Genetic differentiation of *Pseudo-nitzschia pungens* from the Pacific Northwest and the North Sea

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Abstract

Genetic differentiation of *Pseudo-nitzschia pungens* from the Pacific Northwest and the North Sea

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Chair of the Supervisory Committee:
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Using microsatellite data, the population genetic structure of *P. pungens* in the Pacific Northwest and the North Sea was compared. The Pacific Northwest samples showed significant deviations from Hardy-Weinberg and linkage equilibrium whereas the North Sea sample had none. The Pacific Northwest samples were significantly differentiated from the North Sea sample both when considered individually and as a pooled sample, but the individual Pacific Northwest samples were not significantly different from each other. However, clustering algorithms were able to separate the Pacific Northwest samples into two separate populations that exhibited few deviations from Hardy-Weinberg expectations and no deviation from linkage equilibrium. The degree of differentiation between the subpopulations in the Pacific Northwest indicated that there may be cryptic speciation of *P. pungens* as these subpopulations were more divergent than either was from the North Sea sample. Contrasting oceanographic processes in the Pacific Northwest and the North Sea may have contributed to the observed differences in Hardy-Weinberg and linkage equilibrium in the two areas. The sample sites in the Pacific Northwest were

situated in an area of dynamic mixing whereas the sample sites in the North Sea were not. Our study adds to the increasing body of evidence demonstrating cryptic speciation and high genetic diversity in marine phytoplankton.

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CHAPTER 1: INTRODUCTION

1.1 *Pseudo-nitzschia* species

1.1.1 Distribution, reproduction, morphology and identification

Pseudo-nitzschia are chain-forming pennate diatoms (Fig. 1.1) that occur exclusively in the marine plankton and are found throughout the oceans of the world (Hasle 1972, 2002). More than twenty species of *Pseudo-nitzschia* have been described (Hasle and Syvertsen 1997, Bates 2000, Lundholm *et al.* 2003), at least nine of which have been shown to produce measurable amounts of the neurotoxin domoic acid (DA) (Fryxell and Hasle 2003). Species of *Pseudo-nitzschia* commonly found in the Pacific Northwest include *P. multiseries*, *P. pungens*, *P. australis*, *P. fraudulenta*, *P. heimii*, *P. pseudodelicatissima*, *P. delicatissima* and *P. cuspidata* (Horner *et al.* 1997, Bill *et al.* 2006). In the North Sea, all of these species have been observed with the exception of *P. australis* (Hasle *et al.* 1996, Hasle 2002).

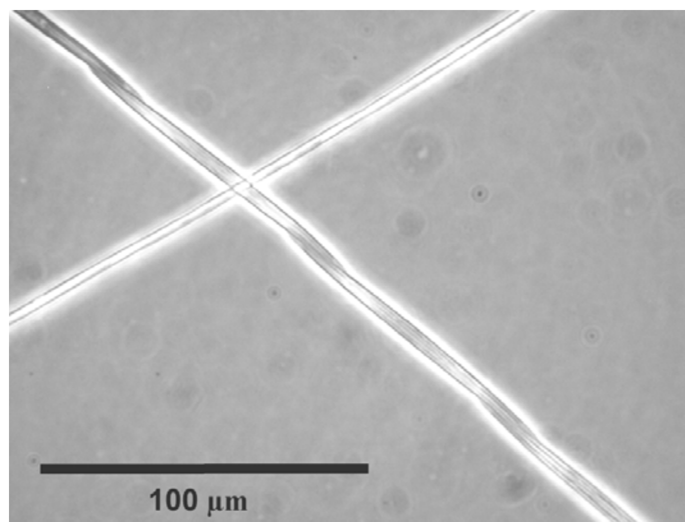

Figure 1.1. Light micrograph of *Pseudo-nitzschia pungens* chains at 400x magnification.

Pseudo-nitzschia cells, like all diatoms, have a silicified cell wall consisting of two halves that fit together like the top and bottom of a pill box (Geitler 1935). Each half consists of the valves, which correspond to the top and bottom of the box, along with several thinner linking structures called girdle elements, together these components are commonly called the frustule (Geitler 1935, Cupp 1943, Round and Crawford 1981, Round *et al.* 1990). Although diatoms occur in many shapes and sizes, they are divided into two overall types, centric and pennate. Geitler (1935) describes centric diatoms as being constructed radially, whereas pennate diatoms possess zygomorphic, or bilateral, structure. Cupp (1943) indicates that the markings on the valve faces of centric diatoms are generally radially arranged while those of pennate diatoms are arranged in connection with a longitudinal line. Cells with bilateral symmetry, i.e. pennate diatoms, can be positioned along three axes (Bold and Wynne 1985): the apical, pervalvar, and transapical axes which correspond to the length, height, and width of the cell (Fig. 1.2).

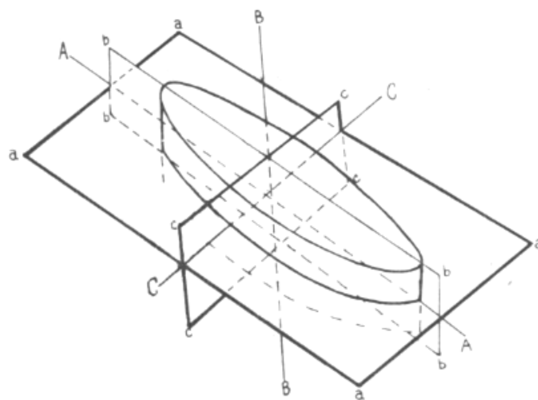

Figure 1.2. From Bold and Wynne (1985) showing the apical (A), pervalvar (B), and transapical (C) axes of a pennate diatom.

Pseudo-nitzschia reproduce primarily by vegetative cell division, as do all other diatoms (Cupp 1943, Round and Crawford 1981). And like other diatoms, vegetative cell division eventually results in the reduction of mean cell size, which can be restored by auxospore formation through sexual reproduction (Geitler 1935, Round *et al.* 1990, Mann 1993). Sexual reproduction has been documented for some species of *Pseudo-nitzschia* in laboratory experiments. The species of *Pseudo-nitzschia* studied by Davidovich and Bates (1998) were shown to be dioecious, meaning that a given clone will produce either "male" or "female" gametes, although on one occasion they did observe sexual reproduction between two clones of the same sex. Additionally, information about sexual reproduction and mating systems is available for six species of *Pseudo-nitzschia*, including *P. pungens* (Chepurnov *et al.* 2005). However, there is no published literature concerning sexual reproduction of *Pseudo-nitzschia* in natural populations.

Many of the *Pseudo-nitzschia* species noted above look similar when viewed with light microscopy, however they can be grouped into three size classes (Hasle and Syvertsen 1997). One class is made up of *P. pungens* and *P. multiseries*, a second of *P. australis*, *P. fraudulenta*, and *P. heimii*, while *P. pseudodelicatissima*, *P. delicatissima* and *P. cuspidata* make up the third class. Although phylogenetically *P. pungens* and *P. multiseries* as well as *P. pseudodelicatissima* and *P. delicatissima* have been shown to be closely related, *P. australis* and *P. fraudulenta* are more distantly related based on the first 872 base pairs of nuclear-encoded large subunit ribosomal DNA (Lundholm *et al.* 2002). These size classes

are easily discernable under light microscopy and the classifications are based strictly on morphology.

It is possible, although sometimes difficult and time consuming, to make further identifications to the species level using electron microscopy or molecular techniques. Hasle *et al.* (1996) provide a review of the characteristics that are used to differentiate species using electron microscopy, examples of which are listed in Table 1.1. Additional morphological characteristics that may be used to differentiate *P. pungens* from *P. multiseries* are listed in Table 1.2 (Hasle 1995). Figure 1.3 shows an electron micrograph of *P. pungens* indicating features listed in Table 1.1. Also, Lundholm *et al.* (2003) have further refined the description of *P. pseudodelicatissima*, based on poroid structure, and described two new species that may have been previously identified as *P. pseudodelicatissima*.

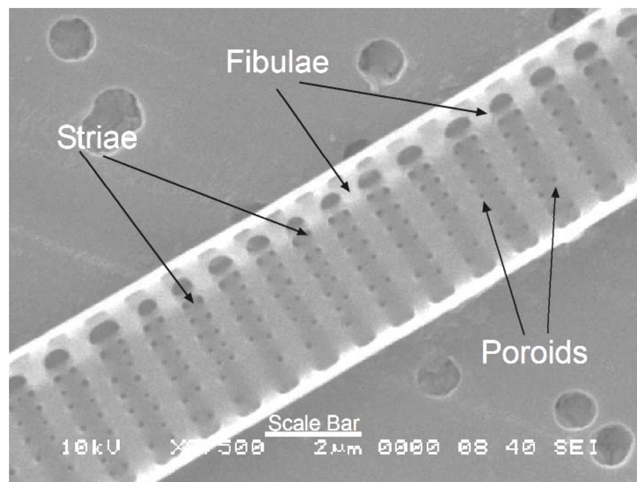

Figure 1.3. Scanning electron micrograph illustrating the components of a *Pseudo-nitzschia pungens* frustule. The scale bar (2 µm) is determined by the software associated with the electron microscope and is based on the magnification, in this case 9,500x.

Table 1.1. Morphometric data for *Pseudo-nitzschia* spp. from Hasle *et al.* (1996).

Species	Apical axis (µm)	Transapical axis (µm)	Fibulae in 10 µm	Striae in 10 µm	Poroids in 1 µm	Band structure Striae in 10 µm
P. pungens	74-142	2.9-4.5	9-15	9-15	3-4	15-19
P. multiseries	68-140	3.4-5.0	10-15	10-15	4-6	19-22
P. australis	75-144	6.5-8.0	13-16	13-16	4-5	19-20
P. fraudulenta	73-117	5-6	19-23	19-23	4-5	35-40
P. heimii	50-78	5-6	14-18	26-28	7-8	~35
P. delicatissima	42-66	1.0-1.5	20-23	~40	10-12	48-52
P. pseudodelicatissima	77-108	1.3-2.0	18-24	32-44	4-5	~44

Table 1.2. Additional features that may be used to differentiate *P. pungens* from *P. multiseries* when using electron microscopy. From Hasle (1995).

Feature	*P. pungens*	*P. multiseries*
Valve face striae		
-Rows of poroids	2	3-4
-Valve ends	Dissimilar	Similar
Proximal mantle	Single or pairs of poroids	Striated as valve face
Band Structure	Single poroids	Striated (2-3 rows of poroids)

It has been shown that differences in ribosomal DNA sequences can be used to differentiate *Pseudo-nitzschia* species (Scholin *et al.* 1994). Species specific fluorescent probes have been designed to target differences in the large-subunit ribosomal RNA and have been used to discriminate *Pseudo-nitzschia* species (Miller and Scholin 1996). Additionally, Manhart *et al.* (1995) found that the internal transcribed spacer region of *P. multiseries* differs significantly from that of *P. pungens* and may also be used for differentiating the two species. Thus, despite the morphological similarities it is possible to differentiate individual cells to the species level.

1.1.2 Potential toxicity

Some species of *Pseudo-nitzschia* can produce the neurotoxin DA, (Horner *et al.* 1997, Fryxell and Hasle 2003), a neuroexcitatory amino acid. Potential DA-producing *Pseudo-nitzschia* species have been observed in both the Pacific Northwest and the North Sea (Hasle 2002, Fryxell and Hasle 2003). However, the reasons for DA production by *Pseudo-nitzschia* are not completely understood. Some of the factors that have been investigated are temperature and salinity (Marchetti *et al.* 2004), trace metal concentrations (Maldonado *et al.* 2002, Wells *et al.* 2005), synergistic interactions with bacteria (Bates *et al.* 2004), and nutrient concentrations (Bates *et al.* 1991, Bates *et al.* 1993, Fehling *et al.* 2004).

1.2 Domoic acid

1.2.1 History of DA and DA events

Domoic acid was first isolated from the red alga *Chondria armata* and extracts from this alga, known in Japan as domoi, showed an anthelmintic effect (Takemoto and Daigo 1958). Prior to a human shellfish poisoning event in 1987, microalgae were not known to produce DA. In Prince Edward Island, Canada, three people died and more than 100 were sickened after they had consumed blue mussels that were tainted with DA (Wright *et al.* 1989, Todd 1993).

The first observed case of DA poisoning on the west coast of North America occurred in Monterey Bay, CA in September 1991 which resulted in the incapacitation and deaths of numerous seabirds (Work *et al.* 1993). In late October

1991, razor clams on the Washington coast were observed to contain levels of DA above the regulatory limit of 20 µg/g shellfish meat (Wekell *et al.* 1994). This prompted the very first razor clam harvest closure due to the measurement of DA in shellfish in Washington State (Horner and Postel 1993, Horner *et al.* 1997).

In May 1998, DA caused California sea lions in Monterey Bay to exhibit aberrant behavior and ultimately resulted in the deaths of over 400 of these sea lions (Scholin *et al.* 2000). In contrast to the 1987 Canada event where the blue mussel (*Mytilus edulis*) was the vector of intoxication (Wright *et al.* 1989), in the 1998 Monterey Bay event planktivorous fish were shown to be the vector (Lefebvre *et al.* 1999). In September of 1998, DA in razor clams on the Washington State coast once again exceeded the regulatory limit (Adams *et al.* 2000, Trainer *et al.* 2001).

Domoic acid levels in razor clams in October 2002 eclipsed the regulatory limit and remained above the limit until November 2003. (Washington State Department of Health, unpublished data, 2003). Along with North America, DA has now been confirmed in shellfish from many areas of the world including New Zealand, Japan, Scotland, France, Spain, and Portugal (see references in Landsberg 2002).

1.2.3 Mode of action and health effects of DA

Domoic acid causes massive depolarization of the neurons, leading to neuronal swelling and cell death (Bates *et al.* 1998). Domoic acid is an excitatory amino acid that is structurally related to glutamic acid and kainic acid (Debonnel *et*

al. 1989, Teitelbaum *et al.* 1990, Baden and Trainer 1993). All three of these are part of a series of dicarboxylic amino acids and some in the series could be involved in pathogenesis or treatment of several neurological or psychiatric syndromes (Debonnel *et al.* 1990).

Glutamic acid is the most common excitatory neurotransmitter in the mammalian nervous system (Hall 1992). Kainic acid, an excitatory amino acid that is obtained from the red alga *Digenea simplex* was used in the past as an anthelmintic and is currently used in neurobiological research (McGeer and McGeer 1979). Kainic acid was shown to have neurotoxic effects with potency 300 times higher than glutamate (Olney 1978). Domoic acid is a water soluble and heat-stable molecule that binds to receptors in the brain, particularly in the hippocampus (Debonnel *et al.* 1989, Sutherland *et al.* 1990). Domoic acid binds to kainate type glutamate receptors with an affinity three times that of kainic acid (Teitelbaum *et al.* 1990) and has been shown to be approximately 10 times more neurotoxic than kainic acid (Novelli *et al.* 1990).

Glutamate receptors are thought to be involved in mechanisms of synaptic plasticity in the brain and associated with functions such as associative memory (Hall 1992, Kennedy and Marder 1992). In rats, DA caused extensive degeneration of hippocampal neurons which was accompanied by severe and long lasting memory impairment (Sutherland *et al.* 1990). Additionally, Teitelbaum *et al.* (1990) found striking similarities between the abnormalities produced in laboratory animals by DA and kainic acid to the effects in people that had ingested DA tainted mussels.

Mild cases of DA poisoning can result in flu-like symptoms. Some of the other symptoms of DA poisoning in humans, ranging from mild to severe intoxication, include headache, dizziness, disorientation, permanent short term memory loss, coma, and death. Todd (1993) provided a review of the 1987 Prince Edward Island event that included details of cases of DA poisoning as well as the steps that were taken to identify the source of the DA. Quick (1992) conducted a retrospective study of the 1991 event on the Washington State coast but could not conclusively show that there were adverse health effects directly related to this DA outbreak. However, tests performed on razor clams that were collected during the 1991 Washington State bloom and canned by local residents showed high levels of DA (John Wekell, National Marine Fisheries Service, pers. comm., 2003).

1.2.4 Economic and cultural impacts of DA

During the past fifteen years the periodic closure of shellfish harvesting on the Washington coast has drastically affected both coastal economics and traditional tribal activities. Coastal communities that depend on shellfish have been greatly impacted by lost revenue realized in both recreational and commercial fisheries (Anderson 1995). Hotel and restaurant operators in coastal communities as well as tourist-oriented businesses are also affected when visits to the coast are reduced due to shellfish harvest closures.

In addition to the economic losses to coastal communities, cultural traditions of coastal Native American tribes have also been impacted. Many of the coastal

tribes use razor clams in their traditional ceremonies as well as for subsistence. Better understanding of the dynamics of toxigenic *Pseudo-nitzschia* spp. blooms including initiation, maintenance, and transport as well as the autecology of individual *Pseudo-nitzschia* species on the Washington coast will help mitigate impacts on coastal tribal communities. This will benefit Native American tribes that depend on shellfish for traditional and subsistence uses in that they will have more information with which to modify or terminate their harvest activities.

In concert with other environmental and oceanographic parameters, genetic information can be used to develop models for the purpose of forecasting toxic blooms of *Pseudo-nitzschia* spp. These models will help managers decide when to open beaches for recreational shellfish collection in addition to aiding tribal and commercial shellfish operators in determining the optimal periods for harvesting their products. Understanding the genetic composition of the potential source populations of toxigenic *Pseudo-nitzschia* spp. will help to mitigate the socio-economic effects of an outbreak.

1.2.5 Sources of *Pseudo-nitzschia* and DA to the Washington coast

The Juan de Fuca eddy has been observed to be a retentive site for phytoplankton, including diatoms of the genus *Pseudo-nitzschia*, as well as a site where high levels of DA have been measured in seawater particulates (Horner *et al.* 2000, Trainer *et al.* 2002). The eddy is a cyclonic cold feature that occurs off the mouth of the Strait of Juan de Fuca on the southern British Columbia shelf that forms

in the spring and declines in the fall (Freeland and Denman 1982). The eddy is apparent in sea surface temperature satellite imagery (Trainer *et al.* 2002). A connection between the eddy and the Washington coast was observed when oil from a tanker spill in the eddy eventually fouled Washington beaches (Venkatesh and Crawford 1993).

The Juan de Fuca eddy is one source of DA and toxigenic *Pseudo-nitzschia* spp. to the coast of Washington State. Additionally, high numbers of *Pseudo-nitzschia* spp. cells have been observed in nearshore upwelling zones (Horner *et al.* 2000). Observations to date indicate that these are the two most likely sources of toxigenic *Pseudo-nitzschia* spp. that impact Washington coastal beaches. However, recent studies indicate that conditions in the eddy region are more favorable for the formation of toxic blooms than those in the nearshore upwelling zone (Trainer *et al.* 2002).

During periods of upwelling-favorable winds (i.e. winds blowing from the north) surface waters exit the eddy to the southeast and flow along the WA shelf (MacFadyen *et al.* 2005). Phytoplankton assemblages, originating from the eddy or the coastal upwelling zone, are kept offshore, via surface layer Ekman drift, with upwelled nutrients contributing to their growth. When winds shift and blow from the south, these assemblages are advected towards the coast as local surface currents respond quickly to shifts in local wind forcing (Hickey 1989). Figure 1.4 illustrates the shoreward advection of eddy spin-off and nearshore upwelling water to the

Washington coast during downwelling favorable conditions (i.e. winds blowing from the south).

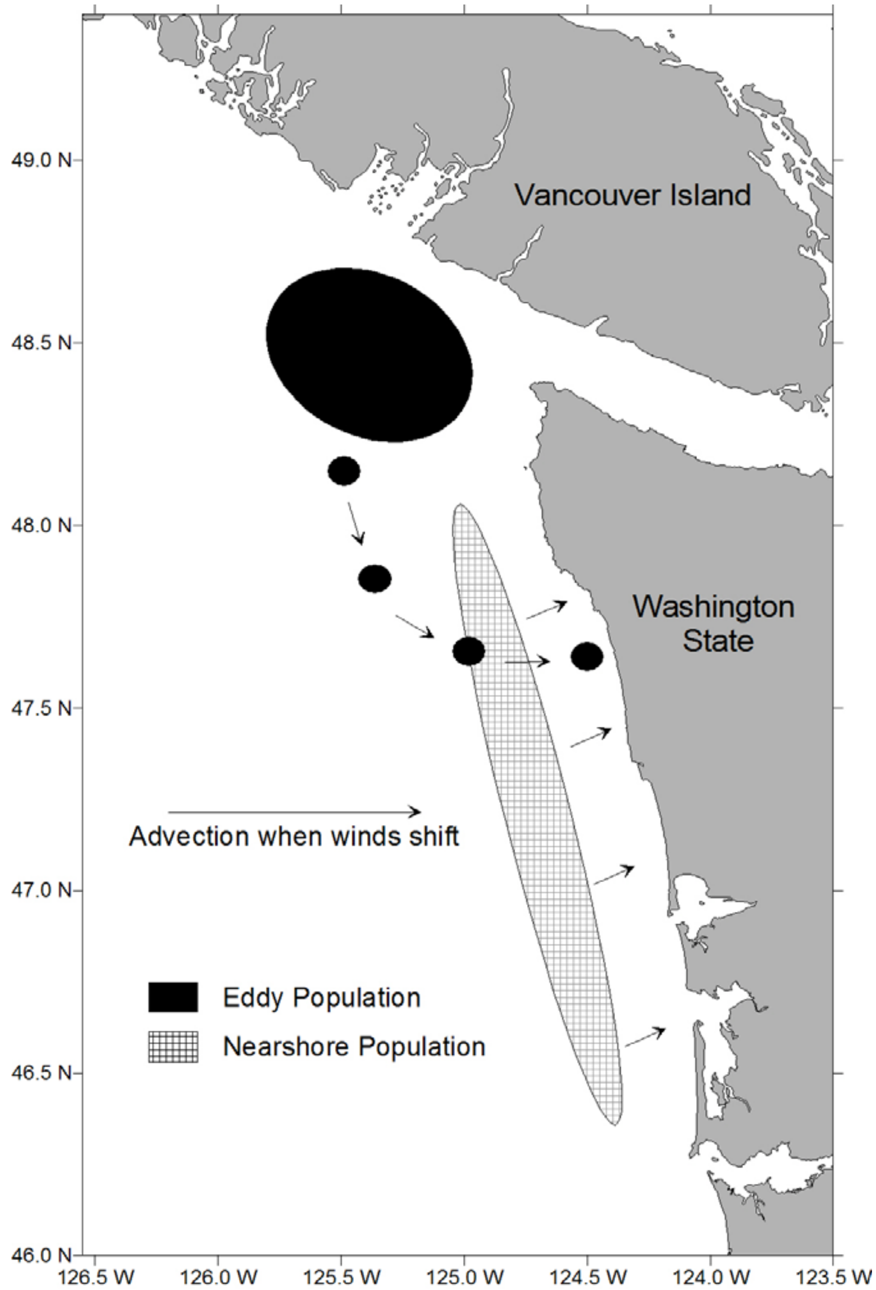

Figure 1.4. Hypothetical sources of *Pseudo-nitzschia* that can impact Washington State coastal beaches. Water masses containing *Pseudo-nitzschia* from the Juan de Fuca eddy (solid black) and nearshore upwelling zone (hatched gray) are advected shoreward after winds shift from a southward to northward direction.

1.5 Molecular methods

1.5.1 Molecular markers and phytoplankton

There are several means for measuring DNA base sequence variation among individuals (Frankham *et al.* 2002). Studies that assessed the genetic diversity of particular phytoplankton species in natural assemblages were performed using isozymes (Gallagher 1980, Chinain *et al.* 1997, and Skov *et al.* 1997), restriction fragment length polymorphisms (Stabile *et al.* 1992, Scholin and Anderson 1996), random amplified polymorphic DNA (Medlin *et al.* 1996), and microsatellites (Rynearson and Armbrust 2000, Rynearson and Armbrust 2004, Evans and Hayes 2004, Evans *et al.* 2004, Evans *et al.* 2005). Because of rapid and cost-efficient screening, co-dominant inheritance and high variability, microsatellites have become the most commonly used marker in population genetics (Balloux and Goudet 2002) and were chosen as the marker for this project. Additionally, microsatellites reveal much higher genetic diversity per locus than markers such as allozymes (Frankham *et al.* 2002).

1.5.2 Microsatellite markers

Microsatellites are short, tandemly repeated sequences of DNA with repeat units between one and five base pairs in length (Jarne and Lagoda 1996). Figure 1.5 shows examples of three types of microsatellite repeats. Pure repeats consist of a specific unit (e.g. CA) repeated in sequence, compound repeats are composed of a

series of one unit (e.g. CA) followed by a series of another unit (e.g. GA), and interrupted repeats are sequences of a specific unit that are separated by other nucleotides.

Pure repeats:	CACACACACACACA
Compound:	CACACACA[GAGAGA]
Interrupted:	[CACA]TT[CACACA]TTCA

Figure 1.5. Types of microsatellite repeats. (Jarne and Lagoda 1996).

Microsatellites are inherited in a Mendelian fashion where, in diploid sexual organisms, one allele is inherited from the mother and another allele is inherited from the father. Microsatellite loci are often highly polymorphic due to variation in the number of repeat units (Bruford and Wayne 1993) which can easily be determined by estimating the size of the entire microsatellite. The high polymorphism of microsatellite markers enables higher statistical power and increased discrimination among genotypes (Halkett *et al.* 2005) making them very useful for population genetic studies. Various population differentiation statistics that are based on the variance in allele frequencies within and among populations can be calculated using microsatellite data (Pearse and Crandall 2004).

1.5.3 Polymerase Chain Reaction

Microsatellite alleles are amplified using the polymerase chain reaction (PCR). The purpose of PCR is to facilitate analysis of a specific DNA sequence by

increasing the number of copies of that sequence and also minimizing the influence of non-target DNA (Mullis and Faloona 1987). Often, only a small amount of DNA is available from a sample and by itself is insufficient for a given analytical procedure. For example, starting with 10^{-6} µg of template DNA, 0.5-1.0 µg of target sequences up to 2 kb in length can be obtained after 30-35 PCR cycles (Sambrook *et al.* 1989).

A single PCR cycle consists of three steps: denaturation, annealing, and extension (Palumbi 1994). During the first step, the template DNA is heat denatured, at approximately 94°C, to form two single DNA strands. Two oligonucleotide primers that have been designed to be complimentary to known sequences that flank the target sequence, are annealed to the single strands of DNA in the second lower temperature step. During the third step, a heat stable DNA polymerase then binds to the template/oligonucleotide combination and creates a DNA strand complimentary to the template (Saiki *et al.* 1988). When complete, a single PCR cycle effectively doubles the number of copies of the sequence of interest.

1.6 Previous population genetic studies with phytoplankton

1.6.1 *Gambierdiscus toxicus*

Using isozyme analysis, Chinain *et al.* (1997) studied the intraspecific variation among isolates of the dinoflagellate *Gambierdiscus toxicus* which causes ciguatera fish poisoning. They found that there was significant genetic variation

among the 19 clones that they analyzed and that their sample was made up of numerous biochemically distinct strains. Interestingly, an isolate collected from the Atlantic Ocean had an isozyme pattern that was very similar to two Tahitian strains while one of these strains appeared to be more distantly related to other strains collected from another Tahitian locale.

1.6.2 *Skeletonema costatum*

Gallagher *et al.* (1980) used allozymes to investigate the genotypic frequencies in winter and summer bloom populations of *S. costatum* in Narragansett Bay, Rhode Island. Individual *S. costatum* cells are morphologically indistinguishable and the presence of distinct populations cannot be detected by routine cell counts. Gallagher *et al.* (1980) examined a total of 457 clones and found that the genotypic frequencies of the populations indicated that they populations were genetically different. They suggested that the annual cycle of succession of *S. costatum* in Narragansett Bay is a function of genetically variable populations and of the appropriate environmental conditions for their growth.

Stabile *et al.* (1992) used restriction fragment length polymorphisms (RFLPs) in chloroplast DNA to study 12 winter strains of *S. costatum*. The 12 strains had identical RFLP patterns but represented eight different allozyme genotypes. They suggested that the lack of correspondence between allozyme patterns and RFLP data might be due to different modes of inheritance of the chloroplast and nuclear genomes in *S. costatum*.

1.6.3 *Pseudo-nitzschia pseudodelicatissima*

Skov *et al.* (1997) studied 10 isolates of *P. pseudodelicatissima* using isozyme analyses. They found that all 10 had different isozyme banding patterns which suggested that *P. pseudodelicatissima* was represented by a large number of clones in their study area. Skov *et al.* (1997) also analyzed one isolate of *P. pungens* and one isolate of *P. delicatissima* and found distinct isozyme banding patterns for each.

1.7 Previous population genetic studies with diatoms using microsatellites

Microsatellite studies have been performed on three diatom species: *Ditylum brightwellii, P. multiseries*, and *P. pungens* (Rynearson and Armbrust 2000, Rynearson and Armbrust 2004, Evans *et al.* 2004, Evans *et al.* 2005). In each of these studies, microsatellite data were used to differentiate populations of each respective species. Interestingly, on similar spatial scales different results were obtained for *D. brightwellii* and *P. pungens*, suggesting that many factors can influence the formation of distinct populations of phytoplankton species.

1.7.1 *Ditylum brightwellii*

Rynearson and Armbrust (2000) developed sets of PCR primers to amplify microsatellite loci in the centric diatom *Ditylum brightwellii*. Using two loci, they found that 22 out of 23 isolates isolated from Hood Canal, WA were unique.

Rynearson and Armbrust (2004) examined the genetic structure of populations of *D. brightwellii* in the Strait of Juan de Fuca and Puget Sound, two connected estuaries. They collected samples at four sites that were all within 100 km of each other. Two of the sites were subjected to extensive daily tidal mixing, while the other two were not. Water properties were measured when *D. brightwellii* was collected from an area of mixing so that the source water at the time of collection could be identified.

Using three microsatellite loci, Rynearson and Armbrust (2004) identified three genetically distinct populations in these estuaries. The Puget Sound populations were genetically different from the Strait of Juan de Fuca populations, and there were also distinct populations observed within the Strait of Juan de Fuca waters. In tests for Hardy-Weinberg proportions (seven samples at three loci) they found that 16 out of 21 tests deviated from expectations, implying clonal selection during asexual reproduction. They suggest that oceanographic properties such as temperature and salinity can influence the structure of a given population and potentially prevent colonization of an area by an exogenous population even at this local scale.

1.7.2 *Pseudo-nitzschia multiseries*

Evans *et al.* (2004) developed nine polymorphic microsatellite markers for *P. multiseries*. With these markers they genotyped 25 field isolates and six isolates that resulted from mating experiments with the field isolates. The field isolates were collected from different parts of the world with 22 collected from eastern Canada,

two from Europe, and one from Russia. They found a substantial degree of genetic variability within the field isolates where 23 of 25 multilocus genotypes were unique. The Russian isolate was the most genetically distinct isolate, contributing 11 new alleles at six loci.

1.7.3 *Pseudo-nitzschia pungens*

Evans *et al.* (2005) used six microsatellite loci to examine the population structure of *P. pungens* in the German North Sea around the islands of Helgoland and Sylt, which are approximately 100 km apart. The purpose of their study was to genotype a large number of *P. pungens* isolates from an open-water region with no obvious barriers to mixing to determine if there was any genetic structure to the *P. pungens* in that area.

During their three sampling periods that spanned 18 months, Evans *et al.* (2005) observed only weak population differentiation both within and between sampling periods. They found that their pooled sample of 464 *P. pungens* isolates from the North Sea did not deviate from Hardy-Weinberg expectations. When this sample was subdivided into three temporally distinct samples, only two out of 18 tests (three samples at six loci) deviated from Hardy-Weinberg expectations. Population differentiation tests indicated that there was weak or no differentiation temporally as well. They concluded that the German North Sea supports a single, largely unstructured population of *P. pungens*.

1.8 Study organism: *Pseudo-nitzschia pungens*

Pseudo-nitzschia pungens was chosen for this study since it is frequently observed in the Pacific Northwest, although it is rarely found in high numbers, and may serve as a model organism to define the population structure of potentially toxic *Pseudo-nitzschia* species in Pacific Northwest waters. Additionally, microsatellite markers were previously developed for this species (Evans and Hayes 2004). In the North Sea, *P. pungens* is the dominant *Pseudo-nitzschia* species off the German coast but it is considered to be non-toxic in this area (Evans and Hayes 2004, Evans *et al.* 2005). In contrast, *P. pungens* has been shown to be weakly toxic in Pacific Northwest waters (Trainer *et al.* 1998). As the genetic diversity of *P. pungens* was previously investigated in the North Sea, a similar study in the Pacific Northwest will provide a comparison of the genetic structure of these potentially toxigenic diatoms from two geographically distant regions.

1.9 Description of sample sites

Pseudo-nitzschia pungens samples were obtained from the waters off Washington State and Vancouver Island, Canada (Fig. 2.1) as well as from various locations in the North Sea (Fig. 2.2). The samples from Washington State and Vancouver Island waters were collected during cruises as part of the Ecology and Oceanography of Harmful Algal Blooms in the Pacific Northwest (ECOHAB-PNW) project. Samples were collected from one site within Barkley Sound in 2004 and from four sites in offshore waters at the edge of the Juan de Fuca eddy region in

2005. Barkley Sound covers an area of approximately 550 km^2, is divided by three channels which are approximately 40, 100, and 150 meters deep, and is influenced by freshwater outflow from Alberni Inlet (Taylor and Haigh 1996). The Barkley Sound sampling site lies in the Imperial Channel (the middle channel) of the Sound where the water depth is approximately 100 meters and which is most exposed to the offshore environment. The four sites in the Juan de Fuca eddy region were within 40 km of each other and were located in over 500 meters of water. The 2005 sites were approximately 110 km west of the coast of Washington State and 115 km south of the Barkley Sound site.

The northern Washington/southern Vancouver Island shelf lies at the northern end of the California Current System, a well described eastern boundary current (Hickey 1979). Surface currents over the shelf are generally southward in the summer and northward in the winter as a result of the large-scale wind patterns. While the ocean variability in the Pacific Northwest is generally large scale (>500 km) and these general current patterns extend shoreward to the shelf break, there are several mesoscale features with altered currents and mixing regimes (Hickey 1998, Hickey and Banas 2003). The Juan de Fuca eddy is one such mesoscale feature where water types from varying origins interact (Hickey and Banas 2003).

The formation of the Juan de Fuca eddy is a result of interaction between effluent from the Strait of Juan de Fuca, southward wind-driven currents along the continental slope and the underlying topography (Hickey and Banas 2003). The Strait of Juan de Fuca is a channel approximately 20 km wide with a large freshwater

source, the Fraser River, at its eastern end that contributes to estuarine circulation in the Strait (Freeland and Denman 1982). Along with output from the Fraser River, circulation in the Strait is influenced by tidal mixing and local wind forcing (Denman *et al.* 1981). The outflowing water from the Strait of Juan de Fuca is well mixed with ocean water and the contrast between the inflowing and outflowing water is small (Freeland and Denman 1982).

The North Sea has a surface area of 575,300 km^2, a volume of 42,294 km^3, and a mean depth of 74 meters (Otto *et al.* 1990). It is open to the Norwegian Sea (Atlantic Ocean) in the north and is bounded by land on the west, south, and east. In addition to inflow from the Atlantic via the Norwegian Sea, there are inputs from the English Channel in the southwest and the Skagerrak in the northeast. *Pseudo-nitzschia pungens* isolates were collected around the German islands of Sylt and Helgoland as well as various locations in Belgian and Dutch waters (Figure 1.7). The sampling sites were all located in the southern part of the North Sea in less than 50 meters of water. The Sylt/Helgoland sites and the Belgian/Dutch sites are approximately 550 km apart.

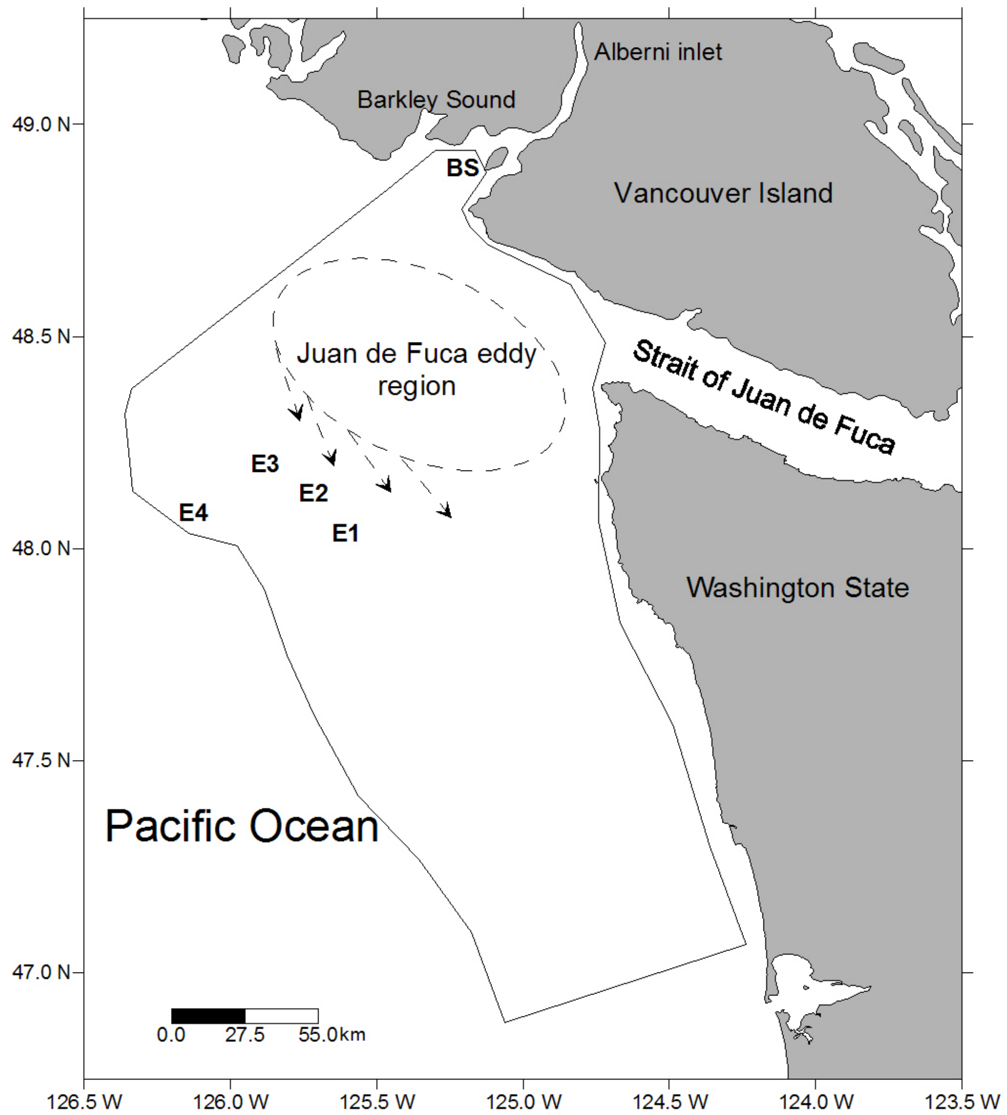

Figure 1.6. Pacific Northwest sample locations. *P. pungens* were collected in 2004 (BS) and 2005 (E1, E2, E3, E4). The approximate location of the Juan de Fuca eddy is noted by the gray ellipse. The dotted arrows illustrate the path of parcels of water that may be ejected from the eddy region. The approximate extent of sampling during ECOHAB-PNW cruises.

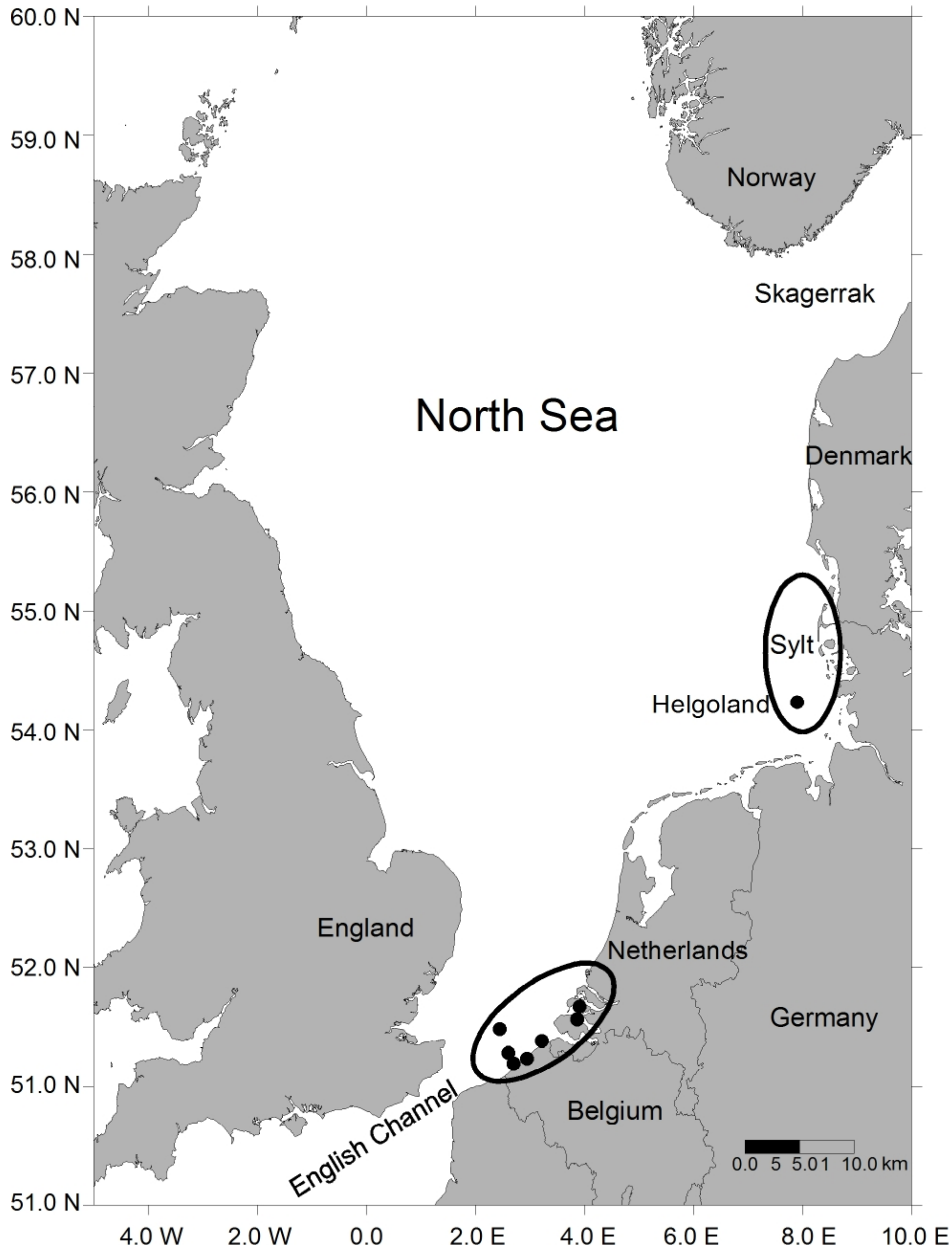

Figure 1.7. North Sea sample locations. A single *P. pungens* isolate was collected from each location in the circled area in Belgian and Dutch waters as well as from the island of Sylt. Fourteen isolates were from the island of Helgoland. The approximate extent of sampling areas from which the North Sea samples were obtained is noted by the ellipses.

In contrast to the Juan de Fuca eddy region, the dominant feature in the dynamics of the North Sea is tidal motion (Otto *et al.* 1990). Damm *et al.* (1994)

describe the general pattern of water mass transport in the North Sea. Water masses that enter the North Sea through the Shetland Passage and the Fair Isle-Shetland passage in the northwestern North Sea flow southward and partially spread into the central North Sea then recirculate through the Norwegian trench back into the Atlantic. Water masses that enter through the English Channel spread into the southern North Sea following the continental coast through the German Bight then move to the north leaving the sea via the Norwegian trench. The residual circulation in the North Sea is anticlockwise and along the continental coast the current is strong up to the Skagerrak where it meets the deeper North Atlantic inflow through the Norwegian Channel (Otto *et al.* 1990).

1.10 Study objectives

The current study had two main objectives. The first was compare *P. pungens* in Pacific Northwest samples to a sample from the North Sea to assess the genetic differentiation of *P. pungens* from these geographically distinct locations. For statistical purposes, the Pacific Northwest *P. pungens* collected from five sites were considered both as individual samples and as a single combined sample. The second objective was to examine the substructure of the Pacific Northwest samples. The degree of differentiation between individual Pacific Northwest samples was determined and tests were performed to assess whether multiple populations of *P. pungens* were present in the combined Pacific Northwest sample.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sample collection

Surface water samples from the Pacific Northwest were collected using a 25 cm diameter, 60 cm long plankton net with 20-µm Nitex mesh and deployed by hand over the side of the R/V Atlantis in September 2004 and September 2005. The net was drawn through the surface water while the ship was on station until color was visible inside the net as water flowed out through the mesh. Additionally, a Seabird SBE 9/11 conductivity, temperature, and depth (CTD) profiler was used to collect water quality data.

Using an upright light microscope (Zeiss Standard RA/18) at 100x magnification, the samples were scanned for *Pseudo-nitzschia* cells that had morphological characteristics consistent with *P. pungens*. If *P. pungens* type cells were present the whole cell (WC) hybridization assay was performed (Miller and Scholin 1996, details are below) to confirm their presence in the water sample. Briefly, the WC assay employs a probe that binds to a conserved sequence of large subunit (LSU) ribosomal RNA and fluoresces under epifluorescent microscopy.

If the WC hybridization assay indicated that *P. pungens* was present in the sample, one to two drops of sample were added to each well of a 48-well plate that contained 0.75 mL of sterile f/2 enriched seawater medium (Guillard and Ryther 1962). If the material in the wells grew to a significant density (e.g. it was visible with the naked eye after approximately 10 days) 0.25 mL was removed from each well using a micropipettor and sterile plastic tip and transferred to the corresponding

well in a new plate containing 0.75 mL of f/2 medium to maintain the sample. A new tip was used for each transfer.

In 2004, the 48-well plates were maintained in an on-deck incubator exposed to ambient light levels with flowing seawater that was drawn from the surface ocean using the flow-through system of the research vessel. Surface water temperatures during the cruise ranged from 10.1°C to 18.5°C. In 2005, the plates were maintained at 12°C on a 12:12 light:dark cycle at photon flux density of approximately 85.0 µEinsteins/m^2/s in a cell culture incubator (Forma Scientific Diurnal Growth Chamber) onboard the ship. Upon returning to port after each cruise, the plates were transferred to culture incubators in the laboratory that employed the same temperature and light conditions as those used onboard the ship in 2005.

DNA extracts from thirteen *P. pungens* isolates from the waters off the island of Helgoland, Germany and two *P. pungens* cultures (one from Helgoland and one from the German island of Sylt) were obtained from Katharine Evans of the Royal Botanic Garden Edinburgh. The *P. pungens* cells were isolated by Stefanie Kühn of the University of Bristol. Sixteen frozen aliquots of *P. pungens* cultures were obtained from Griet Castelyen of the University of Ghent.

2.2 *P. pungens* isolation and culture conditions

Using either a Nikon TMS or Zeiss Axiovert 135 inverted microscope at 200x magnification, individual *P. pungens* cells were isolated from Pacific Northwest samples with 200 µL capillary pipet that was drawn out in the flame of an

alcohol lamp (Fig. 2.1). Drawing out of capillary pipets was accomplished by grasping both ends of the pipet and holding the center of the pipet over the flame. When the glass became pliable, the ends of the pipet were pulled apart and out of the flame. The thin glass between the two ends was broken in the middle and the result was two capillaries that, on one end, have much smaller inner diameters than the original pipet. A fresh drawn out capillary pipet was used for every isolation.

During the isolation process, a single cell or chain of cells (cells in diatom chains are clones) was isolated from each well of the sample plates. The composition of the original sample can be approximated by only isolating a single cell or chain from each well, even though the cells from the original sample had been replicating prior to the isolation procedure. Each cell or chain was rinsed 3 times in sterile f/2 medium to remove exogenous material and placed into an individual well of a 48-well plate containing 0.75 mL of f/2 medium. The plates were maintained in a temperature and light controlled incubator at 12°C on a 12:12 light:dark cycle at a photon flux density of approximately 85 μEinsteins/m^2/s and checked for contamination with other algal species every two days. Cultures that contained visible cells other than *Pseudo-nitzschia* were re-isolated. Once the isolates that were free of contamination grew to a high cell density, they were transferred to glass culture tubes containing 15 mL f/2 medium and maintained using the same conditions as the well plates.

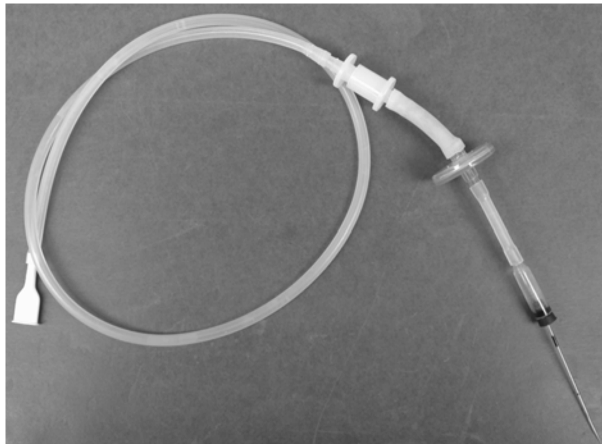

Figure 2.1. Apparatus used for cell isolation. A drawn out capillary pipette (lower right) attached to one end of a piece of silicon tubing and a mouthpiece is attached to the other.

2.3 *P. pungens* identification

Since many *Pseudo-nitzschia* species look similar using light microscopy, a WC hybridization assay using fluorescent positive, negative and *P. pungens* specific (puD1) probes (Oligos, Etc. Wilsonville, OR) developed by Miller and Scholin (1996) was performed to identify *P. pungens* after collection of the original samples onboard ship and in the monoclonal cultures that were developed from these samples. A portion of the sample or culture to be tested was collected onto a filter and fixed at room temperature for 1-2 hours with a modified saline ethanol solution made up of 95% high-grade ethanol (73.5%), distilled water (16.5%), and 25x SET (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris, pH 7.8) buffer (10.0%). The positive, negative, and puD1 probes were added and hybridized to the cells on the filter during an incubation step at 45.0° for 1.5 hours. The puD1 probe targets the large subunit (LSU) ribosomal RNA and is specific to *P. pungens*. The positive control probe (uniC) targets a universally conserved sequence of the small subunit ribosomal RNA

and the negative control probe (uniR) is the RNA-like complimentary sequence of the positive control probe. All three probes were used on each field sample onboard the ship or on a single culture per assay when the monoclonal cultures were being tested to ensure that the probes were binding properly. The excess probe was washed away and the filters were mounted to microscope slides. Epifluorescent microscopy was then used to view the filters, on which *P. pungens* cells fluoresced if they were present. Figure 2.2 shows an example of assay results for a *P. pungens* culture. The fluorescence of *P. pungens* cells is commonly less than that of the positive control (Miller and Scholin 1996). Five of the isolates that tested positive for WC hybridization assay were analyzed with scanning electron microscopy (SEM) to confirm the results.

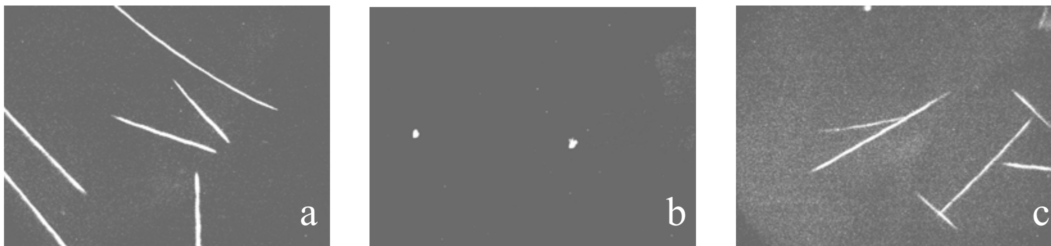

Figure 2.2. Sample images from the whole cell hybridization assay for a *P. pungens* isolate (isolate NA098): (a) positive control, (b) negative control, (c) *P. pungens* specific probe. The bright spots in the negative control are from extraneous material on the sample filter.

2.4 Culture harvesting and storage

Once cultures reached a high cell density, where the growth medium was visibly discolored, approximately 0.5 mL of the culture was transferred to fresh f/2 medium. The remainder of the culture was harvested onto 25 mm Millipore 0.45 μm

HA nitrocellulose filters using a light vacuum (<5 psi). The filter was placed into a 1.6 mL microcentrifuge tube and stored at -80°C. This cycle of transfer and harvesting was repeated at least four times to archive material in the event it was necessary to re-extract DNA from any given isolate.

2.5 DNA extraction

DNA was extracted from the harvested *P. pungens* cultures using Qiagen DNeasy Plant Mini kits with some modifications to the initial steps of the protocol. To prepare the material for the extraction procedure, 500 µL Qiagen lysis buffer, 5 µL RNase A (Qiagen, 100 mg/mL), and 2.5 µL Proteinase K (Qiagen, 20 mg/mL) were added directly to the tube containing the filter with the harvested material. The tubes were then vortexed on a Scientific Industries, Inc. Vortex Genie for 30 seconds at high speed to remove the material from the filters. After centrifugation for 5 minutes at 8000 rpm in an Eppendorf Centrifuge 5415C to pellet the cells, the filters were removed and the tubes were vortexed for two to three seconds to resuspend the material. Following incubation at 65°C for one hour using a VWR Scientific Standard Heatblock, the Qiagen procedure was followed as written until the DNA elution step. DNA was eluted from the spin columns using 70 µL of Qiagen buffer AE and only one elution for each isolate was performed.

2.6 DNA quantitation and standardization

To quantify the DNA extracts, a 96-well plate was prepared by adding 50 µL of Qiagen buffer AE and 0.5 µL PicoGreen (Molecular Probes, Inc.) dye to one well of the plate for every sample that was to be analyzed plus to an extra six wells for standard DNA (lambda DNA, Gibco BRL 252580-010) solutions. Two microliters of each DNA extract and standard DNA solutions were added to individual wells of the well plate. The concentrations of the DNA solutions used for the standard curve were 20, 10, 5, 2, 1, and 0 ng/µL. The well plate was covered with aluminum foil and placed on a platform shaker at medium speed for 5 minutes. A BioTek Instruments, Inc. FLx800 plate reader was used to read the plate at excitation and emission of 485 and 516 nm, respectively. The data were analyzed in Microsoft Excel using the regression analysis tool to determine DNA concentrations. After quantitation, a 100 µL DNA solution with a concentration of 0.2 ng/µL was prepared from each extract.

2.7 Polymerase chain reaction

The polymerase chain reaction (PCR) was performed using a Bio-Rad iCycler to amplify four microsatellite loci (PP2, PP3, PP5, and PP6) using primers and modified conditions developed by Evans and Hayes (2004). The microsatellite core sequences and primer sequences for each locus are listed in Table 2.1. For the current project, it was determined that an annealing temperature of 56°C worked well for all loci. For each 20 µL reaction, magnesium chloride concentrations of 1.5 mM

and 2.5 mM were used for PP2 and PP6, respectively, whereas for PP3 and PP5 2.0 µM was used. In addition to the magnesium chloride each reaction contained 10 mM Tris-HCL, 50 mM KCL, 0.1% Triton X-100 (Mg++ free buffer, Gene Choice), 0.2 mM dNTPs (Epicentre), 75 ng of each primer (Applied Biosystems), 0.5 units of *Taq* polymerase (Gene Choice), and 2 ng DNA. After a three minute initial denaturing step at 94°C, 35 cycles were performed: 1 minute at 94°C (denaturing), 1 minute at 56°C (annealing), and 30 seconds at 72°C (extension). This was followed by a final extension step at 72°C for 20 minutes.

Table 2.1. Microsatellite core and primer sequences from Evans and Hayes (2004). "F" is the forward primer and "R" is the reverse primer for each locus.

Locus	Core sequence	Primer sequence (5' to 3')
PP2	$((GT)_3T)_9N_{16}(GATTGAT)_5$	F: TCCAGACTGGTCCTGCTACC
	$(GAATGAT)_4$	R: CCTGTACGGTATGTGCTCGAA
PP3	$(GT)_4N_{13}(GT)_4N_{37}$	F: ACTGCGTTTGCTCTTTCGAG
	$(GATTT)_7$	R: GCCAGCAAATGGAACAACTT
PP5	$(GT)_2AT(GT)_8AT(GT)_4$	F: GGGTAGTGCTTCGGTTCCT
		R: AGAACTCGCAAATCCGACTG
PP6	$(G(CA)_3C)_2GC(AC)_2(G(CA)_3C)_2$	F: GAGGCGCAACAGTCGTAAAG
	$AC(G(CA)_7C)_2$	R: AATCCCGTGCGTGTAGTTTC

2.8 Microsatellite detection and genotyping

The PCR products for loci PP2, PP5, and PP6 were combined by adding one microliter of each product to 100 µL of water (ACRŌS Organics) while the PCR products for locus PP3 were diluted separately by adding one microliter of PCR product to 200 µL of water. Locus PP3 was analyzed alone as when all four loci were analyzed together, the signal from locus PP3 interfered with the signals of the other three loci. One microliter of each dilution was added to individual wells of a 96-well analysis plate that contained 12 µL of a solution made up of formamide and

the Applied Biosystems (ABI) LIZ-500 internal standard. The plate was placed in a thermocycler and subjected to a temperature of 96°C for six minutes to denature the PCR products. After denaturing, the plate was placed on ice for at least 6 minutes prior to analysis on an ABI 3100 genetic analyzer. If the above dilutions did not give a strong signal with the genetic analyzer, the original PCR products were diluted into smaller volumes of water and reanalyzed. Allele sizes were estimated using the ABI LIZ-500 standard with the ABI GeneScan software. ABI Genotyper software was used to construct multilocus genotypes for each isolate.

2.9 Statistical analysis of microsatellite data

Table 2.2 lists the statistical analyses that were performed using the microsatellite data obtained for the Pacific Northwest and North Sea *P. pungens* samples. The data for the present study were analyzed to determine if there were deviations from Hardy-Weinberg equilibrium (HWE) in the *P. pungens* samples. The detection of deviations from HWE is one of the first steps in the study of population genetics (Rousset and Raymond 1995). Hardy-Weinberg equilibrium in a population assumes that there is random mating, normal Mendelian segregation of alleles, a closed population (i.e. no migration), no mutation, and a large population size (Frankham *et al.* 2002). Each sample was also tested for deviations from linkage equilibrium, which refers to the random association of alleles between loci (Frankham *et al.* 2002). Linkage equilibrium is expected in large, randomly mating populations. Deviations from HWE and linkage equilibrium indicate that processes

Table 2.2. Statistical tests used in the present study.

Test	Software	Assumptions	Methods	References
Deviation from Hardy-Weinberg equilibrium	GENEPOP 3.4	Random mating, normal Mendelian segregation of alleles, equal fertility of parent genotypes, equal fertilization capacity of gametes, equal survival of all genotypes (i.e. no selection), a closed population (i.e. no migration), no mutation, a large population size.	Fisher's exact test with Markov chain	Raymond and Rousset 1995, Frankham *et al.* 2002
Heterozygote deficiency	GENEPOP 3.4	Random mating, normal Mendelian segregation of alleles, equal fertility of parent genotypes, equal fertilization capacity of gametes, equal survival of all genotypes (i.e. no selection), a closed population (i.e. no migration), no mutation, a large population size.	Fisher's exact test using Markov chain	Raymond and Rousset 1995, Rousset and Raymond 1995, Frankham *et al.* 2002
Genotypic differentiation	GENEPOP 3.4	Random sampling of diploid genotypes.	Fisher's exact test using Markov chain	Raymond and Rousset 1995, Goudet *et al.* 1996
F_{ST}	FSTAT 2.9.3.2	Infinite allele model with low mutation rates. The mutation process erases any memory of the prior allelic state.	Permutation tests	Slatkin 1995, Goudet 2001
R_{ST}	GENEPOP 3.4	Stepwise mutation model. No constraints on allele size and the properties of the mutation process do not depend on allele size.	Weighted analysis of variance	Raymond and Rousset 1995, Slatkin 1995, Rousset 1996
Linkage disequilibrium	GENEPOP 3.4	Random mating, no population structure	Fisher's exact test using Markov chain	Raymond and Rousset 1995
$\bar{r}_d$	MULTILOCUS 1.2	Random mating, no population structure	Permutation tests	Agapow and Burt 2000, Frankham *et al.* 2002
Population subdivision	STRUCTURE 2.1	Hardy-Weinberg and linkage equilibrium in each of K possible populations. Based on the variance of pairwise distances between individuals.	Clustering algorithms	Pritchard *et al.* 2000

such as selection, population mixing, non-random mating or clonal reproduction may have occurred. Calculation of *F*-statistics developed by Wright (1951) and estimated using the procedures in Weir and Cockerham (1984) as well as *R*-statistics (Slatkin 1995) were used to differentiate samples and determination of the degree of inbreeding among and between samples.

2.9.1 Descriptive statistics

GENEPOP version 3.4 (Raymond and Rousset 1995) was used calculate basic descriptive population genetic statistics such as allele frequencies, numbers of alleles, observed heterozygosity (H_o), and expected heterozygosity (H_e) for each sample.

Genetic structure was visualized by plotting allele frequencies at each locus for all Pacific Northwest and North Sea isolates. The effective number of alleles (n_e) for each locus was calculated using the formula: $n_e=1/\Sigma\ p_i^2$, where p_i is the frequency of the *i*th allele. The effective number of alleles is the number of alleles that if equally frequent would result in the observed homozygosity (Frankham *et al.* 2002). Comparison of the effective number of alleles to the observed number of alleles will give an indication of rare alleles. Allelic richness, a measure of the number of alleles independent of sample size, per locus for each sample was calculated in FSTAT version 2.9.3.2 (Goudet 2001). The principle behind allelic richness is to allow the genetic diversity of samples to be compared regardless of their respective sample sizes. This is accomplished by estimating the expected number of alleles in a sub-

sample of 2n genes, given that 2N genes have been sampled (where $N \geq n$). In this estimation, "n" is fixed as the smallest number of individuals genotyped at a locus in the samples being compared and "N" is the total number of individuals in a particular sample.

2.9.2 Microsatellite scoring error checking

The program MICRO-CHECKER (Van Oosterhout *et al.* 2004) was used to check for errors in the scoring of the microsatellite alleles. The program helps to detect large allele drop out, the presence of null alleles, and errors in allele scoring due to stutter. Large allele dropout is a result of a phenomenon where shorter alleles are amplified preferentially to larger alleles. Null alleles, in the context of this study, are alleles that are not amplified during PCR due to primer/template mismatch (Wattier *et al.* 1998). Stutter is thought to be caused by incomplete replication of the repeat array by the polymerase during PCR (Luty *et al.* 1990) and may lead to false scoring as homozygotes of heterozygotes with alleles that differ by a single repeat unit.

2.9.3 Hardy-Weinberg and linkage equilibrium

Samples were tested for HWE using an exact test as described in Guo and Thompson (1992) in GENEPOP with Markov chain parameters of 1000 for dememorization number, 300 batches, and 1000 iterations per batch. When the null hypothesis was rejected, the F_{IS} statistic of Wright (1951) estimated following Weir

and Cockerham (1984), was used as an indicator of heterozygote excess or deficit. F_{IS} ranges from -1 to +1 and can be used to assess the degree of departure from HWE. Positive values of F_{IS} indicate a deficit of heterozygotes and negative values indicate an excess of heterozygotes. An additional test for HWE was also performed where the alternative hypothesis was a deficit of heterozygotes using the *U* test described by Rousset and Raymond (1995).

A comparison of F_{IS} values between samples from the Pacific Northwest and the North Sea was conducted in FSTAT to test whether or not the average F_{IS} values for each area were significantly different. To make this comparison, it is necessary to have more than one sample from each area. The first sample group consisted of all of the individual Pacific Northwest samples. To make the second sample group, the North Sea sample was divided into two sub samples; one consisted of the isolates from Belgium and the Netherlands while another consisted of the isolates from Helgoland and Sylt.

Tests for linkage equilibrium between pairs of loci were also performed using GENEPOP with Markov chain parameters of 1000 for the dememorization number, 300 batches, and 1000 iterations per batch. The program MULTILOCUS version 1.2 (Agapow and Burt 2000) was used to calculate $\bar{r}_d$, a measure of linkage disequilibrium, in the combined Pacific Northwest, individual Pacific Northwest, and North Sea samples (Table 3.9). This statistic has a maximum value of one, indicating complete linkage. The program was used to perform 1000 randomizations of the data for each sample where alleles are randomly shuffled among individuals

for each locus independently. A *P*-value is generated from the randomizations that can be used to test whether the observed $\bar{r}_d$ values are significantly larger than expected in randomized populations.

2.9.3 Population differentiation

Tests for genotypic differentiation were performed in GENEPOP using the log-likelihood *G* based exact test as described in Goudet *et al.* (1996). The genotypic differentiation test in GENEPOP employs the null hypothesis that the genotypic distribution is identical across all populations. The statistic F_{ST} (Wright 1951) was estimated following Weir and Cockerham (1984) and pairwise tests of differentiation were performed in FSTAT. Permutation tests were performed in FSTAT, where genotypes were randomized among samples and the significance of the *P*-values from the pairwise tests of differentiation are reported both before and after non-sequential Bonferroni corrections. Values of F_{ST} can range from zero when there is no differentiation and a maximum of one, where subpopulations are fixed for different alleles and can be interpreted as the proportion of genetic variation distributed among subdivided populations (Avise 2004). The value of F_{ST} also indicates the probability that two alleles drawn randomly from the same sub-population are identical by descent relative to the total population, in other words it is the effect of the population subdivision on inbreeding (Frankham *et al.* 2002). For example, where there are low rates of gene flow between subpopulations, F_{ST} values will be high.

The statistic R_{ST} (Slatkin 1995), which is analogous to F_{ST} but employs the assumption that microsatellite alleles mutate under a stepwise mutation model, was also calculated in GENEPOP for population pairs. R_{ST} is the fraction of the total variance of allele size that is between populations (Slatkin 1995). R_{ST} better reflects population differentiation in populations characterized by very low gene exchange, while F_{ST} gives better estimates in cases of high levels of gene flow, although there is no single best estimator (Balloux and Goudet 2002).

2.9.4 Population structure

The software STRUCTURE version 2.1 (Pritchard *et al.* 2000) was used to examine the sub-structure in the Pacific Northwest samples. STRUCTURE employs a Bayesian model-based clustering method using multilocus genotype data to infer population structure and assign individuals to populations. For each isolate, the program calculates the probability for placing that isolate into one cluster or another. The model assumes that the loci are unlinked and at linkage equilibrium within populations. The method also assumes HWE within populations. In essence, the model accounts for the deviations from Hardy-Weinberg or linkage disequilibrium by introducing population structure in an attempt to find groupings that are in equilibrium.

STRUCTURE was run using the ancestry with admixture model for K = 1 through 10, where K is the number of genetically distinct clusters. Using all of the Pacific Northwest isolates, posterior probabilities for each K were calculated after

100,000 burn-in iterations and a run length of 100,000 iterations for each K. The probabilities were then plotted versus K and the K best represented by the data was determined from the point at which the curve leveled off.

For the number of clusters best represented by the data, only individuals with probabilities greater than 0.9 for a specific cluster were retained in that population for subsequent analyses. Microsatellite data from the populations identified by STRUCTURE as well as the microsatellite data from the North Sea were analyzed for HWE, linkage disequilibrium, and population differentiation as previously described.

CHAPTER 3: RESULTS

3.1 Oceanographic conditions at Pacific Northwest sample sites

Table 3.1 shows the surface temperature, salinity, and density data that were collected with the CTD. At station BS, sampled in 2004, it was approximately 3-4°C colder than the stations that were sampled in 2005. Additionally, the salinity at station BS was approximately 1.5 psu lower than at the 2005 stations. The surface temperatures at stations E1 and E2 are similar (within 0.15°C), while the temperatures at stations E3 and E4 are 0.5-0.9°C higher than those at stations E1 and E2. The salinity at all 2005 stations was similar (within 0.07 psu). In 2005, there was a slight density gradient from station E1 to station E2 that resulted from the temperature differences. The density at station BS in 2004 was lower than at the 2005 stations and was most likely due to freshwater inputs to Barkley Sound.

Table 3.1. Surface CTD data for each collection site in the Pacific Northwest.

Station	Collection Date	Temperature (°C)	Salinity (psu)	Density (σ_t)
BS	9/16/2004	12.62	30.44	22.94
E1	9/18/2005	15.80	31.94	23.43
E2	9/18/2005	15.91	31.89	23.37
E3	9/19/2005	16.38	31.91	23.29
E4	9/19/2005	16.70	31.96	23.25

3.2 Collection of isolates

Pseudo-nitzschia pungens was not frequently seen in water samples collected during cruises in 2004 and 2005. In September 2004, *P. pungens* isolates were collected from one location and in September 2005 isolates were collected from four locations which were relatively close together. In 2004, *P. pungens* were observed

in the surface waters at 10 of the 209 stations where samples were collected for *Pseudo-nitzschia* species analyses. It was only possible to obtain isolates from a single site in 2004, as samples from other sites did not survive to the isolation process. A total of 96 isolations were performed from this site which was located in Barkley Sound, Vancouver Island (Table 3.2). In September 2005, 305 total isolations were performed from the four sites on the outer edge of the Juan de Fuca eddy region off the coast of Washington State (Table 3.2). In addition to these four sites, *P. pungens* was observed in the surface waters at seven other sites. Samples from these sites did not survive long enough for isolation of *P. pungens* cells.

Table 3.2. Collection dates, number of *P. pungens* isolated, number of DNA extracts, and the number of isolates genotyped in the Pacific Northwest.

Location	Collection Date	No. Isolated	No. DNA	No. genotyped	% genotyped
BS	9/16/2004	96	24	24	25.0
E1	9/18/2005	40	34	33	82.5
E2	9/18/2005	23	20	20	87.0
E3	9/19/2005	95	77	75	78.9
E4	9/19/2005	22	20	20	90.9

3.3 Identification of isolates

The WC hybridization assay was performed on all of the isolates that grew after transfer to the glass culture tubes. Table 3.2 shows the number of isolates that tested positive for *P. pungens* and that survived to the DNA extraction step for each sample location. Of the 276 *Pseudo-nitzschia* spp. isolates, 175 (63%) were identified as being *P. pungens* by the WC hybridization assay. Scanning electron microscopy was performed on five of these isolates to confirm the results of the WC hybridization assay. Measurements and characteristics (Table 1.1) of the cells

analyzed using SEM conformed to the description of *P. pungen*s in Hasle *et al.* (1996).

3.4 Genotyping of isolates

The low percentage of isolates that were genotyped in relation to the total number of isolates for location BS was partly due to the isolation of species of *Pseudo-nitzschia* other than *P. pungens*. Negative results from molecular probe analyses (i.e. no fluorescence with the puD1 probe) coupled with confirmation by SEM indicated that the negative results were due to species other than *P. pungens*. The low percentage was also partly due to cell death immediately following the isolation procedure or culture death after transfer from the well plate into the glass culture tubes. For locations E1-E4, attrition of isolates was mostly due to cell death immediately following the isolation procedure. After isolates from E1-E4 were growing in well plates, they generally had a high survival rate after transfer to the glass culture tubes. While cell death may be a result of unhealthy cells at the time of isolation, some isolates may not grow in f/2 medium. This can add some bias into the genotyping results from only being able to genotype those isolates that will grow in f/2 medium.

Of the 175 *P. pungens* isolates collected from the Pacific Northwest that survived to the DNA extraction step, 172 (98%) were successfully genotyped at all four loci (Table 3.2). The reason for the exclusion of three isolates from 2005 was the inability to amplify DNA at one or more loci. There was 80% clonal diversity in

Pacific Northwest isolates, that is, 137 out of 172 multilocus genotypes were different. Multilocus genotypes (alleles are indicated as size in base pairs) for all isolates from the Pacific Northwest are listed by sample in the Appendix. In total, there were 27 shared multilocus genotypes. Usually, only two isolates shared a genotype, but there were two instances where a multilocus genotype was shared by three isolates and one instance where four isolates shared a genotype. Station E3 had the most (11) shared multilocus genotypes while stations E1 and E4 had one each. Seventeen multilocus genotypes were observed at more than one station. However, there was no distinct pattern of occurrence for isolates that shared genotypes. For example, genotypes observed at station BS in 2004 were also observed at stations E1, E2, and E3 in 2005. Additionally, one multilocus genotype was observed at all four locations in 2005.

It was only possible to genotype 10 of the 16 isolates received from Belgian and Dutch waters at all four loci. This was likely a result of the samples having thawed during shipping. Also, some of the microcentrifuge tubes in which the cultures were shipped leaked and lost the majority of their contents. Therefore, there was probably not enough of the *P. pungens* culture remaining in these tubes to allow for extraction of an adequate amount of DNA for subsequent analyses. Of the DNA extracts and isolates from the German islands of Helgoland and Sylt, all but one could be genotyped at all four loci. However, it was possible to genotype this lone isolate at three of the four loci. There were no shared genotypes among the North Sea isolates (see the Appendix).

3.5 Microsatellite scoring errors

The results from MICRO-CHECKER indicated that for the combined Pacific Northwest isolates there was no evidence for large allele dropout or scoring errors due to stutter for all loci. For loci PP5 and PP6, however, null alleles may be present due to the general excess of homozygotes for most allele size classes. When each sample was analyzed individually, the presence of null alleles was suggested at locus PP6 for all samples. However, null alleles were suggested at locus PP5 for only the BS and E3 samples. For the North Sea isolates none of the loci showed evidence for large allele dropout, scoring errors due to stutter, or null alleles.

3.6 Analysis of microsatellite data

3.6.1 Descriptive statistics

The numbers of alleles amplified at each locus for the 172 Pacific Northwest isolates ranged from 6 at locus PP6 to 20 at locus PP3 (Table 3.3). In total, 54 alleles were observed over four loci giving an allelic diversity of 13.5 alleles per locus. The effective number of alleles for each locus ranged from 1.54 to 6.23 (Table 3.3).

For the 25 isolates from the North Sea, between 4 (PP6) and 14 (PP3) alleles were amplified at each locus (Table 3.3). The allele size ranges in base pairs (bp)

Table 3.3. Descriptive statistics for Pacific Northwest and North Sea samples. PNW represents all of the Pacific Northwest samples treated as a single sample. Allelic richness is based on a minimum sample size of 20 isolates. An '*' indicates significance (P<0.05), "S.E." the Standard Error, H_o is the observed heterozygosity, H_e is the expected heterozygosity, "W&C" refers to Weir and Cockerham (1984).

Sample		Locus			
		PP2	PP3	PP5	PP6
PNW	Number of isolates	172	172	172	172
	Range (bp)	175-254	173-246	171-199	167-195
	Number of alleles	16	20	12	6
	Effective number of alleles	3.33	6.23	3.09	1.54
	Allelic richness	8.0	11.2	7.4	4.2
	Observed number of genotypes	30	50	27	11
	H_o	0.66	0.77	0.53	0.16
	H_e	0.7	0.84	0.68	0.35
	HWE probability P-value	0.0000*	0.0000*	0.0000*	0.0000*
	HWE S.E.	0.000	0.000	0.000	0.000
	F_{IS} (W&C)	0.064	0.082	0.221	0.557
	Heterozygote deficiency P-value	0.0008*	0.0004*	0.0000*	0.0006*
	Heterozygote deficiency S.E.	0.001	0.000	0.000	0.000
North Sea	Number of isolates	25	25	25	24
	Range (bp)	168-239	192-253	193-201	198-239
	Number of alleles	5	14	5	4
	Effective number of alleles	2.54	6.22	2.22	2.95
	Allelic richness	5.0	13.7	5.0	4.0
	Observed number of genotypes	8	18	7	7
	H_o	0.68	0.92	0.52	0.75
	H_e	0.62	0.86	0.56	0.67
	HWE probability P-value	0.8408	0.809	0.342	0.9064
	HWE S.E.	0.004	0.014	0.007	0.002
	F_{IS} (W&C)	-0.103	-0.076	0.074	-0.114
	Heterozygote deficiency P-value	0.8227	0.7935	0.2042	0.7690
	Heterozygote deficiency S.E.	0.007	0.017	0.005	0.004
BS	HWE probability P-value	0.005*	0.0881	0.0000*	0.0000*
	HWE S.E.	0.002	0.007	0.000	0.000
	F_{IS} (W&C)	0.052	0.12	0.572	0.516
	Heterozygote deficiency P-value	0.2279	0.1274	0.0001*	0.0484*
	Heterozygote deficiency S.E.	0.013	0.009	0.000	0.002

Table 3.3. (cont.).

Sample		Locus PP2	PP3	PP5	PP6
E1	HWE probability *P*-value	0.0003*	0.2419	0.0610	0.0081*
	HWE S.E.	0.000	0.014	0.005	0.001
	F_{IS} (W&C)	0.117	0.107	0.016	0.447
	Heterozygote deficiency *P*-value	0.0112*	0.0162*	0.0959	0.0072*
	Heterozygote deficiency S.E.	0.002	0.004	0.005	0.001
E2	HWE probability *P*-value	0.0522	0.0029*	0.0002*	0.0001*
	HWE S.E.	0.005	0.001	0.000	0.000
	F_{IS} (W&C)	0.138	0.180	0.187	0.718
	Heterozygote deficiency *P*-value	0.2402	0.0112*	0.0081*	0.0000*
	Heterozygote deficiency S.E.	0.010	0.003	0.002	0.000
E3	HWE probability *P*-value	0.0000*	0.0074*	0.0000*	0.0000*
	HWE S.E.	0.000	0.003	0.000	0.000
	F_{IS} (W&C)	0.016	0.017	0.203	0.494
	Heterozygote deficiency *P*-value	0.0223*	0.0249*	0.0007*	0.0333*
	Heterozygote deficiency S.E.	0.004	0.004	0.001	0.003
E4	HWE probability *P*-value	0.0096*	0.2432	0.0331*	0.0004*
	HWE S.E.	0.002	0.0161	0.0038	0.0003
	F_{IS} (W&C)	0.095	0.147	0.139	0.660
	Heterozygote deficiency *P*-value	0.4023	0.0605	0.0285*	0.0005*
	Heterozygote deficiency S.E.	0.014	0.008	0.003	0.000

were slightly offset between the Pacific Northwest and the North Sea for loci PP2, PP3, and PP5, whereas the ranges for locus PP6 did not overlap.

Allele frequencies for each locus are plotted in Figure 3.1 for the Pacific Northwest isolates and the North Sea isolates. In general there were 1-3 alleles that dominated at each locus for each sample. Given the allelic diversity at each locus it would theoretically be possible to differentiate 4.68×10^7 genotypes in the Pacific Northwest and 2.36×10^5 genotypes from the North Sea.

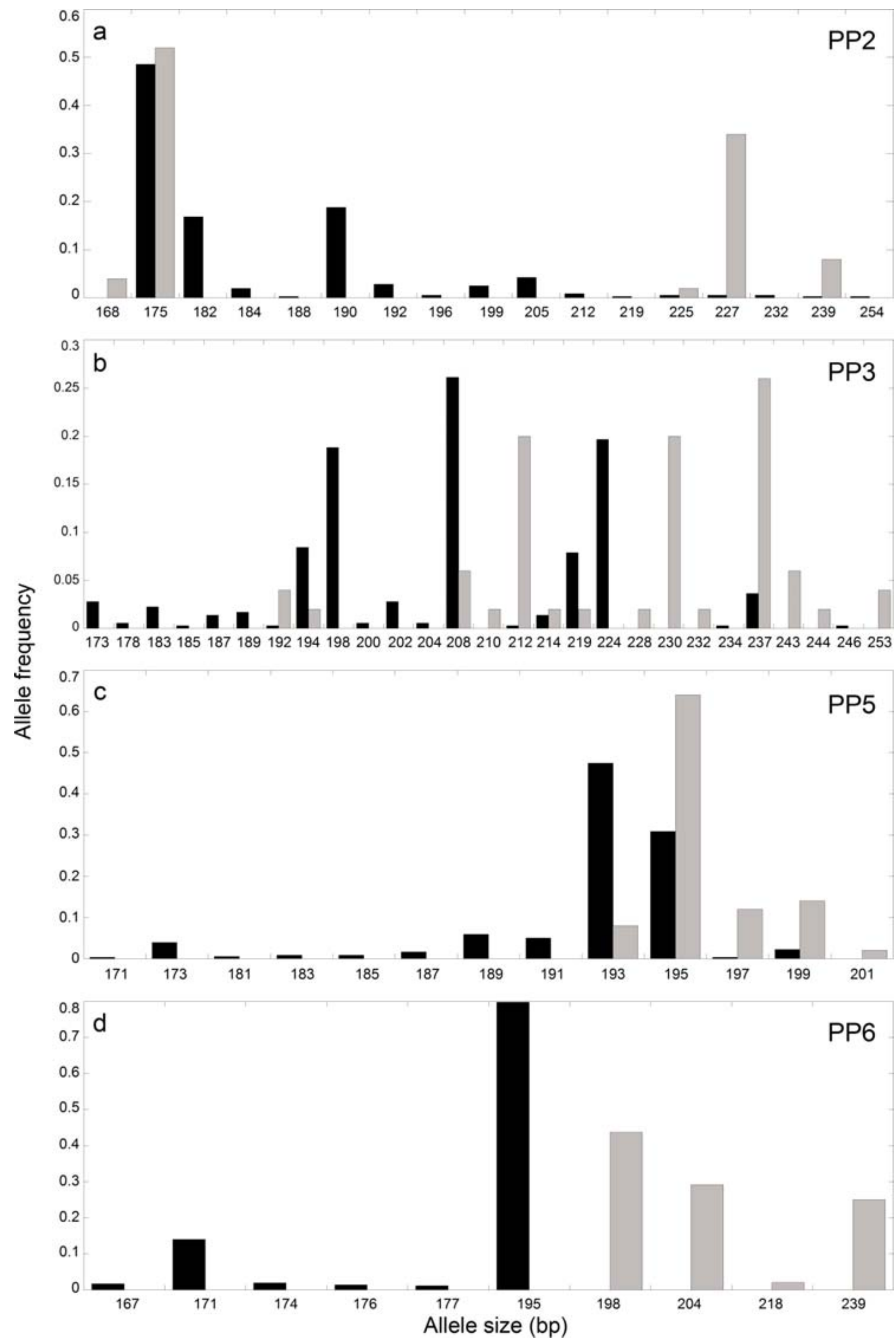

Figure 3.1. Allele frequencies for all Pacific Northwest (black) and North Sea (gray) isolates. (a) locus PP2, (b) locus PP3, (c) locus PP5, (d) locus PP6. Note the different allele size and frequency scales for each locus.

3.6.2 Hardy-Weinberg equilibrium

The observed heterozygosities ranged from 0.16 to 0.77 (mean = 0.53) in the 172 Pacific Northwest isolates when grouped together and 0.52 to 0.92 (mean = 0.72) in North Sea isolates genotyped for this study (Table 3.3). Isolates from the Pacific Northwest deviated from HWE and had a significant deficit of heterozygotes at all loci when considered as a single sample, while none of the loci in North Sea isolates deviated significantly from HWE ($P<0.05$).

When isolates from the five individual Pacific Northwest locations were considered separately, deviations from HWE expectations were detected in all samples at all loci except for one sample at locus PP2, three samples at PP3 and one sample at PP5 ($P<0.05$). However, three of these *P*-values were only slightly smaller than the significance level (0.05, Table 3.3). Heterozygote deficits were detected at locus PP2 in two samples, locus PP3 in three samples, locus PP5 in four samples, and locus PP6 in all samples ($P<0.05$).

The average F_{IS} values for the Pacific Northwest and North Sea groups were 0.176 and -0.065, respectively. The two sided *P*-value for the test for differences in F_{IS} values between regions after 1000 permutations of the data was 0.043, which indicated that the F_{IS} values were significantly different between the North Sea and Pacific Northwest *P. pungens*.

3.6.3 Linkage disequilibrium

In tests for genotypic linkage disequilibrium, or non-random association of alleles, when all of the Pacific Northwest isolates were grouped together all loci exhibited significant linkage disequilibrium ($P<0.05$, Table 3.4). When each of the five Pacific Northwest samples were considered separately 20 out of the 30 locus pair tests were significant ($P<0.05$, Table 3.4). In the North Sea isolates, significant linkage disequilibrium was only detected between loci PP2 and PP3 ($P<0.05$, Table 3.5), and the sample size of 20 isolates may have had an effect on the outcome of this test.

Table 3.4. Linkage disequilibrium for Pacific Northwest isolates. The numbers below the diagonal are *P*-values for each locus pair when isolates from the Pacific Northwest were pooled. The numbers above the diagonal are the number of samples with significant linkage disequilibrium (out of 5). A "*" indicates significance ($P<0.05$)

Locus	PP2	PP3	PP5	PP6
PP2		0	3	5
PP3	0.0282*		3	4
PP5	0.0000*	0.0000*		5
PP6	0.0000*	0.0000*	0.0000*	

Table 3.5. Linkage disequilibrium for North Sea isolates. The numbers below the diagonal are *P*-values for each locus pair for the pooled sample of North Sea isolates A "*" indicates significance ($P<0.05$)

Locus	PP2	PP3	PP5	PP6
PP2				
PP3	0.0142*			
PP5	0.9429	0.6038		
PP6	0.7091	0.8723	0.1384	

The combined and individual Pacific Northwest samples showed higher multilocus linkage ($\bar{r}_d$ values) than would be expected in populations where alleles were exchanged at random (Table 3.6). Conversely, the $\bar{r}_d$ value for the North Sea

sample was much smaller than those for the Pacific Northwest samples and was not significantly larger than that of the randomized populations. Locus pair $\bar{r}_d$ values are listed in the Appendix.

Table 3.6. $\bar{r}_d$ values for combined Pacific Northwest, individual Pacific Northwest, and North Sea samples over all loci. *P*-values determined after 1000 randomizations of the data for each sample and indicate whether the $\bar{r}_d$ values are larger than expected. A "*" indicates significance ($P<0.05$).

Sample	$\bar{r}_d$	*P*-value
Pacific Northwest	0.4496	< 0.0010*
BS	0.6273	< 0.0010*
E1	0.4172	< 0.0010*
E2	0.3939	< 0.0010*
E3	0.4043	< 0.0010*
E4	0.4971	< 0.0010*
North Sea	0.0282	0.2210

3.6.4 Population differentiation

The Pacific Northwest isolates were treated in two ways when comparing them to the North Sea sample. First, all of the Pacific Northwest isolates were considered as a group and compared with the North Sea sample. Both the genotypic test (highly significant) and F_{ST} (0.2431, P = 0.050) indicated significant genetic differentiation between the Pacific Northwest and the North Sea groups over all loci.

Second, genotypic tests were performed and F_{ST} was calculated for the individual Pacific Northwest samples and the North Sea sample. Comparisons between individual Pacific Northwest samples and the North Sea sample indicated significant differentiation at all loci ($P<0.05$, Table 3.7). There was also significant differentiation between some pairs of Pacific Northwest samples: BS was significantly differentiated from all other samples.

Table 3.7. Genotypic differentiation tests performed in GENEPOP. The numbers below the diagonal are *P*-values over all loci. A "*" indicates significance ($P<0.05$). The numbers above the diagonal are the number of single locus tests (out of 4) that were significant ($P<0.05$) for the population pair.

	North Sea	BS	E1	E2	E3	E4
North Sea		4	4	4	4	4
BS	Highly significant		1	2	1	1
E1	Highly significant	0.0106*		0	0	1
E2	Highly significant	0.0086*	0.2831		0	0
E3	Highly significant	0.0010*	0.4101	0.1014		0
E4	Highly significant	0.0173*	0.2123	0.8893	0.0312*	

Pairwise genotypic tests between Pacific Northwest samples showed significant differentiation over all loci ($P<0.05$) between the BS sample and all the other samples as well as between two of the four Juan de Fuca eddy samples. However, the F_{ST} values for all of these pairs are quite low and the pairwise comparisons done in FSTAT were not significant after the Bonferroni corrections (Table 3.8). Every individual Pacific Northwest sample was significantly different from the North Sea sample as indicated by relatively high F_{ST} values and significant *P*-values from both tests of differentiation.

Table 3.8. F_{ST} values for population differentiation tests performed in FSTAT. The numbers below the diagonal are F_{ST} values. The numbers above the diagonal are the per-pair *P*-values for pairwise tests of differentiation. A "*" indicates significance ($P<0.05$), and a "**" indicates significance after non-sequential Bonferroni correction.

	North Sea	BS	E1	E2	E3	E4
North Sea		0.0033**	0.0033**	0.0033**	0.0033**	0.0033**
BS	0.236		0.0333*	0.0333*	0.0133*	0.0630
E1	0.264	-0.002		0.1770	0.3370	0.2930
E2	0.211	0.014	0.005		0.1400	0.8830
E3	0.262	0.004	-0.002	0.012		0.0670
E4	0.194	0.019	0.013	-0.009	0.011	

Population differentiation using R_{ST} showed a pattern similar to that of F_{ST} (Table 3.9). However, for comparisons between the Pacific Northwest and the North Sea samples, R_{ST} was larger than F_{ST}.

Table 3.9. R_{ST} values from population differentiation tests performed in GENEPOP.

	North Sea	BS	E1	E2	E3
North Sea					
BS	0.343				
E1	0.370	-0.019			
E2	0.401	-0.011	0.011		
E3	0.441	0.008	-0.009	0.065	
E4	0.310	-0.024	-0.018	-0.006	0.003

3.6.5 Pacific Northwest population sub-structure analysis

The program STRUCTURE indicated that the Pacific Northwest data set was likely made up of a mixture of two populations (Fig. 3.2). Figure 3.3 shows a bar plot illustrating the two populations identified by STRUCTURE where each vertical bar in the plot corresponds to a single isolate. The proportion of the alleles originating in a given population is represented by the proportion of its vertical bar that is either light or dark colored. These proportions were used to separate the isolates into two populations, PAC1 and PAC2. Isolates with a proportion of the alleles higher than 90% stemming from one population were assigned to that population, while those with intermediate probabilities were excluded from further analyses. Population PAC1 consisted of 29 isolates: six from BS, six from E1, seven from E2, 12 from E3, and six from E4. Population PAC2 consisted of 127 isolates: 18 from BS, 23 from E1, 12 from E2, 60 from E3, and 14 from E4. A total of 16 isolates were

excluded from the analyses with six from E1, two from E2, seven from E3, and one from E4.

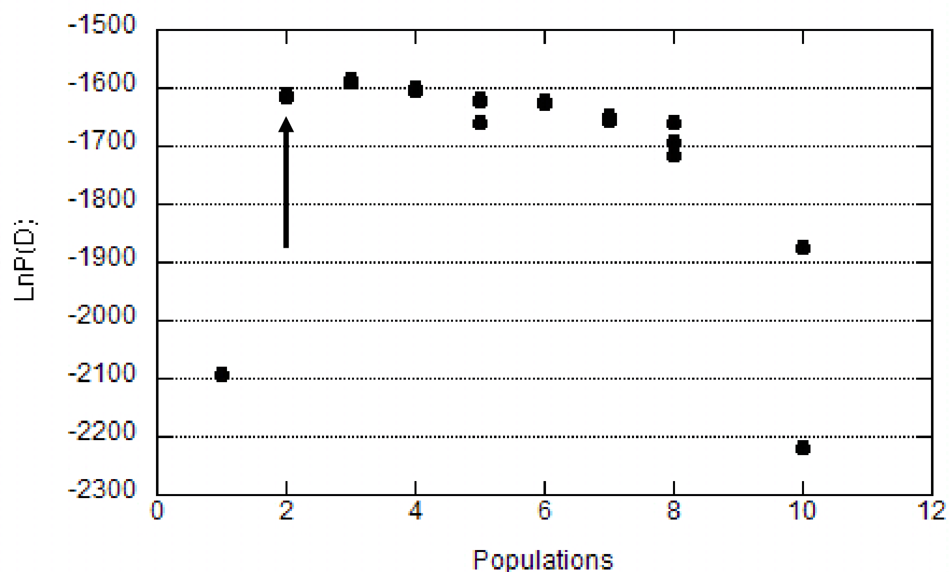

Figure 3.2. Results of STRUCTURE runs for 1-10 populations. The most likely number of populations is indicated by where the curve begins to level off (Arrow). The x-axis is the number of K populations used in the run and the y-axis is the estimated probability that the data are described by specific number of clusters.

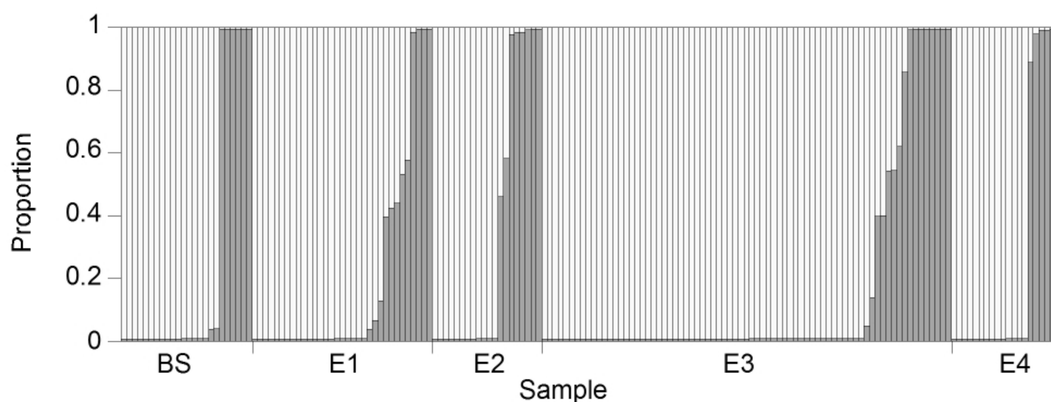

Figure 3.3. Bar plot results from population assignments by STRUCTURE. Each vertical bar is partitioned into K colored segments, in this case 2. Each colored segment represents the estimated membership fraction of that isolate in each of the K populations. The x-axis lists the original samples and the y-axis is the proportion of alleles derived from each population. Light color=PAC1, dark color=PAC2.

PAC1 did not deviate from HWE at loci PP2, PP3, and PP6 (however the test for locus PP6 was borderline), whereas locus PP5 deviated from HWE expectations (P<0.05, Table 3.10). F_{IS} values calculated for with the probability test indicated that there is a deficit of heterozygotes at locus PP5 in population PAC1. Population PAC2 was did not deviate HWE expectations at three of the four loci but there was only one allele present at locus PP6 (P<0.05, Table 3.10) and the HWE probability test could not be performed on this locus.

Table 3.10. Hardy-Weinberg probabilities for populations PAC1 and PAC2 identified by STRUCTURE. A "*" indicates significance (P<0.05). PAC1 consisted of 29 isolates and PAC2 consisted of 127 isolates.

		Locus			
Sample		PP2	PP3	PP5	PP6
PAC1	HWE probability *P*-value	0.6115	0.4039	0.0001*	0.0619
	HWE probability S.E.	0.0146	0.0145	0.0001	0.0033
	F_{IS} (W&C)	-0.030	0.099	0.328	0.114
PAC2	HWE probability *P*-value	0.3646	0.5853	0.2768	One allele
	HWE probability S.E.	0.0069	0.0139	0.0067	One allele
	F_{IS} (W&C)	-0.090	0.028	0.076	One allele

Tests for genotypic linkage disequilibrium in GENEPOP suggested that data did not deviate from random association of alleles between all pairs of loci in both populations PAC1 and PAC2 (Table 3.11). Due to the presence of only one allele at locus PP6 in PAC2, pairwise linkage disequilibrium tests could not be performed with this locus in this population. The P-values for locus pairs PP3 & PP6 and PP5 & PP6 in PAC1 were only slightly greater than the level of significance (P<0.05).

Values of $\bar{r}_d$ calculated over all loci for PAC1 and PAC2 are listed in Table 3.11 and locus pair $\bar{r}_d$ values are listed in the Appendix. After partitioning into two

populations, the $\bar{r}_d$ values are similar to those in the North Sea (Table 3.12) where no significant difference was found between the observed $\bar{r}_d$ value and that of randomized data sets.

Population differentiation in GENEPOP along with pairwise tests of differentiation and calculation of F_{ST} in FSTAT were conducted for populations PAC1, PAC2, and the North Sea sample. All tests indicated that the three populations were distinct (Table 3.13). Additionally, R_{ST} calculated in GENEPOP suggested a high level of differentiation between the population pairs.

Table 3.11. Linkage disequilibrium *P*-values for populations identified by STRUCTURE. Values below the diagonal are for PAC1 and values above the diagonal are for PAC2 "n.p" means that the test was not possible due to the presence of one allele at that locus.

Locus	PP2	PP3	PP5	PP6
PP2		0.9836	0.7369	n.p.
PP3	1.0000		0.2956	n.p.
PP5	1.0000	0.2707		n.p.
PP6	0.7207	0.0666	0.0673	

Table 3.12. $\bar{r}_d$ values for PAC1 and PAC2. *P*-values determined after 1000 randomizations of the data for each sample.

Sample	$\bar{r}_d$	*P*-value
PAC1	0.0446	0.0860
PAC2	-0.0113	0.7850

Table 3.13. Population differentiation between populations PAC1, PAC2, and the North Sea sample. "Highly Significant" is the result given by genepop. An "*" indicates significance after non-sequential Bonferroni correction.

Population Pair		Test result
PAC1 vs. PAC2	Genotypic Test	Highly Significant
	F_{ST}	0.4181
	F_{ST} P-value	0.01667*
	R_{ST}	0.8039
PAC1 vs. North Sea	Genotypic Test	Highly Significant
	F_{ST}	0.2742
	F_{ST} P-value	0.01667*
	R_{ST}	0.6359
PAC2 vs. North Sea	Genotypic Test	Highly Significant
	F_{ST}	0.3542
	F_{ST} P-value	0.01667*
	R_{ST}	0.5397

CHAPTER 4: DISCUSSION

4.1 Collection and identification *P. pungens*

It was not possible to isolate *P. pungens* cells onboard the ship in 2004 and 2005, so the sample had to survive until isolations could be performed at the laboratory in Seattle, WA. An alternate method of sample handling had to be devised, where drops of sample were added to growth medium in 48-well plates, due to conditions that prohibited shipboard isolation. During the 2004 cruise, samples were collected for isolation of *P. pungens* at all of the sites where they were observed, however it was possible to obtain isolates from the Barkley Sound site. With method used to keep the samples viable, it meant that the sample had to survive for at least 15 days prior to isolation. Attrition of the 2004 samples was most likely a result of this method used keep them viable as the majority of the samples did not survive to the isolation step. Maintaining samples in this way was not an ideal method in the cell isolation process because it is best to isolate cells from the sample immediately after the sample is collected.

During the 2005 cruise, samples were collected in a similar fashion, but the success rate was greater. This was probably due in part by the time difference between sample collection and isolation after each cruise. While the sample from 2004 had to survive for 15 days prior to isolation, the four samples from 2005 had to survive only 5-8 days. Cells became noticeably unhealthy if samples had to wait too long between collection and isolation. During the isolation process, the contents of the cells being isolated could be seen leaking out of the frustules as the cells became

unhealthy. For future studies, it will be important to either isolate on board the ship or isolate immediately after returning to port, minimizing the time between collection and isolation. Additionally, methods that eliminate the need for cultured isolates would be extremely useful for diatom population genetic studies. Ruiz Sebastian and O'Ryan (2001) successfully PCR amplified partial small subunit and large subunit rDNA sequences from single dinoflagellate cells. Additionally, Evans *et al.* (2005) stated that in preliminary experiments PCR amplification of microsatellite loci from single *Pseudo-nitzschia* chains has been demonstrated.

Isolates were identified as *P. pungens* using the WC hybridization assay described in Miller and Scholin (1996). This is a very specific assay that has been shown to discriminate between morphologically similar species, such as *P. pungens* and *P. multiseries*. However, the fluorescence signal from *P. pungens* is usually weaker than that of the positive control (Miller and Scholin 1996) (Fig. 2.2), so any isolate that had noticeable fluorescence, weak or strong, was considered to be *P. pungens*. For the 2004 sample, 24 of the isolates were identified as *P. pungens* and for the 2005 samples a total of 151 were identified as *P. pungens* using the WC assay. To confirm the results of the WC assay, two of the positive isolates from 2004 and three of the positive isolates from 2005 were identified as *P. pungens* using SEM. This is a small sample size for confirmation purposes (3.0% of the total number of Pacific Northwest isolates), and in the future a greater percentage should be used. Evans *et al.* (2005) performed SEM on 28 of 464 isolates (6.0%).

There were three isolates that were identified as *P. pungens* using the WC assay for which it was either not possible to obtain genotypes or it was only possible to genotype at 1-2 loci. This could be a result of the cultures in question becoming unhealthy or contaminated in the time between WC assay identification and DNA extraction. Alternatively, these three isolates could have been species other than *P. pungens* and incomplete removal of unbound probe during the wash step of the assay could have resulted in fluorescence of the cells.

Given that the number of isolates per sample ranged from 20-77, test results using individual samples may be affected by low sample size and should be interpreted with caution. When considered as a single sample, the number of isolates from the Pacific Northwest is comparable to the sample size in Evans and Hayes (2004).

4.2 *P. pungens* genotypes

There were no shared genotypes in the 2004 sample, possibly due to the relatively small sample size of 24 isolates. In 2005, the sample sizes ranged from 20-77 and the samples with 20 and 34 isolates each had a single shared genotype. However in sample E3 from 2005 with 77 isolates, 11 genotypes were shared. With eleven shared genotypes, it could be assumed that sample E3 was a good representation of the *P. pungens* community at that specific location. The fact that 86% of the genotypes in this sample were unique indicated that the genotypic diversity of *P. pungens* in the Pacific Northwest was lower than the diversity of the

diatoms *D. brightwellii* (98%) in Puget Sound (Rynearson and Armbrust 2004) and *P. pungens* (98%) in the North Sea (Evans *et al.* 2005). When all of the Pacific Northwest isolates were grouped and considered a single sample, there was an 80% clonal diversity. When the Pacific Northwest samples were pooled, this group was considered to be a good representation of *P. pungens* isolates at the Pacific Northwest sample sites due to the number of shared genotypes that were observed. Temporal structure may not be a factor in the Pacific Northwest sample, as eight of the genotypes that were observed in the 2004 sample were also observed in samples collected in 2005.

Although the North Sea sample only consisted of 25 isolates, the test results for deviations from HWE and linkage equilibrium from this group compared well to larger samples from the same region where no deviations from HWE or linkage equilibrium were detected in a sample of over 400 isolates (Evans and Hayes 2004, Evans *et al.* 2005). Additionally, the size ranges of alleles found for the 25 North Sea samples encompassed the allele size ranges observed by Evans *et al.* (2004) and Evans *et al.* (2005). However due to differences in analytical instrumentation, allele sizes in North Sea isolates analyzed in the present study were smaller by approximately 4 bp for loci PP2 and PP3, 2 bp for locus PP5, and 1 bp for locus PP6. This observation was based on allele sizes provided by Katharine Evans for the North Sea isolates used in the present study that were also genotyped for Evans *et al.* (2005).

It was possible to genotype all of the DNA extracts received from North Sea *P. pungens* at all loci except for one isolate. Despite not being able to genotype this one isolate at all four loci, it was still unique from the other North Sea isolates and was included in the analysis. There were no shared genotypes in the North Sea sample, which was not surprising considering the genetic diversity previously reported for North Sea *P. pungens* (Evans and Hayes 2004, Evans *et al.* 2005).

4.3 Comparison of Pacific Northwest and North Sea samples

4.3.1 Genotypic differentiation, F_{ST} and R_{ST} comparisons

When considered as individual samples and as a single sample, *P. pungens* from the Pacific Northwest appeared to be genetically differentiated from those of the North Sea. Genotypic tests of differentiation, F_{ST}, and R_{ST} values showed that when considered individually and as a single group Pacific Northwest samples were distinct from the North Sea sample. F_{ST} values were all greater than 0.19 when the North Sea sample was compared to the individual Pacific Northwest samples (Table 3.8). Michels *et al.* (2003) observed differentiation between *Daphnia* populations with F_{ST} values ranging from 0.121 to 0.185 and Rynearson and Armbrust (2004) differentiated populations of the diatom *D. brightwellii* where significant F_{ST} values ranged from 0.048 to 0.245. In comparison, Shaw *et al.* (1999) found significant differences between Atlantic and Pacific herring where F_{ST} values ranged from 0.027 to 0.043 and R_{ST} values ranged from 0.220 to 0.279.

The high F_{ST} values, when comparing the individual and combined PNW samples to the North Sea, in the present study were not unexpected results considering that the North Sea sample and the Pacific Northwest samples were collected from different oceans. This difference was readily observed in the frequency distribution of microsatellite alleles from the two locations (Fig. 3.1) which is the reason that R_{ST} is larger than F_{ST}. However on a smaller geographical scale, Rynearson and Armbrust (2004) showed that genetically distinct populations of *D. brightwellii* could be associated with different water mass signatures within the Puget Sound, Washington basin.

4.3.2 Hardy-Weinberg and linkage equilibrium

The individual Pacific Northwest samples deviated from HWE expectations when considered separately and as a single sample, while the North Sea sample did not deviate from HWE expectations. There were a number of possible explanations for the deviations, some of which were more likely than others. Since *P. pungens* multiply primarily by asexual reproduction, it was expected that populations of this diatom would deviate from HWE. The fact that the North Sea sample from the present study did not deviate from HWE could be a result of a small sample size. However, given that Evans and Hayes (2004) and Evans *et al.* (2005) failed to reject the null hypothesis of HWE with a much larger *P. pungens* sample size in the North Sea, this result was interpreted to be representative of prior results from the North Sea. The comparison of F_{IS} between groups from the Pacific Northwest and the

North Sea indicated a deficit of heterozygotes in the Pacific Northwest, suggesting that one or more of the HWE assumptions were violated in the Pacific Northwest.

The North Sea isolates did not exhibit linkage disequilibrium whereas it was evident at some loci in both the individual and combined Pacific Northwest samples. Measures of linkage disequilibrium suggested that in the Pacific Northwest samples there was much stronger disequilibrium present than there was in the North Sea (see the Appendix). This non-random association of alleles, as well as deviations from HWE, could be indicative of processes such as selection, recent mixing of different populations, or population bottlenecks (Frankham *et al.* 2000). It could also be due to asexual reproduction, but not null alleles.

Clonal selection can increase the frequency of adapted genotypes and the subsequent over-representation of some genotypes will result in the appearance of linkage disequilibrium and/or deviations from HWE (Gomez and Carvalho 2000). It was estimated that sexual reproduction occurs in *Pseudo-nitzschia* species on the order of every three years (Davidovich and Bates 1998). After sexual reproduction, given a growth rate of 0.6 d^{-1} (determined from a *P. pungens* isolate that had been in culture for two years at 12°C) and a size reduction of approximately 2.5 µm per month as estimated by Davidovich and Bates (1998), it would take approximately 650 cell divisions to reach the size required for sexual reproduction to take place.

Clonal selection could be acting on *P. pungens* in the Pacific Northwest during the phase of asexual reproduction causing the observed deviations from HWE and linkage equilibrium. If it is assumed that there was a single population of *P.*

pungens in the Pacific Northwest, this clonal selection could be facilitating processes such as population bottlenecks or inbreeding. Since *P. pungens* by nature cannot disperse under their own power, they would be confined to the same water mass in which they were created after sexual reproduction, unless they are dispersed through processes such as turbulent mixing or sinking. If only certain genotypes were capable of adapting to the properties of the water mass in question, clonal selection will eliminate all but the favorable genotypes. Over time, the population of *P. pungens* in that water mass could become inbred. If this were strictly the case, it would be expected that *P. pungens* in the North Sea would be just as likely to suffer from inbreeding as those in the Pacific Northwest. That the North Sea population in Evans *et al.* (2005) did not deviate from HWE or linkage equilibrium suggests that clonal selection resulting in inbreeding or bottlenecks may not be as much of a factor there as it could be in the Pacific Northwest. However, the processes of population bottlenecks and inbreeding seem unlikely in the Pacific Northwest and still need to be tested. The chances are low that a water mass will remain intact in the Pacific Northwest study area long enough that inbreeding can be used to explain the deviations from HWE and linkage equilibrium. Using similar logic the samples that were collected could have come from an inbred population that had been recently advected into the study area.

In the Pacific Northwest clonal diversity in *P. pungens* appears to be maintained at a relatively high level compared to aquatic clonal animals such as rotifers and cladocerans. Gomez and Carvalho (2000) examined the population

structure of rotifers in a temporary pond and found an unexpectedly high number of unique genotypes where 90% of the genotypes they encountered were unique after the parthenogenetic phase. In contrast, Vanoverbeke and De Meester (1997) found between 2-60% unique genotypes in populations of *Daphnia* during a phase of sexual reproduction. In cyclically parthenogenetic rotifers and cladocerans, cloning usually occurs when environmental conditions are favorable and sexual reproduction resulting in the production of resting eggs is triggered by deteriorating conditions (Carvalho 1994). It follows that in temporary environments like seasonal ponds sexual reproduction will occur more frequently with resting egg banks contributing to the genetic diversity of subsequent populations that will inhabit the pond. Gomez and Carvalho (2000) concluded that in intermittent habitats, sexual reproduction resets genetic variation after the parthenogenetic phase. Conversely, in a permanent population prolonged periods of clonal selection deplete genetic variation (Mort and Wolf 1986). After long term clonal selection, departures from HWE have been observed from allozyme analysis in *Daphnia* (Platt and Spitze 2000). Given that the combined Pacific Northwest *P. pungens* sample showed significant departures from HWE and linkage equilibrium, the isolates in this sample may have undergone some degree of clonal selection. Also, although *Pseudo-nitzschia* spp. undergo sexual reproduction, they are not known to have resting stages from which genotypic diversity can be restored as demonstrated for *Daphnia*. Other diatoms have been shown to produce resting spores including the pennate diatom *Eunotia soleirolii* and the centric diatom *Chaetoceros didymium* (Round *et al.* 1990).

The relatively high percentage of unique genotypes in the present study suggested that there was some mechanism operating to maintain the observed genotypic diversity. *Pseudo-nitzschia* cells are known to persist at depth at very low light levels (Trainer *et al.* 1998, Rines *et al.* 2002) so transport of these cells to surface waters via upwelling or vertical mixing may be one possible mechanism that acts to maintain genotypic diversity. Additionally, the northward-flowing California undercurrent, the source of upwelled waters on the Washington coast has been suggested as a means of transport for phytoplankton from the south (Hickey and Banas 2003) and could be another mechanism that introduces new populations of *P. pungens* to the Juan de Fuca eddy region.

Mixing of two different populations could explain both the linkage disequilibrium and the deviation from HWE in the Pacific Northwest sample and also act as a mechanism for maintaining genotypic diversity, provided the admixed populations could mate. The lack of deviation from HWE and linkage equilibrium in the North Sea could be explained by the presence of a single unstructured population of *P. pungens* in that area (Evans *et al.* 2005). Sexual reproduction is one of the main sources of genetic diversity which is then exposed to clonal selection during periods of asexual reproduction (Michels *et al.* 2003). Evans *et al.* (2005) suggested that sexual reproduction plays an important role in the genetic structure of *P. pungens* populations in the North Sea. A likely explanation for the observed deviations from HWE and departures from linkage equilibrium was a lack of sexual reproduction between admixed populations in the Pacific Northwest.

The deviations from HWE and linkage equilibrium could also have been an artifact of the time of sampling. The Pacific Northwest samples could have been far removed from instances of random mating whereas the *P. pungens* in Evans *et al.* (2005) could have been sampled immediately after random mating had occurred. A single bout of random mating will serve to eliminate deviations from HWE, but it will take more than one bout to eliminate linkage disequilibrium (Pfrender and Lynch 2000). The seasonality of sexual reproduction in *Pseudo-nitzschia*, if any, has yet to be documented and sexual reproduction may occur constantly but at low rates. If the Pacific Northwest sample was in fact made up of mixed populations of *P. pungens* it seems that enough random mating either had not occurred since admixture or the populations are incapable of mating. If the populations were incapable of mating, it is unlikely that the observed deviations from HWE and linkage equilibrium were an artifact of sampling.

4.4 Sub-structure of Pacific Northwest isolates

As population mixing was suspected in the Pacific Northwest samples as an explanation for the departures from HWE and linkage equilibrium, the sub-structure of the Pacific Northwest isolates was investigated using the program STRUCTURE. There was a significant deficit of heterozygotes at all loci in the Pacific Northwest samples, both when considered separately and as a single sample. A deficit in heterozygosity could be caused by strong selection against heterozygous genotypes, inbreeding, or a Wahlund effect which is defined as the inclusion of two or more

genetically distinct units into a population sample (Murphy *et al.* 1994). The program STRUCTURE was used to investigate the possibility of a Wahlund effect in the Pacific Northwest sample. Population substructure can cause a Wahlund effect which is known to strongly influence parameter estimates such as *F*-statistics and linkage disequilibrium (Halkett *et al.* 2005)

After running simulations in STRUCTURE that tested for the presence of one to ten populations, the data indicated that there were two distinct populations represented in the Pacific Northwest group of samples (Figure 3.2). In the two populations identified using STRUCTURE, three of four loci in PAC1 and all four loci in PAC2 did not deviate from HWE expectations, although there was only one allele present at locus PP6 in population PAC2.

The populations identified by STRUCTURE were for the most part in HWE and they were in linkage equilibrium for all pairs of loci. Deviations from HWE expectations can be attributed to clonal reproduction within the subpopulations (Avise 2004) as clonal reproduction prevents the reshuffling of alleles among and across loci (Halkett *et al.* 2005). The deviation from HWE at locus PP5 in population PAC1 could also be a result of the relatively smaller size of this population compared do PAC2. Since STRUCTURE assumes linkage equilibrium in populations, some amount of noise in the linkage equilibrium results can be expected as *P. pungens* mainly reproduce asexually and clonal reproduction generates non-random associations between loci (Halkett *et al.* 2005).

Measures of linkage disequilibrium also suggested that there were two distinct populations present in the Pacific Northwest. The $\bar{r}_d$ values calculated for PAC1 and PAC2 were similar to the $\bar{r}_d$ value for the North Sea (Tables 3.6 and 3.12). These low values indicated that there was a weak correlation between pairs of loci in these populations and a random association of alleles. Two randomly mating populations were identified in the combined Pacific Northwest sample suggesting recent admixture of *P. pungens* populations in the Pacific Northwest. It also makes the possibility of population bottlenecks and inbreeding in the original combined Pacific Northwest sample unlikely.

Another possible reason for the lack of HWE and linkage equilibrium in the original Pacific Northwest samples was that there may be some type of cryptic speciation in this area. Cryptic species, also known as sibling species, are morphologically indistinguishable but are reproductively isolated (Avise 2004). The F_{ST} value for PAC1 and PAC2 was higher than comparisons between the North Sea sample and the combined Pacific Northwest sample, PAC1, and PAC2. Pfrender *et al.* (2000) observed pairwise R_{ST} values greater than 0.60 between lake-like and nonlake-like ponds containing *Daphnia* and suggested that there were at least two essentially non-interbreeding forms of *Daphnia* represented that sometimes coexisted in the same pond. The high degree of differentiation in the Pacific Northwest suggested the possibility of two morphologically similar species represented by the PAC1 and PAC2 populations. This situation is possible under the biological species concept (Avise 2004) where these two populations would be

reproductively isolated by some mechanism. Schwenk *et al.* (2004) showed that two morphologically similar species of *Daphnia* previously considered a single species were highly genetically differentiated.

Current techniques employing scanning electron microscopy and molecular probes may not be sufficient for the definitive identification of *P. pungens* in the Pacific Northwest. Although molecular probe analysis identified the isolates used in this study as *P. pungens*, confirmation of the assay was only performed on five of the isolates using SEM. One isolate from PAC1, three isolates from PAC2, and one isolate from the group of intermediate isolates were analyzed using SEM. However, even SEM may not give a definitive species confirmation. Lundholm *et al.* (2003) found substructure in the frustule poroids of isolates that were previously identified as *P. pseudodelicatissima*. Using transmission electron microscopy (TEM), they described the poroid structure and identified three new species. TEM should be performed on a subset of the *P. pungens* isolates from the Pacific Northwest sample to confirm their morphological similarity in finer detail. Also, Schwenk *et al.* (2004) used mitochondrial and nuclear DNA (internal transcribed spacer) markers to identify the different species of *Daphnia*. This type of analysis may be required to establish the presence of cryptic *P. pungens* species in the Pacific Northwest.

4.5 Oceanographic effects on *P. pungens* communities

Populations of *P. pungens* associated with the outflowing Strait water could be mixed with populations of oceanic origin in the Juan de Fuca eddy region.

MacFadyen *et al.* (2005) showed that drifting buoys deployed in the Strait of Juan de Fuca will either exit the area via the northwestward flowing Vancouver Island Coastal Current or become trapped in the Juan de Fuca eddy. Cells trapped in the eddy will remain there for varying lengths of time before spinning off, depending on the retentiveness of the eddy. Those drifters that circuited the eddy did so in 10-12 days.

As the eddy is a semi-permanent feature that starts up in the spring and declines in the fall (Freeland and Denman 1982), mixed populations of *P. pungens* in surface waters may not have sufficient time to undergo sexual reproduction before the surface manifestation of the eddy disappears and the cells are dispersed via prevailing currents. However, even when the surface manifestation of the eddy disappears, doming of the vertical temperature and salinity fields shows that the eddy is still present (MacFadyen *et al.* 2005) and cells may still be retained at depth in the eddy region.

Evans *et al.* (2005) suggest that sexual reproduction played an important role in defining the population structure of *P. pungens* in the North Sea. Oceanographic and environmental conditions (i.e. temperature and light levels) could have an effect on the growth rate of *P. pungens* which, in turn will affect the time that it takes for cells to reach the size range required for sexual reproduction to occur. The dynamic system in the Pacific Northwest may reduce the possibility of contact between *P. pungens* cells in the surface waters diminishing the opportunities for sexual reproduction. The rate of sexual reproduction of *Pseudo-nitzschia* spp. in natural

populations is not well understood, and probably is dependent on local ocean conditions. Seasonality of sexual reproduction in *Pseudo-nitzschia* has not been demonstrated. If the time between instances of sexual reproduction is extended, strains will be subjected to clonal selection during prolonged periods of asexual reproduction resulting in genetic drift.

The samples collected for the present study were from the outer edge of the Juan de Fuca eddy. MacFadyen *et al.* (2005) showed that with changes in local wind forcing, surface drifters transiting the eddy can be ejected and entrained in southward flowing surface currents. Similarly, the *P. pungens* isolated from the area southwest of the eddy region could have been recently ejected. If two (or more) *P. pungens* populations had recently mixed in the Juan de Fuca eddy region, the *P. pungens* community may not have had sufficient time to undergo enough random mating that would eliminate deviations from HWE and linkage equilibrium before being ejected from the eddy. Therefore, the samples collected on the outer edge of the eddy could be composed of two or more distinct populations and would explain the observed deviations from HWE and linkage equilibrium. Additionally, each year after the eddy forms there may be a constant input of new *P. pungens* populations into the eddy community, further confounding the approach of HWE and linkage equilibrium.

Oceanographic conditions in the Pacific Northwest could also have mixed two non-interbreeding cryptic species or these species could coexist in the Juan de Fuca eddy region. That isolates from 2004 and 2005 were partitioned into both

PAC1 and PAC2 suggests that the cryptic species co-exist in the Pacific Northwest. It is conceivable that there is cryptic speciation of *P. pungens* in the Pacific Northwest since species previously identified as *P. pseudodelicatissima* have subsequently been described as being distinct species based on TEM analysis (Lundholm *et al.* 2003). Investigating sexual reproduction of *Pseudo-nitzschia pungens* in the Pacific Northwest with both laboratory experiments and field observations will give better insight into the results of the present study. Mating studies should be performed with isolates from the two populations to confirm that if there are in fact cryptic species, they cannot interbreed.

In contrast, the area in the North Sea from where the isolates for this study were collected is not an area of dynamic mixing of multiple water types. The southern North Sea near the coast of Europe is characterized by inflowing water from the Strait of Dover that follows the continental coast (Otto *et al.* 1990). This area is influenced to a lesser extent by Atlantic water flowing in from the north and Baltic Sea water flowing in from the northeast through the Skagerrak. Estimates referenced in Otto *et al.* (1990) suggest that it could take approximately one year for a parcel of water to make its way through the region. A lack of water mass mixing and a relatively straight flow along the continental coast of the North Sea may be an environment that that is more conducive for *P. pungens* to undergo sexual reproduction, thereby maintaining populations that do not exhibit deviations from HWE and linkage equilibrium. The North Sea environment may allow a population of *P. pungens* to remain intact and undergo random mating as it travels through the

North Sea without the introduction of an extraneous population or cryptic species. The chances of observing deviations from HWE or linkage equilibrium will be reduced in a population that is maintained as a unit as it is carried along by currents, undergoes random mating, and experiences little clonal selection.

4.6 Conclusions

Genotyping a number of isolates comparable to the size of the combined Pacific Northwest sample in this study, from a single location will most likely give a better representation of the population structure at that location. Additionally, development of techniques that will eliminate the need for culturing isolates, such as single cell PCR (de Bruin *et al.* 2003), will make population genetic studies of toxic algae much less labor intensive. Single cell PCR also will eliminate some of the bias in the results from only being able to genotype those isolates that will grow in the culture medium. Also, sequencing of the microsatellite loci from isolates collected in the Pacific Northwest would indicate whether null alleles were present and contributed to the results of the present study.

When taken as a whole, the Pacific Northwest sample deviated from HWE and linkage equilibrium, whereas the North Sea sample did not. It can be concluded that one or more of the assumptions of HWE are being violated in the Pacific Northwest. There were three overall scenarios that could be used to explain the results from the Pacific Northwest samples. First, there was one *P. pungens* population in the area that was far removed from random mating. Second, there was

a recent mixing of two distinct *P. pungens* populations that had yet to exchange genetic material prior to sampling. Third, were actually two species in the area that either co-existed or were recently mixed, with one or both being a different species than that in the North Sea. In comparison, the North Sea population was probably made up of a single, randomly mating population that was subjected to oceanographic conditions that did not influence the structure of the population.

Of the three scenarios, there were most likely either two populations of *P. pungens* or there were cryptic species in the Pacific Northwest. Under these scenarios, data from the Pacific Northwest *P. pungens* compared the most favorably with the North Sea *P. pungens* described in Evans *et al.* (2005), in that each of the two Pacific Northwest populations for the most part did not deviate from HWE and linkage equilibrium. As *P. pungens* are thought to be either non-toxic or weakly toxic, it would be interesting to investigate the DA production capabilities of isolates from the two populations identified in the present study. Toxicity studies could be performed to determine if either of the populations identified in the study produce DA, and if one population produces DA at a higher concentration than the other.

Given the degree of differentiation between the two Pacific Northwest populations, however, it appears that cryptic speciation best explained the population structure observed in the Pacific Northwest sample. To better support the idea that cryptic species exist in the Pacific Northwest, analyses such as TEM, mitochondrial DNA and large subunit ribosomal DNA sequencing, and ITS analyses should be performed on the Pacific Northwest isolates.

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APPENDIX: Multilocus genotypes and $\bar{r}_d$ values

Multilocus genotypes at the Barkley Sound (BS) station.

	Locus			
Isolate	PP2	PP3	PP5	PP6
BS-1	184 / 199	173 / 189	171 / 189	171 / 171
BS-2	175 / 175	198 / 224	195 / 195	195 / 195
BS-3	175 / 175	198 / 224	193 / 193	195 / 195
BS-4	175 / 175	224 / 234	193 / 195	195 / 195
BS-5	175 / 190	219 / 224	193 / 199	195 / 195
BS-6	175 / 190	198 / 208	193 / 195	195 / 195
BS-7	175 / 175	208 / 208	193 / 195	195 / 195
BS-8	175 / 190	224 / 224	193 / 193	195 / 195
BS-9	205 / 205	173 / 194	173 / 185	167 / 177
BS-10	175 / 188	208 / 224	193 / 193	195 / 195
BS-11	192 / 205	173 / 194	173 / 173	167 / 177
BS-12	175 / 190	208 / 208	193 / 193	195 / 195
BS-13	175 / 190	224 / 237	195 / 195	195 / 195
BS-14	192 / 199	189 / 194	173 / 173	167 / 171
BS-15	205 / 212	173 / 183	173 / 173	171 / 177
BS-16	205 / 219	189 / 189	189 / 189	167 / 177
BS-17	175 / 175	198 / 208	193 / 193	195 / 195
BS-18	175 / 182	198 / 198	193 / 193	195 / 195
BS-19	175 / 190	208 / 224	193 / 193	195 / 195
BS-20	175 / 175	198 / 237	195 / 195	195 / 195
BS-21	175 / 182	198 / 208	193 / 195	195 / 195
BS-22	175 / 175	208 / 208	193 / 193	195 / 195
BS-23	175 / 182	208 / 224	193 / 193	195 / 195
BS-24	175 / 190	208 / 237	193 / 193	195 / 195

Multilocus genotypes at station E1.

	Locus			
Isolate	PP2	PP3	PP5	PP6
E1-1	175 / 190	198 / 208	189 / 193	195 / 195
E1-2	175 / 175	208 / 208	193 / 195	195 / 195
E1-3	175 / 182	204 / 224	195 / 195	195 / 195
E1-4	227 / 232	183 / 194	183 / 191	171 / 174
E1-5	184 / 227	173 / 173	187 / 193	171 / 171
E1-6	175 / 182	208 / 208	193 / 193	195 / 195
E1-7	175 / 190	224 / 224	193 / 195	195 / 195
E1-8	175 / 190	192 / 198	173 / 195	171 / 195
E1-9	175 / 175	194 / 208	191 / 193	171 / 195
E1-10	175 / 190	198 / 198	193 / 195	195 / 195
E1-11	184 / 199	194 / 194	183 / 183	171 / 171
E1-12	175 / 182	208 / 224	193 / 193	195 / 195
E1-13	175 / 182	198 / 224	193 / 193	195 / 195

Multilocus genotypes at station E1 (cont.).

	Locus			
Isolate	PP2	PP3	PP5	PP6
E1-14	175 / 190	198 / 237	193 / 193	195 / 195
E1-15	175 / 182	198 / 224	193 / 193	195 / 195
E1-16	175 / 182	198 / 224	193 / 193	195 / 195
E1-17	175 / 182	208 / 208	193 / 195	195 / 195
E1-18	175 / 175	208 / 219	193 / 193	195 / 195
E1-19	175 / 175	194 / 219	189 / 193	171 / 195
E1-20	175 / 190	219 / 224	193 / 195	195 / 195
E1-21	182 / 182	219 / 237	193 / 195	195 / 195
E1-22	190 / 190	173 / 224	189 / 193	171 / 195
E1-23	175 / 190	198 / 224	193 / 193	195 / 195
E1-24	175 / 175	198 / 202	193 / 195	195 / 195
E1-25	175 / 190	198 / 202	193 / 195	195 / 195
E1-26	205 / 205	194 / 194	189 / 191	171 / 171
E1-27	175 / 175	194 / 198	189 / 195	171 / 195
E1-28	175 / 175	208 / 246	193 / 195	195 / 195
E1-29	175 / 175	208 / 224	193 / 195	195 / 195
E1-30	175 / 182	198 / 208	193 / 193	195 / 195
E1-31	175 / 190	208 / 224	193 / 193	195 / 195
E1-32	182 / 182	208 / 224	193 / 193	195 / 195
E1-33	190 / 190	198 / 208	193 / 195	195 / 195

Multilocus genotypes at station E2.

	Locus			
Isolate	PP2	PP3	PP5	PP6
E2-1	192 / 212	187 / 200	181 / 191	171 / 171
E2-2	175 / 182	198 / 208	195 / 195	195 / 195
E2-3	190 / 190	194 / 198	193 / 193	171 / 195
E2-4	175 / 175	198 / 224	193 / 195	195 / 195
E2-5	190 / 199	194 / 198	185 / 199	171 / 171
E2-6	182 / 190	208 / 208	193 / 195	195 / 195
E2-7	175 / 182	208 / 208	193 / 195	195 / 195
E2-8	175 / 190	198 / 219	193 / 193	195 / 195
E2-9	190 / 205	185 / 219	189 / 189	171 / 171
E2-10	182 / 190	198 / 224	195 / 195	195 / 195
E2-11	175 / 182	208 / 208	193 / 193	195 / 195
E2-12	175 / 175	198 / 208	193 / 195	195 / 195
E2-13	182 / 182	208 / 224	193 / 193	195 / 195
E2-14	192 / 205	178 / 194	187 / 189	171 / 171
E2-15	175 / 175	219 / 219	193 / 195	195 / 195
E2-16	175 / 175	208 / 208	193 / 195	195 / 195
E2-17	192 / 199	187 / 198	191 / 191	171 / 174
E2-18	190 / 205	183 / 183	191 / 195	174 / 174
E2-19	184 / 190	194 / 198	193 / 195	176 / 195
E2-20	175 / 182	198 / 214	193 / 195	195 / 195

Multilocus genotypes at station E3.

	Locus			
Isolate	PP2	PP3	PP5	PP6
E3-1	175 / 190	224 / 224	193 / 193	195 / 195
E3-2	175 / 190	198 / 224	193 / 195	195 / 195
E3-3	175 / 190	219 / 237	193 / 195	195 / 195
E3-4	175 / 190	208 / 219	195 / 195	195 / 195
E3-5	175 / 175	198 / 198	193 / 195	195 / 195
E3-6	182 / 190	198 / 224	193 / 193	195 / 195
E3-7	175 / 175	202 / 208	193 / 193	195 / 195
E3-8	175 / 182	224 / 237	193 / 195	195 / 195
E3-9	175 / 175	208 / 214	195 / 195	195 / 195
E3-10	182 / 182	198 / 198	193 / 193	195 / 195
E3-11	182 / 182	198 / 208	193 / 193	195 / 195
E3-12	192 / 225	187 / 194	173 / 173	171 / 176
E3-13	190 / 225	187 / 194	173 / 173	171 / 176
E3-14	175 / 175	202 / 224	193 / 193	195 / 195
E3-15	175 / 182	208 / 237	195 / 195	195 / 195
E3-16	175 / 175	208 / 208	193 / 193	195 / 195
E3-17	175 / 175	208 / 219	193 / 195	195 / 195
E3-18	175 / 190	219 / 224	193 / 193	195 / 195
E3-19	175 / 190	219 / 224	193 / 193	195 / 195
E3-20	175 / 175	194 / 208	187 / 195	171 / 195
E3-21	175 / 205	194 / 208	187 / 195	171 / 195
E3-22	182 / 182	208 / 208	193 / 193	195 / 195
E3-23	192 / 205	183 / 183	189 / 191	171 / 174
E3-24	175 / 190	202 / 208	193 / 193	195 / 195
E3-25	175 / 182	208 / 224	195 / 195	195 / 195
E3-26	175 / 182	214 / 237	193 / 195	195 / 195
E3-27	175 / 190	198 / 208	193 / 193	195 / 195
E3-28	175 / 175	224 / 224	195 / 199	195 / 195
E3-29	182 / 190	208 / 224	193 / 195	195 / 195
E3-30	175 / 182	198 / 224	193 / 195	195 / 195
E3-31	175 / 190	208 / 224	195 / 199	195 / 195
E3-32	175 / 182	208 / 208	193 / 195	195 / 195
E3-33	175 / 190	208 / 224	195 / 199	195 / 195
E3-34	175 / 175	202 / 208	193 / 193	195 / 195
E3-35	192 / 205	194 / 194	189 / 189	171 / 171
E3-36	175 / 182	208 / 224	193 / 195	195 / 195
E3-37	182 / 190	198 / 208	193 / 195	195 / 195
E3-38	175 / 175	198 / 219	195 / 195	195 / 195
E3-39	175 / 182	208 / 224	195 / 195	195 / 195
E3-40	175 / 175	208 / 224	193 / 195	195 / 195
E3-41	175 / 182	214 / 219	193 / 197	195 / 195
E3-42	175 / 182	202 / 224	195 / 195	195 / 195
E3-43	175 / 190	202 / 208	193 / 195	195 / 195
E3-44	175 / 190	198 / 224	193 / 193	195 / 195
E3-45	175 / 175	198 / 237	193 / 193	195 / 195
E3-46	175 / 182	208 / 219	193 / 193	195 / 195

Multilocus genotypes at station E3 (cont.).

	Locus			
Isolate	PP2	PP3	PP5	PP6
E3-47	184 / 192	173 / 194	189 / 191	171 / 171
E3-48	184 / 190	173 / 194	189 / 191	171 / 171
E3-49	175 / 190	224 / 224	193 / 193	195 / 195
E3-50	175 / 190	208 / 224	193 / 195	195 / 195
E3-51	175 / 175	198 / 208	193 / 195	195 / 195
E3-52	175 / 175	198 / 208	193 / 195	195 / 195
E3-53	175 / 175	198 / 208	193 / 193	195 / 195
E3-54	175 / 182	208 / 224	193 / 195	195 / 195
E3-55	175 / 190	219 / 219	193 / 195	195 / 195
E3-56	182 / 190	198 / 198	193 / 193	195 / 195
E3-57	175 / 175	202 / 208	193 / 195	195 / 195
E3-58	175 / 190	198 / 208	193 / 195	195 / 195
E3-59	175 / 175	194 / 208	187 / 195	176 / 195
E3-60	175 / 190	208 / 224	195 / 195	195 / 195
E3-61	182 / 190	208 / 224	193 / 193	195 / 195
E3-62	175 / 192	194 / 198	173 / 193	176 / 195
E3-63	175 / 175	198 / 208	193 / 193	195 / 195
E3-64	182 / 190	224 / 224	195 / 195	195 / 195
E3-65	175 / 199	194 / 208	189 / 195	167 / 195
E3-66	205 / 212	194 / 198	189 / 191	171 / 171
E3-67	175 / 190	214 / 219	191 / 193	195 / 195
E3-68	182 / 190	198 / 208	193 / 195	195 / 195
E3-69	175 / 175	198 / 224	193 / 195	195 / 195
E3-70	175 / 190	208 / 224	193 / 195	195 / 195
E3-71	182 / 190	208 / 208	195 / 195	195 / 195
E3-72	199 / 205	183 / 189	191 / 191	171 / 174
E3-73	175 / 175	208 / 224	193 / 193	195 / 195
E3-74	175 / 175	202 / 208	193 / 195	195 / 195
E3-75	196 / 196	173 / 198	173 / 193	171 / 195

Multilocus genotypes at station E4.

	Locus			
Isolate	PP2	PP3	PP5	PP6
E4-1	175 / 175	208 / 219	193 / 199	195 / 195
E4-2	175 / 182	208 / 208	193 / 195	195 / 195
E4-3	190 / 239	187 / 194	191 / 193	171 / 171
E4-4	190 / 254	198 / 204	191 / 191	171 / 174
E4-5	175 / 182	198 / 237	193 / 195	195 / 195
E4-6	175 / 182	219 / 224	195 / 199	195 / 195
E4-7	190 / 199	194 / 212	187 / 195	171 / 195
E4-8	175 / 175	208 / 237	193 / 195	195 / 195
E4-9	184 / 190	189 / 198	185 / 191	171 / 171
E4-10	175 / 182	224 / 224	195 / 195	195 / 195
E4-11	175 / 190	198 / 224	193 / 195	195 / 195
E4-12	175 / 175	219 / 219	195 / 195	195 / 195
E4-13	175 / 175	224 / 224	195 / 195	195 / 195
E4-14	190 / 190	178 / 183	189 / 189	171 / 171
E4-15	199 / 232	194 / 200	181 / 189	167 / 171
E4-16	175 / 182	198 / 198	195 / 199	195 / 195
E4-17	175 / 175	208 / 224	195 / 195	195 / 195
E4-18	175 / 175	198 / 224	193 / 195	195 / 195
E4-19	182 / 190	198 / 224	193 / 195	195 / 195
E4-20	175 / 182	198 / 208	193 / 193	195 / 195

Multilocus genotypes for the North Sea (NS) sample.

	Locus			
Isolate	PP2	PP3	PP5	PP6
NS-1	175 / 232	210 / 230	193 / 195	198 / 198
NS-2	175 / 232	212 / 237	193 / 195	198 / 239
NS-3	232 / 232	208 / 212	195 / 197	198 / 239
NS-4	175 / 175	212 / 253	195 / 197	198 / 239
NS-5	175 / 232	230 / 237	195 / 201	000 / 000*
NS-6	168 / 232	243 / 253	195 / 195	198 / 204
NS-7	175 / 232	232 / 243	195 / 195	198 / 239
NS-8	175 / 175	212 / 237	197 / 199	198 / 204
NS-9	175 / 239	212 / 230	193 / 195	204 / 239
NS-10	232 / 232	208 / 212	195 / 197	198 / 204
NS-11	175 / 175	237 / 243	195 / 195	198 / 218
NS-12	175 / 175	230 / 230	199 / 199	204 / 204
NS-13	175 / 232	212 / 237	195 / 195	198 / 204
NS-14	175 / 232	237 / 244	195 / 195	198 / 204
NS-15	175 / 175	230 / 230	195 / 195	198 / 204
NS-16	168 / 175	219 / 237	195 / 195	204 / 239
NS-17	175 / 239	212 / 237	195 / 195	204 / 239
NS-18	225 / 232	194 / 237	195 / 197	198 / 239
NS-19	175 / 239	212 / 230	195 / 195	198 / 204
NS-20	175 / 232	230 / 237	195 / 197	198 / 198
NS-21	175 / 175	214 / 230	195 / 199	198 / 239
NS-22	232 / 239	192 / 212	195 / 195	198 / 198
NS-23	175 / 232	208 / 237	195 / 199	204 / 204
NS-24	175 / 232	228 / 237	199 / 199	239 / 239
NS-25	175 / 232	192 / 237	193 / 195	198 / 239

Locus pair $\bar{r}_d$ values. *P*-values were generated after 1000 randomizations of the data and indicate whether the $\bar{r}_d$ values are larger than expected. *P*-values larger than the specified significance level ($P<0.05$) are in bold.

Sample	$\bar{r}_d$ PP2 & PP3	*P*-value	$\bar{r}_d$ PP2 & PP5	*P*-value	$\bar{r}_d$ PP2 & PP6	*P*-value
BS	0.4639	< 0.0010	0.5614	< 0.0010	0.8711	< 0.0010
E1	0.2452	< 0.0010	0.3340	< 0.0010	0.5357	< 0.0010
E2	0.2101	< 0.0140	0.3389	< 0.0140	0.5344	< 0.0140
E3	0.2347	< 0.0010	0.3213	< 0.0010	0.5427	< 0.0010
E4	0.2534	< 0.0090	0.4459	< 0.0090	0.7366	< 0.0090
North Sea	0.1615	< 0.0430	-0.0660	< 0.0430	-0.0314	< 0.0430
Pacific Northwest	0.2608	< 0.0010	0.3800	< 0.0010	0.6062	< 0.0010
PAC1	**-0.0363**	**< 0.7190**	**0.0037**	**< 0.7190**	**-0.0051**	**< 0.7190**
PAC2	**-0.0273**	**< 0.8830**	**-0.0210**	**< 0.8830**	N/A	N/A

Sample	$\bar{r}_d$ PP3 & PP5	*P*-value	$\bar{r}_d$ PP3 & PP6	*P*-value	$\bar{r}_d$ PP5 & PP6	*P*-value
BS	0.4701	< 0.0010	0.5725	< 0.0010	0.6749	< 0.0010
E1	0.2552	< 0.0010	0.4245	< 0.0010	0.6165	< 0.0010
E2	0.1484	< 0.0010	0.3287	< 0.0010	0.6334	< 0.0010
E3	0.2602	< 0.0010	0.3925	< 0.0010	0.5910	< 0.0010
E4	0.2739	< 0.0010	0.3358	< 0.0010	0.6791	< 0.0010
North Sea	**-0.0688**	**< 0.7430**	**0.0107**	**< 0.7430**	**0.1639**	**< 0.6270**
Pacific Northwest	0.2725	< 0.0010	0.4287	< 0.0010	0.6174	< 0.0010
PAC1	**0.0036**	**< 0.4950**	**0.0510**	**< 0.4950**	**0.2036**	**< 0.5170**
PAC2	**0.0124**	**< 0.7830**	N/A	N/A	N/A	N/A