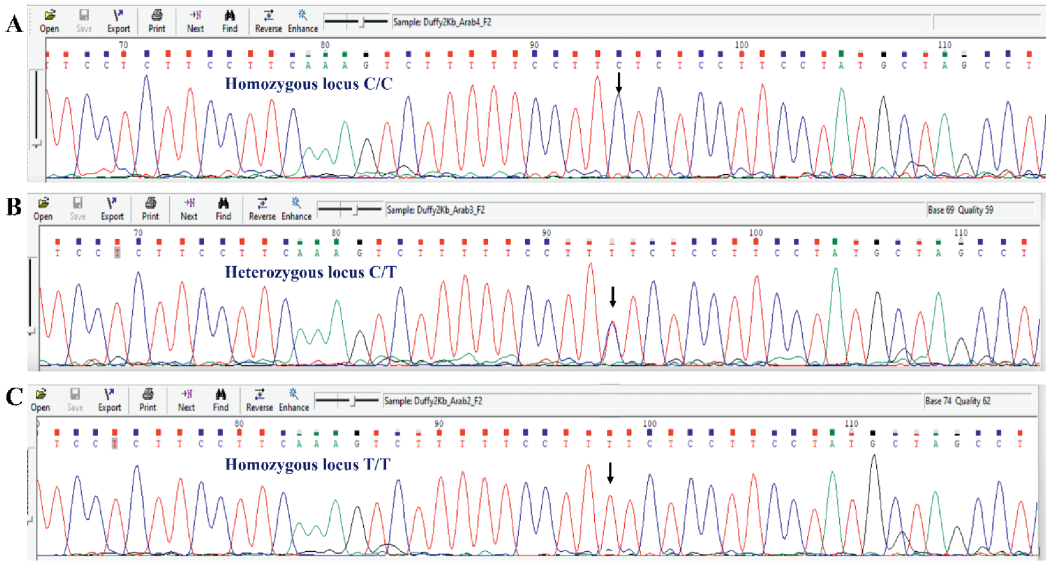

Figure 3: Chromatograms confirming allele-specific PCR rs863002 (C5411T). Chromatogram showing the presence of homozygous C/C (A) and homozygous T/T (C).

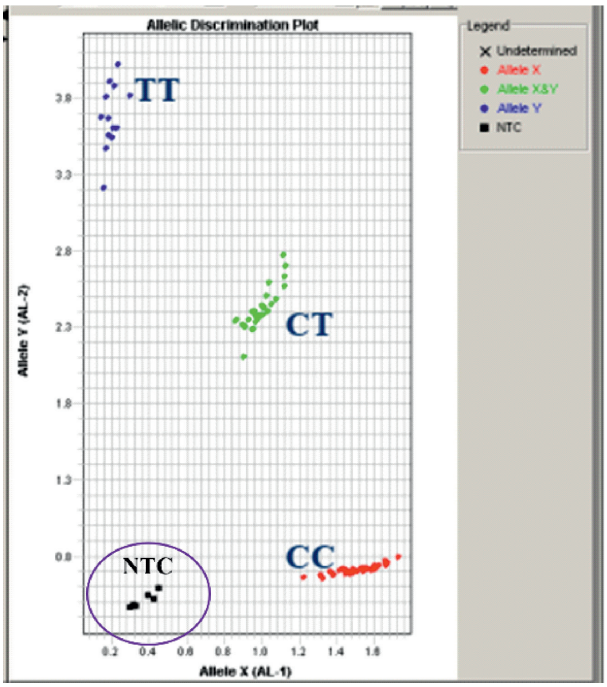

Figure 4: A representative result for the SNP rs863002 (C/T) genotyping using TaqMan SNP assay. The allelic discrimination plot generated by post-real-time PCR assay showed three distinct genotypes viz. C/C, T/T, and C/T.

and TaqMan assay for 242 samples were compared for concordance, and the percentage of identity between the results obtained by the two techniques was found to be 100%. Three genotypes viz., C/C, C/T and T/T were identified by TaqMan assay (fig. 4). The association of the SNP with malaria is not described in the article, as it is part of a study that is under publication.

Discussion

The article described a simple allele-specific PCR technique that can be easily and routinely used in resource-limited lab setups to study SNPs. SNPs are often studied by PCR-RFLP, allele-specific PCR, TaqMan assay, SNaPshot, sequencing, etc, to name a few. However, each technique requires special / added treatment like restriction digestion of PCR product followed by agarose gel electrophoresis in case of RFLP, purification of PCR product post PCR cycles to remove unincorporated primers and dNTPs as in the case of SNaPshot, and sequencing. Such added treatment increases the cost of the assay and time, which in resource-limited situations is often not feasible. The newly modified AS-PCR enables the identification of three genotypes by single-tube PCR assay. The assay can identify the presence of PCR inhibitors as the larger amplicon (539 bp) also serves as an internal amplification control (IC/IAC), thus preventing the need to run a separate PCR control.

The IC assesses the success and reliability of the PCR reaction. In this modified version of AS-PCR, the 539 bp amplicon serving as the IC invariably ensured the success of genotyping. The 539 bp amplicon served two functions: 1) provided a higher concentration of the available target for allele-specific primers for accurate genotyping even in lower available input DNA; 2) it served as the internal reference to verify PCR conditions are optimal for amplification and indicates the presence of inhibitory that can be present in the extracted DNA, serving as the quality control for PCR amplification. Since the assay utilises its own amplicon as the IC, this averted the requirement for an additional IC to check PCR efficiency. This proportionately reduces the competition between allele-specific primer and IC, invariably increasing the chance of required target amplification. The utilisation of IC in the same testing tube is carried out in real-time PCR wherein the target and the IC are differently labelled with fluorophores of a non-overlapping emission spectrum, allowing simultaneous detection of both target and IC in a single tube.

SNPs have been investigated for the identification of SNP/haplotype association with diseases (Lehne *et al*., 2011), plant breeding (Alemu *et al*. 2024), animal husbandry (Zwane *et al*., 2019), and palaeontology (Loureiro *et al*., 2020).

AS-PCR has been used to study SNP in the formerly described areas. For instance, Pham *et al*. (2021) described AS-PCR for the identification of HLA-C* 03: 02 allele in Vietnamese Kinh people. Lee *et al*. (2022) illustrated the identification of SNPs by AS-PCR for the identification of better beef quality, and Kalendar *et al*. (2022) defined PCR assay for high-throughput genotyping and characterisation. The former scientific studies describe canonical AS-PCR methodology. However, the current paper sheds light on improved AS-PCR with higher sensitivity and precision without the need for an internal control target.

SNPs in anthropological research can be used to study and analyse genetic diversity, migration patterns, and population genetics. SNPs emphasise how genetic information flows and can shed light on the links among various populations, the population's structure, and the evolutionary history of humans. They also provide valuable information on how genetic research has advanced our knowledge of human diversity and the intricate relationships between genetic variation, location, and culture.

Resource-constrained AS-PCR procedures can be completed by carefully evaluating the aspects that govern the robustness of allele calling and modifying the experimental design to accommodate the available resources. Simple DNA extraction methods, fine-tuning PCR settings, visualising PCR products with inexpensive techniques such as agarose gel electrophoresis or other alternative detection methods, and ensuring sufficient controls are in place to validate the results are the keys to success.

SNP identification is currently being molecularly analysed using a variety of techniques. Numerous elements, such as the availability and capabilities of technology and the availability of financial and intellectual resources, to name a few, will influence the methods selected by various investigations. When choosing which assay to employ for SNP analysis, several considerations should be considered, including the assay's accuracy, sensitivity, robustness, repeatability, cost, and dependability.

SNP analysis can provide valuable insights into the genetic basis of population-specific health disparities. It is crucial to remember that SNP analysis is only one piece of a complicated puzzle, even though it can help uncover the genetic underpinnings of population-specific health inequalities. Health disparities are complex and impacted by a number of variables, such as lifestyle choices, environmental factors, healthcare access, and social determinants of health. It is essential to combine SNP analysis with other data sets, including socioeconomic, behavioural, and environmental characteristics, to fully comprehend health inequalities and create efficient strategies to address them.

Overall, by optimising PCR conditions, making sure appropriate controls are in place, reducing contamination, and employing straightforward detection techniques, AS-PCR can be modified for usage in situations when resources are scarce. Even if the precise needs could change based on the resources available, AS-PCR can offer a dependable and affordable method for SNP or mutation detection in resource-constrained environments with careful design and optimisation.

In summary, the assay described provides a built-in reference to monitor the success of the PCR reaction, detect potential PCR inhibitors, and ensure the reliability of the genotyping results. It can be a valuable tool for quality control and accurate calling of alleles in PCR-based applications.

Conclusion

SNP are extensively used for SNP genotyping in pharmacokinetics, forensic genetics, and bacterial strain typing, among other applications. The allele-specific SNP technique outlined here is highly reproducible, accurate, sensitive, robust, and affordable. Furthermore, because the assay is based on end-point PCR, it has great promise for use in low-budget investigations or mass studies like epidemiological and surveillance research. Other allele-specific PCR can be developed on similar lines, especially in a set-up where the locus under investigation lacks the presence of restriction sites to identify the variant using end-point PCR easily and without the need for expensive instruments and reagents.

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Ethical Approval

The ethical approval for the study was obtained from the Institutional Ethics Committee, Institutional Committee for Research on Human Subjects, National Institute of Immuno-haematology (ICMR), Mumbai.

Consent

A written consent was obtained from all participants in the study.

Availability of data and material

All data are available from the corresponding author upon request.

Authors' contributions

R.S. performed the experiments. A. G. and G.K. supervised the experiments. R.S. conducted data analysis. R.S. conceptualised the project, A.G. was responsible for the overall supervision and procured funding. R.S. wrote the manuscript. A.G. and G.K. approved the final manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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