Chapter 4: Discussion

The overall objective of this mini-project is to investigate variations within specific genomic regions that may be useful for species identification purposes. Various genomic and mitochondrial regions have been used for this purpose, and in this study the ITS region of six nematode species was sequenced and compared. While the nature of the work is really straightforward, in the beginning stage the project encountered several problems in getting the desired result. This required a lot of time to be spent on optimising nematode isolation methods and DNA extraction protocols, and subsequent PCR and sequencing analysis.

4.1. Sequence analysis and Blast

Six species of plant parasitic nematode was identified based on morphology and genomic DNA of each nematode species was extracted for ITS regions amplification using Fallas-Kaplan universal primer pair. Out of these six nematode species, two species, *Meloidogyne* spp. and *Pratylenchus* spp. produced only one band of ~700bp in size. The phylogenetic tree built from these ITS region sequences showed complete clustering of the two species into their respective clades. The BLAST results for ITS sequence of *Meloidogyne* spp. showed 100% homology with *Meloidogyne incognita* while *Pratylenchus* spp. showed 81% homology with *Pratylenchus pinguicaudatus.* ITS regions of the other four species (*Xiphinema* spp., *Helicotylenchus dihystera*, *Aphelenchus* spp. and *Rotylenchulus reniformis*) were successfully amplified with Fallas-Kaplan universal primer pair and produced two amplicons of ~700bp and ~1163bp in size. Both DNA bands were

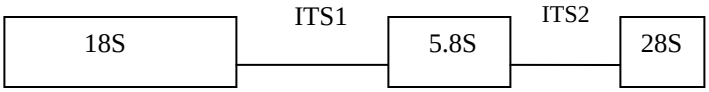
sequenced and its homology level was analysed using BLAST. For the ~700bp amplicon, each species showed high degree of homology (87-88%) with *Meloidogyne arenaria* ribosomal gene family as shown in Table 4.1.

For the ~1163bp amplicon however, there was no significant homology between the obtained ITS sequences of the four nematode species and the database reports, although all of the matching reports do show a small homology (less than 30%) with ITS sequence from other nematode species. The result shows a different length of ITS region as amplified by Fallas-Kaplan universal primer pair and that this primer pair is able to discriminate ITS variants from each other. Figures 4.1 and 4.2 show the map of the ITS regions that exist in an individual nematode.

Table 4.1. Table shows Blast sequence analysis results of *Xiphinema* spp., *Helicotylenchus dihystera*, *Aphelenchus* spp., *Rotylenchulus reniformis* indicating clone homology to *Meloidogyne arenaria.*

Samples	*Meloidogyne arenaria*
Aph3a3	Identities = 644/732 (87%), Gaps = 38/732 (5%) (gb|AY438554.1|)
Rr27a1	Identities = 644/727 (88%), Gaps = 31/727 (4%) (gb|AY438554.1|)
Hd6c2	Identities = 645/727 (88%), Gaps = 31/727 (4%) (gb|AY438554.1|)
Xip9a1	Identities = 644/727 (88%), Gaps = 31/727 (4%) (gb|AY438554.1|)
Xip10a3	Identities = 644/727 (88%), Gaps = 31/727 (4%) (gb|AY438554.1|)

(A)



(B)

	18S	ITS1	5.8S	ITS2	28S
~700bp	185	205	156	111	49
~1163bp	191	481	153	294	43

Figure 4.1: (A) Figure shows the position of 18S, ITS1, 5.8S, ITS2, 28S regions; (B) the difference in length between the two ITS variants.

4.2. ITS heterogeneity

There have been many reports on the polymorphic characteristic of the ITS regions in nematodes and it has been well established over the years. Our results showed that ITS variants could be observed between members of the same species as well as within an individual nematode. From the sequence homology analysis carried out, individual nematodes Xip9 and Xip10 of *Xiphinema* spp. and C4m1 and C4m2 of *Meloidogyne* spp. were found to have intra-specific variation in the ITS sequences. In addition, ITS sequence variant C4m2a2 and C4m2b3 obtained from a single *Meloidogyne* spp. nematode was shown to be intra-individually heterogenic. These results are similar to other reports in different species of nematodes (Mes *et al*., 2004; Nedler *et al*., 2000; Newton *et al*., 1998). The difference in length of the ITS regions may be due to some transposition events that occur between nematode species in which ribosomal gene cluster (including ITSs, 18S, 5.8S and 28S) are either cut out or amplified from two to many copies in different positions

of the nematode genome. After these events, substantial fraction of the expanded fragments is transposed producing a lot of different fragments sizes of these gene clusters. Sometimes incomplete amplification of some ribosomal gene families during the transposition of these transposon elements may affect the phylogenetic trees results (Mes *et al*., 2004; Ward *et al*., 1997). These reports might offer some explanation to the presence of amplicon variants obtained in this study. These changes together with other alterations such as endoreduplication and rearrangements of the entire genome, entire chromosome or portions of chromosomes are even linked with the increase in cell and body size in numerous taxa (Flemming *et al*., 2000; Hugall *et al*., 1999). These genomic events may be related to the variability and diversity of ribosomal cluster including ITSs (Mes *et al*., 2004), which were observed in our study. These data need to be corroborated with further experiments on other genus and species.

The other challenging issue is the level of heterogeneity in some cases of nematode ribosomal gene families. As mentioned in the results, there is a large number of different ITS variants in nematode species. However the level of variability is sometimes low (some species only have sequence differences by just one or two nucleotides). Our results are in correlation with other results that showed low level of variability in ITS variants in nematode species. For instance Newton *et al*. (1998) reported that only a single mutation was identified to distinguish *C. punctata* and *C. cuticei* from the other species of Cooperia genus. In this study, we have observed these kinds of similarities in both ~700bp and ~1163bp DNA bands.

According to Mes *et al*. (2004) in some cases, there are some nucleotide difference in the ITS sequences within a nematode species that caused some of its members to group with other species in a phylogenetics analysis. One of the explanations for this outcome is due to the error rate of PCR amplification. Fidelity of PCR amplification is strictly dependent on the number of PCR jobs (cloning and sequencing), the kind of sequencing procedure done (direct sequencing or indirect sequencing), PCR reagents used (enzyme, $MgCl_2$ or $MgSo_4$, primers) and number of PCR cycles applied especially for those fragments that are more than 1000bp in size (Caddwell and Joyce, 1994). In cloning and sequencing procedure, the *Taq* polymerase used can be considered as a potential agent for increasing error rate due to the fact that *Taq* polymerase was unable to proofread during DNA replication and having high error rate of 10^{-4}. If any error of *Taq* polymerase happens in the initial cycles of PCR, it would increase the production of ITS variants as a result of the exponential nature of PCR amplification. However this error rate is estimated to represent only 10 percent of differences, especially for those fragments that are less than 1000bp provided that all of the manufacturer's instructions to ensure the accuracy of sequencing were adhered to.

4.3. Phylogenetics analysis

Phylogenetic analysis of the ITS sequences of both ~700bp and ~1163bp showed a clear clustering of each morphologically identified nematode species into their respective clades. Phylogenetic tree constructed with ITS sequences of ~700bp illustrates a probable evolutionary relationship of each nematode species. From the tree, *Aphelenchus* spp. and *Xiphinema* spp. was observed to have the closest relationship followed by *Rotylenchulus*

reniformis and *Helicotylenchus dihystera* respectively. Another tree built based on the longer ITS variant showed similar clustering of the nematode species with the tree from the shorter variant except for *Helicotylenchus dihystera* that appeared to be more closely related to *Aphelenchus* spp. and *Xiphinema* spp. compared to *Rotylenchulus reniformis.* This indicates that even though the heterogeneity level of the ITS sequences is low, it is still sufficient to be used to differentiate the nematode at the species level.

On the other hand, in some cases that were discussed by Mes *et al*. in 2004, it was shown that some of the members of a nematode species could end up in a different clade/branch, separated from the rest of its species members. This type of results have been discussed by other researchers in trying to explain how ITS sequences that came from the same origin can be grouped into different species clusters (Nedler *et al*., 2000; Newton *et al*., 1998). The presence of different ribosomal gene cluster variants within an isolate can be associated with polyploidy. It suggests that the variants that exist within individuals can be caused by endoduplicated chromosomes that contains different copies of ITS. The reason for this heterogeneity is complicated to be determined due to lack of sufficient information on this topic. But the clear evidence has been obtained that shows polymorphic ITS variants are associated with polyploidy (Campbell *et al*., 1997). For instance, the phylogenetic tree of four different species that was explained by Mes *et al*. (2004) showed many clusters that was comprised of different species while ITS sequences that came from a single adult nematode of *Cooperia oncophora* lab strain were observed to be dispersed in different clusters in the tree.

In this study, the results obtained showed that ITS region is able to discriminate the different species of plant-parasitic nematodes that were used in this project. Perhaps other molecular markers like mitochondrial DNA or other DNA markers can be used to elucidate some morphological variability that ITS regions could not resolve. We also would suggest that more experiment on different species should be carried out to get stronger evidence on reproducibility of the genomic rearrangements and endoduplications of genes and their effects on the size and composition of ITS sequences in nematodes or other living organisms.