Chapter 4:

A new intraspecific intercross to narrow the bronx waltzer critical region

CHAPTER 4

A NEW INTRASPECIFIC INTERCROSS TO NARROW THE *BRONX WALTZER* CRITICAL REGION

4.1 INTRODUCTION

At the commencement of this project, significant attempts had already been made to identify new markers which could be used to refine the existing *bronx waltzer* map based on the backcross with the inbred strain 101/H (see Section 3.1.3). Since these had so far proved fruitless and evidence was mounting that the strains may harbour a low polymorphism rate within the defined interval, it was decided that the establishment of a new mapping cross with a different inbred strain would be beneficial.

In order for an additional cross to be informative for linkage mapping purposes and allow the size of the candidate region to be reduced, it must fulfil three criteria. First, the phenotype must be distinguishable on the new genetic background with maximum penetrance. Secondly, the strain chosen for the outcross should contain polymorphisms which make it distinguishable from the *bronx waltzer* background within the critical region and finally, it must result in offspring possessing genetic breakpoints between the current flanking markers. The measures taken to ensure the greatest likelihood of these conditions being met are outlined below.

4.1.1 Choosing an outcross strain

At the time of the establishment of this cross, a physical map of the mouse genome had been published and sequence was becoming available. The physical positions of the flanking markers *D5Mit25* and *D5Mit209* were known, and therefore it was possible to isolate markers which mapped between them. These could then be screened against a panel of prepared

genomic DNA samples from a variety of inbred strains in order to determine which had the largest number of informative polymorphisms with *bronx waltzer* within the region, making it a suitable candidate for the outcross. Additionally, this study may provide information regarding the genetic background on which the *bv* mutation first arose, with any strain demonstrating few or no polymorphisms being a potential candidate or close relative of that on which the original *bv* mutation initially arose.

4.1.2 Choosing a breeding strategy

The simplest and most commonly employed breeding strategy for linkage mapping is that of the backcross (see Section 3.1.1.1). This operates on the principle that each mouse resulting from the cross represents a single isolated meiotic recombination event. One complete set of chromosomes are inherited from each inbred parent, making it possible to distinguish at each locus whether the individual has inherited the mutant allele from its F1 parent or that from the wildtype outcross strain. Thus a haplotype map of the recombinant chromosome can be constructed relatively easily.

A second approach sometimes used for mapping is that of the intercross. Here, an outcross is carried out in the same way as for a backcross, but the resulting mice from the F_1 generation are then mated together instead of being crossed back to one of the parental inbred strains. As a result, the F_2 intercross offspring will carry two recombinant chromosomes, as illustrated in Figure 4.1.

The main advantage of this strategy is the occurrence of meiotic recombination events in each parent effectively doubling the number of potentially informative breakpoints in each F_2 animal. However this same feature is also responsible for the major disadvantage of the intercross, since the inheritance of two recombinant chromosomes makes analysis of the data significantly more complex than for a backcross. Each animal carries two

separate haplotypes for each linkage group, making it impossible to assign alleles to one or the other haplotype. At every locus, each allele may have been inherited from either parent. Thus, when generating *de novo* linkage maps from large-scale intercross experiments it is essential to use software designed to carry out multilocus maximum likelihood analysis such as Mapmaker (Lander *et al.* 1987).

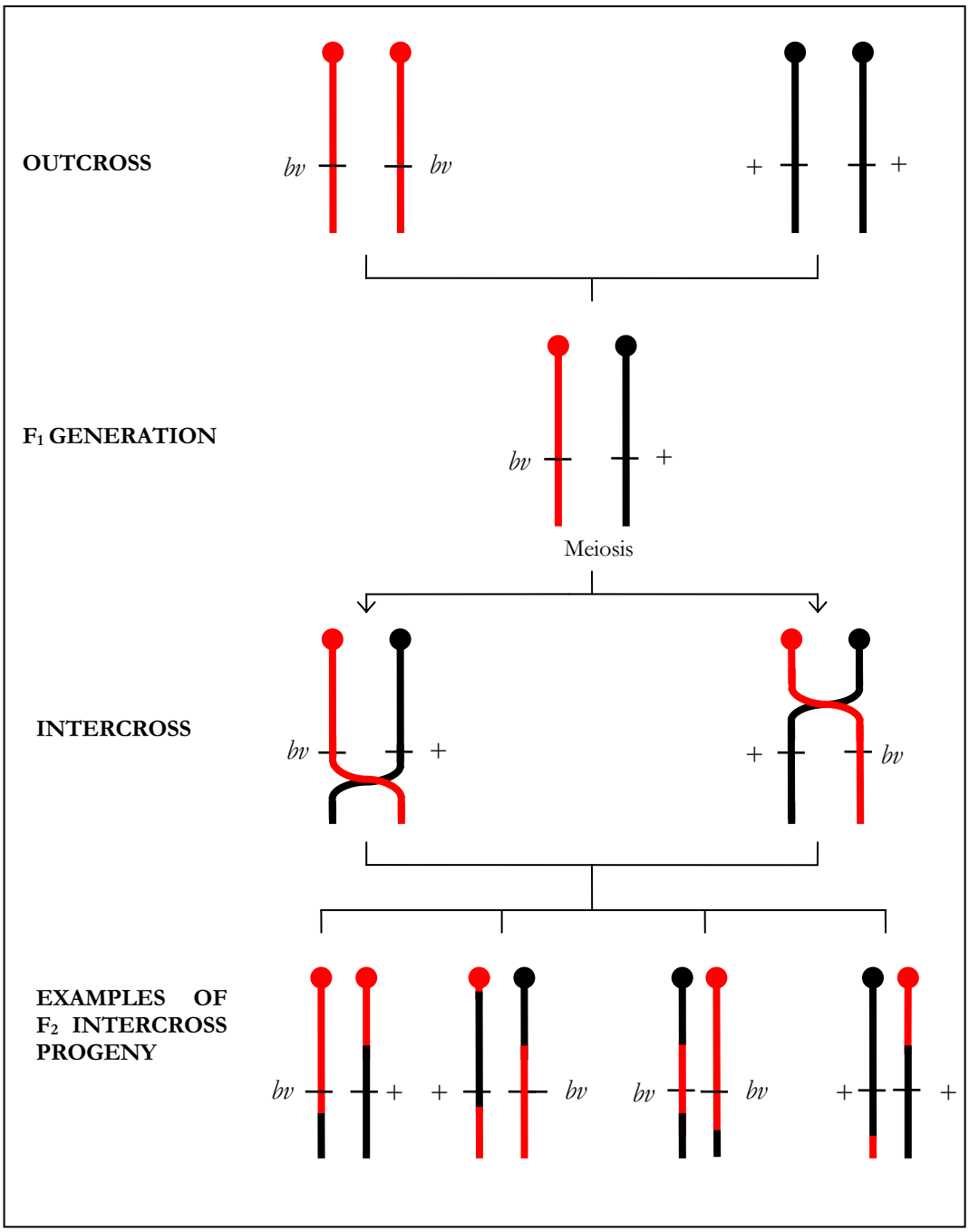

Figure 4.1: Diagram illustrating the breeding strategy employed in the generation of an intercross. Chromosomes derived from *bronx waltzer* are shown in red while those from the selected inbred strain are in black. During gamete formation in the F_1 generation, recombination may occur between the two parental chromosomes at the prophase I phase of meiosis. These F_1 mice are then bred together, producing intercross offspring in possession of two sets of recombinant chromosomes. The expected Mendelian ratios for the F_2 progeny of such a cross are 1:2:1 of wild type, heterozygotes and homozygous mutants.

Despite the difficulties inherent in analysing the data, the intercross strategy is still the most suitable in certain circumstances. The first is in the case of recessive deleterious mutations where animals homozygous for the mutation either die before adulthood or are rendered infertile. Since backcrossing the F_1 generation to an affected mutant is unfeasible, an intercross becomes the only available option for linkage mapping. However, since it is not possible to discern heterozygote carriers from wild type animals, only the affected mutant offspring can be used for analysis.

Although *bronx waltzer* is not deleterious in the homozygous form, the phenotype does have an impact on breeding strategy which needs to be taken into account. Mice homozygous for the mutant *bv* allele exhibit hyperactivity and shaker-waltzer behaviour. This type of behaviour in mice tends to correlate with poor parenting, with pregnancies being irregular and the majority of litters born into a cage with a homozygous parent being trampled or cannibalised, thereby reducing the chances of offspring surviving to adulthood. During general colony maintenance this problem can be bypassed by establishing breeding pairs consisting of a heterozygous female and homozygous mutant male, then removing the male from the cage once a pregnancy has been established. Since the generation of a large number of offspring is crucial to the success of a mapping project, this limits the usefulness of the backcross (see Figure 3.1) for three reasons. Firstly, the number of breeding pairs at the backcross stage is constrained by the availability of mutant males from the original colony which does not always breed efficiently, and also by the fact that only female F_1 animals can be used for subsequent breeding. Secondly, since homozygous mutant males do not breed very effectively the rate at which pups are born can be very irregular and lastly, the rate of production of F_2 offspring is further slowed by the removal of the male from the cage until each litter of pups has been weaned. By contrast, with an intercross strategy (see figure 4.1) the F_1 breeding pairs will both be heterozygous at the *bv* locus and hence manifest no phenotype.

This means that all of the F_1 animals can be used, that pregnancies can overlap with nursing of litters, that breeding will be more predictable and in addition, hybrid vigour might be expected to give larger and more frequent litters.

The second situation where an intercross may be most suitable is when the focus of the mapping has previously been narrowed to a small genomic interval of a few centimorgans or less. At this high level of resolution the data analysis will be much less complex, with only a small fraction of animals expected to show predominantly single recombination events within the region of interest. Under these circumstances, the increased probability of generating a mouse with an informative recombination within the small predefined interval outweighs the difficulties posed by analysis of the data, making an intercross mapping strategy suitable.

With just 1.83cM lying between the established flanking markers, this last set of criteria applies to the current status of the *bronx waltzer* mapping project. Taken in addition to the practical advantages conferred by the breeding strategy, it was decided that the generation of an intercross would be the most beneficial approach for the fine mapping of the *bv* mutation.

4.2 METHODS

Some of the methods employed in this chapter are described in Chapter 2. The following procedures are specific to this chapter.

4.2.1 Inbred strain DNA

Some of the inbred strains tested were already maintained as colonies within the lab and DNA from these was prepared as described in Section 2.2. The remainder of the inbred strain DNA was obtained from the Mouse DNA Resource maintained by the Jackson Laboratory (Bar Harbor, Maine). Table 4.1 shows the inbred strain DNAs analysed and lists their origin.

Mouse Strain	Genealogical Group	Source
101/H	Castle's	IHR Nottingham
129X1/SvJ	Castle's	Jackson Lab
A/J	Castle's	Jackson Lab
BXD-1/Ty	C57 x DBA	Jackson Lab
C58/J	Castle's	Jackson Lab
CE/J	Other	Jackson Lab
DA/HuSn	Swiss	Jackson Lab
DBA/2J	Castle's	Jackson Lab
DDY/Jc1	China/Japan	Jackson Lab
FL/1Re	Castle's	Jackson Lab
LP/J	Castle's	Jackson Lab
NON/LtJ	Swiss	Jackson Lab
RBG/Dn	C57 x DBA x AET	Jackson Lab
ST/bJ	Other	Jackson Lab
SWR/J	Swiss	Jackson Lab
C3HeB/FeJ	Castle's	IHR Nottingham
C57BL/6J	Castle's	IHR Nottingham
BALB/C	Castle's	IHR Nottingham
CBA/Ca	Castle's	IHR Nottingham

Table 4.1: Inbred strains making up the panel of DNAs to be tested for polymorphism with *bronx waltzer* within the critical region. Genealogical groups are taken from Beck *et al.* (2000).

The panel of DNA from a number of inbred strains was tested with markers from across the *bv* critical interval for polymorphism by PCR. Some strains were chosen because colonies of the mice were already maintained within the

lab and thus their employment in a new intercross would be greatly simplified. Others were selected to represent as broad as possible a cross section of the laboratory inbred strains in order to increase the chances of identifying a mouse with a high level of polymorphism with *bronx waltzer*, and also of discovering which strain the mutation may have arisen on. The selection of these strains was informed by the work of Beck and colleagues (2000) to determine the origins of the various laboratory inbred strains, and particularly by the accompanying lineage chart depicting their genealogies

4.2.2 Microsatellite markers

The markers used are listed in Table 4.2. Some of these have previously been mapped onto the *bv*/101 map, while others have been shown to map within the physical interval but do not exhibit polymorphisms on *bv* and 101/H backgrounds.

Marker	Position (Mb)	Forward primer	Reverse primer
D5Mit25	111.27	AACACACCTCCATACTGGTCG	GGCTAACTGAAATTGTTTTGTGC
D5Mit241	111.89	TGTTTATCAGGGTTGGTCTGC	CATATGGGTCATCAGATATCATGG
D5Mit158	112.37	AAAGACGCTGAGGAGTCACTG	CAGGAGACCTTGTAATAAAGGAAA
D5Mit318	112.68	ATATGGGCTTGGCTTTCATG	GACTACACACATCACTCTCTCTCTCA
D5Mit187	113.29	GGACCCACAAGATGGAAAGA	CCTCAGTGGATACATGTTTAAACTT
D5Mit209	114.06	TCTGAGCAAGGTCGTCCAC	CCCTGTCTCAAGATAAAAGCTAGG
D5Mit405	114.18	CAACAACAACAAAAAAAGAAAGATG	CACAGTCCCACATCCACCTA
D5Mit424	117.36	GACTCCTCCTCGCCTTTCTT	AAAATTACATTTGCATCTGGGG

Table 4.2: Markers from within and surrounding the *bv* critical region to be used in the analysis of polymorphisms between various inbred strains and the *bronx waltzer* genetic background. Positions given are taken from Ensembl Build 33.

4.2.3 Phenotyping F_2 intercross offspring

The intercross breeding strategy illustrated in Figure 4.1 was followed, with the resulting generation of F_2 offspring possessing genotypes of either +/+, +/*bv* or *bv*/*bv*. Mice were scored in a similar manner to that described in Section 3.1.1.4 for the generation of the *bv*/101 backcross, their behaviour being assessed and scored according to the presence or absence of a shaker-

waltzer phenotype indicative of vestibular dysfunction. In addition to this, a Preyer reflex test for auditory function was administered with the use of a click box. This was held 30cm directly above the mouse to be tested and a tone-burst of 20kHz and 90 dB SPL emitted while the mouse's response was observed. A positive result was recorded if the animal retracted its pinnae, a negative one if they failed to respond. Mice which exhibited shaker-waltzer behaviour and lacked a Preyer reflex were scored as *bv*/*bv*, with any particular remarks regarding their behaviour being recorded. Animals which behaved normally and exhibited a Preyer reflex were scored as +/?, since it is not possible to phenotypically discern heterozygote +/*bv* animals from wild type +/+ ones. All mice were given an identifier consisting of the letters BC followed by a consecutive number unique to each mouse. This code was used to associate notes taken about each animal with all the tissues later collected.

4.2.4 Tissue collection

At the time of the sacrifice, tissue was collected for DNA preparation as described in Section 2.2. In addition, the inner ears of each mouse were dissected and fixed in a manner suitable for their analysis by scanning electron microscopy. Following bisection of the head and removal of the brain, each half head was placed immediately into fixative (2.5% glutaraldehyde in 0.1M sodium cacodylate and 2mM $CaCl_2$). The inner ear was removed from the skull, the bulla detached and the oval window and the apex of the cochlea were opened up using a sharp needle and fix perfused gently through the resulting holes. The ears were transferred to fresh fixative and placed in rotating stirrers overnight at 4°C to ensure full fixation of the tissue. The samples were washed 6 times for 5 minutes in cocodylate buffer (0.1M sodium cacodylate; 2mM $CaCl_2$) and were then stored at 4°C until required for further analysis.

4.3 RESULTS

4.3.1 Strain selection

As discussed, the choice of inbred strain employed at the outcross step of the intercross breeding strategy is crucial to the success of the linkage mapping project. The strain must have polymorphisms making it distinguishable from the *bronx waltzer* background within the critical interval. As such, the following study was conducted in order to make an informed decision regarding which strain to use.

Each inbred strain genomic DNA sample was used as the template in PCR reactions with primers from markers in and around the *bv* critical region, and the size of the resulting product determined by agarose gel electrophoresis. The results for the markers flanking and surrounding the region are shown in Figure 4.2a and summarised in Table 4.3a. Those for the markers within the region are depicted in Figure 4.2b and summarised in Table 4.3b

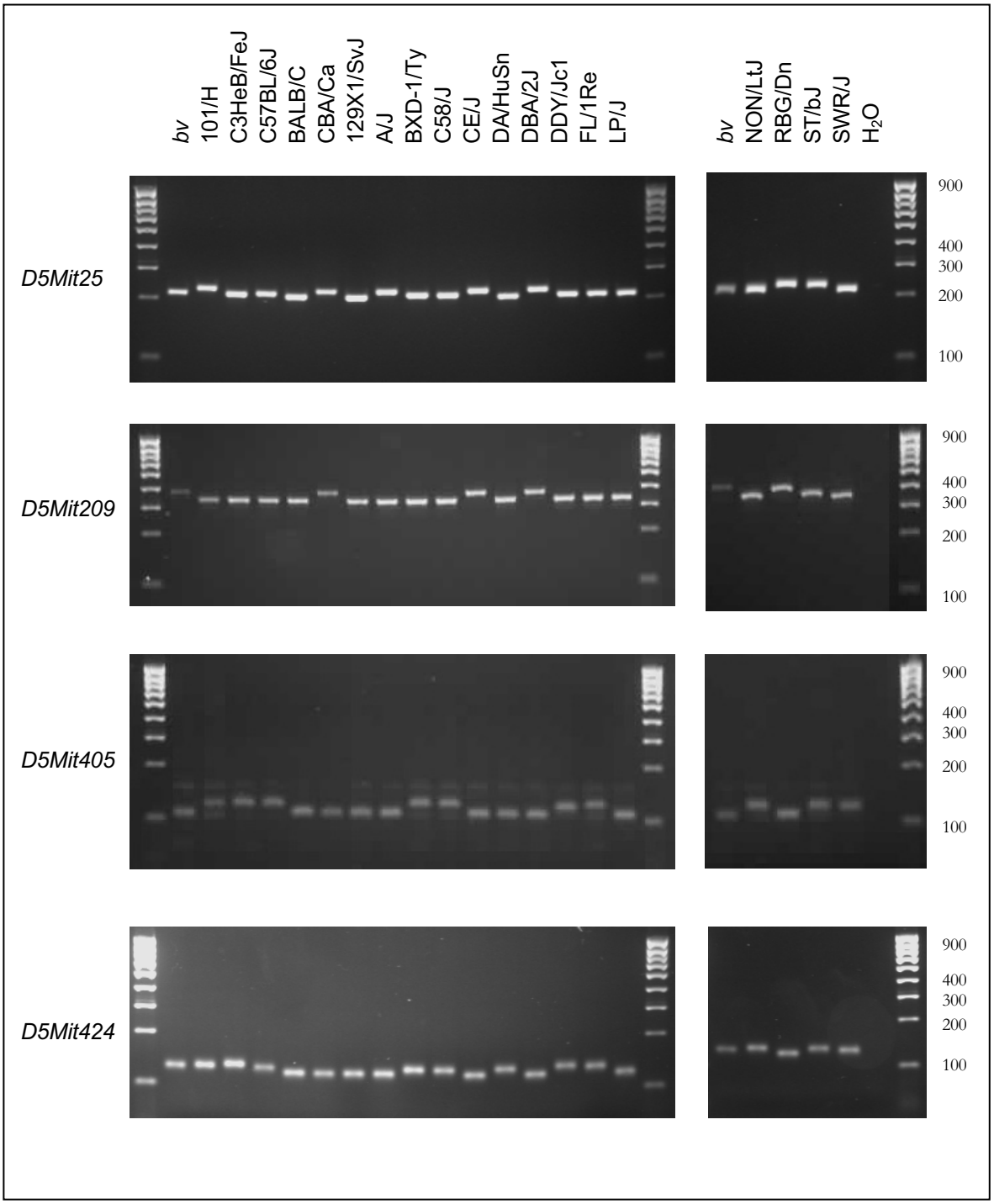

Figure 4.2a: Flanking marker polymorphisms
Agarose gel electrophoresis showing the sizes of fragment amplified when screening polymorphic genetic markers against a panel of DNA from a variety of inbred strains. These markers flank or lie outside the established candidate region.
The marker lane contains 1Kb ladder and the figures given are in base pairs.

Strain	*D5Mit25*	*bv*	*D5Mit209*	*D5Mit405*	*D5Mit424*
101/H	**Y**		**Y**	Y	N
C3H	N		**Y**	Y	N
C57BL/6J	**Y**		**Y**	Y	Y
BALB/C	N		**Y**	N	Y
CBA	**Y**		N	N	**Y**
129X1/SvJ	N		**Y**	N	Y
A/J	**Y**		**Y**	N	Y
BXD-1/Ty	N		**Y**	Y	Y
C58/J	N		**Y**	Y	Y
CE/J	**Y**		N	N	**Y**
DA/HuSn	N		**Y**	N	Y
DBA/2J	**Y**		N	N	**Y**
DDY/Jc1	N		**Y**	Y	N
FL/1Re	N		**Y**	Y	N
LP/J	N		**Y**	N	Y
NON/LtJ	N		**Y**	Y	N
RBG/Dn	**Y**		N	N	**Y**
ST/bJ	**Y**		**Y**	Y	N
SWR/J	N		**Y**	Y	N

Table 4.3a: Summary of flanking marker polymorphisms
Summary of the polymorphism screen of inbred strains for markers flanking the *bronx waltzer* region. A difference in product size when compared to *bv* DNA is indicated by the letter Y, a product size indiscernible from *bv* is indicated by the letter N.
For those strains possessing polymorphic flanking markers on either side of the region the closest markers are highlighted in **BLUE**. Those possessing only one polymorphic flanking marker are shown in **RED**.

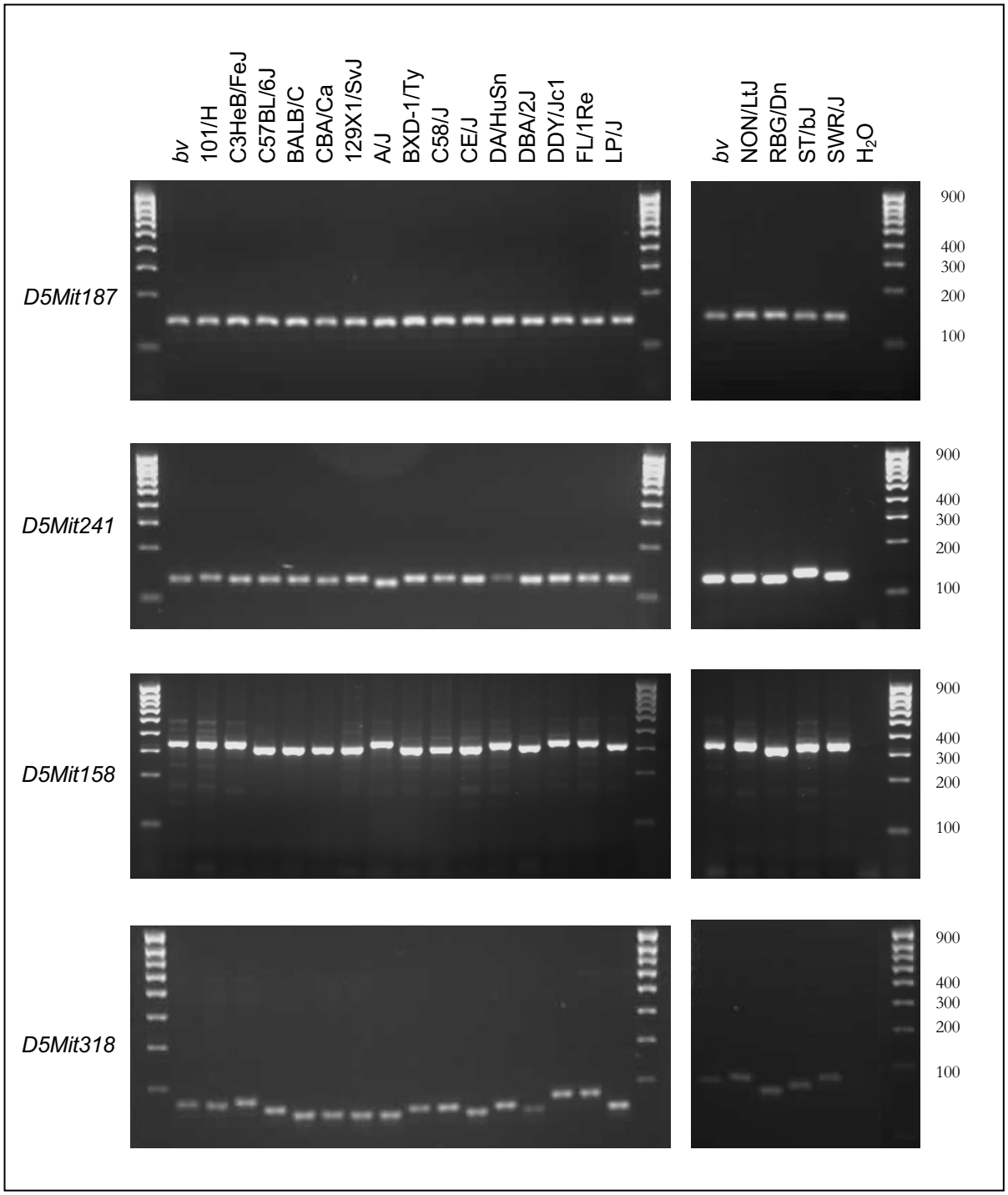

Figure 4.2b: Internal marker polymorphisms
Agarose gel electrophoresis showing the sizes of fragment amplified when screening polymorphic genetic markers against a panel of DNA from a variety of inbred strains.
These markers lie within the critical region, polymorphisms here could be used to narrow the region and therefore cut down the number of candidate genes being considered for *bronx waltzer*.
The marker lane contains 1Kb ladder and the figures given are in base pairs.

Strain	*D5Mit241*	*D5Mit158*	*D5Mit318*	*D5Mit187*	Total
101/H	N	N	N	N	0
C3H	N	N	**Y**	N	1
C57BL/6J	N	**Y**	N	N	1
BALB/C	N	**Y**	**Y**	N	2
CBA	N	**Y**	**Y**	N	2
129X1/SvJ	N	**Y**	**Y**	N	2
A/J	**Y**	N	**Y**	N	2
BXD-1/Ty	N	**Y**	N	N	1
C58/J	N	**Y**	N	N	1
CE/J	N	**Y**	**Y**	N	2
DA/HuSn	N	N	N	N	0
DBA/2J	N	**Y**	N	N	1
DDY/Jc1	N	N	**Y**	N	1
FL/1Re	N	N	**Y**	N	1
LP/J	N	**Y**	N	N	1
NON/LtJ	N	N	**Y**	N	1
RBG/Dn	N	**Y**	**Y**	N	2
ST/bJ	**Y**	N	N	N	1
SWR/J	**Y**	N	N	N	1

Table 4.3b: Summary of internal marker polymorphisms
Summary of the polymorphism screen of inbred strains for markers within the *bronx waltzer* region. A difference in product size when compared to *bv* DNA is indicated by the letter Y highlighted in **BLUE**, a product size indiscernible from *bv* is indicated by the letter N.
The total number of polymorphic markers within the *bv* region for each strain is given in the rightmost column.

4.3.2 An intraspecific intercross

Based on the results of the strain polymorphism assays, the strain CBA/Ca was chosen to be used in the outcross. It was shown to have polymorphisms with *bv* at two useful flanking markers as well as at two internal markers. Other strains gave similar results but as CBA/Ca was already maintained in the lab it was expedient to set up the outcross immediately. For a full discussion of the criteria considered for strain selection see Section 4.4.1.

An outcross was set up between a *bronx waltzer* male homozygous mutant and a CBA/Ca female as the first stage in the intercross breeding strategy (see Figure 4.1). Since the mutation is maintained on an unknown background a single male from the mutant strain was used in the matings to produce all F_1 hybrids, since this limits the number of potential alleles to be inherited by the offspring and makes analysis more straightforward (Silver 1995). These F_1 animals were then intercrossed, giving a total of 233 F_2 progeny, of which 47 were phenotyped as *bronx waltzer* homozygous mutants and the remaining 186 recorded as +/? since heterozygote and wild type animals are indistinguishable. Of the 47 mice recorded as mutants, three were noted as having slightly questionable phenotypes. The mouse identified as BC44 had some head-bobbing and no reaching response but appeared to exhibit a Preyer reflex. Mice BC74 and BC110 showed some head-bobbing but no circling. They lacked a reaching response and writhed on being picked up but had a weak Preyer reflex. On the balance of this evidence, all three mice were recorded as *bv/bv* by phenotype. A pedigree chart illustrating the lineage of the animals used in this mapping project is shown in Figure 4.3.

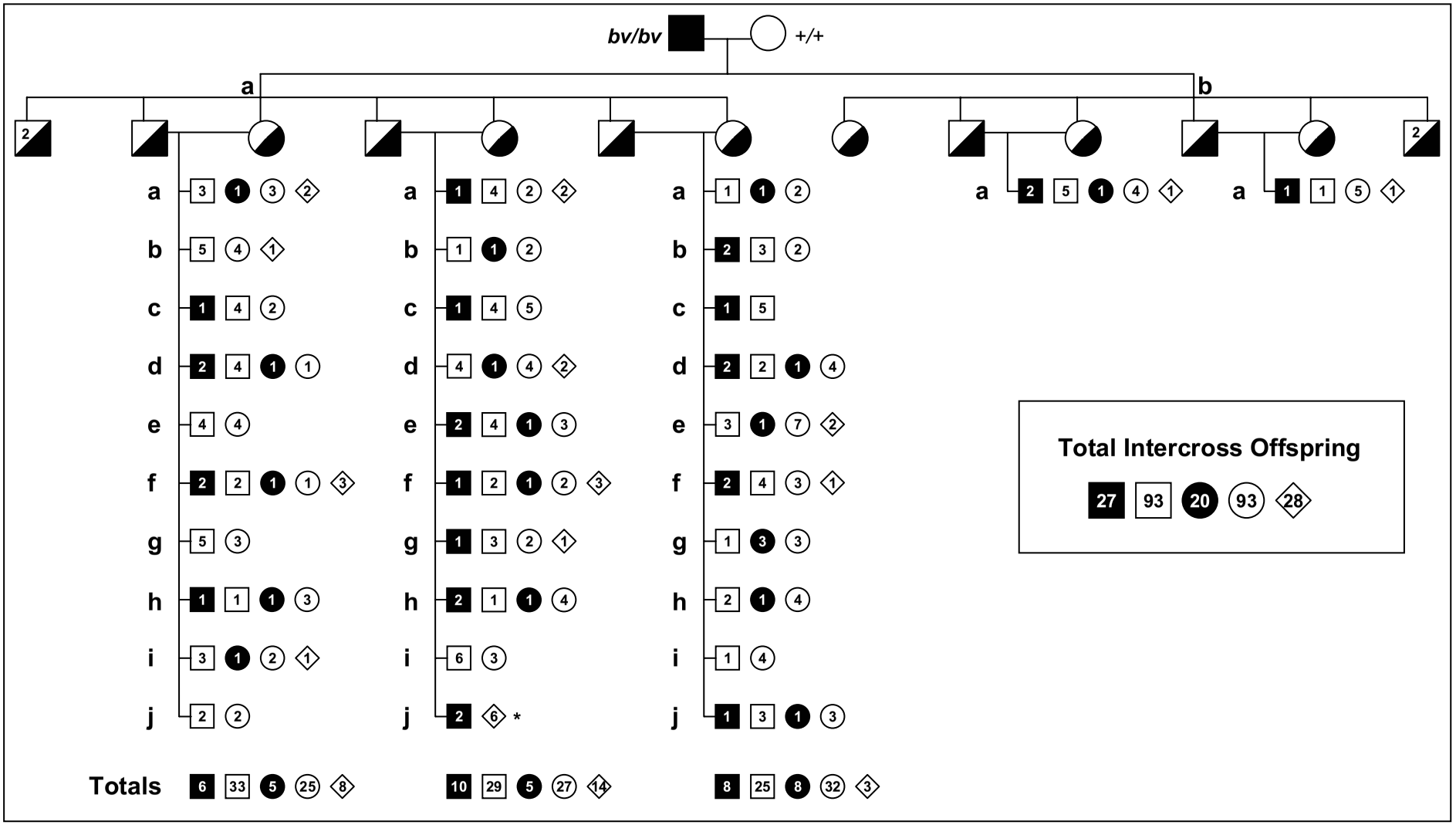

Figure 4.3: Pedigree chart illustrating the lineage of the mice used in the *bv*/CBA/Ca intercross mapping project. Males are represented by squares, females as circles and animals which died before being sexed or phenotyped are shown as diamonds. Animals with mutant phenotypes are shown as filled shapes, controls are unfilled. Known heterozygotes are shown half-filled. The litter marked * was sacrificed at age P12 and was only just beginning to show signs of shaker-waltzer behaviour, therefore only those animals which could be confidently typed as mutants were counted.

4.3.2.1 Statistical analysis

The number of males and females in the F_2 generation was 120 and 113 respectively, which when analysed using the G-test (likelihood ratio test) for goodness of fit (Sokal and Rohlf 1995) gives a value of 0.22 (see Table 4.4a), suggesting a close adherence to the expected ratio of 1:1 (d.f.=1; p=0.64). The same test was used to assess the ratio of control to mutant animals which according to Mendelian ratios is expected to equal 3:1 for this breeding strategy (see Figure 4.1). The observed figures of 186 control mice and 47 mutants give a G-value of 2.92 (see Table 4.4b), which indicates a slight but not significant deviation from the expected ratios (d.f.= 1; p=0.09).

Sex	**Observed frequencies**	**Observed proportions**	**Expected proportions**	**Expected frequencies**	**Ratio**	**G-value (ln L)**	**Adjusted G-value**
	f	$\frac{f}{n}$	$\hat{p}$ and $\hat{q}$	$\hat{f}$	$\frac{f}{\hat{f}}$	$f \ln\left(\frac{f}{\hat{f}}\right)$	2 ln L
Male (p)	120	0.515	0.5	116.5	1.03	3.55	7.1
Female (q)	113	0.485	0.5	116.5	0.97	-3.44	6.88
Sum (n)	233	1	1	233	-	0.11	**0.22**

Table 4.4a: G-test (likelihood ratio test) for goodness of fit applied to the proportion of males to female F_2 offspring obtained in the intercross of *bv* to CBA/Ca. The adjusted G-value allows a p-value to be obtained using chi-square tables.

Phenotype	**Observed frequencies**	**Observed proportions**	**Expected proportions**	**Expected frequencies**	**Ratio**	**G-value (ln L)**	**Adjusted G-value**
	f	$\frac{f}{n}$	$\hat{p}$ and $\hat{q}$	$\hat{f}$	$\frac{f}{\hat{f}}$	$f \ln\left(\frac{f}{\hat{f}}\right)$	2 ln L
Control (p)	186	0.798	0.75	174.75	1.064	11.539	23.078
Mutant (q)	47	0.202	0.25	58.25	0.807	-10.078	20.156
Sum (n)	233	1	1	233	-	1.461	**2.922**

Table 4.4b: G-test (likelihood ratio test) for goodness of fit applied to the proportion of mutant to control F_2 offspring obtained in the intercross of *bv* to CBA/Ca. The adjusted G-value allows a p-value to be obtained using chi-square tables.

4.3.2.2 Genotyping F_2 mice

Since wild type (+/+) and heterozygous (+/*bv*) mice cannot be distinguished from each other, only the homozygous mutant (*bv*/*bv*) F_2 offspring from this intercross can be included in linkage analysis. Genomic DNA was prepared

from tissue samples taken from each of the 47 mutant mice and screened using the flanking markers and markers from within the *bv* candidate region which had been shown to be polymorphic for *bv* and CBA/Ca (see Section 4.3.1). The results of this screen are shown in Figure 4.4. To genotype this cross *D5Mit25* and *D5Mit424* were used as flanking markers, while the markers within the region were *D5Mit158* and *D5Mit318*. As homozygous mutants, these mice will be expected to show a single band of the same size as the *bv* sample. Should any recombination have taken place within the critical region then we would expect to see two bands of different sizes, representing both *bv* and CBA/Ca.

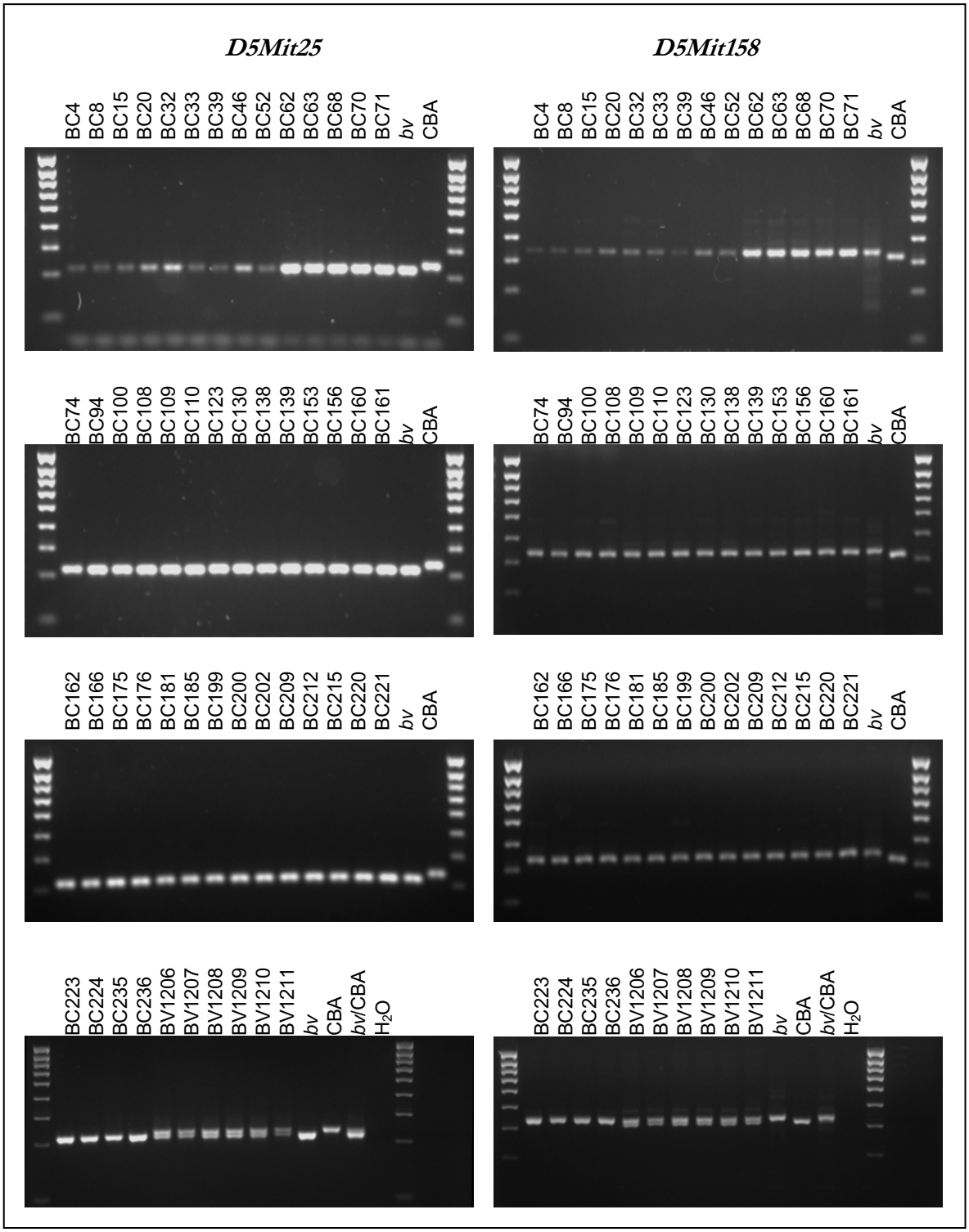

Figure 4.4a: Agarose gel electrophoresis showing PCR products obtained when amplifying polymorphic genetic markers from mutant mice in the F_2 generation of the intercross between *bv* and CBA/Ca. Mutant mice are designated by codes prefixed BC, controls showing the size of fragment expected from each parental strain are given on each row. The final row includes the F_1 parents as heterozygous controls; these are prefixed by the letters *BV*.

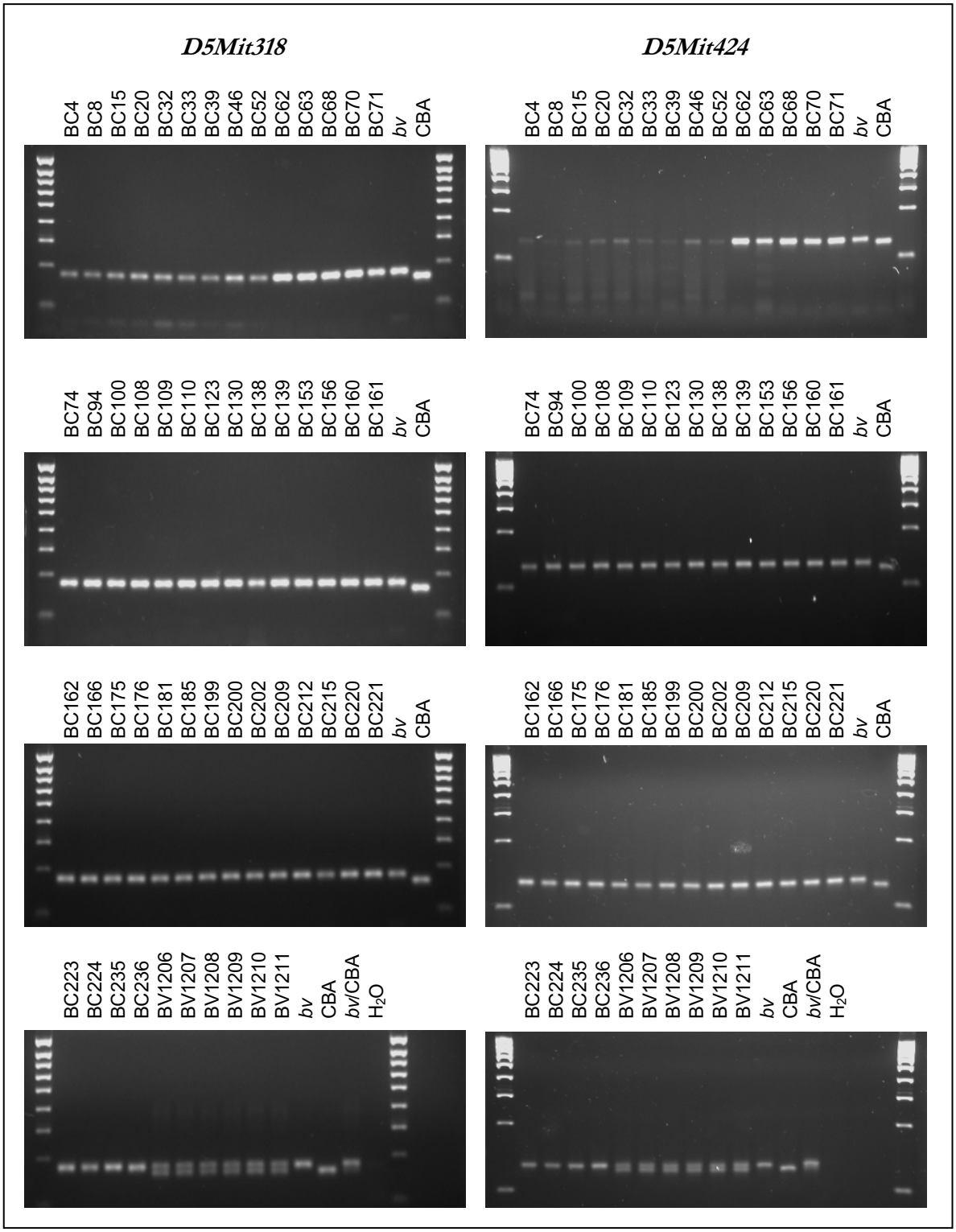

Figure 4.4b: Agarose gel electrophoresis showing PCR products obtained when amplifying polymorphic genetic markers from mutant mice in the F_2 generation of the intercross between *bv* and CBA/Ca. Mutant mice are designated by codes prefixed BC, controls showing the size of fragment expected from each parental strain are given on each row. The final row includes some of the F_1 parents as heterozygous controls; these are prefixed by the letters *BV*.

4.4 DISCUSSION

4.4.1 Strain selection

In order to select a suitable strain with which to outcross the *bronx waltzer* mice, a number of criteria were taken into account. Most importantly, the strain must exhibit polymorphisms within the region already defined by the *bv*/101 backcross map. Therefore strains which gave products distinguishable from those amplified from *bv* DNA with the markers *D5Mit241*, *D5Mit158*, *D5Mit318*, and *D5Mit187* were prioritised. Secondly, it must be possible to type the mice at points either side of the critical interval in order to establish a recombination rate between them, therefore the chosen strain must also possess polymorphisms close to or at the existing flanking markers. Finally, at the time the cross was to be established, it was known that the lab would be relocated in the near future and that any existing crosses could not be transferred. Since the final total of F_2 animals is critical to the probability of obtaining an informative recombination event, it was considered crucial that the breeding scheme began as quickly as possible. Hence priority was given to those strains which were already maintained as colonies within the lab (see Table 4.1).

Of the four markers which lie within the region, three gave polymorphisms with at least one strain, with *D5Mit187* giving the same product size in all the strains tested. Six strains – BALB/C, CBA/Ca, 129X1/SvJ, A/J, CE/J and RBG/Dn – gave polymorphisms with two of the remaining three markers. Eleven strains gave polymorphisms with one of the markers, and the remaining two – 101/H and DA/HuSn were not polymorphic for any of the selected markers. Of the six most polymorphic strains only A/J was also polymorphic with *bv* at the closest flanking markers *D5Mit25* and *D5Mit209*. However, CBA/Ca was an established colony within the lab and demonstrated polymorphisms at the proximal flanking marker *D5Mit25* and

at the more distal marker *D5Mit424*. It was decided that the increased simplicity and rapidity conferred by the use of an easily available strain outweighed the disadvantage of a more distant flanking marker, and hence the intercross was established using a CBA/Ca outcross parent.

4.4.2 The origins of the *bronx waltzer* genetic background

The *bronx waltzer* mutation arose spontaneously in a strain of laboratory mice kept at the Albert Einstein College of Medicine in New York and was first reported in 1979 (Deol and Gluecksohn-Waelsch 1979). The identity of this strain was unreported and the mutation has since been crossed to a variety of different inbred strains, leading to its current maintenance on a mixed genetic background. However, since the continuation of a mutant strain necessitates the selection of the original mutant allele, and since recombination frequencies are low within a small region, it is possible that the genetic material in close proximity to the *bv* locus is still that belonging to the strain on which the mutation arose. Hence an analysis of the polymorphism rates with various inbred strains within the *bv* candidate region may provide information regarding the identity of the original genetic background. This would be useful since it would facilitate the discovery of polymorphic markers.

Taking into account only those genetic markers which are located within the current candidate region and thus lie close to the *bv* allele (see Table 4.3b) we can see that two strains, 101/H and DA/HuSn, show no polymorphisms with the *bronx waltzer* genetic background. However, both of these strains show polymorphisms with *bronx waltzer* at the flanking marker *D5Mit209*, which according to the 101/H genetic map (see section 3.1.2) is located only 0.37cM from the *bv* allele. In addition, these two strains are in all likelihood unrelated or only distantly associated with each other, with 101/H stemming from the Castle's group and DA/HuSn from the Swiss mice (Beck *et al.* 2000). Therefore, since they are unrelated to each other, it is probable that

their apparent similarity to *bronx waltzer* is a consequence of a similar chance rather an indication of a shared genetic heritage. As previously mentioned, the mutation initially arose over 25 years ago and it is not known on which strain the mutation arose on or if the mutation was crossed to other strains in the early years before it was transferred to Nottingham. Over such a large number of generations it is possible that recombinations have occurred even within such a small region, meaning that only DNA extremely closely linked to the *bv* allele would still be that of the original strain. The discovery of markers which are non-recombinant with the mutation may make it possible to acquire more useful data informing the origins of the *bronx waltzer* mouse.

Interestingly, although CBA/Ca was found in this study to be one of the strains most polymorphic with *bv*, in the analysis carried out prior to the establishment of the backcross to 101/H (Bussoli 1996), CBA/Ca was found to be one of the least polymorphic strains with a 31% polymorphism rate in a panel of 32 genetic markers. By contrast, *Mus castaneus* gave an 86% polymorphism rate and 101/H gave 52%. The markers tested in this investigation were taken from a much more extensive region of mouse chromosome 5 since the mutation had yet to be localised at this stage. Therefore it is possible that this apparent discrepancy can be explained by the *bronx waltzer* chromosome 5 comprising some DNA from or closely related to CBA/Ca, but with genetic material from a different strain in close proximity to the locus.

The lack of clarity in these results and the failure to identify a single strain which correlates closely with *bronx waltzer* is probably a reflection and confirmation of its long and complicated breeding history.

4.4.3 Transmission ratio analysis

The transmission of the *bronx waltzer* gene is assumed to be inherited according to Mendelian genetics. As such, in the case of an intercross the F_2

generation would be expected to have a 3:1 ratio of control (either wild type or heterozygote) to mutant mice. The F_2 mice in the present intercross to CBA/Ca were found to deviate slightly but not significantly from this ratio when analysed using a G-test for goodness of fit (Sokal and Rohlf 1995). This adherence to the expected ratios suggests that the *bv* mutation is fully penetrant on the CBA/Ca genetic background, as well as providing some evidence that the *bv* allele is not lethal in its homozygous form. This is an interesting observation since in the previous cross to 101/H (Bussoli 1996; Cheong 2000) and also during the maintenance of the *bronx waltzer* stock on the original unknown background (unpublished observations), the transmission ratio of the *bv* allele has been found to be significantly lower than expected. It was postulated that some of the embryos homozygous for *bv* may be dying prenatally, but a discrepancy between the number of pups recorded at birth and the number still alive at the time of sacrifice led to the suggestion that a disproportionate proportion of *bv* homozygoyte pups were dying at an early age. These animals are often smaller, possibly as a result of their disorientation and lack of co-ordination making it difficult for them to feed properly. As a result, it is possible that the parents identify them as being less "fit" than their littermates and that they are preferentially subject to infanticide. As outlined in Section 4.1.2, the behaviour of *bv* mutants necessitates the removal of the male from the cage once a pregnancy has been established, a strategy which was followed for both the 101/H backcross and for maintenance of the *bronx waltzer* stock. In contrast, since both parents in the intercross breeding strategy are heterozygotes, they are left undisturbed and are both present when litters are born. In general this means that the parents are likely to be less stressed and are hence less likely to cannibalise their offspring (vom Saal and Howard 1982). In addition, levels of infanticide have been shown to vary greatly between strains (Svare *et al.* 1984; Perrigo *et al.* 1993), introducing the possibility that CBA/Ca mice are inherently more likely to keep their offspring after birth. Therefore, the adherence of the transmission ratio to expected frequencies in this case supports the suggestion

in previous studies that the reduction in the number of mutant animals was a result of their being preferentially chewed by the mother, rather than by a lethal effect of the *bronx waltzer* gene.

It is also possible that the *bronx waltzer* phenotype may be subject to a subtle reduction in penetrance or a modification of the phenotype on the CBA/Ca genetic background leading to some mice carrying two copies of the *bv* mutant allele failing to exhibit the behaviour typical of homozygous mutant mice and perhaps having a higher survival rate. However, when analysing the F_2 offspring of this cross, there were very few incidences of mice with indeterminate phenotypes. In the case of a modifier effect being present one might expect to find mice with some but not all of the characteristics of a mutant. For example, they may exhibit mild head-bobbing rather than circling and tail-chasing, or simply fail to show a detectable Preyer reflex. Of the 233 mice included in this cross, only 3 were noted as having slightly unclear phenotypes and all of these were confidently scored as *bv/bv*. Subsequent genotyping analysis showed these mice to be carrying *bronx waltzer* alleles at both flanking markers, thus confirming that their phenotypes were correctly recorded. Hence it is unlikely that the *bronx waltzer* allele is modified on the CBA/Ca genetic background.

4.4.4 Interpretation of intercross data

Of the 47 *bronx waltzer* homozygote F_2 offspring analysed in this study, none had undergone recombination within the critical region. All gave single bands corresponding to the size expected at a *bv* allele at the flanking markers and at each of the internal polymorphic markers. This outcome does not reduce the size of the candidate region for the *bronx waltzer* allele or allow the exclusion of any candidate genes. Recombination events are considered to be relatively random, although some regions recombine at a higher than average rate and are known as "hotspots", while others are less likely to undergo recombination (Steinmetz *et al.* 1987). In order to gauge the relative

recombination rates we might expect in the region surrounding the bv allele we can examine the findings of the previous genetic cross to 101/H (see Chapter 3). In this instance, the genetic distance between the flanking markers *D5Mit25* and *D5Mit209* was found to be 1.83cM. To determine the average recombination rate, we can compare the physical size of mouse chromosome 5 (150Mb, Ensembl Build 33) to its genetic size (92cM, Mouse Genome database) giving a conversion factor of 1cM:1.6Mb, thus the physical size of the region might be expected to be approximately 3.02Mb. The actual physical size is slightly smaller at 2.8Mb, implying that the recombination rate of the region is close to or slightly higher than average. In the panel of 1073 mice, 19 showed recombination within the critical region, equating to one in every 56 meioses. In the present intercross, 47 mutant mice represent 94 meioses since every mouse carries a recombined haplotype from each of its F_1 parents. Hence, should the recombination frequency be consistent with the previous cross, we might have expected to see at least one mouse carrying a recombination within the region. However, different strains exhibit different recombination rates over the same chromosomal region, and in addition the male recombination rate differs from that of females. The lack of recombinant mice arising from this cross is most probably a result of stochastic factors and it is probable that with a larger number of F_2 progeny a recombinant mouse or mice might be found. The scope of the present study was constrained by the imminent relocation of the lab, meaning that the production of litters was brought to a halt prematurely. In order to increase the numbers of F_2 offspring and hence the probability of obtaining recombinations within the region, a new outcross and F_1 intercross could be established. Although the parental animals would be different, the status of CBA/Ca as in inbred strain means that the new parents could be considered identical and while not inbred, *bronx waltzer* DNA in the proximity of the mutation is highly likely to be conserved. This means that data obtained from the F_2 animals generated could be added to those obtained in the present

study, thus increasing its statistical power, although a single recombination event may be all that is required to reduce the size of the region.