Chapter 3: Results

3.1. DNA extraction

Three different methods were used for DNA extraction (Madania *et al*., 2005, G-spin ™ Genomic DNA Extraction kit (for cell/tissue) and QIAamp DNA Min kit (tissue protocol)) with minor modification in all of methods. The results showed that there is interaction between the species and DNA extraction methods being used. Modified Madania *et al*., 2005 method was used to extract DNA from all the nematode species. However we found that only *Meloidogyne* spp. (C4m1 and C4m2) and *Pratylenchus* spp. (Pra8) gave good genomic DNA yield. DNA quantification was done as describe in section 2.4. Genomic DNA of *Aphelenchus* spp. (Aph3), *Rotylenchulus reniformis* (Rr27), *Xiphinema* spp. (Xip9 and Xip10) and *Helicotylenchus dihystera* (Hd6) was extracted using modified QIAamp DNA Min kit (tissue protocol) with good results. However we were unable to extract genomic DNA from *Meloidogyne* spp. and *Pratylenchus* spp. using this kit. Modified G-spin ™ Genomic DNA Extraction kit (for cell/tissue) method was also tested on all the nematode species but failed to extract the genomic DNA from any of them. At the result, we conclude that the genomic DNA of certain nematodes can only be extracted using specific DNA extraction method.

3.2. Polymerase chain reaction (PCR)

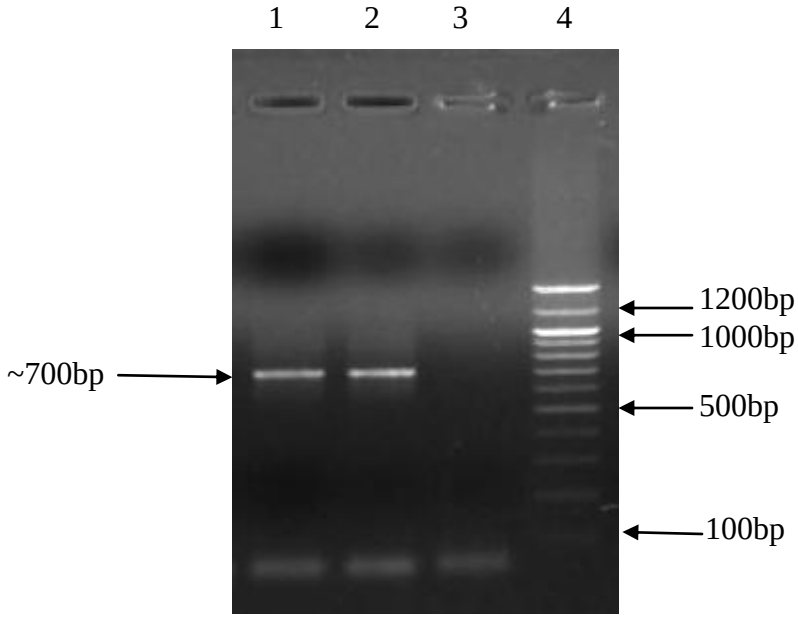


Figure 3.1: Figure shows PCR product of *Meloidogyne* spp. (C4m1 and C4m2) run on 1% agarose gel using Fallas-Kaplan universal primer pair. DNA was extracted using modified Madania *et al*., 2005 method with overnight incubation option.

Lane 1: C4m1

Lane 2: C4m2

Lane 3: Negative control

Lane 4: 100bp DNA ladder (Seegene, USA)

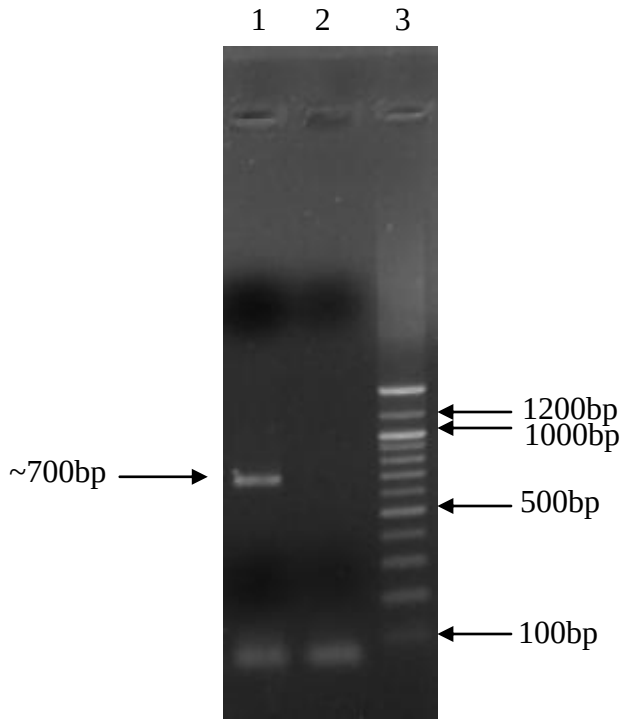


Figure 3.2: Figure shows 1% agarose gel electrophoresis of PCR product amplified from *Pratylenchus* spp. using Fallas-Kaplan universal primer pair. DNA was extracted using modified Madania *et al*., 2005 method and incubated overnight.

Lane 1: Pra8

Lane 2: Negative control

Lane 3: 100bp DNA ladder (Seegene, USA)

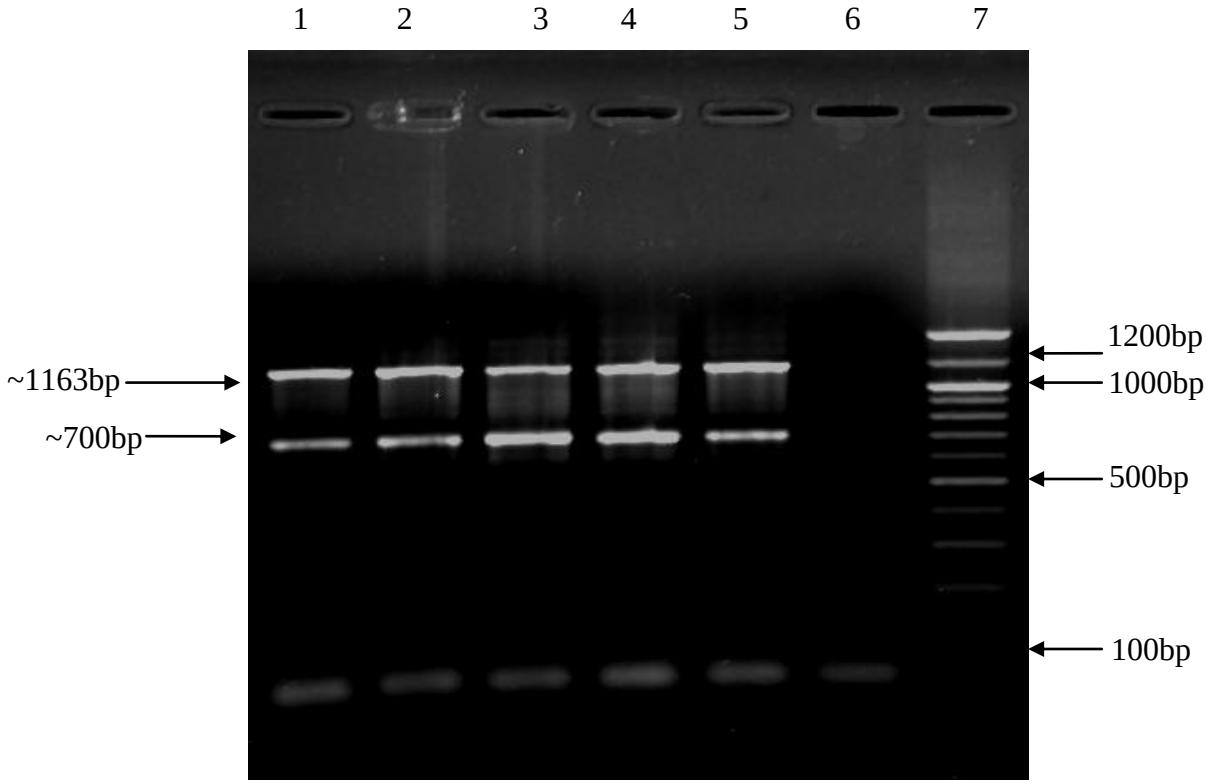


Figure 3.3: Figure shows 1% agarose gel electrophoresis of PCR products amplified from *Aphelenchus* spp. (Aph3), *Helicotylenchus dihystera* (Hd6), *Xiphinema* spp. (Xip9 and Xip10) and *Rotylenchulus reniformis* (Rr27) using Fallas-Kaplan universal primer pair. DNA was extracted with modified QIAamp DNA Min kit (tissue protocol).

Lane 1: Aph3

Lane 2: Hd6

Lane 3: Xip9

Lane 4: Xip10

Lane 5: Rr27

Lane 6 and 7: Negative control and 100bp DNA ladder (Seegene, USA) respectively

3.3. Gel extraction and post gel extraction

The samples that amplified successfully ITS1 and ITS2 regions, were purified by gel extraction. The samples included two bands (~700bp and ~1163bp); both the bands were cut and sequenced.

Gel extraction is a method used to cut off and purify a desired fragment of agarose gel electrophoresis. Final products obtained from gel extraction were more concentrated and purified. Sometimes the fragment size of the sample after gel extraction was reduced because during gel extraction, some of DNA fragment became single-stranded. Therefore the sample was put in 95°C for five minutes and left to cool down naturally at room temperature, returning the DNA back to its double-stranded conformation again.

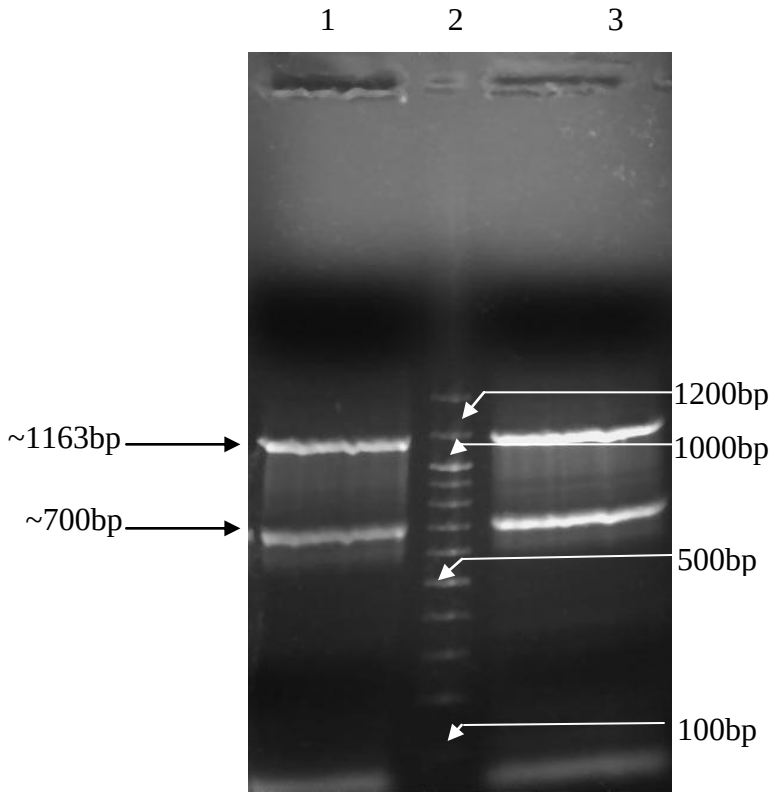


Figure 3.4: Figure shows agarose gel electrophoresis (1%) of amplified ITS1 and ITS2 region to follow gel extraction steps.

Lane 1: Rr27 (*Rotylenchulus reniformis)*

Lane 2: 100bp DNA ladder (Seegene, USA)

Lane 3: Xip10 (*Xiphinema* spp.)

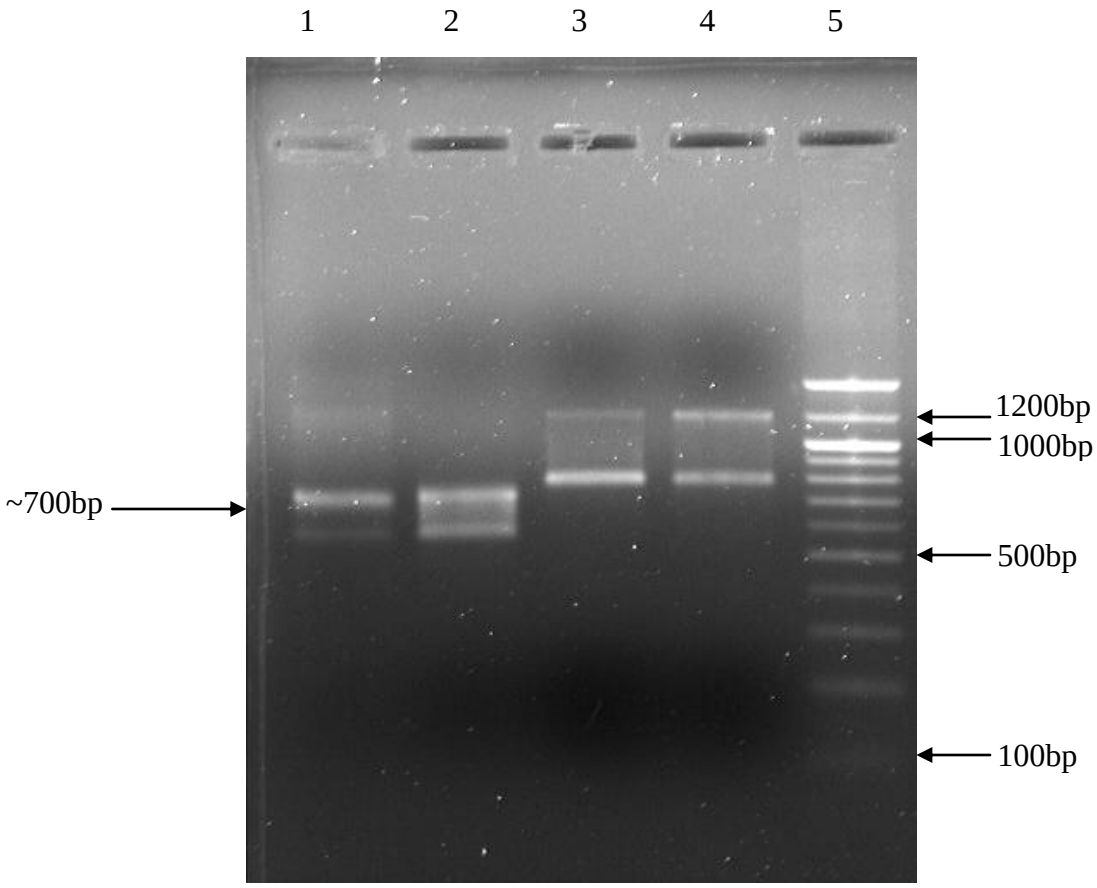


Figure 3.5: Figure shows agarose gel electrophoresis (1%) of PCR product post gel purification to check the presence of the purified DNA of *Rotylenchulus reniformis* (Rr27) and *Xiphinema* spp. (Xip10).

Lane 1: Rr27

Lane 2: Xip10

Lane 3: Rr27 (~1163bp)

Lane 4: Xip10 (~1163bp)

Lane 5: 100bp DNA ladder (Seegene, USA)

3.4. Cloning screening

3.4.1. Blue and white colony screening

After ligation and transformation steps, blue and white colonies were grown on surface of the LB agar plates supplemented with Ampicillin/IPTG/X-Gal. Usually white colonies indicate correct insertion of DNA fragment but sometime they do turn out as false positive result. Colony PCR selection was then done to confirm the correct insertion and to prevent any misidentification before procedding with plasmid extraction step.

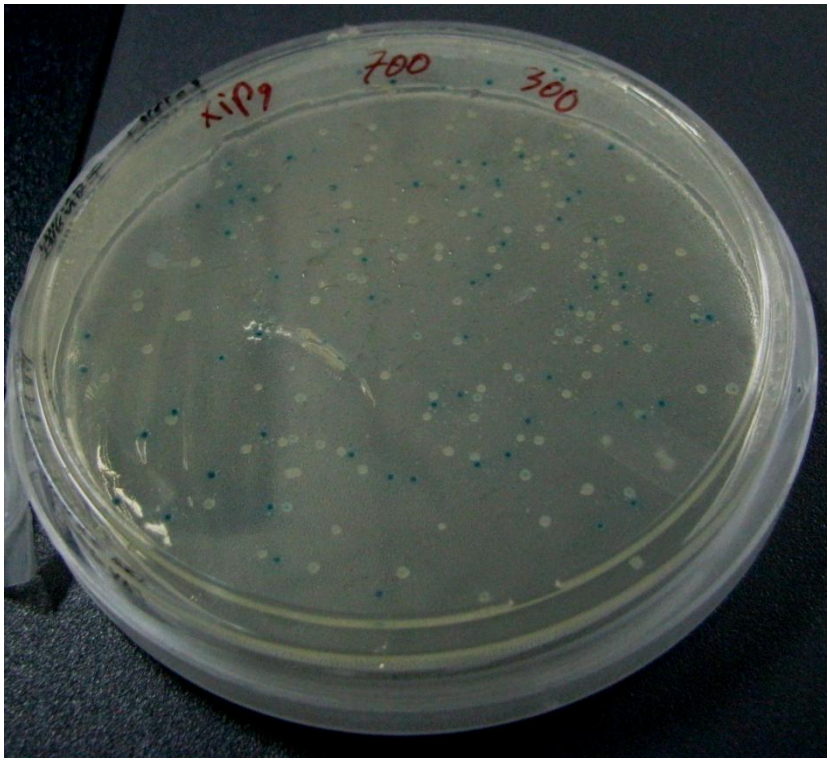


Figure 3.6: Figure shows Ampicillin-resistant white and blue bacterial colonies grown on Ampicillin/IPTG/X-Gal-supplemented LB agar plates.

3.4.2. Colony screening by PCR

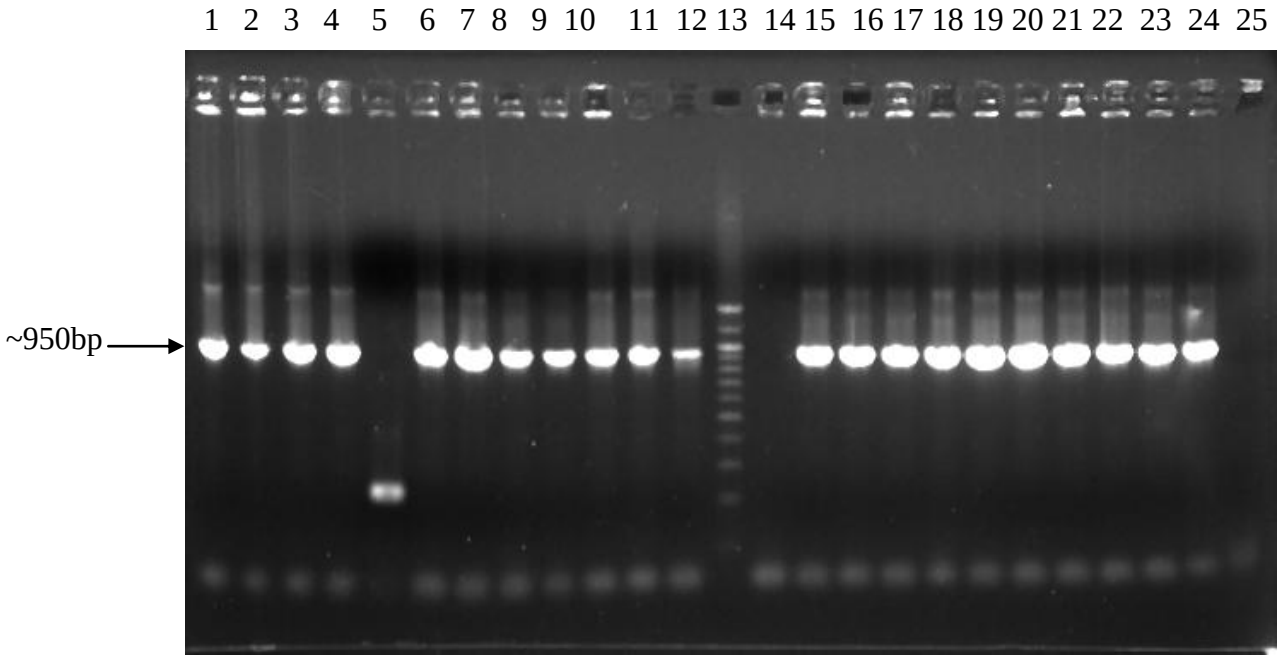


Figure 3.7: Figure shows 1% agarose gel of PCR products clone screening using M13 primers. Samples showing band size of ~950bp were selected for plasmid extraction.

Lane 1-12: PCR product from white colonies of C4m1 (*Meloidogyne* spp.) colonies

Lane 13: 100bp DNA ladder (Seegene, USA)

Lane 14-24: PCR product from white colonies of C4m2 (*Meloidogyne* spp.) colonies

Lane 25: Negative control

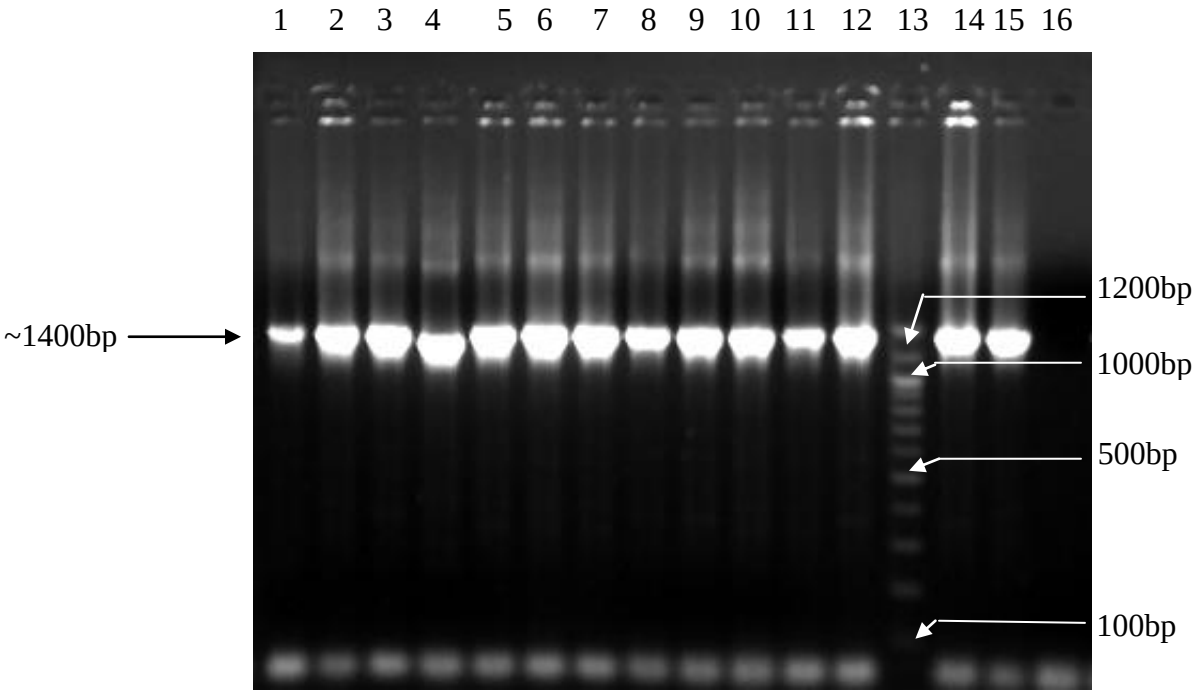


Figure 3.8: Figure shows 1% agarose gel of PCR products for *Helicotylenchus dihystera* clone screening using M13 primers of (cloning of DNA fragment ~1163bp). The samples showing band size of ~1400bp were selected for plasmid extraction step.

Lane 1-12: PCR product from white colonies of Hd6 colonies

Lane 13: 100bp DNA ladder (Seegene, USA)

Lane 14 and 15: PCR product from white colonies of Hd6 colonies

Lane 16: Negative control

3.4.3: Optical density reading for plasmid extraction

Table 3.1: Optical density of plasmid extraction obtained from DNA cloning of fragment ~700bp.

Samples	Concentration(µg/µl)	$Purity_{260/280}$
Pra8c3	1.397	1.76
C4m1d4	3.598	1.85
C4m2a2	3.627	1.84
C4m2b3	4.006	1.88
Xip10a3	5.624	1.85
Xip9a1	3.756	1.83
Rr27a1	3.656	1.86
Hd6c2	2.431	1.77
Aph3a3	1.704	1.80

Table 3.2: Optical density of plasmid extraction obtained from DNA cloning of fragment ~1163bp.

Samples	Concentration (µg/µl)	$Purity_{260/280}$
Xip9a2	2.851	1.82
Xip10a2	1.561	1.76
Aph3a1	3.250	1.88
Hd6d3	2.987	1.78
Rr27a1	2.255	1.75

All the plasmids that had the best qualitative and quantitative values were subjected to restriction enzyme digestion to confirm the insertion.

3.4.4: Restriction enzyme digestion

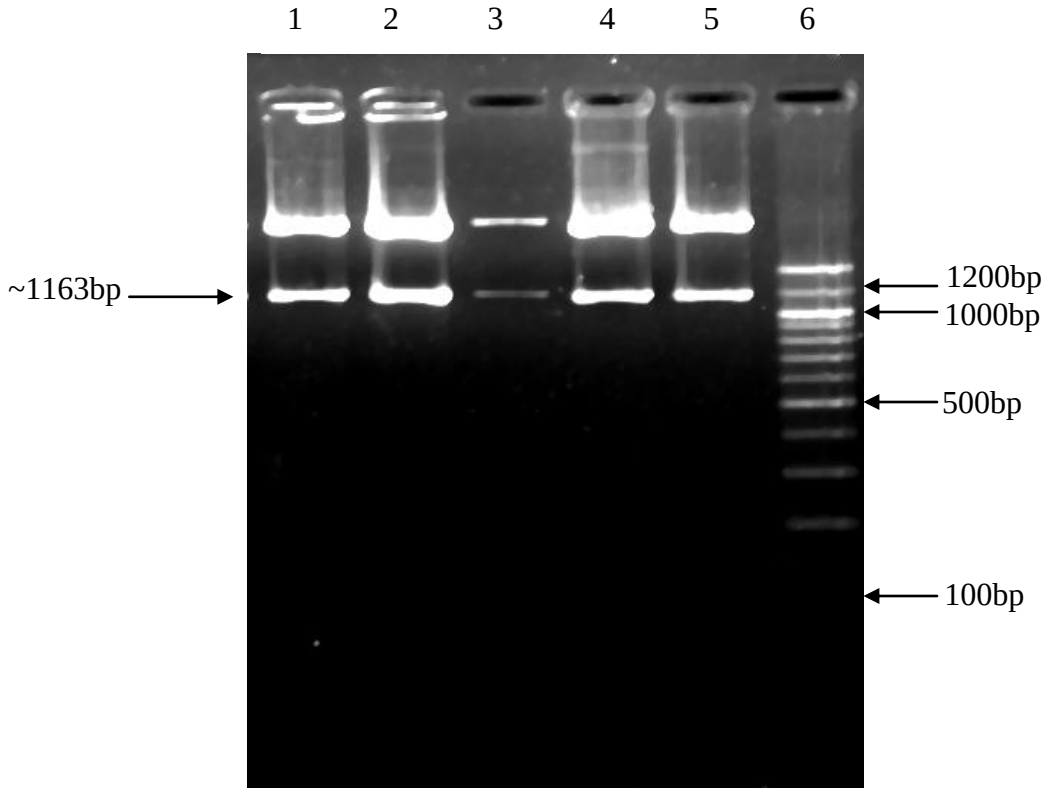


Figure 3.9: Figure shows restriction enzyme digestion result of *Aphelenchus* spp.

Lane 1: Aph3a1

Lane 2: Aph3a2

Lane 3: Aph3b2

Lane 4: Aph3b4

Lane 5: Aph3b5

Lane 6: 100bp DNA ladder (Seegene, USA)

3.5: Samples send to sequencing

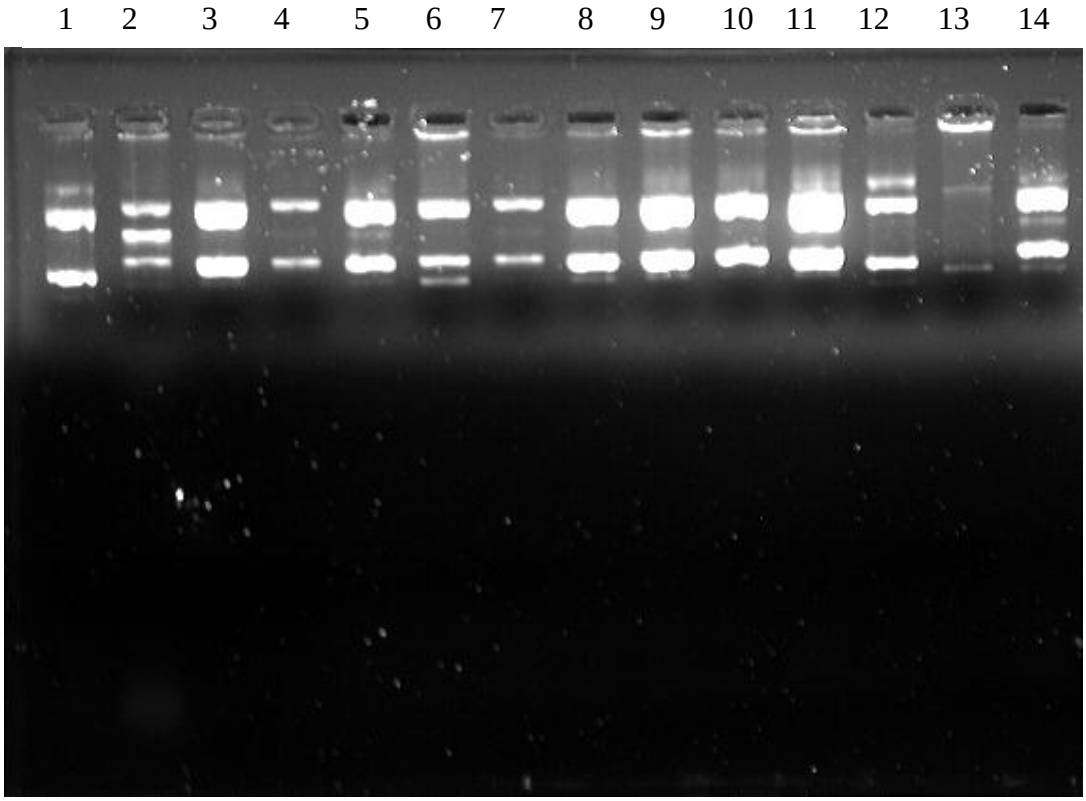


Figure 3.10: Figure shows the samples send to sequencing. The samples that have the correct inserts after restriction enzyme digestion were diluted and sequenced.

Lane 1-8: Pra8c3, Pra8f3, Pra8c5, Pra8b4, Pra8e2, Pra8c2, Pra8f2, Pra8a1 respectively (*Pratylenchus* spp.)

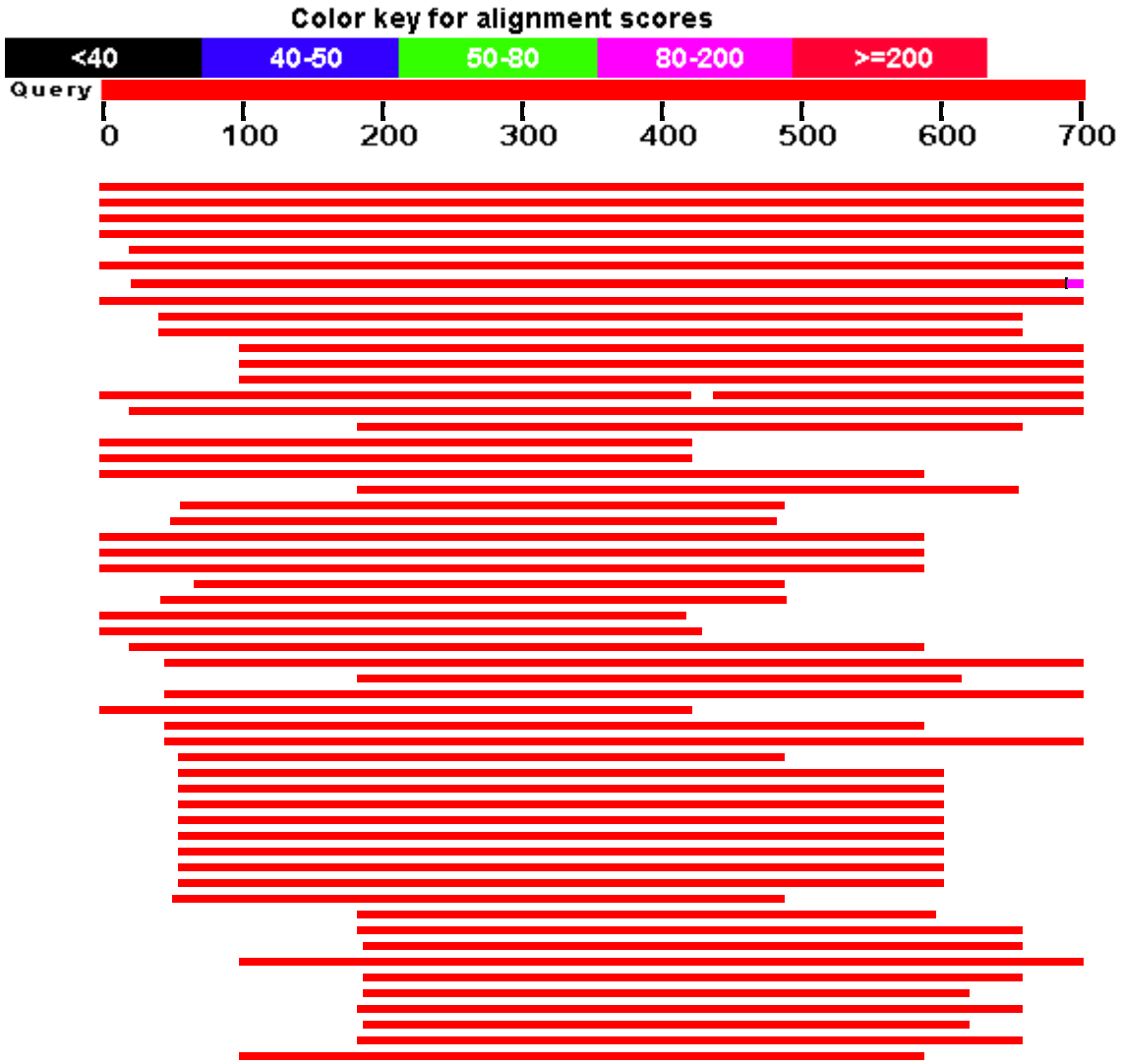
Lane 9 and 10: C4m1d4, C4m1c6 respectively (*Meloidogyne* spp.)

Lane 11-14: C4m2a2, C4m2a1, C4m2b3, C4m2a5 respectively (*Meloidogyne* spp.)

3.6: Sequence homology result

3.6.1: Sequence homology result of clone C4m2a2 of *Meloidogyne* spp. using BLAST

Distribution of 112 Blast Hits on the Query Sequence



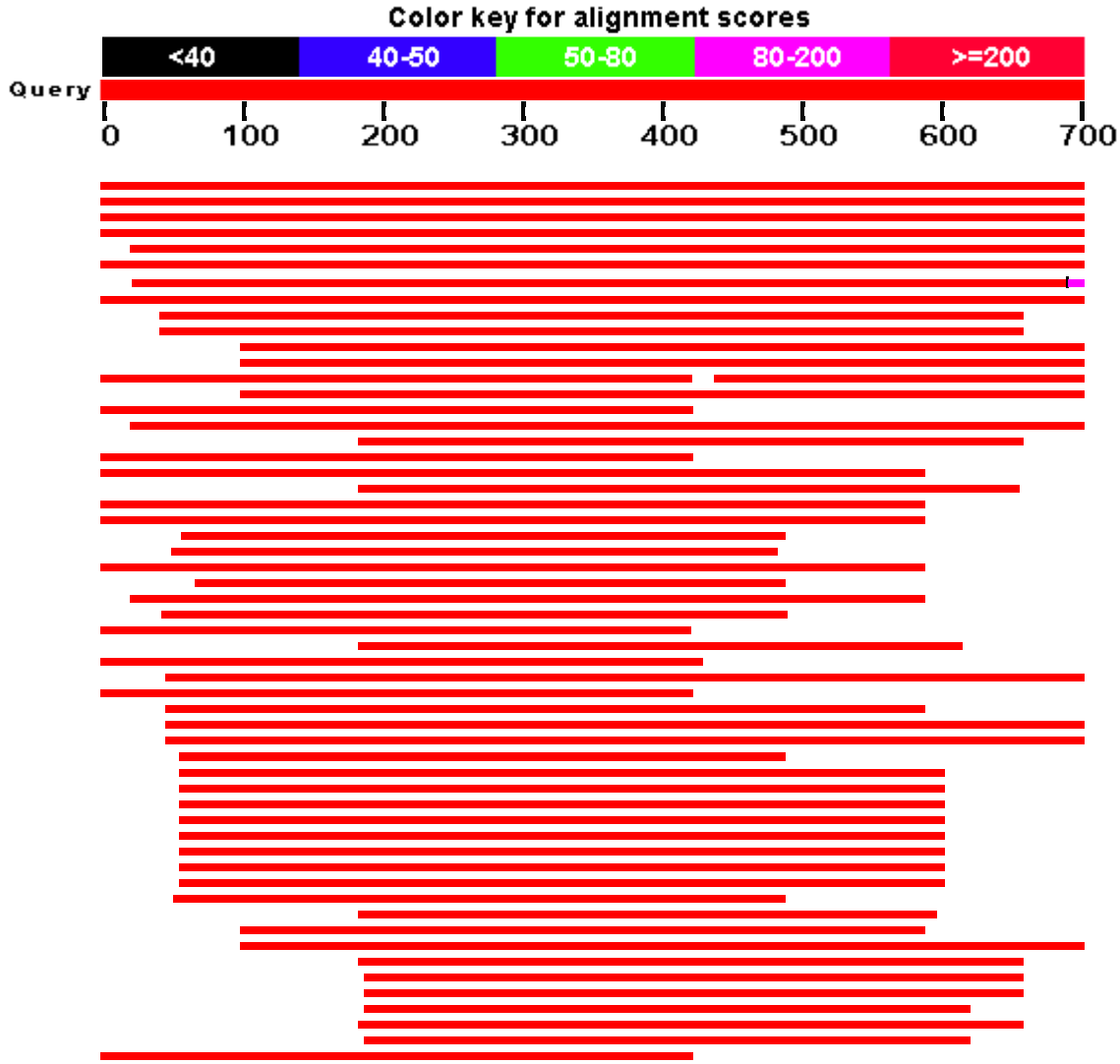
Sequences producing significant alignments:

Accession	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident
FJ534516.1	*Meloidogyne incognita* Isolate C4M2A14 18S…..	1279	1279	100%	0.0	99%

AY438556.1	*Meloidogyne incognita* T1A 18S….	953	953	100%	0.0	91%
AF516723.1	*Meloidogyne incognita* Isolate Adelaide2 18S…..	913	913	100%	0.0	90%

3.6.2: Sequence homology result of clone C4m1d4 of *Meloidogyne* spp. using BLAST

Distribution of 111 Blast Hits on the Query Sequence



Sequences producing significant alignments:

Accession	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident
FJ534516.1	*Meloidogyne incognita* Isolate C4M2A1 18S…..	1295	1295	100%	0.0	99%

AY438556.1	*Meloidogyne incognita* T1A 18S…….	953	953	100%	0.0	91%
AF516723.1	*Meloidogyne incognita* Isolate Adelaide2 18S…..	918	918	100%	0.0	90%

3.7. Phylogenetic tree

3.7.1: Phylogenetic tree of samples with band (~700bp)

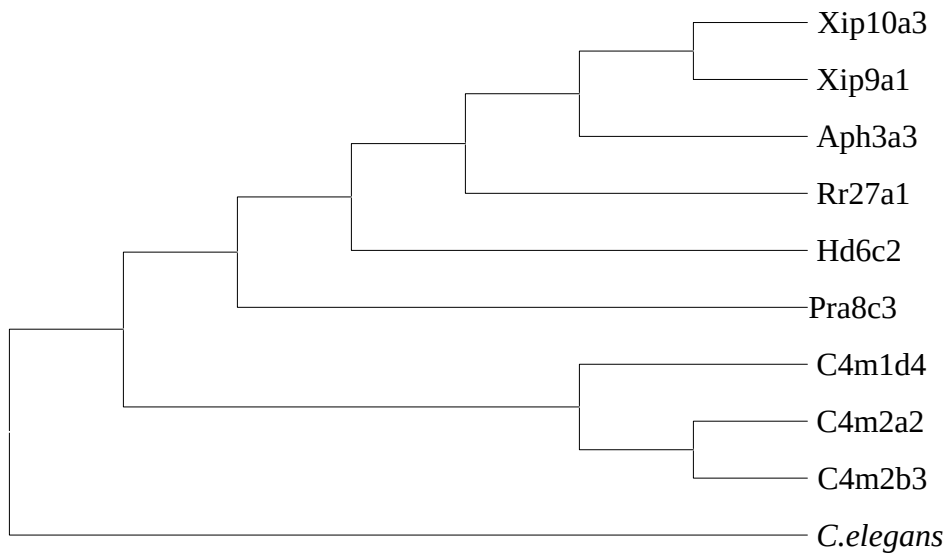


Figure 3.11: Figure shows phylogenetic tree showing the relationship between *Xiphinema* spp. (Xip9 and Xip10), *Aphelenchus* spp. (Aph3), *Rotylenchulus reniformis* (Rr27*), Helicotylenchus dihystera* (Hd6), *Pratylenchus* spp. (Pra8), *Meloidogyne* spp. (C4m1 and C4m2) with band (~700bp) and out-group (*C. elegans*).

3.7.2: Phylogenetic tree of samples with band (~1163bp)

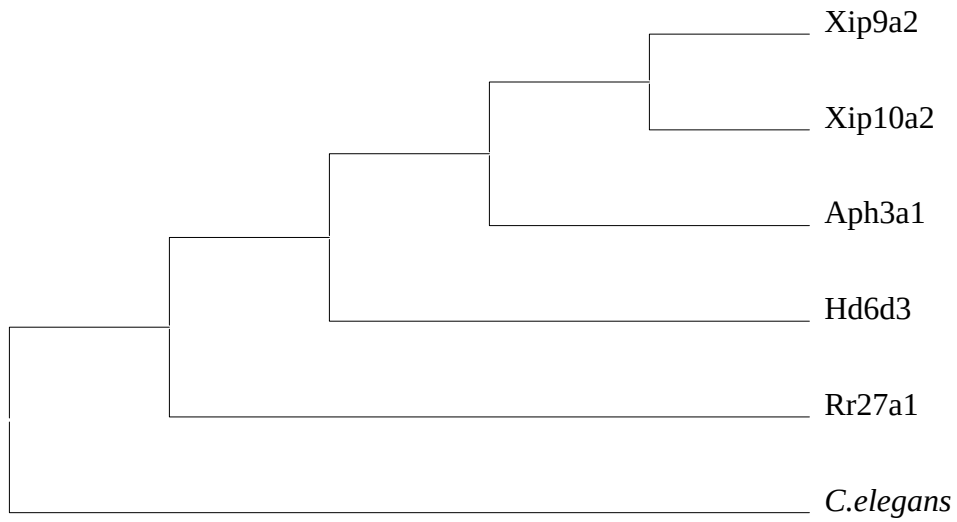


Figure 3.12: Figure shows phylogenetic tree showing the relationship between *Xiphinema* spp. (Xip9 and Xip10), *Aphelenchus* spp. (Aph3), *Helicotylenchus dihystera* (Hd6), *Rotylenchulus reniformis* (Rr27) with band (~1163bp) and out-group (*C. elegans*).