

Population Genetics  
and Tolerance in  
*Nereis diversicolor*

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## Abstract

The genetic basis of adaptation to a gradient of metal contamination in the estuarine polychaete worm *Nereis diversicolor* was studied as a model of adaptive evolution. Amplified Fragment Length Polymorphism (AFLP) analysis was used to investigate patterns of genetic structure in populations of *N. diversicolor* from 5 sites in Cornwall with different levels of copper pollution, including heavily contaminated areas of Restronguet Creek. Recent advances in statistical and computational techniques applied to dominant AFLP data were assessed and used to measure genetic diversity and differentiation within and between populations. Relating adaptive traits that allow species to respond to changes in their habitat at the phenotypic level to changes at the molecular level provides insight into evolutionary responses to environmental change. Toxicity tests confirmed a gradient of tolerance to copper in *N. diversicolor*, corresponding with levels of contamination. Correlation between genetic variation and copper tolerance indicated that genetic structure was the result of selection for tolerant phenotypes rather than isolation by distance. Lower genetic diversity was found in tolerant populations suggesting a bottleneck. Genetic distance between *N. diversicolor* populations was not high enough to indicate separate species. An AFLP genome scan revealed signatures of sympatric speciation, identifying a small number of highly divergent loci linked to adaptive tolerance to copper, against a background of neutral loci, suggesting absence of reproductive isolation. Loci of interest associated with divergent selection were present for both tolerant and non-tolerant phenotypes, reflecting a cost of tolerance in tolerant populations. The population genetics of copper tolerance in *N. diversicolor* provides a model system for exploring rapid evolutionary adaptation to stress in changing environments.

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## Chapter 1 Introduction

### 1.1 *Nereis diversicolor* ecology in estuarine environments

Monitoring short term effects of disturbance events, pollution and anthropogenic pressure, and longer term effects of climate change and sea level rise, requires a thorough understanding of the ecology and dynamics of intertidal habitats and responses to stress. It is estimated that 70% of the global human population lives within 60 km of the coast, plus many of the world's largest cities are connected to the marine environment, either directly or indirectly via rivers and estuaries (Gray, 1991). Aquatic environments play an important role in the provision of ecosystem services, with a range of associated anthropogenic environmental effects, which have subsequently highlighted the need to understand, assess and redress these impacts. As sheltered, flat, productive environments surrounding a waterway, estuaries have always been a popular site of human habitation, transportation, agriculture and industry; with indirect and direct effects on estuarine systems (French, 1997). Monitoring the health of brackish water ecosystems such as estuaries and lagoons is therefore important for environmental impact assessment and coastal management (French, 1997; Townend, 2002).

Estuarine environments involve a complex interplay of physical, chemical and biological processes, influenced by both the marine environment and the fresh water catchment (Townend, 2002). Abundant fauna are supported by the input and cycling of both terrestrial and marine nutrients; including large, dense populations of infaunal invertebrates (Connor *et al.*, 2004). Brackish habitats are characterised by high spatial and temporal environmental variation, so adaptation to estuarine habitats requires tolerance to large fluctuations in abiotic factors such as salinity, temperature and oxygen levels (Abbiati & Maltagliati, 1992; Belfiore & Anderson, 2001; Van Straalen, 2003). While they are often nutrient rich environments, supporting high faunal abundance, temperate estuarine environments generally have low biodiversity and are dominated by a few specialist species that are adapted to a fluctuating environment. Typical estuarine species are polychaetes *Nereis diversicolor* and *Nephtys hombergii*, the burrowing

amphipod *Corophium volutator* and the estuarine clams *Macoma balthica* and *Scrobicularia plana*, of which *Nereis diversicolor* is usually the most common species in muddy estuaries (Murray *et al.*, 2002; Connor *et al.*, 2004).

The distribution of intertidal invertebrates is determined by selection of optimal habitat according to adaptation to a niche, tolerance to limiting factors and ecological interactions such as competition and predation (Levinton, 2001). Biotic interactions such as competition and predation affect the distribution of estuarine benthos. Cover of the macro algae *Fucus vesiculosus* has a negative effect on *Nereis diversicolor*, *Corophium volutator* and *Macoma balthica* abundance, because it creates a physical barrier and lowers redox potential through organic enrichment and prevention of oxygen exchange with the water (Raffaelli, 1999). *Nereis diversicolor* both preys on and competes with *Corophium volutator* (Jensen & Andre, 1993). Invertebrates also fulfil an important role in estuarine food webs as prey for higher trophic level species. On British mudflats *Nereis diversicolor* is considered to be the most important food for large shore birds (Baird *et al.*, 1985), while the amphipod *Corophium volutator* is the main food for several small shorebird species (Evans *et al.*, 1999). When the mud flats are submerged invertebrates provide food for diving ducks and commercially important fish. The estuarine clam *Macoma balthica* is eaten by wading birds, shrimps, crabs and fish and is also killed indirectly by disturbance as *Nereis diversicolor* ingests and moves large quantities of sediment (Hiddink *et al.*, 2002).

Abiotic limiting factors affect the distribution of estuarine invertebrates; including sediment grain size, sorting and organic content; turbidity, oxygen levels and salinity (Evans *et al.*, 1999). There are also interactions between biotic and abiotic limiting factors. The strength of recruitment, competition, predation and physiological tolerance effects on the diversity, abundance and distribution of intertidal benthos is determined by local physical environmental conditions (Dayton, 1971). Substrate stability is considered to be one of the most important factors controlling benthic species diversity and abundance and diversity are thought to increase with increasing substrate stability (Raffaelli & Hawkins, 1996). Estuaries are tidally dominated coastal systems, with flood and ebb tidal currents being the main factor involved in sediment erosion, transport and

deposition (Haslett, 2000), which affects the distribution and dispersal of benthic fauna.

Estuarine organisms also affect their physical environment: deposit feeding by *Macoma balthica* is responsible for biodeposition; burrows increase sediment surface area, permeability and aeration; while mucus secretion by *Nereis diversicolor*, *Corophium volutator* and nematodes stabilises sediment (Murray *et al.*, 2002). Any change in the diversity or abundance of estuarine species would therefore have a significant effect on both the physical habitat and the ecology of the whole ecosystem.

Estuarine species exhibit a range of adaptations to an intertidal habitat with fluctuating salinity and unstable sediment, including osmoregulation and temperature regulation mechanisms, burrowing, sediment stabilisation and benthic development, which avoids eggs and larvae being transported out to sea and allows all life stages to remain in more saline benthic water strata (Green, 1968). The degree and character of fresh and salt water mixing is the main limiting factor for estuarine flora and fauna. Salinity depends on the rate and volume of fresh water input, tidal parameters, basin topography and stratification (Haslett, 2000). Estuarine species must be adapted to cope with large, often rapid salinity fluctuations, ranging from 35 psu at full salinity to 0-3 psu in fresh water. Most marine species cannot tolerate reduced salinity below 10-15 psu so the longitudinal salinity gradient produces ecological zonation (Levinton, 2001). Many brackish water species that occupy a niche because they can withstand lower salinities are outcompeted by other species in marine habitats (Green, 1968; Kristensen, 1988). This introduces the theory that there may be a trade off between tolerance to limiting factors and competitive advantage in less harsh environments, possibly through metabolic costs of tolerance mechanisms that divert energy and resources away from growth and reproduction (Sibly & Calow 1989; Calow & Sibly 1990; Calow & Forbes, 1998; Briggs, 2005; Pook *et al.*, 2009).

The intertidal polychaete worm *Nereis diversicolor* is adapted to life in fluctuating brackish water environments. Many authors have studied the biology and ecology



of *N. diversicolor*; comprehensively reviewed by Scaps (2002). It is widely distributed in the temperate zone, with a geographical range including the Atlantic coast of North America, Europe, the Baltic, Black and Caspian seas and the Mediterranean, as far south as Morocco. It is found in brackish water habitats, in coastal estuaries, lagoons and the low salinity waters that form the majority of the Baltic Sea, where it is often the dominant species in estuarine intertidal mud flats and shallow subtidal sediment.

It forms large, high density populations, for example 961 per m<sup>2</sup> (Chambers & Milne, 1975) and yet competitively defends individual burrows (Kent & Day, 1983). It is omnivorous and exhibits a range of different feeding behaviours, from scavenging and deposit feeding to filter feeding (Fauchald & Jumars, 1979; Reise, 1979; Neilsen *et al.*, 1995). It tolerates a wide range of environmental conditions, including extremes of temperature and hypoxia (Kristensen, 1984, 1988). Most importantly *N. diversicolor* is a euryhaline osmoregulator, able to tolerate a wide range of rapidly fluctuating salinity levels (Hayward & Ryland, 1995), but it tends not to be found in fully marine habitats, where it is outcompeted by larger, more aggressive species such as *Nereis virens* (Kristensen, 1988). *N. diversicolor* is therefore a model example of the benefits of adaptation to a particular environment having associated costs outside that niche.

*N. diversicolor* is adapted to remaining in estuaries. It is gonochoric and reproduces sexually, but without the epitokous breeding form or swarming behaviour observed in marine Nereidids such as *Nereis virens* (Olive & Garwood, 1981; Olive *et al.*, 1986; Scaps, 2002). *N. diversicolor* is semelparous (breeds once and dies) and relatively large eggs are left in burrows following histolysis of the adult female (Olive & Garwood, 1981; Olive *et al.*, 1986). Dispersal of estuarine organisms is more likely in egg, larval and juvenile stages (Bilton *et al.*, 2002). *N. diversicolor* juveniles emerge from the burrow when they are around 6 chaetigers (segments bearing chaetae) in length (Bartels-Hardege & Zeeck, 1990) and disperse in the sediment for up to 4 days before burrowing (Marty & Retiere, 1999). Juveniles of 3 chaetigers in length, which are not capable of burrowing, have been found in plankton samples, probably due to accidental, passive suspension (Marty & Retiere, 1999) but development and dispersal for the

majority of juveniles is benthic. Adults can disperse actively by crawling or swimming short distances to avoid competition or unfavourable environmental conditions (Davey & George, 1986; Ait Alla *et al.*, 2006), or passively through sediment erosion and resuspension in strong tidal currents, which could involve transport over longer distances, but in general dispersal in this species is low.

Low dispersal, combined with geographical isolation of brackish water habitats separated by marine environments means that high levels of differentiation are often found between populations of estuarine species (Bilton *et al.*, 2002). Differences in ecology, morphology and biology have been found between populations of *N. diversicolor* at a range of spatial scales. Sex ratios are biased towards females, with reported proportions of males ranging from 10% in the Thames (Dales, 1950) and 18% in north east England (Olive & Garwood, 1981) to 40% in the Baltic (Bogucki, 1953). Age of maturity and breeding season varies widely between *N. diversicolor* populations over moderate geographical scales (reviewed in Röhner *et al.*, 1997; Scaps, 2002). Age at maturation has been estimated as 12 to 18 months (Kristensen, 1984), 18 to 24 months (Chambers & Milne, 1975), 24 to 36 months (Möller, 1985) and 33 to 42 months (Olive & Garwood, 1981). Breeding seasons around the UK have been recorded as March and June at 2 different locations in the Humber Estuary (Grant *et al.*, 1990), January – March and June – August in the Ythan Estuary in Scotland (Chambers & Milne, 1975), March/April at Blyth on the north east coast (Olive & Garwood, 1981), April/May on the south east coast (Nithart, 1998) and August in both the Avon Estuary and Restronguet Creek in south west England (Hateley *et al.*, 1989; Grant *et al.*, 1990). It is thought that females stop feeding for around 3 months prior to spawning, to avoid predation and maximise reproductive success (Olive *et al.*, 1997; Last & Olive, 1999; Last, 2000). However, eggs can then be reabsorbed and feeding recommences until the next breeding season, possibly mediated by environmental conditions (Olive *et al.*, 1997). Differences in breeding season could be viewed as either a cause or a consequence of temporal reproductive isolation between populations.

Interpopulation morphological differences may be the result of differential adaptation to local environmental conditions. The form, arrangement and number

of hardened scleroprotein paragnaths on the pharynx of Nereididae has been used as a quantitative taxonomic trait and it has been suggested that paragnath patterns in *N. diversicolor* could be associated with salinity, sediment type, or different diets (reviewed by Hateley *et al.*, 1992; Maltagliati *et al.*, 2006; Bakken *et al.*, 2009). While there is debate regarding the cause of this morphological variation, it has been clearly demonstrated that the trait is heritable and therefore may be an evolutionary adaptation to environmental factors (Hateley *et al.*, 1992; Khlebovich & Komendantov, 2002). However, a gradient of paragnath number along the Humber Estuary by Hateley *et al.* (1992) suggested a simple pattern of isolation by distance.

Brackish habitats are characterised by high spatial and temporal environmental variation, so adaptation to estuarine habitats requires general tolerance to large fluctuations in abiotic factors such as salinity, temperature and oxygen levels (Abbiati & Maltagliati, 1992; Van Straalen, 2003). Evolutionary adaptation to extreme habitats is an important factor in the evolution of new species, so there is great interest in stress tolerance in intertidal organisms. Stress applies to any situation where the fitness of individuals and populations is reduced by a change in environmental conditions, either through colonisation of a novel environment or the introduction of new conditions (Van Straalen, 2003; Jha, 2004). Where different populations of the same species are subjected to different levels of stress it provides a useful model for studying stress tolerance mechanisms.

Tolerance to stress can be defined as the ability to prevent, reduce or repair damage (Bickham *et al.*, 2000). Tolerance can develop through acclimation, as the result of individual exposure, or at the population genetic level through selection and adaptation (Luoma, 1977). Many anthropogenic influences on marine environments can be viewed as limiting factors, requiring stress tolerance mechanisms and potentially causing adaptation at the population genetic level in aquatic organisms. Metal pollution in estuaries is a source of environmental stress that can exert differential selective pressure on populations (Bickham *et al.*, 2000; Belfiore & Anderson, 2001). Exposure to metal pollution can affect individuals, populations and communities at morphological, physiological, biochemical and genetic levels (Troncoso *et al.*, 2000; Grant, 2002).

## 1.2 Copper tolerance in *Nereis diversicolor*

Heritable tolerance is a useful model for understanding adaptation as an evolutionary process (Grant, 2002). Differences in tolerance to metals have been demonstrated in natural populations of *Nereis diversicolor*, particularly in heavily contaminated estuaries in South West England (Bryan & Hummerstone, 1971; Bryan & Gibbs, 1983; Grant *et al.*, 1989; Hateley *et al.*, 1989; Briggs, 2005). Bryan and Hummerstone (1971) suggested that copper tolerance in *N. diversicolor* is a heritable trait. Grant *et al.* (1989) and Hateley *et al.* (1989) then demonstrated experimentally that tolerance to copper and zinc are heritable. They found a significant difference in tolerance to copper and zinc between *N. diversicolor* from contaminated and uncontaminated sites in Restronguet Creek and the relatively unpolluted Avon Estuary and offspring of these two populations reared in clean laboratory conditions exhibited the same difference in copper tolerance. Heritable tolerance to zinc was also significant but was less pronounced, which lead Grant *et al.* (1989) to suggest that specific tolerance mechanisms for Cu and Zn evolved separately.

Correlation between genetic variation and environmental factors supports the view that genetic structure is the result of selection for resistant genotypes (Luoma, 1977; Belfiore & Anderson, 2001). Studies by Grant *et al.* (1989), Hateley *et al.* (1989) and Briggs (2005) have found a gradient of tolerance to copper in *N. diversicolor*, which corresponds with levels of copper found in both sediments and *N. diversicolor* tissues. However, metal tolerance in *N. diversicolor* declines outside the most polluted areas, suggesting that copper tolerant are outcompeted by non-tolerant worms in cleaner environments (Grant *et al.*, 1989; Briggs, 2005). In toxicity tests Briggs (2005) found 100% mortality for relatively contaminated sites, including a site with a sediment Cu concentration of 1399  $\mu\text{g g}^{-1}$ , which indicates that tolerant worms are confined to the most contaminated areas by selection against the tolerant phenotype in cleaner areas. Reduced fitness in clean environments implies an associated cost of tolerance, which is characteristic of heritable tolerance mechanisms (Luoma, 1977; Briggs, 2005; Pook *et al.*, 2009).

### 1.3 Tolerance and population genetics

With recent concern regarding anthropogenic effects on the environment, such as climate change, habitat loss and pollution, it has become increasingly important to understand how species respond to these pressures. Such information allows scientists to assess the risk of adverse impacts, predict future effects and address conservation issues. Relating adaptive traits that allow species to respond to changes in their habitat at the phenotypic level to changes at the molecular level provides insight into evolutionary responses to a changing environment (Belfiore & Anderson, 2001). Understanding the effects of anthropogenic impacts on genetic diversity and differentiation in natural populations is one of the central issues in evolutionary and conservation biology but few studies have addressed the effects of chemical contamination on population genetics (Bickham *et al.*, 2000). The population genetics of copper tolerance in *N. diversicolor* offers a model system for exploring evolutionary adaptation to stress.

Population genetics is the study of spatial and temporal genetic variation, within and between populations. An understanding of the mechanism, rate and pattern of evolutionary adaptation is important in assessing the capacity for organisms to respond to anthropogenic effects on their environment. Darwin (1859) identified natural selection as the mechanism of adaptive evolution but it was not until the rediscovery of Mendel's work that the link was made between observable phenotypic traits such as copper tolerance and the genes that contribute to them. Early theoretical works by Haldane (1924), Fisher (1930) and Wright (1931) proposed models of population genetics to explain the effects of dynamic evolutionary forces on changes in the genetic structure of populations over time. Recent advances in biochemical, molecular, statistical and computational techniques have allowed researchers to measure genetic diversity and differentiation directly at the level of proteins and DNA and to fit the data to these models. Thus as part of the neo-Darwinian modern evolutionary synthesis, population genetics is able to make valuable contributions to interdisciplinary studies of evolutionary biology, biogeography, biodiversity, ecology, ecotoxicology and conservation.

## **Genetic diversity**

Population genetics is largely reducible to the study of allele frequencies. Evolution has been defined in terms of allele frequencies, simply as the change in the frequency of an allele within a gene pool (Dobshansky, 1937). Individuals in a population have different genotypes: combinations of alleles which code for phenotypes with varying selective advantages and disadvantages.

An individual diploid, heterozygous organism has 2 different alleles of a gene at a locus on homologous chromosomes, with phenotype generally determined by the dominant allele. Heterozygosity can refer to the proportion of loci in an individual that are heterozygous or the proportion of loci in a population that are potentially heterozygous because more than one allele exists. Expected heterozygosity ( $H_E$ ), sometimes referred to as gene diversity, is the level of heterozygosity expected in a randomly mating population or species (Nei, 1973). The larger the number of alleles and the closer they are to equal frequency, the higher the diversity. Maintenance of heterozygosity is beneficial because it allows populations to retain a reservoir of recessive alleles which may become advantageous under changing environmental conditions. It also avoids deleterious recessive alleles being expressed in homozygotes.

## **Population size**

A population can be defined as a geographically distinct group of individuals that actually or potentially interbreed (Hartl & Clark, 2007). The probability of loci in a population having more than one allele increases with increasing population size and in very large populations almost all loci are polymorphic. The proportion of polymorphic loci in a population is therefore larger than the proportion of heterozygous loci in individuals. Heterozygosity in individuals increases with increasing proportion of polymorphic loci in the population and therefore with increasing population size. It is thought that invertebrates have higher heterozygosity than vertebrates due to larger population sizes. Mean

heterozygosity detected by protein electrophoresis was found to range from 0.041 for mammals to 0.148 for molluscs (Nevo *et al.*, 1984).

The size of populations varies over time due to variable success in changing environmental conditions. Effective population size  $N_e$  is the size of an ideal population with the same heterozygosity as the observed population (Wright, 1931, 1938). In practice this equates to the average number of reproducing individuals that contribute alleles to succeeding generations (Crow & Kimura, 1970). Most natural populations have a much smaller effective population size than observed size. Effective population size may be reduced by unequal sex ratios or reproductive success and by past reductions in population size. Bottlenecks, where a population undergoes a dramatic size reduction, and founder effects, where a new population is started by a small number of migrant individuals, cause reduced genetic variation and a low effective population size (Nei *et al.*, 1975). Carr *et al.* (1995) found low genetic variation in cod, *Gadus morhua*, due to confinement to a small refuge in the last ice age, with an estimated effective population size of  $N_e = 3 \times 10^4$ , compared with  $N_{obs} \approx 10^9$ .

As an extremely common invertebrate with a low generation time and high fecundity *Nereis diversicolor* has very large observed populations. For example 50 worms per  $m^2$  in  $\sim 2 \text{ Km}^2$  of mud in Restronguet Creek alone would be a population of 108 worms. *N. diversicolor* has an unequal sex ratio, although ratios of between 2:1 and 10:1 (Scaps, 2002) will not reduce the effective population size as much as in harem or eusocial structures with more biased sex ratios or reproductive success.

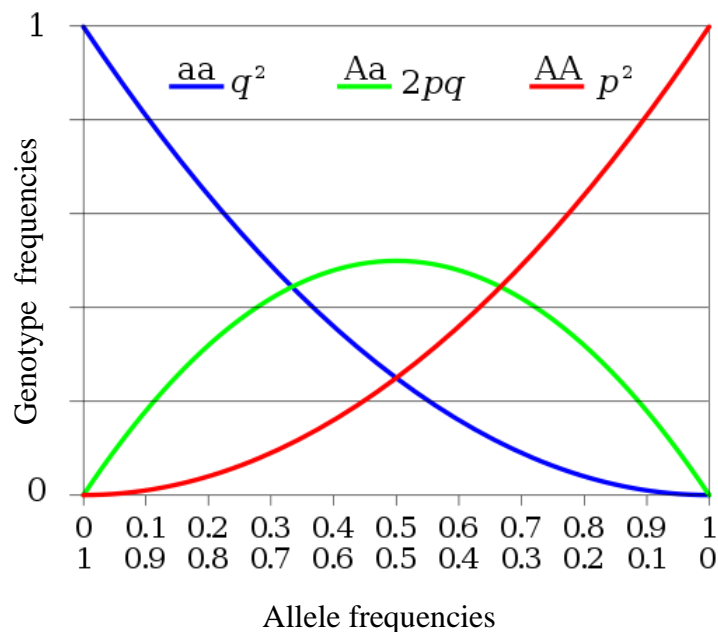
The introduction of metal pollution may have caused a bottleneck, or a founder effect following recolonisation of polluted areas, with an associated reduction in effective population size and heterozygosity. Lower diversity in a sub-population relative to others with the same sex ratio would indicate reduced effective population size due to a bottleneck or founder effect.

## Hardy Weinberg Equilibrium

The Hardy Weinberg principle (Hardy, 1908; Weinberg, 1908) describes the relationship between allele frequencies and genotype frequencies in an ideal population. Genotype frequencies can be predicted from the frequency of alleles at a locus using the formula:

$$p^2 + 2pq + q^2 = 1$$

For a locus with two alleles, dominant A and recessive a, with allele frequencies p and q:  $\text{freq}(A) = p$ ,  $\text{freq}(a) = q$  and  $p + q = 1$ . For a population in Hardy Weinberg Equilibrium  $\text{freq}(AA) = p^2$  for the dominant homozygotes,  $\text{freq}(aa) = q^2$  for the recessive homozygotes and  $\text{freq}(Aa) = 2pq$  for the heterozygotes. This also allows allele frequencies to be calculated from the frequency of dominant and recessive genotypes.



**Figure 1.1: Homozygous and heterozygous genotype frequencies for two alleles A and a under Hardy Weinberg Equilibrium, for a range of allele frequencies from 0 to 1.**

A population will reach Hardy Weinberg Equilibrium in one generation of random mating. When a population is in equilibrium allele and genotype frequencies remain constant over time and genetic variation is maintained. The Hardy Weinberg principle assumes infinite population size, non-overlapping



generations, random mating and no mutation, drift, migration or selection. In practice natural populations rarely reflect these assumptions and the aim of many population genetics studies is to investigate how and why populations deviate from Hardy Weinberg Equilibrium (Hartle & Clark, 2007).

### **Inbreeding**

If an individual mates with a close relative, the offspring may be homozygous for an allele that has identity by descent from a common ancestor (Wright, 1951; Crow & Kimura, 1970). Non random mating between closely related individuals increases homozygosity. Inbreeding in a population changes the genotype frequency, and therefore results in deviation from Hardy Weinberg equilibrium, but does not change the allele frequency.

Inbred populations are characterised by reduced survival, vigour and fertility known as inbreeding depression (Charlesworth & Charlesworth, 1987). In outbreeding populations dominant deleterious mutations are quickly eliminated but recessive mutations are masked by heterozygosity. Inbreeding causes an increase in individuals that are homozygous for recessive deleterious alleles and thus phenotypes with reduced vigour. However, it has been argued that inbreeding increases the rate at which deleterious alleles are eliminated and so effectively speeds up evolution (Crow, 1986). It also favours advantageous recessive alleles and increases phenotypic variation. Positive assortative mating can drive phenotypic divergence, reinforce divergent selection and ultimately lead to speciation.

Random mating between closely related individuals is more likely to occur in small populations. Despite large populations, high numbers of offspring with low dispersal in *Nereis diversicolor* could promote inbreeding between closely related neighbours, leading to inbreeding depression. However, if reduced heterozygosity was simply the result of spatial distribution and low dispersal then tolerant and non tolerant populations would show the same inbreeding effects.

Mating between individuals from 2 genetically different inbred populations can result in hybrid vigour, where the offspring have more advantageous traits than the parents (Crow, 1948). This may be due simply to dominance, because heterozygosity in the hybrid masks deleterious recessive alleles. Alternatively overdominance occurs when the heterozygote has superior fitness to homozygotes for either allele. Heterozygote advantage allows the survival of deleterious recessive alleles, which cause reduced fitness in homozygotes in inbred populations. While F1 hybrids often show hybrid vigour F2 hybrids or backcrosses can suffer from hybrid breakdown, with greatly reduced fitness (Burton, 1986). Hybrid effects could be apparent in a hybrid zone between genetically distinct populations of *Nereis diversicolor* separated by low dispersal or differential tolerance.

In the absence of other evolutionary forces allele frequencies in an inbred population do not change. However, allele frequencies in natural populations vary in space and over time. Four main processes affect the frequency of alleles in a population: mutation, genetic drift, selection and migration.

## **Mutation**

Mutation creates new alleles in a population. This is usually a stochastic process, although the rate of mutation can be increased by mutagens such as radiation and genotoxic chemicals, including arsenic and possibly copper (Jha, 2004).

Mutation rate is often defined as the number of mutations per gene per unit of time. In population genetics mutation rate  $\mu$  is considered to be the rate of new alleles per gamete per generation (Hartl & Clark, 2007). The rate at which mutations occur affects the rate of change in allele frequencies in a population but mutation rates are very low. Experiments on *Caenorhabditis elegans* by Denver *et al.* (2004) estimated the mutation rate to be  $2.1 \times 10^{-8}$  mutations per base pair per generation, for a genome of  $\sim 10^8$  base pairs, giving a haploid genomic mutation rate of  $\sim 2.1$  mutations per genome per generation. Most mutations occur in non coding DNA, are recessive or have a neutral effect on fitness but occasionally a

beneficial mutation in a gene provides scope for evolutionary change. Variation at the genetic level allows selection to act on favourable adaptation at the phenotypic level.

Advantageous phenotypes could also be attributable to heritable epigenetic changes in gene expression that do not involve a change in the underlying DNA sequence. Epigenetic modifications such as transcriptional silencing by DNA methylation of promoter regions could cause transgenerational differences in gene expression, which would not affect AFLP restriction sites in genomic DNA. The idea of epigenetic inheritance questions the assumptions of conventional population genetic models. Extensive epigenetic variation in natural populations could even fill a gap in the Modern Synthesis, explaining complex adaptations and macro evolution despite low mutation rate and rare beneficial alleles. Adaptation through selection of heritable epigenetic variation may be particularly important in small, fragmented populations with low genetic variation (Jablonka & Raz, 2009).

Recurrent mutations or epigenetic changes can maintain a deleterious genotype in a population despite selection against it, with equilibrium reached when the rate of removal by selection balances the rate of replacement by mutation. The frequency of new alleles created through mutation that are retained in a population is determined by drift and selection.

## **Drift**

Allele frequencies in finite natural populations change over time due to random genetic drift, with an associated reduction in heterozygosity (Wright, 1931). The frequency of alleles changes over successive generations due to random sampling in the gametes, which eventually leads to some alleles being lost while others become fixed, with all individuals in a population homozygous for a single allele at a locus. The probability of an allele becoming fixed due to drift is the same as its initial frequency. Populations undergoing genetic drift violate the assumptions of the Hardy Weinberg model because both the allele and genotype frequencies

change over time, although the genotypes will actually be in Hardy Weinberg Equilibrium in each generation, as in Figure 1 (Crow, 1986).

Each generation heterozygosity is reduced by  $1/2N_e$ , where  $N_e$  is the effective population size  $\approx$  the number of reproducing individuals (Wright, 1931). Genetic drift therefore has a greater effect in small populations (Crow & Morton, 1955; Crow & Kimura, 1970). Kerr & Wright's (1954) experiment on *Drosophila* populations of 4 males and 4 females found that in 70% of cases after only 16 generations one of 2 alleles for a particular gene was lost and the other fixed. In populations with greatly reduced effective population size due to a bottleneck or founder effect, drift can outweigh the effects of mutation, migration and selection. However, evolution through drift is random rather than adaptive.

Traditionally it has been thought that phenotypic changes are mostly advantageous, and therefore must be determined by natural selection, so drift only plays a minor part in evolution (Fisher, 1930). However, the neutral theory of molecular evolution (Kimura, 1968) argues that drift is the most important force affecting allele frequencies, as most phenotypic differences between individuals in a population have no adaptive significance and therefore most underlying changes at the population genetic level are the result of random mutation and drift rather than natural selection. This debate has obvious implications for any attempt to link molecular differentiation with adaptive phenotypic traits under selection.

## **Selection**

Natural selection (Darwin, 1859) is considered to be the main driving force of adaptation and evolution. Under selection individuals with advantageous adaptive traits are more successful. Selection pressure causes differential mortality, competitive advantage and reproductive success and determines the relative proportion of phenotypes in a population. Selection acts directly on phenotypes but it also affects both the frequency of alleles and the level of heterozygosity in a population at the molecular level. In a sufficiently large population selection reduces heterozygosity (Li, 1978). Alleles for an adaptive trait can be under

selection while other loci in a genome remain neutral. Non-coding DNA may also be subject to selection if it is linked to coding regions of the genome (Barton, 2000). Selection favours adaptive alleles and eliminates deleterious alleles, although if a deleterious allele is co-dominant heterozygotes may have intermediate fitness.

The selection coefficient  $s$  measures selection pressure opposing the reproductive success of a genotype. For  $s = 0.05$  a genotype would be 95% as fit as the favoured genotype. There is also a measure of relative fitness  $w$ , where  $w = 1 - s$ , or  $s$  can be used as a positive measure of directional selection for adaptive traits.

Haldane (1924) showed that under favourable selection (where  $s$  measures advantage rather than disadvantage) the time required for an allele frequency to change is inversely proportional to the intensity of selection:

$$t = \frac{2}{s} \ln \left( \frac{P_t(1 - P_0)}{P_0(1 - P_t)} \right)$$

Where  $t$  is the number of generations and  $P_0$  and  $P_t$  are the initial and final frequencies of an allele. For example, for weak selection, e.g.  $s = 0.01$  it takes 1840 generations to change an allele frequency from 0.01 to 0.99.

A famous example of the effects of selection is the cryptic peppered moth *Biston betularia* (Majerus, 2009). During the industrial revolution as smoke blackened white lichen on trees the frequency of light and dark colour morphs of the moth changed rapidly under strong selection pressure from birds. This change was too fast to be explained by random genetic drift. The moth has discrete annual generations and it was thought that a single dominant allele was responsible for the melanic phenotype. This allowed Haldane (1924) to use a general selection model to show that if the frequency of melanic moths was 0.02 in 1848 and 0.95 by 1895, the dark form must be one and a half times as fit as the light form, which implies selection pressure  $s \approx 0.33$ .

Copper extraction in Cornwall began in the 1740s. Assuming 1 generation every 2 years in *Nereis diversicolor* (Scaps, 2002), to change an allele frequency from 0.01 to 0.99 in 130 generations would only require a selection pressure of  $s = 0.141$ , half that estimated for the peppered moth.

At the population level directional selection pushes a population towards one end of a phenotypic gradient, while disruptive or divergent selection favours extreme traits, which divides populations. However, selection does not necessarily lead to change: stabilising selection favours intermediate traits and balancing selection allows a range of different advantageous traits. Paradoxically loss of genetic diversity through selection for adaptation to a contaminated environment reduces the potential for adaptation to other sources of stress (Nevo *et al.*, 1986; Bickham *et al.*, 2000).

Interactions between selection pressure and inbreeding, mutation, drift and migration determine the amount of genetic variation, both short term at the population level and long term on an evolutionary scale. In particular directional selection is balanced by immigration, where the flow of less favourable genes can reduce or even prevent adaptation.

## **Migration**

Gene flow is the movement of genes between geographically distinct populations, which has a homogenising effect that counteracts genetic divergence due to drift and selection (Wright, 1931). Migration of gametes or individuals between populations is the most common mechanism of gene flow. The rate of migration or gene flow  $Nm$  is the number of migrants per generation exchanged between sub populations, where  $N$  is the effective population size and  $m$  is the migration rate. Change in allele frequency due to migration is proportional to the rate of migration and to the difference in allele frequency between the donor and recipient populations. Genetic diversity and structure is therefore influenced by an organism's mode of reproduction, development and dispersal (Wright, 1946;

Crisp, 1978; Slatkin, 1985). The dynamic interplay between drift, selection and migration determines population structure.

### **Genetic structure**

Genetic structure is the geographical subdivision of a population into demes and the extent of differentiation between them (Wright, 1931, 1951; Nei, 1972, 1973). Most metapopulations have some form of genetic structure, where there is deviation from panmixia or Hardy Weinberg equilibrium. Diversity between sub populations allows a wider range of traits within a species and a greater capacity for adaptation to different environmental conditions (Wright, 1943). When gene flow between populations is greatly reduced genetic drift or divergent selection can lead to speciation. The relationship between the forces of mutation, inbreeding, drift, selection and migration acting on population structure is therefore important to the understanding of adaptation and speciation. While population structure is determined by the interaction of evolutionary forces it also has a feedback effect in mediating adaptive evolution and speciation (McCauley, 1993). The nature and extent of this role has been the subject of debate

### **Models of population structure**

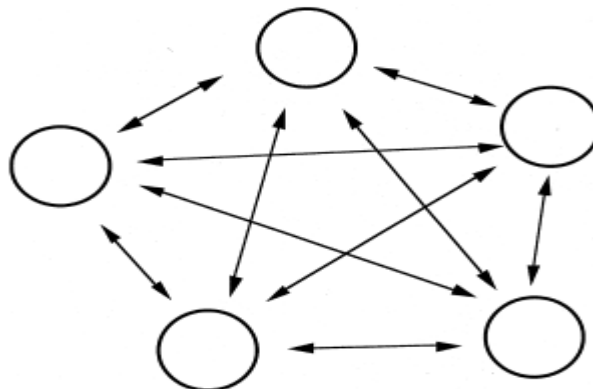
Statistical analysis of differences between populations requires simple models of population structure. Models are idealised extremes of possible structures (Slatkin & Barton, 1989) and so do not necessarily represent the dynamics found in natural populations. Metapopulations may be subdivided into clear sub populations or demes or may appear to be continuous.

Isolation allows differentiation of local sub populations. If a population is distributed continuously over an area that is large compared to the average migration distance of individuals there will be a gradient of differentiation due to isolation by distance (Wright, 1943). A positive correlation between genetic and geographic distance indicates equilibrium between drift and migration, where

replacement of alleles by migration balances loss of alleles due to drift (Malécot, 1955). A pattern of isolation by distance at equilibrium requires both suitable environmental conditions and sufficient time to become established and will therefore be more evident in long established populations than in recently established populations (Slatkin, 1993; Hutchison & Templeton, 1999). Equilibrium is not reached in disturbed habitats characterised by local extinctions and recolonisation or in or fragmented habitats where small populations can undergo stochastic extinctions (McCauley, 1993).

Basic models of population genetics that focus on migration include continuum, stepping stone, lattice and island structures (Slatkin & Barton, 1989). Stepping stone models (Kimura, 1953) are described as 2 dimensional because gene flow is restricted to migration between adjacent sub populations. The confined, elongated shape of rivers and estuaries intuitively suggests a stepping stone structure. Rivers and intertidal shores have also been described as 1 dimensional, linear habitats (Slatkin, 1985; Templeton *et al.*, 1995).

Alternatively island models (Wright, 1951) allow migration to each deme from every other deme.



**Figure 1.2: Wright's island model of gene flow between sub populations.**

Wright's island model (1951) makes a number of assumptions. It does not include mutation or selection. All populations are assumed to have reached equilibrium



between migration and drift. Each population has the same number of individuals and migrants are exchanged with each of the other populations at the same rate. Wright's (1969) infinite island model assumes an infinite number of demes. There is an infinite number of populations and yet all populations are equally likely to exchange migrants with all other populations.

The combination of parameters for gene flow is referred to as  $Nm$  (Number of migrants) under an island or stepping stone model. The island and stepping stone approaches model discrete sub populations or demes, with migration between them, where the parameter  $m$  is the probability that each individual or gamete is an immigrant. Where  $N$  is the effective population size and  $m$  is the migration rate,  $Nm$  is the number of migrants per generation exchanged between sub populations. Neighbourhood size ( $4\pi D\sigma^2$ ) is equivalent under a continuum model. Continuum and lattice models consider dispersal of gametes according to a distribution of dispersal distances, affected by the density of individuals:  $N_b = 4\pi D\sigma^2$ , where  $D$  is density and  $\sigma$  is the standard deviation of the distance moved by each gamete and is equivalent to  $m$ . This is usually referred to as neighbourhood size,  $N_b$ , rather than gene flow,  $Nm$ .

While drift decreases diversity within populations and increases differentiation between them migration has the reverse effect. Very little migration is required to prevent substantial differentiation due to drift. Wright (1931) demonstrated that in an island model of sub populations with  $Nm > 0.5$ , gene flow overrides the effects of differentiation due to drift, whereas with  $Nm < 0.5$  sub populations will tend towards fixation of alleles.

Population structure can also be the result of localised selection on a level that outweighs the homogenising effects of gene flow. The rate of migration between sub-populations affects the level of differentiation due to selection. Haldane (1924) showed that an allele favoured by selection strength  $s$  (where  $s$  measures advantage rather than disadvantage) is maintained at high frequency, against the force of gene flow if  $m < s$ . The higher the selection pressure, the less likely migration is to prevent adaptation.

If gene flow overrides the structuring effects of differentiation due to drift in sub populations with  $Nm > 0.5$ , then in a structured population with  $Nm > 0.5$  the genetic differentiation is likely to be the result of selection rather than drift. Information on the level and pattern of dispersal in *N. diversicolor* is important for understanding the balance of genetic drift, selection and migration (Slatkin, 1985). *N. diversicolor* has low dispersal capabilities (Scaps, 2002) but a low level of migration outweighs the effects of both drift and weak selection so evidence of population differentiation despite migration would indicate strong selection pressure. Selection against non-tolerant worms at contaminated sites is likely to be stronger than selection against tolerant worms at clean sites. Briggs (2005) assessed dispersal by comparing the proportion of tolerant and non-tolerant adult and juvenile *N. diversicolor* in Restronguet Creek during the breeding season. There was no significant difference in the proportion of tolerant and non-tolerant worms among 3 age classes of adult and juvenile *N. diversicolor* at 6 sites. It is likely that this reflects limited migration between sites, although it could also have been the result of complete and instant selection against immigrants at both contaminated and clean sites.

Temporal structure also affects spatial patterns of population differentiation. Local extinction and recolonisation can either increase or decrease differentiation between sub populations (McCauley, 1991). Wright (1940) suggested that extinction and recolonisation would increase genetic differentiation between local populations through founder effects. Bottlenecks and founder effects increase differentiation in two ways: initially diversity is decreased relative to surrounding populations due to reduced population size, which then exaggerates the effects of genetic drift. However, Slatkin (1985) suggested recolonisation could decrease genetic differentiation through gene flow, dependent on whether founders were from a single source population or from multiple surrounding populations and the level of gene flow.

Many statistical analysis methods for population genetics rely on various assumptions regarding these models and their parameters. In order to study the effects of inbreeding, mutation, drift, migration and selection, the interactions

between these factors and their relative contribution to population structure and evolutionary change, statistics must therefore be selected and used with care.

## **Speciation**

If gene flow between sub populations is reduced to the extent where the effects of drift and selection outweigh the balancing effect of migration speciation may occur. Any statement regarding the possibility of speciation must necessarily include a definition of species. A number of species concepts have been proposed, based on morphology, phylogeny, ecology, reproductive isolation or reproductive recognition, but they all face problems such as fossil, asexual and hybrid organisms.

The biological species concept, first postulated by Ray in the 17th century and incorporated into Darwin's work, was seen as a solution to the problems presented by typological and morphological concepts. The most widely used modern definition was suggested by Dobzhansky in 1937 and modified by Mayr in 1942:

“Species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups.”

Because reduced gene flow between populations is such an important factor in speciation the biological species concept, based on reproductive isolation, is often applied in population genetics.

Barriers to gene flow or reproductive isolating mechanisms can be pre-zygotic, preventing fertilisation, or post-zygotic, preventing development of viable or fertile offspring (Dobzhansky, 1937). Pre-zygotic isolating mechanisms are classified as ecological, temporal, ethological, mechanical and gametic, and post-zygotic mechanisms as hybrid inviability, hybrid sterility and hybrid breakdown. Examples are given in Table 1.1.

<b>Reproductive Isolating mechanism</b>		<b>Examples</b>
<b>Pre-zygotic (assortative)</b>		
<b>Ecological</b>	occupy different roles or niches	The mosquito <i>Anopheles gambiae</i> breeds in fresh water while <i>Anopheles merus</i> breeds in salt water (Coetzee <i>et al.</i> , 2000) The fish <i>Hypsoblennius gilberti</i> is found in rocky intertidal and shallow subtidal habitats while <i>H. jenkinsi</i> only inhabits subtidal clam burrows and <i>Serpulorbis</i> tubes (Stephens <i>et al.</i> , 1970)
<b>Temporal</b>	reproduce at different times - seasonal, lunar or circadian cycles	<i>Fucus vesiculosus</i> is fertile May – June and <i>Fucus serratus</i> August – October (Knight & Parke, 1950; Williams, 1996) Synchronised spawning at different times in <i>Montastraea</i> coral species (Knowlton <i>et al.</i> , 1997)
<b>Ethological</b>	behavioural recognition system; sexual signalling, selection or aggression	Frog species have species-specific mating calls and responses (Gerhardt, 1994) Sympatric snapping shrimp, <i>Alpheus</i> , are monogamous and show interspecific aggression (Mathews <i>et al.</i> , 2002)
<b>Mechanical</b>	physiological incompatibility	Many crustaceans and insects have 'lock & key' genitalia e.g. water striders, <i>Gerridae</i> (Arnqvist <i>et al.</i> , 1997)
<b>Gametic isolation</b>	chemical / molecular recognition system	Breeding experiments indicate gametic isolation between molluscs <i>Lacuna marmorata</i> and <i>Lacuna unifasciata</i> (Langan-Cranford & Pearse, 1995) <i>F. vesiculosus</i> and <i>F. serratus</i> have intraspecific gamete recognition systems (Dring, 1992)
<b>Post-zygotic</b>		
<b>Hybrid inviability</b>	offspring are not viable	Hybrids between the urchin <i>Strongylocentrotus droebachiensis</i> and <i>S. purpuratus</i> or <i>S. franciscanus</i> suffer high larval mortality (Levitan, 2002) Few hybrids between high and mid shore <i>Littorina saxatilis</i> morphs survive (Hull <i>et al.</i> , 1996)
<b>Hybrid sterility</b>	hybrid adult is sterile	Plaice, <i>Pleuronectes platessa</i> and flounder, <i>Platichthys flesus</i> interbreed to produce sterile hybrid offspring (Argue & Dunham, 1999)
<b>Hybrid breakdown</b>	F1 hybrids viable and fertile, F2 inviable or sterile	F1 hybrids of genetically differentiated populations of the copepod <i>Tigriopus californicus</i> are vigorous and fertile but F2 hybrid and backcross larvae have high mortality (Burton, 1986)

**Table 1.1: Reproductive isolating mechanisms**

The ecological species concept has recently gained support (Schluter, 2009; Schluter & Conte, 2009; Wolf *et al.*, 2010; Johannesson *et al.*, 2010). According to Van Valen (1976):

“A species is a lineage (or a closely related set of lineages) which occupies an adaptive zone minimally different from that of other lineages in its range”

The ecological species concept focuses on selection as the driving force of speciation; where reproductive isolation is the result of adaptation to contrasting environmental conditions or niches, which could include differential tolerance to limiting factors. Population structure in *N. diversicolor* could be the result of chemical habitat discontinuity, which acts as a physical barrier to gene flow between tolerant and non-tolerant worms. Strong selection pressure can drive and maintain genotypic clines despite high levels of gene flow (Endler, 1973; Schluter, 1996; Nosil *et al.*, 2009).

Disruptive selection occurs in heterogeneous environments and acts to limit gene flow outside the niche or adaptive zone, while competition prevents species occupying the same niche, maintaining species integrity. A competitive advantage in non-tolerant *N. diversicolor* may compound the effects of disruptive selection due to metal tolerance. In this way adaptation to opposing selection pressures can create an ecological barrier to gene flow (Beatty *et al.*, 1998). Hybrids, which are less well adapted to either niche may form a hybrid zone between two species. However, rather than forming a gradient of adaptation, in the case of hybrid breakdown the hybrid zone can act as a barrier to gene flow.

As metal tolerance in *N. diversicolor* declines rapidly outside the most polluted areas (Grant *et al.*, 1989), tolerant and non-tolerant populations are found in close proximity to each other, which suggests that genetic differentiation between adjacent populations is likely to be the result of divergent selection rather than geographic distance. This makes Restronguet Creek a good model for studying the adaptive evolution of tolerance and the potential for speciation.

Recently molecular species concepts have defined taxonomic units based on a threshold of genetic difference in a single mitochondrial 'barcode' gene such as COI (CBOL, 2010; Hebert *et al.*, 2003; Blaxter, 2004). This faces the same problem as the morphological concept: that the chosen markers or threshold may not be appropriate for all taxa (Lorenz *et al.*, 2005) and the question of how different species are remains (Will & Rubinoff, 2004). However, thresholds based on ratios of difference could be used, for example 10 times the average intrapopulation COI haplotype divergence (Witt *et al.*, 2006). An equivalent approach using genome scans of large numbers of molecular markers such as AFLP can set thresholds based on measures of genetic differentiation in order to delimit species.

However, in populations that are in the process of speciation but are not yet reproductively isolated, it could be inferred that selection acts on a few genes for adaptive traits against a background of the homogenising effects of gene flow, characterised by a few highly divergent loci (Beaumont & Nichols, 1996; Wilding *et al.*, 2001). AFLP Genome scans can reveal signatures of speciation, by identifying individual loci with higher than average differentiation, that are associated with adaptive traits, such as tolerance to copper in *Nereis diversicolor*.

#### 1.4 *Nereis diversicolor* Taxonomy

<b>Phylum</b>	Annelida
<b>Class</b>	Polychaeta
<b>Order</b>	Phyllodocida
<b>Family</b>	Nereididae
<b>Genus</b>	<i>Nereis</i>
<b>Species</b>	<i>diversicolor</i>

The taxonomic status and nomenclature of the genus *Nereis* has been an ongoing source of debate and confusion (Breton *et al.*, 2003). *Nereis virens* and *Nereis diversicolor* are now widely referred to as *Neanthes virens* and *Hediste diversicolor*. Scaps (2002) blamed the change on Fong & Garthwaite (1994), although probably erroneously as Abbiati & Maltagliati used *Nereis diversicolor* in 1989 and *Hediste diversicolor* in 1992, possibly following Chambers & Garwood (1992) who argued that *Hediste* is separated from other genera of Nereididae with conical paragnaths by the presence of fused falcigers in posterior neuropodia. This highlights the extent to which taxonomy and phylogeny are based on morphological characteristics and raises the prospect of quantitative molecular methods resolving taxonomic divisions and relationships. Parsimony analysis of 66 morphological characters in Nereidinae by Bakken & Wilson (2005) concluded that the genera *Neanthes* and *Nereis* were not monophyletic and proposed splitting *Alitta virens* and *Hediste diversicolor*. However, many characters had high levels of homoplasy, the overall phylogeny could not be rooted and the authors suggested further analysis using molecular markers.

Scaps (2002) noticed that often molecular biologists prefer *Hediste* while ecologists still use *Nereis* (but stopped short of suggesting a compromise for molecular ecology). From a practical point of view it is perhaps more important for scientists to consistently refer to the same taxonomic unit using the same name, as is the primary aim of the scientific nomenclature system, than to re-assign species to the latest genus or sub-genus based on a subjective choice of criteria. For the sake of consistency and continuity this study refers to all worms formerly known as *Nereis* as *Nereis*, with genus names used in other studies changed where appropriate.

## 1.5 Aims and hypotheses

The aims of the present study were:

- Investigate the effects of an anthropogenic pollutant on the distribution of tolerance in an estuarine species, in order to understand how species respond to environmental limiting factors at the population level.
- Assess the utility and benefits of Amplified Fragment Length Polymorphism analysis and a range of statistical analysis methods applied to AFLP data for studying genetic diversity and differentiation in a non model organism.
- Identify the effects of selection pressure at the population genetic level as an example of divergent adaptive evolution.

To investigate the population level effects of pollution and the potential for speciation as a result of selection for tolerance the following hypotheses were tested:

- Populations at contaminated sites display tolerance to copper.
- Populations at contaminated sites are genetically distinct from populations at clean sites.
- Populations at contaminated sites have lower genetic variation than populations at clean sites.
- Genetic structure corresponds with levels of contamination in a mosaic of polluted and clean habitats, rather than isolation by distance.
- Copper acts as a barrier to gene flow. Tolerance is heritable and tolerant populations persist at contaminated sites with negligible immigration from clean sites.
- Limited gene flow between tolerant and non tolerant populations is high enough to counteract the effects of genetic drift, so interpopulation differentiation is likely to be the result of selection pressure.

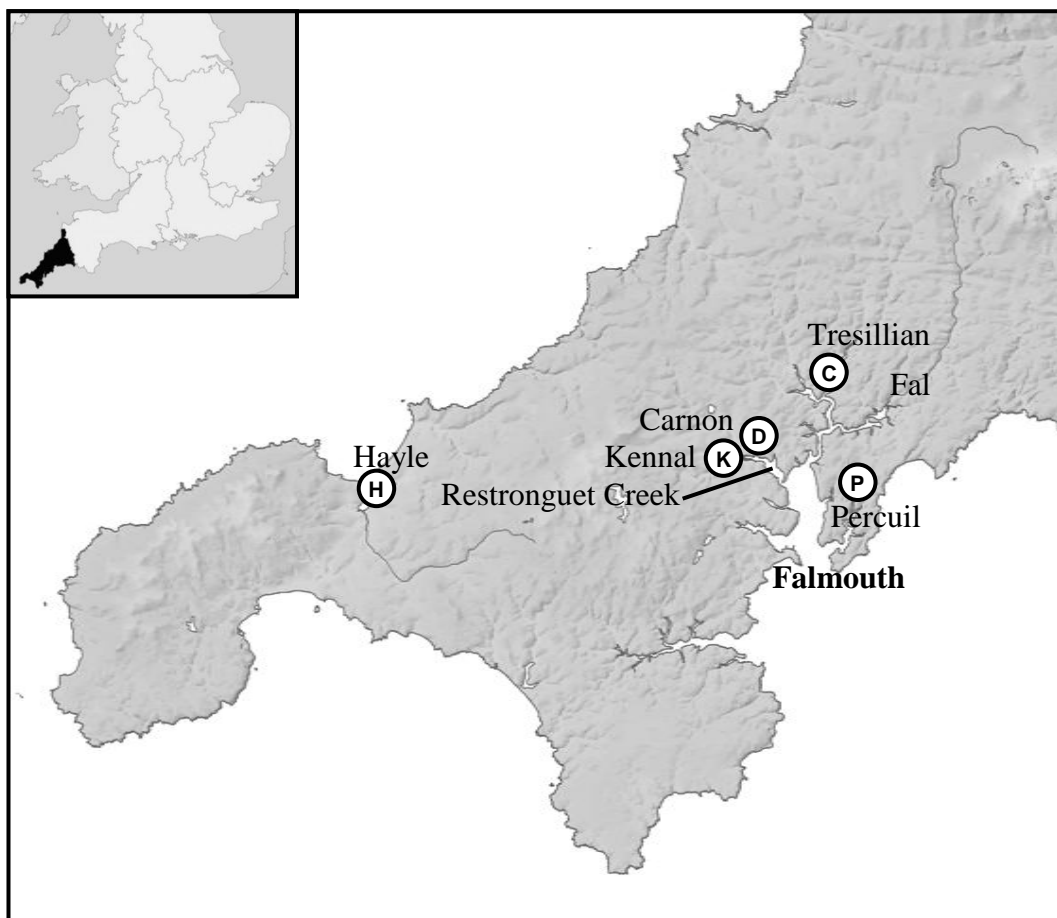


- The fact that tolerant populations have reduced fitness in clean habitats, indicative of a cost of tolerance, is demonstrated by non-tolerance loci that are fixed at clean sites and absent at contaminated sites.
- Selection for tolerant or non tolerant adaptive traits acts on a small proportion of highly differentiated loci in tolerant and non-tolerant populations, which is a signature of divergent selection.

## Chapter 2 Toxicity tests for copper tolerance in *Nereis diversicolor*

### 2.1 Introduction

Gradients of metal contamination affect the biology and ecology of the estuaries, creeks and rivers of Cornwall. This study focussed on sites in South West Cornwall, with 4 sites around Falmouth and 1 at Hayle, which have been assessed in previous research.



**Figure 2.1: Locations of study sites and rivers in Cornwall.**

Environments affected by pollution are often characterised by reduced species richness and diversity, in a community dominated by opportunistic species like *Nereis diversicolor* that are tolerant to a range of stressors (Jennings *et al.*, 2001). The effects of pollution have traditionally been assessed using community

structure indices or the presence of indicator species (Ozoh, 1992b; Begon *et al.*, 1996). Studies of the effects of environmental variables on macrobenthic and meiobenthic community structure in the Fal Estuary have shown that community composition correlates more strongly with levels of metal contamination in sediments than with other environmental factors (Sommerfield *et al.*, 1994a, 1994b; Warwick, 2001). MDS analysis of species composition in estuaries in South West England by Warwick (2001) found that all sites sampled in the Fal Estuary, including heavily contaminated parts of Restronguet Creek (sites K and D in the present study) and the uncontaminated Percuil River (site P), were more similar to each other than to either of 2 other groups, which were based on mud or sand sediment, despite differences in sediment composition within the Fal. Communities in the Fal with low diversity, dominated by opportunistic polychaetes are an indicator of pollution. However, the difference found by Warwick (2001) was largely due to the absence of the usually abundant estuarine amphipod *Corophium volutator* from the whole of the Fal, including uncontaminated areas. This is unexplained, particularly as the few *C. volutator* that have been found in the most heavily contaminated part of the Carnon River contained relatively low levels of copper, indicating regulation, plus *C. Volutator* have shown very high tolerance to copper in tests, with a 168 h LC<sub>50</sub> of 50,000 µg l<sup>-1</sup> Cu (Bryan & Gibbs, 1983). The fact that a few individuals were found high up the Carnon River, above the upper limit of *N. diversicolor*, suggests a distribution mediated by competition and predation interactions (Jensen & Andre, 1993). This shows that the biota of Restronguet Creek is not typical of estuarine environments but does not necessarily reflect the effects of metal contamination.

Increased tolerance to contaminants can be used as a biomarker of pollution (Luoma, 1977). Pollution Induced Community Tolerance (PICT) (Blanck *et al.*, 1988; Millward & Grant, 1995; Grant, 2002) has been used to map contamination. Ogilvie & Grant (2008) used PICT to determine the effects of a gradient of metal pollution on sediment microbial communities in the Fal and Hayle estuaries. They found no consistent relationship between community structure and tolerance, which implies that biodiversity indices would not be a useful indicator of pollution in this case. Tolerance was strongly correlated with levels of copper but

not with other metals, which shows that PICT is a good indicator of pollution levels and can additionally identify the particular pollutant causing a toxic effect.

Tolerant populations of a single species can also be used as biomarkers (Luoma, 1977; Grant, 2002). Tolerant strains of *Nereis diversicolor* have been widely employed as a biomonitor of metal contamination in estuaries (Bryan & Hummerstone, 1971; Bryan & Gibbs, 1983; Grant *et al.*, 1989; Hateley *et al.*, 1989; Virgilio & Abbiati, 2004a; Briggs, 2005; Virgilio *et al.*, 2005). Increased tolerance is direct evidence that a pollutant has exerted selection pressure on a population and demonstrating heritable tolerance implies an evolutionary adaptation with a genetic basis. Luoma (1977) identified four defining attributes of heritable tolerance:

- Tolerance is the result of selection for resistant genotypes. It is heritable and can not be induced.
- It involves specific physiological mechanisms for tolerance to a single toxicant or group of toxicants, rather than just selection for more vigorous phenotypes.
- The fitness of tolerant populations is reduced in clean environments.
- There is a cline of tolerance, with a positive relationship between the levels of pollution and tolerance.

Toxicity tests have been used to explore all of these points. Exposure to low concentrations of copper ( $10 - 30 \mu\text{g l}^{-1} \text{Cu}$ ) in laboratory tests has failed to induce elevated tolerance (Grant *et al.*, 1989). By growing juvenile worms in clean conditions for 4 months before toxicity tests Bryan & Gibbs (1983) showed that tolerance was not lost in the absence of copper. Grant *et al.* (1989) demonstrated heritable tolerance in toxicity tests:  $\text{LT}_{50}$  values for laboratory bred worms were 495 hours for offspring of tolerant worms from site R1 / K and 47 hours for offspring of non-tolerant worms. Small juvenile tolerant worms at contaminated sites also supports heritable tolerance (Hateley *et al.*, 1989; Briggs, 2005).

*Nereis diversicolor* has specific physiological mechanisms for tolerance to copper, involving storage as insoluble granules in membrane bound vesicles in the epidermis and nephridia (Bryan, 1976; Brown, 1982). *N. diversicolor* also exhibits

tolerance to copper, zinc and lead but the mechanism of toxicity and thus the mechanism of tolerance differs between metals (Bryan & Hummerstone, 1971, 1973; Grant *et al.*, 1989; Mouneyrac *et al.*, 2003; Geffard *et al.*, 2005). A population of *N. diversicolor* from the Gannel Estuary studied by Bryan & Hummerstone (1971) contained more than 100 times the level of lead than other populations and yet had low tolerance to copper, which illustrates the fact that different mechanisms are responsible for tolerance to different metals. *N. diversicolor* in Restronguet Creek also show tolerance to zinc, having both lower permeability to zinc and a more efficient excretory system than non-tolerant worms (Bryan & Hummerstone, 1973; Bryan & Gibbs, 1983). Heritable tolerance to zinc found by Grant *et al.* (1989) was significant but less, which suggested that tolerance mechanisms for Cu and Zn evolved separately.

Organisms exposed to a cocktail of contaminants may have genes for a range of different tolerance mechanisms, which could all have genetic markers in AFLP analysis. Sediments in Restronguet Creek contain comparable levels of copper, zinc and arsenic (Bryan, 1976), although the latter are generally less toxic to *N. diversicolor* (Bryan & Gibbs, 1983). Similar levels of zinc are found in *N. diversicolor* in all but most contaminated areas and the toxic effects of zinc are restricted to an area within 1Km of the head of the estuary (Grant *et al.*, 1989). Grant *et al.* (1989) found no significant difference in zinc tolerance between tolerant site R1 / K and mixed site R6 / D and no difference between R6 / D and the unpolluted Avon Estuary, which implies that genetic differences found between worms tolerant or non-tolerant to copper at these sites are unlikely to be confounded by markers for zinc tolerance.

There is evidence that the fitness of tolerant *N. diversicolor* is reduced in clean environments. In toxicity tests Briggs (2005) found 100% mortality for worms from relatively contaminated sites, including a site with a sediment Cu concentration of  $1399 \mu\text{g g}^{-1}$ , which indicates that tolerant worms are confined to the most contaminated areas by selection against the tolerant phenotype in cleaner areas. This may simply be a metabolic cost of the detoxification mechanism. Briggs (2005) and Pook *et al.* (2009) demonstrated a lower growth rate in tolerant worms, compared with non-tolerant worms, under clean conditions. Briggs also

found that small physiological costs and benefits of metal tolerance lead to large behavioural differences in the outcome of intra-specific aggression. When equal sized individuals were paired in clean conditions, only the non-tolerant individual survived in 75% of cases, whereas in moderately high copper concentrations a similar proportion of tolerant individuals were the survivors. This suggests that tolerant worms are excluded from areas with lower contamination through competition and predation by non-tolerant *N. diversicolor*, plus other non-tolerant polychaete species such as *Nephtys hombergii* that are only found at clean sites.

However, rather than defined tolerant and non-tolerant phenotypes, a cline of tolerance has been found, with a positive relationship between levels of pollution and tolerance. Previous toxicity tests carried out by Grant *et al.* (1989), Hateley *et al.* (1989) and Briggs (2005) have found a gradient of tolerance in *N. diversicolor*, from the head to the mouth of Restronguet Creek. In the study by Grant *et al.* (1989) tolerance near the mouth (R22) and in Mylor creek (F10) was slightly higher than for the unpolluted Avon estuary in toxicity tests with  $500 \mu\text{g l}^{-1} \text{Cu}$ , showing a fine scale difference. The level of tolerance generally correlated with levels of copper found in sediments and in *N. diversicolor* tissues.

Within Restronguet Creek worms from site R1 / K in the Kennal River have higher tolerance to copper than those found nearest to the Carnon River, which has higher levels of copper in sediments, due to differences in bioavailability and toxicity. Bioavailability and toxicity of copper are affected by environmental factors such as salinity, pH, temperature, anoxia, sediment composition and organic content (Bryan, 1976; Luoma & Bryan, 1982; Luoma, 1983; Bryan & Langston, 1992). Toxicity of copper to *N. diversicolor* increases with decreasing salinity (Ozoh, 1992a, 1994). Briggs (2005) recorded similar salinity at tolerant site R1 / K and non-tolerant site TM / P, with mean pore water salinity  $\pm 1$  s.e. of  $10.3 \pm 0.6$  at K and  $12.2 \pm 0.9$  at P, which makes them comparable, compared with higher salinity of  $26.2 \pm 0.9$  at site R6 / D further down Restronguet Creek. Because copper is more toxic at lower salinities the selection pressure for tolerance is greatest in low salinity environments higher up rivers (Bryan, 1976). Copper tolerant *N. diversicolor* in Cornwall are subjected to what are thought to be the highest concentrations of copper found in estuaries anywhere in the world.

Levels of copper in Restronguet Creek are highest at the head of the Carnon and Kennal rivers (Grant *et al.*, 1989). Dissolved copper concentrations are typically 600 to 700  $\mu\text{g l}^{-1}$  and up to 900  $\mu\text{g l}^{-1}$  in the Carnon River at the head of Restronguet Creek (Bryan & Gibbs 1983, Bryan & Langston 1992) and copper concentrations in river water of up to 450  $\mu\text{g l}^{-1}$  have been recorded in the River Hayle (Brown, 1977). However, transects in estuaries have shown that *N. diversicolor* tends to avoid the central channel with the highest fresh water influence (Bryan & Gibbs, 1983). Briggs (2005) found pore water copper concentrations  $\pm 1$  s.e. in sediments at the study sites of only 27.7  $\mu\text{g l}^{-1} \pm 9.5$  at site R1 / K, 19.0  $\mu\text{g l}^{-1} \pm 3.1$  at R6 / D and 2.3  $\mu\text{g l}^{-1} \pm 1.5$  at TM / P. Ogilvie & Grant (2008) found almost identical concentrations at these sites but a much higher pore water concentration of 463  $\mu\text{g l}^{-1}$  Cu was measured at site H in the Hayle Estuary. Levels of copper found in sediments are even higher: > 3500 ppm Cu has been recorded in Restronguet Creek sediments (Bryan & Gibbs, 1983). In acute toxicity tests Bryan & Hummerstone (1971) found that the 96h LC50 value of copper for *N. diversicolor* was 2.3 ppm (2300  $\mu\text{g l}^{-1}$ ) for tolerant worms and 0.54 ppm (504  $\mu\text{g l}^{-1}$ ) for non-tolerant worms (Bryan, 1976) so 500  $\mu\text{g l}^{-1}$  Cu was used in the present study.

The concentration of contaminant and type of toxicity test affect the results. Burlinson & Lawrence (2007) used chronic tests with stepwise increases in Cu over 90 days to reveal differences in tolerance that were not shown by acute tests. Other approaches have studied effects of copper on feeding rate (Moreira *et al.*, 2005) and burrowing behaviour (Bonnard *et al.*, 2009). Acute toxicity tests on *N. diversicolor* have mostly been static water tests without sediment (Bryan & Hummerstone, 1971; Bryan & Gibbs, 1983; Grant *et al.*, 1989; Hateley *et al.*, 1989; Briggs, 2005) but tests have been conducted with clean sand added to reduce stress (Burlinson & Lawrence, 2007). As much of the uptake of copper is through ingested sediment (Luoma & Bryan, 1982; Scaps, 2002; Wang, 2002) toxicity tests have also been carried out with contaminated sediment collected from study sites (Briggs, 2005). It is difficult to relate the methods, and thus the results, of laboratory experiments to field conditions (Burton, 1979), although toxicity tests are a simple way to establish the relative levels and distribution of tolerance to copper, in order to relate it to molecular markers.

## 2.2 Study sites

Study sites were chosen based on distributions of copper and copper tolerance in *Nereis diversicolor* determined by previous studies (Bryan & Hummerstone, 1971; Bryan & Gibbs, 1983; Grant *et al.*, 1989; Hateley *et al.*, 1989; Briggs, 2005) The Hayle estuary and the upper parts of Restronguet Creek are heavily contaminated by metals produced by historical mining activity, with levels of copper over 3500 ppm (Bryan & Gibbs, 1983). Copper levels are highest in the Carnon River and decline towards the mouth of Restronguet Creek.

Otherwise The Fal Estuary is fairly typical of temperate, macro-tidal coastal ria systems. It is a funnel shaped estuary with mud flats decreasing in width upstream. Its long length means that compared to other estuaries it has fast tidal currents relative to tidal range (Uncles *et al.*, 2002). It is likely to be hyposynchronous, where the effect of friction between the tidal water and the bed, decreases tidal range, outweighing the effect of margin convergence, which increases tidal range (Dyer, 1997). With lower tidal range further up the estuary, combined with a narrow configuration, upper parts of the estuary have a relatively narrow intertidal zone compared with the large expanses of mud flats nearer the mouth, which limits the area available for colonisation and thus acts as a constraint on population sizes.

Site		Briggs (2005)	Tolerance	OS grid reference
Hayle	H		tolerant	SW548365
Kennal	K	R1	tolerant	SW784388
Devoran	D	R6	mixed	SW803387
St Clements	C		non-tolerant	SW852438
Percuil	P	TM	non-tolerant	SW861362

**Table 2.1: Study site names and locations; predicted tolerance to copper of *Nereis diversicolor* according to previous studies (Bryan & Hummerstone, 1971; Bryan & Gibbs, 1983; Grant *et al.*, 1989; Hateley *et al.*, 1989; Briggs, 2005).**





**Figure 2.2: Maps showing locations of the 5 study sites in South West England: sites K, D, C and P around the Fal Estuary and site H at Hayle.**

The same sites have been used in previous studies by Bryan & Gibbs, 1983; Grant *et al.*, 1989; Hateley *et al.*, 1989 and Briggs, 2005 using alternative site names given in Table 2.1. Briggs (2005) found that the mean percentage organic carbon content  $\pm 1$  s.e. of  $3.60 \% \pm 0.06$  at TM / P was lower than  $4.31 \pm 0.04$  at R1 / K and  $4.52 \pm 0.01$  at R6 / D. This agrees with observed thick mud at K and D and cleaner sand at P in the present study. While Briggs (2005) measured very similar median grain size and proportion of silt at sites R1 / K, R6 / D and TM / P, in the present study site P was observed to have a much higher overall proportion of sand and fine gravel than sites K, D and C. Site H also had sandy sediment with lower organic content. Sites D and C had frequent occurrence of the clam *Scrobicularia plana* and occasional occurrence of the polychaete *Nephtys hombergii*, while site P had abundant amphipods. At sites K and H *Nereis diversicolor* was the only macro fauna found in the sediment. Sites K and P are higher up rivers than the other sites and thus have lower salinity and a narrower intertidal zone (Bryan & Gibbs, 1983; Briggs, 2005).

## 2.3 Methods

Toxicity tests were used to confirm the current distribution of copper tolerant *Nereis diversicolor* in Cornwall, assess any changes since tests carried out by Bryan & Hummerstone (1971), Bryan & Gibbs (1983), Grant *et al.* (1989) and Briggs (2005) and provide a basis for linking genotypes to copper tolerance. In addition the F1 offspring produced by a tolerant / non-tolerant cross from a breeding experiment were tested to investigate inheritance of copper tolerance.

### 2.3.1 Sample collection, transport and animal husbandry

The number of sites visited on each trip was constrained by distance between sites, tides, limiting the number of days that worms were stored for before transportation and availability of environmental cabinets to conduct toxicity tests in. Sample collection for toxicity tests avoided the breeding season in July / August / September. Following higher than expected deaths of adult females during a toxicity test in a sample collected close to the breeding season in June, a second sample was collected from site K in January. 2 samples were also collected from site D in January and October to assess temporal variation in toxicity test results and the reproducibility of bulk segregant analysis.

Adult specimens of *Nereis diversicolor*, > 3 cm in length, were collected by hand at low tide, by digging and sorting through mud at the study sites. Worms from different sites were kept separate. Initially worms were transported in sediment from the study site in large plastic bags, separated from the sediment by sieving with tap water and transferred to clean containers in the laboratory. Later worms were sieved out in Cornwall and transported in clean sand and artificial sea water (ASW) in large plastic storage boxes, which was found to be more successful and less smelly on an 8 hour car journey.

Worms were maintained in a laboratory in 30 cm x 40 cm plastic storage boxes, ~ 50 worms to a box, in 5 cm deep clean sand, covered by a further 15 cm of 17 psu artificial sea water (ASW) made with sea salt mix (TropicMarin) and distilled

water, aerated with air lines from a compressor. Boxes were kept in natural daylight at room temperature, water was changed every few weeks and worms were fed once a week with goldfish flakes (Aquarian).

For both toxicity tests and AFLP analysis experiments were carried out as soon as possible after collection to avoid possible bias due to competition and cannibalism.

### **2.3.2 Breeding experiment**

In order to investigate the distribution of copper tolerance in offspring of tolerant and non-tolerant crosses a breeding experiment was conducted to coincide with the summer breeding season, using 20 females from contaminated site K and 20 females from clean site C. Worms from these 2 sites were assumed to be tolerant and non-tolerant respectively on the basis of previous studies (Bryan, 1976; Bryan & Gibbs, 1983). ~ 1 cm of tissue was cut from the posterior end and used for DNA extraction, for AFLP analysis of population genetics. The female worms were then paired with males from the opposite site, in individual 15 cm x 22 cm plastic boxes with clean sand and ASW. Only 1 pair of worms, an F0 Female from clean site C and an F0 male from contaminated site K bred, producing around 300 offspring in August.

At 11 months old in June and July, 22 pairs of F1 worms were put in separate 15 cm x 22 cm boxes, 3 larger boxes were set up with 10 females and 1 male and the remaining F1 worms were left in 3 original large 30 cm x 40 cm culture boxes ~ 30 – 40 worms to a box. The F1 worms had gametes and the following August, at exactly 2 years old, nearly all simultaneously displayed breeding characteristics by changing colour, followed by hystolysis and death, but no F2 offspring were produced.

100 F1 offspring were used in a toxicity test at 7 months old. Prior to the toxicity test ~ 0.5 cm of tissue was cut from the posterior end of these worms and frozen at -80°C in microcentrifuge tubes for future AFLP analysis.

### 2.3.3 Toxicity tests

Tolerance to copper was measured for samples of 200 worms from each of the 5 study sites, plus 100 F1 offspring of a tolerant / non-tolerant cross, using static – renewal acute toxicity tests. DNA extraction for AFLP analysis was performed on the tails of 30 worms from Hayle that were included in a toxicity test.

All toxicity tests were conducted in 17 psu artificial sea water (ASW) made with salt mix (TropicMarin) and distilled water, containing  $500 \mu\text{g l}^{-1}$  Cu from Analar grade anhydrous copper sulphate. Copper sulphate solution was made with 1.256 g  $\text{CuSO}_4$  in 1 l sterile distilled water ( $\text{sdH}_2\text{O}$ ) and then 10 ml solution was added to a 10 l container of ASW. Tests were performed in environmental cabinets, at  $15^\circ\text{C}$ , in 12 h light / 12 h dark with 1 light tube. Uptake by the organisms can reduce the concentration of the toxin in static water toxicity tests (Ozoh, 1992a), so all water was replaced every 24 hours to maintain the test concentration of Cu.

In pilot studies with 5 – 10 worms in the same plastic box subjected to  $500 \mu\text{g l}^{-1}$  Cu there was high mortality among tolerant worms due to fighting, predation or worms aggregating and becoming entangled. The addition of lengths of plastic tubing did not fully alleviate this problem. It was therefore decided to conduct toxicity tests in individual plastic cups containing 100 ml ASW. This also allowed weight, gender, egg size, presence of parasites and time to death to be recorded and compared for individual worms.

All worms used in site toxicity tests were  $> 150 \text{ mg}$ , while tolerant / non-tolerant cross worms were  $> 50 \text{ mg}$ .  $\sim 50$  worms at a time were removed from sand by sieving and kept in plastic boxes in ASW. All healthy worms extracted were used, to avoid biased selection of individuals within a sample. Individuals were blotted dry on paper and weighed. Each worm was then placed in a clean petri dish and tissue was cut from the posterior section using clean scissors and stored at  $-80^\circ\text{C}$  in a microcentrifuge tube, in case it was required for future AFLP analysis. Tissue from both samples from site D was intended for use in bulk segregant analysis based on the results of the toxicity tests. The size of tissue sample taken was relative to the size of the worm, so that an approximately equal percentage of

tissue was removed. Coelomic fluid was examined under a binocular microscope and the presence and size of eggs and the presence of parasites was recorded.

200 worms plus 'spares' were prepared for each sample. Worms were assigned to individual plastic cups and left to recover in clean ASW for 3 - 5 days, with 1 - 2 water changes, before the test began. Any worms that died, looked sick or produced eggs during the acclimation period were replaced with spares. 5 - 10 controls per sample were kept in clean ASW. Worms were not fed during the experiments.

During the test the production of eggs by any worms was recorded. Worms were checked every 24 hours when the water was changed, death was determined by failure to respond to manual stimulation and time to death was recorded to the nearest 24 hours. Tests were continued until all worms were dead.

## 2.4 Results

Tolerance to copper was measured with toxicity tests on samples of 200 worms from each of the 5 study sites, with 2 separate samples for sites D and K to test reproducibility and investigate possible higher than expected mortality in females from site K in June; plus 100 F1 offspring of a tolerant / non-tolerant cross. Wet weight; gender, defined as female for worms with eggs and male for those without; egg size; presence of parasites and time to death to the nearest 24 hours were recorded for individual worms.

Site / sample		Number	Tolerance	Month	Swapped Parasites	
Hayle	H	200	tolerant	February	4	6
Kennal 1	K1	200	tolerant	June	9	4
Kennal 2	K2	200	tolerant	February	39	0
Devoran 1	D1	200	mixed	February	0	5
Devoran 2	D2	200	mixed	November	0	1
St Clements	C	200	non-tolerant	November	0	7
Percuil	P	200	non-tolerant	June	7	1
K x C Cross	KC	100	?	March	0	0

**Table 2.2: Site name and sample abbreviation for 8 samples, from 5 sites plus F1 offspring of a K x C cross; predicted tolerance; month in which the toxicity test was conducted; number of worms that had to be swapped due to death, sickness or production of eggs during the recovery and acclimation period and the number of worms used in tests in which parasites were observed.**

Notably 39 individuals from tolerant sample K2 had to be swapped for spares during the recovery and acclimation period because they died or looked sick. As worms from Hayle tested at the same time did not suffer the same problem this was probably due to a bacterial problem in the Kennal sample boxes, which could have affected the results for the remaining worms. Parasites, possibly the trematode *Zoogonus rubellus* (McCurdy & Moran, 2004), were observed in a few worms but with no apparent pattern of distribution. The numbers containing parasites were too low to test for associated significant difference in weight or time to death and were found at levels too low to affect population or community structure (Poulin, 1999).

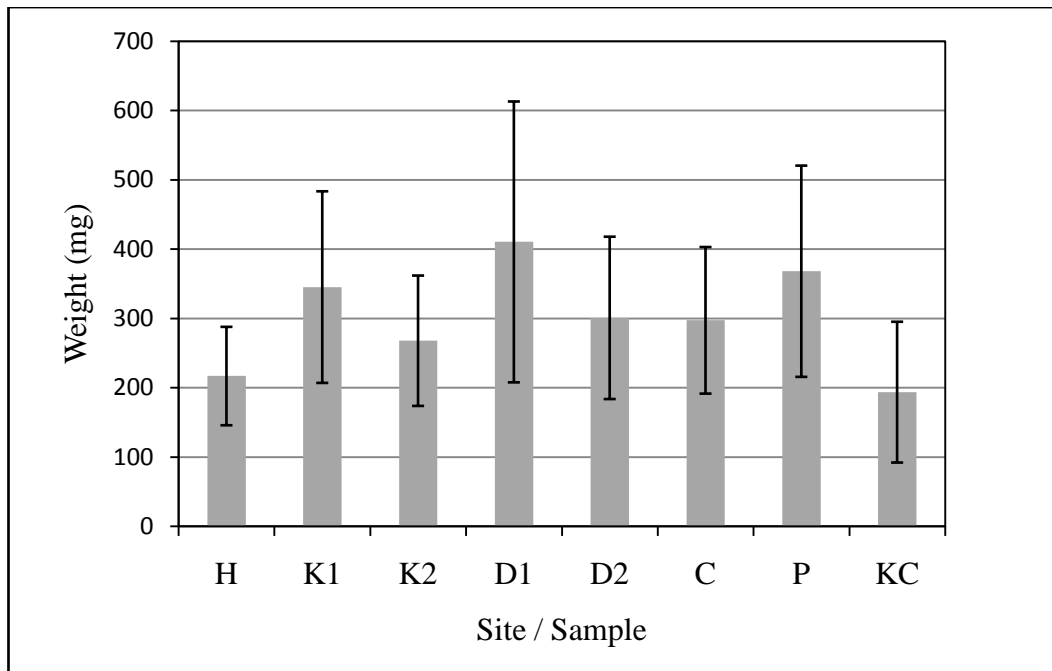
### **2.4.1 Data analysis**

The time to death and weight data for all samples resembled a poisson distribution and were not normally distributed according to a Kolmogorov-Smirnov test ( $p < 0.01$ ), plus there was a significant difference in variances (Levene's test,  $p < 0.001$ ), with variances much larger than the mean. Square root and log transformations only normalised some of the samples and failed to stabilise the variances so non-parametric Kruskal-Wallis tests were used to test for significant difference in time to death and weight between the 8 samples. Non-parametric Spearman rank correlations were used to compare weight and time to death. Z tests were used to compare mean weight or time to death between 2 samples because this test does not require a normal distribution for large samples  $n > 50$  (Fowler *et al.*, 1998). Cross tabulations were used to explore associations between nominal, categorical variables, with chi-square tests to show significant divergence from homogeneity and independence. Post hoc standardised residuals tests were performed, based on the difference between observed and expected frequencies converted to Z-scores, with a Bonferroni correction for multiple pairwise comparisons, to determine the source of any difference. Multiple regression was also used to explore relationships between variables, although the heavily skewed data was not really suitable for regression analysis despite transformation.

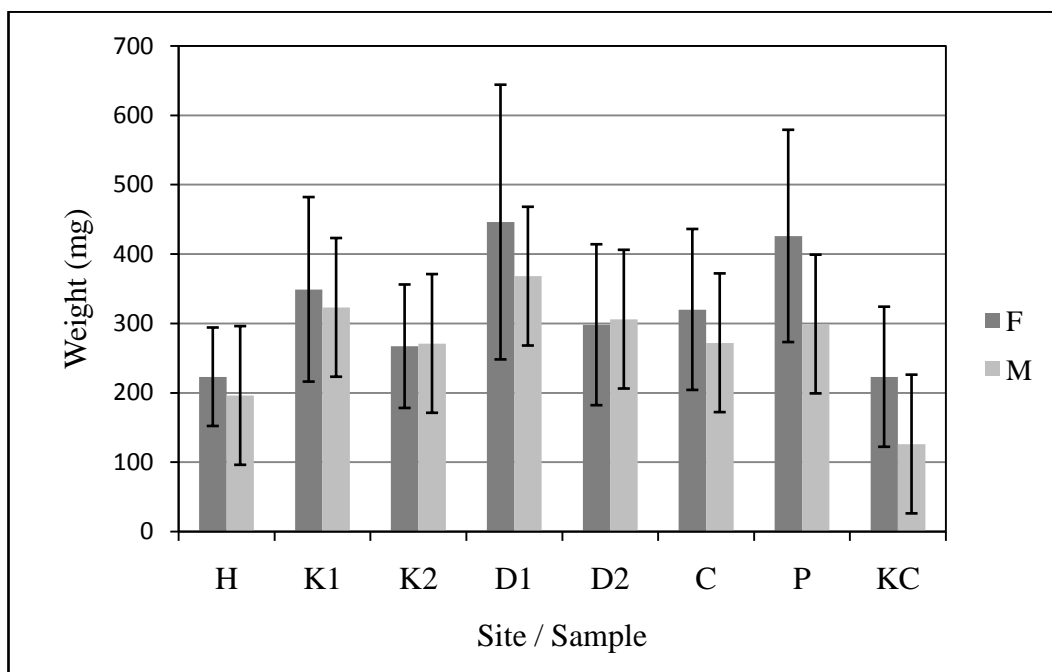
### **2.4.2 Weight**

Smaller worms are more susceptible to copper toxicity, probably due to a higher surface to volume ratio (Ozoh 1992a, 1994; Briggs, 2005). Weight was analysed to assess whether this could have affected the results of toxicity tests. Sexual size dimorphism was also investigated by comparing the weights of worms defined as female and male.





**Figure 2.3: Mean weight (mg)  $\pm$  1 standard deviation for the 8 samples of worms used in toxicity tests (Table A). n = 200 except for KC = 100.**



**Figure 2.4: Mean weight (mg)  $\pm$  1 standard deviation for worms defined as female (F) or male (M), for the 8 samples of worms used in toxicity tests (Table A). n = 200 except for KC = 100.**

There was a significant difference in weight between the 8 samples (Kruskal-Wallis test,  $H = 328$ ,  $df = 7$ ,  $p < 0.001$ ) but the samples appeared to be grouped by time of year rather than tolerance (Figure 2.3; Table 2.2). Figure 1 shows that for both tolerant sample K1 and non-tolerant sample P tested in June and Samples D2 and C tested in November the 2 samples had very similar mean weights. Worms found at site K were larger in June (K1) approaching the breeding season than in February (K2) and worms from site D were smaller in November (D2) after the breeding season than in February (D1). This could be attributable to both larger size and the extra weight of eggs in mature females.

For all 1500 worms there was a significant difference in mean weight between females and worms without eggs defined as male (Z test,  $Z = 2.162$ ,  $P < 0.001$ ). Females had a higher average weight (mean = 317 mg, s.d. = 146) than males (mean = 287 mg, s.d. = 143), which could have been biased by small, immature females grouped with males but agrees with other studies that found females to be larger (Scaps, 2002). However, figure 2 shows that within samples there was a significant difference in weight between males and females in all except for tolerant samples K1 and K2 and mixed sample D2 (Z tests and multiple t test table with a Bonferroni correction,  $p > 0.005$ ), with K2 and D2 having heavier males.

Among the female worms there was a significant difference in mean weight between females with large and small eggs (Z test,  $Z = 2.862$ ,  $P < 0.001$ ). Females with large eggs had a higher average weight (mean = 338 mg, s.d. = 159) than females with small eggs (mean = 279 mg, s.d. = 109). The 124 females that discharged eggs during the toxicity tests also had a higher average weight (mean = 416 mg, s.d. = 162) than those that did not (mean = 303 mg, s.d. = 138) (Z test,  $Z = 4.140$ ,  $P < 0.001$ ), which simply demonstrates that mature females, with large eggs weigh more. The 5 males that discharged sperm during tests also had a high mean weight (mean = 396 mg, s.d. = 160). This is expected for a semelparous species.

Overall there was a significant but very weak negative correlation between weight and time to death ( $r_s = -0.069$ ,  $p < 0.01$ ). Out of the 8 samples 4 showed a significant correlation between weight and time to death but 2 correlations were

negative, for samples K1 ( $r_s = -0.275$ ,  $p < 0.001$ ) and D2 ( $r_s = -0.275$ ,  $p < 0.001$ ), while 2 were positive, for samples H ( $r_s = 0.258$ ,  $p < 0.001$ ) and KC ( $r_s = 0.356$ ,  $p < 0.001$ ). In general weight had no consistent effect on time to death, although the weak positive correlation for samples H and KC, which had the lowest average weight (Figure 2.3), suggests that there may be an effect on smaller worms and thus a higher minimum weight threshold should be used in toxicity tests. However, scatter plots of weight against time to death (not shown) for samples K1 and D2, which had a significant negative relationship between weight and time to death, and D1, which had a lot of the largest worms, were a wedge shape, with both small and very large worms dying first and intermediate weight worms living the longest.

### 2.4.3 Sex ratio and maturity

The presence of sperm could only be clearly determined in a few individuals, so gender was recorded as presence / absence data for eggs and worms without eggs were nominally defined as males. The overall ratio of females to males across all samples was exactly 2:1 but sex ratios and egg size ratios varied between sites:

Site / sample	Month	% Female	% Large eggs
H	February	78%	73%
K1	June	85%	69%
K2	February	76%	84%
D1	February	55%	44%
D2	November	64%	57%
C	November	53%	38%
P	June	55%	58%
KC	March	71%	96%

**Table 2.3: Month in which toxicity tests were performed for each sample; percentage of worms defined as female by the presence of eggs; percentage of female worms that had eggs subjectively defined as large rather than small.**

There was a significant difference in the number of female worms between samples ( $\chi^2 = 93.94$ ,  $df = 7$ ,  $p < 0.001$ ). A post hoc standardised residuals test with a significance level of 0.05 showed that there was a significantly higher than

expected proportion of females in tolerant samples H and K1 compared to D1, D2, C and P and in K2 compared to D1, C and P. Interestingly the F1 KC cross sample had an intermediate proportion of females, which was not significantly different to tolerant or non-tolerant samples. The proportion of females in the KC sample may have been downwardly biased because the 7 month old worms, with a lower average weight than the wild caught samples, may have included immature females without visible eggs.

Egg size was subjectively recorded as small or large. Interestingly in there was an easily distinguishable difference between the 2 sizes in most cases, with very few worms having intermediate sized eggs, which indicated annual cohorts. A few worms had 2 separate sizes of eggs (recorded as large), which may indicate gametogenesis over 2 seasons with failure to completely reabsorb the first seasons eggs (Olive *et al.*, 1997).

Among the female worms there was a significant difference in the number of worms with small or large eggs between samples ( $\chi^2 = 123.12$ ,  $df = 7$ ,  $p < 0.001$ ). A post hoc standardised residuals test with a significance level of 0.05 generally showed a higher than expected proportion of females with large eggs in tolerant H and K samples and small eggs in D, C and P samples as follows:

Sample	H	K1	K2	D1	D2	C	P	KC
Egg size S	KC	KC		H K1 K2 KC	K2 KC	H K1 K2 KC	K2 KC	
L	D1 C	D1 C	D1 D2 C P					H K1 D1 D2 C P

**Table 2.4: Post hoc standardised residuals test comparisons of the proportion of females with small or large eggs. Samples in the top header row had a significantly higher than expected proportion of small or large eggs respectively, compared to the samples listed in the column below.**

The month in which the samples were tested (Table 2.3 / 2.5) does not appear to have affected this ratio. This suggests a possible relationship between proportion of mature females and tolerance, rather than time of year, although there was no significant difference between the tolerant K1 and non-tolerant P samples, both collected in June. There were very few worms with small eggs in the KC cross

sample, which could imply that the proportion of females was not downwardly biased due to misidentification of immature females. There was no relationship between the percentage of females with large eggs and the percentage of females that discharged eggs during toxicity tests.

Sample	Month	% Eggs discharged	Mean TTD		Median TTD (hours)
			(hours)	$\pm$ sd	
H	February	8%	390	245	48
K1	June	40%	303	277	48
K2	February	10%	307	360	60
D1	February	10%	192	177	48
D2	November	7%	229	185	24
C	November	0	0	0	0
P	June	8%	69	37	48
KC	March	0	0	0	0

**Table 2.5: Percentage of female worms that discharged eggs during toxicity tests; mean time at which eggs were first discharged  $\pm$  1 sd; median time to death of individual worms after eggs were first discharged.**

During the toxicity tests 124 females discharged eggs and 5 males discharged sperm. All females that discharged eggs during the toxicity tests had large eggs, which shows that egg size corresponds with maturity. Worms that discharged eggs or sperm generally died  $\sim$  48 hours after eggs were first discharged (Table 2.5), which confirms a semelparous life cycle. A few outliers that survived for hundreds of hours after discharging eggs may have been affected by removal of the tail section prior to the tests rather than normal breeding. The K1 sample tested close to the breeding season in June, in which 40% of females produced eggs during the test, actually had a higher mean and median time to death than K2, in which only 10% produced eggs (Tables 2.5 and 2.6). Overall the 129 worms that discharged eggs or sperm during the tests had a significantly higher mean time to death (mean = 362 hours, s.d. = 306) than those that did not (mean = 326 hours, s.d. = 328) (Z test,  $Z = 1.562$ ,  $p = 0.015$ ). This could mean that mature worms are less susceptible to copper because they are larger or simply that the longer worms survive, the more likely they are to produce eggs. More usefully it demonstrates that the production of eggs or sperm and subsequent death of *N.*

*diversicolor* does not downwardly bias the results of time to death toxicity tests for copper tolerance.

Despite the fact that mature females with large eggs weigh more, exploratory analysis using multiple t test tables with a Bonferroni correction or multiple regression did not reveal any strong relationships between the categorical variables and weight. There was no clear relationship between proportion of females, egg size and the mean weight of samples (Figure 2.3; Table 2.3), which suggests that the number of heavier mature females with large eggs is not the most important factor determining biomass at the study sites.

#### **2.4.4 Time to death**

Surprisingly there was fairly high mortality among the controls, with around a third of the control worms dying in the first 2 weeks of the experiments. Despite daily water changes there was noticeable algal growth and slightly cloudy water in the control containers, but not in the test containers, which suggests that the controls may have suffered from bacterial infections that were suppressed by copper in the test containers. There were also differences in behaviour: test worms everted the proboscis and wriggled in response to copper, whereas controls showed no unusual behaviour before death. Grant *et al.* (1989) disregarded data from toxicity tests unless control mortality was very low. However, as high control mortality in the present study was attributed to bacteria that were suppressed by copper in toxicity tests, conditions were considered to be different enough to ignore the control results.

In the toxicity tests there was a significant difference in median time to death between the 8 samples (Kruskal-Wallis test,  $H = 431$ ,  $df = 7$ ,  $p < 0.001$ ) (Table 2.6).

Across all samples there was a significant difference in mean time to death between females and males (Z test,  $Z = 2.880$ ,  $p < 0.001$ ). Females had a higher average time to death (mean = 361 hours, s.d. = 344) than worms defined as

males (mean = 267 hours, s.d. = 277), which was probably related to higher average weight (Figure 2.4) and therefore lower surface area to volume ratio and lower susceptibility in worms classified as females.

<b>Site / sample</b>		<b>median TTD (hours)</b>	<b>Mean TTD (hours)</b>	<b>±sd</b>
Hayle	H	240	432	406
Kennal 1	K1	456	450	501
Kennal 2	K2	264	445	403
Devoran 1	D1	216	313	269
Devoran 2	D2	192	321	264
St Clements	C	144	154	47
Percuil	P	120	126	25
K x C Cross	KC	168	256	194

**Table 2.6 : Median and mean  $\pm$  1 sd time to death for 8 samples, from 5 sites plus F1 offspring of a K x C cross (Table 2.2), exposed to 500  $\mu\text{g l}^{-1}$  Cu in toxicity tests.**

The 30 worms from tolerant site H used for AFLP analysis had a typical range of lethal times from 96 to 1080 hours, with a median time to death of 252 hours and mean  $\pm$  1 s.d. of  $376 \pm 274$ .

Multiple regression analysis used to explore relationships between time to death and other variables suffered from multicollinearity problems caused by very weak but nevertheless significant relationships between sample, weight and gender variables. Simple regression showed that difference in time to death was accounted for 0.7% by weight, 1.9 % by gender and 13.9 % by sample, increasing to 21.3% by site with replicate samples K2 and D2 and the KC cross data removed (all  $p < 0.01$ ). Population of origin therefore has by far the strongest association with levels of tolerance.

Histograms of time to death for the 8 samples clearly show the difference between tolerant, non-tolerant and intermediate sites:

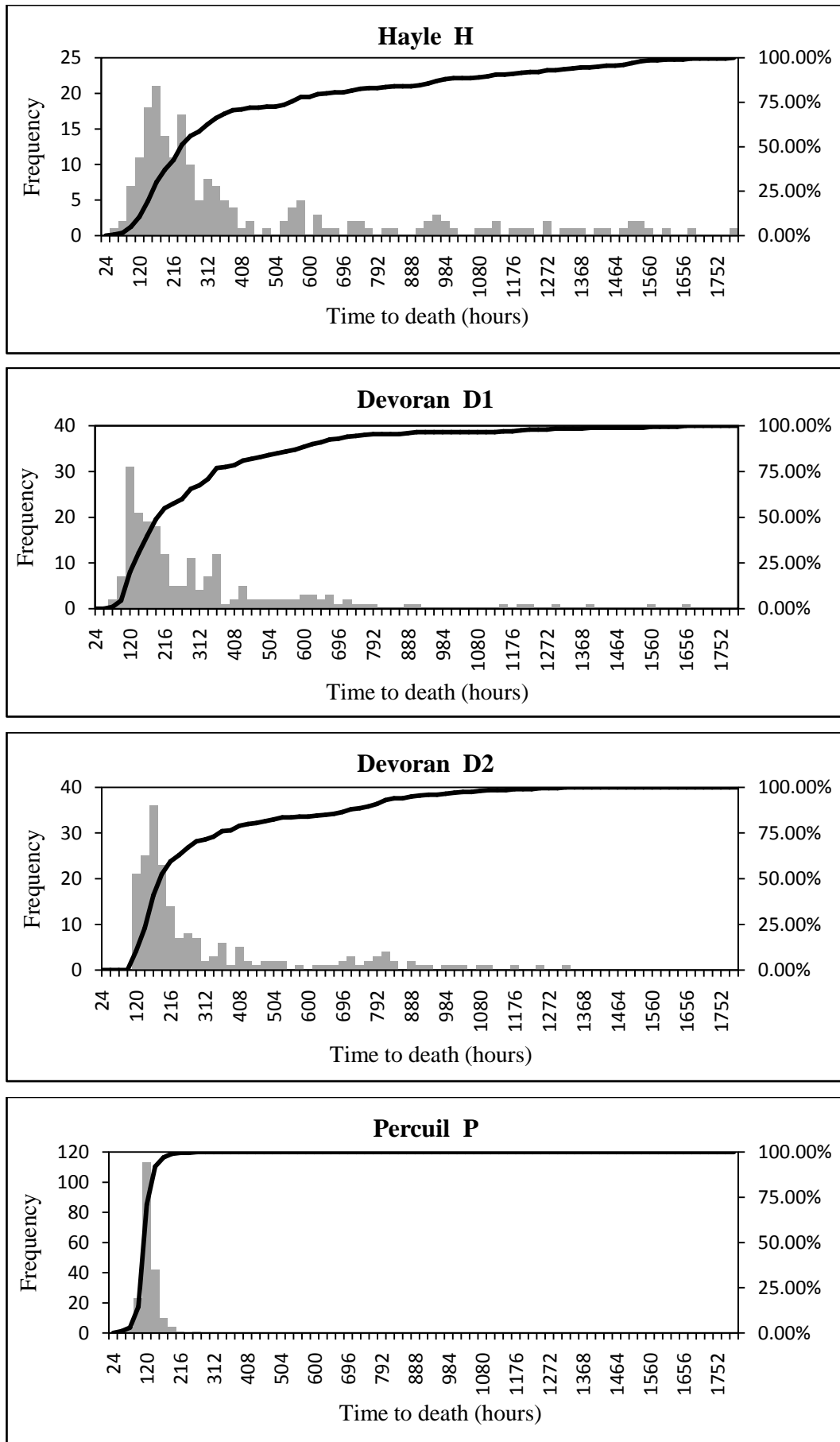


Figure 2.5: Mortality in toxicity tests for 5 sites and K x C F1 cross



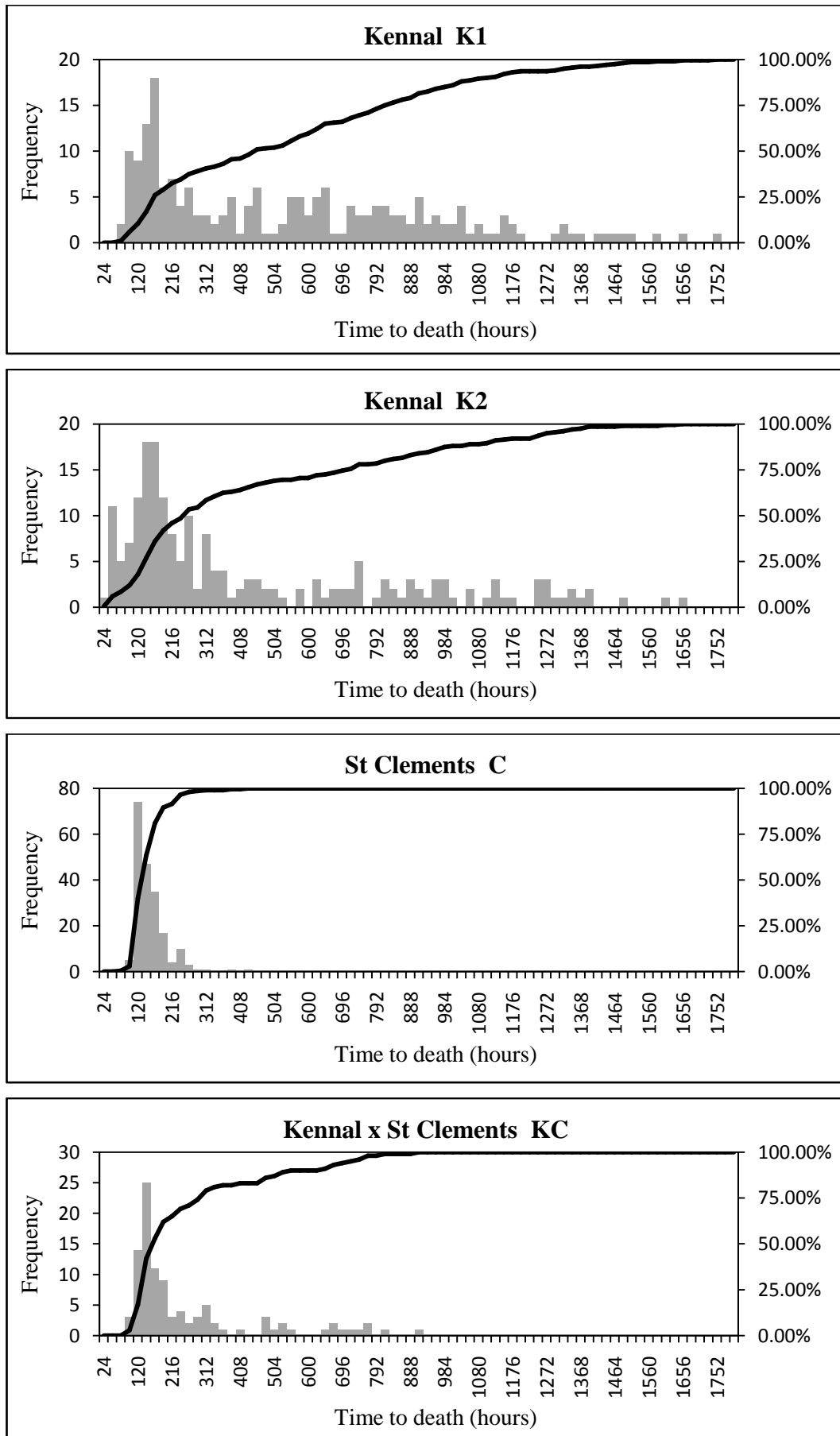
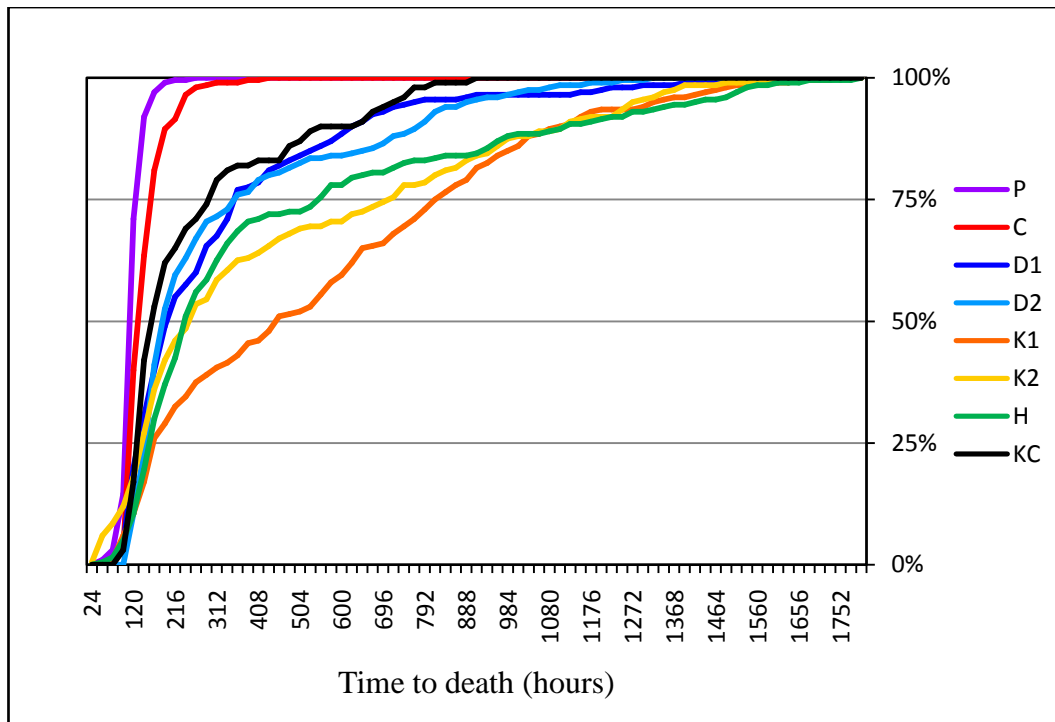


Figure 2.5: Mortality in toxicity tests for 5 sites and K x C F1 cross



**Figure 2.6: Cumulative percentage mortality in toxicity tests for 8 samples, from 5 sites plus K x C F1 cross (Table A). n = 200 except for KC = 100.**

Figures 3 and 4 show that non-tolerant worms from sites P and C nearly all died between 96 and 216 hours. Cumulative mortality at 120 hours was 40% for C and 71% for P, with 100% mortality at 432 and 264 hours respectively, whereas tolerant worms from sites K and H lived for up to 1800 hours.

Similar graphs for the 2 samples from site D shows reproducibility and temporal stability. The smooth line for sample K1, coupled with higher mean time to death in females that produced eggs during the test compared to those that didn't, shows that testing females close to the breeding season in June did not affect the results. However, the bacterial problem that necessitated the replacement of 39 worms in the acclimation phase in sample K2 may have caused the peak at 48 hours and further deaths earlier than expected in that sample.

The most striking result is the distribution of mortality for the F1 offspring of a C x K cross, with a cumulative percentage curve mid way between the curves for non-tolerant site C and tolerant site K. This curve is also very similar to those for site D, which is thought to have mixed or intermediate tolerance.

Rather than a clear distinction between non-tolerant and tolerant sites there appears to be a gradient of tolerance:  $P < C < D < H < K$ . This raises the question of whether some sites such as D have a mixture of non-tolerant and tolerant worms or whether there is a more homogeneous local level of tolerance that varies between sites. Sites with 2 distinct groups of non-tolerant and tolerant worms should have a bimodal distribution of time to death, which is apparent for sample D2 to some extent, though sample D1 has a smoother curve that suggests intermediate tolerance. Time to death for nearby site K, thought to be all tolerant (Bryan & Hummerstone, 1971; Grant *et al.*, 1989; Hateley *et al.*, 1989; Briggs, 2005) also has a skewed distribution, with higher mortality per day earlier in the test between 96 and 216 hours, so it is possible that the histograms for site D and for the C x K cross are a combination of non-tolerant and tolerant distributions. The alternative explanation is that site K has a small proportion of non-tolerant worms.

## 2.5 Discussion

The toxicity test results broadly agreed with the results of other studies on copper tolerance in *Nereis diversicolor* (Bryan & Hummerstone, 1971; Bryan & Gibbs, 1983; Grant *et al.*, 1989; Hateley *et al.*, 1989; Briggs, 2005; Burlinson & Lawrence, 2007), with non-tolerant worms from sites P and C nearly all dying before 216 hours and tolerant worms from K and H living for up to 1800 hours.

Study / Estuary / Site	Cu in Sediment (ppm)	Cu in worms (ppm dry)	Median lethal time (hours)			
			0.5 ppm	1.0 ppm	2.5 ppm	
<b>Bryan (1976)</b>						
Avon	18	20	100	100	100	
Gannel	296	116	128	100	135	
Tamar	509	397	97	93	111	
Hayle	H	712	729	490+	230	260
Restronguet	K	3500	922	490+	299	259
<b>Bryan &amp; Gibbs (1983)</b>						
Restronguet	R1	K	1733	1430	> 400	150 / 250
Restronguet	R4		>3500	630		
Restronguet	R6	D	2540	832		
Restronguet	R13		2170	932		
Restronguet	R22		1785	271		
Tresillian	F1	C	256	84		
Mylor	F10		1117	289		
Mylor	F11		-	243		
Place Cove	F15	P	31	-		
Hayle		H	728	1210		
<b>Briggs (2005)</b>						
Restronguet	R1	K	1360	754	974	
Restronguet	R4		2640	771	202	
Restronguet	R6	D	2210	483	103	
Restronguet	R13		1078	379	92	
Restronguet	R22		753	340	52	
Mylor	F10		675	191	-	
Mylor	F11		456	191	52	
Percuil	TM	P	92	26	71	

**Table 2.7: Comparable data from Bryan, 1976; Bryan & Gibbs, 1983; Briggs, 2005. Average concentration of Cu in sediment and *N. diversicolor* tissues; toxicity to *N. diversicolor* of 3 different concentrations of copper in 50 % sea water at 13-17 °C. Site references are given for original studies, followed by the present study. Comparisons with H and K for Bryan (1976) and C and P for Bryan & Gibbs (1983) only refer to the same river. All other comparisons are the same site, with OS map grid references given in Table 2.1.**

The exact results of toxicity tests on *N. diversicolor* vary between studies though. The 96 h LC<sub>50</sub> value of copper for *N. diversicolor* was found to be 2.3 ppm Cu (2300 µg l<sup>-1</sup>) for tolerant worms and 0.54 ppm (504 µg l<sup>-1</sup>) for non-tolerant worms by Bryan (1976), although the median lethal time of 260 hours given by the same authors for tolerant worms in 2500 µg l<sup>-1</sup> Cu contradicts this (Table 2.7). In the present study using 500 µg l<sup>-1</sup> Cu mortality at 96 hours for non tolerant worms was only 3 % for site C and 14.5 % for site P but this increased to 40% for C and 71% for P at 120 hours, which is fairly close to Bryan (1976).

LT<sub>50</sub> (median) time to death values calculated by Grant *et al.* (1989) were 1407 hours for tolerant site R1 / K and 70 hours for non-tolerant worms from the Avon. Similar values were found for tolerant and non-tolerant worms by Briggs (2005) (Table 2.7). However, in a study carried out in 2002 Burlinson & Lawrence (2007) determined the LT<sub>50</sub> for site R1 / K to be 258 hours in only 400 µg l<sup>-1</sup> Cu. In the present study median time to death of 456 hours for one sample from site R1 / K was similar to that found by Bryan & Hummerstone (1971) and Bryan & Gibbs (1983) but tolerant samples K2 from Restronguet and H from Hayle had lower median lethal times than previous studies of 264 and 240 hours. Samples D1 and D2 from mixed Restronguet site R6 / D and sample P from the clean Percuil River had higher median lethal times than Briggs (2005) (Tables 2.6 and 2.7). Grant *et al.* (1989) found that no worms from tolerant site R1 / K died before 600 hours in 500 µg l<sup>-1</sup> Cu, contrary to median lethal times for Bryan & Hummerstone (1971), Briggs (2005) and the present study, though mean time to death in 1000 µg l<sup>-1</sup> Cu of 100 hours found in the same study by Grant *et al.* (1989) was lower than the median time to death of 299 hours found by Bryan & Hummerstone. Mean rather than median survival times (Table 2.6) would be the best measure of central tendency if the data was more normally distributed (Grant *et al.*, 1989) and may be preferable in any case as they better reflect the range of survival times and appear to give a clearer picture of the gradient of tolerance.

The quantity of available copper could have affected the test results. Bryan & Hummerstone (1971) tested 10 worms in 500 ml of sea water and Briggs (2005) tested groups of up to 17 adult worms in 750 ml ASW. Tests with 1 worm per 100 ml in the present study effectively used twice the volume of water and copper per

worm per day, which could account for the lower lethal times in tolerant populations.

The size of worms used can affect the results. Ozoh (1992a, 1994) showed that juveniles are more sensitive to copper at 300 and 500  $\mu\text{g l}^{-1}$  Cu, under a range of different temperatures and salinities. Briggs (2005) also found that sensitivity to copper is correlated with size, with a gradient of median survival times from adult worms to juveniles, to very small juveniles. However, between these 3 size classes Briggs (2005) found no significant difference in the proportion of different sized worms dying before and after 216 hours, with adult worms from tolerant site R1 / K having a higher mortality rate early in the experiment than juveniles. Low survival time in the largest worms in samples K1, D1 and D2 in the present study could indicate higher stress caused by the small volume test conditions adversely affecting large adult worms. Bryan & Hummerstone (1971) used worms of a similar size, around 300 mg in toxicity tests, which is recommended for future studies.

It could be suggested that if samples D1 and D2 are mixed, the non-tolerant worms, with the lowest time to death, were larger due to a selective advantage. Briggs (2005) and Pook *et al.* (2009) found that tolerant *N. diversicolor* have a lower growth rate under clean laboratory conditions. Differences in weight between the 8 samples from wild populations in the present study (Figure 2.3) did not show a cost of tolerance in terms of reduced size though. Differences were most likely related to cyclical differences associated with breeding season, particularly for differences between samples from the same site. The fact that worms found at site D were smaller in November after the breeding season but of a size likely to be older than 3 months again points to a 2 year life cycle, possibly with continuous growth. Alternatively, small size at Hayle could be attributed to coarse sediment with a lower organic content, for example. A much more comprehensive sampling regime across sites and months would be required to fully investigate the reasons for differences in average weight.

It is known that pollution influences community structure but metal contamination could also affect intraspecific ecological relationships, including sex ratios and

sexual size differences observed in the present study. Fisher's principle that sexually reproducing species will tend towards a sex ratio of 1:1 as an evolutionarily stable strategy (Fisher, 1930) could be overruled by increasingly strong selection against males associated with environmental conditions (Hamilton, 1967; Maynard-Smith & Price, 1973). If copper exerts higher selection pressure on males because they are smaller, and thus particularly on smaller males, this could explain the lower proportion of males at contaminated sites and possibly the lack of significant size dimorphism, with comparatively larger males at tolerant site K. However, this seems unlikely given that there was no overall relationship between size and time to death in adult worms, plus very small juvenile worms of both sexes are found at contaminated sites and tolerate high levels of copper in toxicity tests (Briggs, 2005). Contrary to the present study Pook *et al.* (2009) found that tolerant females weighed less. Comparatively lower weight females at tolerant sites could indicate a cost of tolerance if females at contaminated sites invest less energy in egg production but Pook *et al.* (2009) showed that this is not the case, finding no difference in the energetic investment in egg production between tolerant and non-tolerant females. This could be further investigated with a comparative study of egg size and number.

The ratio of male to female *N. diversicolor* is usually biased towards females but generally varies greatly between areas (reviewed in Scaps, 2002), although differences between studies could be attributable to discrepancies in the identification of males, and a relationship with environmental conditions has been suggested (Bogucki, 1953). Copper has been shown to disrupt sexual characteristics in other invertebrates, for example causing imposex in gastropods (*e.g.* Nias *et al.*, 1993). However, there are not thought to be any adverse effects of copper on egg production in tolerant *N. diversicolor* (Ozoh, 1990) so there is no apparent direct link between copper and sex ratios. Parasites can also cause imposex, as suggested for trematodes in the dog whelk *Nucella lapillus* (Evans *et al.*, 2000), or influence sex ratio in the same way as *Wolbachia* (Wolf *et al.*, 2010) but there was no link between the proportion of females and the low numbers of worms with parasites in the present study.

It is very difficult to establish causal relationships in natural populations affected by multiple biotic and abiotic factors (Burton, 1979; Fowler *et al.*, 1998). Static water toxicity test conditions do not recreate natural conditions as copper levels in estuarine water are much lower: Briggs (2005) found pore water copper concentrations  $\pm 1$  s.e. of only  $27.7 \mu\text{g l}^{-1} \pm 9.5$  at site R1 / K,  $19.0 \mu\text{g l}^{-1} \pm 3.1$  at R6 / D and  $2.3 \mu\text{g l}^{-1} \pm 1.5$  at TM / P. Much of the exposure to copper experienced by natural populations of *N. diversicolor* is through ingested sediments (Luoma & Bryan, 1982; Scaps, 2002; Wang, 2002).

Levels of copper in *N. diversicolor* have a general trend of correspondence with levels found in sediment (Bryan & Gibbs, 1983). Much higher levels of copper have been found in the tissues of other polychaete species, including  $1210 \mu\text{g g}^{-1}$  in *Perinereis cultrifera* at relatively uncontaminated site R22 and  $2227 \mu\text{g g}^{-1}$  in *Nephtys hombergii* at intermediate site R6 / D (Bryan & Gibbs, 1983). Copper levels in *N. diversicolor* of  $271 \mu\text{g g}^{-1}$  at R22 and  $832 \mu\text{g g}^{-1}$  at R6 (Bryan & Gibbs, 1983) suggest that even non tolerant *N. diversicolor* have a higher capacity to prevent uptake or excrete copper compared with other species.

Copper is immobilised in membrane bound vesicles in the epidermis in *N. diversicolor* (Bryan, 1976). This does not fully explain the tolerance mechanism though, because worms from other estuaries have been found with fairly high levels of stored copper but low tolerance in toxicity tests (Bryan, 1976). Experiments by Bryan & Gibbs (1983) to measure the uptake of Cu showed that uptake was proportional to concentration in non-tolerant worms but tolerant worms absorbed Cu most rapidly at low concentrations, indicating an additional binding or exchange mechanism. Total uptake by tolerant worms was also much lower at higher concentrations, showing reduced permeability or excretion.

The gradient of tolerance found in the present study broadly coincides with levels of copper found in sediment and in *N. diversicolor* tissues in previous studies (Table 2.7). These studies found a gradient of tolerance from the head to the mouth of Restronguet Creek, with a general correlation between the level of copper in sediments, the level of copper accumulated in *N. diversicolor* tissues and tolerance. Grant *et al.* (1989), Hateley *et al.* (1989) and Briggs (2005) all



found that the median survival time in toxicity tests was highest for site R1 / K and decreased with decreasing concentration of copper in sediments towards the mouth of Restronguet Creek. The main exception was site R4, which was closest to the most heavily polluted Carnon River. Despite much higher levels of copper in sediment at R4, worms in the adjacent River Kennal at site R1 had higher tissue concentrations of copper and displayed far higher tolerance (Bryan & Gibbs, 1983; Briggs, 2005; Table 2.7). This reflects the fact that bioavailability is an important factor in the toxicity of copper (Luoma, 1983).

It is thought that the toxicity of copper to *N. diversicolor* increases with increasing temperature (Ozoh, 1992a, 1994) and so heat generated by the breakdown of organic matter could increase toxicity at sites with higher organic content. However, chelation by organic ions renders copper less toxic so, despite higher concentrations of Cu, the sediment at the organic rich, muddy site at Kennal may be less toxic than the sandy sediment with a low organic content at Hayle (Bryan, 1976; Brown, 1977; Bryan & Gibbs, 1983). Compared to Restronguet, levels of copper in sediments at Hayle are lower but copper levels in *N. diversicolor* are relatively high (Bryan, 1976; Bryan & Gibbs, 1983; Table 2.7).

The toxicity of copper to *N. diversicolor* increases with decreasing salinity (Bryan & Gibbs, 1983; Ozoh, 1992a, 1994). Bryan & Gibbs (1983) showed that median survival time in both tolerant and non-tolerant worms was correlated with salinity, with Copper having higher toxicity to *N. diversicolor* at lower salinities. This means that selection pressure is greater higher up the river at R1 / K with interstitial salinity of 10.3 than at site R6 / D with salinity of 26.2 (Briggs, 2005), despite higher concentrations of copper in sediments at site D. However, When Briggs (2005) exposed tolerant and non-tolerant worms to sediment from sites with a range of contamination, including R1 / K and R6 / D, at salinity 10 or 17.5, for 4 weeks, there was no clear pattern of effects. Surprisingly non-tolerant worms in 10 psu salinity actually had the highest survival rate in the most contaminated sediments. This suggests that the toxic effects of copper may be sublethal and that the distribution of tolerant worms is determined by a combination of factors.

As the metal contamination has a terrestrial source the most contaminated areas are closest to the head of the estuary. Gradients of contamination and tolerance therefore coincide with salinity gradients. Differential adaptation to low salinity in *N. diversicolor* has been proposed by Smith (1977), characterised by reproductive and developmental differences. However, the study by Grant *et al.* (1989) found a large difference in tolerance to copper between worms from clean and contaminated sites with comparable mean salinity. In the present study copper tolerant site R1 / K and non-tolerant site P were a similar distance up their respective rivers, with similar interstitial salinity (Briggs, 2005) while tolerant site H was much closer to the mouth of the Hayle estuary. This effectively rules out differential adaptation to salinity as a driver of genetic variation in this case.

Parallel evolution of tolerance to similar limiting factors has been proposed for other species, such as the intertidal winkle *Littorina saxatilis* (reviewed in Johannesson *et al.*, 2010). Limited dispersal means that similar tolerance mechanisms may have evolved separately in geographically distant populations of *N. diversicolor* subjected to the same selection pressure. It would be interesting to apply the same set of genetic markers to other metal tolerant populations, although it would be difficult to find a comparable study site with levels of copper as high as those found in Restronguet Creek.

Metal tolerant *N. diversicolor* have been found in the heavily contaminated Pialassa lagoons on the Adriatic coast of Italy. However, Virgilio & Abbiati (2004a) and Virgilio *et al.* (2005) determined the 96h LD<sub>50</sub> and 96h LD<sub>70</sub> for copper to be 340 µg l<sup>-1</sup> Cu and 480 µg l<sup>-1</sup> Cu respectively. This is lower than the 96h LD<sub>50</sub> of 504 µg l<sup>-1</sup> for non-tolerant worms according to Bryan (1976) and the used in the present study. Copper levels at Pialassa are only 11 to 280 µg g<sup>-1</sup> dw (Virgilio & Abbiati, 2004a), comparable to the non-tolerant sites in Restronguet (Table 2.7). Virgilio *et al.* (2005) found no significant difference in time to death between 3 samples from Pialassa, a site 40 Km away on the Adriatic coast and a site 2500 Km away on the Tyrrhenian coast, exposed to 480 µg l<sup>-1</sup> Cu in acute toxicity tests, which suggests that distinct phenotypes or genotypes found at Pialassa are attributable to other metals, such as mercury, in a cocktail of contaminants. Homogeneity of tolerance to other metals in Restronguet Creek

such as zinc (Bryan & Gibbs, 1983; Grant *et al.*, 1989) means that genetic differences are more likely to be related to a specific copper tolerance mechanism.

Studies spanning 30 years have demonstrated temporal stability in levels of copper in sediments and the distribution of copper tolerance *N. diversicolor* over time has generally reflected this. However, Briggs (2005) used 216 hours as a threshold to define tolerance in chi-square analysis of toxicity tests in 500 µg l<sup>-1</sup> Cu. Grant *et al.* (1989) found that no worms from tolerant site R1 / K died before 600 hours, whereas Briggs found that ~15% of worms collected in 2001 from site R1 / K died before 216 hours and would therefore be considered non-tolerant. In the present study mortality before 216 hours was higher for R1 / K, with 32.5 % and 46 % mortality for samples K1 and K2. 216 hour mortality at mixed site R6 / D was also higher in the present study, at 55 % and 59.5 % for samples D1 and D2, compared with 33 – 55 % in Briggs (2005). Mortality for site TM / P was more rapid in the study by Briggs, with 70 % of worms dead by 72 hours, compared with a large increase in mortality up to 71 % between 96 and 120 hours in the present study (Figure 2.6). This suggests that the difference was not due to any consistent methodological discrepancy. In comparison with the results of Hateley *et al.* (1989) for site R13, Briggs (2005) found sediment concentrations of copper to be 20 % lower in 2001 and median time to death of 120 hours rather than 400 hours, possibly indicating a reduction in the range of tolerant worms. Higher mortality before 216 hours and lower median time to death for site R1 / K in the present study, compared to that for worms collected in 1989 by Grant *et al.* and 2001 by Briggs could indicate a reduction in tolerance at this site and possibly the presence of some non-tolerant worms. Alternatively there may be some level of mortality of tolerant worms in tests, due to factors other than copper toxicity, which was coincidentally higher in the present study.

Moderately high concentrations of copper in sediment and tissues but low tolerance in the Tamar estuary (Table 2.7) suggests that there may be an upper threshold of copper toxicity that non-tolerant populations can withstand. Worms considered to be non-tolerant are also found in relatively high levels of copper in Restronguet Creek, indicating that tolerant worms are confined to the most contaminated areas as they are outcompeted by non-tolerant worms in cleaner

areas due to a physiological cost of tolerance (Briggs, 2005). However, the results of the present study agree with other authors that there is a gradient of tolerance, rather than clearly defined tolerant and non-tolerant phenotypes. In the present study non-tolerant site C had a slightly higher level of tolerance than site P, coinciding with levels of copper in sediment, for example. Site D could have a mixture of tolerant and non-tolerant worms or a more homogeneous intermediate level of tolerance. The tolerance mechanism could have a range and possibly escalation of different components or a single, simple mechanism with varying levels of expression and effectiveness. Both cases could be characterised by genotypic differences as the latter would mean differential regulation. In addition, a gradient of tolerance could have an associated gradient of physiological costs, with an additional range of genotypes. A genotypic survey of worms from the 5 study sites was therefore employed to elucidate the genetic basis of phenotypic responses to toxicity tests.

## Chapter 3 Amplified Fragment Length Polymorphism Analysis

### 3.1 Introduction

The study of population genetics requires variable molecular markers to provide information about individual genotypes, allele frequencies in populations and phylogenetic relationships. Amplified Fragment Length Polymorphism analysis (AFLP) (Vos *et al.*, 1995) has been used to address a range of questions in genetics with increasing popularity (reviewed in Mueller & Wolfenbarger, 1999; Bensch & Åkesson, 2005; Bonin *et al.*, 2007; Meudt & Clarke, 2007). Bensch & Åkesson (2005) defined the main areas of molecular ecology research that AFLP analysis can be employed in as genetic diversity, population structure, identification of hybrids, parentage and kinship analysis, phylogenetic reconstruction and Quantitative Trait Loci (QTLs).

#### 3.1.1 Genetic markers

Different characteristics are desirable in genetic markers, depending on the aims of the study (reviewed in Sunnucks, 2000; Bensch & Åkesson, 2005; Meudt & Clarke, 2007). Markers can be proteins, genes or non-coding DNA, with different rates of evolutionary change, levels of linkage and susceptibility to selection. A single marker for a particular gene of interest may be required or multiple loci can be used to analyse genetic diversity.

Markers are either codominant or dominant. Codominant markers can distinguish between diploid organisms that are homozygous or heterozygous by detecting both alleles, which allows direct determination of allele frequencies (Bonin *et al.*, 2007). It can be argued that haploid mitochondrial DNA sequencing products are codominant, because the markers have a range of positive states, as opposed to dominant markers, which are either present or absent (Sunnucks, 2000). Dominant markers cannot distinguish between homozygotes and heterozygotes because a band on a gel could represent a locus that is homozygous or heterozygous for the

band presence allele (Bonin *et al.*, 2007). As a result the accuracy of statistical analysis of dominant markers is reduced compared to co-dominant markers (Lynch & Milligan, 1994; Sunnucks, 2000; Kosman & Leonard, 2005; Bonin *et al.*, 2007). For dominant markers one pair of primers amplify a large number of different loci, while co-dominant marker primers amplify a single targeted product. A range of different markers can be produced using an increasing number of molecular biology methods:

### **AFLP (Amplified Fragment Length Polymorphism Analysis)**

The AFLP technique was first developed by Zabeau & Vos (1993) and Vos *et al.* (1995). Initially it was mainly used by plant biologists (e.g. Ballvora *et al.*, 1995; Thomas *et al.*, 1995) but more recently has gained increasing popularity in studies of animal populations (Bensch & Akesson, 2005). AFLP analysis has been applied to both natural populations and breeding programs of commercially important aquatic species such as shellfish, to assess genetic diversity, trace species or area of origin and identify markers associated with advantageous traits or disease resistance, (e.g. Maldini *et al.*, 2006; Sokolova *et al.*, 2006). It has been employed in conservation genetics to study the causes and effects of population size, structure, phylogeography and gene flow (reviewed in Lucchini, 2003). It has also been applied in studies of evolutionary adaptation to ecological niches, including stress tolerance, to look for signatures of differentiation associated with speciation (Wilding *et al.*, 2001; Kruse *et al.*, 2003; Campbell & Bernatchez, 2004).

AFLP is a dominant marker system. Differences in restriction fragment lengths of homologous DNA sequences are caused by Single Nucleotide Polymorphisms (SNPs), base pair insertions or deletions (INDELs) or microsatellite repeat regions, which create or remove restriction enzyme recognition sites (Wong *et al.*, 2001; Lewin, 2004). In AFLP analysis genomic DNA is digested with restriction enzymes and the resulting DNA fragments are selectively amplified by PCR, using primer sequences corresponding to the restriction site and adapter sequences ligated to the cut ends of the fragment, plus selective bases. Polymorphisms are

detected either by polyacrylamide gel electrophoresis or automated sequencing of labelled fragments.

### **RAPD (Random Amplified Polymorphic DNA)**

RAPD analysis was developed independently by 2 groups in 1990 (Welsh & McLelland 1990, Williams *et al.*, 1990). DNA fragments produced by random PCR amplification of genomic DNA between 2 identical primer sites of arbitrary nucleotide sequence (Williams *et al.*, 1990). Mutations in primer sites determine whether or not a fragment is produced and presence or absence of bands in gel electrophoresis are scored in the same way as AFLP. RAPDs are also dominant markers as it is not possible to distinguish between DNA fragments amplified from a locus that is heterozygous or homozygous for band presence.

### **Isoenzymes**

Isoenzymes (Hunter & Merkert, 1957) were one of the first molecular markers to be discovered and have been widely used in population genetics. **Isozymes** are enzymes that catalyze the same chemical reaction but are coded for by genes located at different loci. **Allozymes** are variant forms of an enzyme with different alleles at the same locus. In Isoenzyme analysis protein is extracted from a sample and separated by gel electrophoresis. The isoenzymes move at different rates, due to amino acid substitutions with different charges, and bands are identified by a staining reaction. Allozyme markers are co-dominant in that both alleles are expressed in heterozygous individuals. Scoring depends on the quaternary structure of the enzyme: monomeric enzymes have 2 bands for the different alleles for heterozygotes but enzymes that form dimers can have 3 bands. Some enzymes have multiple isozymes. These factors vary across taxa so considerable expertise in scoring band patterns is required.

### **Microsatellites**

Microsatellites, also called Simple Sequence Repeats (SSR), Short Tandem Repeats (STR) and Variable Number Tandem Repeats (VNTR), are tandem repeats of short DNA sequence motifs; typically 1 – 10 nucleotides repeated 5 – 50 times (Lewin, 2004). The number of repeats and therefore the length of the microsatellite sequence varies between individuals. New microsatellite markers

are discovered using fluorescently labelled probes for common repeat sequences, which are then cloned, sequenced and primers are developed for the conserved flanking regions. Flanking regions are used as primer sites to PCR amplify microsatellites and polymorphisms are detected by gel electrophoresis. The markers are multiallelic, with different sequence lengths constituting different alleles, highly variable and codominant because they can distinguish between homozygotes with 1 band and heterozygotes with 2 bands. Most microsatellites are dinucleotide (Li *et al.*, 2002), while microsatellites that occur in coding DNA are likely to be trinucleotide and hexanucleotide because they do not cause a frame shift (Toth *et al.*, 2000). Microsatellites have high mutation rates, tend to occur in non-coding DNA and are thought to be selectively neutral (Li *et al.*, 2002).

#### **ISSR (Inter Simple Sequence Repeat)**

ISSR markers (Zietkiewicz *et al.*, 1994) are variable genomic DNA sequences that occur between 2 known microsatellite loci, which can be used as primer sites. ISSRs are dominant markers Like AFLPs.

#### **RFLP (Restriction Fragment Length Polymorphisms)**

RFLP markers (Botstein *et al.*, 1980) are differences in restriction fragment lengths of homologous DNA sequences. RFLP analysis involves digestion of genomic DNA by restriction enzymes and separation of fragments by gel electrophoresis, which are then transferred to a membrane by Southern blotting (Southern, 1975) and labelled hybridization probes are used to detect specific target sequences. The probes are initially developed by digesting DNA, excising and cloning fragments and screening for RFLPs, which are then sequenced. These markers are multiallelic, with different fragment lengths representing different alleles, and are codominant because they can distinguish between homozygotes and heterozygotes, which have 1 or 2 marker bands respectively. ASO (Allele Specific Oligonucleotide) probes offer a simplified version with a dot blot.

#### **CAPS (Cleaved Amplified Polymorphic Sequence)**

CAPS analysis (Konieczny & Ausubel, 1993) involves PCR amplification across a particular restriction site using locus specific primers, followed by restriction enzyme digestion and gel electrophoresis.



### **SNP (Single Nucleotide Polymorphisms)**

SNPs are single base pair variations in DNA sequence at a specific locus (Lewin, 2004). SNPs can be insertions or deletions (indels), which shifts the translation frame, or substitutions. Due to degeneracy in the genetic code synonymous substitutions do not change the amino acid sequence of the protein, while nonsynonymous substitutions can cause a missense change in amino acid sequence or a nonsense stop codon. SNPs occur in coding and non-coding regions of genes and in intergenic regions.

### **Sequencing**

Sequencing of single, orthologous, nuclear DNA (nDNA), mitochondrial DNA (mtDNA) or ribosomal RNA (rRNA) genes using specific or conserved primers is widely used in phylogeny and more recently as barcodes to identify and define molecular species. Gene sequences with greater variation between than within species are also used as barcodes in molecular taxonomy to identify and delimit species (Hebert *et al.*, 2003). The rate of base substitution in the target gene should be fast enough to show difference between populations or taxa and yet slow enough to show evolutionary relationships. Mitochondrial DNA is haploid and therefore has a smaller effective population size and a faster rate of change than nuclear DNA (Lewin, 2004).

### **3.1.2 Comparison of genetic markers**

A summary of the properties of commonly employed markers is given in Table 3.1. There are some properties of genetic markers, such as low financial and time costs, ease of development and accuracy that are desirable in all markers but which may be traded in favour of characteristics appropriate to a particular type of study.

	Single locus	Codominant	PCR	N° loci available	N° loci used	Variation	Species specific	Interspecific comparisons	Reproducibility	Cost
<b>AFLP</b>	No	No	Yes	Many	Many	High	No	No	High	Low
<b>RAPD</b>	No	No	Yes	Many	Many	High	No	No	Moderate	Low
<b>Allozymes</b>	Yes	Yes	No	Moderate	Moderate	Moderate	No	No	Moderate	Moderate
<b>Microsatellites</b>	Yes	Yes	Yes	Many	Moderate	High	Yes	No	Moderate	Moderate
<b>RFLP</b>	Yes	Yes	No	Few	Few	Moderate	No	Yes	High	High
<b>DNA</b>	Yes	Yes	Yes	Few	Single	Low	No	Yes	High	Low
<b>mtDNA</b>	Yes	N/A	Yes	Few	Single	Low	No	Yes	High	Low

**Table 3.1: Summary of the properties of common genetic markers: whether or not they are single or multiple loci, codominant, PCR based; the number of loci available and the number used in studies; level of genetic variation; whether they are species specific and appropriateness of interspecific comparison; reproducibility and cost**

The choice of genetic marker is constrained by the technical difficulty and the associated financial and time demands involved in identifying, developing and using them (Table 3.1). Single Nucleotide Polymorphism (SNP) based markers involve relatively high cost and technical difficulty and require prior sequence information, so are not widely used for non-model organisms (Morin *et al.*, 2004). Isoenzymes are one of the cheapest and easiest molecular markers to develop. Compared to RFLP microsatellites offer a relatively inexpensive method of producing multiple locus specific markers but developing species specific microsatellite primers is time consuming and can be technically difficult. Once microsatellite markers have been developed a large number of related ISSR markers can then be generated for low cost and effort. While the cost per assay may be cheaper for microsatellites than for AFLP the initial development costs make the total outlay much higher.

All of the co-dominant methods require prior sequence information for particular sequences and primer sites of interest, which is generally not available for non-model organisms and involves development time and costs. The main advantage of random amplification RAPD and AFLP techniques is that they do not require any sequence information for the target organism and only require a preliminary survey to select available primer combinations (Mueller & Wolfenbarger, 1999; Bensch & Åkesson, 2005; Meudt & Clarke, 2007).

Polymerase Chain Reaction (PCR) based techniques have largely superseded processes such as RFLP. PCR (Mullis, 1983) is a technique used to replicate DNA to produce many copies of a DNA sequence from a small sample. It is a fast and relatively simple alternative to cloning to produce the large quantities of DNA required by many molecular marker methods. A DNA polymerase enzyme is used to synthesise DNA complementary to a template sequence. Nucleotides are added to the 3'-OH group of a primer, which allows amplification of a specific region of template DNA denoted by the primer sequence. PCR based methods of DNA amplification are better than allozymes and RFLP because they can be applied to relatively degraded samples, only require very small quantities of DNA, and yet can be used to selectively amplify specific regions of DNA. Schirmacher *et al.*

(1998) recommended RAPD for very small animals in preference to allozyme electrophoresis, which requires a large sample of protein.

The ability to analyse a low quality sample is an advantage. DNA based approaches are preferable to allozymes because DNA is more stable than enzymes, which allows extraction from old material and makes sample preparation, preservation and storage easier (Schirmacher *et al.*, 1998; Sunnucks, 2000). As degradation causes DNA sequence to break, the chance of successfully amplifying a fragment is proportional to its length (Frantzen *et al.*, 1998). Because AFLP amplifies shorter fragments than sequenced loci it is less susceptible to DNA degradation. However, the quality and concentration of template DNA and other reagents and the amplification cycling conditions do influence the outcome of PCR based methods. Longer primers used in sequencing mean greater accuracy and reproducibility.

Primers that are transferable between taxa have greater overall utility and cost effectiveness. Microsatellite markers are mostly species specific: motifs, mutation rate, abundance and distribution in exons, introns and intergenic regions varies widely between species (Tóth *et al.*, 2000). Cruz *et al.* (2005) found large differences in microsatellite density between closely related bivalve species, although Slate *et al.* (1998) found some bovine microsatellite loci conserved in deer and sheep. The disadvantage of species specific microsatellites is that new markers must be found and developed for each organism. This also means that they are of questionable use for delimiting species. AFLP and RAPD primers amplify DNA from any organism. However, this means that contamination by non-target organisms such as parasites, symbionts or bacteria is possible, which does not happen with species specific microsatellite markers. Because band homoplasy increases dramatically with increasing taxonomic level (Mechanda *et al.*, 2004) dominant markers are not comparable between taxa in the same way as specific markers with known homology. Data generated from specific sequences that are homologous between taxa are directly comparable and allow meta-analysis.

It is important for results to be easily interpretable and reproducible. Isoenzymes can have slightly different functions or patterns of gene expression in different tissues or developmental stages, which necessitates careful sample selection or comparison of multiple samples within a population. In PCR mismatches between the primer and the template or short fragments of degraded DNA can result in either false positive bands or a reduced amount or a lack of PCR product, giving false negative faint or absent bands. AFLP is generally claimed to have better reproducibility than RAPD (Jones *et al.*, 1997; Mueller & Wolfenberger, 1999; Nybom, 2004). The RAPD technique is reported to be more susceptible to laboratory dependent variation caused by different methods than AFLP and the results can be more difficult to interpret so it needs carefully developed laboratory protocols to be reproducible (Pérez *et al.*, 1998). As AFLP is usually claimed to be highly reproducible between runs an agreed system of recording data would allow results to be compared between different studies of the same species (Meudt & Clarke, 2007).

The most important factor affecting the choice of marker system is the level of genetic diversity and divergence required to answer the question being studied (Bensch & Åkesson, 2005; Bonin *et al.*, 2007; Meudt & Clarke, 2007). The rate of change varies between markers due to differential effects of recombination, mutation or selection. Markers with a higher rate of evolution will have greater variation and a hierarchy of rate of change and sequence diversity determines the usefulness of different markers in different areas of molecular biology. DNA that codes for proteins is much less variable than intergenic DNA and introns simply due to selection pressure to produce a functional protein (Lewin, 2004). In general protein coding regions have much lower heterozygosity per nucleotide than the intergenic sequence that makes up a high proportion of DNA. Mean heterozygosity detected by protein electrophoresis has ranged from 0.041 for mammals to 0.148 for molluscs (Nevo *et al.*, 1984). This means that methods such as RAPD and AFLP that sample DNA as a whole should find higher heterozygosity than allozymes.

Genetic markers used to infer population characteristics must have sufficient variation to detect intraspecific diversity and differentiation. Microsatellites have

high mutation rates and therefore high sequence diversity, which makes them appropriate for studying genetic diversity or similarity between individuals (Li *et al.*, 2002). Microsatellites are considered to be the most rigorous marker for parentage analysis (Sunnucks, 2000; Bensch & Åkesson, 2005). In a paternity study Gerber *et al.* (2000) found that 6 microsatellite loci performed better than 159 AFLP loci. High variability in microsatellites also allows analysis of intraspecific diversity and differentiation over short timescales and small spatial scales (Schlötterer, 2000). For populations with very low heterozygosity, that have undergone a recent bottleneck for example, markers with a high mutation rate such as microsatellites will be more informative than lower mutation rate markers such as allozymes or RFLP.

Interspecific genetic distances should be higher for RAPD and AFLP data than for allozyme data (Van De Zande & Bijlsma, 1995), although Schirmacher *et al.* (1998) found almost identical distances between 3 oligochaete species for RAPD compared to previous allozyme studies. Less diverse ISSRs are better suited to phylogeography or interspecific differentiation. ISSRs are generally less variable than microsatellites but more variable than coding DNA markers, which are used for deep phylogeny. A study of phylogenetic trees based on simulated AFLP data with different levels of divergence by García-Pereira *et al.* (2010) confirmed that the high variability of AFLP markers means that they are not suitable for reconstructing evolutionary relationships over large timescales. Single, relatively conserved sequenced loci are generally used for phylogenetic analysis. Mitochondrial DNA (mtDNA) has a higher nucleotide substitution rate than most nuclear DNA, which means that comparison of nDNA and mtDNA markers can be used to identify hybrids. mtDNA is more suitable for studying genetic divergence in closely related species or between populations of the same species (Nei & Li, 1979). However, Cytochrome C Oxidase I (COI), with the slowest rate of change of the 13 mitochondrial genes, is often used in interspecific studies. Breton *et al.* (2003) found no intraspecific sequence variation in cytochrome c oxidase I (COI) but quite high variation in cytochrome b (cyt b) for *Nereis virens* and *Nereis diversicolor*.

Molecular markers used to infer population structure due to drift or migration should be selectively neutral. Mutations in coding DNA that produce the different alleles of allozymes may be advantageous but are often selectively neutral at the phenotypic level. Microsatellites tend to occur in non-coding DNA (Li *et al.*, 2002) and are generally likely to be selectively neutral. In a study of the abundance and distribution of microsatellites in the genomes of 326 bivalve mollusc species Cruz *et al.* (2005) found they were more common in introns (245 loci/Mb) than in exons (85 loci/Mb). Occasionally microsatellites have a functional role and so can be subject to selection but this is rare (Li *et al.*, 2002), although they may behave like non-neutral markers if they are linked to a gene under selection. Microsatellites are therefore considered more appropriate than AFLP for studies of effective population size and migration (Luikart & England, 1999). However, both Microsatellite and AFLP loci can be surveyed for the effects of selection using statistical tests and any non-neutral loci excluded from the analysis if required.

As this study was interested in detecting loci under selection the AFLP marker system was chosen. Most SNPs occur in non coding sequence and so have no effect on phenotype and can not be subject to selection (Lewin, 2004). AFLP and RAPD analyses detect SNP and microsatellite differences, which mostly occur in selectively neutral non-coding DNA, but the high number of loci surveyed with these methods increases the chances of detecting coding DNA, or markers linked to it, that is subject to selection (Wong *et al.*, 2001; Mariette *et al.*, 2002; Meudt & Clarke, 2007). Using a large number of loci in AFLP also allows statistical identification of outlier loci that are likely to be subject to selection (Beaumont & Nichols, 1996).

The amount of variability in the data depends on both the variation per marker and the number of markers obtained. The number of viable markers is determined by the number of suitable loci available in the genome, of which there are many microsatellites, a moderate number of allozymes and few specific loci suitable for sequencing (Table 3.1). Using a single sequence to estimate diversity has a very high rate of sampling error. Studies using many loci have a higher information content, with statistical power proportional to the number of loci (Bonin *et al.*,

2007). However, compared with using multi-allelic, co-dominant microsatellite markers, AFLP and RAPD analyses have a lower information content due to dominance (Lynch & Milligan, 1994). This can be overcome to some extent by using more AFLP loci to increase the statistical power of genetic diversity and differentiation studies. Lynch & Milligan (1994) suggested sampling 2 to 10 times more individuals with dominant markers in order to achieve the same statistical power as with co-dominant markers and a simulation study by Mariette *et al.* (2002) found that 4 to 10 times more AFLP markers than microsatellite markers were required to accurately estimate genomic and interpopulation diversity.

In their comparison of methods applied to common research questions in molecular ecology Bensch & Åkesson (2005) somewhat subjectively considered AFLP more useful than allozymes, microsatellites, sequencing and SNPs for studies of genetic diversity, population structure, Quantitative Trait Loci (QTLs), identification of hybrids, and shallow phylogenetic reconstruction. AFLPs were only considered to be outperformed by microsatellites for parentage analyses and sequencing of specific loci for deep phylogeny. As this study was primarily interested in the effects of adaptive tolerance on genetic diversity, population structure and loci under selection the AFLP marker system was considered a good choice.



## **3.2 Methods**

### **3.2.1 General methods**

All glass, plastic and other items described as clean were washed in Decon 90, rinsed twice in distilled water and autoclaved if possible. All pipette tips and microcentrifuge tubes were autoclaved and stored in sealed boxes.

Buffers and reagents were made with sterile distilled water (sdH<sub>2</sub>O) and autoclaved if possible. Buffer recipes are given in Appendix 1.

Tissue samples were stored in microcentrifuge tubes in a -80 °C freezer. All DNA solution, enzymes, oligonucleotides, and reagents such as formamide loading dye were stored in a -20 °C freezer. Other reagents were stored according to the manufacturer's instructions.

Microcentrifuge tubes were kept on ice throughout the AFLP procedure.

Local and national safety regulations and procedures regarding the storage, use and documentation of hazardous chemicals and radioactive substances were adhered to.

### **3.2.2 DNA extraction**

DNA extraction protocols using Trizol reagent or a CTAB method failed to produce DNA of sufficient quantity or quality for AFLP analysis. Successful DNA extraction was performed using a QIAGEN DNeasy Tissue kit.

Worms were removed from sand by sieving and all female worms extracted were used, to avoid biased selection of individuals within a sample. They were kept in plastic boxes in 17 psu ASW for at least 1 hour prior to DNA extraction, which allowed sand to be expelled from the gut. Individuals were blotted dry on paper, placed in a clean petri dish and tissue was cut from the middle section using clean

scissors. Frozen samples that had been stored at  $-80^{\circ}\text{C}$  were allowed to defrost but were kept on ice while tissue was cut.

For each sample  $\sim 20$  mg of tissue was placed in a 1.5 ml microcentrifuge tube and 180  $\mu\text{l}$  lysis buffer, plus 20  $\mu\text{l}$  proteinase K were added immediately. Samples were incubated in an oven at  $55^{\circ}\text{C}$  over night and vortexed occasionally. Following tissue lysis 4  $\mu\text{l}$  of  $100\text{ mg ml}^{-1}$  RNase A was added and incubated at room temperature for 2 minutes to remove RNA, which can inhibit downstream enzymatic reactions. Samples were then vortexed for 15 seconds, 200  $\mu\text{l}$  buffer AL was added and mixed by vortexing, and incubated at  $70^{\circ}\text{C}$  for 10 minutes. 200  $\mu\text{l}$  100% ethanol was added to each sample and mixed thoroughly by vortexing. The lysate mixture was loaded into DNA binding spin columns and 2 wash steps were carried out according to the manufacturer's instructions. Elution was performed with 2 x 100  $\mu\text{l}$  of TE elution buffer (10 mM Tris-cl; 0.5 mM EDTA, pH 9) supplied with the kit.

DNA concentration and quality and removal of RNA was checked by electrophoresis on 1% agarose gels stained with  $0.5\text{ }\mu\text{g ml}^{-1}$  ethidium bromide in 0.5x TBE buffer or with a Nanodrop spectrophotometer. DNA concentration from the frozen samples was only  $\sim 5 - 50\text{ ng }\mu\text{l}^{-1}$  compared with  $\sim 80\text{ ng }\mu\text{l}^{-1}$  from fresh samples, so bulked DNA samples were concentrated using sodium acetate and ethanol.

### **3.2.3 AFLP**

AFLP involved digestion of genomic DNA with restriction enzymes, ligation of adapter sequences to the cut ends of the DNA fragments, followed by selective PCR amplification using radioactively labelled primer sequences complementary to the adapter sequence and restriction site, plus selective bases. Polymorphisms in restriction fragment length were detected by polyacrylamide gel electrophoresis and visualised by autoradiography.

### 3.2.3.1 Restriction enzyme digestion

Genomic DNA was digested using 2 different restriction enzymes: *MseI*, a frequent cutter with a 4 base recognition site and *PstI*, a rare cutter with a 6 base recognition site:

#### Recognition and restriction sites

##### ***MseI***

T↓TAA

T | T A A  
A A T | T

##### ***PstI***

CTGCA↓G

C T G C A | G  
G | A C G T C

*PstI* was chosen because it is sensitive to cytosine methylation: it is more likely to cut non-methylated, euchromatic DNA than methylated, heterochromatic DNA, which creates a bias towards coding sequences (Young *et al.*, 1999).

For each sample 10 µl 5x restriction-ligation buffer, 36 µl DNA solution, 2 µl 25 U *PstI* restriction enzyme, 2 µl 10 U *MseI* restriction enzyme (50 µl total reaction volume) were added to a 1.5 ml microcentrifuge tube and incubated in an oven at 37°C for 2 hours.

Initially the volume of DNA solution used for each sample was varied to equalise the concentration of DNA e.g. for DNA bands visualised on an agarose gel, 25 µl DNA solution and 11 µl sdH<sub>2</sub>O were used for stronger bands but for weaker bands a full 36 µl of DNA solution was used. However, this was found to make no difference to the results, probably due to the effects of PCR amplification, so subsequently 36 µl of DNA solution was used for all samples.

### 3.2.3.2 Adapter ligation

#### Adapters

##### MseI

5' GACGATGAGTCCTGAG 3'  
3' TACTCAGGACTCAT 5'

##### PstI

5' CTCGTAGACTGCGTACATGCA 3'  
3' CATCTGACGCATGT 5'

Adapters were ordered as complimentary single strand oligonucleotides. To make the adapter solution forward and reverse oligonucleotides were added to a 1.5 ml tube, using 2.5 µl of each and 95 µl sdH<sub>2</sub>O for 5 µM PstI adapter and 25 µl of each and 50 µl sdH<sub>2</sub>O for 50 µM MseI adapter. The strands were annealed by heating at 95°C for 5 minutes to denature and cooling slowly in a plastic block to renature.

For the AFLP reaction an adapter ligation master mix was made for the number of samples +1, with a 10 µl total reaction volume for each sample, containing 2 µl 5 x restriction-ligation buffer, 1 µl 5 µM PstI adapter, 1 µl 50 µM MseI adapter, 1.2µl 10 mM ATP and 5 µl 5U T4 DNA ligase added just before use. DNA ligase requires ATP to re-form phosphodiester bonds. 10 µl adapter ligation master mix was added to each restriction ligation sample and the tubes were incubated at 37°C for 3 hours.

### 3.2.3.3 PstI primer labelling

#### PstI Primer

5' GACTGCGTACATGCAG + NN 3'

Primers for the rare cutter PstI restriction site were labelled, rather than the MseI primers, to avoid too many bands being visualised. PstI primers, with 2 selective base, were labelled with a  $\gamma^{33}\text{P}$  radionucleotide.

A PstI primer labelling master mix was used. For 48 x 10 µl AFLP reactions enough labelled primer was made for 60 samples (20 µl total reaction volume), with 8 µl sdH<sub>2</sub>O, 4 µl 5x T4 polynucleotide kinase exchange buffer (supplied with PNK), 1 µl 10 U T4 polynucleotide kinase (PNK), 3 µl 200 µM PstI primer (final concentration 300 ng µl<sup>-1</sup>), 4 µl γ[<sup>33</sup>P]-ATP (= 40 µCi). The tube was incubated in a water bath at 37°C for 1 hour.

#### **3.2.3.4 MseI primers**

##### **MseI Primer**

5' GATGAGTCCTGAGTAA + NNN 3'

MseI primers, with 3 selective bases, were supplied as 100 µM stock and made up as 20 µM using 100 µl stock and 400 µl sdH<sub>2</sub>O.

#### **3.2.3.5 AFLP reaction**

An AFLP reaction master mix was made for 60 x 7 µl (420 µl total volume), with 328 µl sdH<sub>2</sub>O, 60 µl 10 x PCR buffer, 6 µl 20mM dNTPs, 20 µl γ[<sup>33</sup>P] labelled PstI primer and 6 µl Amplitaq DNA polymerase (5 U µl<sup>-1</sup>) added just before use.

For each sample 1 µl restricted – ligated template DNA solution, 2 µl of appropriate MseI primer and 7 µl AFLP master mix were added to a thermo strip PCR tube (10 µl total reaction volume).

Touchdown PCR amplification was used to avoid mismatch amplification of nonspecific products. Annealing at higher temperatures at the start ensures that only strictly complimentary sequence is amplified because it prevents mismatches from forming stable duplexes. The temperature can then be reduced for later cycles when the correct product is more abundant. PCR cycling conditions were 11 cycles of denaturing at 94°C for 30 seconds; annealing at 65°C for 30 seconds, decreasing by 0.7°C every cycle; extension at 72°C for 60 seconds; followed by

22 cycles of denaturing at 94°C for 30 seconds; annealing at 56°C for 30 seconds; extension at 72°C for 60 seconds.

A single amplification step was used, rather than pre-selective amplification with 1 selective base followed by selective amplification with up to 4 bases as used in Vos *et al.* (1995) and most other AFLP studies, because a primer + 3 bases alone gave a useful number of bands.

### **3.2.4 Polyacrylamide gel electrophoresis**

Denaturing polyacrylamide gel electrophoresis was used to separate the DNA fragments.

#### **3.2.4.1 Equipment**

0.4 mm gels were run on a Bio-Rad Sequi-Gen GT 38 x 50 cm vertical slab gel electrophoresis unit.

Glass plates, spacers and combs were cleaned thoroughly with hot water and swabbed with 70% ethanol before use. Around every 5 runs the front glass plate only was wiped with Sigmacote silicon solution and then swabbed with 70% ethanol, to prevent air bubbles forming when the gel was poured and to ensure that the gel stuck to the back plate and the front plate could be removed.

#### **3.2.4.2 Gel casting**

2 gel solutions of 100 ml and 50 ml were prepared in glass beakers. The 100 ml solution contained 50 g urea, 10 ml 10x TBE, 11.25 ml 40% acrylamide stock (4.5% acrylamide), sdH<sub>2</sub>O up to 100 ml and these amounts were halved for the 50 ml solution. Gel solutions were heated on low heat and mixed with a magnetic stirrer until the urea had dissolved. Oxygen inhibits polymerisation of acrylamide,

so gel solutions were stirred slowly and gently to avoid air bubbles. 80 µl TEMED and 800 µl 10% APS were added to each solution just before use to polymerise the gel. The 50 ml solution used for the plug at the bottom of the gel had a higher concentration of TEMED & APS so that it polymerised more quickly. The gel injection part of the apparatus was missing (and reportedly ineffective) so the 100 ml gel solution was poured between the plates and any air bubbles removed with an improvised thin plastic hook. The shark tooth comb was inserted with the flat edge facing down, to form a flat surface at the top of the gel and the gel was left to set for 1 hour.

#### **3.2.4.3 Gel running**

2 Litres of 1 x TBE running buffer was made from 10 x TBE stock, with 200 ml TBE, 1800 ml dH<sub>2</sub>O. 500 ml running buffer was poured into the base of the electrophoresis unit, the remaining 1.5 l was poured into the top reservoir, up to 1 cm below the top of the unit, and the comb was gently removed. The gel was pre run at 100 W constant power for 15 minutes to warm it up.

10 µl of formamide loading dye was added to each sample and the PCR tubes were heated to 94°C for 2 minutes in a PCR thermocycler, to denature the DNA, and then immediately put on ice. Heating, formamide in the loading dye and urea in the gel all denature double stranded DNA fragments into single strands with lower molecular weight, which allows higher resolution to 1 nucleotide.

The gel was prepared for loading by washing the buffer over the top of the gel with a plastic syringe, to remove excess urea, and then inserting the shark tooth comb. A Gilson duck billed (flattened) pipette tip was used to quickly load samples into the comb wells. Each gel had 48 lanes. The gel was run at 100 W constant power for around 1 hour 45 minutes.

The gel was then transferred to Whatman blotting paper, covered with cling film and dried on a gel dryer connected to a vacuum pump.

#### 3.2.4.4 Autoradiography

In a dark room the dried gel was placed in a light tight cassette box with a 35 x 43 cm sheet of Kodak Biomax MR scientific imaging film. After 3 days the autoradiogram film was developed in an Xograph developing machine.

#### 3.2.5 Population genetics survey

AFLP was used to carry out a genomic survey of 30 randomly selected individual worms from each of the 5 study sites. Bulk samples of DNA from all 30 individuals were also analysed to look for segregating loci and for comparison with bulk segregant analysis samples for site D. Only female worms were used to avoid the possibility of sex linked differences. 3 primer pair combinations were used. From an initial survey of 1 PstI primer paired with 25 different MseI primers, 3 MseI primers were chosen based on quantity, clarity and reproducibility of bands and polymorphisms. In addition GC triple bonds are harder to break and have more specific annealing properties than double AT bonds, and thus anneal more precisely, so primers with G and C selective bases are preferred.

<b>Primer</b>	<b>Sequence</b>
PstI	GACTGCGTACATGCAG + <b>CC</b>
MseI 42	GATGAGTCCTGAGTAA + <b>AGT</b>
MseI 69	GATGAGTCCTGAGTAA + <b>GCG</b>
MseI 71	GATGAGTCCTGAGTAA + <b>GGA</b>

**Table 3.2: Primers used in population genetics AFLP analysis**

The sampling design was robust to bias due to differences between runs because samples from different sites were run on each gel. A small number of replicates demonstrated 100 % reproducibility of bands between AFLP runs.



### 3.2.6 Bulk segregant analysis

Bulk segregant AFLP analysis (Michelmore *et al.*, 1991; Thomas *et al.*, 1995) was used to look for loci that segregated between tolerant and non-tolerant worms. Assuming mixed tolerance to copper among worms at Devoran (site D / R6) allowed a single site to be used, which avoided genetic variation due to geographic distance. Individual tolerance was determined according to the results of toxicity tests (Chapter 2) and worms were selected from the 2 extremes of tolerance. To test reproducibility and temporal consistency of genetic structure 2 replicate experiments were conducted, with samples D1 and D2 collected on 2 separate occasions in February and November 2008 and subjected to separate toxicity tests.

For each replicate sample individual DNA extractions were performed for 20 tolerant and 20 non-tolerant female worms, from frozen tissue that had been stored at -80°C. Due to low DNA concentration and purity measured using a spectrophotometer only 16 tolerant and 18 non-tolerant DNA samples were used in 1 of the replicates. To bulk the DNA samples a 100 µl restriction enzyme digestion was done with 20 µl restriction-ligation buffer, 4 µl PstI, 4 µl MseI and 4 µl of each of the 18 non-tolerant samples or 4.5 µl of each of the 16 tolerant samples = 72 µl. In the second replicate between 2.5 µl and 4.5 µl of each of the 20 individual DNA samples was used to make up 72 µl of DNA solution in a 100 µl restriction enzyme digestion, to adjust for differences in DNA concentration. AFLP was then carried out as previously described with reagent volumes adjusted.

128 primer combinations of 64 MseI primers and 2 PstI primers were tested on the 4 bulked samples, including the 3 primer pairs used in the population structure analysis.

### 3.3 Results

A graphical representation of the AFLP bands produced, assessment of the performance of the primer combinations used and a brief overview of simple differences in band frequencies between sites are presented here. A range of statistical techniques and software packages that are available for analysis of AFLP data are surveyed in the next chapter.

#### 3.3.1 Data analysis

Contingency tables were used to explore relationships between nominal data categories such as sampling site, primer combination and AFLP band polymorphism presence / absence, with chi-square tests to show significant divergence from homogeneity and independence. Post hoc standardised residuals tests, based on the difference between observed and expected frequencies converted to Z-scores were performed, with a Bonferroni correction for multiple pairwise comparisons, to determine the source of any difference.

ANOVA was used to test for a significant difference in band presence frequency between the 5 sites, with an a posteriori Tukey test to show the source of the difference. Band presence frequency data for individuals from the 5 sites was tested for normality and homogeneity of variances. One set of data for site C was not normally distributed at the 0.05 level using a Kolmogorov-Smirnov test ( $p = 0.002$ ) but the data showed no significant departures from a normal distribution according to a Shapiro-Wilk test ( $p > 0.05$ ), with the discrepancy probably attributable to a high number of individuals with the same frequencies in C. However, as ANOVA is a robust test (Underwood, 1997), and there was no significant difference in variances (Levene's test,  $p > 0.05$ ), ANOVA was considered appropriate (Fowler *et al.*, 1998).

Simple Numerical difference was used to measure distinctions between tolerant (H/K) and non-tolerant (C/P) groups rather than ratios as it avoids division by zero and takes into account the level of presence, ignoring bands such as M71

band 23 that had a ratio of 4:1 between H/K and C/P but had a low number of band occurrences across all sites.

### 3.3.2 Population genetics survey

To assess the extent of genetic variation and differentiation within and between sites in relation to areas of high or low copper contamination AFLP was used to carry out a genomic survey of 30 randomly selected individual worms from each of the 5 study sites:

H	Hayle	tolerant	30 randomly selected individuals
K	Kennal	tolerant	30 randomly selected individuals
D	Devoran	mixed	30 randomly selected individuals
C	St Clements	non-tolerant	30 randomly selected individuals
P	Percuil	non-tolerant	30 randomly selected individuals

**Table 3.3: AFLP sample name, study site, predicted tolerance and number of individuals**

### Tables 3.4 – 3.6: Polymorphic bands from 3 primer combinations

The following tables are a graphical representation of the AFLP bands. The left hand tables are band presence counts for 30 randomly selected individuals from each of the 5 sites, for the polymorphic bands produced by each of the 3 primer combinations. Shading graphically represents the number of bands and does not indicate band intensity.

The right hand tables show band presence / absence for bulked samples from the same 30 individuals from each of the 5 sites, for polymorphic bands produced by each of the 3 primer combinations. Shading indicates relative band intensity within bands and between bands subjectively classified as darker and lighter bands.

**Table 3.4: PstI 1 / MseI M42**

Band	H	K	D	C	P
8	0	1	0	0	0
9	0	0	5	1	3
10	30	24	26	29	29
11	30	28	30	30	30
12	30	30	29	30	30
13	30	30	13	10	0
14	30	29	30	29	30
17	28	30	30	29	29
19	0	4	4	4	1
25	0	0	4	4	29
26	0	0	0	1	0
27	2	2	1	2	0
28	0	0	5	22	5
30	1	6	13	2	0
31	29	29	21	8	1
33	0	0	0	19	25
34	30	30	30	7	5
35	0	1	12	22	25
36	30	30	30	29	29
37	6	14	10	18	9
39	0	0	1	6	0
40	30	30	30	29	29
41	0	0	3	8	0
42	30	30	28	30	30
44	23	24	30	29	30
46	0	2	1	4	1
49	0	0	0	1	0
50	11	4	23	27	30
51	2	2	1	10	4
52	22	14	17	6	0
57	30	30	19	6	5
58	0	1	0	0	0
59	7	6	0	0	0
62	30	25	14	13	4
63	0	0	5	17	14
65	20	22	26	23	25
67	0	10	6	0	0
70	27	27	27	28	14
78	30	30	30	6	4
79	0	0	8	24	29
87	27	30	30	23	14
89	30	30	27	23	30
90	30	30	22	20	21
91	0	0	10	24	29
95	1	1	3	3	0

Band	H	K	D	C	P
8	0	0	0	0	0
9	0	0	0	0	0
10	1	1	1	1	1
11	1	1	1	1	1
12	1	1	1	1	1
13	1	1	1	1	0
14	1	1	1	1	1
17	1	1	1	1	1
19	0	0	0	0	0
25	0	0	0	0	1
26	0	0	0	0	0
27	0	0	0	0	0
28	0	0	0	1	0
30	0	0	1	0	0
31	1	1	1	0	0
33	0	0	0	1	1
34	1	1	1	0	0
35	0	0	1	1	1
36	1	1	1	1	1
37	1	1	1	1	1
39	0	0	0	0	0
40	1	1	1	1	1
41	0	0	0	0	0
42	1	1	1	1	1
44	1	1	1	1	1
46	0	0	0	0	0
49	0	0	0	0	0
50	1	1	1	1	1
51	1	1	1	1	1
52	1	1	1	0	0
57	1	1	1	1	1
58	0	0	0	0	0
59	0	0	0	0	0
62	1	1	1	1	1
63	0	0	0	1	1
65	1	1	1	1	1
67	0	1	1	0	0
70	1	1	1	1	1
78	1	1	1	1	0
79	0	0	1	1	1
87	1	1	1	1	1
89	1	1	1	1	1
90	1	1	1	1	1
91	0	0	1	1	1
95	0	0	0	0	0

**Table 3.5: PstI 1 / MseI M69**

Band	H	K	D	C	P
1	0	0	7	24	30
2	0	0	0	24	30
4	5	9	5	16	11
5	12	9	10	13	19
6	0	0	0	1	0
7	0	0	5	24	24
8	30	30	26	6	4
10	0	0	0	2	0
12	30	30	30	30	27
13	0	0	1	0	0
14	30	30	25	6	8
15	30	30	29	30	30
19	27	19	20	25	29
21	0	0	0	0	2
22	13	28	16	6	4
23	10	7	12	19	21
31	30	30	30	25	18
32	30	30	22	1	2
34	3	0	1	1	0
38	30	30	30	7	1
39	29	28	30	30	30
41	30	30	30	29	30
42	1	0	1	0	0
50	0	3	1	2	0
51	30	30	30	27	30
54	30	30	28	16	30
55	30	30	30	21	30
57	0	0	10	24	30
58	30	30	26	6	0
59	9	13	22	28	28
60	0	0	0	1	0

Band	H	K	D	C	P
1	0	0	1	1	1
2	0	0	0	1	1
4	0	0	0	0	0
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	1	1
8	1	1	1	0	0
10	0	0	0	0	0
12	1	1	1	1	1
13	0	0	0	0	0
14	1	1	1	1	1
15	1	1	1	1	1
19	1	1	1	1	1
21	0	0	0	0	0
22	1	1	1	0	0
23	1	1	1	1	1
31	1	1	1	1	1
32	1	1	1	0	0
34	0	0	0	0	0
38	1	1	1	1	0
39	1	1	1	1	1
41	1	1	1	1	1
42	0	0	0	0	0
50	0	0	0	0	0
51	1	1	1	1	1
54	1	1	1	1	1
55	1	1	1	1	1
57	0	0	0	1	1
58	1	1	1	1	0
59	0	0	1	1	1
60	0	0	0	0	0

**Table 3.6 PstI: 1 / MseI M71**

Band	H	K	D	C	P
3	0	0	13	23	30
4	9	19	10	5	1
5	0	0	11	25	29
6	0	22	27	28	30
7	0	1	13	24	29
8	0	0	0	12	3
9	30	30	29	29	30
13	28	21	20	26	25
14	4	11	14	13	5
15	29	2	11	12	12
16	0	0	5	8	5
18	29	30	29	14	8
20	0	0	0	0	1
22	10	19	22	19	15
23	3	5	2	2	0
24	0	0	2	5	11
30	2	29	28	30	30
31	1	1	2	0	0
37	2	2	19	24	30
39	0	1	7	4	11
40	3	0	4	17	17
41	0	7	0	1	2
42	24	12	12	5	0
43	25	28	24	27	29
44	0	2	0	0	0
49	29	11	5	6	3
50	14	8	8	0	2
51	30	30	30	6	3
52	24	2	7	16	11
57	5	3	0	0	0
60	0	4	3	3	2
65	4	0	0	1	0
66	1	0	0	0	0
68	0	3	2	0	0
69	4	3	4	5	2
70	30	30	30	30	29

Band	H	K	D	C	P
3	0	0	1	1	1
4	0	0	0	0	0
5	0	0	0	1	1
6	0	1	1	1	1
7	0	0	1	1	1
8	0	0	0	0	0
9	1	1	1	1	1
13	1	1	1	1	1
14	0	1	1	1	1
15	1	1	1	1	1
16	0	0	0	0	0
18	1	1	1	1	1
20	0	0	0	0	0
22	1	1	1	1	1
23	0	0	0	0	0
24	0	0	0	0	0
30	0	1	1	1	1
31	0	0	0	0	0
37	0	0	1	1	1
39	0	0	0	0	1
40	0	0	0	1	1
41	0	0	0	0	0
42	1	1	1	0	0
43	1	1	1	1	1
44	0	0	0	0	0
49	1	1	1	1	0
50	1	1	1	0	0
51	1	1	1	0	0
52	1	1	1	1	1
57	0	0	0	0	0
60	0	0	0	0	0
65	0	0	0	0	0
66	0	0	0	0	0
68	0	0	0	0	0
69	0	0	0	0	0
70	1	1	1	1	1

Some polymorphic bands, such as M42 bands 57 and 62 (Table 3.4), have a gradient of band presence counts through sites H, K, D, C, P, while others are split between groups of sites, having a high number of bands in 2 or 3 of the 5 sites and none in the others. Out of the 112 polymorphic bands the difference between the number of bands present in individuals from tolerant sites H + K and non-tolerant sites C + P was 15 or less for 68 bands and 45 or more for 22 bands.

Graphical representation of the 112 polymorphic bands (Tables 3.4 – 3.6) appears to show more polymorphic bands that were complete (present in all 30 individuals) or absent (0 individuals) in sites H and K and more bands that were polymorphic within a site (present in 1-29 individuals) in sites D and C, though this is less apparent for primer M71 (Table 3.6).

<b>MseI Primer</b>	<b>loci</b>	<b>monomorphic</b>	<b>polymorphic</b>	<b>% polymorphic</b>	<b>single presence</b>	<b>single absence</b>
M42	105	65	45	43%	4	1
M69	64	33	31	48%	4	2
M71	78	42	36	46%	2	1
<b>all loci</b>	<b>247</b>	<b>140</b>	<b>112</b>	<b>45%</b>	<b>10</b>	<b>5</b>

**Table 3.7: AFLP results for 30 individuals from each of the 5 sites, produced by 3 primer combinations. Number of loci produced; number of bands that were monomorphic or polymorphic; percentage polymorphism; number of bands with a single presence or absence.**

The number of bands scored from each of the 3 AFLP primer combinations ranged from 64 to 105. M42 had a higher number of clearly defined bands than M69 and M71 over the same distance, particularly at the lower end of the fingerprint. Overall 45% of the scored bands were polymorphic, with little variation between the 3 primer combinations in the percentage of polymorphic bands and no significant difference in the number of polymorphic and non-polymorphic bands among the 3 primer combinations ( $\chi^2$ ,  $df = 2$ ,  $p > 0.05$ ). There were twice as many instances of a single band presence than a single absence within a band.

There was a significant difference in band presence frequency in individuals between the 5 sites (ANOVA,  $F_{4,145} = 5.55$ ,  $p < 0.001$ ). Band presence

frequency was significantly higher for K (mean = 186, s.d. = 3.11) and D (mean = 186, s.d. = 3.51) than for H (mean = 183, s.d. = 2.98) and P (mean = 183, s.d. = 2.77) (a posteriori Tukey test,  $P = 0.05$ ).

site	M42			M69			M71			Total		
	poly	loci	%	poly	loci	%	poly	loci	%	poly	loci	%
<b>H</b>	14	88	16%	9	54	17%	20	65	31%	43	207	21%
<b>K</b>	22	94	23%	8	53	15%	23	69	33%	53	216	25%
<b>D</b>	30	99	30%	19	59	32%	27	71	38%	76	229	33%
<b>C</b>	38	101	38%	25	61	41%	27	71	38%	90	233	39%
<b>P</b>	25	92	27%	14	56	25%	23	70	33%	62	218	28%

**Table 3.8: Percentage variation within sites for each of the 3 primer combinations, calculated as the percentage of bands present that were polymorphic for each site. Bands that were polymorphic across sites were therefore counted as non-polymorphic when present in all 30 individuals within a site and not counted when absent within a site.**

All sites had a lower number of bands present within the site than the total number of bands across all sites. D and C had the highest total numbers of bands present within the site out of 247 possible bands. The percentage of bands present that were polymorphic was also lower within sites than across all sites, suggesting greater variation among than within sites.

	complete bands				polymorphic bands			
	M42	M69	M71	total	M42	M69	M71	total
<b>H</b>	74	45	45	<b>164</b>	14	9	20	<b>43</b>
<b>K</b>	72	45	46	<b>163</b>	22	8	23	<b>53</b>
<b>D</b>	69	40	44	<b>153</b>	30	19	27	<b>76</b>
<b>C</b>	63	36	44	<b>143</b>	38	25	27	<b>90</b>
<b>P</b>	67	42	47	<b>156</b>	25	14	23	<b>62</b>
<b>total</b>	<b>345</b>	<b>208</b>	<b>226</b>		<b>129</b>	<b>75</b>	<b>120</b>	

**Table 3.9: Counts of complete and polymorphic bands within sites for each of the 3 primer combinations. Bands that were polymorphic across sites were counted as non-polymorphic when present in all 30 individuals within a site and not counted when absent within a site.**

There was a weak significant difference in the number of within site bands that were complete or polymorphic between the 3 primer combinations ( $\chi^2 = 6.89$ ,  $df = 2$ ,  $p < 0.05$ ). Differences in the number of complete and polymorphic bands



between sites were significant for M42 ( $\chi^2 = 12.37$ ,  $df = 4$ ,  $p < 0.05$ ) and M69 ( $\chi^2 = 13.84$ ,  $df = 4$ ,  $p < 0.01$ ) but not for M71 ( $\chi^2$ ,  $df = 4$ ,  $p > 0.05$ ).

When all 3 primer combinations were combined there was a significant difference in the number of complete and polymorphic bands between sites ( $\chi^2 = 21.13$ ,  $df = 4$ ,  $p < 0.001$ ). A post hoc standardised residuals test with a significance level of 0.05 revealed that H had a higher than expected number of complete bands compared to D and C, while K had a higher than expected number of complete bands compared to C. D had a higher than expected number of polymorphic bands compared to H, while C had a higher than expected number of polymorphic bands compared to H and K.

	complete bands				absent bands				polymorphic bands			
	M42	M69	M71	total	M42	M69	M71	total	M42	M69	M71	total
<b>H</b>	14	12	3	<b>29</b>	17	10	13	<b>40</b>	14	9	20	<b>43</b>
<b>K</b>	12	12	4	<b>28</b>	11	11	9	<b>31</b>	22	8	23	<b>53</b>
<b>D</b>	9	7	2	<b>18</b>	6	5	7	<b>18</b>	30	19	27	<b>76</b>
<b>C</b>	3	3	2	<b>8</b>	4	3	7	<b>14</b>	38	25	27	<b>90</b>
<b>P</b>	7	9	5	<b>21</b>	13	8	8	<b>29</b>	25	14	23	<b>62</b>
<b>total</b>	<b>45</b>	<b>43</b>	<b>16</b>		<b>51</b>	<b>37</b>	<b>44</b>		<b>129</b>	<b>75</b>	<b>120</b>	

**Table 3.10: Number of polymorphic bands that were complete (present in all 30 individuals), absent (0 individuals), or polymorphic (1-29 individuals) within each of the 5 sites for the 3 primer combinations.**

Further analysis of the polymorphic bands showed that there was a significant difference in the number of within site bands that were complete, absent or polymorphic between the 3 primer combinations ( $\chi^2 = 21.32$ ,  $df = 4$ ,  $p < 0.001$ ). Differences in the number of complete, absent and polymorphic bands between sites were significant for M42 ( $\chi^2 = 31.52$ ,  $df = 8$ ,  $p < 0.001$ ) and M69 ( $\chi^2 = 26.23$ ,  $df = 8$ ,  $p < 0.001$ ) but not for M71 ( $\chi^2$ ,  $df = 8$ ,  $p > 0.05$ ).

When all 3 primer combinations were combined there was a significant difference in the number of complete, absent and polymorphic bands between sites ( $\chi^2 = 51.88$ ,  $df = 8$ ,  $p < 0.001$ ). A post hoc standardised residuals test with a significance level of 0.05 revealed that H and K had a higher than expected number of complete bands, while C had a lower than expected number of

complete bands. K had a higher than expected number of absent bands compared to C and H had a higher than expected number of absent bands compared to both D and C. D had a higher than expected number of polymorphic bands compared to H and K, and C had a higher number of polymorphic bands than all of the other sites except D.

These results for only the polymorphic bands match those for chi square analysis of the number of non-polymorphic and polymorphic bands present within sites, between primers and between sites for all scored loci. This is consistent with higher diversity in sites C and D.

### 3.3.3 Bulk segregant analysis

Bulk segregant analysis was used to identify loci that segregated between tolerant and non-tolerant worms. Tolerant and non-tolerant samples from mixed site D were from the 2 extremes of time to death recorded in toxicity tests. Bulked samples of the 30 individuals from each of the 5 sites were used for comparison.

H	Hayle	tolerant	30 randomly selected individuals
K	Kennal	tolerant	30 randomly selected individuals
D	Devoran	mixed	30 randomly selected individuals
C	St Clements	non-tolerant	30 randomly selected individuals
P	Percuil	non-tolerant	30 randomly selected individuals
D1T	Devoran	tolerant	20 individuals from toxicity test 1 - bulked
D2T	Devoran	tolerant	20 individuals from toxicity test 2 - bulked
D1N	Devoran	non-tolerant	20 individuals from toxicity test 1 - bulked
D2N	Devoran	non-tolerant	20 individuals from toxicity test 2 - bulked

**Table 3.11: Samples, predicted tolerance and number of individuals used in bulk segregant analysis**

Comparison of polymorphic bands from bulked samples with those derived from AFLP analysis of individual worms demonstrated the limitations of bulked samples. In all 3 primer combinations some bands that were present in a high number of individuals were absent or faint in the bulk analysis, particularly at the

top of the fingerprint. The mean ( $\pm 1$  s.d.) within site band presence count for bands that did not show up in the bulk analysis was  $4.03 \pm 3.37$  but this does not constitute a threshold over which bands showed up. There were differences in intensity between ‘darker’ and ‘lighter’ bands throughout the bulk analyses, which did not necessarily correspond with band presence counts in individuals, for example M71 bands 9 – 18 (Table 3.6). There were also differences in intensity within bands, which in cases such as M42 band 35 did represent band presence counts in analysis of individuals. However, M42 band 62 had a noticeable difference in band intensity in the bulk analysis between site D, with a band presence count of 14, and site C, with a band presence count of 13. M71 band 52 appeared lighter for site H, with a band presence count of 24 than for sites C and P, with band presence counts of 16 and 11. In general, though, the bulk analysis did pick up the major differences where bands were split between tolerant and non-tolerant sites.

PstI	MseI	Band	H	K	D1T	D2T	D1N	D2N	C	P	D
1	M42	30	0	0	1	1	1	1	0	0	13
		35	0	0	0	0	1	1	1	1	12
		57	1	1	1	1	1	1	1	1	19
		79	0	0	1	1	1	1	1	1	8
		91	0	0	0	0	1	1	1	1	10
1	M69	1	0	0	0	0	1	1	1	1	7
		14	1	1	1	1	1	1	1	1	25
		57	0	0	0	0	1	1	1	1	10
1	M71	4	1	1	1	1	0	0	0	0	10
		7	0	0	0	0	1	1	1	1	13
		52	1	0	0	0	1	1	1	1	7

**Table 3.12: Bulk segregant analysis. Band presence / absence for 2 tolerant (D1T and D2T) and 2 non-tolerant (D1N and D2N) bulked samples, plus sites H, K, C and P bulks, for the 3 primer combinations used in AFLP analysis of individuals from the 5 sites. Shading indicates relative band intensity within bands. Individual band presence counts for 30 individuals from site D are also shown.**

For the 4 bulked samples, identified as tolerant or non-tolerant according to the results of toxicity tests, out of the 112 polymorphic bands from AFLP analysis with 3 primer combinations, 11 bands showed some level of difference between tolerant and non-tolerant bulks. There was some coincidence between band presence counts in the sample of 30 individuals and the bulked samples from D:

for higher counts bands tended to be present in all bulk samples with a difference in intensity indicating a difference in band count, whereas for the lower counts there was a clearer distinction suggesting a presence / absence split. In all cases except M71 band 52 (H) band presence in the tolerant and non-tolerant bulk samples from D agreed with band presence for the tolerant (H/K) and non-tolerant (C/P) sites.

### 3.4 Discussion

A range of criteria determine the suitability of molecular markers for population genetics studies of non model organisms, including sensitivity to genetic diversity and distance, the number of markers required for data analysis and the technical expertise, time and costs involved. In practice the AFLP technique was found to be technically demanding and suffered from unexplained failures. However, in comparison with other molecular techniques it is relatively simple, rapid and inexpensive (Mueller & Wolfenbarger, 1999; Bensch & Åkesson, 2005; Bonin *et al.*, 2007; Meudt & Clarke, 2007) and in the present study it proved to be highly reproducible, in agreement with Jones *et al.* (1997) and Nybom (2004).

The choice of molecular marker system was constrained by the fact that in genomics *Nereis diversicolor* is a non-model organism. Bleidorn *et al.* (2006) commented that the use of RAPD rather than mtDNA markers in a number of phylogeographic studies has been due to a lack of suitable primers for amplification of variable regions of the mitochondrial genome in annelid species. RAPD and AFLP markers provide a useful alternative when no prior sequence information is available.

The large number of polymorphic AFLP markers is useful for estimating genetic diversity, identifying population structure, and inferring interpopulation relationships, while mtDNA sequences are considered more reliable for phylogenetic reconstruction (Bensch & Åkesson, 2005; Reitzel *et al.*, 2007). A number of authors have recommended using a combination of AFLPs and mtDNA sequences to analyse population structure (Timmermans *et al.*, 2005; Reitzel *et al.*, 2007).

Due to differences in the level of genetic diversity detected by different marker systems the results are generally not comparable. Reitzel *et al.* (2007) stated that the results of RAPD and AFLP studies are not directly comparable, for example. However, the results of AFLP and RAPD studies are often similar. Kruse *et al.* (2003) carried out RAPD and AFLP analysis on 70 individuals from populations of the polychaete *Scoloplos armiger* 2 – 95 Km apart. 4 RAPD primers produced

116 bands, all of which were polymorphic. As in the present AFLP study, no individuals shared the same band profile, which shows that RAPD and AFLP are similar, equally sensitive markers. In the Kruse *et al.* study cluster analysis based on RAPD and AFLP loci showed the same 2 deeply diverged clades of intertidal and subtidal ecotypes but the relationships between individuals within these clades were different. AFLP analysis was based on a subset of only 22 individuals and the number of AFLP loci was not stated, although bootstrap values were much higher than for RAPD.

The main advantage of AFLP analysis is the large amount of data generated. Some AFLP studies exclude bands with a presence frequency of less than 0.05 or 0.01 from data analysis, on the basis that in a sufficiently large population virtually all loci will have more than one allele (Nei, 1973). However, this should not be applied to the relatively small sample populations in AFLP analysis. Other authors exclude bands with a frequency below 0.05 or above 0.95 from calculations of the proportion of polymorphic loci as a margin of error. For this study of 150 individuals this cut off would mean excluding bands with  $\leq 7$  presences or absences. As this threshold is arbitrary and it is unlikely that this level of scoring error occurs the proportion of polymorphic loci was calculated simply as scored.

The percentage of polymorphic AFLP bands varies between studies. This could be attributable to the choice of primers and is not necessarily a comparable measure of diversity between species. In a study of ecotypes of the intertidal winkle *Littorina saxatilis*, adapted to high and low shore conditions, Wilding *et al.* (2001) found that 290 out of 306 loci (95 %) were polymorphic, which is much higher than 45 % in the present study. Other studies have found variation in the number of loci per primer combination, for example Eco + CTC - Mse + CGA yielding 43 polymorphic bands and Eco + CAG - Mse + CGA yielding 80 in Wilding *et al.* (2001). In reviewing RAPD studies on polychaetes Bleidorn *et al.* (2006) commented on the possibility that lack of differentiation in RAPDs could be due to primer choice. This highlights the advantage of using a number of AFLP primer combinations.

The disadvantage of AFLP data is that it is considered to be dominant, as band presence is dominant to band absence. For a particular locus a band will be present for individuals that are homozygous or heterozygous for the band presence allele and absent only for individuals that are homozygous for the band absence allele. A possible way to distinguish between band presence homozygotes and heterozygotes would be to analyse progeny or haploid tissue of each individual with the dominant allele (Clark & Lanigan, 1993). This has worked for tree macrogametophytes (Isabel *et al.*, 1995; Szmidt *et al.*, 1996) but it would be very difficult to extract sufficient DNA for analysis from individual worm eggs. Breeding experiments to produce progeny of specific pairs proved difficult, with only 1 successful F1 cross and no F2 offspring.

The wide range of statistical analyses that can be applied to AFLP data despite the problems associated with dominance, including relatively recent advances in computational techniques, are assessed in the following chapter.

## Chapter 4 Statistical analysis of AFLP data

### 4.1 Introduction

The potential for meaningful statistical analysis of genetic marker data is a consideration when choosing a marker system. Analyses can be affected by the number of loci, comparability between taxa, homoplasy of comigrating bands, assumptions about underlying models and dominance (Bonin *et al.*, 2007; Meudt & Clarke, 2007). The strongest criticism of dominant markers is that dominance limits the information available for statistical analyses, which rely on assumptions and estimation as a result (Sunnucks, 2000; Kosman & Leonard, 2005). However, recent advances in both statistical and computational techniques have allowed a number of software packages to be adapted for dominant data. One of the main aims of this study was therefore a comparison of a range of statistical analysis methods commonly applied to population genetic data and assessment of their suitability for dominant AFLP markers.

In AFLP data band presence is dominant to band absence. For a particular locus a band will be present for individuals that are homozygous or heterozygous for the band presence allele and absent only for individuals that are homozygous for the band absence allele. Because AFLP markers are dominant there is no empirical information regarding the frequency of dominant and recessive alleles or the distribution of heterozygosity (Mueller & Wolfenbarger, 1999; Bensch & Åkesson, 2005; Bonin *et al.*, 2007; Meudt & Clarke, 2007). Many algorithms for calculating genetic diversity and divergence rely on a measure of allele frequency, which can only be estimated for AFLP data (Lynch & Milligan, 1994).

Approaches to the statistical analysis of AFLP profiles can be classified as band based or frequency based (Kosman & Leonard, 2005). Band based methods compare the pattern of band presence (1) and absence (0) between individuals. Allele frequency based methods involve estimates of allele frequencies at each AFLP locus, which are then used in classical population genetics methods to

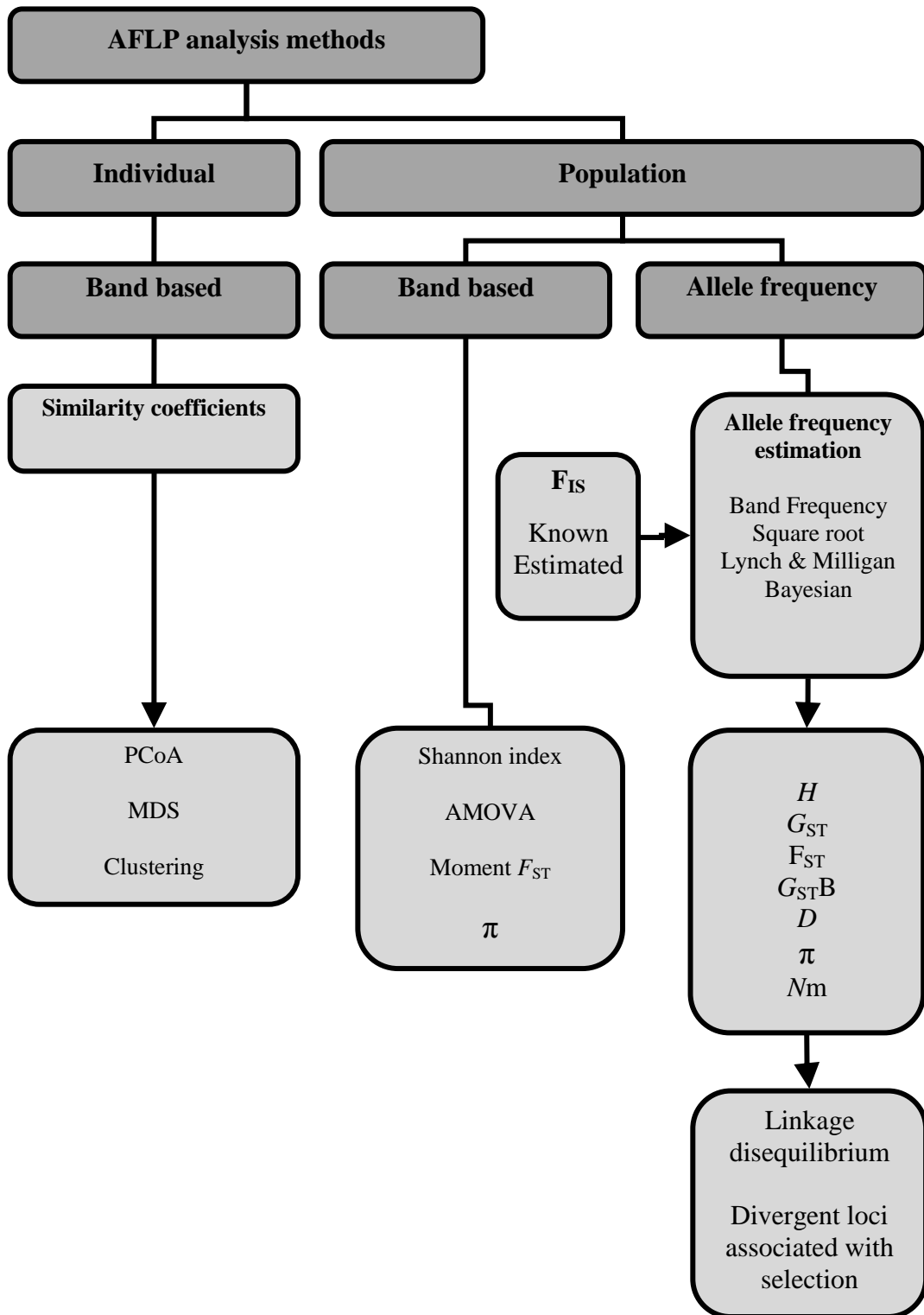


survey genetic diversity or differentiation within and between sample populations (Bonin *et al.*, 2007).

Sunnucks (2000) considered 3 levels of molecular change, which provide information at different levels of population biology, as individual genotypes, allele frequencies in populations and phylogenetic relationships. Selection pressure acting on phenotypes at the individual level has structural effects at the population level. Analysis methods can be classified as individual based, analysing differences between individuals, or population based, analysing differences per marker within and between populations (Bonin *et al.*, 2007; Meudt & Clarke, 2007). In general calculations at the individual level are band based, while calculations of diversity and distance for populations tend to be allele frequency based. The exceptions to this are the Shannon index, AMOVA, Borowsky's (2001) measure of nucleotide diversity and Hill and Weir's (2004) band based method for estimating Wright's (1931)  $F$  statistics, which all analyse band presence frequencies at the population level.

Methods have recently been developed to identify highly divergent outlier loci that may be associated with evolutionary adaptation (Beaumont & Nichols, 1996; Wilding *et al.*, 2001). This approach has been successfully employed in AFLP studies of directional or divergent selection that could lead to sympatric speciation (Wilding *et al.*, 2001; Bonin *et al.*, 2006; Savolainen *et al.*, 2006).

There is a range of mathematical measures available to assess the amount of genetic diversity and differentiation within and between individuals, populations and AFLP loci of interest (Bonin *et al.*, 2007). Some analysis methods are similar and produce similar results (*e.g.*  $F_{ST}$  vs.  $G_{ST}$ ) and they should all reveal the same general pattern of genetic diversity and structure in a data set, but it is important to look at all of them in order to understand the relationships between them and to compare results with the range of different measures used in the literature. Application of a combination of complimentary statistical analysis methods to the AFLP data for *Nereis diversicolor* provides corroboration of estimates of genetic diversity and population differentiation within the study and allows comparison with statistics given in other work.



**Figure 4.1: Summary of statistical analysis methods appropriate for binary, dominant molecular marker data**

## **4.2 Individual level analysis methods**

Individual level statistical analysis methods are all based on the pattern of AFLP band presence and absence between individuals, scored as presence (1) and absence (0).

### **4.2.1 Dissimilarity and distance measures**

Individual level, band based analysis methods are based on pairwise similarity, dissimilarity (1 – similarity) or distance between individuals. A similarity matrix can be created using correlation or covariance (which are not suitable for binary data) or one of a range of similarity coefficients and distance measures based on band presence and absence shared between individuals.

Requirements of further analysis methods should be considered when choosing a measurement. The properties of similarity and distance measures affect their suitability for use in estimates of genetic diversity for dominant molecular marker data (Bonin *et al.*, 2007). Some methods require the input data to have metric and Euclidean properties, for example (Caillez & Kuntz, 1996; Reif *et al.*, 2005). The choice of similarity coefficient or distance measure can also affect the outcome of subsequent multivariate analysis (Jackson *et al.*, 1989; Duarte *et al.*, 1999; Meyer *et al.*, 2004).

**Box 4.1: Similarity coefficients assessed for use with binary AFLP data**

		<b>Individual <i>i</i></b>	
		<b>Band presence (1)</b>	<b>Band absence (0)</b>
<b>Individual <i>j</i></b>	<b>Band presence (1)</b>	<i>a</i>	<i>b</i>
	<b>Band absence (0)</b>	<i>c</i>	<i>d</i>

where  $n = a + b + c + d$

**Jaccard (1908)**

$$\frac{a}{a + b + c}$$

**Czekanowski (1913) / Dice (1945) / Sørensen (1948)**

$$\frac{2a}{2a + b + c}$$

**Ochiai (1957)**

$$\frac{a}{\sqrt{(a + b)(a + c)}}$$

**Sokal and Sneath (1963)**

$$\frac{a}{a + 2b + 2c}$$

**Russell and Rao (1940)**

$$\frac{a}{a + b + c + d}$$

**Simple matching (Sokal & Michener, 1958)**

$$\frac{a + d}{a + b + c + d}$$

**Rogers and Tanimoto (1960)**

$$\frac{a + d}{a + 2b + 2c + d}$$

The Jaccard similarity coefficient measures the number of shared band presences between 2 individuals as a proportion of the total number of bands present in at least 1 of the 2 individuals. It ignores shared band absences and therefore avoids the problem of band absence homoplasy where a shared band absence is due to different mutations (Vekemans *et al.*, 2002). The Czekanowski / Dice / Sørensen coefficient gives more weight to bands present in both individuals and therefore to similarity between individuals. Other coefficients are reducible to simpler coefficients for binary data: for example Gower equates to Jaccard, while Bray-Curtis similarity (Bray & Curtis, 1957), designed for counts of species in ecology, and Nei and Li (1979) genetic dissimilarity are the same as the Czekanowski / Dice / Sørensen coefficient when applied to binary data. Rogers distance, appropriate for allelic informative co-dominant molecular markers, is equivalent to the simple matching coefficient for AFLP data, but only when applied to inbred populations (Reif *et al.*, 2005). The simple-matching coefficient includes double band absences and gives them the same weight as double band presences, which may not be appropriate for cases of frequent band absence homoplasy. However, this coefficient maximizes the amount of information obtained from AFLP profiles. In the Russell and Rao coefficient shared band absence is only included in the denominator and so contributes to dissimilarity. None of these coefficients address the issue of band presence homoplasy due to comigrating bands (Koopman & Gort, 2004).

There are many other, slightly different permutations: Shi (1993) lists the formulae and properties of 39 similarity coefficients that can be applied to binary data. Similarity coefficients are more suitable for binary AFLP data if they are metric. Distance ( $d$ ) between pairs of points ( $i,j,k$ ) is considered to be metric if:

- 1)  $d(i,j) \geq 0$  (non-negativity)
- 2)  $d(i,j) = 0$  if and only if  $i = j$  (identity of indiscernibles)
- 2)  $d(i,j) = d(j,i)$  (symmetry)
- 3)  $d(i,k) \leq d(i,j) + d(j,k)$  (triangle inequality)

All of the similarity coefficients explored in the present study (Box 4.1) are non-negative and have symmetry by including both b and c, which avoids under or

over estimating similarity. All except Russell and Rao have a scale from 0 to 1, where identity ( $b = c = 0$ ) equals 1. This allows conversion of similarity ( $S$ ) to dissimilarity ( $1 - S$ ), which is required by many multivariate methods. The Russell and Rao coefficient has been described as metric (Jackson *et al.*, 1989; Shi, 1993), following Gower & Legendre's (1986) statement that it is metric on a scale of 0 to 1. However, it does not conform to the identity of indiscernibles because when  $b = c = 0$ , similarity is  $a/a+d$  rather than 1. Triangle inequality is desirable because there is much less distortion when a similarity matrix is converted to a dissimilarity matrix if the matrices have the same geometric properties (Shi, 1993). The Czekanowski / Dice / Sørensen and Ochiai coefficients are not metric in this respect.

Euclidean distance is the straight line distance between 2 points positioned on a plane, relative to fixed coordinate axes. For 2 points  $i = (i_1, i_2, \dots, i_n)$  and  $j = (j_1, j_2, \dots, j_n)$  in  $n$ -dimensional Euclidean space, the distance from  $i$  to  $j$  is given by:

$$d(i, j) = \sqrt{(i_1 - j_1)^2 + (i_2 - j_2)^2 \dots + (i_n - j_n)^2}$$

$$\sqrt{\sum_{i=1}^n (i_i - j_i)^2}$$

For binary presence / absence data as in box 4.1 this equates to:

$$\sqrt{b+c}$$

For AFLP data  $i$  and  $j$  are the band presence / absence profiles of 2 individual worms and  $n$  is the number of bands. Euclidean distance ranges from 0 to  $\sqrt{2n}$  and is therefore likely to increase with increasing number of bands, which means that this distance measure can not be compared between studies based on different numbers of loci. Euclidean distance itself is not considered to be an appropriate distance measure for data with zero values and when applied to binary data it does not consider shared band presence, so it is not a suitable measure of dissimilarity for AFLP data.

A Euclidean distance measure is required by some multivariate analysis techniques, such as metric MDS, hierarchical cluster analysis and Analysis of MOlecular VAriance (AMOVA) (Excoffier *et al.*, 1992). For  $n > 3$  dimensional Euclidean space any dissimilarity measure is Euclidean if the resulting distances between all pairs of points in a set of points (dissimilarity matrix) are Euclidean. Detailed definitions and explanations of the mathematical properties of Euclidean dissimilarity measures are given in Gower & Legendre (1986) and Caillez and Kuntz (1996). For the similarity coefficients in Box 4.1 dissimilarity is not Euclidean. However, for all of these coefficients the square root of the dissimilarity is both metric and Euclidean (Gower and Legendre, 1986), so they can be used in multivariate analyses that require Euclidean properties with this transformation.

A number of empirical studies comparing similarity coefficients have produced differing results and concluded that the outcome depends on the relative frequency of presences (1) and absences (0) in the data set (e.g. Gower & Legendre, 1986). In a review of AFLP statistical analysis approaches Bonin *et al.* (2007) recommended testing for correlation between similarity coefficients as per Duarte *et al.* (1999). Weak correlation between band presence based coefficients and those that include shared band absence could indicate frequent band absence homoplasy, in which case a band presence based coefficient should be used.

Dissimilarity coefficients can be calculated for each pair of individuals within a sample and simply averaged to give a basic measure of within population genetic diversity, which can be compared between groups. A pairwise similarity or dissimilarity matrix can also be used in ordination and cluster analysis to define groups and represent similarity within and between groups. The choice of similarity or distance measure affects the outcome of clustering and ordination techniques (Jackson *et al.*, 1989; Duarte *et al.*, 1999; Meyer *et al.*, 2004). In practice a range of similarity indices have been used in ordination and clustering to analyse AFLP data, with very few authors stating why they have chosen a particular measure, which could cause inconsistencies in analysis and comparison of results (Jackson *et al.*, 1989; Duarte *et al.*, 1999; Kosman & Leonard, 2005).

#### 4.2.2 Multi Dimensional Scaling ordination analysis (MDS)

Ordination analysis can be used to create a 2 or 3 dimensional map of multi-dimensional data relationships, with distances between points on ordination plots representing dissimilarities between individuals. There are several ordination methods available to represent multivariate data. Of these both Principal Coordinates Analysis (PCoA) and Principal Component Analysis (PCA) are criticised because they lose distance information when multi-dimensional information is reduced to a 2 dimensional plane (Gower, 1966). In addition PCA was designed for use with Euclidean distance measures and although it has been argued that non-Euclidean similarity measures also produce acceptable results in PCA, it is considered to be the least suitable multivariate technique for binary data (ter Braak, 1985; Jackson *et al.*, 1989). Multiple Correspondence Analysis is appropriate for presence / absence data (ter Braak, 1985) and has been successfully applied in a number of AFLP studies (e.g. Sanchez *et al.*, 1999). However, it is based on a chi-squared distance calculation, which prevents the use of other distance measures, plus it has been criticised for the complexity of both the concept and the algorithm, which limits its implementation and availability in software. MDS and cluster analysis are suitable for AFLP data because they do not require the data to be normally distributed. A further advantage of using MDS and cluster analysis together is that the same dissimilarity matrix is analysed directly in both cases, which creates consistency between the analyses.

In Multi Dimensional Scaling ordination analysis (MDS) the distances between the points on a plot are compared to the original dissimilarities between data points using regression. The goodness of fit of the regression is calculated to give a stress value and the points are rearranged to reduce stress. This process is repeated iteratively until the lowest stress value is achieved and the plot best represents the data. Metric MDS uses parametric linear or curvilinear regression models while non-metric MDS uses non-parametric, monotonic regression. The regression model used to define the relationship between dissimilarity and distance, the stress value calculation method and the distortion algorithm all affect the outcome of MDS, and a range of different methods are available, so it is important to choose one that is appropriate for binary AFLP data.

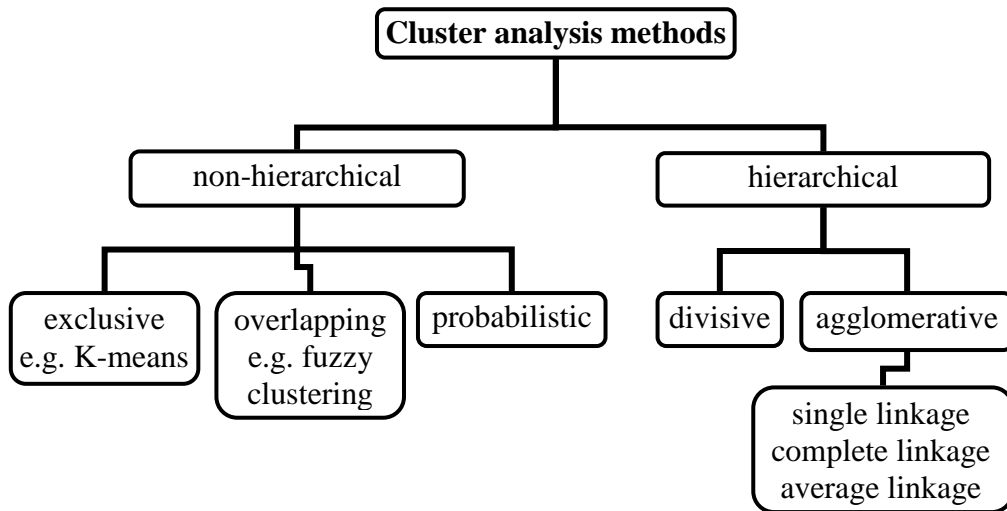


Non-metric MDS is a more appropriate multivariate ordination technique for AFLP data because the underlying model does not have strict linear requirements and allows the use of a dissimilarity matrix created using non-metric similarity coefficients. In non-metric MDS the distances between the points on the plot have the same rank order as the corresponding dissimilarities between individuals (Kruskal, 1964). MDS allows distortion of the 2D plot to best represent rank order distance, which is more appropriate for binary data than PCoA, which uses rotation of the points to best represent variance. Additionally, the scaling feature of MDS reduces the size effects of frequency of band presence for shared presence based similarity coefficients (Jackson *et al.*, 1989). The outcome of MDS is therefore affected by both the measure used for the input dissimilarity matrix and the scaling algorithm itself.

A lot of authors use MDS for AFLP data analysis without stating why a particular similarity coefficient or MDS algorithm was chosen. Different coefficients and different algorithms included in software produce different results, which affects comparisons of MDS between studies. Given that there have been a number of empirical comparisons of the effect of similarity coefficients on clustering methods (Jackson *et al.*, 1989; Johns *et al.*, 1997; Duarte *et al.*, 1999; Meyer *et al.*, 2004) but no equivalent investigations regarding MDS, the present study includes a comparison of similarity coefficients employed in different MDS methods.

### **4.2.3 Cluster analysis**

Cluster analysis classifies individuals into discrete groups. It is affected by both the choice of similarity coefficient used to construct the dissimilarity matrix (Box 4.1) and the clustering algorithm used (Figure 4.2). Cluster analysis methods can be non-hierarchical, where each individual is assigned to a cluster in multi dimensional space, or hierarchical, where the relationships between individuals are shown as a dendrogram (Sneath & Sokal, 1973).



**Figure 4.2: Summary of cluster analysis methods**

K means clustering is a partitional clustering method, which involves choosing a number of clusters,  $K$ , and iteratively assigning individuals to the cluster with the closest centroid. Fuzzy clustering is similar but individuals have a degree of belonging to different clusters. These two techniques are affected by the input number of clusters, produce different results for different runs depending on cluster initialisation and measure variance, so are not recommended for dissimilarity coefficients of binary data. Probabilistic methods attempt to fit the data to a model and therefore depend on the availability of a suitable model.

Hierarchical cluster analysis can be divisive (top down), where the sample is divided into successively smaller clusters, or agglomerative (bottom up), where each individual is assigned to a cluster and the clusters are joined to form successively larger clusters. Hierarchical, agglomerative clustering techniques use different definitions of distance between two clusters. Single linkage is the minimum distance between any one member of each cluster, whereas complete linkage is the maximum distance between any one member of each cluster. Single linkage tends to allow ‘chaining’ whereas complete linkage produces a larger number of evenly sized clusters (Shi, 1993). Average linkage, commonly referred to as Unweighted Pair Group Method with Arithmetic mean (UPGMA) is the mean distance between all members of each cluster. Hierarchical, agglomerative

clustering with UPGMA linkage is considered to be the most appropriate method for binary data and is the most widely used clustering method for AFLP data.

Jackson *et al.* (1989) identified some problems associated with cluster analysis as false clusters found in closely related data; clusters of outliers; tied or similar values producing multiple different dendrograms; size effects, where cluster initiation is based on high presence frequency, and associated chaining, where individuals with lower presence frequency are added to these clusters. Similarity coefficients that emphasise shared similarity such as Jaccard, Czekanowski / Dice / Sørensen and Russel and Rao could encourage cluster formation to be initiated among individuals with the highest band presence frequency, whereas coefficients that include shared band absence, such as simple matching and Rogers and Tanimoto coefficients would allow cluster initiation for high or low band frequencies. However, the study by Jackson *et al.* was based on ecological data for presence / absence of fish species, with a wide range of frequencies. Size effects are much less likely to be a problem for AFLP data, which tends to have a narrower range of band presence frequencies between groups.

Jackson *et al.* (1989) surveyed the effects on cluster analysis of ecological presence / absence data for 6 similarity coefficients: Jaccard, Czekanowski / Dice / Sørensen, Ochiai, Russel and Rao, simple matching, Rogers and Tanimoto; plus Phi and Yule association measures. Estimates of dendrogram dissimilarity showed little difference between the Jaccard and Czekanowski / Dice / Sørensen coefficients and between the simple matching and Rogers and Tanimoto coefficients, indicating some redundancy when these similarity coefficients are applied to binary data.

Statistical analysis in RAPD studies has largely focussed on individual level distance measures and cluster analysis methods. Similar studies by Johns *et al.* (1997) and Duarte *et al.* (1999) produced dissimilarity matrices of RAPD marker data for 8 different similarity coefficients: Jaccard, Czekanowski / Dice / Sørensen, Ochiai, Ochiai II, Russell and Rao, Simple matching, Rogers and Tanaimoto and Anderberg. They compared the coefficients using correlations of genetic dissimilarities and examined their effects on cluster analysis. They found

that despite correlations between distance measures all being higher than 0.86, use of the different similarity coefficients did have some effect on clustering, particularly in grouping individuals with high genetic similarity. This study was repeated for AFLP data by Meyer *et al.* (2004) and produced the same results.

Schmidt & Westheide (2000) tested 5 cluster analysis methods on Nei & Li (1979) distances (equivalent to the Czekanowski / Dice / Sørensen coefficient when applied to binary data) for RAPD loci. UPGMA (unweighted pair group method using arithmetic averages), WPGMA (weighted pair-group method using arithmetic averages), Complete Linkage, Single Linkage (Sneath & Sokal, 1973), and Neighbour Joining (Saitou & Nei, 1987) gave the same results for both inter and intra specific relationships and for both a highly differentiated and a genetically homogeneous species. Kruse *et al.* (2003) also found that for RAPD data UPGMA and Neighbour Joining gave similar results, both with low bootstrap values. While the choice of similarity coefficient may affect results slightly, the choice of clustering method appears to have little effect on cluster analysis.

### 4.3 Individual level results

#### 4.3.1 Comparison of dissimilarity and distance measures

Correlations of distance and dissimilarity matrices were used to compare similarity measures and to look for a discrepancy between shared band presence based measures and those that include shared absence, which could indicate band absence homoplasy.

For all 150 individuals dissimilarity matrices were generated using the Jaccard, Czekanowski / Dice / Sørensen, Ochiai, Sokal and Sneath, Russell and Rao, Simple matching and Rogers and Tanaimoto similarity coefficients, and a distance matrix was generated for Euclidean distance. Dissimilarity and distance matrices were created in R (R Development Core Team, 2007) using the distance function of the ecodist package (Goslee & Urban, 2007) or the ade4 package (Chessel *et al.*, 2004). All matrices were lower triangle, with no diagonal, suitable for further analysis in R.

Pairwise correlations were calculated between the matrices. Despite the fact that the coefficients are based on counts of presence / absence matches and mismatches, they are derived from binary data and are therefore proportions, which require data to be arcsine transformed in order to use a parametric correlation measure. Non-parametric Spearman rank correlations were therefore used to accommodate the data type and to allow comparison with other authors (Jackson *et al.*, 1989; Johns *et al.*, 1997; Duarte *et al.*, 1999; Meyer *et al.*, 2004).

Testing the statistical significance of a simple correlation between distance matrices is not appropriate because distances in a matrix are not independent of each other, as changing one sample would change  $n - 1$  distances to each of the other samples. To overcome the problem of non-independence Mantel permutation tests were used. Both matrices were randomly rearranged and the distribution of correlation test statistics over 1000 permutations was compared to the original test statistic to obtain a p value for significance. Mantel tests were

performed using the mantel function in the vegan package (Oksanen *et al.*, 2007) in R (RDCT, 2007) with a Spearman rank correlation and 1000 permutations.

Mean within site dissimilarity was also calculated as a measure of genetic diversity for dissimilarity matrices for each of the 5 sites generated using the Jaccard, Russell and Rao and Simple matching coefficients. These coefficients were selected to represent the 3 groups of coefficients because Russell and Rao and Jaccard are the simplest measures that do and do not include shared absence respectively and the simple matching coefficient has been used in AMOVA (Excoffier *et al.*, 1992). The dissimilarity data was still not normally distributed after arcsin transformation for proportions (Kolmogorov-Smirnov test,  $p < 0.001$ ), so a non-parametric Kruskal Wallis H test was used to test for significant difference in diversity between the sites.

	mean	s.d.
<b>ED</b>	5.206	1.227
<b>J</b>	0.368	0.081
<b>CDS</b>	0.271	0.064
<b>O</b>	0.271	0.064
<b>SS</b>	0.485	0.095
<b>RR</b>	0.558	0.023
<b>SM</b>	0.331	0.078
<b>RT</b>	0.441	0.094

**Table 4.1: Mean distance or dissimilarity between all 150 individuals from all 5 sites, from matrices created using Euclidean distance (ED), Jaccard (J), Czekanowski / Dice / Sørensen (CDS), Ochiai (O), Sokal and Sneath (SS), Russell and Rao (RR), Simple matching (SM), Rogers and Tanaimoto (RT).**

	ED	J	CDS	O	SS	RR	SM	RT
<b>ED</b>	-	0.999	0.999	0.999	0.999	0.909	1	1
<b>J</b>	0.999	-	1	1	1	0.922	0.999	0.999
<b>CDS</b>	0.999	1	-	1	1	0.922	0.999	0.999
<b>O</b>	0.999	1	1	-	0.999	0.922	0.999	0.999
<b>SS</b>	0.999	1	1	0.999	-	0.922	0.999	0.999
<b>RR</b>	0.909	0.922	0.922	0.922	0.922	-	0.909	0.909
<b>SM</b>	1	0.999	0.999	0.999	0.999	0.909	-	1
<b>RT</b>	1	0.999	0.999	0.999	0.999	0.909	1	-

**Table 4.2: Spearman rank correlations between the 9 distance and dissimilarity matrices (Mantel test,  $p < 0.001$ ).**

The results in Table 4.2 are almost identical to those found by other authors (Johns *et al.*, 1997; Duarte *et al.*, 1999; Meyer *et al.*, 2004) and have same rank order.

The similarity coefficients fall into 3 groups, according to Box 4.1. Jaccard (J), Czekanowski / Dice / Sørensen (CDS), Ochiai (O), and Sokal and Sneath (SS) do not include shared absence (d). Within this group presence / absence mismatches (b and c) are upweighted in Sokal and Sneath, giving higher mean dissimilarity, and shared presence (a) is upweighted in Czekanowski / Dice / Sørensen and Ochiai, which both produce(d) almost identical results with the lowest dissimilarity. The simple matching (SM) and Rogers and Tanaimoto (RT) coefficients include shared absence as similarity, and therefore show lower respective dissimilarity than J and SS. SM and RT behaved in the same way as Euclidean distance. The Russell and Rao (RR) coefficient behaved differently to all of the others because shared absence contributes to dissimilarity. The fact that it only has shared presence as the denominator meant that it was more similar to the J / CDS / O / SS group than to SM / RT.

Strong correlation between band presence based coefficients (J / CDS / O / SS) and those that include shared band absence (SM / RT) indicate that band absence homoplasy is very low (Duarte *et al.*, 1999; Bonin *et al.*, 2007). Correlation between the Jaccard and simple matching coefficients was 0.999, which supports the use of simple matching in AMOVA despite the influence of shared absence.

	SM		J		RR		correlation
	mean	s.d.	mean	s.d.	mean	s.d.	SM vs RR
<b>H</b>	0.204	0.028	0.234	0.031	0.530	0.007	0.272
<b>K</b>	0.235	0.026	0.267	0.028	0.535	0.008	0.405
<b>D</b>	0.301	0.037	0.337	0.039	0.542	0.012	0.703
<b>C</b>	0.313	0.083	0.347	0.086	0.548	0.027	1.000
<b>P</b>	0.243	0.050	0.275	0.054	0.535	0.013	0.522
<b>All</b>	0.331	0.078	0.368	0.081	0.558	0.023	0.909

**Table 4.3: Mean within site dissimilarity for each of the 5 sites using simple matching (SM), Jaccard (J) and Russell and Rao (RR) similarity coefficients. Spearman rank correlations between dissimilarity matrices created using the simple matching and Russell and Rao coefficients (Mantel test,  $p < 0.001$ ).**

As expected the mean dissimilarity within each site (Table 4.3) was lower than the mean dissimilarity for all 150 individuals (Table 4.1). There was a significant difference in within site diversity between the sites for all 3 coefficients according to Kruskal Wallis H tests (Jaccard:  $\chi^2 = 1039$ ,  $df = 4$ ,  $p < 0.001$ ; Russell & Rao:  $\chi^2 = 229$ ,  $df = 4$ ,  $p < 0.001$ ; simple matching:  $\chi^2 = 1067$ ,  $df = 4$ ,  $p < 0.001$ ). H and K had lower within site dissimilarity than D and C.

Among the coefficients Russell and Rao showed the highest mean dissimilarity with the least variation both within and between sites. Dissimilarity was lower in K than in P according to the Jaccard coefficient but the same according to the Russell and Rao coefficient. Correlations between the simple matching and Russell and Rao coefficients showed a weak correlation for H, modest correlations for K and P and strong correlations for D and C. H had the highest number of completely absent bands (Chapter 3, Table 3.10), and therefore the highest shared band absence. Because shared absence is treated as similarity in the simple matching coefficient but contributes to dissimilarity in the Russell and Rao coefficient dissimilarity was higher in H according to Russell and Rao.

### **4.3.2 Comparison of Multi Dimensional Scaling (MDS) ordination methods**

For Multi Dimensional Scaling (MDS) ordination analysis, dissimilarity matrices were created for each site and for all 150 individuals using Euclidean distance and Jaccard, Czekanowski / Dice / Sørensen, Ochiai, Sokal and Sneath, Russell and Rao, simple matching and Rogers and Tanaimoto similarity coefficients (Box 4.1).

MDS was performed in R (RDCT, 2007) for each of these distance measures using the non-metric MDS functions `sammon` and `isoMDS` in the MASS package (Venables & Ripley, 2002), `nmms` in the `ecodist` package (Goslee & Urban, 2007) plus the metric MDS function `cmdscale` in the `stats` package (RDCT, 2007).

`cmdscale` produces classic metric MDS, which uses eigenvalues analysis rather than a stress calculation in a similar way to PCoA (Gower, 1966). `isoMDS` is



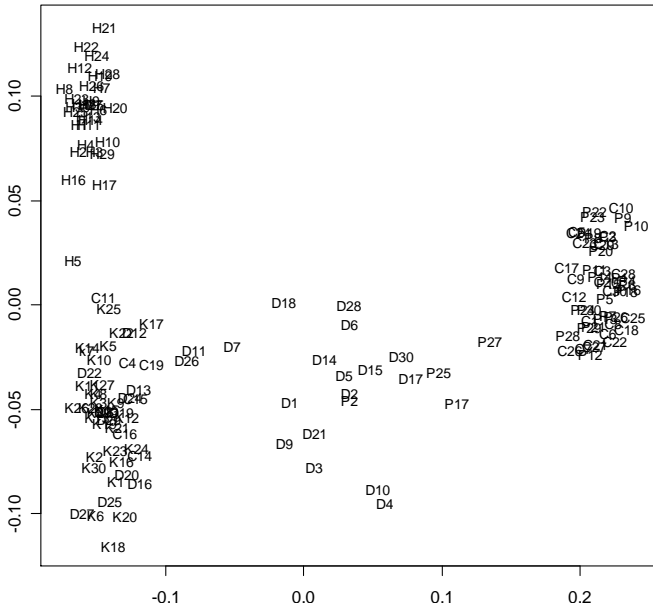
Kruskal's non-metric multidimensional scaling method (Kruskal, 1964). The author of the non-metric MDS function `nmDS` (Goslee & Urban, 2007) also cites Kruskal (1964) but does not give any further information about the algorithm used. Sammon mapping (Sammon, 1969) is very similar to nonlinear metric MDS but it includes an inverse weighting in the stress calculation, which preserves small distances between closely related individuals but tends to throw outliers out into a circle around the edge of the plot. The authors of the `sammon` R function describe it as “non-metric multidimensional scaling” (Venables & Ripley, 2002).

MDS plots for 30 individuals from each site and for all 150 individuals were compared visually to assess the effects of different distance measures and scaling algorithms on MDS analysis of AFLP data.

MDS stress values measure goodness of fit of the plot to the original data and vary in range between MDS methods. Stress values decreased with increasing number of dimensions used in MDS. However, it is difficult to plot more than 3 dimensions and the majority of authors only use 2, so all MDS plots in the analysis were 2 dimensional. Stress values were compared to mean dissimilarity using Spearman rank correlations. This was done for Jaccard, Russell and Rao and Simple matching dissimilarity matrices, for 30 individuals from each of the 5 sites and for all 150 individuals, to assess the effects of sample size and dissimilarity on stress.

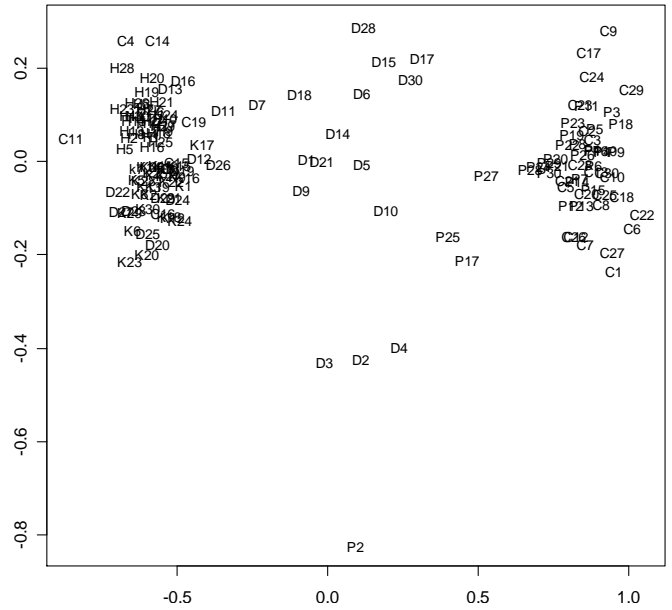
Procrustes analysis was also used to compare MDS plots, using the `procrustes` function in the `vegan` package (Oksanen *et al.*, 2007) in R (RDCT, 2007). This seeks to minimise the sum of squared differences between 2 plots, through scaling, translation, reflection and rotation. The procrustes  $m^2$  statistic is a measure of the difference between the geometric shape, and therefore the configuration of the points, between 2 plots. The significance of  $m^2$  was tested using permutation tests based on 1000 permutations. Pairwise  $m^2$  differences (all  $p < 0.001$ ) were calculated for each combination of the 4 MDS algorithms and 8 dissimilarity measures and the resulting 32 x 32 matrix was used to create an MDS plot of MDS plots.

**cmdscale - Jaccard**



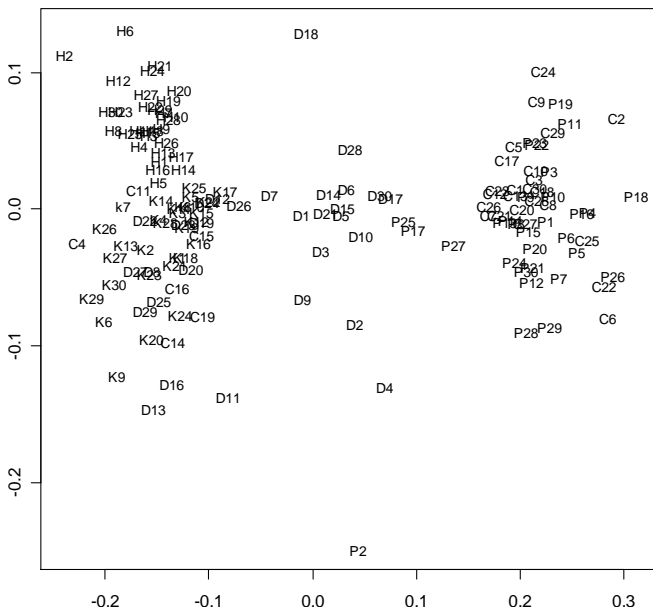
**isoMDS - Jaccard**

stress = 9.180



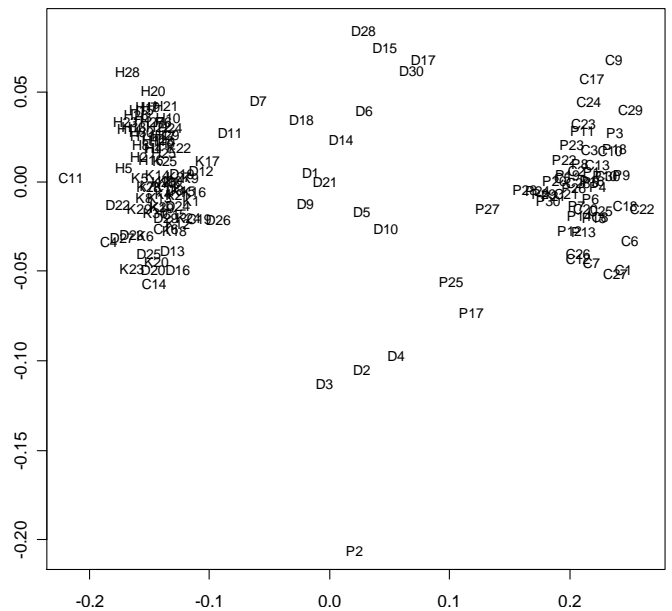
**isoMDS - Russell and Rao**

stress = 11.913



**isoMDS - simple matching**

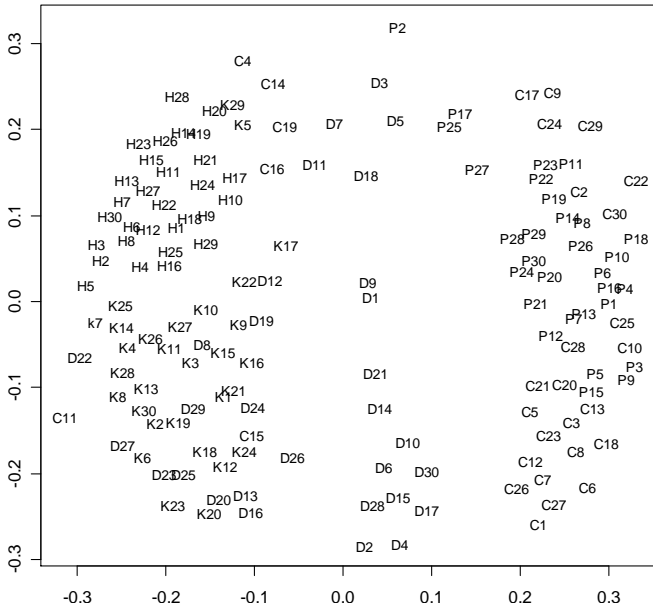
stress = 8.900



**Figure 4.3: MDS ordination plots of all 150 individuals produced with metric cmdscale MDS, from a Jaccard dissimilarity matrix, and non-metric isoMDS, from Jaccard, Russell and Rao and simple matching dissimilarity matrices.**

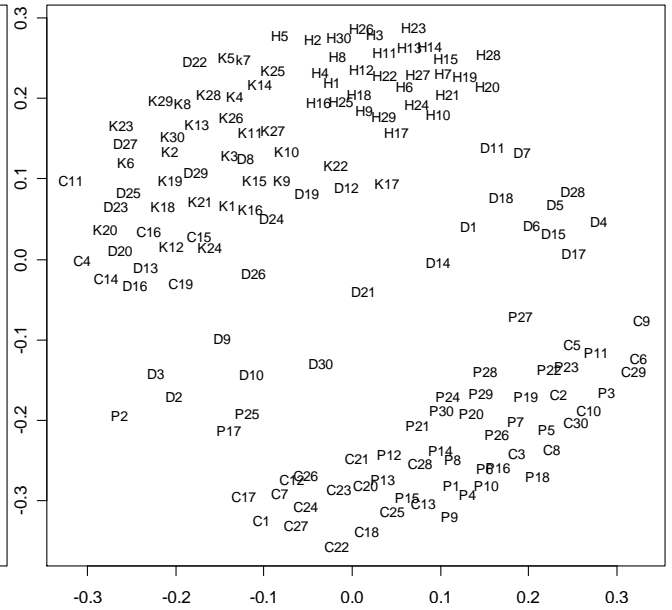
**nmds – Jaccard**

stress = 0.300  $R^2 = 0.738$



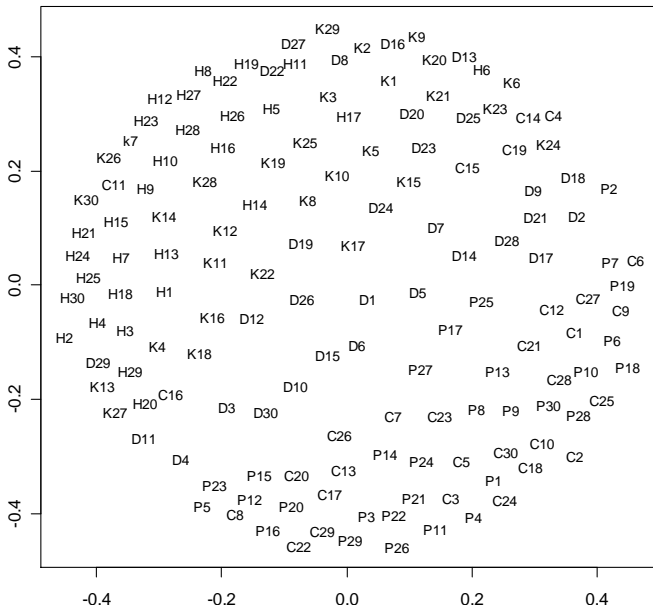
**nmds – Jaccard**

stress = 0.298  $R^2 = 0.745$



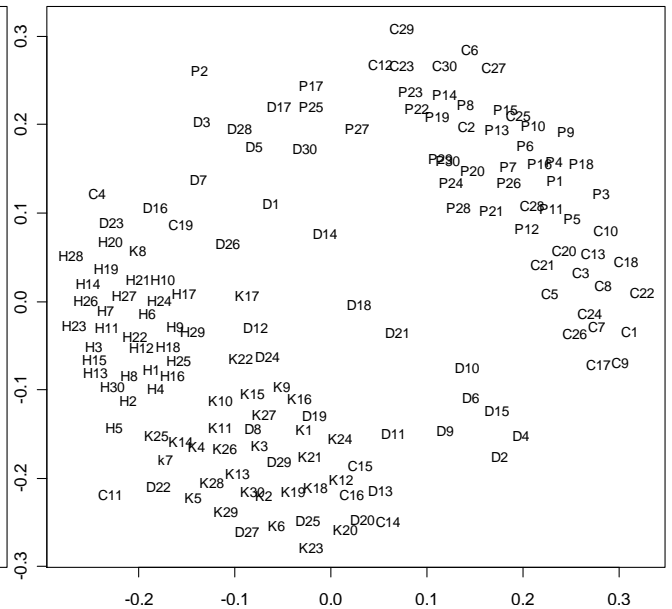
**nmds - Russell and Rao**

stress = 0.425  $R^2 = 0.464$



**nmds - simple matching**

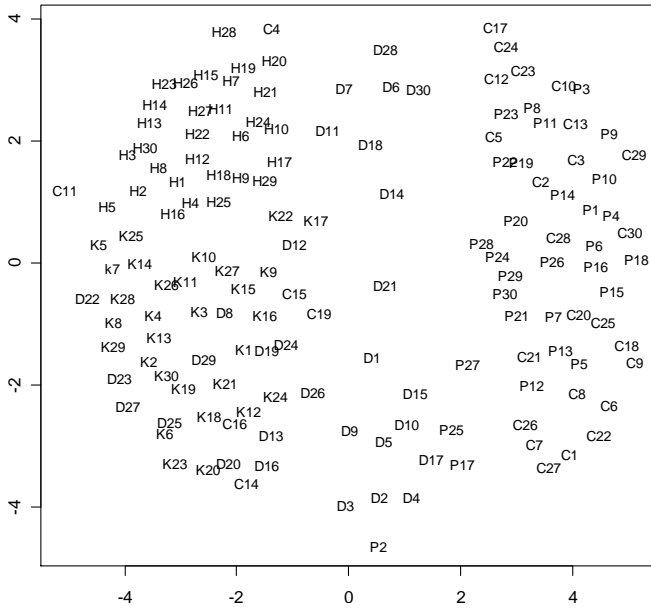
stress = 0.290  $R^2 = 0.756$



**Figure 4.4: MDS ordination plots of all 150 individuals produced with non-metric nmds, from Jaccard, Russell and Rao and simple matching dissimilarity matrices.**

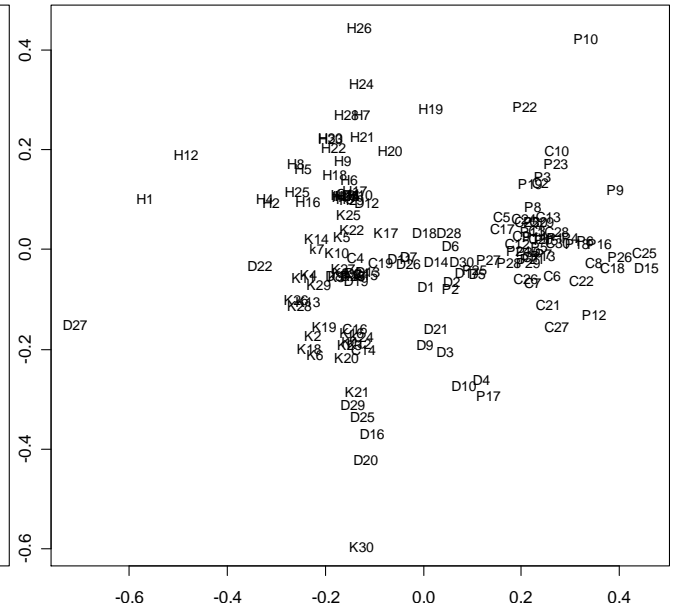
**sammon - Euclidean distance**

stress = 0.093



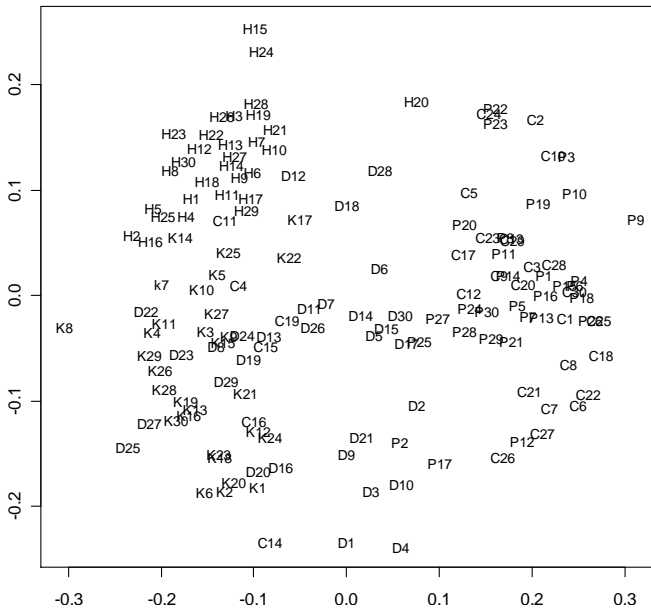
**sammon - Jaccard**

stress = 0.175



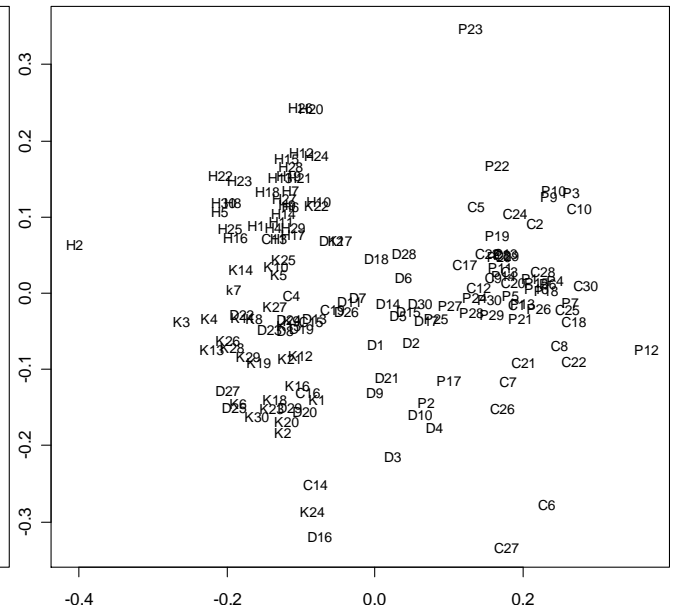
**sammon - Czekanowski / Dice / Sørensen**

stress = 0.110



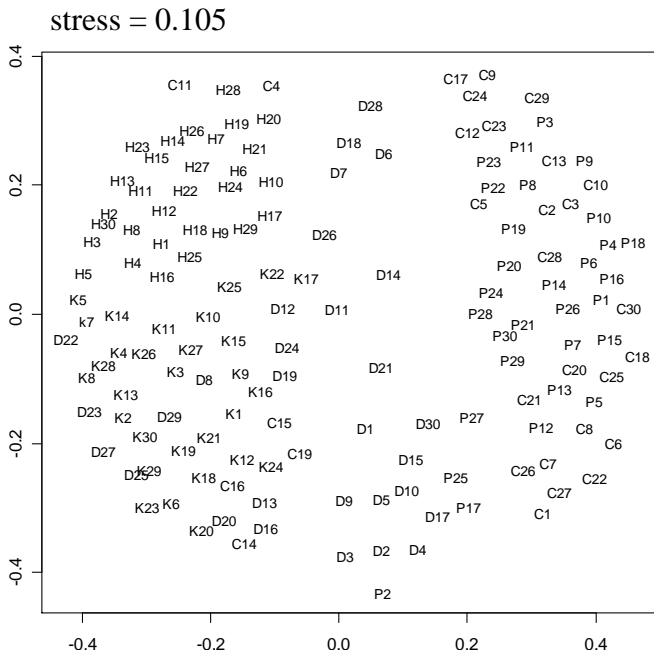
**sammon - Ochiai**

stress = 0.130

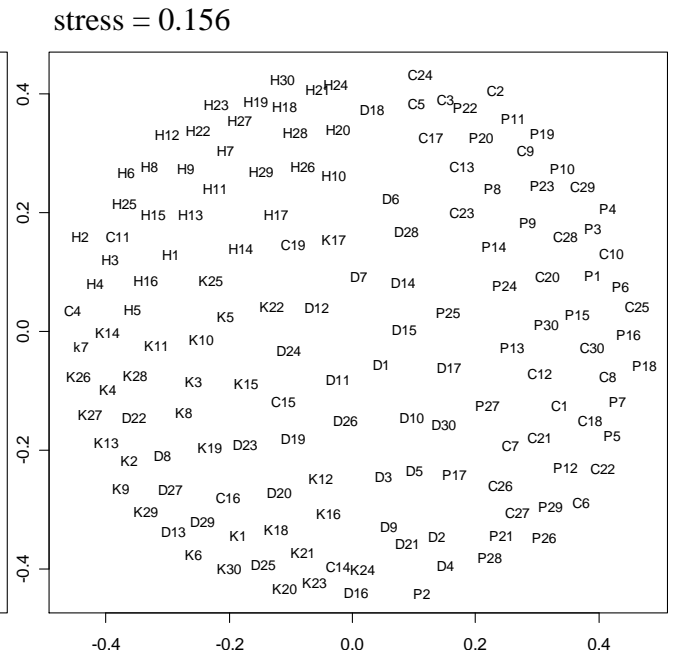


**Figure 4.5: MDS ordination plots of all 150 individuals produced using the sammon MDS algorithm, applied to dissimilarity matrices of 7 different similarity coefficients, plus Euclidean distance**

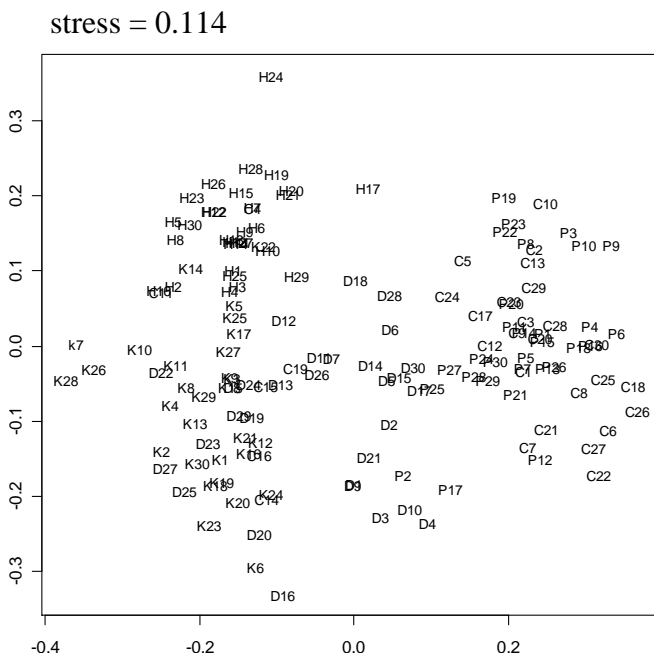
sammon - Sokal and Sneath



sammon - Russell and Rao



sammon -Simple matching



sammon - Rogers and Tanaimoto

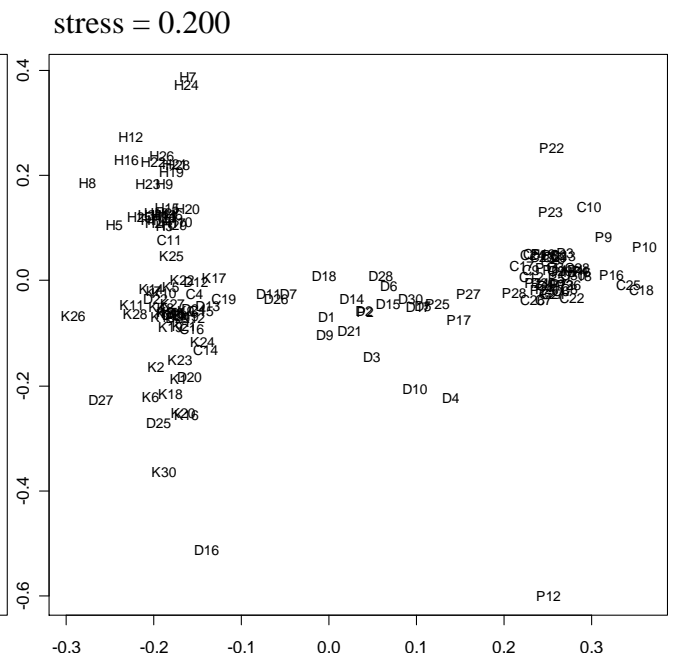


Figure 4.5: MDS ordination plots of all 150 individuals produced using the sammon MDS algorithm, applied to dissimilarity matrices of 7 different similarity coefficients, plus Euclidean distance

For metric cmdscale MDS there was very little observable difference between any of the plots produced using the different dissimilarity measures, so only Jaccard is shown in Figure 1. Compared to non-metric isoMDS, using a parametric algorithm had the effect of emphasising distance between rather than within sites, with the more variable C and D sites more closely grouped and the least polymorphic H and K sites more spread out and further apart from each other.

The plots produced by non-metric isoMDS (Figure 4.3) were all fairly similar to each other, apart from the position of a few outliers, except Russell and Rao, which appeared more spread out (Figure 4.3). The fact that H and K were more spread out compared to D and C in the Russell and Rao plot is consistent with this coefficient treating the high shared absence in H and K as dissimilarity. Stress values ranged from 8.900 for simple matching to 9.453 for Sokhal and Sneath and 11.913 for Russell and Rao.

For non-metric nmds (Figure 4.4) the plots all appeared to be fairly similar, except for reflection or rotation of the whole plot, the position of some outliers and a wider spread of points for Russell and Rao again. Stress values ranged from 0.289 for Czekanowski / Dice / Sørensen and Ochiai to 0.318 for Sokhal and Sneath and 0.425 for Russell and Rao. nmds also gives an  $R^2$  value for goodness of fit, which ranged from 0.761 for Czekanowski / Dice / Sørensen to 0.698 for Sokhal and Sneath and 0.466 for Russell and Rao.

Use of the Sammon algorithm in MDS produced widely differing results for different dissimilarity measures (Figure 4.4). Stress values ranged from 0.093 for Euclidean distance to 0.200 for Russell and Rao. The inversely weighted normalisation factor in Sammon mapping that is intended to preserve relationships between closely related points could be generally affected by distance: the Sokal and Sneath and Russell and Rao matrices had the highest mean dissimilarity (Table 4.1) and appeared to have the most spread out, circular plots. The fact that  $b + c$  distance is upweighted in Sokal and Sneath could explain the similarity to the plot for Euclidean distance. However, the plots for simple matching and Rogers and Tanaimoto contradict the close relationship with Euclidean distance shown by the correlations in Table 4.2. The plot for the Ochiai similarity

coefficient was quite different to that for Czekanowski / Dice / Sørensen, which contradicts other evidence suggesting redundancy. However, almost identical plots and stress values for isoMDS (Figure 4.3) supported the similarity of these 2 coefficients. In general the sammon plots did not represent the correlations between similarity coefficients and there was no obvious relationship between mean dissimilarity, standard deviation or stress and Sammon plot appearance across all distance measures. This coincides with the experience of authors who recently applied the technique to properties of chemical compounds and concluded that “generation of the Sammon map is an uncontrollable objective procedure that ignores the experimental classification” (Ivanenkov *et al.*, 2009).

Treatment of outliers varied with both distance measure and MDS method. According to the dissimilarity matrices P2 was an obvious outlier, with fairly high dissimilarity to all other individuals, including those from its own site. In all of the metric cmdscale plots it appeared towards the centre of the plot, next to D2, despite having higher dissimilarity to D2 than D2 did to surrounding D points. For isoMDS and nmds the position of a few points, including D2 and some of the variable C group, varied but P2 was always at the edge of the plot, far away from all other points. With sammon MDS P2 was variously positioned towards the middle with D2 (Jaccard, Czekanowski / Dice / Sørensen, Ochiai, Simple matching, Rogers and Tanaimoto) or at the edge next to D2 (Euclidean distance, Russell and Rao, Sokal and Sneath,). Some sammon plots also showed apparent outliers that did not feature in sammon plots for other coefficients.

Removing P2 did not change the configuration of the plots for cmdscale. It had no noticeable effect on the variable configurations and stress values for nmds, which suggests that nmds is robust to distortion caused by outliers, whereas removal of P2 lowered the stress value slightly for sammon and isoMDS plots. It did not change the configuration of the plots for isoMDS but it did allow the points to spread out, which is explained by the fact that non-metric MDS displays rank order and not actual distance. Removing P2 changed the shape, configuration and selection of other outliers in sammon plots.

Outliers are difficult to place on a 2D plot and have more variable positions, usually associated with slightly different local minimum values of stress producing different plots, where the configurations of closely related individuals are very similar but the outliers have very different relative positions. nmds automatically repeats the ordination a number of times (default 10) and selects the plot with the lowest final stress. Repeated nmds runs did produce different plots with different minimum stress values for the same similarity coefficient (e.g. Jaccard, Figure 4.4). However, with cmdscale, isoMDS and Sammon, repeating the MDS analysis made no difference to the plot configuration or the stress value. The fact that the stress values were exactly the same should indicate that the global minimum stress had been found. However, it is possible that the algorithms kept finding the same local minimum stress value within the plot, which could account for the unexpected position of known outliers such as P2.

<b>dissimilarity measure</b>	<b>dissimilarity mean</b>	<b>dissimilarity s.d.</b>	<b>isoMDS stress</b>	<b>nmds stress</b>	<b>sammon stress</b>
<b>ED</b>	5.206	1.227	8.935	0.290	0.093
<b>J</b>	0.368	0.081	9.180	0.300	0.175
<b>CDS</b>	0.271	0.064	8.973	0.289	0.110
<b>O</b>	0.271	0.064	8.962	0.289	0.130
<b>SS</b>	0.485	0.095	9.453	0.318	0.105
<b>RR</b>	0.558	0.023	11.913	0.425	0.156
<b>SM</b>	0.331	0.078	8.900	0.290	0.114
<b>RT</b>	0.441	0.094	9.367	0.304	0.200

**Table 4.4: Stress values for non-metric MDS plots of all 150 individuals; for isoMDS, nmds and sammon MDS algorithms; using Euclidean distance (ED) and Jaccard (J), Czekanowski / Dice / Sørensen (CDS), Ochiai (O), Sokal and Sneath (SS), Russell and Rao (RR), Simple matching (SM) and Rogers and Tanaimoto (RT) dissimilarity.**

Stress values have different ranges for different algorithms and so can not be compared between MDS methods. For all 150 individuals there was a strong, positive correlation between mean dissimilarity for the 7 similarity coefficients and stress for isoMDS and nmds (Spearman rank correlations,  $p < 0.01$ ) but not for sammon. There were no significant relationships between standard deviation and stress.



	isoMDS			nmDS			sammon		
	SM	J	RR	SM	J	RR	SM	J	RR
<b>H</b>	23.587	23.799	22.114	0.336	0.344	0.421	0.110	0.112	0.150
<b>K</b>	27.394	27.753	24.800	0.365	0.371	0.420	0.121	0.123	0.149
<b>D</b>	18.161	18.074	19.156	0.336	0.340	0.411	0.106	0.109	0.145
<b>C</b>	0.938	0.183	0.452	0.297	0.304	0.403	0.092	0.095	0.140
<b>P</b>	15.558	15.107	32.563	0.311	0.311	0.418	0.094	0.096	0.147
<b>All</b>	8.900	9.180	11.913	0.290	0.300	0.466	0.114	0.175	0.156

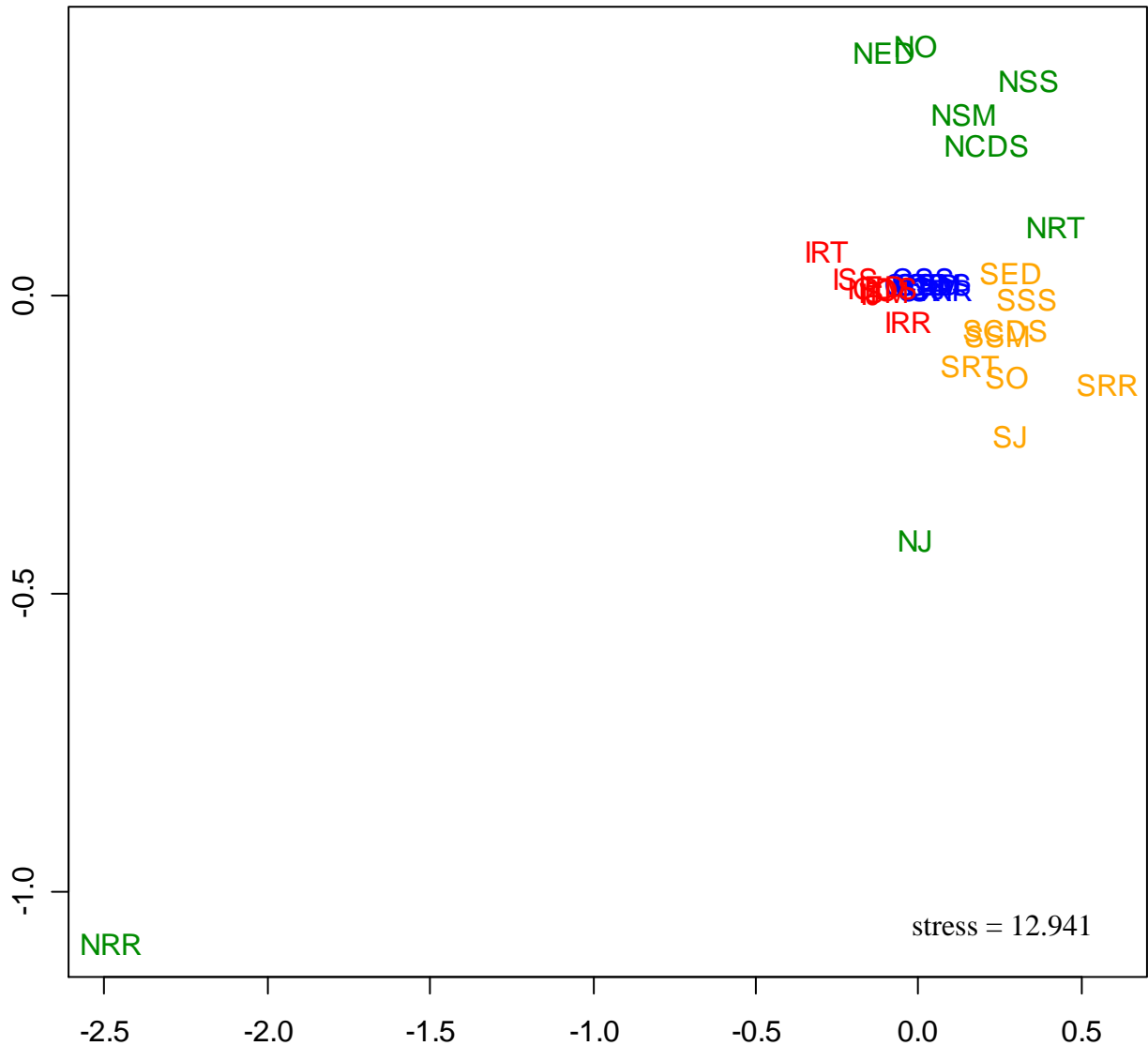
**Table 4.5: Stress values for MDS plots of each of the 5 sites and all 150 individuals; for isoMDS, nmDS and sammon MDS algorithms; using simple matching (SM), Jaccard (J) and Russell and Rao (RR) similarity coefficients.**

For the samples of 30 individuals nmDS and sammon stress values generally increased with increasing mean dissimilarity between the 3 different similarity coefficients:  $SM < J < RR$ . However, there was no obvious overall relationship between stress and the mean or standard deviation of dissimilarity (Tables 4.1 and 4.3). There were some significant relationships between mean or standard deviation of dissimilarity and stress between the 5 sites: all strong negative correlations ( $p < 0.01$ ), where the higher the dissimilarity or variation in dissimilarity was the lower the stress value was, but with only 5 data points these relationships may not be meaningful and there was no overall pattern associated with MDS method or similarity coefficient.

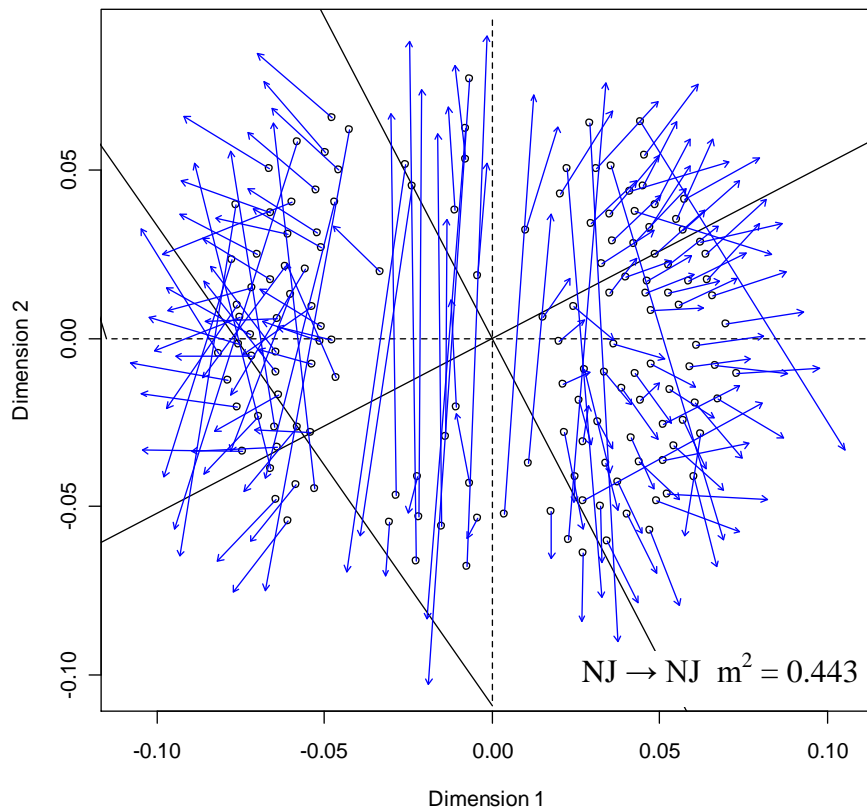
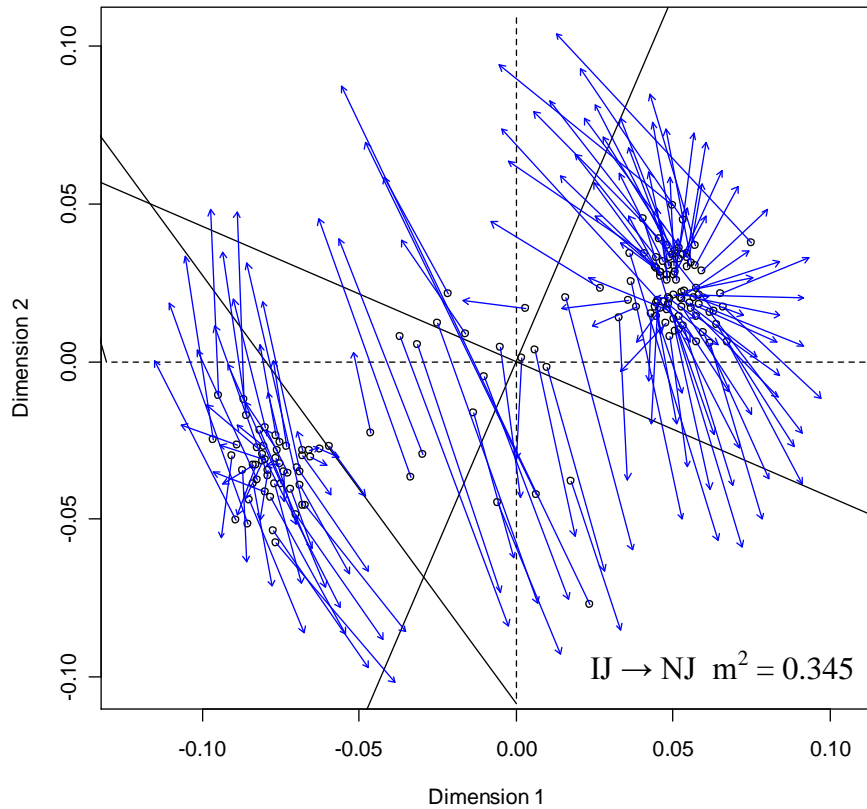
For isoMDS and nmDS all plots of 30 individuals from the same site except C had higher stress values than the plot of all 150 individuals, whereas for sammon all plots except K / SM had lower stress for 30 than for 150 individuals (Table 4.5). This shows that preserving small distances works better for small, closely related data sets in sammon, while nmDS and isoMDS cope better with large, dissimilar, variable AFLP data sets.

All MDS methods suffer from “the curse of dimensionality” (Faith *et al.*, 2006, quoting Bellman, 1961): the larger the data matrix and associated dimensionality, the less variance there is in distances between points. In Sammon the higher stress levels associated with the larger data set and the wider, more even spread of points, with less separation between groups in the 2D plots (Figure 4.5) suggests that the inversely weighted distance normalisation algorithm in Sammon mapping

that preserves small distances is particularly susceptible to this effect. The plots for nmDS were also more spread out with less clearly defined groups compared to cmdscale and isoMDS and looked similar to some of the Sammon plots.



**Figure 4.6: isoMDS plot of procrustes analysis  $m^2$  distances between MDS plots produced by applying cmdscale (C prefix, blue), isoMDS (I prefix, red), nmDS (N prefix, green) and sammon (S prefix, orange) algorithms to Euclidean distance (ED), Jaccard (J), Czekanowski / Dice / Sørensen (CDS), Ochiai (O), Sokal and Sneath (SS), Russell and Rao (RR), Simple matching (SM) and Rogers and Tanaimoto (RT) distance or dissimilarity matrices for all 150 individuals.**



**Figure 4.7: Procrustes plots of MDS of Jaccard dissimilarity for all 150 individuals, showing the difference between 2 MDS plots produced using isoMDS (IJ) and nmds (NJ),  $m^2 = 0.345$ , and between 2 separate runs of nmds (NJ),  $m^2 = 0.443$ . Rotation is shown by the cross hairs and the blue arrows are changes in the position of points between the 2 plots.**

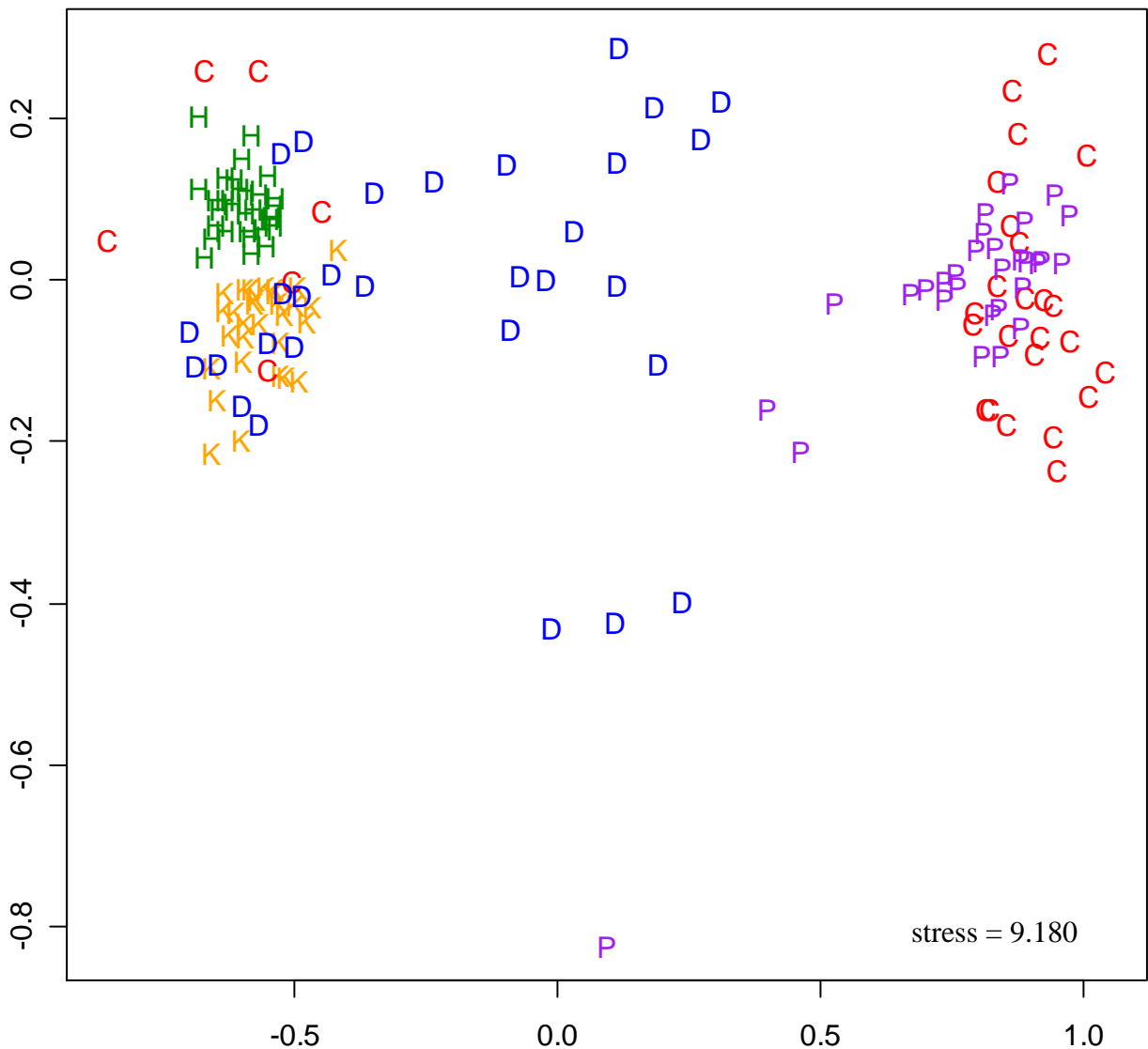
MDS analysis of procrustes  $m^2$  distances between the different MDS algorithms and similarity coefficients grouped the plots by algorithm, with `cmdscale` and `isoMDS` forming tight groups, while `nmDS` and `sammon` were more spread out (Figure 4.6). There was no obvious relationship between mean dissimilarity, stress and difference between the plots. After excluding reflection, rotation, translation and scale, procrustes analysis only compares the remaining geometric shape of the points, so the larger spread of `nmDS` and `sammon` plots was due to greater difference in the relative position of the points. There was no connection with the fact that the original `nmDS` and `sammon` MDS plots appeared more spread out due to scale. The configurations of the `nmDS` and `sammon` plots were therefore affected to a greater extent by the choice of dissimilarity coefficient; in particular Russell and Rao, which was an outlier for all algorithms and caused serious problems for the `nmDS` algorithm. These results were surprising because visually the `nmDS` plots for different similarity coefficients all appeared very similar to each other, while the appearance of the `sammon` plots varied much more widely.

The configuration of `nmDS` plots varies between runs so procrustes differences within and between groups are variable with respect to `nmDS` (Figure 4.7). Comparing replicate `nmDS` plots for the same similarity coefficient to each other gave  $m^2$  values of  $\sim 0.3$  for simple matching (NSM),  $\sim 0.4$  for Jaccard (NJ) and  $\sim 0.6$  for Russell and Rao (NRR). The fact that these differences coincide with difference from the other methods and stress values suggests that `nmDS` has increasing difficulty resolving a configuration for these similarity coefficients.

The peculiar behaviour of the Sammon algorithm, described by the authors of the R function simply as non-metric multidimensional scaling, plus the variation in configuration, spread and stress value ranges between MDS methods demonstrates that care should be taken when selecting a method and also in comparisons of MDS plots in the literature. The fact that for algorithms other than `sammon` the MDS algorithms were not greatly affected by the similarity coefficient used to generate input matrix (except for the Russell and Rao coefficient) suggests that as with cluster analysis the choice of dissimilarity measure is not necessarily important in MDS. The lack of difference between the plots could simply reflect the close correlation of the similarity coefficients for

this data set but it could also show that rank ordering of points in non-metric MDS successfully eliminates the effects of band presence frequency or shared absence homoplasy on different measurements of genetic distance. Further in silico analysis of data sets designed to simulate band absence homoplasy would address this question.

### 4.3.3 Multidimensional scaling ordination analysis



**Figure 4.8: isoMDS plot of a Jaccard dissimilarity matrix of 150 individuals from 5 sites: Hayle (H), Kennal (K), Devoran (D), St Clements (C) and Percuil (P).**

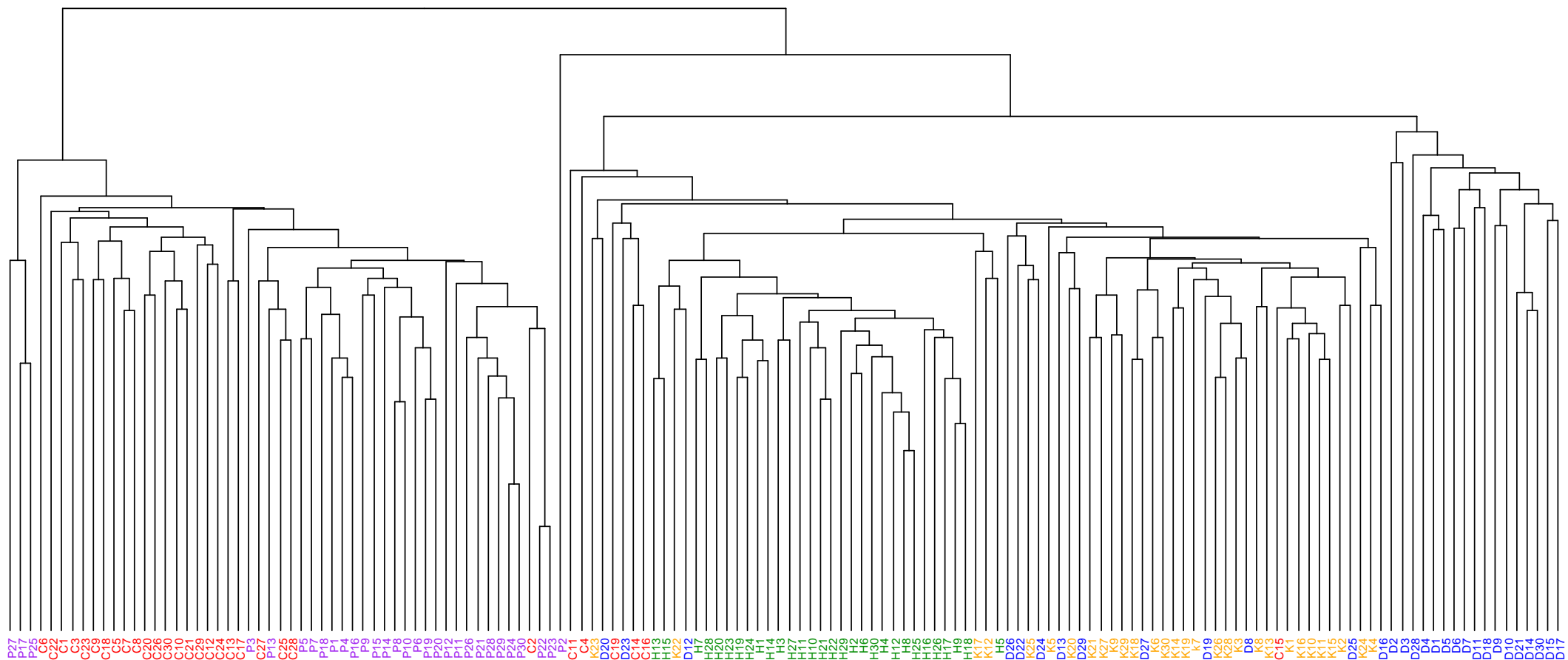
Based on a combination of the performance of the different similarity coefficients and MDS algorithms, their appropriateness for binary AFLP data, plus availability of information about what is ‘in the box’, isoMDS analysis of a Jaccard dissimilarity matrix was selected to represent genetic relationships in *Nereis diversicolor* within and between sites.

The MDS analysis showed clear differentiation by site, with the copper tolerant (H and K) and non-tolerant (C and P) samples forming distinct groups. However, individuals from non tolerant site C were split between the tolerant and non-tolerant groups. Site D, with intermediate copper pollution and tolerance, had the greatest diversity, with some individuals grouped with the tolerant samples from H and K. Mortality in toxicity tests after 216 hours of worms thus considered to be tolerant (Briggs, 2005) was 45 % and 40.5 % for samples D1 and D2 from mixed site R6 / D (Chapter 2, 2.3.4). This coincides with around 40 – 45 % of worms from site D grouped with the tolerant worms in Figure 4.8.

#### **4.3.4 Cluster analysis**

Comparison of hierarchical, agglomerative clustering methods found that single linkage allowed ‘chaining’ to form 2 large clusters, whereas complete linkage produced a larger number of evenly sized clusters. Hierarchical, agglomerative clustering with Unweighted Pair Group Method with Arithmetic mean (UPGMA) linkage produced the plot that most closely resembled the MDS plot in Figure 4.8.

With UPGMA linkage there were small differences between cluster analysis plots for the 9 different dissimilarity measures, with similarity between plot configurations corresponding closely with correlations between the 9 distance and dissimilarity matrices in Table 4.2. All differences were among closely related individuals.



**Figure 4.9: Hierarchical, agglomerative cluster analysis of a Jaccard dissimilarity matrix, with Unweighted Pair Group Method with Arithmetic mean (UPGMA) linkage, for 150 individuals from 5 sites (H – green, K – orange, D – blue, C – red, P – purple)**

There was no evidence of frequency based chaining. There was a significant difference in band presence frequency in individuals between the 5 sites according to ANOVA (Chapter 3, 3.3.2), which can present a problem for cluster analysis. However, the ecological data set of counts of fish species that Jackson *et al.* (1989) used to illustrate this phenomenon had a comparatively much wider range of frequencies. ANOVA compares the ratio of between group variance to within group variance. The result was significant because the variance between groups was greater than the variance within groups but both variances were relatively low and there was actually a low range of AFLP band presence frequencies, so the cluster analysis should not have been affected by size issues.

The grouping of individuals according to site was almost exactly the same as for MDS analysis, which supports the validity and complementary nature of these two methods. 17 individuals from mixed site D (57 %) formed a distinct cluster. Compared to this group tolerant sites K and H were more closely related, despite the large geographic distance between them.

#### **4.3.5 Correlation between genetic and geographic distance**

Mantel permutation tests (Mantel, 1967) with a Spearman rank correlation and 1000 permutations were carried out to test for correlation between genetic and geographic distance at the individual level. The test calculated pairwise correlations between the geographical distance matrix and a matrix of Jaccard dissimilarity coefficients for AFLP. This was done for all 150 individuals from all 5 sites and also excluding site H, to assess the effects of the large geographical distance between H and the other sites.

Map co-ordinates could not be used for geographic distance as *Nereis diversicolor* usually travels by sea, so distance between sites following the coast line was estimated with a piece of string. A 150 x 150 geographical distance matrix was constructed for distances between the sites in Km and converted to a lower triangle, no diagonal matrix using the R function `as.dist` (RDCT, 2007).



	H	K	D	C	P
H	0	141	138	145	137
K	141	0	3	15	16
D	138	3	0	13	13
C	145	15	13	0	20
P	137	16	13	20	0

**Table 4.6: Estimated distances between sites in Km**

Mantel tests showed that there was a modest positive correlation between geographic distance and genetic dissimilarity for all 150 individuals from all 5 sites ( $r = 0.482$ ,  $p < 0.001$ ) but a very weak positive correlation when site H was excluded ( $r = 0.132$ ,  $p < 0.001$ ).

The positive correlation between geographic distance and genetic dissimilarity reported by the Mantel test was mainly due to the fact that it included comparisons between individuals within sites (0 geographic distance) and there was greater similarity within than between sites. This test may be more appropriate for single samples spread out over a gradient of geographical distance. However, the very weak correlation when geographically distant site H was excluded demonstrated that geography only plays a limited part in the genetic differentiation between tolerant and non-tolerant worms. In contrast according to the MDS analysis tolerant site K shares more genetic similarity with tolerant site H, 141 Km away, than it does with mixed site D, 3 Km away.

At the population genetic level a positive correlation between geographic and genetic distance should indicate isolation by distance and equilibrium between drift and migration in long established sub populations (Malécot, 1955; Slatkin, 1993). In a study using Mantel tests of pairwise  $F_{ST}$  values between populations against geographic distance in the freshwater mussel *Pseudanodonta complanata* Skidmore *et al.* (2010) found no significant correlation between geographic and genetic distance ( $r = 0.260$ ,  $n = 12$  populations with 1000 permutations,  $p = 139$ ) but demonstrated isolation by distance after removing populations from a single river noted for anthropogenic introductions ( $r = -0.489$ ,  $n = 11$ ,  $P < 0.001$ ).

#### 4.4 Population level analysis methods

The forces of mutation, drift, selection and migration change allele frequencies in subdivided populations, allowing evolutionary divergence. The structure of genetic differentiation between sub populations can be used to assess the level and pattern of divergence.

Population level calculations of genetic diversity and differentiation analyse differences per marker within and between populations (Bonin *et al.*, 2007). Allele frequency based methods include a number of mathematical measures of genetic diversity and differentiation within and between populations, based on allele frequencies at each locus (Kosman & Leonard, 2005). Because AFLP markers are dominant there is no empirical information available regarding the frequency of dominant and recessive alleles within or between individuals and populations. The allele frequency based approach to AFLP data analysis therefore relies on estimation of allele frequencies (Lynch & Milligan, 1994). Estimation methods can either assume Hardy Weinberg Equilibrium or include additional information regarding the inbreeding coefficient  $F_{IS}$  (Chong *et al.*, 1994).

An invited review of AFLP statistical analysis methods by Bonin *et al.* (2007) listed 5 procedures for estimating allele frequencies as square-root, Lynch & Milligan (1994), Bayesian (Zhivotovsky, 1999), moment-based (Hill & Weir, 2004) and Holsinger *et al.* (2002). However, the moment based method of Hill & Weir is actually a method of calculating genetic diversity and distance statistics. It is based on genotype frequencies, which implies assumptions about allele distributions but does not directly involve a calculation of allele frequencies. The Holsinger approach is another Bayesian method for estimating allele frequencies. Kraus (2000) included simple band frequency per locus as a possible method, though this is only suitable for haploid or self-fertilising species and over estimates allele frequencies when applied to diploid, random mating populations. The 3 main types of procedure for calculating allele frequencies from dominant, diploid data can therefore be classified as square root, Lynch & Milligan and Bayesian methods.

#### 4.4.1 Estimating allele frequencies from AFLP data

##### Square root

The proportion of a population that is heterozygous at a locus is most easily calculated as 1 – homozygosity. If a population is in Hardy Weinberg Equilibrium then the frequency of the recessive null allele at each locus can be estimated as the square root of the frequency of null homozygotes (Clark & Lanigan, 1993). For AFLP data this is the square root of the frequency of absent bands.

e.g. For 11 10 01 00 the frequency of the null allele (0) is  $\sqrt{0.25} = 0.5$

This estimation method is downwardly biased for low frequencies of null homozygotes, particularly in small sample sizes (Lynch & Milligan, 1994). However, in a simulation of RAPD nucleotide divergence run by Clark & Lanigan (1993) there was very little error in estimates of divergence due to this bias.

The square root procedure, assuming Hardy Weinberg equilibrium, can be implemented in genetics analysis software such as Popgene (Yeh *et al.*, 1999) and AFLP-SURV (Vekemans *et al.*, 2002) if no measure of the inbreeding coefficient  $F_{IS}$  is provided.

##### Lynch & Milligan

To address downward bias of null alleles with low frequencies Lynch and Milligan (1994) introduced a method of estimating null allele frequencies by including the variance of the frequency of null homozygotes in the calculation:

$$q = \sqrt{x} \left( 1 - \frac{\text{var}(x)}{8x^2} \right)^{-1}$$

where x is the frequency of the null allele calculated using the square root method.

##### Bayesian

Bayes' theorem expresses the relationship between the conditional probability of event A, given B, and the converse conditional probability of B, given A:

$$P(A | B) = \frac{P(B | A)P(A)}{P(B)}$$

Where:

$P(A|B)$  is the posterior probability of A, given B.

$P(B|A)$  is the posterior probability of B given A.

$P(A)$  is the prior probability of A, with no information about B.

$P(B)$  is the prior probability of B, which acts as a normalizing constant.

In experimental terms it is the posterior probability of a hypothesis, after evidence is observed, in terms of the prior probabilities of the hypothesis, the evidence and the probability of the evidence given the hypothesis.

The Bayesian estimation method assumes that the distribution of allele frequencies ( $p$ ) follows a beta distribution:

$$\varphi(p) = \frac{\Gamma[\theta]}{\Gamma[\theta\bar{p}]\Gamma[\theta(1-\bar{p})]} p^{\theta\bar{p}-1} (1-p)^{\theta(1-\bar{p})-1}$$

(Wright, 1937 in Cabellero *et al.*, 2008), where  $\theta = 4Nm[n/(n-1)]$ ,  $N$  is the subpopulation size,  $n$  is the number of subpopulations,  $m$  is the migration rate,  $\bar{p}$  is the average allele frequency in the total population, and  $\Gamma$  is the gamma function.

The beta distribution is a family of continuous probability distributions with 2 parameters,  $\alpha$  and  $\beta$ . Beta distributions are used in Bayesian statistics as conjugate prior distributions for binomial distributions. A uniform prior distribution is a special case of the beta distribution, with parameters  $\alpha = 1$  and  $\beta = 1$ . Bayesian methods for population level statistics allow the use of different  $\alpha$  and  $\beta$  parameters for beta prior probability distributions. The software AFLP-SURV (Vekemans *et al.*, 2002) offers allele frequency estimation using uniform or non-uniform priors, while DFDIST (Beaumont & Nichols, 1996) and Hickory (Holsinger & Lewis, 2007) allow user specified parameters. DFDIST suggests  $\alpha = 0.25$ ,  $\beta = 0.25$ , while Hickory claims to employ default parameters that give “vague” priors, although these appear to be uniform  $\alpha = 1$ ,  $\beta = 1$ .

**Zhivotovsky (1999)** introduced a Bayesian approach to estimating null allele frequencies, which was shown in simulations to give nearly unbiased estimates of heterozygosity and genetic distance measures including  $F$  statistics. It is considered to be robust to moderate departure from Hardy–Weinberg equilibrium. The Bayesian method also performed best in comparisons of square root, Lynch and Milligan and Bayesian allele frequency estimation applied to empirical AFLP data with known allele frequencies from co-dominant markers (Isabel *et al.*, 1995; Zhivotovsky, 1999). The Zhivotovsky method is used in AFLP-SURV and DFDIST.

**Holsinger *et al.* (2002)** developed a Markov Chain Monte Carlo (MCMC) Bayesian method to estimate allele frequencies which is implemented in the software Hickory (Holsinger & Lewis, 2007).

Loci with no null homozygotes in the sample can still have null alleles in heterozygotes, masked by dominant band presence. Null allele frequencies for complete, monomorphic bands are always zero for the square root method and can not be resolved by the Lynch & Milligan equations but they are estimated by the Bayesian method.

#### **4.4.2 Including known $F_{IS}$ in allele frequency estimates**

Square root and Bayesian estimates of allele frequency can include the inbreeding coefficient  $F_{IS}$  if it is known. In order to estimate nucleotide divergence for RAPD data Chong *et al.* (1994) used an  $F_{IS}$  value from a previous study of co-dominant markers to estimate allele frequency. They defined the expected frequencies of band presence (pp, pq, qp) and band absence (qq) at a locus, where p and q are marker (1) and null (0) alleles, as:

$$f(p-) = 1 - (q^2 + (1-q)qF_{IS})$$

$$f(qq) = q^2 + (1-q)qF_{IS}$$

Iteration was then used to estimate  $q$ . This approach is implemented in Popgene and AFLP-SURV if an  $F_{IS}$  value is provided.

### 4.4.3 Estimating population diversity

#### 4.4.3.1 $S$ Shannon index of phenotypic diversity (Shannon, 1948)

$$S = \sum p_i \log_2 p_i$$

where  $p_i$  is the frequency of band presence at the  $i$ th locus within a population. The Shannon diversity index is a band based measure that gives more weight to band presence than to band absence, which addresses the issue of band absence homoplasy. The application of a general index of diversity to genetic data was advocated by Lewontin (1972). However, this was criticised by Nei (1973) as lacking any interpretable connection with genetic diversity.

#### 4.4.3.2 $H$ Gene diversity (Nei, 1973)

$$H = \frac{\sum_i^n (1 - p_i^2 - q_i^2)}{n}$$

Where  $p_i$  and  $q_i$  are the frequency of presence or absence at each locus  $i$  and  $n$  is the number of loci. An unbiased version, where  $N$  is the number of individuals is:

$$H = \frac{2N}{2N-1} \frac{\sum_i^n (1 - p_i^2 - q_i^2)}{n}$$

Nei's (1973) gene diversity is a measure of the average genetic diversity per locus within a population, and is equivalent to expected heterozygosity ( $H_E$ ) in a random mating population. Nei introduced the term gene diversity on the understanding that heterozygosity was not appropriate for non-random mating

populations, but most authors simply refer to  $H$  as heterozygosity. The maximum possible diversity increases with increasing number of alleles at a locus and tends towards 1 for multiallelic marker systems, with an equal frequency of each allele at a locus having the greatest diversity. For binary data Nei's gene diversity ranges between 0 and 0.5 rather than 1, with 0 being identity and 0.5 being maximum diversity (Kosman, 2003). Complete AFLP bands have 0 gene diversity and bands with an equal frequency of presence and absence have a gene diversity of 0.5. Therefore it is not appropriate to compare measures of gene diversity between dominant and codominant markers.

Nei's gene diversity and the average mismatch dissimilarity  $(b + c / n)$  between individuals within a population are identical measures of diversity per individual for binary data (Kosman, 2003).

Multiallelic markers such as RFLPs, microsatellites and SNPs have a theoretical maximum gene diversity of 1.0, rather than 0.5 for dominant markers. The presence of multiple alleles can provide additional information regarding gene diversity, though rare alleles contribute little to the overall measure of gene diversity.

#### **4.4.4 Estimating population differentiation**

##### **4.4.4.1 $\Phi_{ST}$ AMOVA (Excoffier *et al.*, 1992)**

Analysis of MOlecular VAriance (Excoffier *et al.*, 1992) is a band based method used to estimate  $\Phi_{ST}$ , a measure of genetic differentiation between sub populations. It is similar to ANOVA but uses permutation tests for significance to avoid the issue that binary data is not normally distributed. Genetic diversity is partitioned within and between populations, based on a distance matrix. As it analyses variance AMOVA requires a Euclidean distance measure in order to be strictly rigorous. Excoffier *et al.* (2005) use squared Euclidean distance in their software Arlequin, which may not be appropriate for binary data. Other more

suitable distance measures such as similarity coefficients with Euclidean properties could be used.

A nested AMOVA can be calculated for a hierarchy of levels of population structure. The variance components can then be used to calculate  $\Phi$  statistics for each level to determine, for example, differentiation between groups of sub populations associated with tolerant and non tolerant sites.

The advantage of AMOVA over allele frequency based methods is that it does not rely on as many assumptions about the statistical properties of the data or the underlying model of population structure, although it does assume that loci are independent and mating is random.

#### **4.4.4.2 *F* statistics                      Wright (1951)**

Wright (1951) defined the following relationships with regard to heterozygosity, where  $F$  is the level of fixation, the increase in homozygosity in individuals and populations due to inbreeding or drift:

#### **$F_{IT}$ Overall Fixation index**

$$F_{IT} = \frac{H_T - H_I}{H_T}$$

The mean reduction in heterozygosity per individual, relative to the total population. It is the product of non-random mating within sub-populations and random drift among sub populations:

$$(1 - F_{IT}) = (1 - F_{IS})(1 - F_{ST})$$

This equation is only applicable when there are 2 alleles at a locus (Nei, 1973).



### **$F_{IS}$ Inbreeding coefficient**

$$F_{IS} = \frac{H_S - H_I}{H_S}$$

The mean reduction in heterozygosity per individual due to non-random mating in a population, measured on the scale -1 (all heterozygous) to +1 (all homozygous), with  $F_{IS} = 0$  being Hardy Weinberg Equilibrium ( $p^2 + 2pq + q^2 = 1$ ). It is used as a measure of the amount of inbreeding and deviation from Hardy Weinberg Equilibrium in sub populations. For marker and null alleles A and a at a locus, with frequencies  $p$  and  $q = 1 - p$ , the expected genotype frequencies in a segregating population have the following probabilities (Lynch & Milligan, 1994):

$$PAA = p^2(1 - F_{IS}) + pF_{IS}$$

$$PAa = 2pq(1 - F_{IS})$$

$$Paa = q^2(1 - F_{IS}) + qF_{IS}$$

$F_{IS}$  is also the probability that 2 alleles in an individual are identical by descent. According to Wright's (1934) method of path analysis the probability of identity by descent is  $F_{IS} = 0.5$  for self fertilisation,  $F_{IS} = 0.25$  for mating between siblings and  $F_{IS} = 0.125$  for uncle-neice or cousin mating. In a study of mice, for example, Selander (1970) found high inbreeding of  $F_{IS} = 0.182$

### **$F_{ST}$ Fixation index**

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

$F_{ST}$  is a measure of genetic differentiation. It is the mean reduction in heterozygosity in sub-populations, relative to the total population, due to genetic drift. It measures the proportion of genetic diversity that is due to differentiation

between sub populations, where 0 is no difference and 1 is complete differentiation, with sub-populations fixed for different alleles.

The classic method of estimating  $F_{ST}$  is according to Weir and Cockerham (1984). Weir and Cockerham used alternative notation in their re-definition of  $F$  statistics that are widely used in the literature:  $F \approx F_{IT}$   $\theta \approx F_{ST}$   $f \approx F_{IS}$

#### 4.4.4.3 $G_{ST}$ Gene differentiation (Nei, 1973)

$$G_{ST} = \frac{H_T - \overline{H_S}}{H_T}$$

$G_{ST}$  (Nei, 1973) is an alternative measure of genetic differentiation between subpopulations. Because heterozygosity increases with population size, heterozygosity for the total population of an organism,  $H_T$ , will be higher than the average heterozygosity in sub-populations,  $\overline{H_S}$ .  $G_{ST}$  is the proportion of the heterozygosity in the total population ( $H_T$ ) that occurs between ( $D_{ST}$ ) rather than within ( $H_S$ ) populations.

#### 4.4.4.4 Relationship between $F_{ST}$ and $G_{ST}$

$F_{ST}$  and  $G_{ST}$  differ in that  $F_{IS}$  and  $F_{IT}$  measure the deviations of genotype frequencies from Hardy Weinberg proportions, while Nei's measure uses gene identities (Nei, 1973). For 2 alleles  $G_{ST}$  is equivalent to Wright's  $F_{ST}$  (Nei, 1973). Wright's equation only holds true for 2 alleles so  $G_{ST}$  is recommended for multiple alleles; equivalent to the weighted average of  $F_{ST}$  across all alleles (Weir & Cockerham, 1984). AFLP analysis effectively considers band presence and absence as 2 alleles so  $F_{ST}$  and  $G_{ST}$  are both appropriate. However, unlike  $F_{ST}$ ,  $G_{ST}$  can be applied to more than 2 alleles per locus and to more than 2 populations, which can be of unequal size, which makes it more suitable for multiple sub-populations. It is also claimed that  $H$  is robust to ploidy, the level of mutation,

selection, and migration and the method of reproduction (Nei, 1973; Crow & Aoki, 1984). Due to differences in the calculation method  $F_{ST}$  and  $G_{ST}$  do give different estimates of differentiation, which must be considered when comparing studies.

#### 4.4.4.5 Moment $F_{ST}$ (Hill and Weir, 2004)

Hill and Weir (2004) suggested a moment-based method of estimating  $F_{ST}$  directly from the distribution of genotype frequencies, using the mean and variance of the observed frequency of null homozygotes per locus. This approach assumes Hardy Weinberg equilibrium, independent loci (linkage equilibrium), zero mutation rate and equal genetic distance between populations, which implies an island model. Rather than using the variance of estimated allele frequencies as in the Lynch and Milligan method, using variance within and between genotype frequencies was found to be more robust to low frequencies of null homozygotes. The method also performed better compared to calculations of genetic distance measures based on allele frequencies estimated using the square root method. A comparison of the square root and Hill and Weir procedures applied to AFLP data for groups of pig breeds with high and low polymorphism by Foulley *et al.* (2006) confirmed that the Hill and Weir method is more accurate in the case of low heterozygosity. However, according to Bonin *et al.* (2007) this method is not widely used and it is not implemented in any relevant software.

#### 4.4.4.6 Lynch & Milligan $F_{ST}$ (1994)

$$H_i = 2q_i(1 - q_i) + 2 \text{var}(q_i)$$

Where  $q$  is the frequency of the null allele for the  $i$ th locus. Lynch & Milligan (1994) proposed calculating unbiased  $H$ ,  $F$  and  $D$  statistics by including the variance of the frequency of null alleles in the calculation. Lynch & Milligan calculations of  $H$ ,  $F$  and  $D$  do not necessarily have to be based on allele

frequencies derived by their estimation method. In the software AFLP-SURV Vekemans *et al.* (2002) have included options to estimate allele frequencies using square root and Bayesian methods but not Lynch & Milligan; whereas their  $F$ ,  $H$  and  $D$  calculations do use the Lynch & Milligan method of including variance.

Lynch & Milligan also recommended restricting analysis to AFLP loci with a null homozygote frequency greater than  $3 /$  the number of samples. For 150 samples this would be 0.02, so bands with  $\leq 3$  absences would be excluded from the analysis. The result is a loss of information, bias in the choice of loci for analysis and strong effects of sample size, which could affect estimates of genetic diversity and differentiation (Isabel *et al.*, 1999; Zhivotovsky, 1999). Comparisons of methods including and excluding such loci by (Kraus 2000) revealed only a low level of bias. This method has only been used in a few papers (reviewed in Nybom & Bartish, 2000) and is not implemented in AFLP-SURV.

#### 4.4.4.7 $\pi$ Nucleotide diversity

$$\pi = \sum_{ij} x_i x_j \pi_{ij}$$

Where  $x_i$  and  $x_j$  are the respective frequencies of the  $i$ th and  $j$ th sequences in the population and  $\pi_{ij}$  is the number of nucleotide differences per site between the  $i$ th and  $j$ th sequences. Nei and Li's (1979) measure of nucleotide diversity is the average number of differences per nucleotide site between two randomly chosen DNA sequences. This is analogous to estimating genetic diversity from allele frequency data. It was originally proposed to analyse restriction enzyme fragments of mitochondrial DNA but has been adapted for dominant markers such as RAPD (Borowski, 2001) and AFLP (Innan *et al.*, 1999).

Borowsky's (2001) method of calculating  $\pi$  is band based:

$$\pi = \frac{3\Phi_e}{4m}$$

where  $\Phi_e$  is the proportion of band mismatches (b + c) between two randomly selected individuals, and  $m$  is the number of nucleotides screened. For AFLP data,  $m$  is the total number of bases in both restriction sites, plus the selective extensions. This assumes Hardy Weinberg Equilibrium, an absence of band absence homoplasy and a low value of  $\pi$ .

Clark and Lanigan (1993) suggested a method of calculating  $\pi$  is based on prior estimation of allele frequencies. Innan *et al.*'s (1999) extension of this method for AFLP data assumes Hardy Weinberg Equilibrium, plus a GC content of ~50%. They point out that estimations of nucleotide diversity may not be accurate when there is no information about the frequency of indels and nucleotide substitutions. According to Bonin *et al.* (2007) nucleotide diversity calculations are rarely applied to AFLP data, though it was included in a comparison of methods by Tero *et al.* (2005). The manual for the software AFLP-SURV (Vekemans *et al.*, 2002) states the intention to calculate nucleotide diversity for AFLP data but it has not been implemented yet

#### 4.4.4.8 D Genetic distance (Nei, 1972, 1978)

$$I = \frac{\sum_i^n p_i q_i}{\sqrt{\sum_i p_i^2 \sum_i q_i^2}}$$

$$D = -\ln I$$

where  $p_i$  and  $q_i$  are the frequencies of the  $i$ th alleles in 2 populations. Nei's genetic distance (1972) is defined as the number of allele differences per locus between 2 populations. It is derived from a calculation of normalised genetic

identity ( $I$ ), which is 0 when 2 populations have no alleles in common and 1 when they have the same alleles in the same frequencies. Genetic distance ( $D$ ) is  $-\ln(I)$ . It assumes that all loci have the same neutral rate of mutation, mutation and drift are in equilibrium and effective population sizes are stable.

**Nei's Unbiased genetic distance (1978)** includes a correction for small sample sizes.

**Reynolds' distance (Reynolds *et al.*, 1983)**

$-\ln(F_{ST})$

This distance measure is the equivalent of Nei's distance applied to  $F$  statistics.

#### **4.4.5 Estimating the inbreeding coefficient $F_{IS}$**

Inbred individuals tend to be more homozygous and therefore have more null homozygous band absences than expected by chance. However, the frequency of band absence at a locus can be explained either by the frequency of the null allele or by the level of inbreeding, so it is not possible to estimate both simultaneously (Dasmahapatra *et al.*, 2007). Populations can have different mean  $F_{IS}$  but the same variance (Hartle & Clark, 2007). 2 different approaches have attempted to overcome this circular argument and estimate  $F_{IS}$  from AFLP data.

##### **4.4.5.1 Bayesian $F_{IS}$ Holsinger *et al.* (2002)**

Holsinger *et al.* (2002) developed a Bayesian approach to estimate the inbreeding coefficient  $F_{IS}$ , implemented in the software Hickory (Holsinger & Lewis, 2007). Assuming that  $F_{IS}$  and  $F_{ST}$  are similar across loci and a beta distribution of allele frequencies, it estimates the parameters of this distribution rather than individual allele frequencies and so avoids estimating allele frequencies and  $F_{IS}$  simultaneously. This is one of only a few methods that attempts to estimate the

level of inbreeding and departure from Hardy-Weinberg equilibrium for dominant markers and was thus an important development in the analysis of AFLP data. However, the additional information used to calculate  $F_{IS}$ , derived from differences in genotype distributions at loci with different frequencies, is limited (Hill & Weir, 2004). Estimations of  $F_{IS}$  using this method have been criticised as unreliable (Bonin *et al.*, 2007) and biased (Dasmahapatra *et al.*, 2007) and Holsinger & Lewis themselves advise using it with caution.

#### **4.4.5.2 Iterative $F_{IS}$ Dasmahapatra *et al.* (2007)**

Dasmahapatra *et al.* (2007) suggested an iterative method to estimate  $F_{IS}$ , based on the assumption that wild populations generally have a low proportion of inbred individuals (Marshall *et al.*, 2002 in Dasmahapatra *et al.*, 2007). To solve the circularity problem they simply assumed that at least half of the individuals in a population were outbred ( $F_{IS} = 0$ ) and therefore had allele frequencies in Hardy Weinberg equilibrium. Under this assumption low variance in band absence frequency implied low  $F_{IS}$ , while high variance implied high  $F_{IS}$ . Their FAFLPcalc program is a Visual Basic macro used in Excel, which iteratively searches for the best fit between raw band frequencies and simulated band frequencies with different values of  $F_{IS}$ . So far this method has only been used in 1 other paper (Honnay *et al.*, 2009).

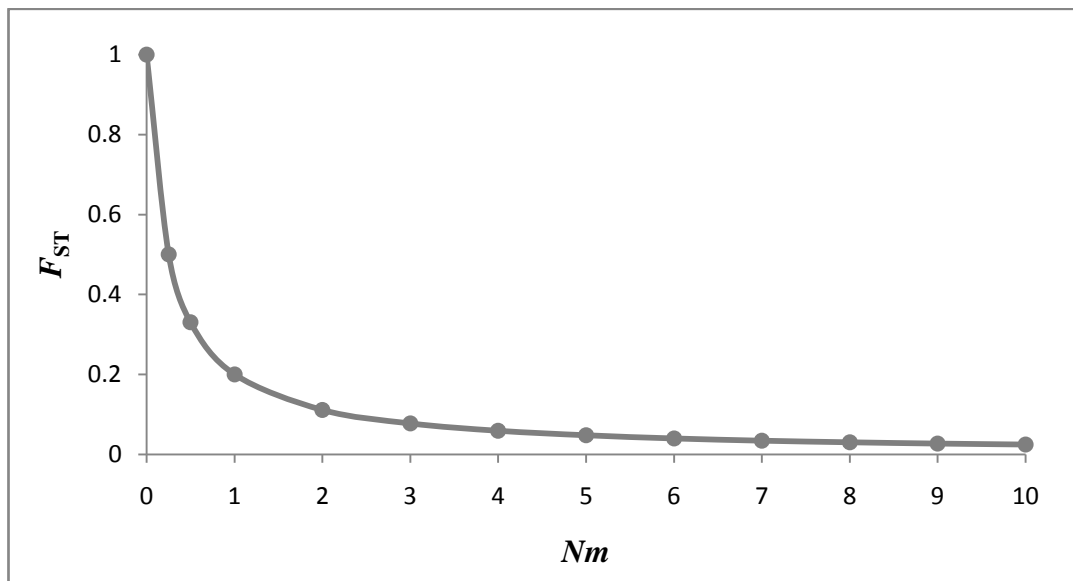
#### **4.4.6 Estimating gene flow $Nm$ from $F_{ST}$**

Where  $N$  is the effective population size and  $m$  is the migration rate,  $Nm$  is the number of migrants per generation exchanged between sub populations, often described as gene flow.

For  $Nm > 0.5$  gene flow is high enough to prevent differentiation due to genetic drift, whereas for  $Nm < 0.5$  sub populations will tend towards fixation of alleles (Wright, 1931). Alternatively structure can be the result of localised selection, on a level that outweighs the homogenising effects of gene flow. If  $Nm > 0.5$

overrides the effects of differentiation due to drift, then if a population has  $Nm > 0.5$  and yet has genetic structure the differentiation is likely to be the result of selection rather than drift.

There is an inverse relationship between gene flow  $Nm$  and the fixation index  $F_{ST}$  as migration between sub populations increases heterozygosity and reduces differentiation (Wright, 1931):



**Figure 4.10: Relationship between fixation index  $F_{ST}$  and gene flow  $Nm$  for an island model of migration.**

$$F_{ST} = \frac{1}{4Nm + 1}$$

Therefore the following equation can be used to estimate gene flow from  $F_{ST}$ :

$$Nm = \frac{1}{4} \left( \frac{1}{F_{ST}} - 1 \right)$$

Estimating  $Nm$  from  $F_{ST}$  has been criticised because the underlying island model and assumptions involved do not reflect real populations (Whitlock & McCauley, 1999). Using  $F_{ST}$  to estimate  $Nm$  only gives an accurate description of the relationship between gene flow and genetic drift if  $F_{ST}$  is robust to the effects of



mutation, selection and number of demes. Crow & Aoki (1984) showed that under a model of island population structure and neutral mutation  $F_{ST}$  is nearly independent of mutation rate and number of demes. Slatkin & Barton (1989) investigated the effects of selection on  $F_{ST}$  and found little difference under different levels of selection, demonstrating that  $F_{ST}$  is robust to violations of island model assumptions. Using  $F_{ST}$  to estimate  $Nm$  is a circular argument, although  $F_{ST}$  has been shown to co-vary with direct estimates of migration (Neigel, 1997) and it is useful for comparison with other studies (Neigel, 2002).

In addition this approach assumes equilibrium between drift and migration in the sub populations, in accordance with Wright's island model (Rousset, 1997). According to Hutchison & Templeton (1999) migration – drift equilibrium is not the case for most natural populations. Malécot (1955) showed that in a stepping stone model of population structure a positive correlation between genetic and geographic distance indicates that populations are in migration – drift equilibrium. According to (Slatkin & Barton, 1989) the relationship between  $F_{ST}$  and  $Nm$  is approximately equivalent in stepping stone and island models. A significant positive correlation between genetic and geographic distance according to a Mantel test could therefore be considered evidence for equilibrium under either model, in which case the equation based on F statistics would be appropriate.

Slatkin & Barton (1989) reviewed 3 methods for estimating the level of gene flow in a population:  $F_{ST}$ , maximum likelihood and rare alleles. The rare alleles method is based on the fact that frequency of private alleles, defined as AFLP bands that only occur in a single sub population sample, should be inversely proportional to gene flow (Slatkin, 1985).

Slatkin & Barton compared these methods using simulated data and found that  $F_{ST}$  and rare alleles gave similar results and showed comparable sensitivity to different levels of selection and population structure, whereas maximum likelihood methods gave biased results when a low number of sub populations were sampled. Due to the practical difficulty in accurately estimating rare alleles for small samples of dominant markers Waples (1987) and Slatkin & Barton (1989) recommended estimating gene flow from  $F_{ST}$ .

#### 4.4.7 $F_{ST}$ simulations to identify loci under selection

Surveying a large number of AFLP loci allows identification of outlier loci that may be of interest (Luikart *et al.*, 2003). Alleles for an adaptive trait under selection are expected to have a higher frequency in a population compared to neutral alleles. Genetic differentiation between sub populations is therefore expected to be higher for loci under disruptive selection. It is possible to identify loci that are potentially under divergent selection by comparing heterozygosity ( $H$ ) within sub populations and genetic differentiation ( $F_{ST}$ ) between populations with a neutral model of evolution. The distribution of  $F_{ST}$  as a function of heterozygosity is relatively robust to population structure, demography and mutation rate (Beaumont & Nichols, 1996; Beaumont, 2005).

The software DFDIST (Beaumont & Nichols, 1996) simulates neutral loci in a subdivided population according to the symmetrical infinite island model, with equilibrium between migration and drift (Wright, 1951). The distribution of  $F_{ST}$  for the empirical data is compared with the simulated neutral  $F_{ST}$  distribution to identify outlier loci. Loci with very low or high  $F_{ST}$ , which fall outside the neutral distribution, are considered to be candidates for selection.

The success of DFDIST in identifying loci under selection depends on the number of such loci in the genome and their average effects, the estimate of genetic differentiation used and the critical probability chosen to detect outliers (Cabellero *et al.*, 2008).

#### 4.4.8 Linkage disequilibrium

Genetic linkage is the non-independent assortment of alleles or markers due to proximity on the same chromosome. In population genetics linkage disequilibrium is the non-random association of alleles or markers at different loci (Luikart *et al.*, 2003). This may be attributable to linkage or to loci on different chromosomes that are functionally related or subject to the same selection pressure. Stabilising selection can cause negative linkage disequilibrium, with loci associated less than

expected by chance, while disruptive selection can increase positive linkage disequilibrium, with loci associated more than expected by chance (Walsh & Lynch, 2007). Recombination in random mating reduces linkage disequilibrium in successive generations, so high disequilibrium can signify a recent bottleneck. However, disequilibrium due to a high number of negative associations in a sample indicates cryptic population subdivision or admixture (Slate & Pemberton, 2007).

Linkage disequilibrium can be measured using contingency tables as the difference between observed and expected distribution of band frequencies (Excoffier *et al.*, 2005). Pairs of AFLP markers show significant linkage disequilibrium if they occur together more or less frequently than expected by chance.

Linkage disequilibrium can be used in association mapping to identify groups of linked loci that are associated with phenotypic traits and potentially the subject of disruptive selection.

<b>Measure</b>	<b>min</b>	<b>max</b>
Shannon	0	lnS
$H$	0	0.5
$F_{ST}, G_{ST}, \Phi_{ST}$	0	1
$F_{IS}$	-1	1
Distance measures	0	1
$\pi$	0	1
$Nm$	0	$25 \times 10^6$

**Table 4.7: Summary of scales of measurement for population level statistics for dominant data**

## 4.5 Population level results

A comprehensive range of estimations of population level diversity, differentiation and distance were calculated for the total sample population of 150 individuals (T), divided into 5 sub populations of 30 individuals for each of the 5 study sites H, K, D, C and P (Chapter 2, Table 2.1). The results of different methods of calculating similar measures of diversity and differentiation were compared and contrasted to assess their suitability for use with dominant AFLP data.

### 4.5.1 Software for population level analyses

There are a large number of free software packages for genetic data analysis, (reviewed by Excoffier & Heckel, 2006; Bonin *et al.*, 2007). A survey of the available software found the following packages to be suitable for the calculations of population level diversity and differentiation required by this study:

	<b>Authors</b>	<b>Available from</b>
<b>Popgene</b>	Yeh <i>et al.</i> (1999)	<a href="http://www.ualberta.ca/~fyeh/index.htm">http://www.ualberta.ca/~fyeh/index.htm</a>
<b>AFLP-SURV</b>	Vekemans <i>et al.</i> (2002)	<a href="http://www.ulb.ac.be/sciences/lagev/aflp-surv.html">http://www.ulb.ac.be/sciences/lagev/aflp-surv.html</a>
<b>Hickory</b>	Holsinger & Lewis (2007)	<a href="http://darwin.eeb.uconn.edu/hickory/software.html">http://darwin.eeb.uconn.edu/hickory/software.html</a>
<b>AFLPcalc</b>	Dasmahapatra <i>et al.</i> (2007)	<a href="http://www.ucl.ac.uk/taxome/kanchon/">www.ucl.ac.uk/taxome/kanchon/</a>
<b>Arlequin</b>	Excoffier <i>et al.</i> (2005)	<a href="http://cmpg.unibe.ch/software/arlequin3/">http://cmpg.unibe.ch/software/arlequin3/</a>
<b>DFDIST</b>	Beaumont & Nichols (1996)	<a href="http://www.rubic.rdg.ac.uk/cgi-bin/MarkBeaumont/">http://www.rubic.rdg.ac.uk/cgi-bin/MarkBeaumont/</a>

**Table 4.8: Software used for population level analyses**

Some have been specifically adapted to accommodate dominant data, e.g. Popgene, while others allow dominant data to be analysed using the settings for haploid data, e.g. Arlequin. Other software packages tested were less successful and either would not run at all under Microsoft Windows Vista, e.g. Genepop (Raymond & Rousset, 1995), or could not be run without crashing, e.g. PCO (Anderson, 2003).

#### 4.5.2 Shannon index of phenotypic diversity

While it is not recommended for use with AFLP data, the Shannon index of phenotypic diversity  $S$  (Shannon, 1948) was calculated using Popgene, for comparison with other studies. This is a band frequency based measure of diversity.

	$S$	s.d.
<b>H</b>	0.070	0.174
<b>K</b>	0.083	0.187
<b>D</b>	0.135	0.231
<b>C</b>	0.165	0.251
<b>P</b>	0.100	0.200
<b>T</b>	0.175	0.251

**Table 4.9: Shannon index of phenotypic diversity  $S$  for 5 sub populations and the total population of 150 individuals (T).**

The results followed the same order of diversity for the 5 sites as individual level analyses,  $T > C > D > P > K > H$ , with sites C and D being the most diverse, while copper tolerant sites H and K had much lower diversity.

Kruse *et al.* (2003) used the Shannon index as a measure of diversity in an RAPD study of sibling species in *Scoloplos armiger*.  $S$  was much higher, ranging from 0.37 to 1.59 within sites. They also used the Shannon index to partition diversity within and between sites and found similar high differentiation for the Shannon index  $H_{ST} = 0.89$  and AMOVA  $\Phi_{ST} = 0.81$ . As  $\Phi_{ST}$  is usually higher than  $F_{ST}$  or  $G_{ST}$  (Bonin *et al.*, 2007 and the present study), the even higher  $H_{ST}$  appears to be the least useful of the band frequency based population level statistics.

#### 4.5.3 $\Phi_{ST}$ AMOVA

The other band based method commonly used in population studies with dominant data is Analysis of MOlecular VAriance (Excoffier *et al.*, 1992), which

partitions variance within and between sub populations to give an estimate of differentiation between the sub populations,  $\Phi_{ST}$ . AMOVA was performed for the 5 sub populations using Arlequin (Excoffier *et al.*, 2005) to measure genetic diversity and differentiation.

A nested AMOVA of 3 hierarchical levels of population structure was also performed, which partitioned the variance between sub populations within groups, between groups and between sub populations. The variance components were then used to calculate  $\Phi$  statistics of differentiation;  $\Phi_{GT}$  between groups within the total population,  $\Phi_{SG}$  between sub populations within groups and  $\Phi_{ST}$  between sub populations within the total population. Significance was based on non-parametric permutation tests with 1000 permutations.

For all 5 sub populations  $\Phi_{ST} = 0.436$  (d.f. = 4,145;  $p < 0.001$ ), with the variation partitioned 56.4% within populations and 43.6% between populations.

	<b>Group 1</b>	<b>HK</b>	<b>HK</b>	<b>HKD</b>
	<b>Group 2</b>	<b>CP</b>	<b>DCP</b>	<b>CP</b>
<b>% Variation between groups</b>		50.75	29.00	42.82
<b>% Variation between sub pops within groups</b>		8.57	21.16	10.46
<b>% Variation within sub pops</b>		40.68	49.83	46.72
<b>Variance between groups (Va)</b>		10.188	5.126	8.073
<b>Variance between sub pops within groups (Vb)</b>		1.721	3.741	1.972
<b>Variance within sub pops (Vc)</b>		8.166	8.808	8.808
<b>Total variance (Vt)</b>		20.076	17.674	18.853
<b><math>\Phi_{GT}</math> Between groups within total pop (Va/Vt)</b>		0.508	0.290	0.428
<b><math>\Phi_{SG}</math> Between sub pops within groups (Vb/Vb+Vc)</b>		0.174	0.298	0.183
<b><math>\Phi_{ST}</math> Between sub pops within total pop (Va+Vb/Vt)</b>		0.593	0.502	0.533

**Table 4.10: Nested AMOVA for sub populations divided into 2 groups: tolerant sites H and K against non-tolerant sites C and P; mixed site D grouped with the non tolerant sites and with the tolerant sites. Percentage variation, variance components and  $\Phi$  statistics of differentiation were calculated in Arlequin with a hierarchy of sub populations, groups and total population. According to permutation tests with 1000 permutations the variance component Va and  $\Phi_{GT}$  between groups was not significant in all 3 cases ( $p > 0.1$ ) but all other values were significant ( $p < 0.001$ ).**

The non-significant value for the variance component and  $\Phi$  statistic between groups can be explained by the small number of demes in each group. The probability of obtaining a value more extreme than the observed value by chance was  $p < 0.001$  in all cases but the probability of obtaining a value equal to the observed value by chance was higher due to the use of permutation tests on small groups, so  $P(\text{rand. value} \geq \text{obs. value})$  was not significant (Excoffier *et al.*, 2005). This is a general problem with performing nested AMOVA on small groups. The other variance components were significant and the  $\Phi$  statistics did follow the same hierarchical rule as Wright's  $F$  statistics, where  $1 - \Phi_{ST} = (1 - \Phi_{SG})(1 - \Phi_{GT})$ . While the non-significant results should be viewed with caution, the test can be considered informative to some extent. However, calculating statistics of genetic differentiation based on partitioning the variance gave slightly different values for  $\Phi_{ST}$  between the 5 sub populations depending on the hierarchical grouping.

There was high (non-significant) genetic differentiation between groups of tolerant and non-tolerant sites. Groups of the 2 tolerant and 2 non-tolerant sites, without the mixed site, had the highest variation between groups and the least variation between sub populations within groups, with differentiation between groups ( $\Phi_{GT} = 0.508$ ) accounting for most of the differentiation between sub populations ( $\Phi_{ST} = 0.593$ ). There was greater variation between groups than within sub populations, whereas when the mixed site was included there was greater variation within sub populations than between groups. There was less difference between the 2 groups and more difference between sub populations within groups when mixed site D was grouped with non-tolerant sites C and P than when it was grouped with H and K, which indicates that site D is more similar to the tolerant sites.

Johnson *et al.* (2005) found comparable results between species in a plant genus, with 35.7% of the total variation between species and  $\Phi_{ST} = 0.615$ . A hierarchical AMOVA on different spatial scales conducted on the sea anemone *Nematostella vectensis* by Reitzel *et al.* (2007) partitioned the variance 24.9% between regions,

45.2% between populations within regions and 29.8% between sub populations within populations.

Because AMOVA is a band based analysis of genotype frequencies the results should not be compared with allele frequency based estimations of genetic differentiation such as  $F_{ST}$  (Excoffier *et al.*, 2005). To illustrate this difference and investigate the extent of deviation between estimates of genetic diversity and differentiation in general a range of methods were compared.

#### 4.5.4 Comparison of genetic differentiation measures

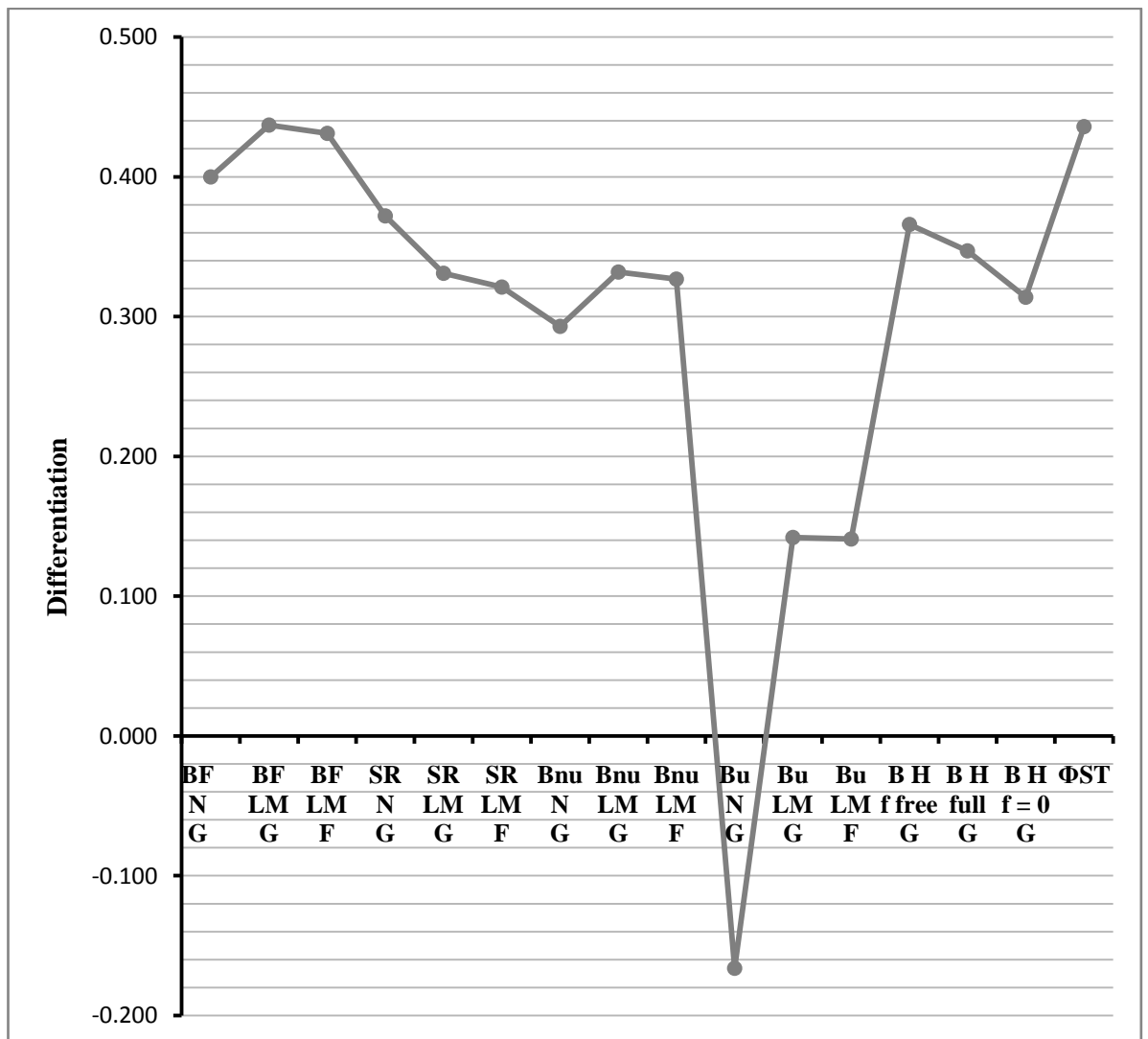
A number of different measures and calculation methods can be used to estimate genetic diversity and differentiation (Figure 4.1). Values of genetic differentiation measures  $G_{ST}$ ,  $F_{ST}$  and  $\Phi_{ST}$  based on different genotype or allele frequency estimation methods were compared. There are discrepancies in the results of these estimations, both between different measures and between values of the same measure based on different allele frequency and differentiation calculation methods, which affects comparisons of measurements between studies. To assess the extent of this difference 16 estimations of differentiation were obtained for 150 individuals in 5 sub populations, according to the following methods:

	<b>Allele frequency estimation method</b>	<b>Calculation method</b>	<b>Software</b>
<b>BF N</b>	Band frequency	Nei (1973)	spread sheet
<b>BF LM</b>	Band frequency	Lynch & Milligan	AFLP-SURV
<b>SR N</b>	Square root	Nei (1973)	Popgene
<b>SR LM</b>	Square root	Lynch & Milligan	AFLP-SURV
<b>Bnu N</b>	Bayesian (Zhivotovsky), non-uniform	Nei (1973)	spread sheet
<b>Bnu LM</b>	Bayesian (Zhivotovsky), non-uniform	Lynch & Milligan	AFLP-SURV
<b>Bu N</b>	Bayesian (Zhivotovsky), uniform	Nei (1973)	spread sheet
<b>Bu LM</b>	Bayesian (Zhivotovsky), uniform	Lynch & Milligan	AFLP-SURV
<b>B H <math>f</math> free</b>	Bayesian (Holsinger)	Holsinger, $f$ free model	Hickory
<b>B H full</b>	Bayesian (Holsinger)	Holsinger, full model	Hickory
<b>B H <math>f = 0</math></b>	Bayesian (Holsinger)	Holsinger, $f = 0$ model	Hickory
<b><math>\Phi_{ST}</math></b>	AMOVA	AMOVA	Arlequin

**Table 4.11: Abbreviations for allele frequency and genetic differentiation estimation methods and software used in analyses.**



Where possible both  $G_{ST}$  and  $F_{ST}$  were calculated using the same method. These methods were chosen because they are freely available in software and therefore reflect the range of statistics that are reported in the literature, although 3 calculations of  $G_{ST}$  according to Nei (1973) were done by hand, based on allele frequencies produced by AFLP-SURV, in order to compare them with Lynch & Milligan calculations for the same allele frequencies. (AFLP-SURV uses the Lynch & Milligan method for  $G_{ST}$  but not for allele frequency estimations).



**Figure 4.11: Estimates of  $G_{ST}$  (G),  $F_{ST}$  (F) and  $\Phi_{ST}$  for 150 individuals in 5 subpopulations, calculated using 16 different methods (Table 4.11).**

All 4 estimates of  $F_{ST}$  were slightly lower than  $G_{ST}$  for the same method. According to Holsinger & Lewis (2007)  $F_{ST}$  should be larger than  $G_{ST}$  because it includes drift over time rather than just among contemporary populations.

Estimates of differentiation were highest for the band based measures:  $G_{ST}$  and  $F_{ST}$  with allele frequency estimates based on band frequencies (BF), plus band frequency variance (AMOVA  $\Phi_{ST}$ ).

The estimates for the Bayesian method with uniform priors (Bu) were all very low and negative for  $G_{ST}$  calculated according to Nei, with  $H_T$  lower than mean  $H_S$ , which is not possible. The Nei calculation used allele frequencies from AFLP-SURV, which produced a positive result for Lynch & Milligan calculations based on the same allele frequencies. It is possible that the Lynch & Milligan correction solved the problem but examination of the allele frequencies suggested an error in data reported by the software. The Bu N method was not included in further analyses.

Other calculations according to Nei were lower than for Lynch & Milligan for the band frequency and Bayesian methods but higher for square root. There was less difference between the square root and Bayesian non uniform values for Lynch & Milligan, which suggests that their correction may have a smoothing effect across methods, but in general there was little difference between all SR and Bnu methods. For the Holsinger Bayesian method  $G_{ST}$  increased with increasing values of  $F_{IS}$  included in the three models:  $f=0 < \text{full model} < f \text{ free}$ , with the full model  $G_{ST}$  similar to that for the Bayesian non uniform Lynch & Milligan method.

A comparison of methods by Bonin *et al.* (2007) based on AFLP data for 13 plant species had comparable results, with high values for the band based methods, very low and in 2 cases negative values for Bayesian uniform Lynch & Milligan and similar values for Bayesian non uniform Lynch & Milligan and Holsinger full model. However, they generally had the highest results for square root  $G_{ST}$  from

Popgene (SR N G), and therefore a larger difference between  $G_{ST}$  and  $F_{ST}$  compared to this study. Most of their species had lower  $F_{ST} < 0.25$ .

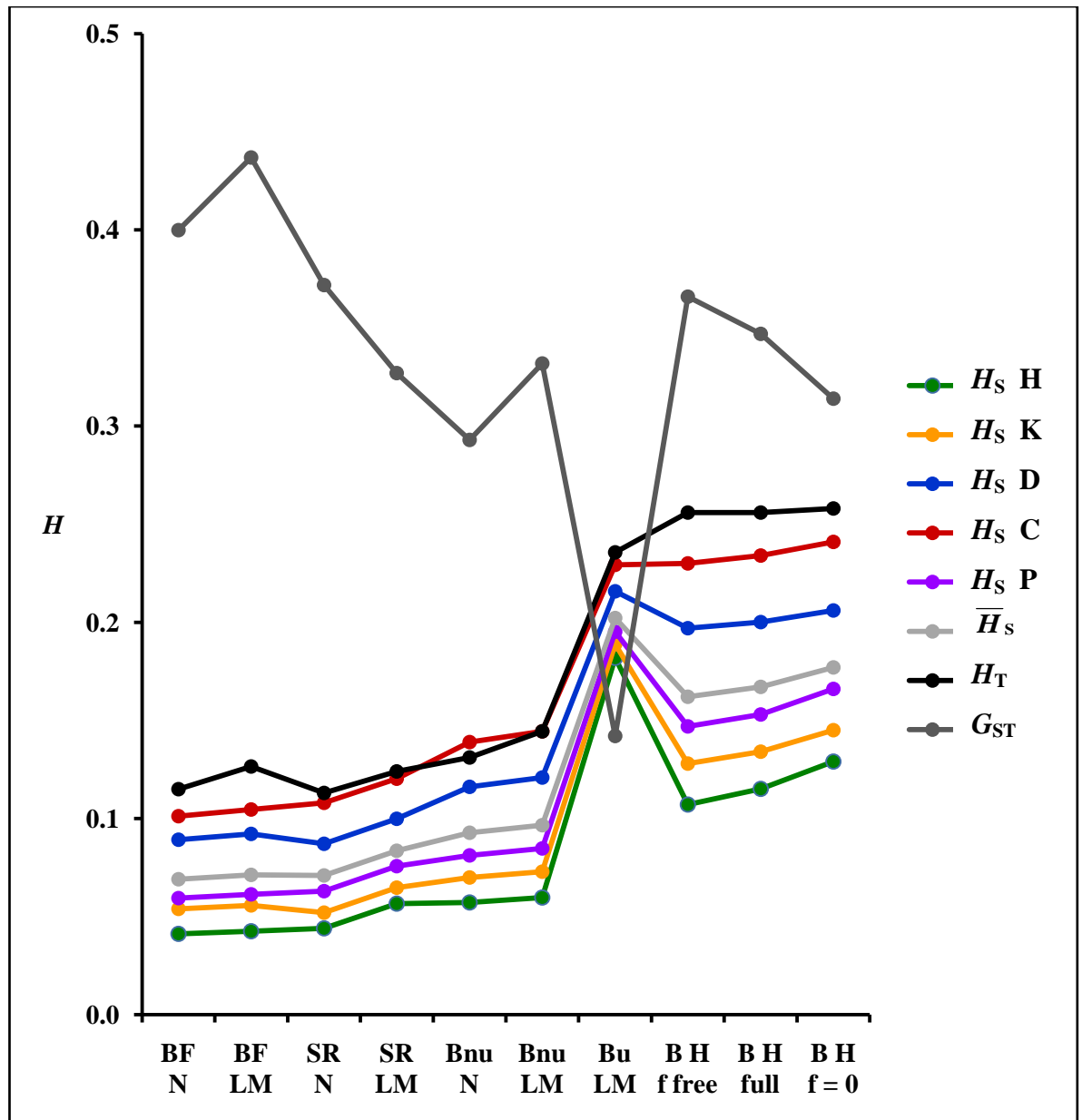
Based on these results and the benefits of  $G_{ST}$  proposed by Crow & Aoki (1984),  $G_{ST}$  was selected over  $F_{ST}$  to perform a more detailed analysis of heterozygosity within and between the 5 sub populations.

#### 4.5.5 Estimation of genetic diversity and differentiation

To analyse genetic diversity and differentiation in the 5 sub populations heterozygosity  $H_S$ , total heterozygosity  $H_T$  and the differentiation estimate  $G_{ST}$  were calculated using 3 different methods, based on 4 different allele frequency estimation methods, with 3 different models for the Holsinger procedure (Table 4.11).

		BF	BF	SR	SR	Bnu	Bnu	Bu	B H	B H	B H
		N	LM	N	LM	N	LM	LM	$f$ free	full	$f=0$
$H_S$	H	0.041	0.043	0.044	0.057	0.057	0.060	0.182	0.107	0.115	0.129
$H_S$	K	0.054	0.056	0.052	0.065	0.070	0.073	0.189	0.128	0.134	0.145
$H_S$	D	0.089	0.092	0.087	0.100	0.116	0.121	0.216	0.197	0.200	0.206
$H_S$	C	0.101	0.105	0.108	0.120	0.139	0.144	0.229	0.230	0.234	0.241
$H_S$	P	0.059	0.061	0.063	0.076	0.081	0.085	0.195	0.147	0.153	0.166
$\overline{H_S}$		0.069	0.071	0.071	0.083	0.093	0.096	0.202	0.162	0.167	0.177
$H_T$		0.115	0.127	0.113	0.124	0.131	0.144	0.236	0.256	0.256	0.258
$G_{ST}$		0.400	0.437	0.372	0.331	0.293	0.332	0.142	0.366	0.347	0.314

**Table 4.12:  $H_S$  for 5 sub populations,  $H_T$  and  $G_{ST}$  calculated using different methods.**



**Figure 4.12:**  $H_S$  for 5 sub populations,  $H_T$  and  $G_{ST}$  calculated using different methods.  $H$  is measured on the scale 0 – 0.5, while  $G_{ST}$  is a proportion measured on the scale 0 – 1.

As in figure 4.11 the band frequency based methods over estimated  $H_T$  and therefore  $G_{ST}$ . The Bayesian method with uniform priors had very high  $H_S$  for all sites compared to  $H_T$  and thus low  $G_{ST}$ .  $H_S$  values for The Holsinger methods had a wider range and were much higher but the proportional relationship between mean  $H_S$  and  $H_T$  was similar to the Square root and Bayesian non uniform methods, giving a similar  $G_{ST}$  value.

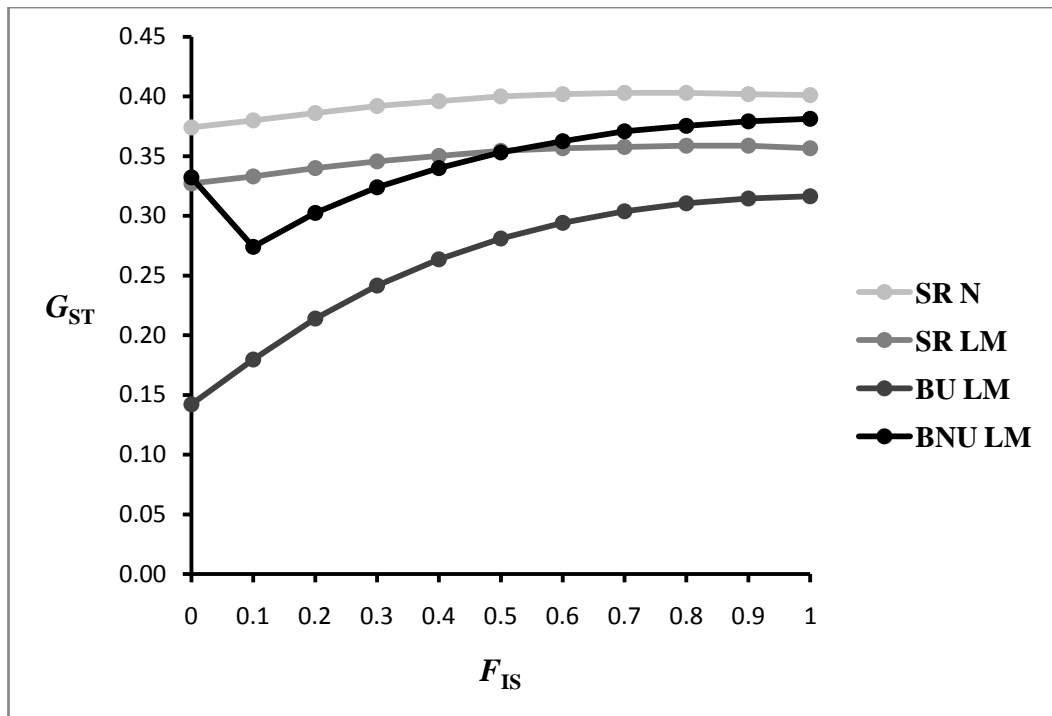
All methods showed the same relative differences in  $H_S$  between the sites and the same order of diversity as other analyses, with site C having very high diversity compared to  $H_T$ . For the Holsinger Bayesian method  $H_S$  decreased with increasing values of  $F_{IS}$  included in the three models:  $f = 0 > \text{full model} > f \text{ free}$ . Including  $F_{IS}$  in the full model had a greater effect on H, K and P than on C and D compared to the  $f = 0$  model.

#### 4.5.6 Including $F_{IS}$ in allele frequency estimates

Known values of the inbreeding coefficient  $F_{IS}$  can be included in allele frequency estimates, using the method of Chong *et al.* (1994). To examine the effects of including  $F_{IS}$  in population level statistics  $G_{ST}$  and  $F_{ST}$  were calculated for 150 individuals in 5 sub populations, for a range of  $F_{IS}$  values from 0 to 1. 4 different methods from Table 4.11 were used, due to availability in Popgene (SR N) and AFLP-SURV (SR LM, Bu LM, Bnu LM), which both include  $F_{IS}$  according to the method of Chong *et al.*. As  $G_{ST}$  is the proportion of  $H$  in the total population ( $H_T$ ) that occurs between rather than within sub populations ( $H_S$ ) it should increase steadily with increasing reduction in  $H$  in sub populations ( $F_{IS}$ ).

$F_{IS}$	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
<b>SR N</b>	0.374	0.380	0.386	0.392	0.396	0.400	0.402	0.403	0.403	0.402	0.401
<b>SR LM</b>	0.327	0.333	0.340	0.345	0.350	0.354	0.356	0.358	0.359	0.359	0.357
<b>Bu LM</b>	0.142	0.180	0.214	0.241	0.264	0.281	0.294	0.304	0.310	0.315	0.316
<b>Bnu LM</b>	0.332	0.274	0.302	0.324	0.340	0.353	0.362	0.371	0.375	0.379	0.381

**Table 4.13:  $G_{ST}$  calculated for a range of  $F_{IS}$  values, using 4 different methods.**



**Figure 4.13:**  $G_{ST}$  calculated for a range of  $F_{IS}$  values, using 4 different methods.

Estimates of  $F_{ST}$  using the same methods matched the pattern for  $G_{ST}$  exactly but were slightly lower (not shown). As in figure 4.11  $G_{ST}$  based on the square root allele frequency estimation method is higher calculated according to Nei (1973) than with the Lynch & Milligan correction and relatively low for the Bayesian method with uniform priors. Including  $F_{IS}$  had a much greater effect on the two Bayesian methods than on the square root methods, although for values of  $F_{IS}$  considered to be high ( $\sim 0.3$ ), the Bayesian results are still lower than the square root results.

In general  $G_{ST}$  increased with increasing  $F_{IS}$  as expected. There appears to be an anomaly at  $F_{IS} = 0$  for the Bayesian method with non uniform priors (BNU LM), confirmed by the fact that for  $F_{IS} = 0.001$   $G_{ST} = 0.259$ . The comparison of methods applied to 13 plant species by Bonin *et al.* (2007) had a similar anomalous result with this method, but only for 1 species with relatively high  $F_{ST}$ . The fact that it did not happen for the other 2 methods in AFLP-SURV, including another Bayesian method, suggests a software problem with the BNU LM method.

$G_{ST}$  for both square root methods curves down slightly towards  $F_{IS} = 1$ , which also happened in Bonin *et al.*'s comparison for some species, while others followed slightly u shaped or irregular patterns. The fact that Popgene and AFLP-SURV produced comparable results for the square root method suggests that this phenomenon is not a software problem. It may be an artefact of the Chong *et al.* method of introducing  $F_{IS}$  into allele frequency estimates.

#### 4.5.6.1 Bayesian $F_{IS}$ (Holsinger *et al.*, 2002)

The MCMC Bayesian approach of Holsinger *et al.* (2002), implemented in the software Hickory (Holsinger & Lewis, 2007) was used to calculate the inbreeding coefficient,  $f$ . Holsinger *et al.* use Weir & Cockerham's (1984) notation, where  $f \approx F_{IS}$  and  $\theta \approx F_{ST}$ . In Hickory v1.1 they give 3 estimates of fixation:  $\theta^I$ , which is equivalent to Wright's  $F_{ST}$ ,  $\theta^{II}$ , which is similar to Nei's  $G_{ST}$ , and their own Bayesian  $G_{STB}$ .  $\theta^I$  should be larger than  $\theta^{II}$  because it includes drift over time rather than just among contemporary populations.

Hickory fits 4 models of the distribution of allele frequencies across loci to the data: a full model, an  $f = 0$  model with zero inbreeding (Hardy Weinberg equilibrium), a  $\theta = 0$  model with zero differentiation between populations and an  $f$  free model. It produces an estimate of  $f$  and in the full model estimates of  $f$  and  $\theta$  affect each other.

The Deviance Information Criterion (DIC) (Spiegelhalter *et al.*, 2002) can be used to choose a model.  $\bar{D}$  is a measure of how well the model fits the data and  $pD$  is an estimate of the number of parameters required to do this. The best model has the lowest DIC and differences in DIC between the models reveal information about the distribution of genetic diversity.

Hickory excludes monomorphic loci from the analysis. Of the 247 loci the 112 polymorphic loci were included in the analysis while the 135 monomorphic loci were excluded.

Analyses were run using the default MCMC sampler values (burn-in = 5000, number of samples = 25000, thinning factor = 5) and the default uniform  $\alpha = 1$  and  $\beta = 1$  parameters for the beta prior probability distribution. The results of replicate runs only varied by  $\sim 0.000001$ , which shows that the Markov chain was converging.

	$\overline{D}$	$\hat{D}$	pD	DIC
<b>full model</b>	1428	1173	255	1683
$f = 0$	1424	1087	338	1762
$\theta = 0$	6119	6020	99	6218
$f$ free	1526	1144	382	1908

**Table 4.14: Model choice criteria for 4 models produced using the Bayesian approach in Hickory (Holsinger & Lewis, 2007).**

The full model had the lowest DIC value and was therefore considered to be the best model. The difference in DIC values between the full model and the  $f = 0$  model of 79 or  $\sim 5\%$  should be evidence of inbreeding. However, most of this difference was accounted for by the difference in pD values, the number of parameters required to fit the model, and the values for  $\overline{D}$  and  $\hat{D}$  are slightly lower for the  $f = 0$  model. According to Holsinger & Lewis this implies that the  $f = 0$  model is as likely as the full model. The very large difference between the full and  $\theta = 0$  models indicates that there are large differences in allele frequency distributions between the 5 populations.

	full model	s.d.	$f = 0$	s.d.	$\theta = 0$	s.d.	$f$ free	s.d.
$\theta^I$	0.571	0.034	0.536	0.021	0.454	0.027	0.592	0.019
$\theta^{II}$	0.406	0.038	0.366	0.021	x	x	0.432	0.022
$H_S$ H	0.115	0.012	0.129	0.006	0.257	0.003	0.107	0.006
$H_S$ K	0.134	0.009	0.145	0.006	0.257	0.003	0.128	0.006
$H_S$ D	0.200	0.007	0.206	0.006	0.257	0.003	0.197	0.007
$H_S$ C	0.234	0.008	0.241	0.005	0.257	0.003	0.230	0.007
$H_S$ P	0.153	0.011	0.166	0.006	0.257	0.003	0.147	0.007
$\overline{H}_S$	0.167	0.008	0.177	0.003	0.257	0.003	0.162	0.004
$H_T$	0.256	0.003	0.258	0.003	0.257	0.003	0.256	0.004
$G_{ST}B$	0.347	0.027	0.314	0.009	x	x	0.366	0.012

**Table 4.15: Population level statistics for 112 polymorphic loci, for 4 models produced using the Bayesian approach in Hickory (Holsinger & Lewis, 2007).**



As expected  $H_S$  was higher for all sub-populations under the  $f = 0$  model than under the full model, which includes the estimate of inbreeding. Sites C and D had higher heterozygosity and less difference in  $H_S$  between the  $f = 0$  model and the full model compared to the other sites, which implies lower inbreeding.

All 3 measures of differentiation were higher for the full model than for the  $f = 0$  model, as the introduction of a coefficient of inbreeding within sub populations meant that more of the calculated  $H_T$  was partitioned between sub-populations.  $\theta^I$  ( $\sim F_{ST}$ ) was larger than  $\theta^{II}$  ( $\sim G_{ST}$ ) (opposite to  $G_{ST}$  /  $F_{ST}$  above). As both of these measures were much larger than  $G_{ST}$  and other measures of differentiation they were not considered in further analyses.

	<b>full model</b>	<b>s.d.</b>	<b><math>\theta = 0</math></b>	<b>s.d.</b>	<b><math>f</math> free</b>	<b>s.d.</b>
<i>f</i> <b>H</b>	0.499	0.291	0.824	0.160	0.502	0.289
<i>f</i> <b>K</b>	0.553	0.297	0.917	0.081	0.497	0.288
<i>f</i> <b>D</b>	0.555	0.292	0.915	0.078	0.504	0.288
<i>f</i> <b>C</b>	0.491	0.276	0.874	0.106	0.501	0.289
<i>f</i> <b>P</b>	0.473	0.301	0.842	0.144	0.510	0.287
<i>f</i>	0.368	0.393	0.924	0.075	0.494	0.291

**Table 4.16: Inbreeding coefficient  $f$  calculated for all 150 individuals and for each sub population of 30 individuals, using the Bayesian approach in Hickory (Holsinger & Lewis, 2007).**

Estimates for sub populations may be unreliable due to small sample size: statistics for the total population were based on 112 polymorphic loci for 150 individuals, whereas sub population estimates were based on between 43 and 90 polymorphic loci for 30 individuals.

As expected the inbreeding coefficients for the sub populations were higher than for the total population. These values did not appear to correspond with the values of  $H_S$  for the 5 sites but  $f$  is applied as a coefficient and the percentage decrease in  $H_S$  between the  $f = 0$  model and the full model was in order  $H > P > K > D > C$ .

	$f$	$H_S$ $f = 0$	$H_S$ full	% decrease
<b>H</b>	0.499	0.129	0.115	10.853
<b>K</b>	0.553	0.145	0.134	7.586
<b>D</b>	0.555	0.206	0.200	2.913
<b>C</b>	0.491	0.241	0.234	2.905
<b>P</b>	0.473	0.166	0.153	7.831

**Table 4.17: Percentage decrease in  $H_S$  between the  $f = 0$  model and the full model for 5 sub populations.**

The inbreeding coefficient 0.368 for the total population was high. However, according to Figure 4.13 this value would not have a great impact on estimates of  $G_{ST}$  if it was included using the Chong method. The difference in  $G_{ST}$  between the 2 Holsinger models  $f = 0$ ,  $G_{ST} = 0.314$  and  $f = 0.368$ ,  $G_{ST} = 0.347$  was closer to the effects of  $F_{IS}$  on  $G_{ST}$  for the Lynch & Milligan square root method than for the other Bayesian methods.

#### 4.5.6.2 Iterative $F_{IS}$ (Dasmahapatra *et al.*, 2007)

The iterative method of Dasmahapatra *et al.* (2007) was also used to estimate  $F_{IS}$ , with the program FAFLPcalc, a Visual Basic macro used in Excel.

	$F_{IS}$
<b>H</b>	-0.121
<b>K</b>	-0.126
<b>D</b>	-0.068
<b>C</b>	-0.041
<b>P</b>	-0.110
<b>Total</b>	-0.007

**Table 4.18: Inbreeding coefficient  $F_{IS}$  calculated for all 150 individuals and for each sub population of 30 individuals, using the Dasmahapatra *et al.* method in AFLPcalc.**

The results were very different to those for the Holsinger method, with negative values indicating more heterozygous individuals than expected under Hardy Weinberg equilibrium and  $F_{IS}$  values for the 5 sites in a completely different order. Higher  $F_{IS}$  in the total population than in sub-populations is unlikely. The discrepancy between the 2 methods of estimating  $F_{IS}$  questions the results.

#### 4.5.7 Genetic distance

Nei's genetic distance (1972) and unbiased genetic distance (1978) were calculated for 150 individuals in 5 sub populations, using Popgene. UPGMA dendrograms were constructed, based on the unbiased (1978) pairwise distances between sub populations, to show the relationships between the populations. A range of values of  $F_{IS}$  were applied according to the method of Chong *et al.* (1994), including the full model  $f$  values for the 5 individual sub populations from Table 4.16, produced using Hickory.

	<b>H</b>	<b>K</b>	<b>D</b>	<b>C</b>	<b>P</b>
<b>H</b>	****	0.984	0.971	0.917	0.885
<b>K</b>	0.016	****	0.982	0.926	0.893
<b>D</b>	0.030	0.018	****	0.961	0.935
<b>C</b>	0.086	0.077	0.040	****	0.981
<b>P</b>	0.122	0.114	0.067	0.020	****

**Table 4.19: Nei's (1972) genetic distance (lower triangle) and genetic identity (upper triangle) between 5 sub populations,  $F_{IS} = 0$ .**

	<b>H</b>	<b>K</b>	<b>D</b>	<b>C</b>	<b>P</b>
<b>H</b>	****	0.985	0.972	0.919	0.886
<b>K</b>	0.015	****	0.984	0.927	0.894
<b>D</b>	0.029	0.017	****	0.962	0.936
<b>C</b>	0.085	0.076	0.038	****	0.982
<b>P</b>	0.121	0.112	0.066	0.018	****

**Table 4.20: Nei's (1978) unbiased genetic distance (lower triangle) and genetic identity (upper triangle) between 5 sub populations,  $F_{IS} = 0$ .**

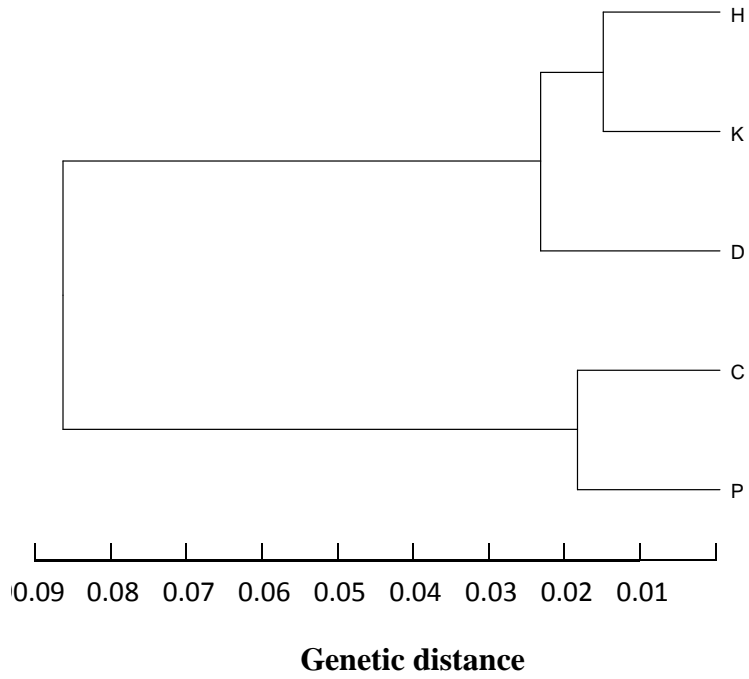
	<b>H</b>	<b>K</b>	<b>D</b>	<b>C</b>	<b>P</b>
<b>H</b>	****	0.983	0.972	0.910	0.878
<b>K</b>	0.018	****	0.986	0.921	0.888
<b>D</b>	0.029	0.014	****	0.957	0.933
<b>C</b>	0.094	0.083	0.044	****	0.985
<b>P</b>	0.131	0.119	0.070	0.015	****

**Table 4.21: Nei's (1978) unbiased genetic distance (lower triangle) and genetic identity (upper triangle) between 5 sub populations, with  $F_{IS}$  from Table 4.16 included for each sub population.**

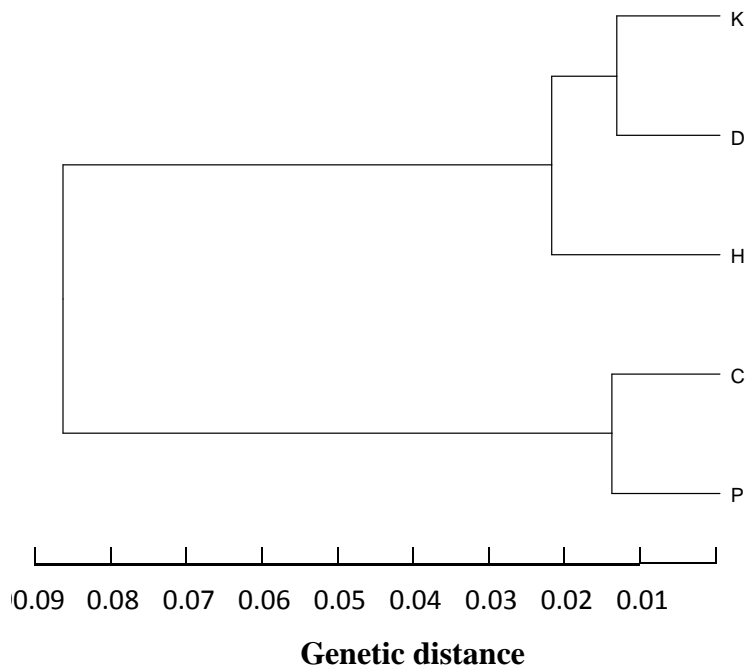
Pairwise distances were very similar for the biased and unbiased distances (Tables 1 and 2), with all distances slightly lower for the unbiased measure. The mean unbiased pairwise distance was  $D = 0.058$ .

According to Thorpe & Solé-Cava (1994) values of Nei's (1978) genetic identity above 0.9 (distance below  $D \approx 0.11$ ) indicate conspecific populations, while identity below 0.8 (distance above  $D \approx 0.22$ ) suggests interspecific differentiation.

In a study of 22 allozyme loci Maltagliati *et al.* (2001) found high unbiased genetic distance ( $D = 0.185$ ) between 2 samples of the nereid polychaete *Perinereis cultrifera*. They suggested that this level of divergence was high enough to indicate sibling species, although it fell in the grey area between the accepted thresholds. The highest unbiased distance in this study,  $D = 0.121$  between polluted site H and clean site P, was almost as high and fell above the threshold for conspecificity.



**Figure 4.14: UPGMA dendrogram based on Nei's (1978) unbiased genetic distance,  $F_{IS} = 0$ .**



**Figure 4.15: UPGMA dendrogram based on Nei's (1978) unbiased genetic distance, with  $F_{IS}$  from Table 1 included for each sub population.**

Figures 4.14 and 4.15 both show the close relationship between the 2 polluted sites and the mixed / intermediate site: H, K and D, and between the 2 clean sites: C and P. This agrees with the individual level cluster analysis (Figure 4.9). Figure 4.14 shows distances under Hardy Weinberg equilibrium,  $F_{IS} = 0$ , while Figure 4.15 includes  $F_{IS}$  values for the 5 individual sub populations. For  $F_{IS} = 0$  (Figure 4.14) the 2 polluted sites H and K had the least genetic distance between them, despite the large geographic distance between these sites. However, with  $F_{IS}$  values for the 5 sub populations included sites K and D were more closely related. The pairwise distance between sub populations decreased for H / D, K / D and C / P and increased for all other pairs, including K / H (Table 4.21). Including the same value of  $F_{IS}$  for each of the 5 sub populations produced the same dendrogram as Figure 2 for  $F_{IS} \geq 0.2$ . To some extent including a coefficient of inbreeding for local sub populations appeared to emphasise genetic differentiation associated with geographic distance, though this did not hold for H / D.

#### 4.5.8 Gene flow between populations

Gene flow  $Nm$  was calculated using the equation of Wright (1951):

$$Nm = \frac{1}{4} \left( \frac{1}{F_{ST}} - 1 \right)$$

Because Wright's F statistics are based on his island model of population structure, using  $F_{ST}$  to calculate gene flow assumes equilibrium between drift and migration. Isolation by distance indicates migration - drift equilibrium. The results of the Mantel test (section 4.3.5) showed a significant positive correlation between genetic dissimilarity and geographical distance ( $r = 0.482$ ,  $p < 0.001$ ). Significant correlations between geographical distance and Nei's unbiased distance, D ( $r = 0.114$ ,  $p < 0.001$ ) and pairwise  $F_{ST}$  between populations ( $r = 0.236$ ,  $p < 0.001$ ) were also positive, although weaker. It could therefore be considered that the migration – drift equilibrium assumption was not violated and the above equation was appropriate.

$Nm$  among all 5 sub populations was calculated for  $F_{ST}$  and  $G_{ST}$ , assuming that  $G_{ST}$  is equivalent to  $F_{ST}$  for AFLP data, derived using the square root method of allele frequency estimation (Table 4.11). For  $F_{ST} = 0.321$   $Nm = 0.529$ , while for  $G_{ST} = 0.372$   $Nm = 0.422$ .

Popgene, which uses the square root method to calculate “ $F_{ST}/G_{ST}$ ”, is the only software for dominant markers that reports an estimate of gene flow. According to Popgene  $Nm = 0.839$ . The manual states that  $Nm$  is calculated from the estimate of  $F_{ST}$  using the method of Slatkin & Barton (1989), who employed Wright’s (1951) equation, as above. However, the results output stated that  $Nm$  was calculated according to McDermott & McDonald (1993), who used the equation:

$$Nm = \frac{1}{2} \left( \frac{1}{F_{ST}} - 1 \right)$$

This form of Wright’s equation, with  $2Nm$  instead of  $4Nm$ , is intended for use in studies of haploid organisms. Popgene offers a choice of calculations for haploid or diploid organisms but uses the same equation for both, so it is likely that there is a bug in the software and the value of  $Nm$  was actually 0.419.

This is comparable with low gene flow associated with low dispersal found in fresh water salamanders (Templeton *et al.*, 1995) and turtles ( $Nm = 0.6$ ) (Souza *et al.*, 2002) inhabiting rivers.

According to Wright (1931) in sub populations with  $Nm > 0.5$ , gene flow overrides the effects of differentiation due to drift, whereas sub populations with  $Nm < 0.5$  will tend towards fixation of alleles. According to Porter (1990)  $Nm > 1$  indicates high gene flow between populations, promoting genetic similarity,  $Nm = 0.5 - 1$  is considered weak gene flow, while for  $Nm 0 - 0.5$  populations are almost completely isolated, with drift far outweighing gene flow.  $Nm \sim 0.5$  for *Nereis diversicolor* is low but only very limited gene flow is required to outweigh drift.  $Nm = 0.5$  is the threshold above which genetic differentiation in a structured

population is likely to be the result of selection rather than drift, so it could be suggested that this is the case based on these results.

Pairwise  $F_{ST}$  was calculated for each pair of populations using Arlequin, which was then used to calculate pairwise estimates of  $Nm$ . Regression of pairwise  $Nm$  values against geographic distance was performed to show the isolation by distance effect associated with a continuous or stepping stone model of gene flow (Slatkin, 1993).

	<b>H</b>	<b>K</b>	<b>D</b>	<b>C</b>	<b>P</b>
<b>H</b>	****	0.730	0.646	0.213	0.112
<b>K</b>	0.255	****	1.462	0.262	0.138
<b>D</b>	0.279	0.146	****	0.627	0.318
<b>C</b>	0.540	0.488	0.285	****	1.887
<b>P</b>	0.691	0.645	0.440	0.117	****

**Table 4.22: Pairwise  $F_{ST}$  (lower triangle) and gene flow  $Nm$  (upper triangle) between 5 sub populations. All pairwise  $F_{ST}$  values were significant,  $p < 0.001$ .**

The pairwise  $F_{ST}$  values have a similar pattern to pairwise genetic distance measured by Nei's D (section 4.5.7), although they most closely resemble the values for D including estimates of the inbreeding coefficient  $F_{IS}$  (Table 4.21, Figure 4.15), with greater differentiation between the 2 tolerant sites H and K than between K and the mixed / intermediate site D, which are closer geographically.

The slope of the regression is a function of dimensionality in the stepping stone model. A slope of -1.0 is expected for a 1 dimensional, linear stepping stone model, while a slope of -0.5 is expected for a 2 dimensional model (Slatkin, 1993). An r value of -0.38 suggests a 2 dimensional model, which may be the case in the radiation of rivers that constitute the Fal estuary.

Black *et al.* (1994) found a similar pattern of gene flow between neighbouring populations in the deep sea tube worm *Riftia pachyptila* inhabiting a linear archipelago of hydrothermal vents ( $r = -0.634$ ).



#### 4.5.9 $F_{ST}$ simulations to identify loci under selection

Population differentiation due to a balance of mutation, drift and migration is characterised by even differentiation across all loci and a spatial pattern of isolation by distance or evidence of a general physical barrier to gene flow, whereas divergence due to divergent selection despite limited gene flow affects a small proportion of loci, with less differentiation in the majority of loci.

The DFDIST package uses simulation to model the distribution of  $F_{ST}$  expected for neutral loci in the absence of selection. The empirical data set is compared with the simulation and loci lying outside the neutral distribution are identified as outliers and thus possibly subject to selection.

DFDIST is a modification of the FDIST program originally written by Beaumont & Nichols (1996), developed specifically for dominant markers. It uses the Bayesian method of Zhivotovsky (1999) to estimate allele frequencies and calculates  $F_{ST}$  according to Weir & Cockerham (1984). There are 4 programs involved: Ddatacal estimates  $H$  and  $F_{ST}$ , Dfdist does the  $F_{ST}$  simulation, pv2 calculates p values and cplot2 gives the probability level quantiles for the simulated  $F_{ST}$  distribution.

**Ddatacal** asks the user to input critical values, plus  $\alpha$  and  $\beta$  parameters for the Bayesian prior probability distribution, and was used with the suggested defaults:

- Critical frequency for the most common allele: 0.999
- P for trimmed mean (weighted by  $H$  between sub populations): 0.3
- Parameters for the Zhivotovsky beta prior distribution: 0.25 0.25

The trimmed mean  $F_{ST}$  excludes the 30% highest and lowest  $F_{ST}$  values for outlier loci, to give an estimate of neutral  $F_{ST}$  (Bonin *et al.*, 2006), which is used as the target average  $F_{ST}$  value in the simulation.

The authors recommend setting the critical frequency to 0.99 to exclude loci where the most frequent allele has a frequency  $\geq 0.99$ . This removed 13 loci with 1

single presence or absence in 150 individuals that had lower than expected  $F_{ST}$ . However, setting the critical frequency for the most common allele to 0.99 gave almost identical values for raw and trimmed mean  $F_{ST}$  of 0.352, so the critical frequency was set to 0.999 to include all 112 loci.

Using these settings the weighted mean  $F_{ST}$  was 0.343 and the trimmed weighted mean was 0.337.

**Dfdist** required 8 user defined parameters to simulate a neutral  $F_{ST}$  distribution:

- Total number of demes: 5
- Number of samples: 5
- Target average Weir & Cockerham  $F_{ST}$ : 0.337
- Sample size: 30
- $\theta$  ( $4Nm\mu$ ) for the metapopulation  $N$ : 0.04
- The number of realisations (loci): 50,000
- Parameters for the Zhivotovsky beta prior distribution: 0.25 0.25
- Maximum allowable frequency across samples of the most common allele: 0.999

Cabellero *et al.* (2008) found that different values of  $\theta$  between 0.004 and 0.1 made no difference to the results, so the suggested default of 0.04 was used.

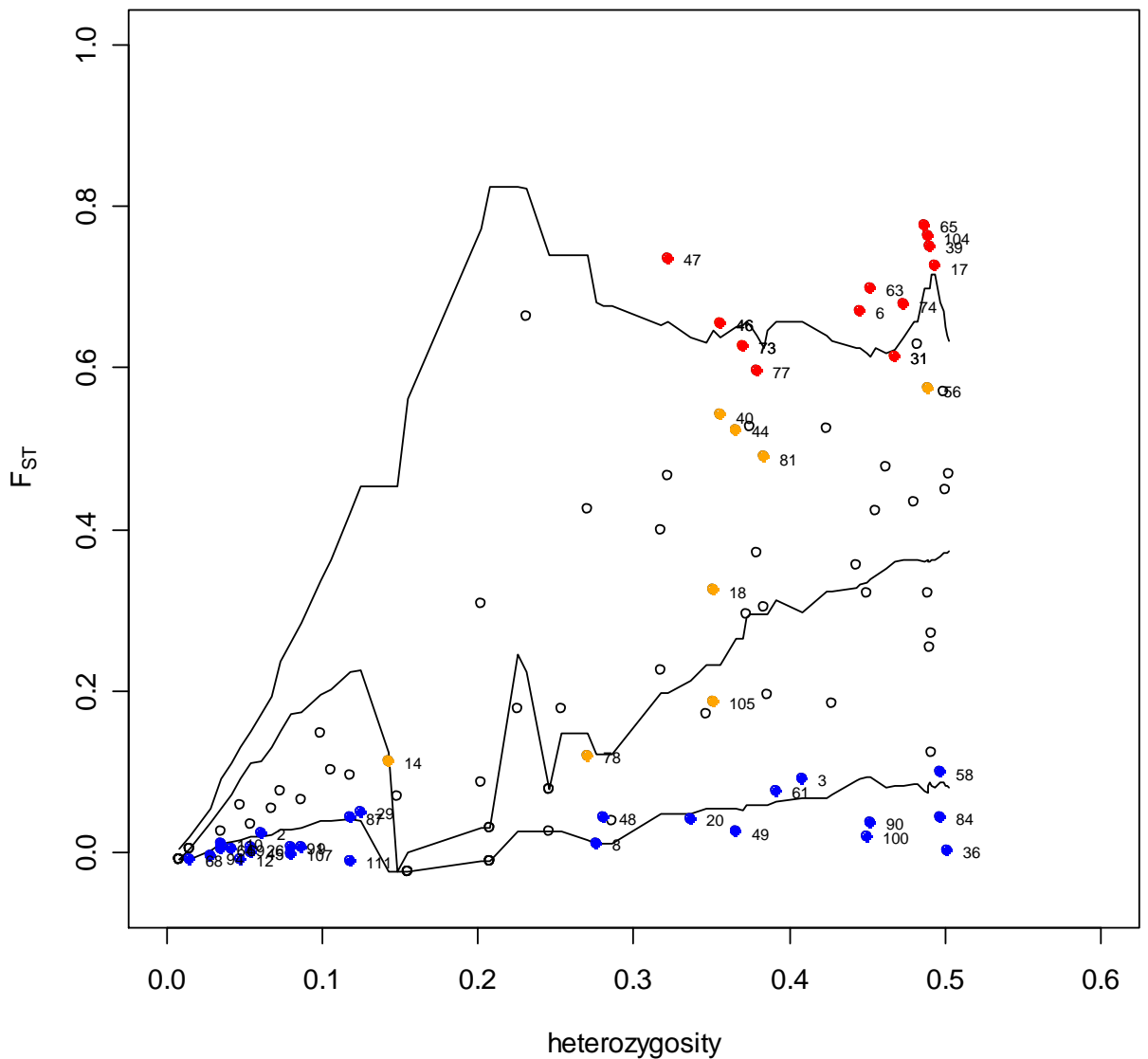
The **Dfdist** simulation was run with the trimmed weighted mean  $F_{ST}$  0.337, calculated by the **Ddatacal** program, as the target average. The trimmed mean  $F_{ST}$  gives the best model of a neutral distribution by excluding the outliers. The average  $F_{ST}$  for the simulation produced by **Dfdist** was 0.300.

**pv2** was run with the default smoothing proportion of 0.04. The confidence intervals for the outliers were based on 50,000 resampled loci from **Dfdist**.

**cplot2** was run with the default smoothing proportion of 0.04 and a critical probability for detecting outliers of 0.95. Most studies that have used **DFDIST** had a 0.95 confidence interval, though the authors recommend 0.99 and Cabellero *et*

al. suggest 0.9995 to avoid false positive results. For 0.9995 only one locus fell outside the neutral distribution, so significance was sacrificed in favour of detecting loci of interest and 0.95 was used.

An R script was used to plot empirical  $F_{ST}$  against heterozygosity, with  $0.95 < p < 0.05$  coloured red and blue respectively, plus 5%, 50%, and 95% confidence intervals for the simulated distribution.



**Figure 4.16:**  $F_{ST}$  as a function of heterozygosity for 112 polymorphic loci, with 5%, 50%, and 95% confidence intervals for a simulated neutral distribution of  $F_{ST}$ . Numbered loci are outliers with lower than expected  $F_{ST}$ ,  $p < 0.05$  (blue), outliers with higher than expected  $F_{ST}$ ,  $p > 0.95$  (red) and additional loci of interest identified by bulk segregant analysis (orange).

12 loci (10%) were identified as outliers with  $p > 0.95$ , having higher  $F_{ST}$  than expected under a neutral model of evolution and so likely to be the subject of divergent selection. Of these 9 loci fell above the 95% confidence limit for the neutral distribution of  $F_{ST}$ .

Using a similar approach Wilding *et al.* (2001) found that 5% of AFLP loci showed greater differentiation than expected, above the 99% quantile, in divergent forms of *Littorina saxatilis*. In a review of 14 AFLP genome scan studies Nosil *et al.* (2009) reported a range of 0.5 – 26% outlier loci, with most studies finding 5 - 10% outliers and 1 - 5% replicated in pair-wise comparisons. 25 - 100% of these outliers were specific to ecotypes and they typically showed high linkage disequilibrium between ecotypes.

#### **4.5.10 Linkage disequilibrium**

A linkage disequilibrium test for non-random association between loci was performed in Arlequin (Excoffier *et al.*, 2005). This test is an extension of the Fisher exact test, based on  $\chi^2$  contingency tables of observed and expected association of AFLP bands for each pair of polymorphic loci (Slatkin, 1994). It is effectively a permutation test, with alternative contingency tables generated using a Markov chain. The default settings of 10,000 Markov chain steps and 1000 dememorisation (burn in) steps were used. The significance level of linkage disequilibrium for each pair of loci is the proportion of possible contingency tables with a probability less than or equal to the observed contingency table. Pairs of AFLP markers were considered to be in significant linkage disequilibrium if they were associated more or less frequently than expected by chance, with loci that occurred together more or less frequently considered to be in positive or negative linkage disequilibrium respectively.

	N° pairs	LD	% LD	N° loci	LD	% LD	N° LD	± s.d.
<b>H</b>	903	84	9.30	43	42	97.67	3.91	2.31
<b>K</b>	1378	150	10.89	53	53	100	5.66	2.48
<b>D</b>	2850	349	12.25	76	76	100	9.18	5.58
<b>C</b>	4005	1068	26.67	90	88	97.78	24.24	17.83
<b>P</b>	1891	397	20.99	62	62	100	12.84	7.07
<b>All</b>	6216	2121	34.12	112	112	100	38.30	22.45

**Table 4.23: Exact test for linkage disequilibrium. Number of pairs of polymorphic loci for each of the 5 sites and for all 150 individuals; number and percentage of AFLP marker pairs with significant linkage disequilibrium ( $p < 0.05$ ); number of polymorphic loci for each of the 5 sites and for all 150 individuals; number and percentage of loci in significant linkage disequilibrium with at least one other ( $p < 0.05$ ) and mean number of loci in significant linkage disequilibrium per polymorphic locus ( $\pm 1$  s.d.).**

In a study of 452 AFLP markers in maize Stich *et al.* (2006) found significant linkage disequilibrium ( $p < 0.05$ ) for about 15% of marker pairs within both of two different groups, which is similar to the intra-site results for *Nereis diversicolor*. Nearly all polymorphic loci were in significant linkage disequilibrium with at least 1 other marker. Percentage linkage disequilibrium between pairs of loci and the number of non-randomly associated markers per locus varied as a function of the number of polymorphic loci.

Linkage disequilibrium is not necessarily a function of distance between loci on a chromosome: it can indicate groups of loci that are functionally linked or subject to the same selection pressure. The appearance of linkage disequilibrium can also be caused by cryptic sub populations or admixture between genotypes with different allele frequencies, which may be the case with the most diverse site C, although it does not explain the lower value for the mixed tolerant and non tolerant site D or the high value for low diversity site P. The higher linkage disequilibrium for all 150 individuals than for separate sites is likely to be negative disequilibrium reflecting genetic differentiation between the sites. There was no evidence of higher linkage disequilibrium due to bottleneck or disruptive selection effects in tolerant sites H and K.

Linkage disequilibrium was further investigated in 20 loci of interest that segregated between tolerant and non-tolerant groups in the bulk segregant analysis

(Chapter 3, Table 3.12) or were identified as likely to be under disruptive selection by the DFDIST analysis (Figure 4.16).

locus	6	14	17	18	31	39	40	44	46	47	56	63	65	73	74	77	78	81	104	105
6	***	+	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	-	+	NS
14	+	***	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	-	+	-
17	+	+	***	-	+	+	-	-	-	-	+	+	+	-	+	-	+	-	+	NS
18	-	-	-	***	-	-	+	+	+	+	-	-	-	+	-	+	-	-	-	NS
31	+	+	+	-	***	+	-	-	-	-	+	+	+	-	+	-	+	-	+	NS
39	+	+	+	-	+	***	-	-	-	-	+	+	+	-	+	-	+	-	+	NS
40	-	-	-	+	-	-	***	+	+	+	-	-	-	+	-	+	-	+	-	NS
44	-	-	-	+	-	-	+	***	+	+	-	-	-	+	-	+	-	+	-	NS
46	-	-	-	+	-	-	+	+	***	+	-	-	-	+	-	+	-	+	-	NS
47	-	-	-	+	-	-	+	+	+	***	-	-	-	+	-	+	-	+	-	NS
56	+	+	+	-	+	+	-	-	-	-	***	+	+	-	+	-	+	-	+	NS
63	+	+	+	-	+	+	-	-	-	-	+	***	+	-	+	-	+	-	+	NS
65	+	+	+	-	+	+	-	-	-	-	+	+	***	-	+	-	+	-	+	NS
73	-	-	-	+	-	-	+	+	+	+	-	-	-	***	-	+	-	+	-	NS
74	+	+	+	-	+	+	-	-	-	-	+	+	+	-	***	-	+	-	+	NS
77	-	-	-	+	-	-	+	+	+	+	-	-	-	+	-	***	-	+	-	NS
78	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+	-	***	-	+	NS
81	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-	+	-	***	-	NS
104	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	-	***	NS
105	NS	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	***

**Table 4.24: Matrix of significant positive (+) and negative (-) linkage disequilibrium between 20 loci of interest for all 150 individuals ( $p < 0.05$ ). Locus numbers are sequential for the 112 polymorphic loci, to match the DFDIST analysis (Figure 4.16). All but 1 associations were non-significant (NS) for locus 105.**

For these 20 markers the mean number of loci in significant linkage disequilibrium per polymorphic locus  $\pm 1$  s.d was  $64.25 \pm 14.54$ , which was much higher and less variable than the average for all loci of  $38.30 \pm 22.45$ , indicating large groups of associated loci. Positive and negative associations clearly split the markers into 2 distinct groups, with positive linkage disequilibrium within groups and negative linkage disequilibrium between groups. Markers 6, 14, 17, 31, 39, 56, 63, 65, 74, 78 and 104 in one group occurred more frequently in tolerant sites H and K, while the other group of markers 18, 40, 44, 46, 47, 73, 77 and 81 occurred more frequently in non-tolerant sites C and P. This does suggest divergent selection for particular combinations of genes.

#### 4.5.11 Loci of interest

Linkage disequilibrium is used in association mapping to identify groups of linked loci that are associated with phenotypic traits and potentially the subject of disruptive selection. Linkage disequilibrium associated with copper tolerance supports the identification of 19 of the 20 loci that segregated between tolerant and non-tolerant groups in the bulk segregant analysis, or were identified as likely to be under disruptive selection by the DFDIST analysis, as loci of interest:

Locus	Band	Bulks / Selection	LD Group	H	K	D	C	P
14	M42 30	Bulks	T	1	6	13	2	0
18	M42 35	Bulks	N	0	1	12	22	25
40	M42 79	Bulks	N	0	0	8	24	29
44	M42 91	Bulks	N	0	0	10	24	29
56	M69 14	Bulks	T	30	30	25	6	8
78	M71 4	Bulks	T	9	19	10	5	1
81	M71 7	Bulks	N	0	1	13	24	29
105	M71 52	Bulks	NS	24	2	7	16	11
31	M42 57	Both	T	30	30	19	6	5
46	M69 1	Both	N	0	0	7	24	30
73	M69 57	Both	N	0	0	10	24	30
6	M42 13	Selection	T	30	30	13	10	0
17	M42 34	Selection	T	30	30	30	7	5
39	M42 78	Selection	T	30	30	30	6	4
47	M69 2	Selection	N	0	0	0	24	30
63	M69 32	Selection	T	30	30	22	1	2
65	M69 38	Selection	T	30	30	30	7	1
74	M69 58	Selection	T	30	30	26	6	0
77	M71 3	Selection	N	0	0	13	23	30
104	M71 51	Selection	T	30	30	30	6	3

**Table 4.25: Band presence counts for 30 individuals from each of the 5 sites, for 20 polymorphic bands associated with tolerant and non-tolerant groups in the bulk segregant analysis (Table 3.12) or identified as likely to be under selection by the DFDIST analysis (Figure 4.16), split into 2 groups of loci in linkage disequilibrium (LD) that were more frequent for Tolerant (T) or non-tolerant (N) sites.**

8 of the 12 outlier loci identified as potentially under selection by DFDIST in Figure 4.16 were fixed for the tolerant sites H and K. Loci 65, 104, 39 and 17 which had particularly high  $H$  and  $F_{ST}$  were fixed in H, K and D, with a low presence in C and P. However, only 2 of the 11 loci identified in the bulk

segregant analysis as segregating between tolerant and non tolerant worms from site D were fixed for the tolerant sites H and K. It is interesting that bands associated with non-tolerant sites were identified as divergent as well as bands associated with tolerance, indicating positive selection for 2 divergent ecotypes.

Loci 31, 46 and 73 were identified in both the bulk segregant and DFDIST analysis. While they did not have high enough  $F_{ST}$  to be considered candidates for selection, a further 4 of the 11 loci identified by the bulk segregant analysis had fairly high  $F_{ST}$  according to DFDIST (Figure 4.16). Despite segregating in the bulks loci 14, 18, 78 and 105 had less clear patterns of association with tolerance across the 5 sites and lower  $F_{ST}$ , plus 105 was not in significant linkage disequilibrium with the other markers, so these loci are of less interest. The consensus of a combination of 3 different analysis methods suggests that loci 31, 46 and 73 are the best candidates for further investigation by sequencing.

Out of the 20 loci of interest there were 6 pairs of bands that occurred consecutively on electrophoresis gels:

Locus	Band	Bulks / Selection	LD Group	H	K	D	C	P
17	M42 34	Selection	T	30	30	30	7	5
18	M42 35	Bulks	N	0	1	12	22	25
39	M42 78	Selection	T	30	30	30	6	4
40	M42 79	Bulks	N	0	0	8	24	29
46	M69 1	Both	N	0	0	7	24	30
47	M69 2	Selection	N	0	0	0	24	30
73	M69 57	Both	N	0	0	10	24	30
74	M69 58	Selection	T	30	30	26	6	0
77	M71 3	Selection	N	0	0	13	23	30
78	M71 4	Bulks	T	9	19	10	5	1
104	M71 51	Selection	T	30	30	30	6	3
105	M71 52	Bulks	NS	24	2	7	16	11

**Table 4.26: Consecutive pairs of bands from the 20 loci of interest.**



Assuming that the range of a 30cm AFLP fingerprint is about 50 – 500 bases; these bands were separated by around 1 – 4 mm, which equates to a difference of around 2 - 6 base pairs between fragment lengths. These pairs of bands could be due to an insertion or deletion polymorphism, a different number of repeats in a microsatellite region or a transposon; making the same sequence slightly longer or shorter in different genotypes. The pairs could be associated with different alleles of the same gene, which may be homozygous for individuals with 1 band and heterozygous for individuals with both bands, which was more apparent in mixed site D. Loci 73 and 74, for example, were both identified as potentially under selection, with individuals having the band associated with either the tolerance or the non-tolerance linkage disequilibrium group, except for some individuals from sites C and D that had both bands.

The coincidence of these results shows that bulk segregant analysis, combined with statistical identification of loci that are potentially under selection and distinct groups with significant linkage disequilibrium, is a useful set of complimentary tools for finding markers of interest in a non-model organism.

## Chapter 5 Discussion

Understanding the relationship between environmental factors, the distribution of phenotypes in a metapopulation and evolutionary change at the molecular level is a central theme in molecular ecology and there is widespread interest in environmental effects on genomic variability and structure in natural populations. A number of population genomics studies have used AFLP genome scans to look for patterns of population structure and signatures of directional or divergent selection associated with environmental gradients, heterogeneous habitats and phenotypic variation (reviewed in Luikart *et al.*, 2003; Nosil *et al.*, 2009). Most of these studies focus on naturally occurring clines, which allow gradual adaptation over long time scales. Evolutionary adaptation to more rapidly changing environments is an increasingly important topic in the light of climate change, sea level rise and even more immediate anthropogenic impacts such as eutrophication and pollution events. There have been infrequent opportunities to investigate strong selection driving rapid evolution in response to recently introduced effects and few studies have addressed the impacts on population genetics of contaminants in aquatic environments (reviewed in Hebert & Luiker, 1996; Beaty *et al.*, 1998; Bickham *et al.*, 2000; Belfiore and Anderson, 2001; Medina *et al.*, 2007). Introduced anthropogenic environmental change can be far more rapid than naturally occurring change, which requires a faster evolutionary response (Stockwell *et al.*, 2003; Hendry, 2004; Medina *et al.*, 2007; Hendry *et al.*, 2008), so differential tolerance to pollution provides an interesting model system to study patterns and timescales of genetic diversity and divergence associated with recent reduction in population size and rapid adaptation in natural populations (Beaty *et al.*, 1998; Bickham *et al.*, 2000; Belfiore & Anderson, 2001; Medina *et al.*, 2007). In addition, genetic markers that are specifically related to stress tolerance can be used as an indicator in biological monitoring (Nevo *et al.*, 1984; Ozoh, 1992b).

Evolutionary adaptation to a new environment has predictable effects at the molecular level. A dramatic reduction in the size of a population due to a genetic bottleneck or founder effect could result in loss of genetic diversity, with reduced heterozygosity within the population (Bickham *et al.*, 2000; Belfiore & Anderson,

2001). Selection for tolerant phenotypes and thus associated genotypes will decrease diversity and the effects of inbreeding and drift will be exaggerated in a small population (Hebert & Luiker, 1996). Rare alleles that were neutral or even deleterious in the ancestral environment may confer a selective advantage in the new environment. Alleles for beneficial adaptive traits will increase in frequency and with divergent selection or directional selection along a cline, genetic differentiation between populations will increase. For example, the frequency of the allele for a reduced number of lateral body armour plates in the stickleback *Gasterosteus aculeatus* is 1% in oceanic populations but up to 100% in freshwater habitats due to strong positive selection in freshwater. It is also thought that selection for this trait has caused widespread, repeated parallel evolution in isolated, locally adapted populations (Colosimo *et al.*, 2005; Barrett *et al.*, 2008; Mäkinen *et al.*, 2008).

In addition to selection pressure genetic diversity and differentiation in natural populations is shaped by mutation, genetic drift, and migration, on different spatial and temporal scales. Rather than the infinitely large panmictic populations that form the theoretical basis for classical models of population genetics, the structure of most natural populations is characterised by isolation by distance to some extent (Wright, 1943). Genetic differentiation between populations involves a balance of selection, drift and migration, with the strength of selection determining the rate of evolution and barriers to gene flow allowing divergence and ultimately speciation (Dobzhansky, 1937; Mayr, 1942). Divergent selection for adaptive traits affects genetic diversity and differentiation and differential tolerance to stress factors can constitute a partial barrier to gene flow, potentially leading to non-allopatric speciation. Heritable tolerance to relatively recently introduced copper pollution in populations of the estuarine polychaete worm *Nereis diversicolor* provides a good model for understanding evolutionary processes involved in rapid adaptation under strong divergent selection (Grant, 2002).

Advances in population genetics techniques have allowed scientists to infer the history of population changes, assess current population status and anticipate the direction of future changes (Belfiore & Anderson, 2001). Molecular markers can

be used to assess population diversity and differentiation, delimit species, provide phylogenetic and phylogeographic information about likely evolutionary relationships and relate phenotypes to environmental variation where morphological and biological analysis fails (Knowlton, 2000; Bonin *et al.*, 2007). Genome scans using the large numbers of loci offered by dominant molecular marker systems like AFLP can be used to investigate the effects of evolutionary adaptation to environmental factors at molecular, individual and population levels (Luikart *et al.*, 2003).

The present study focussed on identifying and characterising adaptive genetic divergence. Differential tolerance to copper was found to correlate with genetic differences between populations. The tolerant populations had reduced heterozygosity, consistent with a bottleneck or founder effect under strong selection. There was high differentiation between the 5 populations, with genetic similarity based on ecotype rather than geographical distance. A combination of analyses revealed a number of outlier loci associated with either tolerant or non-tolerant phenotypes, indicating divergent selection favouring copper tolerance in polluted habitats or competitive ability in clean environments; which is potentially acting as a barrier to gene flow and driving non-allopatric speciation.

A prerequisite for any study of speciation is the definition and identification of separate species. Benthic marine invertebrates often have a small number of conspicuous diagnostic features, which defies morphological taxonomy. Marine organisms with low dispersal and yet very large geographic distributions can turn out to be groups of morphologically similar but genetically distinct species (Palumbi, 1992; Thorpe & Solé-Cava, 1994; Knowlton, 1993, 2000). Morphologically similar sibling species may also occur sympatrically in different ecological niches (Solé-Cava *et al.*, 1985; Kruse *et al.*, 2003; Johannesson *et al.*, 2010). Alternatively, morphotypes thought to be distinct species may be the result of phenotypic plasticity in response to environmental variation (Vrijenhoek, 2009). Many marine invertebrates are asexual and hybridisation is common between sexually reproducing marine invertebrates considered to be morphologically distinct species, which also confounds the biological species concept (Knowlton, 1993, 2000).

Morphological species boundaries can be particularly difficult to determine in polychaetes due to similar interspecific characteristics (Fong & Garthwaite, 1994; Breton *et al.*, 2003; Bleidorn *et al.*, 2006) or intraspecific variation due to phenotypic plasticity (Vrijenhoek, 2009). Rice (1991) cited studies that collectively proposed the existence of cryptic sibling species in 13 different spionid polychaetes. The Nereididae are a diverse and abundant family of polychaete worms, with a wide range of reproductive, developmental and life history characteristics, inhabiting marine and estuarine environments around the world (Fauchald, 1977; Fong & Garthwaite, 1994; Breton *et al.*, 2003). However, with over 500 species in the family (Bakken & Wilson, 2005; Rousset *et al.*, 2007) and a paucity of distinguishing morphological characteristics that is typical of marine invertebrates, assessment of diversity, differentiation and evolutionary relationships within and between species can be difficult.

Using parsimony analysis of 66 morphological characters in 52 species level taxa in the sub-family Nereidinae, which is based on the presence of paragnaths, Bakken & Wilson (2005) decided that the genera *Composetia*, *Neanthes*, *Perinereis* and *Nereis* were not monophyletic, and therefore not supported, with many characters having high levels of homoplasy. It has been suggested that the number, form and arrangement of paragnaths on the pharynx could be a diagnostic and quantitative morphological trait in Nereididae (Hateley *et al.*, 1992; Bakken & Wilson, 2005; Bakken *et al.*, 2009). While paragnaths have been successfully used to delimit some species such as *Nereis japonica* (Fong & Garthwaite, 1994), Hutchings & Reid (1991) found that paragnaths alone were of no use in delimiting *Platynereis* species and Maltagliati *et al.* (2006) found high similarity in paragnaths between *Perinereis cultrifera* and *Nereis diversicolor*.

A molecular phylogenetic analysis of 217 species of Annelida found that the accepted clade Nereidiformia was not monophyletic, with members scattered among 5 different clades (Rousset *et al.*, 2007) (although notably only 3 species of Nereididae were included in this analysis, to represent over 500 nominal species in this family). Measurements of similarity and difference at the molecular level offer a new solution to the problem of taxonomy, species concepts and speciation. However, molecular species definitions that rely on quantitative

analysis of molecular marker data set arbitrary thresholds of genetic difference, which may not be appropriate across all groups of organisms (Solé-Cava & Thorpe, 1991; Thorpe & Solé-Cava, 1994; Hebert *et al.*, 2003; Blaxter, 2004; Witt *et al.*, 2006).

Molecular markers have been widely employed to identify and delimit sibling species on the basis of genetic distance. A number of sibling species have been proposed within the Nereididae (reviewed in Fong & Garthwaite, 1994). *Nereis diversicolor* has previously been considered to be part of a morphological species complex including *Nereis limnicola* and *Nereis japonica* (Fong & Garthwaite, 1994), while a number of authors have suggested that *Nereis diversicolor* is itself a species complex (Röhner *et al.*, 1997; Breton *et al.*, 2003; Audzijonyte *et al.*, 2008). Appendix 3 summarises allozyme studies referred to in the text.

Differences in life history or larval development can be used to distinguish species, although this is not easily applicable to the identification of adults and molecular markers may be more useful in resolving species complexes in this respect. The morphologically similar species complex of *Nereis diversicolor* from Europe, *Nereis limnicola* from the Pacific coast of North America and 2 forms of *Nereis japonica* from Japan inhabit similar brackish environments but are isolated by both geographic distance and reproductive behaviour. *N. limnicola* is a viviparous hermaphrodite, whereas *N. diversicolor* is gonochoric, with demersal larvae. *N. japonica* consists of two different forms with different life history traits: one that undergoes partial epitokal metamorphosis and swarms to produce small eggs and planktonic larvae and one without epitokes that produces large eggs and demersal larvae in burrows, similar to *N. diversicolor*. Fong & Garthwaite (1994) used 10 allozyme loci to separate *N. limnicola*, *N. diversicolor* and the large egg form of *N. japonica*. They confirmed that they were indeed separate species, with average interspecific genetic distance of Nei's (1978)  $D = 0.977$ . Sato & Masuda (1997), Sato (1999) and Sato & Nakashima (2003) later used similar studies to resolve the Asian species complex of 3 forms of *N. japonica*.

The genetic distances between *N. limnicola*, *N. diversicolor* and the different forms of *N. japonica* were considered large enough for them to be separate

species. Some authors have also suggested that *N. diversicolor* is a complex of different species (Röhner *et al.*, 1997; Breton *et al.*, 2003; Audzijonyte *et al.*, 2008), which raises the question of what level of genetic differentiation should be used to delimit species. According to Thorpe & Solé-Cava (1994) values of Nei's (1978) genetic identity above 0.9 (distance below  $D \approx 0.11$ ) indicate populations of the same species, while identity below 0.8 (distance above  $D \approx 0.22$ ) suggests interspecific differentiation.

RAPD markers have been used to investigate sibling species in polychaetes, including *Hesionides areneria* and *Stygocapitella subterranea* (Schmidt & Westheide, 2000). For 27 individuals from 3 populations of *Stygocapitella subterranea* 14 primers produced 335 loci, with 20, 17 and 14 diagnostic loci respectively separating North Sea, US Atlantic and Pacific populations. 5 different methods of cluster analysis agreed on 3 distinct clades. They also compared 65 individuals from 8 internationally distributed populations of *Hesionides areneria* with *H. bengalensis* and *H. Maxima* using 468 RAPD loci. Within *H. areneria* there were no diagnostic loci and cluster analysis showed no large groups or differentiation. Average Nei & Li (1979) genetic distances ranged from 0.33 to 0.41 within populations and 0.36 to 0.44 between populations for *H. Areneria*, while distances between the 3 *Hesionides* species ranged from 0.84 to 0.9. Average Nei & Li (1979) distances in *S. subterranean* ranged from 0.17 to 0.34 within populations and 0.58 to 0.62 between populations, leading Schmidt & Westheide to propose separate *Stygocapitella* species, although they noted that the values were not as high as other typical interspecific distances calculated from RAPD data, for example between the *Hesionides* species or 0.74 between *Nerilla antennata* and *N. mediterranea* (Schmidt & Westheide, 1998). A study of 3 oligochaete species by Schirmacher *et al.* (1998) using 199 RAPD loci found interspecific Nei & Li (1979) distances ranging from 0.85 and 0.86 to only 0.17 between *Enchytraeus variatus* and *E. crypticus*, which were thought to be reproductively isolated. They also found low intraspecific distances in all 3 species, with no 2 individuals separated by more than 0.05.

In the present study mean Czekanowski / Dice / Sørensen distance (which is equivalent to Nei & Li (1979) distance when applied to binary data) between all

150 individuals from all 5 sites was 0.271, with average intrapopulation distances not much lower. This could demonstrate moderately high intraspecific diversity in *N. diversicolor* in comparison with other studies but probably not separate species. However, despite the fact that they are similar dominant marker systems the results of RAPD and AFLP analyses may not be directly comparable.

Allozyme electrophoresis studies have found a varied range of different genetic distances between populations of *Nereis diversicolor* (Fong & Garthwaite, 1994; Abbiati & Maltagliati, 1996; Röhner *et al.*, 1997; Virgilio & Abbiati, 2004b; Virgilio *et al.*, 2005; Virgilio & Abbiati, 2006)(Summarised in Appendix 3). Röhner *et al.* (1997) found Nei's (1972)  $D = 0.265$  between the North Sea and the Baltic, while Abbiati & Maltagliati (1996) found Nei's (1978)  $D = 0.356$  between populations on the Tyrrhenian and Adriatic coasts of Italy. These high values of  $D$  are above the suggested threshold of  $D \approx 0.22$  for separate species (Thorpe & Solé-Cava, 1994).

Other marker systems have found high differentiation between populations of *N. diversicolor*. Cossu *et al.* (2004) found AMOVA  $\Phi_{ST} = 0.469$  between 5 populations around the Tyrrhenian Sea using 5 ISSR markers. Breton *et al.* (2003) found high differentiation in mitochondrial DNA cytochrome b gene sequences: AMOVA was  $\Phi_{ST} = 0.313$  on a national scale and  $\Phi_{ST} = 0.453$  internationally. These values are very similar to the level of differentiation estimated by AMOVA in the present study,  $\Phi_{ST} = 0.436$ . Breton *et al.* (2003) observed a very high 6.6% DNA sequence divergence in cyt b between the NW Atlantic (Canada) and NE Atlantic and Mediterranean samples from France, while Audzijonyte *et al.* (2008) found 4.4 – 7.3 % mtDNA COI sequence divergence between lineages, apparently high enough to indicate separate species (CBOL, 2010).

However, it is possible that high population diversity and differentiation is common in polychaetes and thus the suggested distance thresholds are too low or not applicable. Comparably high intraspecific genetic distances have been found between populations of some other polychaete species, such as  $D = 0.159$  for *Syllis gracilis* (Maltagliati *et al.*, 2000). In a study of 22 allozyme loci Maltagliati *et al.* (2001) found high genetic divergence (Nei's  $D = 0.185$ ;  $F_{ST} = 0.381$ )



between 2 samples of the Nereidid polychaete *Perinereis cultrifera* from a brackish and a marine habitat less than 2 Km apart and thought that they may be sibling species. In contrast Nei's (1978) distance  $D = 0.035$  between populations of *Neanthes succinea* from the Adriatic and Tyrrhenian coasts of Italy was much lower than distances of  $D = 0.272$  to  $D = 0.356$  between comparable populations of *N. diversicolor* (Abbiati & Maltagliati, 1992, 1996). A study of 10 allozyme loci by Fong & Garthwaite (1994) found fairly low Nei's (1978)  $D = 0.001 - 0.046$  for *N. limnicola* from 4 geographically distant sites in California and Oregon but very high differentiation for 2 *N. diversicolor* populations from the German coast of the North Sea separated by only 30 km with  $D = 0.310$ , again above the interspecific threshold, suggesting sibling species in *N. diversicolor*.

Interspecific distances between recognised Nereidid species derived from allozyme markers have been much higher though, ranging from  $D = 0.500$  between *N. limnicola* and *N. japonica* (Fong & Garthwaite, 1994) to  $D = 1.81$  separating *N. diversicolor* and *N. limnicola* (Fong & Garthwaite, 1994) and  $D = 1.180$  separating *N. diversicolor* and *Neanthes succinea* (Abbiati & Maltagliati, 1996). A ratio view of distance thresholds suggests that genetic distance between populations of *N. diversicolor* found by the present study and others is not high enough to declare separate species.

The present study found relatively high differentiation between 5 *N. diversicolor* populations 3 - 145 Km apart, with Nei's (1978)  $D$  between pairs of populations ranging from  $D = 0.015$  to  $D = 0.121$ ,  $F_{ST}$  between pairs of populations ranging from  $F_{ST} = 0.117$  to  $F_{ST} = 0.691$  and differentiation between all populations  $F_{ST} = 0.32$ . 2 of the pairwise genetic distances between populations in the present study fell above the threshold level of divergence for conspecifics ( $D > 0.11$ ) but below the value of genetic distance that indicates interspecific differentiation ( $D > 0.22$ ) (Thorpe & Solé-Cava, 1994). While it is unlikely that any study has revealed sibling species in *N. diversicolor* there is widespread interest in patterns of differentiation and the potential for speciation in this species.

High genetic diversity, high interpopulation differentiation and the apparent complexity of *N. diversicolor* population dynamics has lead researchers to try to

explain spatial and temporal patterns of population structure. Population structure is determined by genetic drift, gene flow, inbreeding, selection and stochastic processes such as mortality and recruitment. Gene flow homogenizes allele frequencies between populations, whereas barriers to gene flow, genetic drift and selection can lead to differentiation. The extent and pattern of genetic diversity and differentiation within and between populations gives an indication of the relative importance of factors contributing to structured populations.

Possible causes of observed diversity and differentiation in *N. diversicolor* have been identified as:

- Genetic drift in isolated populations
- Inbreeding
- Isolation by distance under a stepping stone model of limited gene flow
- Spatial and temporal patchy recruitment
- Larger or longer scale invasions by different evolutionary lineages
- Historical bottleneck or founder events
- Local adaptation to environmental conditions under selection pressure
- Sympatric sibling species?

The global pattern of population structure in *Nereis diversicolor* is of interest as an example of a low dispersal organism adapted to isolated brackish water environments, which could explain the observed structure in the present study. *N. diversicolor* is a euryhaline species able to tolerate a wide range of environmental conditions and yet its range is confined to isolated brackish water habitats by competition outside its ecological niche (Kristensen, 1988). Genetic drift occurs in isolated populations leading to reduced heterozygosity and interpopulation differentiation. In general brackish water species have low heterozygosity and high genetic differentiation between populations due to isolation in estuaries and lagoons separated by full salinity open sea (Abbiati & Maltagliati, 1992; Bilton *et al.*, 2002).

Population size also affects diversity: populations with a larger effective population size tend to have higher heterozygosity while the effects of genetic drift are magnified in small populations (Crow & Morton, 1955; Crow & Kimura, 1970; Kijima & Fujio, 1984). Methods of reproduction, larval development and dispersal therefore affect the level and nature of population structure (Crisp, 1978). Species with planktonic dispersal have a high effective population size and tend to exhibit high genetic diversity within populations and low differentiation between local populations, while species with limited gene flow due to benthic development and dispersal often have low intrapopulation diversity and high interpopulation differentiation due to genetic drift (Kruse *et al.*, 2003). Species adapted to estuarine environments tend not to have pelagic eggs or larvae, which would have a low probability of recruitment to a suitable habitat if they were transported out of the estuary, and so have low dispersal capabilities (Fong & Garthwaite, 1994; Scaps, 2002). *N. diversicolor* produces relatively large eggs in burrows, followed by benthic larval development and dispersal (Dales, 1950; Fong & Garthwaite, 1994; Scaps, 2002). Low dispersal, combined with the physical geographical isolation of brackish water habitats separated by marine environments could lead to highly differentiated populations with low heterozygosity due to genetic drift in relatively small, isolated populations.

Interspecific studies of polychaetes with different levels of adaptations to brackish environments, such as salinity tolerance and mode of larval dispersal, have been used to investigate the influence of biological and ecological characteristics on genetic variation between and within species (Abbiati & Maltagliati, 1996). Abbiati & Maltagliati (1992) used 21 allozyme loci to investigate genetic differentiation between 2 populations of the Nereidid polychaete *Neanthes succinea* from the Adriatic and Tyrrhenian coasts of Italy. *N. succinea* is found in brackish habitats but unlike *N. diversicolor* it is a poor osmoregulator and does not tolerate salinity below 15 psu. It also has a life history more suited to marine habitats, with an epitokous stage and planktonic larvae. Between the 2 populations Nei's  $D = 0.035$  and, based on 7 polymorphic loci,  $F_{ST} = 0.341$ . The inbreeding coefficient  $F_{IS} = 0.012$  was low, while mean heterozygosity was  $H = 0.022$  and  $H = 0.044$  for the 2 populations. Heterozygosity and genetic identity between populations of *N. succinea* were both higher than they had previously found for *N.*

*diversicolor* confined to estuaries (Abbiati & Maltagliati, 1989 in Abbiati & Maltagliati, 1992). In a comparable study Abbiati & Maltagliati (1996) then used 17 allozyme loci to assess genetic diversity and differentiation between 4 populations of *N. diversicolor* from the Adriatic and Tyrrhenian coasts of Italy. Mean heterozygosity within populations was low, range  $H = 0.014 - 0.034$ , but not much lower than they had found for *N. succinea* (Abbiati & Maltagliati, 1992), so the hypothesis of lower heterozygosity in species with more estuarine life history traits was not necessarily supported.

The level of intrapopulation heterozygosity found by Abbiati & Maltagliati for both species was low compared to other marine invertebrates (Solé-Cava & Thorpe, 1991), for example up to 0.148 for molluscs (Nevo *et al.*, 1984). Nóbrega *et al.* (2004) found very high genetic diversity and low differentiation in the ascidian *Phallusia nigra* along 8000 Km of the Atlantic coast, with  $H = 0.28$ ,  $F_{ST} = 0.083$ ,  $Nm = 2.8$ . However, Kyle & Boulding (2000) found that population genetic structure in 4 species of *Littorina* with different developmental traits was not related to dispersal potential. Sato (1999) found lower intrapopulation diversity in the form of *Nereis japonica* with planktonic larvae, while Kruse *et al.* (2003) found higher differentiation between populations of the more mobile subtidal form of *Scoloplos armiger*. This shows that developmental mode and dispersal potential are not necessarily the most important driving force in marine invertebrate population genetics.

Levels of genetic diversity found in *Nereis diversicolor* have been notably variable. Mean observed heterozygosity for 2 *N. diversicolor* populations found by Fong & Garthwaite (1994) were quite different at  $H = 0.023$  and  $H = 0.2$ , the latter being relatively high compared to  $H = 0.050$  for *N. limnicola* and  $H = 0.040$  for *N. japonica*, although lower heterozygosity was expected in *N. limnicola* because it is a largely self fertilising hermaphrodite. However, these values were based on only 9 loci, plus the 2 samples of *N. diversicolor* only consisted of 14 and 26 individuals respectively and only 1 sample of *N. japonica* was examined, which may have allowed sampling errors. Other studies have found similar ranges of observed heterozygosity in *N. diversicolor* populations, for example  $H = 0.012 - 0.052$  (Virgilio *et al.*, 2003),  $H = 0.025 - 0.064$  (Virgilio & Abbiati, 2004b) and

mean observed heterozygosity values were not significantly different between the 8 populations studied by Virgilio & Abbiati (2004b). In contrast Röhner *et al.* (1997) found low observed heterozygosity in 8 populations,  $H = 0.000 - 0.015$ .

Levels of estimated intra-population heterozygosity between  $H \approx 0.05$  and  $H \approx 0.1$  found by the present study had a similar range to previous studies on *N. diversicolor* but were generally higher, and were fairly high compared to other species (Nevo *et al.*, 1984). The results could be due to overestimation of  $H$  for AFLP data, or it could reflect the fact that, compared to AFLP loci, allozymes are highly conserved and so have lower heterozygosity (Lewin, 2004). Differences between the results of allozyme studies could be an artefact of the number and choice of loci. Alternatively, differences in heterozygosity between geographical areas could be attributable to genuine phylogeographic effects.

While a number of allozyme studies have found low heterozygosity, DNA sequence markers have found relatively high genetic diversity coupled with high differentiation between *N. diversicolor* populations. Breton *et al.* (2003) compared large scale population genetic structure of *Nereis virens* and *N. diversicolor* using the mitochondrial DNA cytochrome b gene. It was anticipated that *N. virens* populations would have lower differentiation due to pelagic larval dispersal. *N. virens* from 12 locations in North America, Europe and Japan only had 3 haplotypes, one of which was shared by 89% of individuals, whereas *N. diversicolor* from 1 area in Canada and 2 in France showed much higher diversity, with 12 different haplotypes and no haplotype occurring in more than 1 sample. There was considerable sequence diversity in *N. diversicolor*, with 18.9% of nucleotide sites polymorphic. A preliminary study of mtDNA cytochrome c oxidase I (COI) also found much higher sequence variation for *Nereis diversicolor* than for *Nereis virens*. Breton *et al.* explained this as relatively low diversity in *N. virens* due to a past bottleneck. Alternatively this could be viewed as comparatively high diversity in *N. diversicolor*. Relatively high heterozygosity shows that genetic diversity in *N. diversicolor* is generally not affected by isolation, probably as a result of very large local population sizes.

In addition, only a low level of gene flow between populations is required to balance the effects of genetic drift (Wright, 1931). *Nereis diversicolor* is not biologically restricted to brackish habitats because it can tolerate a wide range of salinity, so limited migration of larvae or adults, and therefore local gene flow between estuaries, is possible. Armonies (1999) noted that juvenile *Scoloplos armiger* and *Arenicola marina* are susceptible to erosion of intertidal sediment and passive transport in the water column, so this is likely to be the case for *N. diversicolor*. Gene flow is also facilitated through limited migration of adults (Scaps, 2002). The average level of gene flow estimated in the present study ( $Nm \approx 0.5$ ) was just high enough to override the effects of genetic drift, which implies an alternative explanation for the observed differentiation between populations.

Population structure associated with reduced heterozygosity in small, isolated populations can also be the result of inbreeding. Deviation from Hardy Weinberg equilibrium and Wright's inbreeding coefficient  $F_{IS}$  can be calculated more easily and more accurately for codominant allozyme markers than for AFLP. Studies of Nereidid polychaetes using codominant markers have generally found negligible levels of inbreeding. Allozyme studies on *Neanthes succinea* (Abbiati & Maltagliati, 1992); *Nereis limnicola*, *Nereis japonica* and *Nereis diversicolor* (Fong & Garthwaite, 1994) have found no overall significant departure from Hardy Weinberg equilibrium, with very low, non-significant values of  $F_{IS}$ . However, Maltagliati *et al.* (2001) found  $F_{IS} = 0.489$  in *Perinereis cultrifera*. Significant deviation from Hardy Weinberg equilibrium was found for 3 loci in only 1 of 8 populations of *N. diversicolor* by Röhner *et al.* (1997), while in the study by Abbiati & Maltagliati (1996) there were 2 cases of departure from Hardy Weinberg equilibrium by a single locus in a single population but mean  $F_{IS}$  values showed no significant heterozygote deficiency in any of the populations overall. Virgilio & Abbiati (2006) found high  $F_{IS}$  values up to  $F_{IS} = 0.651$  within samples but the 6 loci chosen for the study were known to be particularly divergent and possibly associated with local selection, so the estimated level of inbreeding and heterozygote deficiency at these loci was not representative of overall genetic structure.

However, the study of 21 allozyme loci in 8 populations by Virgilio & Abbiati (2004b) found high intrapopulation heterozygote deficiency, with 12 departures from Hardy Weinberg equilibrium out of 86 combinations of loci and a high inbreeding coefficient  $f = 0.391$ , for the same areas where Abbiati & Maltagliati found no inbreeding in 1996. As a result of this discrepancy it is difficult to determine which of the 2 widely different inbreeding coefficients estimated by 2 different methods for AFLP data in the present study is more likely ( $F_{IS} = 0$  vs  $F_{IS} = 0.368$ ). Based on G tests of allozyme data Hateley *et al.* (1992) found no significant departure from Hardy Weinberg equilibrium in 4 populations of worms from Restronguet Creek, including tolerant site R1 / K at Kennal and mixed site R6 / D at Devoran, but they only tested 2 loci.

Virgilio & Abbiati (2004b) suggested that the occurrence of significant  $f$  values spread across most of the analysed loci indicated that either inbreeding or population subdivision was the reason for low heterozygosity, as these processes are expected to operate across all loci, rather than selection, which acts on particular loci associated with adaptive advantage. *N. diversicolor* has very large populations and no known mechanism of sexual selection, so inbreeding seems unlikely, although it could result from mating within high numbers of low dispersal siblings. Agreement by the majority of studies that inbreeding is low suggests that drift or selection or are more likely causes of relatively low heterozygosity and high differentiation found in *N. diversicolor* populations.

Genetic structure in species with low dispersal capabilities is often characterised by isolation by distance (Wright, 1943). Some studies have found very little genetic distance between local populations of *N. diversicolor*. Abbiati & Maltagliati (1996) found zero genetic distance between 2 Tyrrhenian populations in adjacent estuaries 15 Km apart, while Virgilio *et al.* (2003) found  $D = 0.000 - 0.003$  in 5 adjacent lagoons. Virgilio & Abbiati (2004b) found more moderate Cavalli-Sforza & Edwards distances of  $D_C = 0.028 - 0.085$  between 6 moderately geographically distant populations 9 – 175 Km apart around the Adriatic coast (although this measure is not directly comparable with Nei's distance). Abbiati & Maltagliati (1996) found Nei's (1978)  $D = 0.356$  between populations 2,500 Km apart on the Tyrrhenian and Adriatic coasts. This suggests that genetic distance

between *N. diversicolor* populations could simply be related to geographic distance in a pattern of isolation by distance, characteristic of drift in a species with low dispersal.

Abbiati & Maltagliati (1996) found that, while there was little difference in observed heterozygosity between *N. diversicolor* and *N. succinea*, Nei's (1978) unbiased distance between *N. diversicolor* populations ranged from  $D = 0.272$  to  $D = 0.356$  between the 2 Italian coasts, with  $F_{ST} = 0.848$  between the 4 populations, which was much higher than they had found for *N. succinea* (Abbiati & Maltagliati, 1992). This supported their theory regarding high population differentiation in limited dispersal brackish water species, compared with a similar species found in the same areas with more marine developmental and dispersal characteristics. The pattern of genetic distance between the 4 sites was consistent with isolation by distance due to lower dispersal in *N. diversicolor*. There was no difference between the 2 Tyrrhenian coast samples ~ 15 Km apart ( $D = 0.00005$ ) but the authors thought that they were distant enough geographically for common ancestry to be a more likely explanation for genetic identity than gene flow. Contrary to the Abbiati & Maltagliati (1996) study, Röhner *et al.* (1997) found no strong evidence of isolation by distance on an international scale for 8 populations of *N. diversicolor* from Scotland, the Netherlands, Denmark and 5 locations around the Baltic from Germany to Finland. Virgilio & Abbiati (2004b) compared differentiation between 6 *N. diversicolor* populations along the Adriatic coast of Italy, 9 – 175 Km apart, and 2 Tyrrhenian populations 2500 Km away. Superficially they found high differentiation and isolation by distance characteristic of discontinuous distribution and low dispersal. However, as in the present study Multi Dimensional Scaling ordination analysis (MDS) showed 2 distinct clusters, although in this case simply corresponding to the 2 coasts. Cavalli-Sforza and Edward's (1967) genetic distance was  $D_C = 0.068$  between the 2 Tyrrhenian populations,  $D_C = 0.028 - 0.085$  between the 6 Adriatic populations and  $D_C = 0.100 - 0.180$  between the 2 coasts. Mantel tests showed a positive correlation between genetic differentiation and geographic distance only when both coasts were included in the analysis. Differentiation in the Adriatic populations alone



was moderately high ( $\theta = 0.097$ ) but the pattern of genetic structure along the Adriatic coast was not related to geographic distance.

In the present study the 4 Restronguet Creek populations K, D, C and P were separated from each other by 3 – 20 Km and from the Hayle population H by ~140 Km. Nei's (1978) distance ranges were  $D = 0.017 - 0.112$  within the Restronguet area, which is a wider range over a smaller area than was found by Virgilio & Abbiati (2004b), and  $D = 0.015 - 0.121$  between the 2 coasts (Restronguet and Hayle), which does not suggest a relationship with geographic distance. At a geographical distance of 137 Km the differentiation between sites H and P of  $D = 0.121$  could appear to be due to isolation by distance and associated drift but  $D = 0.015$  between sites H and K 141 Km apart did not fit a pattern of isolation by distance. While a Mantel test did show a positive correlation between geographic distance and genetic dissimilarity there was only a very weak correlation when geographically distant site H was excluded, which showed that geography only played a very limited part in the genetic differentiation between tolerant and non-tolerant worms. MDS and cluster analysis confirmed the presence of 2 groups of genotypes, unrelated to geography, with tolerant site K sharing more genetic similarity with tolerant site H 141 Km away than it did with mixed site D and non-tolerant sites C and P 3 – 20 Km away. This coincidence of phenotypes and genotypes appears to be evidence for population structure based on divergent selection for adaptive ecotypes.

However, genetic distance between populations of *N. diversicolor* has been found to be highly variable both spatially and temporally. Separate studies have found genetic distance between populations of *N. diversicolor* on the Tyrrhenian and Adriatic coasts of Italy ranged from  $D = 0.100$  to  $D = 0.180$  (Virgilio & Abbiati, 2004b) and  $D = 0.272$  to  $D = 0.356$  (Abbiati & Maltagliati, 1996). In a study of temporal genetic changes at 4 sites on the Adriatic and Tyrrhenian coasts over a period of 3 years (2001 – 2003) Virgilio & Abbiati (2006) found distances ranging from  $D_C = 0.035$  to  $D_C = 0.195$  and differentiation ranging from  $F_{ST} = 0.017$  to  $F_{ST} = 0.274$  between years. This variation could be attributable to the low number of markers used in allozyme studies (Belfiore & Anderson, 2001).

Small spatial and temporal scale population structure could be determined by stochastic processes such as mortality events and patchy recruitment. Temporal variation in *N. diversicolor* population structure was demonstrated by Virgilio & Abbiati (2006), by sampling 4 sites on the Adriatic and Tyrrhenian coasts of Italy (3 at Pialassa and 1 at Serchio) twice over a period of 3 years (2001 – 2003). Allele frequencies, percentage of polymorphic loci and mean observed heterozygosity at the six allozyme loci (*ALD*, *FH*, *HBDH*, *LDH*, *PGI* and *SDH*) varied considerably both spatially and temporally. Observed heterozygosity at Serchio ranged from  $H = 0.045$  in 2001 to  $H = 0.201$  in 2003. Genetic distance ranging from  $D_C = 0.035$  to  $D_C = 0.195$  and  $F_{ST}$  values ranging from  $F_{ST} = 0.017$  to  $F_{ST} = 0.274$  between years showed significant temporal differentiation within all 4 sites. The combination of local genetic fragmentation and temporal differentiation lead Virgilio & Abbiati (2006) to propose “chaotic genetic patchiness” within estuaries. *Nereis diversicolor* population dynamics are characterised by periodical mass mortality followed by recruitment peaks simply because it is a semelparous organism with a breeding season. Mortality events may additionally be caused by extremes of factors such as tides, weather, predation, disturbance or anthropogenic inputs (Kent & Day, 1983). Sweepstake reproductive success, where a small number of individuals are responsible for patchy recruitment, may be a major process affecting local genetic structure of *N. diversicolor* within estuaries (Virgilio & Abbiati, 2004b, 2006).

The small 10m<sup>2</sup> sampling sites in this study were assumed to be panmictic, but variation in allele frequencies could have been caused by sympatric recruitment of individuals from 2 or more populations. The Wahlund effect of mixed recruitment from different populations was the preferred explanation for parapatric genetic types found by Röhner *et al.* (1997) and complex patterns of genetic structure found in a heavily polluted lagoon environment by Virgilio *et al.* (2003). A subdivided population, with recruitment from 2 different populations could explain the mixed genotypes found at sites C and D in the present study. Toxicity tests appeared to show a cline of tolerance, although the fact that this was the case for all sites implies that a range of tolerance within sites is an artefact of the tests. There was some evidence of possible bimodal peaks in tolerance for 1 sample from site D and interestingly the ~ 40/60 split between worms classified as

tolerant or non-tolerant according to Briggs (2005) fitted the proportions of site D worms grouped with the tolerant site H and K worms or closer to the non-tolerant site C and P worms in MDS analysis. This suggests 2 divergent ecotypes, with either mixed recruitment of both types at sites C and D or possibly an intermediate type or hybrid zone.

Structure due to patchy recruitment over much larger spatial and temporal scales can be viewed as a pattern of invasions by different evolutionary lineages. Deep divergence between such lineages of *N. diversicolor* found in sympatry has lead authors to suggest the existence of separate species (Röhner *et al.*, 1997; Breton *et al.*, 2003; Audzijonyte *et al.*, 2008). In allozyme studies in both the Baltic and the North Sea Röhner *et al.* (1997) found 2 distinct genetic types, A and B, which occurred sympatrically in 4 cases, and suggested that the pattern of local and international differentiation in *N. diversicolor* could be due to the presence of sibling species. There was very high differentiation between the 8 populations ( $F_{ST} = 0.892$ ), which was almost the same as that found by Abbiati & Maltagliati (1996) between the 2 coasts of Italy. Röhner *et al.* (1997) identified the same 3 loci as responsible for differentiation in the study by Fong & Garthwaite (1994) and assigned the 2 populations from that study to type A and B accordingly. UPGMA cluster analysis of Nei's (1972) genetic distance between the 8 populations clearly showed 2 separate groups based on divergence at these 3 loci. Group A contained 2 populations from the North Sea and 1 from the far side of the Baltic in Finland, while the other North Sea population was grouped with the rest of the Baltic samples in group B. Nei's (1972)  $D$  within groups was low,  $D = 0 - 0.004$  for A and  $D = 0 - 0.01$  for B, while distance between the 2 groups ranged from  $D = 0.197$  to  $D = 0.265$ , above the threshold for separate species. Gene flow calculated from pairwise  $F_{ST}$  values was correspondingly high within the 2 groups and very low between the groups.

4 of the sites assigned to group B had a minority of type A alleles. Röhner *et al.* (1997) speculated that heterozygotes at 3 of these sites could be explained by parapatric hybridisation between the 2 types. For the site that only had type A or B homozygotes, and significantly lower heterozygosity than expected under Hardy Weinberg equilibrium, they proposed either the Wahlund effect of admixture between 2 sub-populations or reproductive isolation between sympatric

types as the most likely explanation. The suggestion of hybridisation in 3 mixed type A and B populations but not in a fourth with the same 2 genotypes seems counterintuitive, unless one population had independently developed a reproductive isolating mechanism. This study examined 13 allozyme loci, of which only 5 were polymorphic. Basing the existence of 2 distinct genetic types and possible sympatric speciation on the behaviour of 3 of these loci in a small number of samples highlighted the need for further research with more loci in more populations.

Audzijonyte *et al.* (2008) studied 4 divergent allozyme loci (MDH I, MDH II, PEP, GPT), plus mitochondrial DNA *cytb* and COI gene sequences to survey *Nereis diversicolor* from 31 sites around the Baltic Sea. They found 2 highly differentiated genetic types, as suggested by Fong & Garthwaite (1994) and Röhner *et al.* (1997) and proposed that they should be considered separate species on the basis of reproductive isolation. Type A was dominant in the Northern Baltic while only type B was found on the southern Baltic coasts of Poland, Germany and Denmark. 5 samples were type A only, 6 were type B only but 19 samples contained both types.

Reproductive isolation without hybridisation, in accordance with the biological species concept, can be demonstrated for putative sympatric species by a lack of individuals that are heterozygous for diagnostic markers. Out of a total of 1,157 individuals Audzijonyte *et al.* (2008) found no heterozygotes at any of the 4 allozyme loci. In the intermediate region around southern Finland and Estonia both types were found together, but the absence of heterozygous genotypes indicated a lack of hybridisation between the 2 sympatric genetic types. Audzijonyte *et al.* stated that concordant divergence at 4 diagnostic allozyme loci, over a wide geographical area, plus the complete absence of heterozygotes where the 2 types occurred in sympatry, was sufficient evidence for reproductive isolation and therefore separate biological species. However, Röhner *et al.* (1997) did find heterozygotes. Of the 2 loci common to both studies, MDH I was homozygous but MDH II was heterozygous in 2 individuals at each of 2 sites (4% of each sample). The third allozyme locus considered to be diagnostic of the 2 types by Röhner *et al.* (IDH I), but not used by Audzijonyte *et al.*, also had

heterozygote genotypes. In the Fong & Garthwaite (1994) study the *N. limnicola* and *N. japonica* samples were all monomorphic for the same MDH II allele, while *N. diversicolor* type B shared an IDH I allele with *N. limnicola*. This highlights the danger of using a small number of allozyme loci to lump or split species.

Analysis of 138 COI and 25 cyt b sequences by Audzijonyte *et al.* (2008) did not agree with the proposed division into 2 types. They found 4 distinct, strongly diverged lineages, with 4.4 – 7.3 % COI sequence divergence between them and, while type A were mostly grouped in a single clade, there were 3 type B clades, with  $\Phi_{ST}$  as high as 0.94 between some of the type B sites. There were 8 cases (5%) of individuals with a mismatch between allozyme type and A / B mitochondrial DNA clade assignment. Disagreement between the 2 markers occurred for both sympatric A / B sites and sites where only 1 genetic type was found. According to Audzijonyte *et al.* (2008) lineage mismatch could indicate past mtDNA introgression but that requires fertile hybrids, which contradicts the idea of reproductively isolated biological species, unless speciation was recent.

Virgilio *et al.* (2009) used mitochondrial DNA cytb and COI gene sequences to study phylogeography of *N. diversicolor* across a large proportion of its geographical range, at 16 locations from the NE Atlantic coasts of Europe and Morocco, plus the Mediterranean, Black and Caspian Seas. Their analyses also included the cytb sequences for the NW Atlantic, NE Atlantic and Mediterranean from the study by Breton *et al.* (2003), who had found a 6.6% DNA sequence divergence between Canada and France, and the cyt b and COI sequences for the 2 putative species A and B reported in the Baltic by Audzijonyte *et al.* (2008). Both diversity and differentiation were generally high: for a concatenated cyt b + COI data set of 74 haplotypes only 4 haplotypes were shared by more than 1 population. The COI sequence (345 bp) had 64 haplotypes with 88 polymorphic sites and an average sequence divergence of 6.7% (SE = 0.78%), while the cyt b sequence (290 bp) had 52 haplotypes with 68 polymorphic sites and an average sequence divergence of 4.5% (SE = 0.74%). Sequence divergence in COI > 2% is considered to indicate different species (CBOL, 2010), although this is the subject of extensive debate and may not be a suitable threshold for invertebrate taxonomy.

Different phylogenetic reconstruction methods, using different sequences as an outgroup gave a different arrangement of clades for cyt b, COI and concatenated cyt b + COI sequences but the overall groups were the same. Phylogenetic analyses indicated 5 distinct clades from NE Europe, Portugal and Morocco, the Western Mediterranean, the Adriatic and the Black and Caspian Seas. All trees split the individuals from the Baltic between clades. The best resolved rooted tree, for COI with *Nereis japonica* as outgroup, grouped all of the individuals originally assigned to Baltic A with those from NE Europe, while all but 1 of the Baltic B individuals were split between the Western Mediterranean and Black / Caspian Sea clades, suggesting three different sympatric lineages in the Baltic.

The divergent population structure of *N. diversicolor* at different spatial scales in the Baltic could be explained by multiple historical invasions of the “sea of invaders” (Leppäkoski *et al.*, 2002). Most of the Baltic Sea is a single, huge brackish environment, with greatly reduced salinity due to terrestrial runoff, and so does not experience the same barrier of full salinity found between estuaries as other Seas. However, *Nereis diversicolor* is euryhaline so this should not make a difference to dispersal. The Wahlund effect of more recent, smaller scale patchy recruitment from older lineages could explain the local sympatric occurrence of divergent genotypes in the present study.

The 5 individuals from Severn Estuary on the west coast of the UK examined by Virgilio *et al.* (2009) were scattered throughout the NE Europe clade, grouped with individuals from France, Germany, the Netherlands and Baltic type A. This agrees with the assignment of individuals from East Scotland to type A by Röhner *et al.* (1997). The fact that populations from Northern France, on the other side of the English Channel were also type A (Breton *et al.*, 2003) makes it likely that all populations in the present study would belong to the same NE Europe mtDNA clade. Virgilio *et al.* (2009) showed that relatively conserved mitochondrial gene sequences were a useful marker for investigating the existence of sibling species and ascertaining the large scale genetic structure of *N. diversicolor* at the international level but are of less use for investigating small scale local differentiation.

Variable levels of diversity between local regions could indicate historical colonisation effects, different levels of isolation and drift or selective effects of local environmental conditions. In their study of differentiation over different spatial scales Virgilio & Abbiati (2004b) concluded that large scale differentiation in *N. diversicolor* may have been related to different evolutionary histories of Adriatic and Tyrrhenian populations but small scale structure was more likely to be influenced by adaptation to local environmental conditions. Abbiati & Maltagliati (1996) suggested that differentiation between 4 populations of *N. diversicolor* from an estuary and a canal on the Tyrrhenian coast of Italy, an island off the Tyrrhenian coast and a lagoon on the Adriatic coast could have been due to eco-physiological barriers between the different habitat types resulting in local adaptation, rather than isolation by distance due to genetic drift. However, in the study by Virgilio *et al.* (2009) nucleotide ( $\pi$ ) and haplotype ( $h$ ) diversity was high for the North European coasts ( $n = 35$ ,  $\pi = 0.032$ ,  $h = 0.992$ ) and Black and Caspian Seas ( $n = 21$ ,  $\pi = 0.032$ ,  $h = 0.976$ ) but relatively low for the Adriatic ( $n = 35$ ,  $\pi = 0.002$ ,  $h = 0.524$ ), with 24 out of 35 individuals sharing the same haplotype. The fact that the least diverse Adriatic samples were from 4 different lagoon and estuary habitats in Italy and Croatia which makes local adaptation effects on diversity seem less likely in this case.

Abbiati & Maltagliati (1996) described the pattern of 15 polymorphic allozyme loci in 4 populations of *N. diversicolor* as “peculiar”. 4 loci with alleles that were either monomorphic or absent in different populations ( $F_{ST} = 1$ ) made the highest contribution to very high differentiation of  $F_{ST} = 0.848$ . This agrees with the present study, which also found that a number of loci were either fixed or absent in different populations. The occurrence of some fixed alleles suggests restricted gene flow between populations but could also be loci under selection, or neutral loci hitchhiking: linked with genes that are subject to selection (Barton, 2000).

Röhner *et al.* (1997) also found that their results were characterised by a few highly divergent allozyme loci responsible for most of the difference, with different alleles fixed for different populations. They studied 8 populations of *N. diversicolor* from the Tay estuary in Scotland, an estuary in the Netherlands, a

fjord in Denmark, and from 5 locations around the Baltic from Germany to Finland. The authors did not give any information regarding levels of pollution so it is assumed that all of the study sites were relatively clean. The North Sea is tidal, whereas the Baltic Sea is non-tidal and the Danish fjord had very limited tidal effects but they found no genetic structure associated with habitat type or tidal regime.

For genetic markers to be subject to selection they must be associated with a heritable phenotype that conveys a selective advantage. Rather than looking for a connection between genetic type and environmental factors a number of authors have focussed on linking variation in morphological or physiological traits to evolutionary adaptation to local environmental conditions at the molecular level. Other studies, including the present one, have explored a three way correlation between environmental factors, tolerant phenotypes and genetic variation to support the hypothesis that genetic structure is the result of selection for adaptive traits (Luoma, 1977; Belfiore & Anderson, 2001; Nosil, 2009).

A number of studies have found intraspecific differences in paragnath number and distribution in Nereidids, including *Nereis virens* (Breton *et al.*, 2004) and *Nereis diversicolor* (reviewed by Hateley *et al.*, 1992; Maltagliati *et al.*, 2006). Khlebovich & Komendantov (2002) found that paragnath patterns in *Nereis diversicolor* populations were stable over time and that therefore likely to be a heritable rather than a plastic trait. Hateley *et al.* (1992) demonstrated experimentally that sediment type had no influence on plasticity in the development of paragnaths and that the trait was substantially heritable in crosses. Genetic differences between populations may be the result of either limited gene flow between sites or selection acting on paragnath patterns. It has been suggested that paragnath variation may correspond with environmental factors (Bakken *et al.*, 2009). Intraspecific variation associated with sediment grain size has been demonstrated for *Nereis diversicolor* (Garcia-Arberas & Rallo, 2000) and *Perinereis vallata* (Bakken *et al.*, 2009) and with salinity for *Nereis diversicolor* (Barnes, 1978) and *Perinereis cultrifera* (Maltagliati *et al.*, 2001). However, in a comparison of their own results with other studies of paragnaths



Hateley *et al.* (1992) found no consistent relationship between paragnath pattern and sediment type or salinity for *Nereis diversicolor*. Hateley *et al.* (1992) and Maltagliati *et al.* (2006) both suggested that inter-population differences in paragnath pattern may be adaptations to different diets. However, a gradient of paragnath number along the Humber in the study by Hateley *et al.* also suggested a simple genetic pattern of isolation by distance. They stated that as there was no evidence of a selective advantage, or of reduced heterozygosity associated with strong selection pressure, drift in isolation was more likely, despite the fact that 2 of the estuaries studied were only 2 Km apart. Other intraspecific studies combining paragnath patterns with molecular markers have found much greater, smaller scale genetic differentiation than was indicated by paragnath data alone for *Perinereis cultrifera* (Maltagliati *et al.*, 2001) and *Nereis diversicolor* (Virgilio *et al.*, 2006). This suggests that if paragnath pattern is an example of phenotypic diversity due to drift or divergent adaptation in *Nereis diversicolor* then it is not the strongest influence on genetic differentiation.

Allozyme studies have been used to investigate genetic patterns associated with heritable tolerance to metal pollution in *N. diversicolor* (Hateley *et al.*, 1992; Virgilio *et al.*, 2003; Virgilio & Abbiati, 2004a; Virgilio *et al.*, 2005; Virgilio *et al.*, 2006). Hateley *et al.* (1992) compared variation at 2 allozyme loci, LDH I and LDH II, from sites around the UK in the Humber Estuary, Avon Estuary, plus Mylor and Restronguet Creeks in Cornwall, which are only 2 Km apart. They found high genetic differentiation between estuaries but only weak differentiation within estuaries. LDH I allele frequencies for Restronguet Creek showed higher polymorphism and were significantly different to the other 3 estuaries, while Mylor Creek was significantly different to the other sites at the LDH II locus. Toxicity tests carried out for Restronguet creek, using a Cu concentration of 1.0 mg l<sup>-1</sup>, found no significant difference in time to death between the 2 LDH I phenotypes, and therefore no connection between this marker and copper tolerance.

Virgilio *et al.* (2003) studied the relationship between sediment contamination, tissue contamination and allozyme structure in *N. diversicolor* from the heavily polluted Pialassa lagoons on the Adriatic coast, at 4 sites with different levels of

mercury and 1 clean site. Mercury concentrations in tissues were correlated with a gradient of contamination in sediments, although there were significant differences in concentrations of mercury found in the tissues of individuals both between and within sites. There was no genetic distance between the sites according to Nei's (1978)  $D = 0.000 - 0.003$  but despite the proximity of the sites  $\chi^2$  analyses revealed significant differences between allele and genotype frequencies. There was a low level of genetic differentiation at 6 of the 19 allozyme loci, of which differentiation at LDH, PGI and SDH corresponded with levels of contamination while variation at ALD, FH and HBDH did not. The percentage of polymorphic loci, mean number of alleles per locus and observed heterozygosity were lower at the contaminated sites than at the clean site. Significant departures from HWE were observed for some loci in both contaminated and clean sites, but this was more common for the most contaminated site, with heterozygote deficiency at 3 loci.

In the Virgilio & Abbiati (2004b) study of 8 sites the Pialassa population also had a much lower percentage of polymorphic loci, mean number of alleles per locus and observed heterozygosity compared to the other sites, despite a higher sample size. There was relatively high genetic distance between Pialassa and the other Adriatic sites, shown by an nMDS plot of Cavalli-Sforza & Edwards (1967) distances. The environmental condition of the other sites was not stated though.

Virgilio & Abbiati (2004a) carried out laboratory experiments on *N. diversicolor* exposed to copper stress, from 3 sites ~ 10 Km apart in the contaminated Pialassa lagoons. For each of the 3 sites 2 replicates of 35 individuals were exposed to  $0.34 \text{ mg L}^{-1} \text{ Cu}^{2+}$  (96h LD 50) in acute toxicity tests. The genotypes of dead and surviving individuals after 96 hours were analysed using the 6 allozyme loci found to be of interest in their 2003 study of mercury contamination at Pialassa. There was no significant difference in observed heterozygosity between dead and surviving individuals at any locus. Under laboratory conditions the effect of copper stress on *N. diversicolor* appeared to be related to individual genotypes at ALD and PGI allozyme loci. Individuals from all 3 sites with the genotypes  $\text{ALD}^{100/100}$  and  $\text{PGI}^{102/102}$  had significantly lower mortalities than genotypes homozygous or heterozygous for the alternative allele. This could indicate

sympatric divergence between individuals homozygous for tolerant and non-tolerant genotypes, or stochastic recruitment from different sources, but finding the same pattern of subdivided populations at 3 different polluted sites seems unlikely if one type has a competitive advantage.

To test if genotypic associations with tolerance were consistent over larger spatial scales Virgilio *et al.* (2005) surveyed copper tolerance in *N. diversicolor* from 3 sites on the Adriatic and Tyrrhenian coasts of Italy, including the Pialassa lagoons, a site 40 Km away on the Adriatic coast and a site 2500 Km away on the Tyrrhenian coast. Cavalli-Sforza & Edwards (1967) distances of 0.008 between the 2 Adriatic sites and 0.074 between the 2 coasts were fairly consistent with previous studies on these spatial scales (Abbiati & Maltagliati, 1996; Virgilio *et al.*, 2003; Virgilio & Abbiati, 2004b) and did not appear to be related to levels of contamination. The level of differentiation between the 3 sites was relatively low ( $\theta = 0.047$ ) and the inbreeding coefficient was high ( $f = 0.543$ ), although these statistics were based on only 3 loci. There was no significant difference in time to death between the 3 populations exposed to  $0.48 \text{ mg L}^{-1} \text{ Cu}^{2+}$  (96h LD 70) in acute toxicity tests but this may simply reflect similar levels of copper contamination at all 3 locations. It should be noted that copper levels at Pialassa are 11 to 280  $\mu\text{g/gdw}$  (Virgilio & Abbiati, 2004a) while copper levels at Restronguet are up to 4000  $\mu\text{g/gdw}$  (Bryan & Hummerstone, 1971) and so likely to exert a greater selective effect.

Virgilio *et al.* (2005) investigated the relationship between time to death and genotype at 3 allozyme loci, ALD, PGI and FH. Genotype-tolerance association at the ALD locus was consistent among all 3 populations to some extent, with  $\text{ALD}^{102/102}$  individuals, homozygous for the non-tolerance allele, having the shortest survival times. However, at 1 site heterozygotes had a higher predicted median time to death than individuals homozygous for the tolerant allele, which contradicts the suggested homozygote advantage. In contrast to previous studies there was a reasonable number of both heterozygotes and homozygotes for both tolerance and non-tolerance associated genotypes found at all 3 sites, which does not suggest divergent selection for a particular genotype. There were no significant associations between genotype and time to death for the PGI locus and

the PGI<sup>102/102</sup> allele previously found to be associated with tolerance (Virgilio & Abbiati, 2004a) actually had the lowest predicted median time to death.

Comparison with the Virgilio & Abbiati (2004a) study confirmed a consistent relationship between ALD genotype and copper tolerance but the relationship between tolerance and the PGI locus was not consistent spatially or temporally. Virgilio *et al.* (2006) later found no relationship between concentrations of metals and distribution of ALD genotypes. In the study by Virgilio *et al.* (2003) differentiation at the ALD locus did not correspond with levels of mercury contamination, while the relationship between LDH and SDH loci and mercury contamination found at Pialassa in 2003 was not apparent for copper in the 2004a study. This last discrepancy may be related to different effects of copper and mercury, as the latter directly affects enzyme function as a heavy metal. However, the general lack of agreement between studies questions the usefulness of these allozyme markers as indicators of tolerance to metals.

In the study of *Neanthes succinea* by Abbiati & Maltagliati (1992) 1 out of 21 allozyme loci showed much higher locus specific diversity and differentiation ( $F_{ST} = 0.694$ ) than the others, plus significant deviation from Hardy Weinberg equilibrium. The allozyme PGI has been associated with mercury contamination Virgilio *et al.* (2003). Hvilson (1983) found significant differences in PGI allele frequencies between dead and surviving mussels *Mytilus edulis* exposed to copper while Nevo *et al.* (1981) found that particular PGI genotypes in gastropods *Monodonta turbinata* and *M. turbiformis* were more common in contaminated areas and showed higher tolerance to copper and zinc.

Koehn & Bayne (1989) proposed a heterozygote advantage for enzymes such as PGI that are involved in metabolism, on the basis that heterozygotes require less energy to maintain basal metabolism and therefore have more energy available to respond to stress. Troncoso *et al.* (2000) surveyed 5 polymorphic allozyme loci in the scallop *Argopecten purpuratus* obtained from a commercial hatchery and exposed to copper in acute toxicity tests. They found no difference in allele or genotype frequencies between dead and surviving individuals but there was a significant difference in heterozygosity at 2 loci. For the PGI locus there was a

significant excess of heterozygotes among survivors and a positive relationship between heterozygosity and survival. A general positive relationship between degree of heterozygosity and survival in marine species exposed to mercury was proposed by Nevo *et al.* (1984) and has been found in the caddis fly *Nectopsyche albida* (Benton & Guttman, 1992), for example. However, the results of allozyme studies on other aquatic organisms have not shown a consistent relationship between tolerance to metal contamination and either heterozygote or homozygote advantage (Roark & Brown, 1996). A negative relationship between heterozygosity and tolerance to metal exposure was found in mussels *Mytilus edulis* exposed to copper (Hoare *et al.*, 1994) and higher tolerance to mercury has been exhibited by homozygous individuals in the fish *Gambusia affinis* (Diamond *et al.*, 1989). Virgilio *et al.* (2003) suggested that low heterozygosity associated with tolerance in *N. diversicolor* supported the hypothesis of a selective disadvantage of heterozygotes.

The present study also found lower estimated heterozygosity associated with copper tolerance at the most contaminated sites. Possible effects of both allozyme alleles being expressed in heterozygotes would not be apparent in DNA based AFLP markers though, so it is likely that relatively lower heterozygosity found at some sites was the product of drift or selection rather than a direct result of heterozygote advantage or disadvantage. Some studies have demonstrated rapid accumulation of mutations and an associated increase in genetic diversity in polluted environments due to genotoxic effects (Yauk & Quinn, 1996; Rogstad *et al.*, 2003). Most mutations are deleterious though, which leads to lower population viability (Berckmoes *et al.*, 2005). Pollution is more often associated with reduced genetic diversity, due to selection pressure (Ma *et al.*, 2000; Van Straalen & Timmermans, 2002; Ross *et al.*, 2002) or bottleneck effects of reduced population size (Bickham *et al.*, 2000, Medina *et al.*, 2007). However, studies of population differentiation under selection do not all find associated reduced diversity (Nosil *et al.*, 2009), and this may be an effect of the strength of selection.

Lower heterozygosity found at tolerant sites H and K in the present study could be the result of a bottleneck or founder effect following the introduction of metal pollution and strong selection pressure, possibly reinforced by assortative habitat

selection or mating. The distribution of genotypes in MDS and cluster analysis suggests that comparatively high heterozygosity at sites C and D could be attributable to the Wahlund effect of mixed recruitment, or lower selection pressure and intermediate adaptation. Relatively low heterozygosity at site P, with the lowest tolerance, could be due to isolation by distance: non-tolerant site P and tolerant site K, both with relatively low heterozygosity, were further up a river than the other sites. Rather than divergent selection for ecotypes a study of guppies *Poecilia reticulata* by Crispo *et al.* (2006) found a pattern of isolation by geographic distance and physical barriers to dispersal, with genetic diversity decreasing up rivers. However, tolerant site H in the present study is probably the least isolated geographically, in a wide estuary mouth, close to other less polluted sites, but has the lowest heterozygosity, which is evidence for reduced diversity due to strong selection. Low heterozygosity at non-tolerant site P could therefore be due to the effects of selection for non-tolerance traits.

Estuarine species are often generally characterised by low genetic diversity, due to low dispersal, drift in isolation and selection pressure from stressful fluctuations in natural environmental factors (Crisp, 1978; Abbiati & Maltagliati, 1992; Belfiore & Anderson, 2001) but overall *Nereis diversicolor* displays relatively high genetic diversity. This may have facilitated adaptation to copper pollution where most other species were eliminated but it also presents a paradox. If selection for a particular fitness related trait in a population reduces genetic diversity, and the performance of populations is positively related to genetic variation, then a population with lower variation has reduced ability to respond to other selection pressures. The recent focus of conservation policy on maintaining biodiversity includes the genetic diversity of species. Loss of genetic diversity due to a particular selection pressure reducing the potential for populations to adapt to a range of different environmental changes that they may face in the future, such as climate change, is of current concern (Nevo *et al.*, 1986). However, high differentiation between *N. diversicolor* populations offers a diverse range of phenotypes at the species level, which may have different selective advantages under changing environmental conditions. On wider spatial and temporal scales stochastic recruitment and invasions by diverse, divergent lineages has allowed

colonisation of a range of conditions by phenotypes with different adaptations, ensuring the widespread success of *N. diversicolor*.

Initial divergence between ecotypes can occur over small, non-allopatric spatial scales in response to strong divergent selection for adaptations to different environmental factors (Rice & Hostert, 1993; Schluter, 1996, 2001, 2009; Rundle & Nosil, 2005; Hedrick, 2006; Butlin *et al.*, 2008). Studies using large numbers of molecular markers have found local differentiated populations with divergent phenotypes and corresponding genotypes that segregate according to ecotype (reviewed in Luikart *et al.*, 2003; Nosil *et al.*, 2009). Divergent selection acting in contrasting directions in non-allopatric populations can lead to isolation by adaptation (IBA) and potentially ecological speciation (Rundle & Nosil, 2005; Nosil *et al.*, 2009). Directional or divergent selection can be attributed to abiotic physical and chemical gradients, such as temperature, salinity and pollution, or biotic clines of competition, predation or food preference, and can act on biochemical, physiological, morphological, ecological or life history traits.

AFLP genome scans have found directional or divergent genetic differentiation associated with a gradient of altitude in the frog *Rana temporaria* (Bonin *et al.*, 2006); environmental variables in the pine weevil *Hylobius abietis* (Joost *et al.*, 2007); host plant choice in larch bud moth (Emilianov *et al.*, 2004), the stick insect *Timema cristinae* (Nosil *et al.*, 2008), the leaf beetle *Neochlamisus bebbianae* (Egan *et al.*, 2008) and pea aphids (Via & West, 2008); dwarf and normal ecotypes of lake whitefish *Coregonus clupeaformis* (Campbell & Bernatchez, 2004); wave action and predation resistant ecotypes, with some evidence of reproductive isolation, in the intertidal snail *Littorina saxatilis* (Wilding *et al.*, 2001; Grahame *et al.*, 2006; Butlin *et al.*, 2008) and soil type adaptation, reinforced by the reproductive isolating mechanism of different flowering times in *Howea* palm trees (Savolainen *et al.*, 2006). The present study found strong differentiation between *Nereis diversicolor* populations, with high values of  $D$  and  $F_{ST}$ , based on environmental copper levels and tolerance to copper rather than geographic distance.

Differentiation in RAPD, AFLP and mtDNA sequences between ecotypes of the cosmopolitan polychaete *Scoloplos armiger* is considered high enough for speciation (Kruse, 2003; Kruse *et al.*, 2003; Kruse & Reise, 2003; Kruse *et al.*, 2004; Bleidorn *et al.*, 2006). Sibling species of *S. armiger* have different developmental traits, sperm morphology, larval morphology and ecology (Kruse, 2003; Kruse *et al.*, 2003; Kruse & Reise, 2003; Kruse *et al.*, 2004). An intertidal form has egg cocoons while a subtidal form has pelagic larvae, which was initially thought to be poecilogony - a type of plasticity where a genetically homogeneous species employs different modes of development in different environmental conditions. The intertidal form has also been shown to have higher tolerance to sulphide and hypoxia than the subtidal form (Kruse *et al.*, 2003) and differences between the 2 forms are now thought to be heritable adaptations to intertidal or subtidal habitats (Bleidorn *et al.*, 2006). Genetic differentiation could be explained by a lack of migration between habitat types, selection through predation of egg cocoons in the subtidal and high post settlement larval mortality in the intertidal or reproductive isolation. The two forms occur sympatrically in the North Sea, with no evidence of hybrids, and laboratory breeding experiments have demonstrated reproductive isolation (Kruse & Reise, 2003).

Evolutionary adaptation to local selection pressures affects patterns of genetic structure in natural populations. In the absence of heritable adaptations to selection pressure *S. armiger* would have relatively homogeneous metapopulations characterised by isolation by distance (Wright, 1943). Kruse *et al.* (2003) carried out RAPD and AFLP analysis on *S. armiger* to test the hypothesis of genetic isolation by habitat type against isolation by distance. 1 intertidal and 1 subtidal sample ~ 2 Km apart were collected from each of 4 locations: 3 7 – 16 Km apart, plus a more distant subtidal sample 95 Km away. In UPGMA cluster analysis of Nei & Li (1979) distances for both RAPD and AFLP data the intertidal and subtidal samples formed distinct clusters, indicating less genetic distance between habitats than between sites, including the geographically distant site. For RAPD the Shannon diversity index ranged from 0.37 to 1.59 within sites, which is a wider range and much higher than  $S = 0.175$  in the present study. AMOVA also showed very high intrapopulation variation:  $\Phi_{ST} = 0.81$



compared with  $\Phi_{ST} = 0.436$  in the present study. A hierarchical AMOVA partitioned the variance 81% within sites, -3% (0%) between sites and 22% between intertidal and subtidal habitats. Assuming clean and contaminated sites are habitat types, in the present study the variance was partitioned 41% within sites, 8% between sites and 51% between habitats, showing lower within site diversity but greater differentiation between habitats. Kruse *et al.* (2003) also found higher genetic diversity in the subtidal population, which they attributed to a higher effective population size due to wider dispersal of pelagic larvae. 48 out of 116 bands were only found in subtidal samples but interestingly there were no bands specific to intertidal samples, in contrast to the present study which found loci specific to both tolerant and non-tolerant sites, indicating divergent selection.

On an international scale *cox3-trnQ-nad6* mtDNA phylogeny recovered 5 different clades of *S. armiger* in the NE Atlantic and NE Pacific, with intertidal and subtidal types from the North Sea forming 2 distinct clades (Bleidorn *et al.*, 2006). Tree topology and distances between clades generated by different phylogenetic analysis methods were largely unresolved, but suggested that geographic distance makes a bigger contribution to mtDNA differentiation in this species complex than adaptive evolution underlying the observed morphological, developmental and ecological differences between local ecotypes. This is unsurprising as mtDNA is used for large spatial and temporal scale phylogeny precisely because of the relatively neutral, conserved nature of the mitochondrial genome. Dominant markers such as RAPD and AFLP that sample a large number of loci throughout the nuclear genome are far more likely to detect small numbers of small scale, recent changes, particularly at loci that are subject to selection.

In the absence of reproductive isolation selection can act on a small number of genes for adaptive traits, against a background of the homogenising effects of gene flow, characterised by a few highly divergent loci (Lewontin & Krakauer, 1973; Rice & Hostert, 1993; Beaumont & Nichols, 1996; Wilding *et al.*, 2001). On smaller spatial scales non-allopatric speciation is possible under strong divergent selection, despite gene flow between populations. In a review of

laboratory experiments on the development of reproductive isolation Rice & Hostert (1993) concluded that isolation can rapidly evolve between parapatric or even sympatric populations if divergent selection is strong enough relative to gene flow. This model of speciation is characterised by initial divergence at a few loci maintained by selection, while divergence in the rest of the genome is prevented by gene flow; in contrast to uniform divergence across all loci characteristic of isolation by distance or a physical barrier to gene flow. A signature pattern of differentiation at a small proportion of loci, compared with less differentiation in the majority of loci, indicates divergent selection. Simulation to predict the expected distribution of differentiation across neutral loci, given an average level of divergence between loci can therefore be used to identify outlier loci under selection (Lewontin & Krakauer, 1973; Beaumont & Nichols, 1996). A pattern of linkage disequilibrium at a few loci associated with selective factors, compared with a relative lack of divergence at the majority of loci, could also be considered evidence for the early stages of non-allopatric speciation due to selection. The present study identified ~ 10% of AFLP loci as outliers, with higher  $F_{ST}$  than expected under a neutral model of evolution and so likely to be under divergent selection. This is consistent with a review of 14 AFLP genome scan studies (Nosil *et al.*, 2009) which found a range of 0.5 – 26% outlier loci, with most studies having 5 - 10% outliers and 1 - 5% replicated in pair-wise comparisons. 25 - 100% of these outliers specific to ecotypes and typically high linkage disequilibrium between ecotypes.

A well studied example of non-allopatric speciation under divergent selection is the intertidal wrinkle *Littorina saxatilis*, which displays repeated parallel evolution of clines in heritable shell morphology, associated with vertical gradients of wave action, predation by crabs and crushing, on rocky shores in the UK, Spain and Sweden (Johannesson *et al.*, 1993, 1995; Hull *et al.*, 1996; Wilding *et al.*, 2001; Rolan-Alvarez *et al.*, 2004; Grahame *et al.*, 2006, plus studies reviewed in Butlin *et al.*, 2008 and Johannesson *et al.*, 2010). In the UK *L. saxatilis* has distinct high and mid shore forms, which display partial reproductive isolation due to both assortative mating (Hull, 1998; Pickles & Grahame, 1999) and a high rate of inviable embryos in hybrid zone intermediate shell morphs, suggesting a post-zygotic isolating mechanism (Hull *et al.*, 1996). However, rather than complete

isolation, the pattern of differentiation still shows a signature of the early stages of speciation due to divergent selection, beginning with outlier divergence in a small set of gene loci.

Wilding *et al.* (2001) compared  $F_{ST}$  values for 306 AFLP loci with the distribution of  $F_{ST}$  values expected in the absence of selection, estimated from a simulation model. For high and mid shore samples from 4 sites separated from each other by 4 – 26 Km, with 50 individuals per sample, 15 loci (5%) showed greater differentiation than expected, with  $F_{ST}$  values higher than the 0.99 quantile. Randomisation tests (similar to a Mantel test) showed no relationship between  $F_{ST}$  values and geographic distance but when the 15 divergent loci were removed from the analysis there was a pattern of differentiation associated with distance. Neighbour joining trees of Nei's distance between sites with and without outlier loci agreed with this.  $F_{ST}$  values ranged from 0.0052 between 2 samples of the same type collected from the same site to 0.0230 – 0.0318 between the same ecotype at different sites; 0.0247 – 0.0396 between high and mid shore ecotypes at the same site and up to 0.0633 between different ecotypes from different sites. These values are much lower and less variable than values of  $F_{ST}$  found by the present study.  $F_{ST}$  values were 0.117 between 2 non-tolerant sites, 0.255 between 2 tolerant sites and ranged from 0.488 to 0.691 between ecotypes, reflecting high diversity and differentiation between all sites; particularly between ecotypes, regardless of geography. Tolerant site K is far more similar genetically to tolerant site H 141 Km away than to site P 16 Km away.

Wilding *et al.* (2001) found no fixed differences between the 2 ecotypes, whereas the present study found 2 loci that may be fixed at the 2 tolerant sites and absent at non-tolerant site P and 6 loci that were fixed at site P and absent at the 2 tolerant sites. Outlier loci specific to both extreme ecotypes is good evidence for divergent selection. Heritable tolerance can be associated with a loss of fitness in other respects: a “cost of tolerance” (Luoma, 1977) which creates a competitive disadvantage. This may be the result of pleiotropy, where a change in a single gene affects multiple phenotypic traits, genetic linkage with tolerance genes or an indirect trade-off between adaptive traits and general fitness (Harper *et al.*, 1997; Barton, 2000; Medina *et al.*, 2007). Tolerance mechanisms may require increased

energy expenditure or interfere with other physiological processes such as nutrient uptake (Calow & Forbes, 1998; Briggs, 2005; Pook *et al.*, 2009). Briggs (2005) observed both slower growth and lower aggression in tolerant *N. Diversicolor*. The fact that metal tolerant *N. Diversicolor* are not found in large numbers at clean sites (Bryan & Hummerstone, 1971; Grant *et al.*, 1989; Briggs, 2005) implies selection against tolerant phenotypes in clean conditions, probably as a result of intraspecific competition with non-tolerant *N. Diversicolor* and interspecific interactions under higher biodiversity. Pollution affects the diversity, abundance, distribution and ecological interactions of organisms: pollution tolerant communities have lower species richness and abundance, and thus a lower requirement for competition. The 2 selection pressures of copper and competition driving divergence are absent or reduced in the opposing habitat so this is an example of divergent selection, with different phenotypes favoured by opposing environmental and ecological conditions.

An interesting similarity between *Littorina saxatilis* ecotypes and the present study is that the 2 extremes of the cline are controlled by abiotic and biotic factors respectively and both studies found linkage disequilibrium between loci associated with opposing ecotypes, indicating divergent rather than directional selection. Strong positive or negative linkage disequilibrium among all but 1 of the 20 loci of interest identified by bulk segregant or outlier analysis is further supporting evidence for divergent selection. Among 15 differentiated loci showing linkage disequilibrium for *Littorina saxatilis* ecotypes Grahame *et al.* (2006) found 2 pairs of bands separated in size by a single base pair, which they thought may be allelic. The present study found 6 pairs of consecutive bands among the 20 loci of interest, 4 of which were tolerant / non-tolerant segregating bands. However, hitchhiking by linked loci (Barton, 2000) means that loci of interest are not necessarily functionally involved in adaptation. Divergent effects on linked loci increases with strength of selection (Nosil *et al.*, 2009), which could explain the number of loci with moderately high  $F_{ST}$  in the present study. Grahame *et al.* (2006) did not find a consistent pattern of hybrid zone linkage disequilibrium across shores, and so no firm evidence of loci that are tightly linked in the genome, protecting them from recombination or introgression and facilitating evolutionary adaptation based on a complex set of multiple loci. In the

present study lower linkage disequilibrium at site D, with intermediate tolerance, suggests an intermediate type rather than a hybrid zone, while high linkage disequilibrium at site C could indicate a Wahlund effect of patchy recruitment, which agrees with the picture of genotypes portrayed by MDS analysis.

The extent to which divergent selection can drive differentiation throughout the genome, in loosely linked and unlinked neutral regions, is unclear. Population differentiation with gene flow is characterised by divergence at loci subject to selection but if selection is strong enough to constitute a barrier to gene flow, or an additional reproductive isolating mechanism develops, then non-allopatric divergence would also affect neutral loci. Selection acts against gene flow through mortality or reduced fitness of immigrants (Hendry, 2004). This may be reinforced by assortative mating, either directly by mate choice or through habitat selection, or through development of a post-zygotic isolating mechanism. By acting as a partial barrier to gene flow selection can also promote differentiation through drift in neutral loci (Hendry, 2004; Grahame *et al.*, 2006; Nosil *et al.*, 2009). Nosil *et al.* (2008) found that neutral genetic differentiation was positively correlated with the degree of adaptive divergence in host plant preference between pairs of *Timema cristinae* stick insect populations, for example.

Following the study of outlier loci in *Littorina saxatilis* ecotypes by Wilding *et al.* (2001) a subsequent study by Grahame *et al.* (2006) examined the effects of adaptive divergence on neutral loci, using vertical transects of samples from contiguous high to mid shore habitats on the same shore. Again there was a steep cline in outlier allele frequencies, coinciding with a cline in shell morphology. They also found higher  $F_{ST}$  in non-outlier loci between than within ecotypes, regardless of geographic distance, which is characteristic of a general barrier to gene flow due to assortative habitat or mate choice or post-zygotic isolation. Linkage disequilibrium between outlier loci was high in the middle of the cline with intermediate allele frequencies. Combined with intermediate morphology and intermediate genotypes at differentiated loci in a few individuals, this indicates a hybrid zone between differentiated populations, which potentially acts as a barrier to gene flow in itself through hybrid infertility. However, this pattern

could be consistent with either non-allopatric divergence or secondary contact following allopatric divergence (Rice & Hostert, 1993).

Anecdotally, the fact that only one tolerant / non-tolerant cross was successful in the present study, with no F2 offspring, could indicate a reproductive isolating mechanism, possibly hybrid sterility, but further investigation is required. The large number of loci with lower than expected  $F_{ST}$  found by the present study suggests that reproductive isolation is not the case in *Nereis diversicolor*. The level of gene flow calculated from  $F_{ST}$  between populations was just high enough to override the effects of drift in isolated populations (Wright, 1931) so this appears to be a case of divergence despite gene flow. However, selection acts on islands of differentiation at loci coding for or linked to adaptive traits first. Small scale evolutionary change in populations associated with heritable tolerance to pollution is often rapid, occurring over a few generations (Medina *et al.*, 2007). Studies on genetic change over generations in copepods exposed to toxic stress by Gardeström *et al.* (2006, 2008) have shown that pollution can cause microevolutionary genetic changes in populations over very short timescales. As the strong selection pressure exerted by a pollution event requires rapid evolution over a relatively short time scale there simply may not have been enough time for the slower process of drift in neutral loci.

In the present study of 247 loci around 20 proved to be interesting, potentially subject to selection, with 3 loci consistently identified by a combination of bulk segregant, outlier and linkage disequilibrium analyses the best candidates for further investigation. One of the main advantages of AFLP is the large amount of data generated. Using a much larger number of AFLP loci compared to allozyme studies reduced sampling error and increased the chances of finding loci that consistently segregated with copper tolerance. To be useful as an indicator, associations between genetic markers and tolerance responses should also be consistent in space and time (Diamond *et al.*, 1991; Heagler *et al.*, 1993) and not confounded by other factors (Beatty *et al.*, 1998; Belfiore & Anderson, 2001). Through agreement between toxicity tests carried out at different times, coupled with segregation of loci of interest both in replicate bulk segregant analyses and between study sites, the present study has demonstrated spatial and temporal

stability in associations between AFLP markers and copper tolerance. Future work could include studies on wider spatial scales to investigate the presence of loci of interest in other areas contaminated by metals. Geographic distance or local variation in other environmental variables such as salinity, substrate or tidal regime could be responsible for the observed pattern of diversity and differentiation, and future analyses could be employed to control for this. Segregation studies that look for markers associated with differential tolerance in individuals from the same site can overcome the problem of differences in other variables between sites and in the present study bulk segregant analysis of mixed site D worked well. However, this approach relies on finding a site with intermediate contamination and mixed genotypes and still faces the possibility that different phenotypes could be inhabiting slightly different ecological niches in sympatry, for example different food sources (Schluter, 1996; Campbell & Bernatchez, 2004). In this study agreement between analyses and the strength of the relationship between environmental levels of copper, copper tolerance phenotypes and the distribution of differentiated genotypes implies that divergent selection for tolerance must be the main factor controlling population genetics.

Genetic distance between *Nereis diversicolor* populations is not high enough to indicate separate species and there is little evidence of divergence in neutral loci that would indicate a general barrier to gene flow. However, an AFLP genome scan revealed signatures of speciation, by identifying individual loci that are associated with adaptive tolerance to copper. Highly differentiated outlier loci contrasted with neutral loci is characteristic of non-allopatric divergence despite gene flow in populations under strong, divergent selection pressure. Loci of interest associated with disruptive selection were present for both tolerant and non-tolerant phenotypes, possibly indicating a cost of tolerance in tolerant populations and selection for competitive advantage in non-tolerant populations. There was no strong evidence of isolation by distance, which would be characterised by uniform differentiation across all loci, or general reduced heterozygosity in an isolated brackish water species. Lower diversity at contaminated sites could be the result of a historical bottleneck or founder effect, or the results of strong selection for tolerant genotypes. Toxicity tests confirmed a gradient of tolerance to copper in *N. diversicolor*, which corresponds with patterns

of genetic diversity and differentiation. Thus the clear correspondence between environment, phenotype and genotype, with a pattern of genetic diversity and differentiation that is representative of populations under strong, divergent selection makes heritable tolerance to copper in *Nereis diversicolor* a valuable model for investigating adaptive evolution and the mechanism of non-allopatric speciation in natural populations subjected to anthropogenic selection pressure.



## References

- Abbiati M, Maltagliati F (1989) Relationship between morphological and genetic variability in *Nereis diversicolor* and *N. succinea* (Annelida, Polychaeta). *Second European Symposium of Evolutionary Biology, Roma*, 1.
- Abbiati M, Maltagliati F (1992) Genetic population structure of *Neanthes succinea* (Polychaeta: Nereididae). *Journal of the Marine Biological Association of the United Kingdom*, 72, 511–517.
- Abbiati M, Maltagliati F (1996) Allozyme evidence of genetic differentiation between populations of *Hediste diversicolor* (Polychaeta: Nereididae) from the western Mediterranean. *Journal of the Marine Biological Association of the United Kingdom*, 76, 637–647.
- Ait Alla A, Gillet P, Deutsch B, Moukrim A, Bergayou H (2006) Response of *Nereis diversicolor* (Polychaeta, Nereidae) populations to reduced wastewater discharge in the polluted estuary of Oued Souss, Bay of Agadir, Morocco. *Estuarine, Coastal and Shelf Science*, 70, 633–642.
- Anderson M (2003) *pco: A Fortran Computer Program for Principal Coordinate Analysis*. Distributed by the Author, University of Auckland, New Zealand.
- Argue BJ, Dunham RA (1999) Hybrid fertility, introgression, and backcrossing in fish. *Reviews in Fisheries Science*, 7, 3-4, 137-195.
- Armonies W (1999) Drifting benthos and long-term research: why community monitoring must cover a wide spatial scale. *Senckenbergiana maritima*, 29, 13-18.
- Arnqvist G, Thornhill R, Rowe L (1997) Evolution of animal genitalia: morphological correlates of fitness components in a water strider. *Journal of Evolutionary Biology*, 10, 613-640.
- Audzijonyte A, Ovcarenko I, Bastrop R, Väinölä R (2008) Two cryptic species of the *Hediste diversicolor* group (Polychaeta, Nereididae) in the Baltic Sea, with mitochondrial signatures of different population histories. *Marine Biology*, 155, 599–612.
- Baird D, Evans PR, Milne H, Pienkowski MW (1985) Utilization by shorebirds of benthic invertebrate production in intertidal areas. *Oceanography and Marine Biology Annual Reviews*, 23, 573–597.
- Bakken T, Glasby CJ, Wilson RS (2009) A review of paragnath morphology in Nereididae (Polychaeta). *Zoosymposia*, 2, 305–316.
- Bakken T, Wilson RS (2005) Phylogeny of nereidids (Polychaeta, Nereididae) with paragnaths. *Zoologica Scripta*, 34, 507–547.
- Ballvora A, Hesselbach J, Niewöhner J, Leister D, Salamini F, Gebhardt C (1995) Marker enrichment and high-resolution map of the segment of potato

chromosome VII harbouring the nematode resistance gene Gro1. *Molecular and General Genetics*, 249, 1, 82-90.

Barnes RSK (1978) Variation in paragnath number of *Nereis diversicolor* in relation to sediment type and salinity regime. *Estuarine and Coastal Marine Science*, 6, 275–283.

Barrett RDH, Rogers SM, Schluter D (2008) Natural selection on a major armor gene in threespine stickleback. *Science*, 322, 255–257.

Bartels-Hardege HD, Zeeck E (1990) Reproductive behaviour of *Nereis diversicolor* (Annelida: Polychaeta). *Marine Biology*, 106, 409–412.

Barton NH (2000) Genetic hitchhiking. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 355, 1553–1562.

Beaty BJ, Black WC, Carlson JO, Clements WH, DuTeau N, Harrahy E, Nuckols J, Olson KE, Rayms-Keller A (1998) Molecular and genetic ecotoxicologic approaches to aquatic environmental bioreporting. *Environmental Health Perspectives*, 106, 1395–1407.

Beaumont MA (2005) Adaptation and speciation: What can  $F_{ST}$  tell us? *Trends in Ecology and Evolution*, 20, 435–440.

Beaumont MA, Nichols RA (1996) Evaluating loci for use in the genetic analysis of population structure. *Proceedings of the Royal Society B: Biological Sciences*, 263, 1619–1626.

Begon M, Harper JL, Townsend CR (1996) *Ecology: individuals, populations and communities*. Wiley-Blackwell.

Belfiore NM, Anderson SL (2001) Effects of contaminants on genetic patterns in aquatic organisms: a review. *Mutation Research*, 489, 97–122.

Bellman R (1961) *Adaptive Control Processes: A Guided Tour*. Princeton University Press.

Bensch S, Åkesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? *Molecular Ecology*, 14, 2899–2914.

Benton MJ, Guttman SI (1992) Allozyme genotype and differential resistance to mercury pollution in the caddisfly, *Nectropsyche albida*. I. Single locus genotypes. *Canadian Journal of Fisheries and Aquatic Sciences*, 49, 142–146.

Berckmoes V, Scheirs J, Jordaens K, Blust R, Backeljau T, Verhagen R (2005) Effects of environmental pollution on microsatellite DNA diversity in wood mouse (*Apodemus sylvaticus*) populations. *Environmental Toxicology and Chemistry*, 24, 2898–2907.

- Bickham JW, Sandhu S, Hebert PDN, Chikhi L, Athwal R (2000) Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. *Mutation Research*, 463, 33–51.
- Bilton DT, Paula J, Bishop JDD (2002) Dispersal, genetic differentiation and speciation in estuarine organisms. *Estuarine, Coastal and Shelf Science*, 55, 937–952.
- Black MB, Lutz RA, Vrijenhoek RC (1994) Gene flow among vestimentiferan tube worm (*Riftia pachyptila*) populations from hydrothermal vents of the Eastern Pacific. *Marine Biology*, 120, 33–39.
- Blanck H, Wängberg SÅ, Molander S (1988) Pollution-induced community tolerance — a new ecotoxicological tool. In: Cairns J, Pratt JR (Editors) *Functional testing of aquatic biota for estimating hazards of chemicals ASTM STP 988*, 219–230. American Society for Testing and Materials.
- Blaxter ML (2004) The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 359, 669–679.
- Bleidorn C, Kruse I, Albrecht S, Bartolomaeus T (2006) Mitochondrial sequence data expose the putative cosmopolitan polychaete *Scoloplos armiger* (Annelida, Orbiniidae) as a species complex. *BMC Evolutionary Biology*, 6, 47.
- Bogucki M (1953) Rozród i rozwój wieloszczeta *Nereis diversicolor* (O.F. Müller) w Bałtyku. *Polish Archives of Hydrobiology*, 1, 1–14.
- Bonin A, Ehrich D, Manel S (2007) Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. *Molecular Ecology*, 16, 3737–3758.
- Bonin A, Taberlet P, Miaud C, Pompanon F (2006) Explorative genome scan to detect candidate loci for adaptation along a gradient of altitude in the common frog (*Rana temporaria*). *Molecular Biology and Evolution*, 23, 773–783.
- Bonnard M, Roméo M, Amiard-Triquet C (2009) Effects of Copper on the Burrowing Behavior of Estuarine and Coastal Invertebrates, the Polychaete *Nereis diversicolor* and the Bivalve *Scrobicularia plana*. *Human and Ecological Risk Assessment*, 15, 11 – 26.
- Borowski RL (2001) Estimating Nucleotide Diversity From Random Amplified Polymorphic DNA and Amplified Fragment Length Polymorphism Data. *Molecular Phylogenetics and Evolution*, 18, 1, 143–148.
- Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, 32, 314–331.
- Bray JR, Curtis JT (1957) An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, 27, 325–349.

Breton S, Dufresne F, Desrosiers G, Blier PU (2003) Population structure of two northern hemisphere polychaetes, *Neanthes virens* and *Hediste diversicolor* (Nereididae), with different life-history traits. *Marine Biology*, 142, 707–715.

Breton S, Dufresne F, Desrosiers G, Blier PU (2004) Morphological variation in *Nereis* (*Neanthes*) *virens* (Polychaeta: Nereididae) populations. *Journal of the Marine Biological Association of the United Kingdom*, 84, 983-985.

Briggs A (2005) The Cost and Benefit of Copper Tolerance in *Nereis diversicolor*. PhD thesis, University of East Anglia.

Brown BE (1977) Effects of mine drainage on the river Hayle, Cornwall. A) Factors affecting concentrations of copper, zinc and iron in water, sediments and dominant invertebrate fauna. *Hydrobiologia*, 52, 221-233.

Brown BE (1982) The form and function of metal-containing ‘granules’ in invertebrate tissues. *Biological Reviews*, 57, 4, 621-667.

Bryan GW (1976) Some aspects of heavy metal tolerance in aquatic organisms. In: Lockwood APM (editor) *Effects of pollutants on aquatic organisms*, 7-33. Cambridge University Press.

Bryan GW, Gibbs PE (1983) Heavy metals in the Fal estuary, Cornwall: a study of long term contamination by mining waste and its effects on marine organisms. *Marine Biological Association of the United Kingdom Occasional Publication 2*.

Bryan GW, Hummerstone LG (1971) Adaptation of the polychaete *Nereis diversicolor* to estuarine sediments containing high concentrations of heavy metals. 1. General observations and adaptation to copper. *Journal of the Marine Biological Association of the United Kingdom*, 53, 845-863.

Bryan GW, Hummerstone LG (1973) Adaptation of the polychaete *Nereis diversicolor* to estuarine sediments containing high concentrations of zinc and cadmium. *Journal of the Marine Biological Association of the United Kingdom*, 53, 75-92.

Bryan GW, Langston WJ (1992) Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution*, 76, 89-131.

Burlinson FC, Lawrence FJ (2007) A comparison of acute and chronic toxicity tests used to examine the temporal stability of a gradient in copper tolerance of *Hediste diversicolor* from the Fal estuary, Cornwall, UK. *Marine Pollution Bulletin*, 54, 1, 66-71.

Burton JD (1979) Physico-chemical limitations in experimental investigations. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 286, 443-456.

- Burton RS (1986) Evolutionary consequences of restricted gene flow in the intertidal copepod *Tigriopus californicus*. *Bulletin of Marine Science*, 39, 526–535.
- Butlin RK, Galindo J, Grahame JW (2008) Sympatric, parapatric or allopatric: the most important way to classify speciation? *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363, 1506, 2997–3007.
- Caballero A, Quesada H, Rolán-Alvarez E (2008) Impact of amplified fragment length polymorphism size homoplasy on the estimation of population gene diversity and the detection of selective loci. *Genetics*, 179, 539–554.
- Caillez F, Kuntz P (1996) A contribution to the study of the metric and Euclidean structures of dissimilarities. *Psychometrika*, 61, 241–253.
- Calow P, Forbes VE (1998) How do physiological responses to stress translate into ecological and evolutionary processes? *Comparative Biochemistry and Physiology A*, 120, 1, 11–16.
- Calow P, Sibly RM (1990) A physiological basis of population processes: Ecotoxicological implications. *Functional Ecology*, 4, 283–288.
- Campbell D, Bernatchez L (2004) Generic scan using AFLP markers as a means to assess the role of directional selection in the divergence of sympatric whitefish ecotypes. *Molecular Biology and Evolution*, 21, 945–956.
- Carr SM, Snellen AJ, Howse KA, Wroblewski JS (1995) Mitochondrial DNA sequence variation and genetic stock structure of Atlantic cod (*Gadus morhua*) from bay and offshore locations on the Newfoundland continental shelf. *Molecular Ecology*, 4, 1, 79–88.
- Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: model and estimation procedures. *Evolution*, 21, 550–570.
- CBOL (2010) Consortium for the Barcode of Life, [www.barcoding.si.edu](http://www.barcoding.si.edu)
- Chambers SJ, Garwood PR (1992) *Polychaetes from Scottish Waters. Part 3. Family Nereidae*. National Museums of Scotland.
- Chambers MR, Milne H (1975) Life cycle and production of *Nereis diversicolor* in the Ythan Estuary, Scotland. *Estuarine and Coastal Marine Science*, 3, 133–144.
- Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics*, 18, 237–268.
- Chessel D, Dufour AB, Thioulouse J (2004) The ade4 package I One-table methods. *R News*, 4, 5–10.

- Chong DKX, Yang RC, Yeh FC (1994) Nucleotide divergence between populations of trembling aspen (*Populus tremuloides*) estimated with RAPDs. *Current Genetics*, 26, 374-376.
- Clark AG, Lanigan CM (1993) Prospects for estimating nucleotide divergence with RAPDs. *Molecular Biology and Evolution*, 10, 1096-1111.
- Coetzee M, Craig M, Le Sueur D (2000) Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex. *Parasitology Today*, 16, 2, 74-77.
- Colosimo PF, Hosemann KE, Balabhadra S, Villarreal G, Dickson M, Grimwood J, Schmutz J, Myers RM, Schluter D, Kingsley DM (2005) Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science*, 307, 1928-1933.
- Connor DW, Allen JH, Golding N, Lieberknecht LM, Northen KO, Reker JB (2004) *National Marine Habitat Classification for Britain and Ireland Version 04.05*. Joint Nature Conservation Committee.
- Cossu P, Casu M, Curini Galletti M, Lai T, Maltagliati F, Castelli A (2004) Analisi della struttura genetica di *Hediste diversicolor* (Polychaeta, Nereididae) nel Mediterraneo occidentale mediante marcatori ISSR. *Ecologia. Atti del XIV Congresso Nazionale della Società Italiana di Ecologia (Siena, 4-6 ottobre 2004)*. [www.xivcongresso.societaitalianaecologia.org/articles/Maltagliati-82.pdf](http://www.xivcongresso.societaitalianaecologia.org/articles/Maltagliati-82.pdf).
- Crisp DJ (1978) Genetic consequences of different reproductive strategies in marine invertebrates. In: Battaglia B, Beardmore JA (editors) *Marine organisms: genetics, ecology and evolution*. Plenum.
- Crispo E, Bentzen P, Reznick DN, Kinnison MT, Hendry AP (2006) The relative influence of natural selection and geography on gene flow in guppies. *Molecular Ecology*, 15, 49-62.
- Crow JF (1948) Alternative Hypotheses of Hybrid Vigor. *Genetics*, 33, 5, 477-487.
- Crow JF (1986) *Basic Concepts in Population, Quantitative and Evolutionary Genetics*. WH Freeman & Co.
- Crow JF, Aoki K (1984) Group selection for a polygenic behavioral trait: estimating the degree of population subdivision. *Proceedings of the National Academy of Sciences USA*, 81, 6073-6077.
- Crow JF, Kimura M (1970) *An Introduction to Population Genetics Theory*. Harper and Row.
- Crow JF, Morton NE (1955) Measurement of gene frequency drift in small populations. *Evolution*, 9, 202-214.

- Cruz F, Perez M, Presa P (2005). Distribution and abundance of microsatellites in the genome of bivalves. *Gene*, 346, 241–247.
- Czekanowski (1913) *Zarys metod statystycznych w zastosowaniu do antropologii* [An outline of statistical methods applied in anthropology]. Towarzystwo Naukowe Warszawskie
- Dales RP (1950) The reproduction and larval development of *Nereis diversicolor* O.F. Muller. *Journal of the Marine Biological Association of the United Kingdom*, 29, 321–360.
- Darwin C (1859) *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. John Murray.
- Dasmahapatra KK, Lacy RC, Amos W (2007) Estimating levels of inbreeding using AFLP markers. *Heredity*, 100, 286–295.
- Davey JT, George CL (1986) Species interactions in soft sediments: factors in the distribution of *Nereis (Hediste) diversicolor* in the Tamar estuary. *Ophelia*, 26, 151–164.
- Dayton PK (1971) Competition, disturbance, and community organization: the provision and subsequent utilization of space in a rocky intertidal community. *Ecological Monographs*, 41, 4, 351–389.
- Denver DR, Morris K, Lynch M, Thomas WK (2004) High mutation rate and predominance of insertions in the *Caenorhabditis elegans* nuclear genome. *Nature* 430, 679–682.
- Diamond PM, Newman MC, Mulvey M, Dixon PM, Martinson D (1989) Allozyme genotype and time to death of mosquitofish *Gambusia affinis* during acute exposure to inorganic mercury. *Environmental Toxicology and Chemistry*, 8, 613–622.
- Diamond SA, Newman MC, Mulvey M, Guttman SI (1991) Allozyme genotype and time-to-death of mosquitofish, *Gambusia holbrooki*, during acute inorganic mercury exposure: a comparison of populations. *Aquatic Toxicology*, 21, 119–134.
- Dice LR (1945) Measures of the amount of ecologic association between species. *Ecology*, 26, 297–302.
- Dobzhansky T (1937) *Genetics and the origin of species*. Columbia University Press.
- Dring MJ (1992) *The Biology of Marine Plants*. Cambridge University Press.
- Duarte JM, dos Santos JB, Melo LC (1999) Comparison of similarity coefficients based on RAPD markers in the common bean. *Genetics and Molecular Biology*, 22, 427–432.

- Dyer KR (1997) *Estuaries, A Physical Introduction*. Wiley.
- Egan SP, Nosil P, Funk DJ (2008) Selection and genomic differentiation during ecological speciation: isolating the contributions of host-association via a comparative genome scan of *Neochlamisus bebbianae* leaf beetles. *Evolution*, 62, 1162–1181.
- Emelianov I, Marec F, Mallet J (2004) Genomic evidence for divergence with gene flow in host races of the larch budmoth. *Proceedings of the Royal Society B: Biological Sciences*, 271, 97–105.
- Endler JA (1973) Gene flow and population differentiation. *Science*, 179, 243–250.
- Evans PR, Ward RM, Bone M, Leakey M (1999) Creation of temperate-climate intertidal mudflats: factors affecting colonization and use by benthic invertebrates and their bird predators. *Marine Pollution Bulletin*, 37, 8-12, 535-545.
- Evans SM, Kerrigan E, Palmer N (2000) Causes of imposex in the dogwhelk *Nucella lapillus* (L.) and its use as a biological indicator of tributyltin contamination. *Marine Pollution Bulletin*, 40, 3, 212-219.
- Excoffier L, Heckel G (2006) Computer programs for population genetics data analysis: a survival guide. *Nature Reviews Genetics*, 7, 745–758.
- Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, 1, 47-50.
- Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred for metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131, 479–491.
- Faith J, Mintram R, Angelova M (2006) Targeted projection pursuit for visualising gene expression data classifications. *Bioinformatics*, 22, 2667–2673.
- Fauchald K (1977) The polychaete worms. Definitions and keys to the orders, families and genera. *Natural History Museum of Los Angeles County Science Series*, 28, 85–91.
- Fauchald K, Jumars PA (1979) The diet of worms: a study of polychaete feeding guilds. *Oceanography and Marine Biology Annual Review*, 17, 193–284.
- Fisher RA (1930) *The Genetical Theory of Natural Selection*. Clarendon Press.
- Fong PP, Garthwaite RL (1994) Allozyme electrophoretic analysis of the *Hediste limnicola* – *H. diversicolor* – *H. japonica* species complex (Polychaeta: Nereididae). *Marine Biology*, 118, 463–470.



- Foulley JL, van Schriek MGM, Alderson L, Amigues Y, Bagga M, Boscher MY, Brugmans B, Cardellino R, Davoli R, Delgado JV, Fimland E, Gandini GC, Glodek P, Groenen MAM, Hammond K, Harlizius B, Heuven H, Joosten R, Martinez AM, Matassino D, Meyer JN, Peleman J, Ramos AM, Rattink AP, Russo V, Siggens KW, Vega-Pla JL, Ollivier L (2006) Genetic diversity analysis using lowly polymorphic dominant markers: the example of AFLP in pigs. *Journal of Heredity*, 97, 244–252.
- Fowler J, Cohen, L, Jarvis P (1998) *Practical Statistics for Field Biology*. Wiley.
- Frantzen MAJ, Silk JB, Ferguson JWH, Wayne RK, Kohn MH (1998) Empirical evaluation of preservation methods for faecal DNA. *Molecular Ecology*, 7, 1423–1428.
- French PW (1997) *Coastal And Estuarine Management*. Routledge.
- Garcia-Arberas L, Rallo A (2000) Variations in paragnath number of *Hediste diversicolor* from the Basque coast, in relation to habitat conditions. *Journal of the Marine Biological Association of the United Kingdom*, 80, 733-734.
- García-Pereira MJ, Caballero A, Quesada H (2010) Evaluating the Relationship between Evolutionary Divergence and Phylogenetic Accuracy in AFLP Data Sets. *Molecular Biology and Evolution*, 27, 5, 988-1000.
- Gardeström J, Dahl U, Kotsalainen O, Maxson A, Elfving T, Grahn M, Bengtsson BE, Breitholtz M (2008) Evidence of population genetic effects of long-term exposure to contaminated sediments – a multi-endpoint study with copepods. *Aquatic Toxicology*, 86, 426–436.
- Gardeström J, Gorokhova E, Gilek M, Grahn M, Bengtsson BE, Breitholtz M (2006) A multilevel approach to predict toxicity in copepod populations: Assessment of growth, genetics, and population structure. *Aquatic Toxicology*, 79, 41–48.
- Geffard A, Smith BD, Amiard-Triquet C, Jeantet AY, Rainbow PS (2005) Kinetics of trace metal accumulation and excretion in the polychaete *Nereis diversicolor*. *Marine Biology* 147, 1291-1304.
- Gerber S, Mariette S, Streiff R, Bodenes C, Kremer A (2000) Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. *Molecular Ecology*, 9, 1037–1048.
- Gerhardt HC (1994) The Evolution of Vocalization in Frogs and Toads. *Annual Review of Ecology and Systematics*, 25, 293-324.
- Goslee SC, Urban DL (2007) The ecodist package for dissimilarity-based analysis of ecological data. *Journal of Statistical Software*, 22, 7, 1-19.
- Gower JC (1966) Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika*, 53, 325–328.

- Gower JC, Legendre P (1986) Metric and Euclidean properties of dissimilarity coefficients. *Journal of Classification*, 3, 5–48.
- Grahame JW, Wilding CS, Butlin RK (2006) Adaptation to a steep environmental gradient and an associated barrier to gene exchange in *Littorina saxatilis*. *Evolution*, 60, 268–278.
- Grant A (2002) Pollution tolerant species and communities: intriguing toys or invaluable monitoring tools? *Human and Ecological Risk Assessment*, 8, 5, 955–970.
- Grant A, Hateley JG, Jones NV (1989) Mapping the ecological impact of heavy metals on the estuarine polychaete *Nereis diversicolor* using inherited metal tolerance. *Marine Pollution Bulletin*, 20, 5, 235–236.
- Grant A, Hateley JG, Jones NV (1990) Interpopulation variation in life history and metal tolerance in *Nereis diversicolor*. In: Aldrich JC (editor) *Phenotypic responses and individuality in aquatic ectotherms*, 167–175. JAPAGA.
- Gray JS (1991) Climate change. *Marine Pollution Bulletin*, 22, 169–171.
- Green J (1968) *The Biology Of Estuarine Animals*. Billing & sons.
- Haldane JBS (1924) A Mathematical Theory of Natural and Artificial Selection.
- Hamilton WD (1967) Extraordinary sex ratios. *Science*, 156, 477–488.
- Hardy GH (1908) Mendelian proportions in a mixed population. *Science*, 28, 706, 49–50.
- Harper FA, Smith SE, Macnair MR (1997) Where is the cost in copper tolerance in *Mimulus guttatus*? Testing the trade-off hypothesis. *Functional Ecology*, 11, 6, 764–774.
- Hartl DL, Clark AG (2007) *Principles of Population Genetics*. Fourth edition. Sinauer Associates.
- Haslett SK (2000) *Coastal Systems*. Routledge.
- Hateley JG, Grant A, Jones NV (1989) Heavy metal tolerance in estuarine populations of *Nereis diversicolor*. In: Ryland JS, Tyler PA (editors) *Reproduction, Genetics and Distributions of Marine Organisms. Proceedings of the 23rd European Marine Biological Symposium*, 379–384.
- Hateley JG, Grant A, Taylor SM, Jones NV (1992) Morphological and other evidence on the degree of genetic differentiation between populations of *Nereis diversicolor*. *Journal of the Marine Biological Association of the United Kingdom*, 72, 365–381.

- Hayward PJ, Ryland JS (1995) *Handbook of the Marine Fauna of North-West Europe*. Oxford University Press.
- Heagler MG, Newman MC, Mulvey M, Dixon PM (1993) Allozyme genotype in mosquitofish, *Gambusia holbrooki*, during mercury exposure: temporal stability, concentration effects and field verification. *Environmental Toxicology and Chemistry*, 12, 385–395.
- Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, 270, 313–321.
- Hebert PDN, Luiker M (1996) Genetic effects of contaminant exposure - towards an assessment of impacts on animal populations. *Science of the Total Environment*, 191, 23 - 58.
- Hedrick PW (2006) Genetic polymorphism in heterogeneous environments: the age of genomics. *Annual Review of Ecology and Systematics*, 37, 67–93.
- Hendry AP (2004) Selection against migrants contributes to the rapid evolution of ecologically-dependent reproductive isolation. *Evolutionary Ecology Research*, 6, 1219–1236.
- Hendry AP, Farrugia, TJ, Kinnison, MT (2008) Human influences on rates of phenotypic change in wild animal populations. *Molecular Ecology*, 17, 20-29.
- Hiddink JG, ter Hofstede R, Wolff WJ (2002) Predation of intertidal infauna on juveniles of the bivalve *Macoma balthica*. *Journal of Sea Research*, 47, 2, 141-159.
- Hill WG, Weir BS (2004) Moment estimation of population diversity and genetic distance from data on recessive markers. *Molecular Ecology*, 13, 895–908.
- Hoare K, Beaumont A, Davenport J (1994) Effects of copper exposure during early life stages on heterozygosity in laboratory reared mussel (*Mytilus edulis*) populations. In: Beaumont AR (editor) *Genetics and Evolution of Aquatic Organisms*, 425–434. Chapman & Hall.
- Holsinger KE, Lewis PO (2007) HICKORY: a package for analysis of population genetic data, version 1.1. Department of Ecology and Evolutionary Biology, University of Connecticut.
- Holsinger KE, Lewis PO, Dey DK (2002) A Bayesian approach to inferring population structure from dominant markers. *Molecular Ecology*, 11, 1157–1164.
- Honnay O, Jacquemyn H, Van Looy K, Vandepitte K, Breyne P (2009) Temporal and spatial genetic variation in a metapopulation of the annual *Erysimum cheiranthoides* on stony river banks. *Journal of Ecology*, 97, 1, 131-141.

- Hull SL (1998) Assortative mating between two morphs of *Littorina saxatilis* on a shore in Yorkshire. *Hydrobiologia*, 378, 79–88.
- Hull SL, Grahame J, Mill PJ (1996) Morphological divergence and evidence for reproductive isolation in *Littorina saxatilis* (Olivi) in northeast England. *Journal of Molluscan Studies*, 62, 89–99.
- Hunter RL, Merkert CL (1957) Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science*, 125, 1294–1295.
- Hutchings P, Reid A (1991) The Nereididae from Australia - *Leonnates*, *Platynereis* and *Solomononeis*. *Records of the Australian Museum*, 43, 47–62.
- Hutchison DW, Templeton AR (1999) Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution*, 53, 1898–1914.
- Hvilson MM (1983) Copper-induced differential mortality in the mussel *Mytilus edulis*. *Marine Biology*, 76, 291–295.
- Innan H, Terauchi R, Kahl G, Tajima F (1999) A method for estimating nucleotide diversity from AFLP data. *Genetics*, 151, 1157–1164.
- Isabel N, Beaulieu J, Bousquet J (1995) Complete congruence between gene diversity estimates derived from genotypic data at enzyme and random amplified polymorphic DNA loci in black spruce. *Proceedings of the National Academy of Science of the USA*, 92, 6369–6373.
- Isabel N, Beaulieu J, Thériault P, Bousquet J (1999) Direct evidence for biased gene diversity estimates from dominant random amplified polymorphic DNA (RAPD) fingerprints. *Molecular Ecology*, 8, 477–483.
- Ivanenkov YaA, Bovina EV, Balakin KV (2009) Nonlinear mapping techniques for predicting the pharmacological properties of chemical compounds. *Russian Chemical Reviews*, 78, 5, 503–523.
- Jablonka E, Raz G (2009) Transgenerational epigenetic inheritance: Prevalence, mechanisms, and implications for study of heredity and evolution. *Quarterly Review of Biology*, 84, 131–176.
- Jaccard P (1908) Nouvelles recherches sur la distribution florale. *Bulletin de la Société Vaudoise Des Sciences Naturelles*, 44, 223–270.
- Jackson AA, Somers KM, Harvey HH (1989). Similarity coefficients: measures for co-occurrence and association or simply measures of occurrence? *American Naturalist*, 133, 436–453.
- Jennings S, Kaiser MJ, Reynolds JD (2001) *Marine Fisheries Ecology*. Blackwell.

- Jensen KT, Andre C (1993) Field and laboratory experiments on interactions among an infaunal polychaete, *Nereis diversicolor* and 2 amphipods, *Corophium volutator* and *C. arenarium* – effects on survival and recruitment. *Journal of Experimental Marine Biology and Ecology*, 168, 259–278.
- Jha AN (2004) Genotoxicological studies in aquatic organisms: An overview. *Mutation Research*, 552, 1–17.
- Johannesson K, Johannesson B, Rolán-Alvarez E (1993) Morphological differentiation and genetic cohesiveness over a microenvironmental gradient in the marine snail *Littorina saxatilis*. *Evolution*, 47, 1770–1787.
- Johannesson K, Panova M, Kemppainen P, André C, Rolán-Alvarez E, Butlin RK (2010) Repeated evolution of reproductive isolation in a marine snail: unveiling mechanisms of speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365, 1735–1747.
- Johannesson K, Rolán-Alvarez E, Eken Dahl A (1995) Incipient reproductive isolation between two sympatric morphs of the intertidal snail *Littorina saxatilis*. *Evolution*, 49, 1180–1190.
- Johns MA, Skroch PW, Nienhuis J, Kinrichsen P, Bascur G, Muñoz-Schick C (1997) Gene pool classification of common bean landraces from Chile based on RAPD and morphological data. *Crop Science*, 37, 605–613.
- Johnson EL, Zhang D, Emche SD (2005) Inter- and intra-specific variation among five *Erythroxylum* taxa assessed by AFLP. *Annals of Botany*, 95, 601–608.
- Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van de Wiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevski A, Marmioli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding*, 3, 5, 381–390.
- Joost S, Bonin A, Bruford MW, Després L, Conord C, Erhardt G, Taberlet P (2007) A spatial analysis method (SAM) to detect candidate loci for selection: towards a landscape genomics approach of adaptation. *Molecular Ecology*, 16, 3955–3969.
- Kent AC, Day RW (1983) Population dynamics of an infaunal polychaete: the effect of predators and an adult-recruit interaction. *Journal of Experimental Marine Biology and Ecology*, 73, 185–203.
- Kerr SE, Wright S (1954) Experimental studies of the distribution of gene frequencies in very small populations of *Drosophila melanogaster*: I. Forked. *Evolution*, 8, 172–177.

- Khlebovich VV, Komendantov AY (2002) Temporary variation of paragnath arrangement in *Hediste diversicolor* and *H. japonica* (Polychaeta, Nereididae). *Zoologicheskii Zhurnal*, 81, 503–505.
- Kijima A, Fujio Y (1984) Relationship between average heterozygosity and river population size in chum salmon. *Bulletin of the Japanese Society of Scientific Fisheries*, 50, 4, 603-608.
- Kimura M (1953) Stepping stone model of population. *Annual Report of the National Institute of Genetics, Japan*, 49, 561-576.
- Kimura M (1968) Evolutionary rate at the molecular level. *Nature*, 217, 624–626.
- Knight M, Parke M (1950) A biological study of *Fucus vesiculosus* L. and *Fucus serratus* L.. *Journal of the Marine Biological Association of the United Kingdom*, 29, 439-514.
- Knowlton N (1993) Sibling species in the sea. *Annual Review of Ecology and Systematics*, 24, 189–216.
- Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*, 420, 1, 73-90.
- Knowlton N, Maté JL, Guzmán HM, Rowan R, Jara J (1997) Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panamá and Honduras). *Marine Biology*, 127, 4, 705.
- Knowlton N, Weil E, Weigt LA, Guzman HM (1992) Sibling species in *Montastraea*, coral bleaching, and the coral climate record. *Science*, 330-333.
- Koehn RK, Bayne BL (1989) Towards a physiological and genetical understanding of the energetics of the stress response. *Biological Journal of the Linnean Society*, 37, 157–171.
- Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant Journal*, 4, 403-410.
- Koopman WJM, Gort G (2004) Significance tests and weighted values for AFLP similarities, based on *Arabidopsis* in silico AFLP fragment length distributions. *Genetics*, 167, 1915–1928.
- Kosman E (2003) Nei's gene diversity and the index of average differences are identical measures of diversity within populations. *Plant Pathology*, 52, 533–535.
- Kosman E, Leonard KJ (2005) Similarity coefficients for molecular markers in studies of genetic relationships between individuals for haploid, diploid, and polyploid species. *Molecular Ecology*, 14, 415–424.

- Krauss SL (2000) Accurate gene diversity estimates from amplified fragment length polymorphism (AFLP) markers. *Molecular Ecology*, 9, 1241–1245.
- Kristensen E (1984) Life cycle, growth and production in estuarine populations of polychaetes *Nereis virens* and *Nereis diversicolor*. *Holarctic Ecology*, 7, 249-256.
- Kristensen E (1988) Factors influencing the distribution of nereid polychaetes in Danish coastal waters. *Ophelia*, 29, 127-140.
- Kruse I (2003) Population ecology and genetics of the polychaete *Scoloplos armiger* (Orbiniidae). *Berichte zur Polar und Meeresforschung*, 438, 1-95.
- Kruse I, Reise K (2003) Reproductive isolation between intertidal and subtidal *Scoloplos armiger* (Polychaeta, Orbiniidae) indicates sibling species in the North Sea. *Marine Biology*, 143, 511-517.
- Kruse I, Reusch TBH, Schneider MV (2003) Sibling species or poecilogony in the polychaete *Scoloplos armiger*? *Marine Biology*, 142, 937-947.
- Kruse I, Strasser M, Thiermann F (2004) The role of ecological divergence in speciation between intertidal and subtidal *Scoloplos armiger* (Polychaeta, Orbiniidae). *Journal of Sea Research*, 51, 53-62.
- Kruskal JB (1964) Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika*, 29, 1-27.
- Kyle CJ, Boulding EG (2000) Comparative population genetic structure of marine gastropods (*Littorina* spp.) with and without pelagic larval dispersal. *Marine Biology*, 137, 835–845.
- Langan-Cranford KM, Pearse JS (1995) Breeding experiments confirm species status of two morphologically similar gastropods (*Lacuna* spp.) in central California. *Journal of Experimental Marine Biology and Ecology*, 186, 1, 17-31.
- Last KS (2000) Photoperiodism in the semelparous polychaete *Nereis virens* (Sars). PhD thesis, Newcastle University.
- Last KS, Olive PJW (1999) Photoperiodic control of growth and segment proliferation by *Nereis (Neanthes) virens* in relation to state of maturity and season. *Marine Biology*, 134, 191-199
- Leppäkoski E, Gollasch S, Gruszka P, Ojaveer H, Olenin S, Panov V (2002) The Baltic: a sea of invaders. *Canadian Journal of Fisheries and Aquatic Sciences*, 59, 7, 1175-1188.
- Levinton JS (2001) *Marine Biology: Function, Biodiversity, Ecology*. Oxford University Press.

Levitan DR (2002) The relationship between conspecific fertilization success and reproductive isolation among three congeneric sea urchins. *Evolution*, 56, 8, 1599–1609.

Lewin B (2004) *Genes VIII*. Pearson Prentice Hall.

Lewontin RC (1972) The apportionment of human diversity. *Evolutionary Biology*, 6, 381–398.

Lewontin RC, Krakauer J (1973) Distribution of gene frequency as a test of the theory of selective neutrality of polymorphisms. *Genetics*, 74, 175–195.

Li WH (1978) Maintenance of genetic variability under the joint effect of mutation, selection and random drift. *Genetics*, 90, 349–382.

Li YC, Korol AB, Fahima T, Beiles A, Nevo E (2002) Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Molecular Ecology*, 11, 2453–2465.

Lorenz JG, Jackson WE, Beck JC, Hanner R (2005) The problems and promise of DNA barcodes for species diagnosis of primate biomaterials. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360, 1869–1877.

Lucchini V (2003) AFLP: a useful tool for biodiversity conservation and management. *Comptes Rendus Biologies*, 326, 43–48.

Luikart G, England PE (1999) Statistical analysis of microsatellite data. *Trends in Ecology and Evolution*, 14, 253–256.

Luikart G, England PR, Tallmon D, Jordan S, Taberlet P (2003) The power and promise of population genomics: from genotyping to genome typing. *Nature Reviews Genetics*, 4, 981–994.

Luoma SN (1977) Detection of trace contaminant effects in aquatic ecosystems. *Journal of the Fisheries Research Board of Canada*, 34, 436–439.

Luoma SN (1983) Bioavailability of trace metals to aquatic organisms. *Science of the Total Environment*, 28, 1–22.

Luoma SN, Bryan GW (1982) A statistical study of environmental factors controlling concentrations of heavy metals in the burrowing bivalve *Scrobicularia plana* and the polychaete *Nereis diversicolor*. *Estuarine, Coastal and Shelf Science*, 15, 95–108.

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, 3, 91–99.

Ma XL, Cowles DL, Carter RL (2000) Effect of pollution on genetic diversity in the bay mussel *Mytilus galloprovincialis* and the acorn barnacle *Balanus glandula*. *Marine Environmental Research*, 50, 559–563.



- Majerus MEN (2009) Industrial Melanism in the Peppered Moth, *Biston betularia*: An Excellent Teaching Example of Darwinian Evolution in Action. *Evolution: Education and Outreach*, 2, 1, 63-74.
- Makinen HS, Cano JM, Merila J (2008). Identifying footprints of directional and balancing selection in marine and freshwater three-spined stickleback (*Gasterosteus aculeatus*) populations. *Molecular Ecology*, 17, 3565–3582.
- Maldini M, Marzano FN, Fortes GG, Papa R, Gandolfi G (2006) Fish and seafood traceability based on AFLP markers: Elaboration of a species database. *Aquaculture*, 261, 2, 487-494.
- Malécot (1955) The decrease of relationship with distance. *Cold Spring Harbour Symposium on Quantitative Biology*, 20, 52-53.
- Maltagliati F, Camilli L, Lardicci C, Castelli A (2001) Evidence for morphological and genetic divergence in *Perinereis cultrifera* (Polychaeta: Nereididae) from two habitat types at Elba Island. *Journal of the Marine Biological Association of the United Kingdom*, 81, 3, 411–414.
- Maltagliati F, Massaro L, Cossu P, Castelli A (2006) Morphological differentiation in the ragworm, *Hediste diversicolor* (Polychaeta, Nereididae), as revealed by variation of paragnath number and distribution. *Italian Journal of Zoology*, 73, 255–262.
- Maltagliati F, Peru AP, Casu M, Rossi F, Lardicci C, Curini-galletti M, Castelli A (2000) Is *Syllis gracilis* (Polychaeta: Syllidae) a species complex? An allozyme perspective. *Marine Biology*, 136: 871-879.
- Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27, 209–220.
- Mariette S, Le Corre V, Austerlitz F, Kremer A (2002) Sampling within the genome for measuring within-population diversity: trade-offs between markers. *Molecular Ecology*, 11, 1145–1156.
- Marshall TC, Coltman DW, Pemberton JM, Slate J, Spalton JA, Guinness FE, Smith JA, Pilkington JG, Clutton-Brock TH (2002) Estimating the prevalence of inbreeding from incomplete pedigrees. *Proceedings of the Royal Society B: Biological Sciences*, 269, 1533–1539.
- Marty R, Retière C (1999) Larval-to-juvenile mobility activities of a holobenthic species, *Nereis diversicolor* (O.F.Müller) (polychaeta: Nereidae) – their involvement in recruitment. *Bulletin of Marine Science*, 65, 761–773.
- Mathews LM, Schubart CD, Neigel JE, Felder DL (2002) Genetic, ecological, and behavioural divergence between two sibling snapping shrimp species (Crustacea: Decapoda: *Alpheus*). *Molecular Ecology*, 11, 8, 427-438.

Maynard-Smith J, Price GR (1973) The logic of animal conflict. *Nature* 246, 15-18.

Mayr E (1942) *Systematics and the origin of species from the viewpoint of a zoologist*. Columbia University Press.

McCauley DE (1991) Genetic consequences of local population extinction and recolonisation. *Trends in Ecology and Evolution*, 6, 1, 5-8.

McCauley DE (1993) Genetic consequences of extinction and recolonization in fragmented habitats. In: Kareiva PM, Kingsolver JG, Huey RB (editors) *Biotic Interactions and Global Change*, 217-233. Sinauer.

McCurdy DG, Moran B (2004) Parasitism and soft-bottom community structure: studies in a polychaete–amphipod system. *Journal of the Marine Biological Association of the United Kingdom*, 84, 165–169.

McDermott JM, McDonald BA (1993) Gene Flow in Plant Pathosystems. *Annual Review of Phytopathology*, 31, 353-373.

Mechanda SM, Baum BR, Johnson DA, Arnason JT (2004) Sequence assessment of comigrating AFLP bands in Echinacea – implications for comparative biological studies. *Genome*, 47, 15–25.

Medina MH, Correa JA, Barata C (2007) Micro-evolution due to pollution: Possible consequences for ecosystem responses to toxic stress. *Chemosphere*, 67, 2105-2114.

Meudt HM, Clarke AC (2007) Almost Forgotten or Latest Practice? AFLP applications, analyses and advances. *Trends in plant science*, 12, 3, 106-117.

Meyer AS, Garcia AAF, Souza AP, Souza CL Jr (2004) Comparison of similarity coefficients used for cluster analysis with dominant markers in maize (*Zea mays* L). *Genetics and Molecular Biology*, 27, 83-91.

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease- resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregated populations. *Proceedings of the National Academy of Sciences USA*, 88, 9828–9832.

Millward RN, Grant A (1995) Assessing the impact of copper on nematode communities from a chronically metal-enriched estuary using pollution induced community tolerance. *Marine Pollution Bulletin*, 30, 701-706.

Möller P (1985) Production and abundance of juvenile *Nereis diversicolor* and oogenic cycle of adults in shallow waters of Western Sweden. *Journal of the Marine Biological Association of the United Kingdom*, 65, 603–616.

Moreira SM, Moreira-Santos M, Guilhermino L, Ribeiro R (2005) A short-term sublethal in situ toxicity assay with *Hediste diversicolor* (Polychaeta) for estuarine

- sediments based on postexposure feeding. *Environmental Toxicology and Chemistry*, 24, 8, 201-208.
- Morin PA, Luikart G, Wayne RK (2004) SNPs in ecology, evolution and conservation. *Trends in Ecology and Evolution*, 19, 208–216.
- Mouneyrac C, Mastin O, Amiard JC, Amiard-Triquet C, Beaunier P, Jeantet AY, Smith BD, Rainbow PS (2003) Trace metal detoxification and tolerance of the estuarine worm *Hediste diversicolor* chronically exposed in their environment. *Marine Biology*, 143, 731-744.
- Mueller UG, Wolfenbarger, LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*, 14, 389–39.
- Mullis KB (1983) The unusual origin of the polymerase chain reaction. *Scientific American*, 56–65.
- Murray JMH, Meadows A, Meadows PS (2002) Biogeomorphological implications of microscale interactions between sediment geotechnics and marine benthos: a review. *Geomorphology*, 47, 1, 15-30.
- Nei M (1972) Genetic distance between populations. *American Naturalist*, 106, 283-292.
- Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA*, 70, 3321-3323.
- Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89, 583–590.
- Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Genetics*, 89, 583–590.
- Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution*, 29, 1-10.
- Neigel JE (1997) A comparison of alternative strategies for estimating gene flow from genetic markers. *Annual Review of Ecology and Systematics*, 28, 105–128.
- Neigel JE (2002) Is  $F_{ST}$  obsolete? *Conservation Genetics*, 3, 167–173.
- Nevo E, Beiles A, Ben-Shlomo R (1984) The evolutionary significance of genetic diversity: ecological, demographic and life history correlates. In: Mani GS (editor) *Evolutionary Dynamics of Genetic Diversity. Lecture Notes in Biomathematics*, 53, 13–213.
- Nevo E, Noy R, Lavie B, Beiles A, Muchtar S (1986) Genetic diversity and resistance to marine pollution. *Biological Journal of the Linnean Society*, 29, 2, 139–144.

- Nevo E, Perl T, Beiles A, Wool D (1981) Mercury selection of allozyme genotypes in shrimp. *Experientia*, 37, 1152–1154.
- Nias DJ, McKillup SC, Edyvane KS (1993) Imposex in *Lepsiella vinosa* from Southern Australia. *Marine Pollution Bulletin*, 26, 380–384.
- Nielsen AM, Eriksen NT, Iversen JLL, Riisgård HU (1995) Feeding, growth and respiration in the polychaetes *Nereis diversicolor* (facultative filter-feeder) and *N. Virens* (omnivorous) – a comparative study. *Marine Ecology Progress Series*, 125, 149–158.
- Nithart M (1998) Population dynamics and secondary production of *Nereis diversicolor* in a North Norfolk saltmarsh (UK). *Journal of the Marine Biological Association of the United Kingdom*, 78, 131–143.
- Nóbrega R, Solé-Cava AM, Russo CAM (2004) High genetic homogeneity of an intertidal marine invertebrate along 8000 km of the Atlantic coast of the Americas. *Journal of Experimental Marine Biology and Ecology*, 303, 2, 173-181.
- Nosil P, Egan SP, Funk DJ (2008) Heterogeneous genomic differentiation between walking-stick ecotypes: ‘isolation-by-adaptation’ and multiple roles for divergent selection. *Evolution*, 62, 316–336.
- Nosil P, Funk DJ, Ortiz-Barrientos D (2009) Divergent selection and heterogeneous genomic divergence. *Molecular Ecology*, 18, 375–402.
- Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology*, 13, 1143–1155.
- Nybom H, Bartish I (2000) Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspectives in Plant Ecology, Evolution and Systematics*, 3, 2, 93–114.
- Ochiai A (1957) Zoogeographic studies on the soleoid fishes found in Japan and its neighbouring regions. *Bulletin of the Japanese Society for the Science of Fish*, 22, 526-530.
- Ogilvie LA, Grant A (2008) Linking pollution induced community tolerance (PICT) and microbial community structure in chronically metal polluted estuarine sediments. *Marine Environmental Research*, 65, 2, 87-198.
- Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens HH (2007) vegan: Community Ecology Package, R package version 1.8-8.
- Olive PJW, Fletcher J, Rees S, Desrosiers G (1997) Interactions of environmental temperature with photoperiod in determining age at maturity in a semelparous polychaete *Nereis (Neanthes) virens* sars. *Journal of Thermal Biology*, 22, 489-497.

- Olive PJW, Garwood PR (1981) Gametogenic cycle and population structure of *Nereis (Hediste) diversicolor* and *Nereis pelagica* from North-East England. *Journal of the Marine Biological Association of the United Kingdom*, 61, 193-213.
- Olive PJW, Grant A, Cowin PBD (1986) Decision and choice in polychaete reproductive cycles. In: Porchet M, Andries J-C, Dhainaut A (editors) *Fourth International Symposium on Invertebrate Reproduction*.
- Ozoh PTE (1990) Egg volume regulation in *Hediste (Nereis) diversicolor* eggs: a rapid bioassay. *Water, air and soil pollution*, 49, 231-240.
- Ozoh PTE (1992a) The effects of salinity, temperature and sediment on the toxicity of copper to juvenile *Hediste (Nereis) diversicolor* (O. F. Muller). *Environmental Monitoring and Assessment*, 21, 1, 1-10.
- Ozoh PTE (1992b) The importance of adult *Hediste (Nereis) diversicolor* in managing heavy metal pollution in shores and estuaries. *Environmental Monitoring and Assessment*, 21, 165–171.
- Ozoh PTE (1994) The effect of salinity, temperature and time on the accumulation and depuration of Copper in the ragworm, *Hediste (Nereis) diversicolor* O.F. Müller. *Environmental Monitoring and Assessment*, 29, 155-166.
- Palumbi SR (1992) Marine speciation on a small planet. *Trends in Ecology and Evolution*, 7, 114–118.
- Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295.
- Pérez T, Albornoz J, Dominguez A (1998) An evaluation of RAPD fragment reproducibility and nature. *Molecular Ecology*, 7, 10, 1347-1358.
- Pickles A, Grahame J (1999) Mate choice in divergent morphs of *Littorina saxatilis* (Olivi); speciation in action? *Animal Behaviour*, 58, 181–184.
- Pook C, Lewis C, Galloway T (2009) The metabolic and fitness costs associated with metal resistance in *Nereis diversicolor*. *Marine Pollution Bulletin*, 58, 7, 1063-1071.
- Porter AH (1990) Testing nominal species boundaries using gene flow statistics: The taxonomy of two hybridizing admiral butterflies (Limenitis:Nymphalidae). *Systematic Zoology*, 39, 131-147.
- Poulin R (1999) The functional importance of parasites in animal communities: many roles at many levels? *International Journal for Parasitology*, 29, 903-914.
- R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing. [www.R-project.org](http://www.R-project.org)

- Raffaelli D (1999) Nutrient enrichment and trophic organisation in an estuarine Food web. *Acta Oecologica*, 20, 449-461.
- Raffaelli D, Hawkins S (1996) *Intertidal Ecology*. Chapman & Hall.
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248-249.
- Reif JC, Melchinger AE, Frisch M (2005) Genetical and Mathematical Properties of Similarity and Dissimilarity Coefficients Applied in Plant Breeding and Seed Bank Management. *Crop Science*, 45, 1, 1-7.
- Reise K (1979) Spatial configurations generated by motile benthic polychaetes. *Helgoländer wiss Meeresunters*, 32, 55-72.
- Reitzel AM, Darling JD, Sullivan JC, Finnerty JR (2007) Global population genetic structure of the starlet anemone *Nematostella vectensis*: multiple introductions and implications for conservation policy. *Biological Invasions*, 10, 8, 1197-1213.
- Reynolds J, Weir BS, Cockerham CC (1983) Estimation of the coancestry coefficient: Basis for a short-term genetic distance. *Genetics*, 105, 767-769.
- Rice SA (1991) Reproductive isolation in the *Polydora ligni* complex and the *Streblospio benedicti* complex (Polychaeta: Spionidae). *Bulletin of Marine Science*, 48, 432-447.
- Rice WR, Hostert EE (1993) Laboratory experiments on speciation: what have we learned in 40 years? *Evolution*, 47, 1637-1653.
- Roark S, Brown K (1996) Effects of metal contamination from mine tailings on allozyme distributions of populations of Great Plains fishes. *Environmental Toxicology and Chemistry*, 15, 921-927.
- Rogers DJ, Tanimoto TT (1960) A computer program for classifying plants. *Science*, 132, 1115-1118.
- Rogstad SH, Keane B, Collier MH (2003) Minisatellite DNA mutation rate in dandelions is correlated with leaf tissue concentrations of Cr, Fe, Mn and Ni. *Environmental Toxicology and Chemistry*, 22, 2093-2099.
- Röhner M, Bastrop R, Jürss K (1997) Genetic differentiation in *Hediste diversicolor* (Polychaeta: Nereididae) for the North Sea and the Baltic Sea. *Marine Biology*, 130, 171-180.
- Rolán-Alvarez E, Carballo M, Galindo J, Moran P, Fernández B, Caballero A, Cruz R, Boulding EG, Johannesson K (2004) Nonallopatric and parallel origin of local reproductive barriers between two snail ecotypes. *Molecular Ecology*, 13, 3415-3424.

- Ross K, Cooper N, Bidwell JR, Elder J (2002) Genetic diversity and metal tolerance of two marine species: a comparison between populations from contaminated and reference sites. *Marine Pollution Bulletin*, 44, 671–679.
- Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, 145, 1219–1228.
- Rousset V, Pleijel F, Rouse GW, Erséus C, Siddall ME (2007) A molecular phylogeny of annelids. *Cladistics*, 23, 41–63.
- Rundle H, Nosil P (2005) Ecological speciation. *Ecology Letters*, 8, 336–352.
- Russell PF, Rao TR (1940) On habitat and association of species of anopheline larvae in south-eastern Madras. *Journal of the Malaria Institute of India*, 3, 153–178.
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406–425.
- Sammon JW (1969) A nonlinear mapping for data structure analysis. *IEEE Transactions on Computers*, 18, 401–409.
- Sanchez G, Restrepo S, Duque MC, Fregene M, Bonierbale M, Verdier V (1999) AFLP assessment of genetic variability in cassava accessions (*Manihot esculenta*) resistant and susceptible to the cassava bacterial blight (CBB). *Genome*, 42, 163–172.
- Sato M (1999) Divergence of reproductive and developmental characteristics in *Hediste* (Polychaeta: Nereididae). *Hydrobiologia*, 402, 129–143.
- Sato M, Masuda Y (1997) Genetic differentiation in two sibling species of the brackish-water polychaete *Hediste japonica* complex (Nereididae). *Marine Biology*, 130, 163–170.
- Sato M, Nakashima A (2003) A review of Asian *Hediste* species complex (Nereididae, Polychaeta) with descriptions of two new species and a redescription of *Hediste japonica* (Izuka, 1908). *Zoological Journal of the Linnean Society*, 137, 403–445.
- Savolainen V, Anstett MC, Lexer C, Hutton I, Clarkson JJ, Norup MV, Powell MP, Springate D, Salamin N, Baker WJ (2006) Sympatric speciation in palms on an oceanic island. *Nature*, 441, 210–213.
- Scaps P (2002) A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O.F. Müller) (Annelida: Polychaeta). *Hydrobiologia*, 470, 203–218.
- Schirmacher A, Schmidt H, Westheide W (1998) RAPD–PCR investigations on sibling species of terrestrial *Enchytraeus* (Annelida: Oligochaeta). *Biochemical Systematics and Ecology*, 26, 1, 35–44.

- Schlötterer C (2000) Evolutionary dynamics of microsatellite DNA. *Chromosoma*, 109, 6, 365-371.
- Schluter D (1996) Ecological causes of adaptive radiation. *American Naturalist*, 148, 40-64.
- Schluter D (2001) Ecology and the origin of species. *Trends in Ecology and Evolution*, 16, 372-380.
- Schluter D (2009) Evidence for ecological speciation and its alternative. *Science*, 323, 737-741.
- Schluter D, Conte GL (2009) Genetics and ecological speciation. *Proceedings of the National Academy of Sciences USA*, 106, 9955-9962.
- Schmidt H, Westheide W (1998) RAPD-PCR experiments confirm the distinction between three morphologically similar species of *Nerilla* (Polychaeta: Nerillidae). *Zoologischer Anzeiger*, 26, 277-285.
- Schmidt H, Westheide W (2000) Are the meiofaunal polychaetes *Hesionides arenaria* and *Stygocapitella subterranea* true cosmopolitan species? Results of RAPD-PCR investigations. *Zoologica Scripta*, 29, 1-11.
- Schneider S, Roessli D, Excoffier L (2000) Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.
- Selander RK (1970) Behavior and genetic variation in natural populations. *American Zoologist*, 10, 5366.
- Shannon CE (1948) A mathematical theory of communication. *Bell System Technical Journal*, 27, 379-423 and 623-656.
- Shi GR (1993) Multivariate data analysis in palaeoecology and palaeobiogeography - a review. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 105, 199-234.
- Sibly RM, Calow P (1989) A life-cycle theory of responses to stress. *Biological Journal of the Linnean Society*, 37, 101-116.
- Skidmore R, Leach C, Hoffman J, Amos W, Aldridge D (2010) Conservation genetics of the endangered depressed river mussel, *Pseudanodonta complanata*, using amplified fragment length polymorphism (AFLP) markers. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 20, 5, 560-567.
- Slate J, Coltman DW, Goodman SJ, MacLean I, Pemberton JM, Williams JL (1998) Bovine microsatellite loci are highly conserved in red deer (*Cervus elaphus*), sika deer (*Cervus nippon*) and Soay sheep (*Ovis aries*). *Animal Genetics*, 29, 4, 307-315.



- Slate J, Pemberton JM (2007) Admixture and patterns of linkage disequilibrium in a free-living vertebrate population. *Journal of Evolutionary Biology*, 20, 4, 1415-1427.
- Slatkin M (1985) Gene flow in natural populations. *Annual Review of Ecology and Systematics*, 16, 393-430.
- Slatkin M (1993) Isolation by distance in equilibrium and nonequilibrium populations. *Evolution*, 47, 264-279.
- Slatkin M (1994) Linkage disequilibrium in growing and stable populations. *Genetics*, 137, 331-336.
- Slatkin M, Barton NH (1989) A comparison of three indirect methods for estimating average levels of gene flow. *Evolution*, 43, 1349-1368.
- Smith RI (1977). Physiological and reproductive adaptation of *Nereis diversicolor* to life in the Baltic Sea and adjacent waters. In: Reish DJ, Fauchald K (editors) *Essays on polychaetous annelids*, 373-390. University of South California Press.
- Sneath PHA, Sokal RR (1973) *Numerical taxonomy – the principles and practice of numerical classification*. WH Freeman.
- Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin*, 38, 1409–1438.
- Sokal RR, Sneath PHA (1963) *Principles of Numerical Taxonomy*. WH Freeman.
- Sokolova IM, Oliver JD, Leamy LJ (2006) An AFLP approach to identify genetic markers associated with resistance to *Vibrio vulnificus* and *Perkinsus marinus* in eastern oysters. *Journal of Shellfish Research*, 25, 95-100.
- Solé-Cava AM, Thorpe JP (1991) High levels of genetic variation in natural populations of marine lower invertebrates. *Biological Journal of the Linnean Society*, 44, 65–80.
- Solé-Cava AM, Thorpe JP, Kaye JG (1985) Reproductive isolation with little genetic divergence between *Urticina* (= *Tealia*) *felina* and *U. eques* (Anthozoa: Actiniaria). *Marine Biology*, 85, 279-284.
- Somerfield PJ, Gee MJ, Warwirck RM (1994a) Benthic community structure in relation to an instantaneous discharge of waste water from a tin mine. *Marine Pollution Bulletin*, 28, 363-369.
- Somerfield PJ, Gee MJ, Warwick RM (1994b) Soft-sediment meiofaunal community structure in relation to a long-term heavy-metal gradient in the Fal Estuary system. *Marine Ecology Progress Series*, 105, 79-88.
- Sørensen T (1948) A method of establishing groups of equal amplitude in plant sociology based on similarity of species content and its application to analyses of

the vegetation on Danish commons. *Kongelige Danske Videnskabernes Selskabs Biologiske Skrifter*, 5, 1–34.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*, 98, 503-517.

Souza FL, Cunha AF, Oliveira MA, Pereira GAG, Pinheiro HP, dos Reis SF (2002) Partitioning of molecular variation at local spatial scales in the vulnerable neotropical freshwater turtle, *Hydromedusa maximiliani* (Testudines, Chelidae): implications for conservation of aquatic organisms in natural hierarchical systems. *Biological Conservation*, 104, 119-126.

Spiegelhalter DJ, Best NG, Carlin BP, van der Linde A (2002) Bayesian measures of model complexity and fit. *Journal of the Royal Statistical Society Series B*, 64, 483–689.

Stephens JS, Johnson RK, Key GS, McCosker JE (1970) The Comparative Ecology of Three Sympatric Species of California Blennies of the Genus *Hypsoblennius* Gill (Teleostomi, Blenniidae). *Ecological Monographs*, 40, 2, 213-233.

Stich B, Maurer HP, Melchinger AE, Frisch M, Heckenberger M, Rouppe van der Voort J, Peleman J, Srensen AP, Reif JC (2006) Comparison of linkage disequilibrium in elite European maize inbred lines using AFLP and SSR markers. *Molecular Breeding*, 17, 217–226.

Stockwell CA, Hendry AP, Kinnison MT (2003) Contemporary evolution meets conservation biology. *Trends in Ecology and Evolution*, 18, 94-101.

Sunnucks P (2000) Efficient genetic markers for population biology. *Trends in Ecology and Evolution*, 15, 199–203.

Szmidt AE, Wang X-R, Lu M-Z (1996) Empirical assessment of allozyme and RAPD variation in *Pinus sylvestris* (L.) using haploid tissue analysis. *Heredity*, 76, 412–420.

Templeton AR (1989) The meaning of species and speciation: a genetic perspective. In: Otte D, Endler JA (editors) *Speciation and its consequences*. Sinauer.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, 140, 767-782.

ter Braak CJF (1985) Correspondence analysis of incidence and abundance data: properties in terms of unimodal response model. *Biometrics*, 41, 859–873.

- Tero N, Aspi J, Siikamäki P, Jäkäläniemi A (2005) Local genetic population structure in an endangered plant species, *Silene tatarica* (Caryophyllaceae). *Heredity*, 94, 478–487.
- Thomas CM, Vos P, Zabeau M, Jones DA, Norcott KA, Chadwick BP, Jones JDG (1995) Identification of amplified restriction fragment polymorphism (AFLP) markers tightly linked to the tomato *Cf-9* gene for resistance to *Cladosporium fulvum*. *Plant Journal*, 8, 785-794.
- Thorpe JP, Solé-Cava AM (1994) The use of allozyme electrophoresis in invertebrate systematics. *Zoologica Scripta*, 23, 3-18.
- Timmermans MJTN, Ellers J, Mariën J, Verhoef SC, Ferwerda EB, van Straalen NM (2005) Genetic structure in *Orchesella cincta* (Collembola): strong subdivision of European populations inferred from mtDNA and AFLP markers. *Molecular Ecology*, 14, 2017–2024.
- Tóth G, Gáspári Z, Jurka J (2000) Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Research*, 10, 967–981.
- Townend I (2002) Marine science for strategic planning and management: the requirement for estuaries. *Marine Policy*, 26, 3, 209-219.
- Troncoso L, Galleguillos R, Larrain A (2000) Effects of copper on the fitness of the Chilean scallop *Argopecten purpuratus* (Mollusca: Bivalvia). *Hydrobiologia*, 420, 185–189.
- Uncles RJ, Stephens JA, Smith RE (2002) The dependence of estuarine turbidity on tidal intrusion length, tidal range and residence time. *Continental Shelf Research*, 22, 11-13, 1835-1856.
- Underwood AJ (1997) *Ecological experiments: their logical design and interpretation using analysis of variance*. Cambridge University Press.
- Van de Zande L, Bjilmsma R (1995) Limitation of the RAPD technique in phylogeny reconstruction in *Drosophila*. *Journal of Evolutionary Biology*, 8, 5, 645-656.
- Van Straalen N (2003) Ecotoxicology becomes Stress Ecology. *Environmental Science and Technology*, 37, 324-329.
- Van Straalen NM, Timmermans MJTN (2002) Genetic variation in toxicant-stressed populations: an evaluation of the genetic erosion hypothesis. *Human and Ecological Risk Assessment*, 8, 983–1002.
- Van Valen L (1976) Ecological species, multispecies and oaks. *Taxon*, 25, 233-239.
- Vekemans X, Beauwens T, Lemaire M, Roldan-Ruiz I (2002) Data from amplified fragment length polymorphism (AFLP) markers show indication of size

homoplasy and of a relationship between degree of homoplasy and fragment size. *Molecular Ecology*, 11, 139–151.

Venables WN, Ripley BD (2002) *Modern Applied Statistics with S*. Fourth Edition. Springer.

Via S, West J (2008) The genetic mosaic suggests a new role for hitchhiking in ecological speciation. *Molecular Ecology*, 17, 4334–4345.

Virgilio M, Abbiati M (2004a) Allozyme genotypes and tolerance to copper stress in *Hediste diversicolor* (Polychaeta: Nereididae). *Marine Pollution Bulletin*, 49, 978–985.

Virgilio M, Abbiati M (2004b) Habitat discontinuity and genetic structure in populations of the estuarine species *Hediste diversicolor* (Polychaeta: Nereididae). *Estuarine, Coastal and Shelf Science*, 61, 361–367.

Virgilio M, Abbiati M (2006) Temporal changes in the genetic structure of intertidal populations of *Hediste diversicolor* (Polychaeta: Nereididae). *Journal of Sea Research*, 56, 53–58.

Virgilio M, Backeljau T, Abbiati M (2006) Mitochondrial DNA and allozyme patterns of *Hediste diversicolor* (Polychaeta: Nereididae): the importance of small scale genetic structuring. *Marine Ecology Progress Series*, 326, 157–165.

Virgilio M, Baroncini N, Trombini C, Abbiati M (2003) Relationships between sediments and tissues contamination and allozymic patterns in *Hediste diversicolor* (Polychaeta: Nereididae) in the Pialassa lagoons (North Adriatic Sea). *Oceanologica Acta*, 26, 85–92.

Virgilio M, Fauvelot C, Constantini F, Abbiati M, Backeljau T (2009) Phylogeography of the common ragworm *Hediste diversicolor* (Polychaeta: Nereididae) reveals cryptic diversity and multiple colonization events across its distribution. *Molecular Ecology*, 18, 9, 1980–1994.

Virgilio M, Maci S, Abbiati M (2005) Comparisons of genotype tolerance responses in populations of *Hediste diversicolor* (Polychaeta: Nereididae) exposed to copper stress. *Marine Biology*, 147, 1305–1312.

Vos P, Hogers R, Bleeker M, Reijans M, Van Delee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 23, 4407–4414.

Vrijenhoek RC (2009) Cryptic species, phenotypic plasticity, and complex life histories: assessing deep-sea faunal diversity with molecular markers. *Deep Sea Research Part II: Topical Studies in Oceanography*, 56, 19–20, 1713–1723.

Walsh JB, Lynch M (2007) *Evolution and Selection of Quantitative Traits*. Volume 2. Sinauer.

- Wang WX (2002) Interactions of trace metals and different marine food chains. *Marine Ecology-Progress Series*, 243, 295-309.
- Waples RS (1987) A multispecies approach to the analysis of gene flow in marine shore fishes. *Evolution*, 41, 385-400.
- Warwick RM (2001) Evidence for the effects of metal contamination on the intertidal macrobenthic assemblage of the Fal Estuary. *Marine Pollution Bulletin*, 42, 145-148.
- Weinberg W (1908) Über den Nachweis der Vererbung beim Menschen. *Jahreshefte des Vereins für vaterländische Naturkunde in Württemberg*, 64, 368–382.
- Weir B, Cockerham C (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, 38, 1358–1370.
- Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, 18, 7213-7218.
- Whitlock MC, McCauley DE (1999) Indirect measure of gene flow and migration. *Heredity*, 82, 117–125.
- Wilding CS, Butlin RK, Grahame J (2001) Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. *Journal of Evolutionary Biology*, 14, 611–619.
- Will KW, Rubinoff D (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics*, 20, 1, 47-55.
- Williams GA (1996) Seasonal variation in a low shore *Fucus serratus* (Fucales, Phaeophyta) population and its epiphytic fauna. *Hydrobiologia*, 326/327, 191-197.
- Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18, 22, 6531-5.
- Witt JD, Threlloff DL, Hebert PD (2006) DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology*, 15, 3073–3082.
- Wolf JBW, Lindell J, Backström N (2010) Speciation genetics: current status and evolving approaches. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365, 1717-1733.
- Wong A, Forbes MR, Smith ML (2001) Characterization of AFLP markers in damselflies: prevalence of codominant markers and implications for population genetic applications. *Genome*, 44, 677–684.

- Wright S (1931) Evolution in Mendelian populations. *Genetics*, 16, 97 – 159.
- Wright S (1934) The method of path coefficients. *Annals of Mathematical Statistics*, 5, 161–215.
- Wright S (1938) Size of population and breeding structure in relation to evolution. *Science*, 87, 430–431.
- Wright S (1940) Breeding structure of populations in relation to speciation. *American Naturalist*, 74, 232-248.
- Wright S (1943) Isolation by distance. *Genetics*, 28, 114-138.
- Wright S (1946) Isolation by distance under diverse systems of mating. *Genetics*, 31, 39-59.
- Wright S (1951) The genetical structure of populations. *Annals of Eugenics*, 15, 323-354.
- Wright S (1969) *Evolution and the Genetics of Populations, Vol. 2. The Theory of Gene Frequencies*. Chicago University Press.
- Yauk CL, Quinn JS (1996) Multilocus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site. *Proceedings of the National Academy of Sciences USA*, 93, 22, 12137-12141.
- Yeh FC, Yang R, Boyle T (1999) POPGENE Version 1.31. Microsoft Windows-based Freeware for Population Genetic Analysis. University of Alberta.
- Young WP, Schupp JM, Keim P (1999) DNA methylation and AFLP marker distribution in the soybean genome. *Theoretical and applied genetics*, 99, 5, 785-792.
- Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. *European Patent Office*, 0 534 858 A1, 93/13.
- Zhivotovsky L (1999) Estimating population structure in diploids with multilocus dominant DNA markers. *Molecular Ecology*, 8, 907–913.
- Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, 20, 176-183.

## Appendix 1 Buffers and stock solutions used in AFLP analysis

Stock solutions labelled as 5x are 5 times the normal working strength of the solution. For 1x dilute to 1 part in 5 (i.e., 100 mL of 5x stock and 400 mL water to get a 1x solution).

### **0.5M EDTA (pH 8.0) – disodium ethylenediaminetetraacetate**

186.1g EDTA .2H<sub>2</sub>O

1 Litre dH<sub>2</sub>O

Disodium EDTA will not dissolve until the solution is adjusted to approximately pH 8.0. Add EDTA to 800 mL dH<sub>2</sub>O, add ~ 20g NaOH pellets to adjust pH and stir vigorously on a magnetic stirrer. Use pH meter to monitor pH.

Sterilise by autoclaving

### **TBE buffer**

T – Tris base – hydroxymethyl-aminomethane - buffer - maintains constant pH

B – Boric acid – provides correct ion concentration

E – EDTA - ethylenediaminetetraacetic acid (usually disodium EDTA) – chelates divalent metal cations (e.g. magnesium) required for nuclease activity.

Stock TBE is 5x

Agarose gels - 0.5x TBE (5 mL TBE, 45 mL dH<sub>2</sub>O / 100 mL TBE, 900 mL dH<sub>2</sub>O)

1x TBE (10 mL TBE, 40 mL dH<sub>2</sub>O)

### **5x TBE buffer**

54 g Tris base

27.5 g boric acid

20 mL 0.5M EDTA (pH 8)

Make up to 1 Litre with dH<sub>2</sub>O

Sterilise by autoclaving

### **10x TBE**

For 1 Litre:

108 g Tris

55 g boric acid

40 ml 0.5M EDTA pH 8.0

800 ml sdH<sub>2</sub>O

autoclave

### **5x Restriction-Ligation buffer**

50 mM Tris-HAc adjusted to pH 7.5 with acetic acid

50 mM MgAc

250 mM KAc

25 mM DTT

6 ml H<sub>2</sub>O ???

See Book 2

**10% APS**

10 % = 1 g per 10 ml sdH<sub>2</sub>O

**Storage:** 1 month in fridge

**Formamide loading dye**

98% formamide

1% bromophenol blue

1% xylene cyanol

**Ethidium bromide**

\* Wear gloves & mask

10 mg / mL

1g ethidium bromide

100 mL dH<sub>2</sub>O

Store in a dark container at room temperature

Use 0.5 µg / mL for staining agarose gels

10 mg / mL = 10 µg / µL

For 40 mL gel use 40 x 0.5 µg = 20 µg = 2 µL

**6x Gel loading buffer**

1.5g Ficoll

8.5 mL dH<sub>2</sub>O

25 µL bromophenol blue or orange g dye

(For 1x use 10 mL 6x buffer & 50 mL dH<sub>2</sub>O)

**1x CTAB buffer** (CTAB - Hexadecyltrimethylammonium bromide)**For 100 mL**

2% CTAB (2g)

1.4M NaCl (8.18g)

100 mM Tris-HCl (pH 8.0) (10 mL of 1M stock)

20 mM EDTA (4 mL of 0.5M stock)

1% Polyvinylpyrrolidone (PVP-40) (1 g)

0.2 % 2-mercaptoethanol (200 µl) – **Add to buffer just before extraction**

**Proteinase K**

10 mg/ml stock

1 mg proteinase K in 100 µl dH<sub>2</sub>O

Split into 10 x 10 µl in tubes & store in freezer



**Appendix 2: procrustes analysis  $m^2$  distances between MDS plots produced by applying cmdscale, isoMDS, nmds and sammon algorithms to Euclidean distance (ED), Jaccard (J), Czekanowski / Dice / Sørensen (CDS), Ochiai (O), Sokal and Sneath (SS), Russell and Rao (RR), Simple matching (SM) and Rogers and Tanaimoto (RT) distance or dissimilarity matrices for all 150 individuals.**

		cmdscale								isoMDS								nmds								sammon							
		ED	J	CDS	O	SS	RR	SM	RT	ED	J	CDS	O	SS	RR	SM	RT	ED	J	CDS	O	SS	RR	SM	RT	ED	J	CDS	O	SS	RR	SM	RT
cmdscale	ED	0.000	0.000	0.000	0.002	0.005	0.000	0.001	0.069	0.083	0.068	0.067	0.119	0.072	0.073	0.140	0.263	0.296	0.238	0.267	0.263	0.562	0.253	0.239	0.202	0.217	0.147	0.177	0.229	0.319	0.147	0.111	
	J	0.000	0.000	0.000	0.001	0.004	0.000	0.000	0.077	0.091	0.075	0.074	0.128	0.072	0.081	0.146	0.264	0.299	0.240	0.269	0.260	0.565	0.257	0.240	0.207	0.215	0.148	0.178	0.231	0.317	0.147	0.109	
	CDS	0.000	0.000	0.000	0.002	0.005	0.000	0.001	0.070	0.084	0.068	0.068	0.120	0.069	0.074	0.141	0.264	0.295	0.237	0.267	0.262	0.561	0.253	0.240	0.203	0.217	0.147	0.177	0.229	0.317	0.147	0.111	
	O	0.000	0.000	0.000	0.002	0.005	0.000	0.001	0.070	0.084	0.068	0.068	0.120	0.069	0.074	0.141	0.264	0.295	0.237	0.266	0.263	0.561	0.253	0.240	0.203	0.216	0.147	0.177	0.229	0.317	0.147	0.111	
	SS	0.002	0.001	0.002	0.002	0.003	0.002	0.001	0.089	0.103	0.087	0.086	0.142	0.079	0.093	0.156	0.266	0.306	0.245	0.272	0.258	0.570	0.264	0.240	0.213	0.214	0.149	0.179	0.235	0.316	0.148	0.106	
	RR	0.005	0.004	0.005	0.005	0.003	0.005	0.004	0.099	0.116	0.098	0.096	0.160	0.084	0.103	0.183	0.266	0.298	0.232	0.269	0.254	0.579	0.252	0.231	0.188	0.195	0.125	0.156	0.211	0.282	0.125	0.092	
	SM	0.000	0.000	0.000	0.000	0.002	0.005	0.001	0.069	0.083	0.068	0.067	0.119	0.072	0.073	0.140	0.263	0.296	0.238	0.267	0.263	0.562	0.253	0.239	0.202	0.217	0.147	0.177	0.229	0.319	0.147	0.111	
	RT	0.001	0.000	0.001	0.001	0.001	0.004	0.001	0.079	0.093	0.078	0.077	0.131	0.076	0.083	0.148	0.263	0.302	0.242	0.269	0.259	0.567	0.259	0.240	0.207	0.215	0.148	0.178	0.231	0.318	0.147	0.108	
isoMDS	ED	0.069	0.077	0.070	0.070	0.089	0.099	0.069	0.079	0.013	0.013	0.003	0.003	0.021	0.075	0.002	0.034	0.334	0.341	0.260	0.286	0.303	0.535	0.250	0.307	0.238	0.334	0.247	0.274	0.281	0.425	0.249	0.233
	J	0.083	0.091	0.084	0.084	0.103	0.116	0.083	0.093	0.013	0.013	0.013	0.014	0.023	0.091	0.012	0.036	0.349	0.345	0.296	0.314	0.331	0.518	0.262	0.322	0.267	0.356	0.274	0.303	0.308	0.452	0.273	0.261
	CDS	0.068	0.075	0.068	0.068	0.087	0.098	0.068	0.078	0.003	0.013	0.000	0.000	0.020	0.068	0.002	0.033	0.339	0.341	0.262	0.290	0.308	0.539	0.249	0.307	0.248	0.332	0.246	0.271	0.284	0.423	0.249	0.232
	O	0.067	0.074	0.068	0.068	0.086	0.096	0.067	0.077	0.003	0.014	0.000	0.000	0.021	0.067	0.002	0.034	0.337	0.343	0.261	0.287	0.305	0.541	0.248	0.305	0.243	0.331	0.243	0.269	0.281	0.421	0.247	0.230
	SS	0.119	0.128	0.120	0.120	0.142	0.160	0.119	0.131	0.021	0.023	0.020	0.021	0.105	0.020	0.009	0.034	0.365	0.369	0.311	0.324	0.370	0.533	0.278	0.360	0.307	0.406	0.320	0.342	0.350	0.502	0.321	0.303
	RR	0.072	0.072	0.069	0.069	0.079	0.084	0.072	0.076	0.075	0.091	0.068	0.067	0.105	0.076	0.130	0.032	0.327	0.344	0.250	0.281	0.305	0.559	0.259	0.288	0.244	0.287	0.207	0.224	0.250	0.313	0.212	0.190
	SM	0.073	0.081	0.074	0.074	0.093	0.103	0.073	0.083	0.002	0.012	0.002	0.002	0.020	0.076	0.031	0.031	0.342	0.340	0.265	0.292	0.309	0.539	0.248	0.315	0.247	0.338	0.253	0.278	0.291	0.431	0.256	0.237
	RT	0.140	0.146	0.141	0.141	0.156	0.183	0.140	0.148	0.034	0.036	0.033	0.034	0.009	0.130	0.031	0.031	0.370	0.392	0.342	0.354	0.395	0.544	0.310	0.401	0.347	0.443	0.362	0.381	0.392	0.543	0.366	0.331
nmds	ED	0.263	0.264	0.264	0.264	0.266	0.266	0.263	0.263	0.334	0.349	0.339	0.337	0.365	0.327	0.342	0.370	0.399	0.399	0.422	0.281	0.431	0.679	0.378	0.419	0.353	0.388	0.328	0.342	0.359	0.468	0.314	0.320
	J	0.296	0.299	0.295	0.295	0.306	0.298	0.296	0.302	0.341	0.345	0.341	0.343	0.369	0.344	0.340	0.392	0.399	0.399	0.316	0.440	0.484	0.627	0.317	0.393	0.358	0.387	0.329	0.326	0.414	0.455	0.338	0.369
	CDS	0.238	0.240	0.237	0.237	0.245	0.232	0.238	0.242	0.260	0.296	0.262	0.261	0.311	0.250	0.265	0.342	0.422	0.316	0.295	0.346	0.678	0.218	0.356	0.268	0.335	0.253	0.280	0.310	0.360	0.273	0.278	
	O	0.267	0.269	0.266	0.266	0.272	0.269	0.267	0.269	0.286	0.314	0.290	0.287	0.324	0.281	0.292	0.354	0.281	0.440	0.295	0.396	0.644	0.299	0.478	0.356	0.413	0.337	0.388	0.367	0.452	0.365	0.340	
	SS	0.263	0.260	0.263	0.263	0.258	0.254	0.263	0.259	0.303	0.331	0.308	0.305	0.370	0.305	0.309	0.395	0.431	0.484	0.346	0.396	0.698	0.365	0.333	0.335	0.365	0.295	0.360	0.338	0.440	0.317	0.322	
	RR	0.562	0.565	0.561	0.561	0.570	0.579	0.562	0.567	0.535	0.518	0.539	0.541	0.533	0.559	0.539	0.544	0.679	0.627	0.678	0.644	0.698	0.645	0.673	0.642	0.650	0.638	0.633	0.664	0.724	0.645	0.644	
	SM	0.253	0.257	0.253	0.253	0.264	0.252	0.253	0.259	0.250	0.262	0.249	0.248	0.278	0.259	0.248	0.310	0.378	0.317	0.218	0.299	0.365	0.645	0.364	0.236	0.366	0.278	0.309	0.247	0.356	0.278	0.324	
	RT	0.239	0.240	0.240	0.240	0.240	0.231	0.239	0.240	0.307	0.322	0.307	0.305	0.360	0.288	0.315	0.401	0.419	0.393	0.356	0.478	0.333	0.674	0.364	0.236	0.327	0.255	0.279	0.270	0.368	0.240	0.297	
sammon	ED	0.202	0.207	0.203	0.203	0.213	0.188	0.202	0.207	0.238	0.267	0.248	0.243	0.307	0.244	0.247	0.347	0.353	0.358	0.268	0.356	0.335	0.642	0.236	0.236	0.242	0.242	0.125	0.161	0.061	0.200	0.123	0.224
	J	0.217	0.215	0.217	0.216	0.214	0.195	0.217	0.215	0.334	0.356	0.332	0.331	0.406	0.287	0.338	0.443	0.388	0.387	0.335	0.413	0.365	0.650	0.366	0.327	0.242	0.151	0.151	0.166	0.240	0.249	0.150	0.156
	CDS	0.147	0.148	0.147	0.147	0.419	0.125	0.147	0.148	0.247	0.274	0.246	0.243	0.320	0.207	0.253	0.362	0.328	0.329	0.253	0.337	0.295	0.638	0.278	0.255	0.125	0.151	0.079	0.128	0.177	0.063	0.138	
	O	0.177	0.178	0.177	0.177	0.179	0.156	0.177	0.178	0.274	0.303	0.271	0.269	0.342	0.224	0.278	0.381	0.342	0.326	0.280	0.388	0.360	0.633	0.309	0.279	0.161	0.166	0.079	0.163	0.196	0.098	0.161	
	SS	0.229	0.231	0.229	0.229	0.235	0.211	0.229	0.231	0.281	0.308	0.284	0.281	0.350	0.250	0.291	0.392	0.359	0.414	0.310	0.367	0.338	0.664	0.247	0.270	0.061	0.240	0.128	0.163	0.177	0.133	0.234	
	RR	0.319	0.317	0.317	0.317	0.316	0.282	0.319	0.318	0.425	0.452	0.423	0.421	0.502	0.313	0.431	0.543	0.468	0.455	0.360	0.452	0.440	0.637	0.356	0.368	0.200	0.249	0.177	0.196	0.177	0.185	0.269	
	SM	0.147	0.147	0.147	0.147	0.148	0.125	0.147	0.147	0.249	0.273	0.249	0.247	0.321	0.212	0.256	0.366	0.314	0.338	0.273	0.365	0.317	0.645	0.278	0.240	0.123	0.150	0.063	0.098	0.133	0.185	0.121	
	RT	0.111	0.109	0.111	0.111	0.106	0.092	0.111	0.108	0.233	0.261	0.232	0.230	0.303	0.190	0.237	0.331	0.320	0.369	0.278	0.340	0.322	0.644	0.324	0.297	0.224	0.156	0.138	0.161	0.234	0.269	0.121	

Study		Species	N° loci	N° pops	Scale	H range	D range	F <sub>ST</sub>	F <sub>IS</sub>
Present study - AFLP		<i>Nereis diversicolor</i>	112 / 247		local	0.044 - 0.063	0.015 - 0.121	0.321	0.368?
Abbiati & Maltagliati	1992	<i>Neanthes succinea</i>	7 / 21	2	national		0.035	0.341	
Fong & Garthwaite	1994	<i>Nereis diversicolor</i>	9 / 10	2	local	0.023 - 0.200	0.31		
		<i>Nereis limnicola</i>		4	local		0.001 - 0.046		
		<i>N. limnicola</i> - <i>N. japonica</i>					0.500 - 0.565		
		<i>N. diversicolor</i> - <i>N. japonica</i>					0.786 - 1.056		
		<i>N. diversicolor</i> - <i>N. limnicola</i>					0.775 - 1.181		
Abbiati & Maltagliati	1996	<i>Nereis diversicolor</i>	15 / 17	4	national	0.014 - 0.034	0.000 - 0.356	0.848	
		<i>N. diversicolor</i> - <i>N. succinea</i>		6			1.8		
Rohner <i>et al.</i>	1997	<i>Nereis diversicolor</i>	5 / 13	8	international		0.000 - 0.265 **	0.892	
Maltagliati <i>et al.</i>	2000	<i>Syllis gracilis</i>			local		0.159	0.674	
Maltagliati <i>et al.</i>	2001	<i>Perinereis cultrifera</i>			local		0.185	0.381	0.489
Virgilio <i>et al.</i>	2003	<i>Nereis diversicolor</i>	15 / 19	5	local	0.012 - 0.052	0.000 - 0.003		
Virgilio & Abbiati	2004b	<i>Nereis diversicolor</i>	18 / 21	8	national	0.025 - 0.064	0.10 - 0.180*		
		<i>Nereis diversicolor</i>		2	local		0.068*		
		<i>Nereis diversicolor</i>		6	local		0.028 - 0.085	0.097	0.391
Virgilio <i>et al.</i>	2005	<i>Nereis diversicolor</i>	3	2	local		0.008*		
		<i>Nereis diversicolor</i>	3	2	national		0.074*		
		<i>Nereis diversicolor</i>	3	3	national		0.008 - 0.074*	0.047	0.543
Virgilio & Abbiati	2006	<i>Nereis diversicolor</i>	6	4+4	national	0.045 - 0.201	0.035 - 0.195*	0.017 -	
				4+4	temporal			0.274	

**Appendix 3: Summary of genetic diversity, distance, differentiation and inbreeding found by allozyme electrophoresis studies. Distance measures are Nei (1978), Nei (1972)\* or Cavalli-Sforza & Edwards\*\*. N° loci is the N° polymorphic loci out of total surveyed.**