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Computational Approach to PCR Primer Design for Genetic Analysis of TRAPPC9 Exon 20

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Abstract: Intellectual disability (ID) is a neurodevelopmental disorder defined by an intelligence quotient (IQ) of 70 or below and deficits in at least two domains of adaptive functioning. It is broadly classified into syndromic (S-ID) and non-syndromic forms, with S-ID associated with additional clinical features. This study aims to investigate the molecular basis of syndromic intellectual disability by targeting known genetic contributors such as those involved in Fragile X syndrome, Down syndrome, Prader-Willi syndrome, fetal alcohol spectrum disorders, and autism. Individuals with suspected ID from Tehsil Daska, Pakistan, were recruited for this research. Blood samples were collected, and genomic DNA was extracted using standard protocols. Molecular analyses included gel electrophoresis, primer design using Primer3 Plus, PCR amplification, and Sanger sequencing. Exon 20 of the TRAPPC9 gene, associated with autosomal recessive intellectual disability, was examined; no mutations were detected in this region, indicating that causative variants may lie in other exons or genes. This study highlights the need for broader genetic screening in populations with limited molecular diagnostic access. Identifying causative mutations in ID-related genes may support earlier diagnosis, better genetic counseling, and pave the way for targeted therapeutic strategies.

Keywords: Intellectual disability, Sanger sequencing, BLAST analysis, X-linked mutation, genetic diagnosis, neurodevelopmental disorder, DNA chromatogram, KDM5C, personalized medicine.

Introduction

Intellectual disability (ID) is significant limits and adaptive behavior across conceptual, social, and practical domains. It is complicated neurodevelopment disorder that affects learning, reasoning, communication, and day to day task management. It caused by incomplete or defective mental development (Patel et al., 2020). The term "intellectual disability" encompasses a range of conditions, most of which involve substantial cognitive and behavioral impairments (Carulla et al., 2011). ID typically emerges before the age of 18 and significantly interferes with a person's ability to function independently (Khan et al., 2020). Diagnosis is based on deficits in three key areas:

1. Conceptual skills: including memory, language, reading, writing, and mathematical understanding.
2. Social skills: such as interpersonal relationships, empathy, and communication.
3. Practical skills: including self-care, daily routines, school performance, employment, and money management.

Depending on the severity of functional limitations, ID is commonly classified into four levels: mild, moderate, severe, and profound (Umair et al., 2020).

Worldwide, the prevalence of ID is estimated at around 1% to 3% (Harris et al., 2006). However, access to support services and education remains limited, particularly in low- and middle-income countries where stigma and resource constraints continue to be major barriers (Hodapp et al., 2009). For example, in Scotland, only 4.2% of individuals with ID are

registered with local authorities, compared to 7.1% in Ireland and 4.2% in Canada.

In Pakistan, the burden of intellectual disability is particularly concerning. Studies report that approximately 2.5% of the population is affected, with a substantial proportion from Punjab (55%) and Sindh (28.4%) (National Census, 1998). A study involving over 6,000 children revealed that 1.9% had severe cognitive disability, and 6.5% had mild intellectual disability (Yaqoob et al., 1995). Another study from Karachi estimated the prevalence at 19 per 1,000 individuals (Epidemiol et al., 1998). The severity of ID has also been classified in local populations, with 46.7% of cases being mild, 32.1% moderate, 14.6% severe, and 6.7% profound (Naz et al., 2021).

Contributing factors may include high rates of maternal depression and anxiety, poor prenatal care, and increased consanguineous marriages all of which are prevalent in certain regions of Pakistan (Mirza et al., 2004; Mumford et al., 1996).

Clinical Features and Diagnosis

ID (Intellectual disability) can be subtle at birth, often going undiagnosed until school age, especially in mild case. Early signs include delayed speech, learning difficulties while infants, signs may show feeding difficulties, abnormal head size (macrocephaly or microcephaly), and gross motor delays (Kanungo et al., 2018). Severity impacts daily functioning: mild ID may lead to academic struggles yet some independence, moderate ID has more pronounced social and communication challenges and severe ID necessitate lifelong care, particularly if other impairments are present. Severe ID is defined by IQ scores significant below mean, usually between 20 and 40 ((Hodapp et al., 2009; Jarrold et al., 2000).

Intellectual disability is more than a medical diagnosis it deeply impacts the lives of individuals, families, and communities. Its effects extend beyond cognitive limitations, influencing education, employment opportunities, and quality of life. In countries like Pakistan, where the prevalence is notably high due to factors such as consanguineous marriages and limited healthcare access, understanding and addressing ID is both a medical and societal imperative. By exploring its genetic and environmental roots, we can move toward earlier detection, better support systems, and a future with reduced stigma and improved outcomes for those affected.

MATERIALS AND METHODS

Study Area

The overall study area was chosen, tehsil Daska located in district Sialkot of province Punjab lies in Pakistan. The tehsil lies between Latitude 32.33° North and Longitude 74.35° east as shown in figure 1. The total area of Daska is approximately to 19 square kilometers.

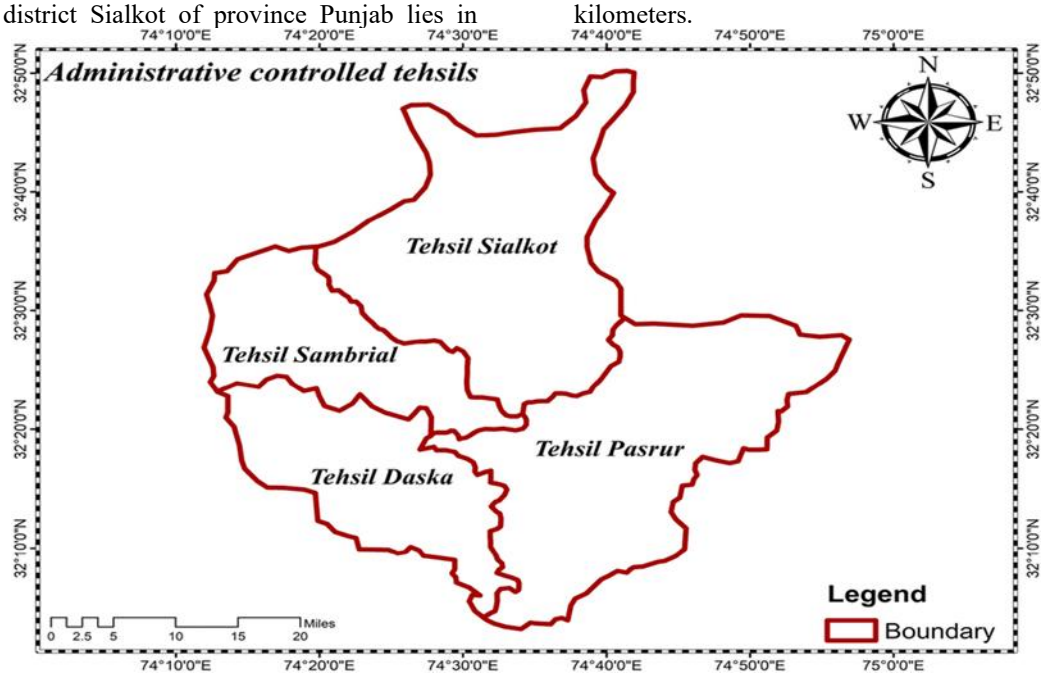

Figure 1 Map of district Sialkot

Examination of the patients

With the guidance of trained psychiatrists, individuals affected by intellectual disability received thoughtful and professional clinical assessments

1.3 Identification and enrollment

Through the special education school system, families with two or more individuals affected by intellectual disability (ID) were identified. Researchers conducted personal visits to these households to collect detailed family histories. Pedigree analysis was performed to investigate the inheritance patterns of ID, using genetic data. A total of 10 families were enrolled in the study, and blood samples were collected from one of them for further genetic analysis

Blood samples

Blood samples were obtained from participating family members via venipuncture of the median cubital vein, using sterile 5 ml and 10 ml disposable syringes. The collected samples were transferred into 50 ml falcon tubes pre-treated with 400 µL of EDTA to prevent coagulation. To ensure sample integrity, all specimens were stored at −20°C until DNA extraction was performed

DNA Extraction

DNA extraction was performed at a high-tech biotechnology laboratory in Lahore following a standardized spin-column protocol. Blood samples were lysed and treated with protease to digest proteins, then subjected to sequential washing steps using specific buffers to remove impurities. The DNA was finally eluted using Buffer AE after incubation and centrifugation, ensuring high-quality genomic material for downstream analysis (Figure 2 and 3).

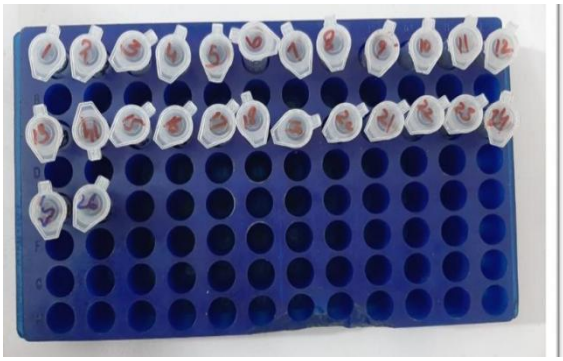

Figure 1 (a) Blood samples with washing buffer preparing for centrifugation (b) Blood samples in centrifugation machine (c) Blood samples in centrifugation machine

Figure 3 (A) Microcentrifuge tube (B) Primer preparations (C) Washing buffer added in centrifuge tubes

Primer designing

A primer is a short synthetic oligonucleotide used in various molecular techniques such as PCR and DNA sequencing. Primers are designed to be complementary to the reverse sequence of the specific region of target DNA to which they will anneal. In this study, PCR primers for exon 20 of the TRAPPC9 gene were designed using the Primer3 Plus software (https://www.primer3plus.com). This tool allows users to input the DNA sequence of interest and customize parameters such as primer length, melting temperature, GC content, and other important features. The primer locations and expected product sizes were then confirmed using the UCSC Genome Browser (https://genome.ucsc.edu).

Steps for primer preparation

Step 1 Added 300 µl of water into lyophilized primers. Obtaining 300 µ l of primers.

Step 2 Primers were prepared in dilutions. Added 10 l of primers and 90 l of injection water (10 µl =1000 picomoles and 0.001 micromoles µm) primers.

1 µl = 100 picomoles

10 µl = 1000 picomoles 1 picomole = 10^6 µm

1000 picomoles = 0.001 µm

After primers preparation, PCR products were prepared.

Table 01 Required reagents for primer preparation

DNA template	3 µl
Forward primer (10 pmol/µL)	1 µl
Reverse primer (10 pmol/µL)	1µl
Master mix	12.5 µl
Nuclear free water	7.5 µl

Table 02 Primer sequence of TRAPPC9 Gene

Exon 20 F.P	GGCAGAAATTGCCCTACGTA
R.P	GTGGGGCCTTTGAAGTGTTA
Product size	394BP

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a widely used molecular technique that allows the amplification of specific DNA segments, producing millions of copies. It is especially useful in detecting genetic mutations and infectious agents by identifying abnormal DNA or RNA in a sample. In this study, exon 20 was amplified using PCR at the Hi-Tech Bio Tech Lab in Lahore. The procedure began with the addition and mixing of all required reagents in PCR tubes. These were then placed in the PCR machine for a run time of approximately 2 hours. The PCR process involves three main steps:

- Denaturation – DNA strands were separated by heating at 94°C for 5 minutes initially, and then for 30 seconds in each cycle.
- Annealing – Primers bound to the target DNA at 54–59°C for 30 seconds, depending on their design.
- Extension – New DNA strands were synthesized at 72°C for 30 seconds, with a final extension of 10 minutes at the same temperature.

This cycle was repeated 30 times to ensure sufficient DNA amplification for downstream analysis (Mullis & Faloona, 1987).

Denaturation

The first step in the PCR cycle is denaturation, where double-stranded DNA is heated to 94–98°C for 20–30 seconds, causing the hydrogen bonds between base pairs to break and separate the strands. An initial denaturation phase at 94°C for 5 minutes ensures complete strand separation. Taq polymerase, a heat-stable enzyme, remains active throughout this high-temperature process.

Annealing
In the annealing step, the reaction temperature is lowered to around 59°C for 30 seconds, allowing primers to bind specifically to the single-stranded DNA templates. The annealing temperature is optimized based on the primers' melting temperatures—in this case, for targeting exon 20 of the TRAPPC9 gene.

Extension
During extension, the temperature is raised to 72°C—the optimal working condition for Taq polymerase. The enzyme synthesizes new DNA strands by adding nucleotides to the annealed primers in the 5' to 3' direction. This process results in the exponential amplification of the target DNA region within a short timeframe

Table. 03 Steps of PCR

Steps	Temperature	Time	No. of cycles
Initial	94°C	5 minutes	
Denaturation	94°C	30 seconds	30cycles
Annealing	54°C	30 seconds	
Extension	72°C	30 seconds	
Final elongation	72°C	5 minutes	

Gel Electrophoresis – Agarose Gel Preparation
To prepare the gel, 2 g of agarose powder was mixed with 100 ml of distilled water and heated in a microwave with TAE buffer until fully dissolved (1–3 minutes), ensuring constant stirring to prevent crystal formation. After cooling the solution for 5 minutes at room temperature, 6 µl of ethidium bromide (EtBr) was added for DNA visualization. The solution was then poured into a gel tray with a comb in place and left to solidify at room temperature for 10 minutes, followed by 10–15 minutes at 4°C for complete setting.

Loading Samples and Running Agarose Gel
After adding loading buffer to each DNA sample, the solidified agarose gel was placed in the electrophoresis chamber and submerged in TAE buffer. A molecular weight ladder was loaded into the first well, followed by careful loading of DNA samples into the remaining wells. The gel was run at 80–150 V until the dye migrated about 75–80% of the way. Once complete, the power was turned off and the gel was removed. DNA bands were visualized under UV light. DNA Sequencing, The amplified DNA samples were sent for commercial sequencing using Sanger's method. The results were analyzed using Chromas software for interpretation.

Data Analysis Tools
Several bioinformatics tools were used to evaluate the sequencing data:

1. VARSEQ for variant analysis across gene panels and genomes.
2. SIFT to predict the impact of amino acid changes on protein function.
3. UCSC Genome Browser to verify sequence positions.
4. CodonCode Aligner for sequence assembly, mutation detection, and editing.
5. Chromas for viewing and editing sequencing chromatograms.

Results
Prior to commencing the study, ethical approval was obtained from the Institutional Review Board of USKT to conduct molecular analyses on blood samples from families affected by intellectual disabilities. Recruitment was facilitated through visits to a specialized school in Daska, where clinical psychologists performed comprehensive assessments of the affected individuals. Diagnoses of intellectual disability were established based on thorough evaluations including speech and motor delays, demographic information (age, gender, height, weight), and detailed prenatal and postnatal histories, complemented by standardized IQ testing.
Behavioral and functional assessments further examined emotional regulation, speech characteristics, feeding and sleep patterns, fatigue, and attentional abilities. These clinical features were systematically documented to support the diagnosis. From an initial cohort of ten families, five were selected for molecular-level investigations, with Family PKID0007 chosen for an in-depth study.

History of Family
This family belongs to city Daska district Sialkot. This family prefers to do cousin marriages. In their family according to pedigree no one found have intellectual disability in their history. But in this family three individuals found have intellectual disability and these are females. The affected individuals of this family show non-syndromic intellectual disability.

Clinical History of Patients
This family comprises three affected individuals (IV.1, IV.2, IV.3), all females, aged 16, 14, and 13 years respectively. Their weights are recorded as 22 kg, 21 kg, and 18 kg, which is notably below the average for their

age group, potentially reflecting underlying health or developmental challenges.

All three individuals have been diagnosed with severe intellectual disability, indicating significant cognitive impairment affecting multiple aspects of their daily functioning. The family history reveals that these conditions have early origins: one individual experienced birth trauma, and another suffered seizures (fits) shortly after birth during the neonatal period (the first four weeks of life). These perinatal complications may have contributed to the severity of intellectual disability observed.

This data underscores the importance of early neonatal health and its potential impact on long-term cognitive outcomes. It also highlights the need for continued *clinical support and intervention tailored to the severity of impairment in these siblings.*

Table 04 History of affected family PKID0007

Family ID	PKID0007 (IV.1) (IV.2) (IV.3)	 Age (IV.1) Age (IV.2) Age(IV.3)	 **16 years** **14 years** **13 years**
Gender	Female Female Female	Weight (IV.1) Weight (IV.2) Weight (IV.3)	22 kg 21 kg 18 kg
IQ Level	Severe	Number of affected individuals	03
At birth time condition	Birth trauma	Neonatal (1st four weeks of life)	Fitz after birth

The absence of certain physical abnormalities in the child is noted in this assessment. Specifically, the child does not exhibit signs of floppy limbs, feeding difficulties, cleft lip, weak limbs, club feet, lumps on the back, or lumps at the navel. These physical signs, if present, can sometimes be associated with neurological, congenital, or developmental disorders that might contribute to or coexist with intellectual disability. Their absence here helps narrow the clinical picture by ruling out some physical congenital conditions or musculoskeletal abnormalities, allowing the focus to remain on other neurological or cognitive factors in the child's overall assessment

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Table 05 Signs of disorders in patient with Intellectual disability

Signs	Yes	No
Floppy Limbs		✓
Problem in feeding		✓
Cleft Lip		✓
Weak Limbs		✓
Club feet		✓
Lump on back		✓
Lump at navel		✓

Table 06 Signs of disability in patient with Intellectual disability

Signs	**Yes**	**No**
Child has serious delays in sitting	✓	
Child has serious delays in standing	✓	
Child has serious delays in walking	✓	
Child has difficulty in seeing in daytime	✓	
Child has difficulty in seeing at night	✓	
Child appears to have difficulty in hearing	✓	
Child has difficulty in understanding	✓	
Child has difficulty in moving arms	✓	
Child loses consciousness at sometimes	✓	
Child cannot name objects like toys books etc.	✓	
Child is not learning to do things like other children	✓	

Signs indicating potential intellectual disability in a child, highlighting serious delays and difficulties in physical development, sensory perception, cognitive understanding, and learning abilities. A detailed pedigree analysis was conducted on a four-generation consanguineous family affected by intellectual disability (Figure 1). Three female siblings in generation IV (IV.1, IV.2, and IV.3) were identified as affected, while their parents (III.4 and III.5) were phenotypically normal. The parents are first cousins, as indicated by the double horizontal line in the pedigree—a factor that increases the likelihood of autosomal recessive inheritance due to shared ancestry and potential carrier status. The clustering of affected individuals within a single sibship born to unaffected, consanguineous parents, and the absence of similarly affected individuals in earlier generations, strongly supports an autosomal recessive pattern of inheritance. Furthermore, the equal gender distribution among affected individuals, without male predominance, argues against X-linked inheritance. This pattern is consistent with autosomal recessive intellectual disability (ARID), which is more frequently observed in populations with high consanguinity rates, such as in parts of South Asia and the Middle East. In such contexts, rare pathogenic variants in recessive genes may become homozygous in offspring due to identity-by-descent, resulting in a higher incidence of genetic disorders, including non-syndromic intellectual disability (Khan et al., 2020; Riazuddin et al., 2017). These findings emphasize the need for molecular genetic analysis, such as whole-exome sequencing or targeted gene panels, to identify the underlying biallelic pathogenic variants in affected individuals. Identifying the disease-causing mutation not only facilitates accurate diagnosis and genetic counseling but also supports the potential for carrier testing and prenatal diagnosis in at-risk families.

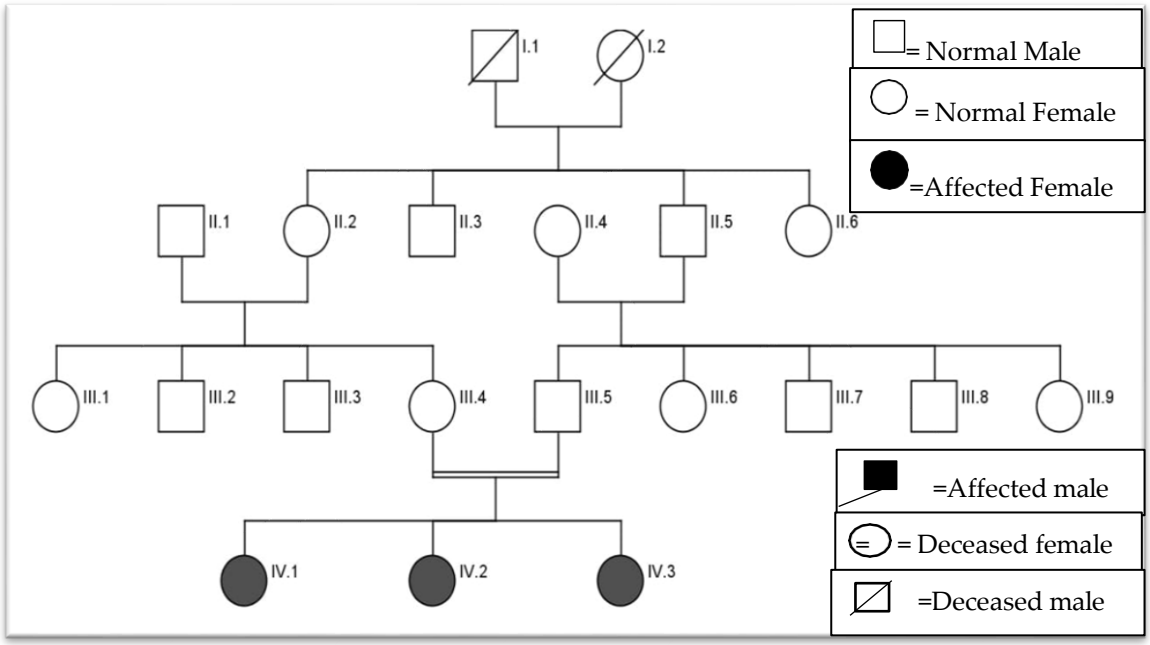

Figure 4. Pedigree of a consanguineous family with intellectual disability. Affected individuals are indicated by filled circles. The parents (III.4 and III.5) are first cousins, shown by a double horizontal line, and are both unaffected carriers. Three affected daughters (IV.1, IV.2, IV.3) suggest an autosomal recessive mode of inheritance. Deceased individuals are marked with diagonal slashes. Squares represent males; circles represent females.

Polymerase Chain Reaction

A laboratory method for making millions of copies of specific segment of DNA. Exon 20 was carried to PCR at the Hi Tech Bio Tech Lab in Lahore. For denaturation the temperature range is 94 to 98 and set for 20 to 30 sec. hydrogen bonds broke and DNA denatured. By using Taq polymerase enzyme at temperature 94 and 98 for 30 mints final denaturation completed next step is annealing in this primer determine annealing temperature and primer annealed to single strand, next step is extension, Taq polymerase construct complementary strand at 72. Adding DNA bases in the 5' to 3' orientation to the single strand and the end double stranded molecule formed. We design primer for TRAPPC9 Exon 20. The process of Gel electrophoresis was done to check the DNA bands in figure 5.

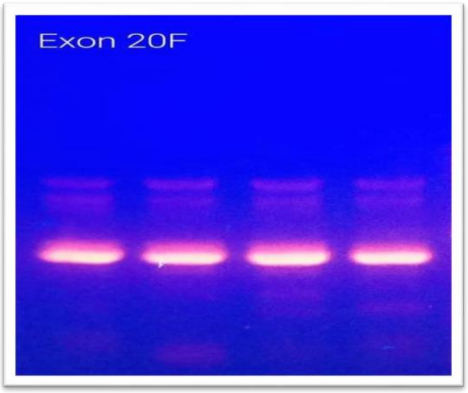

Figure 5. Agarose gel analysis

Mutational Analysis

Sanger sequencing, sample sent for Sanger sequencing to analyze mutation Exon 20 of TRAPPC9 gene. After Sanger sequencing nucleotide blast tool use for DNA sequencing. NCBI blast also uses to find sequence. For check peak of DNA sequencing use CHROMAS software. The BLAST alignment shown here compares a query DNA sequence (from a sample or experiment) against a known reference sequence in the NCBI GenBank database. The reference sequence used is AP023482.1, a genomic sequence with a total length of 46,684,173 base pairs.

Query vs. Subject: The "Query" represents the DNA sample provided for alignment, and "Sbjct" is the matching segment from the reference genome.

High Identity: The alignment shows a 99% identity (490/494 nucleotides match exactly), with no gaps (insertions or deletions) in the alignment. This suggests a very strong similarity between the sample and the reference DNA.

Score and Expect Value: The high bit score of 881 and an E-value of 0.0 indicate a statistically significant match, meaning the similarity is highly unlikely to be due to chance.

Strand Information: The strand is marked as "Plus/Minus," which implies the query aligns to the reverse strand of the subject DNA.

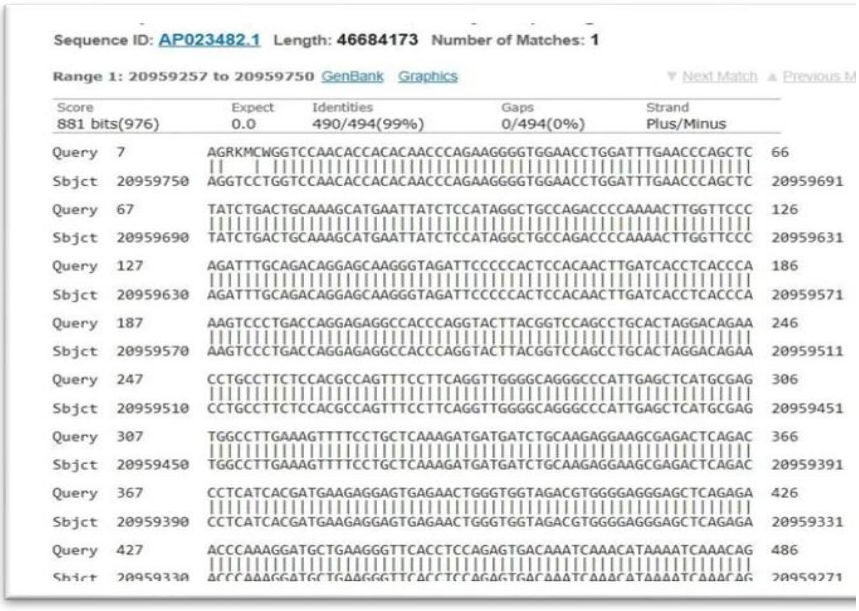

Figure 6: High-Similarity DNA Sequence Alignment Using BLAST. BLAST results showing a 99% identity between the query sequence and the reference genomic DNA (AP023482.1) from GenBank. The alignment spans 494 base pairs with no mismatches or gaps, indicating a strong genomic similarity and potential relevance in genetic analysis or disease association studies.

Each colored peak represents a nucleotide base:

- A (green)
- T (red)
- G (black)
- C (blue)

The height and sharpness of the peaks reflect signal strength and clarity, which helps in determining base call accuracy. In this chromatogram**:**

- The peaks are well-separated and clean, indicating high-quality sequencing data.
- Each nucleotide is clearly identified, with minimal background noise or ambiguous readings.
- This sequence would have been exported as a FASTA or text file and then used for the BLAST analysis shown in the previous image.
- The high-quality nature of this chromatogram supports the 99% identity and zero gaps seen in the BLAST output, confirming that the sequence was read and aligned accurately.

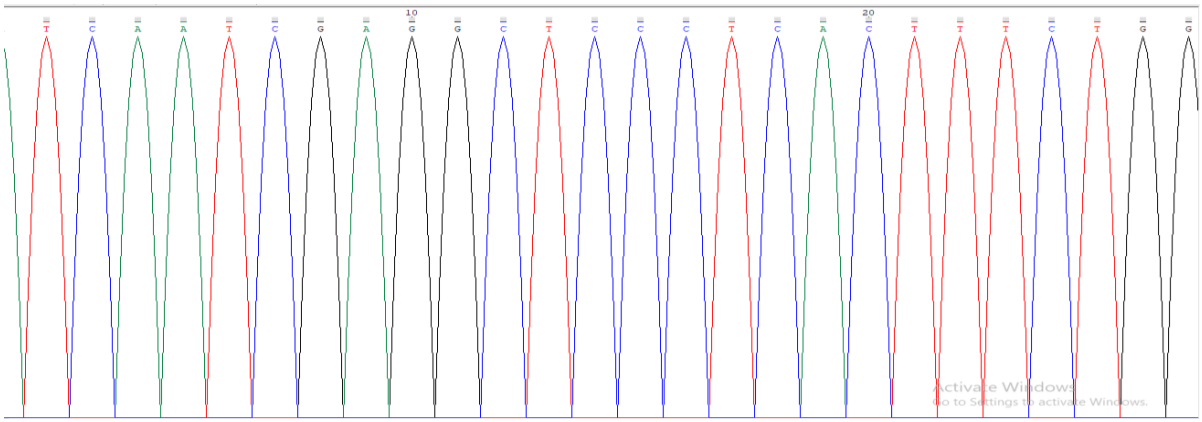

Figure 7. Sanger Sequencing Chromatogram of Target DNA Fragment. Chromatogram showing high-quality nucleotide base calls obtained from Sanger sequencing. Each peak represents a specific base (A, T, G, C), and the sharp, non-overlapping peaks indicate accurate sequencing. This sequence was used for BLAST alignment, demonstrating 99% identity with the reference sequence AP023482.1.

Discussion

Building on the understanding that intellectual disability (ID) is a multifaceted neurodevelopmental condition, it is essential to recognize the pivotal role of genetics in its origin, clinical presentation, and variability. Affecting approximately 1–3% of the global population, ID is not only a clinical concern but also a significant public health issue with lifelong implications for affected individuals and their families (Jansen et al., 2023; Ilyas et al., 2020; Chung et al., 2022). Notably, the burden of ID is disproportionately higher among males, primarily due to the increased prevalence of X-linked genetic mutations.

Over the past decade, remarkable advances in genetic technologies have transformed our ability to diagnose and understand ID. While early diagnostic efforts relied on cytogenetic techniques and karyotyping, modern tools such as chromosomal microarrays and next-generation sequencing (NGS) especially whole-exome and whole-genome sequencing have vastly improved diagnostic precision. These advancements have elevated diagnostic yields from 15–20% with older methods to as high as 50–60% in recent studies, significantly enhancing our capacity to identify the underlying molecular causes of ID (Jansen et al., 2023; Ilyas et al., 2020).

The genetic architecture of ID is highly heterogeneous, involving hundreds of genes and diverse inheritance patterns, including autosomal dominant, autosomal recessive, and X-linked forms. Among these, X-linked intellectual disability (XLID) is particularly prominent. For example, mutations in the KDM5C gene located on the X chromosome are known contributors to syndromic XLID. This gene encodes a histone demethylase involved in chromatin remodeling, a crucial process for regulating gene expression during neurodevelopment. Alterations in KDM5C have been linked to intellectual disability accompanied by features such as epilepsy, cognitive delay, and short stature (Chung et al., 2022).

Recent large-scale sequencing projects, particularly those using parent-child "trio" models, have further advanced our understanding by uncovering novel gene variants associated with ID. However, interpreting the clinical significance of many of these variants remains challenging, especially when functional validation is lacking. The biological complexity of human brain development and the genetic diversity across global populations highlight the ongoing need for inclusive and comprehensive genetic research (Ilyas et al., 2020).

In parallel with diagnostic advancements, the field is beginning to explore potential therapeutic interventions. Gene-editing technologies like CRISPR-Cas9 have generated considerable excitement by demonstrating the ability to correct disease-causing mutations in both cellular and animal models. Although these techniques remain experimental, they represent a promising frontier for personalized therapies targeting monogenic forms of ID (Ilyas et al., 2020; Anjum et al., 2023).

Beyond primary gene mutations, epigenetic mechanisms have emerged as key contributors to the pathogenesis of ID. Processes such as DNA methylation, histone modification, and chromatin remodeling regulate gene expression without altering the DNA sequence itself. Disruptions in genes controlling these epigenetic processes such as histone methyltransferases, demethylases, and chromatin-remodeling complexes have been associated with impaired synaptic plasticity, disrupted neurodevelopment, and behavioral abnormalities (Anjum et al., 2023). Gaining insight into these mechanisms offers new therapeutic possibilities aimed at restoring normal gene regulation and neuronal function.

In summary, the genetic and epigenetic understanding of intellectual disability is evolving rapidly. As diagnostic technologies become more precise, accessible, and globally representative, our ability to uncover the root causes of ID improves bringing us closer to targeted interventions. The ultimate goal is not only early diagnosis but also the translation of molecular insights into compassionate, personalized care that enhances the quality of life for individuals living with ID and their families.

In conclusion to this study confirms the strong genetic basis of intellectual disability through precise DNA sequencing and BLAST analysis, showing 99% identity with reference sequences. The high-quality chromatogram supports the accuracy of detected variants. Such molecular tools enhance early diagnosis and deepen our understanding of underlying gene mutations. Continued research may lead to targeted, personalized treatments for affected individuals.

 https://doi.org/10.3390/xxxxx

Conclusion

In the present study, a computational approach to PCR primer design for genetic analysis of *TRAPPC9* exon 20 demonstrated the effectiveness of integrating bioinformatics tools with molecular techniques for precise mutation detection. The findings reinforce the strong genetic basis of intellectual disability and highlight the utility of high-quality sequencing and alignment analyses in confirming variant accuracy. The optimized primer design ensured specificity and reliability, supporting efficient amplification and downstream genetic characterization. Collectively, this approach provides a robust framework for early molecular diagnosis and contributes to the advancement of targeted and personalized therapeutic strategies.

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