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Absence of *GJB2* gene mutations, the *GJB6* deletion (*GJB6*-D13S1830) and four common mitochondrial mutations in nonsyndromic genetic hearing loss in a South African population

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Abstract

Objective—The purpose of this study was to determine the prevalence of mutations in the *GJB2* gene, the *GJB6*-D13S1830 deletion and the four common mitochondrial mutations (A1555G, A3243G, A7511C and A7445G) in a South African population.

Methods—Using single-strand conformation polymorphism and direct sequencing for screening *GJB2* mutation; Multiplex PCR Amplification for *GJB6*-D13S1830 deletion and Restriction Fragment-Length Polymorphism (PCR-*RFLP*) analysis for the four common *mtDNA* mutations. We screened 182 hearing impaired students to determine the frequency of these mutations in the population.

Results—None of the reported disease causing mutations in *GJB2* nor any novel pathogenic mutations in the coding region were detected, in contrast to the findings among Caucasians. The *GJB6*-D13S1830 deletion and the mitochondrial mutations were not observed in this group.

Conclusion—These results suggest that *GJB2* may not be a significant deafness gene among sub-Saharan Africans, pointing to other unidentified genes as responsible for nonsyndromic hearing loss in these populations.

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Keywords

GJB2; *GJB6*-D13S1830; Molecular diagnosis; mtDNA mutations; Non-syndromic hearing loss; South African population

1. Introduction

Hearing loss (HL) is a common congenital disorder and one of the most distressing disorders affecting humanity. Worldwide, congenital deafness occurs in ~1 in 1000 live births. The aetiologies of hearing loss include both genetic and environmental factors, with genetic factors accounting for over 50%. In 30% of these, a syndrome is implicated. Non-syndromic hearing loss (NSHL) is most often sensorineural. Genetic hearing loss is classified into DFNA (autosomal dominant deafness, ~15–20%), DFNB (autosomal recessive deafness, ~80%), DFN (X-linked deafness, ~1%), and mitochondrial deafness (>1%) [1–5]. Hearing loss is noted to be both phenotypically and genetically heterogeneous [6–10], resulting from single gene mutations or from a combination of mutations in different genes. To date, 134 deafness loci, 57 DFNA loci and 77 DFNB, have been reported, while more than 40 genes for monogenetic NSHL and even more for syndromic HL have been cloned [11].

Mutations in *GJB2*, responsible for DFNB1 and DFNA3, are the most frequent cause of inherited hearing loss [12–16]. There are significant noted population differences in the distribution of *GJB2* alleles in all described populations, such as 167delT among Ashkenazi Jewish, 235delC among Japanese/Chinese, and R143W among Ghanaian populations respectively [4,17,18]. Mutations in *GJB2* have been shown to account for up to 50% of cases of non-syndromic genetic hearing loss among populations in Europe, North America and Asia [16,19]. Findings among Sudanese and Kenyan deaf children [20] document a low incidence of deafness causing *GJB2* variations.

The gene *GJB6* adjacent to *GJB2* on chromosome 13 was first suggested as a possible deafness gene in 1999 [21]. The most common mutation in *GJB6* is a 342-kb deletion, *GJB6*-D13S1830, which causes NSHL when homozygous, or when present on the opposite allele of a *GJB2* mutation. The *GJB6*-D13S1830 mutation occurs in up to 20% of the hearing-impaired USA population and may account for ~10% of all DFNB1 alleles with an extremely wide range based on ethnic origin [14].

Mitochondrial (mtDNA) pathology plays an important role in both inherited and acquired hearing loss, which may occur as the only symptom, or in association with well defined mitochondrial disorders such as MERRF syndrome, the MELAS syndrome, and the Kearns-Sayre syndrome. Nonsyndromic hearing loss due to mitochondrial mutations is uncommon, affecting two genes, the $tRNA^{Ser}$ gene and the 12S rRNA gene. The homoplasmic A1555G mutation in the 12S ribosomal RNA (rRNA) has been associated with aminoglycoside-induced hearing loss and NSHL among many families in various populations [22–30].

The Limpopo Province is the northernmost province of South Africa, covering an area of 123,910 km^2, about 10% total surface area of South Africa. It borders on Botswana in the west, Zimbabwe in the north, and Mozambique in the east (Fig. 1), with a 2001 census

population reported at 5,273,642, of whom 97.3% are of African descent [31]. The prevalence figures on deaf and hard of hearing persons in the Limpopo province, based on the department of health and social services 2004 mid-year estimates [31] indicated that about 44,542 persons in the province were deaf or hard of hearing.

The objective of the current study was to determine the frequency of mutations in *GJB2*, the deletion of *GJB6*-D13S1830 and four common mitochondrial mutations (A1555G, A3243G, A7445G, and T7511C) among the South African children with NSHL.

2. Materials and methods

Ethics approval was obtained from the Committee for Research on Human Subjects (Medical), University of the Witwatersrand and the University of Miami. Informed consent was obtained from the participants 18 years or older or from the parents or guardians with assent from the children.

2.1. Reference population and participants

The reference population for this study was the indigenous African population of the Limpopo Province of South Africa comprising of Venda, Pedi/Northern Sotho, Tsonga (Shangaan) and Swati speaking individuals. The participants included 182 South African hearing impaired students from two schools for the deaf in the province, aged 5–21 years of age, with a male to female ratio of 1:0.6 (Table 1). Of these, 36 had a family history of hearing loss while 146 reported no affected family members. The 63 normally hearing controls were recruited from patients attending the ENT/Head and Neck clinic at the Pietersburg Provincial hospital for surgery of non-otologic conditions (Fig. 2).

2.2. Procedures

A full history was taken and a comprehensive clinical examination performed on each of the participants. The clinical examination included assessment for dysmorphic features as well as urinalysis. Audiometric assessments included tympanometry, transient evoked otoacoustic emissions (TEOAEs), as well as pure tone audiometry. All the participants were noted to have stable sensorineural hearing loss. The exclusion criteria used to rule out syndromic and acquired hearing loss included: stigmata of known syndromes; craniofacial anomalies; signs of neurodegenerative disorders such as neurofibromatosis; history or signs of toxoplasmosis, rubella, cytomegalovirus, herpes, syphilis (TORCH); HIV infections during pregnancy; low birth weight – less than 1500 g; documented low apgar score (less than 4 at 1 min, less than 6 at 5 min); history of anoxic or hypoxic events or prolonged mechanical ventilation; history of hyperbilirubinaemia; history of ototoxic drug use; history of bacterial meningitis and history of head trauma.

2.2.1. DNA isolation and mutation screening—Genomic DNA was extracted from peripheral blood using the salting out procedure as described by Miller et al. [32].

2.2.2. Sequencing techniques for GJB2—Bidirectional sequencing was used to screen genomic DNA for variations in the single coding exon of *GJB2*, as previously described elsewhere [19]) in all 182 participants (primer sequences available on request).

2.2.3. Multiplex PCR Amplification for GJB6-D13S1830—The *GJB6* (D13S1830) deletion was screened using the method described by Wu et al. [33]. Polymerase chain reaction (PCR) was used to amplify DNA fragments simultaneously with each of the three sets of primers in a multiplex state.

2.2.4. Restriction Fragment-Length Polymorphism (PCR-RFLP) analysis for mtDNA mutations (A1555G, A3243G, A7445G, and T7511C)—To detect each of the four mtDNA mutations, PCR was used to amplify mtDNA fragments encompassing the mutation site. This was followed by digestion with a restriction endonuclease that differentially cleaves PCR products containing normal versus mutant sequences. Digestion products were then electrophoresed through 2% agarose gels. The 12SrRNA A1555G and $tRNA^{Ser\ (UCN)}$ A7445G mutations were screened using the method described by Pandya et al. [34]. The T7511C mutation was screened using the method described by Sue et al. [35].

To detect the presence of the A1555G mutation, the PCR fragment was cut with *Bsm*AI. The PCR product of 1605 bp normally yields three bands of sizes 1106, 293, and 206 bp in individuals without A1555G. The A1555G mutation leeds to a lack of the digestion site, yielding two bands of 1399 bp and 206 bp. For the detection of the mtDNA A7445G mutation, the PCR fragment was digested with the restriction enzyme *Xba*I. In unaffected subjects, the digestion normally results in two 400 and 262 bp sized bands. The A7445G mutation leads to the loss of the *Xba*I cutting site, resulting in a single 662 bp size band. To identify the A3243G mtDNA mutation, PCR was performed with the following primers: 5′-GCCTTCCCCCGTAAATGATA-3′ and 5′-AGGTTGGCCATGGGTATGT-3′ using standard PCR conditions. Digestion of the PCR product was carried out using the restriction enzyme *Apa*I. The presence of mtDNA A3243G leads to the cleavage of the 161 bp PCR product into two fragments of sizes 87 bp and 74 bp respectively. The A3243G mutation can further be confirmed by bi-directional sequencing. This mutation can be present at a very low level of heteroplasmy (<10%) and in such cases is difficult to detect by this method.

To screen the T7511C mtDNA mutation, we amplified a 226 bp fragment using primers corresponding to nucleotide positions 7397–7417 “forward” and 7633–7613 “reverse”. The mutant mtDNA creates a novel *Mbo*II restriction site, which can be detected by PCR-RFLP analysis. The wild-type 226 bp PCR product is cleaved into two 196 bp and 30 bp sized fragments respectively, whereas the T7511C mutation leads to cleavage of the PCR product into three fragments of 120 bp, 76 bp and 30 bp [35].

3. Results

Overall, audiology results demonstrated the degree of sensorineural hearing loss to be severe to profound in 22.8% and profound in 75% of the participants, the majority exhibiting flat (70.1%) or sloping (23.4%) audiograms that were mainly symmetrical (81.5%). Low frequency ascending audiograms were found in 6% of the participants, while one subject had a mid-frequency u-shaped audiogram. The rest of the participants had moderate to severe hearing loss. This study did not test for progression of the hearing loss as the participants were not followed up. Among the participants, 112/182 (61.5%) reported a definite pre-lingual onset of hearing loss. Of the rest, 23 (12.6%) reported age at diagnosis

of between 13 and 24 months of age, 10 (5.5%) after 25 months while 37 participants (20.3%) were unsure of the age of onset. A history of consanguinity was reported in 14 participants (7.7% of the study group). When analyzed by language group, the results showed that all these participants were of the Pedi/Northern Sotho language group.

None of the 182 hearing impaired individuals exhibited any of the reported disease causing mutations of *GJB2*, including 35delG, or any other potentially pathogenic *GJB2* mutations (Table 2). Neither was the 342-kb *GJB6*-D13S1830 mutation, nor the four deafness-associated point mutations in mtDNA, A1555G, A3243G, A7445G, and T7511C, detected in any of the study participants. There was, however, a high frequency of two *GJB2* variants, C>T at position g.3318–15 and C>T at position g.3318–34, which occurred in 21.4% and 46.2% of the deaf cohort respectively, and in 35% and 42.6% of a normal hearing control group (n = 63) respectively (Table 2).

4. Discussion

The current study was conducted in a population with a long history of apartheid or “separate development” where inter-racial marriages were previously strongly discouraged, and at one time punishable by law. The studied population groups, especially the Venda [36], and the Pedi/Northern Sotho [37], were in the past reported to practice consanguineous mating widely. As such, this study group was felt to be more representative of the non-admixed genetic pool of indigenous Africans from this region.

4.1. GJB2

Deafness causing *GJB2* variations have been reported in many parts of the world, with marked variation in the reported distribution patterns among different ethnic groups [15,24] with a propensity to occur frequently in some population groups, while seemingly absent in others [17,18,20,38,39]. Until relatively recently, there was very little data on *GJB2* variations among African population groups. The findings of the current study, indicating that *GJB2* does not play a significant role in deafness in this South African population, is not surprising. The *GJB2* mutations 35delG, 167delT, and 235delC, common in Caucasian, Ashkenzi Jewish, and East Asian (Chinese, Japanese and Korean) populations respectively, have been shown to be due to a founder effect rather than a mutational hotspot [40–43]. The same is believed of the R143W mutation which is highly prevalent in the Ghanaian population from Adamarobe village, where it was demonstrated in one study to occur in 21/21 deaf participants [17,38] (Tables 3 and 4). The W24X mutation which is most prevalent in the Indian and Romany (gypsies) populations may also be due to a founder effect.

The 35delG allele of *GJB2*, reported to range from 10% to 20% among Caucasians of northern European descent, was as high as 30–40% in the Mediterranean regions [44,45]. Only one heterozygote for the 35delG mutation was identified among 100 African Brazililians [46]. Whereas 35delG was detected among 5 of 139 Sudanese deaf children, some with a history of consanguinity [40,47], it was not detected among 173 and 190 African American deaf individuals respectively [40,47]. Similar to our findings, the absence of 35delG mutation has also been reported in 406 Kenyan deaf children [20] and in the

Omani population [48]. Neither was it detected in 365 profoundly deaf students in Ghana [17].

A study of *GJB2* variants in a Ghanaian deaf population identified more variants in the C-terminus of the gene compared with findings in other parts of the world [17]. Although a small percentage of carriers of *GJB2* variants within the coding region of *GJB2* was reported, identified among 95/406 Kenyan and 21/139 Sudanese deaf individuals, the 14 variants identified (other than 35delG) were all believed to be benign polymorphisms, since for most of the identified variations an association with ARNSHL could not be made. Of interest is the significantly high occurrence of two variants reported among the South Africans in the current study, namely g.3318–34C>T and g.3318–15C>T. Whereas the prevalence of these variations among Kenyan and Sudanese deaf subjects was 12.7% (g. 3318–34C>T) and 6.45% (g.3318–15C>T) respectively, the current study detected them in 46.2% and 21.4% of the study population respectively. None of the other variants identified in the Sudanese and Kenyan populations were identified in the South African population. The finding of a high frequency of these two variants, g.3318–34C>T (42.6%) and g.3318–15C>T (35%), among the 63 normally hearing control participants strongly suggests that these variations are polymorphisms and do not contribute to the aetiology of the observed non-syndromic SNHL in this population. Our data therefore supports the notion that *GJB2* does not play a significant role in non-syndromic hearing loss in the African population. Because the current study participants may be considered to be more representative of the non-admixed genetic pool of indigenous Africans in this region, the negative findings of this study are significant for deafness research in Southern Africa and indeed for the rest of Africa.

4.2. GJB6

The most common mutation in *GJB6* is the 342-kb *GJB6*-D13S1830 deletion, which causes NSHL when homozygous, or when present on the opposite allele of a *GJB2* mutation. The *GJB6*-D13S1830 mutation, which is most frequent in Spain, France, the United Kingdom, Israel and Brazil (5.9–9.7% of all DFNB1 alleles) is less frequent in the USA, Belgium and Australia (1.3–4.5% of all DFNB1 alleles), and very rare in Southern Italy [13] In Northern Italy, it was found at frequencies similar to those of other European countries [49]. The deletion was also detected in Germany [50], but not in Austria [51], Turkey [19,52,53], China [19], nor among African American populations [16,54]. Similar to studies among other African populations, the 342-kb deletion was not detected in the current study. Since the coding region of *GJB6* was not sequenced, its role in the South African population remains uncertain.

4.3. Mitochondrial DNA mutations

Although the frequency of mitochondrial (mt) genetic hearing loss is unknown, studies suggest that mitochondrial mutations play an important role in inherited and acquired hearing impairment. SNHL is present in 42–70% of individuals with mt disorders which may be syndromic and non-syndromic [55–57]. Mutations in mtDNA were also associated with both aminoglycoside-induced and nonsyndromic hearing loss [42,56].

The A1555G mutation affecting the *MTRNR1* gene, was the first mtDNA mutation to be associated with non-syndromic hearing loss [58], and has been found in 0.5–1.0% of hearing impaired Caucasians [26,58]. It was identified in 0.09% (1/1,161) American [59] and 0% (0/1042) Argentinean [60] populations respectively. A much higher prevalence has been reported among Spanish [23] and Asian patients [61].

A limited number of studies have been performed on the four common mitochondrial mutations in South African deaf individuals (Table 4). The A1555G mutation was identified in one of 106 'Black' control samples [62]. In another study involving a large pedigree of mixed ancestry, 97 South African family members were genotyped and 76 of them were found to be A1555G-positive and are therefore believed to be at risk of developing irreversible hearing loss if exposed to aminoglycosides [63]. This mutation has been found in one South African family [63] and one from Zaire [28].

We report on the analysis of 182 deaf probands for four common mt mutations, A1555G, A3243G, A7445G and T7511C, which are known to cause late onset deafness. Our study involved young subjects from a rural population with few medical records. Because of the widespread use of ototoxic drugs in the region for other medical conditions, it was important to rule out these four mutations as a cause of deafness in the study group. The four *mtDNA* mutations were not detected in our cohort of 182 deaf participants. The mitochondrial genome was not further investigated.

It has been generally noted that concrete health data are often not available to policy makers or managers implementing policy, because appropriate research addressing the priority issues has not been conducted. This is especially true of developing countries where inherited disorders tend to be overshadowed by the disease burden of infectious disease. With the advent of clinical neonatal hearing screening in South Africa, the need for a molecular diagnostic service becomes urgent. The development of molecular diagnostic services for genetic deafness would prevent the frustration of incomplete aetiological diagnosis among deaf individuals, their families and the clinicians who treat them. This can only be done on the background of adequate knowledge of the common deafness genes in local population groups. It is therefore compelling that the deafness genes among African populations be identified.

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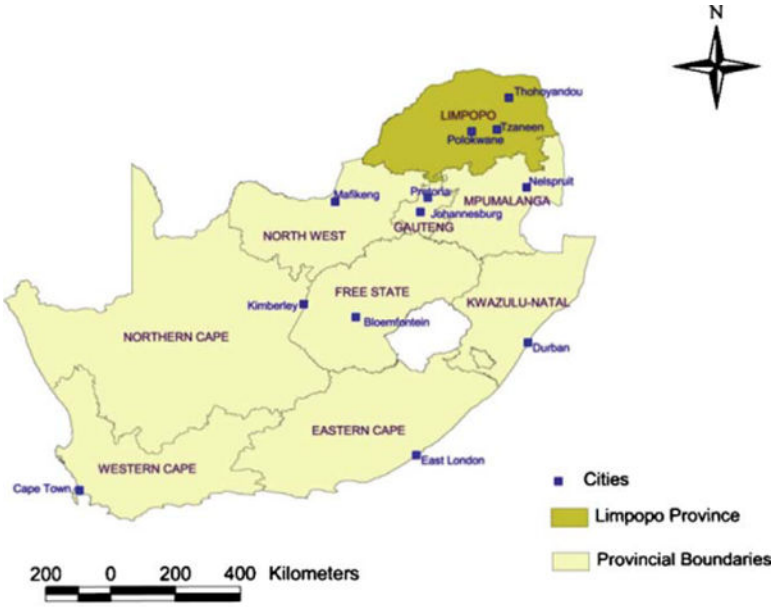

Fig. 1.
Location map of the study area, Limpopo Province, within South Africa.

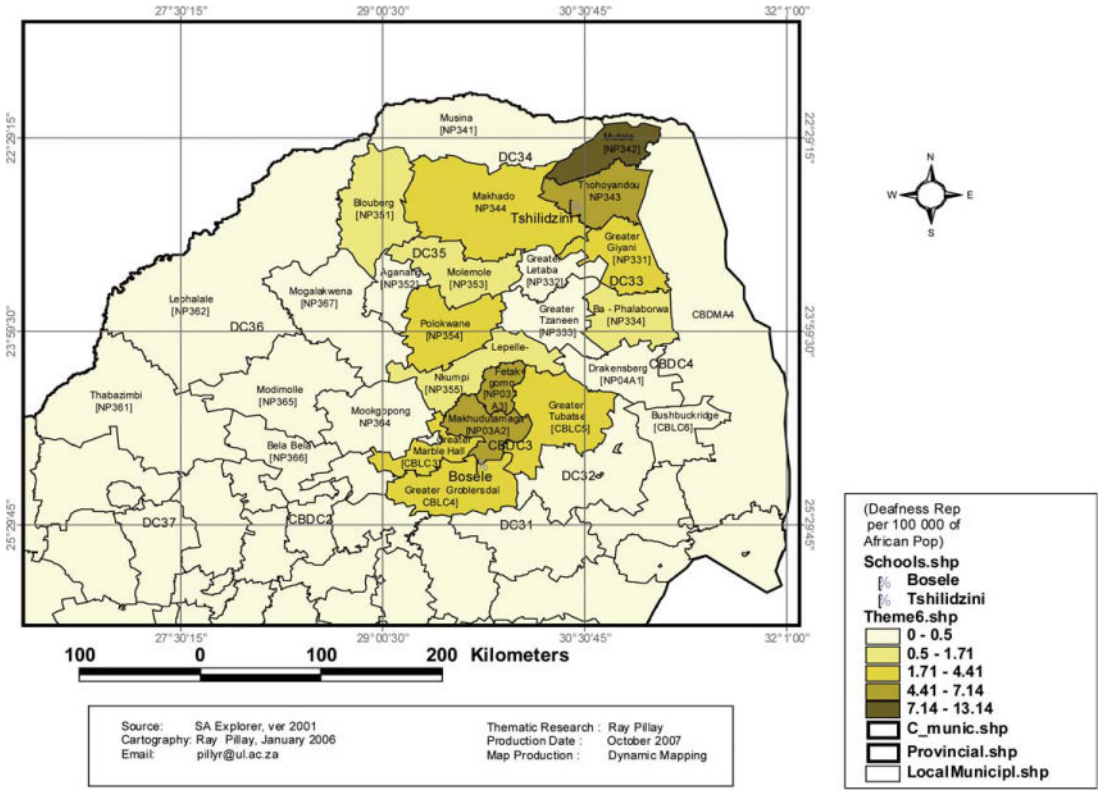

Fig. 2.

Spatial distribution of nonsyndromic hearing loss according to municipality within the Limpopo Province of South Africa normalized to the indigenous black African population from this region.

Table 1

Demographic information of 182 hearing impaired students from the Limpopo Province of South Africa.

Age (years)						**Sex**		**Language group**				**Reported consanguinity (%)**
<5	5–9 (%)	10–14 (%)	15–19 (%)	20+ (%)	Unknown (%)	Male (%)	Female (%)	Venda (%)	Pedi/N.Sotho (%)	Tsonga (%)	Swati (%)	
0	55 (30.2)	72 (39.6)	45 (24.7)	6 (3.3)	4 (2.2)	62 (34.1)	120 (65.9)	84 (46.2)	80 (44.0)	13 (7.1)	5 (2.7)	14 (7.7)

Table 2

GJB2 variations observed in a deaf population and control group from the Limpopo Province of South Africa.

Nucleotide change	Domain	Cohort genotype frequency	Control group genotype frequency	% of cohort (*N*=182)	% of control group (*N*=63)
g.3318–34C>T (heterozygous)	5″-UTR	62	26	34	41
g.3318–34C>T (homozygous)	5″-UTR	22	1	12	1.6
g.3318–15C>T (heterozygous)	5″-UTR	34	17	18.7	27
g.3318–15C>T (homozygous)	5″-UTR	5	5	2.7	8
g.3318–34C>T (heterozygous)/g.3318–15C>T (heterozygous)	5″-UTR	11	6	6	10
g.3318–34C>T (homozygous)/g.3318–15C>T (heterozygous)	5″-UTR	1	0	0.5	0
g.3318–34C>T (heterozygous)/g.3318–15C>T (homozygous)	5″-UTR	0	1	0	1.6

Table 3

GJB2 variations identified among deaf African populations.

Nucleotide change	Domain	Genotype	Affected	Sample size	Ethnic group	Region/country	Study/reference
427T>C		R143W/R143W	21	21	Ghanaians	Adamarobe village/**Ghana**	Brobby et al. [38]
427T>C		R143W/R143W	51				
427T>C		R143W/wt	4				
427T>C, 35insG		R143W/35insG	1				
427T>C, 236T>C		R143W/L79P	1				
427T>C, 608TC>AA		R143W/I203K	1	365	Ghanaians	Ashanti, Central, Eastern, Greater Accra, Upper East, Upper West, Volta and Western regions/ **Ghana**	Hamelmann et al. [17]
427T>C, 641T>C		R143W/L214P	1				
533T>C		V178A/V178A	2				
551G>A		R184Q/wt	1				
589G>T		A197S/wt	1				
427T>C		R143W/wt	1	50	African American	?/North America	Pandya et al. [16]
−35T>G	5′-UTR		1	183	Sudanese	?/**Sudan**	Gasmelseed et al. [20]
−34C>T (homo)	5′-UTR		10 in total	183	Sudanese, Kenyans	?/**Sudan**	Gasmelseed et al. [20]
				406		?/**Kenya**	
			22	182	Pedi, Venda and Tsonga speaking language groups	Limpopo province/**South Africa**	Current study
−34C>T (het)	5′-UTR		65 in total	183	Sudanese, Kenyans Pedi, Venda and Tsonga speaking language groups	?/**Sudan**	Gasmelseed et al. [20]
				406		?/**Kenya**	
			62	182	Pedi, Venda and Tsonga speaking language groups	Limpopo province/**South Africa**	Current study
−15C>T (homo)	5′-UTR		5	182		Limpopo province/**South Africa**	Current study
−15C>T (het)	5′-UTR		38 in total	183	Sudanese, Kenyans Pedi, Venda and Tsonga speaking language groups	?/**Sudan**	Gasmelseed et al. [20]
				406		?/**Kenya**	
			34	182		Limpopo province/**South Africa**	Current study
−6T>A (het)			2	406	Kenyans	?/**Kenya**	Gasmelseed et al. [20]
35delG (homo)	IC1	35del G/35delG	5	183	Sudanese	?/**Sudan**	
35delG		35del G/wt	*?7*	50	African American	?/North America	Pandya et al. [16]

Nucleotide change	Domain	Genotype	Affected	Sample size	Ethnic group	Region/country	Study/reference
249C>G		F83L/wt	1	50	African American	?/North America	Pandya et al. [16]
78C>T	TM1	T26/wt	1				
109G>A		V371I/wt	1	406	Kenyans	?/**Kenya**	
138_142del	EC1	D46_Q48del	1				
		insE/wt					
187C>T		N62/wt	3 in total	183	Sudanese, Kenyans	?/**Sudan**	
				406		?/**Kenya**	
195C>A		Y65X/wt	1				Gasmelseed et al. [20]
310A>C	IC2	R104/wt	1	183	Sudanese	?/**Sudan**	
380G>A		R127H/wt	1				
457G>A	TM3	V153I/wt	2				
478G>A	EC2	G160S/wt	1				
499C>A		V167M/wt	4	406	Kenyans	?/**Kenya**	

African populations were in bold.

Table 4

Reported mutations underlying nonsyndromic hearing loss in African populations.

Gene	Protein	Locus	Mutation	Affected/sample size	References	Ethnic group	Geographical region of origin
GJB2	*Connexin 26*	13q11–12 (DFNB1)	c.35delG	*7/50 (classified P/V)	Pandya et al. [16]	African American	North America
				**1/100	Oliveira et al. [46]	African Brazilians	Brazil
				5/139	Gasmelseed et al. [20]	Sudanese	Northern Africa
			p.R143W	21/21	Brobby et al. [38]	Ghanaian from Adamarobe	West Africa
				51/365	Hamelmann et al. [17]	Ghanaian excl. Adamarobe	West Africa
				1/50	Pandya et al. [16]	African American	North America
			No *GJB2* mutation detected	23/23	Samanich et al. [54]	African American	North America
				406/406	Gasmelseed et al. [20]	Kenyan	East African
				182/182	**Current study**	Venda, Pedi, and Tsonga	South Africa (Limpopo province)
GJB6			**No GJB6-D13S1830 detected**	23/23	Samanich et al. [54]	African American	North America
				50/50 **182/182**	Pandya, 2003 **Current study**	African American Venda, Pedi, and Tsonga	North America South Africa (Limpopo Province)
Mitochondrial genome			mtDNA genotype				
12S rRNA			A1555G	1/106 (control blood)	Bardien et al. [62]	Black population * control blood samples	South Africa (Western Cape Province)
				76/97 (single family)	Human et al. [63]	Mixed Ancestry ** single large family	South Africa (Western Cape Province) South Africa (Limpopo province)
tRNALeu(UUR)			**No A1555G detected No A3243G detected**	**182/182** 23/23	**Current study** Samanich et al. [54]	Venda, Pedi and Tsonga African American	North America
				182/182	**Current study**	Venda, Pedi, and Tsonga	South Africa (Limpopo Province)
ND1			T3308C	2/36 (of a kindred)	Sue et al. [35]	African American	North America
tRNASer (UCN)			**No A7445G detected**	23/23	Samanich et al. [54]	African American	North America

Gene	Protein	Locus	Mutation	Affected/sample size	References	Ethnic group	Geographical region of origin
				182/182	**Current study**	Venda, Pedi, and Tsonga	South Africa (Limpopo Province)
			No T7511C detected	**182/182**	**Current study**	Venda, Pedi, and Tsonga	South Africa (Limpopo Province)

The results of this were in bold.