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# LINKAGE ANALYSIS OF AUTOSMOAL RECESSIVE INHERITED DEAFNESS FOR *MYO15A* AND *HGF* GENES IN FAMILIES FROM DISTRICT DADU (SINDH), PAKISTAN

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## ABSTRACT

Deafness is a partial or complete loss of hearing. It is the most prevalent sensory defect in humans that severely compromises the quality of life and may result in socially isolated Individuals. It is a multi-factorial disorder caused by either genetic or environmental factors or a combination of both. It is estimated that at least 60% of deafness is due to genetic disorders. The prevalence of deafness in Pakistan is 1.6 per 1,000 live births and 70% of hearing loss arises in consanguineous-families, much higher than 1 per 1000 live births worldwide.

**Keywords: Deafness, loci, genetic, linkage**

## INTRODUCTION

The total inability to hear a sound is known as deafness. It is the most common sensory disorder of heterogeneous trait in humans. Deafness is the world's third leading chronic disease in all over the world. (1,2). Hearing loss is a disorder having multifactorial causes one of most common factor is Genetic factor which includes

approximately 60% cases are profound deafness and remaining 40% are of environmental factor (3,4). In Pakistan to date almost 129 loci were determined and 72 genes were identified for the deafness (non-syndromic). The Mutations of *MYO15A*, *HGF* gene each accounts for 3.3-5.12% Recessive deafness (DFNB3 and DFNB39) respectively in the Pakistani population (5).

Hearing loss characterized as conductive, with middle ear anomalies, or sensorineural which is accompanied by only ear malfunction. Non-syndromic deafness accounts for 70% and remaining 30% are Syndromic type of prelingual genetic deafness (6).There are two inherited form of Deafness segregated as monogenic and digenic, most inherited form of deafness are in a monogenic trait (7). The deafness is also a point of differentiation, by the mode of inheritance patterns where Autosomal Recessive inheritance accounts for approximately 80% of cases, autosomal dominant 20%, X-linked 1% and Mitochondrial <1% (4,8).

Very great genetic heterogeneity has been noticed in the type of sensorineural deafness. To date, more than 129 non-syndromic loci have been determined. About 72 loci are of autosomal recessive mode (*DFNB*), while 57 loci in an autosomal dominant mode (*DFNA*). It is believed that approximately 1% of the human protein coding genes are participating in the normal functioning of the inner ear (7).

The different cellular diversities and very small size of the inner ear tissues has posed remarkable challenges to anatomic, physiological, and proteomic studies of human deafness (9).The eccentric methods of biochemistry and physiology are challenging and have been unsuccessful for understanding the mechanisms of the auditory system compared to genetic approaches. These approaches provide a powerful techniques for identifying the essential components of auditory system of transduction and it is more easy way than the mapping of genes because of very great clinical and heterogeneity of deafness (10). Homozygosity mapping was used for highly consanguineous families isolated from different ethnical communities and could be more suitable and fruitful approach for mapping the autosomal recessive non syndromic hearing loss genes (7,11). The Pakistan is one of the most important and richest country for the studying the genetic inherited diseases. Due to its unique culture of consanguineous marriages, approximately more than 60% marriages are cousin marriages and about 80% are first cousin (12). The prevalence of deafness in Pakistan is about 16 per 10

thousand births, it is higher ratio than the whole world's average ratio which is about 10 per 10 thousand births (13, 14).

The study of autosomal recessive hereditary deaf families of district Dadu has allowed advance and extraordinary progress for the mapping of new loci and genes which are responsible for normal functioning of the inner ear.

The majority population of district Dadu is Muslims mainly consist of Sindhi and Balochi. Approximately more than 32 million population of district Dadu having area of almost 8 thousand kilometer sq. bordered by different districts such as larkana on the North side, Nausheroferoze, Nawabshah and Hyderabad districts on the east, Thatta district is on the South and on west malir and lasbela districts of Baluchistan province. The environment of district Dadu is balanced; the land is dry and moist, some areas almost producing a lot of agricultural corps products which are irrigated by Indus River, and Gaj stream fellows across from Kherthar Mountain ranges. Mostly Dadu is surrounded by the rural areas as a result of that Towns like Dadu, Johi, Khiarpur Nathan Shah and Mehar are center of attraction to the villages around them.

The major objectives of current study are to point out the molecular and genetic basis of inherited deafness through the linkage analysis. For the fulfilment of this study, 12 different deaf families with profound hearing loss were in list from various parts of district Dadu. Written agreements were obtained from every study participants; Pedigrees of families were drawn personally by visiting each family after getting informed the consent. The hearing threshold of some unidentified Childs were assessed by the pure tone Audiometry. From the 12 selected families, about 5-10cc blood were collected from each patient and their family members in 50ml falcon tube containing the 400 µl EDTA and were preserved at -80°C. DNA was extracted through inorganic method of Extraction. The Stability of isolated DNA was confirmed through 0.8% Agarose gel Electrophoresis and also Quantified by Spectrophotometry. The DNA was preserved for further process like genotyping for linkage analysis and scanning to known genes. Moreover, the STR Markers of DFNB3 and DFNB39 loci were selected and optimized for their PCR conditions. Linkage analysis of the families showed the two linked family with DFNB39. The remaining 10 deaf families were unlinked to the reported genes and giving in an idea of presence of novel deaf loci in these families.

The current study was plan to determine the molecular basis and genetics of the non-

syndromic autosomal recessive deafness of families from the district Dadu. For the fulfilment of this study, 12 different deaf families with profound hearing loss were in list from various parts of district Dadu. Written agreements were obtained from every participants;Blood samples were collected and treated for DNA extraction. Selected families undergoes exclusion analysis for reported deaf loci, and only two families were found linked. One family linked with DFNB3 and other linked to DFNB39.

## MATERIALS AND METHODS

### Enrollment of Deaf Families

Families having at least two or more deaf individual were selected and Enrolled from different parts of District Dadu. After receiving feedback, detailed history of disease, pedigree was drawn by visiting those families. Informed consent was obtained from all participating deaf individuals and other relatives of deaf families were also included in this research. Enrolled families given the evidences that deafness is an autosomal recessive type of inheritance. The rough pedigree were drawn at first visit of the family. After field work the fair pedigree of enrolled families were drawn in Macromedia FreeHand software.

### Identification

Identification of different deaf families was assessed by pure tone Audiometry test.

### Pedigree Drawing & Analysis

Pedigree drawing was ascertained after visiting to selected families. Rough Pedigree structure were first made on personal visit to family and then the pedigree of enrolled families were appropriately drawn in Macromedia FreeHand. Macromedia FreeHand is a software designed for making pedigrees in a proper manner. It clearly shows the relation between family member, number of living and dead individuals, normal and affected members and all the required information regarding each individual of enrolled family.

### Blood Sample Collection

Blood samples were obtained from 10 selected deaf families of District Dadu. 5-10 ml blood was collected from each patient and their family members in 50ml falcon tube containing 400 µl EDTA and preserved at -80°C for further analysis.

## DNA EXTRACTION

The Extraction of DNA was done by using an Inorganic Extraction method described by Grimberg in 1989 (15). This method consists of 3 major steps:

1. Lysis of RBCs
2. Lysis of WBCs and Digestion of Proteins
3. Salting Out Proteins and Nucleases

**CONFIRMATION AND QUANTIFICATION OF DNA**

There are several ways to quantify the DNA. If the DNA is in pure form we can use a spectrophotometer method which calculate the UV radiations absorbed by the DNA. Other method in which an agarose gel and a fluorescence agent Ethidium bromide in the presence of electric current also used. Two common methods of confirmation and Quantification of DNA; Optical Density Measurement and Agarose Gel Electrophoresis.

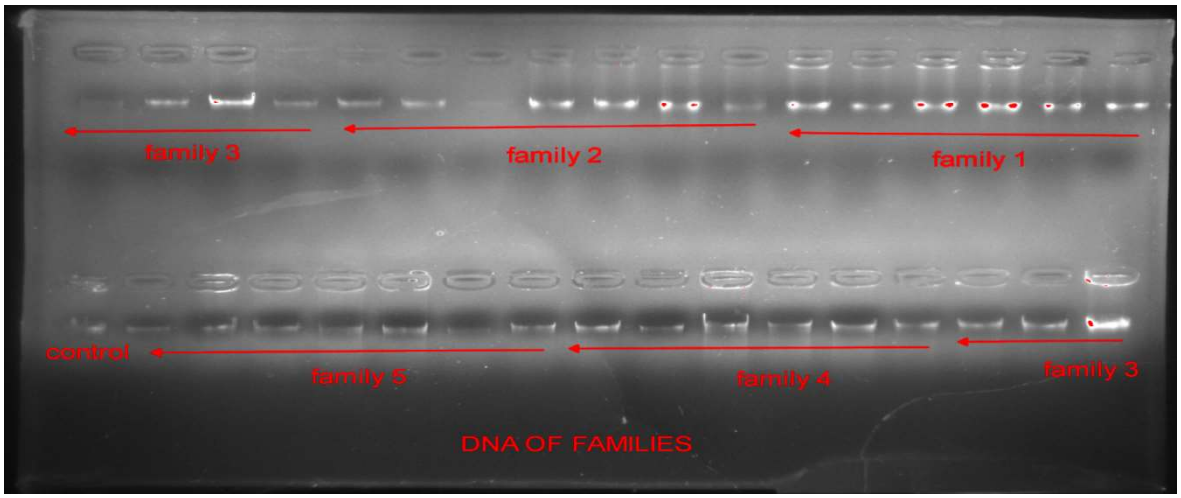


**Figure. 1: Shows the presence of DNA samples of some families in of 0.8 % agrose gel through electrophoresis.**

**OPTIMIZATION OF PRIMERS**

Different PCR programs were used for optimization of given primers, with varying concentration and volume of PCR components. Programs like touchdown 65-55, touchdown 64-54, touchdown 66-56, and Multicemb 57+8 cycle, Multicemb 54+8 cycle, were used to get desired results.

**Agarose gels of optimized primers**

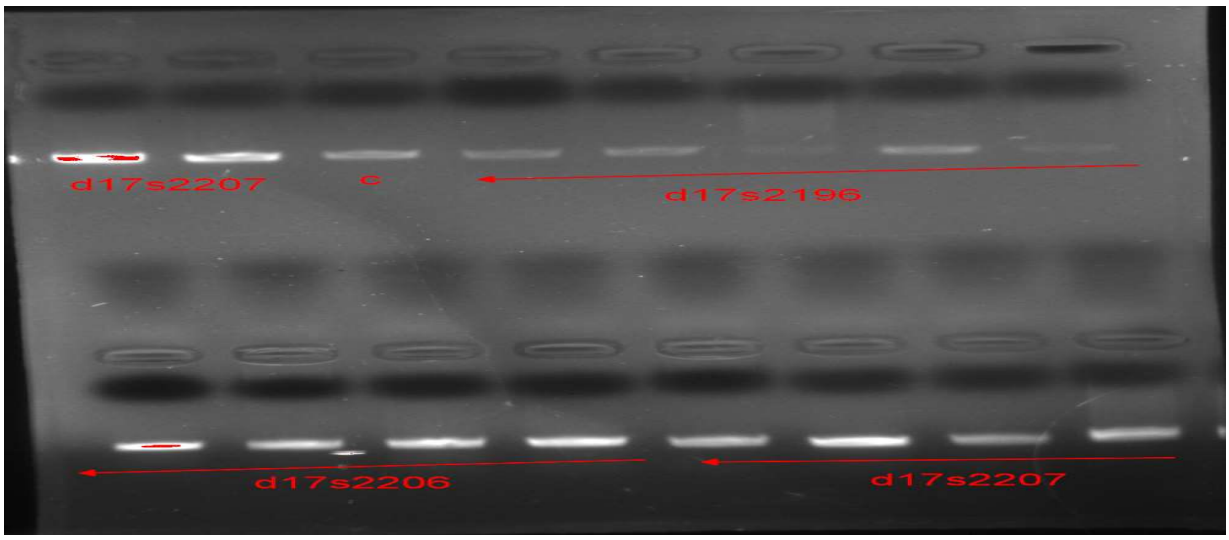


**Figure. 2: Optimized primer of DFNB3 locus.**

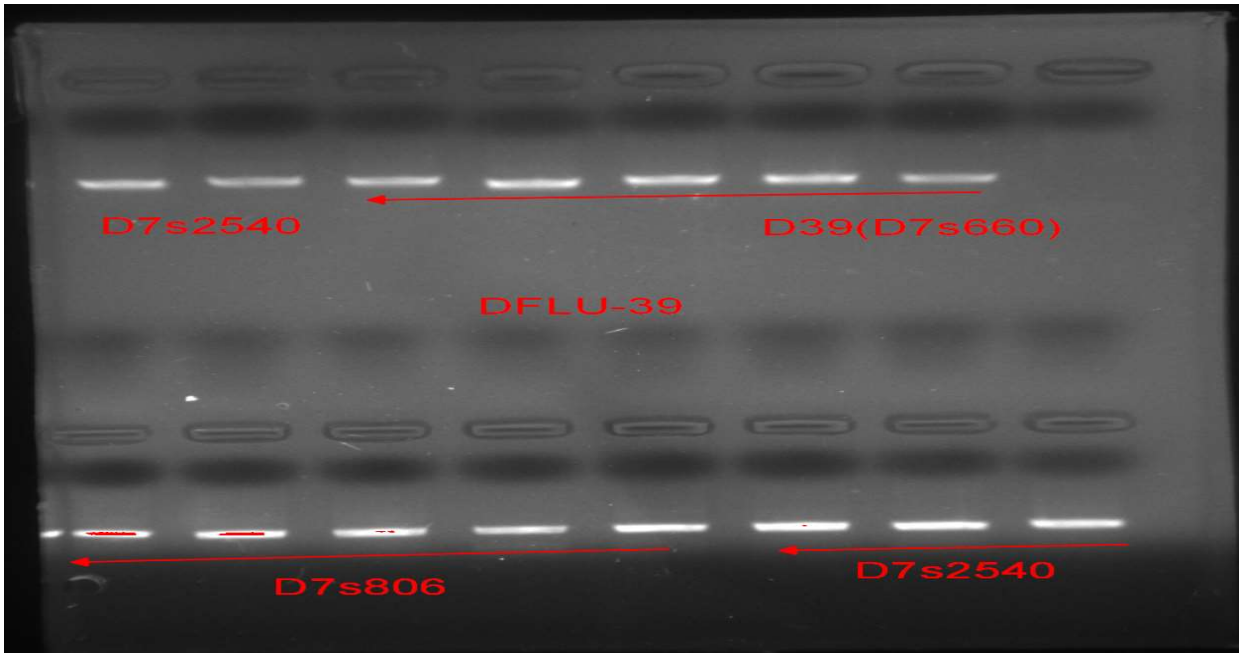


**Figure. 3: Optimized primer for DFNB39 locus.**

## LINKAGE ANALYSIS OF SELECTED LOCI

For linkage analysis three microsatellite markers were used for each selected loci (DFNB3 and DFNB39). These markers were used to check the hetrozygosity and homozygosity in enrolled families. The primers were espicially designed and than cheked for selected markers to make sure they can produce PCR products and amplification. Selected primers (Farward primer and Reverse primers) of each locus having different ASR ranges, ditances (cM) and were fluorescently labelled with different dyes (NED,VIC and FAM). The primers and fluorescent dyes were also used in genotyping and broughted commercially.The selected loci and markers;

**Table 1: Shows the markers with their dyes, distances centimorgan (cM) and ASR length.**

| LOCUS | MARKER | CM | DYE | ASR |
|---|---|---|---|---|
| DFNB3 | D17S2196 | ~44.62 | NED | 139-163 |
| | D17S2207 | ~45 | FAM | 85 |
| | D17S2206 | ~45 | VIC | 141-165 |
| DFNB39 | D7S2540 | ~93.63 | VIC | 180-200 |
| | D7S660 | ~94.87 | FAM | 115-145 |
| | D7S806 | ~97.38 | NED | 190-220 |

## SAMPLE REPARATION FOR ABI 3100 AND 3730 GENETIC ANALYZER

First of all poll 1-1.5 microliter of the sample from the PCR tubes of all families labeled with their names and family number into the 96 wells of polling plate together with 5 microliter of formamide (applied Biosystem). For avoiding the

mixing, the multiplexing of samples was performed in a manner that the PCR amplicons of same size labeled with same fluorophore. Each locus has 3 STR markers, take 1-1.5 microliter from three markers only in one well of polling plate with formamide, thus avoiding overlapping products during analysis the plate must denatured for five minutes at $95^0$C then cooling in ice also required.

**GENOTYPING**

Linkage analysis were done by using a software (Genotyper 4.0 NT). The Genotyper software uses to detect fluorescence of genetic analysis and quantify the size of DNA fragments. It can be showing the results as tubular data or Electro gram or combination of both.

A plate used for genotyping also known as genotyping or polling plate. Polling plate having 96 well which is now ready for the genotyping in a Genetic Analyzer. In Gene scan a sheet are made for the identification of lane number including sample name, file name, dye, internal size standard and contents of each sample.

**HAPLOTYPE ANALYSIS**

Haplotype is set of alleles which are genotyped in the genetic analyzer, representing chromosomal segments of an individual. The genotyped alleles are ordered on basis of the Centimorgan (cM) distance with chromosome. For examining the inheritance pattern of segregating deafness. The locus or gene is said to be linked if the STR markers were positioned in DFNB loci and showing Homozygosity in affected individuals in family. The selected loci was established unlinked when the normal members in family are heterozygous form rather than homozygous.

**RESULTS AND DISCUSSIONS**

Human genetics perform a magnificent duty in genomic era. The presence of the human genomic sequence has solved several dilemmas faced by geneticists in their efforts to understand the basic mechanisms of inherited diseases. Hereditary hearing loss research heterogeneous nature, to date, In Pakistan presently 134 loci and 80 genes have been identified for the inherited type of deafness (non-syndromic). In the population of Pakistan approximately 60% marriages are of consanguineous marriages from which 80% marriages are of first cousins (12). Using linkage analysis in families with inherited deafness from the population of Pakistan, it is calculated that nearly 75% of cases of deafness show autosomal recessive inheritance, 12-24% of cases are autosomal dominant and 1-3% cases are X-linked. The population of Dadu is composed of a mixture of various ethnic groups, came from different areas centuries ago and

having roots to different Pakistani populations. This population is a superb source that can help us for understanding the genetics and molecular basis of hearing and deafness.

Linkage analysis is a commonly used method to locate disease gene in families. This method identifies linkage, which is an aid in narrowing down the intervals in which causative gene reside. Identification of various mutant alleles of selected locus in different families can help us to correlate genotype/phenotype. Linkage analysis has also made a possible mutational study of genes such as *MYO15* and *HGF (16*).

In this Research study, 12 families of prelingual profound deafness were subjected to linkage analysis of given locus (DFNB3 and DFNB39). One family displayed significant or highly suggestive linkage to DFNB3, and other family showed linkage to the DFNB39 locus.

Three STR markers, D17S2196, D17S2207and D17S2206 of locus DFNB3 and D17S660, D17S806, D17S2540 of locus B39 were genotyped on 12 families with deafness.Haplotype of selected deaf families were made in Microsoft Excel/Data Sheet for the determination of inheritance patterns of affected and normal individuals of each family. Two families DFLU-40 and DFLU-42 were linked with DFNB3 and DFNB39 loci and 10 families remained unlinked out of 12 deaf families.

**Table. 2: The status of families.**

| S/No | Family No. | Status of Pedigree DFNB3/B39 | Age ranges of Deaf individuals |
|---|---|---|---|
| 01 | DFLU-27 | Unlinked to the locus DFNB3/B39 | 23-27 years |
| 02 | DFLU-34 | Unlinked to the locus DFNB3/B39 | 23-60 years |
| 03 | DFLU-35 | Unlinked to the locus DFNB3/B39 | 07-18 years |
| 04 | DFLU-36 | Unlinked to the locus DFNB3/B39 | 23-33 years |
| 05 | DFLU-37 | Unlinked to the locus DFNB3/B39 | 35-50 years |
| 06 | DFLU-38 | Unlinked to the locus DFNB3/B39 | 20-25 years |
| 07 | DFLU-39 | Unlinked to the locus DFNB3/B39 | 20-30 years |
| 08 | DFLU-40 | *Linked to the locus DFNB39* | 17-19 years |
| 09 | DFLU-41 | Unlinked to the locus DFNB3/B39 | 05-21 years |
| 10 | DFLU-42 | *Linked to the locus DFNB3* | 14-24 years |
| 11 | DFLU-43 | Unlinked to the locus DFNB3/B39 | 03-17 years |
| 12 | DFLU-44 | Unlinked to the locus DFNB3/B39 | 23-28 years |

**DFNB: for congenital Genes, Non-syndromic deafness. List of the linked and unlinked families.**

## LINKED FAMILIES

### *Family DFLU-40*

This family was collected from Tehsil Johi village Thaheem muhalla johi city, District Dadu. This family was collected from Tehsil Johi village Thaheem Muhalla Johi city Ward no: 1, District Dadu. The family belonged to Thanheem caste, a section of Sindhi tribe.Blood samples were obtained from five family members, including 2 affected and their parents. The affected individuals have their ages between 11 to 27 years. After the collection DNA were

extracted and amplified by using three STR markers of DFNB39 locus or gene *HGF*.Amplified products were checked on 1.2 % agarose gel. Furthermore if the desire product was amplified and results are good, then product is ready for genotyping. The genotyping of amplified samples were carried out on Genetic Analyzer ABI 3130 by Applied Bio systems. The data thus obtained was analyzed to develop the haplotype in order to establish the linkage analysis to recognize alleles and confirm the homozygosity and heterozygosity. This is best and easy way to read the alleles and the inheritance patterns. The family (*DFLU-40)* showed linkage to DFNB39 locus.

The linked family of DFNB39 locus were amplified by these STR markers D17S660, D17S806, and D17S2540, markers showed homozygosity in all the deaf individuals and heterozygosity in parents. For D17S2540 marker father and mother were homozygous with their deaf children and Remaining two STR markers were Heterozygous in entire family members.

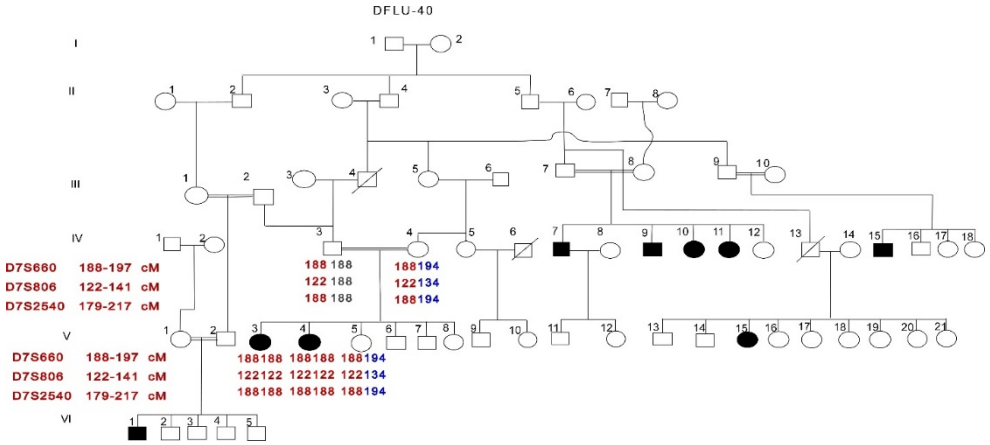


**Figure. 5: Shows DFNB39 loci was linked haplotypes with DFLU-40 Family, Filled squares and circles correspond to affected persons in the family. The red colored digits show diseased allele segregating within the family. Corresponding STR markers associated with the locus are shown on the left side of pedigree.**

## ***Family DFLU-42***

This family was collected from Tehsil Dadu village Haji wadhyo Lund near Pipri canal, District Dadu. The family belonged to Lund caste, a section of Baloch tribe.Blood samples were obtained from six family members including 2 affected and their parents. The first affected individuals have age of 14 and other have age of 24 years. After the blood collection DNA were extracted and amplified by using three STR markers of DFNB3 locus or gene *MYO15A*.Amplified products were checked on 1.2 % agarose gel. Furthermore if the desire product was amplified and results are good, then product is ready for genotyping. The genotyping of amplified samples were carried out on Genetic Analyzer ABI 3130 by Applied Bio systems. The data thus obtained was analyzed to develop the

haplotype in order to establish the linkage analysis to recognize alleles and confirm the homozygosity and heterozygosity. This is best and easy way to read the alleles and the inheritance patterns. The family (*DFLU-42)* showed linkage to DFNB3 locus.

The linked family of DFNB39 locus were amplified by these STR markers D17S2196, D17S2207 and D17S2206, markers showed homozygosity in all the deaf individuals and heterozygosity in parents. For D17S2196 marker father and mother were homozygous with their deaf children and Remaining two STR markers were Heterozygous in entire family members.

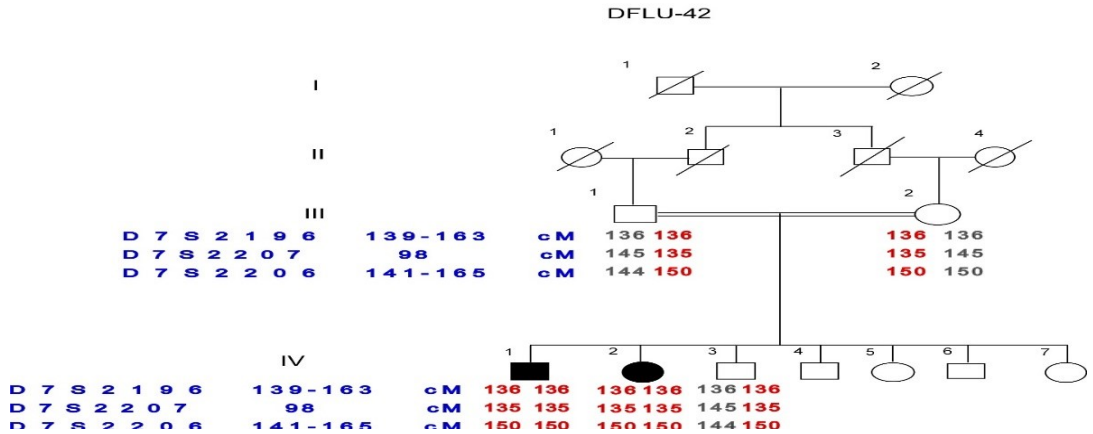


**Figure 6: Shows the DFNB3 loci was linked haplotypes of DFLU-42 Family, Filled squares and circles correspond to affected persons in the family. The red colored digits show diseased allele segregating within the family. Corresponding STR markers associated with the locus are shown on the left side of pedigree.**

## DISCUSSION

The Autosomal Recessive (Non-syndromic) deafness is the most common and serious disorder in the world and accounts approximately more than 70 percent cases (17). The selected loci or genes are specific with population so the Pakistani population is a major source for revealing the secrets of deafness as to date 125 locus of deafness have been determined 63 of them are of DFNB, and 58 loci of DFNA, only 4 loci are X-linked. With the help of Pakistani deaf families 80 genes has been identified and mapped causing autosomal recessive (non-syndromic) deafness. Pursing this approach, we enrolled 12 families segregating hearing impairment belonging to different ethnic groups from diverse areas of Dadu Sindh. One Balochi family was homozygous or linked. This variant is also present in Midwest Us, France and Solvak Caucasians (18, 19). Linkage analysis of all deaf families shows that 2 families were linked. One family showed linkage to DFNB3 locus, while other linked with DFNB39 loci. The other remaining 10

families were unlinked to reported deafness loci.

Linkage analysis was done on the selected 12 families belonging to different areas of district Dadu. Out of twelve families screened for the non-syndromic autosomal recessive locus DFNB3 and DFNB39 only two families DFLU-40 and DFLU-42 was linked. The prevalence of deafness among Pakistani population due to DFNB3 is about 5% (7). The mutation in the gene *Myosin15A* is responsible for DFNB3. Protein Myosin15A encodes *Myo15A* gene (Sellers JR London 2000). The *HGF* gene responsible for the DFNB39 thus, *HGF* is often referred to in the literature as *HGF*/SF. The encoded protein contains a signal peptide, an alpha chain containing four kringle domains, and a beta chain (20).

The District Dadu inbred pedigrees with total 61 subjects 26 affected by prelingual deafness showed that the most probable pattern of inheritance was autosomal recessive. However, their linkage analysis revealed, among the 26 affected subjects, 8 were homozygous, 6 for the novel *MYO15A* gene including 2 normal and 3 for *HGF* gene. Given the extensive consanguinity of the pedigree, there might be at least one more deafness locus segregating to explain the condition in some of the subjects whose deafness is not clearly associated with *MYO15A* mutations, although over looked environmental causes could not be ruled out. (21).

The Ten families (DFLU-27, DFLU-34, DFLU-35, DFLU-36 and DFLU-37, DFLU-38, DFLU-39, DFLU-41, DFLU-43, DFLU-44) remained unlinked to DFNB3 and DFNB39 may be linked to the other known loci, and there is a high probability of reporting new loci/genes due to molecular heterogeneity in our population.

Family size and structure, the number of family members (who agree to participate in the linkage study) and accuracy of clinical data from each participant, all of these play a major role in the success of linkage analysis. In addition, for the analysis to be successful, it is also necessary to have accurate clinical information about each participant.

The unlinked families show that deafness is a complex genetic disorder and there are the chances that many other known loci are involved in the deafness in these families or some novel locus/gene may be involved, which means that it need further study to determine the locus and the gene causing mutation involved deafness in these population, which will help to prevent this genetic disorder in the population of District Dadu Sindh and Pakistan as well.

## CONCLUSION

The prevalence of deafness in Pakistan is 1.6 per 1,000 live births and 70% of hearing loss arises in consanguineous-families, much higher than 1 per 1000 live births worldwide.

## ACKNOWLEDGMENT

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