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BHADURY, PUNYASLOKE

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by

PUNYASLOKE BHADURY

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Punyasloke Bhadury

ABSTRACT

Free-living nematodes are abundant in all marine habitats, highly diverse and can be important ecological indicators for monitoring anthropogenic impacts on the environment. Despite such attributes, nematode diagnostics has traditionally relied on detailed comparison of morphological characters which is often difficult and laborious, and as a result there is an increasing 'black hole' in faunal inventories where the biodiversity of groups such as nematodes is typically underestimated. Molecular methods offer a potentially efficient alternative approach to studying the biodiversity of marine nematode communities, and the main focus of this thesis was to apply molecular ecological tools for improved understanding of nematode diversity in marine and estuarine environments.

Denaturing gradient gel electrophoresis (DGGE) has been evaluated as a novel tool for the identification of marine nematodes and for rapid assessment of their diversity based on amplification of the nuclear 18S rRNA gene. This approach successfully identified nematode taxa based on banding pattern and was also able to detect the most abundant taxa in samples from marine and estuarine environments.

A DNA barcoding approach based on the 18S rRNA gene was applied for the first time in marine nematology, in an attempt to speed up the identification process. The success rate of this approach, across a range of nematode groups, was found to be close to 97%.

A combined morphometrics and molecular approach was also undertaken to investigate cosmopolitanism and cryptic speciation by analysing populations of a cosmopolitan marine nematode, *Terschellingia longicaudata*, from different geographical regions. Results suggest that *Terschellingia longicaudata* is indeed truly cosmopolitan, with a wide geographic distribution. Two haplotypes that were divergent from most *T. longicaudata* were also identified in this study, indicating possible novel cryptic lineages or previously undescribed species of the genus.

The final focus of this thesis was to develop methods for the molecular investigation of nematodes stored in formalin and other organic compounds. The effectiveness of formalin as a short term preservative was first evaluated, since this would allow morphological and molecular work to be conducted on the same specimen. Amplifiable DNA could be routinely obtained from specimens stored in formalin for periods of up to nine days. In addition the effectiveness of other organic solvents for the preservation of both molecular and morphological integrity of marine nematodes was investigated. The final part of this study developed and optimized a novel DNA extraction technique that could be employed to recover DNA from archived formalin fixed marine nematode specimens so as to carry out subsequent molecular analysis such as PCR amplification and sequencing.

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ABBREVIATIONS

APS	Ammonium persulphate
bp	Base pairs
BSA	Bovine serum albumin
DGGE	Denaturing gradient gel electrophoresis
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide 5'-triphosphate
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
E.coli	*Escherichia coli*
g, mg, μg, ng	Gram, milligram, microgram, nanogram
IPTG	Isopropyl thiogalactoside
Kb	Kilobase pair
LB	Luria-Bertani Broth
L, mL, μL	Litre, millilitre, microlitre
Ltd	Limited
M, mM, μM	Molar, millimolar, micromolar
Min	Minute
mt DNA	Mitochondrial DNA
18S rRNA	Nuclear small subunit ribosomal RNA
OD	Optical density
PCR	Polymerase Chain Reaction
rATP	Rat adenosine 5'triphosphate
RCF	Relative centrifugal force
rpm	Revolutions per minute
sec	Second
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N.N',N- tetramethylethylenediamine
T. longicaudata	*Terschellingia longicaudata*
U	units
UV	Ultraviolet
v/v	Volume/volume percentage
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Oral presentation: 'Effectiveness of formalin for short term preservation of marine nematode specimens'.

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1. General Introduction

1.1 Marine nematodes: diversity and ecological importance

The phylum Nematoda is both speciose and biologically as well as ecologically diverse (Chitwood and Chitwood, 1974; Andrássy, 1976; Anderson, 1992; Malakhov, 1994; Lambshead *et al.*, 2003). Nematodes are found at the bottom of deep oceans, in terrestrial soils and inshore muds, in the frozen desert of Antarctica and are present in an incredible numerical abundance. In addition to existing as free living forms in marine and terrestrial environments, they frequently live as parasites within the bodies of plants and other animals including humans (Nickle, 1991; Anderson, 1992; Blaxter and Bird, 1997).

Despite being small and inconspicuous, free living marine nematodes are of fundamental importance in the ecology of seas and estuaries (Austen, 1986; Austen and Warwick, 1989; Coull, 1999; Austen, 2004; Lambshead, 2004). Despite their relatively conserved body plan, many groups of nematodes show appreciable morphological diversity and individual species differ according to their habitats, e.g. species found in fine sediments are short, while those in coarse sands are often either very small or very elongate (Platt and Warwick, 1980). In addition nematodes occupy very different trophic positions in sediments. Many species feed on bacteria, on algae or both; some eat detritus and possibly utilize dissolved organic matter and a considerable number are predators, feeding on other nematodes, oligochaetes, polychaetes, etc. This diversity in feeding is reflected in species diversity, indeed the number of nematode species in most marine habitats is thought to be much higher than that of any other metazoan group (Lambshead and Boucher, 2003; Lambshead, 2004). Therefore nematode abundance in both marine and terrestrial domains is surprisingly high.

1.1.1 Species richness

As a phylum, the Nematoda is highly speciose and possibly hyperdiverse (species richness in excess of 1 million). The deep ocean in particular has been recognised as a potentially hyperdiverse environment. This presumption is based on a series of datasets indicating high local diversity in deep sea sediments, the generality of which is unclear (Grassle and Maciolek, 1992; Groombridge, 1992; Lambshead, 1993; Boucher and Lambshead, 1995; Heywood, 1995). Nematode abundance in marine environments is undoubtedly high and could be in the region of 10^5 to 10^8 animals per square meter. According to Cook *et al.* (2000) nematode abundance in marine sediments declines with depth and distance from continents and is related to food supply and local productivity. Therefore, the highest nematode abundance is generally observed in marshes and marine mud around the coastal regions (Alongi, 1987; Boucher and Clavier, 1990). The pattern of declining abundance with productivity suggests a null hypothesis where species richness and ecological diversity mirrors the basic abundance pattern.

Measuring nematode species richness is often problematic, especially where the region to be assessed is very large, has no biogeographical boundaries and is environmentally diverse. This has therefore limited marine nematode biodiversity research, and the taxonomy of the group is relatively immature in that few of the extant species appear to have been described. In addition, descriptions tend to be clustered in particular biogeographical regions and concentrated in easily sampled habitats such as the littoral. Estimation of global nematode diversity is therefore a difficult task. Nevertheless, Lambshead (2004) has suggested three ways to estimate global diversity based on local sampling. These are (i) by extrapolation methods from known regions to cover unknown regions (May, 1998), (ii) calculation based on total global nematode abundance and (iii) estimating global species richness by generating species accumulation curves along a transect. One of the major problems with the first approach is that marine nematodes from offshore habitats are relatively understudied in any part of the world. For example, British

waters are amongst the most well-known in the world, but only 450 species have been recorded and described so far. This accounts for about 10% of the global described fauna, but is probably an underestimate because many offshore habitats have been under sampled even in the UK. Indeed even systematic surveys of European inshore waters have revealed that 30-40% of species recorded are new to science {e.g. Boucher (1980a) in sublittoral sands in Brittany, Lambshead (1986) in Clyde sandy beaches}, making extrapolations to other regions problematic. In the second method global nematode species richness is estimated by working backwards from total global nematode abundance but this relies on assumptions about the number of individuals per species in nematodes, which are fraught with difficulties (Lambshead, 2004).

Accumulation curves are the most widely used method for estimating global species richness in many organismal groups including rain forest insects (Erwin 1982, 1988) and deep-sea benthic macrofauna (Grassle and Maciolek, 1992). Based on species accumulation with distance, global species richness of marine nematodes has been estimated from a regional data set which covered more than 3000 km of abyssal plain in the north-central equatorial Pacific (Brown, 1998). From these data, the global richness of marine nematodes was estimated to be in the order of 10^7 species (Lambshead, 2004), which was very close to the estimates of Erwin (1988) and Grassle and Maciolek (1992) for tropical rain forest canopy fauna and bathyal deep-sea macrofauna respectively. The curve showed a rapid accumulation of species and then settled down to a linear relationship between species accumulation and distance.

On the other hand, when the species curve was calculated from south to north (degrees of latitude), the species accumulation showed a concave curve with an asymptotic pattern and the calculated global diversity was in the order of 10^5, a difference of two orders of magnitude from the previous estimate (Lambshead, 2004). The reason for this discrepancy may be due to the existence of a latitudinal gradient of organic flux to the seabed which declines from the equator northwards. It is well known that deep sea

nematode abundance and species richness tends to be positively associated with organic flux (Lambshead *et al.*, 2000) and this may be why the species accumulation curves were asymmetric in the central equatorial Pacific. It is also clear from Brown's datasets that nematodes are more speciose in northern stations in comparison to southern stations in the central equatorial Pacific.

In addition to this difference in estimates depending on how species accumulation curves are assembled, datasets such as Brown's have other limitations which are relevant here such as small number of samples, the immature nature of nematode taxonomy (difficult to spot how many species there really are) etc. This study showed for the first time the problem of estimating global diversity from a regional dataset, and some of the drawbacks associated with the estimations taking into consideration such as physical disturbances and flaws in sampling. The species accumulation method is also based on assumptions and therefore the estimations are somehow debatable.

Grassle and Morse-Porteus (1992) have suggested that the high diversity observed in deep sea nematodes forms a small-scale spatio-temporal mosaic, where similar patches with similar species are repeated over large areas, resulting in modest global diversity. As a result, although alpha diversity may be high, beta and gamma diversity may actually be relatively modest (Whittaker, 1970). This hypothesis has been tested to some degree by analysing data sets from smaller, less well-sampled stations (around 1 km diameter) from the North Atlantic abyssal plains. As opposed to the central Pacific, the deep North Atlantic tends to be divided into distinct basins separated into two groups, east and west, by the mid-Atlantic Ridge. The data available include the High Energy Benthic Boundary Layer Experiment (HEBBLE) site off Newfoundland, the Porcupine Abyssal Plain (southwest of the British Isles) and the Madeira Abyssal Plain (Lambshead *et al.*, 2000). While the HEBBLE and Porcupine datasets fit the model the Madeira datasets did not. Because the Madeira site was located in the area of a disturbance caused by turbidity, the

estimated regional species richness, like alpha diversity, appears to be reduced by the impact of their physical disturbance (Lambshead *et al.*, 2001).

The only suitable bathyal data set available for the analysis of regional diversity is from the San Diego Trough (Lambshead *et al.*, 1994). These data produced the lowest estimated regional species richness for all the deep sea data despite having high alpha diversity (Boucher and Lambshead, 1995). This region is, however, apparently anomalous in having unusually low nematode abundance.

Nematode diversity is undoubtedly best understood in coastal regions, and here attempts have been made to determine where species richness is concentrated. Comparing habitats within coastal regions suggests that nematode alpha diversity is significantly lower in intertidal and estuarine stations as opposed to abyssal and bathyal depths where nematode ecological diversity is at its peak (Boucher and Lambshead, 1995). In intertidal sites estimated nematode species richness is comparatively lower than offshore habitats. For example, lowest species richness have been reported in an intertidal mud transect at La Rochelle (J Rzeznik *et al.*, unpublished) and an intertidal mangrove site in Guyana (Ragot *et al.*, 1999) (49 and 76 species respectively). The Clyde sandy beach data (Lambshead, 1986) showed a low species richness in comparison to offshore sites but still relatively high (133) considering that the datasets were collected from a single habitat and the nematode assemblages were exposed to wave action. Relatively high species richness (150-327) has been observed in British estuarine intertidal samples (TJ Ferrero, NJ Mitchell and PJD Lambshead unpublished). However this probably results from the sharp ecological gradient present in such sites and low alpha diversity is typically observed at individual estuarine stations (Boucher and Lambshead, 1995).

The relatively low species richness observed in intertidal compared to deeper-water stations may be a consequence of the nature of the habitat. Physical disturbances such as wave action tend to dominate the intertidal, and it may be that such processes reduce the importance of small-scale patchiness and so lead to relatively low local and regional

species richness (Lambshead and Boucher, 2003). Lambshead (2004) has therefore speculated that fragmentation of intertidal habitats may be instrumental in low species richness in these habitats.

For coastal offshore habitats nematode species richness has been found to be similar to abyssal sites. For example, in the Irish Sea, regional species richness (221) is similar to that seen in abyssal sites (100-281) (TJ Ferrero, unpublished). The highest regional species richness estimates have been recorded in shallow water regions that include a range of different biotopes. A transect study carried out from Dover to Brittany (G Boucher unpublished data) following the English Channel showed high total species richness (922) with high alpha diversity and included nematode habitats such as muddy sand in the bay of Plymouth, coarse sand off North Brittany and in the Bay de Seine, and pebbles in the Dover Straight. Boucher (1997) also found high nematode species richness (702) in the southwest New Caledonia lagoon where three different sediment types are found (Chardy *et al.*, 1988). Interestingly, there is no evidence of a latitudinal influence on estimated coastal nematode regional species richness, replicating the alpha diversity analysis of Boucher and Lambshead (1995). Species richness in Ono Reef lagoon in Fiji (138) and Moorea in Polynesia (56) was similar or sometimes lower than estimated for coastal habitats at higher latitudes. Similar regional diversity patterns with no latitudinal influence have been recorded by Kendall and Aschan (1993) and Ellingsen and Gray (2002) for Norwegian shelf macrofauna.

To conclude it seems that the biodiversity in coastal regions tends to show lower alpha diversity than the deep sea because disturbance tends to predominate over the effects of patchiness, especially in the intertidal regions and estuaries, but a high beta and gamma diversity because of the variety of closely packed, ecologically different habitats. Ellingsen and Gray (2002) also found that beta and gamma diversity was positively associated with environmental variability. To conclude, whilst deep sea nematode species richness undoubtedly seems relatively high, it is difficult to be certain whether it is actually

hyperdiverse and close-packed habitats such as coastal regions contain a higher percentage of global nematode species than some workers have suggested.

1.1.2 Importance of nematodes in marine ecosystems

Being highly speciose and abundant, nematodes play a major role in marine decomposition processes through the direct consumption of detritus, but more importantly, through grazing on, and hence increasing the productivity of, heterotrophic bacteria involved in decomposition (Yeates and Coleman, 1982; Austen, 2004). Nematodes along with other meiofauna mechanically break down detrital particles and cause them to be more susceptible to increased bacterial action (Coull, 1999). Gerlach (1978) also argued that grazing by meiofauna such as nematodes keep bacterial growth in the log phase, and, therefore, the bacteria metabolize faster. Nematodes also aid the recycling of nutrients in the marine environment (Nicholas, 1975), and form an important component of the food chain for other invertebrates and for juvenile fish and shellfish (Gaston, 1992; Austen, 2004). There are reports that nematodes dominate the gut content of bottom feeding juvenile fish (Feller and Coull, 1995; Colombini *et al.*, 1996).

1.1.3 The use of nematodes in biomonitoring

Because of their high abundance in estuarine as well as in coastal habitats, there has been an increase in awareness of the importance of nematodes along with other meiofauna in the functioning of marine systems and their potential role in monitoring anthropogenic impacts in the environment (Coull and Chandler, 1992; Somerfield *et al.*, 1995; Lampadariou *et al.*, 1997; Austen, 2004). This is primarily due to several size-related advantages of the meiobenthos over the macrobenthos. These include their high densities which permit the collection of smaller samples, and shorter generation times combined with a general lack of a planktonic phase in their life cycles, which suggest a potentially shorter response time and therefore higher sensitivity to anthropogenic disturbance (Coull

and Giere, 1988; Heip *et al.*, 1988; Moore and Bett, 1989; Giere, 1993; Warwick, 1993). Moreover meiofaunal components such as nematodes and copepods are abundant and diverse even in habitats which are subjected to natural, physical and chemical stress, and where very few, if any, macrofaunal species remain.

Numerous studies have been conducted, including microcosm and mesocosm experiments, to look at the effects of anthropogenic disturbance on free-living marine nematode communities. Most of these studies have looked into the effects of contamination caused by metals (Warwick *et al.*, 1988; Millward and Grant, 1995; Austen and McEvoy 1997a; Austen and Somerfield, 1997), anti-fouling paints or tributylin tin (Austen and McEvoy, 1997b; Schratzberger *et al.*, 2002), oil-related contamination (Boucher, 1980b; Warwick *et al.*, 1988), organic enrichment (Gee *et al.*, 1985; Olafsson, 1992; Schratzberger and Warwick, 1998), hypoxia (Modig and Olafsson, 1998) and disposal of dredgings and associated contaminants (Schratzberger *et al.*, 2000a, b). Other studies have examined the effects of anthropogenic disturbances on nematode and other meiofaunal communities in natural environments (Marcotte and Coull, 1974; Boucher 1980b; Lambshead, 1986; Sandulli and De Nicola-Giudici, 1990; Warwick *et al.*, 1990; Somerfield *et al.*, 1994; Lampadariou *et al.*, 1997). Experiments in microcosm and mesocosm with natural nematode communities have provided statistically important results regarding the impacts of anthropogenic factors (Austen, 2004). In most of these studies visible changes in the nematode communities were observed due to the impacts of anthropogenic disturbances (Austen and McEvoy, 1997a; Boyd *et al.*, 2000; Gheskiere *et al.*, 2005; Mahmoudi *et al.*, 2005). This led to the conclusion that nematodes along with other meiofauna could be exploited as an effective tool for biomonitoring.

1.1.4 Nematode morphology and taxonomy

At the microscopic level, free living marine nematodes are highly variable morphologically, and no one species can be considered as truly representative. Most adult

nematodes are elongated cylindrical worms, generally 1-2 mm in length, and are sufficiently transparent to allow their internal anatomy to be seen, considerably increasing the number of characters available for identification purposes. Of the 4,000 or so species of free living marine nematodes described, some 450 representing 154 genera have been identified from British waters based on morphological characters (Platt & Warwick, 1983). Free living marine nematodes are usually identified under a compound microscope using a combination of the following key morphological characters (Wieser, 1954; Warwick, 1973; Jensen, 1979; Coomans *et al.*, 1979; Platt 1984, 1985; Vincx, 1986):

- Buccal cavity (Figure 1.1)
- Structure of the amphids (these are specialised sensilla situated laterally on the head)
- Tail shape (Figure 1.2)
- Cuticular patterns, ornamentation, etc (Figure 1.3)

Besides these, special characters such as the presence of gubernacula (male sexual organ), precloacal supplements, structure of the oesophageal bulb, etc. are extensively used by taxonomists for the identification of individual species (Filipjev, 1918; Wieser, 1959; Gerlach, 1963; Riemann, 1966; Rao, 1969; Andrássy, 1976; Ott, 1977; Jensen, 1979; Hope, 1982; Platt and Warwick, 1983; Platt and Warwick, 1988; Castillo-Fernandez and Decraemer, 1993).

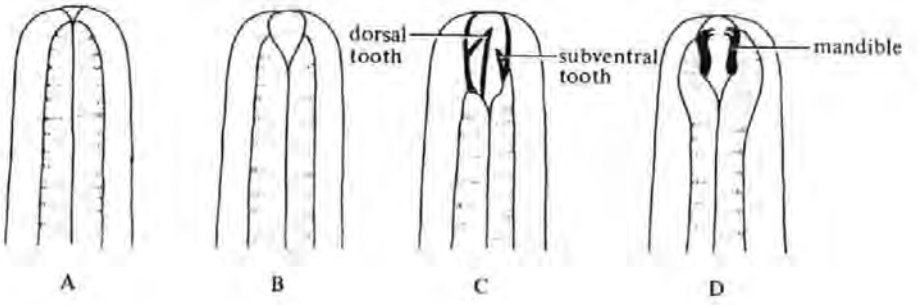


Figure 1.1: Buccal cavities of selected living marine nematodes showing a range of morphologies. A. Minute form; B. Unarmed form; C. Form with fixed teeth; D. Form with moveable mandibles (after Platt and Warwick, 1983).

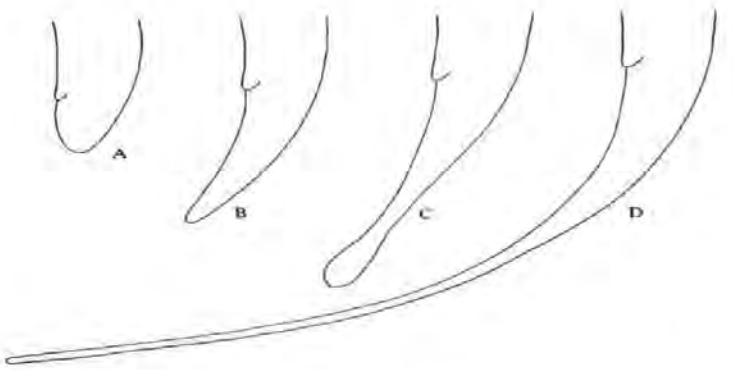


Figure 1.2: Tail shapes of selected marine nematodes. A. Short and round; B. Conical; C. Conico-cylindrical with swollen tip (clavate); D, Elongated, filiform (after Platt and Warwick, 1983).

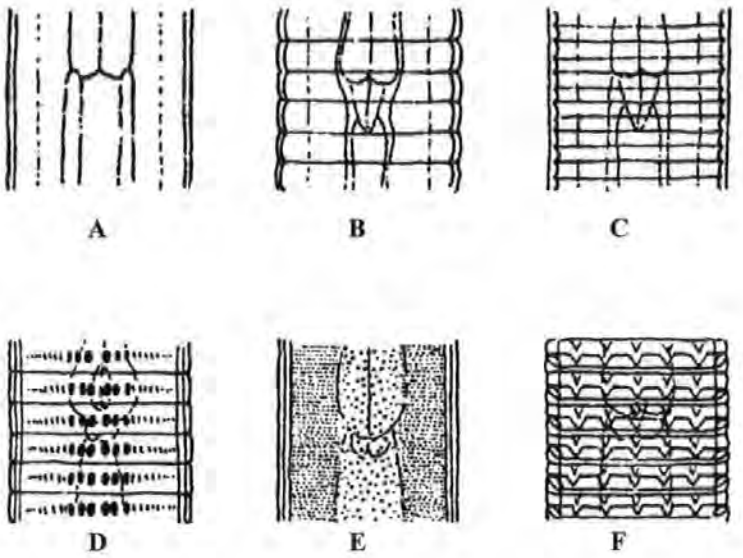


Figure 1.3: Examples of cuticle patterns in the posterior oesophageal region of selected marine nematodes. A. Smooth without dots; B. Coarsely striated, resembling annulation; C. Externally fairly smooth, striations appearing to be the result of deeper structure; D. Transverse rows of dots; E. Covered with fine dots, sometimes irregular laterally, although the surface appears smooth; F. Longitudinal rows of structures (after Platt and Warwick, 1983).

Taxonomists who identify marine nematodes by morphological means often use morphometrics in addition to the qualitative study of the above characters. Morphometric analysis is the quantification of variation in form and typically involves measuring morphological characters such as body length, body diameter, number of cephalic setae, male amphids, etc. It is a useful and important method for the differential diagnosis of genera (Platt and Warwick, 1983; Bett and Moore, 1988; Warwick and Robinson, 2000) and for differentiating between species belonging to the same genus. Because nematode identification involves observation of minute characters, it is often difficult and time consuming for taxonomists to identify them under the microscope. Only specialists in the

field of marine nematology can assign nematodes to particular genera and species, especially in the case of species complexes or assemblages of cryptic or sibling species.

1.1.5 Cosmopolitanism and species delineation among marine nematodes

Marine species are defined as cosmopolitan if they are reported from two or more oceans including connected seas (Sterrer, 1973). Cosmopolitan species have been reported from a wide array of major taxa displaying a broad range of life styles including the phylum Mollusca (Canapa *et al.*, 2003), Echinodermata (Clarke and Downey, 1992) and Porifera (Nichols and Barnes, 2005). Marine invertebrates which have pelagic larval and juvenile stages may in some cases achieve broad geographic ranges through passive dispersal in the water column (Scheltema, 1986), and in some cases genetic studies have revealed that such taxa are genuinely cosmopolitan (Westheide, 1990). On the other hand, it may be expected that meiofaunal species may have relatively restricted geographical ranges because of life-history traits such as a short life cycle, relatively small number of offspring, the general absence of a pelagic larval stage and the relatively limited swimming ability in adults (Giere, 1993; Schmidt and Westheide, 2000).

Free living marine nematodes lack a planktonic phase in their life cycle, and are thought to be dispersed by passive transport in the bedload and water column (Palmer, 1988; Armonies, 1994; Sun and Fleeger, 1994). Tidally induced vertical and horizontal displacements have been observed in nematodes by Rieger and Ott (1971) in the Adriatic Sea, and studies have shown that nematodes closest to the sediment-water interface may be most susceptible to transport (Warwick and Gee, 1984; Eskin and Palmer, 1985). There are also reports of swimming activity in some nematodes (Jensen, 1981; Palmer, 1988), although the role of such active movement in dispersal is unclear, and indeed the dispersal ability of marine nematodes is generally poorly understood, but considered to be relatively low (DePatra and Levin, 1989; Sun and Fleeger, 1994).

Despite these expectations, cases of apparently cosmopolitan taxa have been reported in a number of meiofaunal groups, including Nematoda (Gerlach, 1962), Ostracoda (Hulings, 1971), Tardigrada (Renaud-Mornant and Pollock, 1971), Gnathostomulida (Sterrer, 1973), and Gastrotricha (Hummon *et al.*, 1994). Nevertheless, it is questionable whether populations of several groups of meiofauna such as nematodes reported from the coasts of various continents actually do represent cosmopolitan species. A central point of debate over the presumed cosmopolitan distribution of meiofauna including free living marine nematodes concerns species identification. In particular, critics have questioned the reliability of species identifications from geographically distinct areas especially when made by different investigators using different methods. In fact, careful morphological analysis has shown that some species with a presumed wide geographic range are actually complexes of closely related taxa also termed sibling or cryptic species (Westheide, 1987; Specht and Westheide, 1988; Giere, 1993; Evans, 1994; Warwick and Robinson, 2000).

Sibling or cryptic species are species that are difficult or impossible to distinguish based on morphological characters (Mayr and Ashlock, 1991). Sibling species in the narrow sense often have minute morphological differences that are only noticed once species are recognised for other reasons. In some instances these morphological differences are subtle but diagnostic (Knowlton, 1993). Recent advent of molecular technologies has uncovered cryptic species in various marine invertebrate groups (reviewed in Knowlton 2000). Many of the marine nematode species which have wide geographical distributions may in fact be complexes of such sibling species. A classic example of a new sibling species of marine nematode is *Pontonema mediterranea* described by Warwick and Robinson (2000) based on morphometrics and subtle morphological characters, and cryptic species have been also detected in groups of nematode with socio-economic importance (Chilton *et al.*, 1995; Romstad *et al.*, 1998; Hung *et al.*, 1999). Defining species boundary for sibling species is a significant problem since they are thought to be widespread in the

marine environment (Knowlton, 1993; Coyne and Orr, 2004). Molecular technologies in combination with traditional taxonomy could provide detailed information about the nature of cosmopolitanism and species in marine meiofauna including free-living nematodes, and recent applications of such methods to in marine organisms have proved illuminating (Rogers *et al.*, 1995; Schmidt and Westheide, 2000; Tarjuelo *et al.*, 2001; Véliz *et al.*, 2003; Sponer and Roy, 2002; Lee and ÓFoighil, 2004; Legentil-López and Turon, 2004).

In contrast to the above, recent surveys using highly reproducible techniques (e.g. high resolution video microscopy) have suggested that cosmopolitanism appears to remain a widespread phenomenon among certain meiofaunal groups (Westheide, 1990; Schmidt and Westheide, 2000; Hummon, 1994). Such approaches have relied entirely on observable phenotypic characters, however, and do not exclude the possibility that so-called cosmopolitan species are in fact complexes of morphologically inseparable taxa.

1.1.6 Problems with nematode taxonomy

From the above discussions it is clear that nematodes are highly species rich (Lambshead, 2004) and abundant in marine environments, and these organisms, along with other benthic meiofauna, are potentially important in monitoring anthropogenic impacts. On the other hand it is obvious that nematode taxonomy which is based on careful measurements and comparison of morphological characters is often difficult and laborious and beyond the scope of most ecologists working in the field of marine science. To make matters worse, nematode species can be variable in morphology and the differences between valid species obscured by cryptic diagnostic differences (De Ley *et al.*, 1999). In addition, the global coverage of identified nematode species is highly uneven. In general, northwest European coastlines are reasonably taxonomically described with scattered information from other European coastlines and North America (Lambshead, 2004). Most of the rest of the world is effectively unknown territory. For example, 65% of nominal species collected from Clyde beaches could be named (Lambshead, 1986), but only 38%

from the Irish Sea (TJ Ferrero unpublished), 4% from the deep Norwegian Sea (Jensen, 1988) and just 1% from deep water in the Venezuela Basin (Tietjen, 1984). As a result, ecological studies and surveys of nematode diversity are usually restricted to identifications at genus level in most cases while taxonomic surveys hardly ever approach completeness in identifying all species isolated from all but a few samples.

Given these difficulties, there is a need for applying other technologies such as molecular methods in conjunction with traditional taxonomy to speed up nematode identification and to improve our ability to rapidly assess nematode diversity from marine environments. Such approaches could revolutionise the use of nematodes in biomonitoring, which is currently restricted by the availability of taxonomic expertise, and a coupling of molecular and morphological methods will also aid our understanding of cosmopolitanism and cryptic speciation in nematodes.

1.2 The molecular revolution in nematology

The advent of molecular technologies such as the polymerase chain reaction and DNA sequencing have revolutionised research in biological sciences including marine biology. In the last few years a substantial number of papers have been published in the marine biology literature dealing with issues of species diagnosis, ecology and biodiversity, biogeography and evolution using molecular markers (Lindeque *et al.*, 1999; Donald *et al.*, 2001; Sparagano *et al.*, 2002; Cook *et al.*, 2005; Dawson and Hamner, 2005; Groben and Medlin, 2005; Hackett *et al.*, 2005; Lidie *et al.*, 2005; Van Oppen *et al.*, 2005). Nematology has benefited from these technological developments and some ground-breaking studies on the molecular phylogenetics and evolution have already been published (Blaxter *et al.*, 1998; Kampfer *et al.*, 1998; Aleshin *et al.*, 1998), although such work has been concentrated on the economically important parasitic and soil-dwelling taxa, and molecular investigations of marine nematodes remain very few (Meldal, 2004; Bhadury *et al.*, 2005; Cook *et al.*, 2005; De Ley *et al.*, 2005). Molecular methods also offer

an efficient approach to studying the biodiversity of marine communities. This is true for meiofaunal communities such as marine nematodes where systematic expertise is limited, taxonomy is immature and morphological characters may be inadequate for species identification. Molecular methods may, therefore, offer ways of analysing marine meiofaunal communities including free-living nematodes. The following sections will provide a brief overview of the type of molecular markers used in nematode phylogenetic studies and how the use of molecular markers in combination with other techniques could speed up the process of marine nematode species identification and the rapid assessment of nematode diversity from marine and estuarine environments.

1.2.1 Molecular insights into species identification and diversity assessment

Accurate identification of nematodes from marine and estuarine environments has important implications in studies of systematics (taxonomy and phylogeny), and ecological diversity. Nucleic acid techniques, in particular PCR based methodologies and DNA sequencing (Sanger *et al.*, 1977; Saiki *et al.*, 1985; Mullis and Faloona, 1987) has advanced the identification of marine microbial eukaryotes and macrofauna. To date, these techniques have yet to be applied to marine meiofaunal communities such as nematodes, but have been explored in soil and parasitic nematode identification and diversity studies. The following section provides background on molecular markers that are routinely used in identification and diversity studies of eukaryotes including soil and parasitic nematodes and some of the concepts and techniques that apply.

1.2.1.1 Molecular markers for eukaryote systematics

Genomic DNA sequences evolve at different rates depending on the degree of functional constraint they are subjected to. Non-coding and non-transcribed sequences generally evolve faster than protein coding sequences. In addition mitochondrial DNA appears to evolve faster than nuclear DNA. For addressing issues of systematics and

phylogeny, a segment of DNA that has accumulated changes at a rate comparable to the phyletic events under study is the preferred choice. Generally a rapidly evolving gene is selected for analysing populations or congeneric species. For species identification and diversity studies, a segment of DNA that carries information from the past as well as from the recent history along with very little variation within members of a taxon is most appropriate.

For identification and diversity studies a wide range of molecular markers have been used in different groups of organisms. The most commonly used marker is the nuclear small subunit ribosomal RNA gene, also known as 18S rRNA or the SSU gene. This gene has been used in different groups of organisms such as protists, plankton, molluscs, annelids and polychaetes. The other gene most commonly used for molecular identification is mitochondrial cytochrome c oxidase I also known as COXI. The COXI gene has been proposed as a metazoan target for identification studies based on amplification and sequencing (Hebert *et al.*, 2003). The choice of gene depends on factors such as the presence of conserved and variable regions for primer design, copy numbers in the genome, and suitability for amplification. In addition, other genes such as rbcL and mitochondrial 16S rRNA have also been implemented in the identification of eukaryotic organisms.

In the phylum Nematoda, a number of different genes such as cytochrome c (Vanfleteren *et al.*, 1994), globin (Vanfleteren *et al.*, 1994), RNA polymerase II (Baldwin *et al.*, 1997), heat shock protein 70 (Snutch and Baillie, 1984), ribosomal RNAs and their spacer segments (Aleshin *et al.*, 1998; Blaxter *et al.*, 1998; Kampfer *et al.*, 1998; Dorris *et al.*, 1999) and mitochondrial genes (Hyman and Slater, 1990; Anderson *et al.*, 1993; Blouin *et al.*, 1997; Keddie *et al.*, 1998) have been used for phylogenetic studies. For identification of soil and parasitic nematodes the 18S rRNA gene has been most widely used, and mitochondrial COXI and nuclear 28S rRNA (also known as large subunit

ribosomal RNA or LSU) have been evaluated within some parasitic genera (Floyd *et al.*, 2002; Blaxter, 2004; Powers, 2004; De Ley *et al.*, 2005).

Ribosomal RNA (rRNA) genes are found in all organisms and retain a basic shared function. They are a vital component of the cellular ribosomes, the site of protein synthesis. There are generally four nuclear rRNA genes and two organellar (mitochondrial and chloroplast) rRNA genes. In Eukaryotes, three of the nuclear genes occur in an array starting with the external transcribed spacer region (ETS), followed by the small subunit of the rRNA (18S), the first internal transcribed spacer (ITS-1), the 5.8S gene, the second internal transcribed spacer (ITS-2) and the large subunit (28S) (Figure 1.4). The ETS and both ITS regions contain signals for processing the rRNA transcript (Hillis & Dixon, 1991). They often show intragenomic variation and are usually only used for analyses of intra-specific relationships. The small subunit (18S) is generally the one with the slowest rate of evolution and is therefore used for reconstructing deep phylogenies including the tree of life (Hillis & Dixon, 1991). The large subunit (28S) has some regions that evolve faster than the small subunit and some regions that evolve as slowly as the small subunit. The two smallest units (5S and 5.8S) have been used for phylum level analyses but are too small to be used for robust phylogenetic reconstructions (Halanych, 1991). Organellar rRNAs have higher mutation rates and are therefore used mainly for the inference of relationships of closely related taxa.

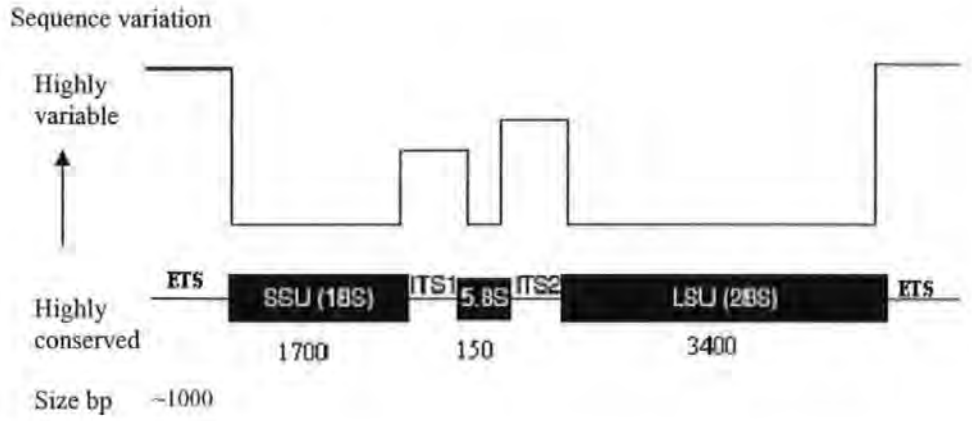


Figure 1.4: The ribosomal RNA (rRNA) cistron. Sizes are approximate and not to scale. The rRNA cistron is present in ~55 directly repeated copies per nematode genome; each cistron comprises the 18S rRNA gene, the internal transcribed spacers1 (ITS1), the 5.8S gene, ITS2 and the 28S rRNA gene. An external nontranscribed spacer (ETS) separates each transcribed cistron. Observed rate of sequence variation has been shown in the diagram and illustrates that the SSU and LSU sequences are the most conserved, followed by the ITS region. The ETS region is the most variable in length and sequence. Additionally the genes are comprised of highly conserved and variable regions (modified from Ellis *et al.* 1986).

In nematodes, they are present in multiple copies per genome and provide a large molar excess of target in polymerase chain reactions compared with single copy genes. *Caenorhabditis elegans* has about 55 sets of rRNA genes (Ellis *et al.*, 1986), and although the number present in other nematodes is not known it is likely to be similar. Each copy of the rRNA cistron is thought to be identical and the copies can be regarded as orthologous.

Nematodes have evolved and diverged over a long period of time and relatively invariant sequences are required for analysis. Therefore, the 18S rRNA gene is the preferred and most widely used marker in nematode studies. The 18S rRNA gene at around 1700 base pairs is easier to amplify and sequence and contains highly conserved domains. Moreover, sequences from this gene have been determined for a large number of soil and parasitic nematode taxa distributed across the phylum (Ellis *et al.*, 1986; Zarlenga *et al.*,

1994 a, b; Fitch *et al.*, 1995; Fitch and Thomas, 1997; Aleshin *et al.*, 1998; Blaxter *et al.*, 1998; Kampfer *et al.*, 1998; Dorris *et al.*, 1999). As a consequence the ribosomal gene has been used extensively in nematode phylogenetic studies (Aleshin *et al.*, 1998; Blaxter *et al.*, 1998; Litvaitis *et al.*, 2000) and has been evaluated for identification and diversity studies (Floyd *et al.*, 2002; Powers, 2004; Blaxter *et al.*, 2005).

1.2.2 Techniques in molecular systematics

One of the first steps towards identification and diversity assessment is the extraction of DNA from fresh, frozen or ethanol preserved specimens or from environmental samples such as sediment or water. Successful DNA extraction and subsequent molecular processes depends on the type of preservative used. For molecular studies specimens are usually preserved in ethanol but small metazoans such as nematodes shrink much faster due to the effects of ethanol preservation and this affects morphology based identification under a microscope. Such effects have also been observed in other metazoans such as polychaete worms and annelids (see Chapter 6). On the other hand specimens preserved with fixative such as formalin maintain their morphological integrity but this preservative is generally seen as compromising subsequent molecular work. Further discussion of the use of formalin-preserved material is found in Chapter 6. The next step following DNA extraction is the amplification of a genomic region based on PCR methodology. A number of approaches and methodologies commonly used in molecular systematics are discussed below, with particular reference to marine systems, and techniques used in this thesis.

1.2.2.1 Amplification and sequencing of a genomic region

Establishment of a PCR approach relies on target sequences that can be detected from information based on similar organisms, and primers conserved in sequences across a range of phyla are often employed in PCR methodology. This approach can then be used

for characterisation of nuclear or mitochondria genomic regions. Subsequently, a small fraction of the amplification product is subjected to cycle sequencing reaction (Murray, 1989), a PCR-based modification of the dideoxy method (Sanger *et al.*, 1977) and then used for sequencing analysis. DNA sequencing is a powerful tool which is widely used for the accurate identification of organisms and for phylogenetic studies. Automated DNA sequencing based on the principle of Sanger *et al.* (1977) is the most widely used method. The combination of these two methods has revealed unexpected diversity in marine microbial eukaryotes such as plankton from deep sea and ocean trenches (Díez *et al.*, 2001a; Massana *et al.*, 2002). These techniques have also been exploited for the identification of soil and parasitic nematodes and have been recently tested for the identification of marine nematodes from British coastal waters (Bhadury *et al.*, 2005; Cook *et al.*, 2005).

1.2.2.2 Molecular Barcoding

Amplification and sequencing of a genomic region have paved the way towards a new concept called molecular barcoding. Molecular barcoding is essentially an identification system which represents ways of discriminating organisms based on the analysis of a small segment of the genome and can be used for rapid species diagnosis (Hebert *et al.*, 2003).This concept has been tested for identification of metazoan organisms and has been very successful over the last few years (Hebert *et al.*, 2003; Janzen *et al.*, 2005; Smith *et al.*, 2005). The concept is discussed in detail in Chapter 4 but essentially involves three stages.

The first stage is the creation of a DNA sequence database for the taxonomic group of interest, from specimens which have been identified using taxonomic keys. The next stage involves amplification and sequencing of the same molecular marker from unidentified specimens within the phylum. Finally, generated sequences are compared with known sequences using phylogenetic approaches and sequences from unidentified

specimens are subsequently assigned to genus and species level. The other option involves bioinformatics analysis where unidentified sequences are compared within databases containing sequences from a wide range of phyla and specimens are subsequently assigned to taxa based on similarity scores. For molecular barcoding the most widely used markers are the nuclear 18S rRNA and 28S rRNA, mitochondrial 16S rRNA and COX1, and internal transcribed spacer regions (ITS1 and ITS2) from the ribosomal RNA cistron (Floyd *et al.*, 2002; Hebert *et al.*, 2003; Powers, 2004; Blaxter *et al.*, 2005). The use of molecular barcoding for nematode identification is relatively limited, but has been implemented in the identification of soil and parasitic nematodes (Duncan *et al.*, 1999; Floyd *et al.*, 2002; Blaxter, 2004; Brito *et al.*, 2004; Powers, 2004). It has also been tested recently on marine nematodes from British waters and some of the initial published results look promising (Cook *et al.*, 2005).

1.2.2.3 Approaches for the rapid assessment of species richness

For rapid studies of the diversity of taxa within a particular environment or sampling area, PCR amplification and sequencing approaches have been applied in a variety of different groups of organisms. For example, in soil nematodes 18S rRNA sequences have been used to define operational taxonomic units (OTUs) and comparisons made with sequence from known taxa to attach taxonomic and ecological attributes. A potentially rapid way of assessing diversity is to extract DNA from environmental samples and subsequently amplify a specific region of genomic DNA using phylum specific or universal primers, followed by sequencing or separation of PCR products by fingerprinting techniques. A wide variety of methods and commercial kits are available for extraction of total DNA from sediment or water samples (Waite *et al.*, 2003; Fortin *et al.*, 2004; Corinaldesi *et al.*, 2005). Diversity can then be assessed using a number of approaches. These include the generation of clone libraries followed by subsequent sequencing, fingerprinting PCR products using denaturing gradient gel electrophoresis (DGGE), and

restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (t-RFLP) studies. These have been discussed in detail in Chapter 3.

1.3 Overview of thesis aims and chapters

The main aims of this thesis are to develop and apply molecular techniques to increase our understanding of the biodiversity of marine nematodes from estuarine and coastal environments. Chapter Two of the thesis will provide information about material and methods that were used in this study. In Chapter Three, a PCR based DGGE technique has been evaluated as a rapid assessment tool for marine nematode diversity. The principal focus of Chapter Four has been to evaluate DNA barcoding whose application should speed up marine nematode identification. The suitability of both nuclear and mitochondrial genomic regions has been thoroughly explored using representative marine nematode taxa from South West England waters. In Chapter Five populations of a supposedly cosmopolitan marine nematode, *Terschellingia longicaudata*, from varied geographical locations have been investigated using both molecular and morphometric methodologies, to examine the level of intra-specific variation in this morphologically defined species, and determine whether it may in fact be a complex of cryptic species. The final section of this thesis evaluates the effectiveness of formalin and other organic preservatives for short term preservation of marine nematode specimens for molecular work without compromising morphological integrity (Chapter Six). Additionally, a novel extraction technique has been optimised and evaluated to recover DNA from archived formalin-fixed marine nematode specimens for subsequent genetic studies.

2. Materials and Methods

2.1 Materials

The following materials were used for molecular work:

Enzymes	Supplier
Accuprime *Pfx* DNA polymerase	Invitrogen UK
DNA Ligase	Promega UK Ltd
Mung bean nuclease	Promega UK Ltd
Pfu DNA polymerase	Promega UK Ltd
Restriction endonucleases	Promega UK Ltd
Taq DNA polymerase	Promega UK Ltd

Chemical	Supplier
Agarose	Promega UK Ltd
Ammonium persulfate (APS)	Sigma-Aldrich UK
Bactotryptone	Difco Laboratories
Custom oligonucleotides (primers)	MWG Biotech UK
Glycerol	Sigma-Aldrich UK
Hydrochloric Acid	Sigma-Aldrich UK
Isopropyl-thiogalactoside (IPTG)	Sigma-Aldrich UK
3-(N-Morpholino)propanesulfonic acid (MOPS)	Sigma-Aldrich UK
Rain X	Halfords (Plymouth, UK)
Sodium Hydroxide	Sigma-Aldrich UK
SYBR Gold Nucleic Acid Stain	Molecular Probes Inc
Tetramethylethylenediamine (TEMED)	Promega UK Ltd
Triton-X	Sigma-Aldrich UK
Urea	Sigma-Aldrich UK

Yeast extract	Difco Laboratories
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	Promega UK Ltd
6x loading dye	Promega UK Ltd
40% (w/v) Acrylamide/Bis-acrylamide (37.5:1)	Sigma-Aldrich UK

Other chemicals were obtained from Sigma, Promega or VWR, and were of molecular biology grade or equivalent. Reagents were stored and handled in accordance with suppliers' recommendations.

Molecular Biology Kits	**Supplier**
GenomiPhi™ Amplification kit	Amersham Biosciences
FastDNA® Kit for Soil	Qbiogene Inc
SoilMaster™ DNA Extraction kit	Epicentre Inc
Powersoil™ DNA Isolation Kit	MoBio Inc
PCR purification kit	Promega
pGEM-T Easy Vector System	Promega
Wizard Miniprep DNA purification system	Promega
Wizard® MagneSil™ GREEN	Promega

Oligonucleotides used in this study

G18F (forward primer)	5'-GCTTGTCTCAAAGATTAAGCC-3'
22R (reverse primer)	5'- GCCTGCTGCCTTCCTTGGA-3'
MN18F (forward primer)	5'-CGCGAATRGCTCATTACAACAGC-3'
Nem_18S_R (reverse primer)	5'-GGGCGGTATCTGATCGCC-3`
NEMF1 (forward primer)	5'-GTGGTGCATGGAATAATAG-3'
23R (reverse primer)	5`-TCGCTCGTTATCGGAAT-3'

23F (forward primer)	5'-ATTCCGATAACGAGCGAGA-3'
18P (reverse primer)	5'-TGATCCWKCYGCAGGTTCAC-3'
D2aF(forward primer)	5'-ACAGTACCGTGAGGGAAAGT-3'
D2aR (reverse primer)	5'-TGCGAAGGAACCAGCTACTA-3'
NC5F (forward primer)	5'-GTAGGTGAACCTGCGGAAGGATCATT-3'
NC2R (reverse primer)	5'-TTAGTTTCTTTTCCTCCGCT-3'
NC1R (reverse primer)	5'-AACAACCCTGAACCAGACGT-3'
16SF1 (forward primer)	5'-AATGGCAGTCTTAGCGTGAG-3'
16SR1 (reverse primer)	5'-AA(AT)ACAACATCGATGTAAAA-3'
MNCOXIF (forward primer)	5'- TTT TTT GGG CAT CCT GAG GTT TAT-3'
MNCOXIR (reverse primer)	5'- TAA ASA AAR AAC ATA ATG AAA ATG -3'
LCO1490F (forward primer)	5'-GGTCAACAAATCATAAAGATATTGG-3'
HCO2198R (reverse primer)	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'
CO2.105CD (forward primer)	5'-CATCAATGATACTGAAGTTATGA-3'
CO2.215R (reverse primer)	5'-CAATTGGTATAAAACTATGATTTGC-3'
mb5F (forward primer)	5'-GGCTGGCTTATTATTAAAATTAG-3'
mb9R (reverse primer)	5'-CAAAGAATAATAAAAAGATACCAA-3'

All the oligonucleotides were obtained from MWG Biotech and were of high purity salt free (HPSF) quality.

2.2 Microbiological methods

2.2.1 Media for microbiology

LB Broth (Luria Bertani)

Yeast extract 5 g L^{-1}

Bactotryptone 10 g L^{-1}

pH Adjusted to 7.0 with NaOH

For LB Agar 15 g L^{-1} of agar was added

SOB media

Yeast extract	5 g L^{-1}
Sodium Chloride (NaCl)	0.5 g L^{-1}
Bactotryptone	20 g L^{-1}

2 mL of 1 M KCl was added to a litre. pH adjusted to 7.0 with NaOH

SOC media

SOC media = SOB media +1/200 volume 2M $MgCl_2$ +1/50 volume 1 M glucose

TfB I (Transformation Buffer I)

RbCl	100 mM
$MnCl_2$	50 mM
KOAc	35 mM
$CaCl_2$	10 mM
Glycerol	15% (v/v)

pH adjusted to 5.8 with 1 M Acetic acid. The solution was finally filter sterilised

TfB II (Transformation Buffer II)

MOPS	10 mM
$CaCl_2$	75 mM
RbCl	10 mM
Glycerol	15% (v/v)

pH adjusted to 6.8 with 1 M KOH. The solution was finally filter sterilised.

All buffers and solutions were made with MilliQ water and were autoclaved or filter sterilised.

2.2.2 Handling and culturing *E. coli*

For plasmid transformations the following strain of *Escherichia coli* was used (genotype included).

XL1-Blue MRF' Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *supE*44, *thi-1*, *recA*1, *gyrA*96, *relA*1, *lac*[F'*proAB lacI*^{q}Z ΔM15 Tn10 (Tet^{R})]

E. coli were handled using standard aseptic techniques as described by Sambrook *et al.* (1989). *E. coli* cells were streaked onto LB agar plates containing ampicillin to achieve single colonies. The plates were incubated inverted overnight at 37°C. Colonies of *E. coli* were maintained (sealed with paraffin) at 4°C for short term storage. Liquid cultures were grown by inoculating a single colony in universal tubes and Erlenmeyer flasks containing LB broth. The cultures were incubated overnight at 37°C with a shaking speed of 225 rpm on an Orbital Incubator SI50 (Stuart Scientific, UK). Cell densities were measured using OD_{600} values in an Eppendorf Biophotometer.

2.2.3 Preparation of chemically competent *E. coli* cells

A single colony of *E. coli* was streaked onto a LB agar plate and incubated overnight at 37°C. A single colony from the plate was then picked and used to inoculate a 5 mL LB broth preculture, which was grown overnight in an orbital shaker at 37°C and 225 rpm. One millilitre of this culture was used to inoculate 50 mL of prewarmed (37°C) LB broth in a 500 mL Erlenmeyer flask. The flask was shaken at 37°C until the cells reached the exponential phase (OD_{600}=0.6-0.8) of growth. The 50 mL culture was again used to inoculate 250 mL LB broth which was incubated at 37°C and 225 rpm until an O.D.= 0.6-1.0 was reached. The cultures were then transferred to four Oakridge centrifuge tubes (Nalgene) and incubated on ice for 10-15 mins. Cells were pelleted at 0°C for 4 min (RCF= 2,500) in an Eppendorf refrigerated centrifuge (5810R), and the pellets washed with 25 mL of ice cold transformation buffer I (TfBI). The cells were divided into two

eppendorf tubes and centrifuged at 0°C for 4 min (RCF= 2,500). The pellets were again re-suspended in 50 mL of ice cold TfBI buffer per tube and incubated on ice for a further 30 minutes. The cells were pelleted at 0°C for 4 min (RCF= 2,500) and the pellets re-suspended in 6 mL ice cold transformation buffer II (TfBII) per tube. Finally the cells were snap frozen in 0.5 mL aliquots in liquid nitrogen and stored at -80°C for future use.

Additionally, sub-cloning efficiency™ DH5α™ *E. coli* competent cells were purchased from Invitrogen Inc for performing routine transformations and stored at -80°C prior to use.

2.2.4 Transformation of chemically competent *E. coli* cells

Chemically competent *E. coli* cells were removed from storage at -80°C and thawed on ice. The thawed cells were mixed and 100 µl aliquots placed into pre-cooled Falcon 2059 polypropylene tubes. Ligated DNA (1-3 µL) (Section 2.3.11; Section 2.3.12) was added to each aliquot and mixed gently. The mixture was left on ice for another 40 mins followed by heat shock treatment at 42°C for 45 seconds in a water bath. The mixture was again placed on ice for 2 min followed by the addition of 900 µL of SOC media (containing 10 mM $MgSO_4$). The cells were shaken (225 rpm) at 37°C for 1 hour before being spread onto LB agar plates containing ampicillin (Section 2.2.6) (in 250 µL aliquots per plate) for plasmid selection. The plates were incubated inverted overnight at 37°C (Gallenkamp Economy Incubator).

2.2.5 Transformation using Invitrogen sub-cloning efficiency™ DH5α™ competent cells

A 50 µL aliquot of chemically competent DH5α cells was thawed on ice for approximately ten minutes. DNA (2-3 µL) was added to the aliquot and stirred gently. The mixture was left on ice for another 30 minutes prior to heat shock treatment at 37°C for 20

seconds. The mixture was left on ice for a further 2 mins followed by the addition of 450 µL of LB medium. The cells were subsequently shaken for an hour at 37°C (225 rpm). Finally 50 µL of the cell suspension was spread onto LB-Ampicillin agar plates containing 15 µL of X-gal. The cell suspension was allowed to soak into the plates in a laminar flow hood before being incubated at 37°C overnight.

2.2.6 Ampicillin stock solution preparation

Ampicillin powder was dissolved in MilliQ water to a final concentration of 50 mg mL^{-1} and was subsequently filter sterilised. The stock solution was stored at -20°C. The stock was added to pre-cooled autoclaved media (temperature below 60°C) to a final concentration of 100 µg mL^{-1}.

2.2.7 Lac selection of plasmids

Luria Bertani agar plates were surface dried in a laminar flow hood and 20 µL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 2 µL of isopropyl-thiogalactoside (IPTG) were added to each plate by spreading over the surface with a sterilised glass spreader. The plates were again surface-dried before transformed *E. coli* cells were spread on the surfaces. After an overnight incubation at 37°C, the X-gal plates were incubated at 4°C to allow the development of blue colour in non-recombinants.

2.2.8 Storage of transformed *E. coli* cells

The recombinant *E. coli* cells were streaked onto LB agar plates with ampicillin selection. Plates were incubated overnight at 37°C before sealing with parafilm, and storing at 4°C for a maximum of four weeks. For long term storage a single recombinant colony was inoculated in 5 mL LB broth containing ampicillin and shaken at 37°C (225 rpm) overnight. 0.5 mL of the culture was added to a cryovial containing 0.5 mL 30%

sterile glycerol (final concentration of glycerol, 15%). The cells were frozen in liquid nitrogen and stored at -80°C.

2.3 Nucleic acid methods

2.3.1 Buffers and solutions

50x TAE Buffer (stock solution)

Tris base	242 g
Glacial Acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

Water added to a final volume of 1 Litre.

1x TAE buffer was prepared by dilution from the stock with MilliQ water for agarose gel electrophoresis and denaturing gradient gel electrophoresis.

0% denaturant (DGGE)

40% Acrylamide stock	15 mL

(acrylamide:bis-acrylamide in the ratio of 37.5:1)

TAE Buffer (50x)	2 mL

MilliQ water to make up a final volume of 100 mL

80% denaturant (DGGE)

40% Acrylamide stock	15 mL
TAE Buffer (50x)	2 mL
Urea	33.6 g
Deionised formamide	32 mL

MilliQ water to make a final volume of 100 mL

2.3.2 DNA extraction from single nematode worm

The DNA extraction method initially proposed by Floyd *et al.* (2002) has been modified for use with marine nematodes. A single nematode worm placed in a 0.5 mL microfuge tube containing 20 µL of 0.25 M sodium hydroxide (NaOH) was frozen overnight at -20°C followed by incubation at 60°C for 12 hours. The tube was then heated to 99°C for three minutes and the solution allowed to cool to room temperature before centrifugation (RCF =16,000; 30 sec). 4µL of 1M hydrochloric acid (HCl), 10µL 0.25M Tris-HCl (pH 8.0) and 5µL 2% Triton X-100 were added to the tube and its contents mixed briefly followed by heating once again to 99°C for three minutes and cooling to room temperature. The extracted DNA was used as a template for PCR amplification.

2.3.3 PCR amplification of the nuclear 18S rRNA gene from extracted DNA

PCR is the *in vitro* amplification of a segment of DNA between two regions of known sequence (Mullis *et al.*, 1986; Scharf *et al.*, 1986; Saiki *et al.*, 1988). Amplification requires the use of primers that are complementary to sequences on opposite strands of the template DNA. The major components of polymerase chain reaction are template DNA, primers (forward and reverse), deoxynucleotide triphosphates (dNTPs) and DNA polymerase enzyme. A series of steps are used for amplifying the target DNA molecule which includes (i) denaturation, where the double-stranded DNA template is separated into single strands; (ii) annealing, where the primers bind to the target sequences on the DNA template; and (iii) extension, where the DNA polymerase extends the primers and copies the DNA template.

For PCR amplification, a nested PCR approach was followed where two pairs of PCR primers were used sequentially to amplify a single locus. The first primer set was used to amplify the locus and the second primer set was used to bind within the first PCR product producing a second PCR product shorter in size than the first one. The logic behind this strategy was that if a non-specific locus were amplified in the first round of

PCR, the probability is very low that it would also be amplified a second time by a second pair of internal primers. Two primers G18F (forward primer) and 23R (reverse primer) were used for nested PCR. The second primer set used in this study were MN18F (forward primer) and Nem_18S_R (reverse primer). For the first primer set, the amplification product size was 1300 bp (approx.) whereas for the second one the product size was 930 bp (approx.). To cover the rest of the fragment two primers namely NemF1 (forward primer) and 23R (reverse primer) were used for amplification. The remaining region of the 18S rRNA gene was amplified using 23F (forward primer) and 18P (reverse primer) (Figure 2.1). The product size in this case was close to 500 bp.

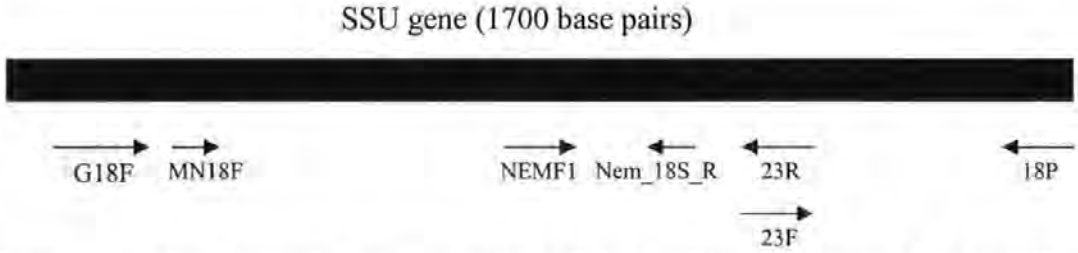


Figure 2.1: Diagrammatic representation of the position of primers for amplification of almost the entire 18S rRNA gene. Positions of the primers are approximate and not to scale.

Each of the primers was diluted to a final concentration of 10 pmol μL^{-1} from 100 pmol μL^{-1}stock. Routine PCRs were conducted with 5 µL of the extracted DNA, 5 µL 10X buffer with $MgCl_2$, 5 µL of 2mM dNTPs, 2 µL of each primer (10 pmol μL^{-1}), 0.5 µL of Taq DNA polymerase (5U/µL) and water to make a total volume of 50 µL for each sample. All samples were stored on ice prior to thermal cycling.

2.3.4 PCR amplification of the mitochondrial 16S rRNA gene from genomic DNA

A small fragment of the mitochondrial 16S rRNA gene of approximately 300 bp was amplified using a step-up PCR methodology. In step-up PCR, the starting annealing temperature is always a few degrees lower than the optimum annealing temperature to promote primer binding and is subsequently adjusted to the optimum annealing

temperature specific for amplification of the marker. Two primers namely 16SF1 (forward primer) and 16SR1 (reverse primer), were used in this study.

2.3.5 PCR amplification of the mitochondrial cytochrome oxidase I gene (COXI) from genomic DNA

A partial fragment (approximately 440 base pairs) from the mitochondrial cytochrome oxidase I gene was amplified using a step-up PCR methodology. Two primers, namely MNCOXIF (forward primer) and MNCOXIR (reverse primer), were used in this study.

2.3.6 Thermal cycler operation

PCR amplifications were carried out in a PTC-100 progammable thermal cycler (MJ Research Inc). The PCR reactions consisted of the initial denaturation at 95°C for 5 min, followed by certain number of cycles of denaturation, annealing and extension and a final extension at 72°C for 5 mins followed by a holding temperature of 4°C. Annealing temperature used for each primer set is shown in Table 2.1. The annealing temperatures used for polymerase chain reaction mediated amplification throughout the study were optimised initially using a gradient block thermocycler (MJ Research PTC-200). Thermal cycle parameters used for denaturing gradient gel electrophoresis are discussed in the next chapter (Section 3.2.4).

Table 2.1: Annealing temperatures used for PCR amplification of specific regions in the nematode genome.

Primers	Genomic region	Thermal cycle parameters
G18F and 23R	18S rRNA	95°C-1 min 51°C-1 min 72°C-2 min — 30 cycles
MN18F and Nem_18S_R	18S rRNA	95°C-1 min 54°C-1 min 72°C-2 min — 37 cycles
NEMF1 and 23R	18S rRNA	95°C-1 min 50°C-1 min 72°C-1 min — 37 cycles
23F and 18P	18S rRNA	95°C-1 min 55°C-1 min 72°C-1 min — 37 cycles
16SF1 and 16SRev	mitochondrial 16S rRNA	94°C-30 sec 42°C-30 sec 72°C-30 sec — 10 cycles 95°C-45 sec 45°C-1 min 72°C-90 sec — 34 cycles
MNCOXIF and MNCOXIR	mitochondrial COXI gene	94°C-30 sec 42°C-30 sec 72°C-30 sec — 10 cycles 95°C-45 sec 45°C-45 sec 72°C-90 sec — 34 cycles

2.3.7 Electrophoresis of DNA

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying DNA fragments. The protocol can be divided into three stages: (1) a gel is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated; (2) the DNA samples are loaded into the sample wells and the gel electrophoresed at a voltage and for a time period that will achieve optimal separation; and (3) the gel is stained or, if ethidium bromide has been incorporated into the gel, visualized directly upon illumination with UV light (Sambrook *et al.*, 1989).

The yield of the PCR products was determined by agarose gel electrophoresis using the Mini sub cell GT system (Bio-rad Laboratories). 5 μL of each PCR product was mixed with 2 μL of 6x loading dye and loaded onto the gel. Electrophoresis was performed for 30 minutes by supplying a steady current of 120 volts. To estimate the size of the DNA fragments, samples were co-migrated with appropriate DNA markers. The following DNA markers (with their sizes) were used:

λ Hind III: 23.1 Kb, 9.4 Kb, 6.5 Kb, 4.3 Kb, 2.3 Kb, 2.0 Kb and 0.5 Kb (Sanger *et al.*, 1982)

Bp ladder: 100 bp-1000 bp in 100 bp increments (Promega, UK)

After the completion of electrophoresis the gel was removed and examined under a UV transilluminator (U.V.P. Inc). Photographs were taken using SYNGENE Gel Documentation System which was attached to a thermal printer.

2.3.8 DNA recovery from agarose gels

PCR products were recovered from agarose gels using a Qiaex gel extraction kit (Qiagen) according to manufacturer's instructions. The final elutions were performed in 20 μL MilliQ water.

2.3.9 Enzymatic digestion of the vector pBluescript SK⁻ for molecular cloning

The plasmid pBluescript SK⁻ was used for cloning. Plasmids were prepared for ligation by digestion with a restriction enzyme for which there is a single restriction site in the polylinker. The restriction enzyme *Eco*RV was used to linearise the plasmid in order to create blunt termini. The following components were added to a 0.5 mL microcentrifuge tube and incubated for 1 hr at 37°C:-

pBluescript SK⁻ (360 ng/μL)	3 μL
10x reaction buffer	1 μL
*Eco*RV	1 μL
MilliQ water	5 μL

The digested plasmid was examined by electrophoresis through a 1% agarose gel for 30 min at 120 volts and observed by transillumination.

2.3.10 Creating blunt termini in DNA

The following components were added to the purified PCR products (recovered from the agarose gel) (Section 2.3.8) in a 0.5 mL micro centrifuge and incubated at 72°C for 30 min to create blunt termini for ligation with pBluescript SK⁻

Purified PCR product	10 μL
2 mM dNTPs	1 μL
10x *Pfu* reaction buffer	1 μL
Pfu DNA polymerase	1 μL

The polished PCR product was used for ligation or stored at 4°C until further use.

2.3.11 Ligation of blunt termini PCR product into digested pBluescript SK⁻

Prior to ligation, the PCR products were tested to see if they contained digestion sites for *Eco*RV. In the absence of such sites the fragments were ligated into the

corresponding digested plasmids i.e., if no *Eco*RV site was found in PCR products the fragments would be cloned into EcoRV digested plasmids. This enabled EcoRV to be added to the ligation reaction to prevent self-ligation of the plasmids. No digestion site was found in any individual PCR products used in this study. The following components were then assembled:

Digested pBluescript SK$^-$	1 μL
10x Ligase reaction buffer	1 μL
10 mM rATP	0.5 μL
PCR product (blunt termini)	5.5 μL
*Eco*RV	1 μL
T4 DNA Ligase	1 μL

Each ligation reaction was incubated for 2-3 hours at room temperature or 15°C overnight. The ligation reactions were then used for transformation or stored at 4°C for future use.

2.3.12 Ligation of PCR products into pGEM-T vector system

In addition to pBluescript SK$^-$, pGEM-T vector (Promega^{TM}) was also used for cloning purposes. The vector is supplied pre-cut with EcoRV and with an additional terminal 3' thymidine on both strands (Figure 2.2). As a result the efficiency of ligations of PCR product into the vector improves through prevention of recircularisation. Additionally, the use of *Taq* DNA polymerase enhances the addition of a terminal adenosine at the 3' end of the PCR product thus making it more compatible with the vector.

M13F T7 promoter

5'---TG TAAAA CGACG GCCAG TGAAT TGTAA TACGA CTCAC TATAG GGCGAATTGG

AC ATTTT GCTGC CGGTC ACTTA ACATT ATGCT GAGTG ATATC CCGCT TAACC

GCCCG ACGTC GCATG CTCCC GGCCG CCATG GCGGC CGCGG GAATT CGAT-T Cloned

CGGGC TGCAG CGTAC GAGGG CCGGC GGTAC CGCCG GCGCC CTTAA GCTA

Insert ATCAC TAGTG AATTC GCGGC CGCCT GCAGG TCGAC CATAT GGGA GAGCT

TAGTG ATCAC TTAAG CGCCG GCGGA CGTCC AGCTG GTATA CCCT CTCGA

CCCAA CGCGT TGGAT GCATA GCTTG AGTATTCTAT AGTGT CACCT AAATA GCTTG GCGTA

GGGTT GCGCA ACCTA CGTAT CGAAC TCATAAGATA TCACA GTGGA TTTAT CGAAC CGCAT

ATCAT GGTCA TAGCT GTTTC C.....3'

TAGTA CCAGT ATCGA CAAAG G

M13R

Figure 2.2: Promoter and multiple cloning sequence site of the pGEM-T vector system.

Ligations were prepared using the pGEM-T vector system according to the manufacturer's instructions. The following components were added in a 0.5 mL tube:

2x Rapid Ligation Buffer	5 μL
pGEM-T	1 μL
PCR product	x μL
T4 DNA Ligase (3U/μL)	1 μL

De-ionised water to make a final volume of 10 μL

Reactions were incubated at room temperature for one hour or overnight at 4°C prior to transformation.

2.3.13 Identifying recombinant clones by colony PCR

Recombinant 18S rRNA clones were identified by a blue/white system of Lac selection on X-gal LB agar plates. The clones containing inserts were further identified by

amplification of the inserted DNA using the primers complementary to regions approximately 300 bp outside the pBluescript SK$^-$ polylinker. The primers used were BS1F forward primer (5'-AAA GGG GGA TGT GCT GCA AGG CG-3') and BS1R reverse primer (5'-GCT TCC GGC TCG TAT TGT GTG-3'). Individual colonies were picked from the transformation plates using sterile loops. Each colony was re-suspended in a 0.5 mL microcentrifuge tube containing 10 µL of MilliQ water. The same loop was used for patching out on LB ampicillin agar plate.

Colonies were propagated overnight at 37°C and subsequently stored at 4°C after the plates were wrapped in parafilm. Colonies resuspended in MilliQ water were boiled for 4 mins and placed on ice. For amplification the following components were added to 10 µL of individual colony suspension to make a final volume of 20 µL:

2 mM dNTPs	2 µL
10x buffer w/$MgCl_2$	2 µL
BS1F primer (100 ng μL^{-1})	0.5 µL
BS1R primer (100 ng μL^{-1})	0.5 µL
Taq DNA polymerase	0.2 µL
MilliQ water	4.8 µL

A positive control of unmodified pBluescript SK$^-$ plasmid and a negative control (no template) to detect any level of contamination were also used in the colony PCR. The thermal cycler parameters used for the colony PCR were 5 min at 94°C for initial denaturation, followed by 25 cycles at 68°C (1 min), 72°C (1 min) and 94°C (1 min). The final annealing phase of 2 min at 68°C was followed by an extension phase for 2 min at 72°C which completed the PCR followed by a holding temperature at 4°C until further use. Each of the colony PCR products (2 µL) was analysed by 1% agarose gel electrophoresis using a 100bp ladder. Plasmids containing the 18S rRNA gene inserts were identified by the increase in size of the amplification products compared to that of control plasmid.

For the pGEM-T Vector system, successful transformations were also identified by colony PCR as described above except that two different primers, namely M13F (5'-TGT AAA ACG ACG GCC AGT-3') and M13R (5'-GGA AAC AGC TAT GAC CAT G-3'), were used for amplification. An annealing temperature of 57°C was used in this case.

2.3.14 Recovery of recombinant plasmid from *E. coli*

2.3.14.1 Promega Wizard Miniprep DNA purification system

Colonies with the inserts which were grown overnight on LB ampicillin plates were used to inoculate 5 mL of LB broth containing ampicillin in sterile universal tubes (30 mL). The cultures were incubated at 37°C with a shaking speed of 225 rpm for 12-15 hours. The cultures were harvested in 1.5 mL microfuge tubes by centrifugation for 5 min (RCF=10,000). The supernatant from each culture was poured off and the tubes blotted on paper towels. Cell resuspension solution (250 µL) was added to each tube to completely resuspend the pellets followed by the addition of 250 µL of cell lysis solution. The tubes were mixed well by inverting each individual tube four times, and tubes were then left to incubate at room temperature for approximately 5 min. Alkaline protease solution (10 µL) was added to each tube and mixed by inversion another four times and tubes were left to incubate at room temperature for 5 min. Wizard Plus SV Neutralization Solution (350 µL) was then added to each tube and mixed immediately by inverting four times. Soon after, the bacterial lysates were centrifuged (RCF=17,000) in a microcentrifuge for 10 min at room temperature. Each lysate was subsequently transferred to the spin column by decantation. Individual spin columns were centrifuged for 1 minute (RCF=17,000). The eluate from the collection tube in the spin column was discarded. Column wash solution (750 µL) was then added to each column and centrifuged for 1 minute at maximum speed. The eluate was again discarded. The process was repeated once more by adding 250 µL of column wash solution in each column. Each column was re-centrifuged for 2 minutes

(RCF=17,000) at room temperature. After completion of centrifugation the columns were transferred to new 1.5 mL microfuge tubes. The elute in the collection tubes was discarded once more. Plasmid DNA in each column was finally eluted by addition of 100 µL of nuclease-free water. The columns were once again centrifuged (RCF=17,000) for 1 minute. Eluted plasmid DNA was stored at -20°C for future use. Prior to DNA sequencing, the concentration of the plasmids was measured in a Biophotometer. The DNA concentration for each sample was calculated from the O.D. value at 260 nm (1 O.D. at 260 nm for double-stranded DNA = 50 ng/mL of dsDNA).

2.3.15 DNA sequencing

To investigate whether the inserts in the plasmid DNA were nematode 18S rRNA, eluted plasmids were subjected to DNA sequencing. The most commonly used sequencing method is the Sanger dideoxy method (Sanger *et al.*, 1977; Avise, 1994; Ferraris and Palumbi, 1996; Hillis *et al.*, 1996). The Sanger method is the basis of automated DNA sequencing and was carried out using both a single capillary sequencer (Applied Biosystems Prism 310) and multi-capillary sequencer (Applied Biosystems Hitachi 3100).

2.3.15.1 Cycle sequencing reaction

Plasmids containing 18S rRNA inserts were prepared for cycle sequencing using the BigDye v2.0 Cycle Sequencing Kit (ABI Ltd) in 200 µL polypropylene PCR tubes by the addition of the following components:

(i) Template DNA (150-300 ng) – 1 µL

(ii) Ready Reaction Mix (supplied by ABI) – 2 µL

(iii) 2.5 × Sequencing Buffer – 3 µL (contains 200 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$)

(iii) Primer (T7 or T3) - 2 µL (3.2 pmol/µL concentration); M13F or M13R in case of pGEM-T.

(iv)Water- to a final volume of 20 µL

The contents of the tubes were briefly centrifuged, and placed in the PTC-100 (MJ Research Inc) thermal cycler to commence the reactions. The thermal cycler was programmed to cycle through the following parameters:

96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min and finally a holding temperature of 4°C.

After the completion of cycle sequencing, templates were prepared for sequence analysis.

2.3.15.2 Cycle Sequencing clean-up for DNA Sequencing (ABI Prism 310)

Removal of unincorporated dye terminators is an important step for successful sequencing. To each 20 µL of cycle sequencing reaction product, 5 µL of 125 mM EDTA and 60 µL of absolute alcohol was added and was mixed well by inverting the tube four times. The tubes were left to incubate at room temperature for 20 min to allow the reaction products to precipitate. After precipitation, tubes were centrifuged (RCF=16,000) for 20 min at 4°C in a refrigerated centrifuge (Eppendorf 5810R). A note of the orientation of the tubes was made to identify the deposition of the product on the tubes. All supernatant from the tubes was discarded and 70 µL of 70% ethanol was added to each tube to wash the pellet. Once again the tubes were centrifuged for 15 min (RCF=16,000) at 4°C. The supernatant from each tube was removed and the pellet dried in a thermal cycler at 95°C (lid open). After drying, 20 µl of template suppression reagent was added to each tube and vortexed. The tubes were heated for 2 min at 95°C. The tubes were kept on ice until required for sequence analysis.

2.3.15.3 Cycle Sequencing clean-up for DNA Sequencing (ABI Hitachi 3100)

Unincorporated dyes were removed using Wizard® MagneSil™ GREEN (Promega Inc) according to manufacturer's instructions. To each 20 µL cycle sequencing reaction product, 180 µL of the Magnesil green particles were added followed by resuspension of

the particles with vigorous shaking. Each reaction was left to incubate at room temperature for 5 minutes. The reaction was mixed by pipetting at 0, 2.5 and 5 minutes. The polypropylene tube was later placed onto the MagnaBot II magnetic separation device to capture the green particles. The liquid portion was removed and discarded. Care was taken to avoid removing the green particles. Each tube was removed from the MagnaBot device and placed on a stand. One hundred μL of 90% ethanol was added to each sample and left to incubate for 5 minutes. Each sample was mixed thoroughly by pipetting at 0, 2.5 and 5 minutes. Once again each tube was placed on the MagnaBot device. The liquid was removed and discarded. The steps were continued for a total of two washes. After the completion of the wash the particles were allowed to air dry for approximately 10 minutes at room temperature. Hi-dye™ formamide (20 μL) was added to the particles and incubated at room temperature for 1-2 minutes. Again the tubes were placed on MagnaBot device to capture the particles. The purified sequencing reactions were transferred to a clean tube for automated DNA sequencing.

2.3.15.4 Sequencing analysis

Samples were analysed using an ABI Prism 310 Genetic Analyzer. Experimental conditions were as follows: Electrophoresis voltage 12 kV, Electrophoresis current 4.0 μA, Laser power 9.9 mW, Gel temperature 50°C, Injection time 60 sec, Run time 150 min. In the case of the ABI Hitachi 3100 Genetic Analyzer following conditions were implemented for sequencing: Electrophoresis voltage 12.2 kV, Electrophoresis current 68.0 μA, Laser power 15.0 mW, Laser current 4.7 A, Oven temperature 50°C, Run time 147 mins (for 16 samples). Sequencing in each case was carried out in both directions. Sequences were checked for quality using the Chromas Pro software. Ambiguous sequences were re-sequenced to resolve ambiguous bases. The results of the sequences were then compared with those of known nematode sequences held on-line at GenBank, EMBL, DDBJ and PDB using the BLAST query engine

(http://www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned using the Clustal-X alignment program using default parameters (Thompson *et al.*, 1997; Jeanmougin *et al.*, 1998).

3. An evaluation of denaturing gradient gel electrophoresis (DGGE) for the study of marine nematode biodiversity

3.1 Introduction

Meiobenthic communities are represented by small metazoans, many of which have short generation times, all year round reproduction and undergo *in situ* benthic development without any planktonic larval stages (Heip *et al.*, 1988; Warwick, 1993; Austen and McEvoy, 1997a). Generally, the communities have high density and high diversity (Austen and McEvoy, 1997a). Free living marine nematodes are the most ecologically important component in marine and estuarine meiobenthos (Austen and Warwick, 1989; Lambshead, 1993; Coull, 1999; Austen *et al.*, 2003; Austen, 2004; Lambshead, 2004) and are useful indicators for environmental monitoring, particularly in relation to marine pollution (Tietjen and Lee, 1984; Lambshead, 1986; Coull and Chandler, 1992; Somerfield *et al.*, 1995; Bongers and Ferris, 1999; Boyd *et al.*, 2000; Ahnert and Schriever, 2001). Despite such attributes marine nematodes remain largely neglected in ecological studies due to the nature of nematode taxonomy. To date there have been limited attempts to apply molecular techniques to facilitate the use of nematode taxonomy in ecological studies. Blaxter *et al.* (2002) and Meldal (2004) used the 18S rRNA gene for barcoding nematodes and molecular phylogenetics respectively, but as yet molecular techniques have not been applied to measurements of diversity in free living marine nematode populations from estuarine and marine benthic habitats.

This introduction provides an overview of the molecular-ecological techniques that are available and routinely used in assessing prokaryotic diversity and in some micro-eukaryotic groups such as plankton. Although numerous studies have been carried out with prokaryotes, almost no studies are available for assessing benthic eukaryotic diversity in organisms such as marine nematodes that play a very important role in the benthic ecosystem. Therefore the main focus of this chapter will be to evaluate nematode diversity

from estuarine and marine environments using molecular approaches. Initial work carried out as part of this thesis (discussed in the following sections) has shown frequent co-amplification of fungal 18S rRNA regions along with nematode 18S rRNA amplicons from two sites in Plymouth Sound. Some of these fungal genera have also been detected in sediment samples from the Plymouth Sound based on PCR-DGGE. Therefore the other focus of this chapter will be to combine microbiological and molecular techniques to investigate whether some of these reported fungal taxa are found in association (existence of any parasitic or symbiotic relationships) with nematodes since bacterial associations with nematodes are well documented (Polz *et al.*, 1999).

3.1.1 Role of molecular techniques

In contrast to the relative lack of application of molecular techniques to meiofaunal groups, such techniques have changed our view of diversity and evolution of microbial life (van Hannen *et al.*, 1998). PCR based methods such as DNA cloning and sequencing, RFLP, DGGE, TGGE, ribosomal intergenic spacer analysis (RISA), automated ribosomal intergenic spacer analysis (ARISA) and other methods such as nucleic acid hybridization are now widely accepted as methods of choice for microbial diversity studies. Some of these techniques have been found to have some drawbacks when used for diversity estimation, however. In the following section a concise overview of these methods is provided, their potential disadvantages assessed, and justification given for selecting and applying DGGE to assess nematode diversity in this study.

3.1.2 PCR based molecular techniques

3.1.2.1 Clone libraries

This approach basically involves PCR amplification of genes from a particular group of organisms or from environmental DNA and subsequent ligation into a plasmid vector so as to generate clone libraries. Individual clones from the library are subject to

direct PCR to re-amplify organismal sequence contained within the plasmid. The PCR product is then digested using restriction enzymes. Resulting fragments of DNA are usually separated by electrophoresis and visualised using DNA specific stains. Cluster analysis is typically then used to identify identical restriction fragment length polymorphism (RFLP) patterns from different clones and a few single representative clones from each RFLP pattern are sequenced and compared to a DNA database of known sequences. Rarefaction curves can be constructed to estimate whether the majority of species in the environment have been sampled and to compare the species diversity of samples taken from different sites and or at different times. Sometimes colonies from the library are directly sequenced to get an idea of composition of the respective phyla. Robust automated DNA sequencing systems have greatly facilitated the screening and analysis of large gene libraries generated by the PCR clone approach.

Although this approach has not been utilised to study the diversity of marine nematode communities, it has been implemented in the assessment of prokaryotic (Wise *et al.*, 1997; Bowman and McCuaig, 2003) and marine micro eukaryotic diversity (Díez *et al.*, 2001a; Massana *et al.*, 2002; Dawson and Pace, 2002; Guillou *et al.*, 2004) and soil nematode diversity (Waite *et al.*, 2003). Being PCR dependent the clone library approach has some drawbacks. For example, there is evidence to show that sampling and preservation methods can influence estimates of species composition in prokaryotic studies (Muyzer, 1998). In addition, the preferential amplification of genomic regions by PCR in some groups lead to an underestimation of species diversity using the clone library approach (Reysenbach *et al.*, 1992). Generation of artefacts such as chimaeric PCR products, deletion errors and point errors along with biases in the cloning process itself can also affect the estimation of species diversity in samples (Muyzer, 1998; Stackebrandt *et al.*, 2000).

3.1.2.2 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a tool that has been used to study microbial diversity and relies on the principle of DNA polymorphisms. In this technique PCR products amplified using specific primer sets are digested with one or more restriction endonucleases and then separated by using agarose or non-denaturing polyacrylamide gel electrophoresis for community analysis (Liu *et al.*, 1997; Tiedje *et al.*, 1999). RFLP banding patterns can then be used to screen clones as mentioned earlier or used to measure community composition. The technique has been tested for assessing diversity in different organisms including soil nematodes (Weidner *et al.*, 1996; Liu *et al.*, 1997; Massana *et al.*, 2000; Waite *et al.*, 2003). Despite these examples RFLP has some drawbacks. The method is useful for detecting structural changes in microbial communites but cannot be used as a measure of diversity or detection of specific phylogenetic groups (Liu *et al.*, 1997). Sometimes banding patterns in diverse communities become too complex to analyze using RFLP method because a single species may have four to six restriction fragments (Tiedje *et al.*, 1999).

3.1.2.3 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a molecular technique that addresses some of the limitations of RFLP (Tiedje *et al.*, 1999). In t-RFLP, PCR amplification of environmental DNA for a region of the DNA is carried out, the only difference being that one primer is labelled with a fluorescent dye. The PCR product is then digested with a restriction enzyme and the resultant DNA fragments separated on a capillary gel-automated sequencing system. Each differently sized fragment generally corresponds to a separate species. In addition to showing species richness, the fluorescence peaks provide an approximate estimate of relative species abundance in the initial sample (Acinas *et al.*, 1997; Liu *et al.*, 1997; Tiedje *et al.*, 1999; Lukow *et al.*, 2000; Tonin *et al.*, 2001). Although the technique can allow analysis of complex communities as well as providing information on diversity

including species richness and evenness as well as similarities between samples (Liu *et al.*, 1997; Casamayor *et al.*, 2002; Buchan *et al.*, 2003), it is thought to be limited by PCR biases which are related to different gene copy number in different organisms. Sometimes incomplete digestion during T-RFLP may also lead to an overestimation of diversity (Osborn *et al.*, 2000).

3.1.2.4 Ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis (ARISA)

RISA and ARISA are similar in principle to RFLP and T-RFLP and provide ribosomal-based fingerprinting of the microbial community. In RISA, the sequence polymorphisms are detected using silver staining whilst in ARISA the forward primer is fluorescently labelled and automatically detected (Fisher and Triplett, 1999). Both these techniques have been implemented in the assessment of microbial diversity (Borneman and Triplett, 1997; Ranjard *et al.*, 2000; Hewson and Fuhrman, 2004) but have some disadvantages. Both the methods provide highly reproducible bacterial community profiles. However RISA requires large quantities of DNA, is relatively time consuming, silver staining is somewhat insensitive and resolution tends to be low (Fisher and Triplett, 1999) but recent development in staining techniques may be useful in future microbial studies (LR Noble *pers comm*). ARISA on the other hand is sensitive and less time consuming, but is still subject to PCR bias (Fisher and Triplett, 1999).

3.1.2.5 Single-strand conformation polymorphism (SSCP) analysis

PCR-based SSCP is technically relatively simple and can be used effectively to display major sequence types over short sequence lengths (usually approximately 100-400 base pairs). SSCP relies on conformational intrastrand differences in DNA of different sequences. In SSCP, PCR products are heat-denatured and plunged into ice prior to electrophoretic separation on a polyacrylamide gel. SSCP has been successfully

implemented to characterise 14 species of parasitic nematodes by analysis of the ITS-2 region of rRNA gene (Gasser and Monti, 1997). The technique has been recently applied to investigate mitochondrial DNA variation and cryptic speciation within the marine nematode *Pellioditis marina* (Derycke *et al.*, 2005). The SSCP technique is known to have some limitations where single-stranded DNA can form more than one stable conformation and therefore one sequence may be represented by more than one band on the gel (Tiedje *et al.*, 1999).

3.1.2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) was first described by Fischer and Lerman (1983) and entails electrophoresis of DNA fragments at high temperature (50-60^{0}C) in a polyacrylamide gel containing a gradient of denaturant (such as urea or formamide) (Myers *et al.*, 1985a, b, c, 1987). The principle of the method is that it allows the separation of DNA fragments of similar length but with different sequences based on the decreased electrophoretic mobility of a partially melted double-stranded DNA within gels containing a linear gradient of DNA denaturants (Muyzer and Smalla, 1998). As the DNA fragment enters the concentration of denaturant where its lowest temperature domain melts, the molecule begins branching and, hence, slows down at a unique position in the gel. This results in separation of fragments with different sequences at different points after the completion of the run. The attachment of a GC-rich segment in the forward primer, called a GC clamp, which never denatures at the conditions chosen for the experiment, allows for a branched-shaped molecule whose shape is anchored as a double-stranded molecule by the GC clamp. Using this strategy, detection of almost all single-base changes in the fragment is possible. The GC clamp was introduced by Myers *et al.* (1985b) during PCR amplification of the DNA fragments. They observed that the addition of guanine and cytosine increased the resolution of detection by roughly 40%. A GC clamp between 40-45 bp in length, as proposed by Sheffield *et al.* (1989), is routinely applied in a number of

studies. The DGGE method has been widely used for analysing diversity in prokaryotes and eukaryotes from different environments by allowing separation of a heterogeneous mixture of PCR amplified genes on a denaturing gel. Individual bands may be subsequently excised, re-amplified and sequenced (Ferris *et al.*, 1996) or challenged with a range of oligonucleotide probes (Muyzer *et al.*, 1993) to give an idea of the composition and diversity of the community.

3.1.2.6.1 Application of DGGE for assessing microbial diversity

DGGE has been used extensively to evaluate prokaryotic microbial diversity from marine and fresh water environments. Riemann *et al.* (1999) studied the bacterial community composition during two consecutive NE monsoon periods in the Arabian Sea by DGGE of rRNA genes. Wieringa *et al.* (2000) studied the depth distribution and diversity of sulphate-reducing bacteria of sandy marine sediment of the Dutch island Schiermonnikoog by DGGE. Similarly, Schafer *et al.* (2001) looked into the microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms by fingerprinting cellular rRNA in a denaturing gel. Kisand and Wikner (2003) combined three molecular methods (DGGE, quantitative DNA-DNA hybridization and a 16S rRNA gene clone library) to estimate the richness of estuarine bacterioplankton consuming riverine dissolved organic matter. Koizumi *et al.* (2003) characterised the depth-related microbial community structure in lake sediment by denaturing gradient gel electrophoresis of amplified 16S rRNA and reverse transcribed 16S rRNA fragments. Bowman *et al.* (2003) studied prokaryotic community activity and structural characteristics in Antarctic continental shelf sediments by DGGE analysis of amplified bacterial 16S rRNA genes. Gillan (2004) studied the effect of an acute copper exposure on the diversity of a microbial community in North Sea sediments by DGGE. Rees *et al.* (2004) evaluated DGGE technique for studying the diversity of the Kenyan soda lake alkaliphiles based on 16S rRNA amplicons. Webster *et al.* (2004) studied the diverse microbial communities that

inhabit Antarctic sponges using the DGGE technique. In a related study, Taylor *et al.* (2004) also explored the relationship between microbial diversity and host specificity in marine-sponge bacterial associations with 16S rRNA and rpoB genes based DGGE. Demergasso *et al.* (2004) applied PCR-DGGE to study the distribution of prokaryotes in the lakes of the Atacama Desert, Northern Chile. Dahllöf and Karle (2005) also used DGGE to study the relative abundance of bacterial species in marine sediment nitrogen fluxes caused by organic enrichment. Postec *et al.* (2005) investigated the microbial diversity of thermophiles inhabiting deep-sea hydrothermal ecosystems through DGGE analysis of 16S rRNA gene.

The application of DGGE to the study of marine eukaryotic diversity is limited to a few groups of organisms, in particular microbial eukaryotes such as plankton. Nűbel *et al.* (2000) studied the phylogenetic diversity and distribution of phototrophic micro-organisms including diatoms along a salinity gradient by PCR-DGGE and sequencing. Díez *et al.* (2001b) applied DGGE to study the diversity of marine picoeukaryotic assemblages using the 18S rRNA gene and compared DGGE with other molecular techniques. Casamayor *et al.* (2002) studied the changes in the diversity of eukaryotic and prokaryotic assemblages in a multipond solar saltern using three different molecular methods including DGGE. Savin *et al.* (2004) used DGGE to evaluate plankton diversity from the Bay of Fundy, Canada using the 18S rRNA gene. Zeidner and Beja (2004) used DGGE to analyse naturally occurring marine oxygenic picophytoplankton using the conserved photosynthetic psbA gene from Red Sea and Mediterranean waters. Work in this thesis has led to a publication by Cook *et al.* (2005) where the diversity of marine nematodes from British waters was investigated by PCR-DGGE of 18S rRNA gene.

The DGGE technique has had a wide application in terrestrial microbiology (Agnelli *et al.*, 2004; da Mota *et al.*, 2005; Laverman *et al.*, 2005), ecology and diversity of fungal communities (Brodie *et al.*, 2003; Marshall *et al.*, 2003; Yergeau *et al.*, 2005), soil and parasitic nematology (Gasser *et al.*, 1996; Gasser *et al.*, 1998; Foucher and Wilson,

2002; Waite *et al.*, 2003; Foucher *et al.*, 2004), microbial dynamics during bioremediation studies (Kirk *et al.*, 2005; Roest *et al.*, 2005; Sercu *et al.*, 2005), food microbiology (Minelli *et al.*, 2004; Rantsiou *et al.*, 2004) and also in diagnostic microbiology (Rasiah *et al.*, 2005). PCR based DGGE has been widely implemented in different groups of organisms and provided vital information towards our understanding of diversity. DGGE has the advantages of being reliable, reproducible, highly sensitive, rapid, easy to set up and relatively inexpensive. Multiple samples can be analyzed concurrently and PCR fragments separated in the gel can be isolated and subsequently sequenced to get an idea of the community composition. In addition, the methodologies are simple and non-radioactive. Because of these advantages, the DGGE technique was selected as a tool for identification and diversity assessment of nematodes in this study.

3.1.2.6.2 Molecular marker selection for DGGE study

Molecular marker selection is one of the most important aspects for the success of DGGE. The 16S rRNA gene is the preferred choice for most prokaryotic studies although several others genes such as nirS (nitrite reductase), pmo-A (coding for the α-subunit of particulate methane monooxygenase), mxa-F (coding for the α-subunit of methanol dehydrogenase) have been also used for functional diversity studies in prokaryotes (Fjellebirkeland *et al.*, 2001; Goregues *et al.*, 2005; Kleikemper *et al.*, 2005). For eukaryotic diversity assessment, the nuclear small subunit ribosomal RNA (18S rRNA) gene has been the preferred molecular marker (Diez *et al.*, 2001b; Waite *et al.*, 2003; Foucher *et al.*, 2004; Cook *et al.*, 2005). The chosen marker must be within a particular size range as DGGE works optimally with fragments from 200 base pairs to 1 Kb (Potts, 1996). The other important aspect is the presence of conserved and variable regions as this is extremely important for designing primers.

For this study, the 18S rRNA gene was chosen as a molecular marker since it can be reliably used for developing primer sets which is a pre-requisite for PCR-dependent

DGGE. The 18S rRNA gene contains variable regions flanked by conserved regions, tends to be species specific, and has been researched extensively for parasitic and soil nematodes (see Chapter 1 and Blaxter *et al.*, 1998; Schlötterer, 1998; Dorris *et al.*, 1999; Foucher and Wilson, 2002; Waite *et al.*, 2003). Moreover several nematode sequences of the 18S rRNA gene are also available from taxa spread widely across the phylum although the sequences are predominantly for terrestrial nematodes (Ellis *et al.*, 1986; Zarlenga *et al.*, 1994 a, b; Fitch *et al.*, 1995; Fitch and Thomas, 1997; Aleshin *et al.*, 1998; Blaxter *et al.*, 1998; Kampfer *et al.*, 1998; Dorris *et al.*, 1999). Existence of 18S rRNA sequences from different nematodes is an added advantage for designing consensus-based primers.

3.1.3 Non-PCR based molecular techniques

3.1.3.1 Nucleic acid hybridization

Nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular ecology (Griffiths *et al.*, 1999; Clegg *et al.*, 2000; Theron and Cloete, 2000). The hybridization techniques can be performed on extracted nucleic acids (DNA or RNA) or *in situ*. Oligonucleotide or polynucleotide probes designed from known sequences can be tagged with a fluorescent marker at the 5' end (Theron and Cloete, 2000). One of the principal limitations of *in situ* hybridization or hybridization of nucleic acids extracted from environmental samples is the lack of sensitivity. The other factor is the copy number of the sequences. Unless the sequences are present in high copy number they are often undetectable.

3.1.3.2 DNA microarrays

In recent years, DNA-DNA hybridization has been used together with DNA micro-arrays to assess microbial diversity from varied environments (Greene and Voordouw, 2003). Microarrays are high throughput systems that provide information on a very large number of genes and on changes in the expression of genes. The microarray has been

found to be a valuable tool in diversity studies since a single array can contain thousands of DNA sequences with high specificity (Cho and Tiedje, 2001). A microarray may contain specific target genes such as nitrate reductase, nitrogenase, etc. to provide functional diversity information or a sample of environmental standards (DNA fragments with less than 70% hybridization) that represent different species found in the environmental (Greene and Voordouw, 2003). Microarrays have been most widely used in prokaryotic diversity studies (Murray *et al.*, 2001; Cho and Tiedje, 2002; Wilson *et al.*, 2002; Taroncher-Oldenburg *et al.*, 2003) rather than studies of eukaryotes. De Santis *et al.* (2005) used microarrays for rapid quantification and taxonomic classification of environmental DNA from both prokaryotic and eukaryotic origins. Microarrays are not subjected to PCR bias; however, it is an expensive method.

3.1.4 General limitations of molecular-based methods

Molecular techniques based on PCR have been used to overcome the limitations of culture-based methods used in microbiology. Nevertheless they have their own limitations. Various problems with nucleic acid extraction methods can result in bias and inconsistencies in diversity estimation. Extraction methods such as bead beating can shear the nucleic acids, leading to problems in subsequent PCR detection (Wintzingerode *et al.*, 1997). Different nucleic acid extraction methods result in different yields of DNA (Wintzingerode *et al.*, 1997). For environmental samples, it is imperative to remove inhibitory substances such as humic acids, which can be co-extracted and generally interfere with PCR analysis. Subsequent purification steps can result in loss of DNA or RNA, again potentially biasing molecular diversity analysis. Preferential amplification of target genes can also result in biased interpretation of diversity. Wintzingerode *et al.* (1997) detailed issues surrounding differential PCR amplification including different affinities of primers to templates, different copy numbers of target genes, hybridization efficiency and primer specificity. In addition, sequences with lower G+C content are

thought to separate more efficiently in the denaturing step of PCR and, therefore, could be preferentially amplified (Wintzingerode *et al.*, 1997). Nevertheless molecular-based methods could provide vital information about the prokaryotic and eukaryotic microbial communities as opposed to traditional methods such as culture-based techniques, microscopy, etc.

3.1.5 Microbial diversity associated within a micro-environment

Almost all environments including soil and sediment harbour a high diversity of microorganisms (Torsvik *et al.*, 1996). Application of molecular technologies using cloning and DGGE has revealed great and previously unknown diversity from different environments (Borneman *et al.*, 1996; Bornemann and Triplett, 1997). High prokaryotic diversity has been observed in association with marine invertebrates. Researchers such as Polz *et al.* (1999) have shown great diversity and heterogeneity of epibiotic bacterial communities on the surface of the marine nematode *Eubostrichus dianae.* Such associations are also well documented in different groups of marine invertebrates e.g. sponges and isopods (Pile *et al.*, 2003; Taylor *et al.*, 2004; Lindquist *et al.*, 2005). Most of these associations are symbiotic in nature and the symbionts are overwhelmingly prokaryotic. On the other hand reported associations between marine invertebrates and micro-eukaryotes are very rare. Zande (1999) reported fungal-marine invertebrate association in a gastropod *Bathynerita naticoidea.* It is thought that the ascomycetes living in the gills of the gastropod may act to detoxify the hydrocarbons and sulfide compounds of the seep environment that the gastropod inhabits, or may be parasites that have infected the gill tissue. There are currently no reports on the diversity and existence of eukaryotic assemblages that are found to be in close association with nematode micro-environments such as their body surfaces.

3.1.6 Aims of this study

The purpose of this chapter was to investigate five main aims:

- To test whether denaturing gradient gel electrophoresis (DGGE) could be used as a tool for identification of marine nematode taxa.
- To investigate the minimum level of marine nematode DNA that could be resolved in a denaturing gel.
- To test whether DGGE could be used as a rapid tool for assessing nematode diversity from estuarine and marine sediments.
- To investigate whether sample size affects the overall interpretation of nematode diversity by DGGE.
- To investigate eukaryotic assemblages that might be present in microenvironments such as the body surface of nematodes from estuarine and marine environments.

3.2 Materials and Methods

3.2.1 Sediment collection

Sediments were collected subtidally using a van Veen grab from muddy and muddy sand substrates in South West England from the Tamar estuary (1-5 m depth) (50° 24' N, 4° 12' W), from Plymouth Sound at Jennycliff (10m depth) (50° 20' N, 4° 08' W) and Plymouth Breakwater (15m depth) (50° 20' N, 4^0 08' W) and off Rame Head (50m depth) (50° 17' N, 4° 17' W) and Cawsand (12m depth) (50° 19' N, 4° 11' W) and also from the North of England at the National Marine Monitoring Programme (NMMP) site off the Humber estuary (70m depth) (54° 00' N, 2° 00' E). All samples were taken from the surface sediment of the grab and one half of each sediment sample was immediately fixed in a storage pot containing 98% molecular grade ethanol (Hayman Limited, England) and the other half in a storage pot containing 4% formalin.

3.2.2 Meiofauna extraction

Prior to processing, sediment samples (100 gm) from each site were fixed overnight in 98% ethanol (molecular grade). Each sediment sample was then washed twice with tap water on a 63 μm sieve to remove finer sediment components, and drive off any alcohol. The washing was continued until the water passing through the sieve became clear. Extraction then followed Somerfield and Warwick's (1996) flotation method, where the sediment containing fauna was suspended in a fluid having a specific gravity similar to that of the nematodes in which the animals remain neutrally buoyant and become suspended whereas the sediment component slowly sinks in the fluid medium. The residue sediment and fauna was concentrated at the edge of the sieve and was saturated with Ludox TM (specific gravity 1.15) before being washed into 100 mL beakers. The mixture of sediment and Ludox was thoroughly stirred and then left for at least two hours to allow the animals to become suspended and separated from the sediment. The supernatant was poured into a 63 μm sieve to collect the fauna. Extracted fauna were washed once again with distilled water and then stored in 98% alcohol. Nematode specimens used for DNA extraction were picked out of the extracted samples using a sterile needle under a binocular stereo microscope (50X) and placed into a cavity block containing approximately 5% glycerol and 10% ethanol. Each specimen was then mounted on a slide (76mm × 26mm) containing a drop of glycerol in the middle and a square cover slip (18mm × 18mm) placed on top. Before mounting, the slides and cover slips were washed in molecular grade alcohol and dried with tissue papers. The cover slip was then sealed with paraffin wax. Only one nematode was mounted on each slide. The slides were labelled and stored before morphological identification. Under a compound microscope each specimen was identified to genus and species level wherever possible based on morphological characters, using pictorial keys for the identification of marine nematodes from North West Europe (Platt and Warwick 1983, 1988). After identification the cover slips were carefully removed from the slides using a sterile scalpel and the specimens individually placed in 0.5 mL PCR

tubes containing 20 µL of 0.25 M NaOH for DNA extraction. Each individual was then subjected to DNA extraction, PCR amplification and DNA sequencing techniques as described in Chapter Two.

3.2.3 Denaturing gradient gel electrophoresis (DGGE)

3.2.3.1 Selection of primers

Three sets of primers were initially selected and designed based on the nematode SSU rRNA molecule for DGGE studies. However, one of the primers sets, 22F forward [5'-GCCTGCTGCCTTCCTTGGA-3'] and 26R reverse [5'-CATTCTTGGCAAATGCTTTCG-3'] (Blaxter *et al.*, 1998) was later abandoned because of apparent co-amplification of fungal ribosomal RNA fragments. Two sets of primers were therefore used for PCR amplification of the nematode 18S rRNA in this study. The primers were (i) G18F forward and 22R reverse and (ii) MN18FGC forward and 22R reverse. The first primer set has been used in previous studies related to nematode phylogenetics (Meldal, 2004; Blaxter *et al.*, 1998) and electrophoretic separation of marine nematodes in a denaturing gel (Cook *et al.*, 2005). A new forward primer was designed in the case of the second primer set so as to selectively amplify nematode 18S rRNA regions and to prevent co-amplification of other eukaryotic ribosomal regions. The forward primer designed in this study was based on a consensus of nematode 18S rRNA sequences held online in GenBank and EMBL databases. Both the forward primers in this study were synthesised with addition of a 40 bp GC clamp at the 5' end to prevent the complete denaturation of DNA (Myers *et al.*, 1985a). The primer sets yielded PCR products of approximately 400 bp and 345 bp respectively. DGGE analysis was carried out using a Bio-Rad Dcode™ mutation analysis system (Bio-Rad, Hercules, CA, USA). For DGGE analysis 0% and 80% stock denaturant solutions were used.

3.2.3.2 Gradient selection and electrophoresis conditions

Two types of gradient namely 25% to 50% and 25% to 60% were prepared (15 mL each) using the 0% and 80% denaturant stocks. The following table details gradient preparation (15 mL volume) from denaturant stocks:

Table 3.1: Denaturing gradients prepared from denaturing stocks for DGGE.

Concentration	20%	25%	30%	35%	40%	45%	50%	60%
80%(Denaturant Stock) (mL)	3.75	4.7	5.6	6.5	7.5	8.4	9.4	11.25
0% (Denaturant Stock) (mL)	11.25	10.3	9.4	8.5	7.5	6.6	5.6	3.75

Electrophoresis was initially carried out at 200 V for 30 minutes followed by an adjustment to 60V for overnight electrophoresis. Following electrophoresis, denaturing gels were stained with SYBR Gold Nucleic Acid stain (1 µL of the stain in 10 mL of 1x TAE buffer) (nucleic acid concentration 10,000X). The staining gel was left for an hour away from light. After an hour, the gel was visualised and recorded using a SYNGENE Gel Documentation System.

3.2.3.3 DGGE band excision, cloning and sequencing

Bands from the denaturing gel were excised, re-amplified, cloned and sequenced when necessary to confirm the identity and homology with nematode ribosomal sequences available online in nucleotide sequence databases. Prominent bands from the denaturing gel were excised using Unicores (1 mm internal diameter, Sigma Aldrich, UK) and placed into 0.5 mL PCR tubes containing 20 µL of MilliQ water. The tubes were left overnight (12-14 hours) at 4°C. PCR reactions were performed in a final volume of 50 µL. For the first set of primers (G18F-22R) the following program was used for PCR amplification: 95°C for 5 min, followed by 36 cycles for 1 min at 95°C, 1 min at 51°C, 2 min at 72°C. A

final annealing temperature of 2 min at 51°C and an extension of 5 min at 72°C completed the PCR. For the second set of primers (MN18F and 22R) the following PCR conditions were implemented: 95°C for 5 min, 36 cycles of 30 sec at 95°C, 1 min at 56°C and 1 min at 72°C and a final extension of 5 min at 72°C. Amplified PCR fragments were cloned using the pGEM-T Easy Vector System. Plasmid DNA containing the inserts was cycle sequenced in both directions using the M13F forward and M13R reverse primers. Sequencing reactions were cleaned following the protocol previously described (Chapter Two) and subsequently sequenced in an ABI Hitachi 3100 Sequencer. Three to four colonies for each clone were sequenced to confirm the sequence identity. Generated sequence data were checked for ambiguities and errors and compared with the nucleotide sequence databases in the BLAST search engine. Sequences were also checked for chimeras using the CHECK CHIMERA program at the Ribosomal Database Project URL (Maidak *et al.*, 1994).

3.2.4 Species separation in a denaturing gel without mung bean nuclease treatment of PCR fragments

Two nematode specimens belonging to different taxa were selected from marine sediment and morphologically identified under the compound microscope as *Thalassironus britannicus* and *Sabatieria sp.* Genomic DNA was extracted from each worm using a modification of the Floyd *et al.* (2002) nucleic acid extraction method as previously described (Chapter Two). Two primers G18FGC and 22R were then used to amplify approximately 400 base pairs from the 5' end of the 18S rRNA gene. Additionally, DNA from the two taxa were mixed together and used as a template for PCR amplification.

The following components were added in each PCR tube for amplification:- dNTPs- 5 μL, template- 5 μL each, primers 2 μL each, 10x *Pfu* buffer- 5 μL, *Pfu* DNA polymerase- 0.5 μL and milliQ water to make up a final volume of 50 μL. The programme used in the thermal cycler for amplification was 2 min at 96°C, 35 cycles of 1 min at 94°C,

1 min at 55°C, 1 min 30 sec at 72°C and finally one cycle of 2 min at 55°C, 5 min at 72°C followed by a holding temperature of 4°C. The PCR products were analysed in 1% agarose gel prior to DGGE analysis. To confirm that the primers were amplifying ribosomal regions of the nematode nuclear genome, amplification products were sequenced in both directions using forward and reverse primers prior to DGGE analysis (G18F and 22R primers). The amplification products were subsequently loaded in a 25% to 60% denaturing gel and underwent electrophoresis at 60V for 16 hours at 60°C.

3.2.5 Species separation in a denaturing gel following mung bean nuclease treatment of PCR fragments

Three nematode specimens belonging to three different taxa were selected from sediment and morphologically identified under a compound microscope as *Sabatieria* sp., *Thalassironus britannicus* and *Enoploides* sp. Genomic DNA was extracted from each worm and subsequently PCR amplified with G18FGC forward and 22R primers. DNA from the three taxa was mixed together and used as a template for PCR amplification using the same set of primers as in the previous experimental set up (Section 3.2.4). Mung bean nuclease treatment of PCR amplicons prior to DGGE removed the artefactual double bands that arise due to the formation of some secondary product as a result of prematurely halted elongation in PCR amplification (Janse *et al.*, 2004). Artefactual double bands could hamper interpretation and analysis of DGGE gels as they may lead to an overestimation of sequence diversity. The mung bean nuclease enzyme ensures degradation of the double stranded DNA from both ends to yield 5'-phosphoryl terminated products (Ardelt and Laskowski, 1971; Kroeker *et al.*, 1976) and thereby removes the artefactual double bands.

3.2.5.1 Mung bean nuclease treatment of the PCR products

PCR amplicons from *Sabatieria* sp., *Thalassironus britannicus* and *Enoploides* sp. were therefore treated with mung bean nuclease before being loaded onto a DGGE gel. The following components were added to the individual PCR product:

PCR product 10 μL

Mung bean nuclease 1 μl (2U/μL)

10x reaction buffer 2 μL

MilliQ water 7 μL

The mixtures were then incubated at 30°C for 30 mins. The amplification products were subsequently loaded in 25-50% and 25-60% denaturing gradients and electrophoresed at 60V for 16 hours at 60°C.

3.2.6 Minimum detectable concentration of nematode DNA in a denaturing gradient gel

To detect the minimum level of marine nematode DNA that could be resolved in a denaturing gradient, a dilution series experiment was performed. Genomic DNA extracted from a single *Thalassironus britannicus* individual was used as template for PCR amplification (G18FGC and 22R). The templates were added in the following order to carry out PCR amplification: 8, 5, 3, 1, 0.5 and 0.1 μL. The PCR products were loaded in a 25% to 60% denaturing gradient for electrophoresis using previous electrophoretic conditions (Section 3.2.5.1).

3.2.7 Application of DGGE for assessment of marine nematode diversity following total nematode extraction from sediment samples

To investigate the potential of DGGE as a tool for diversity assessment, 5 grams of sediment from the sampling stations at Rame Head, Cawsand, Plymouth Breakwater and Tamar estuary (Saltash) previously fixed in 98% alcohol were subjected to total

meiofaunal extraction following the flotation method of Somerfield and Warwick (1996). On average there were 90-100 nematodes from each sample. Subsequently all nematodes from each site were carefully picked under a binocular microscope, placed together in a single sample which was then subjected to total DNA extraction. 18S rRNA gene was selectively amplified using the DGGE primer G18FGC and 22R and following the parameters described earlier in section 3.2.4. The resultant amplicons were separated using DGGE with a 25-60% denaturing urea gradient. Gels were run for 16 hours at 60 volts and then stained with SYBR-Gold. Stained gels were visualised and recorded using the SYNGENE Documentation System. Some of the prominent bands from each site in the gel were excised under a blue light source (DR45 Transilluminator, GRI UK), reamplified and sequenced to verify specificity of the primers. Bands excised and sequenced were assigned unique reference numbers on the gel.

3.2.7.1 Phylogenetic tree construction based on excised band sequences

A phylogenetic tree was constructed from the excised band sequences, 18S rRNA sequences from marine nematode sequences generated in this study and additional nematode 18S rRNA sequences from the GenBank and EMBL databases. GenBank and EMBL sequences used in this study were *Ascolaimus elongatus* (AY85231), *Chromadoropsis vivipara* (AF047891), *Enoplus meridionalis* (EMY16914), *Daptonema procerus* (AF047889), *Metachromadora remanei* (AY854216), *Metachromadora* sp. (AF036595) and *Sabatieiria* sp. (AY854236). Prior to tree construction sequences were aligned in the Clustal-X program using default parameters (Thompson *et al.*, 1997; Jeanmougin *et al.*, 1998). A neighbour-joining tree was constructed with the program MEGA v2.0 (Kumar *et al.*, 2001) using Gamma corrected Kimura distance parameters (Blaxter *et al.*, 1998) and was subsequently validated using 1000 bootstrap replicates.

3.2.8 Morphological analysis of a sediment sample from Saltash (Tamar estuary)

Sediments (5 gm) from the Tamar estuary that had been fixed in formalin were subjected to morphological analysis under a compound microscope. All the meiofauna were extracted from the sediment and washed onto a small 63 μm sieve with a mixture of 10% dilute ethanol and 5% glycerol. The meiofauna were then transferred into a cavity block and washed once again with the same mixture. The cavity block was placed on a warm hotplate (20-30°C) for at least 24 hours to allow the water and ethanol to evaporate leaving the sample material in glycerol. The contents of the cavity block were then mounted on microscope slides as described in section 3.2.2. In addition the slide preparations were permanently sealed with two coats of Bioseal, a xylene-resistant sealant, and identifications were confirmed by several experienced taxonomists.

3.2.9 Total DNA extraction from sediment samples for molecular detection of marine nematodes

One of the main objectives of this study was to evaluate a method for rapid assessment of marine nematode diversity from total DNA extracted directly from estuarine and marine sediments. To extract total DNA from the samples two methods were evaluated in this study.

3.2.9.1 Total DNA extraction from sediment samples using the Macrae et al. (2001) method

A method first described by Macrae *et al.* (2001) and subsequently modified in this study was used to extract total DNA from estuarine and marine sediments. Sediment samples from Jennycliff, Plymouth Breakwater, Rame Head and Tamar estuary were used for DNA extraction. Briefly, 1 gm of sediment was weighed into a 15 mL centrifuge tube (Fisherbrand) containing 0.5 g of glass beads (212-300 microns, Sigma), 0.7 mL of 120 mM extraction buffer (pH 8.0, 30 mM Na_2HPO_4 and 90 mM NaH_2PO_4) and 0.6 mL

phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was added and the tube vortexed for bead beating (10 mins). Samples were then centrifuged for 5 min (RCF=12,000) and the upper aqueous phase removed and collected in a fresh 2 mL screw-top tube. A further 0.5 mL of extraction buffer was added to the original tube and the bead beating and centrifugation steps repeated. After centrifugation, the aqueous phase was pooled with that from the first extraction. An equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and the sample vortexed and centrifuged to remove residual phenol. The upper layer of the tube was carefully removed to a fresh tube and 0.1 volume of 3 M sodium acetate and 0.6 volumes of isopropanol were added to it. The mix was left at room temperature for 10 minutes prior to centrifugation (RCF=12,000) for another 10 minutes. The supernatant was discarded and the DNA pellet washed in 250 µL of 70% (v/v) ethanol. The supernatant was discarded after centrifugation (RCF=12,000) for 5 minutes. The sample was air-dried at 37°C for 30 minutes. 100 µL of MilliQ water was added to resuspend the DNA and the sample stored at -20°C prior to amplifications.

3.2.9.1.1 PCR amplification of DNA samples using MN18FGC and 22R primers and subsequent DGGE analysis

The concentration of total DNA extracted using the modified Macrae *et al.* (2001) method was quantified in a spectrophotometer (Eppendorf Inc). Briefly, 2 µL of template DNA was suspended in 198 µL of MilliQ water in a UV cuvette and measured using the dsDNA (double stranded DNA) program.

DNA samples were subsequently PCR amplified using the MN18FGC and 22R primers. PCR reactions were performed on 0.5 µL aliquots of the extracted DNA and *Pfu* DNA polymerase used for amplification. 1.0 µL of BSA (Bovine Serum Albumin, 10 mg/mL) was added to the PCR reactions. The following program was used for amplification: 2 min at 95°C, 38 cycles of 1 min at 94°C, 1 min at 56°C, 90 seconds at

72°C and finally one cycle of 2 min at 56°C, 30 mins at 72°C followed by a holding temperature of 4°C. DGGE analysis was carried out in a 25% to 60% denaturing gradient. 15 µL of PCR product was loaded into each well of the gel for electrophoresis.

3.2.9.2 Extraction of total DNA using FastDNA® Kit for Soil (Qbiogene Inc)

A second DNA extraction method was tested using the commercially available FastDNA® Kit. Estuarine and marine sediment samples from five sampling stations, Plymouth Breakwater, JennyCliff, Rame Head, Saltash (Tamar estuary) and the NMMP site, were selected for total DNA extraction using the FastDNA® Spin Kit. Approximately 500 mg of sediment from each sampling station was added to Lysing Matrix E tube. 978 µL of Sodium Phosphate buffer and 122 µL of MT buffer were added to each tube. The Lysing Matrix E tubes were centrifuged (RCF=14,000) for 30 seconds. Supernatant from each tube was carefully transferred to a clean tube and 250 µL of PPS (Protein Precipitation Solution) reagent was added to it. The solution was mixed by shaking 10 times. The tubes were centrifuged (RCF=14,000) for 5 minutes to precipitate protein pellets. Supernatant from each tube was transferred to clean 15 mL tubes. 1 mL of Binding Matrix Suspension was added to the supernatant. The Binding Matrix Suspension was handled carefully because it contained Guanidine thiocyanate which is highly toxic and a skin irritant. The suspension was thoroughly resuspended before use. The tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. The tubes were then placed in a rack for 3 minutes to allow settling of the matrix. 500 µL of supernatant from each tube was carefully removed to avoid settled Binding Matrix. The supernatant was discarded. The Binding Matrix was resuspended in the remaining amount of supernatant. Approximately 600 µL of the mixture from each tube was carefully transferred to a SPIN™ Filter and centrifuged (RCF=14,000) for 1 minute. The catch tube was emptied in each case and the remaining supernatant was added to the SPIN™ Filter and spun again.

500 µL of SEWS-M (Salt/Ethanol Wash Solution) was added to the SPIN™ Filter and centrifuged (RCF=14,000) for 1 minute. The flow-through was decanted and the SPIN™ Filter was replaced in the catch tube. The filter was centrifuged (RCF=14,000) for another 2 minutes to dry the matrix of residual SEWS-M wash solution. The SPIN™ Filters were carefully removed and placed in a fresh kit- supplied catch tube. The SPIN™ Filters were air dried for 5 minutes at room temperature. 50 µL of DES (DNase/Pyrogen Free Water) was added to each filter and the matrix was gently stirred with a pipette tip to resuspend the silica for efficient elution of the DNA. The filters were centrifuged (RCF=14,000) for 1 minute so as to transfer eluted DNA to Catch Tubes. The spin filters were discarded and DNA samples were stored in -20°C until further use.

3.2.9.2.1 PCR amplification of total DNA using the G18FGC and 22R primers and DGGE analysis

Prior to PCR amplification, the concentration of total DNA from all the five sites was quantified in a spectrophotometer (see Section 3.2.9.1.1).

DNA from four sites, namely Plymouth Breakwater, JennyCliff, Rame Head and Saltash (Tamar estuary), were used for PCR amplification using the G18FGC and 22R primers. PCR reactions were performed on 0.5 µL aliquots of the extracted DNA and 1 µL of BSA was used in each PCR reaction. DGGE analysis was carried out in a 25% to 60% denaturing gradient and electrophoresis followed previous conditions. 15 µL of PCR amplified DNA from the four sites was loaded into each well in the denaturing gel. Gels were stained using procedures as described earlier (Section 3.2.2.2) and subsequently visualised and recorded. Some of the prominent bands from each site in the gel were excised and sequenced. Bands that were excised and sequenced have been assigned unique reference numbers on the gel.

3.2.9.3 PCR amplification and subsequent DGGE analysis using MN18FGC and 22R primers

A second set of primers, MN18FGC and 22R were tested on the DNA samples from all the sites. PCR amplifications were performed as described previously (Section 3.2.9.1.1) using the same thermal cycle parameters. DGGE analysis was performed in a 25% to 60% denaturing gradient at 60°C for 16 hours at 60V and subsequently visualised. Some of the prominent bands were excised, re-amplified, cloned into pGEM-T vectors and sequenced. Three to four colonies from each clone were sequenced to confirm sequence identity. Selected bands that were excised have been assigned reference numbers on the gel.

3.2.9.4 Phylogenetic tree construction based on excised sequences amplified using MN18FGC and 22R primers

A phylogenetic tree was constructed from excised band sequences and additional partial ssu marine nematode sequences generated in this study as well as sequences from GenBank and EMBL databases. GenBank and EMBL sequences used in this study were *Daptonema oxycerca* (AY854225), *Daptonema normandicum* (AY854224), *Paracanthonchus* sp. (AF047888), *Atrochromadora microlaima* (AY854204), *Dichromadora* sp. (AY854209). Tree construction was carried out following the steps described in section 3.2.7.1.

3.2.10 Influence of sediment sample sizes on the assessment of nematode diversity

The influence of sediment sample sizes on the assessment of nematode diversity was investigated using sediments from the Tamar estuary. Three sample sizes, 0.01g, 0.1g and 1.0g were evaluated. DNA was extracted using the SoilMaster™ DNA Extraction kit following the manufacturer's instructions (Epicentre, Wisconsin, USA). Briefly, four replicates of 0.01g, 0.1g and 1.0g estuarine sediment were used for DNA extraction. Four

replicates of each sample size were weighed and placed into 1.5 mL eppendorf tubes. 250 µL of Soil DNA Extraction buffer and 2 µL of Proteinase K (50 mg/mL) were added to each tube and vortexed briefly for 2 minutes. To increase the yield of DNA the tubes were shaken for 10 min at 37°C. 50 µL of Soil Lysis Buffer was added to each tube and vortexed briefly for 2-3 minutes. The tubes were subsequently incubated at 65°C for 10 minutes. Following incubation, the tubes were centrifuged for 2 min (RCF=1,000). 180 µL of the supernatant from each tube was transferred to new tubes. This was followed by the addition of 60 µL of Protein Precipitation Reagent and thorough mixing by inverting each tube. The tubes were incubated on ice for 8 minutes and then centrifuged at maximum speed for 8 min on a table-top centrifuge. Prior to transfer of the supernatant from the centrifuged tubes directly onto the spin columns, 550 µL of Inhibitor Removal Resin was added to each empty spin column and centrifuged for 1 minute (RCF=2,000) to pack the column. The flow-through was decanted and the columns were placed back into the same collection tubes. Another 550 µL of Inhibitor Removal Resin was added to each packed column and centrifuged once again for 2 minutes (RCF=2,000). The columns were transferred into clean 1.5 mL collection tubes. 100-150 µL of supernatant from each tube (centrifuged for 8 mins previously) was transferred to individual prepared Spin Columns containing the collection tubes. The spin columns were centrifuged for 2 minutes (RCF=2,000). The columns were discarded thereafter and to each collection tube 6 µL of DNA Precipitation solution was added and the tube vortexed for one minute. The tubes were incubated for another 5 minutes at room temperature and then centrifuged for 5 minutes at maximum speed. The supernatant from each tube was carefully decantated. The pellets were washed with 500 µL of Pellet Wash Solution. The tubes were inverted for thorough mixing and then spun for another three minutes at maximum speed in a centrifuge. Once again the supernatant from each tube was carefully decantated. The wash

and spin procedure was repeated once more. The pellets were finally resuspended in 300 µL of TE Buffer (pH 7.5).

To test that there was no bias with the DNA extraction method, two replicates of 0.1 g of sediment from the Tamar estuary were subjected to total DNA extraction using the SoilMaster™ DNA Extraction Kit and Powersoil™ DNA Isolation Kit (MoBio Laboratories, Inc USA). The Powersoil DNA kit relies on bead beating and is effective at removing PCR inhibitors from sediment with high humic acid content through a humic substance removal procedure. DNA was extracted following manufacturer's instructions.

3.2.10.1 PCR amplification and DGGE of amplification products

Four replicates of each of the sample sizes namely 0.01 g, 0.1 g, and 1.0 g were then used for PCR amplification. Amplification was carried out using the primers MN18FGC and 22R. 0.5 µL of template DNA from each replicate was used in the PCR reactions. DNA extracted from sediments using the Powersoil DNA Kit and Soil Master DNA Kit was also subjected to PCR amplification using the same set of primers and amplification parameters. DGGE analysis was carried out in a 25%-50% denaturing gradient and the gel was subsequently stained and photographed. Prominent bands from each replicate were also excised, amplified, cloned and sequenced in order to get an idea about the community structure. Excised bands were assigned reference numbers on the gel.

3.2.11 Investigating eukaryotic assemblages in nematodes from marine and estuarine environments

To investigate the occurrence of non-nematode eukaryotic assemblages in microenvironments such as the nematode body surface, 5 g of sediment from Jennycliff, Plymouth Breakwater, Tamar estuary and Plym estuary were subjected to meiofauna extraction following Somerfield and Warwick's (1996) method described in section 3.2.2. Nematodes (n=16) were randomly picked from each site following meiofauna extraction

using a binocular microscope. Each nematode was placed into 0.5 mL PCR tubes containing 0.25 M NaOH. All the nematodes were subjected to DNA extraction following the modified Floyd *et al.* (2002) protocol.

3.2.11.1 PCR amplification of the 18S rRNA gene for DGGE analysis

All the nematodes were subjected to PCR amplification using the G18SFGC and 22R primers. Briefly, 3 μL of template DNA from each nematode was used in PCR amplification. Prior to DGGE, PCR amplicons were treated with mung bean nuclease according to manufacturer's instructions. Amplicons were separated in 25% to 60% denaturing gradient gels following standard electrophoretic conditions.

3.2.11.1.1 Band excision, amplification and sequencing

Prominent bands in the gel for each site were excised and eluted in 20 μL of MilliQ water. Bands were re-amplified using G18F and 22R primers. Successful PCR amplicons were treated with ExoSAP-IT according to manufacturer's instructions (USB Corporation). ExoSAP-IT utilizes two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase, together in a specially formulated buffer to remove unwanted dNTPs and primers that remain in the PCR product after the completion of amplification process. This is to stop them interfering during sequencing or SNP (single nucleotide polymorphism) analysis. Briefly, 5 μL of PCR product was mixed with 2 μL of ExoSAP-IT in a 0.5 ml PCR tube. The tube was then heated at 37°C for 10 mins followed by 15 mins at 87°C. 2.5 μL of the ExoSAP-IT treated PCR product was cycle sequenced using a BigDye Terminator Kit (Applied Biosystems, Warrington, UK) and cleaned using the Wizard Magnesil™ system (Promega, UK). Sequencing was performed in both directions using the same set of primers in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Generated sequences were then compared with the available eukaryotic sequences held online at GenBank and EMBL using the BLAST search engine.

3.2.11.2 Scanning electron microscopy

Sediments from Jennycliff and Plymouth Breakwater were fixed in 5% formalin prior to electron microscopy. Nematodes from both the samples were subsequently extracted using the protocol of Somerfield and Warwick (1996) (Section 3.2.2) and stored in formalin. Nematodes extracted from both the sites were put into porous pots of type K850 (Emitech Ltd, Kent, UK) to remove the formalin by repeated washes with distilled water and subsequently the specimens were fixed in glutaraldehyde for 30 mins. Dehydration of the samples was achieved by a series of increasing concentrations of ethanol (30% to absolute) according to the following table:-

Table 3.2: Water-ethanol ratio for dehydration process.

Water (%)	Ethanol (%)	Duration (min)
70	30	10
50	50	10
30	70	10
20	80	10
10	90	10
0	100	20

Dehydrated nematodes were then subjected to critical point drying under carbon dioxide in an E3000SII critical point dryer (Poleron, UK) and subsequently coated with gold using a K550 sputter coating (Emitech, UK) to increase the electrical conductivity. The micrographs were taken using a JEOL JSM 5600 Scanning Electron Microscope.

3.2.11.3 Isolation of marine derived fungi from sediments of Jennycliff and Plymouth Breakwater

Sediments from Jennycliff and Plymouth Breakwater were mixed with sterile sea water and a series of dilutions were made. From the dilutions, 0.5 mL volumes were

pipetted onto petri dishes containing fungal media and incubated at 30°C for two weeks. Fungal strains were isolated from plates and subcultured until a pure culture was obtained.

3.2.11.3.1 Ribosomal characterisation of the isolates

Fungal isolates were subsequently characterised by PCR amplification of the partial small subunit ribosomal RNA gene. Two primers, G18F and 22R were used for PCR amplification. Amplified PCR fragments were treated with ExoSAP-IT and cycle sequenced using Big Dye Terminator mix. Sequencing was carried out in both directions in an ABI Prism 3100 sequencer. Sequences were then compared with the available fungal sequences held online at GenBank and EMBL using the BLAST search engine.

3.3 Results

Prior to DGGE analysis for species separation, amplified 18S rRNA genes from nematode worms were sequenced and compared with the available nematode sequences held on line at GenBank and EMBL. All the sequences showed high similarity with the available nematode ribosomal sequences. Sequences found to be chimaeric in this study were discarded and not used in phylogenetic analyses.

3.3.1 Species separation in a denaturing gel without mung bean nuclease treatment of PCR amplicons

The PCR amplicons of the partial 18S rRNA from *Sabatieria* sp. and *Thalassironus britannicus* were subjected to electrophoresis individually and as a mixture in a denaturing gel. Both the taxa separated individually and also in the mixture producing unique banding patterns (Figure 3.1). More than one band was observed in each lane for both taxa in the denaturing gradient. The amplicon separation experiment was re-done following mung bean nuclease treatment of the PCR amplicons.

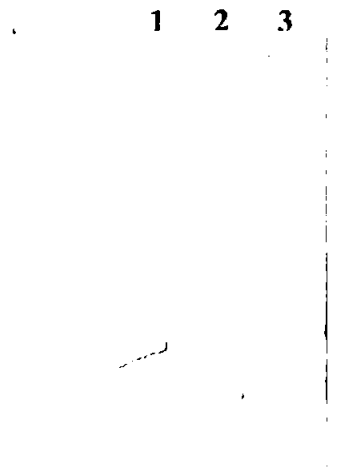
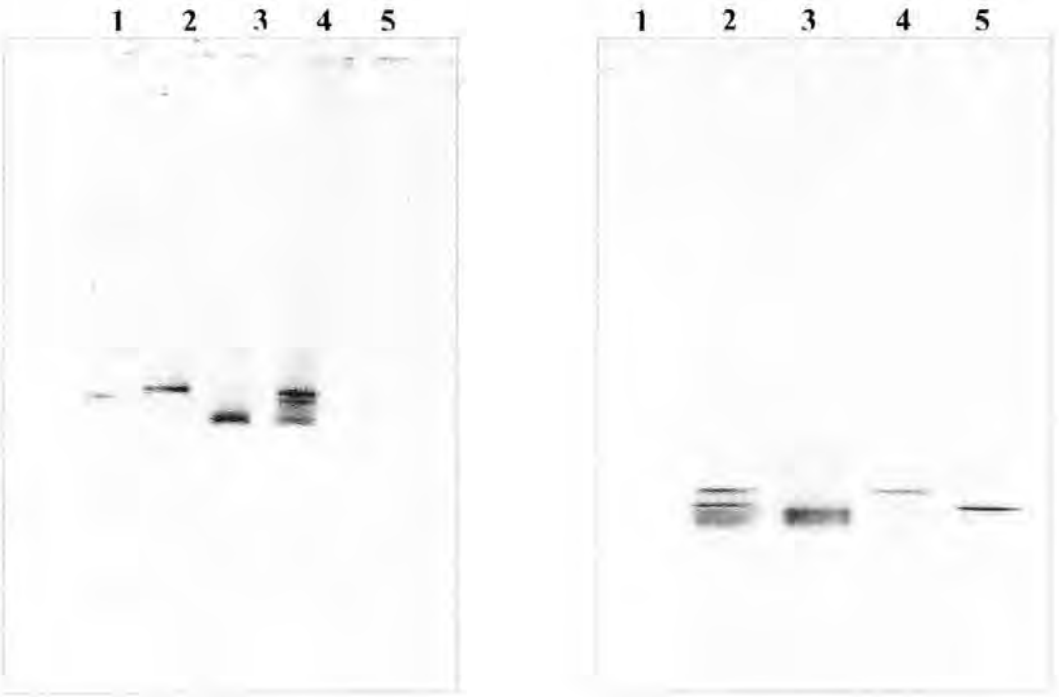


Figure 3.1: Separation of marine nematode taxa in a denaturing gel showing artefactual double band formation; Lane 1: 18S rRNA amplicon from *Thalassironus britannicus*; Lane 2: 18S rRNA amplicon from *Sabatieria* sp. with artefactual double bands; Lane 3: Amplified mixed template DNA from *Sabatieria* sp. and *T. britannicus*.

3.3.2 Species separation in a denaturing gel after post-treatment of PCR amplicons with mung bean nuclease

The PCR amplicons of the 18S rRNA gene of *Sabatieria* sp., *Thalassironus britannicus* and *Enoploides* sp. were subjected to electrophoresis in two denaturing gradients. To detect whether the PCR method led to biased amplification, template DNA from *Sabatieria* sp., *Thalassironus britannicus* and *Enoploides* sp. were mixed and the 18S rRNA gene was amplified. All the PCR amplicons including the mixed PCR product were treated with mung bean nuclease prior to DGGE analysis. The mixed PCR product was loaded in the same gel containing the *Sabatieria* sp., *Thalassironus britannicus* and *Enoploides* sp. amplicons. After the completion of the run it was observed that all the three taxa differentiated successfully in both the denaturing gradients (25 to 50% and 25 to

60%), thereby producing unique banding patterns (Figure 3.2 A and B). The mixed DNA amplicons also showed clear separation in the same gradient and the patterns completely matched to that of *Sabatieria* sp., *Thalassironus britannicus* and *Enoploides* sp. (Figure 3.2 A and 3.2 B). There was no selective amplification in mixed DNA and each taxon differentiated in a denaturing gel generating characteristic banding patterns No additional bands were observed for each taxon in the same gel. The separation of nematode taxa was much clearer in the 25% to 60% gradient than the 25% to 50% gradient.



(A) (B)

Figure 3.2: (A) PCR-DGGE analysis of the 18S rRNA amplicons in a 25% to 60% denaturing gel. Lane 1: *Sabatieria* sp.; Lane 2: *Thalassironus britannicus*; Lane 3: *Enoploides* sp.; Lane 4: Mixed DNA from the three nematodes (PCR amplified); Lane 5: Negative control. (B) PCR-DGGE analysis of the 18S rRNA amplicons in a 25% to 50% denaturing gel. Lane 1: Negative control showing no DNA contamination; Lane 2: Mixed DNA from the three nematodes (PCR amplified); Lane 3: *Enoploides* sp.; Lane 4: *Thalassironus britannicus*; Lane 5: *Sabatieria* sp.

3.3.3 Minimum detectable concentration of nematode DNA in a denaturing gel

The limit of detection of DNA extracted from an individual nematode in a denaturing gel is shown in Figure 3.3. This indicates that the limits of detection are in the region of 0.5 μL of template DNA that represents 2.5% of the DNA extractable from a single nematode specimen. Template concentration is in the range 0.5 to 3.0 ng/μL (n=10, where n= number of specimens), thus giving a limit of detection for genomic DNA of approximately 250 pg (before PCR and DGGE detection).

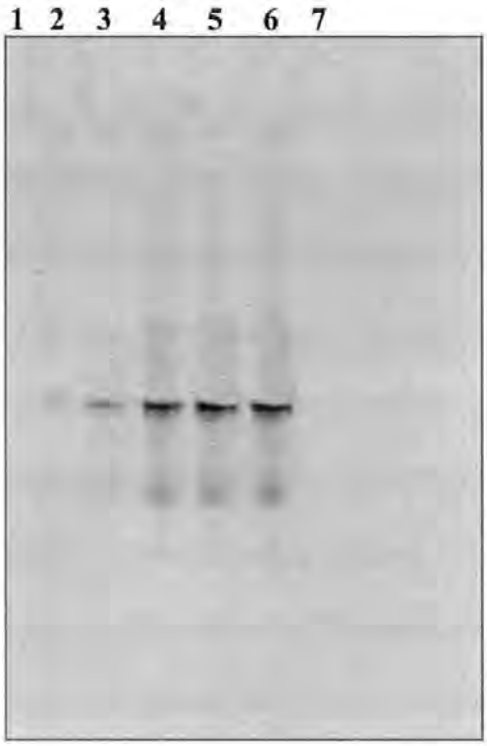


Figure 3.3: DGGE gel showing minimum detectable level of amplified DNA from the following volumes of template from *Thalassironus britannicus*; Lane 1: 0.1 μL; Lane 2: 0.5 μL; Lane 3: 1 μL; Lane 4; 3 μL; Lane 5: 5 μL; Lane 6; 8 μL; Lane 7: Negative.

3.3.4 PCR-DGGE of nematode samples after extraction from estuarine and marine sediments

One of the main objectives of this work was to investigate the potential of DGGE as a tool for assessing marine nematode diversity. PCR/DGGE analysis of nematode samples

taken from Cawsand, Rame Head, Plymouth Breakwater and Saltash (Tamar estuary) is shown in Figure 3.4. The result showed almost similar banding patterns for each of the sites. Approximately 15 bands could be distinguished from each site possibly representing 15 putative taxa. Some of the prominent bands that were excised showed high sequence similarity to the nematode sequences held online at GenBank and EMBL. This indicates that the primer/DGGE system is capable of targeting and resolving 18S rRNA of marine nematodes selected from environmental samples. The phylogenetic placement of the excised sequences suggests that some of them share high sequence similarity with the marine nematode *Terschellingia longicaudata* (Env1), *Sabatieira punctata* (Env2), *Enoplus meridionalis* (Env3), *Thalassironus britannicus* (Env4), *Sabatieira celtica* (Env6), *Metachromadora remanei* (Env7 and Env8) and *Daptonema procerus* (Env9) in the tree (Figure 3.5). The sequence similarities ranged between 97 and 100% for most of the excised and sequenced bands. Some of these sequences have been submitted to the EMBL database and published (Cook *et al.*, 2005) and their accession numbers are AJ867815 to AJ867818.

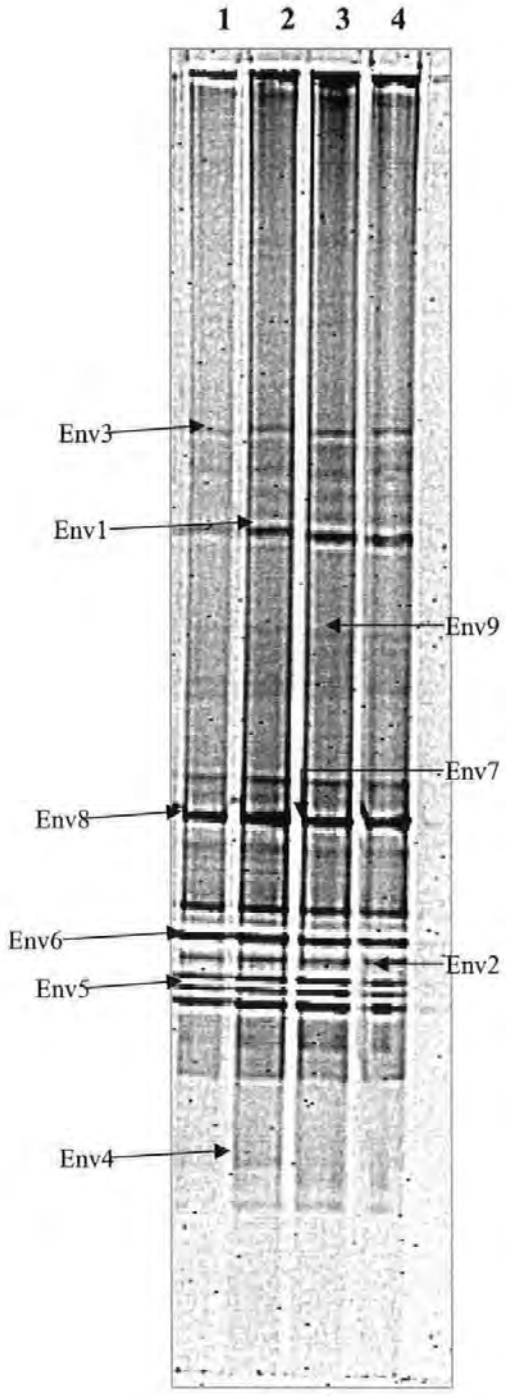


Figure 3.4: Banding patterns of marine nematode communities from four locations; Lane 1: Cawsand; Lane 2: Plymouth Breakwater; Lane 3: Rame Head; Lane 4: Saltash (Arrows indicate bands extracted and sequenced).

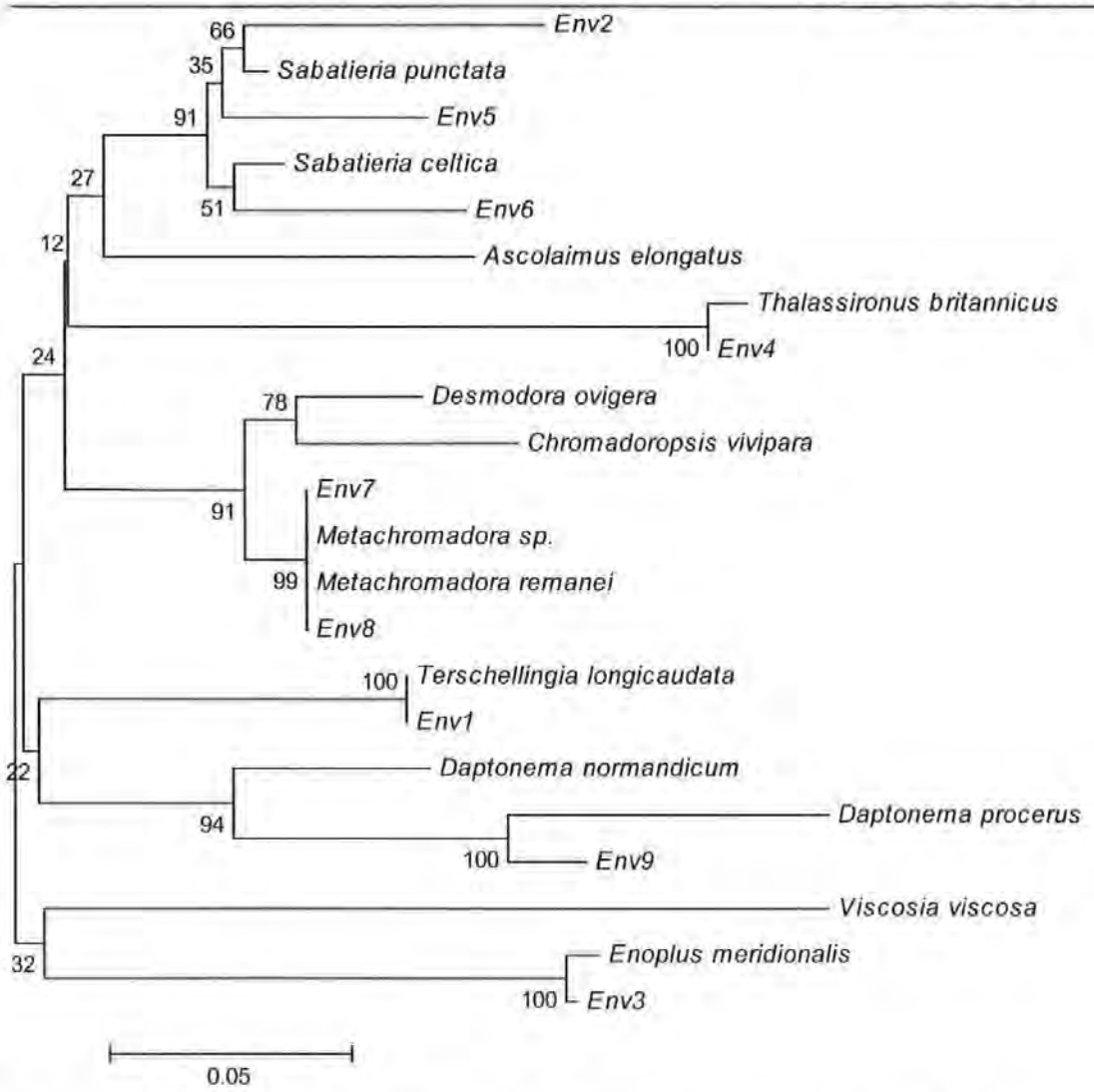


Figure 3.5: Neighbour-Joining tree showing relationship between DGGE bands amplified using G18F and 22R primers (18S rRNA) and most similar sequences of known nematodes. Scale= 0.05 substitutions/site. Numbers beside branches indicate bootstrap values (1,000 replicates).

3.3.5 Morphological analysis of sediment sample from Saltash, Tamar estuary

Morphological analysis of the estuarine sediment sample from Saltash (Tamar estuary) revealed the presence of 25 nematode taxa. Most of the taxa were identified to genus or species level using the pictorial keys. Some of the dominant taxa in the samples were *Terschellingia longicaudata*, *Ptycholaimellus ponticus*, *Sabatieria pulchra* and *Daptonema setosum*. Identified taxa are listed in Table 3.3.

Table 3.3: Nematodes identified from Saltash sample by morphological characteristics. Classification to family is according to Meldal (2004). 'n' denotes number identified.

Taxon	Family/Order	n
Anoplostoma sp. Bűtschli, 1874	Anoplostomatidae	1
Viscosia viscosa Bastian, 1865	Oncholaimidae	3
Microlaimus sp. De Mann 1880	Microlaimidae	1
Molgolaimus tenuispiculum Ditlevsen, 1921	Microlaimidae	1
Atrochromadora sp. Wieser, 1959	Chromadoridae	1
Chromadora sp. Bastian 1865	Chromadoridae	1
Ptycholaimellus ponticus Filipjev, 1922	Chromadoridae	9
Cyatholaimus sp. Bastian 1865	Cyatholaimidae	1
Metachromadora remanei Gerlach 1951	Desmodoridae	1
Metachromadora sp. Filipjev, 1918	Desmodoridae	5
Desmodora pontica Filipjev, 1922	Desmodoridae	2
Sabatieria pulchra (Schneider, 1906)	Comesomatidae	6
Sabatieria celtica Southern, 1914	Comesomatidae	3
Setosabatieria hilarula (De Man, 1922)	Comesomatidae	1
Terschellingia longicaudata De Man, 1907	Linhomoeidae	5
Terschellingia communis De Man, 1888	Linhomoeidae	3
Terschellingia gourbaultae Austen, 1989	Linhomoeidae	1
Sphaerolaimus sp. Bastian, 1865	Sphaerolaimidae	3
Theristus acer Bastian, 1865	Xyalidae	2
Daptonema setosum (Bűtschli, 1874)	Xyalidae	8
Daptonema oxycerca (De Man, 1888)	Xyalidae	5
Daptonema normandicum (De Man, 1890)	Xyalidae	9
Daptonema sp. Cobb, 1920	Xyalidae	7
Axonolaimus paraspinosus Stekhoven and Adam, 1931	Axonolaimidae	4
Odontophora sp. Bűtschli, 1874	Axonolaimidae	5

3.3.6 PCR-DGGE of DNA extracted from sediments using a modified Macrae *et al.* (2001) protocol

The DNA concentrations extracted from Tamar estuary, Plymouth Breakwater, Jennycliff and Rame Head were 75.2 μg/mL, 58.7 μg/mL, 58.4 μg/mL and 60.7 μg/mL respectively. The ribotype diversity was visibly lower in the denaturing gel for DNA from Jennycliff, Plymouth Breakwater, Rame Head and Saltash (Tamar estuary) sediments extracted using a modified Macrae *et al.* (2001) protocol and amplified by MN18FGC and 22R primers (Figure 3.6). This was probably due to inhibition of the PCR amplification or the failure of the extraction method to recover enough DNA from the sediments.

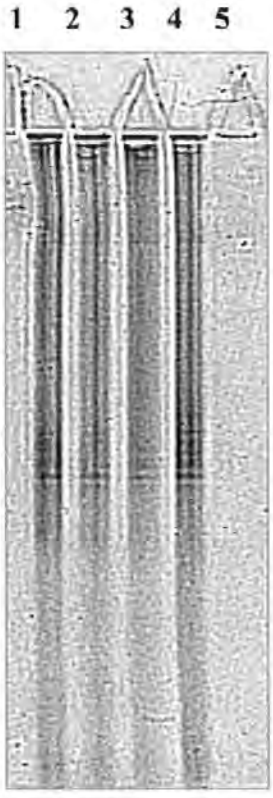


Figure 3.6: PCR-DGGE of total DNA extracted from sediments from four stations using a modified Macrae *et al.* (2001) protocol. Lane 1: Tamar estuary; Lane 2: Plymouth Breakwater; Lane 3: Jennycliff; Lane 4: Rame Head; Lane 5: negative to detect any contamination.

3.3.7 DGGE profiling of DNA extracted from marine and estuarine sediment using FastDNA Spin Kit

The DNA concentration for Tamar estuary, Plymouth Breakwater, JennyCliff, Rame Head and NMMP were 129.9 μg/mL, 45.0 μg/mL, 57.3 μg/mL, 119.4 μg/mL and 45.0 μg/mL respectively. DNA from Jennycliff, Rame Head, Plymouth Breakwater and Saltash (Tamar estuary) sediments amplified by G18FGC and 22R primers generated banding patterns that were characteristic for each site, with certain bands more prevalent at each site (Figure 3.7). Some of the prominent bands that were excised and sequenced were compared in the GenBank and EMBL database using the BLAST search engine. Most of the sequences showed significant similarity (98-99% percent) to that of available nematode ribosomal sequences. However some of the sequences were found to show homology to other eukaryotic ribosomal regions. Four sequences showed similarity with the fungi *Paecilomyces fumosoroseus* (99% similarity), *Rhinocladiella aquaspersa* (98% similarity) and *Syspastospora parasitica* (98% similarity).

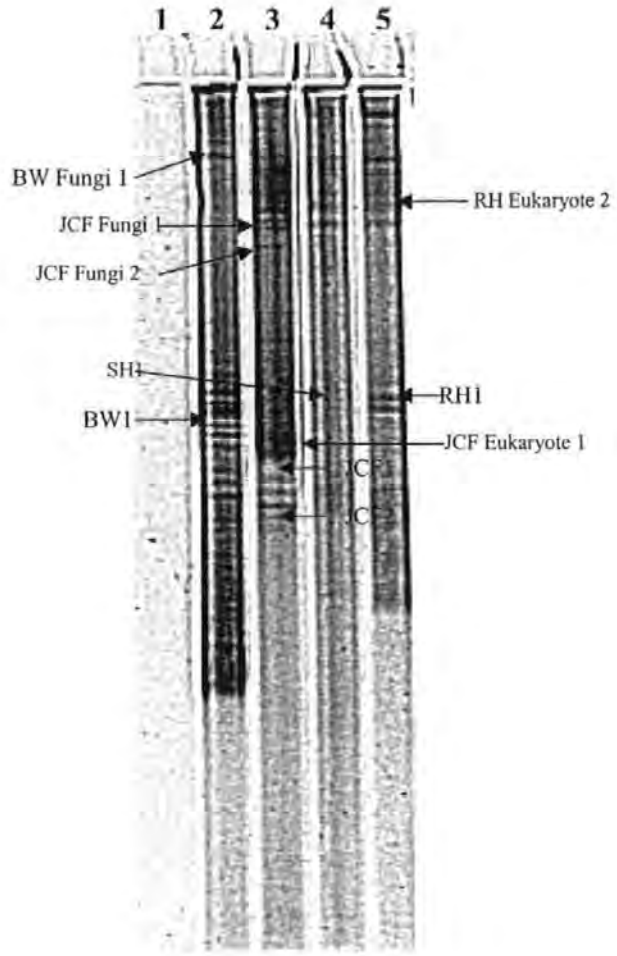


Figure 3.7: Banding patterns of marine nematode communities from five stations amplified using G18FGC and 22R primers; Lane 1: Negative to check for contamination; Lane 2: Plymouth Breakwater (BW); Lane 3: JennyCliff (JCF); Lane 4: Saltash (Tamar estuary) (SH); Lane 5: Rame Head (RH) (Arrows indicate bands that were extracted and sequenced).

Additionally, three sequences showed homologies with an uncultured stramenophile clone IAFDv26 (99% similarity), uncultured marine eukaryotic clone mj223 (99% similarity) and a polychaete *Caulleriella parva* (98% similarity) respectively. The marine nematode sequences generated in this study have been submitted to EMBL under the accession numbers AJ966665 (BW1), AJ966666 (JCF1) AJ969109 (JCF2). Fungal and other eukaryotic sequences reported in this section have been submitted in EMBL and their accession numbers are AJ965493 (JCFFungi1), AJ965494 (JCFFungi2) AJ965671 (BWFungi1), AJ971292 (JCFEukaryote1), AJ971293 (RHEukaryote2).

3.3.8 DGGE pattern of DNA templates amplified using MN18FGC and 22R primers

DNA templates from the above four sites and additionally from the NMMP site off the Humber estuary amplified with the newly designed MN18FGC and 22R primers showed characteristic banding patterns. The ribosomal RNA diversity differed between sites in terms of DGGE banding patterns with certain bands prevalent at each site (Figure 3.8). Approximately 10 bands or ribotypes could be distinguished representing 10 putative taxa for Rame Head, NMMP and Saltash (Tamar estuary) in the gel, whereas for Jennycliff and Plymouth Breakwater the number of bands was between 7 and 8. All of the extracted bands showed high sequence similarity to the available nematode sequences in GenBank and EMBL. Co-amplification of other eukaryotic 18S rRNA genes including fungi was not recorded. The placement of some of the excised sequences in the phylogenetic tree (Figure 3.9) suggests that they share high sequence similarity with *Terschellingia longicaudata* (SH3), *Atrochromadora microlaima* (BW1), *Bathylaimus* sp. (RH1) and *Sabatieria pulchra* (JCF1, BW4 and JCF2). Some of these nematodes have been reported widely by Austen (1986) and Cook *et al.* (2005) from Southwest England in previous studies. Some of the marine nematode sequences reported in this section have been deposited in the EMBL database and the accession numbers are AJ867491 (NMMP1), AJ867492 (JCF2), AJ867493 (SH2), AJ867494 (JCF1), AJ867495 (RH1), AJ867496 (BW2), AJ867497 (NMMP3), AJ867498 (NMMP2), AJ867499 (NMMP4), AJ867500 (RH2), AJ867501 (RH3), AJ867502 (JCF3), AJ868129 (SH1), AJ868130 (BW1), AJ868131 (BW3), AJ868132 (NMMP5), AJ971294 (BW4).

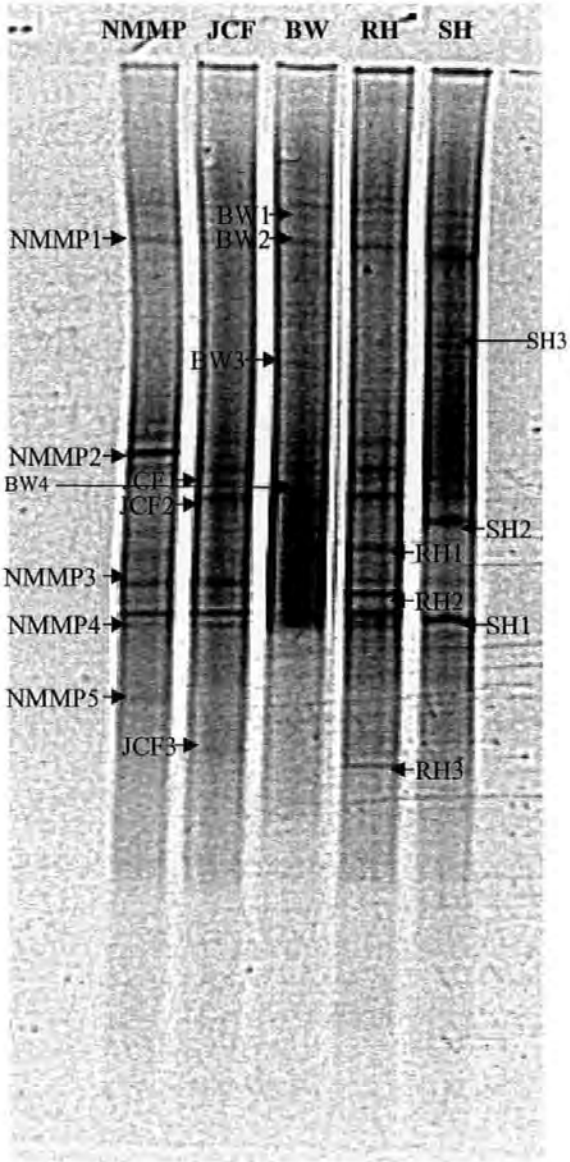


Figure 3.8: Banding patterns of marine nematode communities from five environmental stations amplified using MN18FGC and 22R primers; Lane 1: Negative control; Lane 2: NMMP site off Humber estuary; Lane 3: Jennycliff; Lane 4: Plymouth Breakwater; Lane 5: Rame Head; Lane 6: Saltash (Tamar estuary) (Arrows indicate bands that were extracted and sequenced).

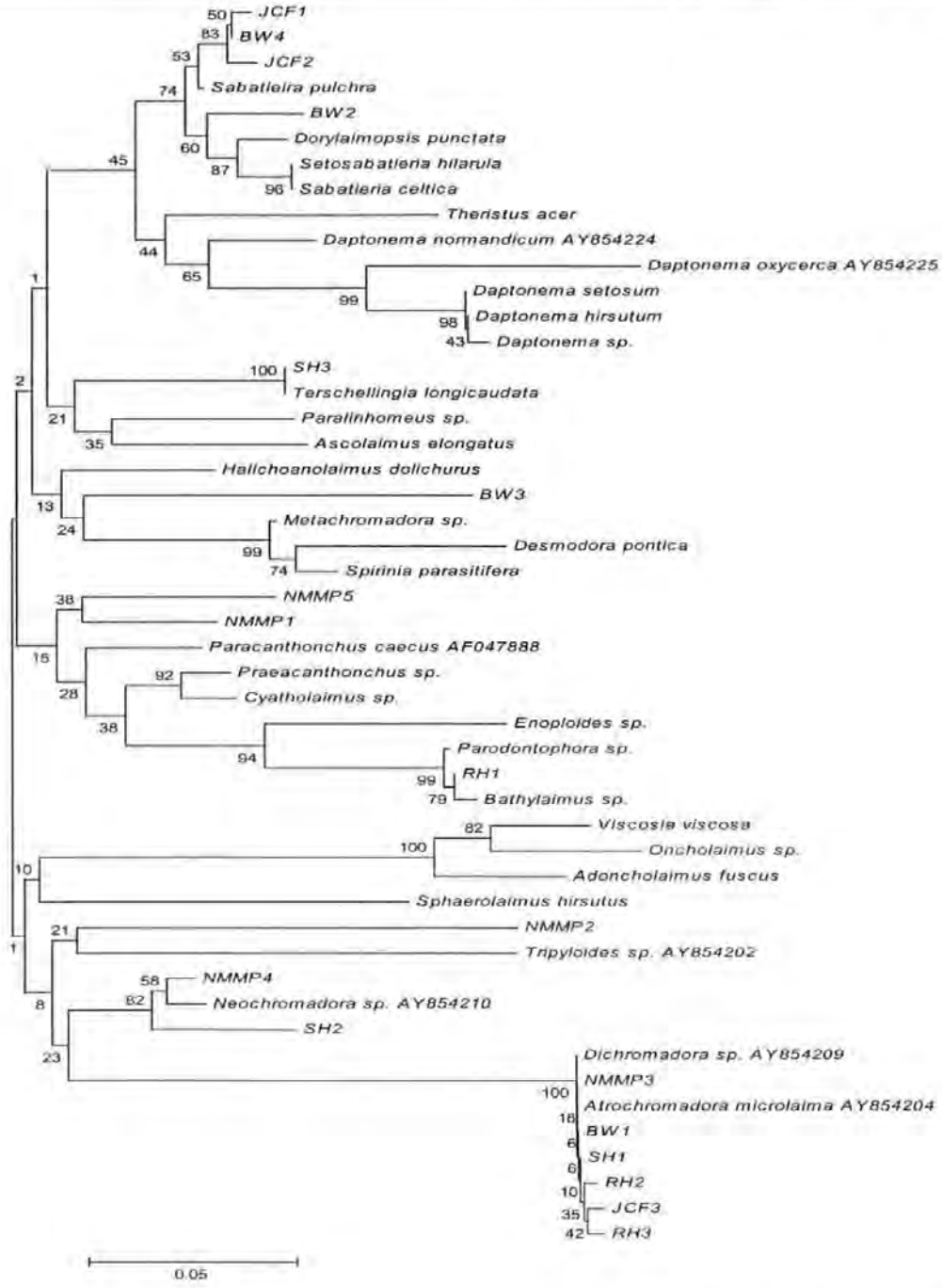


Figure 3.9: Neighbour-Joining tree showing the relationship between the DGGE bands amplified using MN18F and 22R primers and most similar sequences of known nematodes. The distance scale indicates 0.05 substitutions/site. Numbers beside branches indicate bootstrap values (1,000 replicates).

3.3.9 Influence of sediment sample sizes on assessment of nematode diversity

There were characteristic banding patterns for each of the sediment sample sizes (0.01 g to 1.0 g sediment) (Figure 3.10). A graphical representation of the relation between

sample size and number of taxa observed in denaturing gels is plotted in Figure 3.11. The ribotype diversity for 0.01 g sediment replicates was between 8 and 10 taxa while for 1.0 g sediment replicates it was between 16 and 18 taxa. Additionally, replicate 1 of the 0.01 g sediment showed unique band positions compared to the other three replicates. The average ribotype diversity of the 0.1 g sediment replicates was somewhere between 10 and 12. The results of the DNA extraction method comparisons also showed characteristic banding results. The number of visible bands for the 0.1 g sediment extracted using the SoilMaster DNA Kit was somewhere between 10 and 12 whereas using the Powersoil DNA Kit the diversity was approximately between 12 and 15 taxa (Figure 3.10).

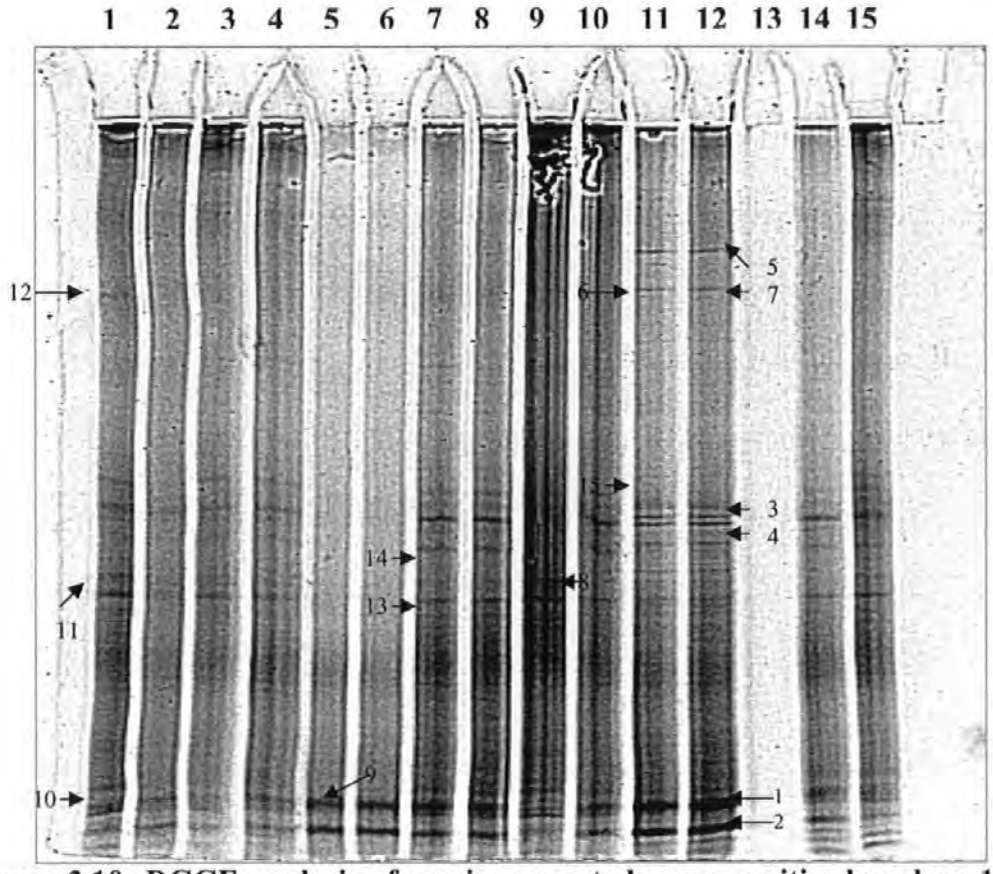


Figure 3.10: DGGE analysis of marine nematode communities based on 18S rRNA amplicons from replicates of different sediment sizes; Lanes 1-4: replicate 1-4 with 0.01 g sediment; Lane 5-8: replicate 5-8 with 0.1 g sediment; Lane 9-12: replicate 9-12 with 1.0 g sediment; Lane 13: negative; Lane 14: 0.01 g sediment sample extracted using the SoilMaster DNA Kit; Lane 15; 0.01 g sediment sample extracted using the PowerSoil DNA Extraction Kit. (Arrows indicate bands that were extracted and sequenced).

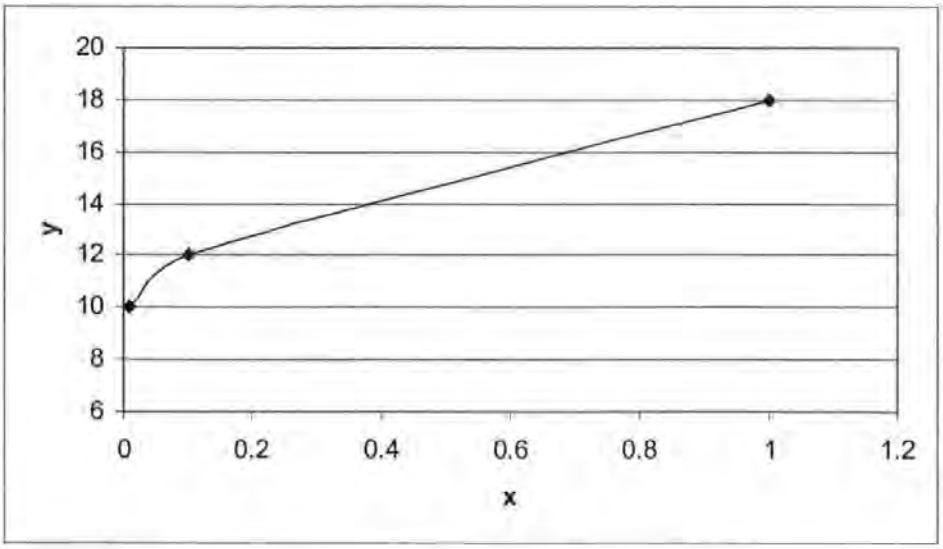


Figure 3.11: Graph showing the relationship between sample size and observed taxa in DGGE gels where x= amount of sediment (g) and y= number of taxa (as represented by number of bands on the gel).

3.3.10 Investigating eukaryotic assemblages in nematodes from marine and estuarine environments

PCR amplicons of putative nematode DNA from each site were electrophoresed in denaturing gels. A significant proportion of the excised bands from Jennycliff and Plymouth Breakwater showed similarities with fungal 18S rRNA sequences in addition to nematode 18S rRNA sequences held online at GenBank and EMBL. The fungal sequences showed homologies with *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes), *Verticillium insectorum* (Deuteromycotina: Hyphomycetes), *Syspastospora parasitica* (Ascomycota: Sordariales) and *Rhinocladiella aquaspersa* (Deuteromycotina: Hyphomycetes) sequences. All the excised bands from the Plym and Tamar estuaries showed sequence similarity with nematode ribosomal sequences (97% to 99% similarity). No fungal sequences were detected in Plym and Tamar estuary specimens. Artefacts were also detected in some cases.

Scanning electron microscope images of nematode worms from Jennycliff and Plymouth Breakwater showed the presence of some secondary structures on their body

surfaces (Figure 3.12). Some of these structures were globular and some of them resembled fungal hyphal attachments.

18S rRNA gene sequencing of the fungal strains from Jennycliff and Plymouth Breakwater sediment samples did not provide any additional information. Only one strain of fungus was isolated from the starch agar media and the ribosomal sequence showed homologies to *Aspergillus* sp. sequences held online in GenBank and EMBL databases.

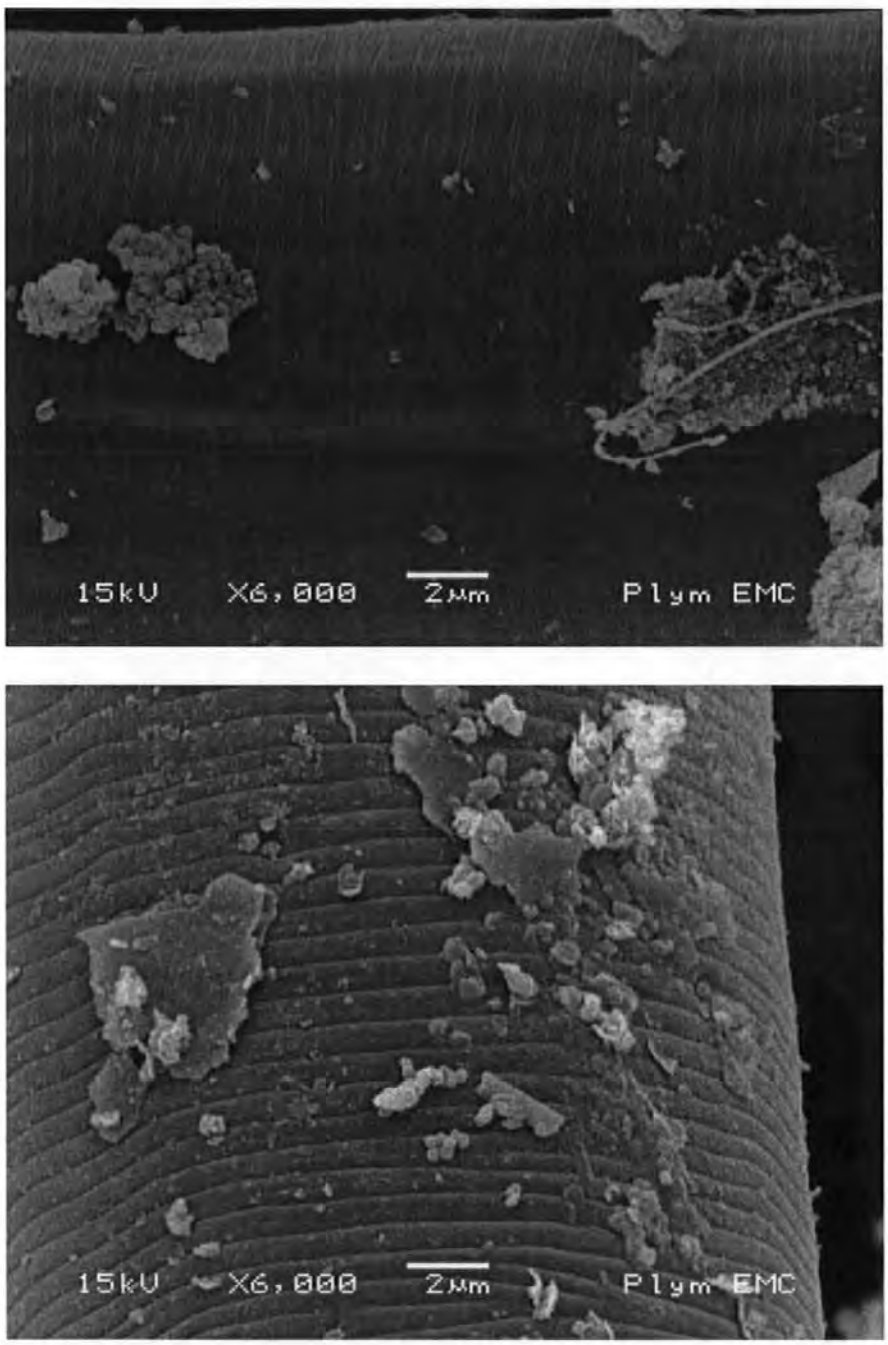


Figure 3.12: Scanning electron micrograph images of nematode body surfaces showing hyphae-like and globular structures.

3.4 Discussion

In this Chapter, denaturing gradient gel electrophoresis was employed to evaluate its potential for identifying individual species of marine nematodes and to explore nematode diversity from estuarine and marine environments as well as to investigate eukaryotic assemblages in nematodes. Throughout this study *Pfu* DNA polymerase was used instead of *Taq* DNA polymerase (except in few cases) for PCR amplifications because of its better proofreading properties (Lundberg *et al.*, 1991; Flaman *et al.*, 1994; Cline *et al.*, 1996). Firstly, from a methodological viewpoint, the separation of ribosomal RNA amplicons from three different taxa individually and as a mixture in denaturing gradients clearly demonstrates that there is no template bias during PCR amplification and amplicons can be separated by DGGE. The unique band position for each taxon could be employed as a molecular tool for identification of marine nematode species. This tool may be particularly useful to characterize deep sea-nematodes, since to date very little is known about them in terms of either genetic diversity or traditional taxonomy. For the nematode diversity studies involving amplification of DNA templates extracted from sediment samples, artefactual double bands have been eliminated by increasing the final extension time period during PCR to thirty minutes.

One of the principal aims of this study was to critically evaluate the DGGE methodology using DNA extracted from marine and estuarine sediments so as to determine the concentration at which target DNA could be amplified to a level sufficient for detection in a denaturing gel. The technique was able to detect PCR products greater than nanogram level where sample had original template of at least 0.25 ng of DNA. In estuarine and marine sediments only a few nematode species tend to be abundant, with many of the species occurring as only a few or even single individuals in an environmental sample (Heip *et al.*, 1985). This is almost certainly the reason that the DGGE analysis of an environmental sample from Saltash (Tamar estuary) and other sites detected only 15 ribotypes or 15 putative taxa. Moreover differences in diversity between sites were not

evident in the denaturing gel. While the total nematodes collected and subsequently electrophoresed from habitats such as the sandy sediment of Cawsand would normally show different ribotype diversity in comparison to that from Rame Head which is characterised by deep sub tidal sediment, the DGGE gel (Figure 3.4) on the other hand showed almost similar ribotype pattern between the sites and presence of additional bands in few sites. When a morphological analysis of half of the sample from Saltash (Tamar estuary) was carried out using traditional taxonomic methods it showed the presence of more than 25 marine nematode taxa. There may be have been some minor differences in terms of species composition within two halves of the same Saltash sample, but it is unlikely that they will be sufficient to explain the considerable difference in numbers of taxa detected using molecular versus morphological methods. Such variation between morphological and molecular datasets indicated that possibly preferential amplification led to biased amplicon production during PCR and DNA from abundant taxa were getting preferentially amplified over other taxa which were represented in very few numbers even though all the worms were carefully extracted from sediments and picked out under a microscope for DNA extraction and subsequent PCR DGGE analysis. Detailed morphological studies carried out previously from Saltash (Tamar estuary) have shown the presence of at least 40 different species of marine nematodes (Austen, 1986). Many of the species occur in low numbers while a few others such as *Terschellingia longicaudata*, *Terschellingia communis*, *Sabatieria pulchra* and *Ptycholaimellus ponticus* form the major part of the nematode community (Austen, 1986). The assumption of preferential amplification of abundant taxa is well supported by the fact that excision of bands from the DGGE gel and subsequent sequencing and phylogenetic analysis revealed the presence of the dominant taxa from these sites. These results show significant similarity with the DGGE results for microbial communities, where the method detects abundant taxa within a sample (Chan *et al.*, 2002; Koizumi *et al.*, 2003). It is evident that the DGGE approach is resolving the dominant species and not the less abundant taxa. The DGGE results for

marine nematodes also show a marked similarity with the soil nematode study where DGGE detects only a small percent of the taxa actually present (Foucher *et al.*, 2004). This conclusion is once again supported by the nematode phylogenetic tree constructed on excised band sequences and known marine nematode sequences, where some of the sequences resolved into groups of known marine nematode sequences which are thought to be dominant in estuarine and marine environments. Such lack of congruity between morphological and molecular methods has been also observed while assessing eukaryotic diversity of other organisms in marine environments (Savin *et al.*, 2004).

The major emphasis of this chapter was to develop and evaluate a method that could be employed rapidly to assess nematode diversity directly from the natural sediment environment without undergoing the time consuming process of extracting all the nematodes from sediments with subsequent PCR amplification and DGGE. Two protocols namely, Macrae *et al.* (2001) and FastDNA® kit that relied on the principal of bead beating, homogenisation and subsequent lysis were applied in this study. DNA extracted by a FastDNA Spin Kit was amplified using the universal primer set and electrophoresed in a denaturing gel. Subsequent band excision and sequencing revealed co-amplification of eukaryotic 18S rRNA genes in addition to marine nematode 18S rRNA amplicons. Amplified fungal 18S rRNA and other eukaryotic 18S rRNA genes were recorded from Plymouth Breakwater and Jennycliff in Plymouth Sound and also from Rame Head. This indicated that the consensus primers initially designed on nematode 18S rRNA sequences were picking up ribosomal regions from other eukaryotes possibly because of the high abundance of the ribosomal gene from these organisms in DNA templates extracted from sediment samples.

As a result, the forward primer was re-designed so that it could selectively amplify nematode 18S rRNA regions from estuarine and marine sediments. A new region around 100 bp inward from the 5' end of the 18S rRNA molecule was selected for consensus primer design where the variable region was flanked by conserved regions. The newly

designed primer was based on an alignment of nematode and fungal sequences from GenBank and EMBL databases. The re-designed forward primer, which is located 100 bp inward from the 5' end of the 18S rRNA gene, along with the reverse primer, was tested on total DNA extracted from sediments for PCR and subsequent DGGE analysis. The amplicons resolved clearly in a 25%-60% denaturing gradient. To evaluate whether the primers were amplifying only nematode 18S rRNA, a number of bands were excised from the gel, re-amplified, cloned and sequenced. All the bands showed high sequence similarity with nematode sequences indicating that the primers as well as the DGGE technique were capable of targeting and resolving 18S rRNA of marine nematodes from environmental sediment samples.

DGGE of PCRs using nematode specific primers on DNA directly extracted from the sediment showed unique ribotype diversity and there was some degree of variation between the sites. For some sites the number of bands visible on the gel was possibly eight while for others it was close to ten and therefore each band in the gel possibly represented a single taxon. This however contrasts with published data available for Rame Head, Jennycliff and Saltash where mean numbers of species were 35, 35 and 18 (Austen and Warwick, 1989; Austen and McEvoy, 1997a, Austen *et al.*, 2003) from 50 gm and 70 gm of sediment. In a previous study, Cook *et al.* (2005) recorded more than 20 different taxa from a sediment sample in the Tamar estuary, South-West England. Despite this, approximately 5-10 species constitute more than 80% of nematode abundance at some of these sites (Austen and Warwick 1989, MC Austen unpublished), such patterns being typical for sub-tidal marine sediments (Heip *et al.*, 1985). It was apparent, therefore that the DGGE approach was once again resolving only the dominant marine nematode species and not the less abundant taxa. This is visible from the phylogenetic tree where some of the sequences resolved into groups of known marine nematode sequences which are dominant in these environments. The results of the DGGE based on direct amplification of total

DNA from sediments showed marked similarity, in terms of failing to resolve the less abundant taxa, to the previous study conducted by Cook *et al.* (2005).

It appears then that the DGGE measure of diversity is rapid but will probably only be effective for monitoring patterns of diversity within the dominant component of the nematode community in estuarine and marine environments. The differences in band intensity in a denaturing gel could also perhaps be used as a surrogate measure of relative abundance and the number of bands used to indicate taxon richness, with a larger number of bands probably being indicative of taxon-rich environments (Sievert *et al.*, 1999; Smalla *et al.*, 2001). In this study the nematode diversity pattern showed similarities with previously observed microbial diversity patterns where the true diversity is probably underestimated in complex communities because taxa that are of low abundance generally go undetected (Muyzer *et al.*, 1993; Holben *et al.*, 2004). However the methodology has been improved in these studies and the new primers developed were more nematode-specific in comparison to the primers used by Foucher and Wilson (2002), Waite *et al.* (2003) and Cook *et al.* (2005) for DGGE studies, or in previous studies related to nematode phylogenetics and soil nematode barcoding (Blaxter *et al.*, 1998; Floyd *et al.*, 2002).

Quantification of organisms by PCR-based methods may result in certain biases and this could have resulted in biased ribotype diversity patterns in DGGE gels from the above studies. PCR biases could be due to the differences in rRNA gene copy number, which may be very important for eukaryotic organisms which may contain up to several thousand copies of the rRNA gene per genome. Moreover during PCR some phylotypes can be preferentially amplified because of preferential priming or differences in elongation rates between amplicons. There also is also evidence that bias in a PCR can occur if the number of cycles is increased, because the amplicons tend to reach equimolar concentration according to the kinetic model (Suzuki *et al.*, 1998). A combination of all these factors can

change the relative concentration of PCR products so that the resulting ribotypes probably do not reflect the actual composition of the native community.

Very little success was achieved when a modified Macrae *et al.* (2001) protocol was used to extract DNA from natural samples. Ribotype diversity was completely different from that observed in environmental samples extracted using the commercially available FastDNA Spin Kit. Although a higher volume of sediment was used (1gm) to extract DNA using the Macrae *et al.* (2001) protocol, the difference in diversity was still not clearly visible in a denaturing gel. The bead beating technique was possibly ineffective in recovering DNA from sediments or it could have also resulted in shearing of DNA. Potential bias in PCR amplification could also have yielded low diversity patterns. Presence of PCR-inhibitory substances in sediment might have also affected the overall the PCR yield (Miller, 2001; Wilson, 1997). There is evidence that humic acids or humic substances co-extracted with nucleic acids strongly inhibit DNA polymerase enzymes. Tebbe and Vahjen (1993) used a commercial preparation of humic acids and found minimum inhibitory concentrations of 0.64, 0.16 and 0.08 $\mu g\ mL^{-1}$ for three *Taq* DNA polymerases. Another reason could have been the loss of nucleic acids in the purification steps, although the spectrometry data do not back up this suggestion

To determine the influence of sediment sample size on nematode diversity assessment four replicates of three sample sizes were subjected to PCR and DGGE analysis. There was an increase in the ribotype diversity between the replicates when the sediment sample size was gradually increased. This result agrees with literature data that shows that diversity is closely linked to the size of the sample, making the analysis of larger or replicated samples critical in establishing taxon diversity (Williams, 1964; Sanders and Hessler, 1969; Browne, 1981; Hutson, 1994; Kendall *et al.*, 2003; Vives and Salicrú, 2005). However, careful analysis of the diversity pattern in the denaturing gel revealed some interesting results. Some of the bands that were visible in the 0.01 g replicates were not evident in 0.1 g and 1.0 g replicates. The conclusion has been supported

by sequencing some of the bands from three sample sizes. For example, Band 11 and 12 from the 0.01 g replicate were not present in the other two sample sizes indicating that rare species go undetected in larger samples possibly because DNA from dominant species tends to get preferentially amplified. This probably suggests that some of the marine nematode taxa present in very low numbers remain undetected when a large sample size is used for diversity studies. It also supports the hypothesis that the PCR technique, which is *in vitro*, most likely amplifies the dominant nematodes preferentially to rare nematodes since these are present in higher number in bigger sediment volumes as compared to smaller volumes. This means there is even less chance that rare species are detected in larger samples. To get a true idea about the nematode diversity from estuarine and marine environments using the DGGE methodology, it is likely to be necessary to include sample sizes of several low volumes such as 0.01 g. Valuable information in terms of nematode diversity might be overlooked if assessments of nematode communities are based on large samples alone. The sediment sample size results showed marked similarities to those from bacterial communities, where replicates of small samples show more variation in genetic community structure than large samples (Santegoeds *et al.*, 1996; Ellingsøe and Johnsen, 2002).

To check that there was no bias during DNA extraction from sediments, two extraction methods using commercial kits were evaluated. The DGGE profiles revealed that nematode diversity assessments were probably dependent on the DNA extraction method used. The ribotype diversity was comparatively higher for samples extracted using the Power Soil method, which employs a humic acid removal procedure, than those using the Soil Master method. Such bias in different DNA extraction methods has been recorded in prokaryotic microbial diversity studies (Kresk and Wellington, 1999; Martin-Laurent *et al.*, 2001; Webster *et al.*, 2003). In conclusion, sediment sample size and DNA extraction methods can affect the apparent abundance and composition of the nematode community

and overall estimate of nematode diversity when assessed by DGGE. PCR bias can also occur.

It was difficult to find or confirm the existence of a relationship between nematodes and other eukaryotes in the assemblages. In this study co-amplification of fungal 18S rRNA was detected frequently through sequencing with primers that had been used previously for nematode phylogenetic studies. Most of this co-amplification was detected in nematodes from Jennycliff and Plymouth Breakwater. No fungal co-amplification was observed in nematodes from the Plym and Tamar estuary sites. Additionally, almost similar fungal homologies were also detected from total sediment DNA templates from Jennycliff and Plymouth Breakwater. Most of the fungal taxa that were detected in this study were found to be entomopathogenic, facultative (can grow in marine and terrestrial environments) and are actively used in microbial pesticides. The presence of fungal assemblages on nematode body surfaces was also hypothesised in this study. These might be similar to prokaryotic microbial assemblages that are found associated with the nematode *Eubostrichus dianae*, for example (Polz *et al.*, 1999). DGGE profiling of nematodes confirmed the presence of these fungi in Plymouth Sound only. Most of the fungal sequences showed similarities with sequences of *Paecilomyces fumosoroseus*, *Verticillium insectorum*, *Syspastospora parasitica* and *Rhinocladiella aquaspersa* held online at GenBank and EMBL databases. At the same time scanning electron microscope images did show some epizoic structures on nematode body surfaces. These structures were mainly globular but in some cases resembled fungal hyphal attachments. To ensure that these fungi were not present in the sediments of Jennycliff and Plymouth Breakwater, fungal cultures were grown and isolated and subsequently identified based on 18S rRNA sequences. Only one strain was successfully grown indicating the difficulties that are usually associated with growing marine derived fungi under laboratory conditions. Although the cultured strain did not conform to any of the previously identified fungal taxa based on 18S rRNA sequences, it was difficult to confirm the absence of taxa such as

Paecilomyces fumosoroseus, *Verticillium insectorum* or *Syspastospora parasitica* in sediment samples in and around Plymouth Breakwater and Jennycliff. Overall there was no clear evidence that fungal assemblages were growing in microenvironments such as the nematode body surface. Clearly more work is needed to investigate and understand the extent of fungal amplification in nematode worms from Jennycliff and Plymouth Breakwater. It may be that parasitic or symbiotic relationships occur between fungi and some nematodes in the waters of Plymouth Sound.

There is also a possibility that the fungal 18S rRNA genes that were getting picked up consistently during PCR amplification were ingested materials or intestinal contents of nematode worms. Thomas *et al.* (1997) have reported such possibilities in a previous study. On the other hand absence of fungal or any other eukaryotic sequences in nematodes from the Plym and Tamar estuaries makes it more speculative whether these fungi were actually part of the gut contents. In another study, R. Floyd *et al.* (unpublished) amplified a wide range of fungi along with nematode 18S rRNA. When isolating DNA of small organisms such as nematodes from complex marine environments it becomes difficult to avoid extracting DNA from a wide range of biological materials and therefore co-amplification is always a possibility. Such problems can be avoided by designing group-specific primers that will selectively amplify organisms of interest rather than co-amplifying regions of other eukaryotic ribosomal RNA. This approach has been adopted in this study where nematode specific primers were designed and evaluated for DGGE studies.

To conclude it seems that currently DGGE is only capable of identifying relatively abundant taxa in an environmental sample. This is suitable for identifying major changes in the species composition between samples, but not for a direct assessment of species richness; thus, the technique is useful as a rapid system for community analysis in a similar manner to the way it is used for microbial ecological studies. The primers that were designed and used in this study were very specific and would work well in detecting

marine nematodes from estuarine and marine environments. Even with several limitations DGGE provides a useful way of detecting changes in communities of marine animals that are small or difficult to identify (including larvae). It has also been shown that selection of sediment sample size and DNA extraction procedures can affect the interpretation of the nematode diversity when assessed by DGGE. In future DGGE could be implemented in combination with small and large volume of sediment samples in order to give a more accurate estimate of nematode diversity.

4. DNA sequence-based approaches to marine nematode identification

4.1 Introduction

Taxonomy forms the basis for much biological research. Recently, for example, much effort has been focused on understanding the biological impacts of climate change (e.g. Caldeira and Wickett, 2003; Lynam *et al.*, 2004; Hays *et al.*, 2005), research which often depends upon taxonomic identifiers to monitor any changes which may be occurring in the biota. These changes are happening at a time when there is a serious crisis in taxonomic expertise throughout the scientific community (Gaston and May, 1992; Daly, 1995; May, 1997; Buyck, 1999; Lammers, 1999; McAllister, 2000; Hopkins and Freckleton, 2002), resulting in the neglect of many highly diverse groups of organisms. This is particularly the case for organisms that live in marine habitats, especially those from benthic sediments. These habitats contain species-rich communities of metazoans including large numbers of nematodes, polychaetes, crustaceans and molluscs (Grassle and Maciolek, 1992; Heip *et al.*, 1985; Coull, 1999; Austen, 2004; Lambshead, 2004). Free living marine nematode worms, which are numerically the most abundant component of the marine meiofauna, are often difficult to identify and require considerable taxonomic expertise (see Chapter 1). Moreover, the existence of cryptic species complexes, whose members may have different functional responses, and difficulties in the identification of juvenile stages, have resulted in the relative neglect of nematodes in many meiofaunal studies (Warwick and Robinson, 2000; Cook *et al.*, 2005). Marine ecologists typically view nematode identification as a laborious and specialist task, beyond the scope of those engaged in routine surveys.

So-called DNA barcoding offers one route to increase the speed of marine nematode species identification in ecological or biomonitoring studies. The following section discusses the rationale and use of DNA barcodes, including their application across different animal phyla. A DNA barcoding approach is then developed and applied, as a

novel way to identify free living nematodes from estuarine and marine environments based on the sequencing of particular regions of the genome.

4.1.1 The concept of DNA barcoding

Genomic identification systems, which represent ways of discriminating organisms based on the analysis of a small segment of the genome, show considerable promise for rapid species diagnosis (Hebert *et al.*, 2003). Indeed genomic identification approaches based on short DNA sequences have become common practice in prokaryotic studies. In the last decade or so, studies involving amplification and sequencing of the 16S rRNA gene have revolutionised microbial research independent of culture-dependent methods (Giovannoni *et al.*, 1990; Fuhrman *et al.*, 1992; Woese, 1996; Pace, 1997; Hugenholtz *et al.*, 1998; Allander *et al.*, 2001). DNA sequence data has led to the establishment of the 'genospecies' concept in bacterial studies where organisms are identified on the basis of sequence identity (Stackebrandt and Goebel, 1994; Cohan, 2002). On the other hand this 'molecularisation' of taxonomy has been considerably slower in multicellular organisms, largely due to serious reservations among the taxonomic community and the existence of morphology-based alternatives (Dunn, 2003; Seberg *et al.*, 2003; see Section 4.1.4).

Within an individual's genome there is enough space for storing information on individual identity and group membership (Blaxter, 2004). For example, it has been estimated that within the human population each unrelated pair of individuals will differ at around 0.1% of their DNA bases (Blaxter, 2004). Such differences within a taxon are largely distributed as clusters in the intronic and intergenic regions of the genome (Blaxter 2004). Thus there are also regions of the genome that are more or less identical between the members of a taxon, but at the same time vary between taxa. These regions of the genome are potentially useful for barcoding as their DNA sequences hold the necessary information from (their past or recent) evolutionary history. Therefore a DNA barcode in the form of sequences carries both specific and taxonomic data for an organism. Like the

machine readable tagging systems that hold necessary information about a product, individual genomic regions hold specific information about the identity and relationship of taxa, and act as barcodes (Hebert *et al.*, 2003; Consortium for the Barcode of Life website).

4.1.2 Molecular markers for DNA barcoding

The choice of molecular marker for barcoding depends on the following factors: (i) the ease of isolation and amplification from a sample (ii) copy number within the genome (iii) presence of conserved flanking sites (iv) variation within and between individuals (v) ease of alignment and further analysis (vi) number of known sequences from identified specimens and (vii) suitability of the marker for barcoding (Blaxter, 2004; Kress *et al.*, 2005).

Researchers like Saccone *et al.* (1999) have argued for the use of mitochondrial genomic regions for barcoding animal taxa on the basis of lack of introns, limited exposure to recombination and haploid nature of inheritance. Hebert *et al.* (2003) have proposed mitochondrial cytochrome c oxidase I gene (COXI) for barcoding metazoan targets for two reasons: The universal primers for this gene can amplify the 5' end of the molecule from almost all animal phyla (Folmer *et al.*, 1994; Zhang and Hewitt, 1997), and COXI has a greater phylogenetic signal than any other mitochondrial gene. Knowlton and Weight (1998) noted that in the COXI gene, third position nucleotides show a high incidence of base substitutions in comparison to other protein-coding genes resulting in a rate of molecular evolution which is three times greater than the 12S or 16S rRNA genes of mitochondria. Cox and Hebert (2001) and Wares and Cunningham (2001) also found that the COXI gene could discriminate not only closely allied species but phylogenetic groups within one species. Although the cytochrome c oxidase I gene has been widely used across different phyla for molecular barcoding (Hebert *et al.*, 2003; Hogg and Hebert, 2004; Lambert *et al.*, 2005), PCR success rates are well below 50% in many groups within the phylum Nematoda (De Ley *et al.*, 2005). Cook *et al.* (2005) were faced with this problem

while trying to amplify COI from marine nematode taxa from British waters. Reasons for such difficulties using universal primers may relate to the emerging evidence that nematode mitochondrial genomes are highly variable and prone to recombination (Lunt and Hyman, 1997), insertional editing (Vanfleteren and Vierstraete, 1999) and multipartitioning (Armstrong *et al.*, 2000). As a consequence, designing cytochrome c oxidase I primers that would work universally across different taxa in this phylum may be genuinely impossible.

The nuclear 18S rRNA has also received great attention as a barcoding locus, this time largely in micro-eukaryotes such as plankton, in fungi and in nematodes (Floyd *et al.*, 2002; Massana *et al.*, 2002; Blaxter, 2004; Powers, 2004; Cook *et al.*, 2005). The 18S rRNA gene generally has a high success rate with PCR but this requires optimization in different phyla. Polymorphism is very rare in the 18S rRNA molecule and the sequence carries high phylogenetic resolution. The LSU gene is also truly universal and is found in every organism and there are universal primer sets that work well across most animal phyla. Intraspecific polymorphism within this molecule is very limited and it apparently performs well in cryptic species identification studies (De Ley *et al.*, 1999; Omilian and Taylor, 2001). In nematodes, the D2/D3 segment (a variable region of the LSU gene) has been widely used for phylogenetic studies and has recently been tested for molecular barcoding in terrestrial nematode taxa and a handful of marine nematode taxa (De Ley *et al.*, 2005).

In contrast to the above, markers such as the internal transcribed spacer region (ITS) have seen limited applications in molecular diagnostic studies, largely due to the nature of the molecule. Of particular concern is the fact that the ITS region often varies by insertions or deletions within an individual, making sequencing very difficult (Elbadri *et al.*, 2002). As a result phylogeny reconstruction based on ITS sequences is often hampered by problems with alignment because of insertions or deletions. Several recent studies in nematodes have revealed the occurrence of multiple ITS haplotypes and high degrees of

polymorphism, making direct sequencing impossible (Hugall *et al.*, 1999). Nevertheless the ITS region has been used for identification of nematodes of socio-economic importance (Powers, 2004). Presently there are more than 30,000 sequences each for the ITS and SSU genes across all taxa and roughly 17,000 sequences representing COI and rbcL genes in various databases (*pers obs*).

4.1.3 Advantages of DNA barcodes

One of the great advantages of DNA barcoding is that a single technique can be applied to all taxa across different phyla. The technique, which involves DNA extraction, PCR amplification of a genomic region and DNA sequencing, can be applied on a large inventory (Figure 4.1). Sequences can be obtained from single specimens irrespective of their life cycle stages, in many cases without affecting morphological identification. Moreover all life stages are amenable to study, as the system depends on genotype not phenotype (Gaston and O'Neill, 2004), and little information is required regarding the taxonomy of the group studied. Similar techniques could also be applied to environmental DNA to detect the presence of certain groups of organisms. Already PCR-cloning-sequencing approaches from environmental DNA have yielded valuable information about the micro-eukaryotes that are found in oceans and deep seas (Massana *et al.*, 2004; Countway *et al.*, 2005).

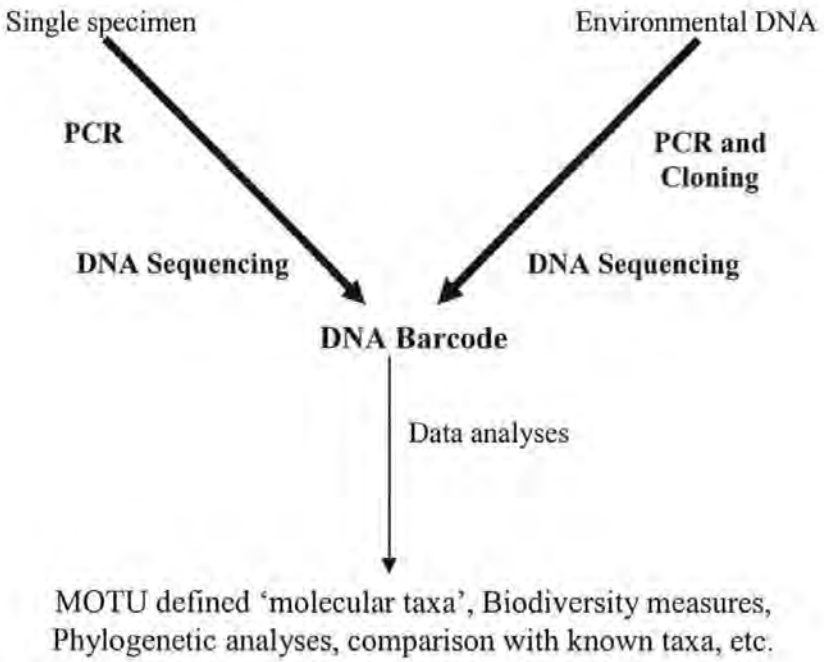


Figure 4.1: Diagrammatic representation of the process of DNA barcoding for taxon identification (modified from Blaxter, 2004).

Barcoding techniques are potentially applicable where traditional methods are unrevealing: identification of eggs for instance, and analysis of stomach contents or excreta may assist in the determination of food web structure. In addition, DNA-based identification could be useful in highlighting specimens that represent undescribed, often cryptic taxa. At the same time sequences generated from specimens could be used to place MOTUs (Molecular Operational Taxonomic Units) within the phylogenetic hierarchy. A specimen's barcode could be compared with existing sequences, and when a close match is found traditional keys and monographs could then be employed to help understand the biological properties of the identified MOTU and their close relatives (Floyd *et al.*, 2002) followed by phylogenetic analyses which generate testable hypotheses of MOTU relatedness. Therefore the success of the MOTU approach is usually dependent upon two factors. Primarily the DNA segment to be used as a molecular barcode should be orthologous between species and secondly it must encompass sufficient variability to allow discrimination between biological species.

4.1.4 Drawbacks of barcoding

As with any other technique or methodology, DNA barcodes have certain drawbacks, some of which are considered here. Often differences in sequences between supposedly conspecific specimens arise, which could be due to either within-OTU variation or methodological error. In terms of the former, multiple copies of particular genes within individuals can differ in sequence. A classic example of this is seen in *Plasmodium falciparum* (malaria parasite) which has multiple different ribosomal RNA cistrons that are differentially expressed over its life cycle (Mercereau-Puijalon *et al.*, 2002). Such a situation can also arise when the nuclear and organellar genomes are heterozygous and therefore the target gene differs in sequence (Blaxter, 2004). It has been also recorded in different organisms such as *Trypanosoma cruzi* (Zingales *et al.*, 1999). On the other hand methodological errors can potentially be rectified by multiple resequencing of the specimen in order to assess both PCR and sequencing-related errors. Generally sequencing directly from PCR products rather than clones eliminates most PCR-introduced error as well as the problem of chimaeric clones which arise from between-amplicon priming. High throughput sequencing technologies that are available nowadays are generally robust as well as accurate and therefore experimental error rates are much lower than formerly.

Another key drawback of barcoding is essentially philosophical, since some taxonomists have questioned whether DNA barcodes for species can actually replace morphological identification and classification systems. It has been argued that DNA barcoding would diminish rather than enhance traditional morphology-based taxonomy. Some researchers have speculated that the amount of genetic divergence in some molecular markers used for barcoding may lead to incorrect species recognition whereas other have raised the question about the increased use of DNA barcodes for phylogeny reconstruction whereas its main objective is actually identification (Kress *et al.*, 2005). Some reviews have widely questioned the overall validity of the DNA barcoding concept (Stoeckle,

2003; Lee, 2004; Will and Rubinoff, 2004). Nevertheless proper implementation of methodologies in DNA barcoding studies along with validation of generated DNA sequences is proving useful in conjunction with traditional taxonomy in order to provide necessary detailed information towards the identification of difficult groups of organisms. Increasingly barcoding approaches based on amplification and sequencing is being applied to different groups of organisms (Blaxter *et al.*, 2005; Chase *et al.*, 2005; Hajibabaei *et al.*, 2005).

To conclude it seems that DNA barcoding is in fact a potentially very useful approach in a range of biodiversity studies especially for groups such as nematodes where traditional taxonomy is immature and relatively difficult. In these groups of organisms DNA barcoding could speed up the process of identification which in turn would assist greatly with biodiversity and biomonitoring studies. At the same time, from a taxonomic point of view care has to be taken to ensure that DNA barcoding is not used as the sole method to identify species, but should be employed in conjunction with morphology based taxonomy and at the same time a good quality control should be in place to ensure that the raw data (DNA sequences) generated from the studies are of highest quality.

4.1.5 DNA Barcoding in practice

The success of prokaryotic studies based on amplification and sequencing of the 16S rRNA gene prompted many researchers to test similar methods on eukaryotic systems. Based on the 18S rRNA gene library and sequencing, an incredible amount of previously underestimated eukaryotic diversity, mainly in planktonic groups, has been unearthed from deep sea, open ocean and deep-sea vent environments (Diez *et al.*, 2001a; Lopez-Garcia *et al.*, 2001; Massana *et al.*, 2002; Moreira and Lopez-Garcia, 2002). In each case novel sequence-defined taxa have been discovered, including some belonging to recently recognized lineages such as stramenopiles and alveolates (Diez *et al.*, 2001a). Incidentally some of these micro-eukaryotes were included with the prokaryotes before the DNA

sequencing approach was undertaken (Diez *et al.*, 2001a; Massana *et al.*, 2002). Similar approaches have been adopted with great success in other eukaryotes such as soil and rhizosphere fungi where morphological identification based on the examination of spores is often problematic (Vandenkoornhuyse *et al.*, 2002) and also in endophytic fungi (Guo *et al.*, 2000).

Implementation of DNA barcoding approaches in the animal kingdom, including soft bodied metazoans, has been comparatively slow when compared to prokaryotes and micro-eukaryotes. Vences *et al.* (2005) demonstrated that the mitochondrial 16S rRNA gene fulfils the requirements for a universal DNA barcoding marker in amphibians. It has been argued that 16S rRNA could be used as a standard DNA barcoding marker for vertebrates as a complement to the COI gene (Vences *et al.*, 2005). Recently, Hebert and colleagues have used the mitochondrial COI gene as a metazoan barcoding target across different phyla. One of his foremost works was the development of a COI profile from 200 closely allied species of lepidopterans, which was 100% successful in correctly identifying subsequent specimens based on COI sequences (Hebert *et al.*, 2003). Similar approaches have been undertaken for biological identification of springtails (Hexapoda: Collembola) (Hogg and Hebert, 2004). Barrett and Hebert (2005) employed the DNA barcoding concept in spiders which contain around 40,000 species and often represent difficulties in species identification. They generated COI profiles for 168 arachnid species which were then used to assign subsequent specimens to the appropriate species with a reported success rate of 100%. Hebert *et al.* (2004a) used similar approaches based on COI profile to identify birds. In that study barcodes were developed for the rapid identification of 260 species of North American birds. As a result four probable new species of birds were discovered. Even in larger organisms, molecular tags have been used to define new taxa e.g. the African forest elephant (Grubb *et al.*, 2000; Roca *et al.*, 2001). Such an approach has also yielded promising results among other groups of organisms e.g. barcoding a micro-alga (Akase *et al.*, 2004), flowering plants (Kress *et al.*, 2005), and chlorophytes (Verbruggen *et al.*,

2005). Molecular barcodes have been also used to reveal cryptic speciation in different groups of organisms, such as the neotropical skipper butterfly *Astraptes fulgerator* (Hebert *et al.*, 2004b) and the mosquito *Anopheles gambiae* (Towson *et al.*, 1999). It has been also applied for assigning unknown life-history stages to adult organisms (Hebert *et al.*, 2004b; Thomas *et al.*, 2005), and in exploratory studies to discover potentially undescribed candidate species (Hebert *et al.*, 2004a; Venter *et al.*, 2004). For the first time a new species of moth from New Guinea has been described based on DNA barcode and morphological characters (Brown *et al.*, 2003).

4.1.5.1 Barcoding in nematology

To date, molecular barcodes have been implemented for soil nematodes and nematodes of socio-economic importance (Floyd *et al.*, 2002; Powers, 2004). Floyd *et al.*, (2002) sequenced the 5' end segment of the 18S rRNA gene to barcode unknown soil nematodes and subsequently developed a molecular operational taxonomic unit (MOTU) scheme where sequences from unknown taxa were compared with sequences from known taxa so as to attach taxonomic and ecological attributes. Recently, De Ley *et al.* (2005) used a combinatorial approach involving morphological vouchering and barcoding of nematodes based on the highly variable D2/D3 segment of the nuclear 28S rRNA gene. Bhadury *et al.* (2005) and Cook *et al.* (2005) have also tested the suitability of the 18S rRNA gene as a marker for barcoding marine nematodes from British waters. Researchers including Tautz *et al.* (2003) have argued for an increased use of nuclear ribosomal RNA genes so as to barcode specimens across different phyla.

4.1.6 Aims of this chapter

Marine ecologists always face a problem while trying to assess the diversity of meiofauna as opposed to macrofauna from estuarine and marine environments. Assessing diversity of meiofaunal groups such as nematodes is extremely difficult, especially when

their taxonomy is immature and requires specialist skills. Thus it is obvious that different organism groups are not equally well determined. Molluscs, annelids, crustaceans are relatively easily identified because they are well documented and well studied. On the other hand some animals, such as free living marine nematode worms, flatworms and nemerteans remain mostly undetermined in a biological sample. The biodiversity of nematodes is often underestimated due to the great similarity between different species (Dorris *et al.*, 1999). Most of them are a couple of millimetres in length and taxonomic identification is based on minute morphological characters. Only a small fraction of nematode species has been described (Lambshead, 1993) and therefore only specialists with extensive taxonomic knowledge can work with this group.

DNA barcoding could be employed to facilitate as well as speed up marine nematode identification for biomonitoring and diversity studies. This concept has worked well for soil and parasitic nematodes as well as in other metazoan phyla (see above), although to date no studies have explored the use of barcodes for the identification of nematodes from estuarine and marine environments. In this chapter, the utility of nuclear and mitochondrial regions as DNA barcodes in marine nematodes is evaluated. Such an approach in turn will help fill up the 'black hole' in many ecological surveys of marine sediments.

The chapter focuses on two main aims:

- To amplify and sequence nuclear 18S rRNA and 28S rRNA genes and mitochondrial 16S rRNA and cytochrome c oxidase I (COXI) genes from representative abundant marine and estuarine nematode taxa from South West English waters so as to create DNA sequence profiles against which unknown individuals can be barcoded.
- To assess the reliability of DNA barcoding in marine nematode identification by assigning unknown specimens to genus and species level when evaluated against known DNA sequence profiles.

4.2 Materials and Methods

For general molecular methods and primer sequences see Chapter 2. For this study only sequences generated by the author, or validated sequences from the GenBank and EMBL databases (from nematode specimens identified morphologically by specialist taxonomists), have been used for phylogeny reconstructions. Other available sequences have not been used due to their possible unreliability. Accession numbers that were used in this study are detailed in the relevant sections.

4.2.1 Sediment collection

Sediments were collected subtidally from muddy and muddy sand sediments in South West England. Details of sampling locations etc. are given in Chapter Three. Additionally sediments were collected for this study from the Plym estuary (Saltram site) in South West England. All samples taken from the surface sediments were collected using a van Veen grab and immediately fixed in storage pots containing 98% molecular grade ethanol (Hayman Limited, England).

4.2.2 Meiofauna extraction and nematode identification

Meiofauna were extracted from sediments following the flotation method of Somerfield and Warwick (1996). Nematode specimens used for DNA extraction were picked from extracted samples and mounted onto slides using standard procedures described in Chapter Three (Section 3.2.2). Some nematodes reduced in size due to the effects of ethanol preservation. Nevertheless, each specimen was carefully identified to genus and species level based on morphological characters under a compound microscope using available keys for the identification of marine nematodes (Platt and Warwick 1983, 1988). After identification, each specimen was carefully removed from its slide and placed in a 0.5 mL PCR tube containing 20 µL of 0.25 M NaOH for DNA extraction.

To test the DNA barcoding concept and its applicability for taxon level identification of nematode specimens based on DNA sequences, sediments from the Tamar and Plym estuaries were fixed in molecular grade ethanol and subsequently subjected to meiofaunal extraction. Forty individuals from each site were randomly selected and placed on slides for taxonomic identification. After taxonomic identification, unique numbers were assigned to each specimen and these were randomised before being subjected to molecular analyses. The identity of individual specimens based on morphological characters and their unique reference numbers are given in Tables 4.1 and 4.2.

Table 4.1: Morphological identifications and corresponding molecular tags for specimens from the Tamar estuary used to test the barcoding concept.

Molecular tag	Morphological ID
Tamar1	*Adoncholaimus fuscus*
Tamar2	*Spirinia parasitifera*
Tamar3	*Sabatieiria* sp.
Tamar4	*Dichromadora* sp.
Tamar5	*Terschellingia longicaudata*
Tamar6	*Praeacanthonchus* sp.
Tamar7	*Enoploides brunettii*
Tamar8	*Metachromadora remanei*
Tamar9	*Sphaerolaimus hirsutus*
Tamar10	*Sabatieria celtica*
Tamar11	*Atrochromadora microlaima*
Tamar12	*Terschellingia longicaudata*
Tamar13	*Terschellingia longicaudata*
Tamar14	*Ascolaimus elongatus*
Tamar15	*Terschellingia* sp.
Tamar16	*Viscosia viscosa*
Tamar17	*Terschellingia longicaudata*
Tamar18	*Sabatieira celtica*
Tamar19	*Setosabatieria hilarula*
Tamar20	*Daptonema setosum*
Tamar21	*Paralinhomeus* sp.
Tamar22	*Sabatieira pulchra*
Tamar23	*Terschellingia longicaudata*
Tamar24	*Desmodora pontica*
Tamar25	*Halichoanolaimus dolichurus*
Tamar26	*Axonolaimus helgolandicus*

Tamar27	*Adoncholaimus* sp.
Tamar28	*Anoplostoma* sp.
Tamar29	*Terschellingia longicaudata*
Tamar30	*Theristus acer*
Tamar31	*Paracanthonchus* sp.
Tamar32	*Neochromadora* sp.
Tamar33	*Metachromadora* sp.
Tamar34	*Cyatholaimus* sp.
Tamar35	*Daptonema normandicum*
Tamar36	*Daptonema oxycerca*
Tamar37	*Terschellingia longicaudata*
Tamar38	*Metachromadora* sp.
Tamar39	*Praeacanthonchus* sp.
Tamar40	*Terschellingia longicaudata*

Table 4.2: Morphological identifications and corresponding molecular tags for specimens from the Plym estuary used to test the barcoding concept.

Molecular tag	Morphological ID
Plym1	*Praeacanthonchus* sp.
Plym2	*Anoplostoma viviparum*
Plym3	*Paracanthonchus* sp.
Plym4	*Daptonema setosum*
Plym5	*Metachromadora* sp.
Plym6	*Sabatieria pulchra*
Plym7	*Terschellingia* sp.
Plym8	*Sphaerolaimus hirsutus*
Plym9	*Theristus* sp.
Plym10	*Metachromadora* sp.
Plym11	*Terschellingia* sp.
Plym12	*Terschellingia longicaudata*
Plym13	*Paralinhomeus* sp.
Plym14	*Sphaerolaimus hirsutus*
Plym15	*Sphaerolaimus* sp.
Plym16	*Axonolaimus helgolandicus*
Plym17	*Metachromadora suecica*
Plym18	*Daptonema* sp.
Plym19	*Sabatieria* sp.
Plym20	*Daptonema hirsutum*
Plym21	*Sabatieira* sp.
Plym22	*Sabatieira* sp.
Plym23	*Enoploides* sp.
Plym24	*Adoncholaimus* sp.
Plym25	*Sphaerolaimus hirsutus*
Plym26	*Adoncholaimus* sp.

Plym27	*Enoploides* sp.
Plym28	*Sphaerolaimus hirsutus*
Plym29	Unidentified Cyatholaimid
Plym30	*Theristus acer*
Plym31	*Metachromadora remanei*
Plym32	*Metachromadora remanei*
Plym33	*Neochromadora* sp.
Plym34	*Sphaerolaimus hirsutus*
Plym35	*Paralinhomeus* sp.
Plym36	*Sphaerolaimus* sp.
Plym37	*Daptonema hirsutum*
Plym38	*Paralinhomeus* sp.
Plym39	*Terschellingia* sp.
Plym40	*Tripyloides* sp.

4.2.3 PCR amplification of the 18S rRNA gene

The 18S rRNA gene in nematodes is approximately 1700 base pairs in length. Four sets of primers were used to amplify almost the entire 18S rRNA gene from 26 marine nematode taxa commonly found in South West English waters. The primers and their respective base positions in relation to the *Caenorhabditis elegans* 18S rRNA gene are given in Table 4.3. Most of these primers have been used previously in nematode phylogenetics and molecular identification studies (Blaxter *et al.*, 1998; Floyd *et al.*, 2002; Meldal, 2004; Bhadury *et al.*, 2005; Cook *et al.*, 2005).

Table 4.3: 18S rRNA primers with their respective base positions in relation to the *Caenorhabditis elegans* 18S rRNA sequence.

Primer name	Position in *C. elegans* sequence	References
G18S4F	30-49	Blaxter *et al.* (1998)
23R	1298-1280	Blaxter *et al.* (1998)
MN18F	111-123	Bhadury *et al.* (2005)
Nem_18S_R	998-1015	Floyd *et al.* (2005)
NEMF1	737-756	P. Bhadury (unpublished)
23F	1280-1298	Blaxter *et al.* (1998)
18P	3' end	Blaxter *et al.* (1998)

4.2.3.1 Cloning and sequencing of the 18S rRNA gene

PCR fragments from the 26 marine nematode taxa were cloned with pBlueScript SK$^-$ vector and the pGEM-T Easy vector system (Promega Inc). Plasmid inserts were sequenced in both directions using the T7 and T3 primers for pBluescript SK$^-$ and M13F and M13R primers for pGEM-T vector respectively. Three to four colonies from each clone were sequenced to confirm the sequence identity. Sequence traces were checked with Chromas Pro software package (Technelysium Pty Ltd) for any ambiguities and /or errors.

4.2.3.2 Phylogenetic analysis of marine nematodes based on 18S rRNA sequences

18S rRNA sequences were aligned in Clustal-X using default parameters (Thompson *et al.*, 1997; Jeanmougin *et al.*, 1998). A phylogenetic tree was reconstructed using neighbour joining analysis and gamma-corrected Kimura distances with MEGA v2.0 (Kumar *et al.*, 2001). The NJ tree was validated using 1,000 bootstrap replicates.

4.2.4 PCR amplification of the D2/D3 segment of nuclear large subunit ribosomal RNA gene (28S rRNA)

The large subunit ribosomal gene in nematodes is approximately 3400 bp in length. A set of primers was used to amplify approximately 780 bp from the D2/D3 expansion segment of the 28S rRNA gene. The primers used in this study were D2a forward and D3b reverse (De Ley *et al.*, 2005). The forward primer corresponds to the 355-375 position while the reverse primer corresponds to the 1006-986 position with the *C. elegans* 28S ribosomal RNA gene. PCR reactions were performed in 0.5 mL tubes containing 4 µL template DNA, 5 µL 10X/$MgCl_2$ buffer, 5 µL 2 mM dNTPs, 2 µL of each of the primers, 1 µL BSA (10mg/mL) and water to a final volume of 50 µL. Thermal cycle parameters were 94°C for 5 min, 38 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 2 min followed by an extension of 10 min at 72°C and final a holding temperature of 4°C.

4.2.4.1 Cloning and sequencing of the partial 28S rRNA gene

Amplified LSU fragments were cleaned with ExoSAP-IT (USB Corporation, USA) according to manufacturer's instructions as mentioned previously (Section 3.2.11.1.1). 2.5 µL of the ExoSAP-IT treated PCR product was cycle sequenced using a BigDye Terminator Kit (Applied Biosystems, Warrington, UK) and cleaned using the Wizard Magnesil™ system (Promega, UK). Fragments were then sequenced in both directions using the same set of primers (D2a forward and D3b reverse). Amplification products which failed to produce good quality sequences were cloned into pGEM-T vector system and subsequently sequenced in both directions with M13F and M13R primers. Sequence traces were checked with Chromas Pro for ambiguities and errors.

4.2.5 PCR amplification and sequencing of the mitochondrial 16S rRNA gene

Two primers were designed in this study based on available nematode mitochondrial 16S rRNA sequences held online at GenBank and EMBL. These were

16SF1 forward and 16SR1 reverse. The forward primer corresponds to the 10839-10858 position and the reverse primer corresponds to the 11167-11151 position with the *C. elegans* mitochondrial genome. PCR and sequencing parameters have been elaborated in Chapter Two (Table 2.1).

4.2.6 PCR amplification and sequencing of the mitochondrial COXI gene

Two sets of primers, namely LCO1490 and HCO2198 (Folmer *et al.*, 1994) and MNCOXIF and MNCOXIR, were used to amplify a partial fragment of the COXI gene. The MNCOXIR and MNCOXIR primers were designed by the author on available parasitic nematode COXI sequences and correspond to the 730-754 and 1170-1147 positions of the mitochondrial COXI gene in *C. elegans*. The primer sets yielded amplification products of 700 bp and 440 bp respectively. For the Folmer *et al.* (1994) primers, the thermal cycle parameters consisted of one cycle of: 1 min at 94°C; five cycles of 1 min at 94°C, 1.5 min at 45°C and 1.5 min at 72°C; 35 cycles of 1 min at 94°C, 1.5 min at 50°C and 1 min at 72°C; and a final cycle of 5 min at 72°C. Amplified fragments were subsequently sequenced with the same set of primers.

4.2.7 PCR amplification and sequencing of the partial ribosomal 18S rRNA gene for molecular barcoding

To test the molecular barcoding concept in marine nematodes, a small fragment from the partial 5' end of the small subunit rRNA gene was selected for amplification. Two primers, namely MN18F and 22R, were used to amplify a fragment of approximately 345 bp. PCR was carried out with an MJ Thermocycler using the following cycling parameters: 95°C for 5 mins, followed by 37 cycles of 95°C for 30 sec, 56°C for 1 min, 72°C for 1 min 30 sec and a final extension of 72°C for 5 min and the PCR tubes were cooled at 4°C. In total, 80 individuals from Saltram (Plym estuary) and Saltash (Tamar estuary) were PCR amplified and sequenced.

4.2.7.1 Phylogenetic analysis to test the reliability of molecular barcodes

Prior to phylogenetic analysis, 18S rRNA sequences from known marine nematode taxa from South West England and selected sequences from the GenBank and EMBL databases were aligned with sequences from Plym and Tamar estuary specimens in the Clustal-X program using the default parameters. GenBank accession numbers of the sequences used in this study were AY854202, AY854209, AY854204, AF047888, AY854210, AY854212, AY854224, AY854225 and AY854238. Neighbour-joining trees were constructed with the program MEGA v2.0 using gamma-corrected Kimura distance parameters (Blaxter *et al.*, 1998). To assess the reliability of NJ trees bootstrap tests were carried out using 1,000 replicates.

4.3 Results

4.3.1 Amplification and sequencing of the 18S rRNA gene

Successful PCR amplification and sequencing were achieved in almost all major marine nematode taxa tested from South West English waters (Figure 4.2). Each taxon possessed a different DNA sequence at the 18S rRNA gene. A distinct pattern of conserved and variable regions was observed in the 18S rRNA molecule among all these taxa. The partial 5' end of the 18S rRNA molecule exhibits a mix of conserved and variable regions which were later tested for molecular barcoding (see below). Almost all sequences showed a similarity of 99% or above when compared with the nematode sequences available online in GenBank and EMBL using the BLAST tool. 18S rRNA sequencing revealed that *Daptonema hirsutum* and *D. setosum* were identical at the 18S rRNA level. The genetic analysis revealed that there may be a systematic problem in *D. hirsutum* and *D. setosum* or a problem during morphological identification of the specimens prior to molecular analysis. Identical sequences were also found for two other nematode species (or 'congeners'), *Setosabatieria hilarula* and *Sabatieria celtica*. Similar observations were also recorded by Meldal (2004) in a separate study. The phylogenetic analysis exhibited

good resolution of the major nematode orders Enoplida, Chromadorida and Monohysterida (Figure 4.3). Most of the species cluster into the genera and families described from morphological studies.

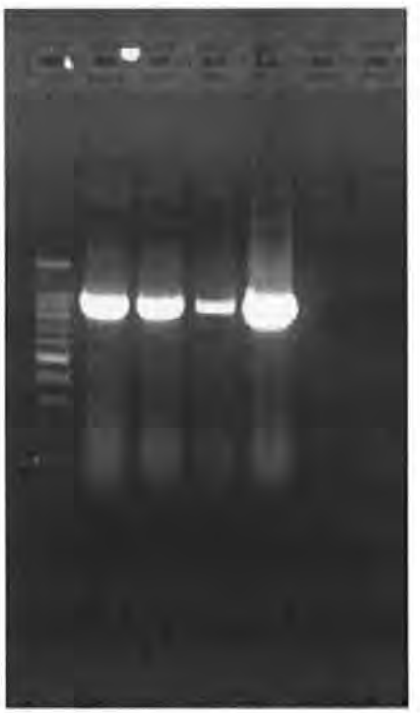


Figure 4.2: Amplified 18S rRNA fragment of approximately 920 bp from different marine nematodes using the MN18F and Nem_18S_R primers. 100 bp ladder at the extreme left.

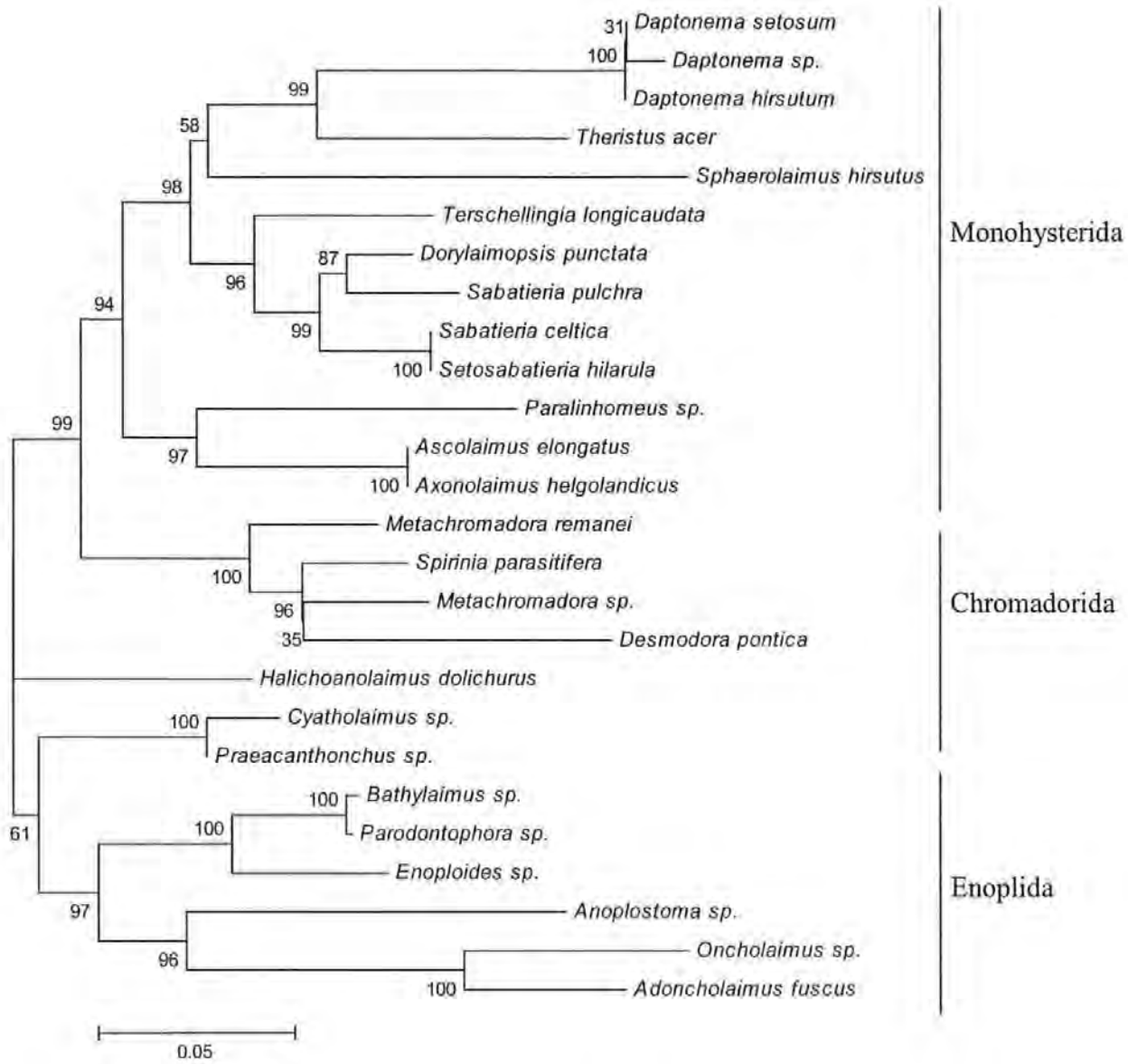


Figure 4.3: Neighbour joining tree with boot strap values (1000 replicates) for twenty six marine nematode taxa from South West England based on 18S rRNA sequences. The scale indicates 0.05 substitutions/site.

4.3.2 Amplification and sequencing of the 28S rRNA gene

Successful PCR amplification and sequences were obtained for ten out of fifteen marine nematode taxa tested, giving a success rate of just over 66%. The D2/D3 expansion segment was highly variable between these taxa. Clearly more optimization is needed for the primers to work in all taxa. The BLAST percentage score was on average 94% for most

of the 28S rRNA sequences. Only one taxon, namely *Adoncholaimus* sp., matched GenBank data with a BLAST percentage identity score of 99%. Given the low amplification and sequencing success rate, the use of 28S rRNA gene for barcoding marine nematodes was abandoned.

4.3.3 Amplification and sequencing of the mitochondrial genomic regions

Amplification and sequencing of two mitochondrial genes, namely 16S rRNA and COXI, were attempted using both nematode-specific and universal primers. In most cases PCR was unreliable and yielded no products. DNA from a single marine nematode taxon, namely *Daptonema* sp. was amplified and sequenced using mitochondrial 16S rRNA primers. For the COXI gene, three taxa were amplified and sequenced using the Hebert *et al.* (2003) primers and nematode-specific primers. These were *Metachromadora remanei*, *Metachromadora* sp. and *Daptonema setosum* respectively. Further work with these genes was abandoned due to the low PCR success rate observed.

4.3.4 Molecular barcoding of marine nematodes based on 18S rRNA sequences

Successful PCR amplification and sequencing was achieved for all eighty individuals from the Tamar and Plym estuaries identified morphologically prior to DNA analyses. All sequences showed similarities of between 97-100% with GenBank and EMBL nematode 18S rRNA sequences. The phylogenetic analysis of the eighty sequences along with known marine nematode 18S rRNA sequences showed clear resolution and most of the sequences were resolved to genus and species level in both the trees (Figures 4.4 and 4.5). In the Tamar estuary only one specimen (Tamar 3) was not assignable to species level in the phylogenetic tree. This was placed within the genus *Sabatieria* on the basis of its 18S rRNA sequence, and indeed was identified as *Sabatieria* sp. based on morphological characters prior to molecular analysis. In the Plym estuary, 6 out of 40 specimens were not readily assignable to species level in the tree. Out of these, three were

assignable to genus level as *Praeacanthonchus* (Plym1), *Terschellingia* (Plym12), and *Sabatieria* (Plym22), and had been identified as such based on morphological characters prior to molecular analyses. The Plym17 specimen was morphologically identified as *Metachromadora suecica*, and indeed clustered with the *Metachromadora* species included in the tree, despite being relatively divergent, differing by seven base pairs from *M. remanei*. Plym19 and Plym 29 clustered with *Atrochromadora microlaima* and *Dichromadora* sp. in the phylogenetic tree.

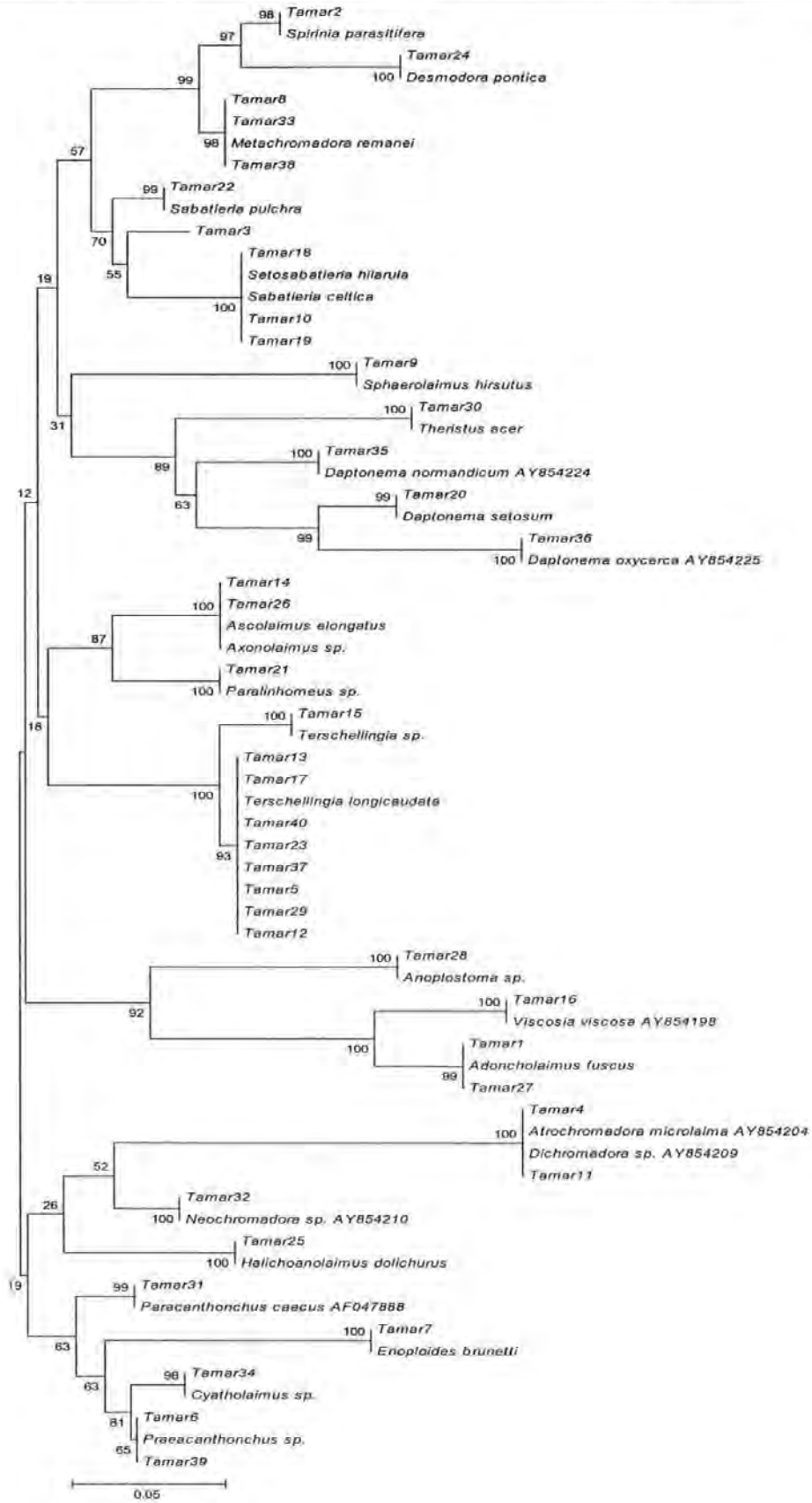


Figure 4.4: Neighbour joining tree with bootstrap values (1000 replicates) showing relationship between Tamar estuary nematode 18S rRNA sequences and sequences from known marine nematodes. The scale indicates 0.5 substitutions/site.

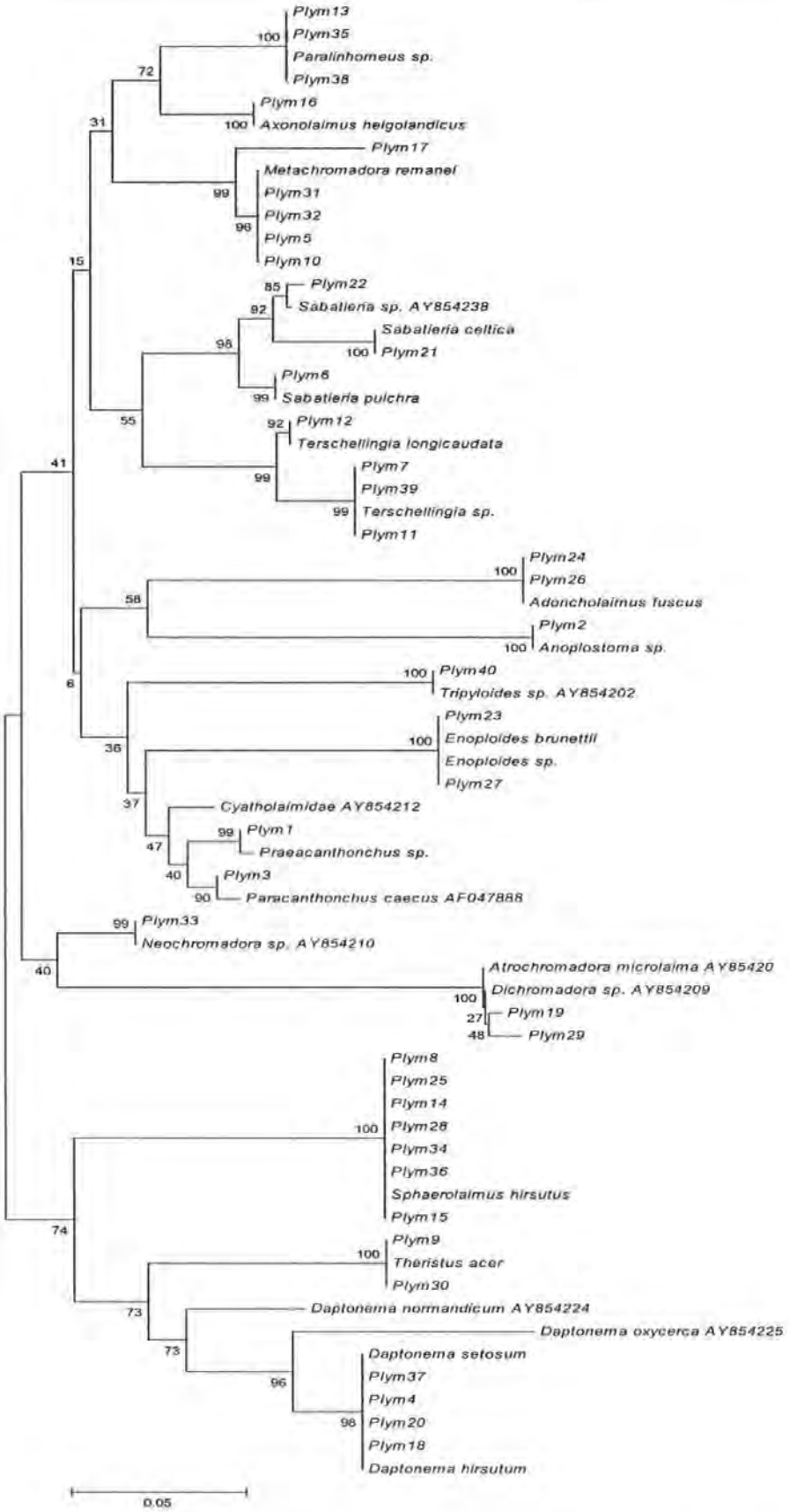


Figure 4.5: Neighbour joining tree with bootstrap values (1000 replicates) showing relationship between Plym estuary nematode 18S rRNA sequences and sequences from known marine nematodes. The scale indicates 0.5 substitutions/site.

4.4 Discussion

The main objectives of this study were to amplify and sequence nuclear and mitochondrial genes from nematode genomes to create DNA sequence profiles useful for the identification of marine nematodes. In total, four genes from both nuclear and mitochondrial genomes were tested in this study. Nuclear 18S rRNA genes were successfully amplified and sequenced from all the taxa tested, and proved to be a valuable marker for barcoding studies. Initially PCR amplification and sequencing was hindered by frequent amplification of fungal 18S rRNA sequences (see Chapter 3). Because most environments harbour a high level of eukaryotic diversity, it becomes difficult to avoid co-extracting DNA from other biological material such as micro-organisms and fungal spores and as a result co-amplification becomes a problem. Therefore, new primer sets were designed and evaluated. The newly designed primers based on available marine nematode sequences were specific and amplified only nematode 18S rRNA sequences (see Chapter 3).

The 18S rRNA gene in marine nematodes contains both highly conserved and variable regions, and there is high inter-specific variation between taxa. Such patterns are common and have been observed across many metazoan phyla (Abouheif *et al.*, 1998). The mix of conserved and variable regions amongst the 18S rRNA molecule makes it suitable for the design of primers to amplify segments of the gene that are variable amongst different species of nematodes. The partial 5' end of the molecule contains a mix of conserved and variable regions which were exploited for molecular barcoding studies. In this study two nematodes (*Setosabatieria hilarula* and *Sabatieria celtica*) were shown to have identical 18S rRNA sequences and this may have arisen through homoplasy or the retention of ancestral polymorphism within the 18S rRNA region for these taxa (Avise, 1994; Manen, 2004; Cook *et al.*, 2005). Homoplasy could arise either due to convergent evolution, evolutionary reversals or due to hybridization but not (by definition) due to common ancestry (Sanderson and Hufford, 1996; Lowrey *et al.*, 2001). Similar results

have also been observed among members of Anoplostomatidae and Enoplidae in the phylum Nematoda (Pegova *et al.*, 2004). Therefore there is always a possibilty that the history of this gene may not actually relect the history of the two species (*Setosabatieria hilarula* and *Sabatieria celtica*). The other possibilities are methodological or sequencing error, although it is unlikely as the 2nd and 3rd amplified segments of the 18S rRNA gene overlap, giving a high confidence in sequence data as effectively the same regions were sequenced several times. DNA amplification and sequencing of other genomic regions, namely 28S rRNA or mitochondrial 16S rRNA, could provide vital information on the relationship between these two species.

PCR and direct sequencing of the highly variable D2/D3 segment of 28S rRNA yielded mixed results. Out of 15 taxa tested, amplification and sequencing was successful for 10 taxa and thus it was clear that further optimisation or designing primers based on other regions of 28S rRNA was required to achieve 100% success with all the marine nematode taxa investigated. De Ley *et al.* (2005) used the same set of 28S rRNA primers and found the PCR success rate was just over 80% among terrestrial and a few marine nematode taxa. An alternative approach, where template DNA from representative taxa was initially amplified using a GenomiPhi amplification kit and subsequently used as templates for D2/D3 amplification was adopted by De Ley *et al.* (2005) to test the concept of barcoding. In this study D2/D3 fragments were directly amplified from low concentration DNA templates (typically 0.5-3 ng/μL) and therefore the GenomiPhi method used by De Ley *et al.* (2005) may improve amplification from marine nematodes. In this study the success rate with the 28S rRNA gene was just above 66%. Most of the sequences obtained were highly variable because of high divergence between taxa, which may create alignment ambiguities and uncertainty in phylogenetic analysis (De Ley *et al.*, 2005). Given such problems, and the relatively low PCR success rate, the use of this gene was abandoned here, although it may prove useful in the future barcoding studies.

In this study two genes present in the mitochondrial genome, namely 16S rRNA and COXI, were also tested for ease of amplification and sequencing from different marine nematode taxa. PCR amplification of 16S rRNA was unsuccessful in all except one out of twenty taxa tested. Similarly for the COXI gene amplification failed in most of the taxa. Only three taxa were successfully amplified and sequenced using the Folmer *et al.* (1994) primers and primers designed as part of this study. Meldal (2004) and Cook *et al.* (2005) also found similar problems while trying to amplify the COXI gene from marine nematodes. Reasons for such failure may relate to the fact that nematode mitochondrial genomes are highly diverse compared to other metazoans, and display unusual properties such as recombination, insertional editing and multipartitioning (Lunt and Hyman, 1997; Blouin, 1998; Keddie *et al.*, 1998; Armstrong *et al.*, 2000; Lavrov and Brown, 2001). Therefore designing phylum-wide primers for mitochondrial genes may be problematic, seriously limiting their future use in barcoding across the phylum.

Based on PCR amplification and sequencing success rates, the 18S rRNA gene proved to be more consistent than other nuclear and mitochondrial genes. The 18S rRNA gene is generally conserved and has a high success rate with PCR. Therefore it has received great attention in recent literature as a barcoding locus (Floyd *et al.*, 2002; Blaxter, 2004; Powers, 2004). In this study, a region of approximately 345 bp from the 5' end of the molecule was selected for barcoding studies and evaluation of its potential to assign specimens to genus and species level. The validity of the technique was evaluated by identifying specimens using traditional taxonomic methods followed by their subsequent randomization, sequencing and inclusion in phylogenetic analysis. Almost all the specimens from the Plym and Tamar estuaries resolved to genus and most of them to species level when compared with representative marine nematode sequences in phylogenetic trees. However there were some exceptions to this. In the Tamar estuary only one specimen (Tamar 3) was not assignable to species level in the phylogenetic tree. This did, however, fall within the genus *Sabatieria* on both molecular and morphological

characters, and may represent a previously unsequenced species of the genus, or indeed a novel cryptic taxon. As stated above, in the Plym estuary, 6 out of 40 specimens were not clearly assignable to species level. Three were correctly identifiable to genus level, however, using 18S rRNA sequences, and indeed these specimens could not be identified further on the basis of morphology, even by accomplished taxonomists. Two specimens from Plym estuary (Plym19 and Plym29) branched with different taxa in the tree although one of them Plym 19 was identified morphologically as *Sabatieria* sp. and the other Plym29 could not be identified but was grouped under the Cyatholaimidae. Misidentification or contamination of DNA could have been responsible for wrong derivation to genus or species level in the tree. The possibility of novel cryptic taxa or sequences from previously undescribed species cannot be ruled out for these two specimens. Therefore, amplification and sequencing of other genomic regions for these two specimens could provide vital information for subsequent assignment to correct genus and species level.

Throughout the study, NJ analysis using gamma-corrected Kimura distances was adopted for tree building following the methodology of Blaxter *et al.* (1998). Additionally, multiple nematode specimens with similar genotypes from the Plym and Tamar estuaries were included for analysis so as to evaluate whether 18S rRNA barcoding marker can resolve specimens (with similar genotypes) correctly to genus and species level in a phylogenetic tree. NJ analysis has been successfully applied as a tree building tool for DNA barcoding studies in the past (Hebert *et al.*, 2003; Blaxter 2004; Ward *et al.*, 2005; Hajibabaei *et al.*, 2006) and was therefore applied in this study. Besides, NJ analysis is fast and thus suited for large datasets and for bootstrap analysis as well as permitting lineages with largely different branch lengths. While the bootstrap values for some of the branch lengths were low in both the trees (Figures 4.4 and 4.5), this was possibly due to the fact that a small fragment of 345 bp from the 18S rRNA molecule was evaluated in the analysis rather than the complete 18S rRNA molecule (1.7 kB). On the other hand, the majority of

the outgroups had bootstrap values of 98 and above, confirming the that specimens were correctly assigned to genus and species level by NJ analysis, and previously confirmed BLAST values (98%-100%) from the sequence data sets strongly support this argument.

Based on 18S rRNA amplification and sequencing, 77 out of 80 specimens were correctly assigned to genus or species level, indicating that the success rate of molecular barcoding using this sequence is close to 97%. At the same time, taxonomic placements of most specimens using molecular data matched those based on morphology. The success rate of the 18S rRNA based DNA barcoding conducted here is slightly lower than that of Hebert *et al.* (2003) where the success rate was 100% based on COXI profiles. Clearly more work is needed to include a larger number of 18S rRNA sequences from marine nematode taxa for the barcoding approach to be more accurate. At the same time traditional taxonomic methods should be continued in order to develop keys for new species of marine nematodes so as to bring a congruency between the two methods (molecular and morphological).

This study shows for the first time the feasibility of developing an 18S rRNA-based identification system for small metazoans such as nematodes that are abundant in the marine and estuarine environments. PCR products were recovered from all the individuals and there was no evidence of any complications with the molecular methods. Moreover, the alignment of the sequences and subsequent phylogenetic analysis was straightforward, as indels and polymorphisms were uncommon for this gene.

Such DNA identification based on 18S rRNA sequences could prove to be useful for marine nematodes and indeed other metazoan groups that form a major part of the meiofauna in the marine environment. The future success of DNA barcoding lies with the development of an 18S rRNA database for identification of marine meiofauna including nematodes. Approaches based on ribosomal sequences could be employed in future for molecular surveys of nematode diversity from marine environments. For more extensive surveys, a cheaper oligonucleotide-hybridization approach could be taken, where the 18S

rRNA PCR products are arrayed on filters or microarrayed on slides and identified by probes derived from diagnostic SSU fragments from known or indicator taxa, chosen for their relevance to the study in question (Jenkins *et al.*, 2004; Steward *et al.*, 2004).

5. Genetic differentiation in the cosmopolitan marine nematode *Terschellingia longicaudata*

5.1 Overview

Many benthic invertebrate species are considered to have a worldwide distribution and are often termed cosmopolitan. A taxon is defined as cosmopolitan if it is reported from two or more oceans including connected seas (Sterrer, 1973). Within the meiobenthos, cosmopolitan species have been reported from a wide array of major taxa displaying a broad range of life styles (Scheltema 1968, 1971, 1988; Rogers *et al.*, 1995; Todaro *et al.*, 1996; Lee and ÓFoighil, 2004). Some marine benthic invertebrates have a broad geographic distribution because of pelagic larval and juvenile stages, which drift for weeks and months in the water column and are thereby dispersed over long distances. On the other hand, many of the supposed cosmopolitan meiofauna living in littoral sediments have no pelagic stages of dispersal, and juveniles, like the adults, are incapable of active swimming. Given such observations, it would be expected that such taxa would have relatively small geographic ranges, and indeed the cosmopolitan nature of some meiofaunal species has been questioned (Gerlach, 1977; Giere, 1993; Schmidt and Westheide, 2000).

A central point of debate over the presumed cosmopolitan distribution of meiofaunal taxa concerns species identification. In particular, critics have questioned the reliability of species identifications from geographically distant areas, especially when made by different investigators using different methods and differing personal opinions to place specimens within a given taxon. Careful morphological analysis has sometimes shown that some species with a presumed wide geographical range are actually composite assemblages of different species (Grassle and Grassle, 1976). In addition, several potentially cosmopolitan species have been identified as cryptic species based on

molecular analyses (Baric and Sturmbauer, 1999; Dawson and Jacobs, 2001; Williams *et al.*, 2001; Quattro *et al.*, 2001; Larsen, 2001).

Equally recent surveys using highly reproducible techniques have confirmed that true cosmopolitanism does appear to occur among certain meiofaunal groups (Hummon, 1994; Todaro *et al.*, 1995; Schmidt and Westheide, 2000). In light of these contrasting results, and the awareness of the possible existence of cryptic species, different approaches to species identification should be combined in studies which attempt to determine the status of supposed cosmopolitan taxa.

Free living nematodes which often dominate the meiofauna include several taxa that appear to have a broad scale cosmopolitan distribution. Marine nematodes generally show little evidence of active dispersal (Palmer, 1988) and probably move by passive dispersal in the bedload and water column (See Chapter 1 and Palmer, 1988; Armonies, 1994; Sun and Fleeger, 1994). Dispersal by other means such as erosion of sediment, or tidally induced vertical and horizontal displacements have also been reported by several researchers in some marine nematode taxa (Rieger and Ott, 1971; Gerlach, 1977; Hagerman and Rieger, 1981; Commito and Tita, 2002), yet it is unclear how such mechanisms could lead to a cosmopolitan distribution. The lack of planktonic phases in the life cycle of nematodes raises the question whether some of the cosmopolitan species that have been reported from different oceans and estuaries actually comprise complexes of cryptic species. Morphological measurements have demonstrated the presence of cryptic species in the marine nematode genus *Pontonema* (Warwick and Robinson, 2000) with a broad geographical distribution. Recently studies conducted by Derycke *et al.*, (2005) have revealed cryptic species assemblages in another marine nematode species *Pellioditis marina* from a small biogeographic region based on molecular datasets. To date, however, no study has investigated cosmopolitanism in marine nematodes using a combination of morphological and molecular techniques.

5.1.1 The model species used in this study

Marine nematodes occur in virtually every marine benthic habitat, and several have a cosmopolitan distribution. One such supposed cosmopolite is the marine and estuarine species *Terschellingia longicaudata* De Man, 1907. It is typically one of the dominant species in soft sediments in inshore waters and is 1.5-1.7 mm in length, a size typical of a wide range of related species (Figure 5.1).

Figure 5.1: Light micrograph of an adult female *Terschellingia longicaudata* (taken at x10 magnification). Note the long tail filament visible at the left-hand side of the picture.

T. longicaudata is reported extensively from British waters in particular from South West England, North East England and parts of Scotland. It has been also reported from many parts of the world's oceans, including the Atlantic coast of France, the Black Sea, the USA (Gulf of Mexico), China (Qingdao province), New Zealand and the Solomon Islands (Sergeeva, 1991; Zhang and Ji, 1994; Burgess *et al.*, 2005; PJD Lambshead *pers comm*).

5.1.2 Aims

The aims of this study were:

(i) To examine and compare the phenotypic and genotypic relationships between populations of the *Terschellingia longicaudata* using a combination of morphometrics and genetic analysis.

(ii) To determine whether molecular or morphological analysis suggest the presence of a cryptic species complex or true cosmopolitanism in *T. longicaudata*.

5.2 Materials and Methods

5.2.1 Sample collection

For morphological and molecular studies populations were collected from the geographically distinct environments described in Table 5.1. Details of the sediment collection from UK waters are available in Chapter 3 (Section 3.2.1). For sites in Bahrain, Mexico and France surface sediments (2-10 cm deep) were sampled by hand and stored either in formalin or alcohol for subsequent analysis. Details of the sediment collection from the Malaysian site are available in Somerfield *et al.* (1998).

Table 5.1: Details of the localities and habitats from where sediments were collected in this study.

Country	Locality	Habitat	Depth	Collector
UK	Tamar estuary	Intertidal	1-5m	P. Bhadury
UK	Rame Head	Subtidal	50m	P. Bhadury
UK	Plym estuary	Subtidal	7-10m	P. Bhadury
UK	NMMP site	Subtidal	70m	Michaela Schratzberger
France	L'orient, Brittany	Coastal area	Intertidal	Melanie Austen
Bahrain	North Tubli Bay	Sheltered bay, extensive mudflats	Intertidal	Melanie Austen
Bahrain	Ras al Barr	Sandy beach	Intertidal	Melanie Austen
Malaysia	Sungai Merbok estuary	Estuarine mangrove vegetation	Intertidal	Paul Somerfield
Mexico	Cancun (Atlantic coast)	Sandy beach	Intertidal	Rachel Jones

5.2.2 Sample processing

Sediments from each location were divided into two parts. One part was fixed in molecular grade ethanol for molecular studies and the other part was fixed in formalin for morphometric studies. For alcohol fixed sediments, specimen extraction and identification was carried out following the protocol described in Chapter Three (Section 3.2.2). Merbok meiofauna were already stored in 4% buffered formalin and *T. longicaudata* specimens were carefully picked from stored meiofauna for subsequent molecular and morphological analyses. Washed meiofauna were extracted from formalin-fixed sediments, following

Somerfield and Warwick's (1998) protocol, and then divided into several cavity blocks, each containing 4 mL of a solution of 5% glycerol and 10% IMS (Industrial Methylated Spirit) and left on a thermostat hot-plate (37°C) overnight. This slow evaporation procedure transfers the fixed nematodes to pure, anhydrous glycerol, a medium suitable for permanent whole mounts (Riemann, 1988). Each nematode was carefully placed on a microscope slide (76 × 26 mm, thickness 1.0 mm, Bluestar) containing a drop of glycerol and subsequently a cover-slip (13 mm diameter, thickness 0, VWR International) was applied and sealed carefully with Bioseal 2, a xylene resistant sealant thereby reducing the risk of squashing the specimens. Specimens were then checked under a compound microscope to confirm their identity prior to morphological measurements.

5.2.3 Molecular analyses

Primers and their sequences used in this study have been described in Chapter Two. For molecular studies fifteen individuals from the Tamar estuary, the Plym estuary, NMMP and Rame Head were used. For Bahrain sites, five individuals from North Tubli Bay and five individuals from Ras al Barr were available for molecular studies. Due to lack of specimens, only five individuals from Brittany (France), three individuals from Merbok (Malaysia) and two individuals from Cancun (Mexico) were used for amplification and sequencing. In this study both nuclear (18S rRNA, ITS1 and ITS2, 28S rRNA) and mitochondrial genes (COXI, COXII, NADH) were targeted for amplification and sequencing.

5.2.3.1 PCR amplification and sequencing of the 18S rRNA gene

Nematodes identified as *Terschellingia longicaudata* from nine geographical regions were carefully taken off slides and individually placed in 0.5 mL PCR tubes containing 0.25 M NaOH. DNA extraction was carried out following a modification of the Floyd *et al.* (2002) protocol. Two primers, MN18F and Nem_18S_R, were used to amplify

approximately 926 bp of the 18S rRNA gene. PCR fragments were subsequently sequenced in both directions using the same set of primers.

5.2.3.2 DNA extraction and PCR amplification from formalinised Merbok samples

Merbok (Malaysia) *T. longicaudata* specimens previously fixed in formalin were subjected to DNA extraction using the modified protocol of Chase *et al.*, (1998) described in Chapter Six (Section 6.2.2.1). Following extraction, genomic DNA from each individual was subjected to amplification of the 18S rRNA gene using MN18F and 22R primers. Amplicons were subsequently sequenced in both directions using same set of primers.

5.2.3.3 Phylogenetic analysis of 18S rRNA sequences

Prior to phylogenetic analysis, 18S rRNA sequences were aligned in Clustal-X using default parameters. Neighbour joining trees were constructed in MEGA. A *T. longicaudata* sequence from Southampton waters (UK) submitted to GenBank (AY854230) was also included in the phylogenetic analysis, together with other authenticated marine nematode sequences generated in this study. NJ trees were subsequently validated with bootstrap analysis of 1000 replicates.

5.2.3.4 PCR amplification of the 28S rRNA gene for Ras al Barr and North Tubli Bay specimens

Material identified as *T. longicaudata* from Bahrain produced 18S rRNA sequences which were highly divergent from remaining *T. longicaudata* material (see results). In the light of this observation, all the specimens from Ras al Barr and North Tubli Bay that were used for 18S rRNA amplification were also subjected to amplification of the D2/D3 expansion segment from the 28S rRNA gene using the same set of primers and thermal cycler parameters as described in Chapter Four (Section 4.2.4), in an attempt to confirm their phylogenetic placement.

5.2.3.5 PCR amplification of the COXI gene

Two methods were implemented to selectively amplify the COXI gene in *T. longicaudata*. The first method involved amplification directly from genomic DNA. In the second method, a GenomiPhi™ Amplification kit (Amersham Biosciences, USA) was used to amplify genomic DNA following the manufacturer's instructions. GenomiPhi™ DNA Amplification Kit offers a simple method for isothermal, representational whole genome amplification. The method employs the unique biochemical properties of Phi29 DNA polymerase, a highly processive enzyme with excellent strand displacement activity. Microgram quantities of high molecular weight DNA are produced overnight from nanogram amounts of starting material. Amplified genomic DNA was subsequently diluted 1,000 to 10,000 times and used as templates for amplification of COXI. In this study two sets of primers were used. They were (i) MNCOXIF and MNCOXIR and (ii) LCO1490F and HCO2198R (Folmer *et al.*, 1994). For PCR amplifications, both *Taq* DNA polymerase and Accuprime *Pfx* DNA polymerase were used.

5.2.3.6 PCR amplification of the mitochondrial cytochrome oxidase subunit II gene (COXII)

Two primers, CO2.105CD forward and CO2.215R reverse (T Powers, *pers comm*), were used to amplify the COXII gene. The following program was used for amplification: 94°C for 2 min, 8 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec followed by 34 cycles of 94°C for 30 sec, 47°C for 1 min, 72°C for 1 min and a final extension temperature of 72°C for 5 min followed by a holding temperature of 4°C.

5.2.3.7 PCR amplification of the ITS1 and ITS2 region

Two primers, NC5F and NC2R, were used to amplify the ITS1 and ITS2 region from the nematode ribosomal RNA gene. Another set of primers flanking the end of the 18S rRNA gene and covering the ITS1 spacer region were also used for amplification.

These were 23F forward and NC1R reverse. For NC5F and NC2R primers an annealing temperature of 51°C was implemented whereas for the 23F and NC1R primers the annealing temperature was adjusted to 55°C. PCR reactions were performed using Accuprime *Pfx* DNA polymerase enzyme.

5.2.3.8 PCR amplification of the NADH dehydrogenase subunit gene

Two primers, mb5F forward and mb9R reverse were also used to amplify a small fragment of the protein-encoding NADH dehydrogenase subunit gene from the mitochondrial genome. PCR reactions were carried out using Accuprime *Pfx* DNA polymerase enzyme. Thermal cycle parameters used in this study were 36 cycles at 94°C for 30 sec, 48°C for 1 min 30 sec, 72°C for 2 min.

5.2.4 Morphometric analysis

Ten individuals from each site in the UK were mounted for morphometric analysis. Fewer specimens were available for morphometric study from non-UK sites and the numbers mounted for North Tubli Bay and Ras al Barr (Bahrain) were eight and five respectively and for Merbok (Malaysia), Cancun (Mexico) and Brittany (France) sites one, two and five specimens were mounted. Morphometric analysis was performed using a compound microscope with camera lucida and interference phase optics. Character lengths were traced directly from the microscopic field of view on to paper, with ×10 magnification for the larger character measurements, such as body length, and ×100 magnification for the smaller characters such as amphids and oesophageal bulb diameter. The character traces were then measured using a ruler, divider or map measurer for curved characters and subsequently calibrated and converted to a millimetre scale (mm). Decisions regarding the choice of characters for analysis were based on recommendations by Platt and Warwick (1983) and, most notably, characters considered diagnostic for this species.

The characters used for morphometrics are detailed in Table 5.2. Morphometric measurements of all specimens are available in Appendix A.

Table 5.2: Characters measured for each *Terschellingia longicaudata* specimen processed.

Males	Females
Body length	Body length
Maximum body diameter	Maximum body diameter
Anal body diameter	Anal body diameter
Tail length	Tail length
Oesophagus length	Oesophagus length
Oesophageal bulb diameter	Oesophageal bulb diameter
Head diameter	Head diameter
Amphid diameter	Amphid diameter
Cephalic seta length	Cephalic seta length
Sub-cephalic seta length	Sub-cephalic seta length
Somatic seta length	Somatic seta length
Cervical seta length	Cervical seta length
Gubernaculum length	Distance from vulva to head
Spicule length	

5.2.5 Data analysis

Multivariate analysis of the morphometric data was performed using the PRIMER software package version 5.1 (Clarke and Warwick, 1994). The software, designed to study changes in biotic communities, has proved to be useful in numerical taxonomy (Warwick and Robinson, 2000). Lower triangular dissimilarity matrices were constructed in PRIMER using the normalized Euclidean distance measure, without prior data transformation. Normalised distance measures are applicable to character ranges such as body length,

maximum body diameter and therefore have been used in this analysis (Clarke and Warwick, 1994). Non-metric multidimensional scaling (MDS), an ordination technique which is robust in representing high dimensional data (indicated by acceptable stress values) was then applied to the dissimilarity data. This ordination technique is based on a complex numerical algorithm which is conceptually simple and makes few model assumptions about the form of the data or the inter-relationship of the samples, and the link between the graphical representation and data is relatively transparent and easy to explain. Additionally it has great flexibility both in the definition and conversion of dissimilarity to distance and its rationale is the preservation of these relationships in the low-dimensional ordination space (Clarke and Warwick, 1994). For each ordination, there were 10 random starts of the MDS in order to allow the program sufficient repetition to find the best solution. MDS ordinations were carried out on together (male and female) using all characters and excluding sexual characters and separately on males and females.

ANOSIM (Analysis of Similarity) was applied to determine the degree and significance of differences between populations based on multiple morphometric characters. The calculation in ANOSIM results in a test statistic (R) which is 1 if all individuals within a population are more similar to each other than to any individual in another population and 0 indicates no difference between populations (and the unlikely value of -1 would occur if all individuals in a population are more similar to individuals in other populations than to any in their own) (Warwick and Robinson, 2000). R is calculated both globally and also between individual pairs of populations. The significance test is then achieved by randomly re-allocating population labels to each specimen, in this case 5000 times, and re-calculating the R statistic. The significance level is then determined by the number of times the value of R in the random simulations exceeds the true measured value, again in both global and pair wise fashion.

SIMPER (Similarity Percentages) was used to investigate the contribution of individual morphological characters to the separation of populations resulting in the

observed clustering pattern (in MDS) or for significant differences between sets of samples (in ANOSIM). It should be noted, however, that SIMPER is an exploratory analysis and not a statistical testing framework (Clarke & Warwick, 1994).

5.3 Results

5.3.1 PCR amplification and sequencing of 18S rRNA gene from *T. longicaudata* specimens collected from different geographic localities

Successful PCR amplification and sequencing were achieved for all morphologically identified *T. longicaudata* specimens collected from nine geographical locations. Approximately 926 bp of the 18S rRNA gene were sequenced from each individual in this study. Most sequences showed 100% homology with the *T. longicaudata* sequence held online at GenBank. The majority of the sequences from Tamar, Plym, Rame Head, NMMP, Brittany, Cancun and all sequences from Merbok (formalin preserved) were identical (Figures 5.2 & 5.5). For Merbok samples only 350 bp of the 18S rRNA gene were amplifiable.

Some samples from a number of localities produced divergent 18S rRNA sequences. Seven individuals from the NMMP site shared a sequence showing 97% homology to that found in the majority of *T. longicaudata* specimens. Four individuals, from two UK sites (Tamar estuary, Rame Head) and Cancun, Mexico, shared another sequence with 96% homology to the commonest genotype (see the alignment Figure 5.3). Sequences from nematodes identified as *T. longicaudata* from sites in Bahrain were very different from other specimens identified as this species, showing <90% homology with the commonest *T. longicaudata* sequence, and indeed having much higher levels of sequence homology with other taxa (see Figure 5.4 and 5.5).

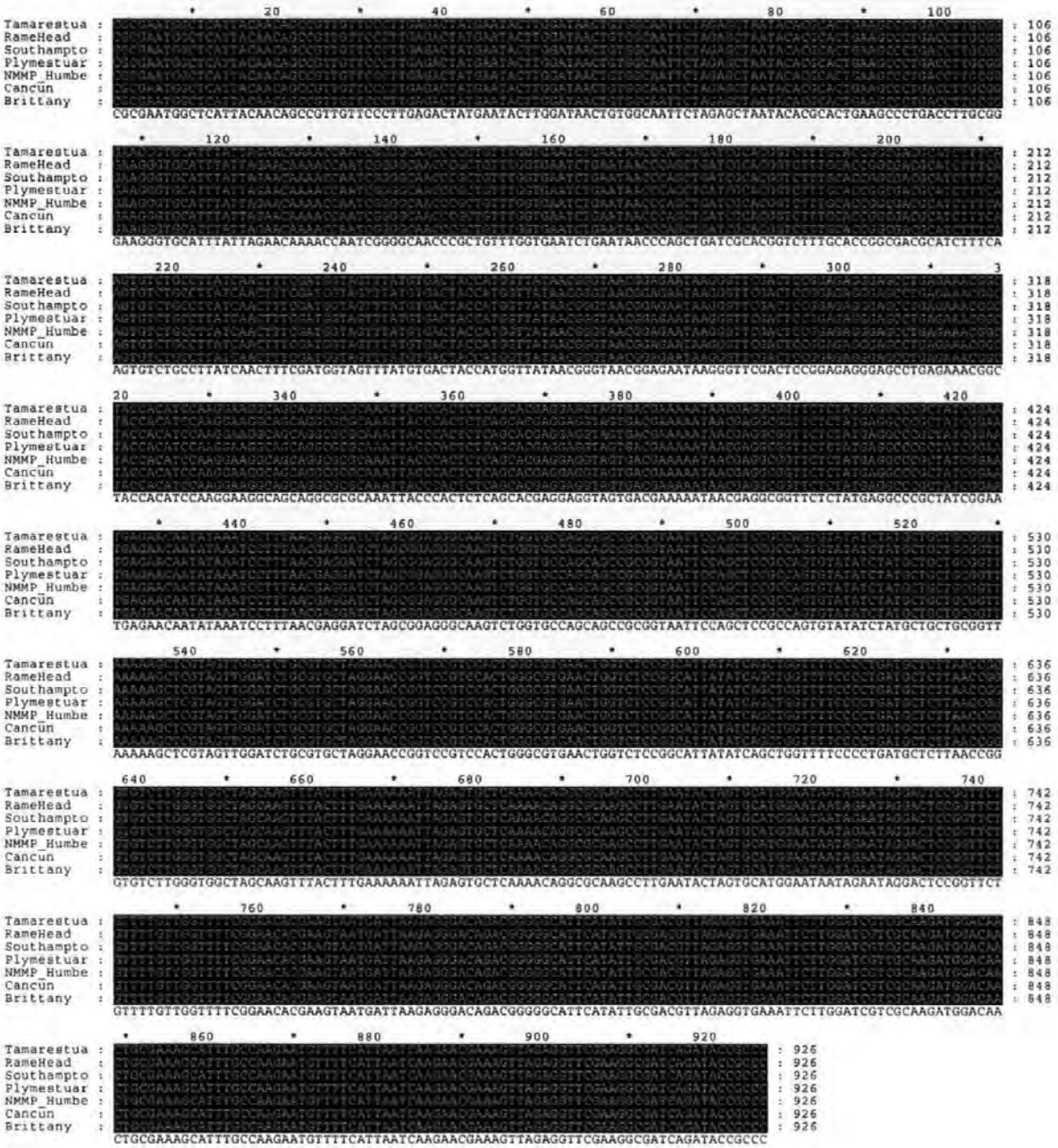


Figure 5.2: Alignments showing similar haplotypes of *T. longicaudata* 18S rRNA sequences from different geographic locations in UK (Tamar estuary, Rame Head, Plym estuary, Southampton, NMMP) and across the globe (Brittany, Cancun).

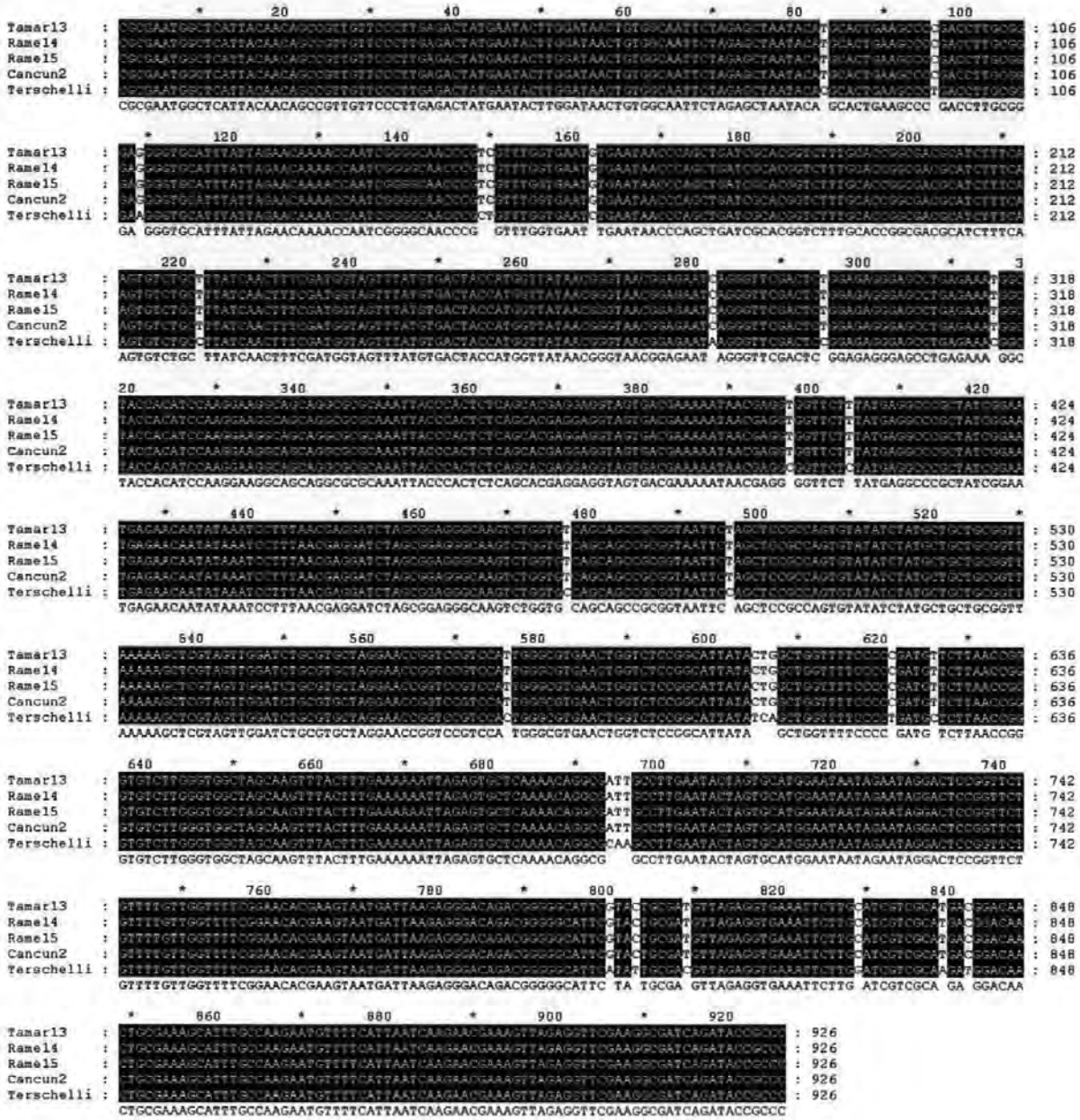


Figure 5.3: Alignment of 18S rRNA sequence (another haplotype) from a single specimen from the Tamar estuary, two specimens from Rame Head and another specimen from Cancun along with *T. longicaudata* sequence showing degree of conserved and variable regions.

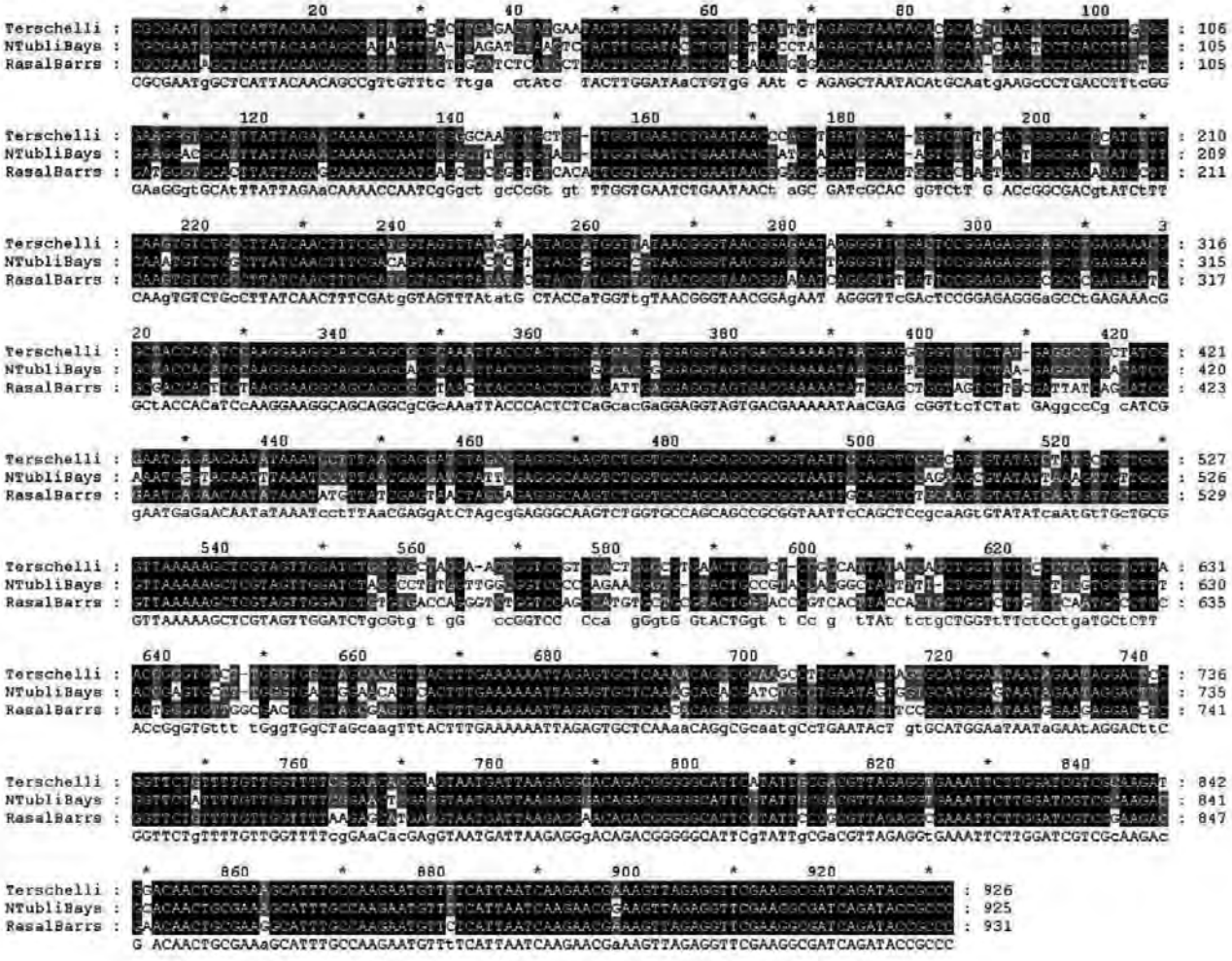


Figure 5.4: Alignments showing high degree of variation between specimens from Ras al Barr and North Tubli Bay in Bahrain and *T. longicaudata* 18S rRNA sequence.

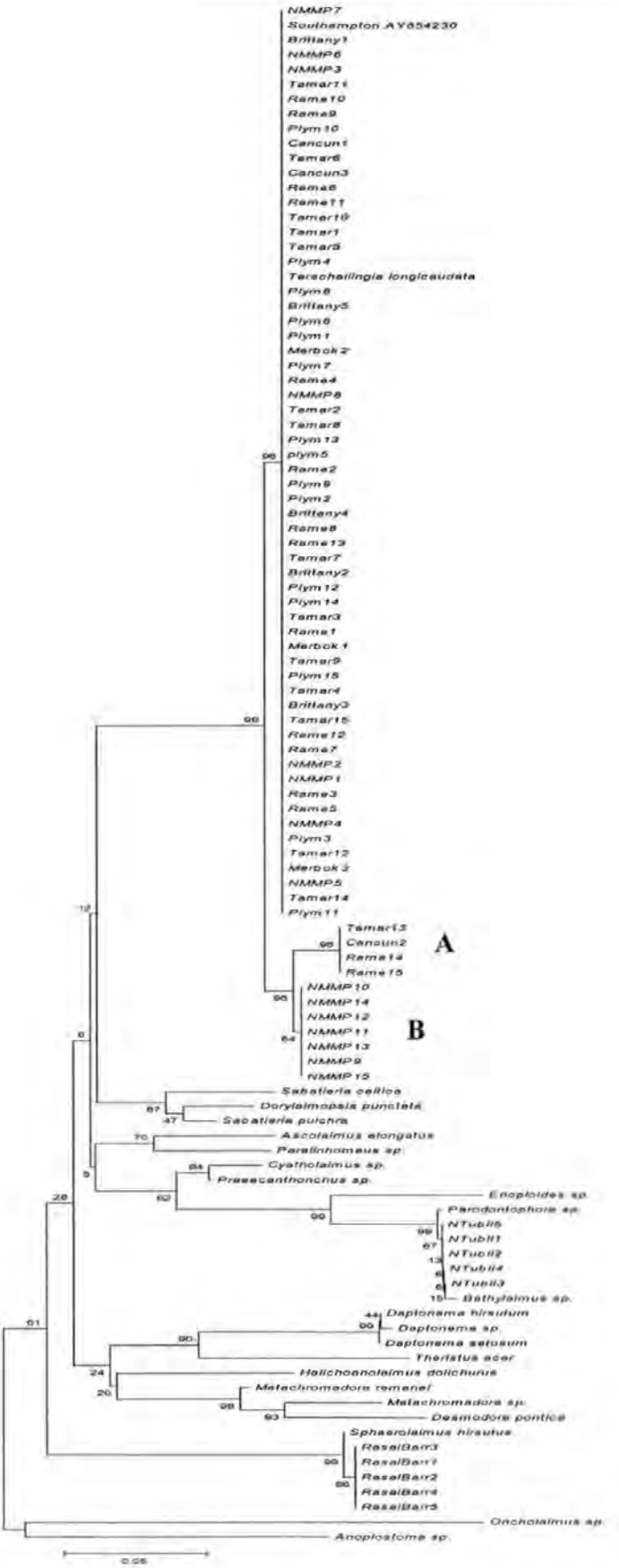


Figure 5.5: Neighbour joining tree of 18S rRNA sequences from populations morphologically identified as *T. longicaudata* and selected additional nematode taxa. The scale bar indicates 0.05 substitution/site. Numbers beside branches indicate bootstrap values (1,000 replicates).

5.3.2 Phylogenetic analysis

The majority of specimens of supposed *T. longicaudata* share a single 18S rRNA sequence, and appear together in the NJ tree (Figure 5.5). However some specimens from Rame, Tamar estuary, and NMMP (UK) and Cancun (Mexico), which had divergent 18S rRNA sequences, form a separate cluster (sequences A & B on the tree). In addition, specimens from North Tubli Bay and Ras al Barr (Bahrain) were segregated from the remaining sequences, appearing in different parts of the tree altogether. Ras al Barr sequences clustered with *Sphaerolaimus hirsutus* whereas North Tubli Bay sequences clustered with those of *Bathylaimus* sp. and *Parodontophora* sp.

5.3.3 Amplification and sequencing of the 28S rRNA gene from Ras al Barr and North Tubli Bay specimens

Amplification for all specimens from the Ras al Barr and North Tubli sites failed in this study.

5.3.4 PCR amplification of the mitochondrial COXI and COXII gene

For the mitochondrial COXI gene, amplification failed in all *T. longicaudata* specimens. Varying annealing temperatures did not produce any amplicons. No amplicons were obtained when genomic DNA was amplified by GenomiPhi^{TM} kit and subsequently used as a template for COXI amplification. As a result amplification and sequencing of this region was abandoned. Similarly no PCR products were obtained for the mitochondrial COXII gene in individuals.

5.3.5 Amplification of the internal transcribed spacer regions

PCR amplification for the spacer regions (ITS1 and ITS2) was erratic in most cases, yielding very little or no products. Wherever amplicons were obtained, sequencing and BLAST searches revealed amplified fungal ITS regions. Some of these amplified fungal

ITS regions were identified as *Filobasidium globisporum*, *Filobasidium elegans* and *Cryptococcus magnus* based on 100% homologies with those held in GenBank and EMBL databases. Most of these fungi have been reported from a variety of environments including aquatic habitats.

5.3.6 Multivariate analyses of the complete character sets for all individuals

The MDS plot for 60 individuals (male and female included and all characters used) from nine geographic locations is shown in Figure 5.6. This may be considered as an average representation of the multivariate information, as the stress value is 0.15 (Clarke and Warwick, 1994). There was considerable overlap between populations, and only a single cluster was observed when all the characters were included, albeit with some outlying specimens such as T1 (male) and T4 (female) from the Tamar estuary (UK), BF4 (female) from Brittany (France), NTBah2 (female) from North Tubli Bay (Bahrain) and P10 (male) from the Plym estuary (UK). The morphological characters that make these specimens markedly different from the rest of the set are absence of setae (subcephalic and somatic) and relatively longer spicules and gubernaculum.

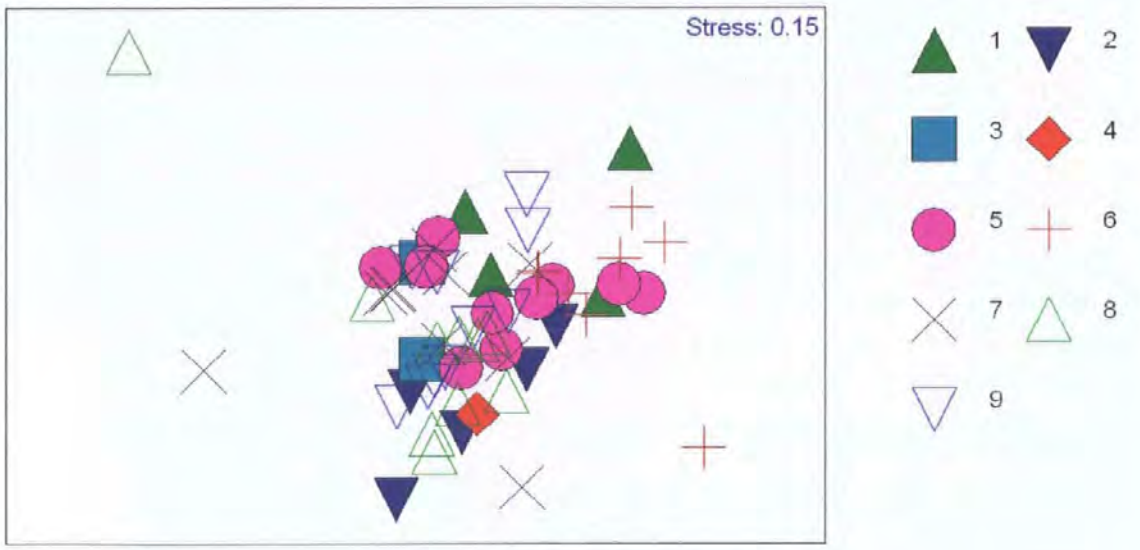


Figure 5.6: Multidimensional scaling (MDS) ordination of *Terschellingia longicaudata* males and females from different geographical locations based on all morphometric characters. Sites as follows: (1) Ras al Barr (Bahrain) (2) Brittany (France) (3) Cancun (Mexico) (4) Merbok (Malaysia) (5) NMMP (UK) (6) North Tubli Bay (Bahrain) (7) Tamar estuary (UK) (8) Plym estuary (UK) (9) Rame Head (UK).

An MDS plot for 60 individuals based on the 12 non-sexual characters (Figure 5.7) had a stress value of 0.16. Only a single cluster was visible even when the sexual characters were removed, although there were some outlying specimens such as T1 and T4 from the Tamar estuary (UK), BF4 from Brittany (France), NTBah2 from North Tubli Bay (Bahrain), RaB2 from Ras al Barr (Bahrain) and P10 from the Plym estuary (UK). The morphological characters that make the outlying specimens different from the rest of the group are absence of sub cephalic, cervical ad somatic setae, relatively longer tail and shorter body length.

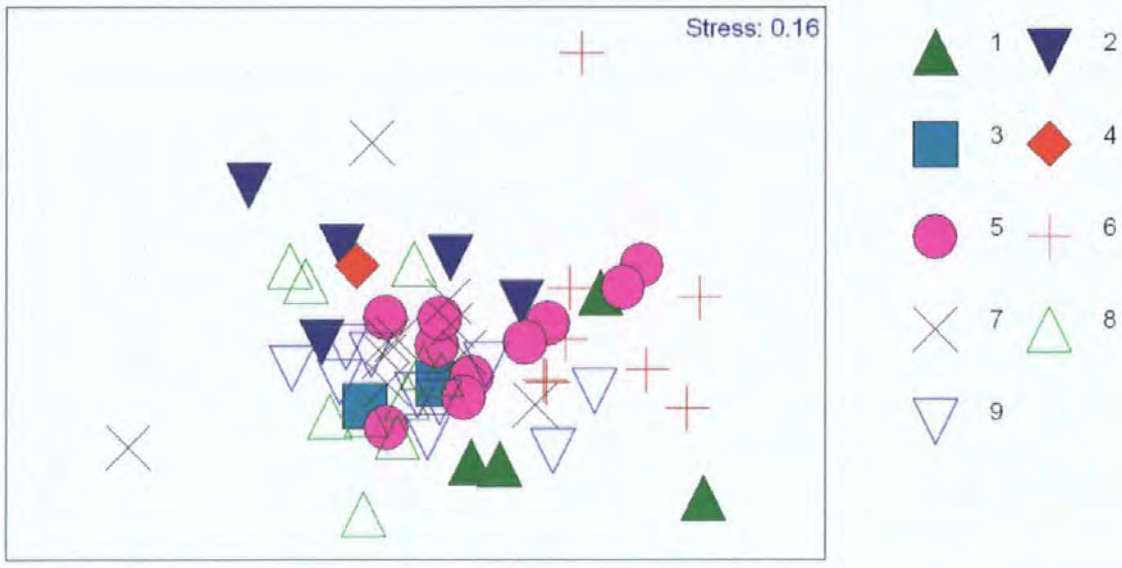


Figure 5.7: MDS plot of males and females from different geographical locations based on non-sexual characters. Sites as follows: (1) Ras al Barr (Bahrain) (2) Brittany (France) (3) Cancun (Mexico) (4) Merbok (Malaysia) (5) NMMP (UK) (6) North Tubli Bay (Bahrain) (7) Tamar estuary (UK) (8) Plym estuary (UK) and (9) Rame Head (UK).

5.3.7 MDS analyses of the complete character sets for males and females

Females

The MDS plot for 44 females from nine geographical locations based on 13 morphometric characters (Figure 5.8) is an average representation of the multivariate information, with a stress value of 0.16. Locations were not clearly seprared but there were some outlying specimens such as NTBah2, RaB2, BF4, T4 and Ra3.

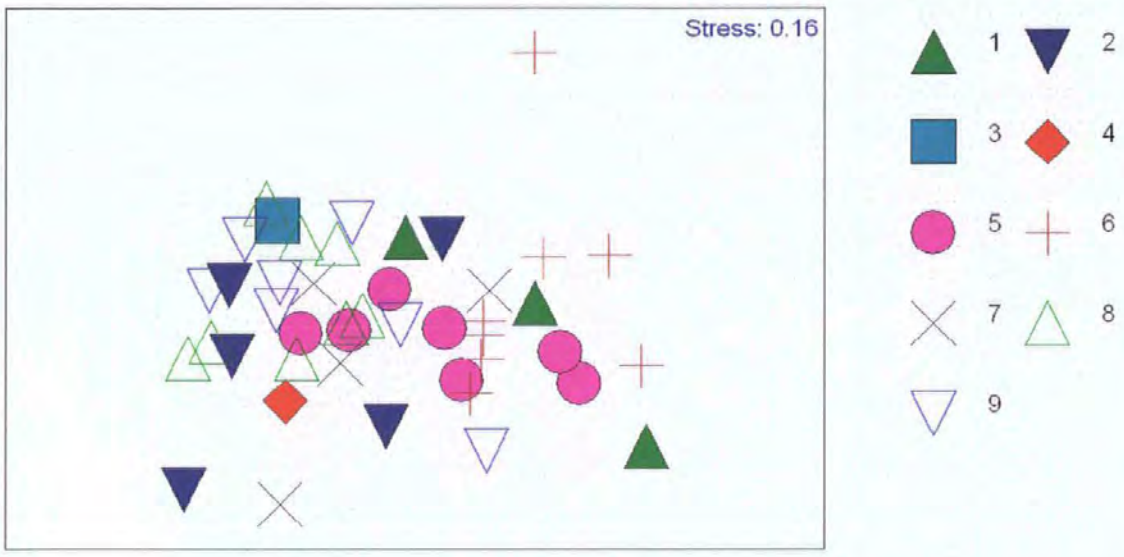


Figure 5.8: MDS plot of females from different geographic locations based on morphometric characters. Numbers correspond to sites as detailed in Figure 5.6

Males

The number of male specimens available for this study was relatively fewer (16 individuals in total) and the MDS ordination had a stress value of 0.12. There was very little difference between the sites although there were outlying specimens such as T1, P10, Ra4 and RaB3 (Figure 5.9).

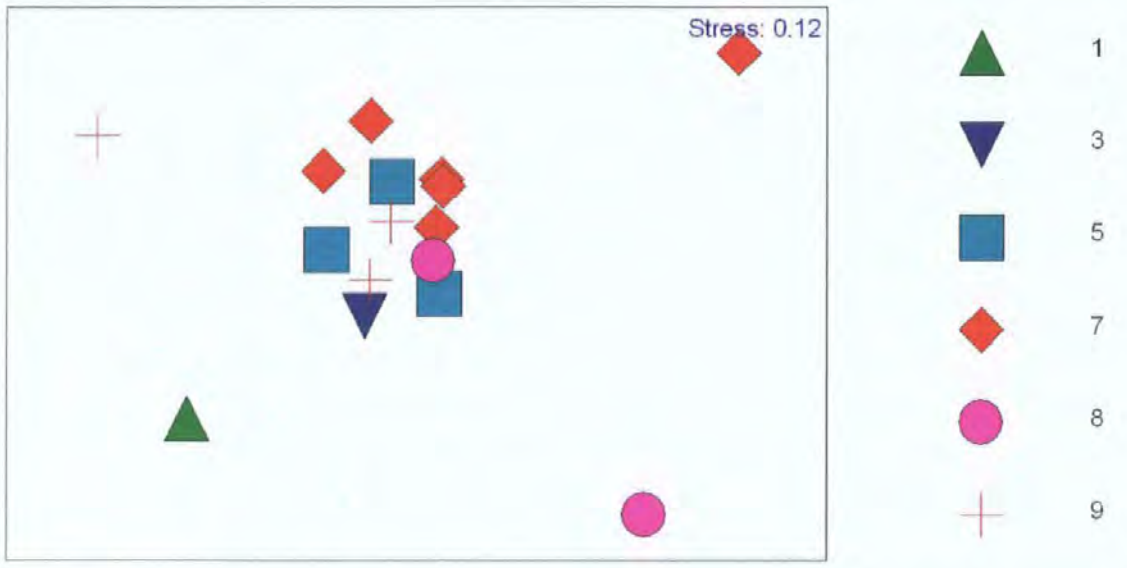


Figure 5.9: MDS plot of male specimens from selected geographic locations based on fourteen characters. Numbers correspond to sites as detailed in Figure 5.6

5.3.8 ANOSIM results for males and females

The results of the ANOSIM for females and males are presented in Tables 5.3 and 5.4. For one way ANOSIM of females, two sites, Merbok and Cancun, were excluded because of the lack of sufficient replicate specimens. The global R statistic in this test was 0.296. Comparison of the values of R from the pair wise tests indicate that specimens were significantly different morphologically between some sites e.g. those from Ras al Barr (site 1 as in Figure 5.6) were significantly different from those in Brittany (site 2 as in Figure 5.6), the Plym estuary (site 8 as in Figure 5.6), and Rame (site 9 as in Figure 5.6) but not significantly different from those at North Tubli (site 5 as in Figure 5.6), NMMP (site 6 as in Figure 5.6) or the Tamar (site 7 as in Figure 5.6). For one way ANOSIM analysis in males, few individuals were available. Here the global R value was 0.091 but there were no significant differences between sites in the pairwise tests. There was probably no difference between male specimens from sites 5 (North Tubli) and 8 (Rame) as indicated by the '0' value in ANOSIM result. Similarly the value of -0.148 possibly indicates that the male populations between sites 5 (North Tubli) and 9 (Rame) are more likely to be

similar to each other than to any of the other populations. Even though there were few replicates at some sites, in no case was a pairwise value of <0.1 reached. Therefore the lack of significant differences between male nematodes at the different sites is likely to be real indicating that male specimens from these sites were almost identical morphologically.

Table 5.3: Summary of results from one-way ANOSIM for female specimens. Value of ANOSIM statistic (R) for test for differences between sites. Number of permutations = 5,000. Global R value was 0.296. R values in bold indicate statistically significant differences ($p<0.05$).

Sites	R
Ras al Barr, Brittany	**0.467**
Ras al Barr, North Tubli	0.262
Ras al Barr, NMMP	0.022
Ras al Barr, Tamar Estuary	0.093
Ras al Barr, Plym Estuary	**0.702**
Ras al Barr, Rame Head	**0.516**
Brittany, North Tubli	0.235
Brittany, NMMP	**0.513**
Brittany, Tamar Estuary	-0.038
Brittany, Plym Estuary	0.164
Brittany , Rame Head	**0.272**
North Tubli, NMMP	0.119
North Tubli, Tamar Estuary	0.156
North Tubli, Plym Estuary	**0.276**
North Tubli, Rame Head	**0.297**
NMMP, Tamar Estuary	**0.358**
NMMP, Plym Estuary	**0.676**
NMMP, Rame Head	**0.531**
Tamar Estuary, Plym Estuary	0.221
Tamar Estuary, Rame Head	0.066
Plym Estuary, Rame Head	0

Table 5.4: Summary of results from one-way ANOSIM for male specimens. Value of ANOSIM statistic (R) for test for differences between sites. Number of permutations = 5,000. Global R value was 0.091.

Sites	R
NMMP, Tamar Estuary	0.031
NMMP, Plym Estuary	0
NMMP, Rame Head	-0.148
Tamar Estuary, Plym Estuary	0.396
Tamar Estuary, Rame Head	0.185
Plym Estuary, Rame Head	0.5

5.3.9 Similarity percentage (SIMPER) results

Similarity percentage calculations were determined on males and females combined firstly including all morphometric characters and then excluding sexual characters, then on only males and only females but including all characters. Merbok was excluded from the analysis because of the limited number of individual specimens. For the females-only analyses, the dissimilarity values between sites were generally low with the lowest value of 6.59% (Cancun, Plym Estuary) and the highest value of 21.49% (Cancun, North Tubli Bay). Despite low dissimilarity values, characters such as body length and distance from vulva to head contributed significantly towards separation of populations between statistically significant sites revealed by the ANOSIM analysis for females. The average dissimilarity values in males were also very low. The characters that contributed towards the separation of male populations between sites were body length and tail length but these results have little importance since there were no significant morphological differences in males between sites in the ANOSIM. Combining males and females and when all the characters were taken into consideration in the analyses the lowest and the highest average dissimilarity values were 12.39% (Brittany, NMMP) and 23.70% (Mexico, NMMP) respectively. The characters that contributed significantly towards separation of the

populations between sites were body length, tail length, distance from vulva to head and spicule length. When the SIMPER analysis was carried out on combined males and females but excluding sexual characters the lowest and highest average dissimilarity values were recorded as 5.79% (Cancun, Plym Estuary) and 18.31% (Cancun, North Tubli Bay) respectively. The characters that contributed towards separation of populations between sites were tail length and body length. The SIMPER analysis results are available in Appendix A.

5.4 Discussion

The main purpose of this study was to investigate populations of the cosmopolitan marine nematode species *Terschellingia longicaudata* from different geographical locations using a combination of morphometric and molecular techniques. The majority of specimens that were morphologically identified as true *T. longicaudata* following the diagnostic characters as described by De Man (1907) and later confirmed by Platt and Warwick (1988) shared a single 18S rRNA sequence. Such a finding suggests that *T. longicaudata* as currently defined is truly cosmopolitan in distribution. In addition, it is worth noting that individual samples come from a wide range of environments, including intertidal mud and shelf sediments at 70 m, low and full salinity waters, so the ecological range of *T. longicaudata* also appears to be genuinely wide.

There were some exceptions to this general pattern. Sequences from four individuals from the Tamar estuary and Rame Head in UK and Cancun in Mexico were identical and differed by 25 bases from the *T. longicaudata* sequence (Figure 5.3). In addition seven specimens from the NMMP site had a sequence that differed by 19 bases.

Molecular data from all specimens collected from Ras al Barr and N Tubli Bay in Bahrain showed very little similarity with *T. longicaudata* sequences (Figure 5.4). Sequences from Ras al Barr showed 99% homology with *Sphaerolaimus hirsutus* 18S rRNA sequence, differing at only 4 base positions. 18S rRNA sequences from North Tubli

Bay showed a 93% match with *Enoploides brunettii*, a species in the Order Enoplida, a quite different phylogenetic lineage of nematodes to that occupied by *Terschellingia longicaudata*, which is in the Order Monohysterida. The presence of these aberrant sequences in specimens from Bahrain, identified as *T. longicaudata*, is intriguing, and has a number of possible explanations.

Throughout the study, NJ analysis using gamma-corrected Kimura distances approach was adopted for tree building following Blaxter *et al.* (1998) methodology. Multiple specimens with similar genotypes from all geographical locations were included for the phylogenetic analysis due to the reasons as mentioned earlier in Chapter Four. While the bootstrap values for some of the branch lengths was low in the tree (Figure 5.5), this was possibly due to the fact that only half of the entire 18S rRNA sequence was included for phylogenetic analysis. On the other hand, outgroups had bootstrap values of 99 and above confirming the fact that genotypes with similar sequences were correctly assigned to *T. longicaudata* using NJ analysis and previously confirmed BLAST values (100%) from the sequences strongly supports this arguement.

In this study all specimens were carefully checked under a compound microscope by experienced nematode taxonomists and confirmed as *T. longicaudata* prior to molecular analyses. Sequences from selected Rame, Tamar and Cancun specimens (A in Figure 5.3) cluster closely with the majority of *T. longicaudata* material, as do those from selected NMMP individuals (B in Figure 5.3), but these sequences differ by 25 and 19 base pairs respectively from the common *T. longicaudata* sequence. The presence of these variant 18S rRNA sequences suggests either that this region of the 18S rRNA is variable in *T. longicaudata*, or that these specimens represent additional members of the genus, possibly previously unrecognized cryptic taxa. It is well known that the genus *Terschellingia* comprises more than 25 species (Gerlach and Riemann, 1973; Austen, 1986). Some of these species in addition to *T. longicaudata* have been reported from different oceans and estuaries and are thus widespread (e.g. *T. communis*) whereas some have restricted

geographical ranges (indeed *T. gourbaultae* is so far reported only from the Tamar estuary). All of these species have smaller oesophageal bulbs than *T. longicaudata*, however, and should have been detected though morphological examination, making it possible that the specimens with divergent 18S rRNA sequences indeed represent previously undetected cryptic taxa.

As discussed above, sequences from Bahrain individuals are highly divergent from *T. longicaudata*. The specimens from Ras al Barr showed 99% homology with *Sphaerolaimus hirsutus* with a difference of 3-4 bases. Both *S. hirsutus* and *T. longicaudata* belong to the Order Monohysterida but they are placed in separate families. Morover *S. hirsutus* is identified on morphological features that are significantly different from *Terschellingia*, e.g. *S. hirsutus* and other members of this genus have a buccal cavity surrounded by a heavily sclerotised buccal capsule which is completely absent in *T. longicaudata*. Whilst contamination of genomic DNA from *S. hirsutus* during experimental work on specimens from Ras al Barr site cannot be ruled out it appears unlikely. In the DNA barcoding study the D2/D3 segment of the 28S rRNA gene was readily amplifiable in *S. hirsutus* but when the same set of primers were used on Ras al Barr specimens no amplicons were obtained. The amplification parameters were changed and repeated several times but no products were obtained.

Sequences from the North Tubli Bay site showed considerable homologies with *Enoploides brunetti* and also with *Parodontophora* sp. These taxa belong to a different order to *T. longicaudata* and are again morphologically very different from the genus *Terschellingia*. It is still unclear why the specimens from North Tubli Bay that were identified as *T. longicaudata* generated sequences which show minimal homology with actual *T. longicaudata* 18S rRNA sequence. Once again contamination cannot be ruled for North Tubli Bay specimens.

Error during sequencing can be ruled out in both these cases, because amplification and sequencing were repeated for all these specimens and the sequence data generated in

this study has been validated extensively. Morphological changes due to ethanol preservation could potentially have led to misidentification of these specimens due to shrinkage, although the characteristic long tail of *T. longicaudata* tends to remain distinct despite this. One possible explanation is that the specimens selected from the Bahrain sites for molecular analyses may have been juveniles, which had not yet developed the adult characters which allow positive identification (Platt and Warwick, 1988), and could inadvertently have been assigned to the wrong genus.

Three mitochondrial genes were targeted in this study but no amplicons were achieved for any of these, which could be due to several reasons. Because amplification failed in *T. longicaudata* it may be that the arrangement of the mitochondrial genome in this species is different from that of previously studied (i.e. parasitic) nematodes. It is well known that nematode mitochondrial genomes are prone to recombination rearrangements and insertional deleting (Blouin, 1998; Keddie *et al.*, 1998; Armstrong *et al.*, 2000; Lavrov and Brown, 2001). The cytochrome oxidase I primers that were used in this study were designed on available parasitic nematode mitochondrial genomic sequences and there is a possibility that there was no match between the primer sites and genomic regions in *T. longicaudata*. This is supported by the fact that genomic DNA from *T. longicaudata* initially amplified by GenomiPhi kit and subsequently diluted for amplification with COXI primers failed to produce any PCR products.

Ironically, Derycke *et al.* (2005) successfully amplified mitochondrial cytochrome c oxidase I gene fragments from different populations in the marine nematode *Pellioditis marina* using the same set of primers that were designed and applied in this study. The success with this marine nematode could be due to its membership of the family Rhabditidae which also includes another well known nematode, *Caenorhabditis elegans*. Therefore there is a possibility that the mitochondrial genome of *Pellioditis marina* matched the primers that were designed on available sequences belonging to members of Rhabditidae as well as other families in the phylum Nematoda. While the cytochrome

oxidase subunit II gene failed to amplify in *T. longicaudata*, the primers successfully amplified around 220 bp in *Oncholaimus* sp., a marine nematode taxon belonging to the order Enoplida. The results make it more likely that the mitochondrial genomic arrangement could be unique in *T. longicaudata*. Use of high fidelity enzymes, and varying annealing temperatures for primers did not increase the success with any of the mitochondrial genes in this study.

Two sets of primers used for amplifying the spacer regions between the nuclear small subunit (18S rRNA) and the large subunit gene (28S rRNA) were also tested in this study. Sequencing results revealed fungal ITS1 and ITS2 regions in all cases instead of nematode internal transcribed spacer sequences and the homologies were 100% in most cases. The primers tested in this study have been used in the past for molecular systematic studies of strongyloid nematodes, which are taxonomically very different from marine nematode groups (Hung *et al.*, 2000; Chilton *et al.*, 2001) yet in the present study there was a considerable problem with extensive co-amplification of fungal spacer regions. As a result the use of these molecular regions was abandoned.

The application of morphometrics on populations collected from different geographical locations suggested that these are morphologically similar, and did not generally point to the presence of cryptic species. MDS plots showed very little differences between the populations, although there were some outlying specimens in the MDS ordination plots. These specimens differed considerably from the rest of the populations based on presence or absence of certain morphometric characters. Generally there was no difference between the populations from UK sites and other global locations. Indeed for sites such as NMMP, Rame and the Tamar Estuary, the majority of the specimens turned out to be *T. longicaudata* based on 18S rRNA sequencing and the MDS seems to be support such interpretation where differences are almost negligible in morphological space. Even when females were used for MDS analysis generally no differences were observed between the sites. For males, the interpretation of the MDS was largely restricted due to

the shortage of replicates for analysis. The ANOSIM values showed statistically significant differences between females from some locations. Very little information was obtained for males, possibly due to the lower number of sites that could be analysed due to the limited number of male specimens at some sites. SIMPER analyses showed that characters such as body length, tail length, oesophagus length, distance between the vulva and head and spicule length contributed towards the population separation that did occur between some sites. The technique was not sufficiently powerful to distinguish potentially cryptic species shown in molecular analyses (A and B) but this may have been due to insufficient replicate specimens of these potentially cryptic species. While Warwick and Robinson (2000) were able to use morphometrics to distinguish population differences and cryptic species assemblages, morphological analyses of specimens from much more widely separated geographical locations in this study did not provide any conclusive evidence of cryptic species in *Terschellingia longicaudata*. Based on the limited datasets available the differences between populations from all the geographical locations were very low indicating the possibility that *T. longicaudata* is a true cosmopolitan species and has a wide geographical distribution ranging from UK to Mexico as well as in Far East Asia such as Malaysia.

To conclude, studies of 18S rRNA in *T. longicaudata* suggest that the species is truly cosmopolitan, since samples from different oceans share the same DNA sequence. In addition, the study points to the possible presence of two previously unrecognised cryptic species. Morphometric analysis of individuals from the same populations (although admittedly not the same individuals) suggests that these cryptic species are morphologically indistinguishable from typical *T. longicaudata* using standard morphometric approaches. Such morphologically indistinguishable species have been reported from a wide range of organisms, including marine meiofauna (Westheide, 1990; Schmidt and Westheide, 2000). Further work is needed to confirm these results, however, which are based on a single genetic locus, where possible intra-specific variability in

nematodes is poorly understood. Interestingly, one of the divergent 18S rRNA sequences reported here is itself widespread (UK & Mexico), suggesting that one of these cryptic forms is also widespread in distribution. Neither of the divergent sequences occurs exclusively within localities either, instead being found together with typical *T. longicaudata* genotypes. Such an observation suggests that the potential cryptic taxa may be genetically differentiated despite being apparently indistinguishable morphologically.

6. Effectiveness of formalin and other organic compounds for the short term preservation of marine nematodes for combined molecular and morphological studies

6.1 Introduction

The development of PCR and automated DNA sequencing in the last two decades has facilitated many areas of research in marine biology. Increasingly molecular tools are applied to address questions of phylogenetics and evolution and genetic variation within and among populations in meiofaunal groups, such as the free living marine nematodes. Since nematode taxonomy relies on microscopic examination of morphological features of specimens, including body shape and relative proportions, thus distortion during specimen fixation should be kept to a minimum. Additionally, molecular and morphological studies need to be integrated to avoid associating sequence data with the wrong taxon; ideally both techniques should be applied to the same specimen.

Traditionally, formalin and its derivatives have been the preferred choice of compound for preserving or fixing marine specimens, including the free-living benthic nematodes, for long term studies because these are inexpensive, effective and low-maintenance preservatives, and they maintain the morphological integrity of specimens (Bucklin and Allen, 2004). Formalin is a 37% aqueous solution of formaldehyde (CH_2O), the most reactive of all the aldehydes. It has been widely used as a principal ingredient in different fluids used for preserving biological and medical samples (Blum 1893, 1894; Jones, 1976; Fox *et al.*, 1995). Museums and scientific institutions all over the world have large collections of animal specimens including marine invertebrates preserved in formaldehyde and its derivatives. A significant proportion of remaining archival specimens in museums were fixed in formaldehyde prior to storage in alcohol (Chatigny, 2000). In the last two decades with the increasing application of molecular technologies the possibility has arisen to use archival specimens in molecular diversity studies. Unfortunately

specimens fixed in formalin for a long period of time are considered to be unsuitable for DNA work due to the effects of this fixative on tissue components, including nucleic acids. In the following section details of the effects of formaldehyde on tissues and nucleic acids are elaborated followed by a section on extraction techniques that are used to recover DNA from formalinised archived tissues and organisms.

Although formalin is a good preservative, it is thought to have direct and indirect effects on tissues and nucleic acids. Almost no systematic studies are available to date exploring the possibility of using formalin and other organic compounds for short term preservation of soft bodied metazoans such as nematodes so as to carry out simultaneous morphological and molecular work. Therefore the focus of this chapter is to investigate whether morphological as well as molecular works could be performed on nematodes through short-term preservation in formalin and other organic compounds. If short term preservation works it would allow both morphological and molecular analysis to be performed on the same individual. The development and optimisation of a technique that could be used to recover DNA from formalin preserved archival nematode worms stored in museums and research institutions for molecular diversity studies is also explored.

6.1.1 Formaldehyde and its effects

6.1.1.1 Mechanism of formaldehyde fixation

French and Edsall (1945) and Walker (1964) were foremost in reviewing the chemistry behind formalin fixation in biological tissues. Formaldehyde acts in the fixation process by combining with the functional groups of certain amino acids, thereby denaturing proteins in tissues. During the primary reaction process the oxygen atoms in formaldehyde undergo hydrogen bonding with primary amines to cross-link proteins. The reactions with the proteins are complex and involve combination with a number of different functional groups. All these reactions are highly dependent on physical factors such as pH, buffers, concentration, temperature, fixation time, etc. (Thompson, 1966;

Crisan and Mattson, 1993; Hamazaki *et al.*, 1993; Koshiba *et al.*, 1993). Some of these reactions are rapid and some slow; some are reversible and others irreversible (French and Edsall, 1945; Freifelder and Davison, 1963; Jackson, 1978; Chaw *et al.*, 1980).

Formaldehyde has been shown to cause the formation of DNA-DNA, DNA-protein and protein-protein hybrids through the formation of cross links (Chaw *et al.*, 1980; Ma and Harris, 1988; Crisan and Mattson, 1993; Chang and Loew, 1994). Some of these cross-links can be partially broken, permitting limited success with PCR, electrophoresis and slot blot experiments (Jackson, 1978; Jackson and Chalkley, 1981; Solomon and Varshavsky, 1985; Orlando and Pardo, 1993). At a neutral pH, formaldehyde can react with three of the bases of DNA: cytosine, guanine and adenine (Fraenkel-Conrat, 1954; McGhee and von Hippel 1975 a, b, 1976 a, b; Neubauer *et al.*, 1992) resulting in the creation of a reactive compound via the methylene group. It is thought that this reactive state can hinder primer annealing, inhibit renaturation, and suppress the replication procedure in PCR (Karlsen *et al.*, 1994). Karlsen *et al.* (1994) also noted that only 2.5% of DNA-protein cross-links need to remain to cause the polymerase enzyme to malfunction after 200 bp. Chang and Loew (1994), however, reported that AT-rich regions of the DNA molecule are more susceptible to reaction with formaldehyde than regions dominated by GC bases and suggested that base composition may influence the success of PCR. To date, the precise effects on nucleic acids of long term exposure of specimens or tissues to formaldehyde are not well characterized. For example, Rumph and Williams (1986) noted that less formaldehyde could be eluted from tissue stored for 100 days than from tissue stored for 50 or 75 days suggesting possible secondary reactions. In addition, the effects of substances such as formic acid, methanol, methylal, methyl formate and polymers of various compositions formed during prolonged formalin storage on DNA remain largely uninvestigated.

6.1.1.2 Nucleotide modifications

As mentioned previously, formaldehyde fixation induces apparent nucleotide substitution in DNA molecules. Karlsen *et al.* (1994) reported that there is a high risk of modification of double stranded DNA molecules when kept exposed to formaldehyde. Wong *et al.* (1998) and Williams *et al.* (1999) detected artificial mutations from partially degraded DNA recovered from formalin-fixed paraffin-embedded tissues. Williams *et al.* (1999) recorded up to one mutation artefact per 500 bases of DNA sequence from formalin-fixed material and through comparison of PCR products amplified by *Taq* DNA polymerase and *Pfu* DNA polymerase found that the error frequency was one per 683 bases and one per 2050 bases respectively. A number of infidelities were also found in a 634 bp rRNA fragment amplified from fixed tissue of the nematode worm *Caenorhabditis elegans* when compared to sequences from unfixed specimens (De Giorgi *et al.*, 1994). The artefacts consisted of single-site mutations where G and T were inserted into the sequence. Chaw *et al.* (1980) using reverse-phase high-pressure liquid chromatography have shown that formaldehyde treated nucleic acids undergo changes and form cross linked nucleosides. Masuda *et al.* (1999) found a heterogeneous increase in the molecular weight of RNAs recovered from formalin-fixed samples and through MALDI-TOF mass spectrometry showed addition of mono-methylol ($-CH_2OH$) to all the four bases at various rates. The modification rate varied from 40% for adenine to 4% for uracil. In addition, some adenines also underwent dimerization through methylene bridging. The authors were able to remove most of the methylol groups from the bases by simply elevating the temperature in formalin-free buffer and restored the template activity of RNA from fixed tissue (Masuda *et al.*, 1999). Conversely France and Kocher (1996) and Bhadury *et al.* (2005) did not find any infidelities while working with formalin-preserved deep sea crustaceans and marine nematodes respectively. Whilst workers should be aware of the possibility of formalin-induced substitutions, their extent, and the influence of factors discussed above on their frequency, are both poorly understood at present.

6.1.2 Fixation and storage conditions and DNA recovery

As discussed above, DNA degradation occurs in specimens or tissues fixed in formaldehyde. Formalin-fixed tissues typically provide low yields of extractable DNA and RNA that exhibit significant degradation (Dubeau *et al.*, 1986; Rupp and Locker, 1988). A study by Shibata (1994) concluded that because of this degradation PCR targets from formalin-preserved samples should be less than 400 bp in length. Goelz *et al.* (1985), however, have reported recovery of DNA fragments of up to 10,000 bp from tissues fixed in 4% neutral-buffered formaldehyde, and Savioz *et al.* (1997) successfully amplified an 838 bp fragment from 46 year old formaldehyde-fixed tissue. The success of obtaining high quality DNA from fixed and embedded tissues appears to be dependent on the following factors (Crisan and Mattson, 1993): (a) the chemical composition of the fixative (b) duration and temperature of fixation (c) size of the specimen and its permeability to the fixative (d) the duration of tissue hypoxia (which is proportional to the amount of DNA degradation) and finally (e) the length of storage time.

One of the problems with archived zoological material is the limited amount of information available regarding the details of fixation which in turn could affect the overall DNA yield. For example, the formaldehyde may or may not have been buffered, fixation time can vary from a few hours to many years and fixation temperatures vary drastically. Indeed, Warén (1983) reported that in some cases boiling formaldehyde solution was used to fix specimens so as to speed up the fixation process, and as Koshiba *et al.* (1993) have shown, higher fixation temperatures result in higher DNA degradation.

Neutral-buffered formaldehyde is a better fixative in terms of DNA preservation than low pH formaldehyde (e.g. Nuovo and Silverstein, 1988; Hamazaki *et al.*, 1993), and there is evidence to show that formaldehyde with low pH or formaldehyde with high formic acid content causes greater degradation of DNA than neutral buffered formaldehyde (Koshiba *et al.*, 1993). Similarly specimens or tissues fixed in non-buffered formaldehyde for prolonged periods give low DNA yields (Rogers *et al.*, 1990; Greer *et al.*, 1991;

Forsthoefel *et al.*, 1992; Hamazaki *et al.*, 1993; Karlsen *et al.*, 1994; Inoue *et al.*, 1996). Karlsen *et al.* (1994) found that DNA could be isolated from tissues fixed in formaldehyde for between 8 and 48 hours, but the extraction failed in tissues fixed for 96 hours, indicating that a longer formaldehyde reaction time can change the DNA extraction capacity. In another study, Inoue *et al.* (1996) extracted DNA from tissues fixed in non-buffered formaldehyde for up to 6 days, but not for 7. Although prolonged storage of specimens in formalin decreases the recovery of DNA, useful DNA has been extracted from tissues up to 85 years old (Shiozawa *et al.*, 1992; Gall *et al.*, 1993; Wang *et al.*, 1994; Pavelic *et al.*, 1996; Shedlock *et al.*, 1997; Chase *et al.*, 1998; Schander and Halanych, 2000).

6.1.3 Common extraction techniques

Optimization of extraction procedures is critical in obtaining DNA usable for PCR amplification from formaldehyde fixed specimens. According to Whittier *et al.* (1999) extraction methods can also differ in their performance with different species. Jackson *et al.* (1990) found compounds such as sodium dodecyl sulphate (SDS) used in DNA extraction protocols could inhibit *Taq* polymerase resulting in lower yields of PCR products. Nevertheless it has been used in several protocols for DNA extraction from archival animal tissues and these have yielded sufficient amounts of PCR products (Shiozawa *et al.*, 1992; De Giorgi *et al.*, 1994; France and Kocher, 1996). Studies involving prolonged digestion of tissues or animal specimens with Proteinase-K give higher yields of DNA than other methods (Shiozawa *et al.*, 1992; Crisan and Mattson, 1993; France and Kocher, 1996; Shedlock *et al.*, 1997). Wang *et al.* (1994) and Shedlock *et al.* (1997) reported that extraction methods involving phenol chloroform could damage, or result in the loss of a large amount of, fragile archival DNA. On the other hand, techniques involving Chelex-100 have resulted in high DNA yield (Walsh *et al.*, 1991; Gill *et al.*, 1992; Wang *et al.*, 1994; Kirby and Reid, 2001).

One of the earliest studies conducted by Shiozawa *et al.* (1992) successfully implemented proteinase K extraction to recover and amplify a 120 bp fragment D loop region of mitochondrial DNA from museum specimens of fish that had been stored for over 66 years. De Giorgi *et al.* (1994) also used proteinase K and phenol chloroform precipitation to investigate the effects of formalin on DNA using both formaldehyde-fixed and fresh material from the nematodes *Xiphinema* sp and *Caenorhabditis elegans*. They were able to amplify and sequence a 643 bp long fragment of the 26S rRNA gene and reported a number of infidelities from formalin-preserved nematode material. Similarly, France and Kocher (1996) performed DNA extractions on formalin fixed, ethanol-preserved deep sea crustaceans using a modification of Shiozawa *et al.*, protocol. They found that nucleotide sequences for mitochondrial 16S rRNA and COXI genes can be recovered from collections of varying age and that these sequences remained unmodified compared with those derived from frozen specimens. Yue and Orban (2001) implemented the Chelex-100 method to recover and successfully amplify DNA from formalinised fish scales. Kirby and Reid (2001) also used this method to amplify and sequence from a larval sandeel (*Ammodytes* sp.) stored in buffered formalin. Shedlock *et al.* (1997) used prolonged soaking of invertebrate as well as vertebrate tissues in GTE buffer (100 mM glycine, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and subsequent proteinase K digestion for DNA extraction and subsequent amplification. They amplified 570 bp of mitochondrial 16S rRNA and 470 bp of Cyt b genes from recovered DNA samples. Dorris *et al.* (2002) also used GTE buffer and proteinase K to recover and amplify DNA from formalin-fixed nematodes. Some researchers have also modified commercially available DNA extraction kits for extracting DNA from archived organisms (Chase *et al.*, 1998).

6.1.4 Contamination

One of the critical aspects of DNA related studies from archived tissues and whole organisms is the extent of contamination affecting the amplification process. Cawkwell and

Quirke (2000) reported that contaminants constitute a serious problem for amplification when the archival DNA is more or less degraded. The combination of low concentration template DNA from formalin fixed tissues or specimens along with numerous amplification cycles and dynamics of primer annealing may contribute to co-amplification of contaminants (Boyle *et al.*, 2004). Boyle *et al.* (2004) also encountered contaminants such as human, chicken, cow, and even limpet DNA getting amplified along with deep sea macrofaunal samples. Similar observations were also recorded by Chase *et al.* (1998) and Schander and Halanych (2003) while amplifying minute deep sea bivalve specimens respectively. Such sporadic contamination could be avoided by using species-specific primers and performing routine extraction and amplification procedures in separate work spaces (Boyle *et al.*, 2004; Bhadury *et al.*, unpublished).

6.1.5 Other organic compounds used for specimen fixation

Given the difficulties discussed above, other organic compounds are typically used for specimen fixation for molecular work. Whilst most of these may have no measurable effects on DNA integrity, their influence on specimen morphology can vary considerably. Molecular grade ethanol is the most common form of compound used for specimen and tissue fixation (Kiesling *et al.*, 2002; Black and Dodson, 2003; Han *et al.*, 2004; Lapegue *et al.*, 2004; Cook *et al.*, 2005). Ethanol preserves specimen by inhibition of cellular enzymes which would otherwise lead to sample degradation. Ethanol is generally considered a good preservative for maintaining specimen and DNA integrity (Post *et al.*, 1993; Dessauer *et al.*, 1996; Takemoto *et al.*, 1995; Flournoy *et al.*, 1996; Koch *et al.*, 1998). For small soft bodied metazoans such as nematodes and polychaete worms, specimens preserved in ethanol tend to deteriorate and shrink rapidly (BHM Meldal *pers comm.*; SNMNH, 2005). Such observations have also been noted for small arthropod specimens and other benthic invertebrates (Morin *et al.*, 2004; NHMUSA, 2005). Moku *et al.* (2004) reported significant shrinkage in the body length of myctophid fish larvae when

preserved in 70% isopropyl alcohol, 70% and 90% ethanol. The shrinking was highest for specimens fixed in isopropanol, followed by 90% ethyl alcohol and 70% ethyl alcohol. Takizawa *et al.* (1994) found that denaturation and dehydration induced by alcohol makes it difficult to obtain accurate measurements of body sizes and body mass values in organisms such as fish larvae. Kruse and Dalley (1990) reported that preserved capelin (*Mallotus villosus*) larvae shrinked much faster when preserved in anhydrous alcohol; there was also differential shrinkage with total length decreasing at a faster rate than standard length. Tucker and Chester (1984) showed that shrinkage was species-specific and that it varied depending on the size of individuals of the same species. Dorris (2000) and Dean and Ballard (2000) noted that extraction of DNA from ethanol preserved nematodes and other invertebrates becomes difficult over time because DNA preserved in ethanol apparently degrades. Such observations were also made by Corthals and Feinstein (2002). Dawson *et al.* (1998) who compared 70% ethanol, lysis buffer, DMSO-NaCl solution, NaCl/CTAB solution and urea extraction solution as preservatives for four classes of marine invertebrates (Anthozoa, Gastropoda, Polychaeta and Scyphozoa). They found DMSO-NaCl to be the most effective method for maintaining integrity of specimens and DNA. Kilpatrick (2002) also reported that a long period in DMSO solution did not apparently affect the integrity of DNA molecules. Further studies by Lee and Beynon (2004) found nail varnish remover (NVR) (a mix of acetone, benzyl alcohol and esters) to be a suitable material for the short-term preservation of whole barnacles for PCR analysis. Fukatsu (1999) reported acetone as a good preservative for insects for molecular studies and proposed that the organic compound maintains the histological integrity of specimens thereby aiding in ultrastructural or taxonomic works. In another study Milton and Venkatesan (1999) used Bouin's fluid in addition to formalin for fixing egg capsules of the aquatic belostomatid bug *Diplonychus indicus*. Mikulski *et al.* (2005) used a mixture of modified saline-ethanol and 10% formalin to fix and store *Karenia brevis* (dinoflagellate) cells for seven months and later successfully labelled those cells with a large subunit

ribosomal RNA probe for flow cytometry analysis. With the exception of the effects reported with ethanol based preservatives, there is no information on how these additional preservatives affect the morphological integrity of soft-bodied animals.

6.1.6 The problems faced in Nematology

Although formalin is a good fixative in terms of specimen integrity, specimens fixed for long time periods are generally thought of as being unsuitable for DNA work (see above). Increasingly in nematology, and indeed many other branches of biology, specimens are being fixed in ethanol for subsequent molecular studies, despite the drawbacks in terms of specimen integrity. At times alcohol preservation of small, soft-bodied metazoans such as nematodes and polychaete worms result in shrinkage of specimens, often making accurate morphological identification impossible (Bhadury *et al.*, 2005; BHM Meldal *pers comm*). Such shrinkage has serious implications in morphometrics where the shape and size of individual animals is extremely important. Additionally, a huge number of marine invertebrates fixed in formalin are currently stored in research collections. These specimens could be exploited for genetic studies through recovery of archival DNA in order to address questions of evolution and population genetics.

There is a lack of systematic investigation into the effects of formalin on metazoan DNA and whether the success of DNA amplification is dependent on the time scale of fixation. Such data would be extremely useful to enable morphological and molecular studies on the same individual. To date most of the studies looking at the effects of formalin on DNA sequences, including infidelities and nucleotide modifications, used standard polymerase enzymes in PCR amplification. Standard *Taq* polymerase enzymes are prone to higher error rates because they lack the proof reading capacity of other polymerases such as *Pfx* and *Pfu* DNA polymerase (Eckert and Kunkel, 1991). Therefore, application and comparison of high fidelity and standard polymerase enzymes on DNA

templates exposed to formalin for a certain time period could provide information regarding nucleotide substitutions and artefacts. Additionally there have been very few studies investigating whether the use of other organic compounds for fixing small soft bodied metazoans such as nematodes would compromise specimen integrity and subsequent molecular analyses.

With these considerations in mind, the purpose of this chapter was to investigate four main aims. These were:

- To investigate whether nematodes preserved in formalin for relatively short periods of time could provide DNA suitable for molecular analyses, and, if so, to determine the duration of this time window (Formalin time series investigation).
- To investigate formalin-induced changes by comparing DNA sequences amplified from formalin and alcohol-fixed nematode worms using high fidelity DNA polymerases.
- To optimise an extraction technique that could be used for recovering DNA from archived formalin-fixed marine nematode specimens.
- To test whether organic preservatives such as acetone, a ketone compound, and butanol, a tertiary alcohol, could be used for nematode worm fixation without affecting the morphological integrity and subsequent molecular analysis.

6.2 Materials and Methods

6.2.1 Formalin time series investigation

6.2.1.1 Sediment fixation and meiofauna extraction

For the purpose of this study, individuals of the cosmopolitan free living marine/estuarine nematode *Terschellingia longicaudata* De Man, 1907 were examined. This species is reported from most of the world's oceans and estuaries, and is typically one of the dominant species in soft sediments in inshore waters (see Chapter Five, Figure 5.1). Twenty five grams of intertidal mud from Saltash (low water neaps) (50° 24' N, 4° 12' W)

on the Tamar estuary, Cornwall (UK) was fixed in 200 mL of 5% unbuffered formalin (Sigma, UK) and stored overnight at room temperature (20°C). The formalin-fixed sediment was subjected to meiofaunal extraction the next day following Somerfield and Warwick's (1996) flotation method. Extracted fauna were then stored in a 50 mL Falcon tube containing 4% unbuffered formalin. *T. longicaudata* specimens (n=6) were picked from the Falcon tube after selected time intervals of 2, 3, 4, 5, 6, 7, 9, 11, 13, 15 and 30 days. Individual specimens were then placed in 0.5 ml PCR tubes containing 20 µL of 0.25 M NaOH for nucleic acid extraction.

6.2.1.2 Nucleic acid extraction and PCR amplification

DNA was extracted using the modification of the method of Floyd *et al.* (2002) described in Chapter Two (Section 2.3.2). The extract was then used for PCR amplification as described below. Two primers, namely G18F forward and 22R reverse were used for PCR amplification of the partial 18S rRNA gene (Blaxter *et al.*, 1998) producing an amplification product of approximately 400bp. PCR reactions were performed on 5 µL aliquots of the extracted DNA as mentioned in Chapter 3 (Section 3.2.3.3). *Pfx* DNA polymerase (Invitrogen, UK) was also used in addition to *Taq* DNA polymerase to amplify individuals picked on the 11^{th}, 13^{th}, 15^{th} and 30^{th} day. P*fx* DNA polymerase is a proofreading enzyme with 3' to 5' exonuclease activity that provides higher fidelity than *Pfu* DNA polymerase and works on complex templates (Cline *et al.*, 1996). From the 11^{th} day until the 30^{th} day *Pfx* DNA polymerase (Invitrogen, UK) was used for PCR amplification to investigate whether there was any template DNA available which would not normally amplify with *Taq* DNA polymerase due to the enzymes lack of processive and high fidelity properties. The PCR reactions were set up according to manufacturer's instructions. 5 µL of each of the PCR products were analysed in 1% (w/v) agarose gels using the Mini sub cell GT systems (Bio-rad Laboratories, USA). A 100 bp ladder (Promega, UK) was used as a size marker.

6.2.1.3 DNA sequencing of PCR amplicons

To check the integrity and identity of the amplicons as well as to investigate any formalin-induced nucleotide substitution PCR products amplified using *Pfx* DNA polymerase from the 11th day specimens were directly treated with ExoSAP-IT according to the manufacturer's instructions (USB Corporation, USA). Sequencing was carried out in both directions (forward and reverse) using an additional nematode specific internal forward primer MN18F and the 22R reverse primer in an ABI Prism 3100 Genetic Analyzer. Additionally, the 18S rRNA gene from *T. longicaudata* specimens preserved in molecular grade ethanol (Hayman Limited, UK) was amplified and sequenced using the same protocol. The generated sequences from formalin-fixed specimens were then compared with those from alcohol-preserved specimens, to check for possible ambiguity and errors, in the Clustal-X alignment program using default parameters (Jeanmougin *et al.*, 1998; Thompson *et al.*, 1997).

6.2.2 Study of long term and short term archived marine nematode materials

Marine nematode worms collected in two previous studies were used for DNA extraction and subsequent molecular analysis, in addition to the materials used in the unbuffered formalin time series experiment. The first of the specimens were collected in 1984, fixed in formalin and mounted in anhydrous glycerol onto slides as part of a study investigating the factors affecting meiobenthic community structure in the Tamar estuary, Southwest England, United Kingdom (Austen, 1986). A number of individual nematodes were mounted on each slide, and some of the nematodes on the slides were morphologically identified as *Atrochromadora microlaima*, *Dichromadora* sp., *Sabatieira pulchra*, *Molgolaimus tenuispiculum*, etc during a previous study (Austen 1986). For the second set of specimens, nematodes were picked from short term bulk meiofauna samples extracted from estuarine sediments of Tamar estuary and stored in buffered formalin at

room temperature for 8-9 months. Some of these nematodes were identified as *Terschellingia* sp., *Dichromadora* sp., and Chromadorid under a compound microscope.

6.2.2.1 Nucleic acid extraction

DNA was extracted from long and short-term archived nematodes using an extended hot lysis protocol, a modification of the method of Chase *et al.* (1998). Nematodes (n=8) were carefully taken off the slides with a sterilised scalpel and placed in 0.5 mL PCR tubes containing 200 µL of ATL tissue lysis buffer from the DNeasy Tissue Kit (Qiagen, Germany). Nematodes (n=5) from the short term bulk meiofauna samples were randomly picked and similarly placed individually in 0.5 mL PCR tubes containing 200 µL of ATL tissue lysis buffer. Nematodes (n=5) from the unbuffered formalin time series experiment that failed to show any amplicons for day 15th and 30th (see results) were also placed individually in ATL buffer. The tubes were incubated at 56°C for 24 hours. Subsequently, 5 µL of proteinase K (50mg mL^{-1}) and an additional 80 µL of the ATL lysis buffer were added to each tube and incubated for another 96 hours at 55°C. The extraction procedure was then completed according to the DNeasy kit (Qiagen Inc) manufacturer's instructions. Finally DNA was eluted in 80 µL of Tris-HCl (buffer pH 8.0). The templates were stored at -20°C until further use.

6.2.2.2 PCR amplification of the 18S rRNA gene from long-term and short-term formalin preserved nematode specimens

Two primers Mn18F forward and 22R reverse were used to amplify the partial 18S rRNA gene in extracted DNA from all the worms. The amplification product size was approximately 345 bp. PCR reactions were performed on 15 µL aliquots of the extracted DNA. 0.5 µL Bovine Serum Albumin (BSA, 10mg mL^{-1}) (Promega Inc) was also added to

each PCR reaction. PCR cycle parameters have been detailed in Chapter 3 (Section 3.2.3.3). PCR products were electrophoresed to check the quality of amplicons.

6.2.2.3 Cloning and DNA Sequencing

PCR products were cloned using the pGEM-T Easy vector system (Promega Inc) according to the manufacturer's instructions. PCR products were cloned into vectors in order to generate high quality sequences. The clone libraries were therefore readily available for future works related to phylogenetics and evolutionary studies. Plasmid DNA containing the inserts was cycle sequenced using BigDye Terminator Kit (Applied Biosystems). Cycle sequencing reactions were cleaned using the Wizard Magnesil™ system (Promega). Sequencing was carried out in both directions using M13F and M13 primers in an ABI Hitachi 3100 Genetic Analyzer. Four to five colonies from each clone were sequenced to confirm the identity of the sequences. The resulting sequences were then compared with those of known 18S rRNA nematode sequences held on-line at GenBank, EMBL, DDBJ and PDB using the BLAST query engine (http://www.ncbi.nlm.nih.gov/BLAST).

6.2.3 Prevention of contamination

Several measures were taken to limit possible contamination during DNA extraction and PCR amplification. DNA extraction, PCR amplification, and cloning were conducted in different rooms, using different pipette sets for each step and sterile filter tips (Biosphere, SARSTEDT). Negative control amplifications (no template DNA) were also carried out during each round of DNA amplification.

6.2.4 Phylogenetic tree construction

Phylogenetic trees were constructed using the sequences from formalin fixed archived nematode specimens and nematodes from bulk meiofauna samples along with

additional marine nematode partial ssu sequences generated in this study. Some partial marine nematode sequences from the GenBank and EMBL databases were also incorporated into the trees. GenBank and EMBL accession numbers used in this study were AY854209, AY854204, AY854238 and AY284713. Prior to phylogenetic analysis, nematode sequences were aligned in Clustal-X using default parameters (Thompson *et al.*, 1997; Jeanmougin *et al.*, 1998). Neighbour-joining trees were constructed with the program MEGA v2.0 (Kumar *et al.*, 2001) using Gamma corrected Kimura distance parameters (Blaxter *et al.*, 1998). To assess the reliability of NJ trees, bootstrap tests were carried out using 1000 replicates.

6.2.5 Evaluation of acetone and butanol for nematode worm fixation

Five grams of sediment from Tamar estuary (Cornwall, UK) were fixed in two 100 mL pots containing 80% acetone and 60% butanol respectively (Hayman Limited, UK). Both the pots were left at room temperature (20-22°C) for a month. Acetone and butanol fixed sediments were subjected to meiofaunal extraction following Somerfield and Warwick's (1996) flotation method. Following extraction, 10 nematode worms from acetone and butanol fixed meiofauna were fixed onto slides for morphological analysis. Subsequently all the worms were carefully transferred to 0.5 mL PCR tubes containing 0.25 M NaOH. DNA was extracted following the modified Floyd *et al.* (2002) protocol. Subsequent PCR amplifications were carried out using the G18F and 22R primers following similar parameters described earlier. BSA (10mg/mL) (Promega Inc) was used in all the PCR reactions.

6.3 Results

6.3.1 Formalin time series experiment

The electrophoresis images clearly show that there is little apparent inhibition of PCR amplification in individuals fixed in formalin for up to 9 days (Figure 6.1). Visible

changes in the quantity of 18S rRNA gene amplicons were observed in individuals fixed in formalin from day 11 onwards until day 30. PCR amplification was apparently inhibited for all the nematodes from day 11 onwards as evident by the absence of PCR amplicons and the presence of highly smeared DNA with distinct primer dimers (Figure 6.1). Standard *Taq* DNA polymerase was used for PCR amplification of nematodes from all the above days.

There was partial amplification success for individuals fixed for 11 days, which produced some amplicons with Accuprime *Pfx* DNA polymerase enzyme (Figure 6.2). However, there were no visible PCR products in case of 13 and 15 day individuals or those fixed for 1 month.

18S rRNA PCR amplicons from 11 day nematodes (n=5) that were amplified using Accuprime *Pfx* DNA polymerase were sequenced and compared with the sequences from absolute alcohol preserved nematodes (n=5) amplified using standard *Taq* DNA polymerase. Sequences from formalin preserved specimens showed no apparent ambiguity or error when aligned with sequences from alcohol preserved specimens in a ClustalX alignment program using default parameters. In other words, the sequences were all identical. Indeed all the *T. longicaudata* 18S rRNA gene sequences generated in this study showed 100% similarity with the *T. longicaudata* sequence held on line in GenBank.

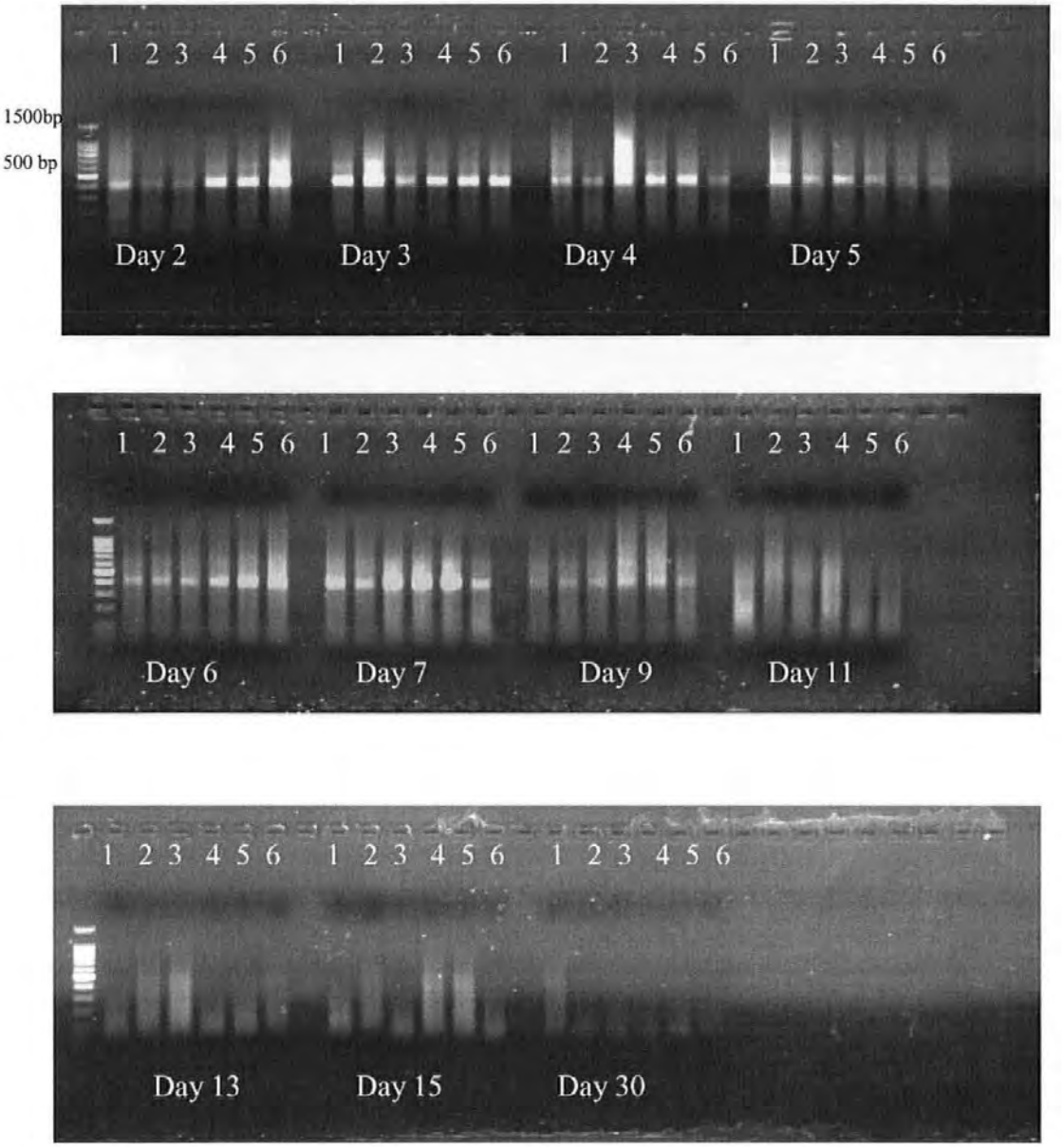


Figure 6.1: Gel showing partial 18S rRNA gene amplification products of *T. longicaudata* specimens extracted from formalin after 2, 3, 4, 5, 6, 7, 9, 11, 13, 15 and 30 days and a 100 bp ladder in the left hand side of image.

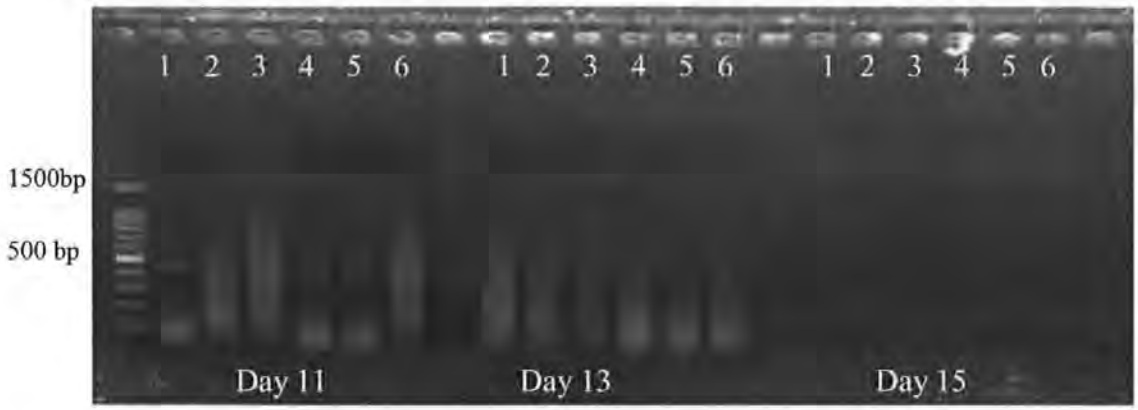


Figure 6.2: Gel showing results of PCR amplification of 18S rRNA gene after 11, 13 and 15 days using Accuprime *Pfx* DNA polymerase. Limited amplification is visible in some of the day 11 samples, but no amplification can be detected after longer time periods No amplification was observed from specimens fixed for one month. 100 bp ladder at left hand side of the gel.

6.3.2 Amplification of recovered DNA from long-term and short-term formalin preserved nematode specimens

Successful PCR amplicons of the 18S rRNA gene were achieved on recovered template DNA from long term (Figure 6.3) and short-term archived nematode specimens using ribosomal primers. All the sequences showed similarities between 97% and 100% with GenBank and EMBL nematode sequences (Table 6.1 and 6.2). The phylogenetic placement of the long term archived nematode sequences in the tree (Figure 6.4) suggests that some of them are close to *Dichromadora* sp., *Atrochromadora microlaima* and *Sabatieria* sp., etc based on sequence simmilarities. This show a significant degree of correspondence to the morphological identification as the dominant taxa were originally identified as *Atrochromadora microlaima*, *Sabatieria pulchra*, *Dichromadora* sp., *Molgolaimus tenuispiculum* from slides (Austen, 1986). Phylogenetic analysis of the sequences from short term archived meiofauna samples suggests that the specimens are close to *Dichromadora* sp., *Atrochromadora microlaima*, *Terschellingia longicaudata* etc (Figure 6.5). Some of these worms had been identified as *Terschellingia* sp.,

Dichromadora sp., Chromadorid based on morphological features prior to nucleic acid extraction. Nematodes from the unbuffered formalin time series experiment did not produce any amplicons in this study.

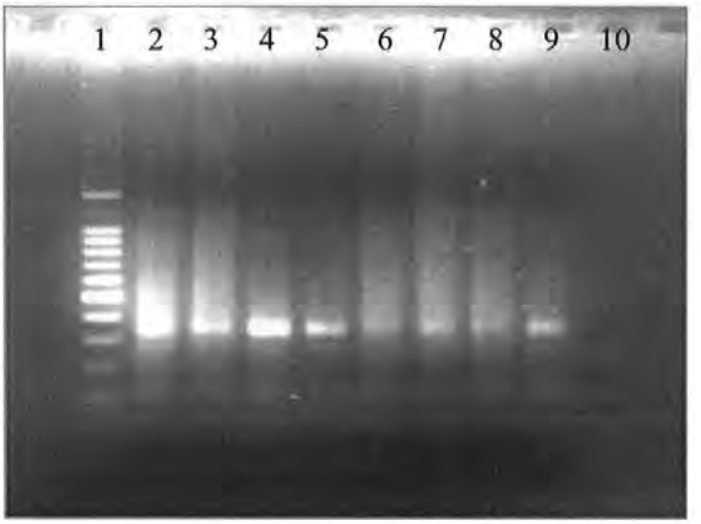


Figure 6.3: Gel image showing amplified nematode 18S rRNA gene fragments from long-term archived marine nematode worms; Lane 1: 100 bp molecular marker; Lane2 to 9: PCR products (350 bp approx); Lane 10: Control amplification without any template DNA.

Table 6.1: DNA sequences from short-term archived nematode worms picked up from bulk meiofauna samples with closest BLAST search results.

Band	Closest BLAST match	% similarity	Reference
FormalinSeq1	*Terschellingia longicaudata*	97	Meldal (2004)
FormalinSeq2	Nematode 18S rRNA JCF1	99	Bhadury *et al.*, (2005)
	Nematode 18S rRNA SH1		Meldal (2004)
	Dichromadora sp.		
	Atrochromadora microlaima		
FormalinSeq3	Nematode 18S rRNA BW4	100	Bhadury *et al.*, (2005)
FormalinSeq4	Chromadorid	96	Helder *et al.*, (2004)
	Chromadoropsis vivipara		
			Aleshin *et al.*, (1998)
FormalinSeq5	Nematode 18S rRNA JCF1	99	Bhadury *et al.*, (2005)
	Nematode 18S rRNA SH1		Meldal (2004)
	Dichromadora sp.		
	Atrochromadora microlaima		

Table 6.2: DNA sequences from long-term archived nematode specimens collected from Tamar estuary with the closest BLAST matches.

Band	Closest BLAST match	% similarity	Source
ArchTamar1	Uncultured nematode band JCF1	100	Bhadury *et al.*, (2005)
	Uncultured nematode band SH1		Meldal (2004)
	Dichromadora sp.		
	Atrochromadora microlaima		
ArchTamar2	Chromadorid	97	Helder *et al.*, (2004)
	Chromadoropsis vivipara		Aleshin *et al.*, (1998)
ArchTamar3	Chromadorid	97	Helder *et al.*, (2004)
	Chromadoropsis vivipara		Aleshin *et al.*, (1998)
ArchTamar4	*Sabatieira* sp. 210-BHMM	99	Meldal (2004)
	Uncultured nematode band SH1		Bhadury *et al.*, (2005)
ArchTamar5	*Adoncholaimus fuscus*	100	Meldal (2004)
ArchTamar6	*Spirinia parasitifera*	100	Meldal (2004)
ArchTamar7	Uncultured nematode band JCF1	100	Bhadury *et al.*, (2005)
	Uncultured nematode band SH1		Meldal (2004)
	Dichromadora sp.		
	Atrochromadora microlaima		
ArchTamar8	Uncultured nematode band JCF1	100	Bhadury *et al.*, (2005)
	Uncultured nematode band SH1		Meldal (2004)
	Dichromadora sp.		
	Atrochromadora microlaima		

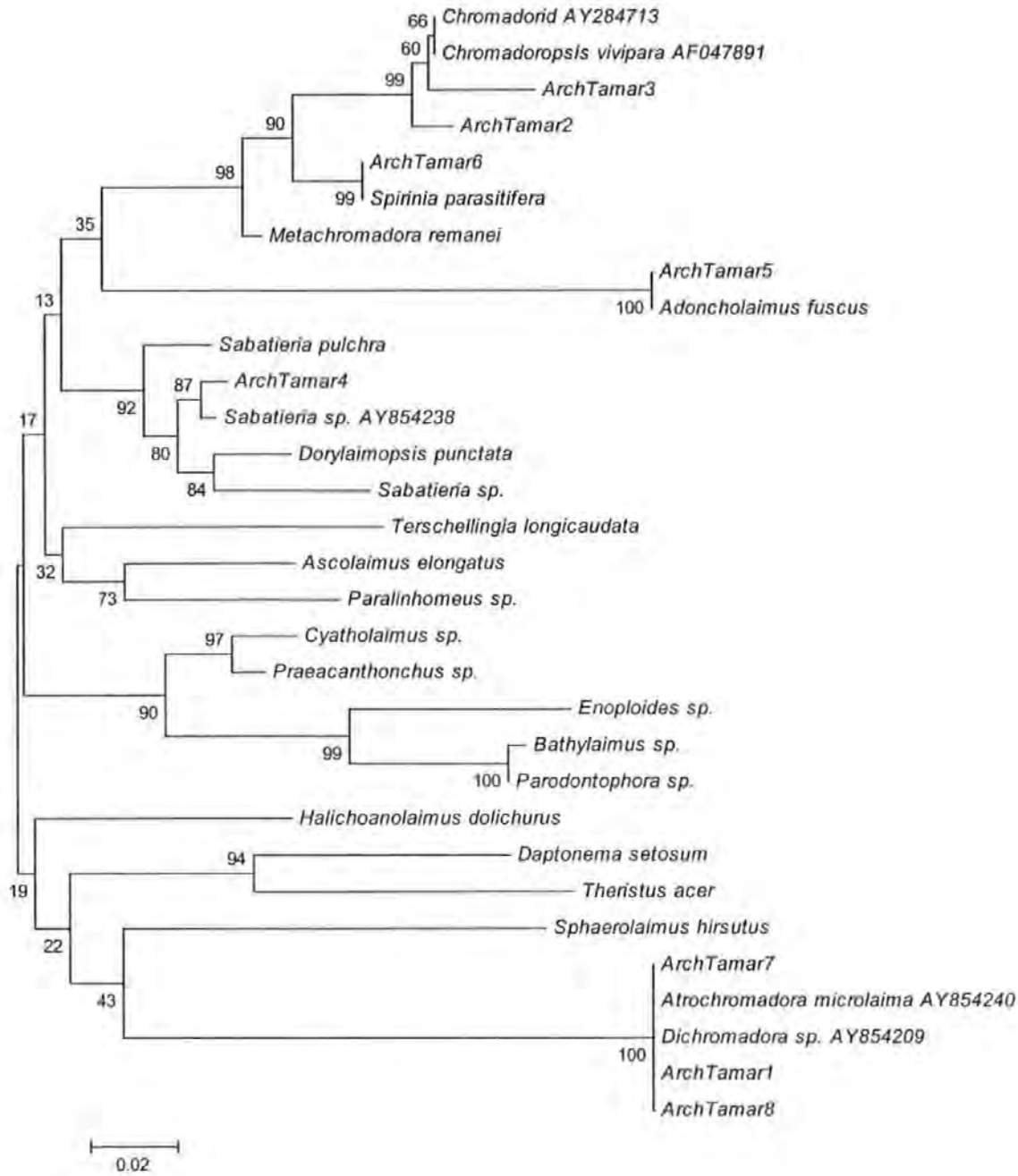


Figure 6.4: NJ tree with 1000 bootstrap replicates showing relationship between long-term archived marine nematode sequences and most similar sequences of known nematodes. The scale indicates 0.02 substitutions/site. Arch Tamar indicates sequences slide mounted archived marine nematode from the Tamar estuary.

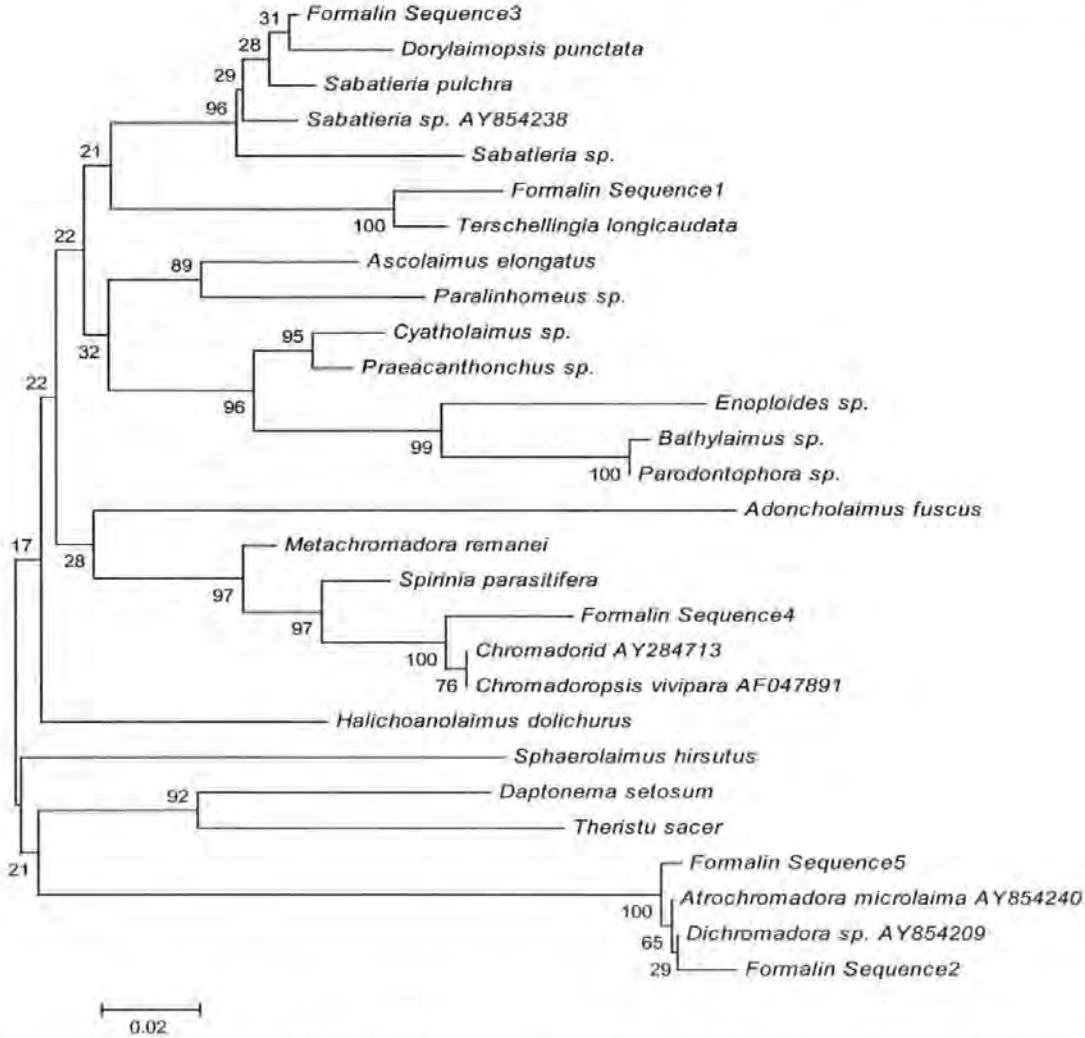


Figure 6.5: NJ tree with 1000 bootstrap replicates showing relationship between short-term archived nematode sequences and most similar sequences of known nematodes. The scale indicates 0.02 substitutions/site. Formalin sequence indicates nematodes from formalin fixed bulk meiofauna samples.

6.3.3 Evaluation of acetone and butanol for nematode worm fixation and molecular analysis

In this study the effectiveness of other organic compounds such as acetone and butanol was also evaluated. Nematode worms fixed in acetone showed very little shrinkage compared to the worms fixed in butanol (Figure 6.6). Morphological characters such as the position of gubernaculum, amphid, tail shape and cuticular patterns were also well preserved for acetone preserved worms. On the other hand butanol fixed worms shrank in size affecting the overall morphological integrity of the specimens. Nevertheless, 18S

rRNA gene from worms preserved in acetone and butanol was successfully amplified using nematode ribosomal primers (Figure 6.7).

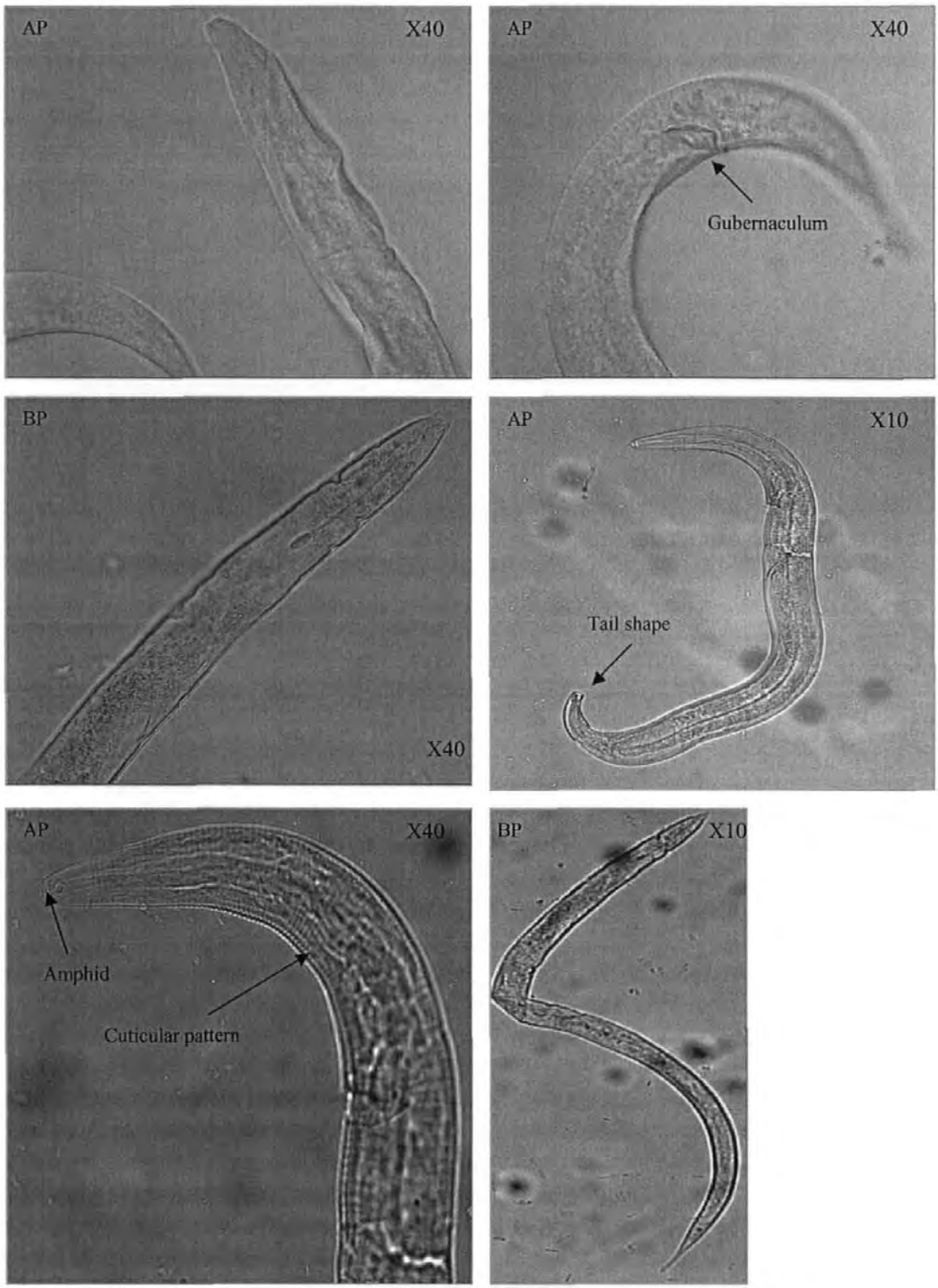


Figure 6.6: Images of nematode worms fixed in acetone and butanol under low and high resolution. (AP indicates acetone preservation; BP indicates butanol preservation).

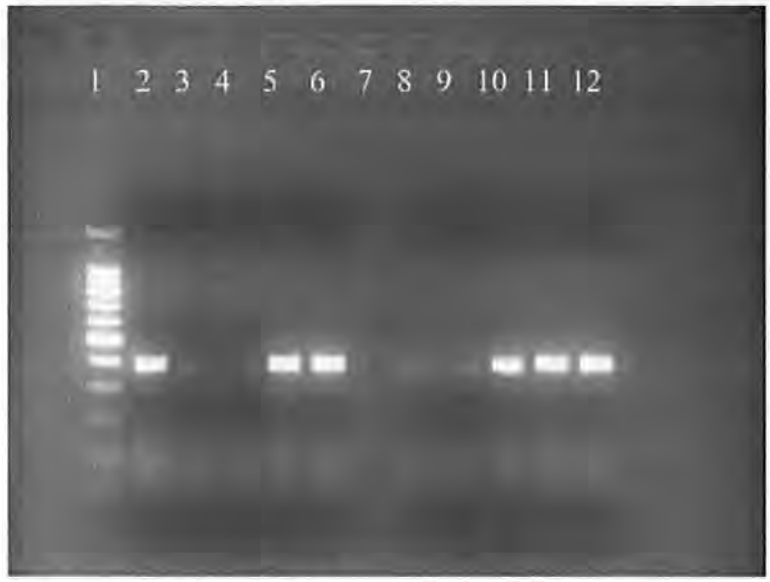


Figure 6.7: Gel of nematode 18S rRNA gene amplified using G18F and 22R primers. Lane 1: 100 bp molecular marker; Lane 2 to 6: Amplified DNA from nematode worms fixed in acetone; Lane 8 to 12: Amplified DNA from nematode worms fixed in butanol.

6.4 Discussion

The primary objective of this work was to investigate whether unbuffered formalin could be used as a fixative agent for short periods of time, without inhibiting or otherwise compromising subsequent molecular analyses. This study shows that formalin can be used to fix small invertebrates such as marine nematodes for molecular biological work for a short period of time. This would allow both morphological and molecular work to be conducted on the same individual, providing it is done relatively rapidly after collection. It has been shown that the average PCR yield after up to nine days in formalin was relatively high, with a rapid drop-out subsequently and no visible PCR products from day 11 onwards. Researchers such as Ben-Ezra *et al.* (1991) have shown that the formalin fixation procedure lowers the success of polymerase chain reaction and this probably could have been the reason why no PCR products were obtained from nematode DNA from day 11 onwards when amplified with *Taq* polymerase. When proof-reading and high fidelity *Pfx* DNA polymerase was used, in some instances it was possible to obtain PCR products from

specimens preserved in formalin for upto 11th day but not for 15th and 30th days. This possibly indicated that template DNAs from nematodes preserved in formalin for 15 and 30 days might have cross-linked and therefore did not amplify in the presence of high fidelity *Pfx* DNA polymerase enzyme. One of the major reported problems of formaldehyde fixation is that it induces apparent substitutions in nucleotides. In this study no such modification or substitutions were observed when nuclear 18S rRNA sequences from 11 day worms amplified by *Pfx* DNA polymerase were compared with alcohol preserved worms amplified by *Taq* DNA polymerase. Similar observations were also noted in a study conducted by Thomas *et al.* (1997). France and Kocher (1996) also found that nucleotide sequences from formalin fixed collections of varying age remain unmodified when compared with those derived from frozen specimens. Similarly, Shiozawa *et al.* (1992) and Bucklin and Allen (2004) did not encounter sequence modification or damage-induced sequence artefacts in formalin preserved specimens of trouts and zooplankton respectively. On the other hand researchers like De Giorgi *et al.* (1994) while working with formalin fixed nematodes, found that although PCR products appeared to be of the correct length, sequencing revealed misincorporated, or missing, nucleotides; purines in particular were affected. However in that study *Taq* DNA polymerase was used for amplification and there is evidence to show that *Taq* polymerase tends to misincorporate nucleotides during amplification (Eckert and Kunkel, 1991; Hultman *et al.*, 1991). Hamazaki *et al.* (1993) have argued that formalin fixation does have an effect on DNA and PCR amplification. They found that lambda phage DNA fixed in buffered formalin showed incomplete digestion on restriction endonuclease treatment. Lambda phage DNA fixed in unbuffered formalin showed poor PCR amplification due to degradation of DNA during fixation. Although, the lambda phage DNA fixed for three months showed some amplification there was no amplification from tissues kept in unbuffered formalin for longer than 6 months (Hamazaki *et al.*, 1993). In this study, no PCR products were obtained from nematode worms that were exposed to unbuffered formalin for one month. It is difficult to conclude

whether any formalin induced substitution occurred during this study. Perhaps a detailed study involving amplification of more genomic regions in nematodes and sequencing of several individuals may throw some light on DNA modification due to formalin exposure. The application of high fidelity *Pfx* DNA polymerase for PCR amplification and sequence comparisons were novel, yet there was no conclusive evidence of nucleotide mismatch or substitution.

One of the major emphases of this chapter was to develop and optimise a DNA extraction protocol that could be applied to recover DNA from archived marine nematodes for subsequent genetic diversity studies. A modified Proteinase K digestion methodology was implemented and tested on marine nematodes collected as part of a study in Tamar estuary approximately 20 years ago. Successful 18S rRNA amplicons were obtained for all the worms. All the sequences showed similarity of between 97%-100% when compared with known nematode sequences held online at GenBank and EMBL databases. The DNA extraction technique was also tested on nematodes picked from bulk meiofauna stored for a short time period in buffered formalin for 8-9 months. In this case successful amplification products and sequences were generated for all the worms. This indicated that possibly DNA degradation was much slower in nematodes exposed to formalin and was therefore available for PCR amplification. It could be that the chemical modifications caused by buffered formalin on DNA molecules were temporary and were therefore reversible by proteinase-K treatment. It was relatively difficult to conclude whether there was any nucleotide modification due to formalin exposure from DNA sequences from long-term and short-term archived nematode specimens. DNA sequences from slide-mounted worms and nematode worms from bulk meiofauna showed 97-100% similarity with GenBank and EMBL nematode ribosomal sequences. Moreover the BLAST results and taxonomic data for these worms seem to suggest that the sequences were probably assigned correctly in the phylogenetic tree and in BLAST queries. However when the same methodology was applied to nematodes collected from the unbuffered formalin time series experiment they

failed to produce any amplicons. There is evidence to show that tissues or specimens preserved in unbuffered formalin for prolonged periods generally become unsuitable for DNA extraction and subsequent molecular applications (see paper by Inoue *et al.*, 1996). This is because nuleic acid degrades and undergoes chemical alteration and some of these chemical alterations are irreversible (Chaw *et al.*, 1980; Chang and Loew 1994; Inoue *et al.*, 1996). This was the reason why no amplicons were obtained from nematode specimens that were preserved in unbuffered formalin as part of the formalin time-series experiment. This study shows the importance of buffering formalin when used for long term preservation of meiofauna specimens.

Several factors were taken into consideration in order to successfully carry out DNA extraction from archived nematodes. Firstly, the primers that were used in this study were nematode-specific which prevented co-amplification of contaminants if any were present in this study. In a separate study Dorris *et al.* (2002) also used nematode-specific primers to avoid contamination when amplifying approximately 150 bp of 18S rRNA gene from bulk terrestrial nematode samples stored in formalin. Application of group- or phylum-specific primers could help to prevent the problem of co-amplification of contaminants when archived DNA samples are used in molecular ecology studies. Finally all the steps including DNA extraction and PCR amplification were carried out in separate places to limit contamination.

In this study successful PCR amplicons were obtained from archival nematodes preserved in buffered formalin for long-term and short–term periods. For both long-term and short-term archived nematodes, a proteinase-K digestion method was implemented, as there is some evidence that this can remove the inhibitors which interfere with the activity of the PCR reactions (An and Fleming, 1991). It could be that the proteinase K digestion method along with the purification steps was able to reverse creation of the mono-methylol groups that might have formed with the DNA bases due to formalin exposure. Recovery of DNA and further amplification was successful in short-term and long-term archival

nematodes. In contrast, in the formalin time series experiment no PCR amplicons were obtained from nematodes picked up from day 13 onwards. This could once again be due to the cross-links that might have formed in presence of unbuffered formalin. Clearly more work is needed to investigate and also to see if longer PCR fragments (above 400 bp) could be also amplified from formalin-preserved nematode specimens.

Effectiveness of other organic compounds for fixation of small soft bodied metazoans such as nematodes was also tested in this study. The ketone based compound propanone, commonly known as acetone, and a tertiary alcohol, butanol, were tested for fixing marine nematode worms. Worms fixed in butanol and acetone were readily amplified with 18S rRNA primers indicating that there was no inhibitory effect of the compounds on the amplification process and that DNA did not degrade during the fixation process. Worms fixed in acetone preserved and maintained all the morphological characters necessary for taxon identification under a compound microscope but butanol fixed worms were relatively shrunken in size. Fukatsu (1999) also reported acetone as a better preservative than ethanol for fixing insects and that it maintained all the necessary histological structures of the insects necessary for identification. It was also shown that molecular analysis was successful in insects that were stored in acetone for up to three years (Fukatsu, 1999). In addition, recently a DMSO (Dimethyl sulfoxide) salt solution based preservation technique has been tested on nematode worms and has shown promising results (PJD Lambshead, *pers comm*). Presently there is not enough data testing the effectiveness of acetone for long term preservation of soft bodied metazoans such as nematodes and clearly more work is needed to test the suitability of this organic compound.

To conclude, in future, small soft-bodied invertebrates such as nematodes could be stored in unbuffered formalin for short periods possibly for 7 days but perhaps up to 2 weeks. This would allow taxonomists to carry out morphological and morphometric analyses followed by the application of molecular investigations on the same individual.

Such coupling of morphological and molecular analyses is critical in many groups where identification requires close examination, particularly to avoid assigning sequence data to the wrong organism. The modified DNA extraction technique described in this chapter could be also employed to investigate the genetic diversity of archived nematode specimens provided they were stored in buffered formalin for a prolonged period of time but the technique needs further modification so as to become cost effective and less time consuming.

7. Final Discussion and Future work

In comparison to other areas of marine biology, the application of molecular techniques to meiofaunal communities and in particular marine nematodes are still limited despite the potential of these tools for the improved understanding of biodiversity. In this study molecular ecological tools were applied for the first time to obtain a better understanding of nematode diversity, and to speed up the process of nematode identification from estuarine and marine environments. Problems in the assessment of nematode diversity, and species identification were addressed in detail for the first time in this study and a better understanding gained regarding the nature of cosmopolitanism in one genus at least.

Nematodes are highly abundant in estuarine and oceanic waters and require specialist taxonomic skills for their identification. The relatively immature nature of nematode taxonomy along with their high species diversity has partly led to a neglect of this group by many ecologists working in marine and estuarine environments. A wide range of molecular tools were evaluated during this study, in an attempt to find molecular solutions to some of these problems.

Firstly, DGGE was evaluated as a novel method for the identification of nematodes from marine environment. The technique showed clear separation of 18S rRNA amplicons from different nematode taxa in a denaturing gel with the generation of a characteristic banding pattern for each taxon. These banding patterns could be potentially exploited to identify nematodes from estuarine and marine environments; and in particular this rapid and relatively cheap technique may prove useful for the identification of deep sea nematodes which are particularly under-investigated. Such an approach has already proved useful in the identification of soil nematodes (Foucher and Wilson, 2002).

One of the main focuses of this thesis was to evaluate a molecular technique that could be implemented as a rapid tool for assessing nematode diversity from marine and estuarine environments. DGGE was the obvious choice because of its technological advantages as well as its varied application in the estimation of diversity of prokaryotes as well as in other marine micro-eukaryotes. DGGE was employed to test its suitability for assessing nematode diversity from marine and estuarine sediments. Two methods, one involving extraction of total nematodes from sediments followed by DNA extraction and the other where total community DNA was extracted directly from sediments were employed. The resulting DNA was subjected to PCR amplification with 18S rRNA primers and subsequent electrophoresis in denaturing gradients. The ribotype diversity was in both cases found to be relatively similar across sites, since the method only detected the most abundant taxa present in the samples. This was confirmed by morphological analysis of sediment samples where the number of taxa detected was much higher than that estimated using DGGE. Nevertheless tentative phylogenetic affiliations of some of the bands were determined by subsequent sequencing and phylogenetic analysis. Thus DGGE was able to provide an assessment of nematode diversity from marine and estuarine environments and a picture of community composition (Cook *et al.*, 2005), but is apparently restricted to providing information on the most abundant taxa. In the light of this finding, DGGE should be applied with caution in diversity studies, and only used when it is appropriate to the question being asked. In particular, studies which aim to assess the total species richness of a site must continue to use other, complementary approaches. The assessment of nematode diversity by DGGE conducted here mirrored prokaryotic and other eukaryotes studies where only the abundant taxa were detected from the environment (Muyzer *et al.*, 1993; Foucher *et al.*, 2004; Holben *et al.*, 2004; Savin *et al.*, 2004). Especially studies conducted in soil nematodes have shown inconsistency between morphological entities and PCR based DGGE (Waite *et al.*, 2003; Foucher *et al.*, 2004). The importance of sample size and its effect on subsequent estimates nematode diversity was also assessed. It became

clear that to get a detailed idea of nematode diversity several small volumes of sediment were needed for DNA extraction and subsequent DGGE. Additionally the primers that were designed and evaluated here were nematode-specific and have an added advantage over other primer sets that were used in previous nematode identification and diversity studies (Foucher and Wilson 2002; Waite *et al.*, 2003; Cook *et al.*, 2005).

During the course of this study co-amplification of fungal 18S rRNAs was frequently encountered while trying to amplify individual nematodes. Some of the amplified fungal 18S rRNA fragments were dominated by *Paecilomyces fumosoroseus*, *Verticillium insectorum*, *Syspastospora parasitica* and *Rhinocladiella aquaspersa*. In particular, co-amplification of the above fungal species was found to be frequent from two locations namely Jennycliff and Breakwater close to Plymouth. Using a combination of microbiological and molecular techniques the possibility of an association between fungi and nematodes was investigated. Electron micrograph images revealed some secondary hyphae-like structures as well as globular formations on the body surface of nematodes collected from Jenny Cliff and Breakwater. Studies conducted by Polz *et al.* (1999) have shown the presence of bacterial communities growing on the body surfaces of nematodes with a possibly symbiotic relationship. Such associations have been also observed in other marine organisms (see Chapter Three). No conclusive evidence was found in this study regarding the prevalence of fungal taxa in sediment samples or in close association with nematodes. Clearly more work is needed to get a better idea whether any relationship exists between fungal communities and nematodes in the marine environment or whether these amplified fungal DNAs constitute part of the gut contents in nematodes or merely represents some sort of contamination.

Since nematodes are relatively difficult to identify under a microscope and typically require confirmation by an experienced nematologist, one of the aims of this study was to develop a technique that could speed up the process of nematode identification from estuarine and marine environments for biomonitoring or biodiversity studies. In this study

a novel methodology based on amplification and sequencing of a region of the genome was evaluated for the first time for the identification of marine nematodes. The genomic identification system, also known as DNA barcoding, was evaluated via amplification and sequencing of a small region of the nematode 18S rRNA gene (around 345 bp). The success rate of 18S rRNA-based amplification and sequencing in the assignment of unidentified nematode specimens to genus and species level was found to be close to 97%. Although this success rate was slightly lower than that achieved by Hebert *et al.* (2003) the technique was still able to assign unidentified nematode specimens to genus or species level. In a recent meeting in 2005 researchers agreed in principle to use DNA barcoding in addition to morphology based method to speed up identification of organisms from different habitats including marine environments, supporting the importance of this molecular based methodology (details in Barcoding of Life website). In this study other genomic regions such as the nuclear 28S rRNA gene and mitochondrial 16S rRNA and COXI genes were also evaluated for DNA barcoding. While amplification failed in almost all the representative marine nematode taxa tested for the 16S rRNA and COXI gene, there was partial success with the 28S rRNA gene. Clearly more work is needed to resolve amplification failures for mitochondrial genes in marine nematodes. Such failure in amplification of nematode mitochondrial genes is well documented (Meldal, 2004; Cook *et al.*, 2005). At the other end of the spectrum mitochondrial genes have worked well in parasitic nematodes and have been also evaluated as barcoding loci (see review by Powers, 2004). In order to successfully amplify and sequence mitochondrial genes in marine nematodes an approach based on designing consensus primers specific to order or family level may prove to be profitable. At the same time there is a need to sequence mitochondrial genomes from marine nematode taxa to get an idea about the genome organisation which in turn would create a platform for future molecular ecological and population genetic studies. Similarly with the 28S rRNA gene further optimisation and

selection of different regions within the molecule needs to be undertaken to investigate the gene's potential as a barcoding marker.

Unlike many meiofaunal species, some nematodes are thought to have a worldwide distribution and are considered to be cosmopolitan. Until now, only a handful of studies have investigated cosmopolitanism and cryptic speciation among marine nematodes (Warwick and Robinson, 2000; Derycke *et al.*, 2005). None of these studies combined morphological and molecular techniques to address issues of cosmopolitanism but used separate morphological (Warwick and Robinson, 2002) or molecular techniques (Derycke *et al.*, 2005). In this study a combination of morphological and molecular techniques was used for the first time to investigate populations of an ostensibly cosmopolitan marine nematode, *Terschellingia longicaudata*, from different geographic locations. Populations from different geographical localities were examined by amplification and sequencing of the 18S rRNA gene. Three different haplotypes were revealed. The first haplotype consisted of the majority sequences from morphologically defined *T. longicaudata* specimens from locations across UK, France, Mexico and Malaysia. The second haplotype occurred in a few sequences from two sites in the UK and a single site in Mexico and differed from the majority sequence by 25 bases, whilst the third haplotype consisted of sequences from a site in the UK and differed from the actual majority *T. longicaudata* sequence by 19 bases. Given the degree of sequence conservation normally reported in the 18S rRNA gene within taxa, it is possible that these sequences represent two additional cryptic species of *Terschellingia*. Interestingly, at least one of these is also apparently cosmopolitan, and both co-occur with the most common taxon suggesting that they may be genetically divergent, despite their apparent morphological similarity.

Sequences from Bahrain showed very little similarity with those of *T. longicaudata* and were in fact closer to taxa belonging to either completely different families or orders. This result was entirely unexpected, and may have resulted from the misidentification of these specimens.

The morphometric data did not provide any additional information about the populations from different geographical locations besides confirming the fact distinct geographical morphotypes could not be recognised. This study did suggest that, based on 18S rRNA sequences and morphology *T. longicaudata* is apparently a truly cosmopolitan species since samples from different oceans share the same DNA sequence. As well as being geographically cosmopolitan, this species apparently has a wide ecological range, being found in a range of coastal and near shore locations. Morphometric analysis supports the fact that these taxa are morphologically indistinguishable from typical *T. longicaudata* using standard morphological techniques. This study is the first to collate both phenotypic and genotypic data in an attempt to understand cosmopolitanism and cryptic speciation in marine nematodes. Molecular approaches have also shown the occurrence of cosmopolitan species among other benthic organisms (Westheide *et al.*, 2003). At the same time this study also raises the question of how benthic organisms such as marine nematodes with low dispersal capabilities can have cosmopolitan distribution. More work is needed with nuclear and mitochondrial markers for better understanding of cosmopolitanism. Studies conducted by Derycke *et al.* (2005) on a supposedly cosmopolitan marine nematode, *Pellioditis marina*, have shown cryptic lineages based on mitochondrial cytochrome c oxidase and nuclear ITS regions. It would be interesting to investigate whether 18S rRNA sequences from different populations of the *Pellioditis marina* show identical or different haplotypes in future studies. This would then give a better idea about the concept of cosmopolitanism and cryptic species assemblages based on multiple molecular markers.

During the course of this study it became apparent that nematode specimens that were fixed in absolute alcohol for molecular analyses shrink rapidly and lose their morphological integrity. This in turn causes problems in identification which is typically based on careful observation of minute morphological characters under a compound microscope. In order to maintain their morphological integrity, marine nematodes are usually fixed in formalin but such specimens are usually seen as being useless for

molecular analysis due to formalin-induced changes in DNA. For the first time the possibility of combined morphological and molecular analyses of nematode specimens based on short-term preservation in unbuffered formalin was explored in this study. Novel specimen preservation for a short time period (up to 9 days) was developed allowing morphological and molecular analyses to be conducted on the same specimen. The development of this methodology would therefore give some time for taxonomists to preserve sediments from offshore locations in unbuffered formalin and bring them back to the laboratory for morphological and molecular work. This study explored the possibility of using other organic compounds for nematode storage besides the conventional compounds such as ethanol and formalin. Acetone and butanol were evaluated for their effectiveness in maintaining morphological integrity of nematodes without hindering subsequent molecular work. Whilst both of these compounds worked well at the molecular level it became clear that acetone was a better preservative than the other since it maintains the morphological integrity of specimens. Studies by Lee and Beynon (2004) have also explored the possibilities of using other organic compounds for preservation of marine organisms which could be evaluated for nematode preservation as possible alternatives to acetone. Considerable progress has been made towards utilising cryogenic methods for nematode preservation (PJD Lambshead *pers comm*).

In this study a novel technique based on an extended hot lysis methodology was developed and optimised for the recovery of DNA from formalin-preserved archived nematode specimens which are stored in research organisations and are currently seen as being unavailable for molecular analyses. The technique that was developed in this study was able to successfully recover and amplify 18S rRNA fragments from archived nematode specimens. In almost all the cases, amplified fragments of approximately 345 bp could be amplified using nematode specific primers, enough to provide phylogenetic resolution and tentative affiliations to known taxa. The technique was validated on a wide variety of specimens as well as on *Terschellingia longicaudata* from Merbok in Malaysia

which was investigated as part of the cosmopolitanism and cryptic species study. This methodological development would be useful in future in order to address a wide range of issues in systematics and population biology, including the study of temporal changes in population structure.

Future work

The techniques and approaches evaluated here provide a valuable framework for the future application of molecular systematics in marine nematology that will speed identification of marine nematodes for future biomonitoring and biodiversity surveys.

In addition, the future of molecular identification and diversity assessment of nematodes from marine and estuarine environments will depend on the development of high throughput hybridisation analysis using microarrays or 'DNA phylochips'. For fingerprinting nematode communities from estuarine and marine environments, the use of a DNA macro-array would be an important extension of the approaches used in this study. Microarrays are high throughput systems that provide information on a large number of genes and changes in the expression of genes. On the other hand a DNA macroarray has been used for studying the dynamics of microbial communities and functional genes from various environments (Jenkins *et al.*, 2004; Steward *et al.*, 2004). An appealing feature of hybridisation-based methods such as DNA arrays is that discrimination among sequences is primarily a function of their overall similarity to probes of known sequence and it reflects true diversity of communities from environmental samples. Initial studies with DNA macroarrays towards the identification of nematode communities from estuarine environments have been promising (P Bhadury, unpublished) although more works are needed towards the development of a cost-effective DNA macroarray. Additionally, clone libraries and sequences of the 18S rRNA gene from representative nematode taxa now exist that could be exploited towards the development of this concept. In addition, the development of methodologies based around mass spectrometry for screening single

nucleotide polymorphisms (SNPs) (Haff and Smirnov, 1997) may be useful for rapid screening of PCR amplicons from environmental samples.

The future study of cosmopolitanism and cryptic speciation in marine nematodes depends on the use of multiple loci from the nematode genome in conjunction with morphometric methods for better understanding of these concepts. The time is right to vigorously pursue the decoding of the mitochondrial genomes of marine nematodes and indeed technologies such as the genomic amplification kits are gradually becoming available that could provide information about the arrangement of the mitochondrial genome at the molecular level.

Nematodes are an extremely important component of the benthic meiofauna and play a vital role in marine ecosystem and are thought to be an extremely useful biomonitoring tool for determining the anthropogenic impacts on marine environments. This study has shown the effectiveness of implementing molecular techniques into marine nematology and its clear potential for better understanding of marine nematodes in their environment.

Appendix A

Morphometrics measurements of populations of *Terschellingia longicaudata* collected from different geographical locations as follows: RaB: Ras al Barr, BF=Brittany, Mex= Cancun, NMMP= NMMP site off Humber estuary, NTBah= North Tubli Bay, T= Tamar estuary, P= Plym estuary, Ra= Rame Head. Characters included are as follows OL= oesophagus length, OBD=oesophageal bulb diameter, HD= head diameter, TL= tail length, BL= body length, MBD=maximum body diameter, AD= amphid diameter, ABD= anal body diameter, CS= length of cephalic seta, SCS= length of sub-cephalic seta, SS= length of somatic seta, SP= spicule length, GB=gubernaculum length, CerS= length of cervical seta, VtoH= distance between vulva to head.

	RaB1	RaB2	RaB3	RaB4	BF1	BF2	BF3	BF4	BF5	Mex1	Mex2	Merbok1	NMMP1	NMMP2	NMMP4	NMMP5
OL	0.14	0.009	0.11	0.08	0.11	0.12	0.14	0.15	0.1	0.1	0.14	0.12	0.1	0.14	0.11	0.14
OBD	0.023	0.023	0.026	0.02	0.021	0.031	0.033	0.034	0.031	0.025	0.026	0.031	0.023	0.03	0.024	0.028
HD	0.016	0.013	0.015	0.013	0.012	0.018	0.018	0.018	0.018	0.014	0.016	0.018	0.012	0.018	0.014	0.015
TL	0.44	0.22	0.34	0.42	0.46	0.46	0.42	0.29	0.26	0.39	0.48	0.38	0.22	0.36	0.41	0.3
BL	1.32	0.9	1.45	1.21	1.38	1.46	1.65	1.14	1.11	1.54	1.67	1.21	0.85	1.49	1.44	1.42
MBD	0.031	0.031	0.028	0.027	0.028	0.04	0.044	0.05	0.038	0.031	0.032	0.048	0.033	0.038	0.03	0.039
AD	0.007	0.008	0.007	0.007	0.008	0.01	0.008	0.01	0.008	0.009	0.008	0.009	0.006	0.009	0.009	0.008
ABD	0.018	0.016	0.019	0.018	0.018	0.025	0.022	0.03	0.023	0.022	0.023	0.025	0.018	0.02	0.02	0.022
CS	0.006	0.006	0.007	0.005	0.004	0.004	0.003	0.002	0.004	0.003	0.004	0.004	0.005	0.003	0.004	0.005
SCS	0.006	0.007	0.007	0	0	0	0.004	0.005	0	0.004	0.005	0	0	0.003	0.004	0.004
SS	0	0.003	0	0	0.004	0.005	0.005	0	0.004	0.004	0.006	0	0	0.005	0	0
SP	0	0	0.03	0	0	0	0	0	0	0.032	0	0	0	0	0.045	0.04
GB	0	0	0.013	0	0	0	0	0	0	0.018	0	0	0	0	0.025	0.023
CerS	0.008	0.007	0.007	0	0.003	0	0.006	0	0.005	0.006	0.005	0.007	0	0.003	0.004	0.004
VtoH	0.53	0.41	0	0.43	0.62	0.59	0.73	0.45	0.41	0	0.75	0.54	0.42	0.58	0	0

	NMMP6	NMMP7	NMMP8	NMMP9	NMMP10	NTBah1	NTBah2	NTBah3	NTBah4	NTBah5	NTBah6	NtBah7	NTBah8	T1	T2	T3
OL	0.1	0.12	0.11	0.12	0.14	0.085	0.08	0.08	0.08	0.07	0.07	0.08	0.09	0.14	0.12	0.13
OBD	0.024	0.025	0.025	0.022	0.029	0.022	0.025	0.015	0.026	0.02	0.02	0.025	0.022	0.04	0.03	0.03
HD	0.011	0.013	0.013	0.015	0.013	0.015	0.012	0.012	0.015	0.016	0.012	0.015	0.014	0.028	0.016	0.017
TL	0.31	0.28	0.38	0.36	0.44	0.38	0.39	0.3	0.36	0.33	0.21	0.28	0.29	0.46	0.37	0.35
BL	0.91	1.09	1.54	1.07	1.54	1.05	1.05	1.12	1.16	1.03	1	1.16	1.09	1.64	1.52	1.39
MBD	0.025	0.028	0.032	0.025	0.033	0.035	0.038	0.03	0.03	0.032	0.026	0.032	0.029	0.042	0.038	0.042
AD	0.007	0.009	0.008	0.008	0.01	0.008	0.006	0.009	0.007	0.008	0.005	0.007	0.008	0.009	0.006	0.009
ABD	0.018	0.018	0.023	0.018	0.022	0.015	0.029	0.014	0.017	0.016	0.017	0.018	0.018	0.017	0.019	0.021
CS	0.004	0.003	0.005	0.003	0.005	0.005	0.009	0.008	0.005	0.008	0.005	0.003	0.004	0.006	0.003	0.004
SCS	0	0.004	0.006	0.003	0.007	0	0	0	0	0	0.005	0.006	0.005	0.007	0.003	0.003
SS	0	0	0	0.003	0.004	0	0.008	0	0	0	0.003	0	0	0.003	0	0.003
SP	0	0	0	0	0.051	0	0	0	0	0	0	0	0	0.037	0	0.051
GB	0	0	0	0	0.018	0	0	0	0	0	0	0	0	0.019	0	0.024
CerS	0	0	0.004	0.003	0.005	0.005	0	0.005	0	0.004	0	0.005	0.005	0.007	0.005	0.006

	T4	T5	T6	T7	T8	T9	T10	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Ra1	Ra2	Ra3	Ra4	Ra5
OL	0.12	0.14	0.11	0.13	0.13	0.13	0.12	0.12	0.1	0.14	0.12	0.13	0.12	0.13	0.1	0.15	0.13	0.14	0.12	0.13	0.11	0.14
OBD	0.041	0.031	0.022	0.027	0.028	0.032	0.03	0.028	0.038	0.031	0.038	0.032	0.028	0.025	0.028	0.027	0.025	0.025	0.032	0.031	0.025	0.028
HD	0.017	0.015	0.011	0.018	0.015	0.015	0.015	0.018	0.018	0.015	0.019	0.017	0.018	0.016	0.015	0.013	0.014	0.012	0.016	0.014	0.015	0.015
TL	0.26	0.38	0.32	0.31	0.37	0.35	0.47	0.36	0.44	0.55	0.38	0.41	0.46	0.46	0.29	0.45	0.57	0.47	0.42	0.24	0.17	0.33
BL	1.4	1.21	1.16	1.34	1.48	1.45	1.45	1.47	1.39	1.61	1.55	1.53	1.44	1.76	1.4	1.4	1.5	1.43	1.69	0.88	0.82	1.19
MBD	0.051	0.04	0.027	0.034	0.037	0.042	0.037	0.03	0.038	0.035	0.045	0.036	0.038	0.031	0.031	0.038	0.036	0.03	0.045	0.028	0.03	0.031
AD	0.006	0.007	0.008	0.009	0.01	0.009	0.01	0.009	0.009	0.009	0.01	0.01	0.009	0.008	0.01	0.008	0.011	0.008	0.007	0.008	0.009	0.007
ABD	0.018	0.018	0.018	0.015	0.023	0.02	0.021	0.023	0.027	0.021	0.025	0.021	0.021	0.023	0.025	0.02	0.023	0.022	0.023	0.016	0.018	0.02
CS	0.004	0.005	0.005	0.004	0.004	0.004	0.003	0.004	0.004	0.004	0.005	0.007	0.004	0.004	0.004	0.005	0.007	0.004	0.004	0.004	0.005	0.005
SCS	0	0.004	0.005	0.005	0.006	0.004	0.004	0.003	0.006	0.005	0.003	0	0	0.006	0.005	0.005	0.005	0.004	0.005	0.006	0.005	0.004
SS	0.004	0.003	0.004	0.003	0.004	0.005	0.004	0	0.003	0	0	0.004	0	0	0.002	0.005	0	0.003	0	0	0	0
SP	0	0.032	0	0.04	0	0.042	0.039	0	0	0.031	0	0	0	0	0	0	0.45	0.028	0	0	0.019	0
GB	0	0.017	0	0.018	0	0.022	0.021	0	0	0.018	0	0	0	0	0	0	0.021	0.015	0	0	0.014	0
CerS	0.005	0.003	0.005	0.004	0.006	0.005	0.004	0.007	0	0.004	0	0.007	0	0.006	0.005	0.005	0.007	0.005	0.006	0.007	0.003	0.005

	Ra6	Ra7	Ra8	Ra9	Ra10
OL	0.15	0.16	0.14	0.15	0.14
OBD	0.027	0.033	0.032	0.025	0.03
HD	0.012	0.018	0.015	0.015	0.017
TL	0.42	0.39	0.41	0.32	0.47
BL	1.43	1.54	1.58	1.5	1.47
MBD	0.032	0.043	0.042	0.035	0.042
AD	0.01	0.009	0.008	0.009	0.008
ABD	0.023	0.028	0.022	0.02	0.025
CS	0.006	0.005	0.004	0.005	0.006
SCS	0.004	0.004	0.005	0.005	0.004
SS	0	0	0.004	0.005	0.004
SP	0	0	0	0.033	0
GB	0	0	0	0.019	0
CerS	0.004	0.007	0.005	0.005	0.007

Lowest and highest SIMPER (Similarity Percentages) value based on female characters. Groups designate geographical sites as detailed in Figure 5.6.

Groups 3 and 8

Average dissimilarity = 6.59

	Group3	Group8				
Species characters	Average Abundance	Average Abundance	Average Dissimilarity	Dissimilarity/ Standard Deviation	Contribution%	Cumulative%
Body length	1.67	1.49	3.37	2.55	51.15	51.15
Tail length	0.48	0.41	1.25	1.19	18.99	70.14
Vulva to Head	0.75	0.68	1.21	1.24	18.39	88.53
Oesophagus length	0.14	0.12	0.36	1.65	5.45	93.98

Groups 3 and 6

Average dissimilarity = 21.49

	Group3	Group6				
Species characters	Average Abundance	Average Abundance	Average Dissimilarity	Dissimilarity/ Standard Deviation	Contribution%	Cumulative%
Body length	1.67	1.08	11.28	8.31	52.46	52.46
Vulva to Head	0.75	0.47	5.36	4.74	24.94	77.40
Tail length	0.48	0.32	3.14	2.52	14.60	92.00

Lowest and highest SIMPER (Similarity Percentages) value based on all characters (male and female included). Groups designate geographical sites as detailed in Figure 5.6.

Groups 2 and 6

Average dissimilarity = 12.39

	Group2	Group6				
Species characters	Average Abundance	Average Abundance	Average Dissimilarity	Dissimilarity/ Standard Deviation	Contribution%	Cumulative%
Body length	1.35	1.08	5.65	1.43	45.57	45.57
Vulva to Head	0.56	0.47	2.65	1.48	21.36	66.93
Tail length	0.38	0.32	2.19	1.62	17.66	84.60
Oesophagus length	0.12	0.08	0.97	2.16	7.84	92.44

Groups 3 and 6

Average dissimilarity = 23.70

Species characters	Group3 Average Abundance	Group6 Average Abundance	Average Dissimilarity	Dissimilarity/ Standard Deviation	Contribution%	Cumulative%
Body length	1.61	1.08	11.03	7.42	46.56	46.56
Vulva to Head	0.38	0.47	8.21	2.63	34.65	81.21
Tail length	0.44	0.32	2.44	1.61	10.29	91.50

Lowest and highest SIMPER (Similarity Percentages) value based on characters excluding sexual ones (male and female included). Groups designate geographical sites as detailed in Figure 5.6.

Groups 3 and 8

Average dissimilarity = 5.79

Species characters	Group3 Average Abundance	Group8 Average Abundance	Average Dissimilarity	Dissimilarity/ Standard Deviation	Contribution%	Cumulative%
Body length	1.61	1.51	3.11	1.64	53.69	53.69
Tail length	0.44	0.44	1.68	1.39	29.04	82.73
Oesophagus length	0.12	0.12	0.47	1.42	8.16	90.88

Groups 3 and 6

Average dissimilarity = 18.31

Species characters	Group3 Average Abundance	Group6 Average Abundance	Average Dissimilarity	Dissimilarity/ Standard Deviation	Contribution%	Cumulative%
Body length	1.61	1.08	13.51	6.22	73.77	73.77
Tail length	0.44	0.32	3.03	1.57	16.56	90.34

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