Chapter 5:

Assessment of genes located within the candidate region

CHAPTER 5

ASSESSMENT OF GENES LOCATED WITHIN THE CANDIDATE REGION

5.1 INTRODUCTION

The aim of this chapter is to investigate the relative probability of each gene within the critical region being responsible for the *bronx waltzer* phenotype. Each of the 52 genes which have been mapped to the 2.6Mb region between the flanking markers established in Chapter 3 is considered to be a candidate gene. Based on the approximation by the Mouse Sequencing Consortium of 30,000 genes in 2.5Gb euchromatic DNA, the average gene density of the mouse genome is estimated to be 12 per megabase (Waterston *et al.* 2002). The *bronx waltzer* candidate region with 20 genes per megabase is thus relatively very gene-rich and it would be unwieldy to carry out functional studies on each of these genes. Hence it was considered necessary to prioritise for further investigation those genes which were thought to have the highest likelihood of causing the balance and hearing defects observed in mice homozygous for the *bronx waltzer* mutation. This prioritisation was carried out by assessing each gene in a number of different ways, as described below.

5.1.1 What makes a good candidate gene?

Before analysing each potential candidate gene it is important to set out what we are looking for in such an analysis. The functioning of the mechanisms required for hearing has yet to be fully elucidated and it is therefore very difficult to completely rule out any gene since it may play an as yet undefined role in the ear. However, it is possible to pick out characteristics which may suggest a potential role in the ear and hence warrant further investigation. The

factors to be considered in making this decision are outlined in the following paragraphs.

5.1.1.1 A potential role in pathways known to be required for hearing

Through a combination of the cloning of genes responsible for various types of genetic deafness and tissue-specific gene discovery, some of the molecules and mechanisms required for hearing function have been characterised. Of those genes which have been implicated in sensorineural deafness like that seen in the *bronx waltzer* mutants, the roles which they play allow them to be grouped into a number of categories which are summarised in Table 5.1. With these groupings in mind, candidate genes can be assessed as to whether their known or predicted functions might be involved in similar processes. For example, given the importance of maintaining ion gradients in the inner ear for sound transduction to take place (see Section 1.1.2.2) and the discovery of a number of deafness genes involved in ion transport, a gene manifesting as an ion transporter or channel might make an interesting candidate. Similarly, with the knowledge that stereocilia require highly-organised actin bundles to maintain the shape so crucial to their function, any molecule exhibiting a potential role in cytoskeletal organisation or interaction would stand out as one warranting further investigation. The association of several genes shown to be involved in actin bundling, as well as a large number of unconventional myosins – molecular motors known to interact with actin – with forms of genetic deafness lends weight to this proposition.

However, *bronx waltzer* possesses a uniquely specific phenotype which makes it unlikely that such generic pathways should be seriously disrupted. In the organ of Corti only the inner hair cells are affected, and these begin to develop at E17.5 as expected but almost immediately degenerate and most are reabsorbed shortly after birth. A similar series of events can be observed in the maculae and cristae of the vestibular system, where hair cells begin to develop but fail to mature and are reabsorbed into the epithelium. This

suggests that the mutated gene lies downstream of the cell fate specification of hair cells into IHCs and OHCs, and since the outer hair cells appear normal, it must represent a factor necessary only for the correct differentiation or maintenance of inner hair cells and vestibular hair cells, rather than for general hair cell development or function. Even so, it is still possible that the phenotype may result from a mutation in a part of a more widely expressed gene which is transcribed only in the cell types affected, or indeed in a cell-type specific regulatory element associated with such a gene.

Table 5.1: Biological roles of identified genes for human nonsyndromic sensorineural hearing loss (Adapted from Friedman and Griffith 2003)
NSHL, nonsyndromic hearing loss
[a]Only one mutant allele of this gene has been reported in one family segregating NSHL. An inconsequential variant may be in disequilibrium with the actual mutant allele in a closely linked gene.
[b]In the absence of functional clues, proteins are categorised as having an unknown function

Biological role	**Gene product**	**Gene symbol**	**NSHL locus/location**	**Phenotypes of allelic mutations**	**Mouse model (gene symbol)**
Adhesion	cadherin 23 protocadherin 15	*CDH23* *PCDH15*	*DFNB12*/10q21-q22 *DFNB23*/10q21-q22	Usher syndrome 1D Usher syndrome 1F	waltzer (*v*) Ames waltzer (*av*)
Cytoskeleton	diaphanous 1 espin	*DIAPH1*[a] *ESPN*	*DFNA1*/5q31 *DFNB36*/1p36	- -	- jerker (*je*)
Enzyme	Transmembrane protease, serine 3	*TMPRSS3*	*DFNB8*, *B10*/21q22.3	-	-
Extracellular matrix	cochlin α2(XI) collagen otoanchorin α-tectorin	*COCH* *COL11A2* *OTOA* *TECTA*	*DFNA9*/14q12-q13 *DFNA13*/7q22.1 *DFNB22*/6p12.2 *DFNA8,A10,A12,B21*/ 11q22-q24	- Type 3 Stickler syndrome, OSMED - -	- *Col11a2*$^{-/-}$
Gap junction	connexin 26 connexin 30 connexin 31 connexin 43	*GJB2* *GJB6*[a] *GJB3* *GJA1*	*DFNB1,A3*/13q12 *DFNB1,A3*/13q12 *DFNA2*/1p34 n.a./6q21-q23.2	Keratodermia Clouston's syndrome - -	*Gjb2*$^{-/-}$ *Gjb6*$^{-/-}$ *Gjb3*$^{-/-}$ -
Ion channel, transporter	Potassium channel NQ4 pendrin	*KCNQ4* *SLC26A4*	*DFNA2*/1p34 *DFNB4*/7q31	- Pendred syndrome	- *Pds*$^{-/-}$
Integral membrane protein (predicted)	Transmembrane inner ear-expressed Transmembrane cochlear-expressed gene 1	*TMIE* *TMC1*	*DFNB6*/3p14-p21 *DFNB7*, B11, A36/ 9q13-q21	- -	spinner (*sr*) Beethoven (*Bth*), deafness (*dn*)

Biological role	**Gene product**	**Gene symbol**	**NSHL locus/location**	**Phenotypes of allelic mutations**	**Mouse model (gene symbol)**
Motor	myosin IA	*MYO1A*	*DFNA48*/12q13-q15	-	-
	myosin IIA	*MYH9a*	*DFNA17*/22q13	-	-
	myosin IIIA	*MYO3A*	*DFNB30*/10p12.1	-	-
	myosin VI	*MYO6*	*DFNA22,B37*/6q13	-	Snell's waltzer (*sv*)
	myosin VIIA	*MYO7A*	*DFNB2,A11*/11q12.3	Usher syndrome 1B	shaker 1 (*sh1*)
	myosin XVA	*MYO15A*	*DFNB3*/17p11.2	-	shaker 2 (*sh2*)
	prestin	*PRES*	n.a./7q22.1	-	*Pres*$^{-/-}$
	myosin heavy chain 14	*MYH14*	*DFNA4*/19q13.33	-	-
Macromolecular organiser	harmonin	*USH1C*	*DFNB18*/11p15.1	Usher syndrome 1C	-
Neuron/synapse	otoferlin	*OTOF*	*DFNB9*/2p22-p23	-	-
	whirlin	*WHRN*	DFNB31/9q32-q34	-	whirler (*wi*)
Tight junction	claudin 14	*CLDN14*	*DFNB29*/21q22	-	-
Translation	12S rRNA	-	mitochondrial gene	-	-
	tRNA-Ser(UNC)	-	mitochondrial gene	Myoclonic epilepsy, ataxia	-
Transcription regulator	Eyes absent 4	*EYA4*	*DFNA10*/6q22-q23	-	-
	Pou domain, class 4, factor 3	*POU4F3*	*DFNA15*/5q31	-	*Brn3c*$^{-/-}$, dreidel (*ddl*)
	Transcription factor, CP2-like 3	*TFCP2L3*[a]	*DFNA28*/8q22	-	-
	Pou domain, class 3, factor 4	*POU3F4*	*DFN3*/Xq21.1	-	*Pou3f4*$^{-/-}$, sex-linked fidget (*slf*)
Unknown function[b]	crystalline, mu	*CRYM*	n.a/16p13.11-p12.3	-	-
	DFNA5	*DFNA5*[a]	*DFNA5*/7q15	-	-
	stereocilin	*STRC*	*DFNB16*/15q21-22	-	-
	wolframin	*WFS1*	*DFNA6,A14,A38*/4p16	Wolfram syndrome (DIDMOAD)	-

5.1.1.2 Expression in the inner ear during development

Since the *bronx waltzer* phenotype is restricted to the sensory patches of the inner ear and is visible from E17.5, the point at which the hair cells begin to develop (see Section 1.2.3.6), it might be expected for the mutated gene to be expressed at this time point in the tissues affected. Specifically, one might anticipate expression of the gene in normal inner hair cells and vestibular hair cells at E17.5. However, it is also possible that the gene may be expressed not in these cells themselves but in cells nearby which have an inductive influence over them. For example, the supporting cells and pillar cells of the organ of Corti may secrete some factor necessary for the proper maintenance of inner hair cells, or conversely may produce an inhibitory signal which would normally be switched off.

Since the bronx waltzer phenotype so specifically affects IHCs which have abundant afferent innervation and not OHCs which are associated primarily with efferent nerve fibres, it is important to consider the possibility of the mutation affecting primarily neuronal tissue. However, although cochlear and vestibular neurons eventually degenerate in the absence of hair cells (Schuknecht 1974, pp 333), it has been shown that hair cells are able to survive even when the development of neurons is inhibited (Fritzsch *et al.* 2005) and that normal growth of nerve fibers can occur in the absence of hair cells (Fritzsch *et al.* 2005). Thus it is unlikely that the IHC degeneration seen in the *bronx waltzer* cochlea is a result of disruption to their innervation, unless the mutation manifests in a dominant negative manner, actively triggering the degeneration of IHCs.

It should also be considered that a gene may cause developmental defects in hearing in the absence of any detectable expression within the ear itself. An example of such a gene is the transcription factor *Hoxa1*. Murphy and Hill (1991) found it to be expressed in the neuroectoderm and mesoderm of the developing mouse hindbrain, adjacent to site of ear formation. No expression

was observed in the otic placode, the otic pit or the otocyst, and yet mice homozygous for a targeted deletion of the gene were found to have malformed inner ears, in addition to altered rhombomeres 4 and 5 of the hindbrain (Lufkin *et al.* 1991; Chisaka *et al.* 1992). This was thought to be due to the inductive influence of the neural tube on the morphogenesis of the ear (Gallagher *et al.* 1996; Noramly and Grainger 2002). However, this type of gross ear abnormality contrasts sharply with the highly tissue-specific nature of the *bronx waltzer* phenotype and it is unlikely that a remotely expressed gene could exert such fine control over different cell types. Thus it is relatively certain that the *bv* gene would not be expected to act in this manner, and will instead be found to be expressed within or in close proximity to the affected tissues of the organ of Corti and vestibular sensory patches.

5.1.1.2.1 Sources of expression data

For some previously characterised genes the expression patterns may have already been published, although it is important to verify that the experiment was carried out at an appropriate time point and that the authors looked for expression in the ear and at an appropriate time point since for many genes the expression may have only been characterised in a small selection of adult tissue types.

A second source of expression data exists in the form of searchable libraries or databases containing details of genes or ESTs which have been demonstrated to be expressed in a relevant tissue type. In this case, examples include the Washington University Inner Ear Protein Database (Thalmann and Thalmann) which lists proteins identified from the tissues and proteins of the guinea pig inner ear by 1D- and 2D-Polyacrylamide Gel Electrophoresis (PAGE), and the Morton human Fetal cochlear cDNA library (Robertson *et al.* 1994) which holds sequences of ESTs identified by a combination of subtractive hybridization and differential screening strategies. Other cDNA libraries of interest include the full-length mouse subtracted inner ear library

prepared by Beisel *et al.* (Beisel *et al.* 2004; Pompeia *et al.* 2004)) and the EST database resulting from the screening of the cDNA population from mouse Organ of Corti ranging from P5 to P13 to include the maturation of hair cells and onset of hearing (Pompeia *et al.* 2004). In addition, a number of microarray studies have identified genes or ESTs differentially expressed in the mouse inner ear (Chen and Corey 2002), mouse cochlea (Morris *et al.* 2005) or the rat vestibular epithelium (Cristobal *et al.* 2005).

The expression of individual genes, including those which have only been predicted, can also be investigated directly using techniques such as *in situ* hybridisation, Northern blotting and RT-PCR. For each of these techniques it is necessary only to have access to the sequence of the gene and a sample of the tissue to be tested.

5.1.1.3 The eye and the ear

Another indicator of a potential role in the ear may be when a gene has a known function or expression in the eye. Common features in their development have led to the suggestion that the eye and ear share a shared evolutionary origin, with the sensory receptors in both organs having evolved from a common ancestral ciliated cell (Popper and Fay 1997). This proposition is supported by evidence gathered in *Drosophila* by Jarman *et al.* (1994) who showed that *Atonal* (*Ato*) is responsible not only for the establishment of chordotonal precursors and controlling the differentiation of the modified chordotonal organs in the antenna which form Johnston's organ, the auditory organ of the fly, but is also the proneural gene for photoreceptors in the compound eye. Niwa *et al.* (2004) subsequently examined whether there exist any similarities in the conditions necessary for the formation of these different sensory organs. The development of all three organs was found to be initiated by the expression of *decapentaplegic* (*DPP*), with all of them failing to form when its signalling was blocked by overexpression of the inhibitor *Daughters Against DPP* (DAD). In addition, all

three organs form simultaneously and were each shown to rely on the presence of the hormonal factor ecdysone at an early third instar larval stage for proper development to take place, suggesting that ecdysone represents a temporal initiation signal common to the eye, Johnstone's organ and the chordotonal organ. This conservation of early developmental function in sensory organ formation suggests that there once existed a primitive sensory organ precursor which would differentiate according to the identity of its segment of origin.

A second reason to consider genes expressed in the eye as candidates for a deafness phenotype is the existence of single gene disorders which affect both the ear and the eye. The most widespread of these are the various forms of Usher Syndrome (Keats and Corey 1999) which are characterised by congenital sensorineural deafness and progressive retinitis pigmentosa. The syndromes are clinically variable and genetically heterogenous, with some but not all of the genes responsible having been identified. Many of these interact with each other to form the transmembrane complex which binds stereocilia bundles together (Ahmed *et al.* 2003) but for others the functions are unclear. The fact that a number of genes are necessary for proper development and maintenance of both the ear and the eye points to some overlapping of gene function. In addition, the mouse models for Usher Syndrome such as shaker-1 (*Myo7a*) and waltzer (*Cdh23*) exhibit hearing and vestibular defects show only minor abnormalities in the retina. Libby *et al.* (2003) showed that mice with mutations in *Cdh23* have abnormal retinal function when assessed by electroretinography but no anatomical abnormality could be detected, and mice with mutations in *Myo7a* have recently been shown to have abnormally distributed melanosomes in the retinal pigmented epithelium (Gibbs *et al.* 2004). Hence it is feasible that a gene affecting both the ear and the eye may initially manifest as solely a hearing phenotype when its function is disrupted in the mouse and retinal function is not specifically investigated as in the case of *bronx walter*. It has been suggested that the genes may play different roles in

the two species or that a level of functional redundancy may exist in the mouse. Other important differences include environmental factors such as light exposure since laboratory mice are generally kept in relatively dim light conditions, and also the lifespan of the mouse, which may not be long enough for the progressive degeneration of the retina to become measurable (Zheng *et al.* 2005). More significantly, the diversity in severity of phenotype in humans suffering from Usher Syndrome caused by the same mutation suggests a strong genetic or stochastic modifying effect (Petit 2001), thus one might expect variation to occur when the mutation manifests in a different species.

5.1.2 Assessing genes with known or predicted functions

Of the 52 genes annotated within the candidate region for *bronx waltzer*, 31 have been previously characterised. For these genes a certain amount of information is available making it possible to assess their candidacy to an extent based on the published literature. For some, a function may be fully elucidated and a mouse model may already exist. In these cases the possibility of their being responsible for the *bronx waltzer* phenotype can be investigated by enquiring as to whether the mouse model exhibits similar behaviour and establishing whether the function of the gene correlates with the phenotype of the *bv* mice. Other genes may have less information available and may have been identified only by homology of their protein domains to other, better characterised genes. These can be assessed and prioritised based on their possible function and on that of related family members but are harder to rule out since they may perform an as yet unreported function within the ear.

5.1.3 Assessing novel and predicted genes

The remaining 21 uncharacterised genes within the critical region have little or no published information associated with them. They may have been identified by gene-prediction programs based on conserved structures such as

splice sites and homology to known genes as well as the existence of ESTs which imply that the region is transcribed (Mathe *et al.* 2002; Brent and Guigo 2004). As a result there may be no information concerning their function, although some may have predicted protein domains based on their homology to known genes which may provide clues as to their function. In addition, since their sequence is known they can be compared to those inner ear derived cDNA libraries which have made EST sequences available for searching to establish whether or not they have been found to be expressed in the expected tissues.

5.2 METHODS

5.2.1 Data mining of inner ear expression databases

A number of studies have been carried out with the intention of identifying genes expressed in inner ear tissues. These have ranged from the characterisation of proteins isolated from tissues and fluids within the ear to the construction of cDNA libraries from specific inner ear tissue samples, and also the use of microarray technologies to identify differentially expressed genes. Some of these data are available in a searchable format, and these have been used to derive evidence for the expression of some of the *bronx waltzer* candidate genes in the tissue one might expect the causative agent for the mutation to be found. The databases examined, their origins, locations and the methods used to search them are summarised in Table 5.2

The Washington University Inner Ear Protein Database (Thalmann and Thalmann) and Inner Ear Gene Expression Database (Chen and Corey 2002) were designed to be searched using gene IDs and descriptions and thus novel genes could not be included. The Human Cochlea EST database (Robertson *et al.* 1994) was queried using translating BLAST (tBLASTn) in order to compensate for differences between the mouse and human gene sequences, while the mouse Organ of Corti EST database (Pompeia *et al.* 2004) was queried directly using cDNA sequences and BLASTn (Altschul *et al.* 1990).

Resource	Construction	Reference	Search procedure
Washington University Inner Ear Protein Database	Isolation and identification of proteins in guinea pig inner ear tissues and fluids by PAGE	http://oto.wustl.edu/thc/innerear2d.htm (Thalmann and Thalmann)	Online list searched for Gene IDs and descriptions. Unknown genes not included.
Inner Ear Gene Expression Database	Microarray screen of mouse cochleas at P2 and P32	http://www.mgh.harvard.edu/depts/coreylab (Chen and Corey 2002)	Database downloaded and searched locally with gene IDs and descriptions. Unknown genes not included.
Human Cochlea EST database	Human fetal cochlear cDNA library subtracted against total human fetal brain RNA	http://hearing.bwh.harvard.edu/estinfo.HTM (Robertson *et al.* 1994) Also at http://neibank.nei.nih.gov	Online list searched for Gene IDs and descriptions of orthologues. EST database queried using tBLASTn with protein sequences of all candidate genes to account for variation in human and mouse sequence
Organ of Corti EST database	cDNA library constructed from mice between P5 and P13	http://neibank.nei.nih.gov (Pompeia *et al.* 2004)	Online list searched for Gene IDs, RefSeq IDs and Accession numbers. EST database queried using BLASTn with cDNA sequences of all candidate genes.

Table 5.2: Origins, locations and methods of interrogation of publicly available resources describing genes showing expression in inner ear tissues

5.2.2 Expression studies using inner ear cDNA

As a means of ascertaining whether the genes lying within the critical region for *bronx waltzer* are expressed within the ear, studies were carried out using cDNA prepared from wild type inner ear tissue. Successful amplification of a fragment of the expected size from this material can be taken as an indication that the gene may be expressed in the tissue of interest.

5.2.2.1 Obtaining inner ear cDNA

The inner ear capsules were dissected out from a single litter of ten C3HeB/FeJ mice at age P0, with care being taken to remove as much extraneous tissue as possible. These were kept briefly in ice cold RNase-free Phosphate Buffered Saline (PBS) before being homogenised and incubated at room temperature for 5 minutes. RNA was prepared from this tissue as described in Section 2.6, including the optional DNase treatment to remove any genomic DNA contamination. Reverse transcription was then performed as described in Section 2.7.

5.2.2.2 Primers designed for amplification from cDNA

In order to make certain that any products amplified from the cDNA material were truly representative of an mRNA molecule isolated from inner ear tissue rather than from genomic DNA contamination, primers were designed in adjacent exons to span the intervening intron. In this manner, products resulting from cDNA and from genomic DNA can easily be distinguished by their differing sizes, with the genomic product being larger as a result of the inclusion of the intron which will have been spliced out in a cDNA template. This strategy is illustrated in Figure 5.1 and the primers designed for each of the genes within the *bv* region are given in Table 5.3. Two exceptions to this strategy were genes consisting of only one exon where the genomic and cDNA expected product lengths will be the same, and genes with no introns

small enough to be suitable for PCR amplification where no genomic band will be observed. These are indicated in Table 5.3.

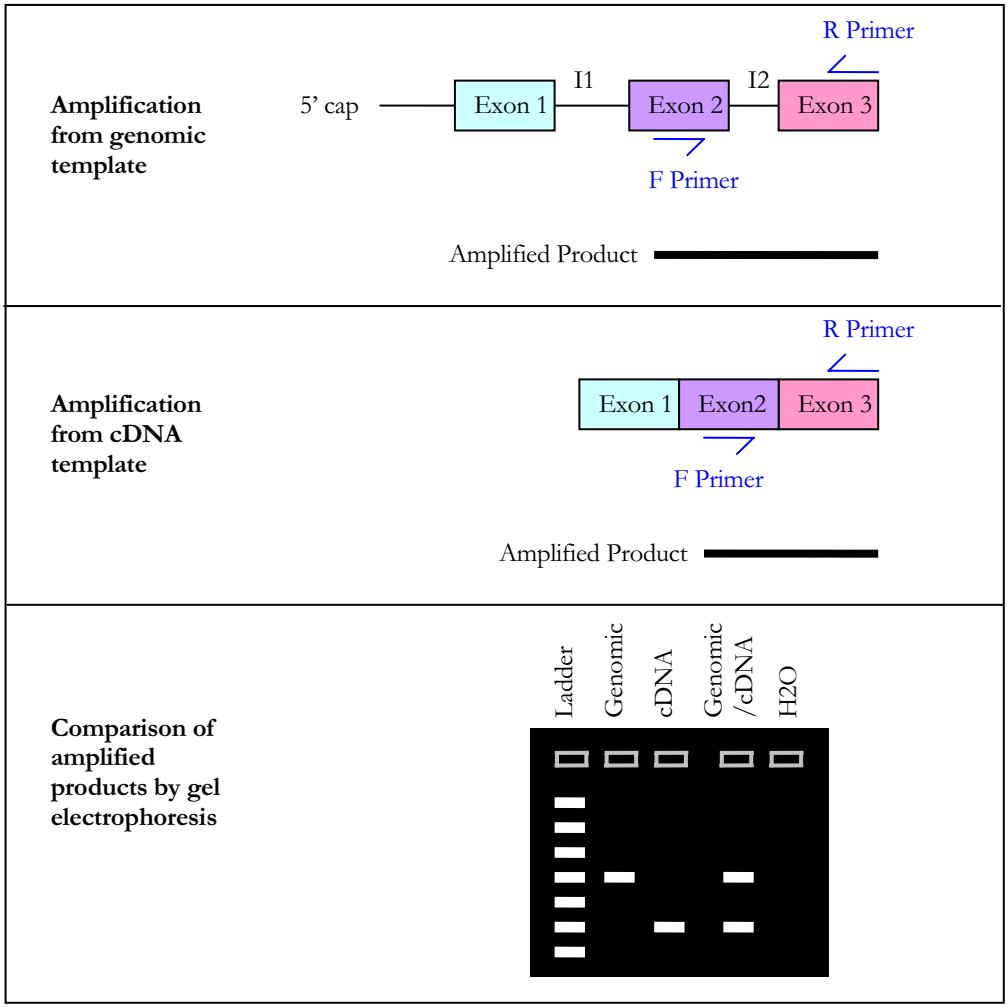

Figure 5.1: Diagram illustrating the strategy used in the design of primers to amplify fragments of candidate genes using cDNA obtained from P0 inner ears as the PCR template. The different sized products amplified from genomic DNA and cDNA as a result of the splicing out of Intron 2 allow the origin of the template to be confirmed by agarose gel electrophoresis.

Gene ID	Notes	Exons	Intron size	F primer	R primer	Expected Genomic size	Expected cDNA size
Acacb		4-5	144	GCTGATCATAGACATTGCCAAG	AAGTTTGGGGTTTTCCGAAG	226	82
Foxn4		4-5	89	TGAGCCCTATAGGCAACCAC	TGGGTAGCTGGGGAGTACAG	204	115
NM146163		3-4	139	GGGGAAGAAGGAAGGCTATG	CAAGCAGTTGGAGCACTTTG	237	98
Kctd10		4-5	546	CTTACGAGCCCTTCTGCAAG	CTGTTGTAGAGCAGCTTCACG	649	103
Ube3b		10-11	248	TGCGCAGTTCTCAGACAATC	CGAAGCATGTCATGGGACTC	377	129
Mmab		5-6	242	CCAGTGCATGCTACAGGAC	GGAGCTGGCTGGAGTACTTG	385	143
Mvk		4-5	284	CCTTGGAGCAACTGGAGAAG	GCCAGACACACAGAGTAGGC	495	211
E41930	2 exons, large intron	1-2	16366	GAGGACTCAGAACCCACCAG	TGGGGAAGATCTTGATGGAC	16545	179
Trpv4		8-9	229	GTAAGTTTGGGGCTGTGTCC	CTGTGAAGAGCGTGATGACC	400	171
Gltp	All introns too large	3-4	2060	GACCCAGCCAAGTTCAAGAC	GCCAGCCATGGTACTTCTTC	2301	241
NM029992		7-8	169	AACAGGAGAACCTGCTGAGG	CTGTTGAGCTGCGCGTTG	193	24
Git2		5-6	109	GAACAGGGAATCTTGAGACCTG	ATCTTGTGTGCCTGGGTCAG	276	167
NM175120	Only 1 exon	n/a	0	CGGGACACACAACAAACAAC	TTCTGACCAATCGTGGAAGG	238	238
Q9D1S6		2-3	611	TCGGACATTACTGCACCTTG	CCATGGACGTGTTGTGGTAG	802	191
NM181075		1-2	587	TTCAGTGATGCTGCGCTTAG	AAAGCCTCCAAGGGTCACTG	849	262
NM026263		6-7	453	GACACTTTGTCTATGGGACACG	GCTCTTGCTCTTCTGAGCTG	582	129
Q8C864	Only 1 exon	n/a	0	CTACAGCCCTCCACAAAAGC	TTCTCTGTTTCCTCCCTTGC	483	483
Oasl2	All introns too large	2-3	1209	AAGGTTCAGTCCCGGAAGAC	GGGCGAGTTTTCACAAAATG	1403	194
Oasl1	All introns too large	3-4	1516	CTCATCTTCACGATCCAGACC	AGTGTTTGACCAACCGAAGG	1745	229
NM028211		3-4	174	TTCGGACCACTCCTGAGTTC	GCTGCATCTTTGCCTTTTG	288	114
Tcf1		5-6	110	GCGGCCTTACACCAAGTATC	GGTAGCGAGGCCATGATAAG	298	188
E44804	Only 1 exon	n/a	0	ATGACGAAGGGAACGTCATC	TCCCGGTAGTGTTTCGTCTC	296	296
Usmg3		6-7	426	TCTCTGTGTTGCCATGATCG	GATTGTCAGCTGGCTGTGTG	590	164
Rpl37	Only 1 exon	n/a	0	ATGACGAAGGGAACGTCATC	TCCCGGTAGTGTTTCGTCTC	181	181
Acads		5-6	212	GCTTGGATCACCAACTCCTG	TCCTCAAAGATGAGGTTAGCTG	397	185
NM175352	All introns too large	1-2	3901	CTGGCAAGGAGGAGAAGAAG	GGCTTGGCAATCTCAAAGAG	4184	283
NM175403		3-4	81	GGACATCTTTGACCGTGTCG	GTGCACAGACTTTGGGATTG	245	164
Calbp1		5-6	208	ACTGATGGGCCCTAAACTCC	CACGTCTCGGATAATTTCCTC	398	190
Pop5		4-5	161	CAATGCCTACACTGGAGTCG	CTTCGTCAGTGCAATTCTGC	385	224

Gene ID	Notes	Exons	Intron size	F primer	R primer	Expected Genomic size	Expected cDNA size
Rnf10		5-6	74	CTGTGGACACATTTTCTGCTG	ATTCACCCACTTGGATTTGG	300	226
D5Ertd33e		6-7	87	CATTGGTGAGGTCATTGCAG	AGAGTGAATGGCCACAATCC	250	163
Dnclc1	All introns too large	2-3	1679	TGTCGGAAGAGATGCAACAG	AACAGAAGAATGGCCACCTG	1899	220
Sfrs9		3-4	286	CGTACAGAAGGACGGAATGG	GGCTTTGGTATGGCGAGTC	489	203
NM029645	All introns too large	1-2	5271	CGGTAATCGAGCATCTGGAG	GGGGCCACAAAGTATTCCTC	5521	250
Q8C4H9	Only 1 exon	n/a	0	TGTTTTTGACTCCTCCCTTTG	TAGTGGGTTCAGCGGGTAAG	250	250
15E1	2 exons, large intron	1-2	1257	GCGAGTACGACCAGTGCTTC	GAGTTTTCAGGCTTTTCTTTGC	1444	187
Cox6a1		1-2	131	CAGCGTCTCGGGTCTCTC	GGTCTCTCGTGCTCTTCGTG	324	193
Msi2h		4-5	99	GCGAATACTTCGGCCAGTTC	GATTGCGCCAGCACTTTATC	232	133
Q9D2I7	Only 1 exon	n/a	0	ATTCACATCCCACGAAGGAG	AAAGAGAGGTGGGTGTGGTG	177	177
Pla2g1b	All introns too large	2-3	1051	GTGGCAGTTCCGCAATATG	CCCGGAGCATGAGTAGGAG	1283	232
Sirt4		3-4	418	ACCGGACGTCGTTTTCTTTG	AAGGCAGCAACTCTCCACAG	663	245
NM133915		3-4	95	GGGCAGCAACCTCTCTGAAC	CGGAACTCTCCAGTTCATCC	364	269
Gcn1l1		3-4	219	AGTTGCTGCCTCGAGTGG	CAGCACGTCCACCTCCTC	383	164
NM198163		4-5	82	CCGAGTCCTTTGTCAACGTC	AGAGCTGGATCCCCATCTG	244	162
Q8R1D2	All introns too large	3-4	788	TGTCAGAGATCGAGCAGAGC	GCCTCTTGAGATCGTTGAGG	1028	240
Rpl29	Only 1 exon	n/a	0	AACCACGGTCGCAAAGATAC	TTAGGGTGAGCGATGAAAGC	235	235
Cit		8-9	270	TGAAGCTAGCCCTGGAAAAG	GAGTTGACGACTCCTTTCTGC	525	255
Prkab1		3-4	255	GGACCTGCCTGAAGGAGAG	TTCAAAGTCAGTTTTCTTCACTTG	391	136
NM177759		7-8	579	CAGGTGATCCAGAAGAAGTCG	TTCCTGAGGATTTCCATCTTG	728	149
Q9D4T7	2 exons, large intron	1-2	1389	GGCCTTCATATCCCACAATG	ACAGGTGGGAGCAGTTTCAC	1618	229
Hspb8	2 exons, large intron	2-3	5916	AGAGAAGCAGCAGGAAGGTG	CCTGGTTGTCTTGAGGAAGC	6113	197
NM026886		5-6	179	ATCTCGAAGCCGGAAGTCTC	CCCTGGCTGACAGGAAGC	424	245
Lnfrg	Positive control	3-4	103	CCACGTGGATGATGACAACTAC	CTGATGCAGAAGCCAGCTC	298	195
Actb	Positive control	5-6	125	ATCCATGAAACTACATTCAATTCCAT	ACCGATCCACACAGAGTACTTGCGC	332	207

Table 5.3: Primers used for the PCR amplification of fragments from genes which lie within the candidate region for *bronx waltzer*. Primers are located in adjacent exons and designed to amplify across the intervening intron, giving different sized products when genomic and cDNA templates are used. Exceptions to this strategy, such as genes with only one exon or introns too large for PCR amplification are indicated in the Notes column.

5.2.2.3 PCR from cDNA

Although the PCR method used here was very similar to the standard protocol described in Section 2.4.1, the PCR cycles used to amplify from genomic and cDNA templates differed in important ways. The cDNA PCR cycle (Table 5.4a) had a higher number of total cycles to account for the limited amount of template material available, while the genomic PCR cycle (Table 5.4b) included a longer extension time to take into account the large size of some of the included exons. The PCR programmes used are given in the tables below.

Step	PCR phase	Temperature	Time
1	Initial denaturation	95°C	2 minutes
2	Denaturation	95°C	30 seconds
3	Annealing	55°C (~2°C below T_M of primers)	30 seconds
4	Extension	72°C (ReddyMix™)	30 seconds
5	Thermocycling	Repeat steps 2-4, 39 times	–
6	Cooling	4°C	Indefinitely

A

Step	PCR phase	Temperature	Time
1	Initial denaturation	95°C	2 minutes
2	Denaturation	95°C	30 seconds
3	Annealing	55°C (~2°C below T_M of primers)	30 seconds
4	Extension	72°C (ReddyMix™)	60 seconds
5	Thermocycling	Repeat steps 2-4, 29 times	–
6	Cooling	4°C	Indefinitely

B

Table 5.4: PCR conditions used for amplification from cDNA (A) and genomic DNA (B). The differences between the two programmes are highlighted in red.

5.3 RESULTS

5.3.1 Assessment of previously characterised genes

A summary of the published information on each of the 31 characterised genes is presented in Table 5.5, including relevant expression data, known or predicted functions and any characterised models. Those genes marked by an asterisk have been selected as having a potential function or evidence which suggests they be involved in the *bv* phenotype and are discussed in greater detail in Section 5.4.

Table 5.5: Summary of published literature relating to previously characterised genes annotated within the described critical region for the *bronx waltzer* locus.
Gene locations were obtained from Mouse Ensembl Build 33 (http://www.ensembl.org/Mus_musculus/). Entrez Gene entries can be retrieved at http://www.ncbi.nlm.nih.gov/entrez/.
Genes marked with an asterisk were selected for further studies (see Section 5.4.3)

Mouse gene	Entrez Gene	Human orthologue	Entrez Gene	Expression information	Function or protein domains	Model	Phenotype	Key references
Acacb Acetyl-coenzyme A carboxylase beta	100705	ACACB/ ACC2	32	Heart and skeletal muscles. Localises to mitochondria.	Control of fatty acid oxidation in muscle tissue	$Acacb^{-/-}$ knockout mice	Higher fatty acid oxidation rate. Reduced storage of fat in adipose tissue.	(Oh *et al.* 2005)
Foxn4 * Forkhead box protein N4	116810	FOXN4	121643	Retina, ventral hindbrain, spinal cord, dorsalmidbrain	Transcription factor controlling retinal development by activation of Math3, NeuroD1, and Prox1	$Foxn4^{-/-}$ knockout mice	Retinal dysplasia	(Li *et al.* 2004) (Gouge *et al.* 2001)
Myo1h * Myosin 1h	231646	MYO1H	64004	Utricles (weaker than other myosins tested)	Myosin head (motor domain) Calmodulin-binding region ATP/GTP-binding site motif	-	-	(Dumont *et al.* 2002)
Kctd10 * potassium channel tetramerization domain-containing 10	330171	KCTD10	83892	-	Voltage-gated potassium channel activity	-	-	-
Ube3b * ubiquitin protein ligase E3B	117146	UBE3B	89910	Highly expressed in chick basilar papillar following noise exposure	Targeting of proteins for proteolytic degradation	-	-	(Gong *et al.* 2003) (Lomax *et al.* 2000)
Mmab Methylmalonic aciduria type B homolog	77697	MMAB	326625	Liver and skeletal muscle	Catalyses synthesis of the coenzyme adenosylcobalamin	Human methylmalonic aciduria	Deficient metabolism of some amino acids, resulting in a high level of acid in the blood	(Dobson *et al.* 2002)

Mouse gene	Entrez Gene	Human orthologue	Entrez Gene	Expression information	Function or protein domains	Model	Phenotype	Key references
Mvk Melavonate kinase	17855	MVK	4598	-	Conversion of mevalonic acid to 5-phosphomevalonic acid	Human mevalonic aciduria	Disorder of the cholesterol/isoprene biosynthetic pathway. Accumulation of mevalonic acid	(Schafer *et al.* 1992)
Trpv4 * transient receptor potential cation channel V4	63873	TRPV4	59341	Many tissues, including inner-ear hair cells, sensory neurons, and Merkel cells.	Osmotically activated cation-selective channel	$Trpv4^{-/-}$ knockout mice	Impaired pressure sensation and thermal responsiveness	(Suzuki *et al.* 2003) (Lee *et al.* 2005)
Gltp Glycolipid transfer protein	56356	GLTP	51228	Expressed in all tissues examined. Highest in cerebrum, lowest in liver and heart muscle.	Selectively accelerates intermembrane transfer of glycolipids	-	-	(Lin *et al.* 2000)
Git2 * G protein-coupled receptor kinase-interactor 2	26431	GIT2	9815		Regulation of golgi organisation, actin cytoskeletal organisation and paxillin localisation	-	-	(Mazaki *et al.* 2001)
Q9D1S6	-	ANKRD13 ankyrin repeat domain 13	88455	-	Leukotriene B4 type 2 receptor Ubiquitin interacting motif Ankyrin	-	-	-

Mouse gene	Entrez Gene	Human orthologue	Entrez Gene	Expression information	Function or protein domains	Model	Phenotype	Key references
Oasl2 2'-5'-oligoadenylate synthetase like protein 2	23962	OASL	8638	Most tissues, highest in primary blood leukocytes and other hematopoietic system tissues, colon, and stomach.	Involved in antiviral activity of interferons. Human orthologue found to interact with thyroid hormone receptors	-	-	(Eskildsen *et al.* 2003) (Lee *et al.* 1995)
Oasl1 2'-5' oligoadenylate synthetase-like 1	231655	OASL	8638	Most tissues, highest in primary blood leukocytes and other hematopoietic system tissues, colon, and stomach.	Not active in antiviral activity. Human orthologue found to interact with thyroid hormone receptors	-	-	(Eskildsen *et al.* 2003) (Lee *et al.* 1995)
Tcf1 Hepatocyte nuclear factor 1-alpha	21405	TCF1	6927	Predominantly in liver and kidney	Transcription factor for genes expressed exclusively in the liver	$Tcf1^{-/-}$ knockout mice Linked to MODY3 in humans	Defect in bile acid transport, increased bile acid and liver cholesterol synthesis, and impaired high-density lipoprotein metabolism.	(Shih *et al.* 2001)
Usmg3 Upregulated during skeletal muscle growth 3	83678	PSL4 Presenilin-like protein 4	121665	-	Membrane protein Peptidase activity	-	-	(Grigorenko *et al.* 2002)
Rpl37 60S ribosomal protein L37	67281	RPL37	6167	All tissues examined	Structural constituent of ribosome	-	-	(Su and Bird 1995)

Mouse gene	Entrez Gene	Human orthologue	Entrez Gene	Expression information	Function or protein domains	Model	Phenotype	Key references
Acads Acyl-CoA dehydrogenase, short-chain specific	11409	ACADS	35	Brain, particularly hippocampus	Fatty acid beta-oxidation. Regulation of theta oscillations during sleep	Acads mutant BALB/cByJ mice	Organic aciduria. Upregulation of Glo1 (involved in the detoxification of metabolic by-products)	(Tafti *et al.* 2003)
Cabp1 * Calcium-binding protein	29867	CABP1	9478	Brain, retina	Competitively inhibits calmodulin binding to CAMK2 May be involved in neuronal signal transduction	-	-	(Yamaguchi *et al.* 1999)
Pop5 Processing of precursor 5, ribonuclease P	117109	POP5	51367	-	Subunit of ribonucleaseP Required for 5'-end maturation of tRNAs	-	-	(Hartmann and Hartmann 2003)
Rnf10 Ring finger protein 10	50849	RNF10	9921	-	Ring finger motif, implies protein-protein interactions. Ubiquitin-protein ligase activity.	-	-	(Seki *et al.* 2000)
Dnclc1 * Dynein light chain 1, cytoplasmic	56455	DNCL1	8655	Wide variety of tissues Localizes to edge of postsynaptic differentiations, along cytoskeletal structures, and the edge of Golgi apparatus.	Inhibits the activity of neuronal NO synthase, implying regulatory role in numerous biologic processes	Drosophila dlc1 mutants	Pleiotropic morphogenetic defects in bristle and wing development and female sterility	(Dick *et al.* 1996) (Jaffrey and Snyder 1996)

Mouse gene	Entrez Gene	Human orthologue	Entrez Gene	Expression information	Function or protein domains	Model	Phenotype	Key references
Sfrs9 Splicing factor, arginine/serine rich 9	108014	SFRS9	8683	All tissues tested, highest expression in pancreas, followed by kidney, placenta, and heart.	Involved in mRNA formation Modulator of SMN exon 7 inclusion (causative agent for proximal spinal muscular atrophy)	-	-	(Stoss *et al.* 1999)
Cox6a1 Cytochrome c oxidase polypeptide VIa-liver	12861	COX6A1	1337	Brain, particularly cerebellum. Primary visual cortex	Subunit of COX, terminal enzyme of the respiratory chain. Probable housekeeping gene	-	-	(Wong-Riley *et al.* 2000)
Msi2h Musashi homolog 1	76626	MSI1	4440	Predominantly in brain	neural RNA-binding protein	-	-	(Good *et al.* 1998)
Pla2g1b Phospholipase A2 precursor	18778	PLA2G1B	5319	Many tissues, primarily secreted from pancreas	Catalyzes the release of fatty acids from glycero-3-phosphocholines Protection of membranes from oxidative injury is an important function	-	-	(Seilhamer *et al.* 1986) (van Kuijk and Dratz 1987)
Sirt4 NAD-dependent deacetylase sirtuin 4	75387	SIRT4	23409	Many tissues	Role in epigenetic gene silencing May function via mono-ADP-ribosylation of proteins	-	-	(Frye 1999)
Pxn * Paxillin alpha	19303	PXN	5829	All tissues tested, localises to focal adhesions	Scaffolding protein involved in actin organisation Role in fibronectin and integrin signalling	Paxillin$^{-/-}$ mice	Homozygous lethal	(Hagel *et al.* 2002)

Mouse gene	Entrez Gene	Human orthologue	Entrez Gene	Expression information	Function or protein domains	Model	Phenotype	Key references
Gcn1l1 General control of amino-acid synthesis 1-like 1	231659	GCN1L1	10985	Ubiquitous expression	S. cerevisiae GCN1 required for activation of protein kinase GCN2, leading to upregulation of aa synthesis during starvation	-	-	(Marton *et al.* 1997)
Rpl29 60S ribosomal protein L29	19944	RPL29	6159	Many tissues	Heparin/heparan sulfate-binding peptide. Possible pseudogene (human orthologue is on Chr3)	-	-	(Liu *et al.* 1996)
Cit * Citron protein (Rho-interacting, serine/threonine kinase)	12704	CIT	11113	Strongest in testis. Also keratinocytes, brain, spleen, lung, kidney.	Essential for cytokinesis in specific neuronal precursors	Citron-K$^{-/-}$ knockout mice	Grow slowly, severely ataxic, die prematurely from seizures. Development of CNS affected due to altered cytokinesis and massive apoptosis.	(Di Cunto *et al.* 2000)
Prkab1 5'-AMP-activated protein kinase, beta-1 subunit	19079	PRKAB1	5564	All tissues tested, strongest in liver	Plays a role in hormonal and nutrient-derived anorexigenic and orexigenic signals and in energy balance	-	-	(Minokoshi *et al.* 2004) (Thornton *et al.* 1998)
Hspb8 Heat-shock protein beta-8	80888	HSPB8	26353	Many tissues, most abundant in skeletal muscle, heart, and placenta. Not found in blood.	Neuromuscular function	Human motor neuropathy type II	Distal muscle atrophy	(Kappe *et al.* 2001)

5.3.2 Gene expression in the inner ear – published data

Of the four databases searched, three yielded data suggesting that clones contained within the collections represent transcripts of genes within the *bronx waltzer* region. These results are given in Table 5.6. The fourth database used – the Washington University Inner Ear Protein Database – was queried for all the known genes within the region but did not yield any positive results and is not included in the table.

Table 5.6: Results obtained from screening publicly available databases of inner ear ESTs.
The Inner Ear Gene Expression (IEGE) database is the result of a microarray study and the results represent clones which showed expression in the inner ear and match the candidate gene by sequence homology. Since the database is searched by gene description, only previously characterised genes could be screened and novel genes are therefore left blank.
The human fetal cochlea and mouse organ of Corti data sets are the result of cDNA library construction and sequencing. The results represent clones within the libraries which show strong sequence homology to the candidate gene. In each case the letter N indicates a negative result in querying the database. The human fetal cochlea library was screened by description and by tBLASTn, while the mouse organ of Corti library was screened by description and by BLASTn to allow inclusion of uncharacterised genes.
Genes which show homology to sequences within more than one database are highlighted in **red**, those which show homology to sequences in only one database are highlighted in **blue**, and those showing no homology are shown in **black**.

Gene ID	Alternate names	Inner Ear Gene Expression database (Chen & Corey)	Human fetal cochlea cDNA library (Morton)	Mouse organ of Corti cDNA library (Pompeia)
Acacb	Acc2, Accb	N	N	N
Foxn4		N	N	N
NM146163	Myo1h 4631401O15Rik	N	N	N
Kctd10		N	N	N
Ube3b		similar?	AW021415 BI492368	N
Mmab		N	N	N
Mvk		W50181, AA109721	N	gi98d12
E41930		N	N	N
Trpv4		N	N	gi119c11, gi215a11
Gltp		AA388875	BI495579 BI495580	gi62b04
NM029992	A930031F18Rik		N	gi147h04
Git2		N	N	N
NM175120			N	N

Gene ID	Alternate names	Inner Ear Gene Expression database (Chen & Corey)	Human fetal cochlea cDNA library (Morton)	Mouse organ of Corti cDNA library (Pompeia)
Q9D1S6	1100001D10Rik		BI494434, BI494435	gi212h02, gi14c04
NM181075			N	gi43g02, gi17h10, gi106f11, gi203c07
NM026263			N	N
Q8C864			N	N
Oasl2		N	N	N
Oasl1		N	N	N
NM028211			N	gi170c01, gi193e04
Tcf1		N	N	N
E44804			N	gi100d09, gi193e09, gi193e11, gi259b02
Usmg3		N	N	gi33c10, gi97c07, gi138f10
Rpl37		AA015377, AA013942, AA529915	N	gi180f07, gi159c04, gi159a04, gi76c07, gi10g11, gi51f12, gi149a05
Acads		N	N	gi178g07
NM175352			N	N
NM175403	2410014A08Rik		BI496418, BI496417	gi107f05, gi184g06, gi210g08
Cabp1		AA111277, W63874	AW023490, AW023596	N
Pop5			N	N
Rnf10		N	N	gi135c05, gi220e05
D5Ertd33e			N	gi172d11
Dnclc1		AA497776, AA184581, AA106442, AA260435, AA260435	AW020245, BI491303, BI491360, BI494005, BI494038, BI494999, N66476	gi140d06, gi185e09, gi186e09, gi251g06
Sfrs9		N	AW021515, AW022794, AW022564, BI492475, BI496917, H91554, N67420	gi131d02
NM029645			N	N
Q8C4H9			N	N

Gene ID	Alternate names	Inner Ear Gene Expression database (Chen & Corey)	Human fetal cochlea cDNA library (Morton)	Mouse organ of Corti cDNA library (Pompeia)
15E1_mouse			N	N
Cox6a1		L06465, AA529006, AA472081	BI495001, BI495002	gi101d12, gi133d04, gi136f09, gi199a01,gi199b11, gi204b08
Msi2h		D49654	AW022564, H88010	N
Q9D2I7			N	N
Pla2g1b		N	N	N
Sirt4		N	N	N
NM133915	Paxillin alpha	AA172749, W34244	AW023219	gi135e01
Gcn1l1		N	N	N
NM198163			BI494830, BI497452, H89152	gi06f02
Q8R1D2	2210403N09Rik		N	gi13f06
Rpl29		AA271049, X05021, AA073078, AA038751, AA182248, AA103915	N	gi169g01, gi164c07, gi75d07, gi121c02, gi189g07, gi219f12, gi12d07, gi255g10
Citron		U39904	BI492898, AW022177	N
Prkab1		AA098332, W71242, AA137821	N	gi105g11, gi130e06
NM177759			N	N
Q9D4T7	4930562A09Rik		N	N
Hspb8			N	N
NM026886	1500001A10Rik		N	N

5.3.3 Gene expression in the inner ear – RT-PCR data

Primers in adjacent exons of candidate genes were designed to amplify the intervening intron, giving different sized products when PCR was performed with genomic DNA and inner ear cDNA templates. Primers which gave the expected product sizes can be taken as an indication that the gene to which they are designed is represented as an mRNA transcript in the tissue obtained from P0 inner ears. Any which give a product of expected size with a genomic template but fail to produce a product from the inner ear cDNA library may indicate that the gene is not expressed in the ear at stage P0, making it less likely that they would be responsible for the *bv* phenotype.

For some genes, such as those with only one exon or large introns, it was not possible to design primers which would give differently sized products when amplified from genomic DNA and cDNA to provide confirmation that the cDNA product was genuine. However, none of the other cDNA amplifications gave products of the size expected from a genomic template, suggesting that the cDNA used was free of genomic DNA contamination. This should mean that any bands amplified from the cDNA are truly representative of an mRNA molecule extracted from inner ear tissue.

The agarose gel images resulting from this experiment are presented in Figures 5.2a-d and the sizes of the expected and observed products are given in the accompanying Tables 5.7a-d. The results for each gene are summarised in Table 5.8.

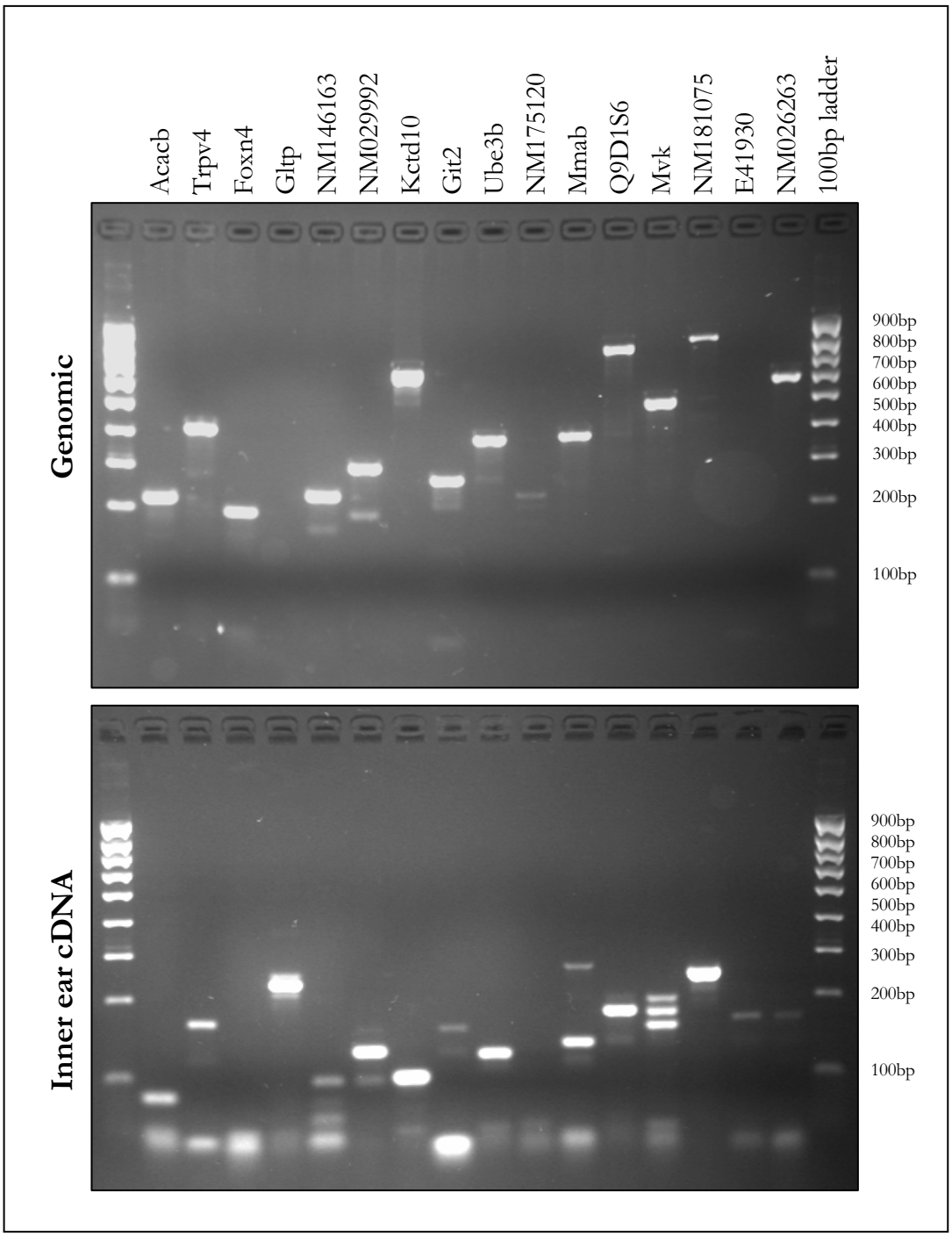

Figure 5.2a: Agarose gel electrophoresis showing the sizes of PCR products amplified from genomic DNA and inner ear cDNA templates using primers designed within adjacent exons of candidate genes. The differing sizes of genomic and cDNA products reflects the splicing out of introns in the cDNA material and serves as confirmation that a positive result represents a true transcript rather than genomic DNA contamination.

GENE	Expected genomic size (bp)	Observed genomic size (approx bp)	Expected cDNA size (bp)	Observed cDNA size (approx bp)
Acacb	226	220	82	80
Trpv4	400	400	171	170
Foxn4	204	190	115	Negative
Gltp	2301	Too large	241	240
NM146163	237	230	98	100
NM029992	193	290 (190)	24	130 (100)
Kctd10	649	650	103	100
Git2	276	270	167	170
Ube3b	377	380	129	130
NM175120	238	230	238	Negative
Mmab	385	380	143	140 (270)
Q9D1S6	802	800	191	180
Mvk	490	490	211	160 (200, 180)
NM181075	849	850	262	260
E41930	16545	Too large	179	180
NM026263	582	600	129	180

Table 5.7a: Comparison of expected and observed sizes of fragments obtained by PCR amplification using either genomic DNA or inner ear cDNA as template. The expected sizes are based on sequence data, while observed sizes are taken from the gel images shown in Figure 5.2a. Genomic fragments marked as "Too large" are beyond the scope of the PCR protocol used which will only amplify fragments up to approximately 1000bp. cDNA fragments marked as "Negative" gave no product on amplification. Observed sizes which differ significantly from expected sizes are highlighted in red.

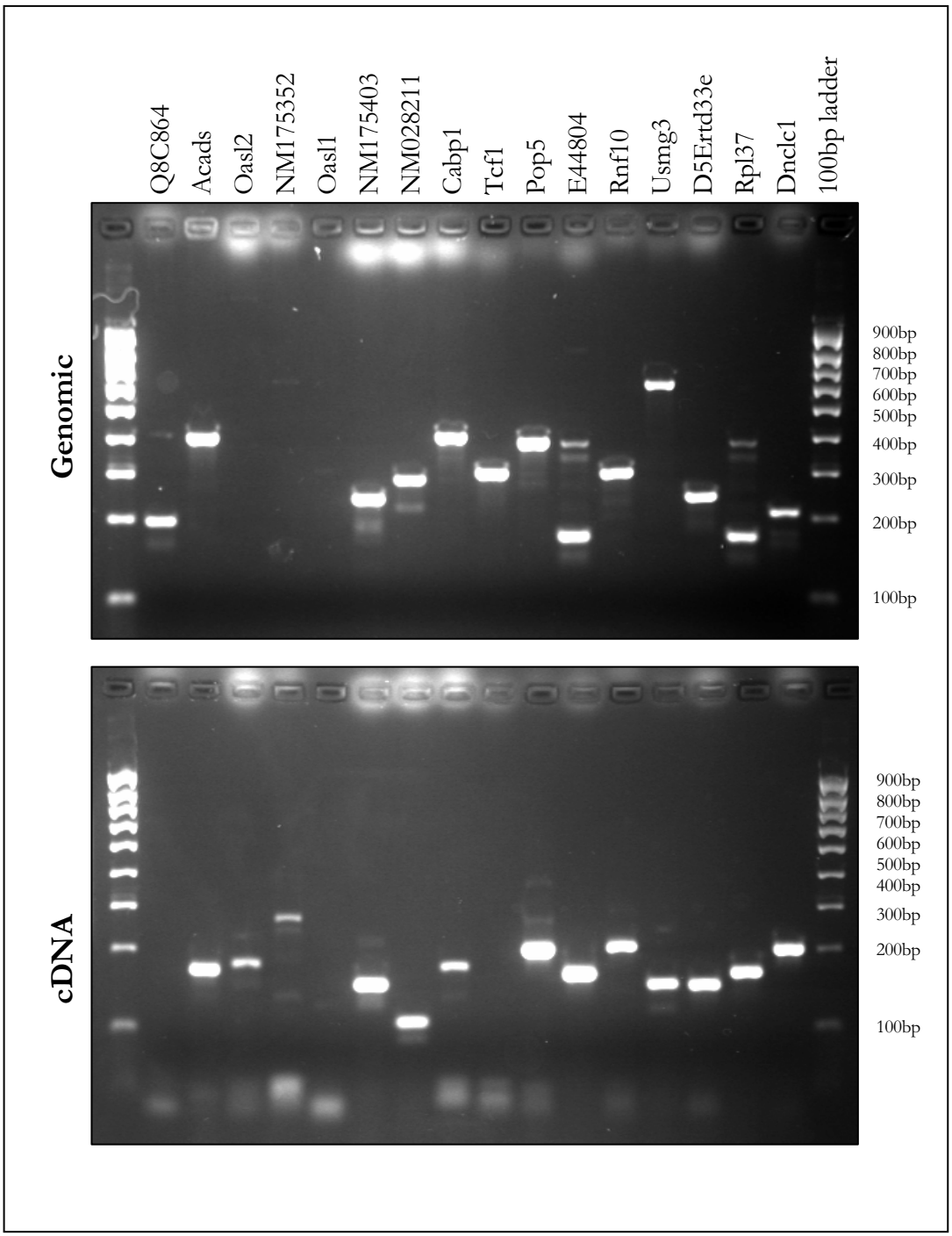

Figure 5.2b: Agarose gel electrophoresis showing the sizes of PCR products amplified from genomic DNA and inner ear cDNA templates using primers designed within adjacent exons of candidate genes. The differing sizes of genomic and cDNA products reflects the splicing out of introns in the cDNA material and serves as confirmation that a positive result represents a true transcript rather than genomic DNA contamination.

GENE	Expected genomic size (bp)	Observed genomic size (approx bp)	Expected cDNA size (bp)	Observed cDNA size (approx bp)
Q8C864	483	190	483	Negative
Acads	397	400	185	190
Oasl2	1403	Too large	194	200
NM175352	4184	Too large	283	300
Oasl1	1745	Too large	229	Negative
NM175403	245	230	164	160
NM028211	288	280	114	120
Cabp1	398	400	190	190
Tcf1	298	300	188	Negative
Pop5	385	380	224	220
E44804	181 (400)	180	181	180
Rnf10	300	300	226	230
Usmg3	590	600	164	170
D5Ertd33e	250	250	163	160
Rpl37	181 (400)	180	181	180
Dnclc1	1899	210	220	220

Table 5.7b: Comparison of expected and observed sizes of fragments obtained by PCR amplification using either genomic DNA or inner ear cDNA as template. The expected sizes are based on sequence data, while observed sizes are taken from the gel images shown in Figure 5.2b. Genomic fragments marked as "Too large" are beyond the scope of the PCR protocol used which will only amplify fragments up to approximately 1000bp. cDNA fragments marked as "Negative" gave no product on amplification. Observed sizes which differ significantly from expected sizes are highlighted in red.

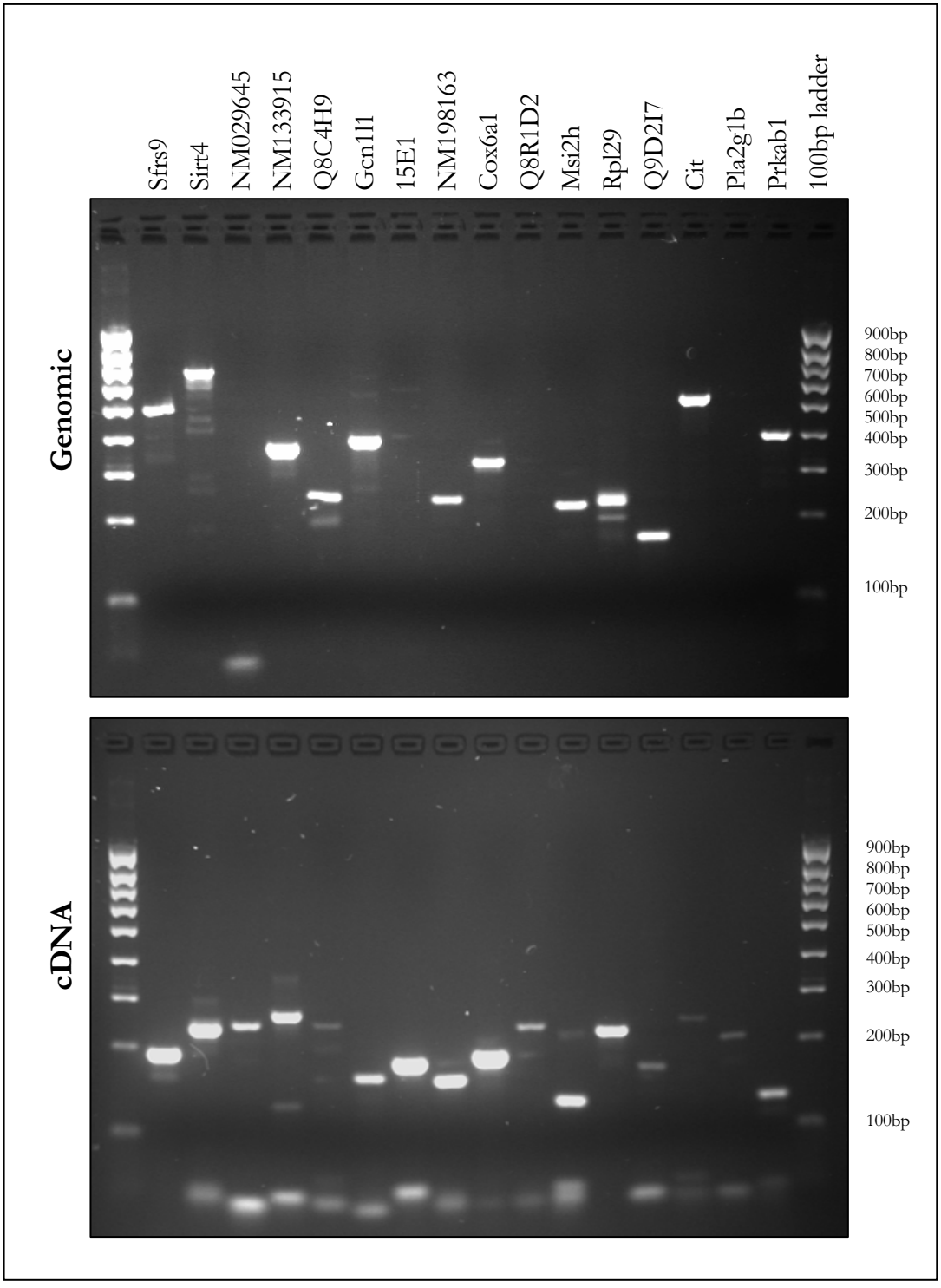

Figure 5.2c: Agarose gel electrophoresis showing the sizes of PCR products amplified from genomic DNA and inner ear cDNA templates using primers designed within adjacent exons of candidate genes. The differing sizes of genomic and cDNA products reflects the splicing out of introns in the cDNA material and serves as confirmation that a positive result represents a true transcript rather than genomic DNA contamination.

GENE	Expected genomic size (bp)	Observed genomic size (approx bp)	Expected cDNA size (bp)	Observed cDNA size (approx bp)
Sfrs9	489	500	203	190
Sirt4	663	700	245	240
NM029645	5521	Too large	250	250
NM133915	364	350	269	270
Q8C4H9	250	250	250	250
Gcn1l1	383	380	164	160
15E1	1444	Too large	187	180
NM198163	244	240	162	160
Cox6a1	324	330	193	190
Q8R1D2	1028	Too large	240	250
Msi2h	232	230	133	130
Rpl29	235	240	235	240
Q9D2I7	177	180	177	180
Cit	525	530	255	250
Pla2g1b	1283	Too large	232	230
Prkab1	391	400	136	140

Table 5.7c: Comparison of expected and observed sizes of fragments obtained by PCR amplification using either genomic DNA or inner ear cDNA as template. The expected sizes are based on sequence data, while observed sizes are taken from the gel images shown in Figure 5.2c. Genomic fragments marked as "Too large" are beyond the scope of the PCR protocol used which will only amplify fragments up to approximately 1000bp. cDNA fragments marked as "Negative" gave no product on amplification. Observed sizes which differ significantly from expected sizes are highlighted in red.

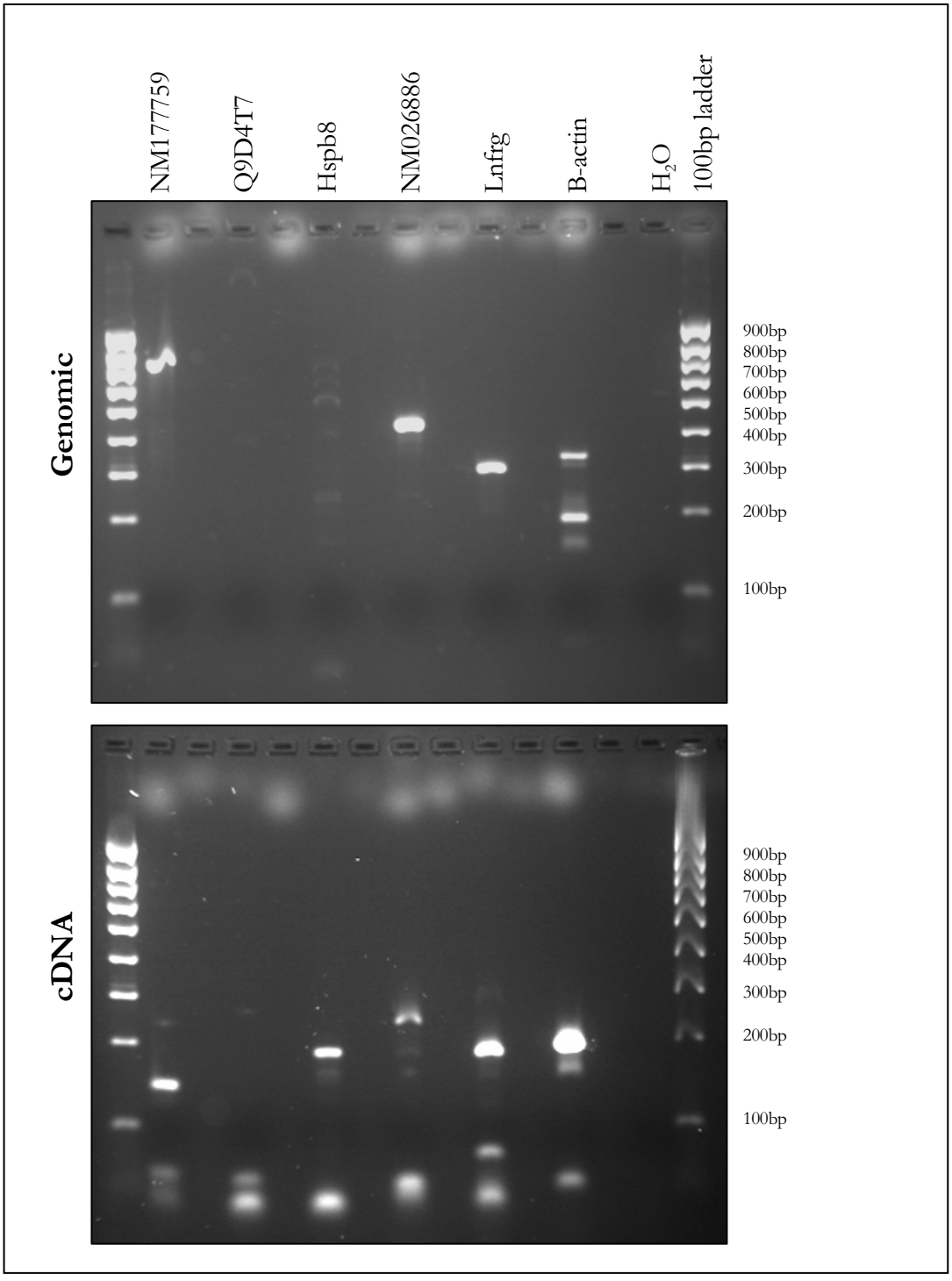

Figure 5.2d: Agarose gel electrophoresis showing the sizes of PCR products amplified from genomic DNA and inner ear cDNA templates using primers designed within adjacent exons of candidate genes. The differing sizes of genomic and cDNA products reflects the splicing out of introns in the cDNA material and serves as confirmation that a positive result represents a true transcript rather than genomic DNA contamination.

GENE	Expected genomic size (bp)	Observed genomic size (approx bp)	Expected cDNA size (bp)	Observed cDNA size (approx bp)
NM177759	728	750	149	150
Q9D4T7	1618	Too large	229	Negative
Hspb8	6113	Too large	197	190
NM026886	424	430	245	250
Lnfrg	298	310	195	190
B-actin	332	330 (180)	207	200

Table 5.7d: Comparison of expected and observed sizes of fragments obtained by PCR amplification using either genomic DNA or inner ear cDNA as template. The expected sizes are based on sequence data, while observed sizes are taken from the gel images shown in Figure 5.2d. Genomic fragments marked as "Too large" are beyond the scope of the PCR protocol used which will only amplify fragments up to approximately 1000bp. cDNA fragments marked as "Negative" gave no product on amplification. Observed sizes which differ significantly from expected sizes are highlighted in red.

GENE	Notes	Genomic result	cDNA result	Outcome
Acacb		✓	✓	**Positive**
Foxn4		✓	No band	**Negative**
NM146163		✓	✓	**Positive**
Kctd10		✓	✓	**Positive**
Ube3b		✓	✓	**Positive**
Mmab		✓	(✓)	**Positive**
Mvk		✓	(✓)	**Positive**
E41930	Large intron	No band	✓	**Positive**
Trpv4		✓	✓	**Positive**
Gltp	Large intron	No band	✓	**Positive**
NM029992		(✓)	✗	**Inconclusive**
Git2		✓	✓	**Positive**
NM175120	Only 1 exon	✓	No band	**Negative**
Q9D1S6		✓	✓	**Positive**
NM181075		✓	✓	**Positive**
NM026263		✓	✗	**Inconclusive**
Q8C864	Only 1 exon	✗	No band	**Inconclusive**
Oasl2	Large intron	No band	✓	**Positive**
Oasl1	Large intron	No band	No band	**Inconclusive**
NM028211		✓	✓	**Positive**
Tcf1		✓	No band	**Negative**
E44804	Only 1 exon	✓	✓	**Positive**
Usmg3		✓	✓	**Positive**
Rpl37	Only 1 exon	✓	✓	**Positive**
Acads		✓	✓	**Positive**
NM175352	Large intron	No band	✓	**Positive**
NM175403		✓	✓	**Positive**
Cabp1		✓	✓	**Positive**
Pop5		✓	✓	**Positive**
Rnf10		✓	✓	**Positive**

GENE	Notes	Genomic result	cDNA result	Outcome
D5Ertd33e		✓	✓	**Positive**
Dnclc1	Large intron	(✓)	✓	**Positive**
Sfrs9		✓	✓	**Positive**
NM029645	Large intron	No band	✓	**Positive**
Q8C4H9	Only 1 exon	✓	✓	**Positive**
15E1	Large intron	No band	✓	**Positive**
Cox6a1		✓	✓	**Positive**
Msi2h		✓	✓	**Positive**
Q9D2I7	Only 1 exon	✓	✓	**Positive**
Pla2g1b	Large intron	No band	✓	**Positive**
Sirt4		✓	✓	**Positive**
NM133915		✓	✓	**Positive**
Gcn1l1		✓	✓	**Positive**
NM198163		✓	✓	**Positive**
Q8R1D2	Large intron	No band	✓	**Positive**
Rpl29	Only 1 exon	✓	✓	**Positive**
Cit		✓	✓	**Positive**
Prkab1		No band	✓	**Positive**
NM177759		✓	✓	**Positive**
Q9D4T7	Large intron	No band	No band	**Inconclusive**
Hspb8	Large intron	No band	✓	**Positive**
NM026886		✓	✓	**Positive**
Lnfrg	Positive control	✓	✓	**Positive**
Actb	Positive control	(✓)	✓	**Positive**

Table 5.8: Summary of the data resulting from amplification of fragments of candidate genes from genomic DNA and inner ear cDNA templates. In the Genomic result and cDNA result columns a green tick (✓) indicates the obtained band was of the expected size, a bracketed tick indicates that bands of other sizes were also obtained and a red cross (✗) indicates that the band obtained was not of the expected size.
Where no band was obtained in the genomic sample, this is often explained by the large size of the intervening intron which prevented amplification by PCR.
Where no band was obtained in the cDNA sample but amplification in the genomic sample was successful, this is interpreted as a negative outcome and highlighted in **red**. Where a genomic band was also absent or where the cDNA product was not of the expected size, this is interpreted as an inconclusive outcome and highlighted in **blue**.

5.4 DISCUSSION

5.4.1 Analysis of inner ear expression data

5.4.1.1 Inner ear expression databases

The searching of four inner ear gene expression databases gave positive results in three, with none of the *bronx waltzer* candidate genes matching those described in the Washington University Inner Ear Protein Database (Thalmann and Thalmann). This is a relatively small database comprising 52 genes compiled by PAGE purification of fluids and tissues from the guinea pig inner ear, with identification of proteins carried out by amino acid sequencing, mass spectrometry and immunohistochemistry. This method is not especially sensitive and it might be expected that genes expressed at low levels could be missed, thus it is not remarkable that none of the 52 genes investigated here were found to be included.

The Inner Ear Gene Expression Database (Chen and Corey 2002) resulted from a microarray study where cochlear mRNA was extracted from mice at ages P2 and P32 and hybridised to Genechip oligonucleotide arrays. The effects of the *bronx waltzer* mutation can be observed from the beginning of hair cell development at E16.5 and hair cells can be seen to be degenerating until P1, after which point the observations are of disorganisation and missing hair cells. It is therefore possible, though not certain, that the gene responsible for the *bronx waltzer* phenotype may be down-regulated or even turned off by P2 and thus would not be present within this database. The microarrayed samples were associated with known genes and ESTs by sequence homology and it is these descriptions and gene IDs which are searchable in the database. However, since the data dates from 2001, many new transcripts have been reported and some gene IDs have altered, making effective querying of the database difficult. Therefore a negative result when screening this database may be less informative and the total number of genes found to be positive

might be expected to be lower than for the databases queried on the basis of sequence homology.

The Mouse organ of Corti EST database (Pompeia *et al.* 2004) and the Morton Human Fetal Cochlea EST database (Robertson *et al.* 1994) both take the form of cDNA library sequences which have been matched to published gene sequences using BLAST (Basic Local Alignment Search Tool). These matches can be searched by gene description, but it is also possible to directly screen the EST sequences by BLAST. This second approach greatly augments the sensitivity of the screen, since it allows the inclusion of novel genes which cannot be identified by description and bypasses the problem of fluidity in gene IDs. The limitations incurred are those found when using any sequence homology approach, namely that conserved regions and gene families may lead to false matches. This is particularly problematic when BLASTing against ESTs because the sequences are by nature relatively short and thus the differences between similar sequences which may emerge in longer sequence reads are not apparent. When searching the Mouse organ of Corti EST database this problem could be avoided by using a high cut-off value since the query sequence and database sequences originate from the same organism and might be expected to show a high level of homology in the case of a true match. When querying the Human Fetal Cochlea EST database, divergence between the two organisms necessitates the use of lower cut-off in order to detect orthologues. This carries the risk of identifying a different member of the same gene family, although one could argue that the expression of a close family member within the inner ear should contribute towards the potential candidacy of a gene. One solution to this problem would be to query the EST database not with the mouse protein sequences but instead with their human orthologues. However, very few orthologues have been directly confirmed by means other than sequence similarity, thus introducing the same uncertainty as regards conservation of function. In addition, this approach would not allow the inclusion of novel genes since few of these possess verified

orthologues. Once again, in the case of these EST libraries the age of the analysed tissue must be taken into account. The Mouse Organ of Corti ESTs were derived from tissue of mice between P5 and P13, once again introducing the possibility that the *bv* gene may have been turned off or down-regulated in the samples studied if it required only for the maintenance of hair cells. The tissue used in the preparation of the Human Fetal Cochlea Library was obtained from foetuses between the ages of 16 and 22 weeks. Although the maturation of the hearing process has not been widely studied in humans, it is thought that cochlear anatomy is grossly mature by 22-26 foetal weeks but that hair cells may continue to mature beyond this stage (Larsen 1997). Thus the mRNA molecules represented in this library are likely to represent those present in the developing ear at the appropriate stage.

Given the advantages and drawbacks of the different databases as outlined above, it was considered when evaluating these data that greater significance should be given to those genes which are represented in more than one of the databases screened.

5.4.1.2 RT-PCR data

Of the 52 candidate genes tested for expression in P0 inner ear tissue by PCR amplification, 42 gave a positive result where bands of the expected size were observed in both the genomic and cDNA amplifications. Two – *Mmab* and *Mvk* – were probably positive, with additional bands other than that of the expected size being observed. This may be a reflection of other gene family members, repeat regions or alternative transcripts. However, since the question being addressed here is the presence or absence of a specific band, its presence even in the company of other bands can be interpreted as a positive result. Five genes gave an inconclusive result, with two of these – *Oasl1* and Q9D4T7 – being primers which gave no product with either genomic or cDNA templates where the genomic product was too large for amplification. It is thus possible that these represent a negative result, but may

also be the result of primer failure. The remaining three inconclusive results were those where the bands obtained in either the genomic or cDNA assay were not of the expected size. This may represent non-specific amplification, but since each of these genes – NM029992, NM026263 and Q8C864 – are novel predictions, it is also possible that their coding regions have not been correctly annotated and that the observed sizes are representative of the actual gene product. Only three genes were found to be negative, giving a genomic product of the expected size but no product when amplified from the cDNA sample. These genes – *Foxn4*, NM175120 and *Tcf1* - represent the most interesting data from this study. Given that the great majority of genes tested were found to be present in the cDNA sample, the apparent absence of these genes can be interpreted as significant evidence that they are not expressed in the P0 inner ear.

The 42 genes which were found to be positive in the course of this simple expression screen compare to the total of 26 genes which were identified as being positive in at least one of the published gene expression databases (see Table 5.5). This discrepancy is most likely to be a reflection of the comparative impurity of the starting material. Although care was taken when dissecting out the inner ears for RNA extraction to remove as much extraneous tissue as possible, it is likely that a small amount of brain and muscle tissue was processed with it. This would result in mRNA populations from these tissues being represented at low levels in the cDNA sample which was later screened with gene-specific primers, thus increasing the likelihood of a positive result. Since the amplification from cDNA was not quantitative, it is not possible to infer whether a positive band suggests that a gene is either strongly or weakly represented in the sample. In any case, this would not be informative since a gene required only in a very specific cell type would appear to exhibit low level expression but could still play an important role in ear development, while a ubiquitously expressed muscle gene might show a

high level expression despite the relatively low number of muscle cells in the total tissue.

In order to rectify the issue of contamination with other tissue, more of the inner ear could be dissected away before processing. Since it is expected the *bv* gene should be detectable in inner hair cells during development, it might seem sensible to generate cDNA purely from the organ of Corti. However, this procedure is not a simple one. The mouse organ of Corti is extremely small at P0, making it very difficult to dissect and also necessitating the collection of tissue from a large number of mice in order to obtain sufficient RNA to carry out the study. By way of an example, in the construction of the mouse organ of Corti library, Pompeia *et al.* (2004) finely microdissected OCs from mice aged P5 to P13 and even with the larger size of the ear at this age, four hundred ears were required to obtain sufficient tissue. Another solution would be to carefully dissect the organ of Corti from a small number of ears and use an RNA amplification technique such as T7-based linear amplification (Van Gelder *et al.* 1990; Baugh *et al.* 2001) in order to generate sufficient material. This was the approach used by (Morris *et al.* 2005) in their study of genes differentially expressed in the lateral wall, organ of Corti and spiral ganglion. If sufficient time and expertise were available, this method would have been employed and the PCR screen repeated using the same sets of primers with the new material as template.

5.4.2 Assessment of candidacy based on the evidence gathered

The various sources of information described in this chapter are intended to allow the prioritisation of candidate genes for further investigation. To this end, the data available for each gene are summarised in Table 5.11 in order to simplify the process of comparison. In addition, a scoring system has been developed to allow a relatively objective method of selecting those genes with the most evidence in their favour for inclusion in further studies. The criteria for scoring are described in Table 5.9. The allocation of points was weighted, with stronger evidence contributing +1 or -1 point depending on whether it strengthened or weakened the case for the gene in question, while less strong evidence contributed +0.5 or -0.5 points. For example, when assessing the published literature available for a given gene, information concerning a mouse model was weighted more strongly than that arising from a human disorder or other model organism since disruption of the gene in the mouse might be expected to give a phenotype more closely resembling that of *bronx waltzer*. In the case of the inner ear cDNA screen, negative results were rare and positive ones may have been the result of contamination with other tissue as discussed in Section 5.4.1.2. Therefore negative results were considered to have more significance and were weighted +1, while a positive result contributed +0.5 points.

Data source	Criteria	Points
Published literature	Expression described in the ear or retina	+1
	Involvement in pathways known to be important in hearing	+1
	Gene function extensively studied and no evidence reported of involvement in hearing	-1
	Knockout mouse model lacking a hearing or vestibular phenotype	-1
	Human condition or other model organism not showing a hearing or vestibular abnormality	-0.5
Inner ear gene expression databases	Represented in no queried database	0
	Represented in one queried database	+0.5
	Represented in more than one queried database	+1
Inner ear cDNA PCR screen	Inconclusive result	0
	Positive result	+0.5
	Negative result	-1

Table 5.9: System used for the allocation of points to candidate genes from the *bronx waltzer* region in order to quantify the evidence for and against their being responsible for the phenotype.

These scores were then used to establish a candidacy rating ranging from "Very low" to "Very high" using the cut-off values shown in Table 5.10. The points awarded to each candidate gene along with their total scores and assigned level of candidacy are given in Table 5.11.

Total points	Candidacy rating
≥ 2.5	Very high
1.5 – 2	High
1	Medium
0 – 0.5	Low
≤ -0.5	Very low

Table 5.10: Cut-off values used in the assignment of candidacy ratings to genes from the *bronx waltzer* region

Table 5.11: Summary of functional and expression data available for genes situated within the *bronx waltzer* candidate region. Genes assigned a candidacy rating of either "Very high" or "High" are highlighted in red. For a full explanation of the weighted scoring system employed see Section 5.4.2

Gene	Description	Summary of relevant published data	Representation in inner ear expression databases	Inner ear cDNA PCR screen	Total points	Candidacy rating
Acacb	Acetyl-coenzyme A carboxylase beta	Involved in control of fatty acid oxidation in muscle tissue (-1) Knockout mice do not have a hearing or vestibular phenotype (-1)	-	Positive (+0.5)	-1.5	Very low
Foxn4	Forkhead box protein N4	Expressed in the retina and brain (+1) Transcription factor involved in activation of Math3 (+1) Knockout mice do not have a hearing or vestibular phenotype (-1)	-	Negative (-1)	0	Low
NM146163 (*Myosin 1h*)	Myosin 1h	Expressed in utricles (+1) Many other non-conventional myosins have roles in the inner ear (+1)	-	Positive (+0.5)	2.5	Very high
Kctd10	potassium channel tetramerization domain-containing 10	Voltage-gated potassium channel activity (+1)	-	Positive (+0.5)	1.5	High
Ube3b	ubiquitin protein ligase E3B	Highly expressed in chick basilar papillar following noise exposure (+1) Targets proteins for degradation.	Chen & Corey (+1) Morton	Positive (+0.5)	2.5	Very high
Mmab	Methylmalonic aciduria type B homolog	Involved in amino acid metabolism (-1) Human disorder results in high level of acid in the blood (-0.5)	-	Probably positive (+0.5)	-1	Very low
Mvk	Melavonate kinase	Involved in cholesterol metabolism (-1) Human disorder results in accumulation of mevalonic acid (-0.5)	Chen & Corey (+1) Pompeia	Probably positive (+0.5)	0	Low
E41930	Ensembl predicted gene	n/a	-	Positive (+0.5)	0.5	Low
Trpv4	transient receptor potential cation channel V4	Expressed in many tissues, including inner ear hair cells (+1) Osmotically activated cation-selective channel (+1) Knockout mice do not have a hearing or vestibular phenotype (-1)	Pompeia (+0.5)	Positive (+0.5)	2	High

Gene	Description	Summary of relevant published data	Representation in inner ear expression databases	Inner ear cDNA PCR screen	Total points	Candidacy rating
Gltp	Glycolipid transfer protein	Widespread expression. Selectively accelerates intermembrane transfer of glycolipids (-1)	Chen & Corey (+1) Morton Pompeia	Positive (+0.5)	0.5	Low
NM029992	Novel gene	n/a	Pompeia (+0.5)	Inconclusive (0)	0.5	Low
Git2	G protein-coupled receptor kinase-interactor 2	Involved in regulation of golgi organisation, actin cytoskeletal organisation and paxillin localisation (+1)	-	Positive (+0.5)	1.5	High
NM175120	Novel gene	n/a	-	Negative (-1)	-1	Very low
Q9D1S6	Novel gene	Predicted protein domains include Leukotriene B4 type 2 receptor, Ubiquitin interacting motif, Ankyrin	Morton (+1) Pompeia	Positive (+0.5)	1.5	High
NM181075	Novel gene	n/a	Pompeia (+0.5)	Positive (+0.5)	1	Medium
NM026263	Novel gene	n/a	-	Inconclusive (0)	0	Low
Q8C864	Novel gene	n/a	-	Inconclusive (0)	0	Low
Oasl2	2'-5'-oligoadenylate synthetase like protein 2	Important in anti-viral activity of interferons (-1) Human orthologue found to interact with thyroid hormone receptors	-	Positive (+0.5)	-0.5	Very low
Oasl1	2'-5' oligoadenylate synthetase-like 1	Inactive in 2'-5' oligoadenylate synthesis, but function not yet described Human orthologue found to interact with thyroid hormone receptors	-	Negative (-1)	-1	Very low
NM028211	Novel gene	n/a	Pompeia (+0.5)	Positive (+0.5)	1	Medium
Tcf1	Hepatocyte nuclear factor 1-alpha	Transcription factor, found abundantly in liver Knockout mice do not have a hearing or vestibular phenotype (-1)	-	Negative (-1)	-2	Very low
E44804	Ensembl predicted gene	n/a	Pompeia (+0.5)	Positive (+0.5)	1	Medium

Gene	Description	Summary of relevant published data	Representation in inner ear expression databases	Inner ear cDNA PCR screen	Total points	Candidacy rating
Usmg3	Upregulated during skeletal muscle growth 3	Membrane protein with peptidase activity	Pompeia (+0.5)	Positive (+0.5)	1	Medium
Rpl37	60S ribosomal protein L37	Structural constituent of ribosome (-1)	Chen & Corey (+1) Pompeia	Positive (+0.5)	0.5	Medium
Acads	Acyl-CoA dehydrogenase, short-chain specific	Involved in fatty acid beta-oxidation (-1) Knockout mice do not have a hearing or vestibular phenotype (-1)	Pompeia (+0.5)	Positive (+0.5)	-1	Very low
NM175352	Novel gene	n/a	-	Positive (+0.5)	0.5	Low
NM175403	Novel gene	n/a	Morton (+1) Pompeia	Positive (+0.5)	1.5	High
Cabp1	Calcium-binding protein	Expressed in brain and retina (+1) May be involved in neuronal signal transduction	Chen & Corey (+1) Morton	Positive (+0.5)	2.5	Very high
Pop5	Processing of precursor 5, ribonuclease P	Subunit of ribonucleaseP, required for 5'-end maturation of tRNAs (-1)	-	Positive (+0.5)	-0.5	Very low
Rnf10	Ring finger protein 10	Ring finger motif, implies protein-protein interactions. Ubiquitin-protein ligase activity	Pompeia (+0.5)	Positive (+0.5)	1	Medium
D5Ertd33e	Novel gene	n/a	Pompeia (+0.5)	Positive (+0.5)	1	Medium
Dnclc1	Dynein light chain 1, cytoplasmic	Inhibits the activity of neuronal NO synthase, implying regulatory role in numerous biologic processes Drosophila mutants have pleiotropic morphogenetic defects.	Chen & Corey (+1) Morton Pompeia	Positive (+0.5)	1.5	High
Sfrs9	Splicing factor, arginine/serine rich 9	Involved in mRNA formation (-1)	Morton (+1) Pompeia	Positive (+0.5)	0.5	Low

Gene	Description	Summary of relevant published data	Representation in inner ear expression databases	Inner ear cDNA PCR screen	Total points	Candidacy rating
NM029645	Novel gene	n/a	-	Positive (+0.5)	0.5	Low
Q8C4H9	Novel gene	n/a	-	Positive (+0.5)	0.5	Low
15E1	Novel gene	n/a	-	Positive (+0.5)	0.5	Low
Cox6a1	Cytochrome c oxidase polypeptide VIa-liver	Subunit of COX, terminal enzyme of the respiratory chain. Probable housekeeping gene (-1)	Chen & Corey (+1) Morton Pompeia	Positive (+0.5)	0.5	Low
Msi2h	Musashi homolog 1	neural RNA-binding protein (-1)	Chen & Corey (+1) Morton	Positive (+0.5)	0.5	Low
Q9D2I7	Novel gene	n/a	-	Positive (+0.5)	0.5	Low
Pla2g1b	Phospholipase A2 precursor	Involved in protection of membranes from oxidative injury	-	Positive (+0.5)	0.5	Low
Sirt4	NAD-dependent deacetylase sirtuin 4	Role in epigenetic gene silencing	-	Positive (+0.5)	0.5	Low
NM133915 (*Paxillin*)	Paxillin alpha	Required at focal adhesions for actin organisation (+1) Knockout mice do not have a vestibular or hearing phenotype (-1)	Chen & Corey (+1) Morton Pompeia	Positive (+0.5)	1.5	High
Gcn1l1	General control of amino-acid synthesis 1-like 1	Required for activation of protein kinase GCN2, leading to upregulation of aa synthesis during starvation (-1)	-	Positive (+0.5)	-0.5	Very low
NM198163	Novel gene	n/a	Morton (+1) Pompeia	Positive (+0.5)	1.5	High

Gene	Description	Summary of relevant published data	Representation in inner ear expression databases	Inner ear cDNA PCR screen	Total points	Candidacy rating
Q8R1D2	Novel gene	n/a	Pompeia (+0.5)	Positive (+0.5)	1	Medium
Rpl29	60S ribosomal protein L29	Ribosomal subunit (-1)	Chen & Corey (+1) Pompeia	Positive (+0.5)	0.5	Low
Cit	Citron protein (Rho-interacting, serine/threonine kinase)	Involved in Rho/Rac signalling (+1) Knockout mice do not have a hearing or vestibular phenotype (-1)	Chen & Corey (+1) Morton	Positive (+0.5)	1.5	High
Prkab1	5'-AMP-activated protein kinase, beta-1 subunit	Plays a role in hormonal and nutrient-derived anorexigenic and orexigenic signals and in energy balance (-1)	Chen & Corey (+1) Pompeia	Positive (+0.5)	0.5	Low
NM177759	Novel gene	n/a	-	Positive (+0.5)	0.5	Low
Q9D4T7	Novel gene	n/a	-	Inconclusive (0)	0	Low
Hspb8	Heat-shock protein beta-8	Neuromuscular function (-1)	-	Positive (+0.5)	-0.5	Very low
NM026886	Novel gene	n/a	-	Positive (+0.5)	0.5	Low

5.4.3 Selection of candidate genes for further analysis

Of the 54 genes examined, twelve scored greater than 1.5 points using the weighted scale and obtained a candidacy rating of either "High" or "Very high". Of these twelve, three were the novel genes Q9D1S6, NM175403 and NM198163 which each scored 1.5 points. Their points were accrued via expression data which was not dissimilar from that of many of the known genes, with the difference being that the lack of specific literature meant that no negative points were assigned to them. Therefore these genes were not initially prioritised but may be investigated at a later date. The remaining nine genes in the "High" or "Very high" candidacy categories were *Myo1h, Kctd10, Ube3b, Trpv4, Git2, Cabp1, Dnclc1, Paxillin* and *Citron*. These genes were all selected for further analysis. One other gene, *Foxn4*, was included in later studies. At the time the assessment was carried out this gene appeared to represent an interesting candidate but in September 2004, data regarding the phenotype of a knockout mouse (Li *et al.*, 2004) was published and personal communication confirmed that the mouse did not exhibit any hearing or vestibular phenotype.

Taken together, these ten genes represent a manageable subset of genes from within the *bronx waltzer* candidate region for which further investigation is justified on the merit of the evidence considered. All ten of them have been previously described to some degree, with none of the novel genes having been selected. It is very possible that the *bronx waltzer* gene could be as yet undescribed, however it is very difficult to select which of the 23 uncharacterised genes to investigate when there is so little information to go on. It would be beneficial in this case to carry out more detailed expression studies using sections or whole mount in situ hybridisations to determine whether they are expressed in the expected tissues of the inner ear. This work

was beyond the scope of the current project, but should be considered in further pursuance of the *bv* gene.