Chapter 3

Experimental Results

Introduction

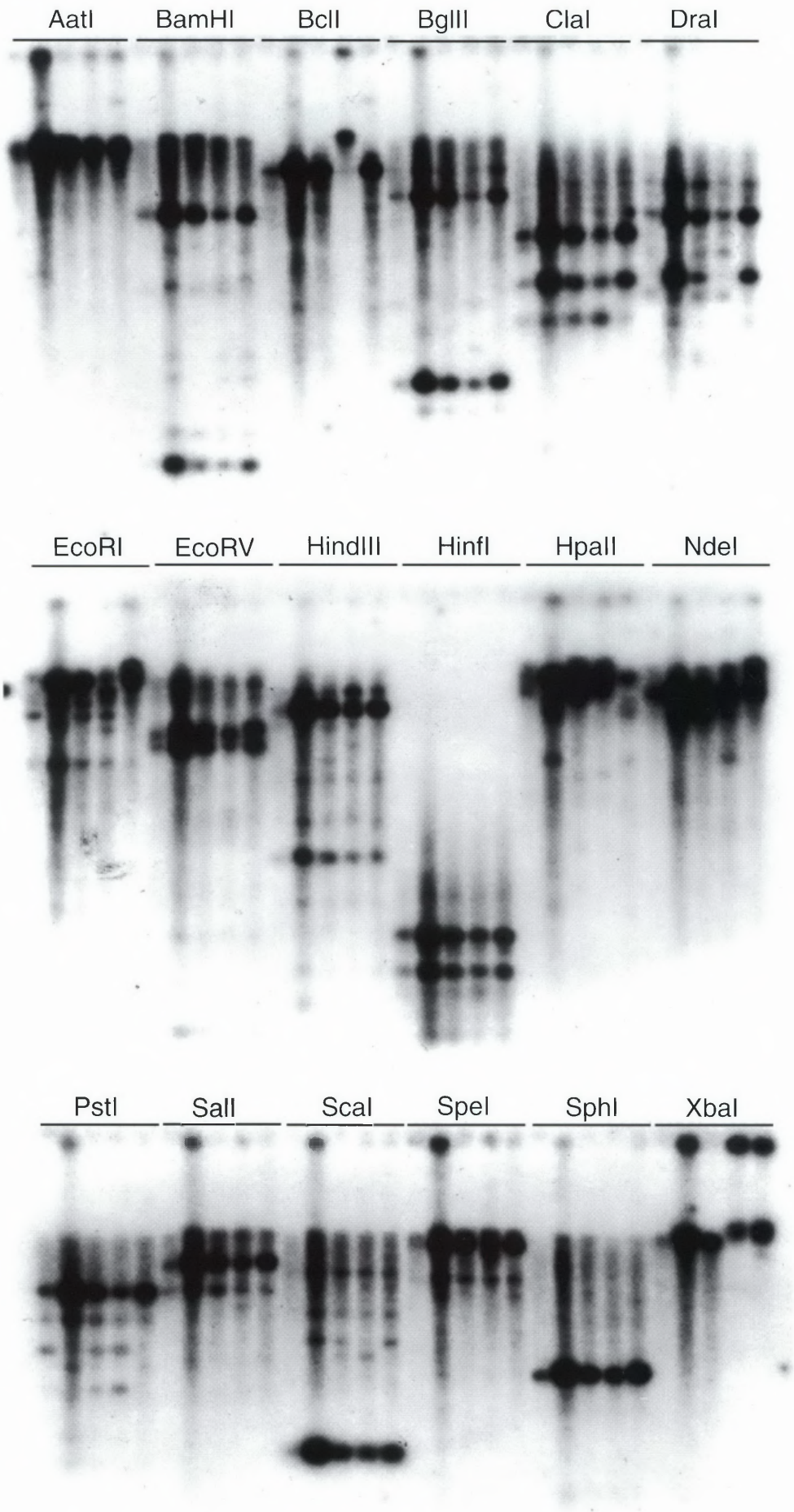
The cloning of the *T. colubriformis* β-tubulin gene *tcb-1* and the initial restriction fragment length polymorphism (RFLP) analysis to identify β-tubulin gene polymorphisms associated with benzimidazole (BZ) resistance in *T. colubriformis* were conducted by Dr. Warwick Grant of CSIRO Division of Animal Health. The *tcb-1* gene was obtained by screening an EMBL3 *T. colubriformis* genomic library under low stringency conditions with a *C. elegans* β-tubulin gene *tub-1* (Gremke, 1986). A 12kb SalI fragment from one (clone 1.5a) of the five positive clones obtained from the library screening was subcloned into the Bluescript plasmid vector. This clone was given the designation pWG21.

Dr. Grant digested the pWG21 clone with a number of restriction enzymes, blotted and probed the DNA digests with the *C. elegans tub-1* probe. Restriction fragments from the pWG21 clone that hybridised strongly to the *tub-1* probe were assumed to contain β-tubulin coding sequence because the *C. elegans* probe consisted largely of coding sequence. The intensity of hybridisation of the *T. colubriformis* DNA with the *tub-1* probe indicated that the bulk of the coding region of the *T. colubriformis* β-tubulin gene was on either a 2.9kb PstI fragment or two ScaI fragments, one of 1.1kb and the other 1.8kb in size. A restriction map to determine the order of these fragments and the relationship to the β-tubulin gene they represented was not constructed at this time.

Dr. Grant observed that the 1.1kb ScaI fragment showed the most intense hybridisation of the three fragments to the *tub-1* probe. Consequently the 1.1kb ScaI fragment was gel purified and used as a probe on genomic southerns to identify RFLPs associated with BZ resistance in *T. colubriformis*. (Figure 6) Five strains of *T. colubriformis,* McMaster susceptible, McMaster resistant, Kybebolite susceptible, Kybebolite resistant and New England Resistant *Trichostrongylus* (see Materials and Methods), were used in the RFLP analysis.

DNA from each of the five strains was digested with 18 restriction enzymes and the digests run on 20x10cm gels using 30 well combs, blotted and probed with

Figure 6. RFLP analysis of BZ resistant and susceptible strains of *T. colubriformis* DNA from five strains: (left to right) Kybebolite susceptible, Kybebolite resistant, New England resistant *Trichostrongylus*, McMaster susceptible and McMaster resistant were each digested with 18 different restriction enzymes. The digests were run on 0.8% agarose gels, blotted and probed with a 1.1kb ScaI fragment from the pWG21 clone. Each consecutive set of five lanes represents digests of the five strains with the same enzyme. RFLPs can be seen in the McMaster resistant strain in the ClaI digests, the McMaster susceptible lane in the DraI digests and the McMaster resistant strain in the EcoRI digests.


AatI
BamHI
BclI
BglII
ClaI
DraI
EcoRI
EcoRV
HindIII
HinfI
HpaII
NdeI
PstI
SalI
ScaI
SpeI
SphI
XbaI

the 1.1kb ScaI fragment. Using this probe RFLPs were observed when genomic DNA was digested with the enzymes ClaI, DraI and EcoRI.

In all three cases one or more bands appeared to have been deleted from the resistant strains when compared to the number of bands hybridising in the susceptible strains. For the other 15 enzymes RFLPs were not identified and only one or two major hybridising bands were observed for all five strains. Dr. Grant's results have been shown here as they form the basis of this project.

The southerns showing RFLPs were repeated to clarify and confirm Dr. Grants results and are shown in this chapter. All results reported hereafter were obtained by the author as part of this thesis.

Confirmation that the pWG 21 Clone Contained a β-tubulin Gene

The initial results obtained by Dr Grant indicated that RFLPs between BZ susceptible and resistant strains could be detected using a 1.1kb ScaI fragment from the pWG21 clone and that this fragment hybridised strongly to the *C. elegans* β-tubulin gene *tub-1,* suggesting that the pWG 21 clone contained β-tubulin coding sequence. Before repeating the RFLP analysis it was confirmed that pWG21 did contain a β-tubulin gene by obtaining some sequence from the *T. colubriformis* fragments that hybridised to the *C. elegans tub-1* gene and comparing this sequence to other known β-tubulin genes. The order of the fragments shown to hybridise to the *tub-1* probe was determined by restriction enzyme analysis (Figure 7).

A 2.9 kb PstI fragment from pWG21 (that was shown by Dr Grant to hybridise to the *C. elegans tub-1* probe) was subcloned into the Bluescript SK+ plasmid vector and given the designation pWG34. Restriction mapping of pWG34 showed that the 1.1kb ScaI fragment which detected the RFLPs between resistant and susceptible strains of *T. colubriformis* was located within the PstI fragment and that the 1.8kb ScaI fragment that also hybridised to the *C. elegans tub-1* β-tubulin gene overlapped the PstI fragment and adjoined the 1.1kb ScaI fragment (Figure 7). The PstI fragment contains only one DraI site.

A combination of nested deletion clones and subcloned fragments were used to obtain 1431 bp of sequence from regions along the 2.9kb pWG34 clone. All subclones were sequenced from the KS and SK plasmid sequencing primers to determine the position of these clones within the gene. A custom made primer

was used to obtain the carboxy-terminal variable sequence of the β-tubulin gene (see Materials and Methods).

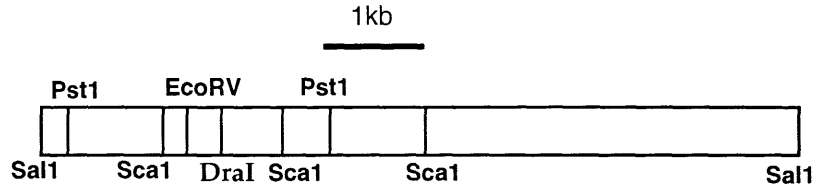


Figure 7. Restriction map of the pWG 21 clone. The Pst fragment was subcloned (pWG34) and used to obtain sequence.

Comparison of the sequence obtained from the pWG34 clone with other β-tubulin genes showed that the pWG34 clone did indeed contain coding sequence of a β-tubulin gene but the clone did not contain the whole gene. Sequence from one end of the cloned PstI fragment started at amino acid 45 and extended in a 3' direction into the gene. Sequence from the opposite end of the PstI fragment did not show any homology to any β-tubulin coding sequence. However, the pWG34 clone did contain the carboxy-terminal variable region of the gene with a stop codon. The nucleotide sequence obtained from the pWG34 clone is shown in Figure 8.

When the deduced coding sequence was compared to the *Haemonchus contortus* isotype 1 gene (Kwa *et al.*, 1993) (Figure 9) the boundaries of six introns were identified. All of the six introns identified in *T. colubriformis* were in identical positions to introns identified in the *H. contortus* isotype 1 β-tubulin gene. The *H. contortus* isotype 1 β-tubulin gene was reported to contain nine introns. The *T. colubriformis* gene may also contain nine introns as an additional introns may be present in the unsequenced regions. A schematic diagram showing the regions for which sequence was obtained and the position of the introns identified is given in Figure 10.

The coding sequence obtained from the *T. colubriformis* clone showed a striking degree of homology to the *H. contortus* isotype 1 β-tubulin gene. The 3' terminal amino acid sequence was completely conserved which is most unusual when comparing sequences for this region between species of invertebrates (Burns & Surridge, 1990).

Region 1

GAA AGA ATT AAT GTG TAT TAC AAT GAG GCA CAT ggtatgtgaatgtcttgtttttgcttacgttatttac
ctagacatctctgttaacttatgattgtcttttctgagctgattcagctatttcacgatacacgttcttagttggtgtgacattattatgtattggtgtgaa

Region 2

caatggaattcgatactgttttca GGA GGC AAA TAT GTT CCA CGT GCT GTT CTT GTT GAT CTC GAA CCC GGA ACA ATG GAT TCC GTT CGT TCT GGA CCG TAC GGG
CAG TTG TTC CGT CCG GAT AAT TAC GTG TTC GGC CAG TCA GGA GCG CGT AAC AAC TGG GCA AAG GGC CAC TAT ACT GAG

Region 3

C CAA CTA ACG CAT TCC TTG GGA GGA GGT ACT GGA TCC GGT **ATA AAC** ACT CTG CTT ATC TCA AAA ATT CGT GAA GAG TAC CCG GAC AGA ATT ATG
GCT TCG TTC TCT GTT GTT CCA TCA CCT AAG GTA cag

Region 4

TCC GAC ACT GTT GTG GAG CCC TAC AAT GCT ACT TTA TCA GTC CAT CAG CTG GTA GAG AAT ACC GAT GAA ACA TTC TGC ATC GAT AAC GAA GCT CTG TAT
GAT ATC TGC TTC CGC ACC TTG AAA CTC ACA AAT CCA ACC TAT GGA GAT CTC AAC CAT CTT ggtaaattgtttagtttggtcagattttatctacatatt
ttacatacataaatacagacagctatcatagtgtccattcgatctatgaatgcaattttta GTG TCT GTC ACA ATG TCT GGT GTC ACG ACT TGC CTC CGA TTC CCA

Region 5

TTC CCT CGT CTT CAC TTC TTC ATG CCC GGT TTT GCC CCA TTG TCT GCA AAG GGT GCC CAA GCA TAT CGT GCC TCA ACA GTC GCT GAG CTT ACA CAG CAG
gtatgtgggccggttttctcgcattcccacgt tttactctcctcagatttataatacacatatttttttag ATG TTC GTA GCC AAG AAC ATG ATG GCT GCG TGT GTA CCT CGT CTA
GGT CGC TAT CTT ACC GTT GCG GTC ATG TTC CGT GGT CGC ATG AGC ATG CGG

Region 6

ctggtatacgtctgaactgttggtagagtccaattctgttctgaactgttttggatgagctcatattttcagaaacgctgaagcagactgcggatcattcgatgccctcgcaat
ttttcgaagcttacgccatgttattacagGAA GTA GAC GAC CAG ATG ATG TCG GTG CAG AAC AAG AAC TCG TCA TAT TTC GTG GAA TGG ATT CCA AAC AAC GTG AAG
ACT GCT GTT TGT GAC ATT CCT CCT CGT GGA CTG

Region7

TAC ACT GGT GAG GGC ATG GAC GAG ATG GAG TTC ACA GAA GCT GAG TCG AAC ATG AAT GAT CTG ATC TCC GAA TAC CAA CAG TAC CAG
gtgtgtgatctctttctttctcgagttctcctatgcttgatgtctgcgggacatttttt ttcctacatatatttcacgtcttaatttttccag GAA GCT ACT GCT GAT GAC ATG GGC GAT
CTC GAT GCA GAA GGT GGA GAA GAG GCG TAC CCA GAA GAG TAG

Figure 9. Alignment of the coding sequence obtained from the *T. colubriformis* pWG34 clone (top line of sequence in bold) and the *Haemonchus contortus* β–tubulin gene *gru-1* (Kwa *et al.*, 1993) Gaps in the *T. colubriformis* gene are the unsequenced regions of the gene.

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      5     10    15    20    25    30    35    40    45    50    55    60    65    70    75    80    85    90    95

ATG CGT GAA ATC GTT CAT GTG CAA GCC GGT CAA TGC GGC AAC CAG ATC GGA TCA AAG TTC TGG GAA GTG ATC TCT GAT GAG CAC GGT ATC CAG CCC
Met Arg Glu Ile Val His Val Gln Ala Gly Gln Cys Gly Asn Gln Ile Gly Ser Lys Phe Trp Glu Val Ile Ser Asp Glu His Gly Ile Gln Pro

100   105   110   115   120   125   130   135   140     145   150   155   160   165   170   175   180   185   190
                                              GAA AGA ATT AAT GTG TAT TAC AAT GAG GCA CAT GGA GGC AAA TAT GTT CCA CGT GCT GTT
GAT GGA ACA TAC AAA GGA GAA TCA GAT CTG CAA TTA GAA AGG ATC AAT GTG TAC TAC AAT GAA GCA CAT GGA GGC AAG TAT GTT CCA CGT GCT GTT
Asp Gly Thr Tyr Lys Gly Glu Ser Asp Leu Gln Leu Glu Arg Ile Asn Val Tyr Tyr Asn Glu Ala His Gly Gly Lys Tyr Val Pro Arg Ala Val>

195   200   205   210   215   220   225   230   235   240 245   250   255   260   265   270   275   280   285
CTT GTT GAT CTC GAA CCC GGA ACA ATG GAT TCC GTT CGT TCT GGA CCG TAC GGG CAG TTG TTC CGT CCG GAT AAT TAC GTG TTC GGC CAG TCA GGA
CTT GTT GAT CTC GAG CCT GGA ACG ATG GAC TCC GTT CGT TCT GGA CCG TAT GGA CAG CTT TTC CGT CCA GAT AAT TAC GTG TTT GGC CAG TCA GGA
Leu Val Asp Leu Glu Pro Gly Thr Met Asp Ser Val Arg Ser Gly Pro Tyr Gly Gln Leu Phe Arg Pro Asp Asn Tyr Val Phe Gly Gln Ser Gly

290   295   300   305   310   315   320   325   330   335   340   345   350   355   360   365   370   375   380
GCG GGT AAC AAT TGG GCG AAG GGC CAC TAT ACT GAG GGA GCC GAG CTA
GCG GGT AAC AAT TGG GCG AAG GGC CAC TAT ACT GAG GGA GCC GAG CTA GTT GAT AAC GTA TTA GAC GTT GTC CGC AAA GAA GCT GAA GGT TGT GAT
Ala Gly Asn Asn Trp Ala Lys Gly His Tyr Thr Glu Gly Ala Glu Leu Val Asp Asn Val Leu Asp Val Val Arg Lys Glu Ala Glu Gly Cys Asp>

385   390   395   400   405   410   415   420   425   430 435   440   445   450   455   460   465   470   475   480
                  C CAA CTA ACG CAT TCC TTG GGA GGA GGC ACT GGA TCC GGT         ACT CTG CTT ATC TCA AAA ATT CGT GAA GAG TAC CCG
TGC CTT CAG GGC TTC CAA TTG ACG CAT TCA CTT GGA GGA GGC ACT GGA TCT GGA ATG GGC ACT TTG TTA ATT TCA AAA ATT CGT GAA GAG TAC CCT
Cys Leu Gln Gly Phe Gln Leu Thr His Ser Leu Gly Gly Gly Thr Gly Ser Gly Met Gly Thr Leu Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro>

485   490   495   500   505   510   515   520   525   530   535   540   545   550   555   560   565   570   575
GAC AGA ATT ATG GCT TCG TTC TCT GTT GTT CCA TCA CCT AAG GTA TCC GAC ACT GTT GTG GAG CCC TAC AAT GCT ACT TTA TCA GTC CAT CAG CTG
GAT AGA ATT ATG GCT TCG TTC TCC GTT GTT CCA TCA CCC AAG GTA TCC GAC ACT GTC GTA GAA CCC TAC AAT GCT ACC CTT TCC GTC CAT CAA CTG
Asp Arg Ile Met Ala Ser Phe Ser Val Val Pro Ser Pro Lys Val Ser Asp Thr Val Val Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu

580   585   590   595   600   605   610   615   620   625   630   635   640   645   650   655   660   665   670
GTA GAG AAT ACC GAT GAA ACA TTC TGC ATC GAT AAC GAA GCT CTG TAT GAT ATC TGC TTC CGC ACC TTG AAA CTC ACA AAT CCA ACC TAT GGA GAT
GTA GAG AAC ACC GAT GAA ACA TAC TGT ATT GAC AAC GAA GCT CTG TAT GAT ATC TGC TTC CGC ACT TTG AAA CTC ACA AAT CCA ACC TAT GGA GAT
Val Glu Asn Thr Asp Glu Thr Tyr Cys Ile Asp Asn Glu Ala Leu Tyr Asp Ile Cys Phe Arg Thr Leu Lys Leu Thr Asn Pro Thr Tyr Gly Asp>
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675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765

CTC AAC CAT CTT GTG TCT GTC ACA ATG TCT GGT GTC ACG ACT TGC CTT CGA TTC CCA

CTC AAC CAC CTT GTG TCT GTC ACA ATG TCT GGT GTC ACG ACC TGC CTT CGA TTC CCT GGA CAG CTG AAT GCT GAT CTT CGC AAG TTA GCC GTG AAC

Leu Asn His Leu Val Ser Val Thr Met Ser Gly Val Thr Thr Cys Leu Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu Arg Lys Leu Ala Val Asn

770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860

CCT CGT CTT CAC TTC TTC ATG CCC GGT TTT GCC CCA CTG TCT GCA AAG GGT GCC CAA GCA TAT CGT GCC TCA ACA GTC GCT GAG

ATG GTT CCA TTC CCT CGT CTT CAC TTC TTC ATG CCC GGT TTT GCT CCA CTG TCT GCA AAG GGT GCT CAA GCA TAT CGC GCT TCG ACA GTT GCT GAG

Met Val Pro Phe Pro Arg Leu His Phe Phe Met Pro Gly Phe Ala Pro Leu Ser Ala Lys Gly Ala Gln Ala Tyr Arg Ala Ser Thr Val Ala Glu

865 870 875 880 885 890 895 900 905 910 915 920 925 930 935 940 945 950 955 960

CTT ACA CAG CAG ATG TTC GAT GCC AAG AAC ATG ATG GCT GCG TGT GAT CCT CGT CAT GGT CGC TAT CTT ACC GTT GCG GCT ATG TTC CGT GGT CGC

CTT ACA CAG CAA ATG TTC GAT GCA AAG AAC ATG ATG GCT GCC TGT GAT CCT CGC CAT GGA CGT TAT CTT ACG GTC GCT GCT ATG TTC CGT GGT CGT

Leu Thr Gln Gln Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Asp Pro Arg His Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg

965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050 1055

ATG AGC ATG CGA GAA GTA GAC GAC CAG ATG ATG TCG GTG CAG AAC AAG AAC TCG TCA TAT TTC GTG GAA TGG ATT CCA AAC AAC GTG AAG ACT GCT

ATG AGC ATG CGA GAA GTA GAT GAT CAG ATG ATG TCC GTG CAG AAC AAG AAC TCA TCA TAT TTC GTG GAA TGG ATT CCA AAC AAC GTT AAG ACT GCC

Met Ser Met Arg Glu Val Asp Asp Gln Met Met Ser Val Gln Asn Lys Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn Asn Val Lys Thr Ala

1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150

GTT TGT GAC ATT CCT CCT CGT GGA CTG

GTT TGT GAC ATT CCT CCT CGT GGA CTG AAA ATG GCG GCT ACC TTC GTG GGT AAC TCG ACT GCT ATC CAG GAG CTG TTC AAG CGT ATT TCG GAG CAA

Val Cys Asp Ile Pro Pro Arg Gly Leu Lys Met Ala Ala Thr Phe Val Gly Asn Ser Thr Ala Ile Gln Glu Leu Phe Lys Arg Ile Ser Glu Gln>

1155 1160 1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245

TGG TAC ACT GGT GAG GGT ATG GAC GAA ATG GAG TTC ACA GAA GCT GAG TCG AAC ATG AAT

TTC ACT GCC ATG TTC CGA CGC AAA GCT TTC CTT CAT TGG TAC ACT GGT GAG GGT ATG GAC GAA ATG GAG TTC ACA GAA GCT GAG TCG AAC ATG AAT

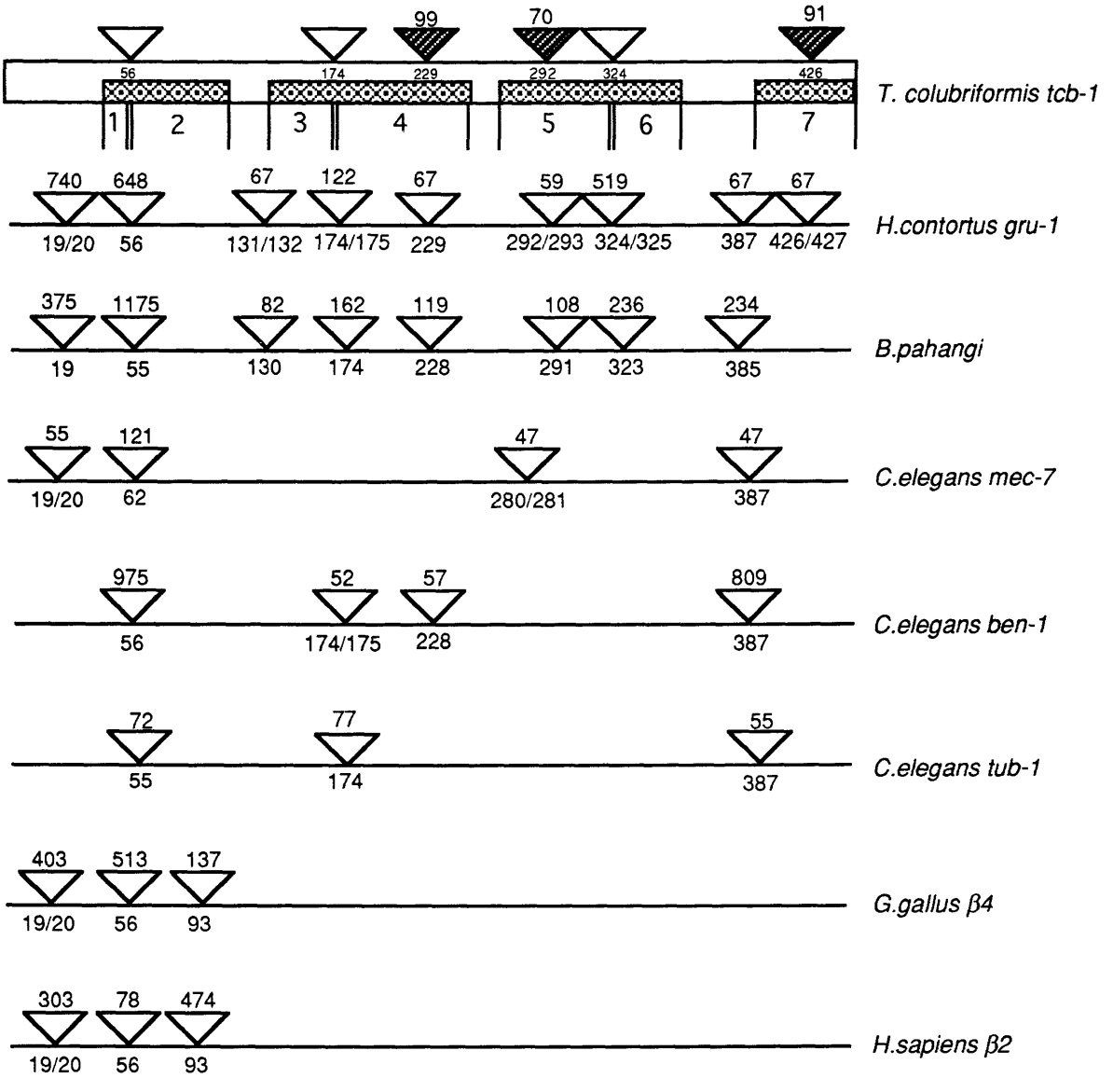
Phe Thr Ala Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly Glu Gly Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn

1250 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330 1335 1340

GAT CTG ATC TCC GAA TAC CAA CAG TAC CAG GAA GCT ACT GCT GAT GAC ATG GGC GAT CTC GAT GCA GAA GGT GGA GAA GAG GCG TAC CCA GAA GAG TAG

GAC CTT ATC TCC GAA TAC CAG CAG TAC CAG GAA GCT ACC GCT GAC GAT ATG GGC GAT CTC GAT GCA GAA GGT GGA GAA GAG GCA TAT CCC GAG GAG TAA

Asp Leu Ile Ser Glu Tyr Gln Gln Tyr Gln Glu Ala Thr Ala Asp Asp Met Gly Asp Leu Asp Ala Glu Gly Gly Glu Glu Ala Tyr Pro Glu Glu ***


99
70
91
56
174
229
292
324
426
1
2
3
4
5
6
7
T. colubriformis tcb-1
740
648
67
122
67
59
519
67
67
19/20
56
131/132
174/175
229
292/293
324/325
387
426/427
H.contortus gru-1
375
1175
82
162
119
108
236
234
19
55
130
174
228
291
323
385
B.pahangi
55
121
47
47
19/20
62
280/281
387
C.elegans mec-7
975
52
57
809
56
174/175
228
387
C.elegans ben-1
72
77
55
55
174
387
C.elegans tub-1
403
513
137
19/20
56
93
G.gallus β4
303
78
474
19/20
56
93
H.sapiens β2

RFLPs between BZ Susceptible and Resistant Strains of *T. colubriformis*.

Having confirmed that pWG 21 contained a β-tubulin gene and determined that the 1.1kb ScaI fragment contained tubulin sequence from amino acid 55 to aa 324 of the gene, the RFLP digests were repeated to confirm and clarify Dr. Grant's initial observations.

The results for the ClaI and DraI digests are shown in Figure 11. Hybridisation to the EcoRI blot were not as clear as the DraI and ClaI digests. The bands in the EcoRI blot were of high molecular weight and not clearly resolved on 0.8% agarose gels. However it was still clear that a lower molecular weight band seen in the McMaster susceptible lane was absent in the McMaster resistant strain (refer to Figure 6).

For both the DraI and ClaI digests a reduction in the number of bands hybridising in the resistant strain compared to the susceptible strain was observed (Figure 11). The bands hybridising in the resistant strains were a subset of the bands hybridising in the susceptible strain but were of lower intensity in DNA extracted from the susceptible population than the DNA extracted from the resistant population. No new bands were observed in the BZ resistant strains implying that resistant individuals were already present in the BZ naive McMaster susceptible population. Both the KRT and NERT strains also had a subset of bands that were present in the McMaster susceptible strain.

As mentioned in Chapter 1, clonal strains of parasitic nematodes of sheep can not be cultured. Therefore "strains" in this case are composed of individuals of different genotypes and the degree of genetic diversity in the McMaster susceptible strain used was unknown. The genomic DNA used for these southerns was prepared from aliquots of larvae from these strains and therefore contains the DNA from many individual worms of differing genotype: any particular strain might therefore have a number of different alleles at any given locus. The observations made in the genomic southerns therefore, could be explained in one of three ways.

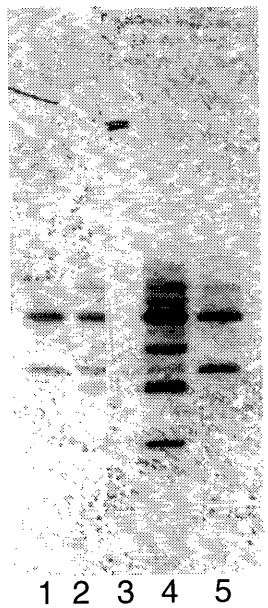
(1) If the ScaI fragment is locus specific then the bands that hybridise in a susceptible population represent alleles at the *tcb-1* locus and the intensity of hybridisation is indicative of the allele frequency of particular *tcb-1* alleles in the population. In this case selection by BZ results in the elimination of individuals carrying particular BZ susceptible alleles leaving only individuals carrying a particular resistance allele. The number of bands present in the susceptible

Figure 11. Southern blots showing the RFLPs detected in BZ susceptible and resistant strains of *T. colubriformis* by the 1.1kb ScaI fragment (used by Dr. Grant in the original RFLP analysis shown in Figure 6)

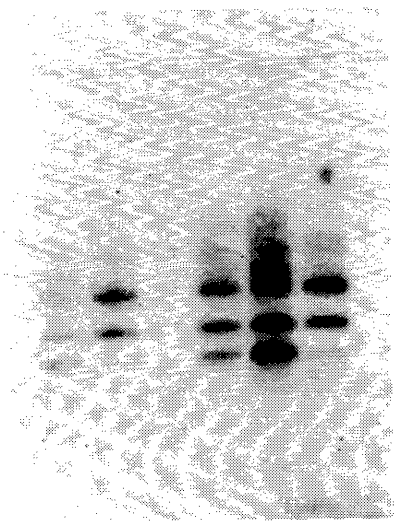
(a) *T. colubriformis* DNA digested with DraI. Lane 1. Kybebolite resistant. Lane 2. New England resistant strain. Lane 3. Blank.
Lane 4. McMaster susceptible. Lane 5. McMaster resistant

(b) *T. colubriformis* DNA digested with ClaI .Lane 1. Kybebolite susceptible strain. Lane 2. Kybebolite resistant strain. Lane 3. blank Lane 4. New England resistant strain. Lane 5. McMaster susceptible. Lane 6. McMaster resistant

(a)



(b)



population (eight) is an estimate of the maximum number of alleles present at the *tcb-1* locus. The minimum possible number cannot be determined as one allele might account for more than one band in the Southern. For example, the allele present in the pWG21 clone has a DraI site in the 1.1kb ScaI fragment and would therefore account for two bands in the genomic southern.

(2) If the Sca1 probe is not locus specific then each band seen on Southerns could represent a distinct β-tubulin locus. Selection by BZs must then result in the deletion of a particular BZ susceptible β-tubulin locus from the genome of particular worms, causing a reduction in the number of hybridising bands seen in southerns of DNA from resistant worms.

(3) The bands detected on genomic southern blots could represent a combination of loci and alleles, with the intensity of hybridisation of the individual bands influenced by (a) the degree of conservation between loci (the most intense bands being the *tcb-1* locus and fainter bands being other β-tubulin loci) and (b) the frequency of each allele in the population (the most prevalent alleles giving the greater intensity of hybridisation). If either hypothesis (2) or (3) were correct then to arrive at the results seen in the resistant populations would require the elimination of multiple loci and alleles.

In order to determine if any one of these hypotheses was correct the following steps were necessary:

Firstly, it was necessary to determine if the locus represented by the pWG21 clone was a multi-allelic locus and whether multiple alleles contributed to the RFLPs seen with the DraI and ClaI digests. Assuming the ScaI fragment was not locus specific, the genomic Southerns were reprobed with other fragments from the pWG21 clone in an attempt to determine which bands were associated specifically with the *tcb-1* locus. These results are discussed in the next section.

Secondly, the bands that remained in the resistant population were readily detected in DNA extracted from the susceptible strain and it was thought that a resistance allele should be present in a genomic library constructed from the susceptible strain that would give rise to the bands seen in both susceptible and resistant strains on genomic southerns. The locus could then be identified by obtaining sequence information from this clone. Additional clones were obtained from the genomic library. These were digested with the DraI and hybridised to the ScaI probe along side genomic DNA digested with DraI. In this way a clone was identified which gave identical sized hybridising bands to those seen in resistant strains of worms.

If the *tcb-1* locus was found not to be associated with BZ resistance then it would be necessary to identify other β-tubulin loci in *T. colubriformis* and determine if any of these were associated with BZ resistance. Consequently the genomic library was rescreened to isolate representative clones of other β-tubulin loci to use as probes if the *tcb-1* locus was found not to be associated with BZ resistance. The results of these experiments are described in the following sections.

Does the *tcb-1* Locus Account for the RFLPs Observed between Resistant and Susceptible Strains?

Since β-tubulin genes are so highly conserved in coding sequence the bands observed in the RFLPs could have been due to the 1.1kb ScaI fragment hybridising to multiple β-tubulin loci in the genome. With the aim of identifying a locus specific probe the genomic southerns were probed with other subclones derived from the pWG21 clone to determine if the same RFLPs were observed with the additional probes. Three fragments were subcloned from the pWG 34 clone (a 2.9kb Pst fragment). The 1.1 kb ScaI fragment (pWG36) mentioned above, a 800bp ScaI-PstI fragment (pWG37, a fragment of the 1.8kb ScaI fragment) and a 1.4 kb Pst-EcoRV fragment (pWG35) (see Figure 12).

The pWG35 clone contained coding sequence from the 5' end of the gene from aa45 through to aa175. The pWG35 clone overlapped with the pWG36 (1.1kb ScaI fragment) clone by approximately 500bp. This 500bp overlap contained coding sequence from codon 55 to codon 175 and intron sequences. The pWG36 clone (1.1kb ScaI fragment) extends from within an intron at the 5' end of the gene into coding sequence at aa55 and extends to aa324 with some intron sequences. The pWG37 clone (800bp ScaI-Pst fragment) extended from aa 345 to beyond the stop codon and into the 3' flanking sequence and therefore contained the 3' variable coding region of this β-tubulin locus.

The DraI digests of the McMaster susceptible and McMaster resistant strains were reprobed with the pWG35 5' clone and the pWG37 3' clone (Figure 13). In both cases a subset of the bands seen in the Southerns with the pWG36 probe were identified. pWG35 accounted for seven of the eight bands seen in the McMaster susceptible strain and one of the bands seen in the resistant population. The 3' pWG37 clone hybridised to two bands in the susceptible population and a single band in the resistant population (refer to Figure 12 for a summary of southern data).

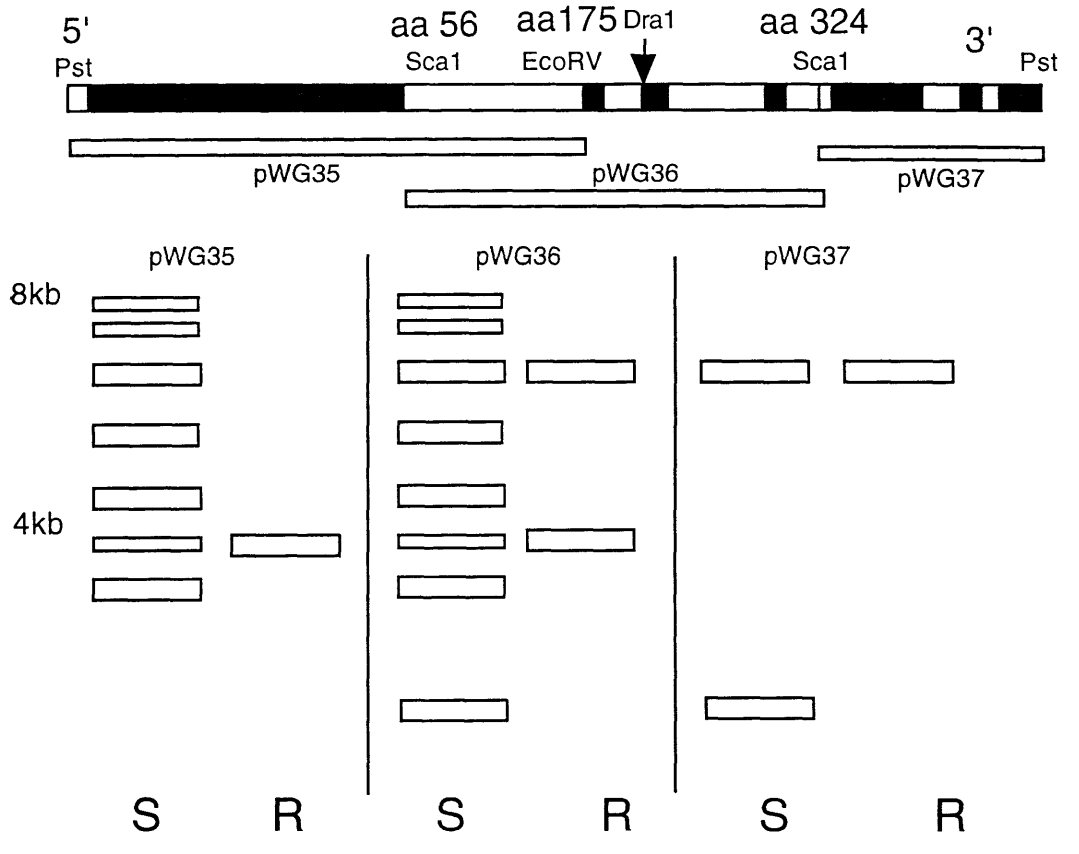
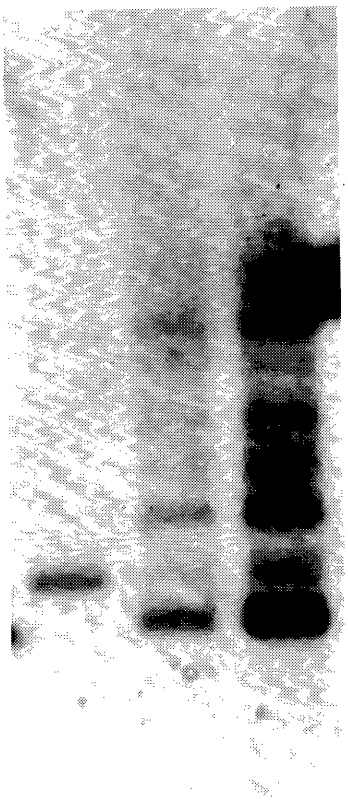


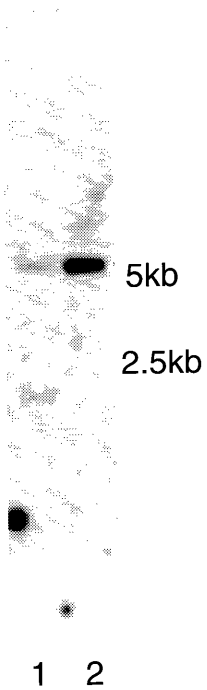
Figure12. Schematic diagram of southern blots using three probes subcloned from the pWG21 clone. The two strains compared were McMaster susceptible (S) and McMaster resistant (R). The probes used on the individual southerns are shown under the restriction map. Amino acid positions are indicated above the restriction map. The pWG36 clone was used in the initial RFLP analysis by Dr. Grant.

Figure 13. Southern blots of DNA extracted from McMaster susceptible and McMaster resistant strains of *T. colubriformis* digested with DraI and (a) probed with pWG35 which contains a PstI -EcoRV fragment containing coding DNA from aa44-175 as well as intervening non-coding sequence. Lane 1. McMaster resistant. Lane 2. McMaster susceptible (250ng of DNA) Lane 3. McMaster susceptible (500ng of DNA).
(b) probed with pWG37 which contains coding sequence from aa348 with intervening sequences through to 3' flanking sequence. Lane 1. McMaster susceptible. Lane 2. McMaster resistant.

(a)



(b)



All the bands seen in the DraI RFLP can be accounted for by the three different fragments which contain varying proportions of coding sequence. This could mean either (a) none of the fragments are locus specific and therefore this experiment does not provide any evidence that the *tcb-1* locus is solely involved in generating the RFLPs or (b) that all three probes are locus specific and therefore resistance is due to selection of a particular allele at the *tcb-1* locus. In other words these southerns did not clarify which hypothesis might be correct. They did, however, indicate there were multiple polymorphic DraI sites 5' of the β-tubulin gene since it was the most 5' probe (pWG35) that detected the greater number of polymorphic bands and no additional bands were detected with either of the probes. The 3' probe only detected two of the bands in the susceptible population.

Since the susceptible strain clearly contains the bands remaining in DNA extracted from the resistant strain after BZ selection it was considered to be highly probable that a clone representing the resistance allele would be present in a genomic library prepared from the susceptible strain. The library was rescreened in order to identify such a clone.

All three probes used in the southern analysis contained some sequence that would be conserved between loci. However, only the pWG36 clone contained over 50% coding sequence, whereas the pWG35 clone contained mostly intron sequence and the pWG37 clone contained mostly intron and 3' noncoding sequence. Therefore it was unlikely that the pWG35 and pWG37 clones detected all the β-tubulin loci present in the *T. colubriformis* genome. If these probes did detect all loci, this southern data would then suggest that resistant worms had only one β-tubulin locus whereas susceptible worms contained multiple loci and that all loci in susceptible worms were identical in nucleotide sequence and most of these loci were deleted from the genome of susceptible worms when BZ selection was applied to the population. Both of these conclusions were considered highly improbable.

Since the aim of the work was to develop an assay for resistance and the southern data clearly showed that bands that remained in DNA extracted from the resistant population could be readily detected in DNA extracted from the susceptible population it was considered highly probable that the genomic library would contain a clone for the resistance allele. The library was rescreened in order to identify such a clone.

Rescreening the Genomic Library in Order to Clone the Resistance Allele

Two probes were used to screen the genomic library. The pWG 36 clone and the 1.8kb ScaI fragment (this fragment was gel purified after digesting the pWG34 clone with DraI) which contains the 3' variable end and flanking sequence from the pWG21 clone (see Figure 7 for the restriction map.).

Filters were hybridised in a formamide buffer at 42°C and washed under moderate stringency conditions in order to detect additional clones from the *tcb-1* locus. These conditions also allowed the cloning of β-tubulin clones that possibly represented other β-tubulin loci in *T. colubriformis* because of the high degree of sequence homology between the different β-tubulin loci (if this degree of homology was not present between loci additional library screenings at lower stringency would have been conducted in order to clone the additional genes).

For each screening four genome equivalents of clones were probed. Since four β-tubulin loci have been mapped in *C. elegans* it was postulated that *T. colubriformis*, also a nematode, might have three to five loci and therefore one might expect 16 positives per library screening (screening four genome equivalents per library screening).

From three library screenings 75 positives were picked. These were spotted out as dilution series (see Figure 5 in Materials and methods) and hybridised again with the pWG36 and 1.8kb ScaI probes. Instead of plaque purifying all 75 clones a subset of 26 clones that showed varying degrees of hybridisation intensity with these two probes were selected. That is; some clones hybridised strongly to both probes while others hybridised only weakly with one or other of the probes. It was considered that twenty six clones would be enough to cover the possibility that four different β-tubulin loci existed.

The 26 clones were likely to be partially overlapping clones of a number of β-tubulin loci. To streamline the characterisation of these clones a number of steps were taken. Firstly the clones were separated into groups according to similarity of restriction digests with SalI. Secondly a differential hybridisation experiment comparing hybridisation of macroplaques of each lambda clone with a number of different probes was conducted. Finally hybridising fragments from each group were subcloned and a custom made primer used to obtain sequence from the variable 3' end of the loci cloned.

Firstly the 26 clones were separated into different groups according to similarity or dissimilarity of restriction digest pattern when digested with the enzyme SalI. SalI was used as the only sites for this enzyme are in the cloning site of the lambda vector and therefore the insert is released from the lambda arms. The

SalI group	Lambda cloneR	5' PstI-EcoRV pWG	ScaI pWG	3' ScaI-Pst pWG	PstI-SalI3' flanking	Tcb-2 cDNA	cDNA coding	cDNA3' end
1	1.1	+++	+++	+++	+++	-	+++	+
1	1.5a	++	+++	+++	+++	-	+++	-
1	1.7	+++	+++	++	+++	-	+++	+
1	2.3	+	+++	++	+++	-	++	+
1	2.7	+++	+++	+++	+++	-	++	+
1	2.9	+++	+++	+++	+++	-	++	+
1	3.1*	+++	+++	+++	+++	-	++	+
1	3.14	+++	+++	+++	+++	-	++	+
2	2.2*	+	+++	-	+	-	-	-
2	1.16	+	+++	-	+	-	-	-
3	2.1	-	+	+	+	-	++	+
3	3.3	-	+	+	+	-	+	-
3	3.9	-	-	+	+	-	++	+
3	3.11	-	-	-	F	-	++	+
3	3.1		-	-	+	-	++	+
4	2.2	+	++	-	-	-	-	-
4	3.2	-	+	+	++	+++	+++	+++
4	3.7	+	+++	-	-	-	-	-
4	3.17	+++	+++	-	-	-	-	-
4	2.4	+++	+++	-	+	-	-	-
4	1.2	+	+++	-	+	-	-	-
5	3.5	+	++	+	++	+++	+++	+++
5	3.6	-	-	-	+	-	-	-
5	1.19	-	+	+	+	+	++	+++
6	3.8	+	+++	-	-	-	-	-
7	2.12	-	+	+	+	-	-	-

TABLE 7: Results of the macroplaque experiment. Phage were spotted out onto plates and grown overnight (see materials and methods). Duplicate lifts were taken of each plate. Lifts were probed separately with 5 probes from the *tcb-1* locus and with a cDNA clone which coded for a second *T.colubriformis* β-tubulin gene designated *tcb-2*. Hybridisation of the probes was scored according to the following:
- no detectable hybridisation on overnight exposure with double screens
+ detectable hybridisation but very weak
++ medium hybridisation signal
+++ very strong hybridisation signal

lambda arms run as a 23kb and 8kb fragments on agarose gels and any additional fragments are generated by SalI sites present in the insert. On this basis the clones could be separated into seven groups (Table 7)

Secondly a differential hybridisation experiment was conducted using macroplaques of each of the lambda clones. Macroplaques of each clone were hybridised to the three probes used previously in the RFLP analyses. These were the 5' Pst- EcoRV fragment , the 1.1 kb ScaI fragment and the 0.8kb Sca-Pst (Figure 12). In addition the macroplaques were screened with the 3' end of a *T. colubriformis* cDNA provided by Le Jambre and Lenane. Results are recorded in Table 7.

Clones that hybridised strongly to all three of the clones were assumed to be additional clones from the *tcb-1* locus. Clones that showed different hybridisation patterns could have been either partially overlapping clones of the *tcb-1* locus or had the potential to represent different loci.

Correlation of DraI Fragments from Lambda Clones and DraI Fragments that Hybridise in Genomic DNA

Representatives from each of the seven classes were digested with Dra1 and probed with pWG35 and pWG36 to determine if any correlation could be made between the bands that hybridised from lambda clones to bands that hybridised in genomic southerns.

Lambda clones representing the *tcb-1* locus (Group 1 of the of the macroplaque experiment-see Table 7) showed different size DraI fragments when probed with the pWG35 clone (Figure 14b). Multiple DraI fragments in genomic DNA (see southern data - Figure 13) also hybridised to pWG35 suggesting that alleles from the *tcb-1* locus have multiple DraI polymorphisms 5' of the *tcb-1* locus. In addition pWG35 only hybridised with clones that were in the *tcb-1* class and no other class. In contrast, pWG36 hybridised, with varying degrees of intensity, to fragments from all the classes (pWG36 having a 500bp overlap with the pWG35) (see Figure 14a). The strongest signal for the pWG36 clone was observed for all the clones from the *tcb-1* group. In addition pWG36 hybridised to two fragments in each of the group one clones, one of the bands (approximately 5kb in size - see Figure 14a) was the same size for each of the *tcb-1* clones, showing that the DraI site present within the gene is conserved in all the clones form the *tcb-1* locus. The 5kb DraI fragment that hybridised for

Figure 14. Southern blots of lambda DNA digested with DraI and probed with

(a) pWG 36 (1.1 kb ScaI fragment, the same fragment used to detect RFLPs between BZ susceptible and resistant strains of *T-.colubriformis*).
Lane 1: Lambda clone 2.3
Lane 2: Lambda clone 2.2*
Lane 3: Lambda clone 3.3
Lane 4: Lambda clone 3.2
Lane 5: Lambda clone 1.19
Lane 6: Lambda clone 2.12
Lane 7: Lambda clone 3.8
Lane 8: Lambda clone 2.7
Lane 9: Lambda clone 2.9
Lane 10: Lambda clone 3.1*
Lane 11: Lambda clone 3.1

Underneath the photograph is the macroplaque group to which the clones were assigned (see Table 7).

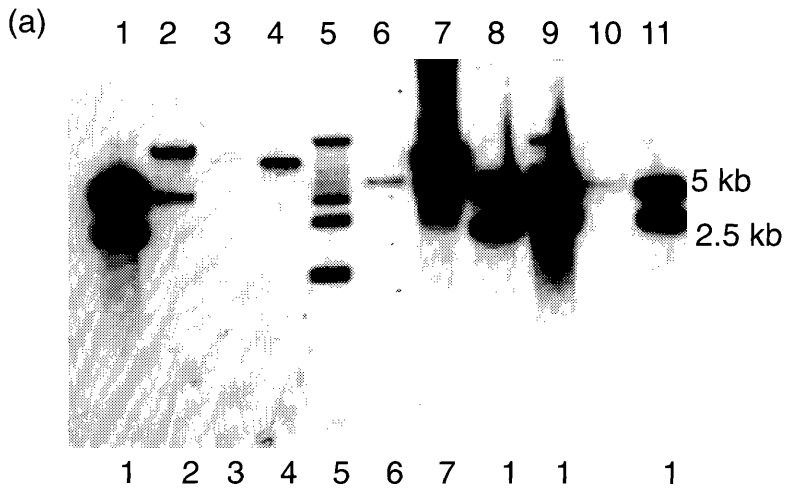
(b) pWG 35 (1.4kb PstI-EcoRV fragment which extends from aa44 through to aa175 of the *tcb-1* gene).

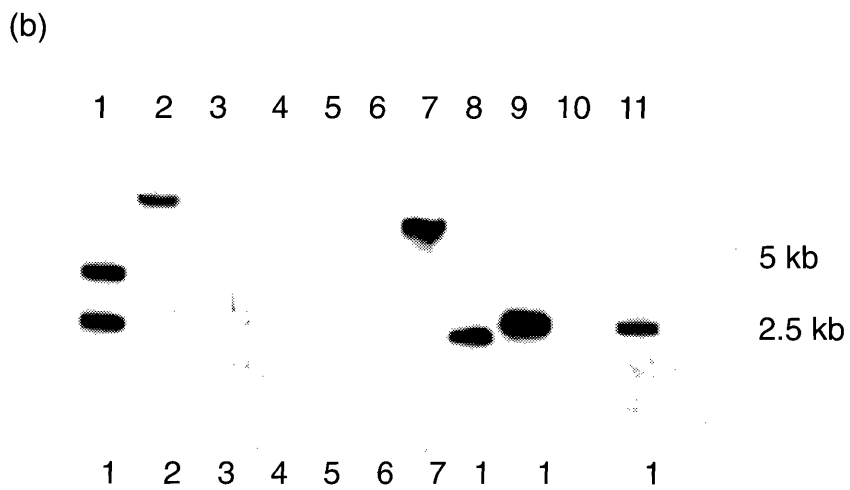
The Lane order is the same for (a) and (b) as listed under (a). Clones were selected from the groupings as shown in Table . Four clones from group one (which contained lambda clones that were considered to be additional clones from the *tcb-1* locus) and one clone from each of the remaining six groups were selected at random. The group numbers are shown under the blot.

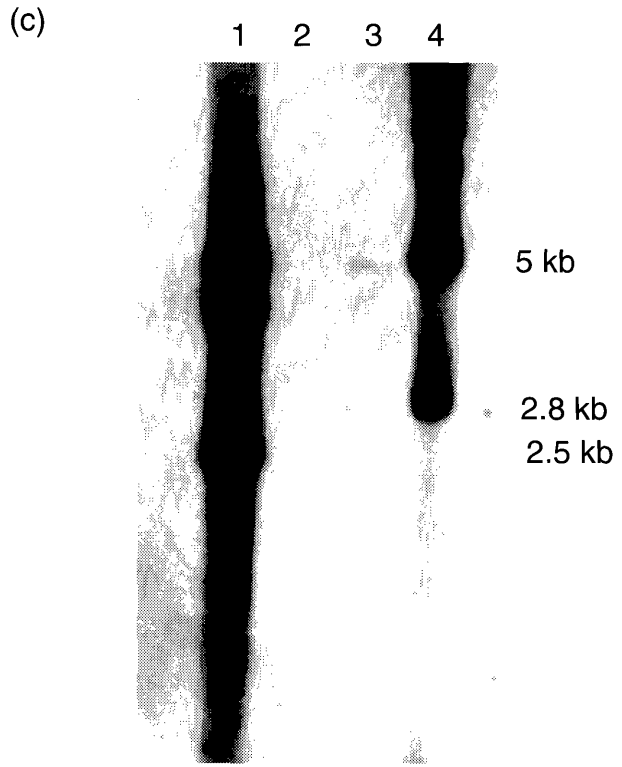
(c) Lambda clones 2.3 and 2.9 from group 1 (clones representing the *tcb-1* locus) and genomic DNA from the *T. colubriformis* McMaster resistant strain digested with DraI and probed with pWG36.

Lane 1: Lambda clone 2.3
Lane 2: Blank
Lane 3: McMaster resistant genomic DNA- bands are faint.
Lane 4: Lambda clone 2.9

This southern showed that the Lambda clone 2.9 when digested with the enzyme DraI had bands that hybridised to the pWG36 clone that were the same size as bands that hybridised in genomic DNA from the McMaster resistant strain when digested with DraI and probed with pWG36 (1.1 kb ScaI fragment used in the RFLP analysis.). It was concluded that this clone contained a putative BZ resistance
tcb-1 allele. In addition it was concluded that the Lambda clone 2.3 represented a susceptibility allele for the *tcb-1* locus.


(a)
1 2 3 4 5 6 7 8 9 10 11
5 kb
2.5 kb
1 2 3 4 5 6 7 1 1 1


(b)
1 2 3 4 5 6 7 8 9 10 11
5 kb
2.5 kb
1 2 3 4 5 6 7 1 1 1


(c)
1 2 3 4
5 kb
2.8 kb
2.5 kb

all of the clones from the *tcb-1* locus contained sequence from the 3' end of the gene as it did not hybridise to pWG35 which contains the 5' end of the gene. These observations were all consistent with the genomic southern data summarised in Figure 12 since in the genomic southerns the pWG35 clone hybridised to multiple DraI fragments in the susceptible population and only one band in the resistant population. Whereas pWG37 which contained the 3' end of the *tcb-1* locus hybridised to only two bands in the susceptible population one of which was the same size as the only band that hybridised with this probe in the resistant population (suggesting that the majority of 3' ends of *tcb-1* alleles were represented by this band). Since clones from the *tcb-1* locus showed differing DraI sized fragments at the 5' end of the gene but had the same sized DraI fragment at the 3' end of the gene, this locus could account for at least some of the polymorphic bands shown to be associated with BZ resistance in the initial RFLP analysis and the conclusion was made that selection for resistance was occurring at the *tcb-1* locus.

Most importantly, two clones, clone 2.9 and 3.1, from the *tcb-1* locus possibly represented a resistance allele as they showed two hybridising bands that were similar in size to the hybridising bands seen in genomic southerns of resistant strains of worms. In order to confirm this a genomic southern was repeated with lambda DNA of two clones from group 1, the 2.9 clone which was the putative resistance allele and 2.3 which was another group 1 clone that showed a different hybridisation pattern to 2.9, were digested with Dral and run side by side with genomic DNA from resistant worms digested with DraI. The southern was probed with pWG36. Results are shown in Figure 14c.

The hybridising bands from the 2.9 lambda clone were shown to be exactly the same size as those Dra I fragments that hybridised in genomic DNA from resistant worms. Therefore this clone was considered to be a putative resistance allele.

Identification of Additional β-tubulin Loci

The limited comparison of restriction fragments containing β-tubulin sequences and hybridisation of macroplaques did not convincingly distinguish additional alleles from the *tcb-1* locus from different loci as it was not possible to distinguish partially overlapping clones from clones that represented distinctly different loci. Representatives were selected from each macroplaque group (Table 7) for which nucleotide sequence was to be obtained. Clones were selected on the following criteria (i) they had different restriction patterns when

Figure 15. SalI restriction digests of lambda clones from which sequence for the *tcb-1*, *tcb-2, tcb-3* and *tcb-4* 3' variable sequences were obtained showing the different SalI digests of the four loci.

Lane 1. SPP-1 markers (size in kb 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36.)

Lane 2 clone 1.5a (*tcb-1.1*)

Lane 3. clone 2.9 (*tcb-1*)

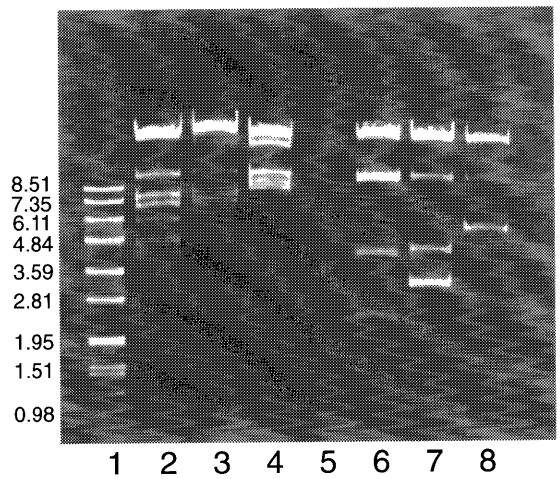
Lane 4. clone 2.3 (*tcb-1*)

Lane 5. blank

Lane 6. clone 2.1 (*tcb-3*)

Lane 7. clone 3.5 (*tcb-2*)

Lane 8. clone 2.12 (*tcb-4*)


8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.51
0.98
1 2 3 4 5 6 7 8

digested with the restriction enzyme SalI (Figure 15); (ii) they showed a different hybridisation pattern in the macroplaque experiment (Table 7) and (iii) they did or did not hybridise specifically with the probes from the *T. colubriformis tcb-2* cDNA clone (Table 7). For example, the lambda clone 2.12 had a different SalI digest pattern with very faint hybridisation to both pWG36 and to the 1.8kb ScaI fragment in the macroplaque experiment and completely different restriction enzyme banding pattern to all the other groups and was selected for sequence analysis. Clones that hybridised specifically to the cDNA probe, namely 3.2 from group 4 and 3.5 from group 5 were strong candidates for representing a different locus to the *tcb-1* locus.

Two additional clones to the original pWG21 were selected from group one as these appeared to be alleles of the *tcb-1* locus because they hybridised to all three probes from the pWG34 clone and had similar SalI digest patterns but they had different sized DraI fragments that hybridised to pWG35 (5' end of the tcb-1 locus) (Figure 14b). Both clones had DraI fragments that correlated in size to Dra1 bands seen in genomic southerns of the susceptible strain. The 2.9 clone had DraI fragments that were the same size as DraI fragments seen in DNA from the resistant strain (Figure 14c). Since these two clones alone could account for four out of the eight bands seen in the RFLP analysis using DraI, it was important to confirm that both these clones were alleles of the *tcb-1* locus.

All the lambda clones selected for sequencing were digested with SalI, as this released the insert from the lambda arms, blotted and probed with the pWG36. SalI fragments that hybridised to pWG36 were subcloned into the Bluescript SK+ plasmid.

A primer was designed specifically to sequence the 3' ends of the β-tubulin genes. The sequence used corresponded to amino acids 401-406 which is a highly conserved region of all β-tubulin genes (Table 4). The consensus sequence used was based on *C. elegans*, *H. contortus* and *B. pahangi* sequences. The primer ends in methionine which has only one codon and a third base change in this codon would change the amino acid therefore this primer should be virtually universal for β-tubulin genes. Results of subcloning and sequencing are summarised in Table 8 .

TABLE 8: Lambda clones from which 3' variable sequence was obtained

Group	lambda clone	Size of SalI fragments	Subclone name	3' sequence	Locus
1	1.5a	8 kb	pWG21	yes	1
1	2.3	8.5 kb	pWG23	yes	1
1	2.9	8 kb	pWG22	yes	1
2	1.16	7 kb		no	not determined
3	2.1	4.5 kb	pWG30	yes	3
4	3.2	> 8 kb		yes	2
5	3.5	3.6 kb	pWG24	yes	2
6	3.8	4 kb		no	not determined
7	2.12	5 kb	pWG31	yes	4

Sequence was obtained from representatives of five out of the seven macroplaque groups (Table 7). Where sequence was obtained in the forward direction a primer specific to the particular clone was designed that allowed sequencing in the reverse direction. Sequence was obtained for three group-1 clones and for clones from groups 3, 4, 5 and 7.

Four different 3' end sequences were identified from the five groups that primed successfully with P1. The sequences for Groups 4 and 5 were virtually identical as expected as these were the clones that hybridised to the cDNA clone. The individual sequences are represented in Figure 16a .

The amino acid sequences for the four 3' ends are listed in Figure 16b. The sequences extend from what corresponds to amino acid 418 in other β-tubulin sequences. The variable terminal peptide begins at amino acid 430. These sequences will now be referred to as *tcb1-tcb4* as noted in Figure 16a.

Sequence analysis confirmed that the clones thought to represent alleles from the *tcb-1* locus on the basis of macroplaque data and southern data were virtually identical at the 3' end except for two nucleotide differences. One of the nucleotide differences was a silent third base substitution in the aspartic acid residue at aa 432 and the other change was in the intron found at the 3' end of the gene.

Surprisingly two of the 3' end sequences, *tcb-1* and *tcb-2*, in *T. colubriformis* had identical terminal amino acid sequences to two β-tubulin genes that have ben

Part A Nucleotide sequence of the carboxy terminal end of four putative β-tubulin loci from *T. colubriformis*

T. colubriformis tcb1 (Lambda clone 1.5a)

CTGATCTCCGAATACCAACAGTACCAGgtgtgatctctttctttctcgagttctcctatgcttgatgtctgcggggacatttttttcctacatatatttcacgtcttaattttccagGAAGCTACTGCTGAT
GACATGGGCGATCTCGATGCAGAAGGTGGAGAAGAGGCGTACCCAGAAGAGTAGagatccactgtgttacgactccctttttttctgtgtcaatgcgaaatacacattggttgcgttgttttcaaagaaagctgt
ttactgtgac*atataat*gaaggtga

T. colubriformis tcb2 (Lambda clone 3.5)

CTGGTGTCCGAATACCAACAATATCAAGAAGCAACTGCTGACGAAGAGGGTGAAATGGAAGGTGCCGTTGAGAATGACACTTACGCTGAAGAATAGtgcagcagtcgcccgatccgctactcattacatttctt
gcagctatcgtactgcgcatattgtaacgtttctctcaacggctaatctcaatgcactttctttttgtgattttaatatgttctatacttgatgctaatattt

T. colubriformis tcb3 (Lambda clone 2.1)

CTGGTTTCTGAATACCAACAATATCAGgtgcagtatttcgctgcaattcaaaaacaactactgcgctggttttatcgttcgtcaactttgtacatttcatataagtgtagaacataaatctgtactacgtata
atggggcacatgttcgatttgtctccagccaaagcaaccgaactgggtcaccgctccacgaggatgggggacaaccacccaggtgtccagtggagcagcccgtaggagcgtggacactgctacctccggcggtc
tgacccactttgggtgtctgtttgaaggattaatcgcagctgggaaagagtgaactttcacgaatctacttcccatcaagactcaagactggtttcagGAAGCAACAGTCGATGAAGAAGCCACAGAGACATTT
GAGACTGAGTGAaatcggcgtagttcgtcgatcgaggtgtcagcttctcgcttcattcgttgcccagcttcctttttttcctcggaatatcacaag**aataaa**caacatlcttttctgttatcaggtat

T. colubriformis tcb4 (Lambda clone 2.12)

CTGGTGTCTGAGTATCAACAATATCAGgttgtggatgtagctgagtggtcccaaagaagatctaggatctaatggtatttagGAGGCGACACGAGACGATGAAGGTGAATTCGATGAACACGATCATGACGTC
GAGG**AATAAa**gctgttgaaatacttaaacggcaaccctcaatgacccgtagca

Part B Amino Acid sequence of the carboxy terminus

T. colubriformis tcb-1	LISEYQQYQEATADDMGDLDAEGGEEAYPEE
T. colubriformis tcb-2	LVSEYQQYQEATADEEGEMEGAVENDTYAEE
T. colubriformis tcb-3	LVSEYQQYQEATVDEEATETFETFETE
T. colubriformis tcb-3	LVSEYQQYQEATRDDEGEFDEHDHDVEE

Part C

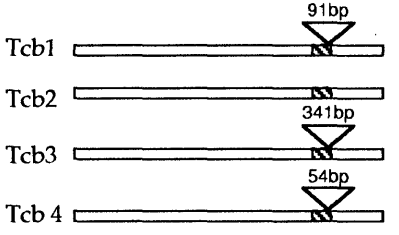


Figure 16: Part A shows the nucleotide sequence obtained from the plasmids pWG 34, pWG24, pWG30, and pWG31 The coding DNA in uppercase and the noncoding DNA in lowercase. Sequence begins at lysine amino acid 418 .The putative polyadenylation signals are in bold and intron splice site consensus sequences are underlined. Part B shows the amino acid sequence after translation of the coding DNA highlighted in part A. Part C shows the position of the sequence within the gene and shows the position and size of introns . The hatched region is the conserved sequence

cloned in *H. contortus*. At the nucleotide level silent third base substitutions are present. The sequences for *tcb-3* and *tcb-4* have not been reported in other nematodes. Burns and Surridge (1990) observed that a phenylalanine or tyrosine residue was conserved in the majority of β-tubulin genes vertebrate or invertebrate The *tcb-4* clone could be the *T. colubriformis* homologue of the *C. elegans mec-7* gene as there is a conserved phenylalanine residue in the C-terminus which is conserved in most vertebrate β-tubulin genes.

Three of the four sequences *tcb-1*, *tcb-3* and *tcb-4* contained an intron at aa 426. The intron sizes were 90bp, 56bp, and 351bp respectively. Both clones representing the *tcb-2* locus did not contain an intron in this position. It has been reported that the presence of an intron at this position (aa 426) in the isotype 1 gene of *H. contortus* was specific to that nematode (Kwa *et al.*, 1993). However, it is also present here in three of the four putative β-tubulin loci from T. *colubriformis*.

A putative consensus polyadenylation signal was found in the *tcb-3* and *tcb-4* sequences. The polyA signal in *tcb-4* (2.12) coincides with the last codon and stop signal of the coding region. This was also observed for the *tub-1* locus in *C. elegans* (Kwa *et al.*, 1993). The polyadenylation signal for *tcb-3* (2.1) is 83 bp after the stop codon. A consensus polyadenylation signal for *tcb-1* was not found. However, the polyA signal for the homologous H. *contortus* gene occurs 69bp after the stop signal. At this position in *tcb-1* is the sequence AAAGAAA.

Sequence was not obtained from the clones in groups 2 and 6. The most likely reason for this is that the 3' ends of the gene were not present in these clones. This explanation seems more probable than sequence divergence of the primer position due to the high conservation of this region in other β-tubulin genes.

The CLUSTAL V program (Higgins & Sharp, 1988; Higgins & sharp, 1989) was used to obtain multiple alignment of the four 3' sequences obtained from *T. colubriformis* with 3' sequences of other nematode β-tubulin genes. The nematodes *H. contortus, C. elegans* and *Brugia malayi* were included in the alignment. The results indicated that the *tcb-1* locus was most similar in sequence to the *gru-1* locus in *H. contortus* and *ben-1* in *C. elegans* both of which have been implicated in BZ resistance in the respective nematodes. The similarity of the *tcb-2, tcb-3* and *tcb-4* sequences with other β-tubulin loci from the different nematodes suggests that isotypic classes exist in nematodes as has

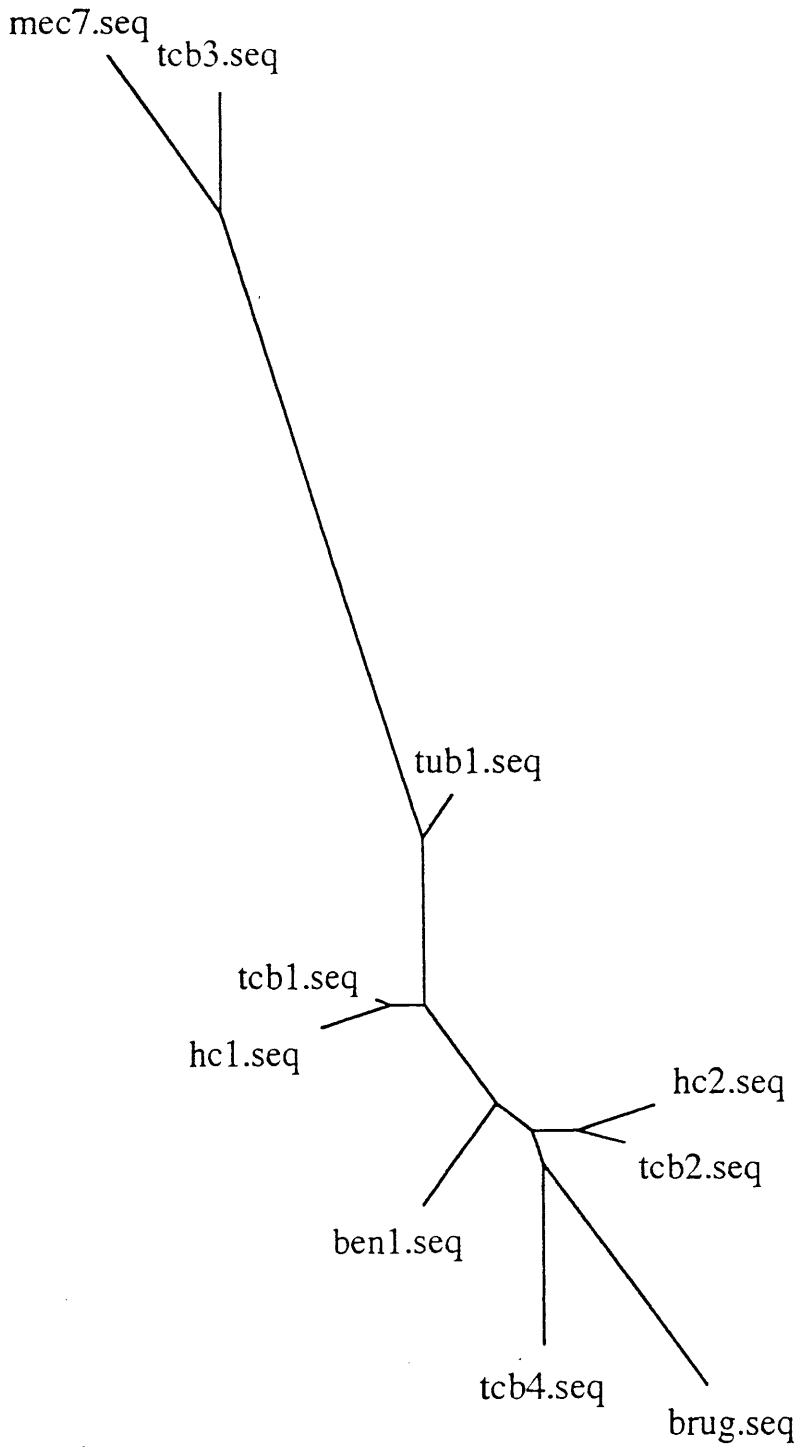
Figure 17. The top part of the figure shows the multiple sequence alignment of the 3' variable regions of β-tubulin genes from *C. elegans, Brugia pahangi , H. contortus and T. colubriformis* obtained from the CLUSTAL V program (Higgins and Sharp, 1988) using the default parameters. The lower part of the figure shows the amino acid sequences of the carboxy termini.

Sequence alignment from about amino acid 418 in B-tubulin genes from *C. elegans*, *T. colubriformis*, *H. contortus* and *B. pahangi*

```
mec7  A-------GCAACATGAACGAT-CTTGTTTCGGAATACCAACAATACCA-GGAGGCAGCTGCTGATGAAGATGCCGCCGAAGCGTTCGA---CGGAGAG
tcb3  T-------CGAATATGAACGAC-CTGGTTTCTGAATACCAACAATATCA-GGAAGCAACAGTCGATGAAGAAGCGACAGAGACATTTGA---GACTGAG
tcb1  CTGATCTCCGAATACCAACAGTACCAGGAAGCTACTGCTGA---TGACATGGGCGATCTCGATGCAGAAGGTGGAGAAGAGGCGTAC---CCAGAAGAG
tcb2  CTGGTGTCCGAATACCAACAATATCAAGAAGCAACTGCTGACGAAGA---GGGTGAAATGGAAGGTGCCGTTGAGAATGACACTTACG---CTGAAGAA
tcb4  CTGGTGTCTGAGTATCAACAATATCAGGAGGCGACACGAGACGATGA---AGGTGAATTCGA---------TGAACACGATCATGACG---TCGAGGAA
tub1  CTCATCTCAGAATACCAACAATACCAAGAAGCAACTGCCGAAGACGACGTCGACGGATACGCTGAGGGAGAAGCTGGAGAGACTTACGAATCGGAGCAA
ben1  CTTGTCTCAGAATACCAGCAATACCAGGAAGCAACTGCAGA---GGAAGATGGAGAACTTGATGGAACTGATGGAGA-------TGC---T-----GAA
hc1   CTTATCTCCGAATACCAGCAGTACCAGGAAGCTACCGCTGA---CGATATGGGCGATCTCGATGCAGAAGGTGGAGAAGAGGCATAT---CCCGAGGAG
hc2   CTGGTATCTGAGTACCAACAATATCAAGAGGCAACTGCTGATGATGA---GGGTGAAATGGAAGGTGCCGTTGAAAACGACACGTATG---CAGAGGAG
brug  TTGGTGTCCGAATATCAACAATATCAGGATGCGACGGCTGATGAAGA---AGGTGATCTTCAGGAAGGTGAATCGGAATACATTGAAC---AGGAAGAG
```

T. colubriformis Tcb-1	tcb 1	LISEYQQYQEATADDMGDLDAEGGEEAYPEE
H. contortus Isotype 1	hc 1	LISEYQQYQEATADDMGDLDAEGGEEAYPEE
C. elegans tub-1	tub 1	LISEYQQYQEATAEDDVDGYAEGEAGETYESEQ
B. pahangi	brug	LVSEYQQYQDATADEEGDLQEGESEYIEQEE
C. elegans ben-1	ben 1	LISEYQQYQEATAEEDGEMEGTDEDAE
T. colubriformis tcb-2	tcb 2	LVSEYQQYQEATADEEGEMEGAVENDTYAEE
H. contortus Isotype 2	hc 2	LVSEYQQYQEATADDEGEMEGAVENDTYAEE
T. colubriformis tcb-3	tcb 3	LVSEYQQYQEATVDEEATETFETE
C. elegans mec-7	mec 7	LVSEYQQYQEAAADEDAAEAFDGE
T. colubriformis tcb--4	tcb 4	LVSEYQQYQEATRDDEGEFDEHDHDVEE

Figure 18. An unrooted tree showing distances in nucleotide sequences generated from the dendogram obtained from the CLUSTAL V analysis shown in Figure 17. The distances were obtained by using the CLUSTAL V alignment in the PHYLIP program DNAdist. The tree was plotted using the program Treetool.


mec7.seq
tcb3.seq
tub1.seq
tcb1.seq
hc1.seq
hc2.seq
tcb2.seq
ben1.seq
tcb4.seq
brug.seq

been observed for vertebrate β-tubulins. A unrooted tree to show nucleotide distance was constructed using the DNAdist program in the PHYLIP package (Figure 18)

Developing an Assay for BZ Resistance

Three pieces of information suggested it would be worthwhile investigating the potential to use the sequence polymorphisms observed in the two *tcb-1* alleles as markers of BZ resistance.

(1) Southern data using DraI digests of genomic DNA and the three probes from pWG21 suggested that there is a reduction in allelic diversity at this locus after selection by BZ.

(2) Digestion of lambda clones with DraI and probing with the same probes shows that the clones from this locus have the same size 3' DraI fragments and different size 5' hybridising fragments as observed in genomic southerns. Most significantly, the lambda clone 2.9 has the same size DraI hybridising fragments as the fragments that hybridise in genomic DNA extracted from BZ resistant larvae.

(3). The homologous locus in *H. contortus* had been implicated in BZ resistance (Roos *et al.*, 1990).

Three approaches were taken to determine if the sequence polymorphisms in the 2.9 allele in particular could serve as marker for BZ resistance. All approaches involved using the polymerase chain reaction. They were heteroduplex analysis, restriction enzyme site differences in PCR products and a nested PCR assay with modified primers to detect specific alleles at the *tcb-1* locus.

Heteroduplex Analysis

Heteroduplex analysis is applied to identical length PCR products. Using non-denaturing gel electrophoresis homoduplex and heteroduplex molecules of DNA can be resolved. The technique is sensitive enough to resolve heteroduplex DNA mismatches containing a single base pair mismatch (see Materials and Methods).

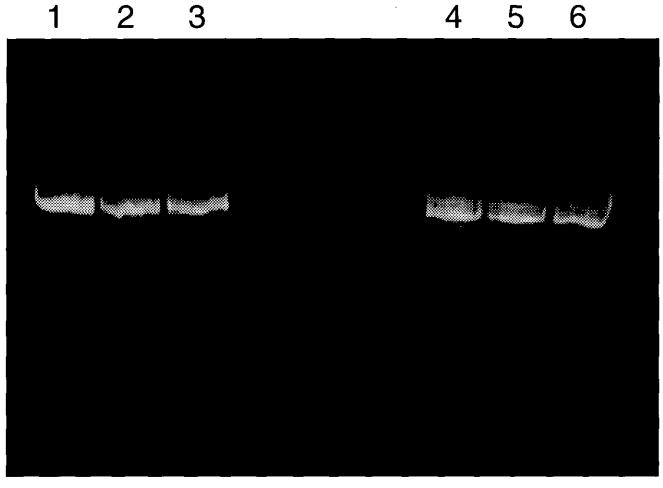
Heteroduplex analysis was first applied to the lambda clones from the *tcb-1* locus (Figure 19a). The primers P1 and LM7 were used in the polymerase chain reaction to generate a 362bp product from aa401 into the noncoding region 3' of the stop signal.

Figure 19. Heteroduplex analysis of PCR products amplified from

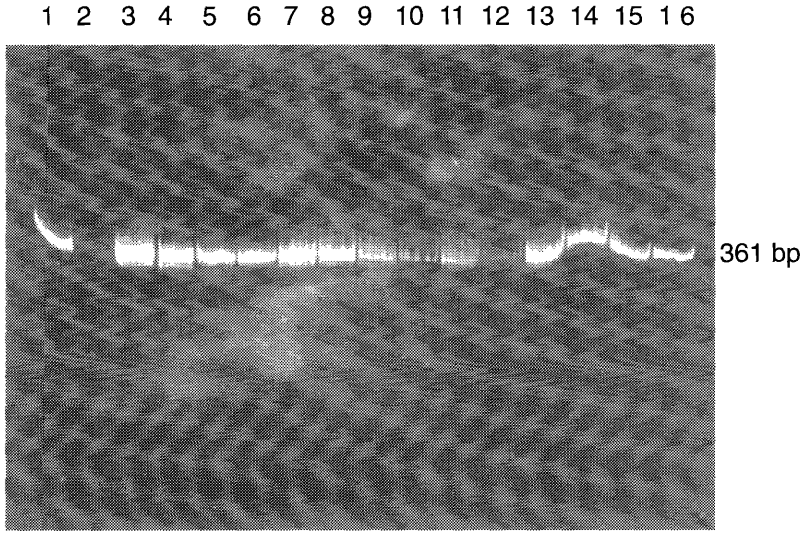
(a) lambda DNA clones. Putative resistance clones were 1.5a and 2.9 and the susceptible alleles 2.3, 2.7 and 1.7. The sequence of 3.14 was not determined but the results of the heteroduplex experiment indicates the sequence is identical to 2.9 and 1.5a, the putative resistance clones.
Lane 1. 1.5a (control)
Lane 2. 1.5a with 3.14
Lane 3. 1.5a with 2.9
Lane 4. 1.5a with 2.7
Lane 5. 1.5a with 2.3
Lane 6. 1.5a with 1.7

(b) lambda DNA clones (2.3, 2.7 and 1.7 putative susceptible alleles and 2.9 the putative resistance allele) and products from individual larvae (susceptible S1 and S5 and resistant R1 and R5)
Lane 1. 2.3 with 2.7
Lane 2. MDE control
Lane 3. 2.7 with 2.9
Lane 4. 2.3 with 2.9
Lane 5. 1.5a with R1
Lane 6. 1.5a with R5
Lane 7. 1.5a with S1
Lane 8. 1.5a with S5
Lane 9. S1 with R1
Lane 10. S5 with R1
Lane 11. R5 with S1
Lane 12. R5 with S5
Lane 13. 2.7 with R1
Lane 14. 2.7 with S1
Lane 15. 1.7 with 2.3
Lane 16. 1.7 with 2.7

(a)



(b)



Products from these clones were mixed in pairwise combinations, denatured and reannealed and run on MDE gels. Results of the heteroduplex analysis from the lambda clones showed that three *tcb-1* clones had 3' sequences that formed a homoduplex with the 2.3 product and that two clones formed homoduplexes with the putative resistance clone 2.9 and that the PCR products from the 2.3 and 2.9 alleles formed a heteroduplex on MDE gels (Table 9).

The products from the pWG22 and pWG23 clones were then mixed in pairwise fashion with the P1-LM7 products of individual susceptible and resistant larvae. The results obtained were not very satisfactory as the homoduplex and heteroduplex products were not well resolved. This was probably due to the difference in concentrations of PCR products obtained from individual larvae and plasmid. It was difficult to get an adequate amount of DNA in a volume that was possible to load on the gel. However, there was an indication that the 2.9 product formed a homoduplex with the product generated from resistant larvae (Figure 19b).

TABLE 9: Heteroduplex Analysis of Lambda Clones from the *tcb-1* locus

	tcb-1	**1.7**	**2.3**	**2.7**	**2.9**	**3.14**
tcb-1	Hom	Het	Het	Het	Hom	Hom
1.7		Hom	Hom	Hom	nd	nd
2.3			Hom	Hom	Het	nd
2.7				Hom	nd	nd
2.9					Hom	nd
3.14						Hom

Restriction Enzyme Site Differences

In order to obtain a better assay the sequences from the 2.3 and 2.9 alleles were compared for restriction enzyme site differences using the Macvector program. The sequences varied in a restriction site for NlaIII. This would generate four fragments, 9bp, 36bp, 144bp and 170bp in size when PCR products from the 2.9 allele were digested with NlaIII and three fragments, 9bp 36bp and 314bp when PCR products from the 2.3 allele were digested.

The PCR product amplified between the primers P1 and LM7 from individual susceptible and resistant larvae were digested with this enzyme and resolved on

8% acrylamide gels. Products were not clearly resolved after digestion with the restriction enzyme so whether there was a difference between susceptible and resistant larvae could not be determined.

The low resolution of NlaIII digests and heteroduplex analysis of PCR products from larvae may have been due to the high conservation of the P1 primer allowing priming from multiple loci and the reaction therefore may not have been specific for the *tcb-1* locus. A second primer pair specific for *tcb-1* and internal to P1 and LM7 were designed and employed in a nested PCR reaction (Figure 20) in combination with an allele specific PCR reaction described in the following section.

Nested PCR and Restriction Site Generating PCR

Gasparini *et al.* (1992) reported a technique for discriminating between alleles at a locus in which an artificial restriction site is introduced into the PCR product that allows otherwise undetectable polymorphisms to be detected (Restriction site generating PCR or RG-PCR). The primer sequence contains a mismatch that will generate a novel restriction site in one allele but not the other, thereby allowing discrimination between the PCR products from each allele following digestion with the appropriate enzyme. Primers were designed to detect both nucleotide differences present between 2.3 and 2.9 clones (Figure 21). Therefore, in any individual worm the 2.3 and/or the 2.9 allele can be detected. This is an advantage over PCR assays for specific alleles as these usually have the discriminating polymorphism as the 3' nucleotide in the primer and absence of the allele is detected as the absence of a PCR product. False negatives are possible in such a case. In contrast with the RG-PCR a product is obtained for both alleles and detection is achieved by the presence or absence of a restriction site.

The primer to detect the 2.9 allele contains a mismatch which generated a HpaII site in the PCR product. A second primer was designed for the 2.3 allele such that an EcoRV site would be generated in the PCR product if that allele was present in the worm (Figure 21). Therefore if both alleles were present in one worm, that is the worm is a heterozygote for these two alleles, then both the alleles could be detected (Figure 20).

The assay was initially applied to the McMaster susceptible and McMaster resistant strains that were also used in the original RFLP analysis. Products were initially amplified using the P1 and LM7 primers. Products were diluted and an aliquot added to the second reactions containing the primers WG5 and WG6 the primers that distinguished between the two alleles. Aliquots of this second reaction were then digested separately with HpaII and EcoRV and the products

Figure 20. A schematic diagram of the allele specific PCR assay. DNA is amplified initially in the first reaction using a pair of primers that flank the region of interest where, in this case P1 is a primer that would anneal to multiple β-tubulin loci and the primer LM7 is a locus specific primer. An aliquot of this reaction is then mixed with the allele specific primers (WG5 and WG6) for the second reaction. Aliquots of products from the second reaction are digested separately with EcoRV (E) and HpaII (H). Digests are run on 8% polyacrylamide gels with an aliquot of the second reaction as an undigested (U) control. Size markers are also run on the gel.

PCR Assay for two alleles at the *tcb-1* locus

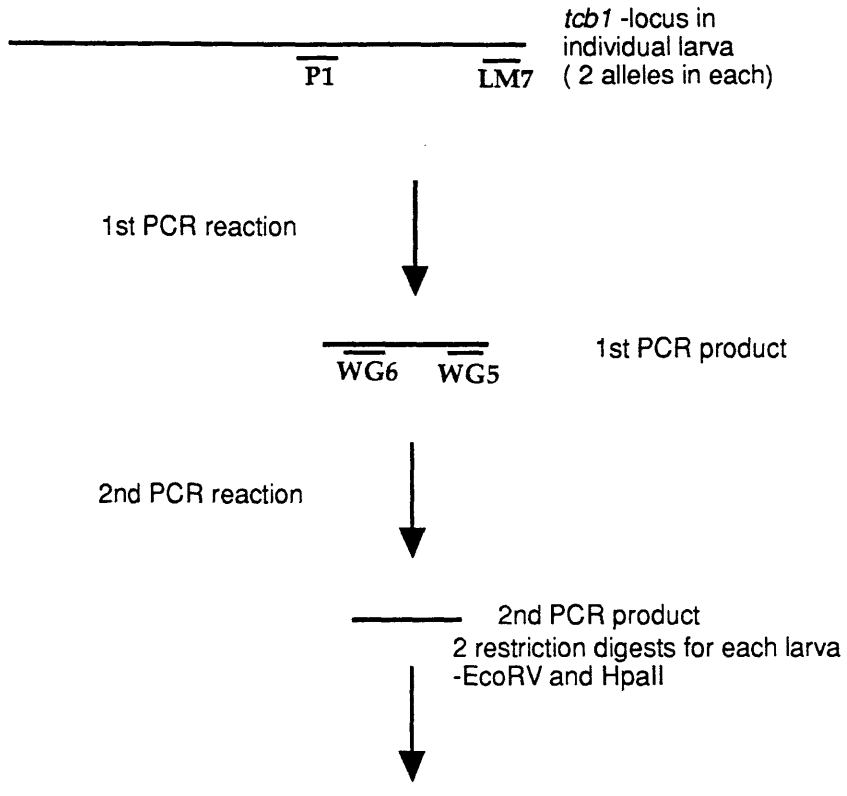
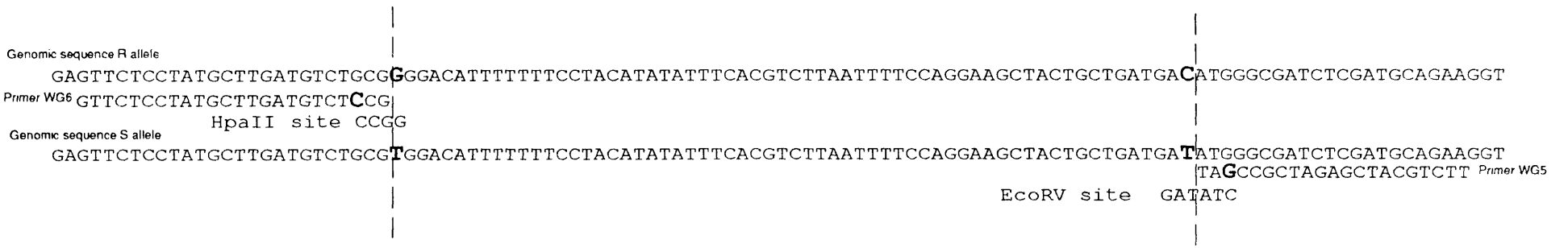


Figure 21. Restriction Site Generating PCR. This is a diagram showing the positions of nucleotide substitutions between the putative resistance and susceptible alleles. The allele specific primers WG5 and WG6 are shown with the mismatches shown in bold. Examples of the products obtained on amplification of the resistant and susceptible alleles are shown in the lower half of the diagram. A HpaII site is created only if the resistant allele is present in the worm and an EcoRV site is only created if the susceptible allele is present in the worm.

Restriction Site Generating PCR - (RSG-PCR)



Products from each allele:

HpaII site

R allele GTTCTCCTATGCTTGATGTCTCCGGGGACATTTT....

S allele GTTCTCCTATGCTTGATGTCTCCGTGGACATTTT....

no HpaII site

no EcoRV site

....GATGACATCGGCGATCTCGATGCAGAAGGT R allele

....GATGATATCGGCGATCTCGATGCAGAAGGT S allele

EcoRV site

were run on 8% acrylamide gels. The genotypes were scored according to Figure 22. Examples of the genotypes observed are shown in Figure 23. The results of the analysis are shown in Table 10. The susceptible population showed a mixture of genotypes whereas the resistant population showed overwhelmingly only one genotype and that was homozygous for the putative resistance allele. This genotype was also seen in the susceptible population but at a much lower frequency.

For many susceptible larvae an additional band was observed that did not cut with either HpaII or EcoRV. This was seen at a very high frequency in the susceptible population. This was initially taken as evidence for additional polymorphism at these two sites and perhaps it therefore represented an additional susceptible allele at the *tcb-1* locus. This band was always observed as a heterozygote with either the HpaII allele or the EcoRV allele no homozygotes were observed in the 225 individuals tested from the susceptible population. Analysis of the data obtained for goodness of fit between susceptible and resistance alleles and Hardy-Weinberg equilibrium showed extreme disequilibrium due to this additional putative susceptible allele (Figure 24a). Given this apparent extreme disequilibrium in an apparently unselected population it then became important to determine if this band was (a) an artefact of the PCR assay with the mismatched primers causing incorrect incorporation of nucleotides or (b) incomplete digestion of PCR products or (c) whether there truly was a third allele at this locus.

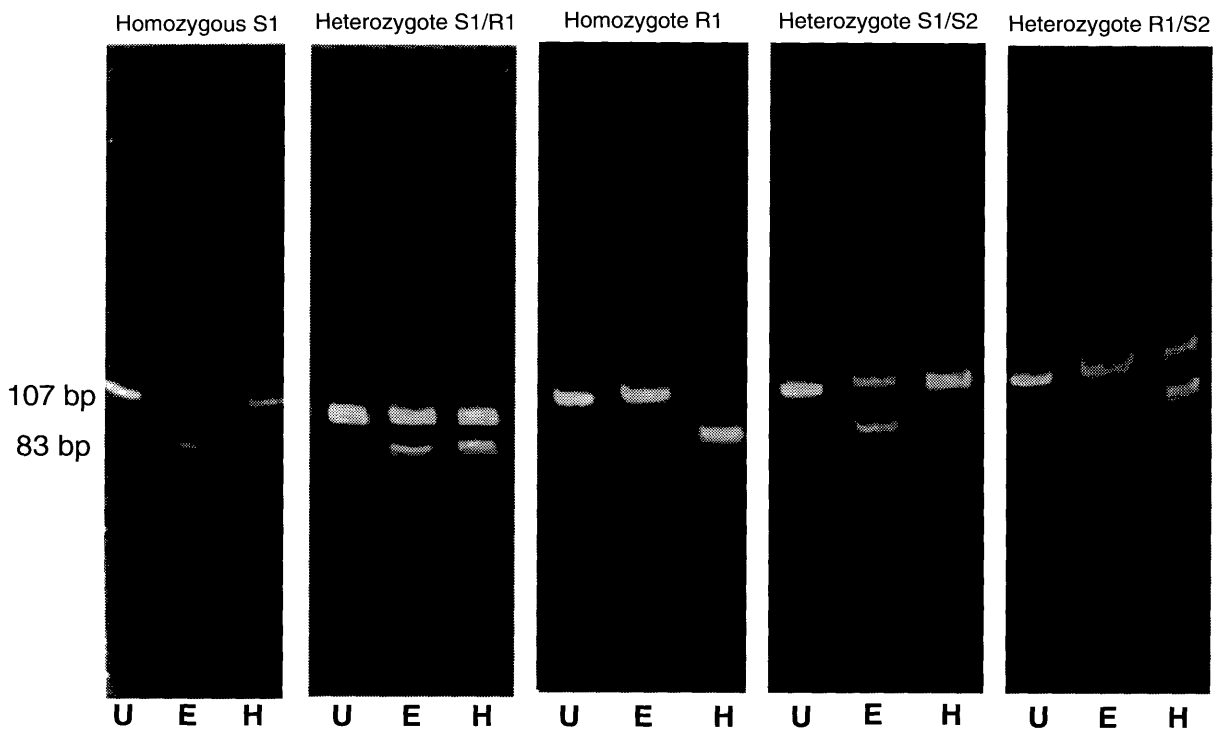
The mismatches in the pWG5 and pWG6 primers were very close to the 3' ends of the primers. An artefactural band may have arisen if the mismatch in the primers interfered with the correct incorporation of the first few nucleotides from the template DNA. For the HpaII site not to be correctly created only the single nucleotide associated with the resistance allele which is the first nucleotide to be incorporated in the PCR product from the pWG6 primer (see Figure 21) needed to be different as the other three nucleotides that form the restriction site at the 3' end of the primer sequence and so would be present in all PCR products. In contrast three of the six nucleotides that form the EcoRV site are amplified from the DNA of the individual larva being assayed (the other three nucleotides for the EcoRV site are in the primer and would be incorporated into all PCR products), therefore any of the three sites immediately amplified from the template DNA could be altered either by misincorporation in the PCR reaction or sequence polymorphism in the population that would prevent the creation of an EcoRV site in the product.

Figure 22. Schematic diagram showing scoring system for individual larvae after restriction enzyme digestion of the PCR products and electrophoresis on 8% polyacrylamide gels.

GEL

Uncut EcoRV HpaII	ENZYME DIGEST	GENOTYPE
	Complete cleavage with EcoRV. No cleavage with HpaII	Homozygous allele S1
	Cuts with both enzymes to give cut and uncut	Heterozygous allele S1 and allele R1
	Complete cleavage with HpaII. No cleavage with EcoRV.	Homozygous allele R1
	No cleavage with HpaII. Uncut and cut with EcoRV.	Heterozygous allele S1 and S2
	No cleavage with EcoRV. Cut and uncut with HpaII.	Heterozygous allele R1 and S2
	No cleavage with either enzyme.	Homozygous allele S2

Figure 23. Shows an example of each of the patterns observed for individual larvae after amplification of DNA with the primers WG5 and WG6 and restriction enzyme digestion. Each sample was digested separately with EcoRV (E) and HpaII (H). The restricted products and undigested (U) samples were then electrophoresed on 8% acrylamide gels.


Homozygous S1
Heterozygote S1/R1
Homozygote R1
Heterozygote S1/S2
Heterozygote R1/S2
107 bp
83 bp
U E H U E H U E H U E H U E H

STRAIN	GENOTYPE						TOTAL # SCORED
	S1/ S1	**S1/ R1**	**R1/ R1**	**S1/ S2**	**R1/ S2**	**S2/ S2**	
McMaster susceptible	3	43	13	148	18	0	225
McMaster resistant	2	5	103	1	8	0	119
Arthursleigh	0	1	30	0	9	0	40
Badgerys Creek	0	0	41	2	5	0	48

TABLE 10:
Allele frequencies in one BZ susceptible population and three different resistant populations.

In order to characterise this additional band PCR products from worms displaying this band were subcloned and sequenced across the polymorphic sites. Since homozygotes were not observed for this band products from worms having this band in combination with either the EcoRV digested product or the HpaII digested product were subcloned and sequenced. PCR products from four worms that displayed the uncut product were sequenced. When the sequence was analysed there was no evidence of additional sequence polymorphism. However, the products that should have cut with EcoRV and that would have been scored as the susceptible allele did not always cut to completion and therefore the additional band was an artefact of the assay itself. In order to use this assay to detect the BZ susceptible allele unambiguously this problem could be addressed by (a) altering the primer so that a different enzyme site was generated or (b) not using a restriction digest to detect this allele at all since it is the resistance allele that is the allele of crucial significance. Since no additional polymorphism linked to susceptibility was detected at this site any product not digested with the HpaII enzyme would be scored as a susceptible allele (c) PCR products could be purified before the restriction enzyme digest. This last option would increase the expense of such an assay.

The data were rescored with the additional band being grouped with the susceptible allele. When the new numbers were retested for goodness of fit to Hardy-Weinberg equilibrium no disequilibrium for the resistance and susceptible alleles was observed in the susceptible population (Figure 24b).

The resistant population was also tested for Hardy-Weinberg equilibrium and also displayed some disequilibrium due to a higher than expected homozygous susceptible frequency (figure 24c). To test whether the polymorphism associated with the resistance allele in the McMaster populations was restricted to those particular strains the assay was applied to an additional two independently derived BZ resistant strains, Badgery's Creek resistant and Arthursleigh, of *T. colubriformis*. The results are shown in Table 10. The PCR fragments were amplified successfully in these two strains and both the polymorphisms characterised for the McMaster strains were observed in these strains as well as the artefact band. The polymorphism associated with resistance in the McMaster strain also appeared to be associated with resistance in these additional strains of BZ resistant *T. colubriformis*. This is preliminary evidence that this polymorphism could be used to test for BZ resistance in all Australian field strains of *T. colubriformis*.

Figure 24. Calculation of Hardy-Weinberg equilibrium for three alleles (a) at the *tcb-1* locus and two alleles (b) at the *tcb-1* locus.

(a)

Chi square test for goodness of fit between the susceptible and resistance alleles and the Hardy-Weinberg equilibrium.

McMaster susceptible population with three alleles

	S1S1	S1S2	S1R1	S2S2	S2R1	R1R1
exp	43.2	72.9	38.25	30.6	31.95	8.325
obs	3	148	43	0	18	13
$(obs-exp)^2/exp$	37.4	77.4	0.59	30.6	6.09	2.6

Chi square with two degrees of freedom=154.71. Reject at the 5% level of significance

(b)

McMaster susceptible population with two alleles at the *tcb-1* locus.

	S1S1	S1R1	R1R1
exp	148.5	67.5	9
obs	151	61	13
$(obs-exp)^2/exp$	0.042	0.63	1.78

Chi- square with one degree of freedom= 2.45 not significant at the 5% level.

McMaster resistant population with two alleles

	S1S1	S1R1	R1R1
exp	0.71	17.6	100.67
obs	3	13	103
$(obs-exp)^2/exp$	7.37	1.2	0.05

Chi-square with one degree of freedom= 8.62 Reject at the 5% level of significance.

Chapter 4

General Discussion

Current assays for the detection of anthelmintic resistance rely on the phenotypic expression of resistance and are relatively insensitive (Martin *et al.*, 1989). Phenotypic assays can only detect resistant homozygotes if resistance is recessive and the heterozygous carriers of recessive resistance genes cannot be detected. Consequently by the time the resistance allele can be detected in a phenotypic assay the allele frequency in the population is already high due to the presence of heterozygotes.

Shoop (1994) recently suggested that the way in which the term resistance was defined should be carefully considered. The accepted definition of resistance is taken as the ability to survive treatment at the recommended dose rate. The dose rate recommended by the manufacturer for broad spectrum anthelmintics is set at the rate that will remove the most tolerant of the target species and is often far in excess of the dose that would kill the most sensitive of the target species; for example, the recommended dose rate for ivermectin is 200μg/kg but only 20μg/kg is required to be 95% efficacious against drug naive *H. contortus*. Therefore it is necessary to be able to detect resistance in target species at much lower levels than the recommended dose rate because species that were initially susceptible to drug treatment that are found to be resistant to the recommended dose rate have reached extremely high levels of resistance rendering a class of compounds useless for control of that organism. Shoop (1994) stated that assays that were easily interpreted and sensitive enough to measure resistance at levels far below the point at which the manufacturers recommended use level would fail would be invaluable as they would allow the implementation of some alternative measure (such as a narrow spectrum anthelmintic or a drug rotation strategy) to remove the resistant population before a broad spectrum anthelmintic was rendered ineffective. Jackson (1993) recently commented that even though computer models have been developed to predict the long term impact of control strategies the biology of the ecosystem is not fully understood and more sensitive assays would go along way in helping to define the real effects of the control strategies currently in use on the development and management of drug resistance. This work presents just such a sensitive assay for benzimidazole resistance in the sheep parasitic nematode *T. colubriformis*.

The RFLP Analysis

Initially an RFLP analysis was conducted using a *T. colubriformis* β-tubulin probe pWG36 (which contained mostly coding sequence from the middle of the gene and some intron sequences) to determine if polymorphisms could be detected that would discriminate BZ susceptible strains from BZ resistant strains of worms. RFLPs were identified using this probe when genomic DNA was digested with DraI and ClaI (Figure 11). In both cases a reduction in the number of hybridising bands in the resistant populations was observed when compared with the number of bands that hybridized in the susceptible populations. The RFLPs seen in the DraI digests were the most dramatic with eight bands hybridising in the McMaster susceptible population and only two or three bands hybridising in the resistant populations. Also the bands that hybridised in the three independently selected resistant strains were the same size and all of the bands seen in the resistant populations could be detected in the susceptible strain.

The DraI RFLP results were further investigated by probing genomic DNA with two additional probes from the *tcb-1* locus: a 5' probe (pWG35) and a 3' probe (pWG37) (Figure 12). It was considered highly probable that both probes were locus specific as pWG35 consisted mainly of a large intron located between amino acid 55 and 56 and some coding sequence and the 3' probe pWG37 contained the 3' variable end of the gene and 3' flanking sequence. The pWG 35 5' probe hybridised to seven out of eight bands seen in DNA of the BZ naive strain (McMaster susceptible) and only one band in the resistant strain (McMaster resistant) whereas the 3' probe hybridised to only two bands in the BZ susceptible strain and one band in the resistant strain. These results indicated that there was a high degree of polymorphism for DraI sites in the 5' region of the tubulin locus whereas the 3' region of the gene showed very little polymorphism in DraI sites. Since it was considered that the pWG35 clone was locus specific it was concluded that there were multiple alleles of the *tcb-1* locus present in the susceptible population and that selection by BZ reduced the polymorphism of the *tcb-1* locus in resistant populations by removing (killing) individuals from the population that carried susceptible alleles. By counting the number of bands that hybridise in the southerns probed with the various probes an estimate of the number of *tcb-1* alleles can be obtained. There is a maximum of seven alleles (this is a maximum as one allele could account for two bands if there is a DraI site in the region covered by the probe) at the *tcb-1* locus in the susceptible population when DNA from the susceptible strain is probed with the 5' probe pWG35.

The PCR assay, detects two nucleotide differences in two cloned alleles (2.3, a putative susceptibility allele and 2.9 a putative BZ resistance allele) of the *tcb-1* locus (Figure 21). When individual worms are screened using the allele specific PCR assay, only two alleles are detected, either 2.3 or 2.9. These data, combined with the observation that pWG37 detects at most two DraI alleles, implies that there is much more limited polymorphism at the 3' end of the gene. This apparent inconsistency could be reconciled in the following way; the nucleotide polymorphisms used to identify the 2.9 allele is tightly linked to resistance because firstly, the 2.9 genomic clone contained DraI fragments that were the same size as the DraI fragments that hybridised in genomic southerns of DNA extracted from the resistant strain (McMaster resistant) when probed with the *tcb-1* subclones pWG35, pWG 36 and pWG37. Secondly almost 100% of individual worms from three independently selected resistant strains when screened for the 2.3 and 2.9 alleles were homozygous for the nucleotide polymorphisms identified in the 2.9 allele. It was concluded therefore that the nucleotide polymorphisms (identified by the creation of a HpaII site in PCR products amplified from individual worms) at the 3' end of the gene and the DraI sites detected at the 5' end of the gene (all resistant populations had the same Dra I bands) in resistant strains are both tightly linked to resistance.

The DraI RFLP bands seen in the resistant strains can also be detected in genomic southerns of DNA extracted from the BZ naive strain (McMaster susceptible) and the PCR assay also detects the 2.9 (R) nucleotide polymorphisms in a high proportion of individual worms from the susceptible population. The conclusion was made that individuals from the susceptible population that carried the 2.9 nucleotide polymorphisms were in fact carrying the resistance determining factor and that no new mutation needed to occur in this allele for BZ resistance to arise in the population. The alternative hypothesis is that a new mutation arose during selection by BZ in some individuals that carried the allele with that particular combination of DraI sites and the 2.9 nucleotide polymorphisms and therefore there would be a subset of individuals detected in the susceptible population carrying the 2.9 polymorphisms that would not be carrying the resistance determining factor and the PCR assay would be overestimating the allele frequency for resistance. It is not possible to test if individual worms from the susceptible population that carry the polymorphisms associated with resistance detected in the PCR assay also have the particular DraI fragments also associated with resistance as the worms are too small to carry out individual southerns. However if a new mutation was required to give rise to BZ resistance then presumably this could occur in any

tcb-1 allele and therefore independently selected populations of BZ resistant worms would show different banding patterns or different combinations of DraI bands in the RFLP analysis and therefore the 2.9 polymorphisms would not necessarily be associated with resistance in all independently selected strains. This was not observed. The observations made here are that independently selected BZ resistant strains have the same DraI sites at the 5' end of the *tcb-1* locus and the same nucleotide polymorphisms at the 3' end of the gene and the conclusion is that the allele carrying the BZ resistance factor (which is identified by this particular combination of DraI sites and the 2.9 nucleotide polymorphisms) existed in Australian worm populations a long time before they were exposed to BZs. The fact that resistance to BZ arose rapidly in the field and that BZ resistant strains can be readily selected in the laboratory lend support to this conclusion. Roos *et al.* (1990) conducted a laboratory selection experiment where a BZ naive strain of *H. contortus* was selected over seven generations and the population was resistant after only two rounds of selection. In addition Roos *et al.* (1990) demonstrated that reduction in the number of β-tubulin hybridising bands and an increase in drug binding to tubulin extracted from the selected populations (pmol [^{3}H]MBZ bound /mg of protein) were correlated with the increase in BZ resistance of the population. In addition, southern blots of DNA from of individual adult worms, which is possible in *H. contortus* as they are bigger, from the susceptible and resistant populations were probed with the *gru-1* β-tubulin probe. Susceptible individuals contained multiple hybridising bands whereas resistant individuals all contained a single 11kb hybridising band that was also detected in some susceptible worms but usually in combination with other bands. The conclusion from that study was that an allele for resistance was present in the BZ naive population before exposure to BZ took place and that no new mutation was necessary for BZ resistance to develop.

The RFLP data indicated that there were probably multiple susceptibility alleles (alleles that were polymorphic for DraI sites at the 5' end) at the *tcb-1* locus but the PCR assay detects only one susceptibility allele. The conclusion was made that the nucleotide polymorphism detected in the putative susceptibility allele is linked to all susceptibility (S) alleles and that the susceptibility alleles that are different, with respect to DraI sites, cannot be distinguished by using the PCR assay.

The Allele Specific PCR Assay

This assay is an allele specific PCR assay that detects nucleotide polymorphisms that are associated with an allele at a particular β-tubulin locus implicated in BZ

resistance. The assay is a genetic assay that can detect the resistance allele in individual nematodes. Since it is a genetic assay detecting a particular allele at a particular locus, expression of the resistance phenotype is not a prerequisite for successful detection of the resistance gene. Not only can homozygous resistant individuals be detected when they are present even at very low frequencies but heterozygous carriers of recessive resistance alleles can also be detected. Therefore a true estimate of allele frequency for the resistance allele can be determined as well as any small shift in allele frequencies due to selection by drug treatment.

The PCR assay proved to be very versatile as all stages of the nematodes life cycle, eggs, third stage larvae (fresh or frozen) which have a tough cuticle and adults, are amenable to being assayed. In addition enough material was available from any of the life stages for the assay to be repeated. No phenotypic assay available today for the detection of drug resistance in parasitic nematodes offers this degree of accuracy, repeatability, versatility or sensitivity.

In addition, sequence data obtained from this project allowed speciation between *H. contortus* and *T. colubriformis* to be incorporated automatically into the assay. This is particularly relevant to the field situation as infection by these two nematodes often occurs simultaneously. Therefore eggs obtained directly from faecal samples can be used in the assay without further culturing and visual differentiation of larvae.

The PCR assay was designed utilising a nested PCR strategy to minimise false positives and increase sensitivity while allowing simultaneous detection of two alleles (Rand S) in individual larvae. When this assay was applied to susceptible strains a mixture of genotypes was observed including a significant proportion of worms heterozygous and homozygous for the resistance allele. This confirms the RFLP data where the bands seen in the resistant population were readily detectable in DNA extracted from the susceptible population as well as the heteroduplex data where products from the susceptible nematodes formed both homoduplexes and heteroduplexes with DNA amplified from the cloned resistance allele. Varying phenotypes with respect to drug binding to tubulin extracted from individual worms from a susceptible population of *H. contortus* have also been observed (Lacey, 1988) which implied the presence of homozygous and heterozygous genotypes for BZ resistance in the unselected population. These data provide a genetic basis for Lacey's observations.

The resistance allele detected in this assay was cloned from a lambda genomic library and shown to contain DraI restriction fragments that corresponded to

DraI fragments seen in genomic Southerns of DNA extracted from three independently derived resistant strains (Kybebolite resistant, New England Resistant *Trichostrongylus* (NERT) and McMaster resistant) of *T. colubriformis*. Heteroduplex analysis of PCR products from the 3' end of this allele showed the product from this genomic clone formed homoduplexes with the products amplified from individual resistant worms and a mixture of homoduplex and heteroduplex conformations with products amplified from susceptible worms. The heteroduplex results indicated that resistant larvae contained only one sequence in this region and were therefore homozygous for that particular region of sequence whereas susceptible larvae contained a variant sequence and were either homozygous or heterozygous for the polymorphisms present in this region reflecting the mixture of alleles present at this locus before drug selection .

An additional allele from this locus also cloned from the same lambda genomic library was shown to contain DraI fragments that corresponded to DraI fragments seen in Southerns of DraI digested genomic DNA extracted from susceptible strains of worms. The 3' end of this allele was sequenced and shown to be identical to the sequence of the C-terminal region of the putative resistance allele except for two silent nucleotide changes, one in an intron and the other a silent third base change in amino acid. These sequence polymorphisms were responsible for the heteroduplex conformations observed between PCR products amplified from the 3' region of the R allele and the same region in the putative susceptible allele.

The assay was successfully applied to a number of independently derived resistant strains (McMaster resistant laboratory selection, Badgery's Creek resistant *Trichostrongylus* and Arthursleigh) confirming the prediction from the RFLP data that the bands seen in two additional independently selected BZ resistant strains (KRT and NERT) carry the same R allele as those seen in the laboratory selected resistant strain (McMaster resistant). It would be useful to compare both the biochemical assays and the PCR assay but it is difficult to make direct comparisons for each of the strains used in this project between the PCR assay, the tubulin binding assay and the larval development assay as results are reported for some strains using one of the assays but then not the other assay. The McMaster susceptible strain and the Arthursleigh strain have been compared using the tubulin binding assay and the larval development assay (LDA). Using the tubulin binding assay which measures the amount of radiolabelled drug bound per unit measure of protein, the McMaster susceptible strain bound considerably more radiolabelled drug than the 8TBZ Badgery's

creek resistant strain (97.7 pmole/mg of crude protein and 18.7pmole/mg crude protein respectively). LD_{50} values determined using the LDA for McMaster susceptible was 0.09μM for Thiabendazole and 1.7μM for the Arthursleigh strain. .

Kwa *et al.*(1993) have published the sequence of a β-tubulin BZ resistance allele from *H. contortus*. When the sequence of this β-tubulin gene was compared to the sequence obtained from the pWG 21 clone in this study a striking degree of homology was observed. All intron/exon boundaries are conserved but more significantly the 3' carboxy terminal amino acid sequence is identical between these genes. Thus there is functional homology between *tcb-1* in *T. colubriformis* and *gru-1* in *H. contortus*. The implication of this is that the mechanism of resistance is identical in these two species. Since both species are often present simultaneously in the host and the same locus has been implicated in BZ resistance and the sequence of these genes is so similar, then by designing primers that amplify a region including an intron (introns are in the same position but vary in length and sequence between the species) speciation can be included in the assay while simultaneously assaying for BZ resistance status in both species.

Population Genetics

The McMaster susceptible and McMaster resistant strains were tested for Hardy-Weinberg equilibrium between the resistant and susceptible alleles. The allele frequencies in the unselected strain, McMaster susceptible, were 0.81 and 0.19 for the susceptible and resistant alleles respectively and the strain was in equilibrium for these two alleles. This suggests that the resistance allele was present in the BZ naive population as a natural polymorphism and had no deleterious effect on either homozygote or heterozygote worms. In other words there appears to be no fitness penalty associated with the resistance allele in this strain. The allele frequencies for the two alleles had changed dramatically for the BZ selected population with the resistance allele having an allele frequency of 0.92 and the susceptible allele a frequency of 0.08. This strain, however, was not in equilibrium for the two alleles as the number of homozygous susceptibles (three individuals out of 125 tested) detected was higher than expected. This disequilibrium may be due to sample size if the true population allele frequency was much lower than the allele frequency estimated from the sample tested or there may be some individuals where a cross over event has occurred between the polymorphism and the resistance mutation and drug selection has increased the number of these individuals in the population.

Technical Considerations

In this study three assay protocols were explored for suitability as assays for the detecting the BZ resistance allele. These were (i) heteroduplex analysis, (ii) restriction enzyme site differences and (iii) detection of individual alleles using restriction enzyme site generating PCR. Any of the three would be adequate assays for the detection of the resistance allele after some modifications to optimise the reaction conditions as a technical problem was encountered with each of the assays investigated. Poor resolution of bands was a problem for the heteroduplex analysis and the restriction enzyme digests with NlaIII. Both assays were applied to DNA amplified directly from parasite material in one amplification reaction using a primer pair, one of which hybridised to multiple β-tubulin loci. The sequence of this primer coupled with a low melting temperature (58°C) may have resulted in multiple products being amplified in the PCR reaction. Non-specific products would interfere with both heteroduplex analysis and restriction enzyme digestion and would be seen as poor resolution of bands on gels. The problem was encountered with DNA amplified from parasite material as heteroduplexes formed between PCR products from cloned material were readily resolved on the MDE gels (Figure 19). The problem of resolution for both the heteroduplex analysis and the digestion with NlaIII may have been overcome by replacing the primer that could anneal to multiple loci with a new locus specific primer with a higher melting temperature. Alternatively a nested PCR protocol could have been applied to both protocols but neither the heteroduplex analysis or the digestion of PCR products with NlaIII were pursued as each protocol involved a particularly expensive reagent; the heteroduplex analysis required the MDE gel solution which cost $35.00 per gel or per 20 samples and the NlaIII enzyme was a new enzyme in limited supply that cost 13¢ per unit compared to 9¢ and 2¢ per unit for HpaII and EcoRV respectively, the enzymes used in the assay finally applied to field strains of *T. colubriformis.*

To overcome the problem of amplification of non-specific PCR products a nested PCR strategy was applied to the restriction site generating PCR protocol. Products were amplified directly from parasite material using the same primers (P1 and LM7) as used for the first two assays. These primers flank the region containing the nucleotide polymorphisms of interest, and by carrying out this first PCR reaction the concentration of the specific target was increased. An aliquot of the first reaction was then added to second amplification reaction including a second set of locus specific primers modified to detect individual alleles from the *tcb-1* locus. The PCR products from this second reaction were

then digested separately with the restriction enzymes HpaII (which detected the polymorphism associated with BZ resistance) and EcoRV (which detected the nucleotide polymorphism associated with BZ susceptibility). The restriction digests were then electrophoresed in 8% non-denaturing polyacrylamide minigels. As presented in Chapter 3 a PCR product that did not cut with either enzyme was observed at a very high frequency and always in combination with one or other of the characterised alleles, never alone (this would have been scored as a homozygote for an uncharacterised allele). The first round PCR products from worms displaying this band were subcloned and sequenced across the restriction enzyme sites. In all cases the nucleotides for the susceptible allele were present therefore the products that did not cut with either enzyme from the second PCR reaction should have cut with EcoRV. Removal of excess nucleotides and primers from the second reaction before digestion may overcome the problem of incomplete digestion by EcoRV. It would be preferable to minimise the number of steps in the assay. Therefore since the HpaII digest reliably detects the resistance allele, the detection specifically of the polymorphism associated with the susceptible allele need not be included in the assay.

Functional Significance of the Polymorphisms Detected in the PCR Assay

The sequence obtained for the resistant and susceptible alleles of the *tcb-1* locus extended from amino acid 416 beyond the stop codon at amino acid 447 into the non-translated flanking region of the gene. This region also contains a 99bp intron positioned between aa425 and 426. One function attributed to this region of β-tubulins is the regulation of microtubule stability. Microtubule associated proteins such as Tau and MAP2 enhance microtubule stability, Padillo *et al.* (1993) proposed that in the absence of MAPS, the carboxy terminus interferes with the GTP binding site on the β-tubulin molecule causing MT instability and isoforms with different sequences at the C-terminus could result in different dynamic properties. Truncation of the carboxy terminal domain of yeast β-tubulin resulted in temperature sensitive growth, reduced rate of nuclear division and hypersensitivity to TBZ (Matsuzaki, Matsumoto & Yahara, 1988).

The polymorphism detected for the BZ resistant allele in *T. colubriformis* is a nucleotide difference in an intron at the 3' end of the gene. This polymorphism is not located within the intron splice sites nor does it occur in any sequence that may correspond to a lariat consensus sequence within the intron. The polymorphism that is associated with the susceptible allele is a third base change

that does not alter the amino acid sequence. Hence neither polymorphism that is detected with this assay has an obvious functional significance.

Amino acid substitutions associated with BZ resistance that may have functional significance have been identified in the fungus *Aspergillus nidulans*. Jung *et al.* (1992) have analysed 18 different BZ resistant *Aspergillus nidulans* mutants of which two mutants showed aa 200 substitutions resulting in a change from the wild type for BZ resistance status. One mutant had *benA15* had an alteration at aa6 which resulted in resistance to BZ. Whereas the second mutant, benA16, had an alteration at aa165 which resulted in supersensitivity to BZ. These mutant alleles showed no other substitution from the wild type allele. Of the other 16 BZ resistant mutants characterised, amino acid substitutions were found at aa 198-200. No alleles contained more than one of these substitutions but the hypothesis put forward was that each of these amino acid positions is involved in forming the BZ binding site. A phenylalanine to tyrosine substitution at amino acid position 167 in *N. crassa* was shown to result in BZ resistance.

Kwa *et al.* (1994a) recently observed that there was an amino acid substitution, phenylalanine (susceptible) for tyrosine (resistant), in a BZ resistance allele at aa position 200 in *H. contortus*. This substitution was found to be consistent for BZ resistant strains from Europe, Africa, Britain and Australia. Therefore the amino acid 200 substitution may have functional significance. However, the genomic clones for the susceptible and resistant alleles of the *tcb-1* locus cloned in this project from *T. colubriformis* had phenylalanine at aa 200. In order to confirm that the substitution is not present in resistant worms this region should be cloned directly from resistant worms and sequenced. If the clones for both the susceptible and resistance alleles were fully sequenced amino acid substitutions might be identified that could possibly have functional significance.

In order to test for functional significance of an amino acid substitution an expression system is required to compare the phenotype of the wild type allele and the altered allele. The expression system in *C. elegans* first described by Mello (1992) has been used by Grant (1992) to express parasite genes. Grant transformed *C. elegans* with the putative BZ resistance allele and obtained expression of that gene consistent with the allele being BZ resistant. Kwa *et al.*(1994b) has also used this system to express both the *H. contortus* resistance and susceptible alleles in *C. elegans* and obtained the expected phenotype for the respective alleles which is strong evidence that the amino acid 200 substitution has functional significance for BZ resistance in *H. contortus*. Lubega et al.(1993) have taken a different approach more amenable to biochemical analysis

by expressing the nematode β-tubulin protein in *E. coli*. They have observed that other factors in addition to expression of the particular resistance allele of β-tubulin in *E. coli* are required for the decrease in drug binding as seen in whole protein extracts .

Are Additional Loci Involved in BZ Resistance?

This thesis presents evidence that one β-tubulin locus can account for the BZ resistance status in the four resistant strains examined. Two lambda clones obtained from the genomic library could account for four out of the eight bands seen in genomic southerns of DNA extracted from the susceptible population and one of these clones had two bands which were the same size as the only two bands that hybridised in DNA extracted from three independent resistant strains. The implication of this was that BZ resistance was due to the selection of a particular allele at the *tcb-1* locus. Furthermore an allele specific PCR assay designed to detect the resistance allele showed that this allele was present at a low frequency in the BZ naive population but was virtually the only allele remaining at the *tcb-1* locus in BZ selected strains of *T. colubriformis*.

Roos *et al.* (1990) has demonstrated that strains of *H. contortus* that are highly resistant to BZ have a particular allele present at the isotype 1 locus. However, in addition to a particular allele occurring at the isotype 1 locus, a second locus (isotype 2) appears to be deleted from the genome of highly resistant worms but that a particular allele is always present at the isotype 1 locus even in the highly resistant worms.

The involvement of additional β-tubulin loci in BZ resistance in *T. colubriformis* was not investigated in this study. However, clones for an additional three *T. colubriformis* β-tubulin loci (including a sequence homologue of *H. contortus* isotype 2 gene) have been described in this thesis. These could be used as probes to investigate the involvement of other β-tubulin loci in *T. colubriformis*.

Sequence Comparisons

β-tubulin genes are amongst the most highly conserved genes in nature. Most eukaryotes contain multiple β-tubulin genes except yeast which has only one β-tubulin gene. These genes are highly conserved between loci within and between species. The sequence of the 3' terminal 30 amino acids of β-tubulin loci have been shown to be useful in distinguishing the different β-tubulin loci in various species. In vertebrates, for example, the locus from which these alleles are

derived has been defined by sequencing the 3' end region of these genes and three different isotypic classes have been defined for vertebrates on the basis of sequence conservation of the different loci between species. Comparison of the 3'end sequences of the β-tubulin clones isolated from the *T. colubriformis* library with the 3' ends of β-tubulin genes from other nematodes shows, as for other organisms, that the sequences for particular β-tubulin loci are more highly conserved between species and that the sequences within a particular species are divergent. As with vertebrates therefore, nematode β-tubulin genes can be divided into four different isotypic classes. This is the first example of isotypic classes for invertebrate β-tubulin genes. In addition, for the first time a specific function (benzimidazole resistance) can be assigned to one of the isotypic classes.

The partial sequencing of a 2.9kb Pst fragment (pWG34) was conducted in order to confirm that the fragments used as probes in the RFLP analysis did in fact contain a β-tubulin gene. Analysis of the 1431bp obtained from this fragment confirmed that the clone we had isolated did contain a β-tubulin gene. When this sequence was compared to the sequence for a β-tubulin gene implicated in BZ resistance in *H. contortus* the nucleotide sequence was found to be 85% homologous in the coding region and the C-terminal sequences of these genes had identical amino acid sequences. This is the first incidence reported of this degree of homology between β-tubulin genes for two species of invertebrates.

In addition the positions of six introns were identified in the *T. colubriformis* sequence and these were also 100% conserved with the position of introns in the *H. contortus* sequence. The entire sequence for introns was not determined for this clone. However 3 introns were fully sequenced. Comparison of the introns in the same position in *H. contortus* showed that in both cases the *T. colubriformis* introns were larger. This difference in intron length has allowed the speciation of these two nematodes to be incorporated into the PCR assay. There was a low degree of homology of regions of sequence in the introns between these two species.

Sequence beyond the stop signal was also obtained for the *T. colubriformis* clone. This region of sequence was compared to the *H. contortus* sequence in this region and again a moderate degree of conservation was identified in this region. The *H. contortus* gene had a consensus polyadenylation signal 69bp beyond the stop codon. The sequence AAGAAA was present in the *T. colubriformis* at precisely this position. The consensus sequence for

polyadenylation signal in vertebrates is AATAAA. Sheets *et al.* (1990) have studied the effect of point mutations on efficiency of polyadenylation of vertebrate mRNAs and shown that the sequence AAGAAA can function as a polyadenylation signal in vertebrates but the efficiency is reduced to 6.0% of the wild type. The sequence TATAAA functioned with 17% of the efficiency compared to the consensus sequence in their experiments. This sequence is present 199bp beyond the stop signal in the *T. colubriformis tcb-1* sequence. Therefore there are two sequences that could act as polyadenylation sites for the *tcb-1* locus. A cDNA clone representing the *tcb-1* locus could be obtained and the 3' end sequenced to determine if either of these sequences acted as polyadenylation signals.

Sequence was obtained from an additional three clones that showed differences in restriction enzyme fragment pattern and variation in the degree of hybridisation to three probes subcloned from the pWG 35 fragment. A primer that hybridised to a very highly conserved region of sequence in all β-tubulin genes was used to obtain sequence towards the 3' end of each gene. Once the sequence was obtained in one direction primers were obtained that allowed the sequence for the opposite orientation to be determined. Three divergent sequences were obtained from these three clones. One of the sequences (pWG24) showed 100% homology to a cDNA clone characterised in this laboratory by Le Jambre and Lenane (unpublished data). The sequence for an homologous gene in *H. contortus*, designated the isotype 2 gene, has been published by Geary *et al.* (1992). These homologous genes also show 100% homology in amino acid sequence at the C-terminus as does the isotype 1 gene to the *T. colubriformis tcb-1* sequence. The *tcb-3* and *tcb-4* sequences are unique and homologous genes have not been cloned from other parasitic nematodes.

The sequences for *tcb-3* and *tcb-4* sequences contained introns at the same position as the *T. colubriformis tcb-1* β-tubulin clone. The *tcb-2* sequence did not contain an intron in this region. All three introns identified varied in length and showed no sequence homology to each other.

A consensus polyadenylation signal (AATAAA) was identified for the *tcb-3* and *tcb-4* clones. The *tcb-3* polyadenylation signal was located 83bp beyond the stop signal. The signal for *tcb-4* coincided with the stop signal itself. The polyadenylation signal for the *tub-1* gene in *C. elegans* also coincided with the stop signal.

The CLUSTALV program was used to compare the homology of these C-terminal sequences with β-tubulin genes from other nematodes. The comparison

included the four *C. elegans* β-tubulin sequences and the sequences from *Brugia malayi* (Guenette *et al.*, 1990) and *H. contortus* (Geary *et al.*, 1992; Kwa *et al.*, 1993). No other nematode sequences were present in the genebank database. The results of the CLUSTAL analysis showed that the tcb-*1* gene responsible for the RFLPs observed between BZ susceptible and resistant strains of *T. colubriformis* and the homologous isotype 1 gene from *H. contortus* also implicated in BZ resistance most resembled the *C. elegans ben-1* gene which is the locus demonstrated to be uniquely susceptible to BZs in this nematode (Driscoll *et al.*, 1989). The unrooted tree for which phylogenetic distances were calculated placed the *ben-1* gene almost midway between the *tcb-1* and *tcb-2* loci. Perhaps the sequence similarities between the *tcb-1* /isotype 1 and *tcb-2* /isotype 2 loci with the *ben-1* gene may help to explain the observations of Kwa *et al.* (1993) where selection of a particular isotype 1 allele and the deletion of the isotype 2 gene are required for expression of high levels of BZ resistance in *H. contortus*. The *tcb-3* clone most resembled the *mec-7* gene which gives rise to the 15pf microtubules unique to the six touch receptor cells in *C. elegans*. The *mec-7* product is more sensitive to inhibition by colchicine than the other β-tubulin products in nematodes and in this respect more closely resembles microtubules from higher eukaryotes than the other microtubules present in the nematode. Vertebrate tubulins have been shown to have a conserved phenylalanine residue in the C-terminal sequence (Burns & Surridge, 1990). Both the *mec-7* gene and the *T. colubriformis tcb-3* gene have the highly conserved aromatic residue phenylalanine in the same position in the C-terminal variable sequence whereas the remaining nematode β-tubulins do not have this conserved phenylalanine. It is highly probable therefore, that the *T. colubriformis tcb-3* clone represents the homologue of the *C. elegans mec-7* gene. The *tcb-4* sequence appeared to most closely resembled the *B. pahangi* β-tubulin sequence but these were not as closely related as the *H. contortus* genes which is not surprising since *B. pahangi* is a microfilarial parasite and not as closely related. None of the parasitic 3' sequences were clearly related to the 3' sequence of the *C. elegans tub-1* gene. The function of this gene in *C. elegans* has not been determined as mutations in this gene appear to be either silent (meaning the gene is redundant) or lethal (making the gene essential for viability).

Future Work

This thesis describes an assay that detects a nucleotide polymorphism that is tightly linked to BZ resistance. This assay can be used to genotype individual

worms for BZ resistance. This assay, after some optimisation of reaction conditions could be used for the routine diagnosis of BZ resistance in *T. colubriformis*. however before this assay was applied routinely an essential study would be to correlate the PCR assay with the phenotypic assays such as the faecal egg count assay, the egg hatch assay or the larval development assay to determine the allele frequencies at which the phenotypic assays detect the presence of the resistance allele or at what allele frequency should alternative worm control measures be introduced to reduce the increase in the frequency of worms carrying the resistance allele. The PCR assay could also be used to "tag" particular strains of worms (an alternative to radiolabelling of larvae) for use in population genetic studies. For example; tagged strains could be used to determine establishment rates of incoming larvae. Also the effects of refugia on the development of resistance could be examined by artificially seeding pasture with genetically tagged strains and monitoring the development of resistance or changes in allele frequencies with different control strategies

It would be beneficial to sequence in entirety the two alleles cloned from the *tcb-1* locus in order to identify amino acid changes that may have functional relevance to BZ resistance. This difference could then be used to identify resistant worms rather than detecting a linked marker. Additionally it would be of interest to determine if *T. colubriformis* strains from other countries contained the nucleotide polymorphisms shown to be associated with resistance in Australian strains as it might be possible that these polymorphisms may be unique to Australian strains of worms.

Only the 3' ends of the additional β-tubulin genes were sequenced in this project and it would be of interest to obtain the full sequence of these genes. However the clones of these additional loci could be used as probes, or the sequence of these clones used to design locus specific primers, which could be used to investigate the role of these different loci in the development of BZ resistance.

Conclusions

This thesis describes the characterisation of the β-tubulin gene family in *T. colubriformis* and the role of a particular β-tubulin locus (*tcb-1*) in BZ resistance and the subsequent development of a PCR based assay that can be used to genotype individual *T. colubriformis* for a marker associated with a BZ resistance allele. This is the first genotypic assay for the detection of a drug resistance allele in this species of nematode and one of only two (the other detects BZ resistance in *H. contortus*) genotypic assays for the detection of a

drug resistance gene in parasitic nematodes of sheep. The assay detects a polymorphism in a β-tubulin allele shown to be involved in BZ resistance in an RFLP analysis. Sequence analysis of this allele revealed striking sequence homology to a β-tubulin allele shown to be involved in BZ resistance in the closely related nematode *H. contortus* suggesting that these genes have functional homology. Comparison of these sequences with other nematode β-tubulins suggested that the gene responsible for BZ resistance in these parasitic nematodes was closely related to the *ben-1* gene in the free-living nematode *C. elegans*, the product of which has been shown to be responsible for BZ susceptibility in that nematode.

When the assay was applied to a BZ naive strain (McMaster susceptible) the resistance allele could be readily detected and the allele frequency of the resistance allele was shown to be in Hardy-Weinberg equilibrium. This suggests that the resistance allele was present in the unselected population as a natural polymorphism and that no deleterious effects were associated with the resistance allele. The assay was also applied successfully to a number of independently selected BZ resistant populations implying that the same allele was selected in each population.

Clones for an additional three *T. colubriformis* β-tubulin genes were obtained. Sequence analysis of the variable 3' ends of these genes showed that the sequence of the loci vary within *T. colubriformis* but are highly conserved between closely related nematodes. This degree of homology between species has only been reported for vertebrates and this is the first example of conservation of isotypic classes for β-tubulins in invertebrates.

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