Genetic studies of deafness in Pakistan

By

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CIIT/FA09-PBS-001/ISB

PhD Thesis

COMSATS Institute of Information Technology
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Genetic studies of deafness in Pakistan

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In partial fulfillment

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(Biosciences)

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It is certified that Ms. **Sobia Shafique, CIIT/FA09-PBS-001** has carried out all the work related to this thesis under my supervision at the Department of Biosciences, CIIT, Islamabad and the work fulfills the requirement for the award of PhD degree.

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DEDICATION

My Loving Parents,

My Wonderful Husband

&

My Precious Son

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Sobia Shafique

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ABSTRACT

Genetic studies of deafness in Pakistan

The current study was conducted in a cohort of 32 families to identify the genetic factors causative of autosomal recessive non-syndromic hearing loss (ARNSHL) in these families. Frequently mutated deafness genes, *GJB2* and *MYO15A* were initially screened. The *GJB2* and *MYO15A* screening excluded 12 ARNSHL families, which were further analyzed by homozygosity mapping using whole genome single nucleotide polymorphism (SNP) array genotyping. Known ARNSHL genes residing in the homozygous regions were sequenced for the identification of genetic defect in the families. This resulted in the identification of mutations in different candidate genes in 30 families. Along with 12 recurrent mutations that were detected in 21 families, 9 novel mutations were also identified in 9 different families (*GJB2* (c.598G>A, p.Gly200Arg); *MYO15A* (c.9948G>A, p.Gln3316Gln; c.3866+1G>A; c.8767C>T, p.Arg2923* and c.8222T>C, p.Phe2741Ser), *TMC1* (c.362+18A>G), *BSND* (c.97G>C, p.Val33Leu), *TMPRSS3* (c.726C>G, p.Cys242Trp) and *MSRB3* (c.20T>G, p.Leu7Arg)). Among the 21 novel and recurrent mutations, 10 (48%) were missense changes, 4 (19%) nonsense variations, 3 (14%) intronic changes, 2 (9%) splice site mutations and 2 (9%) were frameshift changes. 50% of the families carried *GJB2* mutations while *MYO15A* was the second most frequent (13%) cause of ARNSHL in these 30 families. Of the 32 families the remaining two excluded families, were exome sequenced, which resulted in the identification of putative ARNSHL novel genes. Interestingly, three variants c.193G>A (p.Ala65Thr), c.633+1G>C (p.?) and c.32C>G (p.Ser11*) in three different genes *TSPEAR*, *CCNI2* and *PCDHGA10*, segregated with ARNSHL in one of the family. In another family a variant c.2910C>A (p.Asp970Glu) was detected in *ATP10B* that segregated with the phenotype. As the genetic screening resulted in the identification of genetic defect in all the 32 families, the present study therefore increases the spectrum of deafness causing mutations and adds novel genes to the existing data, which could lead to the identification of population specific deafness genes causative of ARNSHL. In addition, these results provide detailed genetic information that has potential diagnostic implication in the establishment of a cost effective and time efficient deafness gene mutation screening scheme such as molecular inversion probe analysis.

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LIST OF ABBREVIATIONS

ABR	Auditory Brainstem Response
ADNSHL	Autosomal Dominant Non-Syndromic Hearing Loss
Arg	Arginine
ARNSHL	Autosomal Recessive Non-Syndromic Hearing Loss
ATP	Adenosine Tri Phosphate
BAM	Binary Sequence Alignment/Map format
bp	Base Pair
BLAT	Blast Like Alignment Tool
BOA	Behavioral Observation Audiometry
BSND	Barttin CLCNK-type Chloride Channel Accessory Beta Subunit
Ca^{+}	Calcium Ion
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
cM	Centi Morgan
DAPT	Difluorophenylacetyl-alanyl- phenylglycine-t-butyl-ester
dB	Decibel
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
EOAE	Evoked Otoacoustic Emission
EVS	Exome Variant Server
Ex	Exon
GJB2	Gap Junction Protein Beta 2
HCl	Hydrochloric Acid
HI	Hearing Impairment
HL	Hearing Loss
HOPE	Have your Protein Explained
Hz	Hertz
IHC	Inner Hair Cell
K^{+}	Potassium Ion
Kb	Kilobases
KCl	Potassium Chloride
KCl	Potassium Chloride
LB	Lauria Broth
LDH	Lactate Dehydrogenase
Leu	Leucine
LOH	Loss of Heterozygosity
LOD	Logarithm of Odds
M	Molar
Mb	Megabases
mg	Milligram
$MgCl_2$	Magnesium Chloride
MIP	Molecular Inversion Probe
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MSRB3	Methionine Sulphoxide Reductase B3

MYO15A	Myosin XVA
Na^+	Sodium Ion
NaCl	Sodium Chloride
ng	Nanogram
NGS	Next Generation Sequencing
OHC	Outer Hair Cell
OMIM	Online Mendelian Inheritance in Man
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Log of Hydrogen Ion Concentration
PhyloP	Phylogenetic Profiling Conservation Score Analysis
PolyPhen-2	Polymorphism Phenotyping v2
qPCR	Quantitative polymerase chain reaction
RBC	Red Blood Cell
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
Rpm	Revolution Per Minute
rs	Reference SNP
RT	Reverse Transcription
RP	Retinitis Pigmentosa
SDS	Sodium Dodecyl Sulphate
siRNA	Small interfering RNA
SIFT	Sorting Intolerant From Tolerant
SLC26A4	Solute Carrier Family 26 Member 4
SM	Scala Media
SNHL	Sensorineural Hearing Loss
SNP	Single Nucleotide Polymorphism
SRT	Speech Reception Threshold
STR	Short Tandem Repeat
STS	Sequence Tagged Site
ST	Scala Tympani
SV	Scala Vestibuli
T	Thymine
Taq	Thermophilus Aquaticus
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	Tetra Ethyl Methyl Ethylene Diamine
TM	Melting Temperature
TMC1	Transmembrane Channel-Like Gene 1
TMIE	Transmembrane Inner Ear-expressed
TMPRSS3	Transmembrane Protease, Serine 3
U	International Unit for Enzyme Activity
UV	Ultra Violet
VNTR	Variable Number of Tandem Repeat
VRA	Visual Reinforcement Audiometry
WBC	White Blood Cell
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
α	Alpha

β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre
°C	Degree Celsius

Chapter 1

Introduction

1. Introduction

1.1. Anatomy and Function of the Ear

The human ear is an intricate sensory organ that consists of both an auditory and a vestibular system. The auditory assembly is divided into three functional regions: outer ear, middle ear, and inner ear (Figure 1.1).

1.1.1. The Outer Ear

The outer ear consists of the visible fleshy appendage (auricle or pinna) and the external auditory canal. The auricle channels the sound waves into the S-shaped external auditory canal, which is part of the bony tube called the external auditory meatus. The widening of the diameter of the auditory canal along its length results in amplification of the sound waves. The external auditory canal extends from the auricle to a thin epithelial double layered partition called the tympanic membrane. The middle and inner ear are physically separated by the tympanic membrane by forming a barrier between the external auditory canal and the middle ear (Figure 1.1). Hair and ceruminous glands (sebaceous glands) are embedded in the skin near the entrance of the external auditory canal, which prevents foreign particles from reaching the tympanic membrane. The wax secreted by these ceruminous glands also keeps the tympanic membrane soft and waterproof (Møller, 2006).

1.1.2. The Middle Ear

The middle ear is an air-filled chamber consisting of the tympanic cavity. It is separated from the inner ear by a bony partition containing the vestibular window and the cochlear window. Three auditory ossicles are attached to the tympanic cavity by ligaments: malleus (hammer), incus (anvil), and stapes (stirrup) (Figure 1.1). The malleus passes vibrations to the incus, which passes them to the stapes. The auditory ossicles thus transmit the vibrations across the tympanic cavity to the vestibular window. The vibrating medium for the travelling sound waves changes from air (middle ear) to liquid (inner ear) as the vibrations are propagated across the vestibular window. The sound waves are amplified about 20 fold as they move from the large surface area of the tympanic membrane to a much smaller surface area of the vestibular window (Møller, 2006).

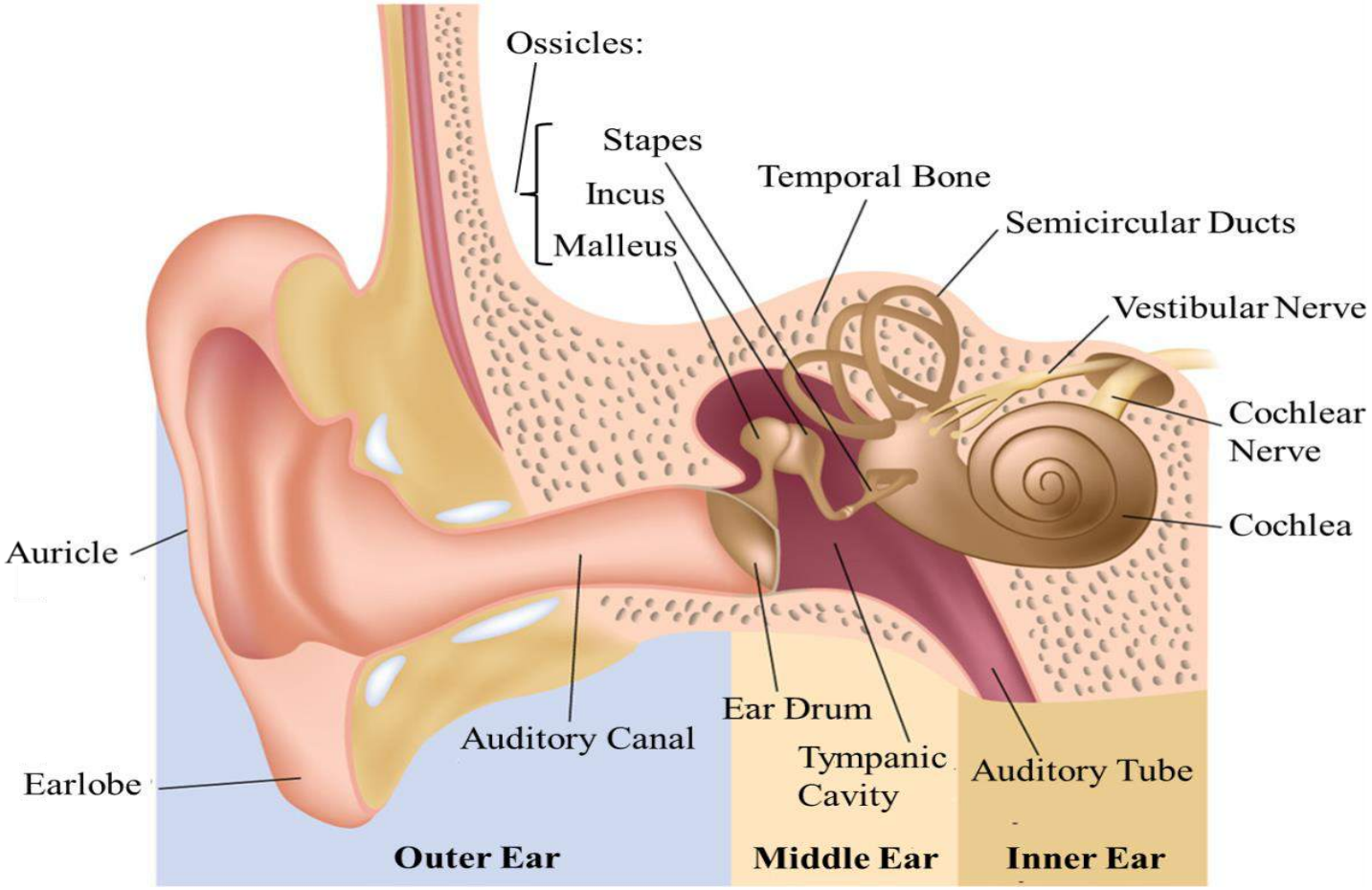

Figure 1.1. Anatomy of a human ear differentiated into the three functional regions: outer ear, middle ear, and inner ear (http://www.yournursingtutor.com/wpcontent/uploads/2012/06/9727799_l.jpg).

1.1.3. The Inner Ear

The inner ear consists of the vestibular system (involved in maintaining equilibrium) and the auditory system (involved in hearing), which are the two main functional units of the balancing and hearing processes. The bony and the membranous labyrinth along with the hair cells are important structures of the inner ear as these are involved in equilibrium and the hearing process.

1.1.3.1. Bony labyrinth

The bony labyrinth structurally and functionally consists of three major areas: the vestibule, the semicircular canals and the cochlea. The vestibule is the central portion and contains the vestibular or oval window, into which the stapes fit at one end and the cochlear or round window is present on the opposite end (Figure 1.2). The osseous or bony labyrinth is composed of a dense bone that surrounds and protects the membranous labyrinth. The bony labyrinth is filled with perilymph and is lined by periosteum. Perilymph resembles the extra cellular fluid due to its high Na^+ and a low K^+ concentration. The tubular chambers of the membranous labyrinth are filled with endolymph, which is rich in K^+. This difference in the ionic composition of the two fluids creates a potential difference required for the mechanoelectrical transduction of sound across these structures.

1.1.3.2. Membranous labyrinth

The membranous labyrinth lies within the bony labyrinth and inside the vestibule and is composed of two connected sacs: utricle and saccule (Figure 1.3). The utricle and saccule contain receptors that are responsive to gravity and linear motion. The three bony semicircular canals positioned at nearly right angles to each other are sensitive to angular acceleration and deceleration of the head. The snail-shaped cochlea is coiled two and a half times around a bony axis called modiolus (Figure 1.3). Spiral lamina (Figure 1.3) is a thin bony plate, which projects out from the modiolus and divides the cochlear canal into two chambers. The upper chamber is scala vestibule and starts with the oval window and runs along the vestibule, while the lower chamber is scala tympani, which ends at the round window (Figure 1.3). Both these chambers are filled with perilymph. The triangular middle chamber called the cochlear duct or scala media lies between the scala vestibuli and the scala tympani

and is filled with endolymph (Møller, 2006).

Within the cochlear duct is a specialized structure called the spiral organ or organ of corti (Figure 1.3), which lies above the basilar membrane. The organ of corti includes the extra cellular matrix called the tectorial membrane, supporting cells, neurons and hair cells. The tips of these hair cells are embedded in the tectorial membrane, which forms a gelatinous canopy over them (Figure 1.3).

1.1.3.3. Hair cells

The sensory hair cells originate from the ectoderm and are located in the cochlea. These hair cells are surrounded by the supporting cells (Figure 1.3), and are made of actin-filled microvilli called stereocilia (Figure 1.4), which are arranged in a V shape on the hair cells at different heights. The tip stretches from the upper end of the shorter stereocilium to the nearest taller stereocilium, which functions in opening of the transduction channels; resulting in the depolarization of the hair cells. The swaying of the stereocilia back and forth makes the hair cells hyperpolarized.

There are two kinds of hair cells, the outer hair cells (OHCs) and the inner hair cells (IHCs).

1.1.3.3.1. Outer hair cells

There are roughly 12,000 OHCs arranged in 3-4 rows. These cells are cylindrical in shape. Although there are far more OHCs than IHCs they only receive roughly 5% of the distribution of the nerve fibers from the VIII cranial nerve.

1.1.3.3.2. Inner hair cells

IHCs are pear shaped sensory cells in the cochlea, which function in signal transduction. There are roughly 3000-3500 IHCs, which are connected to roughly 95% of the distribution of the nerve fibers from the VIII cranial nerve. When IHCs are damaged there is a severe to profound hearing loss (Møller, 2006; Pickles *et al.*, 1984).

1.2. Sound Wave Transduction

Sound waves are characterized by their frequency (measured in Hz) and intensity (measured in dB). The frequency is directly related to the sound pitch while the

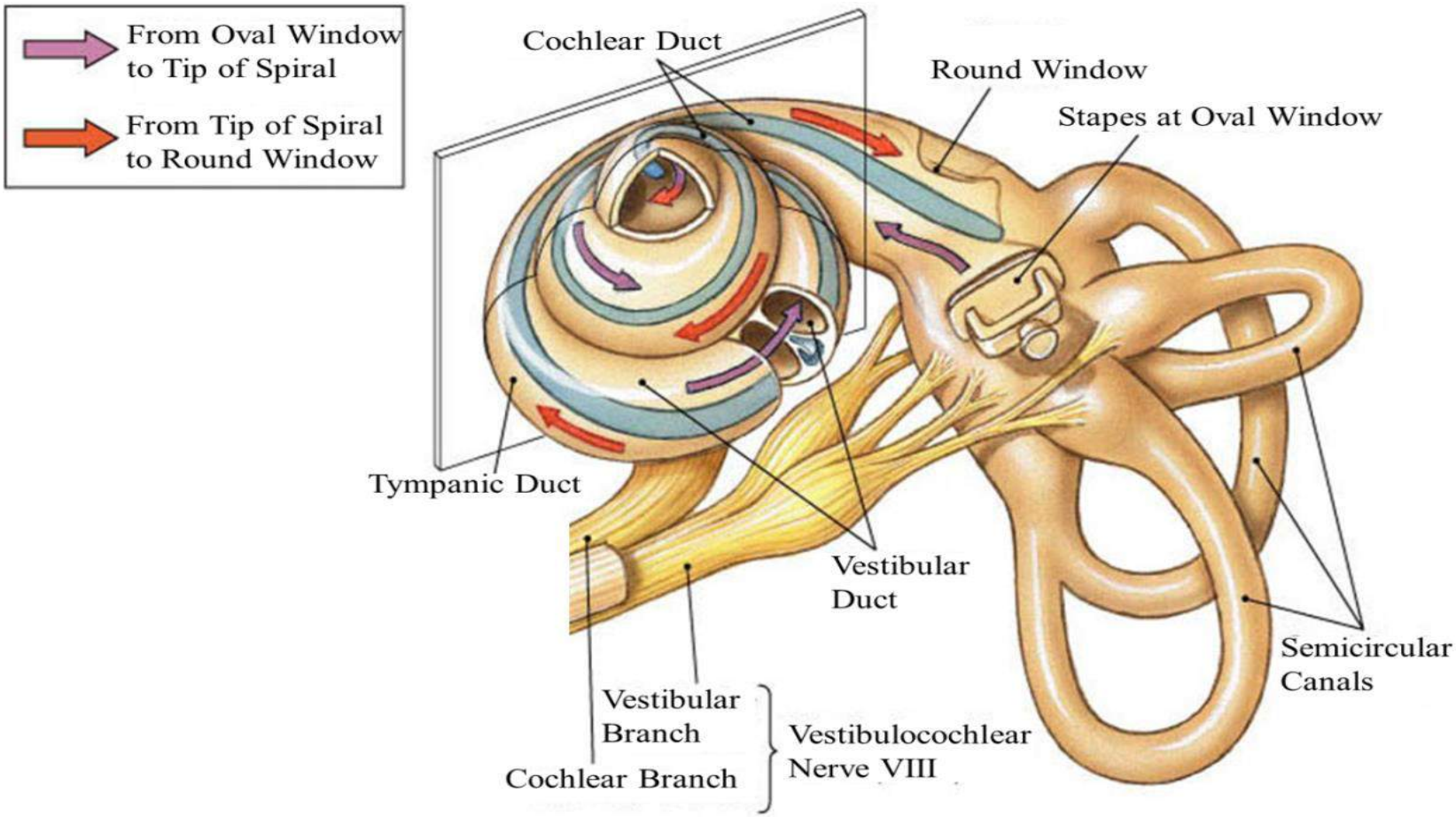

Figure 1.2. The cochlear structures showing the movement of sound wave from the vestibular to the cochlear window (http://droualb.faculty.mjc.edu/Lecture%20Notes/Unit%205/cochlea_I.jpg).

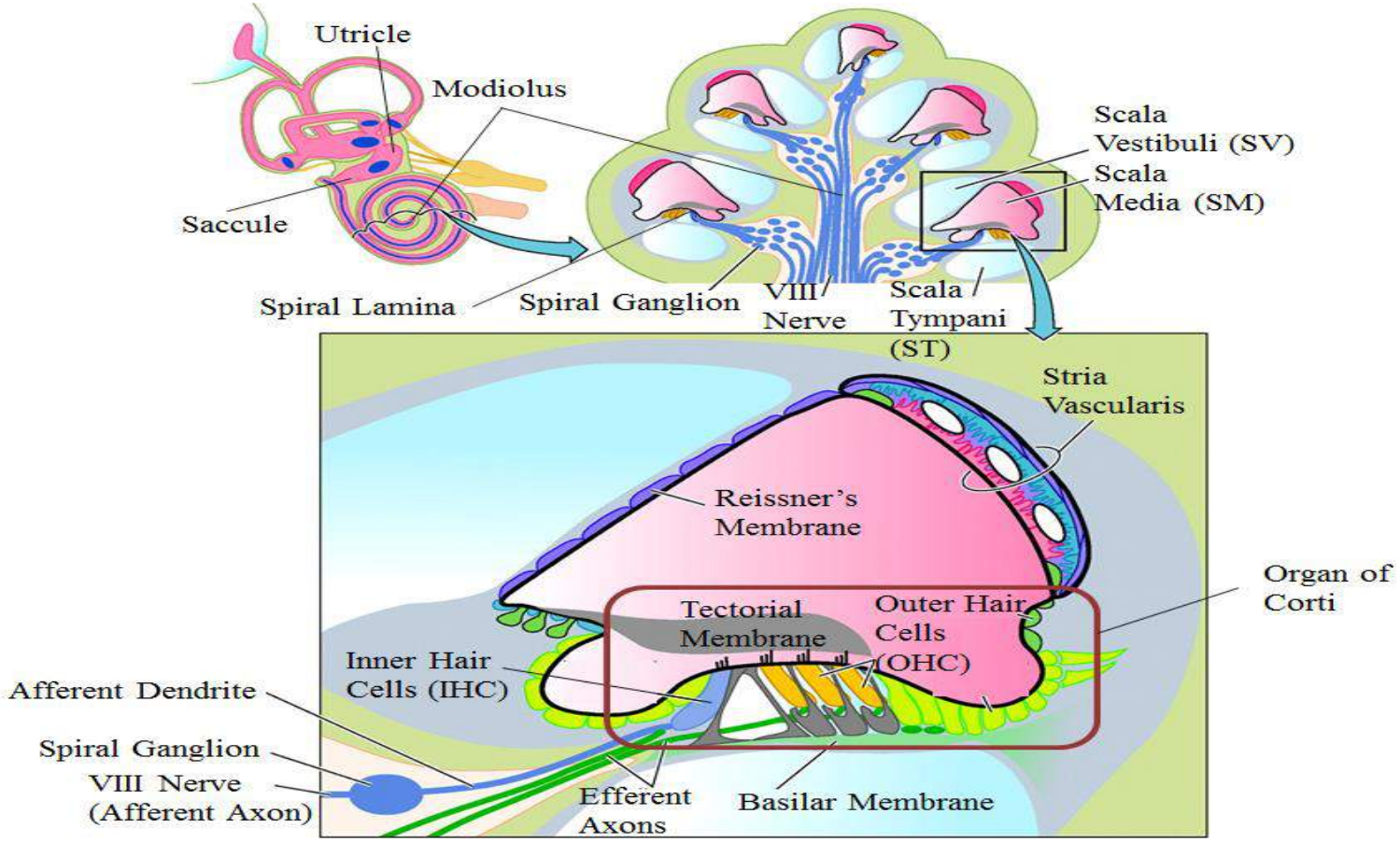

Figure 1.3. Cross-section of cochlea showing scala media (SM) sandwiched between scala vestibuli (SV) and scala tympani (ST). The organ of corti is shown with inner hair cells (IHC) and outer hair cells (OHC), which are responsible for the amplification of sound waves (modified and adapted from Zdebik *et al.* (2009).

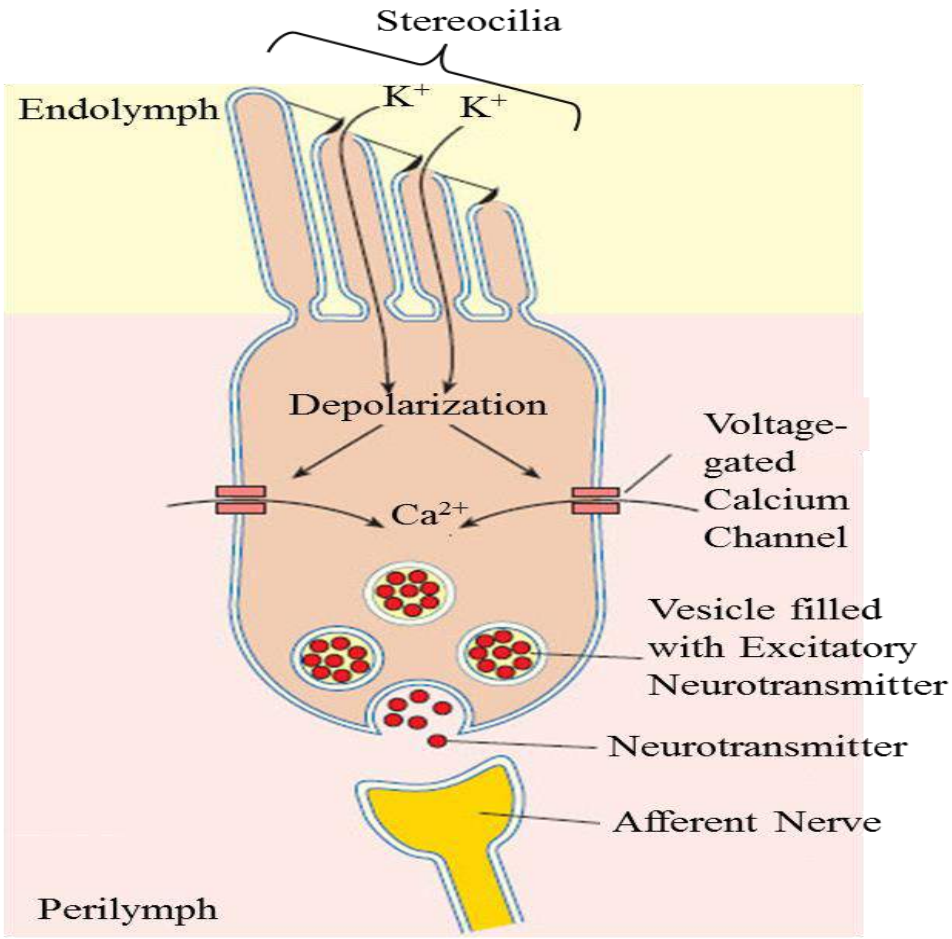

Figure 1.4. The depolarization of the hair cells occurs with the influx of K^+, which cause the voltage-gated calcium channels to open. The incoming Ca^{2+} result in the release of the neurotransmitter (http://labspace.open.ac.uk/file.php/5630/SD329_1_017i.jpg).

amplitude of the sound waves is related to the intensity or the loudness. A barely audible sound at the threshold of hearing has an intensity of zero dB. Every increase of 10 decibels (dB) indicates a tenfold increase in sound intensity. The human ear can easily differentiate between the intensity range of 0.1 dB to 0.5 dB, while the human hearing threshold lies between the minimum and maximum range of 0 dB and 120 dB, respectively. For proper characterization of hearing loss, audiometry is performed along with a detailed clinical assessment by a physician (Kochhar *et al.*, 2007).

As the sound waves pass through the external auditory canal, they cause vibrations of the tympanic membrane, these vibrations are transmitted through the auditory ossicles, which amplifies it approximately 20 fold as it reaches the stapes, which is located within the vestibular window. The vibrating auditory ossicles create varying pressure on the vestibular window resulting in the generation of waves in the perilymph passing onto the scala vestibuli and reaching the scala tympani. The successive waves of the perilymph within the scala tympani results in the to and fro motion of the cochlear window into the tympanic cavity.

The vibrations then travel along the length of the basilar membrane resulting in the movement of the organ of corti present on top of the basilar membrane and containing the IHCs and the OHCs. The cilia of the IHCs and OHCs are embedded in the tectorial membrane. This tugging of the tectorial membrane causes deflection within the hair cells stereocilia, triggering the opening and closing of the ion channels. These gated channels located at the tips of the stereocilia in turn allow the influx of K^+ present in the endolymph resulting in the depolarization of the hair cells anchored in the basilar membrane. This stimulation and excitation of the sensory cells opens up the voltage gated channels and results in the influx of Ca^{2+}, which cause the release of the neurotransmitter from the receptor cell and leads to the excitation of the sensory endings of the cochlear nerve. Cochlear sensory neurons in the vestibulocochlear nerve (VIII) synapses with neurons in the medulla oblongata, which project to the midbrain; thus the action potential travels through an afferent nerve fibre (Figure 1.4) from the hair cell to the brain. Neurons in this area in turn project to the thalamus, which finally sends axons to the auditory cortex of the temporal lobe resulting in the translation of the auditory sensations as sound (Hudspeth, 1989; Møller, 2006; Pickles *et al.*, 1984). The sounds of variable intensities are distinguished on the basis of changes in the OHCs length. In case of low intensity sound waves, the impulses

are carried by the afferent nerve of the OHCs to the brain, which in turn signals the OHCs to shorten its length thus tugging the tectorial membrane and causing depolarization of the IHCs leading to sound perception. In the case of high intensity sound waves the above process leading to the deflection of the IHCs stereocilia results in sound perception. However, the vigorous movement of the organ of corti can cause damage to the stereocilia, therefore to protect the stereocilia, OHCs length is increased, which in turn pushes the tectorial membrane away.

The cochlear duct has the property to distinguish between a range of frequencies. It has the capacity to resonate sound waves of variable frequencies at different locations along its length. The length of the hair cells along with their respective stereocilia undergo a gradual increase from the base to the apex of the cochlear duct. In addition, the distance between the inner and outer hair cells increases gradually from the base towards the apex. Therefore, the basilar membrane undergoes maximum displacement at the base upon receiving a sound of higher frequency; while it undergoes maximum displacement at the apex, which is caused when a sound of lower frequency is received (Figure 1.5).

1.3. Hearing Loss

Hearing loss/ hearing impairment or deafness is the inability to hear sound in one or both ears partially or completely. It can be a permanent or a progressive loss of hearing. Hearing loss is known to be the most common congenital sensorineural disorder affecting 1 in 1000 children. 4% of people aged younger than 45 years are affected by hearing loss (Estivill *et al.*, 1998); while 50% people above the age of 80 years experience hearing loss (Huang and Tang, 2010; Nadol, 1993). 278 million people worldwide suffer from hearing loss (Morton and Nance, 2006; Smith *et al.*, 2005). Early onset of hearing loss adversely affects the development of the child in terms of speech acquisition, which further influences their social interaction. Quality of life deteriorates equally in the case of early and late onset hearing loss thus having a significant impact upon the affected individual.

1.4. Characterization and Etiology of Hearing Loss

Hearing loss is characterized on the basis of five major factors: type, cause, onset, association and degree (Figure 1.6). Another reason of hearing loss could be the

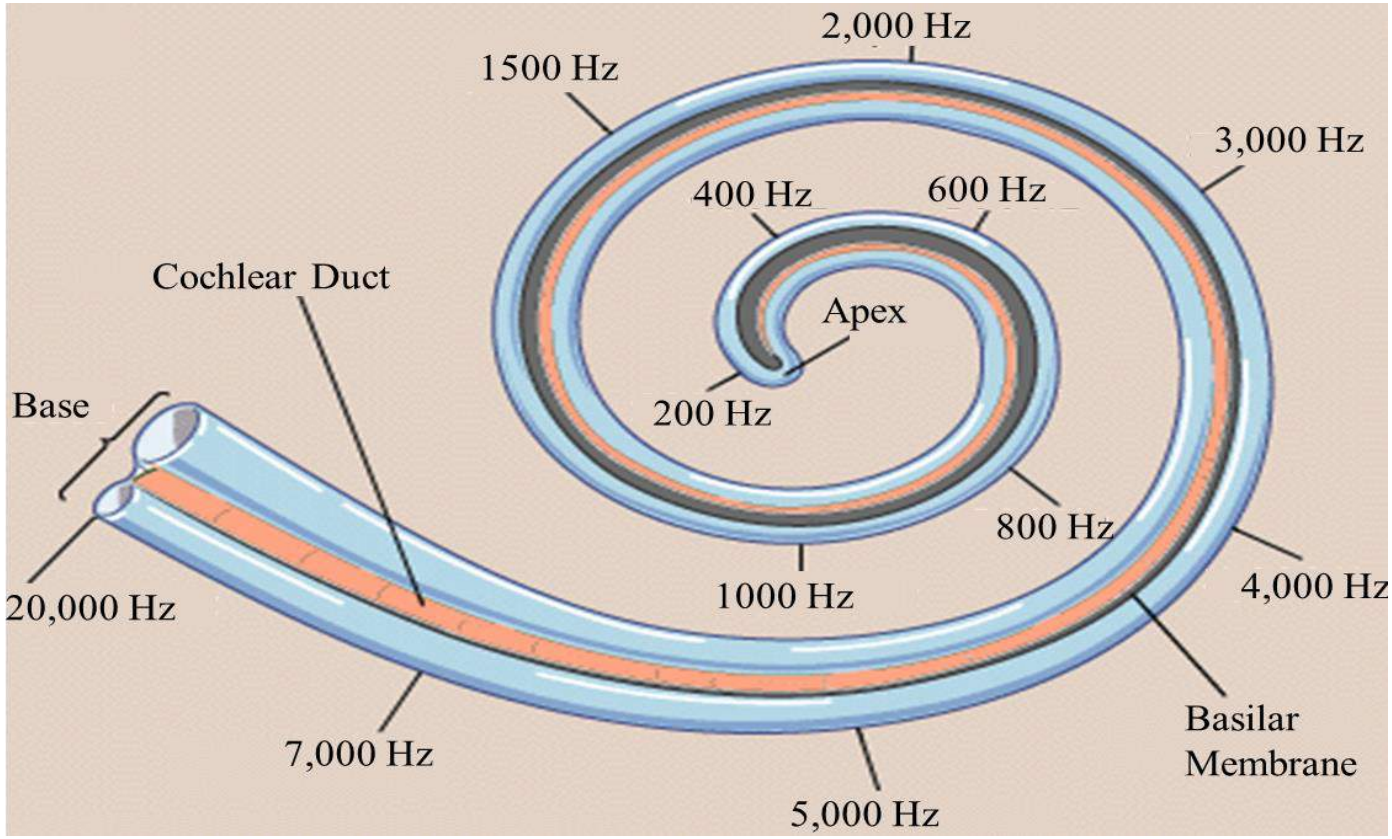

Figure 1.5. The types and lengths of the transverse basilar fibres distinguish between the sounds of different frequencies within the organ of corti (http://media-2.web.britannica.com/eb-media/98/14298-004-99934987.gif).

central auditory dysfunction, this happens when the auditory cortex is damaged due to trauma, disease, hereditary factors and tumors.

1.4.1. Hearing Loss by Type

1.4.1.1. Conductive hearing loss

Conductive hearing loss is caused by the sound waves not being transmitted from the outer ear to the middle or inner ear including the ear canal, ear drum, ossicles and middle ear. Due to this, sound waves do not reach the stapes or the oval window thus causing hearing loss. Treatment for this type of hearing loss is surgical such as draining fluid in the middle ear or repairing a punctured eardrum or replacement of the ossicles.

1.4.1.1.1. External ear

Any foreign object blocking the ear canal can also reduce the amount of sound reaching the inner ear. The most common cause is cerumen (ear wax); however, it rarely causes significant hearing loss as the ear canal must be fully blocked to cause any significant change in hearing. If the ear wax is pressed against the ear drum it impedes the movement of the ear drum hence the vibrations are not passed onto the middle ear. Infections of the external ear will also affect hearing as infections can cause swelling and release of debris, which may block the pathway.

1.4.1.1.2. Tympanic membrane

Small perforations in the tympanic membrane do not cause significant hearing loss unless it results in the spread of infection to the middle ear. Large perforations reduce the flexible surface area, thus causing an impairment in the transmission of vibrations resulting in hearing loss.

1.4.1.1.3. Middle ear

Infections and cholesteatoma are disrupting pathological processes of the middle ear, which can often cause hearing loss. An example would be acute otitis media that can lead to temporary hearing loss. As the infection subsides, there may still be persisting loss of hearing depending on the severity of the infection.

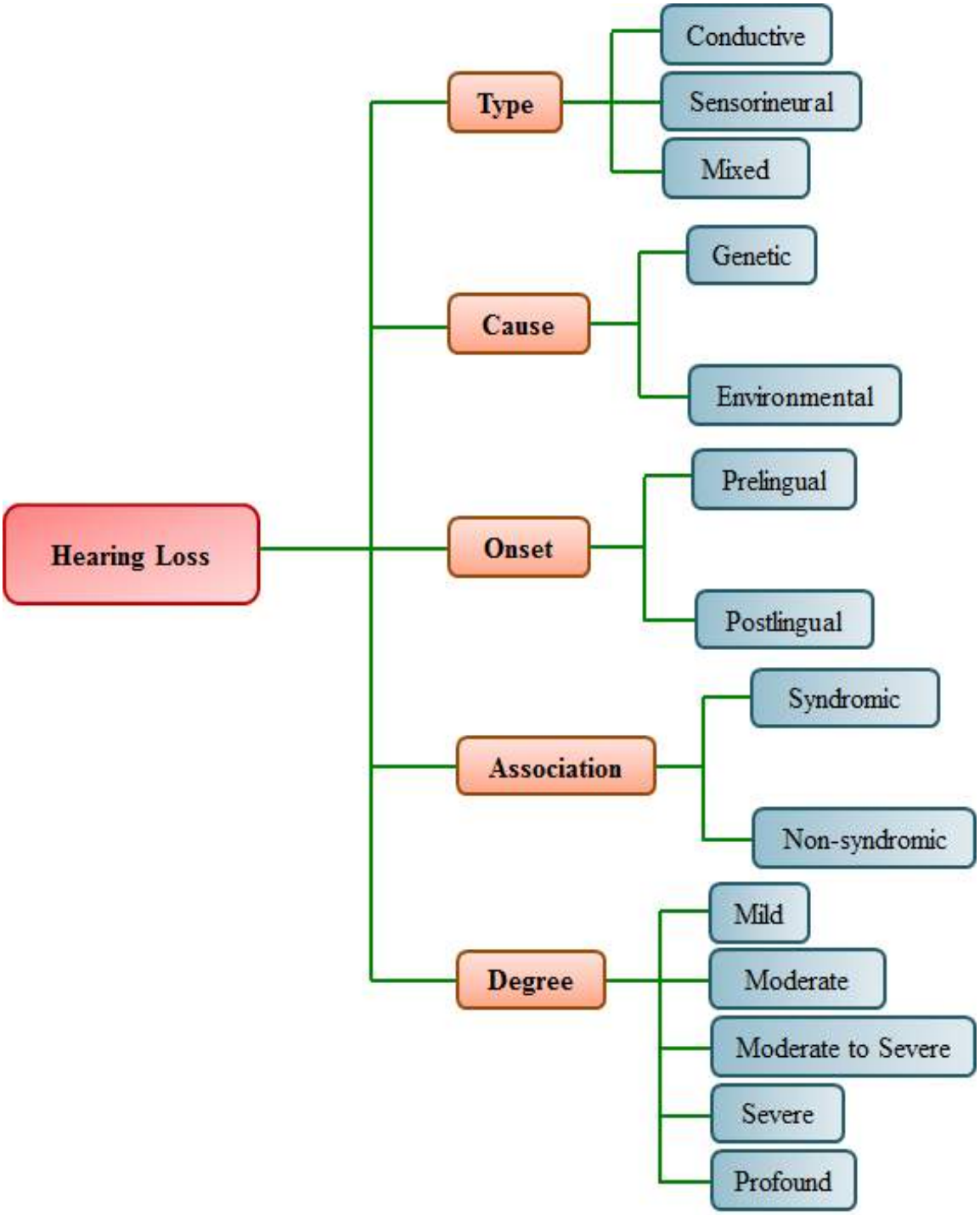

Figure 1.6. Classification of hearing impairment on the basis of type, cause, onset, association and degree.

1.4.1.2. Sensorineural hearing loss

Sensorineural hearing loss (SNHL) is caused by the damage to the cochlear structure or any afferent or efferent nerve pathway. SNHL can be inherited, which could occur independently without any other disease, or as part of an inherited syndrome.

1.4.1.3. Mixed hearing loss

This occurs when both conductive and sensorineural hearing loss are seen in the same patient. It is possible that the patient first develops conductive hearing loss and then also develops sensorineural hearing loss (Møller, 2006) (Figure 1.6).

1.4.2. Hearing Loss by Cause

Genetic factors account for 50% of hearing loss cases while the other half is caused by environmental factors (Toriello *et al.*, 2004) (Figure 1.6). The environmental factors include prenatal infections caused by TORCH organisms including toxoplasmosis, rubella, cytomegalovirus and herpes; and exposure to ototoxic drugs or trauma. Postnatal infections in the form of bacterial meningitis such as *Streptococcus pneumoniae*, *Haemophilus influenza* and *Neisseria meningitides* can also result in environmental hearing loss in children. Variable, fluctuating sensorineural hearing loss is associated with neonatal asymptomatic cytomegalovirus (CMV) infection (Roizen, 2003). Hearing loss in adults is usually due to environmental factors in combination with genetic factors such as exposure to loud noise. The hearing loss with advancing age is called presbycusis and is marked by reduced sound perception (Konings *et al.*, 2009).

1.4.3. Hearing Loss by Onset

The onset of hearing loss can be prelingual or postlingual (Figure 1.6), the onset of the former is before the development of speech. All congenital hearing loss is prelingual; but not *vice versa*. Postlingual hearing loss develops after the development of speech.

1.4.4. Hearing Loss by Association

Genetic causes can either lead to non-syndromic or syndromic hearing loss (Figure 1.6). Syndromic hearing loss has a pleiotropic effect; where it is associated with other

phenotypic traits as well as abnormalities of the ear. Whereas non-syndromic hearing loss is associated with defects of the middle or inner ear only (Birkenhager *et al.*, 2007).

1.4.5. Hearing Loss by Degree

Hearing loss can also be categorized by severity or degree of loss. It can be mild (26-40 dB), moderate (41-55 dB), moderate to severe (56-70 dB), severe (71-90 dB) and profound (90 dB) (Kochhar *et al.*, 2007). Loss of sound perception of varying intensities determines whether the hearing loss is designated as low frequency (<500 Hz), middle frequency (501-2000 Hz), or high frequency (>2000 Hz).

1.5. Clinical Diagnosis of Hearing Loss

Hearing loss is classified by audiometry, which comprises of behavioral testing and pure-tone audiometry.

1.5.1. Behavioral Testing

Behavioral testing involves two main techniques: behavioral observation audiometry (BOA) and visual reinforcement audiometry (VRA). BOA is used in children upto the age of six months. VRA is used in children of ages six months to two and a half years. It can produce a reliable audiogram; however, it is still dependent on the skills of the tester and the maturity of the child.

1.5.2. Pure-tone Audiometry

Pure-tone audiometry (air and bone conduction) assesses the lowest intensity of sound a person can hear as a function of frequency. In air conduction testing, octave frequencies of 125, 150, 500, 1000, 2000, 4000, and 8000 Hz are used. Speech reception thresholds (SRT) and speech discrimination scores are also tested. Sound is frequencies of 125, 150, 500, 1000, 2000, 4000, and 8000 Hz are used. Speech reception thresholds (SRT) and speech discrimination scores are also tested. Sound is delivered through earphones in the air conduction audiometry. This determines the condition of the external ear, middle ear, and inner ear. In bone conduction audiometry, a vibrating tuning fork is placed on the mastoid bone or the forehead. Octave frequencies of 250, 500, 1000, 2000 and 4000 Hz are used. This directly

determines the condition of the inner ear and the function of the cochlea as it bypasses the external and the middle ear. From these data an audiogram is constructed on which frequency (Hz) is on the horizontal axis and the sound intensity (dB) is on the vertical axis. In addition, speech audiometry measures the ability of a person to hear and understand spoken sentences.

1.5.3. Auditory Brainstem Response

This is used to test the inner ear and brain pathways involved in hearing. It involves the placement of electrodes on the head and recording the electrophysiological responses, which originate from the VIII cranial nerve in response to certain sounds. It correlates best with hearing sensitivity range of 1000-4000 Hz.

1.5.4. Evoked Otoacoustic Emission

EOAEs are sounds generated by the cochlea, these are measured by inserting a probe with a microphone and a transducer in the external auditory canal. EOAE is used in ears that are capable of only hearing 40-50 dB (Kochhar *et al.*, 2007).

1.6. Hereditary Hearing Loss

Hearing loss and other genetic diseases are becoming frequent particularly due to consanguineous marriages and lack of knowledge about disease inheritance patterns. Various research groups in Pakistan and worldwide are working on the genetics of hearing loss. The most common form of hearing loss is ARNSHL, which will be the main focus of the current study. Recent advancements in molecular biology techniques provide useful tools to the molecular geneticists to locate the mutated genes, which are the root cause of genetic disorders. Genetic etiology of hearing loss can be grouped into two categories: syndromic hearing loss and non-syndromic hearing loss. Of the many different forms of inheritance, ARNSHL is the most severe in its phenotype. It is non-progressive, severe to profound, either congenital or postlingual. On the other hand, dominant deafness is progressive and does not affect speech development due to its postlingual nature (Van Camp *et al.*, 1997).

1.6.1. Syndromic Hearing Loss

Syndromic deafness is responsible for about 30% of prelingual deafness in

combination with other clinical features (Van Camp *et al.*, 1997), 700 such genetic syndromes are known. The disease can follow a recessive, dominant, X-linked, or mitochondrial mode of inheritance. Till date, 45 loci have been mapped and 44 genes have been identified in the case of syndromic deafness (Hereditary Hearing Loss Homepage, URL: http://hereditaryhearingloss.org/). Some examples of syndromes with a dominant mode of inheritance include Waardenburg, Brachio-oto-renal, Stickler and Neurofibromatosis 2.

1.6.1.1. Autosomal dominant syndromic hearing loss

Waardenburg syndrome (WS) follows an autosomal dominant mode of inheritance and it causes sensorineural hearing impairment as well as pigmentary abnormalities of the skin, hair (white forelock) and eyes (heterochromia iridis). Branchio-oto-renal, Stickler, etc. are other syndromes that follow a dominant inheritance pattern (Toriello *et al.*, 2004). Six different genes have been identified for WS (Hereditary Hearing Loss Homepage, URL: http://hereditaryhearingloss.org/).

1.6.1.2. Autosomal recessive syndromic hearing loss

Usher, Pendred, Jervell, Lange-Nielsen, etc. syndromes are recessively inherited syndromes. Usher syndrome is the most common of these syndromes and has an autosomal location. Affected individuals suffer from congenital sensory defects and later on develop retinitis pigmentosa (RP) (Toriello *et al.*, 2004). Usher syndrome accounts for 50% of the deaf-blind and 3-6% of congenital blindness cases (Boughman and Fishman, 1983). In total, 17 loci have been mapped and 11 genes have been identified for Usher syndrome (Hereditary Hearing Loss Homepage, URL: http://hereditaryhearingloss.org/).

Pendred syndrome is the second most common syndrome that follows a recessive mode of inheritance and accounts for ~4-10% of inherited hearing loss. The symptoms of this syndrome include congenital severe to profound sensorineural hearing loss and goiter (Fraser, 1965), in addition the vestibular function is also impaired. The causative gene is solute carrier family 26 member 4 (*SLC26A4*) (MIM#605646), which encodes the anion transporter pendrin (Everett *et al.*, 1997).

1.6.1.3. X-linked syndromic hearing loss

Alport, Mohr-Tranebjaerg, etc. are the syndromes that follow an X-linked mode of inheritance. In Alport syndrome the collagen type IV alpha-5 (*COL4A5*) (MIM#303630) gene produces a defective type IV collagen (Barker *et al.*, 1990; King *et al.*, 2002). The syndrome causes infection of the kidney and progressive sensorineural hearing loss (Toriello *et al.*, 2004).

1.6.2. Non-syndromic Hearing Loss

Non-syndromic hearing loss is usually due to abnormalities of the inner and/or middle ear and is found in 70% of hereditary cases (Van Camp *et al.*, 1997). Non-syndromic hearing loss can have an autosomal recessive, autosomal dominant, X-linked, and mitochondrial mode of inheritance, which contribute to 80%, 18%, 1-3% and <1% of the cases, respectively (Morton, 1991). Standard locus nomenclature for non-syndromic autosomal recessive deafness starts with DFNB, while for autosomal dominant it is DFNA and DFN for X-linked. Till date 140 loci and 91 genes have been identified, which are believed to cause non-syndromic hearing loss. Among them 76 recessive (Table 1.1), 55 dominant, 5 X-linked, 2 Modifier, 1 Y-linked and 1 AUNA loci have been mapped (Hereditary Hearing Loss Homepage, URL: http://hereditaryhearingloss.org/). Except for a few exceptions, prelingual deafness is commonly found in autosomal recessive and X-linked inheritance. Autosomal dominant non-syndromic hearing loss (ADNSHL) is a heterogeneous disorder, which is characterized by postlingual, late onset and progressive sensorineural hearing loss with certain exceptions. Postlingual hearing loss is multifactorial and involves environmental and genetic factors (Bayazit and Yilmaz, 2006). In the current study, we have focused on ARNSHL families and have identified the causative genes using techniques such as homozygosity mapping (whole genome SNP microarray analysis) and Exome sequencing.

Mutated genes that have been found to cause various forms of non-syndromic hearing loss and follow a recessive inheritance pattern are given in Table 1.1 (Hereditary Hearing Loss Homepage, URL: http://hereditaryhearingloss.org/).

1.7. Techniques Used to Identify the Causative Genes

1.7.1. Homozygosity Mapping

Homozygosity mapping is a commonly used technique for identifying the disease causing loci/genes in familial genetic studies.

Homozygosity based linkage analysis uses highly polymorphic genetic markers that have a known chromosomal location. Genotyping different markers in individuals results in the construction of a haplotype of alleles, which they inherit from both parents. These haplotypes are further analysed to find the causative locus containing the mutated gene. Significance of linkage is determined by calculating logarithm of odds (LOD) score. LOD score of 3.3 is considered as a significant evidence for linkage of the marker to the disease locus (Dawn Teare and Barrett, 2005; Lander and Botstein, 1987; Strachan *et al.*, 2011; Woods *et al.*, 2006).

In consanguineous marriages, 10% of the genome is homozygous and these mostly harbor the causative mutated gene. Therefore, it is best to analyze consanguineous families with affected and non-affected individuals using homozygosity mapping to identify the mutations in disease causing genes in autosomal recessive disorders (Dawn Teare and Barrett, 2005; Lander and Botstein, 1987; Strachan *et al.*, 2011; Woods *et al.*, 2006).

Single Nucleotide Polymorphisms (SNPs) are scattered throughout the genome and it is estimated that 1 SNP is present at an interval of 200-300 bp and overall 3.2 million SNPs are spread over the human genome. The use of high-resolution single nucleotide polymorphism (SNP) microarrays has provided geneticists with a powerful tool for genetic studies as SNPs are abundant and spread more evenly over the genome as compared to microsatellite markers (Chitkara *et al.*, 1994; Sachidanandam *et al.*, 2001; Strachan *et al.*, 2011).

This technique is very powerful in mapping the disease genes in consanguineous families with inherited disorders and has shown good results in Pakistan (Ahmed *et al.*, 2011; Micheal *et al.*, 2014; Rafiq *et al.*, 2010), India (Ganapathy *et al.*, 2014; Nirmalan *et al.*, 2006), Saudi Arabia (Al-Qahtani *et al.*, 2010; Imtiaz *et al.*, 2011; Ramzan *et al.*, 2013), Turkey (Kalay *et al.*, 2007; Seco *et al.*, 2014) and also in non-

Table 1.1. Loci and genes involved in causing autosomal recessive non-syndromic hearing loss

Locus	Gene	OMIM	References
DFNB1	*GJB2*	121011	Guilford *et al.*, 1994b; Kelsell *et al.*, 1997
DFNB2	*MYO7A*	276903	Guilford *et al.*, 1994a; Liu *et al.*, 1997; Weil *et al.*, 1997
DFNB3	*MYO15A*	602666	Friedman *et al.*, 1995; Wang *et al.*, 1998
DFNB4	*SLC26A4*	605646	Baldwin *et al.*, 1995; Li *et al.*, 1998
DFNB5	-	*600792	Fukushima *et al.*, 1995
DFNB6	*TMIE*	607237	Fukushima *et al.*, 1995; Naz *et al.*, 2002
DFNB7/11	*TMC1*	606706	Jain *et al.*, 1995; Kurima *et al.*, 2002; Scott *et al.*, 1996
DFNB8/10	*TMPRSS3*	605511	Bonne-Tamir *et al.*, 1996; Scott *et al.*, 2001; Veske *et al.*, 1996
DFNB9	*OTOF*	603681	Chaib *et al.*, 1996a; Yasunaga *et al.*, 1999
DFNB12	*CDH23*	605516	Bork *et al.*, 2001; Chaib *et al.*, 1996b
DFNB13	-	*603098	Mustapha *et al.*, 1998a
DFNB14	-	* 603678	Mustapha *et al.*, 1998b
DFNB15/ 72/95	*GIPC3*	608792	Ain *et al.*, 2007; Charizopoulou *et al.*, 2011; Rehman *et al.*, 2011
DFNB16	*STRC*	606440	Campbell *et al.*, 1997; Verpy *et al.*, 2001
DFNB17	-	*603010	Greinwald *et al.*, 1998
DFNB18	*USH1C*	605242	Ahmed *et al.*, 2002; Jain *et al.*, 1998; Sottile *et al.*, 1990
DFNB19	-	$	**
DFNB20	-	*604060	Moynihan *et al.*, 1999
DFNB21	*TECTA*	602574	Mustapha *et al.*, 1999
DFNB22	*OTOA*	607038	Zwaenepoel *et al.*, 2002
DFNB23	*PCDH15*	605514	Ahmed *et al.*, 2003b
DFNB24	*RDX*	179410	Khan *et al.*, 2007
DFNB25	*GRXCR1*	613283	Schraders *et al.*, 2010a
DFNB26	-	*605428	Riazuddin *et al.*, 2000
DFNB27	-	*605818	Riazuddin *et al.*, 2000
DFNB28	*TRIOBP*	609761	Riazuddin *et al.*, 2006b; Shahin *et al.*, 2006
DFNB29	*CLDN14*	605608	Wilcox *et al.*, 2001
DFNB30	*MYO3A*	606808	Walsh *et al.*, 2002
DFNB31	*WHRN*	607928	Mburu *et al.*, 2003; Mustapha *et al.*, 2002
DFNB33	-	*607239	Medlej-Hashim *et al.*, 2002
DFNB35	*ESRRB*	602167	Ansar *et al.*, 2003a; Collin *et al.*, 2008
DFNB36	*ESPN*	606351	Naz *et al.*, 2004
DFNB37	*MYO6*	600970	Ahmed *et al.*, 2003a
DFNB38	-	*608219	Ansar *et al.*, 2003b
DFNB39	*HGF*	142409	Schultz *et al.*, 2009
DFNB40	-	*608264	Delmaghani *et al.*, 2003
DFNB42	*ILDR1*	609739	Aslam *et al.*, 2005; Borck *et al.*, 2011

Table 1.1. cont.

Locus	Gene	OMIM	References
DFNB44	*ADCY1*	103072	Ansar *et al.*, 2004; Santos-Cortez *et al.*, 2014
DFNB45	-	*612433	Bhatti *et al.*, 2008
DFNB46	-	*609647	Mir *et al.*, 2005
DFNB48	*CIB2*	605564	Ahmad *et al.*, 2005
DFNB49	*MARVELD2*	610572	Ramzan *et al.*, 2005; Riazuddin *et al.*, 2006a
DFNB49	*BDP1*	607012	Girotto *et al.*, 2013
DFNB51	-	*609941	Shaikh *et al.*, 2005
DFNB53	*COL11A2*	120290	Chen *et al.*, 2005
DFNB55	-	*609952	Irshad *et al.*, 2005
DFNB59	*PJVK*	610219	Delmaghani *et al.*, 2006
DFNB61	*SLC26A5*	604943	Liu *et al.*, 2003
DFNB62	-	*610143	Ali *et al.*, 2006
DFNB63	*LRTOMT*/ *COMT2*	612414	Ahmed *et al.*, 2008; Du *et al.*, 2008
DFNB65	-	*610248	Tariq *et al.*, 2006
DFNB66/67	*LHFPL5*	609427	Kalay *et al.*, 2006; Shabbir *et al.*, 2006; Tlili *et al.*, 2005
DFNB68	-	*610419	Santos *et al.*, 2006
DFNB71	-	*612789	Chishti *et al.*, 2009
DFNB73	*BSND*	606412	Riazuddin *et al.*, 2009
DFNB74	*MSRB3*	613719	Ahmed *et al.*, 2011; Waryah *et al.*, 2009
DFNB76	*SYNE4*	615535	Horn *et al.*, 2013
DFNB77	*LOXHD1*	613072	Grillet *et al.*, 2009
DFNB79	*TPRN*	613354	Li *et al.*, 2010; Rehman *et al.*, 2010
DFNB80	-	$	Ali Mosrati *et al.*, 2013
DFNB81	-	$	Rehman *et al.*, 2011
DFNB82/32	*GPSM2*	609245	Walsh *et al.*, 2010
DFNB83/47	-	*609946	Hassan *et al.*, 2006
DFNB84	*PTPRQ*	603317	Schraders *et al.*, 2010b
DFNB84	*OTOGL*	614925	Yariz *et al.*, 2012
DFNB85	-	*613392	Shahin *et al.*, 2010
DFNB86	*TBC1D24*	613577	Ali *et al.*, 2012; Rehman *et al.*, 2014
DFNB88	*ELMOD3*	615427	Jaworek *et al.*, 2013
DFNB89	*KARS*	601421	Basit *et al.*, 2011; Santos-Cortez *et al.*, 2013
DFNB90	-	$	Ali *et al.*, 2011
DFNB91	*SERPINB6*	173321	Sirmaci *et al.*, 2010
DFNB93	*CABP2*	607314	Tabatabaiefar *et al.*, 2011
DFNB96	-	*614414	Ansar *et al.*, 2011
DFNB98	*TSPEAR*	612920	Delmaghani *et al.*, 2012
DFNB101	*GRXCR2*	615762	Imtiaz *et al.*, 2014
DFNB102	*CLIC5*	607293	Seco *et al.*, 2014

Table 1.1. cont.

Locus	Gene	OMIM	References
[&]	*OTOG*	604487	Schraders *et al.*, 2012
[&]	*EPS8*	600206	Behlouli *et al.*, 2014

DFNB, Deafness, Non-syndromic, Recessive; *, These OMIM# are for the loci for which genes have not yet been identified; [$], No OMIM# has been assigned to the DFNB80, DFNB81, DFNB90 loci; **, Not published yet, however presented at The Molecular Biology of Hearing and Deafness Meeting, Bethesda, October 8-11, 1998 (Green *et al.*, abstract 108); [&], No locus has been assigned to the gene

consanguineous families in The Netherlands (Astuto *et al.*, 2002; Collin *et al.*, 2011), Japan (Kondo *et al.*, 2004) and other populations (Aller *et al.*, 2010; Hildebrandt *et al.*, 2009).

1.7.2. Next-Generation Sequencing

Next-generation sequencing (NGS) is a fast and highly accurate DNA sequencing method. This technique can produce large amounts of data by sequencing numerous DNA strands in parallel. The technique is faster and cheaper as compared to the Sanger sequencing method (Gilissen *et al.*, 2011; Metzker, 2010). The major steps involved in NGS include target selection, enrichment, sequencing, imaging, and finally alignment of the sequence data to the reference genome. Target enrichment strategies can provide an enriched sequence for specific regions or fragments of a chromosome (target NGS), whole exome sequencing (WES), or whole genome sequencing (WGS) (Majewski *et al.*, 2011; Metzker, 2010).

1.8. Genetic Counseling

Molecular genetic counseling is the process of providing information of the identified mutations to the families and individuals regarding their disease status and its management. The determination of the carrier status is very important for spouse selection in case of hereditary genetic disorders. When a therapeutic intervention is possible the affected family is referred to a medical consultant for further management (Bowles Biesecker and Marteau, 1999).

Diseases that are inherited recessively e.g. ARNSHL have severe phenotypes, whereas diseases inherited dominantly are milder in nature. An autosomal gene mutation affects males and females equally; however, X-linked disorders in males produce a more severe phenotype. This is because males are hemizygous, where the affected males usually have normal parents with the mother being a carrier (Avraham and Kanaan, 2012; Idan *et al.*, 2013). Genetic counseling can be very accurate for well-known genotype-phenotype correlations; however, it is much harder in heterogeneous or dominant cases. In addition, the absence of informative family history and pathogenic mutations as in the case of *de novo* mutations could pose a challenge to the genetic counselors (Mahdieh *et al.*, 2010).

Genetic counseling services are not well established in Pakistan, therefore consanguineous marriages occur at a high rate, making the genetic diseases highly prevalent in the Pakistani population. In addition, the availability and trend to seek prenatal diagnosis is non-existant. Genetic counseling in Pakistan is urgently needed to manage the further spread of genetic disorders and educate people to change the prevailing social practices. It is important to understand that it is easier to prevent a disease than treat it later.

1.9. Therapeutic Interventions for Hearing Loss

The impairment associated with genetic diseases such as hearing loss can be countered by using oral supplements such as magnesium and antioxidants in adults (Choi *et al.*, 2014); while the use of vitamin A minimizes the risk of hearing loss in children (Wieringa *et al.*, 2012). Cochlear implants are the most common rehabilitation option for the severe to profound hearing loss patients. The success of this approach is dependent on factors such as the age at which the patient is implanted and his/her residual hearing. The success and performance of cochlear implant is thought to be poorer where hearing loss is caused by neural damage of the auditory cortex (e.g., caused by cytomegalovirus, meningitis, auditory neuropathy) (Pyman *et al.*, 2000) as compared to those which are known to primarily effect the hair cells (e.g. ARNSHL), *e.g.* mutations in the gap junction protein beta 2 (*GJB2*) (MIM#121011) or gap junction protein beta 6 (*GJB6*) (MIM#604418), which affect the cochlea and lead to ARNSHL. Several studies assessed speech perception after cochlear implants in children with *GJB2* mutations, improved speech and enhanced auditory perception results were observed in this *GJB2* group (Sinnathuray *et al.*, 2004a; Sinnathuray *et al.*, 2004b).

Recent work is more focused on developing a method to correct mutated genes using highly targeted techniques such as gene therapy. This is an advanced and expensive measure, which is difficult and impractical to implement in developing countries.

Gene therapy can be employed to drive the expression of a down-regulated or a missing gene by inserting a normal gene into the cells. To transport the gene into the inner ear, viruses such as retro, (Bedrosian *et al.*, 2006) adeno (Husseman and

Raphael, 2009), adeno associated (Bedrosian *et al.*, 2006; Iizuka *et al.*, 2008; Lalwani and Mhatre, 2003) and lenti (Bedrosian *et al.*, 2006), etc. are used. The adeno associated (AAVi) and lenti viruses are associated with minimum toxicity and prolonged expression, however, lenti viruses are preferred as they can carry a larger insert. However, the use of viral vectors is associated with toxicity and immunogenicity issues. Another effective and safe *in vitro* approach is to use small molecules such as DAPT (Difluorophenylacetyl-alanyl- phenylglycine-t-butyl-ester) in the inner ear; where they inhibit the gamma-secretase and increase the expression of the atonal homolog 1 (drosophila) (*ATOH1*) (MIM#601461) gene, thus generating new hair cells (Crawford and Roelink, 2007; Zhao *et al.*, 2011). Nanoparticles are a safer choice to deliver the genetic therapeutic agents to the affected cells, but their efficiency regarding specificity needs to be improved (Buckiova *et al.*, 2012).

Small interfering RNA (siRNA) work by blocking the expression of the complementary nucleotide stretch (Vickers *et al.*, 2003); while microRNA is a short stretch of RNA which binds to the complementary sequence or the target RNA and either leads to the blocking of protein synthesis or target degradation (Ambros, 2004). It has been documented that the siRNA inhibition of NADPH oxidase 3 (*NOX3*) (MIM#607105), which is involved in the generation of reactive oxygen specie within the inner ear, attenuates the hearing loss by protecting the hair cells (Mukherjea *et al.*, 2010).

The best route to administer the therapeutic drug is via the round window membrane, which acts as a barrier to restrict the transfer of molecules to the inner ear (Goycoolea, 2001; Jero *et al.*, 2001b). However, the permeability of the round window membrane needs to be increased in order to effectively deliver the therapeutic agent. This can be achieved by soaking the therapeutic agents i.e. vector/small molecule into gelatin sponge (Jero *et al.*, 2001a).

Mouse models have proven to be useful for studying hearing loss genes and hundreds of mouse models have been created to model human genetic hearing impairment. The mouse genome bears similarity to the human genome as both have ~30,000 genes, while only <1% of the mouse genome lacks those regions, which have no genetic similarity with human genetic makeup. Moreover, there lies a physiological resemblance between the mouse and the human cochlea. Gene replacement therapy in

transgenic mice helps to identify the functions of the novel genes and to characterize the genes within the pathways of the hearing mechanism (Avraham, 2003). The function of *ATOH1* has been characterized using transgenic mice; it was found that this gene is responsible for hair cell differentiation (Bermingham *et al.*, 1999). In addition, overexpression of *GJB2* has been shown to treat deaf mice lacking *GJB6* (Ahmad *et al.*, 2007). Another study has shown the successful treatment of hearing loss in knockout mice by AAV1 mediated delivery of vesicular glutamate transporter 3 (*VGLUT3*) (MIM#607557) gene (Akil *et al.*, 2012). The identification of the ARNSHL genes: otoancorin (*OTOA*) (MIM#607038), transmembrane inner ear-expressed (*TMIE*) (MIM#607237), transmembrane channel-like protein 1 (*TMC1*) (MIM#606706), myosin VI (*MYO6*) (MIM#600970), POU domain class 3, transcription factor 4 (*POU3F4*) (MIM#300039), POU domain class 4, transcription factor 3 (*POU4F3*) (MIM#602460) and eyes absent 4 *EYA4* (MIM#603550), has been done using orthologous mice genes: *Otoa*, *Tmie*, *Tmc1*, *Myo6sv*, *Pou3f4*, *Pou4f3*, and *Eya4*, respectively (Hereditary Hearing Loss Homepage, URL: http://hereditaryhearingloss.org/).

1.10. Prevention of Hearing Loss

Knowledge of disease genetics has advanced rapidly, therefore families of individuals with hearing impairment are keen to undergo molecular diagnosis to prevent the future occurrence of the disease in their family members (Boudreault *et al.*, 2010; Parker *et al.*, 2000; Withrow *et al.*, 2009). Using molecular diagnosis, a better understanding of the etiology of hearing impairment can assist in preimplantation techniques such as the use of embryonic cells; in addition prenatal diagnosis can be conducted using the fetal cells obtained from the amniotic fluid or the chorionic villi (Morton and Nance, 2006; Nance, 2003). In order to assist this, the needed information includes phenotypic features, which along with the inheritance pattern, type and ethnic background information assist in the genetic testing for ARNSHL in consanguineous families. However, screening of the frequently mutated candidate genes in hearing loss i.e. *GJB2*, *SLC26A4*, *CDH23*, *TMC1*, myosin XVA (*MYO15A*) (MIM#602666) and otoferlin (*OTOF*) (MIM#603681) was not impossible using Sanger sequencing and Molecular Inversion Probes (MIPs) but was extremely cumbersome and expensive. Additionally, the causative mutation in 66% of the

patients of Middle Eastern origin with inherited hearing loss could not be identified by screening these genes; as in these families it could either be a new variation residing within the same gene or a variation in a novel gene (Brownstein *et al.*, 2011). This problem can be efficiently solved by NGS or WES, which employs the parallel sequencing of hundreds of deafness genes and has thus resulted in facilitating genetic diagnostic of hearing loss and other disorders; and has provided an ideal solution to determine the causative mutations (Brownstein *et al.*, 2011; Rehm, 2013; Shearer *et al.*, 2010).

It must be pointed out that it is best to take preventive measures against genetic diseases rather than treating the patients after the onset of the disease especially in the underdeveloped countries.

1.11. Molecular Genetics of Hearing Loss

The most frequently associated genes in ARNSHL are *GJB2*, *MYO15A*, *TMC1*, *SLC26A4*, *OTOF* and *CDH23*. Each of these genes have been found to contain more than 20 different mutations, most of which have been reported in consanguineous families (Hilgert *et al.*, 2009a). Mutations in *GJB2* are the most common cause of ARNSHL, which can account for upto 50% of the cases in the Mediterranean region (Estivill *et al.*, 1998; Zelante *et al.*, 1997), while mutations in a number of other genes have been shown to cause deafness in the remaining cases. In the Pakistani population mutations in *MYO15A* account for 5% of recessive deafness (Friedman *et al.*, 2002). Described below are the various genes linked to hearing loss in Pakistani patients, which have been grouped into functional categories that will help in understanding their role in the hearing process.

1.11.1. *GJB2*

Ion homeostasis is maintained by the connexins including the *GJB2, GJB3* and *GJB6* (Li *et al.*, 2014; Loeza-Becerra *et al.*, 2014), in addition ion channels including *SLC26A4* and other genes such as *TMPRSS3* are also involved in this process. Mutations in these genes have been known to cause hearing loss (Hilgert *et al.*, 2009b). Gap junction beta 2 (*GJB2*) encodes the protein connexin 26 (Cx26). *GJB2* has one coding and one non-coding exon. The coding exon harbors the maximum number of the known *GJB2* mutations. This protein is important for the recycling of

ions (K^+ and Ca^{2+}), second messengers (cAMP, cGMP, IP_3), and other small molecules (glucose, siRNA). Where the ions (K^+ and Ca^{2+}), are involved in the generation of action potential in the hair cells. This exchange of K^+ and Ca^{2+} between the cytoplasm of adjacent cells is vital for physiological events such as cell differentiation, cell growth, etc. The connexons or hemichannel are formed by the oligomerisation of six subunits of the connexin protein. These proteins reside in the plasma membrane of the neighboring cells adjacent to each other to create the gap junction channel. The *GJB2* and *GJB6* are expressed in the supporting cells lining the cochlear epithelium; however, the network of gap junction channels is present in the epithelial and the connective tissue cells (Mese *et al.*, 2008).

GJB2, *GJB3*, and *GJB6* are known to cause syndromic and non-syndromic forms of hearing loss. Where the severity varies from moderate to severe and depends upon the homozygous or heterozygous status of the variation (Snoeckx *et al.*, 2005). More than 220 mutations have been reported in *GJB2* (Hilgert *et al.*, 2009a), where 35delG, is the most frequent *GJB2* mutation, which is found in 70% of the cases (Snoeckx *et al.*, 2005); while compound heterozygous mutations of the *GJB2* and *GJB6* (Pandya *et al.*, 2003) account for 8% of the total *GJB2* hearing loss. Cochlear implants in children carrying *GJB2* mutations have resulted in improvement of hearing (Vivero *et al.*, 2010).

1.11.2. *SLC26A4*

Solute carrier family 26 member 4 (*SLC26A4*) is the second major contributor of genetic hearing loss and has been shown to be responsible for ARNSHL (DFNB4) and Pendred syndrome (PS). 140 different mutations have been reported in *SLC26A4* causing hearing loss (Hu *et al.*, 2012). The SLC26A4 is expressed in the inner ear, kidney and thyroid. Like Cx26, this protein is found in the epithelial cells and the supporting cells within the cochlea where pendrin is responsible for exchanging Cl^-, HCO_3^-, OH^-, I^- and formate (Yoshino *et al.*, 2006). Disease causing mutations within the *SLC26A4* may lead to Pendred syndrome with enlarged thyroid in adults and progressive hearing loss along with enlarged vestibular aqueduct (EVA). While in the case of non-syndromic hearing loss i.e. DFNB4 phenotype consists of enlarged aqueduct only (Reardon *et al.*, 2000). Usually mutations within the promoter region of *SLC26A4* or in the transcription activator *FOX11*, result in DFNB4 or Pendred

syndrome (Yang *et al.*, 2007). SLC26A4 null mice have reduced potential within the cochlea as a result of oxidative stress and the resultant reduced mechanotransduction leads to deaf mice (Singh and Wangemann, 2008).

1.11.3. *TMPRSS3*

Transmembrane protease, serine 3 (*TMPRSS3*) gene comprising of 13 exons encodes the protein type II transmembrane serine protease 3. This protein has a transmembrane domain near the N-terminus (residues 1-69), a low density lipoprotein receptor A domain (residues 72-108), a scavenger receptor domain that is rich in cysteines (residues 109-205) and a C-terminal serine protease domain (residues 217-449). Mutations in *TMPRSS3* have been shown to be responsible for ARNSHL (DFNB8/10) and result in congenital bilateral profound hearing loss. This gene is expressed in the cells of the stria vascularis and organ of corti. 16 mutations have been reported in this gene, which result in the disruption of the proteolytic activity of the protein (Fasquelle *et al.*, 2011). The protein is reported to be involved in the activation of the epithelial sodium channel (ENaC) of the inner ear and is thus thought to regulate the sodium homeostasis within the endolymph of the cochlea (Guipponi *et al.*, 2002).

1.11.4. *MYO15A*

The proteins which are involved in the development of the hair cells are functionally characterized into cytoskeleton proteins, scaffolding proteins, adhesion and motor proteins. These proteins interact and link to the actin filaments that are cross-linked within the hair cell stereocilia, where they maintain the structural integrity of the stereocilia. The five unconventional myosins, IIIA, VI, VIIA, IX and XVA fall in the category of the motor proteins and are involved in deafness. These unconventional myosins bind and hydrolyze ATP; and using the energy thus released move the actin filaments (Krendel and Mooseker, 2005; Mermall *et al.*, 1998; Mooseker and Cheney, 1995). Myosin XV is responsible for the delivery of the scaffold protein whirlin to the stereocilia tips leading to the elongation and formation of the stereocilia bundle. The stereocilia bundle is very important for the release and conduction of the neurotransmitters to the auditory nerve (Belyantseva *et al.*, 2005; Delprat *et al.*, 2005).

MYO15A consisting of 66 exons encodes myosin, which has a N-terminal domain (residues 1-1,223), a conserved motor head domain (residues 1,224-1,899), the neck region (residues 1,909-1,942) and a C-terminal tail domain (residues 2,066-3,497) (Garcia-Alvarez *et al.*, 2003; Kalay *et al.*, 2007). Mutations in *MYO15A* linked to the DFNB3 locus cause mostly severe to profound hearing loss (Hilgert *et al.*, 2009b), these mutations are spread over the motor head and tail domains of *MYO15A* (Belyantseva *et al.*, 2003).

Overall, 50 mutations have been reported in *MYO15A* (Figure 1.7) primarily in the motor domains followed by the tail domains (2 MyTH4 domains, 2 FERM domains and a putative SH3 domain) (Fattahi *et al.*, 2012) (Figure 1.7).

1.11.5. *TMC1*

Transmembrane channel-like gene 1 (*TMC1*) encodes a transmembrane protein that is expressed in the neurosensory hair cells of the mouse cochlea. Mutations in *TMC1* cause dominant (DFNA36) as well as recessive hearing loss (DFNB7). Congenital severe to profound hearing loss is caused by the inactivating recessive mutations; while if a dominant mutation is present in the *TMC1* then hearing loss has late onset and is progressive (Kitajiri *et al.*, 2007). The exact molecular function of *TMC1* is yet to be determined, while according to a recent report, *C. elegans TMC1* encodes a sodium sensor that functions in salt chemosensation. When expressed in mammalian cell cultures, the *C. elegans TMC1* generated a cationic conductance in the presence of high extracellular sodium thus indicating its role as an ionotropic sensory receptor (Chatzigeorgiou *et al.*, 2013).

1.11.6. *MSRB3*

Methionine sulphoxide reductase B3 (*MSRB3*) gene consists of six exons and encodes the methionine sulphoxide reductase B3 protein. Mutations in *MSRB3* have been shown to be responsible for ARNSHL (DFNB74). The presence of endoplasmic reticulum localization signal (residues 1-31) is present at the N-terminal while the rest of the peptide encodes the methionine sulfoxide reductase (Msr) domain, which preserves the biological activity of proteins by catalyzing the reduction of the methionine sulphoxide to methionine. Mouse *MSRB3* is expressed in the inner and outer hair cells in the organ of corti and vestibular sensory epithelium (Ahmed *et al.*,

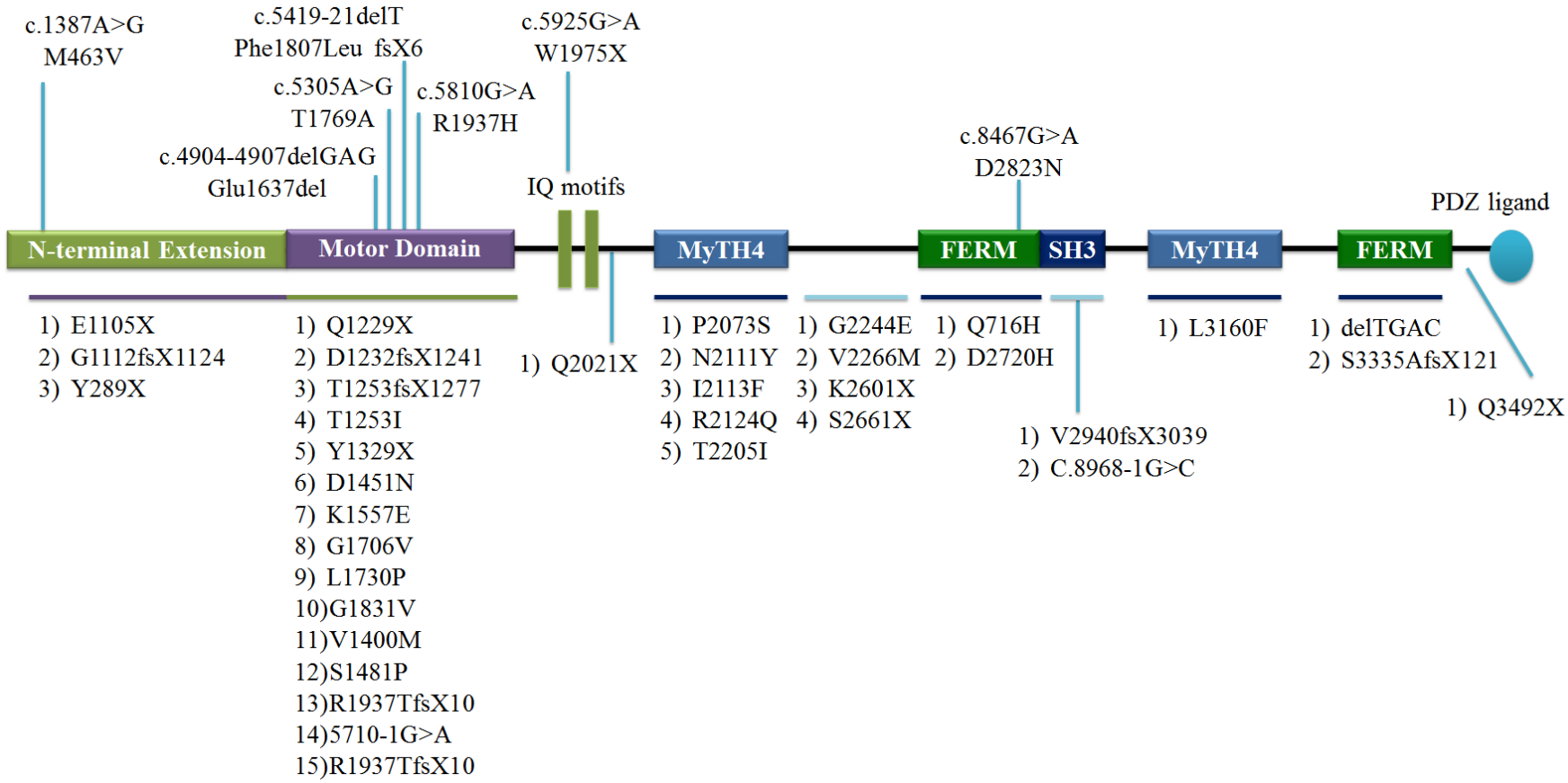

Figure 1.7. The mutations concentrating in the motor and tail domains of the *MYO15A* gene (Fattahi *et al.*, 2012).

outer hair cells in the organ of corti and vestibular sensory epithelium (Ahmed *et al.*, 2011).

1.11.7. *BSND*

Barttin CLCNK-type chloride channel accessory beta subunit (*BSND*) encodes the barttin protein, which is an important constituent of the chloride channels CLCNKA and CLCNKB, in the inner ear and the kidney. Chloride channels and the barttin protein form heteromers, which recycle K^+ in the inner ear and reabsorbs the salt within the kidneys. Biallelic mutations in this gene have been shown to be responsible for Bartter syndrome and ARNSHL (DFNB73) (Riazuddin *et al.*, 2009).

1.12. Importance of Conducting Genetic Analysis in the Pakistani Population

The region where Pakistan is located used to be inhabited by the Indus Valley civilization. The inhabitants of the Indus Valley consisted of numerous races with diverse cultures, as they had been subsequently ruled by Aryans, Persians, Greeks, Arabs, Turks and Mongols. The migrants from the neighboring countries (Iran, Afghanistan and Central Asian States) also influenced the culture and added genetic diversity to the races. With the independence of Pakistan in 1947, four provinces came into existence, these were the Punjab, NWFP (North West Frontier Province, later named Khyber Pakhtunkhwa), Sindh and Balochistan. The eastern, northern and western borders of the state are shared with India, China, Afghanistan and Iran, respectively. The provinces of Pakistan are inhabited by the descendants of the above mentioned races. Therefore, Pakistan being a multi-racial nation is distributed into a number of ethnic groups such as Baloch, Burusho, Pathan, Punjabi, Sindhi, Brahui, Kashmiri, etc. (Mohyuddin *et al.*, 2001). These ethnic groups have their own distinct languages, culture, ancestry and physical appearance (Qamar *et al.*, 2002).

Higher rate of inbreeding in isolated populations results in an increased occurrence of inherited disorders. Excessive inbreeding leads to the appearance of homozygous mutations in future generations and also results in an increase in the number of individuals carrying mutations in genes in a heterozygous state. When the mutation carrying individuals intermarry, the number of affected individuals in the successive generations also increases as offspring of consanguineous parents inherit identical

stretches of chromosomes from their parents (Lander and Botstein, 1987; Woods *et al.*, 2006).

Though a lot of work has been conducted on the genetics of hearing loss, a large number of families still remain unscreened. With the advancement in technology, the process of genetic analysis is becoming easier thus enhancing the researchers ability to identify the genetic causes in families. The data being accumulated could be used to counsel the affected and normal individuals of these families, suffering from inherited disorders. In addition, the functional characterization of some of the suitable variants has made therapeutic interventions possible for a few families. By educating people to engage in out of family marriages, will not only widen the gene pool but will dilute the mutant alleles as well.

In Pakistan, profound bilateral deafness occurs at 1.6 per 1000. 70% of the hearing loss cases are severe and congenital in the Pakistani population. This higher than normal number of cases is due to the high rate of consanguineous marriages (60%) (Elahi *et al.*, 1998). This makes the Pakistani population ideal for conducting genetic studies. The high prevalence of hearing loss, with its recessive form being more frequent has led to the identification of a number of novel loci/genes in consanguineous Pakistani families. A comprehensive list of loci/genes involved in causing non-syndromic hearing loss in the Pakistani populations is presented in Table 1.2.

1.13. Thesis Outline

The aim of this thesis was to identify the genetic causes of ARNSHL in the Pakistani population. Homozygosity mapping was performed for the known ARNSHL genes by employing genome-wide high-density SNP microarray analysis. The homozygous regions harboring previously known deafness genes were identified in most of the families and subsequent Sanger sequencing of known genes identified novel as well as recurrent mutations in 30 hearing loss families. In 2 families splice-site mutations were functionally characterized using *in vitro* minigene experiment. The causative variants in 2 ARNSHL families that remained unsolved were further analyzed by employing NGS technique. On the basis of the current study, genetic counseling services can be offered to the families with identified mutations to reduce the burden of disease in the future generations. Due to the extensive analysis of the current study,

another benefit could be the establishment of a cost-efficient, allele-specific analysis of frequently occurring variants in combination with other reported mutations in the Pakistani population.

Table 1.2. Loci and genes involved in causing non-syndromic hearing loss in the Pakistani population

Locus	Gene	OMIM	References
DFNB1	*GJB2*	121011	Guilford *et al.*, 1994b; Kelsell *et al.*, 1997
DFNB2	*MYO7A*	276903	Guilford *et al.*, 1994a; Liu *et al.*, 1997; Weil *et al.*, 1997
DFNB3	*MYO15A*	602666	Friedman *et al.*, 1995; Wang *et al.*, 1998
DFNB4	*SLC26A4*	605646	Baldwin *et al.*, 1995; Li *et al.*, 1998
DFNB7/11	*TMC1*	606706	Jain *et al.*, 1995; Kurima *et al.*, 2002; Scott *et al.*, 1996
DFNB8/10	*TMPRSS3*	605511	Bonne-Tamir *et al.*, 1996; Scott *et al.*, 2001; Veske *et al.*, 1996
DFNB16	*STRC*	606440	Campbell *et al.*, 1997; Verpy *et al.*, 2001
DFNB18	*USH1C*	605242	Ahmed *et al.*, 2002; Jain *et al.*, 1998; Sottile *et al.*, 1990
DFNB20	-	*604060	Moynihan *et al.*, 1999
DFNB21	*TECTA*	602574	Mustapha *et al.*, 1999
DFNB22	*OTOA*	607038	Zwaenepoel *et al.*, 2002
DFNB23	*PCDH15*	605514	Ahmed *et al.*, 2003b
DFNB24	*RDX*	179410	Khan *et al.*, 2007
DFNB25	*GRXCR1*	613283	Schraders *et al.*, 2010a
DFNB26	-	*605428	Riazuddin *et al.*, 2000
DFNB28	*TRIOBP*	609761	Riazuddin *et al.*, 2006b; Shahin *et al.*, 2006
DFNB29	*CLDN14*	605608	Wilcox *et al.*, 2001
DFNB35	*ESRRB*	602167	Ansar *et al.*, 2003a; Collin *et al.*, 2008
DFNB36	*ESPN*	606351	Naz *et al.*, 2004
DFNB37	*MYO6*	600970	Ahmed *et al.*, 2003a
DFNB38	-	*608219	Ansar *et al.*, 2003b
DFNB39	*HGF*	142409	Schultz *et al.*, 2009
DFNB42	*ILDR1*	609739	Aslam *et al.*, 2005; Borck *et al.*, 2011
DFNB44	*ADCY1*	103072	Ansar *et al.*, 2004; Santos-Cortez *et al.*, 2014
DFNB45	-	*612433	Bhatti *et al.*, 2008
DFNB46	-	*609647	Mir *et al.*, 2005
DFNB47	-	*609946	Hassan *et al.*, 2006
DFNB48	*CIB2*	605564	Ahmad *et al.*, 2005
DFNB49	*MARVELD2*	610572	Ramzan *et al.*, 2005; Riazuddin *et al.*, 2006a
DFNB49	*BDP1*	607012	Girotto *et al.*, 2013
DFNB51	-	*609941	Shaikh *et al.*, 2005
DFNB55	-	*609952	Irshad *et al.*, 2005
DFNB62	-	*610143	Ali *et al.*, 2006
DFNB63	*LRTOMT/ COMT2*	612414	Ahmed *et al.*, 2008; Du *et al.*, 2008
DFNB65	-	*610248	Tariq *et al.*, 2006

Table 1.2. cont.

Locus	Gene	OMIM	References
DFNB66/67	*LHFPL5*	609427	Kalay *et al.*, 2006; Shabbir *et al.*, 2006; Tlili *et al.*, 2005
DFNB68	-	*610419	Santos *et al.*, 2006
DFNB15/ 72/95	*GIPC3*	608792	Ain *et al.*, 2007; Charizopoulou *et al.*, 2011; Rehman *et al.*, 2011
DFNB73	*BSND*	606412	Riazuddin *et al.*, 2009
DFNB74	*MSRB3*	613719	Ahmed *et al.*, 2011; Waryah *et al.*, 2009
DFNB79	*TPRN*	613354	Rehman *et al.*, 2010
DFNB81	-	[$]	Rehman *et al.*, 2011
DFNB86	*TBC1D24*	613577	Ali *et al.*, 2012; Rehman *et al.*, 2014
DFNB88	*ELMOD3*	615427	Jaworek *et al.*, 2013
DFNB89	*KARS*	601421	Basit *et al.*, 2011; Santos-Cortez *et al.*, 2013
DFNB90	-	[$]	Ali *et al.*, 2011
DFNB96	-	*614414	Ansar *et al.*, 2011
DFNB101	*GRXCR2*	615762	Imtiaz *et al.*, 2014
DFNM1	-	*605429	Riazuddin *et al.*, 2000

DFNB, Deafness, Non-syndromic, Recessive; DFNM, Deafness, Non-syndromic, Modifier; *, OMIM# has been provided for the loci for which genes have not been identified; [$], No OMIM# has not been assigned to the DFNB80, DFNB81, DFNB90 loci

Chapter 2

Material and Methods

2. Material and Methods

2.1. Ethics Committee Approval

32 families with hearing loss were recruited for the present study from different regions of Punjab and Khyber Pakhtunkhwa. Of these only one family suffered from syndromic hearing loss; the rest were non-syndromic and had an autosomal recessive pattern of inheritance (ARNSHL). Families with more than two affected members were selected for further analysis, all these had severe to profound hearing loss (Table 2.1). The current study conformed to the tenets of the Helsinki declaration and was approved by the Department of Biosciences, Ethics Review Board, COMSATS Institute of Information Technology, Islamabad, Pakistan and the Radboud University Medical Centre Nijmegen, The Netherlands. All patients, their normal hearing family members and ethnically matched control individuals were informed about the purpose of the study and written consent was taken before recruitment and sampling. Informed written consent of minors was obtained from their guardians.

2.2. Identification and Diagnosis of Hearing Loss Families

Frequent visits to special schools of handicapped children and disability centers led to the identification of the different hearing loss families. In order to determine the level of hearing loss audiometery was performed on a few members from each family including normal and affected. The complete family history was obtained from these families. The parents were interviewed in detail regarding the prenatal, perinatal and postnatal history of the affected members of the families to rule out environmental hearing loss, and only the families with genetic basis of hearing loss were recruited for the current study. A pedigree was drawn to determine the pattern of inheritance and to observe the consanguinity within the family.

2.3. Collection and Blood Sampling of Hearing Loss Families

After informed consent was obtained blood was drawn from all the available family members comprising of affected and normal individuals. Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) were used to collect the blood samples and were kept at 4 °C till further use. Blood samples of ethnically matched control individuals were also collected in EDTA tubes.

Table 2.1. Characteristics of 32 Pakistani families diagnosed with autosomal recessive non-syndromic hearing loss

Family ID	Mutated Gene	Consanguinity	Sampled Individuals	Affected Individuals	Analysis Method
DFR3	*MYO15A*	First cousin marriage in different loops	18	5	VNTR marker analysis
DFR4	*TSPEAR, CCNI2, PCDHGA10*	First cousin marriage in 2nd generation	17	5	Exome sequencing
DFR5	*ATP10B*	First cousin marriage in 2nd generation	12	5	Exome sequencing
DFR10	*GJB2*	Consanguinity	5	2	Sanger sequencing
DFR18	*MSRB3*	Consanguinity in different loops	19	7	SNP based array
DFR19	*GJB2*	Consanguinity	9	5	Sanger sequencing
DFR20	*HGF*	First cousin marriage	15	3	SNP based array
DFR22	*TMC1*	Consanguinity in different loops	18	8	SNP based array
DFR23	*MYO15A*	First cousin marriage in different loops	10	4	SNP based array
DFR24	*TMPRSS3*	First cousin marriage in 1st and 2nd generation	13	5	SNP based array
DFR27	*GJB2*	First cousin marriage	20	6	Sanger sequencing
DFR28	*MYO15A*	First cousin marriage	6	2	VNTR marker analysis
DFR33	*GJB2*	First cousin marriage	9	3	Sanger sequencing
DFR34	*GJB2*	First cousin marriage	13	4	Sanger sequencing
DFR35	*GJB2*	No consanguinity reported	6	2	Sanger sequencing
DFR37	*HGF*	First cousin marriage in 3rd generation	8	3	SNP based array
DFR39	*SLC26A4*	First cousin marriage	9	3	SNP based array
DFR43	*GJB2*	First cousin marriage	8	4	Sanger sequencing

Table 2.1. cont.

Family ID	Mutated Gene	Consanguinity	Sampled Individuals	Affected Individuals	Analysis Method
DFR45	*GJB2*	First cousin marriage	12	3	Sanger sequencing
2DF	*GJB2*	First cousin marriage in 3rd generation	26	7	Sanger sequencing
7DF	*BSND*	First cousin marriage in 2nd generation	10	6	SNP based array
8DF	*GJB2*	First cousin marriage	13	4	Sanger sequencing
9DF	*GJB2*	First cousin marriage in different loops	12	4	Sanger sequencing
11DF	*TMC1*	First cousin marriage in 2nd generation	7	3	VNTR marker analysis
13DF	*MYO15A*	First cousins marriage in different loops	11	7	VNTR marker analysis
19DFS	*TMC1*	First cousin marriage	14	5	SNP based array
25DFS	*GJB2*	First cousin marriage in 2nd generation	5	4	Sanger sequencing
26DF	*TMIE*	First cousin marriage	18	4	SNP based array
32DFS	*GJB2*	First cousin marriage in different loops	18	6	Sanger sequencing
37DFS	*GJB2*	First cousin marriage in 2nd generation	4	2	Sanger sequencing
38DFS	*GJB2*	First cousin marriage	9	3	Sanger sequencing
39DFS	*GJB2*	First cousin marriage	4	1	Sanger sequencing

Note: Disease onset and severity in all families was congenital and severe to profound, respectively.

ID, identification number; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats

2.4. Genomic DNA Extraction from Blood

gDNA extraction was carried out using standard phenol-chloroform/organic method (Sambrook *et al.*, 2001) summarized as follows. Red blood cells (RBC) were lysed followed by removal of their debris. The supernatant containing the white blood cells (WBC) was incubated overnight with proteinase K to extract the gDNA. The digested proteins were then removed by extraction with phenol/chloroform and isoamyl alcohol. Isopropanol and sodium acetate were used to precipitate the gDNA followed by a 70% ethanol wash. The gDNA was then re-suspended in 1X TE (pH 8) buffer and stored at -20 °C for further use. To quantify the gDNA, a known concentration of λ DNA-*Hin*D-III marker along with the gDNA was electrophoretically separated on 1% agarose gel. The gel was documented by obtaining an image using an UV-transilluminator gel documentation system and the Bio Cap MW (ver 11.01) software (Vilber Lourmat, Marne-La-Vallee Cedex1, France).

2.5. *GJB2* Screening

Prior to whole genome single nucleotide polymorphism (SNP) analysis, a total of 32 families were pre-screened for mutations in *GJB2*. One affected member from each loop within a family was selected to be screened using allele specific PCR in order to determine the mutation residing in *GJB2*, followed by Sanger sequencing; and *in silico* bioinformatics analysis of the variation.

2.5.1. Sanger Sequencing

The sequencing was performed by designing primers using the Exon Primer application from UCSC genome browser database (http://genome.ucsc.edu) and Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primer sequences along with their respective product sizes and annealing temperatures are given in the Table 2.2. The following summarizes the sequencing strategies of all the candidate genes. The exons and their exon intron boundaries of the genes were amplified using a thermal cycler (Gene AMP, PCR System 9700, Applied Biosystems) using the following conditions: 0.25 mM dNTPs, 1X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 mM $MgCl_2$, 0.5 μM forward and reverse primer, 2.5U *Taq* polymerase (Fermentas, Life Sciences, Ontario, Canada) and 50ng of DNA. The amplification of DNA was carried out by initial denaturation at 94 °C

Table 2.2. PCR primers for *GJB2*

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
GJB2 Ex-1	F-GGGCTCAAAGGAACTAGGAG	496	60
GJB2 Ex-1	R-GGACGTGTGTTGGTCCAG		60
GJB2 Ex-2I	F-CCAGACTCAGAGAAGTCTCCC	505	58
GJB2 Ex-2I	R-TGTGTAGGTCCACCACAGG		58
GJB2 Ex-2II	F-ACCGGAGACATGAGAAGAAG	465	58
GJB2 Ex-2II	R-CTTGACAGCTGAGCACGG		58

ID, identification number; Temp, temperature; bp, base pairs

for 5 min, followed by 35 cycles of denaturation, annealing and extension for 35 sec at 94 °C, 58 °C and 72 °C, respectively. A final extension was done at 72 °C for 7 min. The amplified samples were separated on 2% agarose gel and DNA bands documented using UV-transilluminator gel documentation system. Following amplification, purification was performed using GeneJET TM PCR purification kit (Fermentas, Life Sciences, Ontario, Canada) and sequencing was done using the dye-termination chemistry (BigDye Terminator, ver 3 on a 3730 or 2100 DNA analyzer; Applied Biosystems). The analysis of the sequencing data were performed using the VectorNTI Advance 11 software (Invitrogen Life Technologies, Carlsbad, CA).

2.5.2. Segregation Analysis

In case a pathogenic variant was found, the segregation analysis of the mutation within the families was performed using Sanger sequencing except that restriction fragment length polymorphism (RFLP) was performed for two genes i.e. *TMPRSS3* and *MSRB3* to identify the causative mutations within DFR24 and DFR18. The enzyme *Acil* (Fermentas, Life Sciences, Ontario, Canada) was used for the *TMPRSS3* mutation while, the enzyme *TseI* (Fermentas, Life Sciences, Ontario, Canada) was used for the *MSRB3* mutation. The primer sequences, annealing temperatures and the PCR product sizes along with the enzymes are given in Table 2.3 and 2.4. For digestion, 3U/µl and 10 U/µl of the enzymes *Acil* and *TseI*, respectively were used alongwith 2 µl of enzyme buffer and 8-10 µl of the amplified product. The digestion mixture was incubated overnight and the product was separated the following morning on 2% agarose gel. After the segregation analysis, the allele frequency of the novel variations was determined in a cohort of healthy control individuals.

2.5.3. *In Silico* Analysis of the Variations Residing in the Candidate Genes

To analyze the pathogenic effect of the mutation residing within a gene, Alamut Biointeractive online software (http://www.interactive-biosoftware.com) was used. The output includes the phyloP (Phylogenetic Profiling conservation score analysis) score, which gives the conservation of the amino acid across several species; indicated by Grantham distance; allele frequencies were calculated and also obtained from the databases i.e. dbSNP and Exome Sequencing Project. Sorting intolerant from tolerant (SIFT: http://sift.jcvi.org; (Sim *et al.*, 2012)) and Polymorphism phenotyping

Table 2.3. PCR primers for *TMPRSS3*

Primer ID	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
TMPRSS3 Ex-1I	F-GATCACTCCCTGATGCAAAG	437	57
	R-TGGCTTCTGAAAGATGGAAC		
TMPRSS3 Ex-1II	F-CTCCTTGGCATTGTGATAGG	437	57
	R-GGACTCCCTCTCACCATTTAG		
TMPRSS3 Ex-2	F-ACCGTATGACCAAGATGCAC	361	57
	R-TCTAGGGAAGTGCAGGTGTC		
TMPRSS3 Ex-3	F-TTCTCTGCAAAGAGGGGAG	330	57
	R-TCCAGTAATTAAGGCTGGGC		
TMPRSS3 Ex-4	F-GGGGACAGTTGTTAGTGTTGC	249	57
	R-AAGGGTCAGGGTTGGCTTC		
TMPRSS3 Ex-5	F-TGCCTATGGTCTCAGGGTTC	286	57
	R-CGTTAAAGCACCCAATAGTGC		
TMPRSS3 Ex-6	F-ACATCCCCCATGTACAATCC	293	57
	R-CATCACAAATCCAGCAGGTG		
TMPRSS3 Ex-7	F-GTGTGACCTCATCCTCATGG	388	57
	R-ATGACACTGCTCCCCTCCTC		
TMPRSS3 Ex-8	F-CCCTTGCAGCACTTGTCTTAG	395	57
	R-CTTCTCACCACCCAAAGCAG		
TMPRSS3 Ex-9	F-GACCAATGTTGAGTTCAGCC	674	57
	R-AGCCACATTGTCCAGGATAC		
TMPRSS3 Ex-10	F-TCCTCAGAGGCAGAAGCATAG	279	57
	R-CCCATGGGAACATCACAATG		
TMPRSS3 Ex-11	F-TGTTGCGACACACCAGAGAG	400	57
	R-CTTGAGCAAATTTCTTCTCCAC		
TMPRSS3 Ex-12	F-GTCCACAGAAAGCAATCTCG	380	57
	R-AGCACAAGCGTCTGACACC		
TMPRSS3 Ex-13	F-GTCATCATGTTGGACGGATG	663	57
	R-AGACCCCTGGAGAGAAAACC		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

Table 2.4. PCR primers for *MSRB3*

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
MSRB3 Ex-1	F-CTCATATTTGGACTCGGCTG	328	60
	R-AGGCGCAGAATGGAATC		
MSRB3 Ex-2	F-GTGCATGTGTTCAGTTCCAG	238	57
	R-CTGAGGGAAGCCCACAG		
MSRB3 Ex-3	F-CAGCTGCAGTTCAGGTAAGC	552	57
	R-CCTAAATCGCTGTTACTTCCAC		
MSRB3 Ex-4	F-GACCCTTCTGCATTTTCTGG	734	57
	R-TGCTTGCCAAAATCTCTTTC		
MSRB3 Ex-5	F-TATTACCTCCCCTCCCAACC	260	57
	R-AGGTGGGACTCACATTTTGG		
MSRB3 Ex-6	F-TTTTCCTTCAGCTTTTGACC	401	57
	R-AACATAGGCATCCATCGGAC		
MSRB3 Ex-7	F-CCTTCATTTCTCTACAGGCTGG	395	57
	R-TCAATAGCACAAAACTGCCC		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

v2 (PolyPhen-2: http://genetics.bwh.harvard.edu/pph2; (Adzhubei *et al.*, 2010)) were used for analyses of the missense changes. The online tool used to evaluate the effects on splicing was NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/; (Brunak *et al.*, 1991)). While the online tool, SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP-4.0/) was used to predict the presence and location of the signal peptide cleavage sites (Petersen *et al.*, 2011). In addition, the online tool, have your protein explained (HOPE) (http://www.cmbi.ru.nl/hope/input) was used to predict the three dimensional structural changes at the protein level (Venselaar *et al.*, 2010). The exome variant server (EVS) and an in-house exome database (Human Genetics, Radboud University Medical Centre) were also searched for the presence of putative pathogenic variants.

2.5.4. Minigene Construction and Splicing Assay (for c.362+18A>G mutation in *TMC1* and c.9948G>A mutation in *MYO15A*)

A plasmid containing the genomic region encompassing exons 3-5 of *RHO* inserted at the *EcoRI/SalI* sites in the pCI-NEO vector was used for *in vivo* splicing assays (Gamundi *et al.*, 2008). The plasmid was adapted from the Gateway cloning technology (Invitrogen Life Technologies, Carlsbad, CA) and the experiment performed according to the manufacturer's protocol. PCR amplified fragments of wild-type and mutant *TMC1* exon 8, along with flanking intronic sequences were generated with the following primers, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCtgggtcctaatgttgactgc-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCggatttagaaaatcaatatcaggg-3', these contain the attB1 and attB2 sites necessary for Gateway cloning. Similarly, amplified fragments of wild-type and mutant *MYO15A* exon 61 were generated using primers, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCttccccaggagaaatggag-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCcagcttgctgggaaggac-3'.

Recombinant vectors were employed for transfection of HEK293T cells that were incubated at 37 ^{o}C for 24 hours. RNA was then isolated from the transfected cells and reverse transcribed into cDNA and sequenced. Forward primer 5'-cggaggtcaacaacgagtct-3' and reverse primer 5'-aggtgtaggggatgggagac-3' were used to amplify and sequence the amplified cDNA fragments along with the flanking *RHO* sequences present in the vector.

2.6. *MYO15A* Screening

Following the *GJB2* screening and analysis; *MYO15A* was screened in 16 unsolved families using microsatellite markers with heterozygosity ~70% (D17S1843, D17S2196, D17S783 and D17S1824) and in families in which haplotype analysis showed compatibility with genetic linkage, Sanger sequence analysis was performed for *MYO15A*.

2.6.1. CA-repeat Marker PCR and Analysis

The information regarding the specific markers was obtained from UCSC genome browser database (https://genome.ucsc.edu) and ordered from Invitrogen, Inc., Carlsbad, CA. The size range of the PCR product as well as the cytogenetic location of markers was obtained from UCSC genome browser database (https://genome.ucsc.edu), Marshfield Medical Center database and Human Genome Database. For the *MYO15A* gene and locus, annealing temperatures and the flanking markers used for homozygosity mapping are mentioned in Table 2.5 and 2.6. The microsatellite markers were designed by adding a forward and reverse M13 tail. The forward sequence added was 5'-TGT AAA ACG ACG GCC AGT-3' and the reverse sequence was 5'-CAG GAA ACA GCT ATG ACC-3'. These markers were amplified in two steps. The first PCR reaction was performed using standard conditions, with annealing temperature mentioned in Table 2.6. The second PCR was conducted using 1 µl of the amplified PCR product as template. Unlabeled reverse primer was complementary to the M13 tail while the forward primer was fluorescently labeled. The dyes used were FAM (blue), VIC (green), or NED (yellow). Thermal cycling of this mixture was done with initial denaturation at 94 °C for 3 min, followed by 10 cycles including denaturation and annealing at 94 °C and 56 °C, respectively for 20 sec and extension at 72 °C for 45 sec. A final extension was performed at 72 °C for 6 min. From each reaction, 2 µl of the PCR product was mixed with formamide and *liz* internal lane standard. A volume of 10 µl of the above mixture was sent to the core sequencing facility of the Department of Human Genetics, Radboud University, Nijmegen Medical Centre, Nijmegen, The Netherlands. The electrophoretic data were then analyzed using a Gene mapper software package (ver. 4.0). The varied allele sizes resulted in the construction of haplotypes, which determine the presence of linkage (shared homozygous stretches of DNA that were present in all affected

Table 2.5. PCR primers for *MYO15A*

Primer ID	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
MYO15A Ex-1	F-AGCTGCTCACTCCTTTCCAG	368	58
	R-CATCACACCCCTCACAGATG		
MYO15A Ex-2_1	F-CTCCAAGTCCCTGAGCCC	580	58
	R-AAGGGGAGTTTGCGGGAG		
MYO15A Ex-2_2	F-GGCCATCAACTGGCTCAC	581	58
	R-AAGGAGACGAGTACCCCGAC		
MYO15A Ex-2_3	F-CCCTACGACTACTACCACCCC	564	58
	R-GAGGGACAGCTTGGACCTG		
MYO15A Ex-2_4	F-ATGGATGACATCGCCGAG	567	58
	R-CATGCCAGCCAGCTTGTAG		
MYO15A Ex-2_5	F-GTCGGAGAAGAAGCCCATC	575	56
	R-GTGACCAAGCTCGCCTCC		
MYO15A Ex-2_6	F-CTACTAGCCTTCCCAGGGC	451	56
	R-GTCTCTGAGTCCTCGGGTTC		
MYO15A Ex-2_7	F-CACTCGGGCTGTGAAGC	566	58
	R-ACGCAGCCAGTGGGTATG		
MYO15A Ex-2_8	F-CTCCCAAGGATGTCACTCCC	451	58
	R-TCAGGCTGTCTCTCTTGCC		
MYO15A Ex-3	F-CTCCCCAACAATGGTAGCAG	295	58
	R-TGAAAATGTCCTGGGAGGTG		
MYO15A Ex-4	F-GGTCTCCTAGCTGGTTGGTG	329	58
	R-TCTGGCATTCTAGGCAGAGG		
MYO15A Ex-5	F-ATCTGTCCGGATGGAAACAG	393	56
	R-CAGTGGCCTCCAACCTGTAG		
MYO15A Ex-6-7	F-TTGGCTGTTTGGCTGGATAC	590	58
	R-TGGAACTCAGCCTCCTCATC		
MYO15A Ex-8	F-TCCTGGAGAGAGTGGTGGTC	239	56
	R-CTAGGACAGGCCTTTGGATG		
MYO15A Ex-9-10	F-CTTGGGAAGGCTCATGTCTG	493	56
	R-TTGACGTCTACCTAGCCCTACC		
MYO15A Ex-11	F-GAGCCTCACCCATCACAGAG	391	58
	R-AAACTCACCCTCCCCAAATC		
MYO15A Ex-12	F-AGGCCACCACACTACTGGTC	406	58
	R-AAAGATGAACAACCCCAAAGG		
MYO15A Ex-13	F-TCCCAAAGTGCTGGGACTAC	349	57
	R-TGACCCAGGGACAGAGAGAG		
MYO15A Ex-14-15	F-CTTCTCCATGATCCCACTCC	489	58
	R-GGTCCATGAAGGGAGGATG		

Table 2.5. cont.

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
MYO15A Ex-16	F-GTGAGCTGAGGGCCTGTG	314	58
	R-TCAGAATTGCCCTTAAACAACC		
MYO15A Ex-17	F-GTTCTGGGAGGTATGCAAGC	397	57
	R-TGAATAGTAGGTCGGGTGTCC		
MYO15A Ex-18	F-GGGAAGCTGTGAGTTGTTCC	398	58
	R-TTTGCTCAAGGTCACAGAGC		
MYO15A Ex-19	F-CCCAGGTTGGTCATGAGAAG	383	56
	R-CCACCCTTGTCTTTGTCTGG		
MYO15A Ex-20-21	F-TTCCTCCTCATTTCGGTCTC	440	58
	R-AAGGTCACACAGCATGGG		
MYO15A Ex-22	F-CCTCTCAGGACCTGCATCTG	317	58
	R-CTGCCTGGGTTGTGTATTCC		
MYO15A Ex-23	F-CTCGGGTAGGTCTCATAGCC	259	56
	R-GATGGCAGTCTCTAGCTTGG		
MYO15A Ex-24	F-CCAGCATCCTCTCTTCAACC	387	58
	R-CGAGCCTGACCATAGGTCTC		
MYO15A Ex-25	F-GGCCTCTCTACCTTTTGGTC	230	56
	R-CTCCTCTTGGGTGCTAAGTG		
MYO15A Ex-26-27-28	F-ACCTCCACACAACAGGCAAG	685	58
	R-AGGCTCATACCCAACACCAC		
MYO15A Ex-29	F-CCTGTCAGTCCAGCTCTGTC	185	56
	R-AGGGGCTAAGAATTAGAAGGTG		
MYO15A Ex-30	F-ACATGGGAGGGACATGAGAG	486	58
	R-GCCCAGTAGAAATGCAGAGC		
MYO15A Ex-31	F-GAGTAGCCTGGGCCTTTCTC	395	58
	R-CAGCTCCAGAACATCCAGTG		
MYO15A Ex-32-33	F-CCAGGGAAGTGAGGCTACAG	685	58
	R-AGTTCAGGGCTCAGCCTGTC		
MYO15A Ex-34-35	F-CACTGAATACCAGGGTGCAG	558	58
	R-ATGACCCTGGCTGTGAGAAG		
MYO15A Ex-36-37-38	F-GAAGAGGCCAGTGTCAGGTG	657	58
	R-ACAATAGTGCCCTGGAGCAG		
MYO15A Ex-39-40	F-AGCTCCACAACCCATCTCTG	598	58
	R-CGCTTCAACCCAGTAAGCTG		
MYO15A Ex-41-42	F-GGACTTCAGGCCAGTCTCTG	593	58
	R-GGGCTCAGGGACATAGACAC		
MYO15A Ex-43-44	F-ACTCTAGCCTGGGGGACAAC	666	58
	R-TAAAACAGCTGCCCATCCTC		

Table 2.5. cont.

Primer ID	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
MYO15A Ex-45	F-TTGCCACTCACCCTAGTATAGTC	297	58
	R-CAGAGAAGCAGAGCAAGGAG		
MYO15A Ex-46-47	F-TCATCCATTTCTGGGTTTCAG	600	58
	R-CTTCAATGGCTTGGCACAG		
MYO15A Ex-48	F-GGGCAGGACAGGATCAGAAG	291	58
	R-AGGGAGATCCCTGTTGCTG		
MYO15A Ex-49-50	F-AGGAAGGATGTGAGGAGCAG	497	56
	R-GCTGGACCTTAGGAGACCTG		
MYO15A Ex-51	F-CCCCTTAGTCACAAGACAAGAC	319	58
	R-TTATCCCCACTCGCCTCAC		
MYO15A Ex-52-53	F-TCTGGGTCCAGTCAGCTAGG	664	58
	R-GAGAACAAGTTTGTTGGCCC		
MYO15A Ex-54-55	F-TGTGTCCCCTTTCTGTTCTG	534	58
	R-TGATAGATGGGGAAACTGAACC		
MYO15A Ex-56	F-GTGCCCACCCTGTTCTTATG	222	58
	R-CCTCCTGGAGCATGGACAC		
MYO15A Ex-57_1	F-CCCAAGTGTTGTTCCCAGAC	400	58
	R-CAGACATCTTCCCCAGGTAGG		
MYO15A Ex-57_2	F-CCTACCTGTTGTGAGGCAGAG	439	58
	R-GGTTCCCACTCATTGACCTC		
MYO15A Ex-58	F-TTGTGGAGAGAATGCAGTGG	340	56
	R-GGATTACAGGTGCCTGCC		
MYO15A Ex-59	F-CAGGAGACAAGGGCTGTCC	214	58
	R-CTGGAGCCTGGGCTGTC		
MYO15A Ex-60	F-AGAAGGACAGAGGTCAAGCC	236	58
	R-AAATCTGGGTGGAGGGC		
MYO15A Ex-61	F-TCACCTGGGTAGCAGATTGG	331	57
	R-AGTCTGATTCACAGGCCCAG		
MYO15A Ex-62	F-CATGCATGTCCCCAGGTC	271	58
	R-TGAGAGGGCAGGGTTGC		
MYO15A Ex-63	F-ACAGTGAGGATTGCCTGAGC	257	58
	R-ATGACCACCCTCCTCAGC		
MYO15A Ex-64	F-GAAGCTATGCAGTTCAGGGC	268	58
	R-TGGGGACCAGGGAACAG		
MYO15A Ex-65	F-TGGTTGAGACTATCCTCGCC	271	58
	R-GACCTGACCTATCTTGGAGCC		

Table 2.5. cont.

Primer ID	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
MYO15A Ex-66	F-CAAGGTAAGAGCTGGGGAAG	240	58
	R-TTGATCCTGAGAGGTTCAGTG		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

Table 2.6. CA repeat analysis, microsatellite markers for *MYO15A*. The distance (cM) and percentage heterozygosity of the markers along with the product length is given

Marker	**cM**	**Heterozygosity (%)**	**Length (bp)**
D17S1843	41.12	0.70	177-197
D17S2196	44.62	0.81	317-341
D17S783	47	0.72	170
D17S1824	49.67	0.83	204

bp, base pairs

Note: Annealing temperature for all the markers was 58 °C

members of the family in question).

2.7. Homozygosity Mapping using Whole Genome SNP Microarray

11 families with affected individuals were mapped for homozygosity using Illumina HumanOmniExpress whole genome single nucleotide polymorphism (SNP) microarray (>700K SNPs) or the Illumina Human Linkage-12 panel (Affymetrix, Santa Clara, CA). The genotyping was performed according to the manufacturer's protocols and consisted of the following steps:

2.7.1. Sample Preparation

The concentration of the selected gDNA of affected and normal individuals from each loop within the families was first visually quantified by electrophoretically separating the gDNA dilution with λ DNA-*Hin*D-III marker and the concentration was re-confirmed with a nanodrop spectrophotometer (Isogen, Life Science, De Meern, The Netherlands). The final concentration was adjusted to 48ng/µl for each sample and 100ng DNA was sent to the Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany, for the SNP microarray analysis where the genotyping was performed according to the manufacturer's protocols.

2.7.2. Digestion of Samples by Restriction Enzymes

The samples were digested in a 96 well plate using the restriction enzyme *NspI* and a digestion mastermix containing AccuGENE® Water 11.5 µl, NE Buffer 2 (10X) 2 µl, BSA (100X; 10 mg/ml) 0.2 µl and *NspI* (10 U/µl) 1 µl. The plate was placed in a thermal cycler at 37 ºC for 120 min and then at 65 ºC for 20 min; the samples were then stored at 4 ºC till further use.

2.7.3. Ligation

The ligation mastermix consisted of Adaptor NspI (50µM) 0.75µL, T4 DNA ligase buffer (10X) 2.5µl and T4 DNA ligase (400U/µl) 2µl; 5.25µl of this ligation mastermix was aliquoted to the previously digested DNA and ligated at 16 ºC for 180 min followed by 70 ºC for 20 min. The samples in the 96 well plate were then diluted with 75 µl of ultrapure water.

2.7.4. Polymerase Chain Reaction

The DNA was amplified using a PCR and a mastermix containing: 39.5 µl of ultrapure water, TITANIUM *Taq* PCR Buffer (10X) 10 µl, GC-Melt (5M) 20 µl, dNTP (2.5 mM each) 14 µl, PCR Primer (100 µM) 4.5 µl, TITANIUM *Taq* DNA Polymerase (50X) 2 µl. 90 µl of this mastermix was then poured per well containing 10 µl ligated DNA. This was followed by amplification as follows: stage1 (denaturation): 94 ºC for 3 min (1 cycle), stage 2 (annealing): 94 ºC for 30 sec, 60 ºC for 45 sec and 68 ºC for 15 sec, 30 cycles were performed in total with a final extension for 7 min at 78 ºC.

2.7.5. Fragmentation

DNaseI was used to fragment the amplified DNA. The mastermix containing 5 µl fragmentation buffer (DNaseI buffer) was added to a 96 well plate containing 45 µl of the amplified and purified PCR products. This was followed by the addition of 5 µl diluted fragmentation reagent to the mixture of buffer and sample. The samples were then fragmented at 37 ºC for 35 min and 95 ºC for 15 min.

2.7.6. Labeling

The enzyme terminal deoxynucleotidyl transferase (TdT) was used to label the DNA at the 3' end of the fragmented DNA. The mastermix used to label the DNA contained 14 µl TdT Buffer (5X), 2 µl terminal deoxynucleotidyl transferase (TdT) (30 mM) and 3.5 µl TdT enzyme (30 U/µl). A volume of 19.5 µl of the reaction mixture was aliqouted per well of the 96 well plate. Samples were labeled at 37 ºC for 4 hours and 95 ºC for 15 min followed by targeted hybridization.

2.7.7. Targeted Hybridization

For hybridization, the mixture containing MES (12X; 1.25 M) 12 µl, Denhardt's Solution (50X) 13 µl, EDTA (0.5 M) 3 µl, HSDNA (10 mg/ml) 3 µl, Human Cot-1 DNA (1 mg/ml) 3 µl, Tween-20 (3%) 1 µl, DMSO (100%) 13 µl and TMACL (5 M) 140 µl was heated at 95 ºC for 10 min, followed by a holding step at 49 ºC. The hybridized samples were further analysed by loading them on the Illumina HumanOmniExpress whole genome single nucleotide polymorphism (SNP) microarray (>700K SNPs) (Affymetrix, Santa Clara, CA). Afterwards washing,

staining and scanning of the chips was performed. The GeneChip scanner 3000 7G (Affymetrix, Santa Clara, CA) was used.

2.7.8. Data Analysis

The genotype calls were obtained using the Affymetrix Genotyping Console (version 2.0). The online tool, Homozygosity Mapper (http://www.homozygositymapper.org/) was used to analyze the data and determine the common homozygous intervals in the affected individuals, which were lacking in the normal individuals. All the homozygous regions of at least 1Mb were further analyzed and the mapping of the data were performed using the Human Genome Build hg19, release Feb. 2009.

2.7.9. Candidate Gene Analysis and Mutation Screening

The known candidate genes residing within the homozygous regions were screened for the potential known or novel pathogenic variants in the hearing loss families. The genes were screened using Sanger sequencing. The sequencing primers were designed using the Exon Primer tool from UCSC genome browser database and Primer3 and were amplified using standard conditions. The sequences, annealing temperatures and PCR product sizes are given in Table 2.3, 2.4 and 2.7-2.12. The amplification was followed by purification and sequencing; the procedures are summarized in section 2.5.1; while the segregation and *in silico* analysis is described in section 2.5.2 and section 2.5.3.

2.8. Exome Next Generation Sequencing

The probands of the families DFR4 and DFR5 were sent for Exome NGS. Homozygosity mapping excluded the possibility of disease causing mutations in known loci/genes in family DFR5 therefore it was analysed further by NGS. In the family DFR4, identified homozygous regions did not harbor the known hearing loss loci/genes, therefore this family was also analysed by NGS. A SureSelect^{XT} Human All Exon v.2 Kit (50Mb) from Agilent Technologies, Inc. (Santa Clara, CA) was used to capture all exons of ~21,000 genes according to the manufacturer's protocol on a 5500xl Genetic Analyzer platform from Life Technologies (Bleiswijk, Netherlands).

Table 2.7. PCR primers for *TMC1*

Primer ID	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
TMC1 Ex-1	F-CGACAAGAGCAAAACCTCTG	703	58
	R-TTACTGGGTGACATGCTGTG		
TMC1 Ex-2	F-GGGAGGGGAGTAATAGCTTG	392	58
	R-ATCCCAGTACCCTTAGGTGG		
TMC1 Ex-3	F-CCAGCATGGAACATTCACAG	589	56
	R-CCAACTGCTTTCCTCCATTC		
TMC1 Ex-4	F-TGCATTCCTTGGCTATTCTTG	445	58
	R-AAACCCAGGCCTCATTCTG		
TMC1 Ex-5	F-TTCAACCCCTTTGACCTGAG	383	58
	R-AAAGGGAAAGCCCCAAAAG		
TMC1 Ex-6	F-AAAATCTTTGCATGGCATTG	395	58
	R-TTGCTGAACTTGATTCCTCC		
TMC1 Ex-7	F-ATCACGATGTGGAGAATTGC	429	56
	R-GCATCATCAGATTAAGGCTCTC		
TMC1 Ex-8	F-GAGCTAAATATCTGATACCTGCC	265	56
	R-GAAAATCAATATCAGGGAAAGTG		
TMC1 Ex-9	F-GCAGACCTGGTAAATTCAAATG	257	58
	R-CCCCTTTAGAAAAGAACCAGTG		
TMC1 Ex-10	F-GCTGCCAGAGAGACATTTCC	281	58
	R-CCAGAAACTGACAAAGGCATC		
TMC1 Ex-11	F-AAAAAGGACCAATGCCTCAC	380	56
	R-CATGGGCACCATTTTGAAG		
TMC1 Ex-12	F-CATGGAGACCCAAAGAGTCC	325	58
	R-ATTCAGCCTGACCCAGGAG		
TMC1 Ex-13	F-AATCAACATGGCAGCTGAAAC	393	56
	R-TCCAATCCCCAAATTAATCC		
TMC1 Ex-14	F-TTGCTTCTCCACTTCAACACTC	371	56
	R-TTGGTAGGCAGAAACCATGAG		
TMC1 Ex-15	F-TACTTGCCATCGTTTGTTGG	495	56
	R-AAGGGCAGGATAGGGGATAG		
TMC1 Ex-16	F-CCAAAATTCTGGCAAAAAGC	339	58
	R-GAGATTTCAAAGGCATTTCTGG		
TMC1 Ex-17	F-CTCAAGTTTGCCAGAAATGC	428	56
	R-CCAGCACACAGTCAACATTC		
TMC1 Ex-18	F-TTGCAGTCTTCAAGCCAATAC	326	58
	R-TCCCCCTCTGTGAGAAACAC		
TMC1 Ex-19	F-AATTGAAACCCTAGCCATGC	367	58
	R-GCTTTGCAAAACAGAGAGACAC		

Table 2.7. cont.

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
TMC1 Ex-20	F-GCAGTGTGACTTTGTTATGGAG	370	58
	R-GAATGACCATTCCCACCTC		
TMC1 Ex-21	F-TGAGGTCAAAGTGTCAGCAAG	387	58
	R-GCAGAACCCTTAGGGAGAGTG		
TMC1 Ex-22-23	F-GCTTAATGTTCATTCCCATGC	474	58
	R-TTGTGGAATGACTCGCTCAC		
TMC1 Ex-24	F-GATTTCTGGCCACCTCATTC	679	58
	R-GTGGTTGGGGAGACAGGTAG		
	*R-CCTTAATTGGCCAGCATGAC		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; *R, reverse sequencing primer; bp, base pairs

Table 2.8. PCR primers for *HGF*

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
HGF IVS4	F-GATGTTTATGGCCGAGAGGA	500	59
	R-GGCTTTAAGAGAGACAAGTGAGG		
HGF Ex-5	F-TCAGCAAATTCACAGGCTCA	517	59
	R-TAGTTGCATTTGCACGAACA		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

Table 2.9. PCR primers for *SLC26A4*

Primer ID	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
SLC26A4 Ex-1	F-TTCCTCTTCTCCTCCCCATGTG	598	58
	R-CTCTCCCCTCGTCCTGTTTT		
SLC26A4 Ex-2	F-AGACCCGAAGGTTCTCAGGT	646	58
	R-GGGCTCCTAGAACCCACTCT		
SLC26A4 Ex-3	F-AAGCATGGTAAGCACTTCAGG	398	58
	R-TGAAGGGTAAGCAACCATCTG		
SLC26A4 Ex-4	F-TTGCATCATCATAAAGGCAAA	286	58
	R-TGCACTTAATATAGCCAAAACACT		
SLC26A4 Ex-5	F-CCTATGCAGACACATTGAACATTTG	442	58
	R-TGAGCCTTAATAAGTGGGGTCTTG		
SLC26A4 Ex-6	F-GGTTTCTATCTCAGGCAAACAT	270	58
	R-ATTGTTTCTGGAATGAACAGTGACC		
SLC26A4 Ex-7-8	F-CATGGTTTTTCATGTGGGAAGATTC	553	58
	R-GGAGTATCAGTGAAATGAAGCTTGT		
SLC26A4 Ex-9	F-TCATGTCTAATATGTGACTGAGCAGA	347	58
	R-TCCAACCCCTTCTTTAGCTG		
SLC26A4 Ex-10	F-AAATACTCAGCGAAGGTCTTGC	250	58
	R-CGAGCCTTCCTCTGTTGC		
SLC26A4 Ex-11-12	F-ACACATCCAGTGAGCTGGAA	453	58
	R-GTGATATGGCAGGAAGCAT		
SLC26A4 Ex-13	F-TGTAATTTGTTTGTGGATCATTG	539	58
	R-GGAGAGCACAGCAGTAGAGGA		
SLC26A4 Ex-14	F-CACCAGAATGATGGGCTCTT	267	58
	R-GCATTTTCTCCCTTTGGCTA		
SLC26A4 Ex-15	F-GCTCCTCTGAGCAACTGTGA	297	58
	R-TAGGGCCTATTCCTGATTGG		
SLC26A4 Ex-16	F-TTGAGAAATAGCCTTTCCAGAT	250	58
	R-GACCCTCTAACTGCTCTCATCA		
SLC26A4 Ex-17	F-CTCCTTGATGTCTTGCTTACCA	442	58
	R-CCCATGTATTTGCCCTGTTG		
SLC26A4 Ex-18	F-TGAATGCTACTGAATTATGGGC	183	58
	R-AGATAGGAGAAAGGGCTTACGG		
SLC26A4 Ex-19	F-GGTAGGGTGTGCCCTGTAGT	472	58
	R-ACTGAGGCTCCATGAAGTTAT		
SLC26A4 Ex-20	F-GAGAAGCACCAGGAAAGC	291	58
	R-GCATTTGGGGGAATTATGTT		

Table 2.9. cont.

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
SLC26A4 Ex-21	F-CACCTAAGATGAGTAGCAGTAAGCA	265	58
	R-TGCTTCTCTTTCAATGGCTTAATA		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

Table 2.10. PCR primers for *TMIE*

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
TMIE Ex-1	F-AATGATGCTCGCTGACTACC	318	58
	R-GTAAGGGATCAAACAAGCTCC		
TMIE Ex-2	F-GAGTGTTTGCTGAGACCTGG	258	58
	R-GGTGCTGTGTTCCCACTG		
TMIE Ex-3	F-CATTCCTTGGGTCTCTGAAC	261	58
	R-AGCAGGACTGAGGAACTGG		
TMIE Ex-4	F-ACCCCAGGACCTTGTCTC	249	58
	R-CAAATGAGCTCAGAGTCCAAC		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

Table 2.11. PCR primers for *BSND*

Primer ID	**Primer Sequences 5'-3'**	**Product Size (bp)**	**Annealing Temp. (°C)**
BSND Ex-1	F-GCTGAACTACAGCCACCC	306	58
	R-ATACAGGCACAGTGTCCAGC		
BSND Ex-2	F-CCTGAGGGGACAGTAGCC	225	58
	R-CTCAGTCAGGGGAGACTGG		
BSND Ex-3	F-ACATACCCAAAGCAAACAGG	479	56
	R-CCACTGCCCTCTCCTTAC		
BSND Ex-4	F-GGAGCTCTGAGTCCAATGAG	1151	58
	R-AGGAATGGGAAACCTGACTG		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

2.8.1. Library Preparation

The DNA samples were sheared to produce 150-200 bp long stretches. Blunt ends were phosphorylated at the 5'-ends. Klenow fragment of DNA polymerase was used to add adenine overhangs to the blunt ends. The ligated adaptors with fragments were amplified. Finally these adaptors were hybridized to the enriched biotinylated ribonucleic acid (RNA) probe library. The streptavidin coated magnetic beads were used to extract the hybridized fragments and the RNA probes were then digested; while the enriched DNA was obtained for further use. The DNA sequencing was performed using the Life Technologies SOLiD TM sequencer. This system uses five universal sequencing primers, which anneal to the adaptors, followed by the ligation to an eight nucleotide probe. This probe contains two color-coded bases along with a fluorescent label attached at the 3'-end. From this 3'-end, three bases and the florescent label are cleaved resulting in the emission of fluorescence to detect the incorporated bases. This process is repeated ten times after which, denaturation and then removal of initially synthesized oligonucleotide takes place. Each of the universal primers used is offset by one base and the whole procedure including probe annealing, ligation, fluorescence detection and cleavage of fluorescent label to detect the remaining bases is repeated till the complete DNA template sequence was decoded by recording the fluorescence produced. Variants were identified by aligning to the human genome (hg19) using LifeScope™ Genomic Analysis Software v2.5.1 (Bleiswijk, Netherlands). The causative mutation was identified from the vast numberof variants by filtering the Exome sequencing data. Calling of single nucleotide changes on the aligned sequence, was based on DiBayes algorithm; while minor insertions and deletions were identified using SOLiD Small Indel Tool.

2.8.2. NGS Data Filtration

The data filtration process involved many steps aimed at narrowing down the exonic region. Initially thousands of variants were obtained with most of them being present in the canonical splice sites, while a few were located in the exonic sequences. The first step involved excluding all variants found in dbSNP (v132) and an in-house database. The dbSNP (v132) and in-house database consisted of 177 exomes based on healthy controls and patients with extremely rare disorders. In order to reduce the number of variants (non-synonymous and altered transcripts), the minimum cut off

value of the variant sequence reads was set to 10. Since the focus of the current study was on ARNSHL, the data were further filtered to retain only the homozygous changes (with 80% variant calls) and compound heterozygous (with 20-80% variant calls). The next filtration step was to validate the number of the variant reads, which were automatically generated in the output file by investigating the Binary Sequence Alignment/Map format (BAM file). The considerable difference regarding the number of reads between the automated output file and the BAM file reduced the number of variants. With mostly non-synonymous variants being causative of recessive disease and also a few compound heterozygous.

2.8.3. Variant Screening

The exonic variants residing within the largest homozygous regions were prioritized. The variants with a frequency of less than one in the dbSNP and the homozygous variants with more than 80% variant calls were retained. In addition variants with Grantham distance and phyloP score of greater than 80 and 2, respectively were further analysed. Based upon these criteria, 699 variants were obtained in the family DFR4 while, 555 variants were obtained in the family DFR5. The pathogenic variants from the Exome data were prioritized based upon their presence in the homozygous regions obtained after the whole genome SNP microarray analysis. Therefore, four homozygous variants residing within the homozygous regions were shortlisted in the family DFR4 while, four homozygous variants residing within the homozygous regions and two compound heterozygous variants outside of these regions were further analysed in the family DFR5. The validation of the variants was performed by Sanger sequencing of the selected variant within the families. The sequences of primers, PCR product sizes and the annealing temperatures are given in Table 2.12. The segregation of Exome data and further analysis is mentioned in sections 2.5.1, 2.5.2 and 2.5.3, respectively. A list of selected variants and their attributes along with pathogenicity scores determined by various softwares is given in Table 2.13 and 2.14.

Table 2.12. PCR primers for variant validation after Exome sequencing and analysis

Primer ID	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
CCNI2	F-TCGCTCAAAATCAGGAGGTG	657	58
	R-CAGCCAAGTCCTCAAGTCTC		
MYOT	F-TCACAAGGTCAGGAGATCAAG	689	60
	R-TGTGACGTTGTGTATCCAGG		
TRIOBP	F-CTTCCATGGACTCTCTGCAC	583	58
	R-TTTTGGCACCCTAACCCTTC		
TSPEAR	F-TGATGCCCCATACATGTTGG	540	58
	R-TCCCTCGATTGCAGGAATTG		
PCDHGA10	F-TTCAACTACACAAGCCCCAC	328	60
	R-AGATGTTGCCCACGAAAGAG		
TACC2	F-CAAAAATGTCCAGCAAATGG	390	56
	R-TACCATCCTTTGAGGGGAAG		
TIGD6	F-AGGCAAGAGGAAAGGTGATG	645	58
	R-ATGTTCTTGAGGCAGTGTGG		
ATP10B	F-ACATGCATTTCCAAGGTTGC	549	58
	R-CCACTTCATCCCCTAGTTGC		
IDE	F-GGGTAGATCAACTCCTCTGC	615	60
	R-ACTGCTCTGCTCCTTAAACG		
DIAPH1	F-ACCTGCTCCTGGGGATAGTA	336	56
	R-GCTTCTCCAGGCAAGGGA		
TBC1D24	F-GGGACCTGGTGAACAAGTAC	557	56
	R-GTGTCTTTTCAGCCATGCAC		

ID, identification number; Temp, temperature; F, forward primer; R, reverse primer; bp, base pairs

Table 2.13. The validated variants and their *in silico* prediction within the family DFR4 are listed

Gene (RefSeq ID)	Protein Function	cDNA Change	Amino acid Change	PhyloP	Grantham Distance	SIFT	Mutation Taster	PolyPhen-2	Zygosity	Segregation in Family
CCNI2 (NM_001039780)	Regulation in cell cycle	c.633+1G>C	-	4.81	0	-	Unknown	Unknown	Homozygous	Segregating
MYOT (NM_006790)	Muscle contraction	c.78G>A	p.Ser26Ser	0.633	0	-	Polymorp-hism	Unknown	Homozygous	Not Segregating
TRIOBP (NM_001039141)	Actin modification	c.3709C>G	p.Leu1237Val	0.04	32	Tole-rated	Polymorp-hism	Unknown	Homozygous	Not Segregating
TSPEAR (NM_144991)	Cell adhesion	c.193G>A	p.Ala65Thr	-0.20	58	Tole-rated	Polymorp-hism	Unknown	Homozygous	Segregating
PCDHGA10 (NM_018913)	Cell adhesion	c.32C>G	p.Ser11*	-0.44	0	Delet-erious	Disease Causing	Probably Damaging	Homozygous	Segregating

Table 2.14. The validated variants and their *in silico* prediction within the family DFR5 are listed

Gene (RefSeq ID)	Protein Function	cDNA Change	Amino acid Change	PhyloP	Grantham Distance	SIFT	Mutation Taster	PolyPhen-2	Zygosity	Segregation in Family
TACC2 (NM_206862)	Microtubule cytoskeleton organization	c.8015C>G	p.Pro2672Arg	2.63	103	Tolerated	Disease Causing	Unknown	Homozygous	Not Segregating
TIGD6 (NM_001243253)	Regulation of transcription	c.305G>A	p.Arg102Gln	-0.12	43	Tolerated	Polymorphism	Not Damaging	Homozygous	Not Segregating
ATP10B (NM_025153)	ATP biosynthetic process	c.2910C>A	p.Asp970Glu	-0.36	45	Tolerated	Polymorphism	Unknown	Homozygous	Segregating
IDE (NM_004969)	Hormone and cellular proteolysis	c.636A>T	p.Lys212Asn	-0.92	94	Tolerated	Disease Causing	Unknown	Homozygous	Not Segregating
DIAPH1 (NM_005219)	Microtubule cytoskeleton organization	c.1964C>G	p.Pro655Arg	5.05	103	Deleterious	Disease Causing	Unknown	Heterozygous	Not Segregating

Chapter 3

Results

3. Results

In the current study, genetic analysis was performed using different techniques on a total of 32 ARNSHL Pakistani families. Initial screening of *GJB2*, *MYO15A* and whole genome SNP array analysis followed by sequencing led to the identification of nine novel mutations; which were identified in nine different families (*GJB2* (c.598G>A, p.Gly200Arg); *MYO15A* (c.9948G>A, p.Gln3316Gln; c.3866+1G>A; c.8767C>T, p.Arg2923* and c.8222T>C, p.Phe2741Ser), *TMC1* (c.362+18A>G), *BSND* (c.97G>C, p.Val33Leu), *TMPRSS3* (c.726C>G, p.Cys242Trp), *MSRB3* (c.20T>G, p.Leu7Arg)). Furthermore, 12 recurrent mutations were detected in 21 other families. The Exome sequencing of two excluded families revealed pathogenic novel variants in novel genes (Table 3.1). The detailed results are given below:

3.1. Clinical History and Evaluation

Medical histories were obtained from all individuals in the families in order to exclude environmentally caused hearing loss. Investigations were conducted to determine goiter, hair pigmentation, skin pigmentation, vertigo, night blindness, myocardial infarction, goitre, kidney anomalies and the presence of infectious diseases such as typhoid, meningitis, mumps, rubella, chronic otitis media, and antibiotic/drug use. This information not only helped the families to be clearly categorized but also clearly differentiated between the syndromic from non-syndromic individuals. Personal histories were also taken about severe injuries, respiratory infections or auditory infections, acoustic trauma, drugs or medication, and trauma to the head. The parents were asked about the onset of hearing loss to confirm its congenital nature. Developmental histories were taken regarding different milestones including when the child started walking, to identify motor difficulties, speech development, mental performance in school and German measles infection in the mother during the first trimester of the pregnancy. Finally the family pedigree was drawn indicating hearing loss in siblings and the hearing status of parents and extended family, such as grandparents, uncles, aunts and cousins. The families were mostly sampled from Punjab province while a few families were sampled from Khyber Pakhtunkhwa province of Pakistan.

Pure tone audiometry for bone and air conduction was carried out on affected and

Table 3.1. Characteristics of 32 Pakistani families diagnosed with autosomal recessive non-syndromic hearing loss

Family ID	Mutated Gene	Identified Mutation	Consanguinity	Sampled Individuals	Affected Individuals	Analysis Method
DFR3	*MYO15A*	c.8222T>C (p.Phe2741Ser)	First cousin marriage in different loops	18	5	VNTR marker analysis
DFR4	*TSPEAR*, *CCNI2*, *PCDHGA10*	c.193G>A (p.Ala65Thr), c.633+1G>C (p.?), c.32C>G (p.Ser11*)	First cousin marriage in 2nd generation	17	5	Exome sequencing
DFR5	*ATP10B*	c.2910C>A (p.Asp970Glu)	First cousin marriage in 2nd generation	12	5	Exome sequencing
DFR10	*GJB2*	c.598G>A (p.Gly200Arg)	Consanguinity	5	2	Sanger sequencing
DFR18	*MSRB3*	c.20T>G (p.Leu7Arg)	Consanguinity in different loops	19	7	SNP based array
DFR19	*GJB2*	c.231G>A (p.Trp77*)	Consanguinity	9	5	Sanger sequencing
DFR20	*HGF*	c.482+1991_2000delGATGATGAAA (p.?)	First cousin marriage	15	3	SNP based array
DFR22	*TMC1*	c.1114G>A (p.Val372Met)	Consanguinity in different loops	18	8	SNP based array
DFR23	*MYO15A*	c.9948G>A (p.Gln3316Gln)	First cousin marriage in different loops	10	4	SNP based array
DFR24	*TMPRSS3*	c.726C>G (p.Cys242Trp)	First cousin marriage in 1st and 2nd generation	13	5	SNP based array
DFR27	*GJB2*	c.71G>A (p.Trp24*)	First cousin marriage	20	6	Sanger sequencing
DFR28	*MYO15A*	c.8767C>T (p.Arg2923*)	First cousin marriage	6	2	VNTR marker analysis
DFR33	*GJB2*	c.71G>A (p.Trp24*)	First cousin marriage	9	3	Sanger sequencing

Table 3.1. cont.

Family ID	Mutated Gene	Identified Mutation	Consanguinity	Sampled Individuals	Affected Individuals	Analysis Method
DFR34	*GJB2*	c.71G>A (p.Trp24*)	First cousin marriage	13	4	Sanger sequencing
DFR35	*GJB2*	c.231G>A (p.Trp77*)	No consanguinity reported	6	2	Sanger sequencing
DFR37	*HGF*	In-4: c.482+1986_1988delTGA (p.?)	First cousin marriage in 3rd generation	8	3	SNP based array
DFR39	*SLC26A4*	c.1337A>G (p.Gln446Arg)	First cousin marriage	9	3	SNP based array
DFR43	*GJB2*	c.231G>A (p.Trp77*)	First cousin marriage	8	4	Sanger sequencing
DFR45	*GJB2*	c.231G>A (p.Trp77*)	First cousin marriage	12	3	Sanger sequencing
2DF	*GJB2*	c.71G>A (p.Trp24*)	First cousin marriage in 3rd generation	26	7	Sanger sequencing
7DF	*BSND*	c.97G>C (p.Val33Leu)	First cousin marriage in 2nd generation	10	6	SNP based array
8DF	*GJB2*	c.35delG (p.Gly12Valfs*2)	First cousin marriage	13	4	Sanger sequencing
9DF	*GJB2*	c.35delG (p.Gly12Valfs*2)	First cousin marriage in different loops	12	4	Sanger sequencing
11DF	*TMC1*	c.362+18A>G (p.Glu122Tyrfs*10)	First cousin marriage in 2nd generation	7	3	VNTR marker analysis
13DF	*MYO15A*	c.3866+1G>A (p.?)	First cousins marriage in different loops	11	7	VNTR marker analysis
19DFS	*TMC1*	c.100C>T (p.Arg34*)	First cousin marriage	14	5	SNP based array
25DFS	*GJB2*	c.35delG (p.Gly12Valfs*2) c.439G>A (p.Glu147Lys)	First cousin marriage in 2nd generation	5	4	Sanger sequencing

Table 3.1. cont.

Family ID	Mutated Gene	Identified Mutation	Consanguinity	Sampled Individuals	Affected Individuals	Analysis Method
26DF	*TMIE*	c.241C>T (p.Arg81Cys)	First cousin marriage	18	4	SNP based array
32DFS	*GJB2*	c.71G>A (p.Trp24*)	First cousin marriage in different loops	18	6	Sanger sequencing
37DFS	*GJB2*	c.377_378insATGCGGA (p.Arg127Cysfs*85)	First cousin marriage in 2nd generation	4	2	Sanger sequencing
38DFS	*GJB2*	c.380G>A (p.Arg127His)#	First cousin marriage	9	3	Sanger sequencing
39DFS	*GJB2*	c.380G>A (p.Arg127His)#	First cousin marriage	4	1	Sanger sequencing

Note: Disease onset and severity in all families was congenital and severe to profound, respectively.

ID, identification number; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeat; #The pathogenicity of this mutation is controversial

unaffected members of the family by trained professionals with frequencies ranging from 250-8000Hz. All families in this study were consanguineous (Table 3.1) and all patients in these families were diagnosed with severe to profound congenital hearing loss.

3.2. Results of *GJB2* Screening

Sequence analysis of *GJB2* in the current cohort identified 16 families with mutations in this gene (Table 3.1; Table 3.2), which segregated with hearing loss in the families. A recurrent nonsense mutation c.71G>A (p.Trp24*) was the most common and was found in five families (31%); which are DFR27 (Figure 3.1 (i) and (ii)), DFR33 (Figure 3.2 (i) and (ii)), DFR34 (Figure 3.3 (i) and (ii)), 2DF (Figure 3.4 (i)) and 32DFS (Figure 3.5 (i)). This variant was followed by another nonsense variant, c.231G>A (p.Trp77*) that was identified in four families (25%); which are DFR19 (Figure 3.6 (i) and (ii)), DFR35 (Figure 3.7 (i) and (ii)), DFR43 (Figure 3.8 (i) and (ii)) and DFR45 (Figure 3.9 (i) and (ii)) (Table 3.2). The variant c.380G>A (p.Arg127His) was found in the families 38DFS and 39DFS; while the variant c.35delG (p.Gly12Valfs*2) was found in another two families 8DF and 9DF (Table 3.2). The compound heterozygous variant c.35delG (p.Gly12Valfs*2) and c.439G>A (p.Glu147Lys) was found in only one family 25DFS; while the last variant found in one family 37DFS was c.377_378insATGCGGA (p.Arg127Cysfs*85) (Table 3.2). In addition to the above mentioned six recurrent *GJB2* mutations, a novel homozygous change c.598G>A (p.Gly200Arg) was found in one consanguineous family DFR10 (Figure 3.16 (i) and (ii); Table 3.3); as predicted by the HOPE server the larger side chain of the mutant residue arginine might affect the proper folding of the cysteine rich domain. In addition, the residue is present in the conserved region of the protein (Figure 3.16 (iii)).

The number of the affected and the total sampled individuals per family in the cohort of the 16 families in which *GJB2* mutations were found, is mentioned in Table 3.1.

3.3. Results of *MYO15A* Screening

Out of the nine novel mutations (Table 3.3) identified in the current study, four were present in *MYO15A*, of which two were splice site mutations (c.9948G>A and c.3866+1G>A), one was a nonsense (c.8767C>T) and one a missense mutation

Table 3.2. Spectrum of recurrent *GJB2* mutations in Pakistani families with autosomal recessive non-syndromic hearing loss

Mutation identified (Protein change)	**Type of Mutation**	**No. of Families**	**No. of Affected Members**	**Frequency in EVS**
c.71G>A (p.Trp24*)	Nonsense (homozygous)	5	26	Absent
c.231G>A (p.Trp77*)	Nonsense (homozygous)	4	14	AA=0/AG=1/GG=4299
c.35delG (p.Gly12Valfs*2)	Frameshift (homozygous)	2	8	Absent
c.35delG (p.Gly12Valfs*2) c.439G>A (p.Glu147Lys)	Missense (compound heterozygous)	1	3	Absent
c.380G>A (p.Arg127His)[#]	Missense (homozygous)	2	4	AA=0/AG=26/GG=4274
c.377_378insATGCGGA (p.Arg127Cysfs*85)	Frameshift (homozygous)	1	2	Absent

As reference sequence NM_004004.5 was employed.

EVS, exome variant server; [#]The pathogenicity of this mutation is controversial

DFR27 (M: *GJB2*, c.71G>A; p.Trp24*)

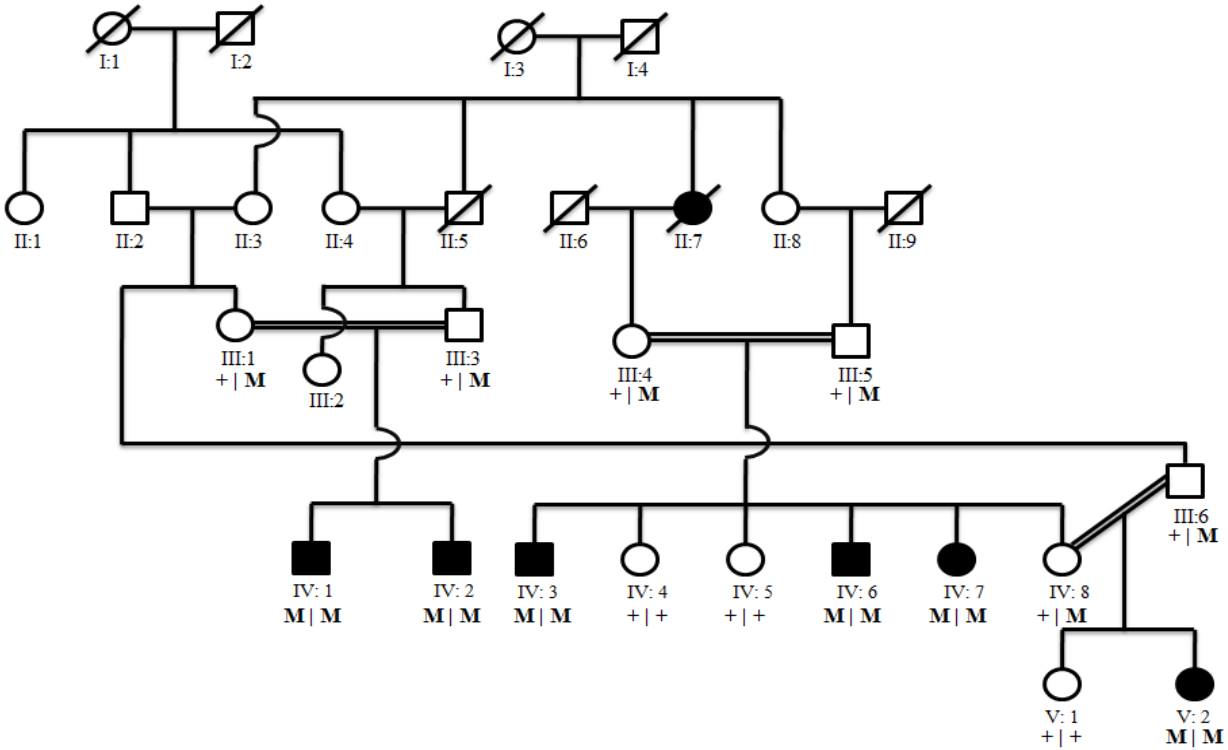

Figure 3.1 (i). Pedigree of family DFR27. The circles represent females while squares are for males. The filled circles indicate affected females and filled squares represent affected males. The diagonal lines across the symbols indicate the deceased individuals. The double line in the pedigree shows consanguineous marriage. Generations are represented by Roman numerals while individuals within a generation are symbolized by Arabic numerals. In addition the segregation of the *GJB2* mutation is also shown in the pedigree. The +|+ represents homozygous ancestral, M|M is for homozygous variant and +|M is for heterozygous carriers.

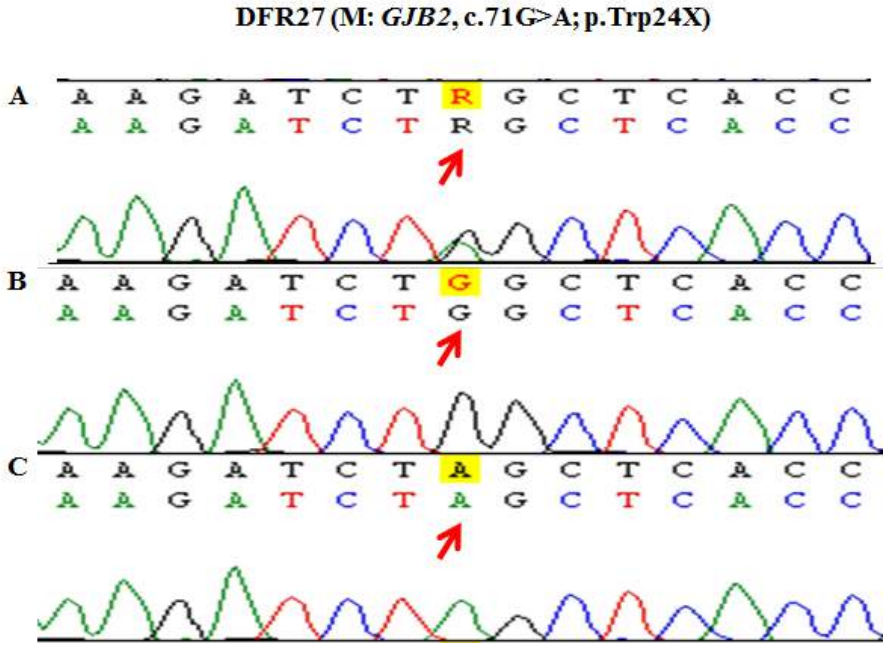

Figure 3.1 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper panel is for heterozygous variation; **B)** the middle figure is for homozygous ancestral and **C)** the bottom one is indicating the homozygous variant.

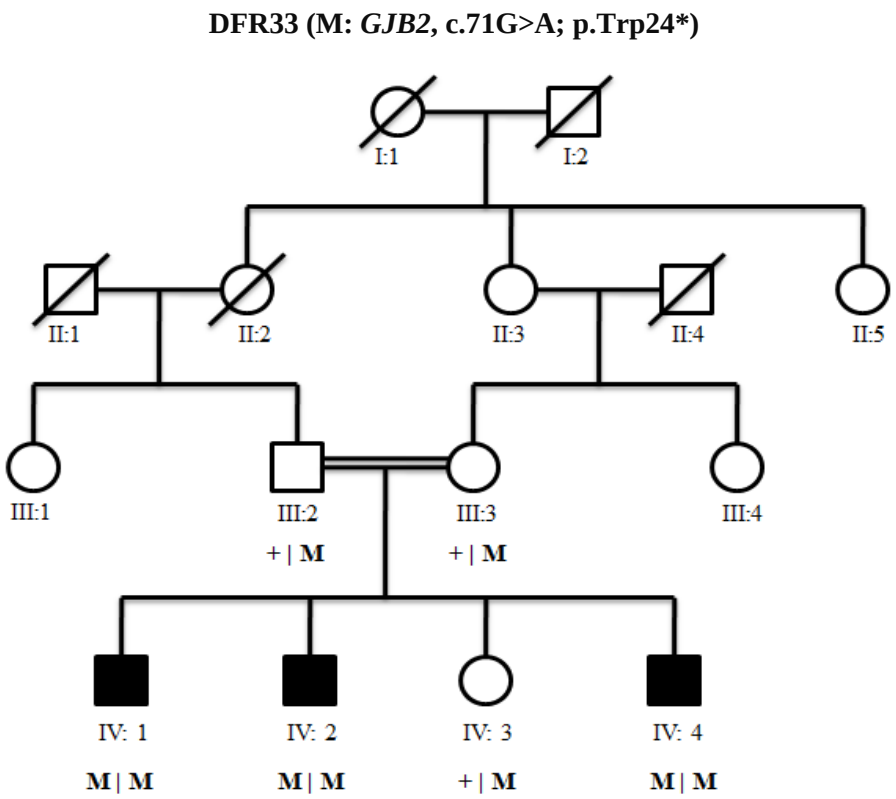

Figure 3.2 (i). Pedigree of family DFR33. For a description of the symbols see legend of Figure 3.1 (i).

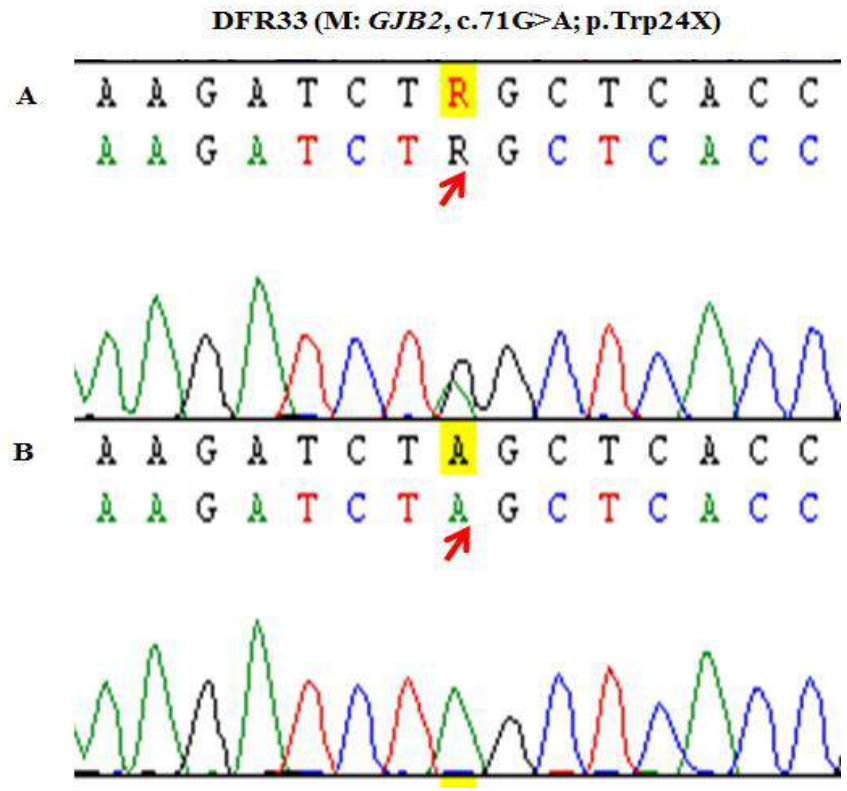

Figure 3.2 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper figure is for heterozygous variation and **B)** the bottom one is indicating the homozygous variant.

DFR34 (M: *GJB2*, c.71G>A; p.Trp24*)

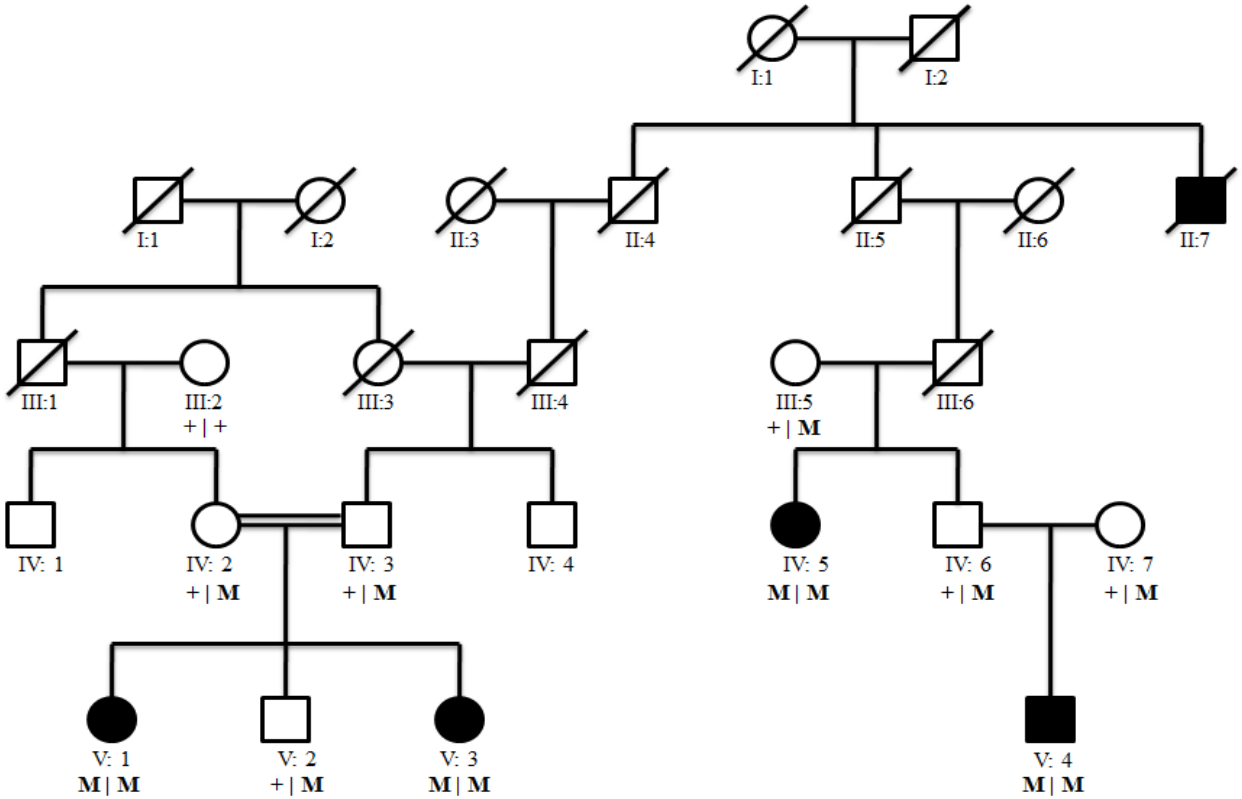

Figure 3.3 (i). Pedigree of family DFR34. For a description of the symbols see legend of Figure 3.1 (i).

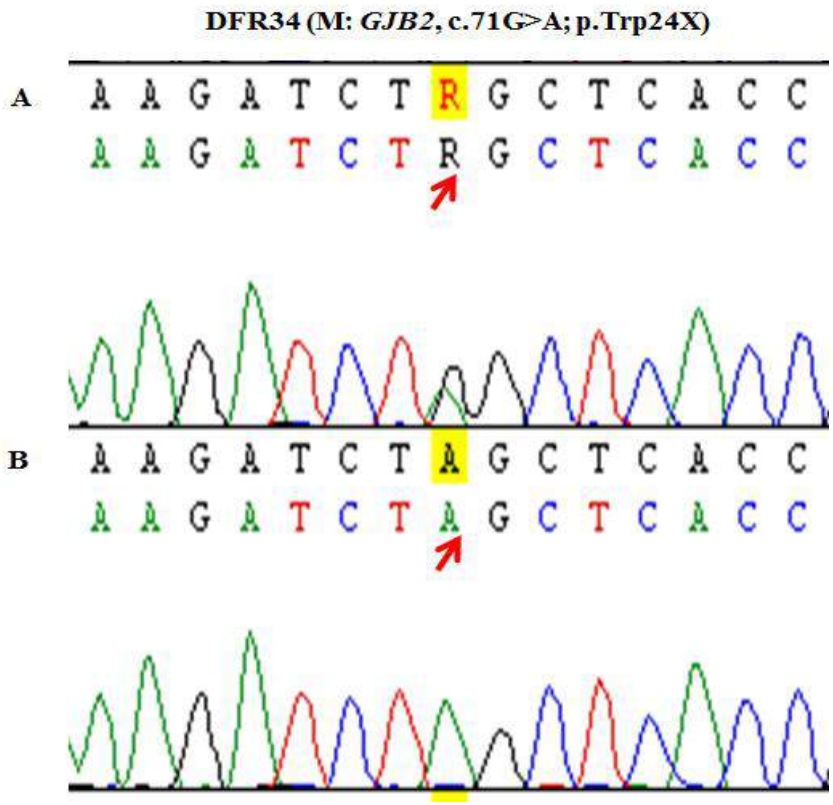

Figure 3.3 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper panel is for heterozygous variation and **B)** the bottom one is indicating the homozygous variant.

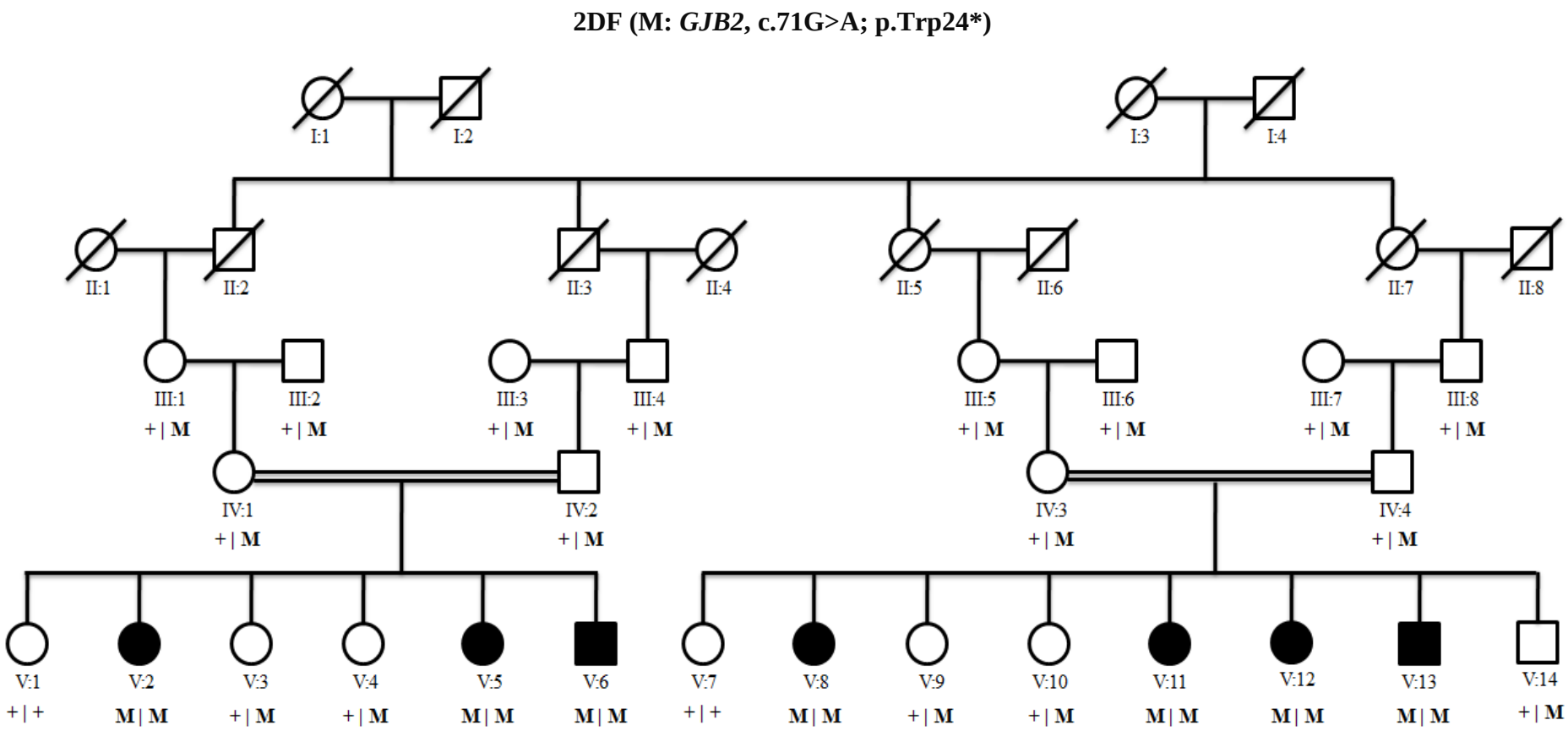

Figure 3.4. Pedigree of family 2DF. For a description of the symbols see legend of Figure 3.1 (i).

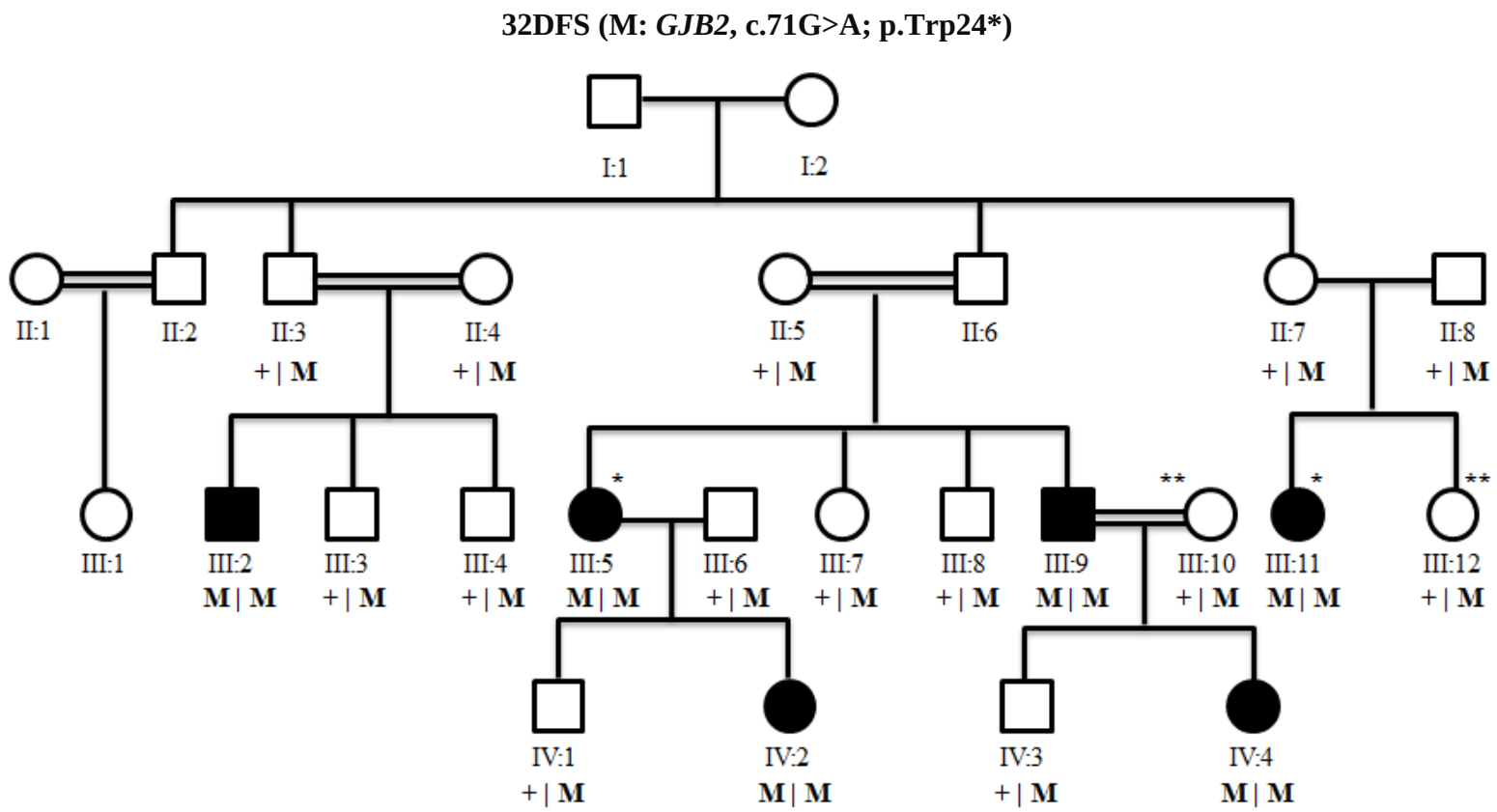

Figure 3.5. Pedigree of family 32DFS. For a description of the symbols see legend of Figure 3.1 (i).

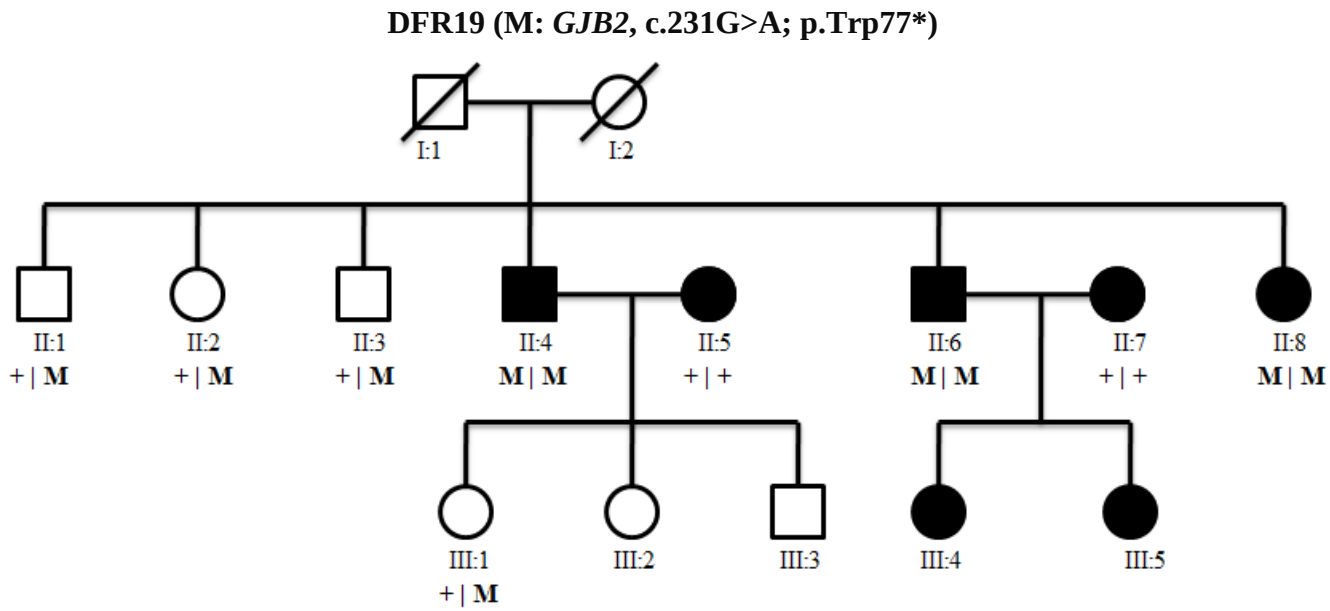

Figure 3.6 (i). Pedigree of family DFR19. For a description of the symbols see legend of Figure 3.1 (i).

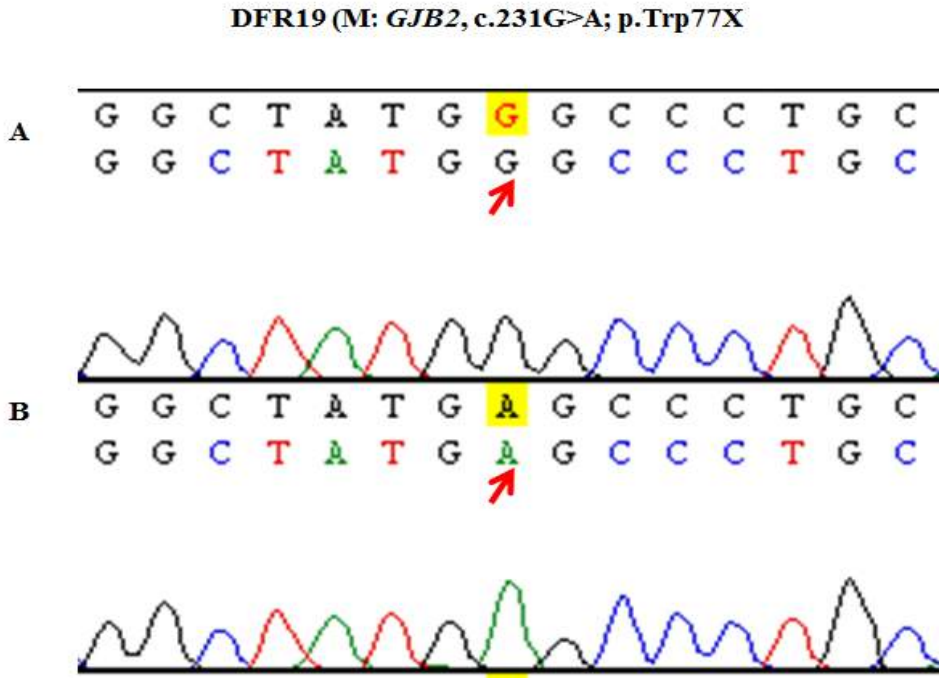

Figure 3.6 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper figure is for heterozygous variation and **B)** the bottom one is indicating the homozygous variant.

DFR35 (M: *GJB2*, c.231G>A; p.Trp77*)

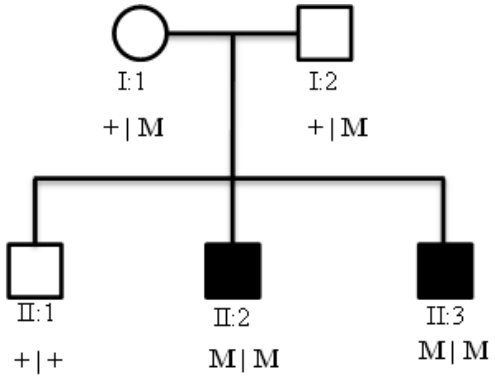

Figure 3.7 (i). Pedigree of family DFR35. For a description of the symbols see legend of Figure 3.1 (i).

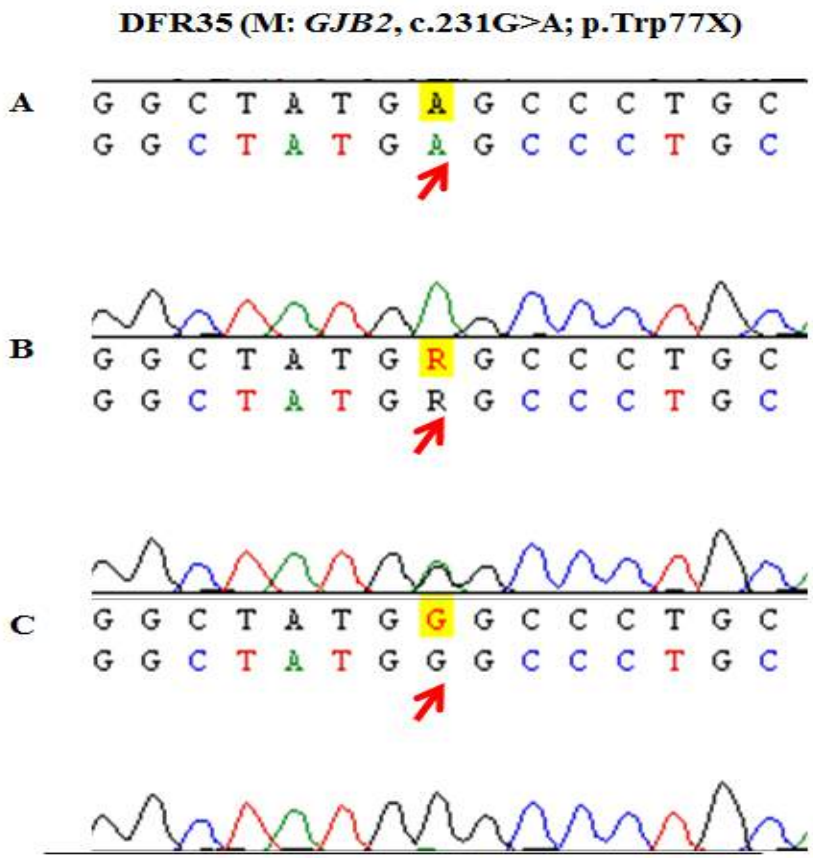

Figure 3.7 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper figure is for homozygous mutant variant; **B)** the middle figure is for heterozygous variation and C) the bottom one is indicating the homozygous variant.

DFR43 (M: *GJB2*, c.231G>A; p.Trp77*)

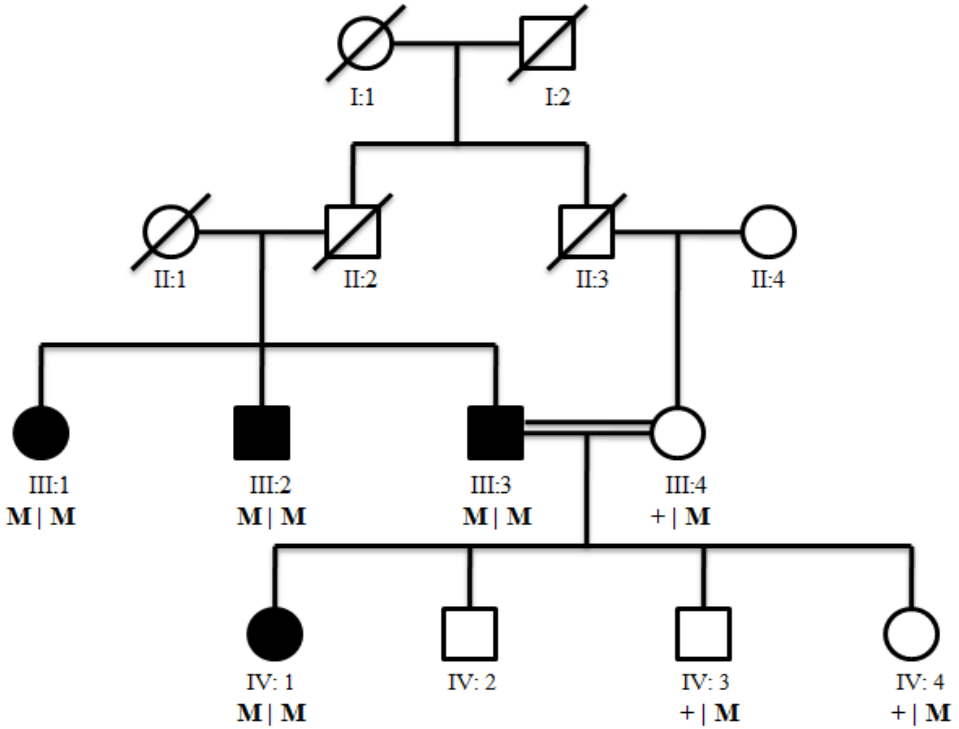

Figure 3.8 (i). Pedigree of family DFR43. For a description of the symbols see legend of Figure 3.1 (i).

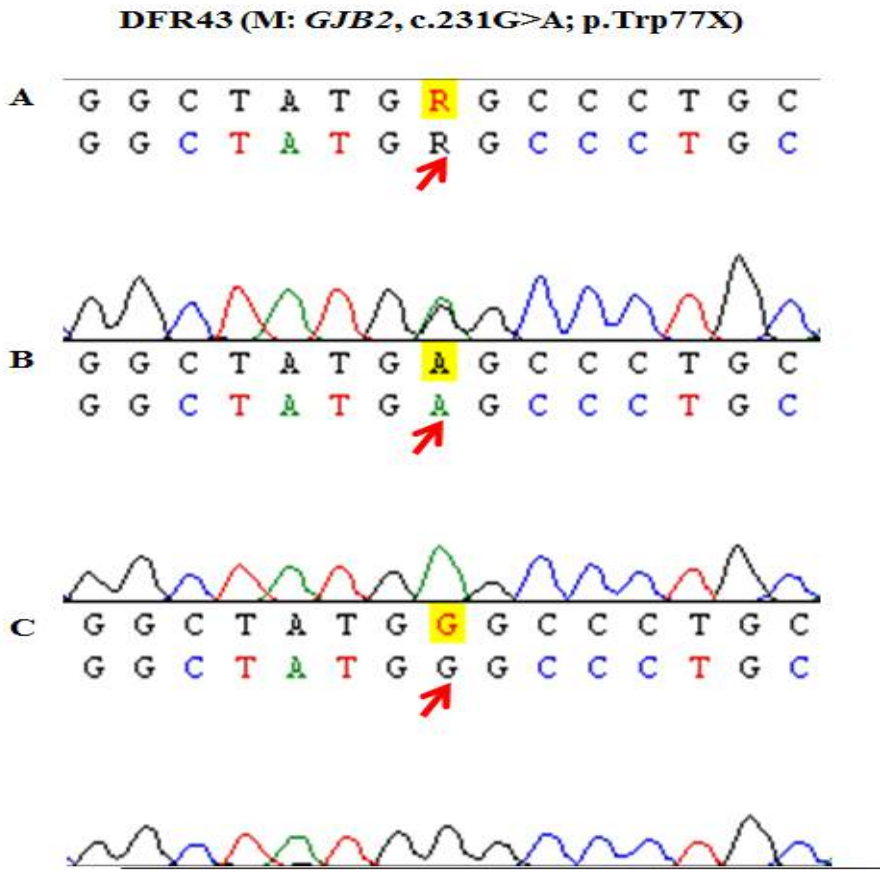

Figure 3.8 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper figure is for heterozygous variation; **B)** the middle figure is for homozygous variant and **C)** the bottom one is indicating the homozygous ancestral.

DFR45 (M: *GJB2*, c.231G>A; p.Trp77*)

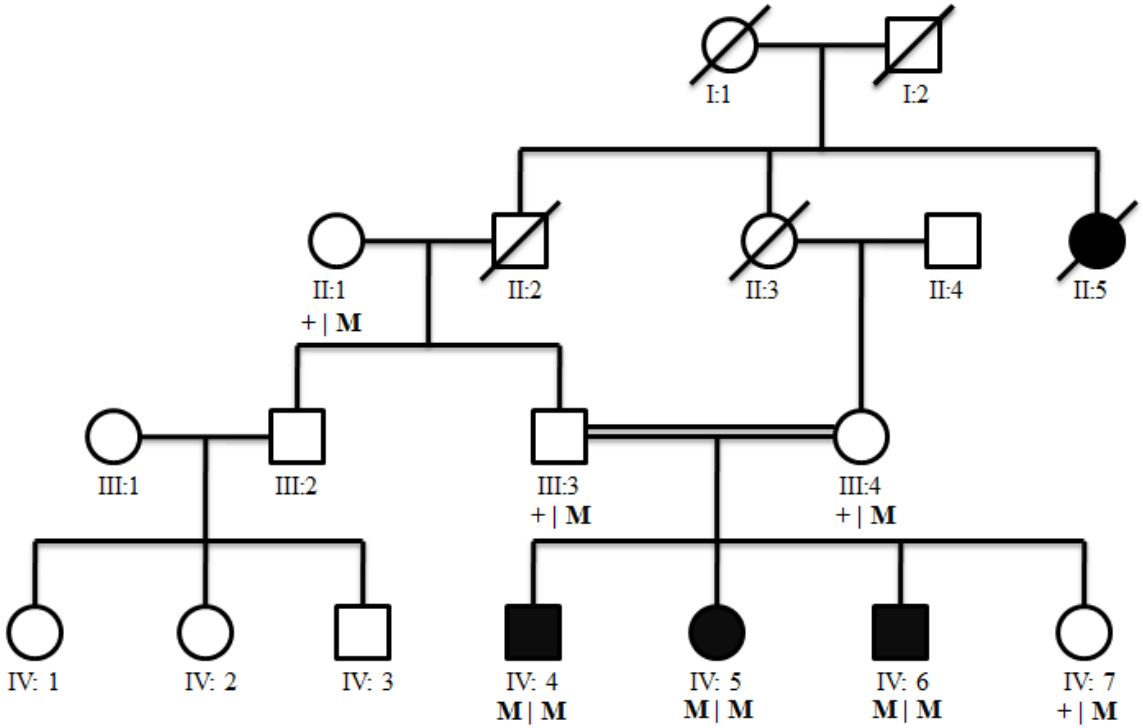

Figure 3.9 (i). Pedigree of family DFR45. For a description of the symbols see legend of Figure 3.1 (i).

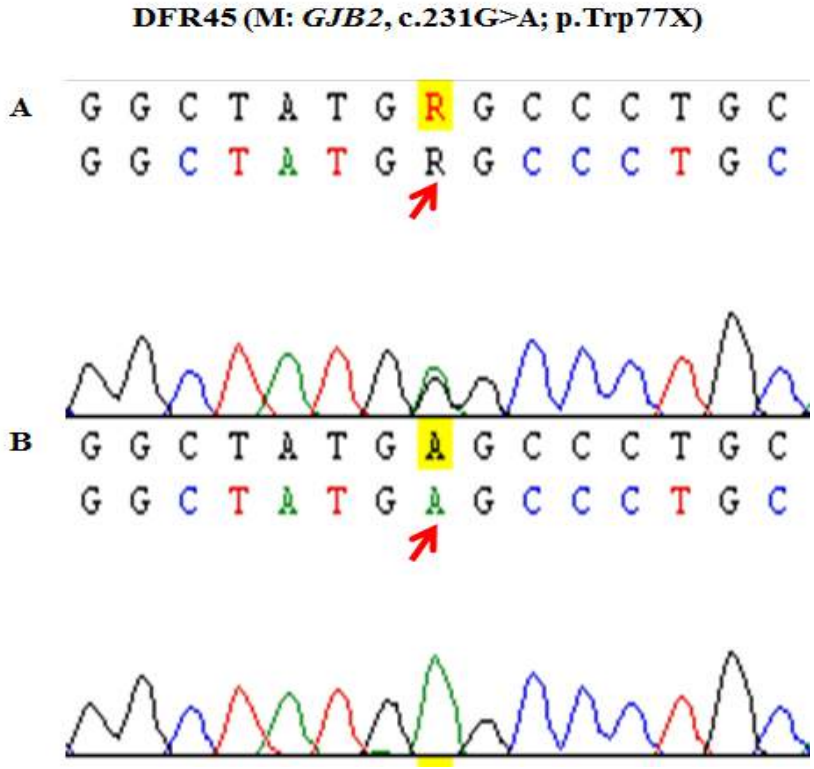

Figure 3.9 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper figure is for heterozygous variation and **B)** the bottom one is indicating the homozygous variant.

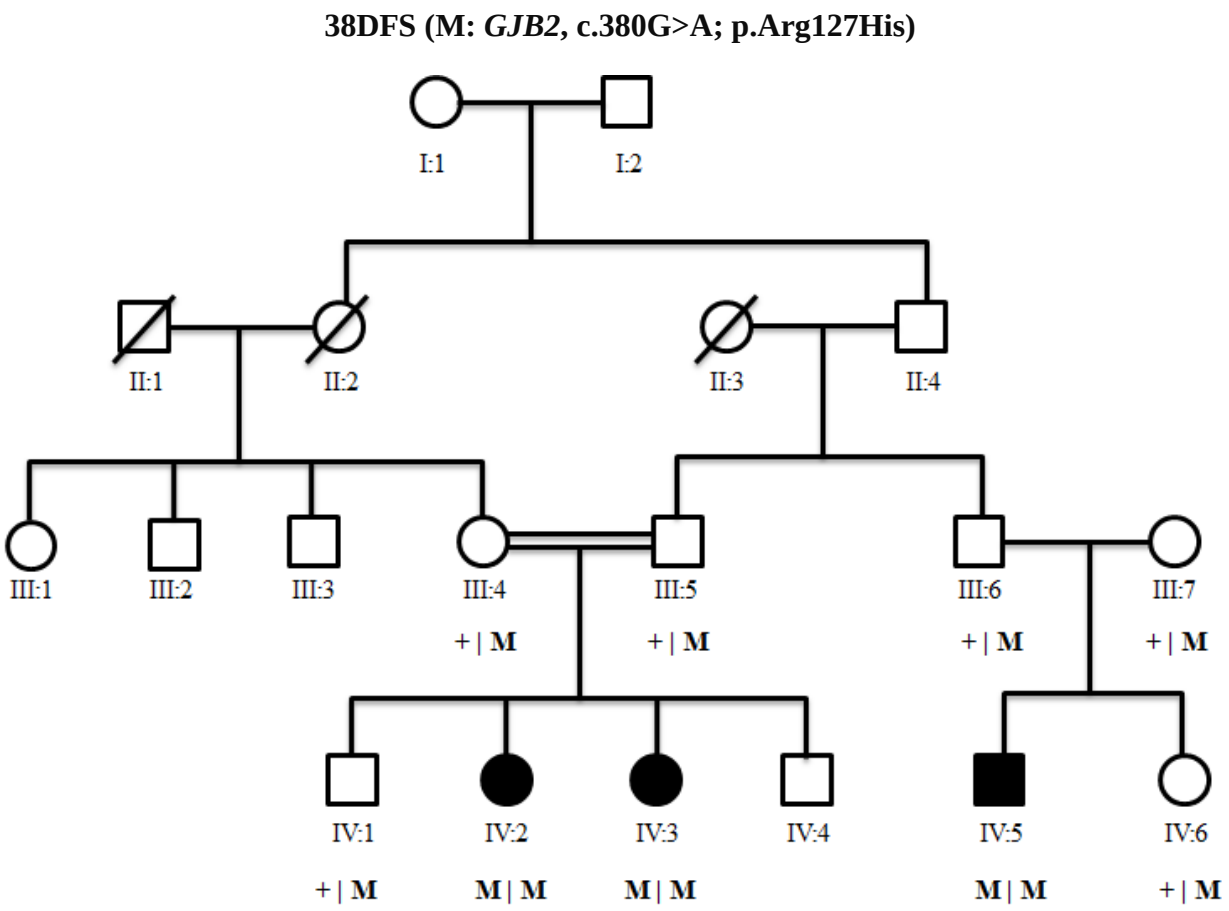

Figure 3.10. Pedigree of family 38DFS. For a description of the symbols see legend of Figure 3.1 (i).

39DFS (M: *GJB2*, c.380G>A; p.Arg127His)

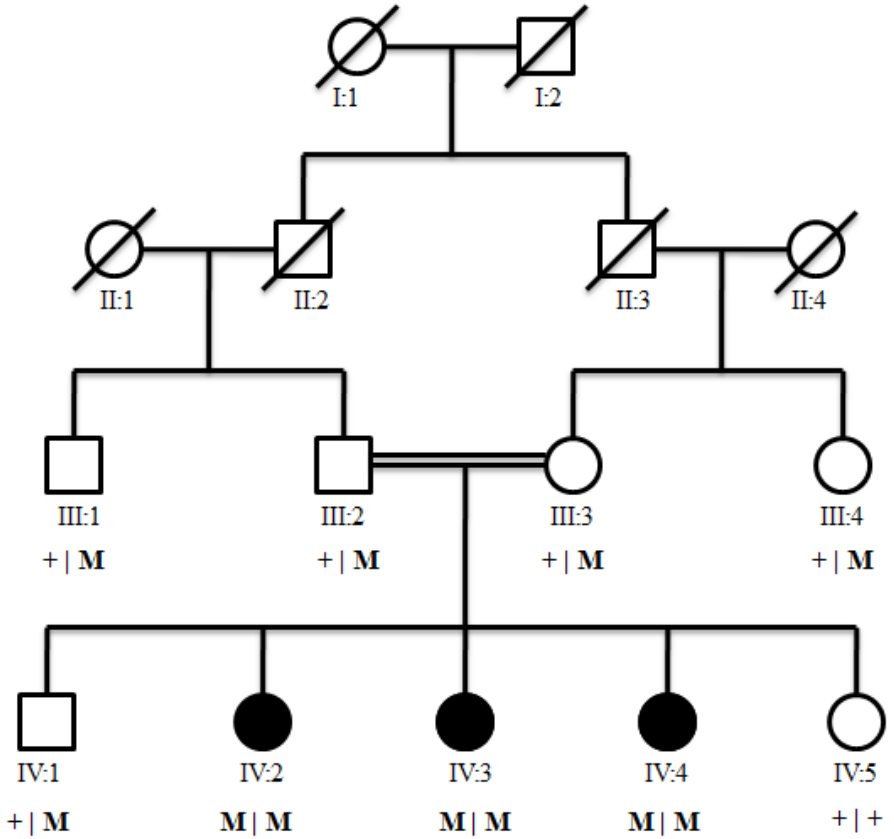

Figure 3.11. Pedigree of family 39DFS. For a description of the symbols see legend of Figure 3.1 (i).

8DF (M: *GJB2*, c.35delG; p.Gly12Valfs*2)

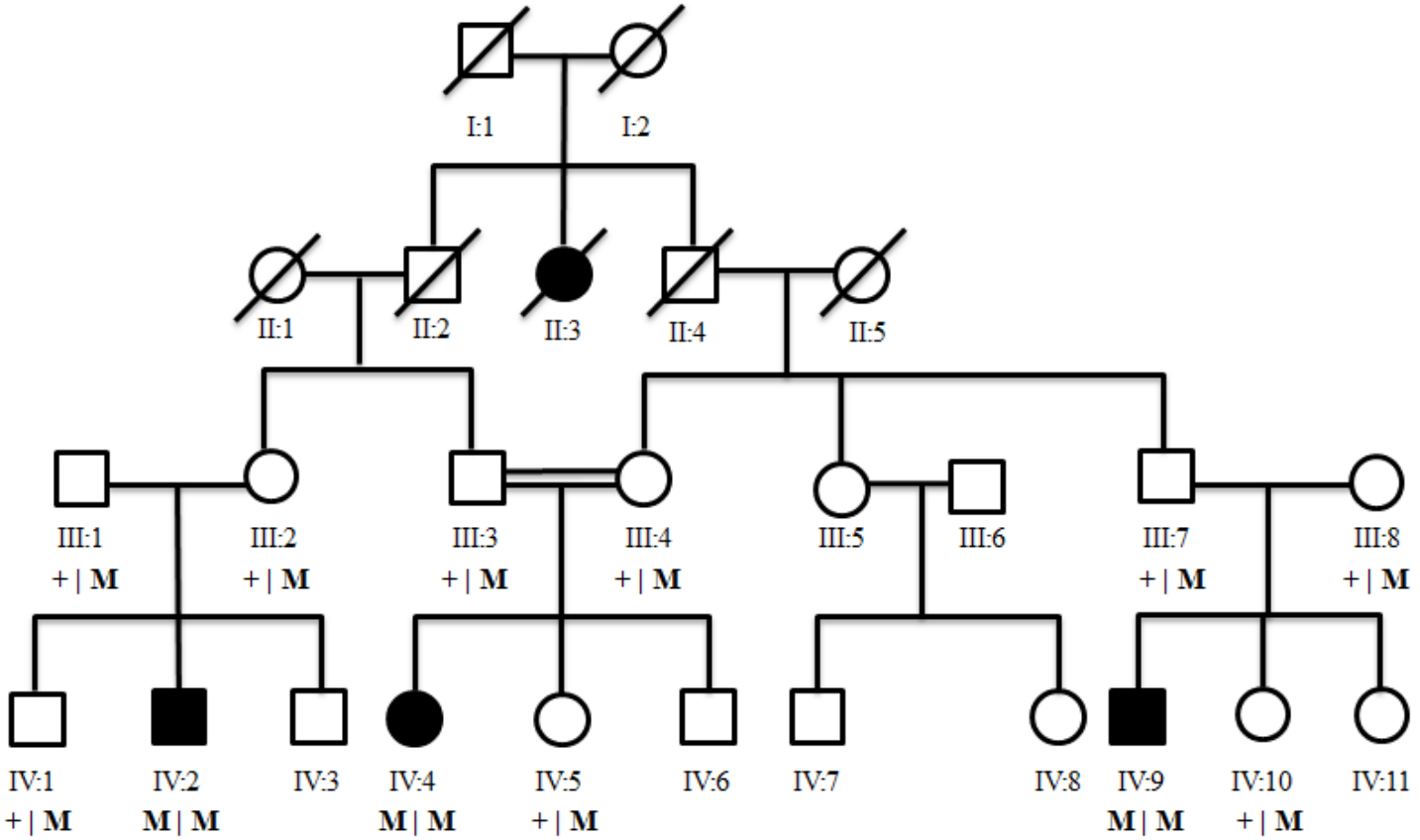

Figure 3.12. Pedigree of family 8DF. For a description of the symbols see legend of Figure 3.1 (i).

9DF (M: *GJB2*, c.35delG; p.Gly12Valfs*2)

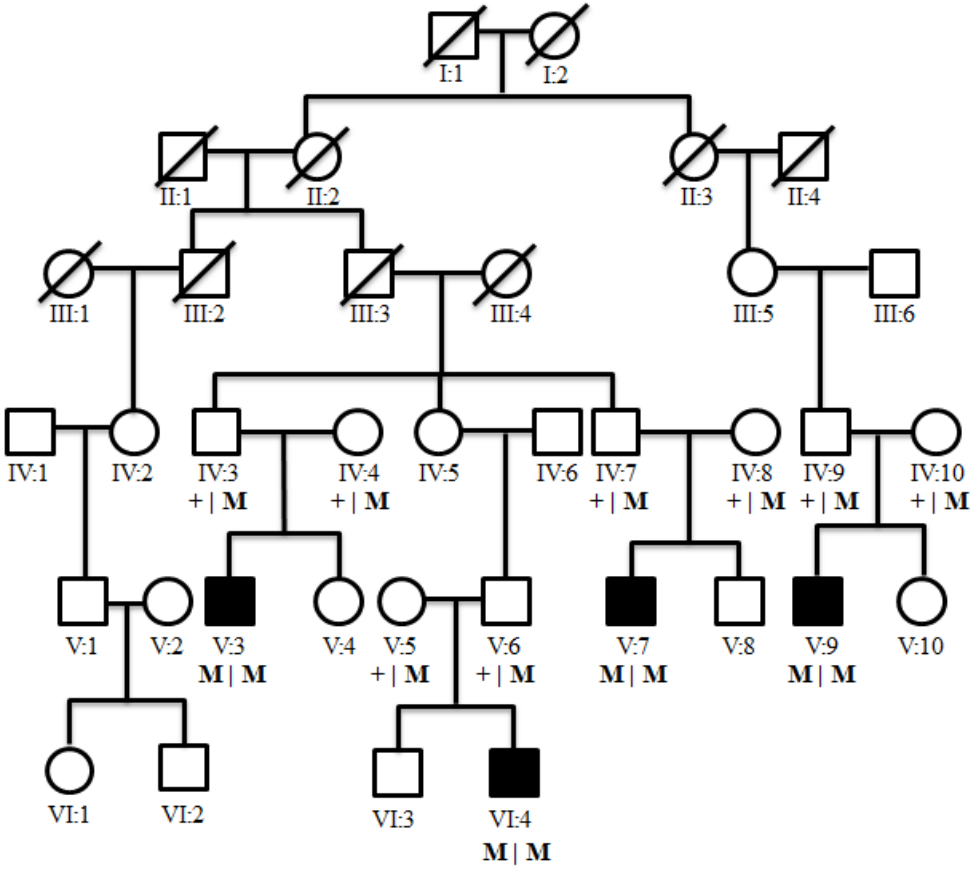

Figure 3.13. Pedigree of family 9DF. For a description of the symbols see legend of Figure 3.1 (i).

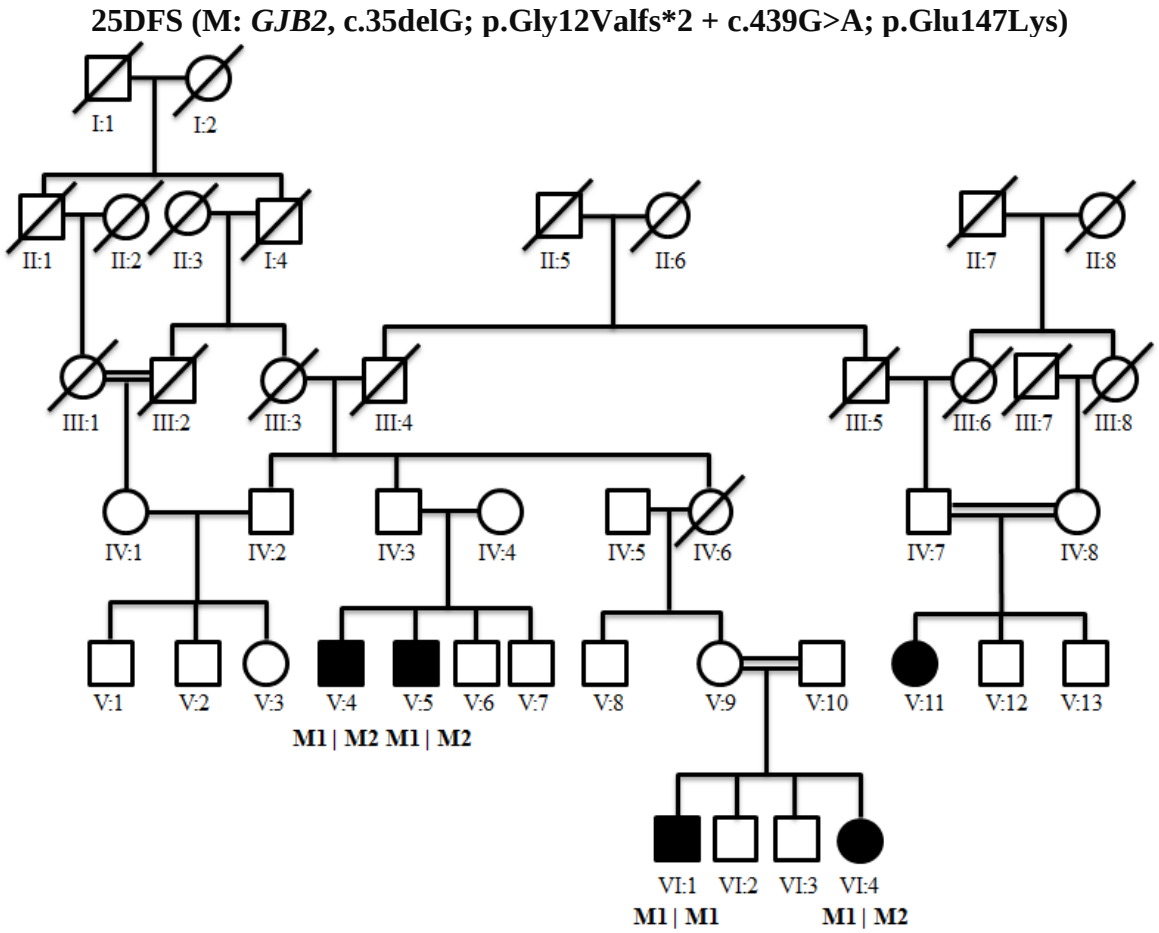

Figure 3.14. Pedigree of family 25DFS. For a description of the symbols see legend of Figure 3.1 (i).

37DFS (M: *GJB2*, c.377_378insATGCGGA; p.Arg127Cysfs*85)

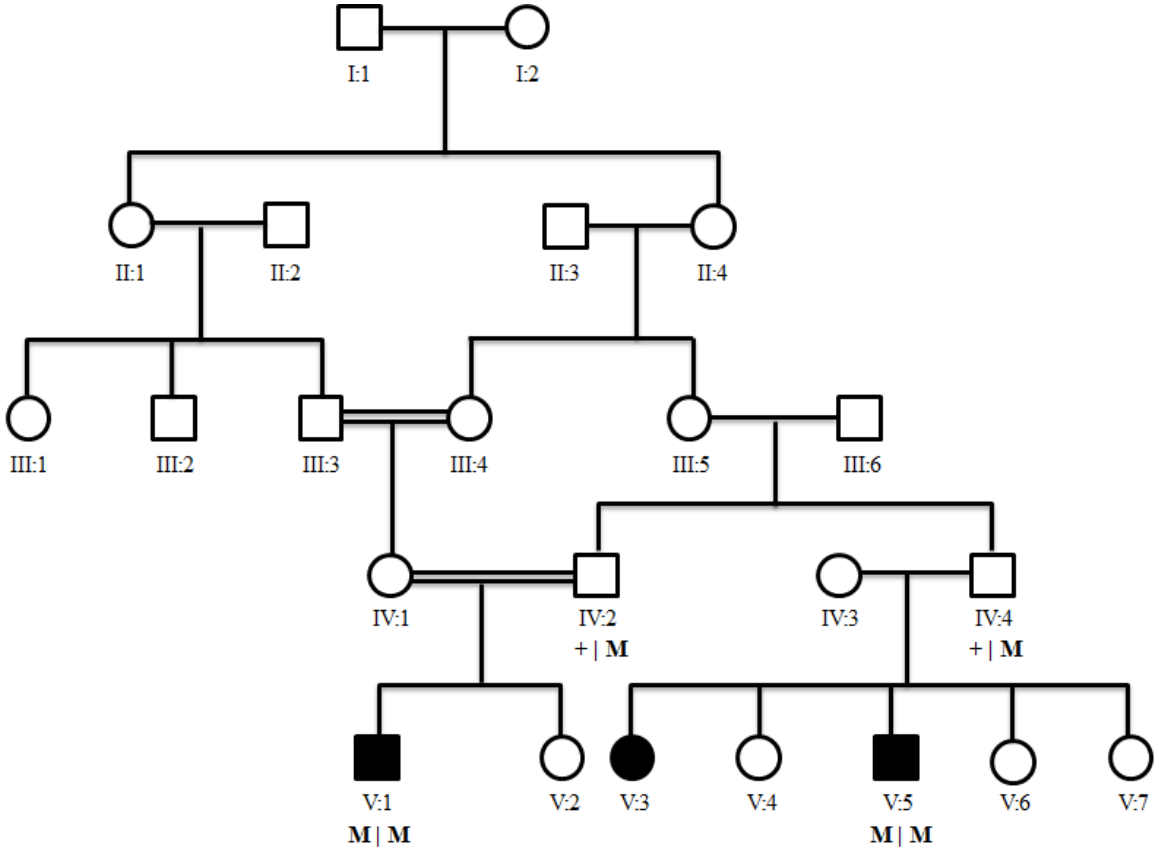

Figure 3.15. Pedigree of family 37DFS. For a description of the symbols see legend of Figure 3.1 (i).

DFR10 (M: *GJB2*, c.598G>A; p.Gly200Arg)

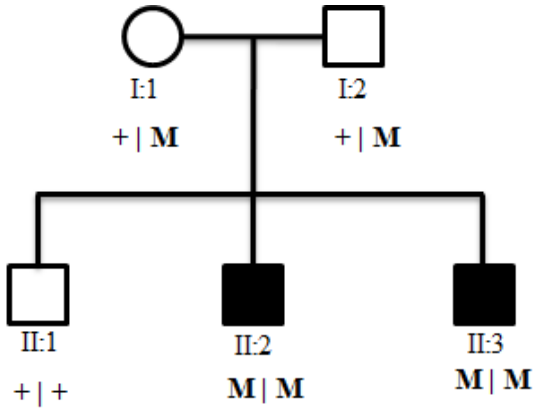

Figure 3.16 (i). Pedigree of family DFR10. For a description of the symbols see legend of Figure 3.1 (i).

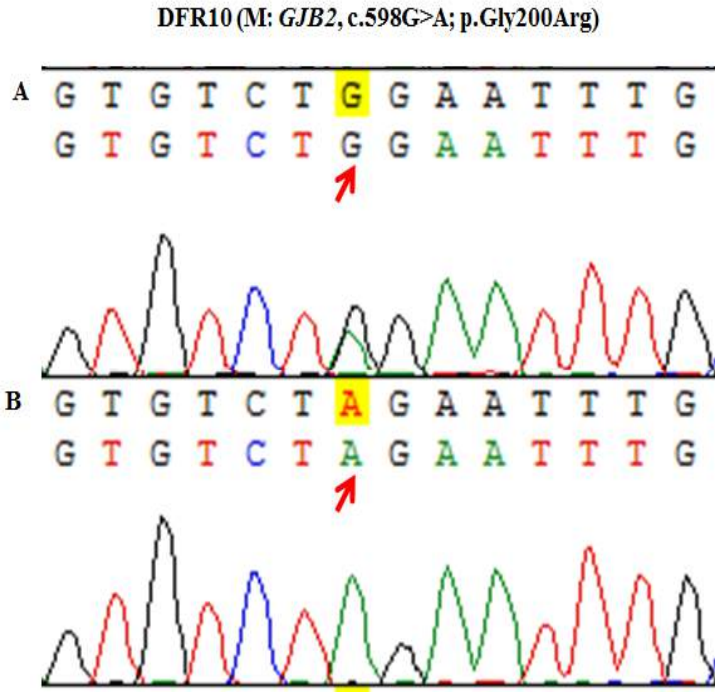

Figure 3.16 (ii). Sequencing chromatogram of *GJB2* variant. **A)** The red arrow in the upper figure is for heterozygous variation, **B)** while the bottom one is indicating the homozygous variant.

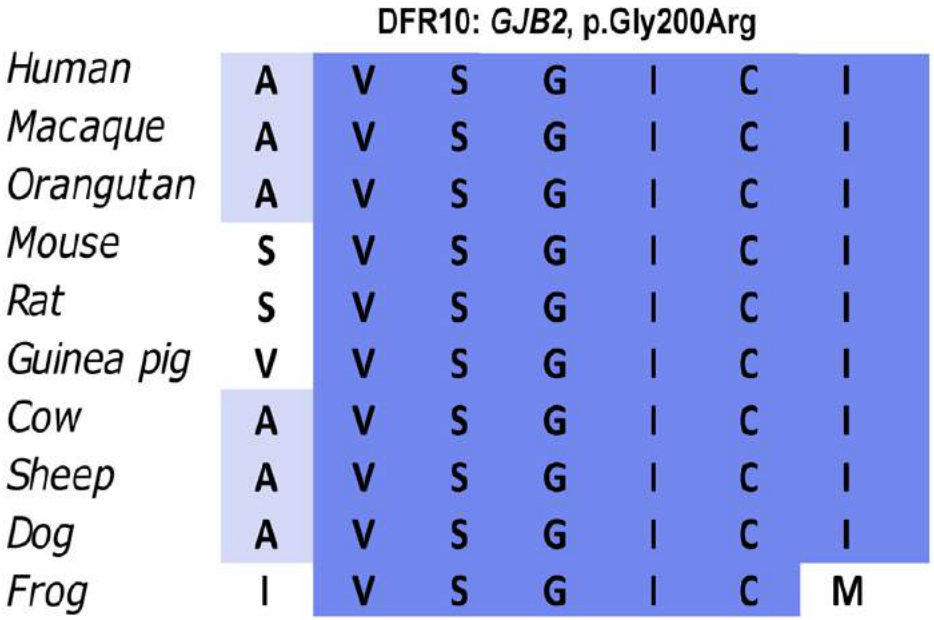

Figure 3.16 (iii). Amino acid sequence conservation of p.Gly200 across 9 species. Multiple-alignment of the corresponding stretches of protein sequences across different species. The blue color shading represents the intensity of conservation, where the dark blue shading represents highly conserved stretch while the light blue shading denotes moderate conservation of residues across different species.

Table 3.3. Novel mutations identified in known genes for autosomal recessive non-syndromic hearing loss in the current study

Family ID	**Size of homozygous regions (Mb)**	**Chr.**	**Flanking SNPs**	**Chr. position (in hg19)**	**Candidate gene (Acc. No.)**	**Mutation (Predicted protein change)**	**PhyloP; SIFT; PolyPhen-2**	**NetGene2**	**Frequency in EVS**
DFR10	ND	13	ND	ND	*GJB2* (NM_004004.5)	Ex-2: c.598G>A (p.Gly200Arg)	3.43; Deleterious; Damaging	NA	Absent
DFR23	ND	17	ND	ND	*MYO15A* (NM_016239.3)	Ex-61: c.9948G>A (p.Gln3316Gln)	NA	Abolition of splice site	Absent
13DF	ND	17	ND	ND	*MYO15A* (NM_016239.3)	In-5: c.3866+1G>A (p.?)	NA	Abolition of splice site	AA=0/ AG=1/ GG=6051
DFR28	ND	17	ND	ND	*MYO15A* (NM_016239.3)	Ex-50: c.8767C>T (p.Arg2923*)	NA	NA	TT=0/ TC=1/ CC=6266
DFR3	ND	17	ND	ND	*MYO15A* (NM_016239.3)	Ex-45: c.8222T>C (p.Phe2741Ser)	4.97; Deleterious; Damaging	NA	Absent
11DF	ND	9	ND	ND	*TMC1* (NM_138691.2)	In-8: c.362+18A>G (p.Glu122Tyrfs*10)	NA	NA	Absent
7DF	8.40	1	rs1242330; rs7521242	53,396,842-61,803,889	*BSND* (NM_057176.2)	Ex-1: c.97G>C (p.Val33Leu)	1.09 Deleterious; Damaging	NA	Absent

Table 3.3. cont.

Family ID	(Size of homozygous regions (Mb)	Chr.	Flanking SNPs	Chr. position (in hg19)	Candidate gene (Acc. No.)	Mutation (Predicted protein change)	PhyloP; SIFT; PolyPhen-2	NetGene2	Frequency in EVS
DFR24	3.49	21	rs2838063; rs881969	42,929,129-46,421,694	*TMPRSS3* (NM_024022.2)	Ex-8: c.726C>G (p.Cys242Trp)	-0.28 Deleterious; Damaging	NA	Absent
DFR18	2.92	12	rs6581511; rs11176432	64,278,102-67,207,064	*MSRB3* (NM_001031679.2)	Ex-4: c.20T>G (p.Leu7Arg)	3.76; Tolerated; Possibly Damaging	NA	Absent

Acc. No., accession number of reference sequence; Chr, chromosome; Ex, exon; EVS, exome variant server; In, intron; SNPs, single nucleotide polymorphisms; Ref, references

(c.8222 T>C). In the family DFR23 (Figure 3.17 (i)), the c.9948G>A variant changes the last nucleotide of exon 61 and is predicted to affect the splice donor site. The splice donor site in the reference sequence has a high confidence score of 94%, which is reduced to a score of 24% in the mutant. To determine the effect of the c.9948G>A mutation on splicing, a minigene experiment was performed. This showed correct splicing of the wildtype *MYO15A* exon 61, while the c.9948G>A mutation almost completely abolished the normal splicing (Figure 3.17 (ii)). The variant found in the family 13DF (Figure 3.18 (i)) was another splice site mutation. NetGene2 predicted the abolition of the splice donor site of exon 5 as a result of the c.3866+1G>A mutation in intron 5 of *MYO15A*. The nonsense mutation c.8767C>T (p.Arg2923*) in the family DFR28 (Figure 3.19 (i)) was novel and predicted to lead to the synthesis of a truncated protein, while the missense mutation c.8222T>C, found in the family DFR3 (Figure 3.20 (i)) leads to the substitution of serine for phenylalanine (p.Phe2741Ser) at amino acid position 2741 (Figure 3.20 (ii)) that resides in the conserved region of the protein (Figure 3.20 (iii)). This missense change was predicted to be deleterious by SIFT and PolyPhen-2 (Table 3.3).

3.4. Results of *TMC1* Candidate Gene Screening

Haplotype analysis of STR-markers flanking *TMC1* showed genetic linkage of family 11DF with the disease. Since sequence analysis of *TMC1* revealed a novel intronic mutation, c.362+18A>G (Table 3.3), this family was not further analyzed by SNP-array genotyping. The variant segregated with hearing loss in the family 11DF (Figure 3.21 (i)) and was predicted to create a novel splice donor site with a similar confidence score as the original splice donor site. To determine the effect of the c.362+18A>G mutation on splicing, a minigene experiment was conducted. This revealed correct splicing of the wild-type *TMC1* exon 8, while the c.362+18A>G mutation resulted in a 17 bp extension of exon 8 (Figure 3.21 (ii)), leading to a frameshift and a premature stop codon (p.Glu122Tyrfs*10).

3.5. Results of Analysis of Whole Genome SNP Array and Candidate Gene Sequencing with Novel Variants

The families that remained unsolved post *GJB2* and *MYO15A* screening were sent for Illumina HumanOmniExpress whole genome single nucleotide polymorphism (SNP)

DFR23 (M: *MYO15A*, c.9948G>A; p.Gln3316Gln)

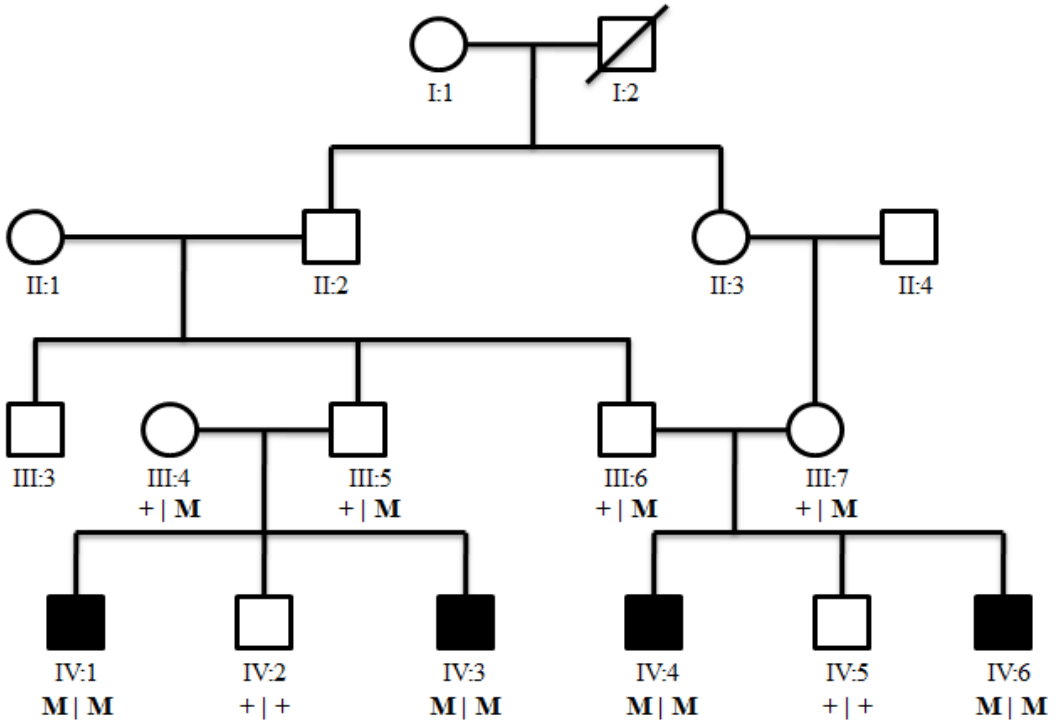

Figure 3.17 (i). Pedigree of family DFR23. For a description of the symbols see legend of Figure 3.1 (i).

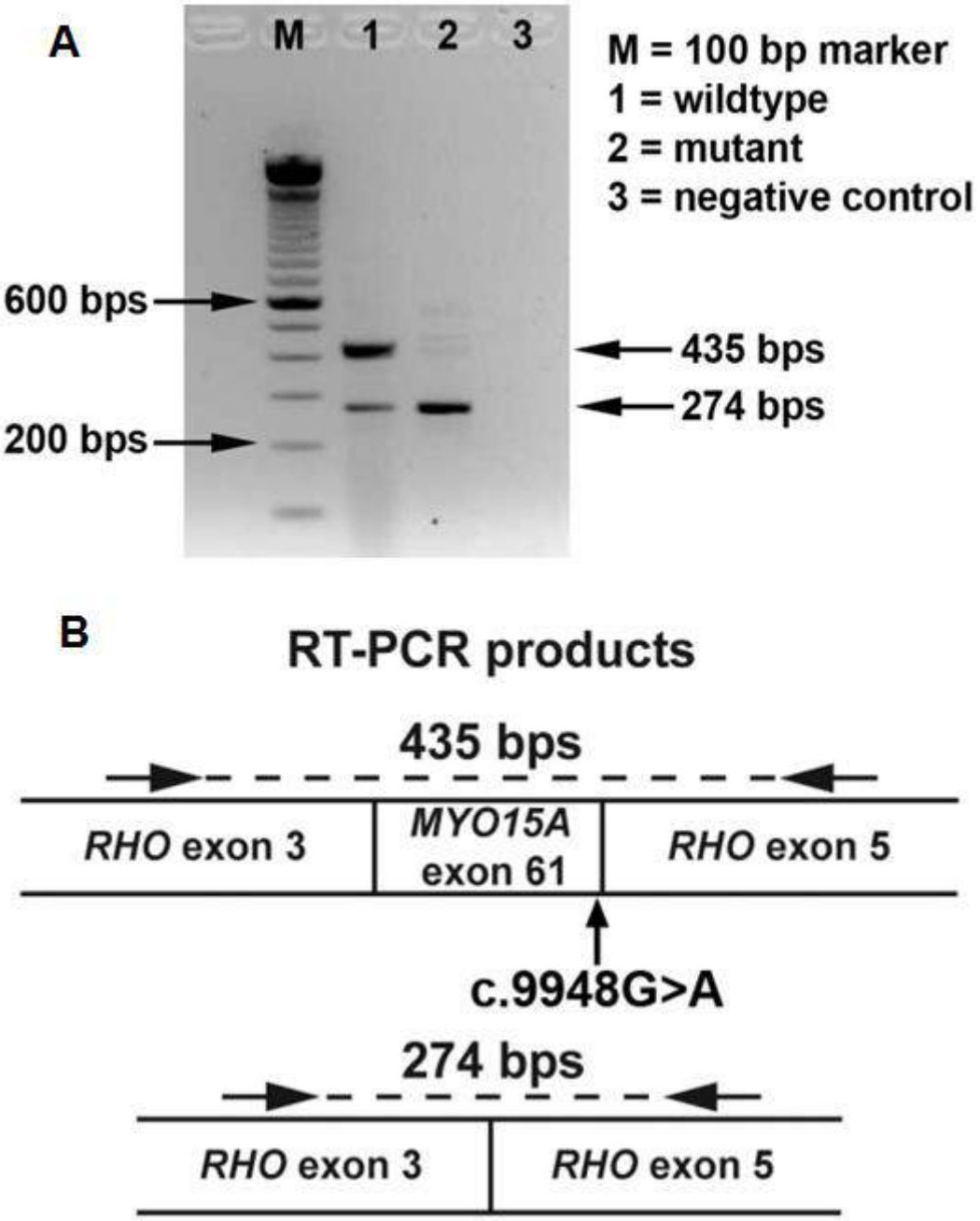

Figure 3.17 (ii). Effect of *MYO15A* c. 9948G>A using a minigene approach. **A)** An agarose gel containing RT-PCR products detected from HEK293T cells transfected with the wildtype and mutant minigene construct and **B)** a schematic representation of the identified splicing products. The RT-PCR products were verified by sequence analysis. The c.9948G>A mutation leads to skipping of exon 61.

13DF (M: *MYO15A*, c.3866+1G>A; p.?)

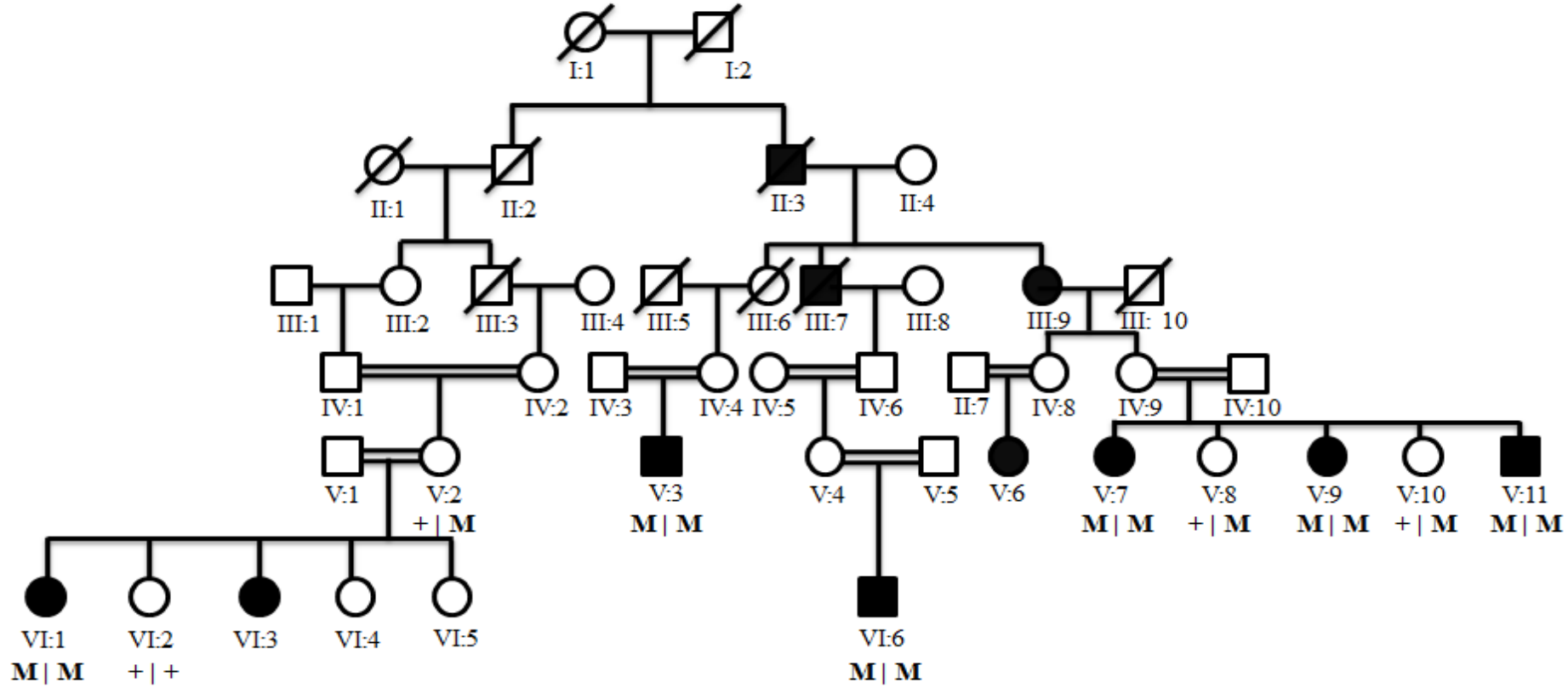

Figure 3.18. Pedigree of family 13DF. For a description of the symbols see legend of Figure 3.1 (i).

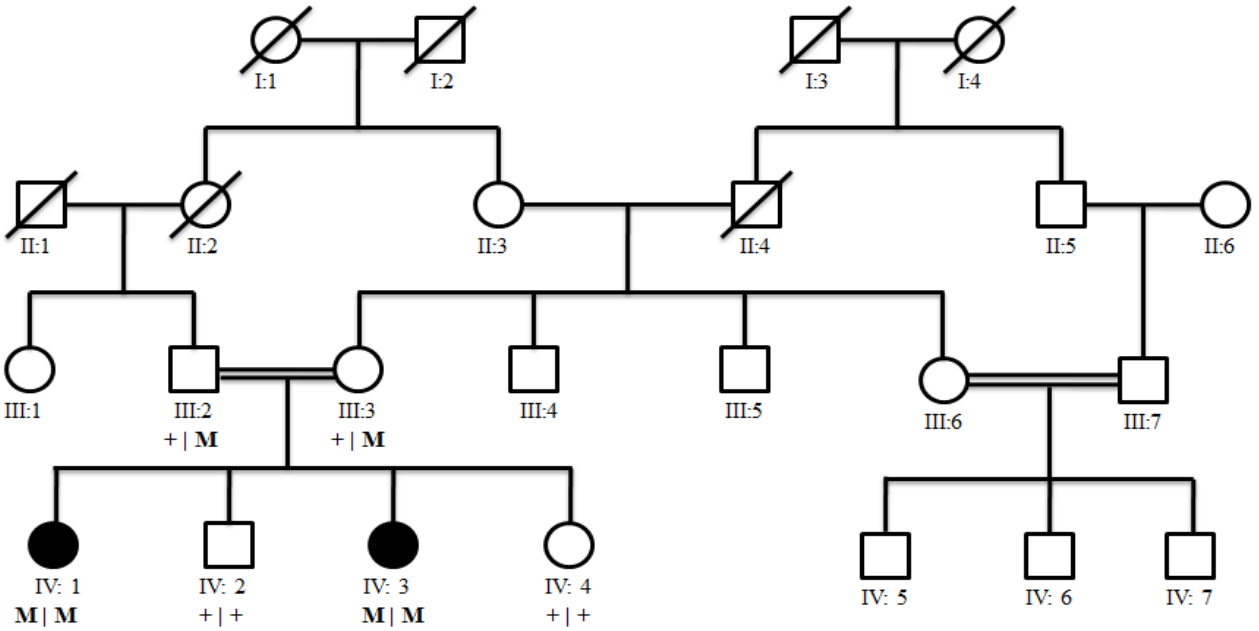

Figure 3.19. Pedigree of family DFR28. For a description of the symbols see legend of Figure 3.1 (i).

DFR3 (M: *MYO15A*, c.8222T>C; p.Phe2741Ser)

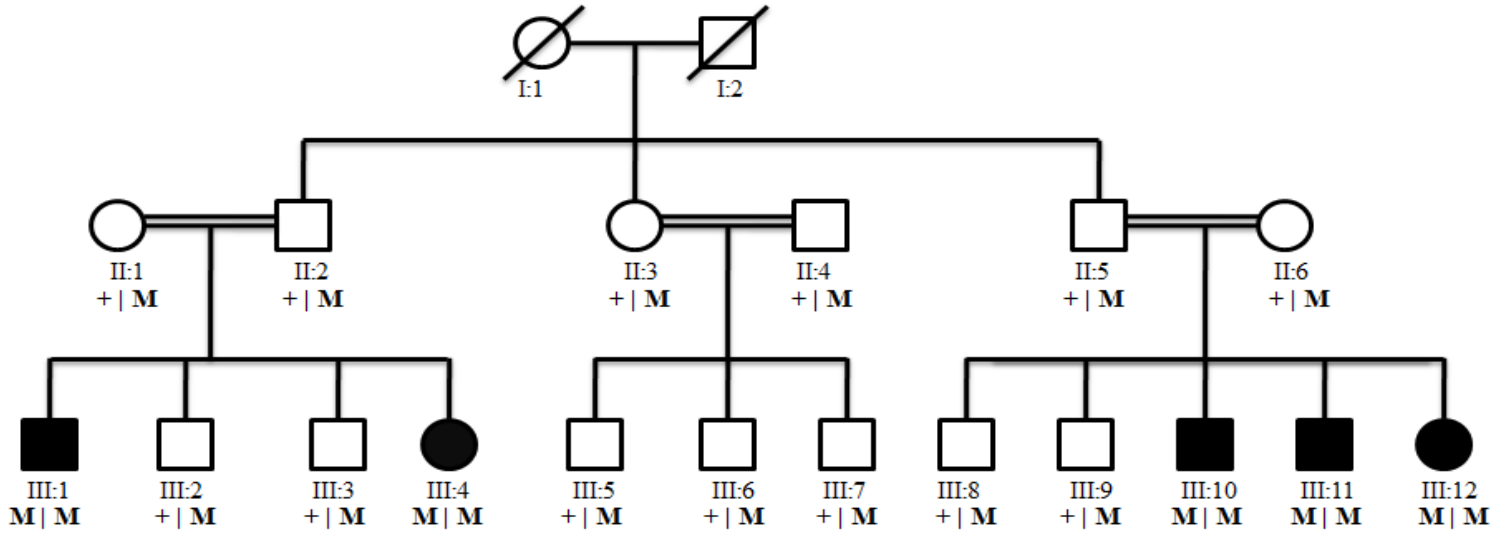

Figure 3.20 (i). Pedigree of family DFR3. For a description of the symbols see legend of Figure 3.1 (i).

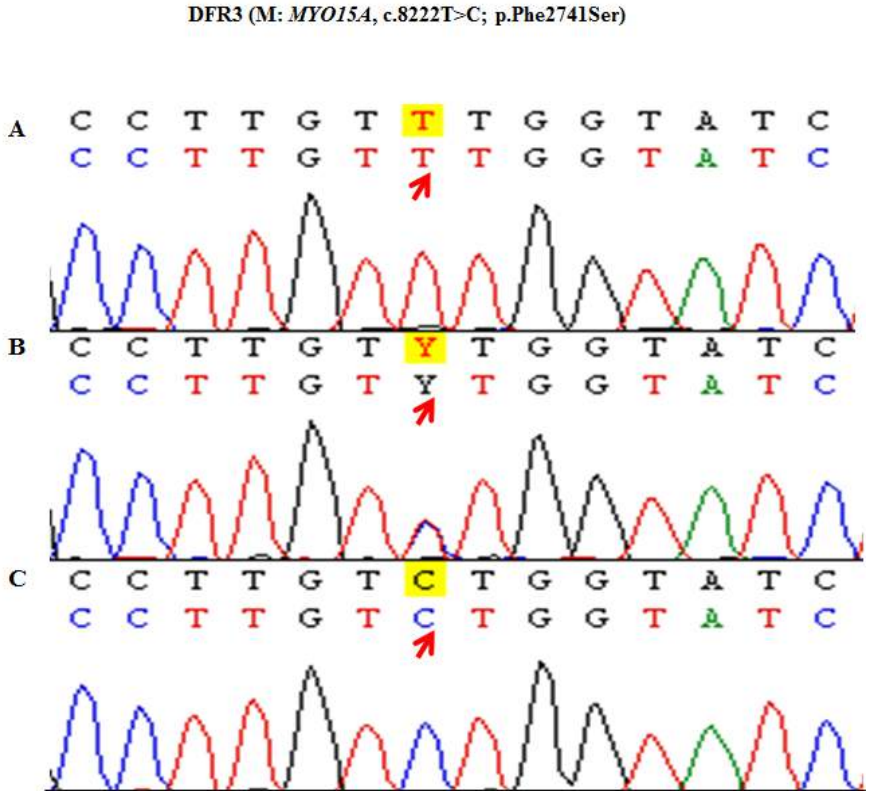

Figure 3.20 (ii). Sequencing chromatogram of *MYO15A* variant. **A)** The red arrow in the upper figure is for wild type; **B)** the middle figure is for heterozygous variation and **C)** the bottom one is indicating the homozygous variant.

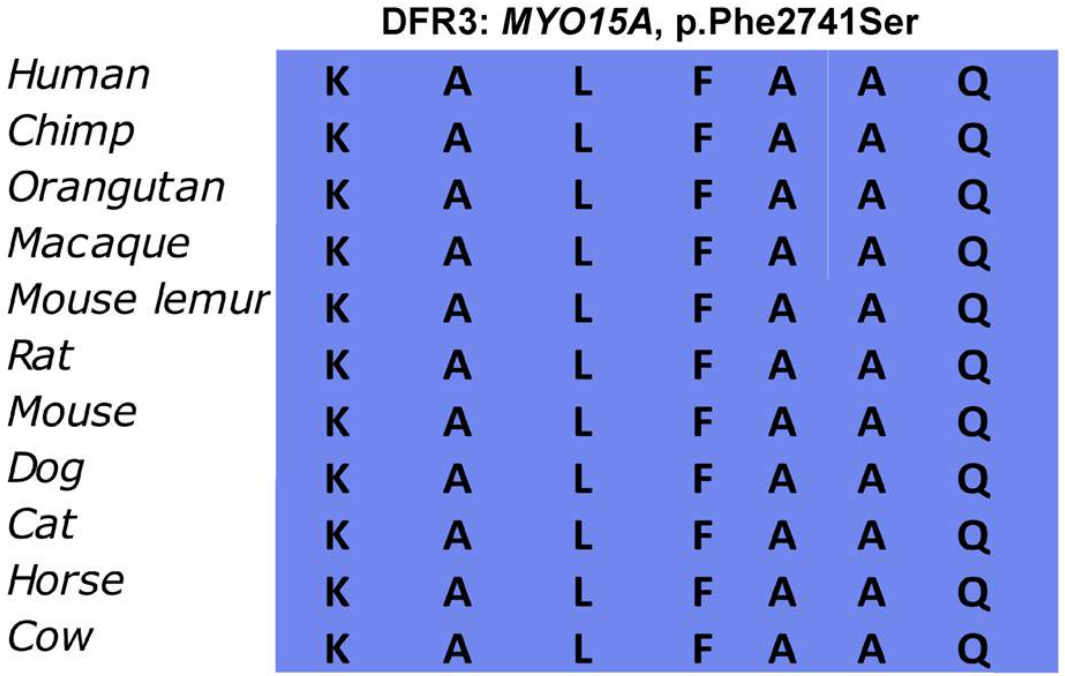

Figure 3.20 (iii). Amino acid sequence conservation of p.Phe2741 across 11 species. Multiple-alignment of the corresponding stretches of protein sequences across different species. The blue color shading represents the intensity of conservation, where the dark blue shading represents highly conserved stretch while the light blue shading denotes moderate conservation of residues across different species.

11DF (M: *TMC1*, c.362+18A>G; p.?)

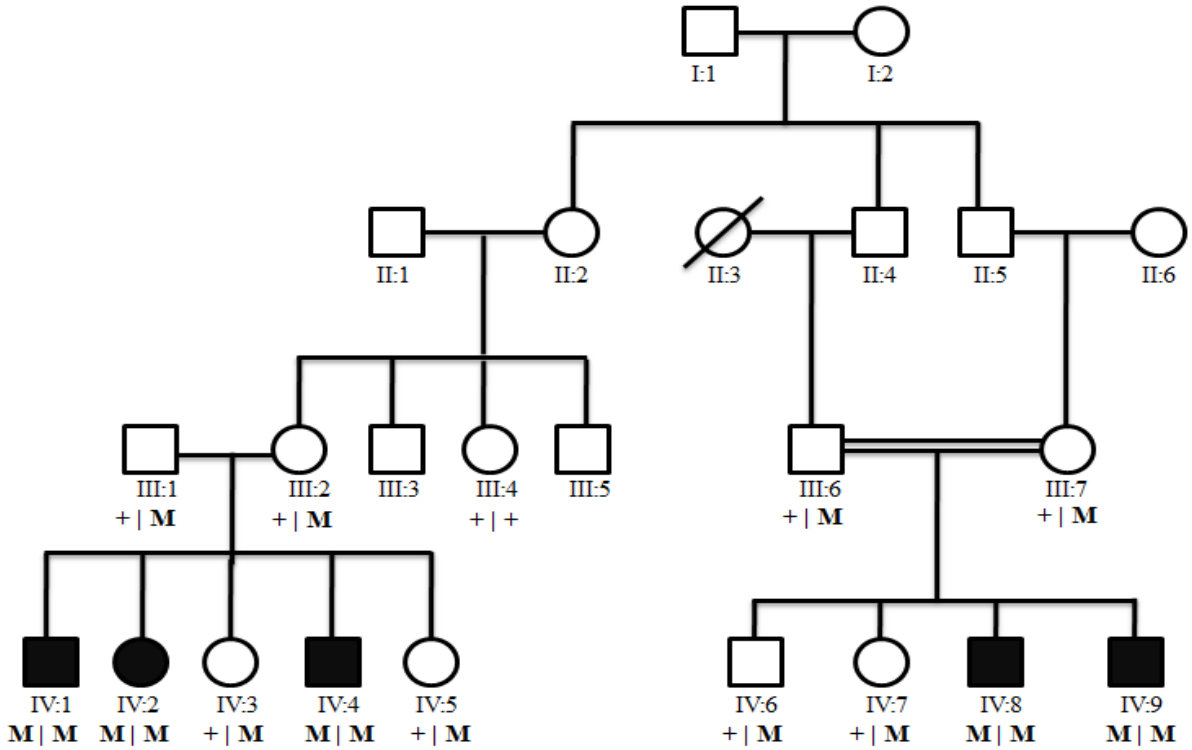

Figure 3.21 (i). Pedigree of family 11DF. For a description of the symbols see legend of Figure 3.1 (i).

microarray analysis (>700K SNPs). The online tool, Homozygosity Mapper (http://www.homozygositymapper.org/) was used to analyze the data and determine the common homozygous intervals in the affected individuals, which were lacking in the normal individuals. All the homozygous regions of at least 1Mb were selected and the candidate genes were sequenced. In the family DFR24 (Figure 3.22 (i)), a homozygous region of 3.49 Mb was obtained on chromosome 21 (Figure 3.22 (ii)), harboring *TMPRSS3* (MIM_605511). Sanger sequencing revealed a novel homozygous missense mutation that co-segregated with hearing impairment in family DFR24, c.726C>G (p.Cys242Trp) (Figure 3.22 (iii)) and was predicted to be probably damaging by PolyPhen-2 and deleterious by SIFT (Table 3.3). This mutation is located in the peptidase S1 domain and the HOPE server predicted the abolition of the catalytic activity of TMPRSS3 (Figure 3.22 (v)). In addition the amino acid substitution is present in a region of the protein that is conserved across different species and therefore probably affects the core structure of the peptidase domain (Figure 3.22 (iv)).

In family DFR18 (Figure 3.23 (i)) SNP microarray data analysis revealed a 17.6 Mb homozygous region on chromosome 12 flanked by SNPs rs7978381 and rs7976686 (Figure 3.23 (ii)), a LOD score of 2.7 was calculated for this region. The known deafness gene, *MSRB3* (MIM_613719), in the region was subsequently sequenced, revealing a novel homozygous nucleotide substitution c.20T>G (p.Leu7Arg) in exon 4 (Table 3.3). The leucine at position 7 is located in the signal peptide of the MSRB3 protein and as a result of the substitution by arginine this signal peptide loses its function as predicted by SignalP 4.0. Furthermore, the Leu7 residue is non-conserved across species (Figure 3.23 (iii)). The mutation abolishes a *TseI* restriction site, which allowed the segregation analysis of this variant in the family and controls to be checked by restriction digestion. This variant segregated in the family but was also found heterozygously in 3 out of 178 ethnically matched control alleles.

Homozygosity mapping data analysis of family 7DF (Figure 3.24 (i)) using whole genome SNP array revealed another novel missense mutation in *BSND* (MIM_606412), c.97G>C (p.Val33Leu). The novel variant was predicted to be deleterious by SIFT and PolyPhen-2 (Table 3.3) and Val33 is conserved across species (Figure 3.24 (ii)).

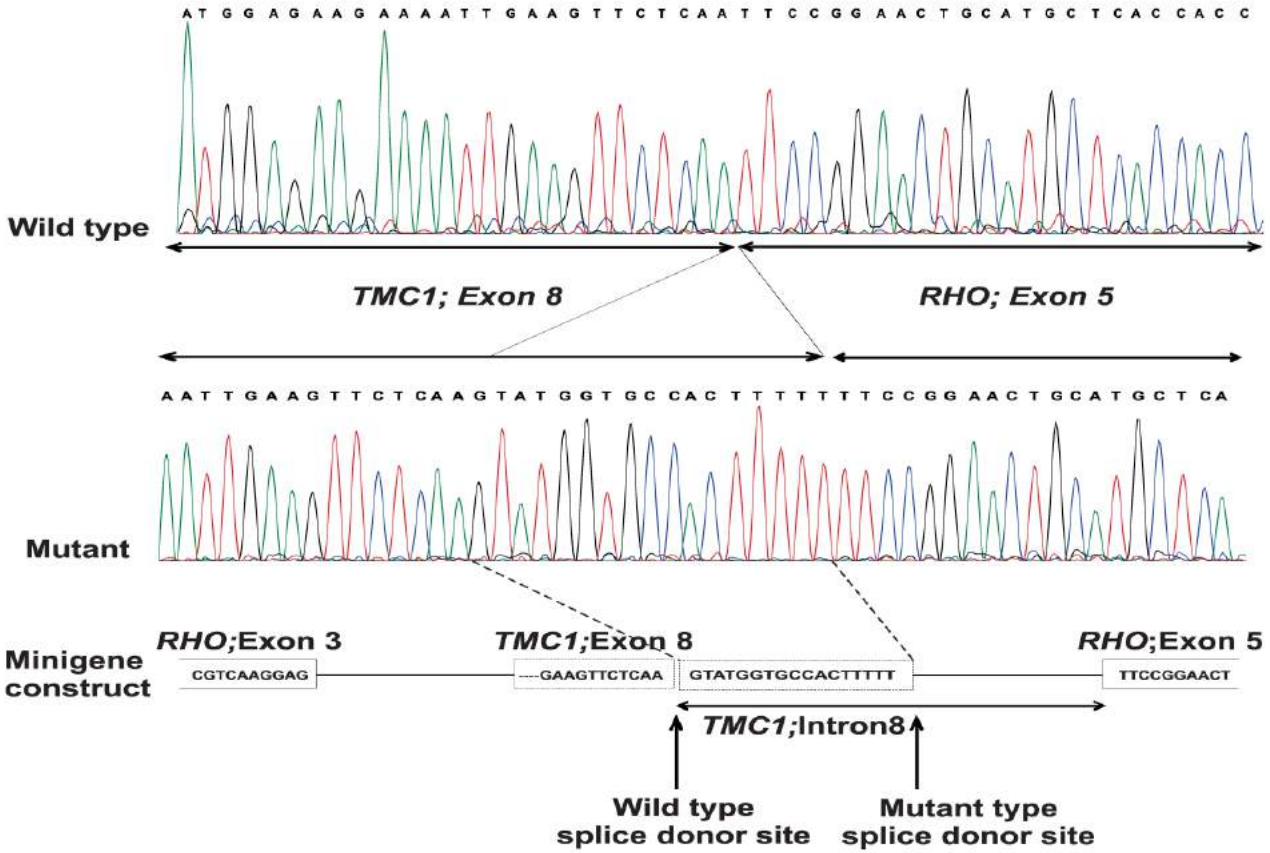

Figure 3.21 (ii). Effect of *TMC1* intronic mutation c.362+18A>G using a minigene approach. Electropherogram of the partial cDNA sequence of RNA derived from cells transfected with the pCI-NEO with either the mutant or wildtype *TMC1* exon 8. The mutation leads to the insertion of 17bp at the 3'end of exon 8, which can be predicted to result in a premature stop codon in exon 9 (p.Glu122Tyrfs*10).

DFR24 (M: *TMPRSS3*, c.726C>G; p.Cys242Trp)

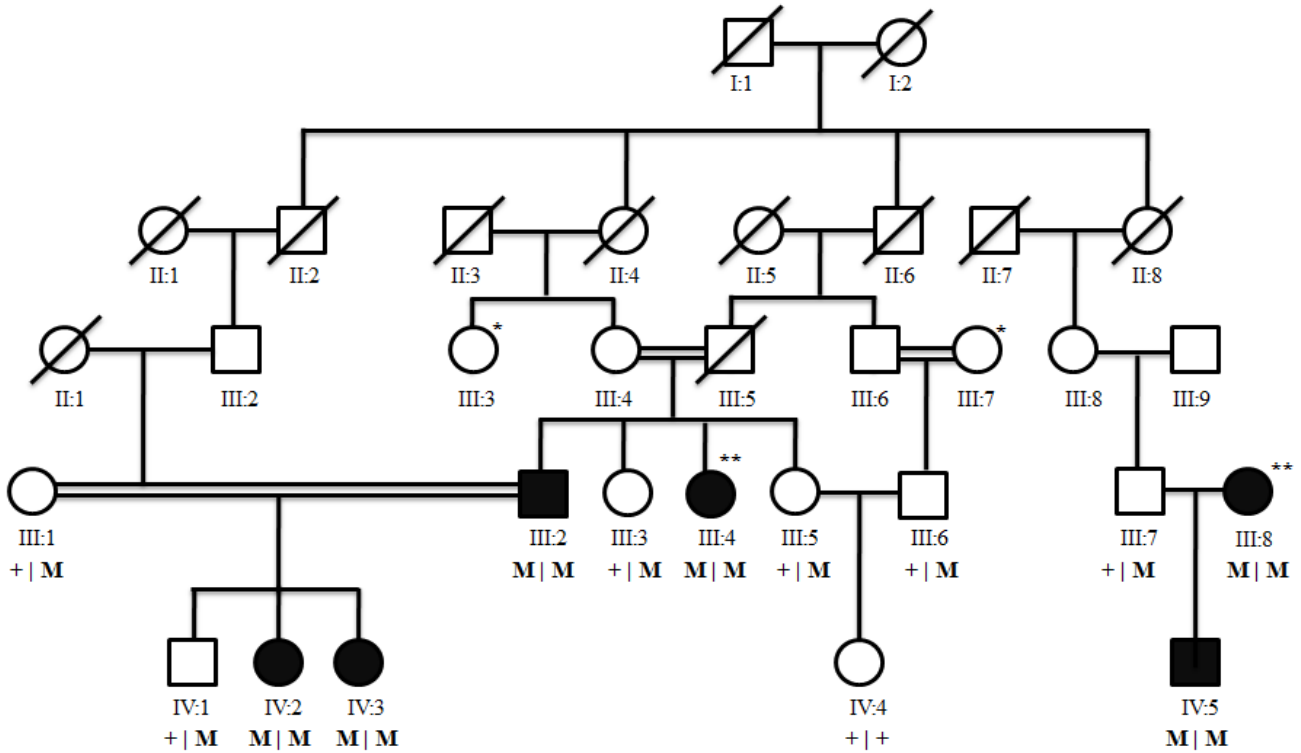

Figure 3.22 (i). Pedigree of family DFR24. For a description of the symbols see legend of Figure 3.1 (i).

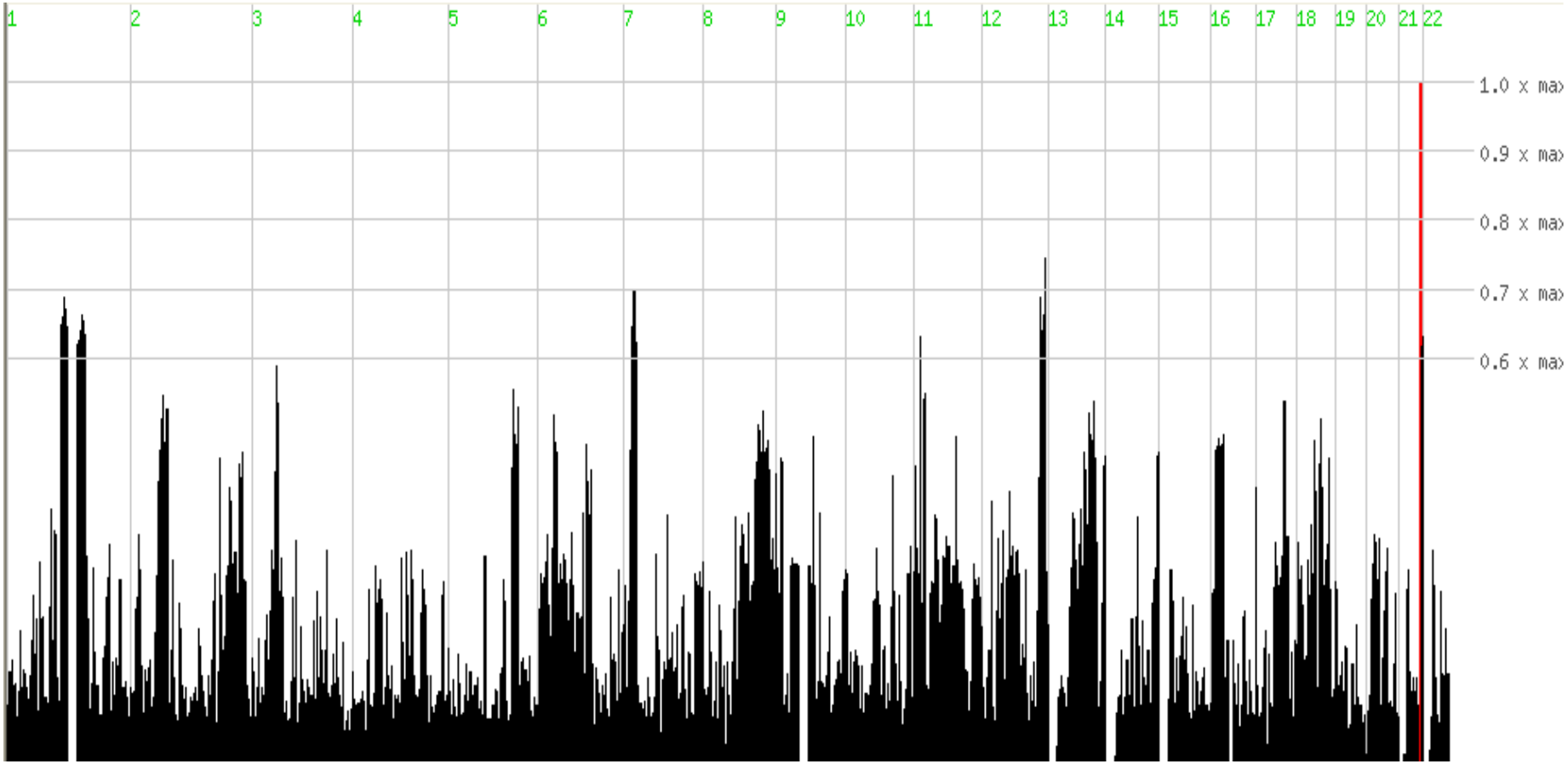

Figure 3.22 (ii). The Homozygosity Mapper plot showing homozygous region in red color in family DFR24.

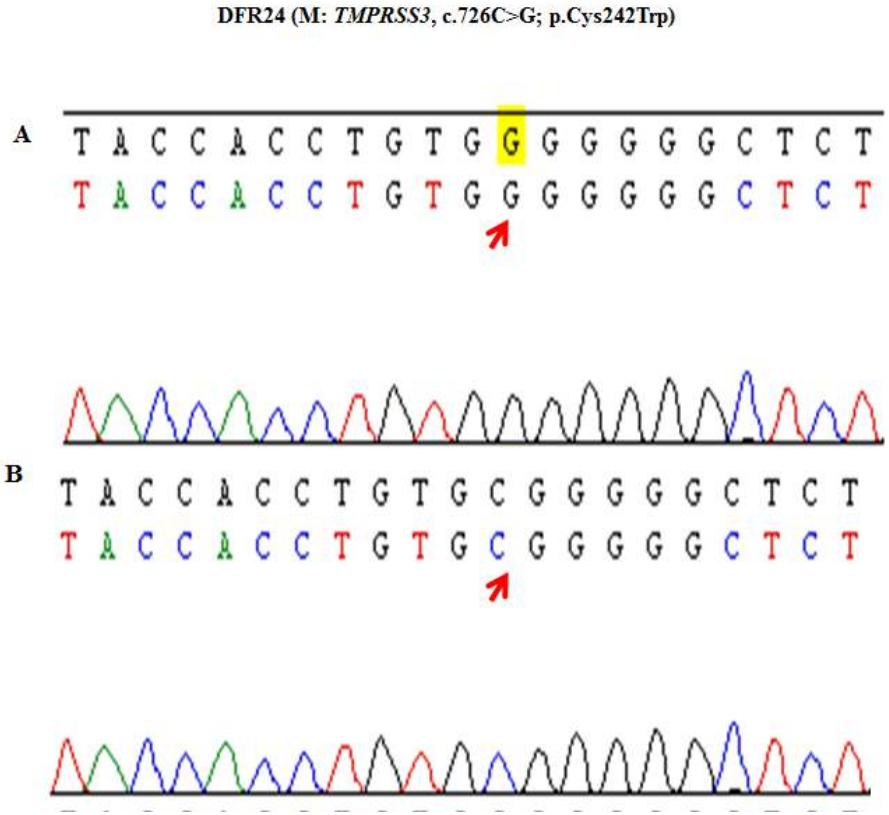

Figure 3.22 (iii). Sequencing chromatogram of *MYO15A* variant. **A)** The red arrow in the upper figure is for homozygous mutant variant; **B)** and the bottom one is indicating the homozygous ancestral.

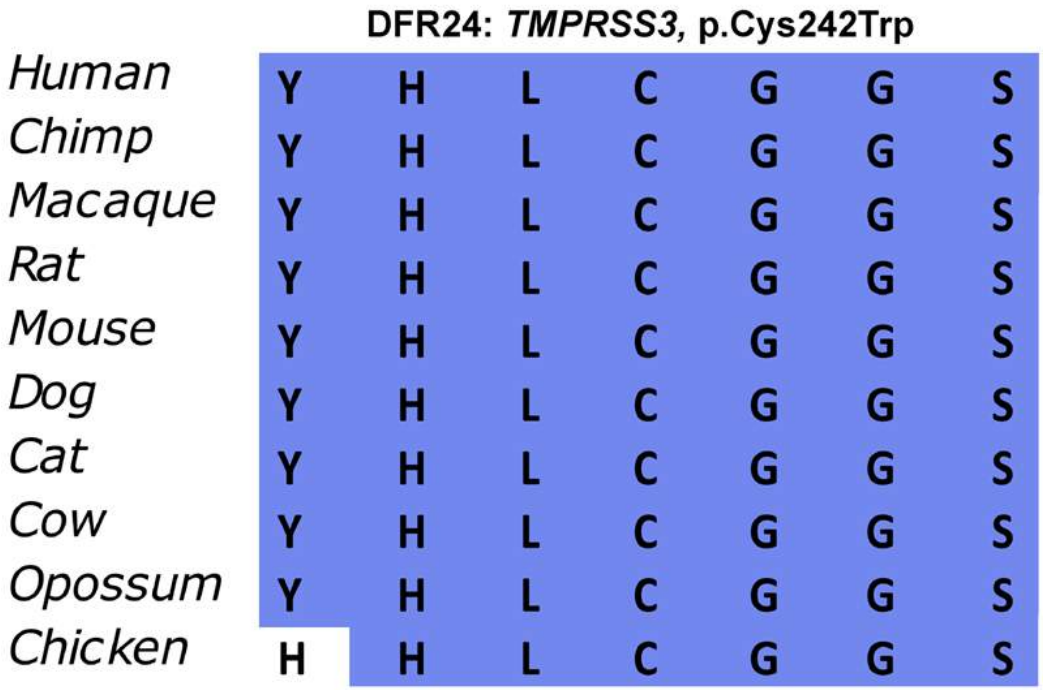

Figure 3.22 (iv). Amino acid sequence conservation of p.Cys24 across 10 species. Multiple-alignment of the corresponding stretches of protein sequences across different species. The blue color shading represents the intensity of conservation, where the dark blue shading represents highly conserved stretch while the light blue shading denotes moderate conservation of residues across different species.

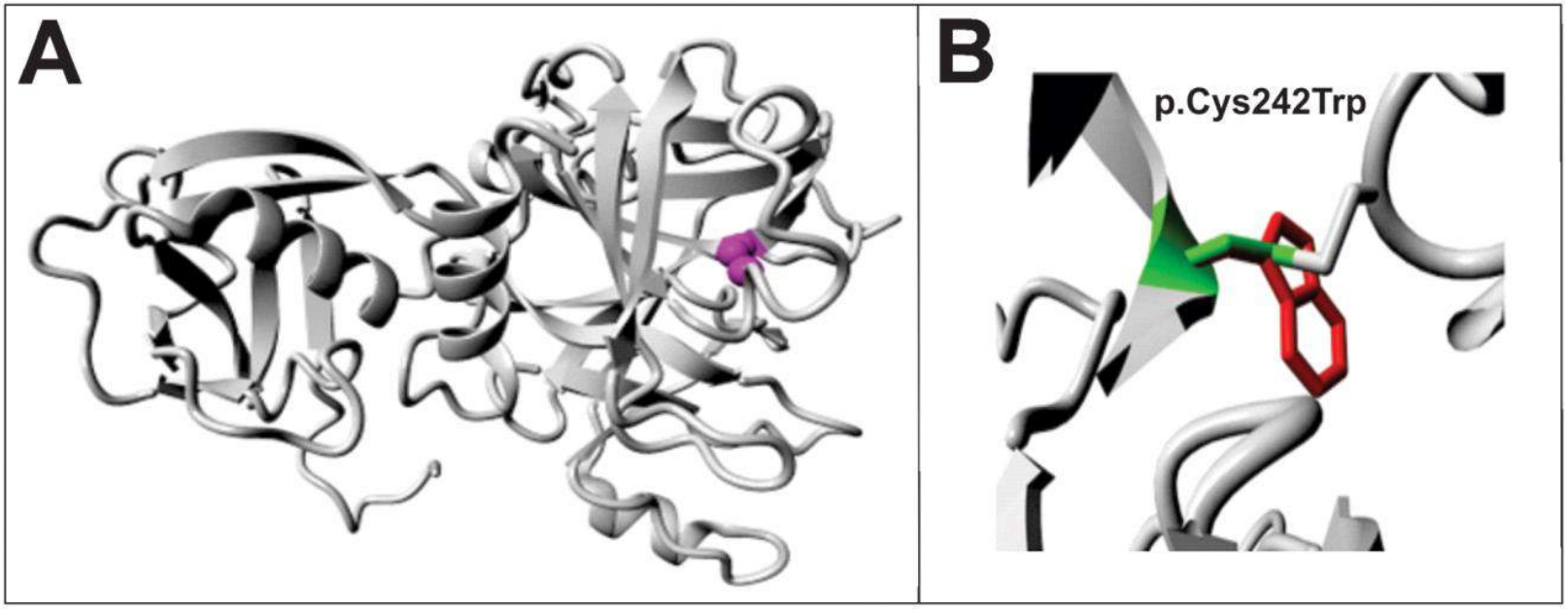

Figure 3.22 (v). Predicted effect of mutation c.726C>G (p.Cys242Trp) on the three dimensional structure of TMPRSS3. **A)** Wild type protein structure with an intact disulphide bridge showing position of the mutated residue (magenta). **B)** Close-up view of the structure showing the wild type residue cysteine (green) and the mutant residue tryptophan (red). In case of the mutant residue there will be no disulphide bridge at this position.

DFR18 (M: *MSRB3*, c.20T>G; p.Leu7Arg)

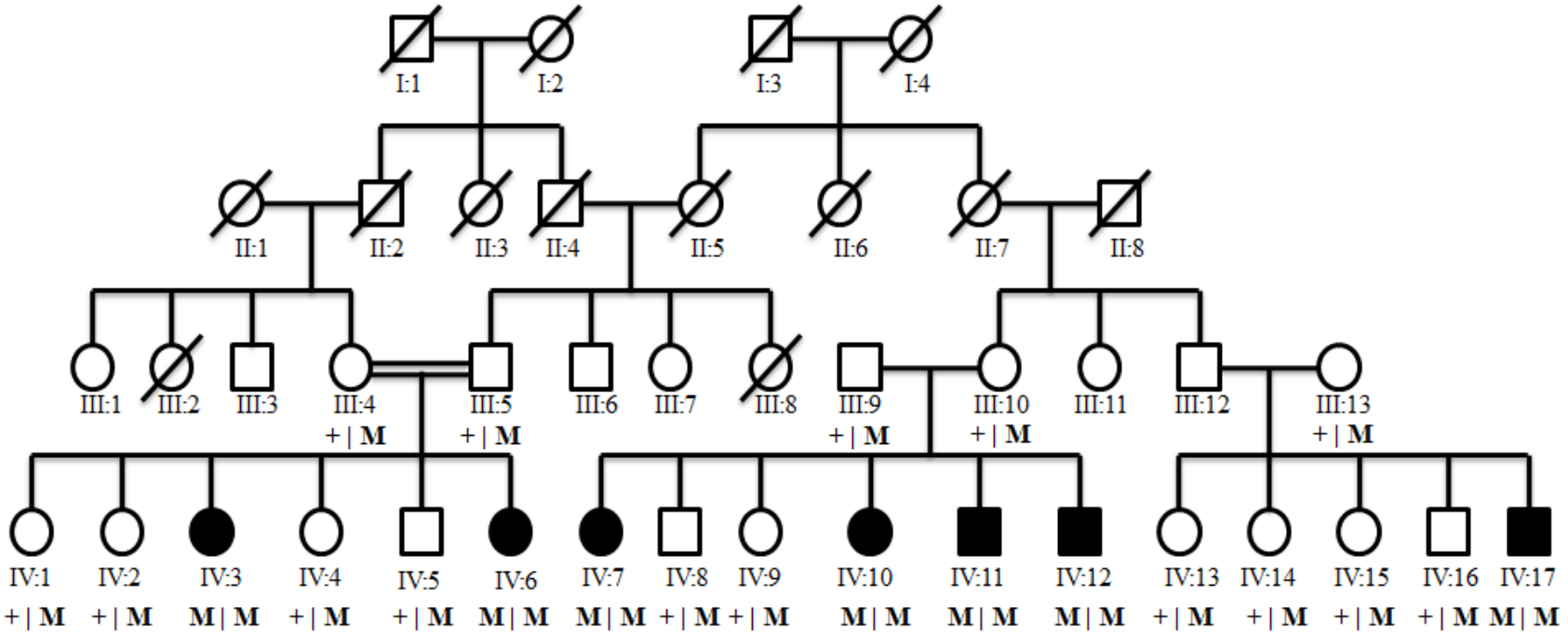

Figure 3.23 (i). Pedigree of family DFR18. For a description of the symbols see legend of Figure 3.1 (i).

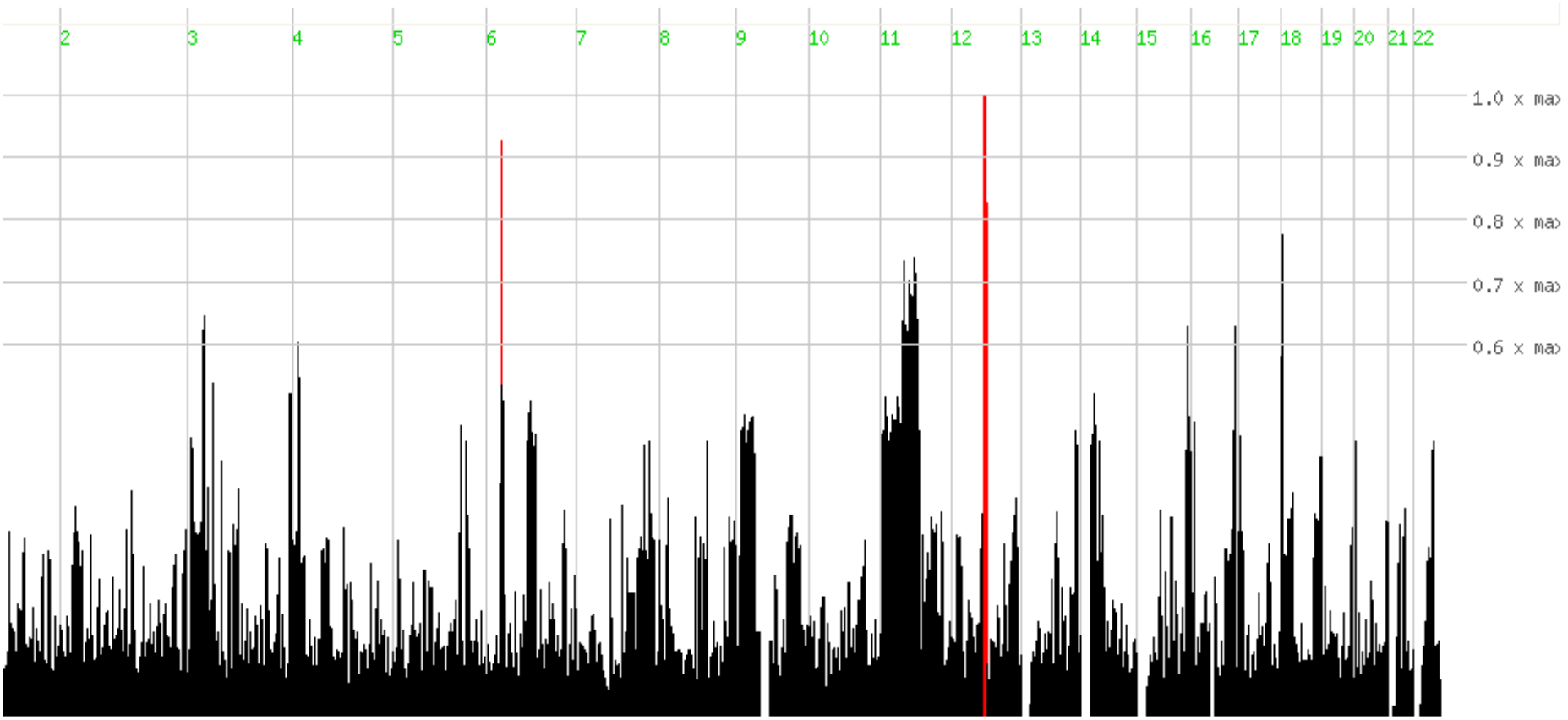

Figure 3.23 (ii). The Homozygosity Mapper plot showing homozygous region in red color in family DFR18.

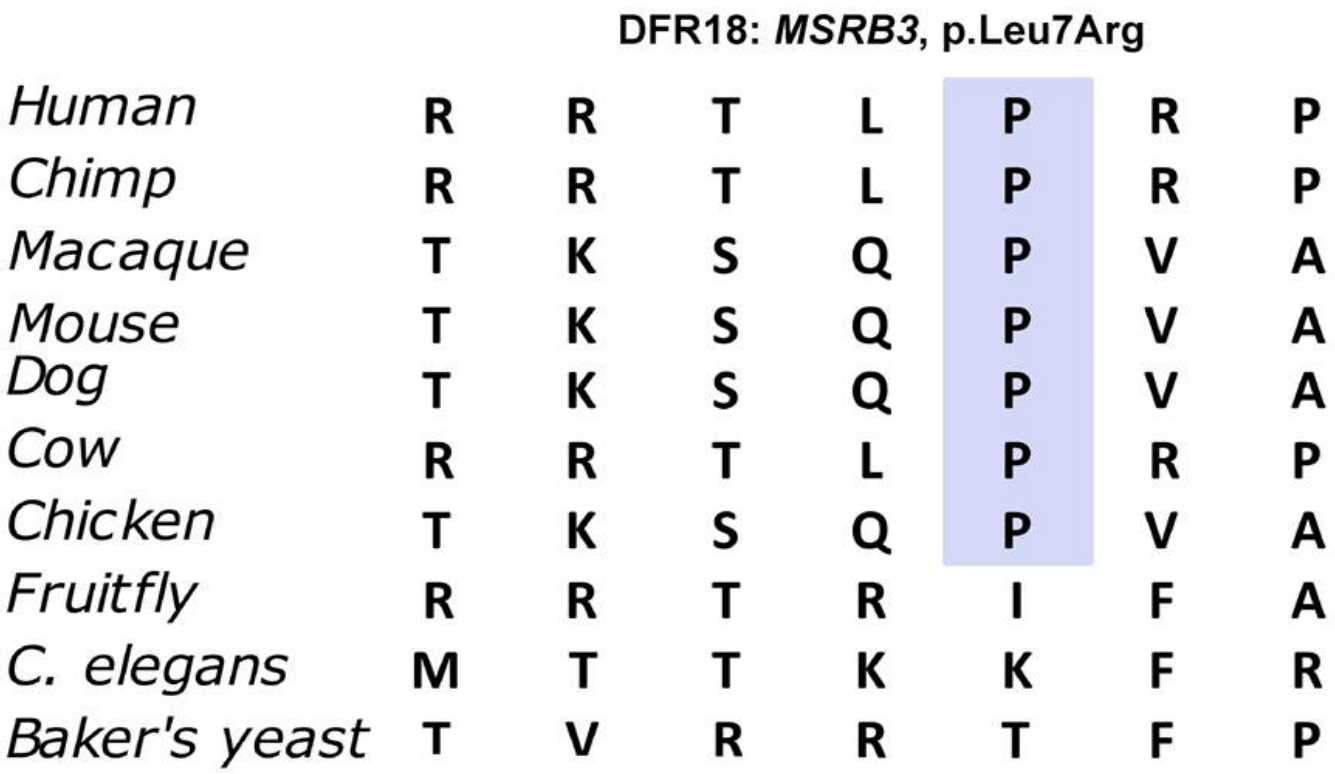

Figure 3.23 (iii). Non-conserved residue of p.Leu7 across 10 species. Multiple-alignment of the corresponding stretches of protein sequences across different species. The blue color shading represents the intensity of conservation, where the dark blue shading represents highly conserved stretch while the light blue shading denotes moderate conservation of residues across different species.

3.6. Results of Analysis of Whole Genome SNP Array and Candidate Gene Sequencing Revealing Recurrent Mutations

The homozygous intervals in the affected members of the families obtained from Illumina HumanOmniExpress whole genome single nucleotide polymorphism (SNP) microarray followed by analysis with Homozygosity Mapper (http://www.homozygositymapper.org/) revealed recurrent variants as well (Table 3.4). Two families DFR22 (Figure 3.25 (i)) and 19DFS (Figure 3.26 (i)) revealed 9.23 and 11.96 Mb homozygous regions on chromosome 9, respectively. The gene *TMC1* located in these homozygous regions was sequenced and recurrent mutations were identified in the respective families (c.1114G>A; p.Val372Met (Santos *et al.*, 2005) and c.100C>T; p.Arg34* (Kitajiri *et al.*, 2007). The families DFR20 (Figure 3.27 (i)) and DFR37 (Figure 3.28 (i)) revealed homozygous regions of 26.06 and 37.85 Mb, respectively. The identified mutations in these families in the homozygous region containing *HGF* have been already reported as deletions (c.482+1991_2000delGATGATGAAA and c.482+1986_1988delTGA (Schultz *et al.*, 2009). The family DFR39 (Figure 3.29 (i)) revealed a 29.83 Mb homozygous region harboring the candidate gene *SLC26A4*. Sequencing identified the mutation (c.1337A>G; p.Gln446Arg (Reardon *et al.*, 2000). Another recurrent variation was encountered after sequencing of the candidate gene lying in the 26.52 Mb region in the family 26DF (Figure 3.30 (i)). Sequence analysis of the candidate *TMIE* gene identified a non-synonymous recurrent missense mutation c.241C>T (p.Arg81Cys) (Naz *et al.*, 2002).

3.7. Results of Exome Analysis

The families with undetermined pathogenic variants were sent for Exome sequencing, these two families were DFR4 and DFR5.

3.7.1. DFR4

DFR4 was a family with a highly inbred left loop (DFR4 a) and an outbred right loop (DFR4 b). The family DFR4 had (Figure 3.31 (i)) a total of 17 sampled individuals out of which, five were affected and 12 were un-affected individuals. The audiometries of the affected individuals (V:4-6, V:7, V:13) in comparison to the normal individuals demonstrated a profound hearing loss. Genotyping of the

7DF (M: *BSND*, c.97G>C; p.Val33Leu)

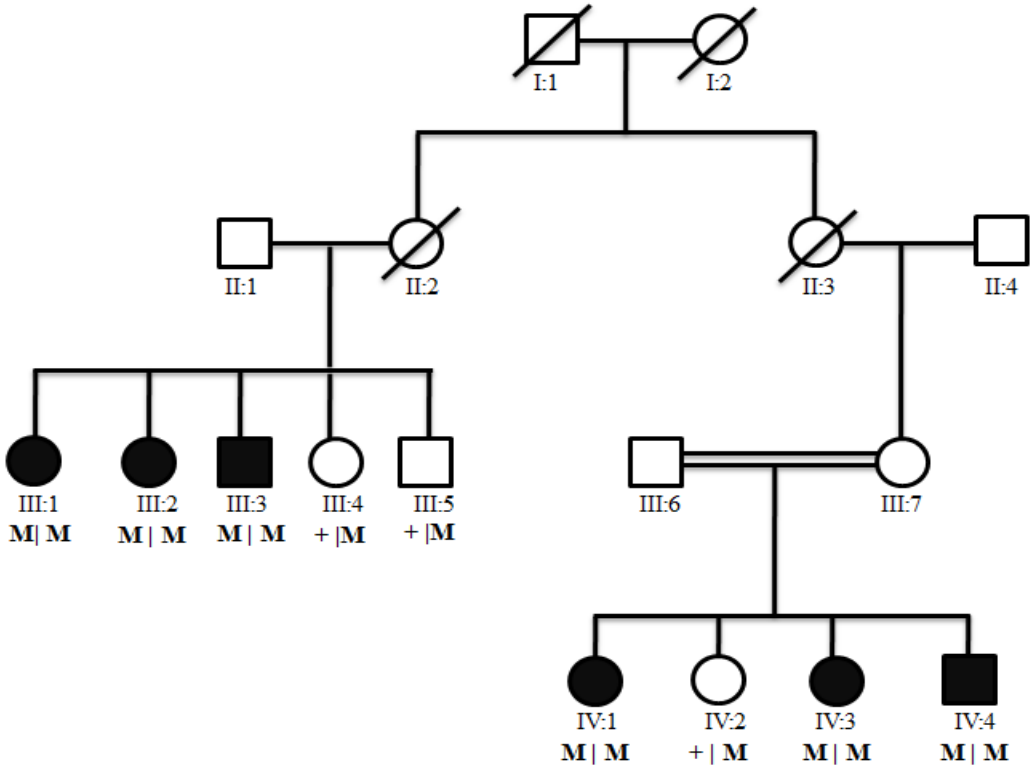

Figure 3.24 (i). Pedigree of family 7DF. For a description of the symbols see legend of Figure 3.1 (i).

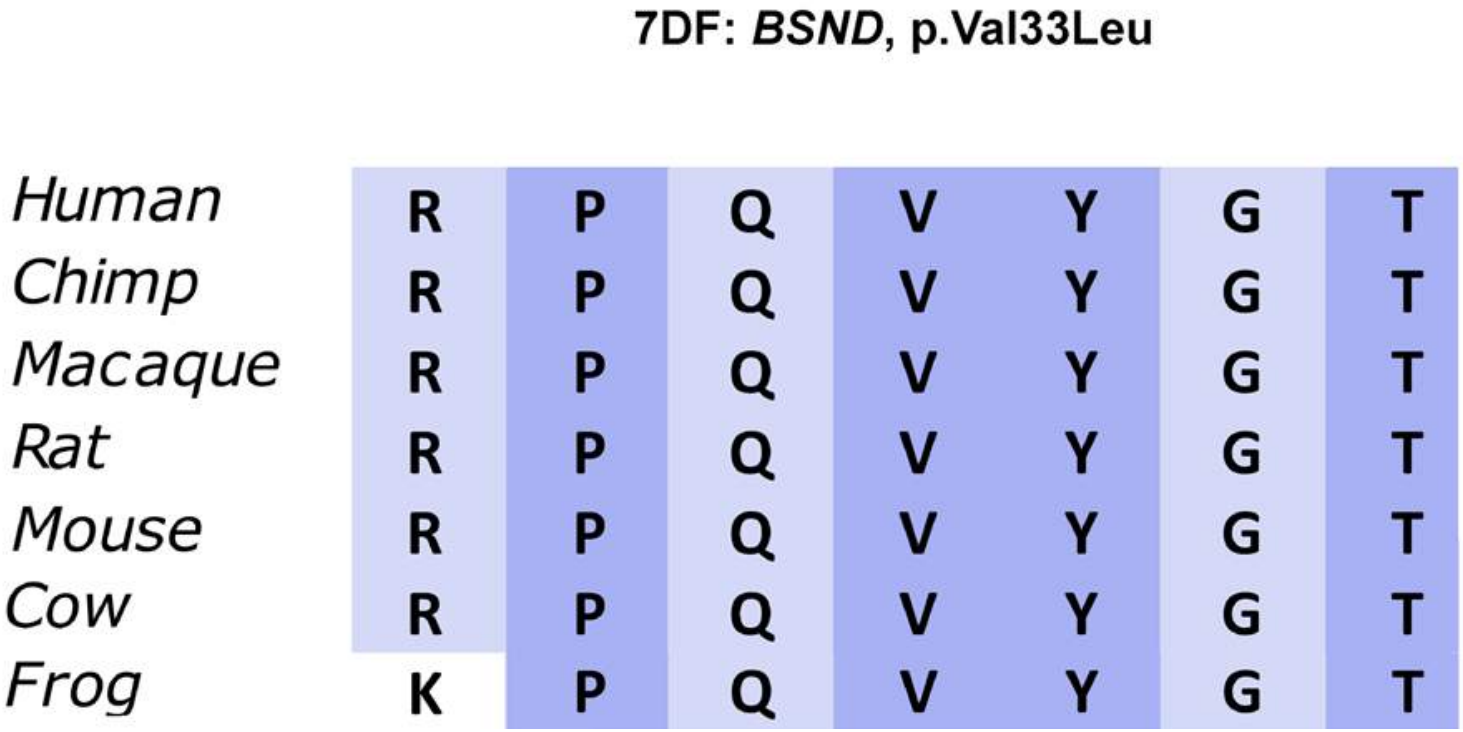

Figure 3.24 (ii). Amino acid sequence conservation of p.Val33 across 7 species. Multiple-alignment of the corresponding stretches of protein sequences across different species. The blue color shading represents the intensity of conservation, where the dark blue shading represents highly conserved stretch while the light blue shading denotes moderate conservation of residues across different species.

Table 3.4. Recurrent mutations in known autosomal recessive non-syndromic hearing loss genes in 6 Pakistani families

Family ID	Size of Homozygous Region (Mb)	Chr.	Flanking SNPs	Chr. Position (In hg19)	Candidate Gene	Mutation (Predicted protein change)	Frequency in EVS	References
DFR22	9.23	9	rs4275319; rs2295861	68,513,625-77,745,424	*TMC1* (NM_138691.2)	Ex-15: c.1114G>A (p.Val372Met)	AA=0/ AG=2/ GG=6501	Santos *et al.*, 2005
19DFS	11.96	9	rs10867845; rs10867778	72252269-84222230	*TMC1* (NM_138691.2)	Ex-7: c.100C>T (p.Arg34*)	Absent	Kitajiri *et al.*, 2007
DFR20	26.06	7	rs10485886; rs2073791	78,057,840-104,122,989	*HGF* (NM_000601)	In-4: c.482+1991_2000delGATGATGAAA (p.?)	Absent	Schultz *et al.*, 2009
DFR37	37.85	7	rs13234819; rs104869	44,342,969-82,197,469	*HGF* (NM_000601)	In-4: c.482+1986_1988delTGA (p.?)	Absent	Schultz *et al.*, 2009
DFR39	29.83	7	rs2285507; rs10253693	92,989,228-122,825,956	*SLC26A4* (NM_000441.1)	Ex-11: c.1337A>G (p.Gln446Arg)	Absent	Reardon *et al.*, 2000
26DF	26.52	3	rs304838; rs536036	30806674-57326883	*TMIE* (NM_147196)	Ex-3: c.241C>T (p.Arg81Cys)	Absent	Naz *et al.*, 2002

Acc. No., accession number of reference sequence; Chr, chromosome; Ex, exon; EVS, exome variant server; In, intron; SNPs, single nucleotide polymorphisms

DFR22 (M: *TMC1*, c.1114G>A; p.Val372Met)

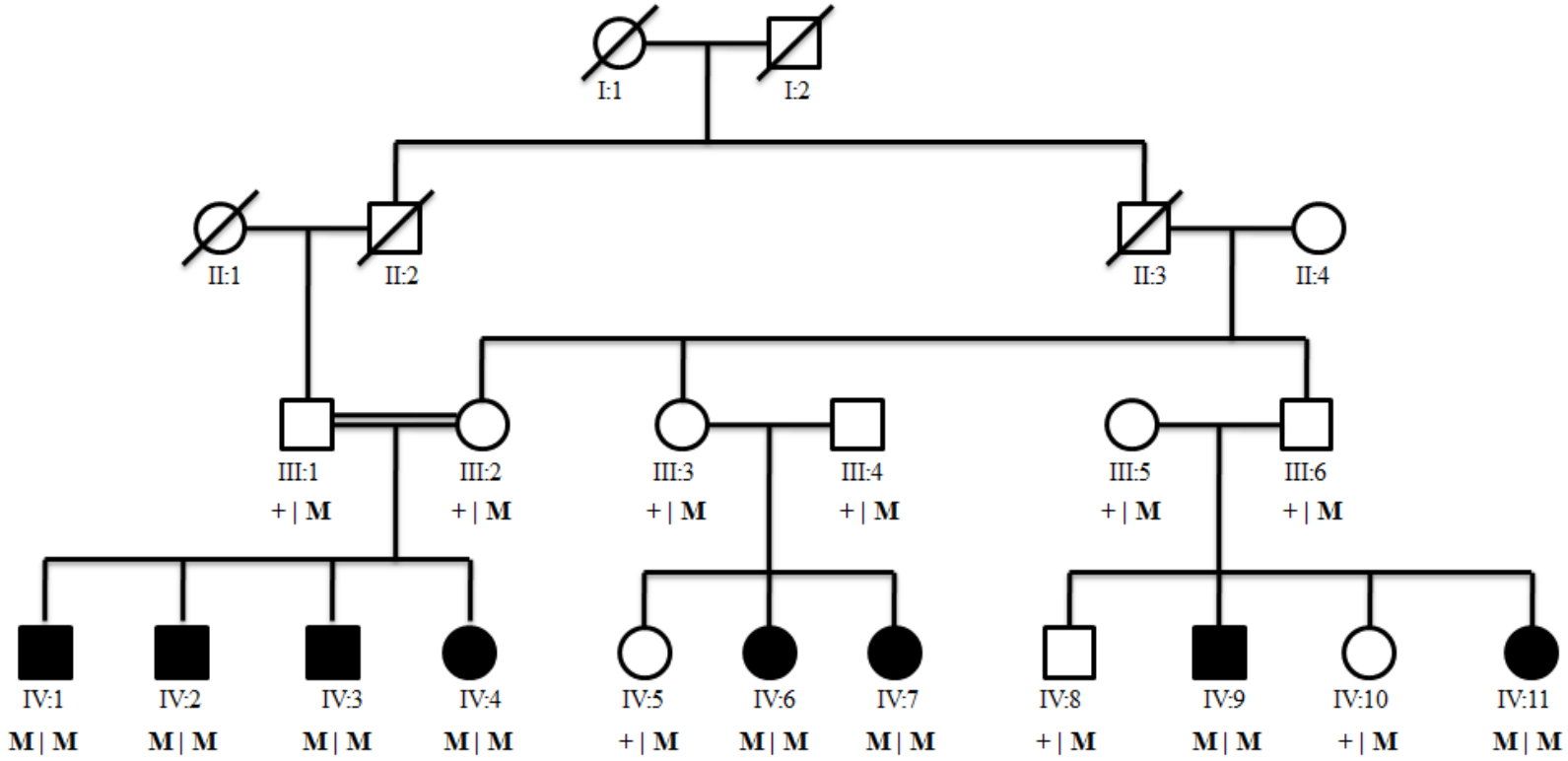

Figure 3.25 (i). Pedigree of family DFR22. For a description of the symbols see legend of Figure 3.1 (i).

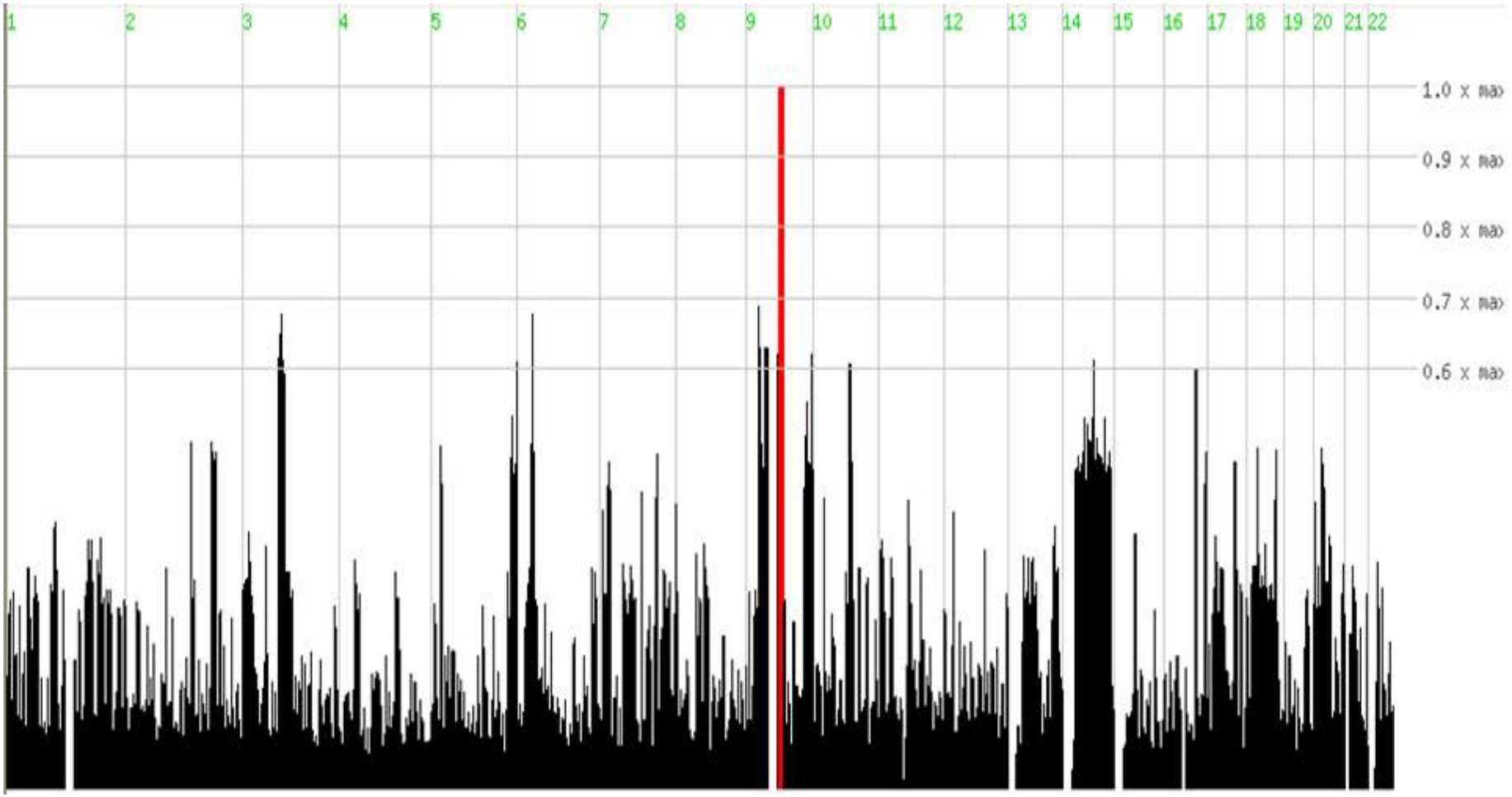

Figure 3.25 (ii). The Homozygosity Mapper plot showing homozygous region in red color in family DFR22.

19DFS (M: *TMC1*, c.100C>T; p.Arg34*)

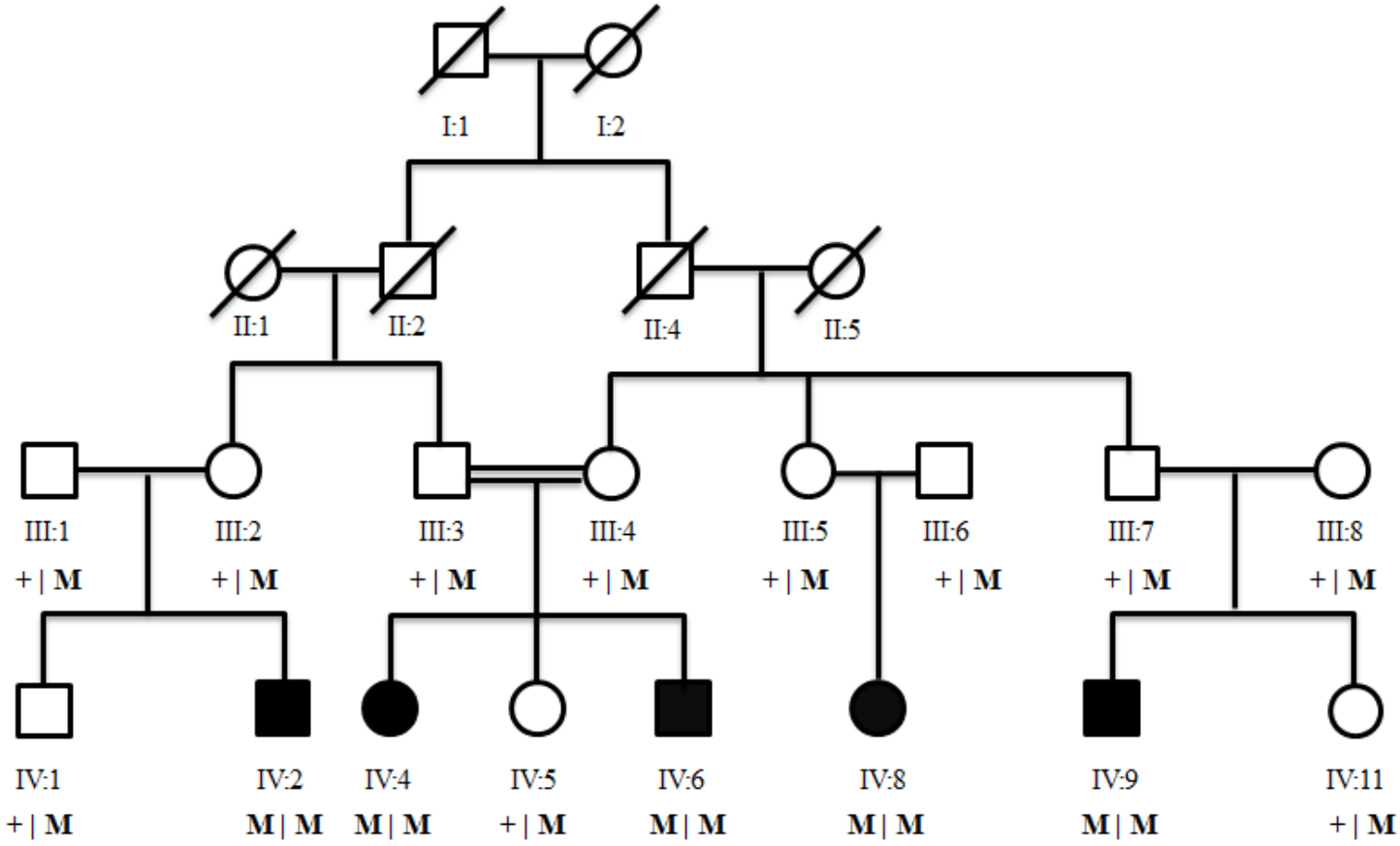

Figure 3.26. Pedigree of family 19DFS. For a description of the symbols see legend of Figure 3.1 (i).

DFR20 (M: *HGF*, c.482+1991_2000delGATGATGAAA; p.?)

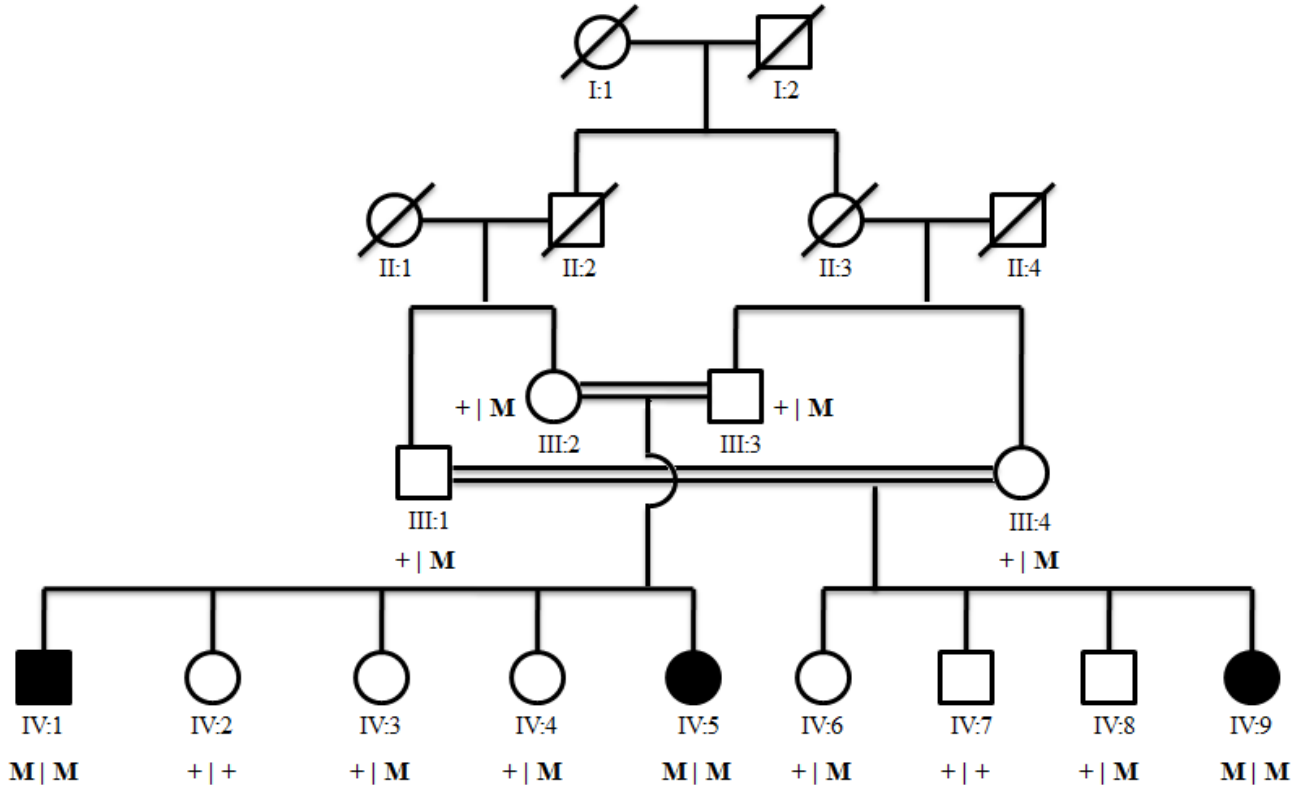

Figure 3.27 (i). Pedigree of family DFR20. For a description of the symbols see legend of Figure 3.1 (i).

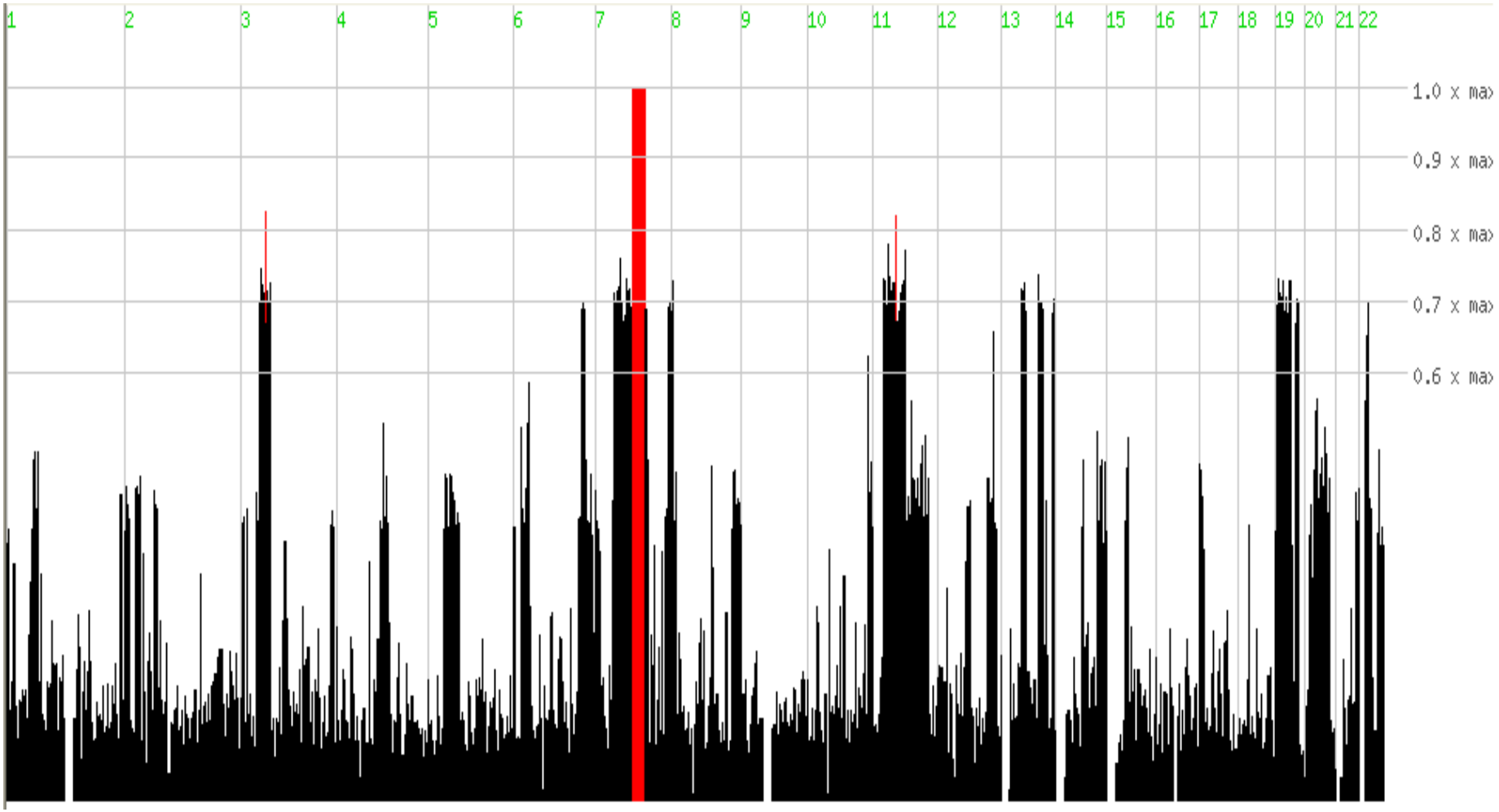

Figure 3.27 (ii). The Homozygosity Mapper plot showing homozygous region in red color in family DFR20.

DFR37 (M: *HGF*, c.482+1986_1988delTGA; p.?)

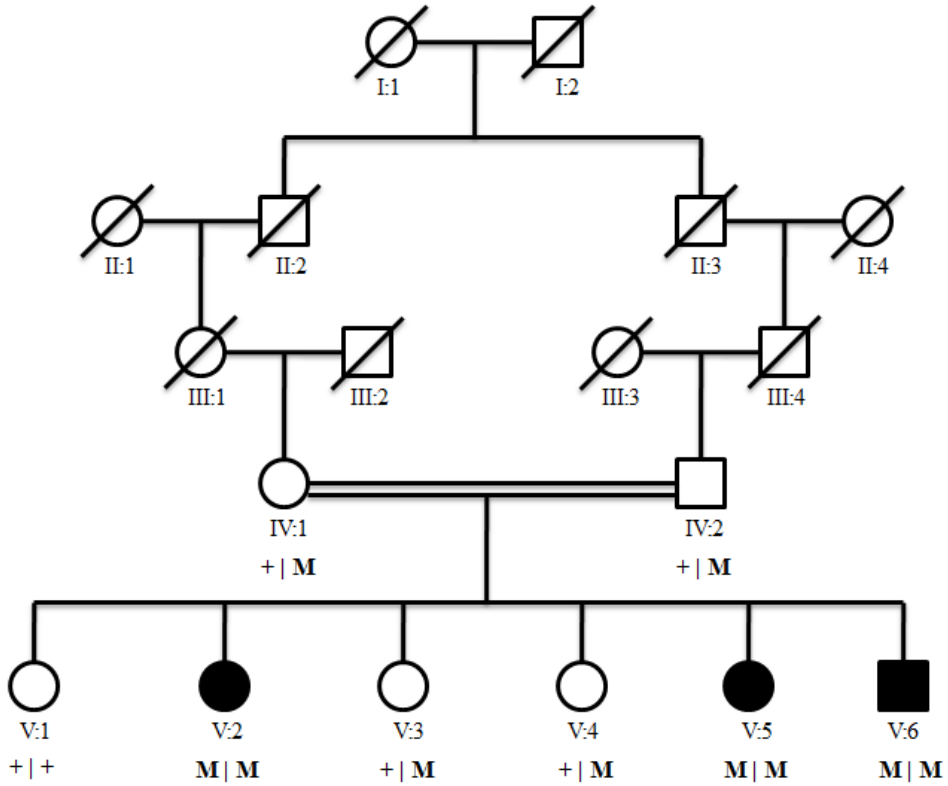

Figure 3.28 (i). Pedigree of family DFR37. For a description of the symbols see legend of Figure 3.1 (i).

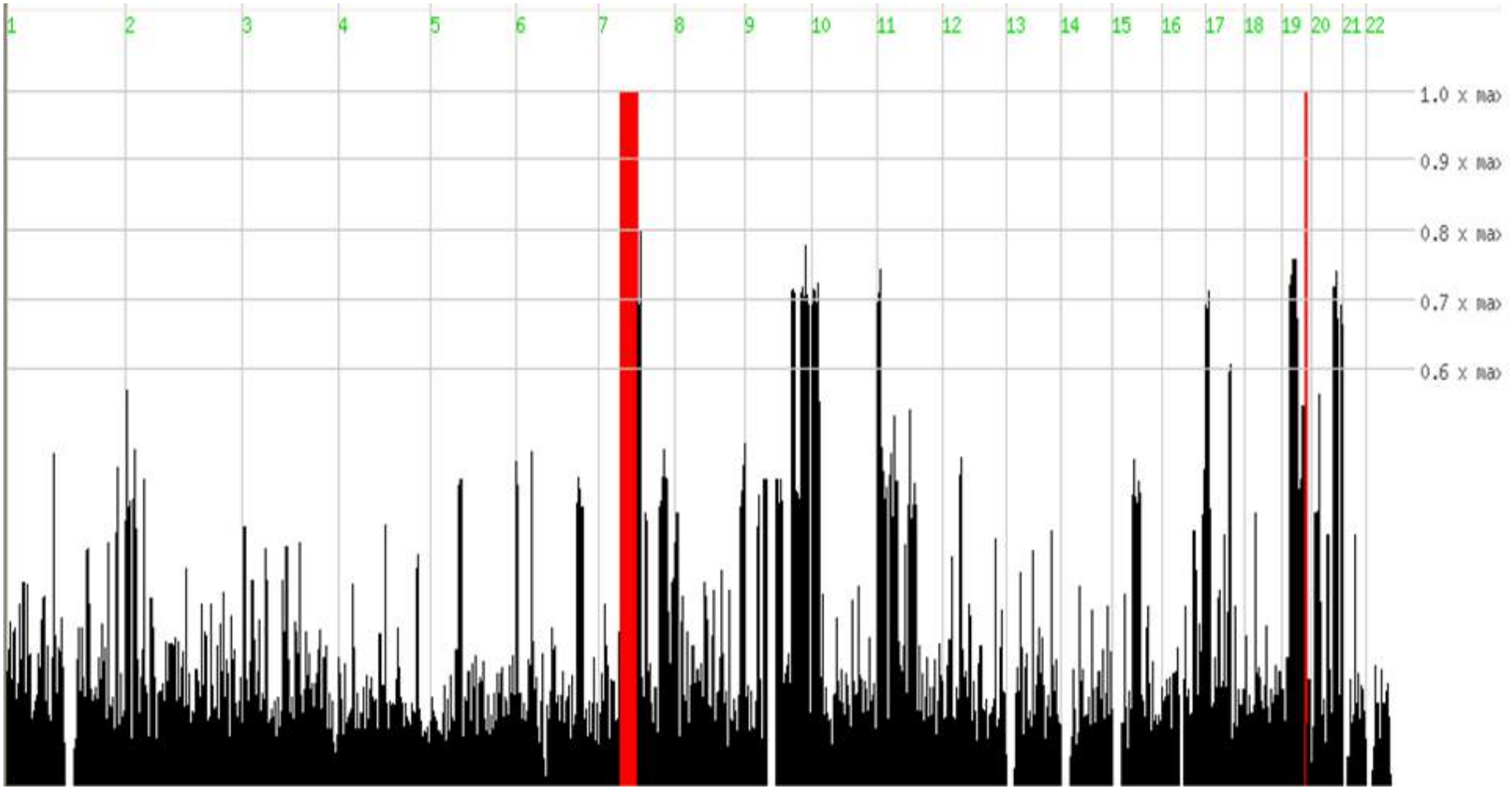

Figure 3.28 (ii). The Homozygosity Mapper plot showing homozygous region in red color in family DFR37.

DFR39 (M: *SLC26A4*, c.1337A>G; p.Gln446Arg)

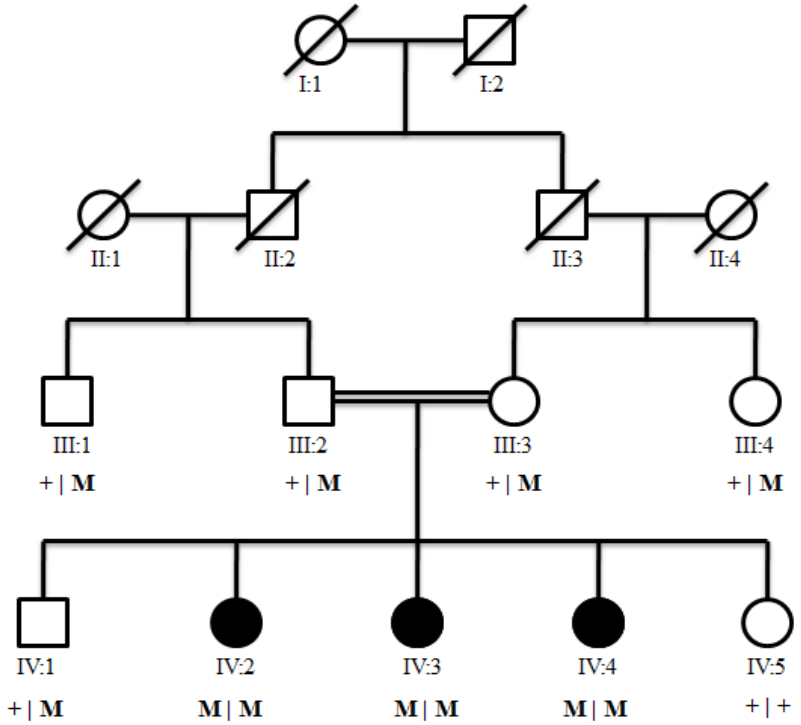

Figure 3.29. Pedigree of family DFR39. For a description of the symbols see legend of Figure 3.1 (i).

26DF (M: *TMIE*, c.241C>T; p.Arg81Cys)

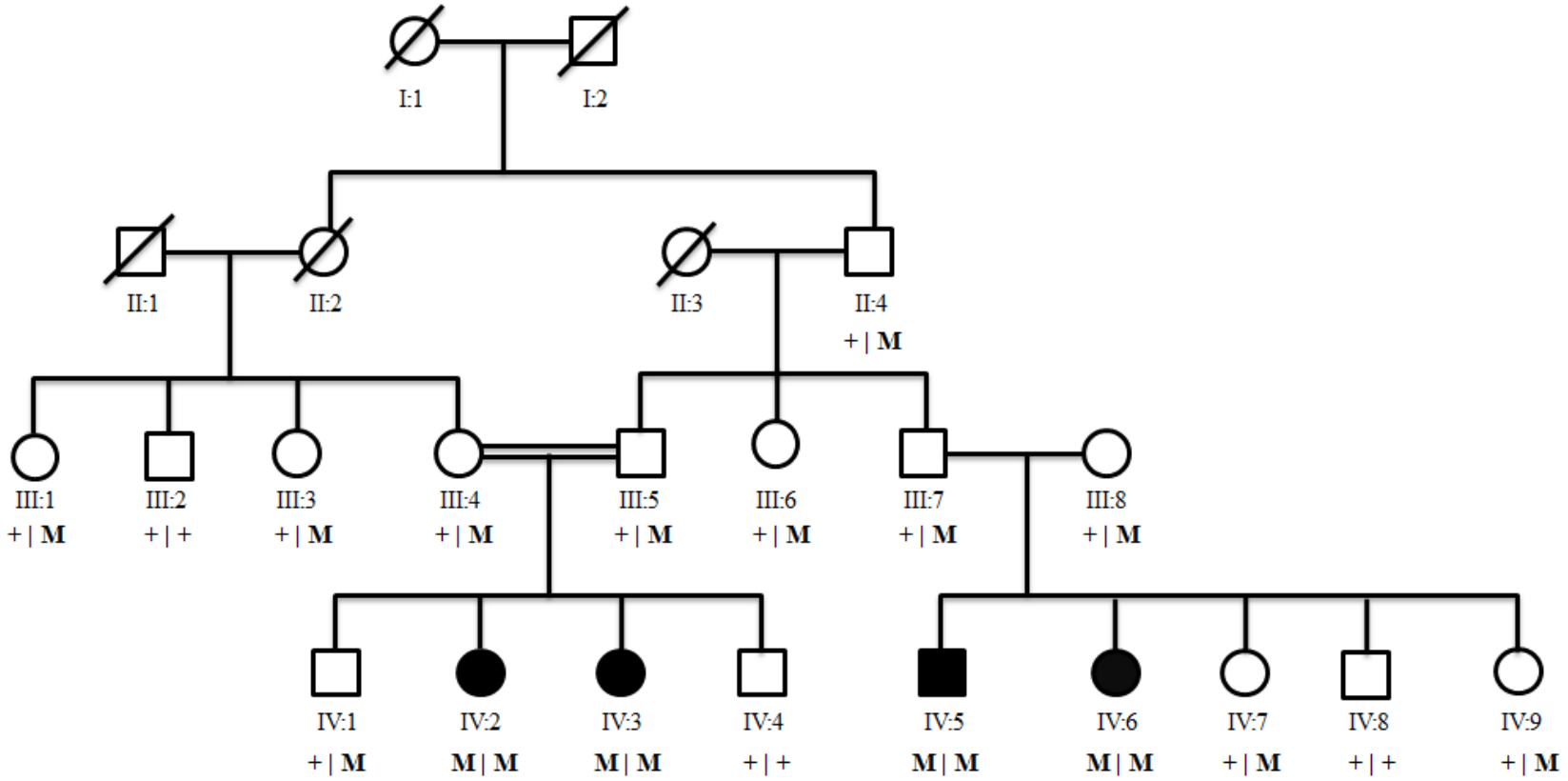

Figure 3.30. Pedigree of family 26DF. For a description of the symbols see legend of Figure 3.1 (i).

individuals (V:4-6) using Illumina HumanOmniExpress whole genome single nucleotide polymorphism (SNP) microarray analysis revealed multiple homozygous regions on chromosome 5. After region refinement through microsatellite markers analysis the homozygosity was again observed only in the inbred left loop (DFR4 a) while the outbred right loop (DFR4 b) did not show homozygosity of the respective regions. There was no gene associated with deafness in these regions and due to the large number of genes in these regions, individuals of the family were sent for Exome sequencing to identify the causative variants. The Exome data were filtered and the potential variants were selected on the basis of their relevance in causing hearing impairment. The phyloP >2.0 and Grantham distance >80 were selected, which resulted in narrowing down the variants to only five homozygous variants. The segregation in the family was validated using Sanger sequencing. Among the five variants, three were found to segregate in the left loop. The three variants were as follows: *TSPEAR* (c.193G>A; p.Ala65Thr), *CCNI2* (c.633+1G>C) and *PCDHGA10* (c.32C>G; p.Ser11*).

3.7.2. DFR5

The family DFR5 had a highly inbred right loop (DFR5 d) while the remaining loops (DFR5 a, b and c) were outbred. In the family DFR5, (Figure 3.32 (i)) a total of 12 individuals were sampled. Out of these individuals, five were affected and seven were un-affected individuals. The audiometries of the affected individuals (IV:1, IV:5, IV:8, IV:9, IV:12) in comparison with the normal individuals demonstrated profound hearing loss. Multiple regions on chromosomes 5 and 10 were identified when genotyping the individuals (IV:1, IV:5, IV:8, IV:9, IV:12 and IV:15) was performed using Illumina HumanOmniExpress whole genome single nucleotide polymorphism (SNP) microarray analysis. These regions had no known deafness gene and as there were a large number of genes in these regions therefore the samples of the individuals were sent for Exome sequencing. The Exome data were filtered and the potential variants were selected on the basis of their relevance in causing hearing impairment. The phyloP score of greater than 2 and Grantham distance of greater than 80 were selected, which resulted in narrowing down the variants to only five homozygous variants. The segregation was validated using Sanger sequencing, which identified only one of the variant to segregate with the disease in the right loop (DFR5 d), this

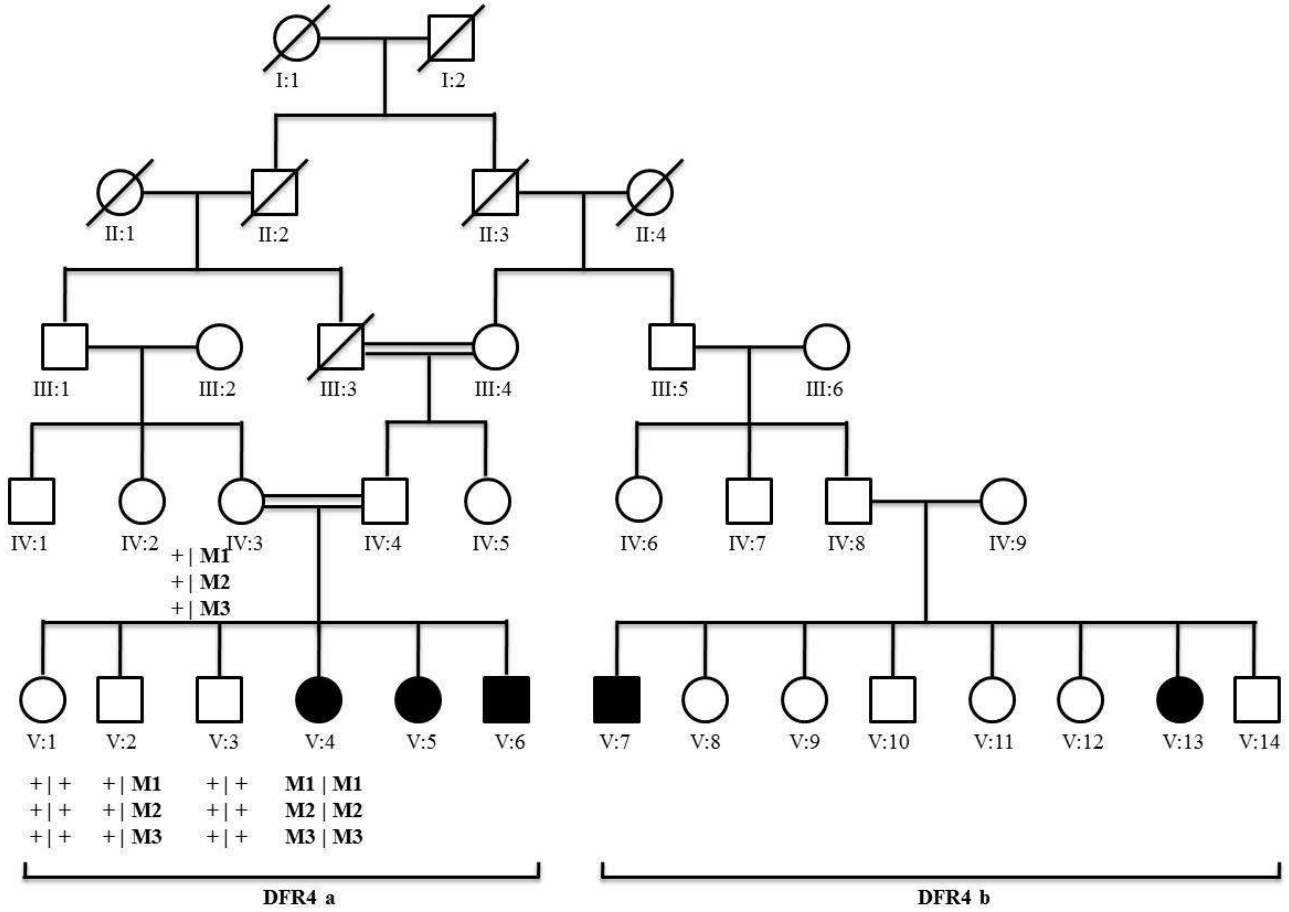

Figure 3.31. Pedigree of family DFR4. For a description of the symbols see legend of Figure 3.1 (i).

DFR5 (M: *ATP10B*, c.2910C>A; p.Asp970Glu)

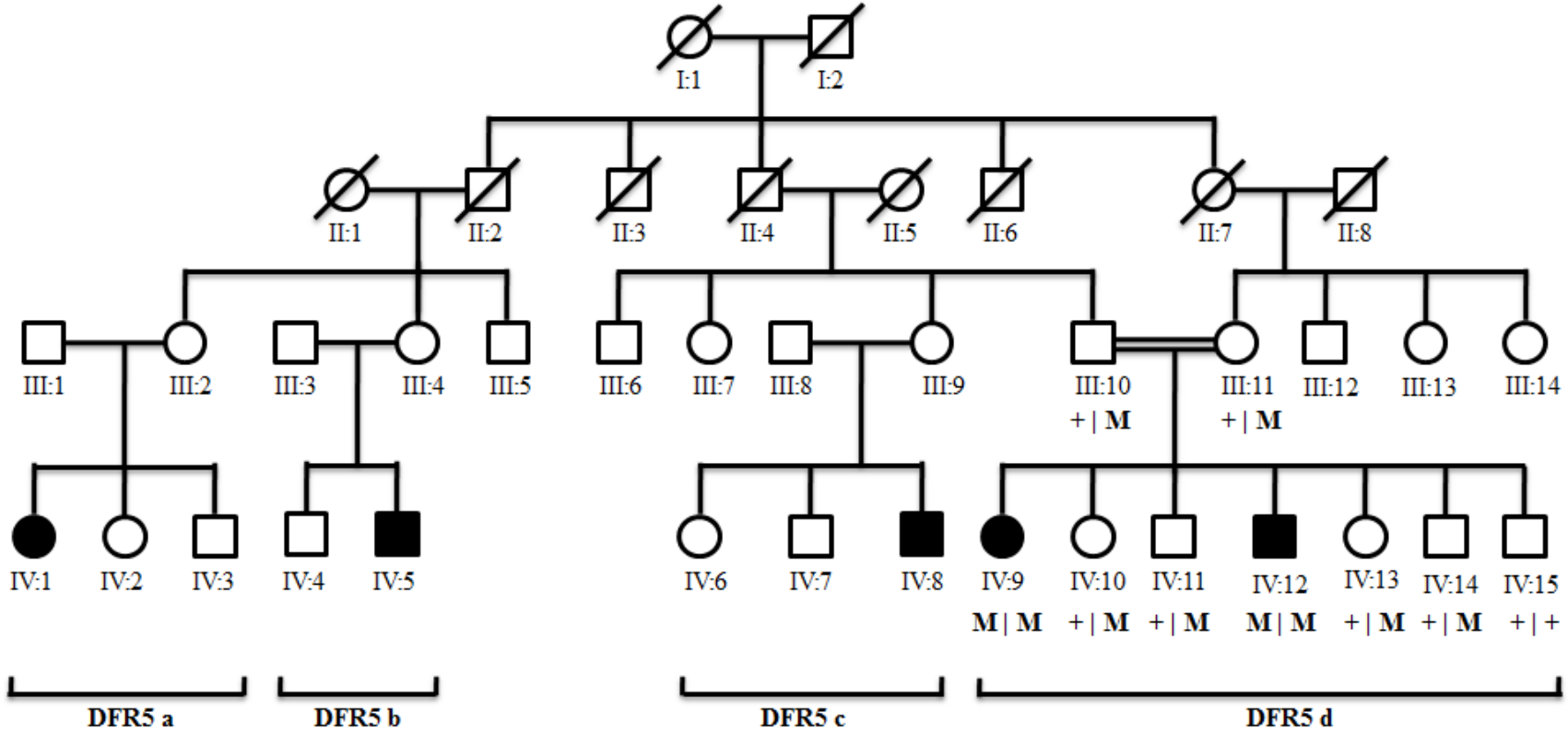

Figure 3.32. Pedigree of family DFR5. For a description of the symbols see legend of Figure 3.1 (i).

was in *ATP10B* (c.2910C>A; p.Asp970Glu).

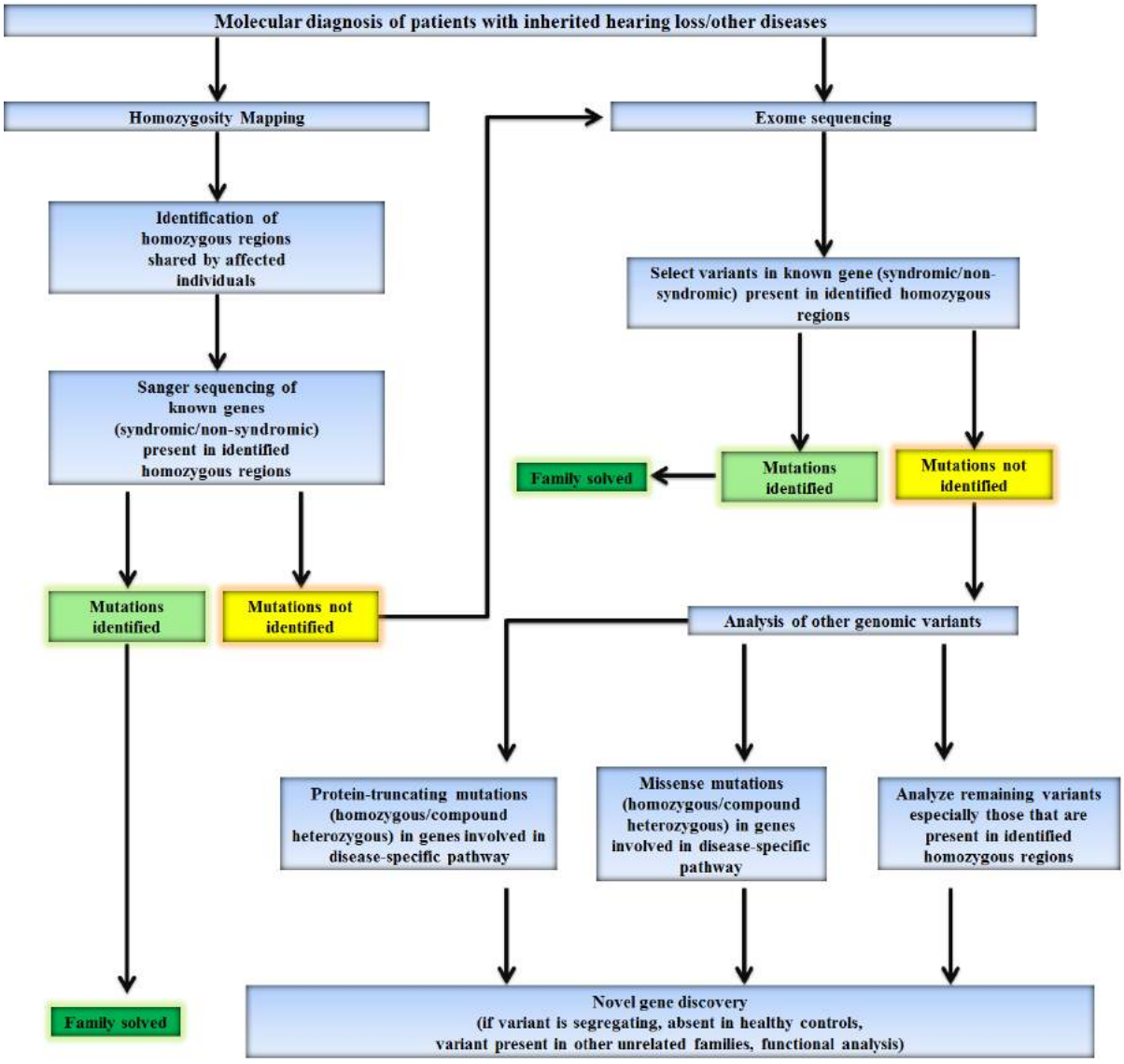

Figure 3.33. A Flow chart representation of the proposed steps in the identification of genetic causes of inherited hearing loss in Pakistani population.

Chapter 3

Discussion

4. Discussion

In a cohort of 32 autosomal recessive non-syndromic hearing loss (ARNSHL) families, genetic defects were identified in 30 families in 9 candidate deafness genes; *GJB2*, *MYO15A*, *TMC1*, *BSND*, *TMPRSS3*, *MSRB3*, *HGF*, *SLC26A4* and *TMIE*. In these genes, previously identified as well as novel mutations were detected, the latter were identified in *GJB2*, *MYO15A*, *TMC1*, *BSND*, *TMPRSS3* and *MSRB3*.

GJB2 was the most frequently mutated gene in these families as it was found to be the disease causative gene in 16 families. In *GJB2* the only identified novel mutation c.598G>A (p.Gly200Arg) affects a residue in the cysteine-rich domain that is involved in the formation of disulphide bonds (Minarik *et al.*, 2003). Although the effect of glycine on the disulphide bond formation cannot be predicted, the mutant residue arginine may affect the proper folding of the cysteine-rich domain because of the larger size of the arginine side chain.

In the current study *GJB2* mutations were found to be the most common, followed by *MYO15A* mutations. The overall frequency of the two most common nonsense variants c.71G>A (p.Trp24*) and c.231G>A (p.Trp77*) of *GJB2* in Pakistani deafness patients was calculated from the current panel of 32 families with 125 affected persons as well as previously reported studies of Santos *et al.* (2005) (430 deaf patients), Bukhari *et al.* (2013) (70 deaf persons) and Yoong *et al.* (2011) (65 deaf patients) (Table 4.1). Using these data, the frequency was found to be 6.1% for p.Trp24* and 4.3% for p.Trp77*. The variant p.Trp24* is also prevalent at a high frequency in the Indian population (Mani *et al.*, 2009), which is not unusual as both the countries shared a common history and close proximity (Qamar *et al.*, 2002). The third recurrent mutation p.Gly12Valfs*2 found in the current study has previously been reported from Northern areas of Pakistan (Bukhari *et al.*, 2013) (Table 4.1) and also in Caucasian and Turks (Snoeckx *et al.*, 2005). Of the recurrent mutations two families had the *GJB2* variant c.380G>A (p.Arg127His), which was found to segregate with hearing loss and was present homozygously in all the 4 affected members. Other studies have also reported deafness patients carrying this mutation in a homozygous state (Bukhari *et al.*, 2013; Minarik *et al.*, 2003; Padma *et al.*, 2009).

Based on the higher than normal carrier frequency in patients as well as controls,

Table 4.1. Mutations identified in known autosomal recessive non-syndromic hearing loss genes within the Pakistani population

Gene	RefSeq ID	Nucleotide Variant	Protein Variant	Phenotype	No. of Families	No. of Patients	References
GJB2	NM_004004.5	c.380G>A	p.Arg127His	ARNSHL	19 2	38 4	Santos *et al.*, 2005, Current Study
GJB2	NM_004004.5	c.71G>A	p.Trp24*	ARNSHL	4 1 2 5	5 1 4 26	Yoong *et al.*, 2011, Bukhari *et al.*, 2013, Santos *et al.*, 2005, Current Study
GJB2	NM_004004.5	c.457G>A	p.Val153Ile	ARNSHL	13	36	Santos *et al.*, 2005
GJB2	NM_004004.5	c.231G>A	p.Trp77*	ARNSHL	5 4	10 14	Santos *et al.*, 2005, Current Study
GJB2	NM_004004.5	c.35delG	p.G12Vfs*1	ARNSHL	1 2	1 8	Bukhari *et al.*, 2013, Current Study
GJB2	NM_004004.5	c.269T>C	p.Leu90Pro	ARNSHL	2	4	Santos *et al.*, 2005
GJB2	NM_004004.5	c.493C>T	p.Arg165Trp	ARNSHL	2	4	Santos *et al.*, 2005
GJB2	NM_004004.5	c.35delG c.439G>A	p.Gly12Valfs*2 p.Glu147Lys	ARNSHL	1	4	Current Study
GJB2	NM_004004.5	c.377_378insATGCGGA	p.Arg127Cysfs*85	ARNSHL	1	2	Current Study
GJB2	NM_004004.5	c.167delT	p.Leu56Argfs*26	ARNSHL	1	2	Santos *et al.*, 2005
GJB2	NM_004004.5	c.95G>A	p.Arg32His	ARNSHL	1	2	Santos *et al.*, 2005
GJB2	NM_004004.5	c.358-360delGAG	p.Glu120del	ARNSHL	1	2	Santos *et al.*, 2005
GJB2	NM_004004.5	c.79G>A	p.Val27Ile	ARNSHL	1	2	Santos *et al.*, 2005

Table 4.1. cont.

Gene	RefSeq ID	Nucleotide Variant	Protein Variant	Phenotype	No. of Families	No. of Patients	References
GJB2	NM_004004.5	c.341A>G	p.Glu114Gly	ARNSHL	1	2	Santos *et al.*, 2005
GJB2	NM_004004.5	c.598G>A	p.Gly200Arg	ARNSHL	1	2	Current Study
GJB2	NM_004004.5	c.104T>G	p.Ile35Ser	ARNSHL	1	1	Bukhari *et al.*, 2013
SLC26A4	NM_000441.1	c.269C>T	p.Ser90Leu	ARNSHL	5 5	15 15	Anwar *et al.*, 2009, Park *et al.*, 2003
SLC26A4	NM_000441.1	c.716 T>A	p.Val239Asp	ARNSHL	16 1 2	3 3 8	Anwar *et al.*, 2009, Park *et al.*, 2003, Khan *et al.*, 2013
SLC26A4	NM_000441.1	c.170C>A	p.Ser57*	ARNSHL	1 1	3 3	Anwar *et al.*, 2009, Khan *et al.*, 2013
SLC26A4	NM_000441.1	c.1363A>T	p.Ile455Phe	ARNSHL	1 2	3 6	Anwar *et al.*, 2009, Park *et al.*, 2003
SLC26A4	NM_000441.1	c.1337A>G	p.Gln446Arg	ARNSHL	10	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.71G>T	p.Arg24Leu	ARNSHL	2	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.1667A>G	p.Tyr556Cys	ARNSHL	2	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.2145G>T	p.Lys715Asn	ARNSHL	2	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.304+2T>C	p.Met103Lysfs*4a	ARNSHL	1	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.416G>T	p.Gly139Val	ARNSHL	1	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.694G>A	p.Val231Met	ARNSHL	1	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.164+1027del24368ins7	p.?	ARNSHL	1	3	Anwar *et al.*, 2009

Table 4.1. cont.

Gene	RefSeq ID	Nucleotide Variant	Protein Variant	Phenotype	No. of Families	No. of Patients	References
SLC26A4	NM_000441.1	c.1115C>T	p.Ala372Val	ARNSHL	1	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.1341+3A>C	p.Gly439Valfs*19a	ARNSHL	1	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.1692_1693insA	p.Cys565Metfs*8	ARNSHL	1	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	c.1264-477_2090-4927del11202	p.?	ARNSHL	1	3	Anwar *et al.*, 2009
SLC26A4	NM_000441.1	IVS8+4A>G	p.?	ARNSHL	1	3	Park *et al.*, 2003
SLC26A4	NM_000441.1	1863delT	p.?	ARNSHL	1	3	Park *et al.*, 2003
MYO15A	NM_016239.3	c.8158G4C	p.Asp2720His	ARNSHL	4	16	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.3685C>T	p.Gln1229*	ARNSHL	1	8	Liburd *et al.*, 2001
MYO15A	NM_016239.3	c.8486G >A	p.Gln2716His	ARNSHL	1	6	Liburd *et al.*, 2001
MYO15A	NM_016239.3	c.8821_8822insTG	p.Val2940fs*3034	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.9478C4T	p.Leu3160Phe	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.10474C4T	p.Gln3492*	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.3313G>T	p.Glu1105*	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.3334delG	p.Gly1112fs*1124	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.386611G4A	p.Thr1253fs*1277	ARNSHL	1	4	Nal *et al.*, 2007

Table 4.1. cont.

Gene	RefSeq ID	Nucleotide Variant	Protein Variant	Phenotype	No. of Families	No. of Patients	References
MYO15A	NM_016239.3	c.4176C4A	p.Tyr1392*	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.4669A4G	p.Lys1557Glu	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.5117_5118GC4TT	p.Gly1706Val	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.5189T4C	p.Leu1730Pro	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.6061C4T	p.Gln2021*	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.6731G4A	p.Gly2244Glu	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.6796G4A	p.Val2266Met	ARNSHL	1	4	Nal *et al.*, 2007
MYO15A	NM_016239.3	c.1185dupC	p.E396fs*431	ARNSHL	1	3	Bashir *et al.*, 2013
MYO15A	NM_016239.3	IVS4+1G>T	p.1232fs*1241	ARNSHL	1	4	Liburd *et al.*, 2001
MYO15A	NM_016239.3	c.8222T>C	p.Phe2741Ser	ARNSHL	1	5	Current Study
MYO15A	NM_016239.3	c.3866+1G>A	p.?	ARNSHL	1	7	Current Study
MYO15A	NM_016239.3	c.9948G>A	p.Gln3316Gln	ARNSHL	1	4	Current Study
MYO15A	NM_016239.3	c.8767C>T	p.Arg2923*	ARNSHL	1	2	Current Study
TMPRSS3	NM_024022.2	c.1219T>C	p.Cys407Arg	ARNSHL	5	10	Lee *et al.*, 2012
TMPRSS3	NM_024022.2	c.310G>A	p.Glu104Lys	ARNSHL	1	7	Lee *et al.*, 2012
TMPRSS3	NM_024022.2	c.310G>T	p.Glu104Stop	ARNSHL	1	6	Lee *et al.*, 2012
TMPRSS3	NM_024022.2	c.208delC	p.His70Thrfs*19	ARNSHL	1	6	Lee *et al.*, 2012
TMPRSS3	NM_024022.2	c.767C>T	p.Ala256Val	ARNSHL	1	5	Lee *et al.*, 2012
TMPRSS3	NM_024022.2	c.1273T>C	p.Cys425Arg	ARNSHL	1	4	Lee *et al.*, 2012

Table 4.1. cont.

Gene	RefSeq ID	Nucleotide Variant	Protein Variant	Phenotype	No. of Families	No. of Patients	References
TMPRSS3	NM_024022.2	IVS 4-6 G>A	p.?	ARNSHL	1	4	Scott *et al.*, 2001
TMPRSS3	NM_024022.2	c.157G>A	p.Val53Ile	ARNSHL	1	4	Scott *et al.*, 2001
TMPRSS3	NM_024022.2	c.726C>G	p.Cys242Trp	ARNSHL	1	5	Current Study
TMC1	NM_138691.2	c.1114G>A	p.Val372Met	ARNSHL	1 1	2 8	Santos *et al.*, 2005, Current Study
TMC1	NM_138691.2	c.100C>T	p.Arg34*	ARNSHL	Case-control 1	1 5	Ben Said *et al.*, 2010, Current Study
TMC1	NM_138691.2	c.2004T>G	p.Ser668Arg	ARNSHL	2	4	Santos *et al.*, 2005
TMC1	NM_138691.2	c.830A>G	p.Thr277Cys	ARNSHL	1	2	Santos *et al.*, 2005
TMC1	NM_138691.2	c.1334G>A	p.Arg445His	ARNSHL	1	2	Santos *et al.*, 2005
TMC1	NM_138691.2	c.2035G>A	p.Glu679Lys	ARNSHL	1	2	Santos *et al.*, 2005
TMC1	NM_138691.2	c.536-8T>A		ARNSHL	1	2	Santos *et al.*, 2005
TMC1	NM_138691.2	c.362+18A>G	p.Glu122Tyrfs*10	ARNSHL	1	3	Current Study
TMIE	NM_147196	c.241C>T	p.Arg81Cys	ARNSHL	2 1	4 3	Santos *et al.*, 2006, Naz *et al.*, 2002
TMIE	NM_147196	c.92A>G	p.Glu31Gly	ARNSHL	1	2	Santos *et al.*, 2006
TMIE	NM_147196	c.250C>T	p.Arg84Trp	ARNSHL	1	3	Naz *et al.*, 2002
TMIE	NM_147196	IVS1-2_98delAGCCCAGinsC	p.?	ARNSHL	1	3	Naz *et al.*, 2002

Table 4.1. cont.

Gene	RefSeq ID	Nucleotide Variant	Protein Variant	Phenotype	No. of Families	No. of Patients	References
TMIE	NM_147196	c.274C>T	p. Arg92Trp	ARNSHL	1	3	Naz *et al.*, 2002
TMIE	NM_147196	c.241C>T	p.Arg81Cys	ARNSHL	1	4	Current Study
MSRB3	NM_001031679.2	c.265T>G	p.Cys89Gly	ARNSHL	6	12	Ahmed *et al.*, 2011
MSRB3	NM_001031679.2	c.55T>C	p.Arg19*	ARNSHL	2	4	Ahmed *et al.*, 2011
MSRB3	NM_001031679.2	c.20T>G	p.Leu7Arg	ARNSHL	1	7	Current Study
HGF	NM_000601	c.482+1986_1988delTGA	P.?	ARNSHL	36 1	36 3	Schultz *et al.*, 2009, Current Study
HGF	NM_000601	c.482+1991_2000delGATGATGAAA	P.?	ARNSHL	1 1	5 3	Schultz *et al.*, 2009, Current Study
HGF	NM_000601	c.495G>A	p.Ser165Ser	ARNSHL	1	3	Schultz *et al.*, 2009
BSND	NM_057176.2	c.35T>C	p.Ile12Thr	ARNSHL	1 4	3 25	Iqbal *et al.*, 2011, Riazuddin *et al.*, 2009
BSND	NM_057176.2	c.97G>C	p.Val33Leu	ARNSHL	1	6	Current Study

Padma *et al.* (2009) suggested that it is unlikely that this variant has a pathogenic role in deafness. However, Matos *et al.* (2010) have proposed that this mutation is likely to be pathogenic in tandem with other unknown genetic or environmental factors. Identification of 4 patients with homozygous mutation p.Arg127His out of 125 in the current study and previously reported 9 heterozygous cases and 1 compound heterozygous case in 70 deaf patients by Bukhari *et al.* (2013) and 18 heterozygous cases and 1 homozygous occurrence in a panel of 19 families by Santos *et al.* (2005) (Table 4.1), demonstrates that this is the most frequently occurring mutation in Pakistani deafness families and therefore becomes important in carrier screening and genetic counseling of Pakistani deaf patients. Among the families with *GJB2* mutations, 35% (60/173) individuals were found to carry the mutation in a heterozygous state while the same number of affected individuals carried these mutations in a homozygous state. Therefore, as a preliminary screening of deafness families the sequencing of this gene is suggested in Pakistan.

In the current study *MYO15A* was found to be the second leading cause of deafness in the Pakistani population, but still no recurrent mutation was identified in this gene and all identified mutations were novel. A total of four nonsense mutations were identified, two in *GJB2* (Table 3.2), one in *MYO15A* (Table 3.3) and one in *TMC1* (Table 3.4); while three frameshift mutations were identified, two in *GJB2* (Table 3.2) and one in *TMC1* (Table 3.3), these nonsense and frameshift mutations are likely to cause nonsense mediated decay (NMD) because they are either present in the middle or near the 5'end of the genes.

Of the two splice site, a nonsense and a missense mutation in *MYO15A* (Table 3.3), the c.9948G>A (p.Gln3316Gln) mutation affects the last nucleotide of exon 61 and changes the consensus splice site sequence. Based on the results of a minigene assay, performed in the current study, the c.9948G>A mutation is expected to lead to skipping of exon 61, which would result in frameshift and NMD can be expected to occur for the transcribed mRNA. The second splice site mutation c.3866+1G>A is a canonical splice site change and is predicted to remove the splice donor site of exon 5. An alternative splice site is predicted at position +99 in intron 5, if this alternative site is used it would lead to a frameshift and a premature stop codon (encoded by nucleotide 2-4 of intron 5). Both splice site mutations are thus predicted to lead to

frameshift and NMD could occur for mRNA transcripts. The two other novel mutations, c.8767C>T (p.Arg2923*) and c.8222T>C (p.Phe2741Ser), found in two different families, are located in the region of the gene that encodes the tail region of *MYO15A* and are likely to cause a loss of function of this region. The p.Arg2923* mutation is present in the SH3 domain, which is involved in the myosin XVa and whirlin interactions. These two proteins are involved in the elongation of the stereocilia (Belyantseva *et al.*, 2005; Weng *et al.*, 1995). Most of the previously reported mutations of *MYO15A* causing congenital severe to profound deafness were found in the motor head and the tail domains (Belyantseva *et al.*, 2003; Hilgert *et al.*, 2009). Collectively these results indicate that the motor head and tail regions of *MYO15A* are essential in the hearing process and any mutation in these regions is thus critical (Anderson *et al.*, 2000).

In Pakistan previously a total of 18 different mutations including 8 missense (p.Gln2716His, p.Lys1557Glu, p.Gly1706Val, p.Leu1730Pro, p.Gly2244Glu, p.Val2266Met, p.Asp2720His, p.Lys3160Phe), 5 nonsense (p.Gln1229*, p.Tyr1392*, p.Gln2021*, p.Gln3492*, p.Glu1105*), 4 frameshift (p.Thr1253fs*1277, p.Gly1112fs*1124, p.Val2940fs*3034) and 1 splice site (IVS4+1G>T) (Table 4.1) have been found in the motor and FERM domains of MYO15A and are known to cause profound deafness (Liburd *et al.*, 2001; Nal *et al.*, 2007); while duplication of cytosine (c.1185dupC; p.Glu396fs*431) has been reported in exon 2 with less severe hearing loss (Bashir *et al.*, 2012).

In the current study, after the *GJB2* and *MYO15A* candidate gene analysis homozygosity mapping analyses was performed in the remaining 12 Pakistani families with autosomal recessive hearing loss. This allowed the identification of causative mutations in 10 families, of these six families had recurrent mutations *TMC1* (c.1114G>A, p.Val372Met; c.100C>T, p.Arg34*), *HGF* (c.482+1991_2000delGATGATGAAA; c.482+1986_1988delTGA), *SLC26A4* (c.1337A>G, p.Gln446Arg) and *TMIE* (c.241C>T, p.Arg81Cys) and four families had novel mutations *TMC1* (c.362+18A>G), *BSND* (c.97G>C, p.Val33Leu), *TMPRSS3* (c.726C>G, p.Cys242Trp) and *MSRB3* (c.20T>G, p.Leu7Arg).

TMC1 encoding a transmembrane protein is expressed in the neurosensory hair cells of the mouse cochlea (Kitajiri *et al.*, 2007). Genetic analysis of deafness panel in the

current study resulted in the identification of two recurrent (c.100C>T; p.Arg34* and c.1114G>A; p.Val372Met) (Table 4.1) and a novel mutation (c.362+18A>G). By combining the reported data of *TMC1* from Pakistan by Santos *et al.* (2005) and the current panel, p.Val372Met mutation is found to be the most frequent with 8 affected individuals in the family DFR22. Another frequent *TMC1* mutation reported from Pakistan is p.Arg34*, it leads to a truncated protein and thus probably causes congenital severe to profound hearing loss (Ben Said *et al.*, 2010; Hilgert *et al.*, 2008; Kitajiri *et al.*, 2007; Sirmaci *et al.*, 2009). Ben Said *et al.* (2010) have previously described p.Arg34* to be an old founder mutation found in several populations including the Pakistani population. It is likely that the family described here carries the mutation on the same founder haplotype. Also a novel intronic mutation in *TMC1*, c.362+18A>G was identified in the current study, which creates a novel splice donor site and the insertion of 17 nucleotides, as demonstrated by the minigene experiment.

Barttin CLCNK-type chloride channel accessory beta subunit (*BSND*) encodes the barttin protein, which is a vital subunit of the chloride and voltage-sensitive Ka (CLCNKA) and Kb (CLCNKB) channels in the inner ear and the kidney. Chloride channels and the barttin protein form heteromers, which function in the recycling of K^+ in the inner ear and salt reabsorption in the kidneys (Estevez *et al.*, 2001; Hayama *et al.*, 2003; Janssen *et al.*, 2009; Kramer *et al.*, 2008; Rickheit *et al.*, 2008; Waldegger *et al.*, 2002). As predicted by *in silico* analysis, the currently identified novel *BSND* mutation p.Val33Leu residing in the transmembranal region might result in the malfunctioning of chloride channels CLCNKA and CLCNKB, however, in the current family there was no renal involvement. Previously in this gene c.35T>C (p.Ile12Thr) mutation has been reported by Iqbal *et al.* (2011) and Riazuddin *et al.* (2009) (Table 4.1).

The *TMPRSS3* is co-expressed with ENaC within the organ of Corti, stria vascularis and spiral ganglions (Guipponi *et al.*, 2007; Guipponi *et al.*, 2008). Hearing loss caused by mutations in *TMPRSS3* is bilateral and severe to profound with no defects of the middle ear and the vestibular system (Fasquelle *et al.*, 2011). Weegerink *et al.* (2011) reported that mutations within the various domains of TMPRSS3 might affect the protease activity, which ultimately results in hearing loss with variable phenotypes. Molina *et al.* (2013) showed that TMPRSS3 is responsible for the

outflow of K^+ from the IHCs. Mutations in *TMPRSS3* have also been shown to cause progressive hearing loss with a postlingual onset (Weegerink *et al.*, 2011). The novel *TMPRSS3* mutation c.726C>G (p.Cys242Trp) in exon 8, identified in the current study, affects the serine protease domain as predicted by 3D modeling. Previously another mutation in the same exon of *TMPRSS3*, c. 647G>T (p.Arg216Leu) has been shown to result in a failure of the protein to undergo proteolytic cleavage resulting in the inactivation of the sodium channel (Elbracht *et al.*, 2007). Out of the total 16 *TMPRSS3* mutations known so far, the most frequently reported mutation in the Pakistani cohort is the c.1219T>C (p.Cys407Arg) (Table 4.1). This mutation occurs within the active site of the TMPRSS3 protein and is predicted to be deleterious by SIFT and probably damaging by PolyPhen-2 (Lee *et al.*, 2012).

The first mutation identified in *MSRB3* was c.265T>G (p.Cys89Gly), which was reported in six families with ARNSHL (Ahmed *et al.*, 2011) (Table 4.1). The novel homozygous variant p.Leu7Arg (c.20T>G) in *MSRB3* identified in the current study is located in the mitochondrial signal sequence and may result in mislocalization of the protein. Ahmed *et al.* (2011) have previously shown the importance of the mitochondrial isoforms when they found a mutation c.55T>C (p.Arg19*) to be the causative factor of hearing impairment. This mutation also resides in the signal sequence for mitochondrial localization. However, the variant c.20T>G (p.Leu7Arg) was also found in a heterozygous state in 3 out of 89 (1.7%) ethnically matched controls. Thus currently it is uncertain whether this variant is the cause of hearing impairment in the family and further studies are necessary for a definite conclusion on the pathogenic effect of this variant.

Hepatocyte growth factor (HGF) is a mitogen secreted from the cultured hepatocyte cells and functions as a growth factor and is also involved in various signaling pathways (Hayashi *et al.*, 2001; Kmiecik *et al.*, 1992; Miyazawa *et al.*, 1998; Nakamura *et al.*, 1989; Sakata *et al.*, 1996; Zhang and Vande Woude, 2003). This protein has a signal peptide, an alpha chain with four kringle domains and a beta chain domain (Miyazawa *et al.*, 1991). *HGF* has been shown to be responsible for ARNSHL (DFNB39) and previously 3 mutations have been reported in *HGF* causing hearing loss. Schultz *et al.* (2009) while studying murine conditional *HGF* knockout of cochlear tissues, observed profound hearing loss while the ubiquitous overexpression

of *HGF* resulted in deterioration of outer hair cells and progressive hearing loss. In the current study two recurrent mutations, c.482+1991_2000delGATGATGAAA and c.482+1986_1988delTGA were identified. Both of these intronic deletions alter the splice acceptor site of exon 5 and are likely to result in skipping of exon 5.

SLC26A4 is the second major cause of ARNSHL worldwide (Kitajiri *et al.*, 2007), out of a total of 140 mutations in this gene reported worldwide, 18 mutations were found in the Pakistani population. The second most recurrent mutation of *SLC26A4* in the Pakistani population is c.1337A>G (p.Gln446Arg), which was also found in the current cohort of ARNSHL families. The most frequent *SLC26A4* mutation reported from Pakistan is c.716T>A (p.Val239Asp) (Anwar *et al.*, 2009; Khan *et al.*, 2013; Park *et al.*, 2003) (Table 4.1). These mutations lead to congenital severe to profound hearing loss (Reardon *et al.*, 2000).

Transmembrane inner ear (*TMIE*) gene has 4 exons and is expressed in the developing inner ear (Mitchem *et al.*, 2002), the function of this gene is not yet known. Eight mutations have been reported in *TMIE* worldwide (Hilgert *et al.*, 2009), mutations in this gene (DFNB6) mostly cause severe to profound hearing loss (Naz *et al.*, 2002; Santos *et al.*, 2006). TMIE protein has an extracellular domain (residues 28-57), helical domain (residues 58-78) and a cytoplasmic domain (residues 78-156) (UniProt Home: http://www.uniprot.org/uniprot/Q8NEW7). Studies suggest that the protein is localised in the plasma membrane (Gleason *et al.*, 2009; Shin *et al.*, 2010). A study by Karuppasamy *et al.* (2011) suggests that the tmie protein might be involved in signal transduction and cell trafficking within the auditory system. The mutation c.241C>T (p.Arg81Cys) in *TMIE* is a recurrent mutation and alters a highly conserved amino acid residue. This mutation found in the current panel has already been reported in Pakistani population by Naz *et al.* (2002) and Santos *et al.* (2006) (Table 4.1). The mutation c.241C>T resides within the cytoplasmic domain and is predicted to be deleterious by SIFT and probably damaging by PolyPhen-2 analysis.

These 10 autosomal recessive hearing loss consanguineous families in which the pathogenic mutations reside in disease causing genes were identified using homozygosity mapping. These families constituted 31% of the total panel of hearing loss families thus showing that homozygosity mapping is an effective technique to detect pathogenic mutations residing in genes implicated in autosomal non-syndromic

recessive hearing loss in the Pakistani population. Exome sequencing of the excluded families was another valuable technique used in the current study for the identification of novel genes, which have not been previously shown to be associated with ARNSHL. Initial homozygosity mapping facilitated in filtering the probable pathogenic variants obtained from Exome sequencing. As there is a high rate of consanguineous marriages in the Pakistani population therefore homozygosity mapping in combination with Exome sequencing can be effective in the molecular diagnosis of hereditary disorders.

Exome sequencing was performed on DFR4 and resulted in the identification of one already reported ARNSHL gene and two putative ARNSHL novel genes in the current study. In family DFR4 (a highly inbred left loop (DFR4 a) and an outbred right loop (DFR4 b)), three variants c.193G>A (p.Ala65Thr), c.633+1G>C (p.?) and c.32C>G (p.Ser11*) in the genes thrombospondin-type laminin G domain and EAR repeats (*TSPEAR*), cyclin I family, member 2 (*CCNI2*) and protocadherin gamma subfamily A, 10 (*PCDHGA10*), respectively segregated with ARNSHL in the left loop (DFR4 a).

The novel variant c.193G>A (p.Ala65Thr) in *TSPEAR* results in a missense change from alanine to threonine at position 65. The Grantham distance of 58 indicates small physiochemical difference between alanine and threonine in addition the phyloP score of -0.20 is also suggestive of non-conserved nucleotide. The variant lies within the protein domain Laminin G, thrombospondin-type, N-terminal. SIFT revealed the variant to be tolerated, and Mutation Taster indicates it to be a polymorphism, dbSNP report it to be a rare variant with a frequency of 0.001.

TSPEAR mutation has already been shown to be responsible for ARNSHL (DFNB98) and result in congenital bilateral profound hearing loss in the Iranian population, where they identified a variant in exon 10 (c.1726G>T+c.1728delC), which results in frameshift (p.V576Lfs*37) (Delmaghani *et al.*, 2012). This gene is reported to be expressed in the murine cochlear tissues, where the alternate splicing of *TSPEAR* has been shown to result in two transcripts. The encoded isoforms have a signal peptide and a thrombospondin-type laminin G (TSP) domain, and seven tandem repeats called EAR (epilepsy-associated repeat) because of which these proteins belong to the EAR protein family. These proteins include four members of the leucine-rich, glioma-

inactivated (Lgi) subfamily *i.e.* Lgi (1-4); Vlgr1 (Very large G protein-coupled receptor 1) and Tspear. Lgi1 is involved in the formation of a complex with α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Fukata *et al.*, 2006; Fukata *et al.*, 2010), which is required to transmit the auditory signals across the synaptic junctions (Parks, 2000). Mutations in *LGI1* have been shown to result in auditory dysfunctioning and speech inability in mouse model (Chabrol *et al.*, 2010; Charizopoulou *et al.*, 2011; Morante-Redolat *et al.*, 2002; Yagi *et al.*, 2005). While Vlgr1 is required for the normal development of the stereocilia (Mcgee *et al.*, 2006) and mutations in this gene have been reported to cause audiogenic seizures and type II Usher syndrome (USHII) (Skradski *et al.*, 2001; Weston *et al.*, 2004). Out of the six above mentioned EAR proteins, *LGI1* and *VLGR1* have been shown to be involved in audiogenic seizures and hearing loss in mice as well as humans. Though *TSPEAR* has also been reported to cause hearing loss in humans, the pathogenic mechanism is not clear yet.

In addition to *TSPEAR*, in family DFR4 two other variants were also found to segregate with the phenotype, therefore the possibility of their being disease causative cannot be excluded. Of these two the variant c.633+1G>C in *CCNI2* is a substitution that is located in the splice donor site of intron 3. The consequence of this change is not predictable, but skipping of exon 3 is very likely, in addition the high phyloP score of 4.81 also indicates the possible pathogenic role of this variant. The cytogenetic location of *CCNI2* is 5q31.1 encoding a cyclin-I2 (40622Da) of 369 amino acids belonging to the cyclin family. CCN12 has three predicted functional domains, cyclin-N domain, cyclin-like domain, cyclin A/B/D/E domain. Though till date no functional studies have been performed, however, according to GO annotations *CCNI2* has been involved in the regulation of cyclin-dependent protein serine/threonine kinase activity (Gene Ontology: http://amigo.geneontology.org/amigo/term/GO:0000079). Moreover, this gene has not been specifically related to any disorder except for a study by Taneera *et al.* (2013) in which they reported aberrated expression of CCN12 in the pancreatic islets in type 2 diabetes patients. In the current deafness family DFR4, the three affected children carry this variant homozygously, which was inherited from their carrier parents.

The third variant c.32C>G (p.Ser11*) in *PCDHGA10* in family DFR4 is a nonsense substitution in exon 1a resulting in a premature stop codon. This nonsense variation is likely to cause nonsense mediated decay (NMD) as it is present near the 5' end of the gene. The phyloP score of -0.44 is suggestive of non-conserved nucleotide. The cytogenetic location of *PCDHGA10* is 5q31, encoding two isoforms; one being 936 amino acid long and the second has 850 amino acids.

PCDHGA10 is a cell adhesion protein (Uniprot: http://www.uniprot.org/uniprot/Q9Y5H3) and is likely to be involved in the establishment of the connections among neurons within the brain as *PCDHG* genes are highly expressed in the brain. Wang *et al.* (2002) have previously shown that the deletion of the *PCDHG* region results in neural collapse in the case of late embryonic mice. *PCDHGA10* belongs in the superfamily of transmembrane glycoproteins, cadherins that bind Ca^{2+} and also belong to the subfamily of non-classical cadherins, the protocadherins, which further constitute three clusters that are alpha, beta and gamma (Gumbiner, 1996; Shapiro and Colman, 1999; Suzuki, 1996; Takeichi, 1995; Tepass, 1999; Uemura, 1998; Wu *et al.*, 2001). There are 22 proteins within the gamma gene cluster called the *PCDHG*; all of these 22 members differ from each other on the basis of a variable exon, which expresses the N-terminal peptide along with extracellular and transmembrane regions. This is followed by 3 exons, which express a constant C-terminal cytoplasmic region that is common among all the members (Wu and Maniatis, 1999).

The non-classical cadherins *i.e.* cadherin-23 (*CDH23*) and protocadherin-15 (*PCDH15*) are involved in the formation of filaments between the stereocilia, known as the tip links that facilitate interaction among the stereocilium thus causing the deflection of the hair cells and opening of the transduction channels. This results in the influx of extracellular Ca^{2+}, which causes the release of the neurotransmitter from the receptor cell and leads to the excitation of the sensory endings of the cochlear nerve. Cochlear sensory neurons in the vestibulocochlear nerve (VIII) synapses with neurons in the medulla oblongata, which project to the midbrain; thus the action potential travels through an afferent nerve fibre from the hair cell to the brain (Ahmed *et al.*, 2006; Alagramam *et al.*, 2001; Gillespie and Muller, 2009; Kazmierczak *et al.*, 2007; Siemens *et al.*, 2004; Sollner *et al.*, 2004). The mutations in *CDH23* and

PCDH15 are known to cause Usher syndrome, DFNB12 and DFNB23 (Ahmed *et al.*, 2008; Ammar-Khodja *et al.*, 2009; Astuto *et al.*, 2002; Jaijo *et al.*, 2012; Schultz *et al.*, 2011).

Another family DFR5 with a highly inbred right loop (DFR5d) and three outbred loops (DFR4 a, b and c) was also Exome sequenced. A putative ARNSHL novel gene ATPase, class V, type 10B (*ATP10B*) with an identified variant c.2910C>A (p.Asp970Glu) in exon 19 segregated with the phenotype in the right inbred loop (DFR5 d) only.

The variant p.Asp970Glu in *ATP10B* results in a missense substitution from aspartic acid to glutamic acid at position 970. The Grantham distance of 45 indicates small physiochemical difference between the two amino acids. The phyloP score of -0.36 is suggestive of a non-conserved nucleotide. The variant lies within the protein domain ATPase, P-type, phospholipid-translocating, flippase, SIFT revealed the variant to be tolerated and Mutation Taster indicates it to be a polymorphism.

The cytogenetic location of *ATP10B* is 5q34 encoding a probable phospholipid-transporting ATPase VB of 165391 Da (1461 amino acids), which is expressed in the brain. *ATP10B* belongs to the subfamily of P4-type ATPases that hydrolyze the ATP to transport the aminophospholipids across the phospholipid membrane by forming vesicles (Paulusma and Oude Elferink, 2005; Suzuki *et al.*, 1997). Takatsu *et al.* (2011) showed that *ATP10B* belongs to the class of P4-ATPases that require CDC50 proteins for their exit from the endoplasmic reticulum lumen to the other parts of the cell in order to perform their respective functions. According to a recent study by Demos *et al.* (2014) mutation in another member of ATPase family, *ATP1A3* (ATPase Na+/K+ transporting, alpha 3 polypeptide) is causative of CAPOS syndrome that includes phenotypic features such as cerebellar ataxia, visual degeneration and sensorineural deafness. This Sodium/potassium-transporting ATPase subunit alpha-3 is involved in cation transport and the maintenance of the electrochemical gradient (Demos *et al.*, 2014). *ATP1A3* belong to the P family of the ATPases and the mutation in this gene is the only ATPase mutation reported till date to cause syndromic hearing loss.

Though *in silico* analysis indicated the mutations residing in the putative ARNSHL

novel genes to be likely pathogenic but *in vitro* and *in vivo* studies are yet to be performed to confirm the predicted pathogenicity.

In the current study mutations were identified in 9 genes (*GJB2*, *MYO15A*, *TMC1*, *BSND*, *TMPRSS3*, *MSRB3*, *HGF*, *SLC26A4* and *TMIE*) therefore the mutation spectrum of these 9 genes was also reviewed in the Pakistani population. Till date the genetic data of 593 patients and 241 families with ARNSHL have been described for these genes in Pakistani patients (Table 4.1). *GJB2* remains the most frequently mutated gene with a frequency of 29% from 174 hearing loss patients in 71 families (Figure 4.1). *MYO15A* (25 families with 107 affected persons) and *SLC26A4* (60 deafness families with 101 affected persons) with frequencies of 18% and 17%, respectively, are the other two most frequently mutated genes after *GJB2* causative of hearing loss in Pakistan (Figure 4.2). In the case of *SLC26A4* and *MYO15A* it can be predicted that *SLC26A4* is more frequent in small nuclear families with one or two affected individuals while *MYO15A* is more frequent in inbred families.

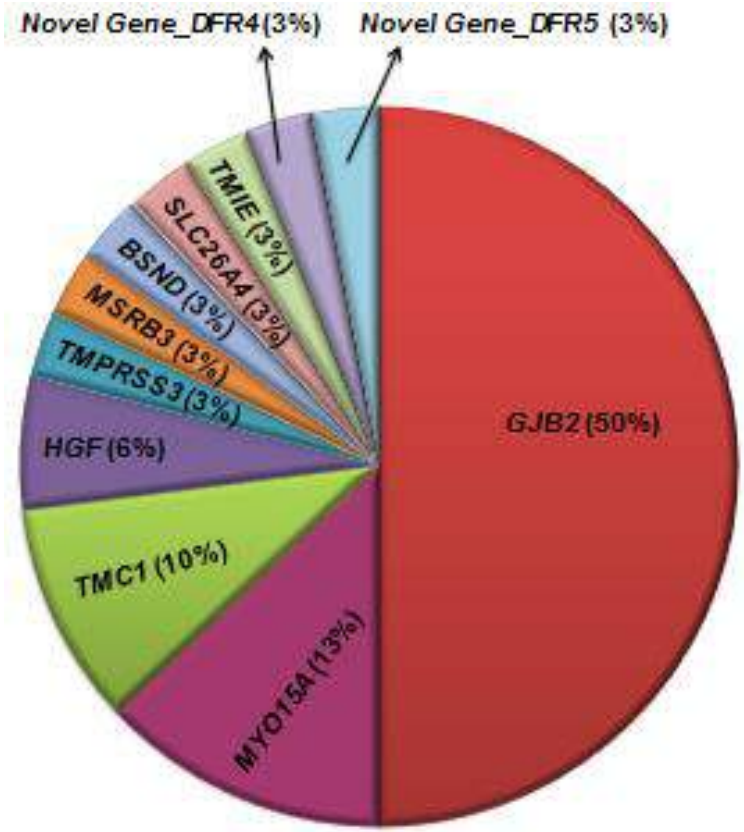

Figure 4.1. Mutations identified in Pakistani ARNSHL families in the current study.

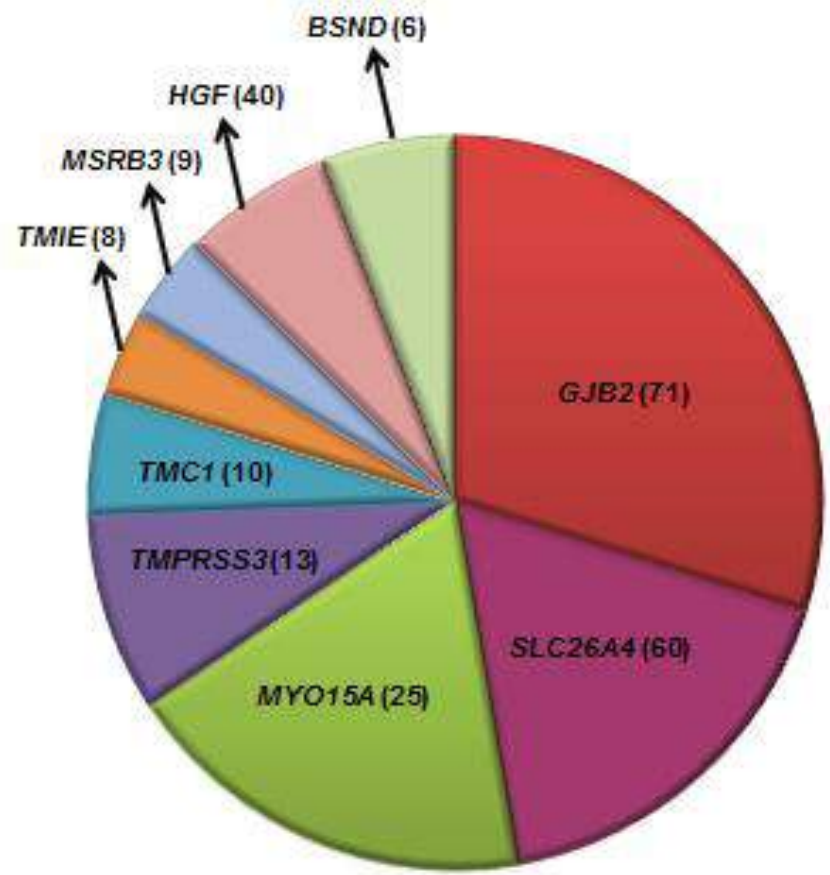

Figure 4.2. Occurrence of mutations in ARNSHL Pakistani families. Numbers of families with mutations in respective genes are indicated between parentheses.

Conclusion

In conclusion, in the present study 50% (16/32) of the families were found to carry causative mutations in *GJB2* followed by *MYO15A* (13%, 4/32) and *TMC1* (9%, 3/30). According to the reviewed literature of Pakistani ARNSHL families *GJB2* remains the most frequently mutated gene followed by *MYO15A* and *SLC26A4*. Based on these data it is therefore suggested that as an initial step for the genetic diagnosis of hearing loss, *GJB2* should be analyzed first in Pakistani patients and if this gene is excluded then analysis should be further extended to *MYO15A* and *SLC26A4*. Homozygosity mapping has proved to be an effective approach to determine the mutated genes in consanguineous families in this study. Identification of novel mutations in known deafness genes as well as putative novel genes in the current study highlights the fact that although a large number of deafness genes and mutations have already been identified but the full mutation spectrum for ARNSHL is still undefined and there is still a need to thoroughly screen different populations to identify additional ARNSHL genes. This can help in the proper management and diagnosis of the disease. Genetic counseling of the families is important to better inform couples about the risk of transferring hearing impairment to their offspring. On the basis of the current work a cost effective deafness gene mutation screening method such as molecular inversion probe analysis can be devised to save time and money invested in mutation detection protocols.

Chapter 5

References

5. References

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