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Revealing the distinct habitat ranges and hybrid zone of genetic sub-populations within *Pseudo-nitzschia pungens* (Bacillariophyceae) in the West Pacific area

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ABSTRACT

Genetic sub-populations (clades) of cosmopolitan marine diatom *Pseudo-nitzschia pungens* might have distinct habitats, and their hybrid zone is suspected in higher latitude area of the West Pacific area, however, it is still unrevealed because of technical difficulties and lack of evidences in natural environments. The aim of this study is to investigate the habitat characteristics of each clade of *P. pungens* on geographical distribution with the habitat temperature ranges of each clade and to reveal their hybrid zone in the West Pacific area. We employed the 137 number of nucleotide sequences of *P. pungens* and its sampling data (spatial and temporal scale) originated from the West Pacific area, and used field application of qPCR assay for intra-specific level of *P. pungens*. Only two genotypes, clade I and III, were identified in the West Pacific area. Clade I was distributed from 39 to 32.3°N, and clade III were from 1.4 to 34.4°N. The estimated habitat temperature for the clade I and clade III ranges were 8.1–26.9 °C and 24.2–31.2 °C, respectively. The latitudinal distributions and temperature ranges of each clade were significantly different. The qPCR assay employed, and results suggested that the hybrid zone for clade I and III has been observed in the southern Korean coasts, and clade III might be introduced from the Southern Pacific area. The cell abundances of clade III were strongly related with the higher seawater temperature and warm current force. This study has defined distinct habitat characteristics of genetically different sub-populations of *P. pungens*, and revealed its hybrid zone in natural environment for the first time. We also provided strong evidences about dispersion of the population of clade III to higher latitude in the West Pacific area.

1. Introduction

After speciation, or sufficient evolutionary changes for one species to become two or more sub-species, each population may continue to co-habitat and interact (Barton and Hewitt, 1989; Harrison, 1990). A hybrid zone is an area where the ranges of two interbreeding species meet. Classically, the studies of ecological habitats and hybrid zones on sub-populations are highly important to improve autecological perspectives and species evolution. Many biologists have emphasized the evolutionary importance of hybridization that it plays a "key role in race formation" (Grant, 1981), therefore often treated hybrid zones as "windows on the evolutionary process" (Harrison, 1990) and "natural laboratories" (Hewitt, 1988). The hybridization could form new evolutionary lines that are isolated from the ancestral types and are therefore free to evolve in new evolutionary directions (Stebbins, 1959). Each sub-population of species might take possession of different ecological habitats with different adaptation strategies in natural environments (Daehler, 2001; Skóra et al., 2015). In many instances, hybridization appears to occur at ecotones or boundaries between different habitats (Harrison, 1993), however, it is very challenging to estimate the exact habitat ranges of populations in opened marine environment, especially in micro-organism studies.

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Because of uncertain and/or undistinguishable phenotypes, genotype concept is commonly used to discriminate cryptic species and sub-population in many micro-organism species, and it has been assumed as an integrated system adapted to the ecological habitats and/or niches of each population (Schluter, 2001). *Pseudo-nitzschia pungens* is one of the most cosmopolitan and common marine diatom species (Bates et al., 1998; Hasle, 2002; Lelong et al., 2012; Trainer et al., 2012) which has a potential neurotoxin, domoic acid that causes amnesic shellfish poisoning (Bates et al., 1998; Rhodes et al., 1996; Trainer et al., 1998). This diatom had significant genetic differentiation in the ITS region, and could therefore be separated into three distinct genotypes (clades) in the phylogenetic tree (Casteleyn et al., 2008; Kim et al., 2015; Lim et al., 2014). Each clade appears to have their own distribution (Casteleyn et al., 2008; Casteleyn et al., 2010; Kim et al., 2015; Lim et al., 2014). Previous studies have reported that clade I populations were distributed in world-wide temperate zone while clade II were specifically in Pacific Northwest area, and clade III was found only in the tropical warm-temperate area, and showed different growth traits in laboratory experiments (Kim et al., 2015). Therefore, it has been assumed that each clade might have a distinct ecological habitat, whilst no field study has been carried out to support this hypothesis.

Until 2008, there were only five ITS sequences of *P. pungens* in the West Pacific region, two belonged to clade III (Vietnam; DQ166533 and DQ062665, unpublished), and the others corresponded to clade I (Japan; Casteleyn et al., 2008). In 2014, 47 ITS sequences of clade III information were reported from the vicinity of Peninsula Malaysia west coast and northern coats of Borneo Island (from 1.38 to 6.52°N) (Lim et al., 2014). In 2015, four clones belonging to clade III and seven clones of clade I were reported during the summer season in Korean coasts and East China Sea. Clade III was relatively abundant at higher latitudes (from 31.6 to 34.4°N) as reported previously (Kim et al., 2015). Thus, the authors assumed that isolates of clade III might be migrated from the Southern Pacific via warm current towards the northern area, and that a hybrid zone between clade I and clade I and III had been observed in this area. However, evidences were not enough to prove this hypothesis, since the number of isolates of *P. pungens* from the higher latitudes were limited, and there was no substantial method to quantify the abundances of each clade in the hybrid zone proposed. Generally, *Pseudo-nitzschia* species are difficult to identify morphologically (Antonella and Luca, 2013; Hasle et al., 1996), and it is impossible that to distinguish their clades under light microscope level (Casteleyn et al., 2008; Churro et al., 2009; Kim et al., 2015). Alternatively, several qPCR methods have been developed to measure the abundance of *Pseudo-nitzschia* species (Andree et al., 2011; Delaney et al., 2011; Fitzpatrick et al., 2010). Recently, a qPCR assay was developed to detect and quantify *P. pungens* at intra-specific level that enables to clarify their geographical distribution and origin of each clade, however, it has not yet applied to ecological field studies (Kim et al., 2017).

This study aimed to identified the habitat ranges of each clade of *P. pungens* focusing on latitudinal distribution and habitat temperature ranges, and revealed the possibility of their hybrid zone in the West Pacific area. To address this, we established new *P. pungens* strains, all the ITS sequences of *P. pungens* with sampling information to compare the differences between habitat ranges of each clade in the West Pacific area. Also, a recently developed qPCR assay was applied to analyze the seasonal occurring patterns of each clade in suspected hybrid zone.

2. Methods

2.1. Strains and DNA extraction

The strains of *Pseudo-nitzschia pungens* from 46 sites of Korean coasts (2010–2011; monthly), 10 sites of East China Sea (August 2011), one site of Philippines (October 2011), two sites of China and Malaysia (September 2014) and four sites of Palau (September 2014) (Fig. 1A, Table S1). Seawater samples were collected using a Van Dorn sampler (Wildlife supply company, MI, USA), then single cells or single colonies of *P. pungens* were isolated and transferred into individual wells of a 96-well plate filled with 200 μL of f/2 medium (Guillard, 1975) with a Pasteur pipette (Hilgenberg, Germany) under an inverted microscope (IX71; Olympus, Tokyo, Japan). The *P. pungens* cells of log phase from each well were transferred into a culture 50 mL flask (SPL, Daegu, Korea) containing 25 mL of sterile F/2 medium and maintained at 20 °C, with a 12:12 light:dark (L:D) photoperiod by cool-white fluorescent lamps. The fifteen mililiters of each culture were harvested when they reached at stationary growth phase, then, genomic DNA were extracted. The procedures of extraction were carried out using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Finally, a total 76 numbers of genomic DNA samples were extracted.

2.2. Phylogenetic tree

Polymerase chain reaction (PCR) was performed with forward PnITSF and reverse PnITSR primer set (Kim et al., 2015) to amplify the ITS2 region for 76 numbers of *P. pungens* genomic DNA samples. The total twenty μL of reaction mixtures were amplified in 1× Ex Taq Buffer that containing 0.1 μg template DNA, 0.3 μM of each primer, 0.25 μM of deoxynucleoside triphosphate and 1 unit of TaKaRa Ex Taq. The PCR and sequencing steps were done according to Kim et al. (2015). The acquired 76 sequences of the ITS region together with our 66 sequences of *P. pungens* (obtained from GenBank, https://www.ncbi.nlm.nih.gov/genbank/) were aligned with BIOEDIT 7.1.3.0 software (North Carolina State University), then maximum likelihood (ML), neighbor-joining (NJ) and UPGMA (unweighted pair-group method with arithmetic algorithm) trees were constructed with MEGA 6.06 software (1000 replicates) to confirm the clade alignment of each strain. Best model was computed as Kimura-2-parameter (Kimura, 1980) in MEGA 6.06. The two sequences, LC194953 and LC194954, were used for representing clade II of *P. pungens* and two *P. multiseries* sequences (AY257844 and DQ445651) were used as an outgroup. After obtaining the trees, genetic diversities of intra-population and inter-population was computed on clades level by MEGA 6.06.

2.3. Environmental samples for qPCR assay

The environmental seawater samples were collected at the 36 sites of Korean coasts in July 2008 to determine the hybrid zone of *P. pungens* in the West Pacific area (Fig. 1B, Table S1). Afterwards, intensively periodic sampling was carried out from August 2009 to November 2010 at selected 10 sites of the southern Korean coasts (Fig. 2). During this periodic sampling, surface seawater temperature (SST), salinity, DO, pH and conductivity were recorded with a YSI 650 Multi-Parameter Display System (YSI Inc., Yellow Springs, OH, USA), and nutrients (nitrite, nitrate, ammonium, phosphate and silicate) were measured by standard techniques using an automated flow injection system (QuikChem 8000, Lachat Instruments, Inc., Milwaukee, USA). Also, the environmental seawaters were collected from two oceanic regions (the Straits of Korea and East China Sea) which are influenced by The Kuroshio warm current effectively and the three sites of the southern Pacific area (at coasts of Philippines, Malaysia and Palau) (Fig. 1A, Table S1) to understand the migration of clade III population via warm current. At each sampling sites, the pelagic seawaters were collected from 50 cm depth using a Van Dorn sampler, and 300–1000 mL of each water sample was filtered through the ISOPORE membrane filters (Millipore, Cork, Ireland) of 3-μm pore size and 47-mm diameter. The filters were placed into 2-mL micro-tubes (Axygen Sciences,