Biogeography of the Genus *Sargassum* (Heterokontophyta: Phaeophyceae) and the Phylogeographic Patterns of *Sargassum* spp. in Northwest Pacific

CHEANG, Chi Chiu

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

The Chinese University of Hong Kong

May, 2009

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

Thesis/ Assessment Committee

Professor Norman Ying-Shiu Woo (Chair)

Professor Put O. Ang, Jr. (Thesis Supervisor)

Professor Ka-Hou Chu (Thesis Supervisor)

Professor Li-Wen Jiang (Committee Member)

Professor Wendy Nelson (External Examiner)

Abstract:

The biogeographical pattern of the brown macroalgal genus *Sargassum* as well as the phylogeography of selected *Sargassum* spp. along NW Pacific coast were elucidated using analytical biogeographical and comparative phylogeographical tools. The NW Pacific can be divided into 47 Operational Geographical Units (OGUs) based on geographical boundaries such as major river mouth and island groups. Multidimensional scaling techniques based on the species composition of *Sargassum* species within each OGU was then applied and three main biogeographic clades were recognized. The highest species richness was found in southern China and southern Japan. The degrees of compositional difference of *Sargassum* species among OGUs are most positively correlated with the combined effect of the mean lowest winter sea surface temperature (LWSST), latitude (which reflected photoperiodicity) and mean annual salinity, compared to lower correlation with geographical distance and the Euclidean distance among OGUs, generated by differences in their environmental parameters. Results of the regression tree analysis reveal the mean LWSST to be the most critical parameter in structuring biogeographical patterns of *Sargassum* in this region.

Two allopatrically distributed varieties of *S. hemiphyllum*, v. *chinense* and v. *hemiphyllum*, are genetically distinct in terms of their internal transcribed spacer 2 (ITS2) and Rubisco spacer. The genetic break between these two varieties, with v. *chinense* distributed in southern Chinese coast and v. *hemiphyllum* in Japan and Korea, is situated in a region that includes Bohai, Yellow Sea and East China Sea, all of which were heavily influenced by the Yangtze and Yellow Rivers in China. An introgression of the mitochondrial (Mt) genome from v. *chinense* to v. *hemiphyllum*, possibly mediated by the Kuroshio Current, is evident based on the Mt marker

TrnW_I spacer. Hybridization between the two varieties may still be ongoing since the concerted evolution of ITS2 is not yet saturated in the Korean population located geographically in-between the distribution of the two varieties. In contrast, no variation in ITS2 and Rubisco spacer is revealed in *S. muticum*, including the native Asian populations and introduced populations in Europe and North America. There is a fixed one-nucleotide difference in the TrnW_I spacer, between the population in eastern Japan and all the other populations examined. This finding supports the earlier suggestion that the source of the introduced *S. muticum* populations is western and central Japan (Seto Inland Sea), where the germlings of *S. muticum* have been associated with the Pacific oysters previously introduced for farming in Canada, UK and France in earlier years.

To investigate the effect of freshwater outflow from Yangtze and Yellow Rivers in eastern China in shaping the genetic population structure of *Sargassum* spp., a comparative phylogeographic study was conducted on four closely related *Sargassum* species showing either continuous (*Sargassum thunbergii* and *S. muticum*) or discontinuous (*S. hemihyllum* and *S. fusiforme*) distribution patterns along the Chinese coast. The results showed discontinuously distributed species to exhibit more haplotypes (e.g. four in TrnW_I spacer) among their populations than those with continuous distribution (two in TrnW_I spacer) pattern. Little or no population differentiation is revealed in species with a continuous distribution. Their occurrences in the brackish Bohai region may be attributed to the presence of inherited physiochemical traits that allow them to tolerate lower salinity waters in estuaries. The discontinuously distributed species, however, exhibited a deep genetic divergence among populations, as revealed by various genetic markers. There are two main lineages of *S. fusiforme* based on ITS2 and TrnW_I sequences, but the

geographical region associated with this genetic break between the two lineages in eastern and southwestern Japan is different from that of *S. hemiphyllum*. Analysis of molecular variance (AMOVA) results indicate that the maintenance of the population structure of *S. fusiforme* appears not to be correlated with the outflow of the two rivers. For *S. hemphyllum*, reduced salinity as the suspected genetic barrier was investigated directly in the laboratory to elucidate its effect on the growth and survival of *S. hemiphyllum* var. *chinense*. Statistically significant difference was observed in the relative growth rate (calculated based on wet weight) of branches cultured under different salinities, with the optimal growth under salinity level of 33 ppt. The lethal limit of vegetative growth was between 0 and 10 ppt. Germlings cultured in 15 ppt attained the highest survivorship. The optimal growth of the germlings occurred at 25 ppt, while the lowest lethal limit was within the range of 0 ppt and 5 ppt. Germlings reared under low salinity were deficient in rhizoid development, making them highly unlikely to grow into large thallus in the natural environment with strong waves. Compared with the optimal and lethal salinity level of *S. mutium*, the lethal limits of both vegetative branches and germlings of the two species are comparable. The optimal growth of branches of *S. muticum* occurred under salinity level of 27 ppt, in contrast to the optimal salinity level of *S. hemiphyllum* at 33 ppt. This could have explained the absence of *S. hemiphyllum* in brackish water and support the suggestion that river discharge serves as a barrier for the exchange of genetic materials among its populations.

Results from this dissertation research provide the first comprehensive phylogeographic information on a major group of marine algae in NW Pacific. These also provide an insight into a better understanding of evolutionary history of marine organisms in this part of the world.

摘要:

本論文利用「分析生物地理學」和「比較親緣地理學」的方法，闡明大型褐藻屬馬尾藻(*Sargassum*)，在西北太平洋的生物地理分佈及親緣地理分佈。首先根據地理邊界如河口、島嶼等，把西北太平洋地區劃分為四十七個「地理學運算單位」（geographical operational unit），就單位之間馬尾藻屬各品種出現的相似度建構多元尺度（multidimensional scaling），並與由運算單位之間的地理距離及環境參數計算出來的相似度作「相關分析」(correlation analysis)。分析結果指出馬尾藻在西北太平洋海岸有三個主要「生物地理分佈支」，而中國南邊與日本南邊則擁有最高的物種豐富度(species richness)；運算單位之間的馬尾藻相似度差距，跟冬天平均最低海面溫度、緯度和每年平均鹽度的綜合作用有最大的相關系數，比起馬尾藻相似度與地理距離的相關系數；以及馬尾藻相似度與所有環境系數綜合作用的相關系數為高。「回歸樹分析」(regression tree analysis)顯示冬天海面平均最低溫度，是構成馬尾藻地理分佈最重要的環境參數。

馬尾藻屬的半葉馬尾藻(*Sargassum hemiphyllum*)是西北太平洋海岸的優勢種，共有兩個變種——半葉變種及中國變種，它們分別分佈於日本、南韓地區及南中國地區，以渤海、黃海和東海等受黃河、長江影響的區域作邊界。本論文根據葉綠體染色體內的雙磷酸核酮糖羧化脢(*Rubisco*)大小單位的間隔序列區，及細胞核內第二內轉錄間隔區(ITS2)，發現半葉變種和中國變種有明顯的基因區別，這個基因區別與地域分佈有關。從粒線體染色體內TrnW和TrnI的間隔序列區，顯示有粒線體基因滲入(introgression)的痕跡，這可能是由南向北經西太平洋黑潮(Kuroshio Current)傳遞。由未完成的第二內轉錄間隔區的協同進化(concerted evolution)，可看出這兩個變種的邊界種群——南韓南面地區，仍有雜交的情況發生。

相反地，另一西北太平洋優勢種海黍子(*Sargassum muticum*)，無論於西北太平洋本土原生地，或是北美西岸及歐洲海岸等入侵地，都未錄得*Rubisco*間隔序列區和第二轉錄間隔區內的變異。日本東部海岸的海黍子，在TrnW和TrnI的間隔序列區上，與其他地方的種群有固定的基因差距，相距為一個鹼基對。這証明海黍子的入侵源頭，為日本西南部至瀨戶內海地區；估計海黍子的繁殖幼體，依附在太平洋的牡蠣外殼上，由於早年加拿大、英國及法國等，均從日本進口太平洋牡蠣的養殖籽苗，於是海黍子可能因此而被意外地引入北美西岸及歐洲海岸。

為了解黃河、長江的大量淡水流注對馬尾藻進化的影響，本論文利用親緣地理分析比較四種馬尾藻的進化歷史。鼠尾藻(*Sargassum thunbergii*)和海黍子(*S. muticum*)連續分佈在日本、南韓和中國沿岸，而半葉馬尾藻(*S. hemihyllum*)及羊棲菜(*S. fusiforme*)則常見於日本、南韓到南中國一帶，卻不見於渤海、黃海等受黃河、長江淡水影響的地區，呈現間斷的分佈模式。分析結果指出呈現不間斷分佈的鼠尾藻和海黍子，都顯示較少的單型(haplotypes)，種群分化現象較輕微；可能由於這兩個品種擁有能忍受低鹽環境的特性，所以可於渤海、黃海等

河口地區出現。呈現間斷分佈的半葉馬尾藻及羊棲菜，除了展現較多不同的單型外，種群間也有很明顯的趨異(divergence)。雖然半葉馬尾藻與羊棲菜都分別有兩個主要譜系(lineage)，但羊棲菜的兩個譜系分佈於日本東邊及西南邊，這與半葉馬尾藻的兩個譜系分佈不同(見上文)。分子方差(AMOVA)的結果顯示，黃河、長江的淡水流注，與維持半葉馬尾藻譜系的分佈有關，但與羊棲菜譜系的分佈無關。

由於馬尾藻的進化可能與河口淡化有關，本論文利用實驗室養殖方法，測定低鹽度對半葉馬尾藻的中國變種在生長及生存上的影響。養殖在不同鹽度的營養小枝，在相對生長率上有顯著分別，它的最佳生長鹽度在33 ppt，而致死界限則在0 ppt 到 10 ppt 之間。至於繁殖幼體生存率最高的鹽度為15 ppt，最佳生長鹽度是25 ppt，而致死界限則在0 ppt 和 5 ppt 之間。在低鹽的環境下(5 ppt)，繁殖幼體的假根未能正常發育，這可能是半葉馬尾藻在河口低鹽的野生環境中不能固定在基質上的原因，所以河口流注可能成為兩個變種之間基因流動的障礙。另外，從半葉馬尾藻和海黍子的最佳生長和致死界限相比可見，無論是營養小枝或繁殖幼體，半葉馬尾藻和海黍子的致死界限都在10 ppt以下，而海黍子營養小枝的最佳生長鹽度為27 ppt，比半葉馬尾藻的33 ppt為低，故此半葉馬尾藻不能於低鹽環境出現，而海黍子則見於渤海、黃海等地。

本論文是太平洋西北地區內第一篇，有關海洋主要藻類的親緣分佈研究，本論文之結果有助我們更深刻地了解此區海洋生物的進化歷史。

Acknowledgement

I am grateful to my supervisors, Profs. P. O. Ang and K. H. Chu, for their patience, guidance and assistance throughout my whole study period. They have not just taught me how to practically conduct a scientific research, inspired me how to think critically, but also granted me a vision on how a good scientist should be. I hope that I will carry on as a scientist for my career and share what I gained from them to other people. My thanks should extend to the collaborators who help in collecting samples. Without them, the studies in this thesis will not be possible.

Special thanks come to all the labmates and friends who assist me, through various ways, in completing the thesis. They includes technicians and supporting staffs, Henry K. C. Cheung, Ben H. B. Yeung, Y. H. Yung, Albert C. P. Li, labmates, K. H. Choi, Z. W. Xu, Corsica, S. L. Kong, Celia, M. M. Choi, Choyee L. S. Choi, T. P. Lin, W. C. Ma, Jovy M. C. Tam, Winnie S. Y. Wong, Samantha M. W. Lee, Chris H. L. Fung, Atwood Y. M. Chiu, Y. W. Sin, F. F. Yeung, Anton Y. T. Tsang, Y. M. Tsang, Y. H. Lam, Connie K. Y. Ng, L. M. Tsang, Benda Y. M. Lee and L. T. Wu.

The last but not the least, I have to thank my wife Shirley C. C. Suen. Without her encouragement and spiritual support, this piece of work would not be finished. She has been helping me in different ways, from the spiritual aspect such as comforting me during my tough period and discussing critically with me my study, to the most practical way like collecting specimens with me aboard in a dangerous rocky shore. No appropriate words exist in the world to express how I am grateful and appreciate on all she did for me. The only words that I could say are "Thank you!".

Contents

List of Tables

List of Figures

Chapter One: General Introduction

1.1 Biogeography of macroalgae

Biogeography of macroalgae has long been a subject of great interests to many biologists (e.g. Setchell 1931a, b, 1933, van den Hoek 1984, Lüning 1990 and references cited). Based on the differences in the composition of the algal communities found in these localities, Lüning (1990) has identified 23 phycogeographic regions, such as the cold temperate Northwestern Pacific and tropical Indo-west Pacific regions, around the world's coastal zones. The highest diversity of marine macroalgae is found in the temperate seas, in contrast to the trend of many marine organisms such as corals (Veron 2000) that achieve their highest diversity in the tropics (Silva 1992). Bolton (1994), however, noted that this distribution pattern is not a general trend for marine benthic algae. A number of algal hotspots, e.g. South Australia, Japan, and the Philippines, are present. Seawater temperature pattern and other localized historical events may have contributed to the formation of these hotspots (Bolton 1994).

1.2 Historical and ecological biogeographies

Historical and ecological biogeographies are the two main streams of current biogeographical studies (Myers & Giller 1988). Historical biogeography refers to the past events that led to the present biogeography, while ecological biogeography focuses on the role of present ecological environment in shaping the current biogeographical patterns observed. Ecological biogeography appears to be the prominent stream in explaining the biogeographical pattern of marine macroalgae,

whereas studies on algal historical biogeography are more limited (Lindstrom 1987, 2001).

1.2.1 Historical biogeography

Historical biogeography has undergone revolutionary changes in the last few decades because of the emergence of various subdisciplines such as vicariance biogeography, cladistic biogeography and phylogeography. Methodologies and approaches applied in these fields have advanced significantly through critical and comprehensive debates and discussion (Crisci 2001). The lack of a standard approach, however, continues to hinder the development of historical biogeography in accounting for the present patterns of phycogeography. Lindstrom (2001) adopted vicariance biogeographic approach and analyzed pairs of sister species inhabiting separately the North Pacific and the North Atlantic Oceans. She revealed that the opening and closing of the Bering Strait over the last 8 Myr led to several vicariant events that accounted for the divergence of the sister species investigated (Lindstrom 2001). This approach, however, may only be applicable to regions where sister species are present.

Lüning (1990) comprehensively reviewed the possible explanations of the present macroalgal distribution pattern by correlating this with both the historical events and the contemporary environmental conditions. He looked into the details of phycogeography in different parts of the world and provided insights on how geological event(s) in various geographical regions, such as the Arctic Ocean, could have contributed to current macroalgal biogeography. The historical biogeographic component of his approach, although comprehensive, is still limited by the lack of

empirical evidences. More direct evidences, such as those obtained by molecular phylogenetic or comparative phylogeographic studies, would certainly help to consolidate his ample accounts of current phycogeography.

1.2.2 Ecological biogeography

In contrast with historical biogeography, ecological biogeography appears to be the dominant approach taken to explain the present day phycogeography. Many ecological factors have been proposed to be the driving force in structuring algal communities. Some of these included seawater temperature (van den Hoek 1984, Peters & Breeman 1993, Schils & Wilson 2006), salinity (Russell 1987a, Cheang *et al.* 2008), irradiance (Lüning & Dring 1979), photoperiodicity (Terry & Moss 1980, Cunningham *et al.* 1993), desiccation tolerance (Schonbeck & Norton 1978) and tidal amplitude (Silva 1992). Many of these factors do not just individually affect the biogeography of marine macroalgal species, but also in a synergistic manner (Norton 1977a).

1.2.2.1 Sea surface temperature

Sea surface temperature has been considered to be the prime factor in structuring the present global biogeography of marine algae (Lüning 1990) as it affects their growth, reproduction and survival (Breeman 1988). For example, Orfanidis *et al.* (1999) found that seawater temperature influenced the growth, reproduction and survival of two red algae, *Eupogodon spinellus* (C. Agardh) Kützing and *Eupogodon planus* (C. Agardh) Kützing. The optimal temperature for the growth of these two species was 25°C and both of them survived at temperatures between 8 and 30°C. Reproduction of gametophyte, however, only occurred at 20°C

in *E. planus*. The distinct geographical distribution boundaries of these two species, when compared with the isotherm of sea surface temperature (SST), was found not to be determined by the growth or survival limits, but by the reproductive requirements of *E. planus* in the Mediterranean and the Canary Islands. Breeman (1988) also found that the temperature regime in the edge populations corresponds to the lethal limits and the lowest thermo-requirements of almost all of the 60 algal species investigated in the North Atlantic Ocean. All these studies showed that those studied algae evolved differential responses to temperature (Breeman & Pakker 1994, Pakker *et al.* 1994, Breeman *et al.* 2002).

1.2.2.2 Salinity

Salinity has been shown to influence the survivorship as well as the growth of algal species (Bäck *et al.* 1992, Strømgren 1994), which in turn, may affect their biogeography. *Caloglossa leprieurii* (Montagne) G. Martens var. *leprieurii* and var. *angusta* in Australia demonstrated various ranges of tolerance to different salinity levels. *C. leprieurii* var. *angusta*, which was found in brackish water, demonstrated a narrower and lower salinity tolerance than var. *leprieurii* (Mosisch 1993). These two variants may represent two different ecotypes of this species (Mosisch 1993). Similarly, interspecific difference of salinity tolerance was observed in the brown algal genera of *Sargassum* and *Fucus*. The pelagic species, such as *S. natans* (Linnaeus) Gaillon, was more stenohaline than the intertidal species like *S. pteropleuron* Grunow in Florida, USA (Hanisak & Samuel 1987). On the other hand, *Fucus vesiculosus* Linnaeus normally found in the maritime environment decayed in brackish water and *F. ceranoides* Linnaeus, which is found in estuarine area, died at full salinity (34 ppt) (Khfaji & Norton 1979). In South America, distinct genetic

difference in various marine organisms, e.g. sea urchin and shrimp, has been detected between the two sides of the mouth of Amazon River (Gusmão *et al.* 2000, Lessios *et al.* 2001). These findings suggest that, from the evolutionary standpoint, salinity may, on top of its ecological effect, act also as one of the agents of natural selection on marine algae. The effect of salinity was postulated to be more critical especially for those algae inhabiting the estuarine environment (Russell 1987a).

1.2.2.3 Irradiance and photoperiodicity

Solar irradiance serves two functions for the benthic macroalgae (Lüning 1990), one as the source of energy, while the other, as an environmental signal. The amount of irradiance undoubtedly affects the distribution of the macroalgae, which rely on the solar energy for photosynthesis. Many algal species have minimum light requirements in order to survive (Lüning 1990) and the deepest range of distribution of algae like *Halimeda* was determined by this requirement (Hillis-Colinvaux 1986).

Similar to many terrestrial plants, day length serves as an environmental signal in marine macroalgae for the onset of various developmental activities such as the formation of new frond for *Laminaria hyperborea* (Gunnerus) Foslie in winter (Lüning 1986). The presence of photoperiodic ecotypes in different geographical populations among species, like *Scytosiphon lomentaria* (Lyngbye) Link (Lüning 1980), may reflect the importance of photoperiod in affecting macroalgal distribution. This factor was found to be effective only when it interacts with the temperature (Breeman 1988).

1.2.2.4 Desiccation tolerance and tidal amplitude

Desiccation tolerance is one of the most severe problems for the intertidal macroalgae. The fundamental niches (the upper to the lower tidal zones) of two co-existing *Fucus* species, *F. serratus* and *F. vesiculosus*, were found to be significantly different from their realized niches (the lower tidal zone only). The time of emergence at low tide was found to be the prime factor attributing to this difference (Schonbeck & Norton 1978). Due to their high surface area to volume ratio, the sheet-like algae like *Ulva* and *Porphyra* were more vulnerable to desiccation (Lüning 1990). They would thus usually grow in dense population so that the underneath layer could remain moist (Lüning 1990). In contrast to desiccation that may affect the vertical distribution of algae, the amplitude of the tide among different geographic regions was postulated to affect the geographical distribution of macroalgae. The higher tidal amplitude was hypothesized to create a more heterogeneous environment for a diverse algal assemblage to evolve (Silva 1992).

1.2.2.5 The relative importance of environmental factors

Among the factors mentioned, factors like irradiance and desiccation tolerance mainly affect the vertical distribution of the macroalgal species (Schonbeck & Norton 1978). In contrast, the seawater temperature and salinity have been postulated to be two of the most significant factors in affecting the biogeography of marine macroalgae (Russell 1987b, Breeman 1988).

1.3 Phycogeography in NW Pacific

1.3.1 Present environmental factors: seawater temperature and salinity

In NW Pacific, three phycogeographical zones have been recognized based on their temperature regimes (Lüning 1990). These are the cold temperate NW Pacific, the warm temperate Japan and the Indo-West Pacific tropical regions (Fig. 1.1). This difference in temperature regimes is primarily a result of the interaction between two main oceanic currents, the cold Oyashio current that flows from the northern Bering Sea to the south through the Sea of Japan and the warm Kuroshio current that originates from the tropical eastern Philippines and flows north to southern Japan via eastern Taiwan and the Ryukus (Lüning 1990, Briggs 1995). Lüning (1990) indicated that the seawater temperature is warmer and the latitudinal gradient change is steeper in NW Pacific than in the NW Atlantic Ocean. Adey and Steneck (2001) also pointed out that seawater temperature is higher in the western Pacific than in the western Atlantic. The overall effect of this is NW Pacific being thermally a highly fluctuating region. This temperature variation may induce certain stress on marine organisms and thus could become a major structuring force behind the biodiversity and distribution patterns of the marine flora (and also fauna) in this region.

Aside from SST, the fluctuation of seawater salinity, which is induced by the discharge of the Yangtze (Changjiang) and Yellow Rivers, is also a characteristic of the coastal area of NW Pacific (Senjyu *et al.* 2006). The effect of the Yangtze River extends as far east as the Tsushima Strait between the Korean Peninsula and Kyushu, Japan. Salinity of the coastal areas in this region decreases over summer and is related to the large outflow from the river (Senjyu *et al.* 2006). The Bohai Sea, an

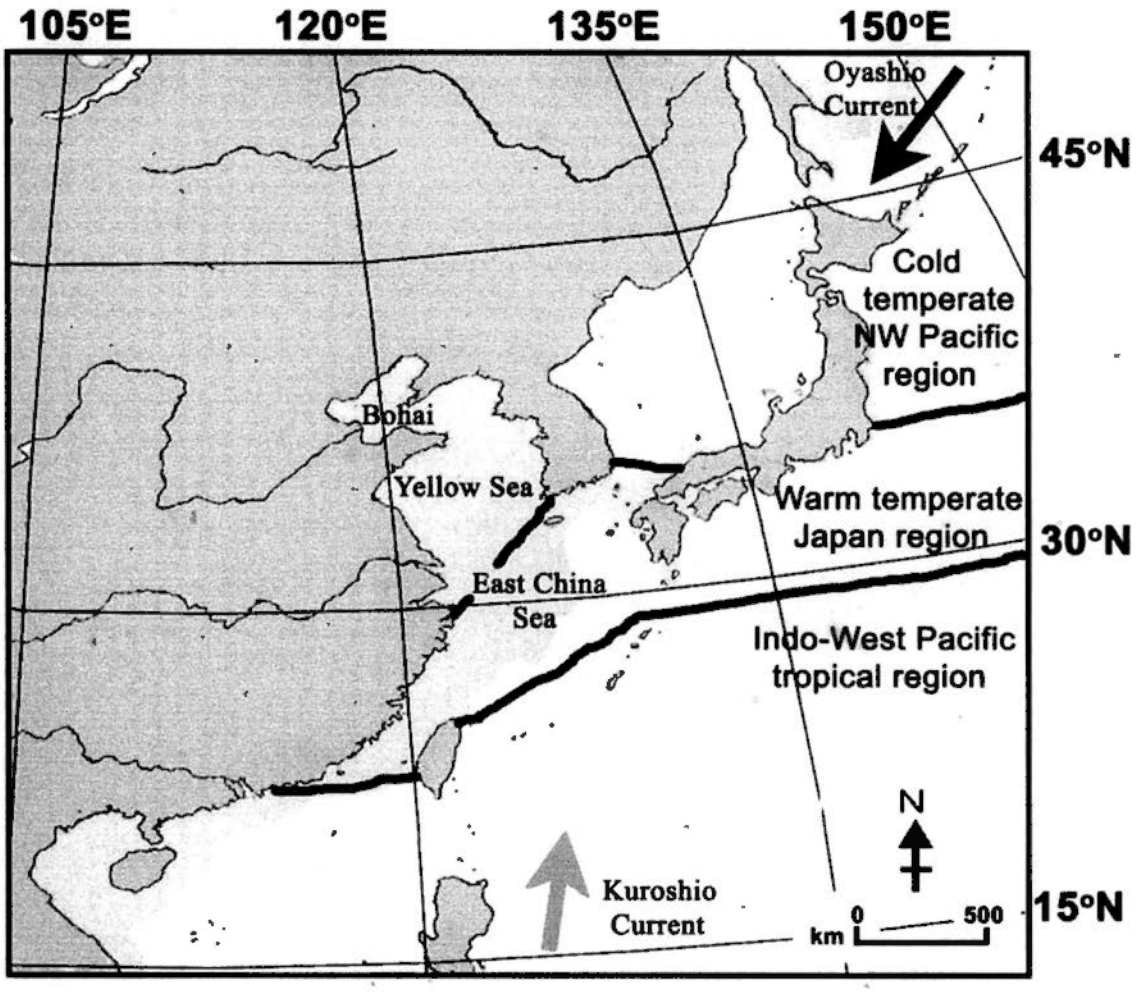

Figure 1.1 Map of NW Pacific showing the three phycogeographical regions (After Lüning 1990). The gray region refers to the possible extent of the area exposed during the period of lowering of sea level in the Pleistocene (After Xu & Oda 1999).

enclosed gulf situated in northeastern China (Fig. 1.1), is a region being affected by the discharge of the Yellow River as well (Sündermann & Feng 2004). The water body over the continental shelf of northeastern China, encompassing the Bohai Sea, Yellow Sea and East China Sea, is an integral area affected by both Yangtze and Yellow Rivers and maintains a salinity of <30 ppt especially during the summer wet season (Cheang 2003).

1.3.2 Past geological event

No major tectonic movement was recognized in NW Pacific during Miocene to Holocene (Briggs 1995). Instead, fluctuations in sea level throughout Pleistocene to Holocene (Xu & Oda 1999, Voris 2000) were postulated to significantly affect the phylogeographic pattern in this part of the world (Briggs 1995). The continental shelf in northeastern China was believed to be exposed during the period of low sea level (Xu & Oda 1999). In addition to a period (19.5-10.5 kyrs B. P.) of heavy freshwater discharge in the "paleo-coast" of the continental shelf region during late Pleistocene (Xu & Oda 1999), the lowering of sea level was suspected to create fragmentation in the ancestral populations such that diversification or genetic population differentiation was detected in several marine fishes (Liu *et al.* 2006, 2007). Similarly, genetic population differentiation was also observed in the brown alga, *Sargassum hemiphyllum* (Turner) C. Agardh (Cheang *et al.* 2008). Two genetically distinct varieties of *S. hemiphyllum* could be recognized which could have originated from the fragmentation of their distribution range during the Pleistocene. The influx from the Yellow and Yangtze Rivers was hypothesized to be the dispersal barrier that maintains the differentiation of these two varieties even after the glacial period.

1.4 Approach to decipher the underlying forces

To decipher the possible cause(s) underlying the present biogeography of marine macroalgae, the structure and pattern of how the macroalgae are distributed could be assessed using various approaches.

1.4.1 Analytical biogeography

In contrast with the conventional narrative approach to explain a biogeographical pattern, the hypothetico-deductive approach is more objective and desirable to infer the cause(s) of biogeography (Myers & Giller 1988). Various methods, which could be categorized into the field of analytical biogeography, have been applied not just to objectively synthesize the biogeographical data of many living organisms (Porzecanski & Cracraft 2005, Proches 2005), but also to sort out the most significant factor out of a range of environmental parameters that are related to the biogeographical patterns (Clarke & Ainsworth 1993). Some of these methods have been introduced to phycological studies, e.g. cladistic biogeography (Garbary 1987, Lindstrom 2001) and multivariate clustering (van den Hoek 1984, Carballo *et al.* 2002, Báez *et al.* 2004, Bolton *et al.* 2004). Multivariate clustering and related ordination methods, which analyze the information of species occurrence in different geographical regions by sophisticated statistical analyses in order to reveal biogeographical pattern (Warwick *et al.* 1990), have seldom been used in phycological studies (*cf.* Carballo *et al.* 2002, Báez *et al.* 2004) and yet are powerful in identifying the underlying influential factors in biogeography (Warwick *et al.* 1990, Clarke & Ainsworth 1993).

1.4.2 Molecular biogeography and comparative phylogeography

As independent evidence, molecular information provides a new horizon in understanding the biogeography of living organisms (Palumbi 1997). This approach offers a higher resolution of the biogeographical pattern (genetic population structure), and provides insight on the factors restricting gene flow and leading to speciation (Palumbi 1997). Phylogeography, which is the study of the intraspecific genealogy based on molecular data, serves as the connection between the phylogeny and the demography of geographical populations within a single species (Avise 2004). Comparative phylogeography, which focuses on the congruence or incongruence of phylogeographic patterns among closely related species, is also of paramount significance in understanding the general pattern of the formation of biogeographical patterns among different organisms in an evolutionary context (Bermingham & Martin 1998).

1.4.2.1 Genetic markers in algal phylogeography

Various methods have been utilized for genetic analysis of macroalgae at various systematic levels. On one hand, allozyme analysis (e.g. Benzie *et al.* 2000) and restriction fragment length polymorphisms (RFLP) of nuclear ribosomal intergenic spacer (e.g. van Oppen *et al.* 1994) have been used to elucidate the phylogenetic relationships among species. Random amplified polymorphic DNAs (RAPDs) (e.g. van Oppen *et al.* 1996, Ho *et al.* 1995) and amplified fragment length polymorphisms (AFLP) (Kusumo & Druehl 2000), on the other hand, were performed to investigate the intra-specific variation and large-scale biogeographical population variation.

Sequence divergences of various genes in nuclear and chloroplast genomes have been commonly utilized in phylogenetic studies of macroalgae (e.g. Freshwater *et al.* 1999, McCourt *et al.* 1999, Serrão *et al.* 1999, Yotsukura *et al.* 1999, Phillips & Fredericq 2000, Stiger *et al.* 2003, Phillips *et al.* 2005). For nuclear genome (nDNA), sequence divergences of internal transcribed spacer 1 (ITS-1) and 2 (ITS-2) and subunit 5.8S of ribosomal RNA were used to solve systematic problems from genus to subspecies levels (Serrão *et al.* 1999, Yotsukura *et al.* 1999, Stiger *et al.* 2000, 2003). The ITS2 divergence of the *Sargassum* species in subgenus *Bactrophycus* demonstrated the affinity of the section *Phyllocystae* to the subgenus *Sargassum* rather than to the subgenus *Bactrophycus*, suggesting the suitability of ITS2 as the genetic marker at the subgenus and sectional levels (Stiger *et al.* 2000).

Ribulose-1-5-bisphosphate carboxylase/oxygenase (*Rbc*/*Rubisco*) gene in the chloroplast genome is also commonly used to evaluate genetic diversity from the level of order to populations in a large geographical scale (Freshwater *et al.* 1999, Phillips & Fredericq 2000, Sasaki *et al.* 2001). *Rbc* is a protein-coding gene with three regions. The middle region, called *Rbc*L-S spacer, between the two protein-coding regions, *Rbc*S and *Rbc*L, is evolutionarily more variable compared to the protein-coding regions due to its non-coding nature. The *Rbc*L gene is used for the study of the relationship among samples at the higher taxonomic levels such as order and genus (McCourt *et al.* 1999, Sasaki *et al.* 2001). In contrast, *Rbc*L-S spacer was shown to be suitable for the levels of species to populations (Phillips & Fredericq 2000, Phillips *et al.* 2005). Based on the analysis of divergence of *Rbc*L-S spacer (Phillips & Fredericq 2000), different species of *Sargassum* in the Gulf of Mexico, when compared with those in the Pacific basin, were found to cluster as a single

biogeographical unit rather than in a way predicted by their taxonomic classification. The study of Phillips and Fredericq (2000) suggests that *Rbc*L-S spacer is a suitable genetic marker in the phylogeographical study of *Sargassum*.

Mitochondrial markers have recently been used to assess the algal population genetic variability due to their relatively rapid evolutionary rate (e.g. Voisin *et al.* 2005, Andreakis *et al.* 2007, Engel *et al.* 2008). Separation of the Atlantic and Indo-Pacific lineages of an invasive Rhodophyte *Asparagopsis* spp. due to the emergence of the Isthmus of Panama was inferred based on the mitochondrial Cox2-Cox 3 intergenic spacer, in addition to two markers in different genomes. Two cryptic species inside *A. taxiformis* (Delile) Trevisan de Saint-Léon were recognized based on this mitochondrial marker (Andreakis *et al.* 2007). Voisin *et al.* (2005) also revealed cryptic diversity within the introduced range of an NW Pacific originated brown macroalgal species, *Undaria pinnatifida* (Harvey) Suringar, by utilizing the two intergenic noncoding mitochondrial loci, the spacer regions between *atp8* and *trnS* genes as well as *trnW* and *trnI* genes. Various invasive patterns, such as the multiple source introductions in New Zealand and the putative secondary relay in Melbourne and Argentina, were thus inferred (Voisin *et al.* 2005). These highly informative loci located in the mitochondria genome remain valuable tools for future phylogeographical studies, especially in brown algae in which direct transferring of the "universal" primer was not successful (Engel *et al.* 2008).

1.5 The Genus *Sargassum*

The brown macroalgal genus *Sargassum* C. Agardh (Fucales, Phaeophyceae) is one of the most suitable candidates for the study of the factor(s) governing or affecting the marine biogeography in NW Pacific. It is one of the most species rich genera in algae (Yoshida 1983), which consists of 577 recognized species (Guiry & Guiry 2008 in AlgaeBase). *Sargassum* is usually one of the major components of marine floristic environment and is a dominant macroalgal genus found from the intertidal to the subtidal and from tropical to temperate regions in NW Pacific (Yoshida 1983, Phillips 1995). It is ecologically important as it always forms dense underwater algal stand (Phillips 1995) that serves as nursing and feeding grounds for different kinds of marine life worldwide (Ornellas & Coutinho 1998, Taylor 1998). An understanding of factor(s) governing the distribution of this structurally and functionally important component of marine coastal flora will likely shed light on the structuring force(s) responsible for the distribution patterns of the marine flora or even of the entire coastal realm observed in this region.

Up to 25, 59 and 141 *Sargassum* species were recorded in Korea, Japan and China respectively in NW Pacific (Kang 1966, Yoshida 1998, Tseng & Lu 2000). Among the widely distributed species, some have a discontinuous distribution and are not found in the region of the Gulf of Bohai and the Yellow Sea, a region that is heavily affected by the two largest rivers in China, the Yellow and Yangtze Rivers (Cheang 2003). A clear divergence between two varieties of *Sargassum hemiphyllum*, allopatrically distributed in Japan, Korea region and the southern Chinese region, was demonstrated by the PCR-RFLP data of Rubisco spacer (Cheang *et al.* 2008). This genetic break is located between the regions from

Zhejiang Province in China to Cheju Island in Korea. This location is within the Bohai to Yellow Sea region that is heavily affected by the two Chinese rivers. While fragmentation of the ancient population due to lowering of the sea level was believed to have initiated the divergence of the two varieties, the huge freshwater discharge from the two rivers and its associated adverse effects on the physiological ecology of *Sargassum* may have sustained this allopatric divergence until now (Cheang *et al.* 2008). This environmental stress, most probably the brackish environment associated with the freshwater discharge of the rivers, may exert similar effect on those other *Sargassum* species, such as *S. fusiforme*, that also demonstrate a discontinuous distribution pattern and are absent in the region of Bohai and Yellow Seas.

In contrast, some commonly found *Sargassum* species in NW Pacific, like *S. thunbergii* and *S. muticum* (Yoshida 1998, Tseng & Lu 2000), possess almost continuous distribution pattern and are present along the coast of Bohai (Fig. 1.2). How these species respond to the hyposaline environment in Bohai Sea region, compared to those other species like *S. hemiphyllum* which are absent from the region, remains unclear.

The difference between the two patterns of distribution, continuous and discontinuous, among different *Sargassum* species offers a golden opportunity to examine if the river associated factors, most probably reduced salinity, played a role in the genetic differentiation of populations among *Sargassum* species. Should there be a salinity-associated effect on the genetic differentiation of populations, one may predict that those species occurring inside the Gulf of Bohai would not exhibit genetic population differentiations as that seen in *S. hemiphyllum*. The hyposaline

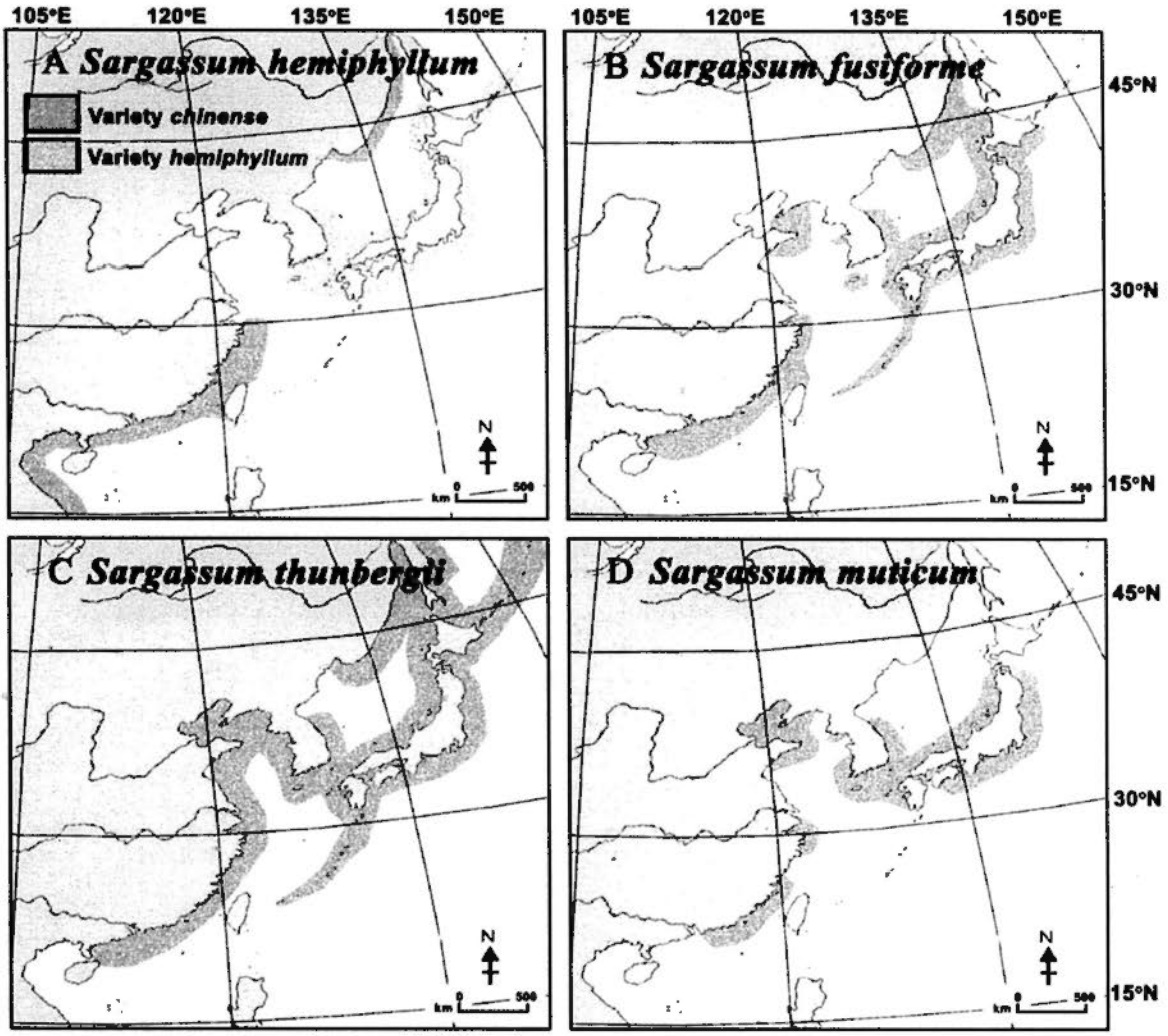

Figure 1.2 The distribution of *Sargassum hemiphyllum* (A), *S. fusiforme* (B), *S. thunbergii* (C) and *S. muticum* (D) in NW Pacific. A and B refer to species with discontinuous distribution that do not extend to Bohai Sea, while C and D refer to those with continuous distribution. The data on the distribution ranges of these species were compiled from literature reviewed in Chapter Two.

environment would not have posed as a dispersal barrier for these species as they are able to survive under reduced salinity. On the other hand, those populations with discontinuous distribution would have differentiated or evolved independently between the northern part (Korean and Japanese coasts) and the southern part (Chinese coast) of this hyposaline environment affected by the Yellow and Yangtze Rivers. Examination of the physio-ecological tolerances of the species with different distribution ranges could provide direct evidence on whether these species are able to survive under the adverse condition caused by the environmental parameters tested (e.g. Hanisak & Samuel 1987, Orfanidis *et al.* 1999). Results from this type of experimental evaluation can be integrated with information obtained from comparative phycogeographical studies to provide a more comprehensive understanding of driving forces underlying the biogeographical patterns of macroalgae.

1.6 Structure of the dissertation

This dissertation is aimed at elucidating the biogeographical pattern of the brown macroalgal genus *Sargassum* as well as the phylogeography of selected *Sargassum* spp. in the northwestern Pacific coast. The methods in analytical biogeography and comparative phylogeography were adopted with a view to decipher the possible environmental factors affecting the biogeography and phylogeography of *Sargassum* in NW Pacific. Comparative phylogeographic patterns of four species with two similar distribution patterns defined by the presence of the Yangtze and Yellow rivers were analyzed and the information on the shared phylogeographic pattern was used to shed light on the general and/or unique evolutionary history of these *Sargassum* spp. Additional experiments to assess the

effect of reduced salinity on the growth and survival of vegetative branches and germlings of *S. hemiphyllum* were also carried out to verify whether reduced salinity could serve as a selecting force for this *Sargassum* species. The importance of this environmental factor in structuring the phylogeographical pattern of this species was then evaluated.

This dissertation is divided into seven chapters. A general account of the biogeography of *Sargassum* and the possible underlying environmental driven forces are given in Chapter One. The data on the occurrences of different *Sargassum* species in operational geographical units (OGU) in NW Pacific were analyzed in Chapter Two using multivariate statistical tests in order to elucidate the biogeographical pattern of *Sargassum* in NW Pacific. Phylogeographical approach was utilized to investigate the genetic population structures of *Sargassum hemiphyllum* and *S. muticum* and the results are presented in Chapters Three and Four respectively. In Chapter Five, a comparative phylogeographic approach was conducted to compare the population genetic structures between species (*S. muticum* and *S. thunbergii*) with a continuous distribution pattern along Bohai region and those species (*S. hemiphyllum* and *S. fusiforme*) which have a discontinuous distribution pattern. In Chapter Six, salinity as one of the important environmental factors influencing algal biogeography was evaluated by testing its effects on the growth and survivorship of vegetative branches and germlings of *S. hemiphyllum* var. *chinense*. Finally, based on information on the population genetic structures of selected *Sargassum* species, historical evidence of past geological events and direct experimental results showing eco-physiological adaptation of *S. hemiphyllum*, a

synthesis of the possible ecological factors and/or evolutionary events affecting the biogeography of *Sargassum* in NW Pacific is given in Chapter Seven.

Chapter Two:
Analytical Biogeography of *Sargassum* in the NW Pacific

2.1 Introduction

The global distribution patterns of macroalgae have been examined by various workers over the years (e.g. van den Hoek 1984, Lüning 1990 and references cited) and different phycogeographic regions have been recognized based on the compositional differences among the algal communities in these localities (see Lüning 1990 for review). Temperature is believed to be the primary structuring factor of global algal distribution (Lüning 1990) while many other factors have also been proposed to be the driving force in structuring algal communities, including photoperiod (Terry & Moss 1980, Cunningham *et al.* 1993), tidal amplitude (Silva 1992) and salinity (Russell 1987a, Cheang *et al.* 2008).

In the NW Pacific, three phycogeographic zones, the cold temperate NW Pacific, the warm temperate Japan zone, and the Indo-West Pacific tropical regions, have been proposed based on temperature regimes (Lüning 1990). This difference in temperature regimes is mainly a result of the interaction between two main oceanic

currents, the cold Oyashio current and the warm Kuroshio Current (Lüning 1990, Briggs 1995). The temperature regimes formed may induce certain stresses on marine organisms and thus could become a major structuring force behind the biodiversity and distribution patterns of the marine flora (and also fauna) in this region.

To elucidate the underlying structuring forces on algal biogeography, the conventional descriptive and narrative approach in documenting and analyzing the phycogeographic data appears highly dependent on the researchers' own experiences and expertise (Báez *et al.* 2004). Given these limitations, a more objective and systematic approach is desirable in order to integrate the considerable quantity of phycogeographic information available. Various methods in analytical biogeography have been applied to objectively analyze the biogeographic data of algae, e.g. cladistic biogeography (Garbary 1987, Lindstrom 2001) and multivariate clustering (van den Hoek 1984, Carballo *et al.* 2002, Báez *et al.* 2004). Multivariate clustering and related ordination methods are powerful in identifying the underlying influential factors in biogeography (Warwick *et al.* 1990, Clarke & Ainsworth 1993), but have seldom been used for this purpose in studies of macroalgae (*cf.* Carballo *et al.* 2002, Báez *et al.* 2004).

It is hardly practical to attempt to obtain comprehensive distribution information on all algal species for multivariate analyses. However, it may be possible to use dominant species, especially those which are widely distributed. The genus *Sargassum* C. Agardh (Fucales, Phaeophyceae) consists of 577 recognized species (Guiry & Guiry 2008 in AlgaeBase) and is one of the most species rich genera in algae (Yoshida 1983). *Sargassum* is usually one of the major components of marine floristic environment, which forms a dense underwater algal stand (Phillips 1995) that serves as nursing and feeding grounds for different marine lives (Ornellas & Coutinho 1998, Ng 2009), from the intertidal to the subtidal and from tropical to temperate regions in the NW Pacific (Yoshida 1983, Phillips 1995). While four subgenera (*Sargassum*, *Bactrophycus*, *Anthrophycus* and *Phyllotrichia*) are currently recognized within the genus *Sargassum* based on differences in the arrangement and orientation of their laminas (leaves) with respect to their central axis (Phillips 1995, Stiger *et al.* 2003), only subgenera *Bactrophycus* and *Sargassum* are present in the NW Pacific. Since the genus *Sargassum* provides a relatively large number of species as biogeographic variables, it is a good candidate for the multivariate analysis of algal biogeography, thereby enabling a more reliable result to be obtained.

The objectives of this study are therefore (a) to analyze the biogeographic distribution of the genus *Sargassum* in the NW Pacific and the associated pattern of distribution of environmental parameters, with a view to identifying the environmental factors best correlated with the algal distribution patterns; and (b) based on this information, to elucidate the potential underlying cause(s) of the distribution to help shed light on the main community-structuring forces responsible for the biogeographic patterns of the marine flora observed in NW Pacific.

2.2 Materials and Methods

2.2.1 Distribution data of *Sargassum*

The taxonomic information on *Sargassum* is better documented for the NW Pacific (Tseng 1983, Yoshida 1998) than for other parts of the world. *Sargassum* materials have been extensively collected in Japan, Korea and China in the last few decades. Cross verifications have been carried out by various phycologists, e.g. CK Tseng and T. Yoshida, through a series of taxonomic workshops (Abbott 1985-1999). So despite problems on taxonomic identification of *Sargassum* in many other regions, this problem is not serious and taxonomic identification of *Sargassum* materials from this region should at least be consistent.

The literature on the records, checklists and ecological studies of *Sargassum* in NW Pacific region were reviewed as part of the effort to compile the distribution data of this genus. An attempt was made to collate the most comprehensive information on *Sargassum* distribution in different areas of the region, including northern Vietnam (Nguyễn *et al.* 1993) Taiwan (Shen & Fan 1950, Chiang 1960, Chen 1986, Huang 1990, Yang 1994, 1995, Wang & Chiang 2001, Huang 2002, Tsai *et al.* 2004, Hwang *et al.* 2004), mainland China including Hong Kong and Macau (Setchell 1931a, b, 1933, 1936, Tseng 1983, 1996, Kitayama *et al.* 1995, Tseng & Lu 2000, Zhou *et al.* 2001, Wang 2003, Lu & Tseng 2004), Japan (Okamura 1928, Tokida & Masaki 1959, Yoshida 1983, 1998, Umezaki 1984a, b, Ohta & Ninomiya 1990, Tokuda *et al.* 1994, Kitayama 1996, 1998, Kawai 1997, Kurihara & Iima 1999, Sato & Wada 2000, Yoshida *et al.* 2000, Ishizuka & Tanaka 2004, Konishi & Hayashida 2004), South Korea (Kang 1966, Kim Y. H. *et al.* 1995, 1997, 2004, Nam *et al.* 1996, Kim & Park 1997, Lee J. W. *et al.* 1997, 2000, Lee S. Y. *et al.* 1997, Lee W. J. *et al.* 1997, Kim K. Y. *et al.* 1998, Lee & Kim 1999, Kim & Kim 2000, Yoo 2003a, b, c, Choi & Kim 2004, Kim B. J. *et al.* 2004, Kim M. K. *et al.* 2004) and Russia (Klochkova 1998, Kashenko 1999, Galysheva 2004, Kafanov *et al.* 2004). Information on the west coast of North Korea, however, was not available since there were too few ecological surveys and reports of *Sargassum* for that area.

Operational geographic units (OGU) were defined (Báez *et al.* 2004) based on groups of islands and the current political boundaries. The latter was the basic unit on which most records and checklists of algae were based (Fig. 2.1). The size range of OGU varied from tens of kilometers (mainly for remote and isolated islands) to hundreds of kilometers. The presence/absence data of various species of *Sargassum* were compiled for each OGU.

The taxonomy of *Sargassum* has gone through several major revisions or partial revision in the past decades (e.g. Setchell 1931a, b, 1933, 1936, Womersley 1954, Yoshida 1983, Tseng 1985). Some confusion of synonymies as used or reported in different biogeographic records or studies is inevitable. To address this problem, the taxonomic nomenclature adopted in this study was based entirely on the online database AlgaeBase (Guiry & Guiry 2008). All records in the literature were checked for their synonyms and converted to the valid basionyms as listed in the database. Questionable species records, for instance species name with incorrect or no authority, were excluded from the analysis. Species currently with confusing taxonomic status were combined to form species-complexes. Since the recognition of subgenus is less problematic than the species identification. The subgeneric grouping of each species compiled in the dataset was also identified based on the review of

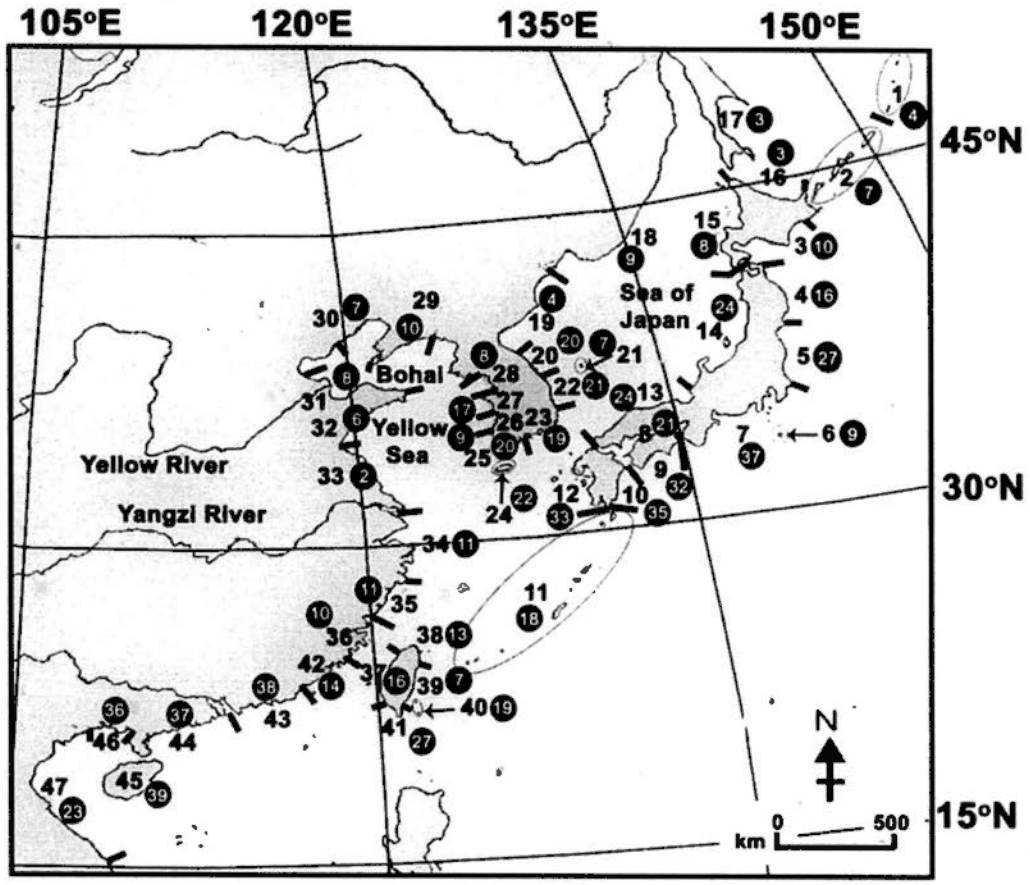

Figure 2.1 Map of NW Pacific showing the location of the 47 operational geographic units (OGUs) analyzed. Refer to Appendix 2.1 for the name of these OGUs. The numbers in circles represent the species richness of *Sargassum* in each OGU.

Phillips (1995) and the phylogenetic work of Stiger *et al.* (2003), in order to get rid of the problem of species identification. The total number of species and the number of species in each subgenus were then determined for each OGU.

2.2.2 Statistical analysis on *Sargassum* community structure

The occurrence of species/species complex in each OGU was organized in the form of presence (1) /absence (0) data. The presence/absence species dataset and the dataset showing the number of species in each subgenus within each OGU were used to calculate the pairwise Jaccard coefficient and Bray-Curtis similarities (Bray & Curtis 1957) respectively using the computer program PRIMER v.6.0 (Clarke & Gorley 2006). Pairwise Jaccard coefficient is one of the most suitable proximity coefficients for binary data (Shi 1993), whereas Bray-Curtis similarities are preferred for ecological data (Clarke *et al.* 2006). Pairwise biogeographic distance matrices among OGUs were calculated. Hierarchical cluster analysis utilizing between-group linking method was employed to visualize the structure of the biogeographic data. "SIMPROF" test in PRIMER v.6.0 was carried out to provide statistical significance of the branching pattern in the dendrogram of cluster analysis through permutation of the dataset 10,000 times.

2.2.3 Geographical matrix, environmental data and their analysis with community data

As an analogous analysis with the Mantel test which is based on the geographical distances and the pairwise genetic distance (Rousset 1997), the analy "RELATE" was carried out to test the correlation between the geographical distances and biogeographical matrices, and to infer the likelihood of applying the theory of "isolation by distance" (Wright 1943) to explain the biogeography. The pairwise geographical distances between OGUs were calculated among the centroids of all OGUs by Google Earth®. The distances were formatted as a distance matrix across each OGU, which was then compared with the biogeographic distance matrix using the analysis "RELATE" in PRIMER v.6.0. This analysis is a Spearman rank correlation to test whether the degree of compositional difference of biogeographic data is positively correlated with the geographical distance among OGUs.

Various environmental parameters, which were identified to be critical in affecting algal distribution, were compiled from different sources. The latitude of centroid point (LAT) as an indicator of photoperiodicity, the average sea surface temperature (Ann SST) and the annual average salinity (SAL) were obtained from the coastal and marine environmental database (Fautin 2007). The mean lowest SST

in the winter (WinLSST) and the highest SST in the summer (SumHSST) between 1984 and 1998 were acquired from the satellite images of National Oceanic and Atmospheric Administration (NOAA) SST database (http://www.osdpd.noaa.gov/PSB/EPS/SST/al_climo_mon.html). The tidal amplitude was modified after Le Provost *et al.* (1995). All these parameters were averaged for each OGU. These data, representing the major environmental factors that potentially affect *Sargassum* species distribution, were normalized, converted into Euclidean distance across OGUs and used in the analysis "RELATE" as described above.

"BEST" analysis from PRIMER v.6.0 was utilized to isolate the most significant environmental parameter(s) influencing the biogeographic dataset, while "LINKTREE" analysis, a non-parametric regression tree analysis, was employed to further dissect the differential effect of these parameters. A bifurcating tree was then obtained with the nodes representing the various environmental criteria identified by "LINKTREE" analysis. "SIMPROFF" test with 1000 permutations was carried out to assess the significance of the branching pattern.

2.3 Results

2.3.1 The biogeographic dataset

In the 47 OGUs defined, a total of 151 species/species complexes of *Sargassum* were recorded, nine of which were recognized to the variety level (Appendix 2.1). Incorrect authorities were found in the records of five species (~3%), and records with correct authorities were used in the analysis of the species concerned. This compilation also yielded 15 species complexes (10%), constituting 30 *Sargassum* species in total.

2.3.2 Geographical distributions of species and subgenera

On average, 17 species were present in each OGU. The Guangdong, Hainan and Guangxi region of China (OGUs 44, 45, 56) and the southern Honshu, southern Shikoku and Kyushu region of Japan (OGUs 7, 9, 10, 12) were the two regions with the highest species richness (Fig. 2.1). In contrast, OGUs with the lowest species richness were mainly present in the Bohai and Yellow Sea region (OGUs 29, 30, 31, 32, 33) and the Hokkaido and Russian region (OGUs 1, 2, 3, 15, 16, 17, 18). When converted into per unit coastline, the richness appeared highest in the regions of southern China, Taiwan and Korea (Fig. 2.2).

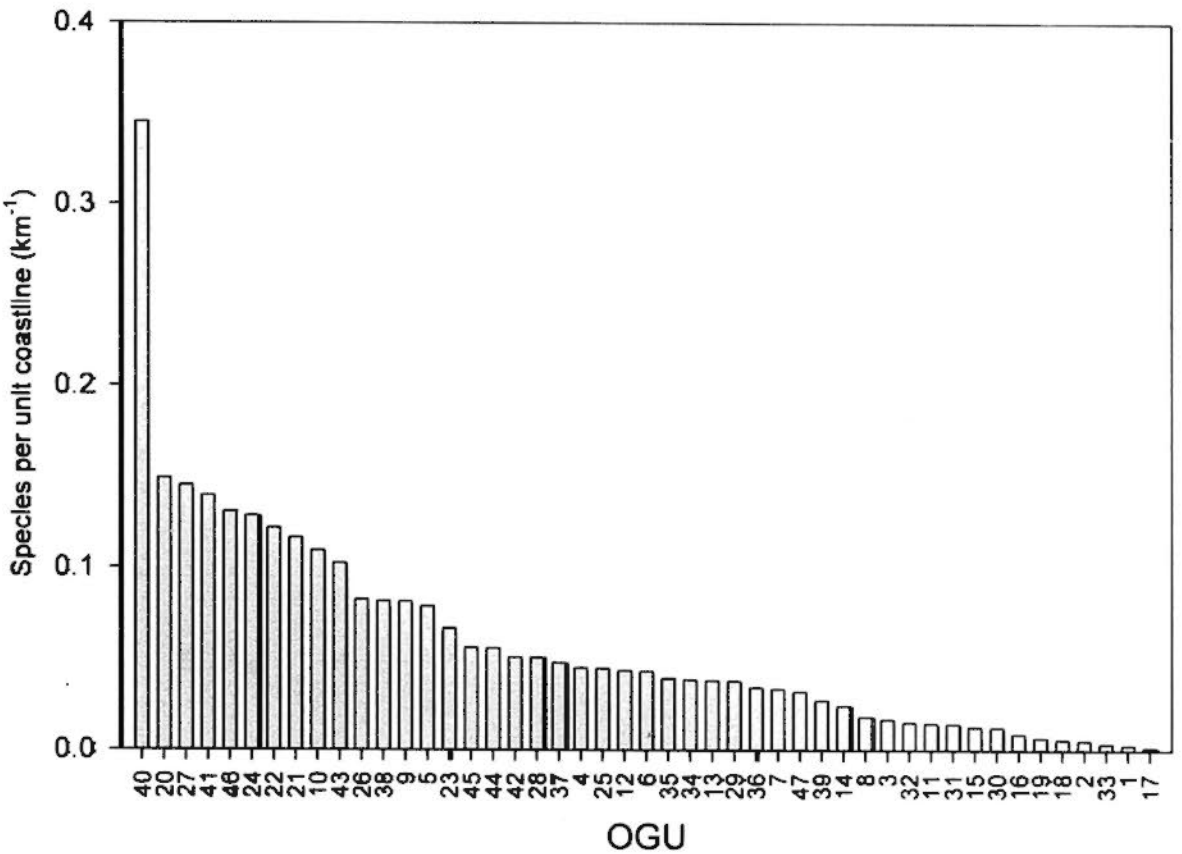

Figure 2.2 Richness per unit coastline across different OGUs. Refer to Fig. 2.1 for the geographical location of these OGUs.

With respect to the distribution of *Sargassum* species, each species was reported in five OGUs on the average. Almost half of the species (47.7%) were recorded only in one OGU (Fig. 2.3) and about 70.0% were distributed in less than five OGUs.

The top nine most widely distributed species were all found in over 20 OGUs (Fig. 2.4). *Sargassum thunbergii* (Mertens ex Roth) Kuntze, which was recorded in 37 OGUs, was the most widely distributed species. *Sargasssum horneri* (Turner) C. Agardh (35 OGUs) and the *S. siliquastrum* (Turner) C. Agardh / *S. tortile* (C. Agardh) C. Agardh species complex (31 OGUs) were widely distributed in the NW Pacific except in the tropical region such as in OGUs 45, 46 and 47 (distribution map not shown).

Of the 151 species reported, 111 (73.51%) belong to the subgenus *Sargassum* and 25.83% (39 species) to *Bactrophycus*, while one species could not be assigned to any subgenus. Members of *Bactrophycus* were found almost throughout the NW Pacific (46/47 OGUs), but a higher number was concentrated in the northern NW Pacific (Fig. 2.5). Members of the subgenus *Sargassum* occurred in 32 OGUs that ranged from tropical to temperate areas except the boreal region and Bohai Gulf. A total of 72 species were found in only one OGU (Fig. 2.3) and many of these were

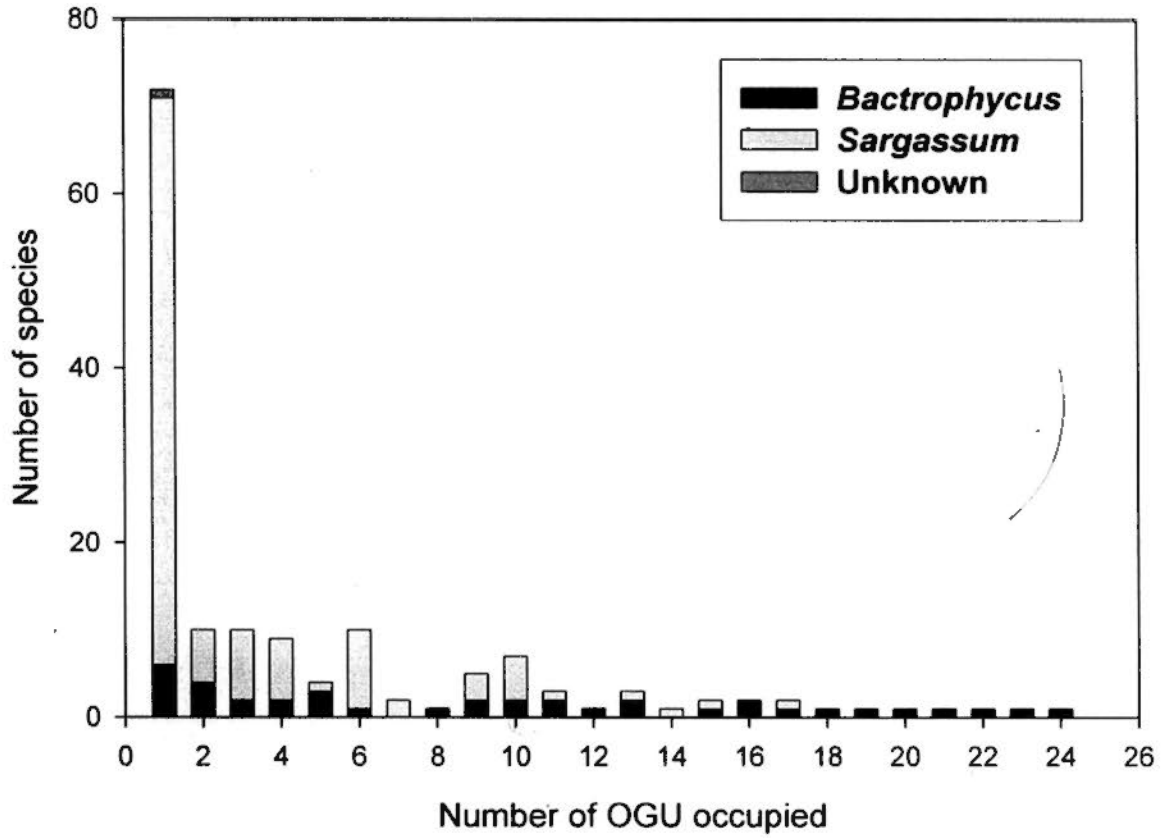

Figure 2.3 Frequency distribution of the number of species in each of the two subgenera (*Bactrophycus* and *Sargassum*) recorded in different numbers of OGUs.

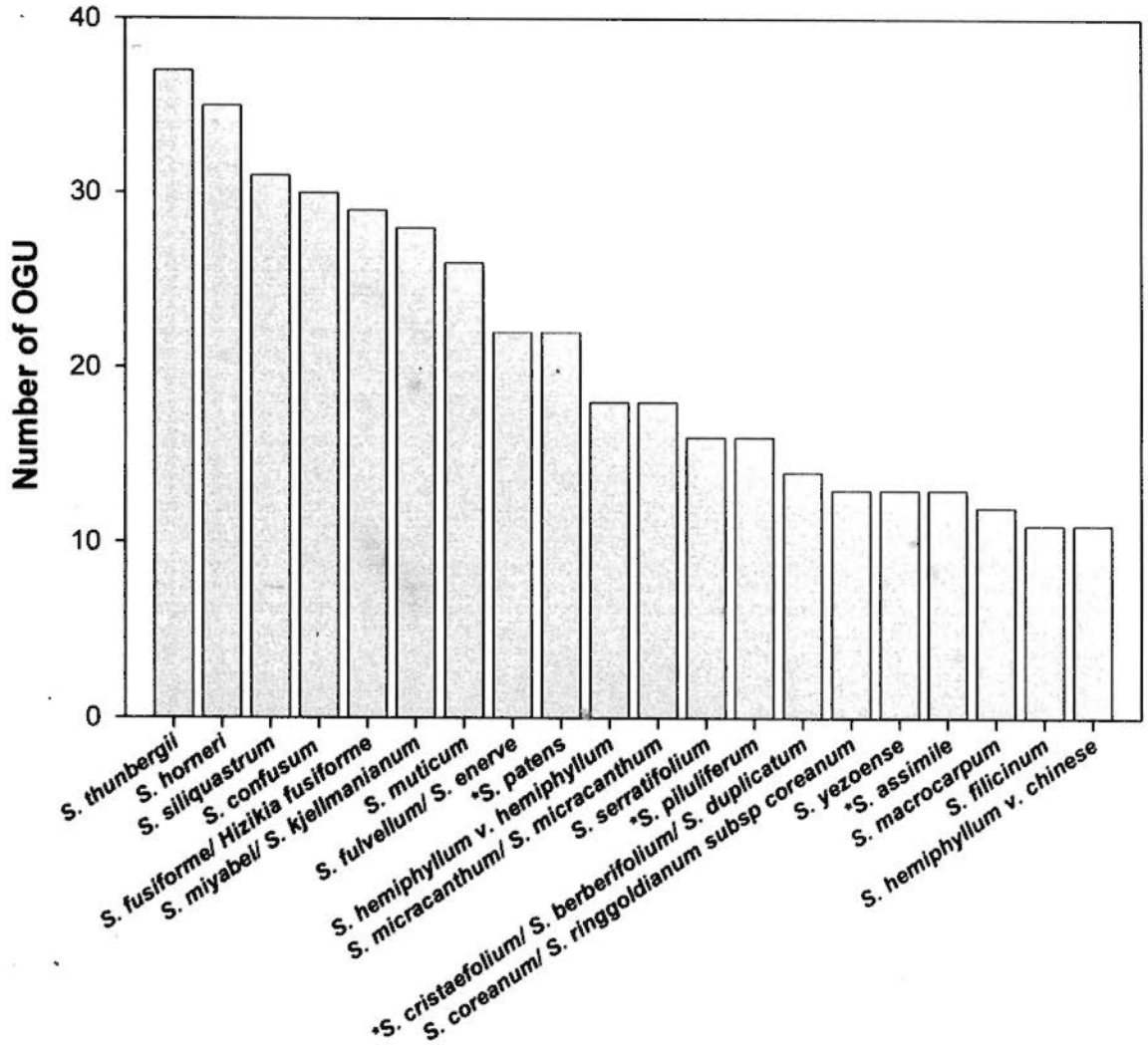

Figure 2.4 The number of OGUs recording the top 20 most widely distributed *Sargassum* species in the NW Pacific. Species marked * belong to the subgenus *Sargassum*, others to the subgenus *Bactrophycus*

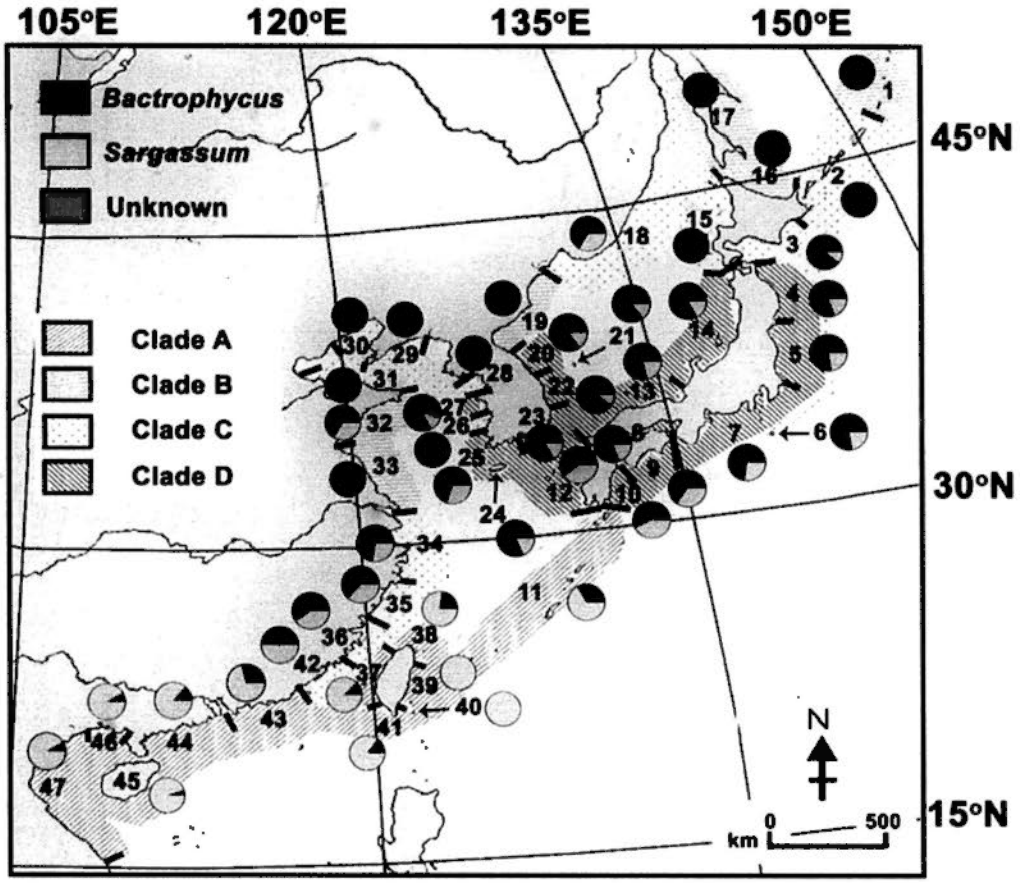

Figure 2.5 The distribution of the subgenera of *Sargassum* in the NW Pacific. The four biogeographic clades identified in cluster analysis are labeled in different hatching patterns.

distributed in the southern NW Pacific (Fig. 2.5). In contrast, members of *Bactrophycus* exhibited a wider span of distribution from 1 to 37 OGUs. Sixteen out of the top 20 widely distributed species belonged to *Bactrophycus* (Fig. 2.4), far more than the four species that belonged to the subgenus *Sargassum*.

2.3.3 Compositional differences of species and subgenera across OGUs

The results of the cluster analyses based on subgenera or species datasets were essentially consistent. There were four statistically significant biogeographic clades recognized based on the subgenera dataset (Fig. 2.6). Clade A corresponded to the tropical region, including the coast of southern mainland China, Taiwan and Okinawa (Fig. 2.5). Clade B corresponded to the area that included central China (OGUs 32 &33), northern Japan (OGUs 1, 16 & 17) and the east coast of North Korea (OGU 19). Clade C covered a wide range of areas, including Russia (OGU 18), northern Japan (OGUs 2, 3 & 15), the Bohai Gulf (OGUs 26, 28-31) and the Chinese coast along the East China Sea (OGUs 34-36, 42). This clade was the sister group of clade D that corresponded mainly to the Korean coast and both the east and west coasts of Japan (Fig. 2.5).

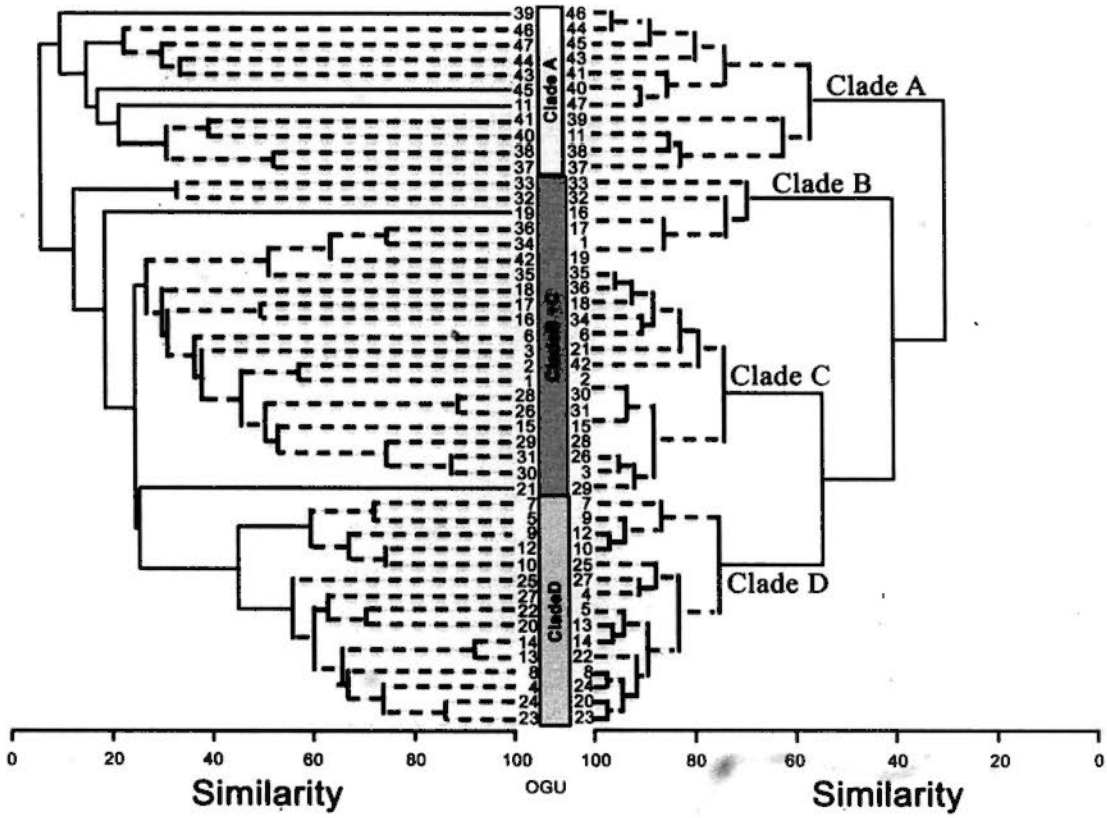

Figure 2.6 Dendrograms generated by species (left) and subgenera (right) datasets. Solid lines refer to the branches with significant statistical support ($P < 0.05$, SIMPROFF Permutation= 1000

The dendrogram generated by the species dataset demonstrated a generally similar branching pattern but with a higher resolution (Fig. 2.6). The same OGUs were clustered in either clades A or D, although the levels of similarity at which these OGUs were clustered in some cases were slightly different. For example, the coastal OGUs of Taiwan, except the most distantly related east coast (OGU 39), were clustered first with OGU 11 (Okinawa) and OGU 45 (Hainan). This group was further grouped with the Chinese coast along the South China Sea to form clade A (Fig. 2.6). Clades B and C, however, were not distinct. While the same OGUs were grouped in clade C as in the cluster generated from the subgenus dataset, some OGUs (i.e. OGUs 1, 16 & 17) that belonged to clade B in the clustering generated from the subgenus dataset were now grouped with clade C.

2.3.4 Factors affecting the biogeography of genus *Sargassum*

The degrees of compositional difference of *Sargassum* species among OGUs were positively correlated with the geographical distance and the environmental difference across OGUs (Table 2.1). The R-value of these two tests were similarly much lower than that generated by "BEST" analysis (Table 2.1). Based on "BEST" analysis, the correlation between species compositional difference and the combined effect of the mean lowest SST in winter, latitude and annual salinity was most

Table 2.1 The global R-values, i.e. Spearman rank correlation coefficients, between the biogeographic matrix based on species or subgenera, and the distance matrices generated by geographical distances (GeoMatrix) and the environmental factors (EnvMatrix). Results of "Best" Analysis show the combination of environmental factors that gave the highest R values. $P < 0.001$, $N = 1081$ for all R-values.

	Biogeographic matrix[1]	
	Species	Subgenera
GeoMatrix	0.550	0.495
EnvMatrix	0.472	0.519
"BEST" with the combinations of environmental factors[1] showing the highest R-value	0.667 (winLSST + LAT + SAL)	0.668 (winLSST + SAL)
	0.665 (winLSST + LAT)	0.665 (winLSST)
	0.637 (winLSST + sumHSST + LAT + SAL)	0.658 (winLSST + LAT + SAL)

[1] LAT: latitude of the centroid of OGU, AnnSST: the average annual sea surface temperature, SAL: average annual salinity, WinLSST: the average lowest Sea Surface Temperature (SST) in winter between 1984 and 1998, SumHSST: the average highest SST in summer between 1984 and 1998.

significant ($R = 0.667$, $P < 0.001$), followed by the combined effect of mean lowest winter SST and latitude and that of mean lowest winter SST, mean highest summer SST, latitude and annual salinity (Table 2.1).

Similar results were obtained in the correlation test using the subgenera dataset as the biogeographic matrix (Table 2.1). The mean lowest SST in winter, latitude and annual salinity were the main components in the best-correlated combinations, while the combined factors of mean lowest winter SST and annual salinity yielded the highest R-value ($R = 0.668$, $P < 0.001$).

The biogeographic matrices based on the species and subgenera datasets were successively correlated with all the environmental parameters by "LINKTREE" (Tables 2.2 & 2.3). Although the sequences of the serial correlations were different between the datasets, the environmental parameters distinguishing the biogeographic clades A and D were substantially consistent. The average lowest SST in winter was the main determining factor in separating clade A, except OGU 11, from other OGUs. The mean lowest SST during winter was higher than 19 °C in all OGUs of clade A, while it was lower than 17 °C in all other OGUs (Fig. 2.7, Tables 2.2 & 2.3).

Table 2.2 Information on the nodes in "LINKTREE" analysis on species data. Refer to Fig. 2.7 for the linkage trees based on species dataset, * indicates nodes involved in distinguishing clade A; # nodes involved in distinguishing clade D.

Species dataset

Node	R-valve	B%	Factor(s) and the criteria[1]
A*#	0.89	95	WinLSST(°C)>19(<17)
B#	0.56	64	SumHSST(°C)>30(<28)/ MeanSST(°C)>25.4(<24.7)/ SAL(ppt)>34.6(<34.5)
C#	0.44	59	LAT(°)<47.8(>47.8)
D#	0.39	48	WinLSST(°C)<5(>6)
E	0.82	61	MeanSST(°C)<13.8(>15.4)/ LAT(°)>37.8(<35.8)
F	0.70	39	WinLSST(°C)>5(<4)/ SumHSST(°C)<26(>26)
G	0.67	32	TIDE(cm)>10(<5)
H	0.57	18	SumHSST(°C)<17(>22)/ MeanSST(°C)<6.4(>11.4)/ LAT(°)>44.3(<43.3)
I#	0.97	87	SAL(ppt)<31.3(>31.4)
J#	0.83	49	LAT(°)<30.3(>32.3)
K	0.78	10	TIDE(cm)<130(>135)
L#	0.52	29	WinLSST(°C)<16(>16)
M#	0.59	27	SAL(ppt)<33.3(>33.6)
N	0.52	17	SAL(ppt)<33.9(>34)
O	0.51	19	WinLSST(°C)>7(<6)
P	0.52	5	TIDE(cm)<80(>100)
Q	0.52	4	SAL(ppt)<33.8(>33.9)
R	0.79	3	TIDE(cm)>25(<15)
S	0.92	11	TIDE(cm)<5(>30)
T	0.75	5	SumHSST(°C)<26(>27)/ TIDE(cm)<40(>50)/ LAT(°)>34.8(<33.3)

[1] Symbols as in Table 2.1.

Table 2.3 Information on the nodes in "LINKTREE" analysis on the subgenus data. Refer to Fig. 2.7 for the linkage trees based on subgenera datasets, * nodes involved in distinguishing clade A; # nodes involved in distinguishing clade D.

Subgenus dataset

Node	R-valve	Factor(s) and the criteria[1]
A*#	0.74	WinLSST(°C)>19(<17)
B#	0.47	LAT(°)<47.8(>47.8)
C#	0.43	WinLSST(°C)<6(>7)
D	0.82	SumHSST(°C)<26(>26)
E	0.74	LAT(°)>35.8(<33.3)
F	0.31	WinLSST(°C)>2(<0)
G	0.76	LAT(°)>37.8(<35.8)
H	0.93	SumHSST(°C)<26(>26)
I	0.84	MeanSST(°C)<13.8(>16.3)/ TIDE(cm) >10(<5)/ WinLSST(°C)>6(<6)
J	1	TIDE(cm)<50(>125)/ WinLSST(°C)<4(>6)
K	0	SAL(ppt)<32.8(>34)/ WinLSST(°C)<2(>4)
L#	0.49	SumHSST(°C)<28(>30)/ MeanSST(°C)<24.7(>25.4)/ SAL(ppt)<34.5(>34.6)/ WinLSST(°C)<17(>17)
M#	0.42	TIDE(cm)>120(<100)
N#	0.64	SAL(ppt)<33.3(>33.6)
O	0.73	MeanSST(°C)<20.3(>21.6)
P	0.55	TIDE(cm)>100(<80)
Q	0.77	SumHSST(°C)<22(>25)/ LAT(°)>39.8(<38.3)
R	0.78	SumHSST(°C)<25(>26)
S	0.34	SumHSST(°C)<27(>27)
T	0.38	MeanSST(°C)>18.5(<17)/ WinLSST(°C)>10(<9)/ LAT(°)<35.8(>36.3)
U	1	TIDE(cm)<30(>40)
V	1	SumHSST(°C)<26(>27)/ TIDE(cm)<40(>50)/ LAT(°)>34.8(<33.3)
W	0.78	LAT(°)>35.3(<27.3)/ SAL(ppt)<32.6(>33.7)/ MeanSST(°C)<16.1(>21.4)/ WinLSST(°C)<11(>14)/ SumHSST(°C)<26(>27)
X	0.83	SAL(ppt)<34.4(>34.5)
Y	0.77	TIDE(cm)<60(>70)/ LAT(°)<22.8(>23.8)/ Mean SST(°C)>24.8(<24.6)
Z	0.76	MeanSST(°C)<26.6(>26.7)
AA	1	LAT(°)>22.8(<21.3)/ WinLSST(°C)<19(>20)/ MeanSST(°C)<24.8(>25.3)

[1] Symbols as in Table 2.1.

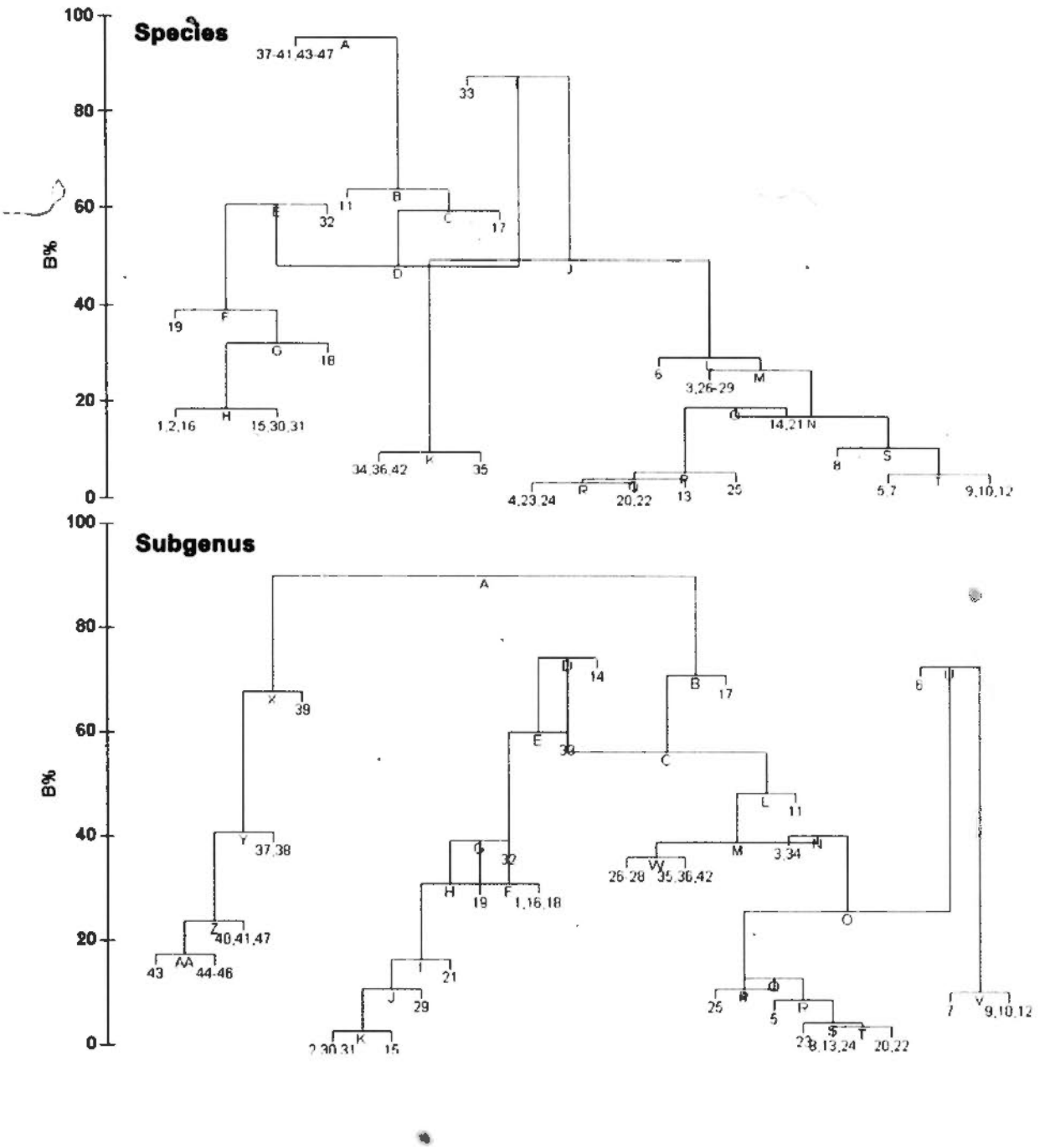

Figure 2.7 Bifurcating tree of "LINKTREE". The numbers in the branches correspond to the OGU, and the letters in the nodes to the environmental criteria (see Tables 2.2 & 2.3) that contributed to the bifurcation.

In both linkage trees, OGUs 21 and 6 were grouped together with clade D in the analysis of the species and the subgenus datasets respectively (Fig. 2.7), while OGU 27 was not grouped with other members of clade D. The environmental parameters resolving clade D were almost the same for the two datasets. These included the mean lowest SST in winter being between 16°C (7°C for the subgenus dataset) and 17°C, the mean highest SST for the summer being lower than 28°C, the mean SST each month being lower than 24.7°C, the mean annual salinity being within 33.6 ppt and 34.5 ppt, and the latitude being lower than 47.8°N. There was an additional constraint of latitude being higher than 32.3° in the linkage tree generated by the species dataset, and the tidal amplitude being less than 100 cm for the subgenus dataset. In contrast to clades A and D, there were no distinct environmental parameters associated with the biogeographic clades B and C.

2.4 Discussion

2.4.1 Validity of the dataset

The validity of utilizing the rich amounts of unexplored phycological information to analyze algal biogeography has been called into question because of the problem associated with the consistency of algal identification by various researchers with different taxonomic expertise, and the different levels of sampling

efforts involved in different studies from different localities (Lewis 1990). The taxonomic status of *Sargassum* as a genus is well established. Materials from the NW Pacific that formed the basis of the present study were identified by experts from the region that worked closely together. This should have improved the consistency of species identification and alleviated the chances of potential misidentification. Moreover, recent taxonomic re-evaluation of members of the subgenus *Bactrophycus* using molecular tools (eg. Oak *et al.* 2002, Phillips *et al.* 2005) further strengthens the validity of the taxonomic status of these species. The standardization of nomenclature, utilizing the web database AlgaeBase, should also have helped resolve the problem of synonymy, thus enhancing the reliability of the database used in this study.

To deal with the problem of sampling efforts, Lewis (1990) proposed two ways to obtain the "stable information" of a particular region or taxon. The first approach is to comb the collection records for a particular locality over time and look for the point where the cumulative number of taxa recorded reaches a plateau. This approach resembles the technique used to obtain optimal sample size in ecological studies, which has been widely discussed in ecological literature. The second approach is to assess the number of times the taxa have been reported. This will indicate whether a

certain taxon sampled was consistently recognized in the region. These approaches, which involve the repeated sampling of a locality, represent an ideal. However, they are not always feasible for logistical or financial reasons. However, the problem is reduced to a manageable compass if, instead of looking at the whole flora, only dominant algal genera are considered. In the case of *Sargassum* in the NW Pacific, large amounts of information have been collected in recent years by various workers, and comprehensive reviews, theses and monographs have been published based on these materials (e.g. Kang 1966, Yang 1995, Yoshida 1998, Tseng & Lu 2000). There is good reason to believe that these *Sargassum* records are both comprehensive and accurate. On the other hand, a large number of new species have been reported from the region, especially from localities around southern China, perhaps prematurely (e.g. Tseng & Lu 2000). Lack of repeated records of these new species could be a problem and this problem has been addressed in the present study.

Instead of going back to the original collecting sites to verify the species record, especially those of new species [i.e. Approach 2 of Lewis (1990)], newly described species from the region were noted from the dataset and analyses were carried out separately with or without these new species. Forty-four out of the 151 species/species complexes in NW Pacific were species first described by Tseng and

Lu less than two decades ago (reviewed in Tseng & Lu 2000). Most of these species (42/44) belong to the subgenus *Sargassum*, and 40 of these plus two other new species under *Bactrophycus* were found only in one OGU. None of these 44 species have been reported elsewhere since they were first described.

While the validity of these species may need further verification using other tools, e.g. molecular techniques, their exclusion did not change the general results of multivariate analyses either at the subgenera or species levels (data not shown). The question of the taxonomic validity of these species was therefore not a critical one as far as the purpose of the present study was concerned. All data analyses in the present study and the subsequent discussion about the results, unless otherwise stated, were therefore based on the original data set that included these new species.

Nevertheless, the finding that the subgenus *Sargassum* was highly localized in the NW Pacific should be treated with caution, since 42 of the species recorded in only one OGU (around 60%) were species described as new. Most of these were in OGUs 44, 45 and 46 (southern China). Of the remaining 30 species that occurred in only one OGU, 15 were distributed in other areas outside the geographical coverage of this study. For instance, *S. cymosum* C Agardh, which was recorded in northern

Vietnam, was also recorded to be widely distributed in tropical areas of the Atlantic, Indian and Pacific Oceans (Guiry & Guiry 2008). Other species, like *S. tenue* J. Agardh (in east Guangdong, OGU 43) and *S. virgatum* (Mert.) C. Agardh (in north Vietnam, OGU 47), are commonly found in the Indo-West Pacific region.

2.4.2 Species richness and distribution of *Sargassum* in NW Pacific

The records of the present study encompass almost one fourth of the total number of species of *Sargassum* (577 species, Guiry & Guiry 2008), indicating that the NW Pacific is a *Sargassum* rich area. This result is consistent with the findings by Phillips (1995), albeit at a finer resolution, showing that both China and Japan are rich in *Sargassum* species.

Based on the results of both total richness and richness per unit coastline, two high diversity areas of *Sargassum* in NW Pacific can be identified. Southern Japan and southern China exhibit higher total species richness than the rest of Japan and China respectively. If species richness is standardized on a per unit coastline basis, southern China remains the region with the highest richness. The Taiwan region also has comparably high species richness. In the NE, on the other hand, Korea becomes

the region with the highest richness instead of southern Japan (Fig. 2.2). Phillips (1995) mentioned that the subgenus *Sargassum* has either not been recorded in the Korean checklist or has been omitted from it. This present study supplies this missing information, and has found records of *S. yendoi* Okamura et Yamada under the subgenus *Sargassum* along the Korean shore (Kang 1966, Oak *et al.* 2002).

The geographical areas covered by clades B and C are relatively large, encompassing areas as far north as the Kurile Islands in northern Japan (OGU 1) and as far south as southern Fujian in mainland China (OGU 42). There is also no distinct environmental constraint that delimits these two clades, in contrast to clades A and D (Fig. 2.7, Table 2.2 & 2.3). This suggests that clades B and C constitute a composite biogeographic clade rather than a real biogeographic region regulated by specific environmental constraints. The grouping of some distantly situated OGUs (e.g. OGUs 1 & 42) into these two clades may simply be spurious, and may be due to unrelated but similar biogeographic properties associated with these OGUs, e.g. relatively low species richness (Fig. 2.1) and the high proportion of members of the subgenus *Bactrophycus* in these localities (Fig. 2.5).

The four biogeographic clades identified in this study (Figs. 2.5, 2.6) are different from the phycogeographic zones proposed by Lüning (1990) for the NW Pacific (Fig. 2.8). While the areas covered by clade A correspond to those of the tropical phycogeographic zone, the cold and warm temperate zones (western Pacific boreal and Japan zones respectively in Briggs 1974) were not matched by any biogeographic patterns based on *Sargassum*. Instead of a distinct boreal zone, clade B+C in this study is more heterogeneous, including not only regions of the boreal area but also other non-boreal regions like the Bohai Gulf and the central Chinese coastal areas. The areas associated with clade D in this study correspond to the warm temperate zone (Fig. 2.8), but include more Japanese areas such as Honshu, Shikoku and Kyushu as well as most of the Korean coast.

This discrepancy in mapping the biogeographic zones of the NW Pacific may have originated from differences in the dataset on which these different studies were based. Lüning (1990)'s division of phycogeographic regions was mainly based on a summary of previous works of van den Hoek (1975, 1984) and Michanek (1979). The major geographical areas of concern in some of these works were the Atlantic Ocean only (van den Hoek 1975). The algal group under study, on the other hand, was also restricted only to the Rhodophyta (van den Hoek 1984) or was not

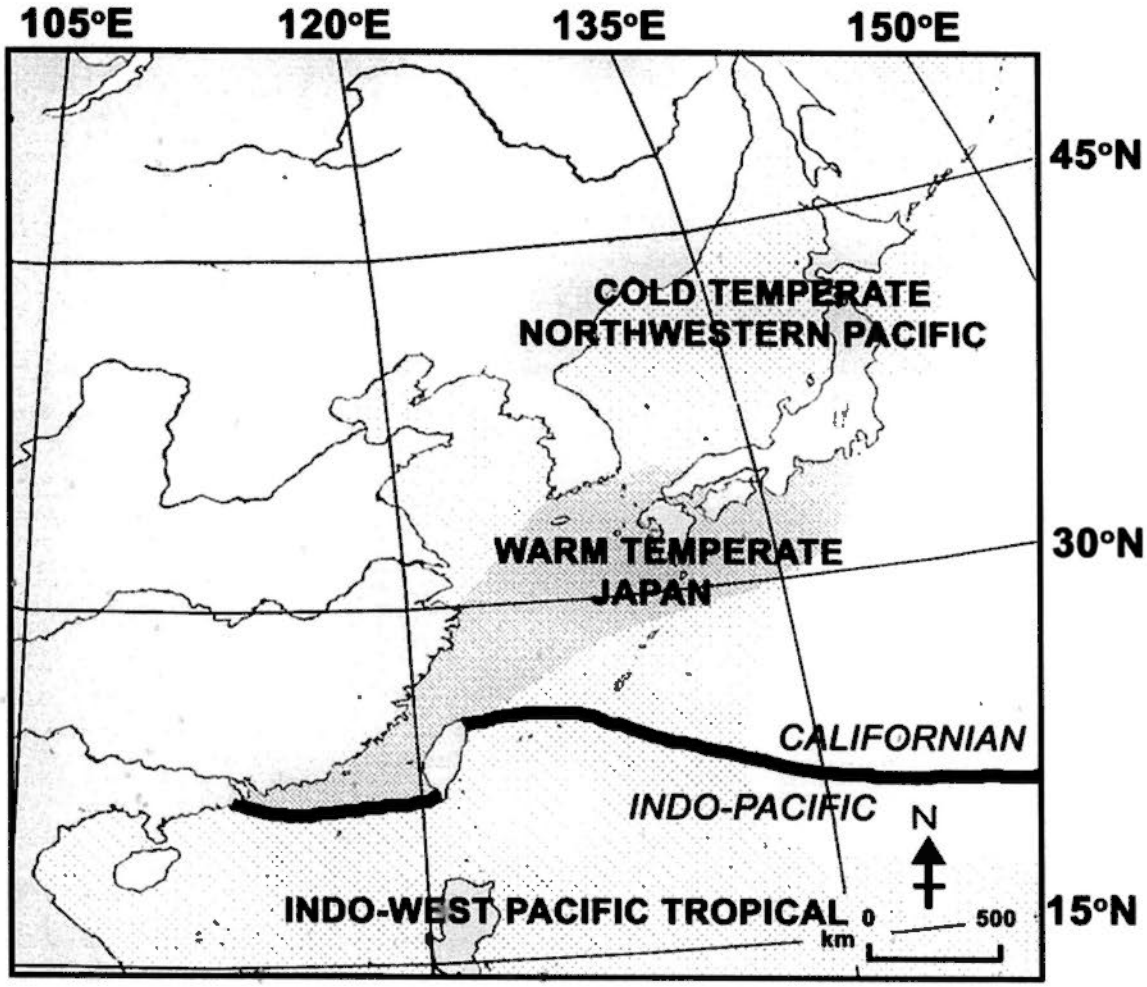

Figure 2.8 The three phycogeographic zones in bold and hatches proposed by Lüning (1990) after Briggs (1974). The boundary between the two thermogeographic regions (names in italics above and below the solid line) proposed by Adey and Steneck (2001) are also shown.

mentioned at all (Michanek 1979). The results of the present study, which was based on an important ecological member of Phaeophyceae, may provide a sounder basis for generalizing the present algal biogeography in NW Pacific.

2.4.3 Geographical distances and environmental parameters as explanations of the observed biogeographic patterns

The compositional difference among different OGUs was not well correlated with their geographical distances. Geographical distances alone may not be a good explanation of the compositional difference observed. The result of the overall correlation with environmental matrix also suggests that the distribution of *Sargassum* is not well correlated with the combined effect of the environmental parameters considered in this study. The average lowest SST in winter, latitude and annual salinity differences taken together, however, show a much higher correlation with the biogeographic patterns. This implies that other factors such as tidal amplitude might exert little effect on the composition of *Sargassum* species in the NW Pacific.

Temperature has long been recognized as a major factor that affects the global biogeography of benthic algae (Lüning 1990, Adey & Steneck 2001). It likely plays a

similar crucial role in structuring the biogeographic pattern of *Sargassum* shown in this study. The separation of clade A associated with the winLSST in "LINKTREE" analysis (Fig. 2.7, Tables 2.2 & 2.3) illustrates the importance of this factor on the biogeography of *Sargassum*. The geographical boundary between clade A and clade B (Fig. 2.5) also appears to coincide with the boundary between the two main thermogeographic zones, the Indo-Pacific and Californian Zones suggested by Adey and Steneck (2001) for the NW Pacific (Fig. 2.8), underlining the importance of temperature in affecting *Sargassum* flora in this region. Though few examples were available for the NW Pacific (e.g. Taki & Ogishima 1997, Shen *et al.* 2004), the biogeography of other marine organisms such as coral (Middlebrook *et al.* 2008), zooplankton (Southward *et al.* 1995) and marine snails (Tomanek & Somero 1999) have also been shown to be regulated by this ecologically important factor in other parts of the world.

The mean lowest SST in the winter was suggested to have potentially a more profound effect on *Sargassum* biogeography than the mean highest SST in the summer and the mean monthly SST. This may be related to the life history of *Sargassum*. Many species of *Sargassum* grow actively from late autumn (Murase *et al.* 2000, Ang 2006) to early summer (Koh & Shin 2004, Shimabukuro *et al.* 2007)

and reproduce from spring (Ang 2006) to early and mid-summer (Murase *et al.* 2000, Shimabukuro *et al.* 2007). Comparable to the 20 °C winter and 25 °C summer isotherms proposed by Stephenson (1948) as the delimiting condition of tropical and warm temperate biogeographic zones, the differentiating conditions of 17°C and 19°C detected in this study (Table 2.2) may represent the optimal growing or lethal temperatures of different *Sargassum* species.

Van den Hoek (1982) has suggested that the lowest winter temperature might correspond to the northern lethal boundary of several *Cladophora* species. Although *Cladophora* and *Sargassum* belong to different algal groups, the northern lethal boundary concept may be equally applicable to members of the tropical subgenus *Sargassum*. None of them were found in the boreal region (Fig. 2.5) and many of them have been shown not to be able to survive under cold temperature (Hwang *et al.* 2004). In contrast, it has been asserted that members of *Bactrophycus* are eco-physiologically incapable of invading the tropical area (Phillips 1995). This assertion is only partially supported by this present study, since the distributional range of different species under the subgenus *Bactrophycus* varies widely in NW Pacific covering 1 to 37 OGUs (Fig. 2.3). These species may vary in their adaptive capability, e.g. being eurythermal to a wide range of temperature regimes in this

region. *Sargassum thunbergii*, which is the most widely distributed species of *Bactrophycus* (Fig. 2.4), was found to grow optimally under a wide range of temperature regimes between 15°C and 25°C (Haraguchi *et al.* 2005). Other species investigated by Haraguchi *et al.* (2005), consisted of five *Bactrophycus* and two *Sargassum* species, showed a relatively narrower range of optimal growth between 15°C to 20°C and 20°C to 25°C respectively. Among the *Sargassum* species treated with the lowest temperature (10 °C) in another study (Baba 2007), species of *Bactrophycus* generally possessed higher relative growth rate (RGR) of 10%, compared to the RGR of <5% for two species under the subgenus *Sargassum*. Other studies testing the effect of culturing temperature on the growth of *Sargassum* species (e.g. Ogawa 1994, Ogawa *et al.* 1995), however, were restricted to testing only single rather than several species. Further studies are therefore needed to test the hypothesis on the role of temperature in delimiting the distribution range of other *Sargassum* species.

Latitude and salinity seem to be less significant in determining the biogeography of *Sargassum* than the mean lowest SST in winter. These variables appear only in the later part of the serial correlation in the "LINKTREE" analysis (Fig. 2.7, Tables 2.2 & 2.3). The photoperiod, which varies with latitude and hence

would be an immediate physiological factor reflected by latitude, has been shown to be important in the reproduction of some macroalgae (Terry & Moss 1980, Cunningham *et al.* 1993). Salinity has also been shown to affect algal biogeography (Wilkinson *et al.* 1995). Since both factors (a mean annual salinity of within 33.6 and 34.5 ppt and a latitude higher than 32.3°N) were detected to be imposing an environmental constraint on the formation of clade D, they should still be important in setting the *Sargassum* biogeographic pattern. They may be playing a role at a finer and/or localized scale, as they appear to do with other forms of marine life (Engle & Summers 1999).

2.4.4 The interplay between environmental and historical factors

To properly account for the present distribution pattern of *Sargassum* species, their evolutionary histories should also be taken into consideration. Phillips (1995) suggested that the NW Pacific region was not a "water accumulation area", so that the high diversity of *Sargassum* species here may be explained by a recent radiation, rather than the accumulation of species transported by the oceanic current. The large species number of the subgenus *Bactrophycus* (Fig. 2.5) may provide evidence to suggest that the NW Pacific is the center of diversity for this subgenus and its recent radiation in this region (Phillips 1995).

Though several phylogenetic studies have been conducted at the subgenus level of *Sargassum* (Oak *et al.* 2002, Stiger *et al.* 2003, Phillips *et al.* 2005), none has yet provided information about the radiation of *Bactrophycus*. The phylogenetic relationship among the members of the subgenus *Sargassum* has not yet been fully elucidated (Stiger *et al.* 2003), but the monophyletic status of either the subgenus *Sargassum* or *Bactrophycus* has been recognized (Stiger *et al.* 2003, Phillips *et al.* 2005). Another subgenus, *Arthrophycus*, which occurs only in Australia, is a sister group of *Bactrophycus*. These two form clades that are parallel to the subgenus *Sargassum* (Phillips *et al.* 2005). The divergent time between the subgenera *Bactrophycus* and *Sargassum* was not considered in these phylogenetic works. Phillips and Fredericq (2000) recognized a Gulf of Mexico/Caribbean cluster within the subgenus *Sargassum*, with species in the Pacific as the sister group (Fig. 2.9). This divergence, as suggested by these authors, could be timed by the closure of the Isthmus of Panama (mid-Pliocene, ~3 Mya, Briggs 1995). Assuming a similar and consistent evolutionary rate over time, the divergence of *Bactrophycus* and *Arthrophycus* is probable to have taken place earlier than 3 Mya (mid-Pliocene), since this splitting occurred earlier than the branch-off of the Gulf of Mexico/Caribbean cluster (Phillips & Fredericq 2000). The radiation of *Bactrophycus* may have begun after the separation from *Arthrophycus*, and probably

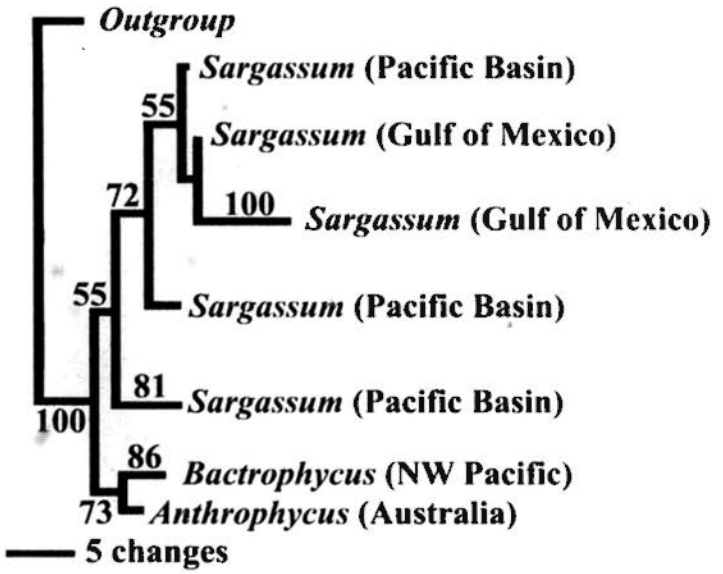

Figure 2.9 The phylogeny of subgenera within the genus *Sargassum*. Numbers on the node refer to the bootstrap value in 1000 replicates (Modified after Phillips & Fredericq, 2000)

occurred during the late Pliocene to Pleistocene. The evolution of the subgenus *Sargassum* might have occurred in the Miocene or even earlier, since this subgenus split basally in the phylogenetic tree (Phillips & Frederieq 2000).

Given that the subgenera *Bactrophycus*, *Arthrophycus* and *Sargassum* are mainly distributed in the NW Pacific, Australia and Pacific tropical region respectively, the most parsimonious explanation for their present distribution is that a common ancestor of *Arthrophycus* and *Bactrophycus*, which may have sympatrically diverged from the subgenus *Sargassum*, once occurred in the tropical western Pacific. This common ancestor spread as far north as the NW Pacific and as far south as Australia and its immediate vicinity. During the middle to late Miocene, a drop in the sea level by ~170 m (Lincoln & Schlanger 1991) may have resulted in the fragmentation of the distribution range of this common ancestor, leading to the independent evolution of *Bactrophycus* and *Arthrophycus* in their respective hemispheres. As no species closely related with *Bactrophycus* nor with *Arthrophycus* was present in the tropical area, the common ancestor in this region might have become extinct for some reason, leaving these disjunctly distributed but phylogenetically more related subgenera in the northern and southern hemispheres.

A further scenario, based on paleo-climatic and oceanographic evidence, might be advanced. The most probable region in the NW Pacific from which *Bactrophycus* radiated is the Sea of Japan and its immediate vicinity, as Lüning (1990) has speculated for algae in general. The Sea of Japan was isolated during the last glacial period when the sea level dropped. As an area heavily affected by the cold paleo-Oyashio current (Oba & Murayama 2004), the Sea of Japan must have been a stressful area for antecedent members of *Bactrophycus*. Only those that survived the adverse environment of the Sea of Japan would have propagated through the last glacial maxima. After the inter-glacial rise of sea level, these cold tolerant species of *Bactrophycus* would have followed the Oyashio current southwards to invade the Yellow Sea. A few of these species might still have retained their ability to adapt to a warmer climate, and so were able to reach the warmer southern Chinese coast (e.g. OGUs 44, 45 and 46). As a result, members of *Bactrophycus* exhibit a differential eurythermal trait, allowing them to be more extensively distributed in the NW Pacific. In contrast, members of the subgenus *Sargassum* did not exhibit this eurythermal trait allowing them to invade the colder part of the NW Pacific, and their distribution ranges have therefore been restricted to the warmer region of the NW Pacific and the Tropics (Fig. 2.3).

The present ecological environment, characterized above all by its different temperature regimes, supports the postulated past distribution pattern. Both the Oyashio and the Kuroshio Currents would have played important roles in maintaining the thermal regimes in NW Pacific (Lüning 1990). The geographical region covered by clade A appears to coincide with the region affected most by the Kuroshio Current. The two high diversity regions in Japan-Korea and southern China-Taiwan also indicate the respective center of diversity for the subgenera *Bactrophycus* and *Sargassum* (Fig. 2.5), while the region covering the Yellow Sea could be regarded as a transitional zone for the two subgenera in the NW Pacific. This notion is consistent with the proposal of Adey and Steneck (2001) based on their thermo-model on benthic macroalgae, that there are significant transitional areas in the world, but is in conflict with the classic model of discrete zoographical zones proposed by Briggs (1974). The center of diversity for the subgenus *Sargassum* may extend further south from southern China and Taiwan to include the tropical region around the South China Sea.

Chapter Three:

Phylogeography of *Sargassum hemiphyllum*

3.1 Introduction

Sargassum hemiphyllum (Turner) C. Agardh is a common species found in the marine floristic assemblages throughout the NW Pacific (e.g. Umezaki 1984b, Yokoyama *et al.* 1999, Sato & Wada 2000, Ang 2006). This species is distributed in Japan (Yoshida 1983), Korea (Kang 1966), the Chinese coast of the East and South China Seas (Tseng & Lu 2000) and Vietnam (Ajisaka *et al.* 1997). It is ecologically important as it serves as one of the major components of coastal *Sargassum* bed (Yokoyama *et al.* 1999) that supports a variety of other marine species (Taylor 1998, Ng 2009).

This species exhibits extensive intra-specific morphological variation, which was sufficient for the recognition of two varieties *S. hemiphyllum hemiphyllum* and *S. h. chinense* (Ajisaka *et al.* 1997). The leaves of var. *hemiphyllum* are smaller than those of var. *chinense* (Cheang *et al.* 2008). These two varieties exhibit allopatric distribution in which var. *hemiphyllum* is distributed in the Japanese and Korean coasts, and var. *chinense*, the southern Chinese coast and Vietnam (Ajisaka *et al.*

1997, Cheang *et al.* 2008). A more in-depth study of the morphological differences between the two varieties found leaf size to follow a gradient that increases from the south to the north along the NW Pacific (Cheang 2003).

This gradual change of morphology along a latitudinal gradient, however, could not be explained by the genetic population structure as distinct genetic differentiation was detected between the two varieties (Cheang 2003, Cheang *et al.* 2008). Three haplotypes were revealed based on the sequencing data of Rubisco spacer of the plastmid genome. Two slightly different haplotypes belonging to the specimens of var. *hemiphyllum*, and one distantly related haplotype from the specimens of var. *chinense* were detected (Cheang *et al.* 2008).

The development of these two genetically distinct lineages (varieties) were postulated to be related to the formation of glacial refugia associated with the sea-level fluctuation during the Quaternary Period. One possible refugium is the East China Sea basin, which remained an almost enclosed marine region during the glacial period, surrounded by the continental Chinese coast and the Ryukyu Island chain (Ota 1998). Further south is the South China Sea basin, which was disconnected from the East China Sea basin by the land bridge formed in the Taiwan

Strait. The divergence of the two varieties was believed to be maintained by the huge freshwater discharge from the Yellow and Yangtze Rivers in China (Cheang *et al.* 2008), from where a genetic break of the *S. hemiphyllum* populations existed between the regions from Zhejiang Province in China to Cheju Island in Korea.

While PCR-RFLP study of the amplicon of Rubisco spacer revealed only two haplotypes out of the three reported (Cheang *et al.* 2008), more sequencing data on other specimens were needed to confirm the result of PCR-RFLP study. The genetic difference between the two varieties need to be further ascertained by other genetic markers, since the gene tree generated based on a single locus will not necessarily reflect the actual species tree (Pamilo & Nei 1988). There are also inconsistencies between the phylogenetic relationships revealed by nuclear and cytoplasmic markers in some plant species (reviewed by Rieseberg & Soltis 1991), which may be caused by reasons like "chloroplast capture" (Soltis & Kuzoff 1995) or differential rate of incomplete lineage sorting between the genes in different genomes (Comes & Abbott 2001). The application of more markers in other genome(s) (e.g. nuclear DNA), which may experience different selective forces, would provide more information on the genetic difference revealed. This may provide deeper insights in understanding the evolutionary history of this species.

Recently, the application of the mitochondrial markers reveals the population genetic differentiation at a higher level of resolution compared to the use of plastid and nuclear markers (Engel *et al.* 2008). The mitochondrial intergenic *Trn*W_I spacer has proven to be one of the effective markers in revealing the genetic population structure of the brown alga *Undaria pinnatifida* (Harvey) Suringar (Voisin *et al.* 2005). This marker may be suitable for the phylogeographical study of *S. hemiphyllum* to provide a higher resolution, compared to the plastid marker Rubisco spacer (Cheang *et al.* 2008), to reveal any population infrastructure within the two varieties. On the other hand, more conserved markers such as nuclear marker ITS2 (Stiger *et al.* 2000) could assist in clarifying the taxonomic status of the two varieties and provide insight on whether these two varieties are in fact two phylogenetic species.

This study, thus, aimed at 1) verifying the taxonomic status of the two varieties of *Sargassum hemiphyllum* based on the sequence divergence of nuclear marker ITS2; 2) ascertaining sequence divergence between the two varieties with a larger sampling size (cf. Cheang 2003) based on the sequence data of *Rbc*L-S spacer gene in chloroplast DNA, and 3) determining any finer genetic population structure within each variety based on mitochondrial marker, *Trn*W_I spacer. The elucidation of the

finer genetic population structure of *S. hemiphyllum* could provide more insights on the evolutionary history of this species, which in turn could also provide further understanding about the evolution of the marine flora in NW Pacific.

3.2 Materials and Methods

3.2.1 Sampling, preservation and DNA-extraction of *S. hemiphyllum*

In order to obtain a more comprehensive sampling of *Sargassum hemiphyllum* in NW Pacific, specimens from additional localities were collected either by snorkeling or sampling during low tide, on top of the specimens already collected and reported in Cheang *et al.* 2008 (Fig. 3.1, Table 3.1). The specimens were collected 1 – 2 m apart to avoid collecting individuals from the same mother plant, as *Sargassum* germlings are known to have a short dispersal distance (Kendrick & Walker 1995). About 1 g of samples from the new branches without epiphytes were cleaned with brushes and subsequently preserved in 95% ethanol or silica gel until DNA extraction (Cheang 2003). Two to three vouchers haphazardly selected from each population were air dried and deposited in the Simon F. S. Li Marine Science Laboratory Herbarium, the Chinese University of Hong Kong. Genomic DNA of these specimens was extracted by modified cetyltrimethylammonium bromide

Table 3.1 Collection details of *S. hemiphyllum* populations examined in this study. Acronyms for the collection sites are also given. Refer to Fig. 3.1 for location of these collection sites.

Localities	Acronym	Date of collection	Collectors
1. Seto Marine Biological Laboratory of Kyoto University, Wakayama Prefecture, Japan (135°20'E, 33°41'N)	JpWY	22 Mar 01	M. Sato
2. Shishikui, Tokushima Prefecture, Japan (134°18'E, 33°33'N)	JpTS	7 Apr 07	C.C. Cheang & P. O. Ang
3. Sukumo, Kochi Prefecture, Japan (132°42'E, 32°54'N)	JpSM	6 Apr 07	C.C. Cheang & P. O. Ang
4. Kawatana, Nagasaki Prefecture, Japan (129°52'E, 33°03'N)	JpNS	19 Apr 07	N. Murase
5. Sungsan, Cheju Island, Korea (126°56'E, 33°27'N)	KrCJ	14 Feb 01	J. H. Kim
6. Hoatian, Pin Tan, Fujian Province, China (119°47'E, 25°40'N)	CNFJ	25 May 01	Y. Zhang
7. Kwangyinting, Penghu, Taiwan (119°33'E, 23°34'N)	TWPH	24 Mar 02	C.C. Cheang & P. O. Ang
8. Sai Kung, Hong Kong, China (114°16'E, 22°22'N)	HKSK	26 Feb 02	F. F. Yeung
9. Naozhoudao, Zhanjiang, Guangdong Province, China (110°37'E, 20°55'N)	CNZJ	14 May 01	C.C. Cheang & P. O. Ang

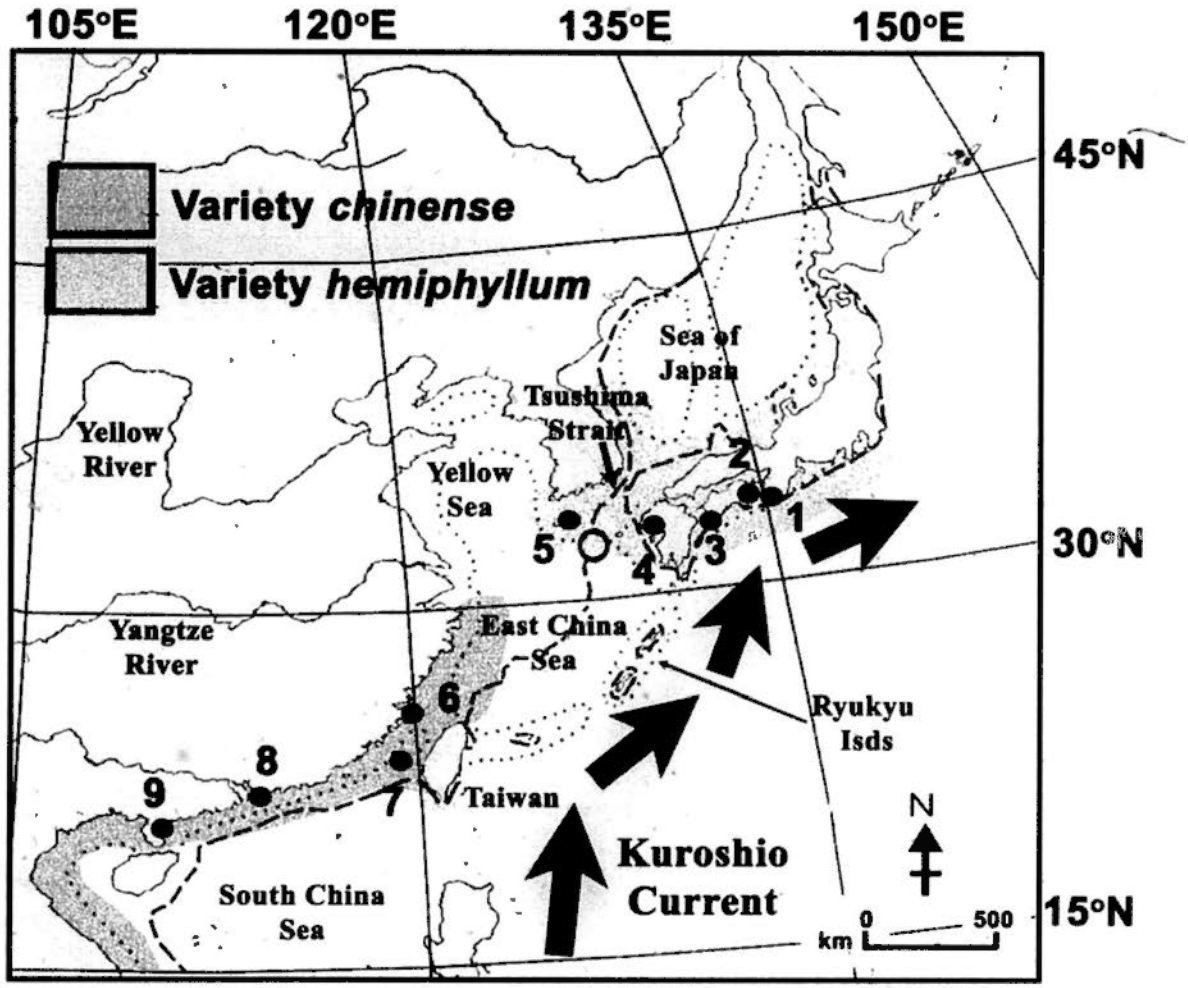

Figure 3.1 Distribution of *S. hemiphyllum* in northwestern Pacific. Dotted and dashed lines refer to the "Paleo-coast" during the early and late Pleistocene respectively (Ota 1998), while open circle indicates the location of the mouth of the "Paleo-Yellow River" (Xu & Oda 1999). Sampling sites are indicated in numbers. Refer to Table 3.1 for details of the sampling information.

(CTAB) method (Protocol two of Ho *et al.* 1995). Briefly, 1 g of tissue from the tip of new branches was detached and washed by brushes to avoid impurities as much as possible. The tissues were ground to fine powder in liquid nitrogen by a sterilized pestle and mortar and then transferred to 700 µL CTAB extraction buffer (1% CTAB; 10 mM ethylenediaminetetraacetic acid [EDTA]; 50 mM tris(hydroxymethyl) aminomethane [Tris] [pH 8.0]; 0.7 M sodium chloride; 2% mercaptoethanol; 1% polyvinylpolypyrrolidone [PVPP]). After two 700 µL chloroform:isoamyl alcohol (24:1) remixing to get rid of the organic impurities, extracts were purified twice by 70% ethanol precipitation methods and then dissolved in 200 mL Mili-Q water. The extracted genomic DNA was further purified by GENECLEAN II Kit (BIO 101 Inc., CA, USA), following the manufacturer's instructions (Yoshida *et al.* 2000).

3.2.2 Polymerase chain reaction (PCR) and direct sequencing

The spacer between Rubisco large and small subunits in plastid DNA, spacer between TrnW and TrnI in mitochondrial DNA and ITS2 region in the nuclear genome of all DNA samples were amplified through PCR (profile: initial 3 min at 96°C, 32 cycles of 40 s at 96°C, 30 s at 55°C 50 s at 72°C, final 3 min at 72°C) using the thermocycler, Mastercycler Gradient (Eppendorf®, Hamburg, Germany). The primers used are listed in Table 3.2, while the amplifications consisted of 0.25 µM of

Table 3.2 The primers and the PCR profile used for the three genetic markers: nuclear ITS2, plastid Rubisco spacer and mitochondrial TrnW_I spacer.

Markers	Forward/ Backward	Sequences
ITS2	F	ITS2FC2*: 5'-TTGTCGGGGAGGAGGAGG-3'
	R	25BR2-Reverse[1]: 5'-TCCTCCGCTTAGTATATGCTTA-3'
Rubisco spacer	F	M1F[2]: 5'-GACCTTTAAAAGCAGCTTTAGAT-3'
	R	M1R[2]: 5'-CCCCATAGTTCCCTAATACGCATT-3'
TrnW_I spacer	F	TrnW-I-F[3]: 5'-GGGGTTCAAATCCCTCTCTT-3'
	R	TrnW-I-R[3]: 5'-CCTACATTGTTAGCTTCATGAGAA-3'

* modified from the sequence (GenBank Accession number: AY150006) of *Sargassum hemiphyllum* (Oak *et al.* 2002)

[1] based on Yoshida *et al.* (2000).

[2] based on Cheang (2003).

[3] based on Voisin *et al.* (2005).

each of the primers, 1 x supplied PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each of dNTP, 0.025 units/μl of *Taq* polymerase (GE Healthcare Life Sciences, Chalfont St. Giles, United Kingdom) and 1 μl of DNA template. All concentrations were given as final concentrations in 25 μl amplifications. Successful PCR was checked by running the amplicon in 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide under X-ray exposure. The QIAquick PCR purification kit (QIAGEN, Hilden, Germany) was used to purify the PCR products obtained. In cases where sub-bands occurred in the gel, the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) was used instead to re-dissolve the PCR product from the target band so that the product could be isolated from the undesirable sub-bands. The amount of reagents and the PCR product added were based strictly on the instructions of the kits. Purified PCR products were subjected to commercial sequencing (Macrogen Inc., Seoul, South Korea) in which BigDyeTM terminator cycling conditions were employed through automated sequencer ABI3730XL (Applied Biosystems, Foster City, CA, U.S.A.). Twenty haphazardly selected specimens were sequenced twice in order to verify the results. Sequences obtained, together with those sequences of *S. hemiphyllum* available from GenBank, were visually edited and aligned using the software MEGA ver. 4 (Tamura *et al.* 2007). New sequences obtained, if any, were deposited to GenBank.

The heteroplasmy of the mitochondrial markers in *Fucus serratus* Linnaeus was documented (Coyer *et al.* 2004). The problem of heteroplasmy could hinder the applicability of the mitochondrial marker in phylogenetic reconstruction or in the inference of demographic history (Posada & Crandall 2002). This problem was not significant in the present study, since all the sequences demonstrate a clear single peak in their chromatograms (data not shown).

3.2.2 Cloning of PCR product

Besides possessing the two allelic copies in the heterozygous individual as a nuclear marker, ITS2 is also known for its intragenomic variation due to the hundreds or even thousands of ribosomal operons that are tandemly distributed throughout the genome (Dover 1982). This leads to the problem of multiple copies during single PCR amplification of ITS2 region (e.g. Harris & Crandall 2000). In cases of the failure of direct sequencing due to multiple copies, amplicons of ITS2 of each individual were cloned by the TA-cloning system (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instruction. In short, amplicons were ligated to plasmid vector pUC18 under 16°C overnight, and the mixture was then transformed into XL10-Gold® Ultracompetent cell (Stratagene, La Jolla, USA). The transformed cells were allowed to be heat-shocked and inoculated in ampicillin

agar plate for 16 hour so that the cell with both the inserted PCR amplicon and the ampicillin resistant gene could survive and form colony under the selective agent. Five to eight clones were picked for each PCR reaction (individual). The same procedure for PCR reaction and direct sequencing were performed except that two universal primer M-13 and RV (Takara Biotechnology Co., Ltd., Dalian, China) were used in PCR.

3.2.3 Statistical treatments

Basic parameters of population such as sequence divergence, nucleotide diversity and haplotype diversity as well as the pairwise Φ_{st} difference among populations were calculated. Analysis of molecular variance (AMOVA) was carried out to demonstrate the partition of genetic variance among populations and between varieties, and to test if the partition is statistically significant. All these calculations were done using the software Arlequin ver. 3.1 (Excoffier *et al.* 2005).

Neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) trees were generated by PAUP 4.0 Beta (Swofford 2000), and the Bayesian inference (BI) the program MrBayes v.3.12 (Ronquist & Huelsenbeck 2003), in order to analyze the phylogenetic relationships among populations. Akaike information

criterion implemented in Modeltest ver. 3.7 (Posada & Crandall 1998) was applied, *a priori*, to estimate the appropriate substitution model to be used in the NJ, ML and BI methods. TVM + I, GTR and HKY models were found to be the optimal model for ITS2, the Rubisco spacer and TrnW_I spacer respectively. For NJ, MP and ML methods, significance of the branching was assessed by 1000 bootstrapping replicates, in each of which 100 random taxa were added and the tree was heuristically searched by tree bisection-reconnection branch swapping method (Felsenstein 1985). For BI, two independent Markov-chain-Monte-Carlo searches with random starting points were conducted for each data set until the divergence between two runs became small and stationary. Trees were sampled every 100 cycles in at least 1,500,000 generations with the burn-in value set to the later third-fourth of the sampling trees in which consensus was reached for the two parallel runs. *Posteriori* possibility was then calculated from the sampled trees to illustrate the statistical confidence for the BI tree. *Sargassum thunbergii* (Mertens ex Roth) Kuntze and *S. muticum* (Yendo) Fensholt were designated as outgroups, since they were all phylogenetically closely related (Stiger *et al.* 2003, Phillips *et al.* 2005). All the sequences of *S. hemiphyllum* available from GenBank were included in the trees as well.

Based on the Rubisco spacer sequence, the divergence time of the major diversifications was estimated based on simple *p*-distance using the method of molecular clock (Sarich & Wilson 1973). A Chi-square test to test the difference between the log-likelihood values of the molecular-clock constrained and unconstrained trees (with outgroup sequences) (Andreakis *et al.* 2007) was performed and no significant difference was found ($-\ln L_{constrained}$: 476.19, $-\ln L_{unconstrained}$: 473.73, df: 138, $p > 0.05$), suggesting that the constancy of the evolutionary rate throughout the tree could be assumed (Andreakis *et al.* 2007). The molecular clock based on Rubisco spacer was calibrated by the time of the closure of the Isthmus of Panama (mid-Pliocene, ~3 Mya; Briggs 1995). The Gulf of Mexico/Caribbean cluster of *Sargassum* spp. was characterized by a distinct difference of 10 base pairs from other *Sargassum* species in the Pacific, while the difference of six more steps was present for the most distantly related species inside the cluster (Phillips & Frederieq 2000). As the spacer region possessed 159 bp (Phillips & Frederieq 2000), the average substitution rate for the Rubisco spacer region was estimated to be 2.10% - 3.35% per Mya. This range of evolutionary rate was used to estimate the time of the major diversifications occurred in the tree of Rubisco spacer generated for *S. hemiphyllum*.

The number of haplotypes for the three markers (genotype for nuclear marker) was recorded for each sampling locality. Computer software TCS ver.1.13 (Clement *et al.* 2000) was utilized to generate the TCS haplotype network that demonstrates the relationship among haplotypes. Only the sequencing data from the TA cloning were subjected to the construction of TCS haplotype network.

3.3 Results

Based on ITS2, Rubisco spacer and TrnW_I spacer, 62, 142 and 93 sequences were obtained respectively with two, three and four haplotypes identified possessing five, six and 12 polymorphic sites (mostly transversion) (Table 3.3, Fig. 3.2). The variability of ITS2 was the least (sequence divergence = 0.0052) while that of TrnW_I spacer the highest (sequence divergence = 0.054) (Table 3.3). The haplotype (h) and nucleotide (π) diversities were the highest in TrnW_I spacer (h = 0.75, π = 0.026), followed by Rubisco spacer (h = 0.65, π = 0.0099) and ITS2 (h = 0.42, π = 0.0037). The number of sequences obtained for each population and its respective h and π are shown in Table 3.3. In general, the genetic variations (ITS2: h = 0.00, π 0.00; Rubisco spacer: h = 0.47, π = 0.0016; TrnW_I spacer: h= 0.75, π = 0.026) within the northern populations, consisting of Japan and Korean specimens, were relatively higher than those (ITS2 & Rubisco spacer: h = 0.00, π = 0.00; TrnW_I

Table 3.3 Genetic variability of *S. hemiphyllum* based on the markers, ITS2, Rubisco spacer and TrnW_I spacer. Refer to Table 3.1 for the acronyms used to represent the collection sites.

		ITS2	Rubisco spacer	TrnW_I spacer
Overall	n (n_h)	62 (2)	142 (3)	93 (4)
Northern Pop.		18 (1)	85 (2)	54 (4)
	JpWY	3 (1)	20 (1)	13 (4)
	JpTS	1 (1)	8 (1)	N.A.
	JpSM	10 (1)	19 (1)	13 (1)
	JpNS	3 (1)	14 (2)	12 (1)
	KrCJ	1 (1)	24 (1)	12 (1)
Southern Pop.		44 (1)	57 (1)	39 (2)
	CNFJ	15 (1)	18 (1)	17 (2)
	TWPH	11 (1)	14 (1)	7 (1)
	HKSK	14 (1)	14 (1)	8 (1)
	CNZJ	4 (1)	11 (1)	7 (2)
IDS/ $T_S/T_V(s)$		2 / 0 / 3 (5)	0 / 0 / 6 (6)	0 / 2 / 10 (12)
SeqDi		5/573 = 0.0052	6/297 = 0.020	12/221 = 0.054
h ± SD	Overall	0.42 ± 0.048	0.65 ± 0.015	0.75 ± 0.013
π ± SD		0.0037 ± 0.0023	0.0099 ± 0.0058	0.026 ± 0.014
Northern Pop.		0.00 ± 0.00	0.47 ± 0.032	0.62 ± 0.046
		0.00 ± 0.00	0.0016 ± 0.0015	0.015 ± 0.0086
	JpWY	0.00 ± 0.00	0.00 ± 0.00	0.74 ± 0.091
		0.00 ± 0.00	0.00 ± 0.00	0.027 ± 0.016
	JpTS	1.00 ± 0.00	0.00 ± 0.00	N.A.
		0.00 ± 0.00	0.00 ± 0.00	
	JpSM	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	JpNS	0.00 ± 0.00	0.51 ± 0.10	0.00 ± 0.00
		0.00 ± 0.00	0.0017 ± 0.0018	0.00 ± 0.00
	KrCJ	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Southern Pop.		0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.027
		0.00 ± 0.00	0.00 ± 0.00	0.0023 ± 0.0022
	CNFJ	0.00 ± 0.00	0.00 ± 0.00	0.44 ± 0.10
		0.00 ± 0.00	0.00 ± 0.00	0.0020 ± 0.0021
	TWPH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

HKSK	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CNZJ	0.00 ± 0.00	0.00 ± 0.00	0.48 ± 0.17
	0.00 ± 0.00	0.00 ± 0.00	0.0022 ± 0.0024

n: number of sequences obtained in this study; n_h: number of haplotypes revealed; SD: Standard deviation; IDS: Indel site; T_S: Site of transition; T_V: Site of transversion; *s*: Polymorphic site; SeqDi: Sequence divergence; h: haplotype diversity; π: nucleotide diversity.

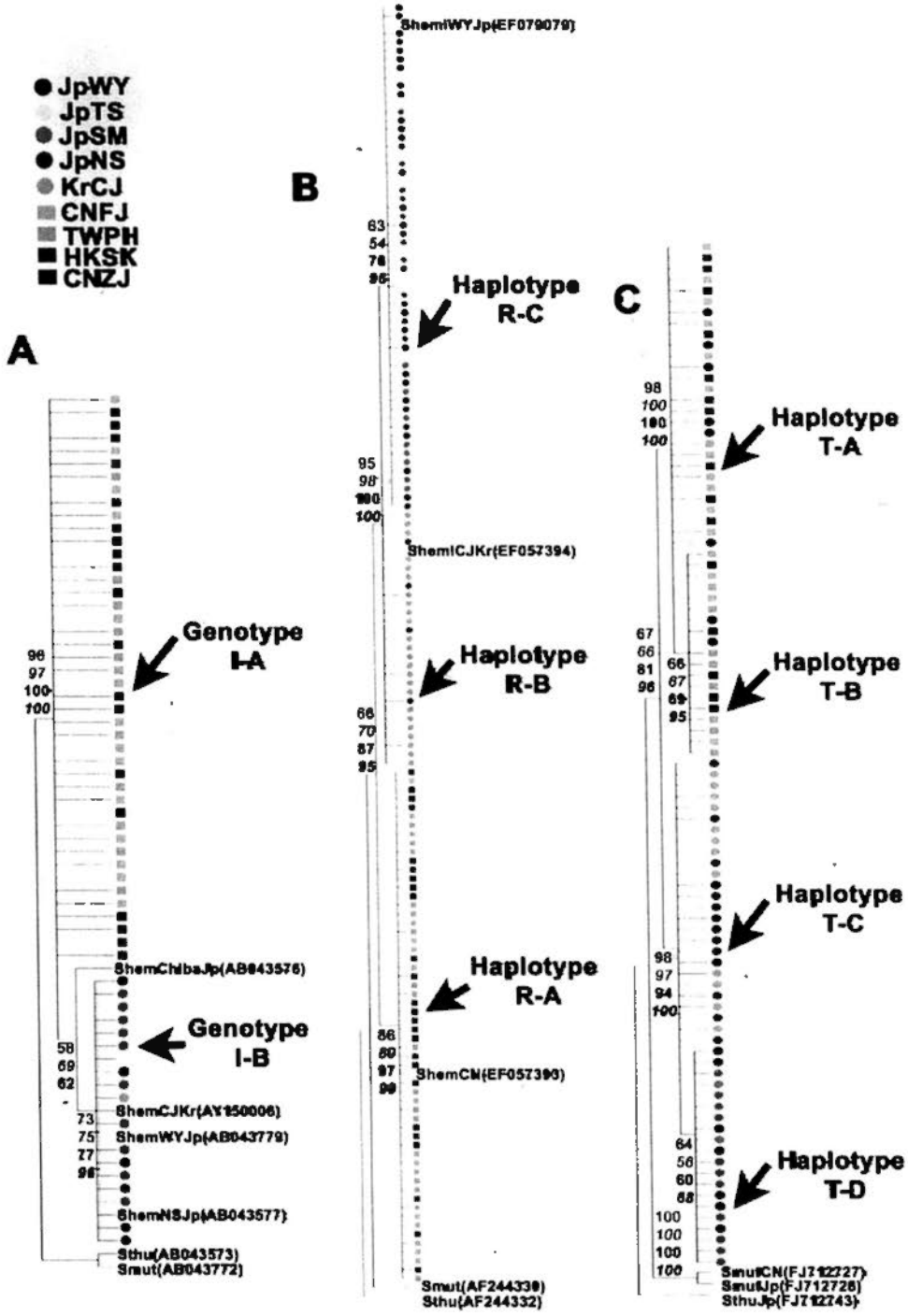

Figure 3.2 Neighbor-joining (NJ) tree illustrating the relationship among different populations of *S. hemiphyllum* based on (A) ITS2, (B) Rubisco spacer, and (C) TrnW_I spacer. The same topology of the trees was obtained for the maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) approaches. Bootstrap values (1000 replicates for NJ, MP and 100 for ML) for NJ (regular), MP (italic) and ML (bold) methods and the posteriori probabilities (bold and italic) of BI method are indicated at the nodes. Available sequences from GenBank are included in the trees for ITS2 (*S. muticum*: AB043772; *S. thunbergii*: AB043573; *S. hemiphyllum*: AB043576-7, AB043779, AY150006), Rubisco spacer (*S. muticum*: AF244330; *S. thunbergii*: AF244332; *S. hemiphyllum*: EF057393-4, EF079079) and TrnW_I spacer (*S. muticum*: FJ712727; *S. thunbergii*: FJ712742). Square and circle symbols refer to the populations of varieties *chinense* and *hemiphyllum* respectively.

spacer: h= 0.50, π = 0.0023) within the southern Chinese populations. The population of Wakayama, Japan (JpWY) demonstrated the most number of haplotypes (4) and the highest genetic diversity (h= 0.74, π = 0.027) among all the populations based on the marker TrnW_I spacer, while many populations (e.g. JpSM & TWPH) exhibited one haplotype and no genetic diversity based on all the three markers.

In concordance with the number of haplotypes/genotypes, there were two, three and four lineages in the phylogenetic trees corresponding respectively to the haplotypes/genotypes of ITS2, Rubisco and TrnW_I spacers (Fig. 3.2). Sequences of *S. hemiphyllum* were clustered into a single clade with a high confidence in all the three trees, while a clear reciprocal monophyletic relationship is evident between the two varieties based on ITS2 and Rubisco spacer.

The ITS2 sequences from individuals of the Korean and Japanese populations that were regarded as variety *hemiphyllum* were grouped into a subclade inside the larger clade of *S. hemiphyllum* consisting of the Chinese populations belonging to variety *chinense* (Fig. 3.2). The two ITS2 genotypes (lineages) that differed by five base pairs could be recognized (Fig. 3.3). Genotype I-A (GenBank accession: FJ712722), to which no identical ITS2 sequence could be found in GenBank, was

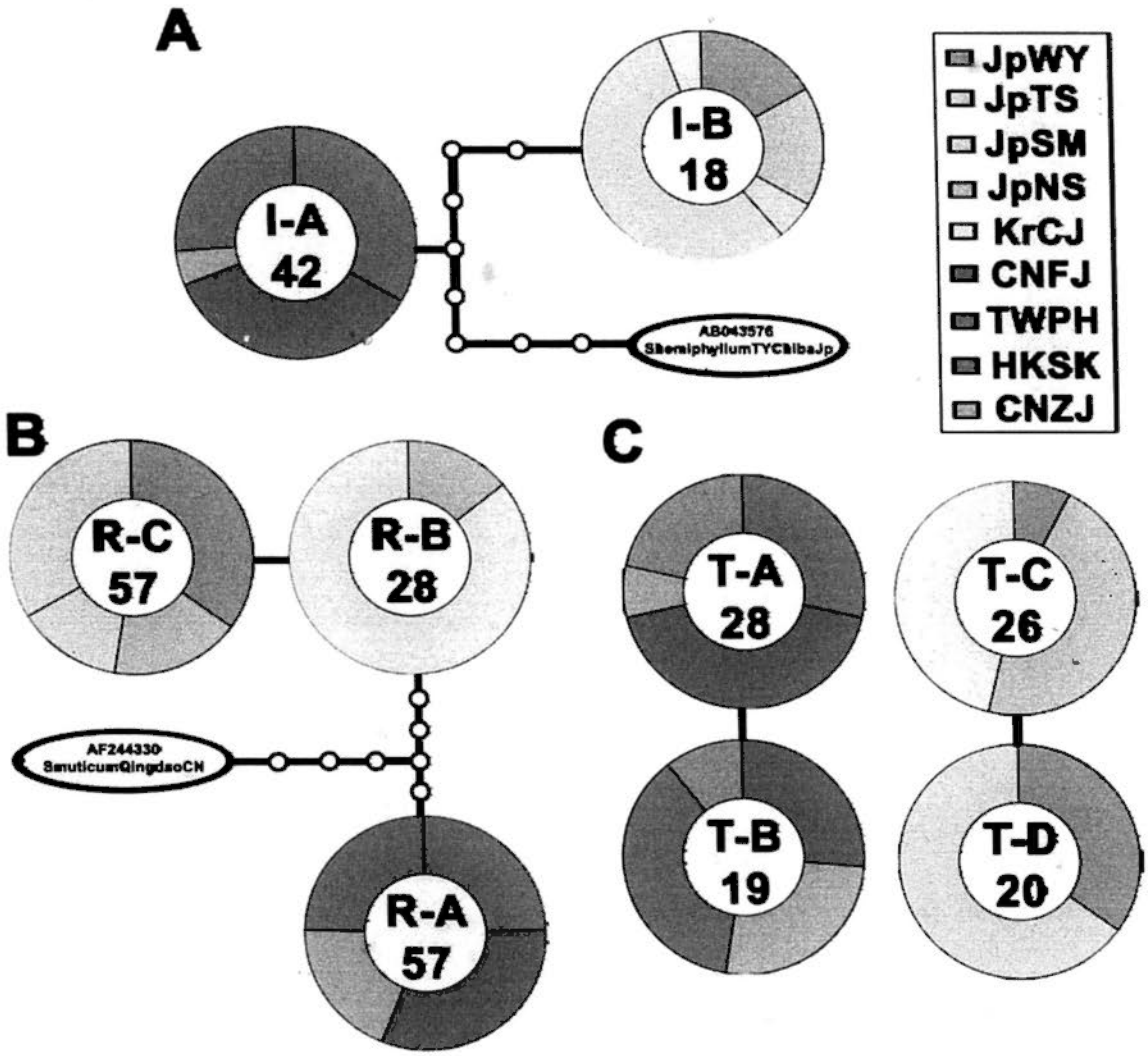

Figure 3.3 The TCS haplotype networks based on markers A) ITS2, B) Rubisco spacer and C) TrnI_W spacer. Sampling localities of the haplotype/genotype are indicated by the portions of the doughnut chart, while the name of the haplotypes/genotypes and the number of sequences are shown inside the chart. Each node represents a missing haplotype possessing one nucleotide change. Sequences of *S. thunbergii* (AB043573, AF244332) and *S. muticum* (AB043772, AF244330) are not shown, as they are not linked to the network. GenBank ITS2 sequences of *S. hemiphyllum* from JpNS (AB043577), JpWY(AB043779) and KrCJ (AY150006) are identical to genotype I-B, while Rubisco spacer sequences of *S. hemiphyllum* EF057393 from China, EF057394 from KrCJ and EF079079 from JpWY are the same as haplotypes R-A, R-B and R-C respectively. Refer to Table 3.1 for the acronyms used for the sampling localities.

found in the specimens from all the southern Chinese populations (CNFJ, TWPH, HKCK & CNZJ) (Fig. 3.4) representing variety *chinense* (Genotype I-A). Genotype I-B, in contrast, was identical to all the available sequences in GenBank, and was identified as belonging to variety *hemiphyllum* (Fig. 3.3). All these specimens came from the populations of the Korean and Japanese coasts (JpWY, JpTS, JpSM, JpNS & KrCJ) (Fig. 3.4). Although the sequence of *S. hemiphyllum* in Chiba of Japan (AB043576) (Stiger *et al.* 2003) was closely related to both genotypes I-A and I-B (Fig. 3.2) and differed from them in 6 and 9 steps respectively (Fig. 3.3), this sequence appeared problematic as it contained seven ambiguous base pairs out of the total of 573 bp as well as four polymorphic sites completely different from the sequences of all *S. thunbergii*, *S. muticum* and *S. hemiphyllum*.

For Rubisco spacer, the two varieties could be distinguished easily as each of them formed a single cluster with high statistical support (Fig. 3.2). As the sequence divergence was estimated to be 1.89% (3/159 bp) between the two varieties based on this marker, the divergent time of these varieties was estimated to be 0.56 Mya - 0.90 Mya. A subclade, consisting of almost all the Japanese populations, was found within the cluster of variety *hemiphyllum* in the tree and existed as a sister group with the specimens from Korean and some sequences of JpNS (Fig. 3.2). This subclade was

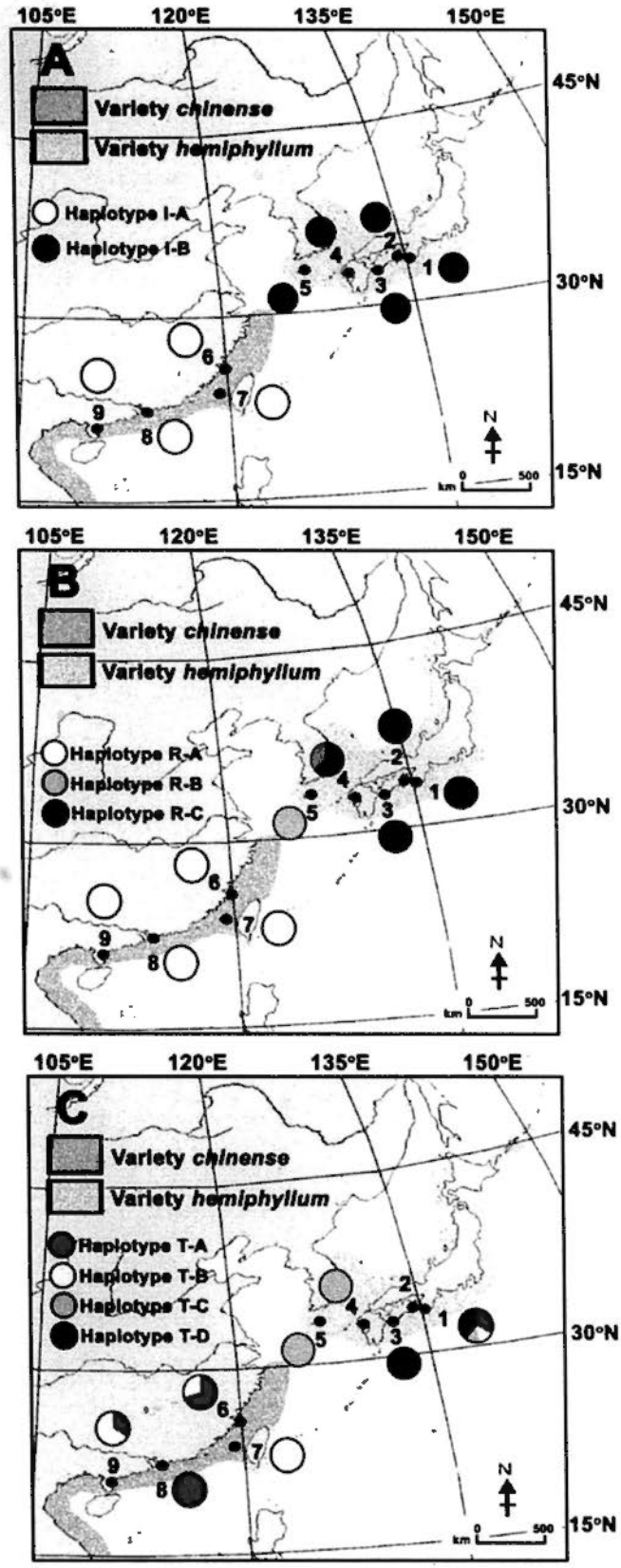

Figure 3.4 The distribution of the haplotypes/genotypes identified based on A) ITS2, B) Rubisco spacer and C) TrnW_I spacer. Refer to Table 3.3 for the number of haplotypes obtained in each population, and refer to Fig. 3.3 for the relationship among the haplotypes/genotypes.

estimated to have formed around 0.19 Mya – 0.30 Mya, given that the divergence of the Japanese populations within the subclade and the Korean populations outside the subclade was 0.63% (1/159 bp).

The three lineages corresponding to the three haplotypes revealed were consistent with those three (GenBank accession: EF057393, EF057384 and EF079079) obtained earlier by Cheang *et al.* (2008). The haplotype R-A, possessed by all the Chinese populations of this study (Fig. 3.4), was identical to EF057393, which was also collected from China (Cheang *et al.* 2008). The haplotype R-B that is the same as EF057384 based on a sample from Cheju, Korea, differed from haplotype R-A by five base pairs (Fig. 3.3). This haplotype was found mainly in Cheju Island of Korea, but also in Nagasaki of Japan (JpNS) (Figs. 3.2 & 3.3). The haplotype R-C, which was represented by the sequence EF079079 from Wakayama, differed from haplotypes R-B and R-A by one and six steps respectively (Fig. 3.3). This haplotype occurred entirely in the Japanese populations and was the major haplotype in Japan (Fig. 3.4).

Four lineages revealed could be identified in the TrnW_1 spacer tree (Fig. 3.2). They were grouped into two main clades, in which haplotypes T-A (FJ712723) and

T-B (FJ712724), consisting mainly of the Chinese populations, formed one clade while haplotypes T-C (FJ712726) and T-D (FJ712725), comprising the Korean and Japanese populations, formed the other clade. These two main clades possessed such a high sequence divergence (12 bp) that they could not be connected in the TCS network (Fig. 3.3). One base pair difference each was observed between the T-A/T-B and T-C/T-D pairs (Fig. 3.3). Haplotypes T-A and T-B were found mainly in the Chinese coast and they were distributed in areas of HKSK (100%), CNZJ and CNFJ, and areas of TWPH (100%), CNZJ and CNFJ, respectively. Both haplotypes also appeared in the JpWY population in Japan (Fig. 3.4). In contrast, haplotypes T-C and T-D could only be found in Japan and Korea. Haplotype T-C was associated with KrCJ (100%), JpNS (100%) and JpWY populations, whereas haplotype T-D was found in JpSM (100%) and JpWY only.

Regarding the population structure, the result of AMOVA suggested that a majority of genetic variation was attributed to between-varieties variance, indicating that the two varieties were indeed genetically distinct (Table 3.4). Both the "within variety, among populations" and the within population variations are much smaller, though statistically significant, and increased with the variability of the markers (Table 3.4). The result of F_{ST} pairwise differences among populations agreed well

Table 3.4 Results of AMOVA based on the three markers

Marker	Source of Variance	df	% of variation	Φ	P
ITS2	Between varieties	1	100.00	63.871	0.0068[a]
	Within variety, among populations	7	0.00	0.000	1.000[a]
	Within population	53	0.00	0.000	<0.001[b]
Rbc spacer	Between varieties	1	94.05	178.199	0.0098[a]
	Within variety, among populations	7	5.24	15.403	<0.001[a]
	Within population	126	0.71	2.545	<0.001[b]
TrnW_I spacer	Between varieties	1	74.18	167.368	0.027[a]
	Within variety, among populations	6	15.35	51.478	<0.001[a]
	Within population	81	10.47	41.191	<0.001[b]

[a] Random value larger than or equal to the observed value.

[b] Random value smaller than or equal to the observed value.

with that of AMOVA in exhibiting the differentiation of populations between varieties. Two distinct groups (the Japanese-Korea populations and Chinese populations) corresponding to the two varieties could be observed based on ITS2 data, and yet the Korean population could further be distinguished (F_{ST}= 0.955 - 1.000, p< 0.001) from the Japanese (except JpNS with a slight affinity to KrCJ, F_{ST}= 0.719, p< 0.001) and Chinese populations based on Rubisco spacer data (Table 3.5). The pattern of the genetic difference among populations based on TrnW_I spacer data seems to be a bit different from those based on ITS2 and Rubisco spacer. Almost all the populations, except JpNS-KrCJ, HKSK-JpWY, CNFJ-HKSK, CNFJ-CNZJ and TWPH-CNZJ pairings, were significantly different. The southern populations remained relatively homogenous compared to the northern populations in Japan and Korea, though there were significant pair-wise differences among the Chinese populations, such as HK-TW pair (F_{ST}= 1.000, p< 0.001) and HKSK-CNZJ pair (F_{ST}= 0.686, p <0.05). The northern populations in Japan and Korea were much more fragmented; for example, the JpSM was completely different from JpNS as well as KrCJ (F_{ST}= 1.000, p< 0.001). Population JpWY was less similar to the other Japanese populations (F_{ST}= 0.544 - 0.592, p< 0.001) but more similar to the populations in China (F_{ST}= 0.245 0.335, p< 0.05 or even N.S.) in TrnW_I spacer as compared to the other two markers (Table 3.5).

Table 3.5 F_{ST} pairwise differences among populations based on A) ITS2; B) Rubisco spacer, and C) TrnW_I spacer. P value was generated by 1023 times of permutation (* P<0.05, ** P<0.001). For acronyms used to represent the populations, refer to Table 3.1.

A.	JpWY	JpTS	JpSM	JpNS	KrCJ	CNFJ	TW	HK
JpTS	0.000							
JpSM	0.000	0.000						
JpNS	0.000	0.000	0.000					
KrCJ	0.000	0.000	0.000	0.000				
CNFJ	1.000*	1.000*	1.000**	1.000*	1.000			
TW	1.000*	1.000	1.000**	1.000*	1.000	0.000		
HK	1.000**	1.000	1.000**	1.000*	1.000	0.000	0.000	
CNZJ	1.000*	1.000	1.000**	1.000*	1.000	0.000	0.000	0.000

B.	JpWY	JpTS	JpSM	JpNS	KrCJ	CNFJ	TW	HK
JpTS	0.000							
JpSM	0.000	0.000						
JpNS	0.364*	0.250	0.396*					
KrCJ	1.000**	1.000**	1.000**	0.719**				
CNFJ	1.000**	1.000**	1.000**	0.966**	1.000**			
TW	1.000**	1.000**	1.000**	0.961**	1.000**	0.000		
HK	1.000**	1.000**	1.000**	0.961**	1.000**	0.000	0.000	
CNZJ	1.000**	1.000**	1.000**	0.955**	1.000**	0.000	0.000	0.000

C.	JpWY	JpSM	JpNS	KrCJ	CNFJ	TW	HK
JpSM	0.592**						
JpNS	0.544**	1.000**					
KrCJ	0.544**	1.000**	0.000				
CNFJ	0.335*	0.979**	0.976**	0.976**			
TW	0.321*	1.000**	1.000**	1.000**	0.592*		
HK	0.245	1.000**	1.000**	1.000**	0.160	1.000**	
CNZJ	0.260*	0.990**	0.985**	0.985**	0.225	0.167	0.686*

Direct sequencing data for the population of KrCJ and some of the individuals in JpNS was unsuccessful. Amplicons of 14 KrCJ specimens and five specimens of JpNS were successfully subjected to TA cloning (Table 3.6). The number of alleles revealed per specimen was in most cases larger than two (Table 3.6), indicating that there were intragenomic variations (i.e. variable repetitive region) in the ITS2 region of *S. hemiphyllum* rather than simply the heterozygous state of specimens. A total of 39 different alleles (GenBank accession: FJ712744 - FJ712782) were revealed (Fig. 3.5). Although there was a large number of allele per individual, a clear star-shape TCS haplotype with the sequences of variety *hemiphyllum* as the most abundant haplotypes was revealed (Fig. 3.5). This suggests that a majority of the ITS2 copies in KrCJ and JpNS were the sequence of variety *hemiphyllum*, albeit with many variants in one to three mutation event(s). A deletion of seven base pairs was recorded in one of the clones of KrCJ2 specimens, ant this haplotype could not be linked to the main TCS network (Fig. 3.5). It is worthwhile to note that there were at least two most parsimonious pathways (five steps), which were able to connect the haplotypes between the two varieties of *S. hemiphyllum* (Fig. 3.5). Some haplotypes such as two of the clones of JpNS8, though were not identical, were closely related to the sequences of the variety *chinense* (TWPH20, GenBank accession: FJ712722, in Fig. 3.5).

Table 3.6 List of specimens subjected to cloning with the total number of the clones and the haplotypes revealed. Refer to Table 3.1 for the acronyms used for the collection sites.

Specimen	n_{Clone}	n_h
KrCJ2	3	3
KrCJ3	3	2
KrCJ9	9	7
KrCJ10	4	2
KrCJ14	6	4
KrCJ15	3	1
KrCJ16	3	2
KrCJ17	1	1
KrCJ18	13	6
KrCJ19	4	3
KrCJ20	4	3
KrCJ21	6	4
KrCJ22	6	3
KrCJ23	3	2
JpNS3	1	1
JpNS4	2	2
JpNS6	5	3
JpNS7	5	4
JpNS8	3	3

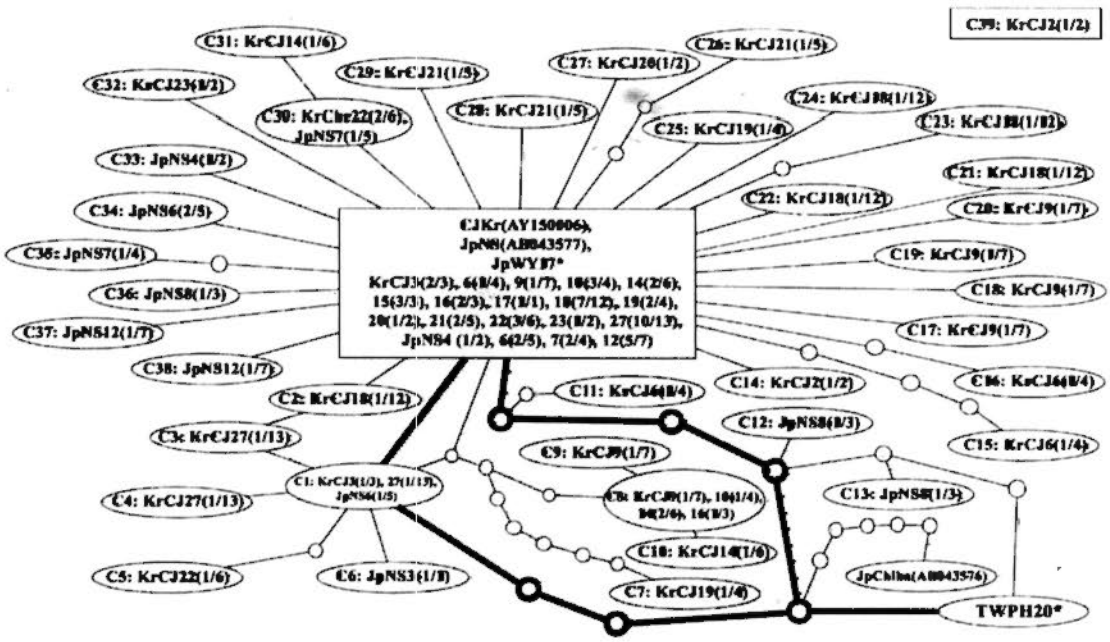

Figure 3.5 The TCS networks of clones of ITS2 amplicons amplified from KrCJ and JpNS populations. Sequences of varieties *hemiphyllum* and *chinense* (FJ712722), labeled with asterisks, and three GenBank available sequences AB043576, AB043577 and AY150006 are included as references. The thick lines represent the two most parsimonious pathways connecting the sequences of the two varieties. One of the clones of KrCJ2 is not connected to the main framework, because of a seven-step deletion event. Refer to Table 3.1 for the acronyms used for the sampling localities. (GenBank accessions of clones C1 – C39: FJ712744 – FJ712782)

3.4 Discussion

3.4.1 Variability of the three genetic markers

Engel *et al.* (2008) compared eight markers in the mitochondria genome, together with the Rubisco spacer in the plastid genome, for their performance in analyzing the phylogeny and phylogeographical pattern of nine laminarian and three fucoid species. The latter included no *Sargassum* species. Some markers were found to be suitable for within-family phylogenetic studies, while others were good at investigating intra-specific phylogeography. The TrnW_I spacer was shown to be one of the suitable markers to investigate the variation at the intraspecific level (Engel *et al.* 2008). Andreakis *et al.* (2007) also found that the mitochondrial *cox*2-3 spacer changed four and 14 times faster than the plastid Rubisco spacer and the nuclear LSU region, respectively, in the red algae *Asparagopsis* spp. The result of the present study agrees well with these findings and clearly shows that TrnW_I spacer exhibited the highest variability among the three markers considered (Table 3.3). Comparable to the result of Andreakis *et al.* (2007), the sequence divergence of TrnW_I spacer was 10 times higher than that of the nuclear ITS2 region, and 2.5 times higher than that of plastid Rubisco spacer.

The number of haplotypes revealed in the present study, however, was relatively fewer than those revealed in previous studies on other marine macroalgae. For example, there were four, 25 and 26 haplotypes in *Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon for the markers Rubisco spacer, LSU region and *Cox*2-3 spacer respectively, while there were one, 10 and 12 haplotypes for the same three markers respectively in *Asparagopsis armata* Harvey (Andreakis *et al.* 2007). The low number of haplotypes in *S. hemiphyllum*, as compared with that in the other algae, may be attributed to the difference in evolutionary rates in different taxonomic groups as well as among different genetic markers considered. Nevertheless, the detection of more haplotypes (four) in TrnW_I spacer compared to two in ITS2 and three in Rubisco spacer in this study indicates that the TrnW_I spacer is indeed able to reveal a finer-scale phylogeographic pattern in *S. hemiphyllum* to demonstrate a more recent evolutionary history of the species. The present study, thus, affirms the applicability of using mitochondrial TrnW_I spacer in studying the phylogeography of *Sargassum* species.

3.4.2 Genetic population structure

Whether the populations of a species were genetically structured or homogenous depends on the gene flow among populations, genetic drift and natural

selection (Freeland 2005). For the connected populations with sufficient gene flow, the genetic diversity is usually high while the population differentiation is low (Freeland 2005). In contrast, isolated populations with no gene flow possess low diversity and high population differentiation. The population of *S. hemiphyllum* appears to belong to the latter case as there is low genetic diversity (Fig. 3.3) and significant population structure (Fig. 3.5), where a majority of the genetic variance was found at the level of varieties for all the three markers (Fig. 3.4).

Germlings of *Sargassum* spp. have very restricted dispersal ability. Over 98% of the germlings of *S. spinuligerum* Sonder occurred within the range of 1 m beside the parent plants (Kendrick & Walker 1995), while the vast majority of the offspring of *S. muticum* were found within two to three meters of the parent plants (Deysher & Norton 1982). However, dispersal of *Sargassum* spp. may take the form of drifting weeds, as demonstrated to be possible for *S. muticum* (Deysher & Norton 1982) or *S. horneri* (Turner) C. Agardh (Komatsu *et al.* 2007). Drifting *Sargassum* individuals can become fertile (Norton 1977a). This specific mechanism may provide a chance for the dispersal of *Sargassum* individuals to moderate or longer distances, the degree of which depends on various factors such as water current (Norton 1992). Despite the seemingly isolated populations with low gene flow, the possibility of

long dispersal of *S. hemiphyllum* could not be completely ruled out (see sections 3.4.4 & 3.4.5).

3.4.3 The divergent time of the two varieties (lineages)

Sargassum hemiphyllum var. *chinense* and var. *hemiphyllum* examined in this study were shown to be genetically distinct based on ITS2 and Rubisco spacer (Fig. 3.2). On the other hand, there was a clear divergence between the Japanese-Korean (haplotypes T-C and T-D) and Chinese lineages (haplotypes T-A and T-B) based on TrnW_I spacer, although some specimens of the Chinese lineage were found to be mixed with those in JpWY (see further discussion in section 3.4.4). The reciprocal monophyly found at least in ITS2 and Rubisco spacer, which is associated with high pairwise F_{ST} values, could be attributed to a historical isolation by a relatively old barrier, in accordance with the framework postulated by Zink (2002) in inferring population history. It is also reasonable to assume that the divergences demonstrated, based on the three markers, may represent a single vicariant event that occurred in the evolutionary history of *S. hemiphyllum*. The divergence of the two varieties, however, was not detected in several earlier phylogenetic works on the genus *Sargassum* (Phillips & Fredericq 2000, Oak *et al.* 2002, Stiger *et al.* 2003, Phillips *et al.* 2005). Moreover, the time of divergence within genus *Sargassum*, which could

have helped to time the divergence of the two varieties found in this study, was not estimated in these studies for the two markers used (Rubisco spacer and ITS).

Calculated based on the Rubisco spacer sequences of Phillips and Fredericq (2000), the time of divergence (0.56 – 0.90 Mya) between the two varieties of *S. hemiphyllum* was found to lie between the early and middle Pleistocene, or in terms of a finer geological scale, the pre-Illinoian period in North America or the Cromerian complex in Europe. The oxygen isotopic-based eustatic sea level estimate (Miller *et al.* 2005) indicated a drop in sea level ranging from 50 m to 90 m below the present level during 0.56 – 0.90 Mya, with a maximum of over -120m reaching the level of the last glacial maximum (Miller *et al.* 2005). This drop in the sea level during the Pleistocene could lead to the exposure of land mass and the fragmentation of the marine habitat in NW Pacific (Voris 2000), which in turn caused the divergence of marine taxa that inhabited these areas (Liu *et al.* 2006, 2007, Tsang *et al.* 2008). Different genetic lineages between the Chinese and Japanese populations, similar to that shown in the two varieties of *S. hemiphyllum* in this study, were revealed in other marine species in NW Pacific such as fishes (Liu *et al.* 2006, 2007), shellfish (Yokogawa 1997) and barnacle (Tsang *et al.* 2008). Liu *et al.* (2007) proposed three putative refugia zones, the Sea of Japan, Yellow Sea and South China

Sea, in NW Pacific to account for the three corresponding lineages of the fish *Chelon haematocheilus* [Temminck & Schlegel, 1845] that diverged during the Pleistocene.

Instead of three refugia, the allopatric distribution of the two *S. hemiphyllum* varieties (Fig. 3.1) and their genetic distinctness may suggest two ancient refugia for this species. These refugia could be situated in southern and northern NW Pacific. The north refugium could be the region of the East China Sea and/or the Sea of Japan (Fig. 3.1). This region was suspected to be an enclosed to semi-enclosed marine region during the early Pleistocene due to the formation of land bridges connecting Taiwan all the way to the northern part of the Ryukyu Island chain (Ota 1998). This refugium could have harbored the ancestral populations of the variety *hemiphyllum*. In contrast, the ancestors of var. *chinense* could have originated from the southern refugium in the area of the South China Sea that was isolated from the East China Sea by the land mass formed between continental China and Taiwan (Ota 1998).

The divergence of haplotypes R-B and R-C of Rubisco spacer within variety *hemiphyllum* was estimated to occur in the late middle Pleistocene (0.19 Mya – 0.30 Mya). The distributions of these two haplotypes appear allopatric within Japanese and Korean populations (Fig. 3.4), with both haplotypes found in JpNS. This may

indicate that haplotype R-B may be derived around the area of Korea and its vicinity while haplotype R-C could have evolved in the Japanese region during the glacial oscillation in late middle Pleistocene. Towards the late Pleistocene, the sea level dropped to as low as -120 m compared to the present sea level (Voris 2000). The extensive East China basin was exposed, leading to the eastward expansion of the "paleo-coast" to as far east as the Ryukyu (Ota 1998). The large volume of discharge of the paleo-Yellow and paleo-Yangtze Rivers (Fig. 3.1) and its associated attributes such as low salinity and high sedimentation in the region of eastern East China Sea near the Tsushima Strait (Xu & Oda 1999) could have contributed to the fragmentation of the populations of var. *hemiphyllum*, since those attributes such as soft substratum do not favor the establishment of *Sargassum* species. This ecological barrier may block the dispersal between the much shrunken Yellow Sea and the Sea of Japan refugia, giving rise to further divergence of the two subclades based on the Rubisco spacer within variety *hemiphyllum*. Nevertheless, the above interpretation of the evolutionary history of *S. hemiphyllum* should be viewed with caution since some of the assumptions underlying molecular clock approach in estimating divergence time were called into question (Ayala 1997).

Besides the lowering of sea level as the initial putative cause of the divergence of the two varieties of *S. hemiphyllum*, the present ecological environment may help to reinforce/maintain the effect of this separation, resulting in their observed allopatric distribution. One possible factor that contributed to this reinforcement was proposed to be the freshwater discharge from the Yellow and the Yangtze Rivers in China (Cheang *et al.* 2008). The effect of the Yangtze River was found to extend as far east as the Tsushima Strait between Korea and Japan (Senjyu *et al.* 2006). There was an evident Yangtze Ring Haline Front developed in the Yellow and East China Seas during spring and throughout summer due to the freshwater discharge from the Yangtze River (Park & Chu 2006). This hyposaline condition, as well as the unstable substratum developed from sedimentation in the coast nearby are believed to exert adverse effect on the establishment of *Sargassum*, which in turn create a dispersal barrier preventing the contemporary contact between the two varieties or even between the two lineages within the variety *hemiphyllum*.

3.4.4 Introgressive hybridization

Haplotypes T-A and T-B of TrnW_I spacer, which originated in southern population and are believed to be the haplotypes of variety *chinense*, are also found in the specimens of JpWY that possess variety *hemiphyllum* haplotype in Rubisco

spacer (Table 3.7). These specimens may represent hybrids between varieties *hemiphyllum* and *chinense*, resulting from the cross of the individuals of variety *chinense* with those of variety *hemiphyllum*, leaving behind the haplotype of variety *chinense* in the population of variety *hemiphyllum*. This process is known as the introgressive hybridization (Anderson 1953).

Introgressive hybridization, which is the incorporation of the genes of one species into the populations of another species, has been documented in many marine species (e.g. moon jelly, Schroth *et al.* 2002, cichlid fish, Schelly *et al.* 2006, corals, Willis *et al.* 2006). These organisms were suspected to be recently diverged either allopatrically or sympatrically throughout the Pliocene to Pleistocene. While lineage sorting is incomplete and the reproductive isolation has not yet developed during the relatively short course of separation, either a recent or present secondary contact allowed the two recently diverged lineages to merge again so that the mitochondrial genome could be introgressed from one species to another. This situation is highly likely to happen in the two varieties of *S. hemiphyllum*, which diverged during the early to middle Pleistocene.

Table 3.7 List of the potential hybrids identified based on various markers and their corresponding clades labeled in Fig. 3.2. All specimens are from Wakayama, Japan (JpWY).

Specimens	Rbc spacer	TrnW_I spacer
JpWY5	R-C	T-B
JpWY6	R-C	T-B
JpWY7	R-C	T-A
JpWY8	R-C	T-A
JpWY9	R-C	T-A
JpWY10	R-C	T-A
JpWY11	R-C	T-A
JpWY12	R-C	T-A

Another piece of evidence that supports introgressive hybridization in *S. hemiphyllum* is the multiple copies of the ITS2 amplicons obtained during its amplification. Oak *et al.* (2002) and Stiger *et al.* (2003) both utilized ITS2 as the genetic marker in resolving the phylogeny within the subgenus *Bactrophycus* of genus *Sargassum*. None of them, however, has reported the problem of multiple copies of PCR products when amplifying the ITS2 region. Along with the fact that most of the specimens in this present study could yield unambiguous sequences through direct sequencing, this may suggest that a major haplotype dominates in most of the populations, whereas the individuals that could not be successfully sequenced in particular populations may consist of a pool of copies that differed from the major haplotype by a slight variation in their sequences (Fig. 3.5).

These different copies of ITS2 may have been unified or are in the process of being unified into a single type under the influence of the "molecular drive" of concerted evolution (Dover 1982), which is the conversion of multiple copies to a single type of DNA fragment in a species during evolution through the processes of unequal crossing-over and biased gene conversion (Dover 1982, Palumbi 1994). Although this homogenizing force leads to a single type of copies in various multigene families such as the ribosomal operons that contain the ITS region (Dover

1982), intragenomic variation of ITS region was still documented in various marine taxa such as crustaceans (Chu *et al.* 2001, Harris & Crandall 2000), sponge (Nichols & Barnes 2005), corals (LaJeunesse & Pinzón 2007), diatoms (Alverson & Kolnick 2005), seagrass (Valeria Ruggiero & Procaccini 2004) and seaweed (Serrão *et al.* 1999, Famà *et al.* 2000, Coyer *et al.* 2001). This is similar to the situation observed in the present study on *S. hemiphyllum*. One of the possible reasons accounting for the amplification of multiple copies of ITS2 is introgressive hybridization with closely related species during contemporary contact (Serrão *et al.* 1999, Coyer *et al.* 2001). In brown algal genera *Fucus* Linnaeus and *Macrocystis* C. Agardh, the rate of homogenization of concerted evolution was thought to lag behind the rates of recent and rapid radiation of the species and/or the rates of hybridization between two closely related species (Serrão *et al.* 1999, Coyer *et al.* 2001). It would not be surprising that this scenario could also happen in *Sargassum*, which is a member of the Phaeophyceae (brown algae) as well.

3.4.5 Area of hybridization and directionality of dispersal

The occurrence of putative hybrids in JpWY suggests that the southern Pacific coast of Honshu, Japan could be a hybrid zone of the two varieties of *S. hemiphyllum*. The introgression of the mitochondrial haplotype of var. *chinense* into those of var.

hemiphyllum but not *vice versa* indicates that the introgression is unidirectional from south to north. The dispersal from the southern var. *chinense* to the northern var. *hemiphyllum* populations was probably mediated by the Kuroshio Current, which originates from the eastern Philippines, headed to eastern Japan via eastern Taiwan, Ryukyu (Okinawa) and eastern Shikoku of Japan (Fig. 3.1).

Instead of germlings that disperse in a short range (Kendrick & Walker 1995), drifting thalli are more plausible means of transport with the Kuroshio Current. In addition to the observation of large mats of drifting *S. horneri* in the East China Sea, direct evidence of the transportation of the drifting *S. horneri* was also documented from the Zhejiang Province (the mouth of Yangtze River) to the eastern Kyushu of Japan (Komatsu *et al.* 2007). This transportation is likely to be mediated by the combined effect of the coastal current (most probably the Yangtze Ring Haline Front due to the freshwater discharge from the Yangtze River, Park & Chu 2006) and the Kuroshio Current. *Sargassum hemiphyllum* was also found in the drifting *Sargassum* assemblage right off the exact locality, i.e. Wakayama of Japan (Komatsu *et al.* 2007), where potential hybridization is recognized in this study, and southern Kyushu and Shikoku between March to July (Ohno 1984). A small number of the detached thalli of var. *chinense* from southern China could be transported occasionally to JpWY by

drifting along with the current, producing gametes that hybridize with var. *hemiphyllum* in JpWY. A similar situation was revealed in other marine species such as the intertidal barnacle *Tetraclita japonica* [Pilsbry, 1916], in which putative hybrids could be found in the southern Pacific side of Honshu, Japan (Tsang *et al.* 2008), an area that is geographically close to JpWY. A hybrid zone was also proposed for two species of *Mytilus*, *M. trossulus* [Gould, 1850] and *M. galloprovincialis* [Lamarck, 1819], in Hiura (Inoue *et al.* 1997), which is near Wakayama (JpWY).

The occurrences of multiple copies of ITS2 in the specimens of KrCJ and some of those of JpNS may suggest that the region near southern Korea and western Kyushu could be another hybrid zone. The hybrid produced here, however, might not be the hybrid between the two varieties. Rather, it may be the hybrid of the two lineages (haplotypes R-B and R-C) revealed based on the Rubisco spacer. These two lineages were undoubtedly more closely related than the two varieties of *S. hemiphyllum* and hybridization between the two lineages is highly probable.

Not much information was obtained so far for marine hybrid zone in NW Pacific Ocean, though the genetic population structure of some marine species in this

area has been elucidated (*cf.* Chiang *et al.* 2001, Zhang *et al.* 2006, Liu *et al.* 2006, 2007). The potential hybrid zone revealed in various marine organisms such as bivalve (Inoue *et al.* 1997), barnacle (Tsang *et al.* 2008) and *S. hemiphyllum* in this study may suggest a common hybrid zone of marine taxa in NW Pacific, from where the recently differentiated lineages have their secondary contact through transport by the Kuroshio Current. This postulation remains to be tested by carrying out more phylogeographic work on other marine organisms in the future. The application of nuclear marker in association with cytoplasmic markers appears critical in determining any hybridization event of the marine taxa, and in turn, the exact hybrid zone in NW Pacific Ocean.

Chapter Four: Phylogeography of invasive species *Sargassum muticum* in both its native and introduced ranges

4.1 Introduction

Biological invasion as a serious crisis is becoming increasingly recognized worldwide (Ruiz *et al.* 1997) and many macro and microalgae are involved (Boudouresque & Verlaque 2002). These non-indigenous organisms, by definition (Williamson & Fitter 1996), exert adverse effects and cause, in most cases, a permanent change in the recipient community. A phylogeographic approach is a powerful tool to infer their invasive history (Geller 1996, Geller *et al.* 1997), which in turn could provide information needed for a better management strategy to deal with them (Sakai *et al.* 2001). Phycological studies utilizing this approach have helped to reveal cryptic diversity of various invasive or introduced marine algae such as *Undaria pinnatifida* (Harvey) Suringar (McIvor *et al.* 2001, Voisin *et al.* 2005, Uwai *et al.* 2006a, Andreakis *et al.* 2007) as well as their invasive histories (Durand *et al.* 2002, Uwai *et al.* 2006b).

Sargassum muticum (Yendo) Fensholt is one of the most well known invasive species in the world (Rueness 1989, Fernández 1999, Karlsson & Loo 1999). However, there has been little information about the genetic variability of its invasive or native populations. *Sargassum muticum* was previously known as *S. kjellmanianum* Yendo forma *muticus* (see Yoshida 1983 for details), and was later

raised to the species level by Fensholt (1955). Among the three closely related taxa, *S. muticum* forma *longifolium* (Tseng & Chang) Yoshida (originally as *S. kjellmanianum* f. *longifolium*) was postulated as an ecological variant that occurred mainly in northern China (Critchley 1983a); *S. kjellmanianum* Yendo was suggested to be conspecific with *S. miyabei* Yendo and was thus transferred to *S. miyabei* (Yoshida 1978). *S. kjellmanianum* forma *muticus* (*S. muticum*), which is found mainly in the warm current affected Japanese coast, was suggested to differ morphologically from *S. kjellmanianum* (*S. miyabei*), which occurs in the cold current region, in its basal leaves and vesicles (Yendo 1907). *Sargassum kjellmanianum* f. *muticus* possesses larger leaves and muticous vesicles, when compared with the mucronate vesicles of *S. kjellmanianum* (Yendo 1907, Critchley 1983a). Okamura (1928) also revealed two forms of *S. kjellmanianum* sensu Okamura, which differ in their leaf sizes, and ascribed this to be the main difference between *S. kjellmanianum* Yendo and its forma *muticus*. This view, however, was rejected by Yoshida who considered all the specimens in Okamura's study to belong to *S. kjellmanianum* Yendo forma *muticus* (Critchley 1983a). By far, the most important character that distinguishes the two species is that *S. muticum* is monoecious and *S. miyabei* is dioecious, or more precisely, androgynous (Yoshida 1978). This character, however, could only be examined in the laboratory when the reproductive structure is well developed.

Sargassum muticum was first found introduced into the Pacific Northwest of North America in 1944 (Scagel 1956) and in the Atlantic coast of Europe in 1973 (Critchley *et al.* 1983). The suspected vector that carried *Sargassum muticum* across the oceans was the commercially imported Pacific oyster, *Crassostrea gigas*

[Thunberg 1793] that originated from Japan (Critchley & Dijkema 1984, Goulletquer *et al.* 2002, Mineur *et al.* 2007, for alternative view refer to Grizel & Héral 1991). Though eradication was attempted during its early colonization in southern England (Critchley 1983a), the expansion of its distribution range appears to continue (Harries *et al.* 2007a). Throughout the introduced range, the local marine floral communities were altered with (Harries *et al.* 2007b) or without (Karlsson & Loo 1999) substantial harmful effect on the recipient algal assemblages, and some stability was apparently achieved several years after introduction (Critchley 1983a, Critchley *et al.* 1990). While Japan is the only suspected source population of *S. muticum* in North America, there were two possible ways of introduction for the European populations since oysters were first imported into Europe (France) from both Japan and British Columbia of Canada (Grizel & Héral 1991). Canada, however, was generally believed to be the source of introduction in Europe as there was an uncontrolled transport of the Pacific oysters from British Colombia to northwestern France by air in the late 60s to early 70s (Farnham *et al.* 1973).

In the past decades, many researchers had focused on the basic biology and ecology of *S. muticum* (e.g. Deysher & Norton 1982, Espinoza 1990, Hwang & Dring 2002), through which to decipher the reason behind the rapid expansion of this species (Norton 1976, Andrew & Viejo 1998, Steen & Rueness 2004). Numerous eco-physiological characteristics, such as the eurythermal, euryhaline nature of the species (Norton 1977b, Hales & Fletcher 1989), timing of its reproduction during summer, competitive advantage offered by the higher growth rate of its germlings compared to that of other fucoid species (Steen & Rueness 2004), the capabilities for it to self fertilize and to retain its germlings on drifting parental thallus (Karlsson &

Loo 1999), were found and thought to aid this species in expanding its distribution range. A conceptual model, which integrated all these possible factors, has been synthesized to account for its expansion (Fernández 1999). However, all these attributes of *S. muticum*, whether they were valid or not, stemmed from the assumption that a single phylogenetic species or lineage was associated with all the introduced populations. This assumption needs to be verified by examining the genetic variability of *S. muticum* at its population levels. The aim of this study was therefore to assess the genetic diversity within this species, with emphasis on its native range and with a view to understand its invasive history, revealing any cryptic lineage(s) in the introduced populations.

In contrast to the ecological information, there has been limited information on the genetic variability of this famous invader (Stiger *et al.* 2003, Phillips *et al.* 2005, Zhao *et al.* 2008a). In the phylogenetic studies of the genus *Sargassum*, Stiger *et al.* (2003) and Phillips *et al.* (2005) found intraspecific variability of a few specimens of *S. muticum* from different localities based on ITS2 in the nuclear genome and *Rubisco* spacer in the plastid genome, respectively. The two specimens studied by Stiger *et al.* (2003) came only from Japan, while the two specimens studied by Phillips *et al.* (2005) were collected separately from Qingdao, China and San Francisco, USA. The sample sizes of these studies were obviously far from being comprehensive for an in-depth assessment of the genetic variability of *S. muticum* (Muirhead *et al.* 2008). A recent study assessing the intraspecific genetic diversity of *S. muticum* in Qingdao, China, documented a higher genetic variability among populations than within population based on ISSR and RAPD (Zhao *et al.* 2008a). The study, however, is only restricted to one province of China. The two

markers used were anonymous genotypic markers, which are unable to provide the information of dominance and have relatively low precision (Sunnucks 2000).

Markers from organelle genomes are widely applied in phylogeographic studies of marine algae. They have been successful in examining the genetic diversity as well as in identifying cryptic invasion in various groups of macroalgae (e.g. Zuccarello *et al.* 2002, Gurgel *et al.* 2004, Andreakis *et al.* 2007). This study, thus, utilized one marker from each of the organelle genomes, namely *Rubisco* spacer in CpDNA (Phillips *et al.* 2005) and *Trn*W_I spacer in MtDNA (Voisin *et al.* 2005), as well as the nuclear marker, ITS2 (Stiger *et al.* 2003), in assessing the intraspecific genetic diversity of *S. muticum*. Insights on the invasion history of this species could thus be gained which could contribute to an understanding of biological invasion in general.

4.2 Materials and Methods

4.2.1 Sampling, preservation and DNA-extraction of *S. muticum*

The native and introduced distribution ranges of *Sargassum muticum* were first compiled based on literature review (Kang 1966, Yoshida 1983, 1998, Phillips 1995, Kashenko 1999, Stæhr *et al.* 2000, Tseng & Lu 2000, Loughnane & Stengel 2002, Gałysheva 2004, Harries *et al.* 2007a). All records listing *S. kjellmanianum* Yendo f. *muticus*, *S. kjellmanianum* sensu Okamuna and *S. kjellmanianum* sensu Tseng et Chiang were considered to be *S. muticum* (Yoshida 1998). Specimens of *S. muticum*, which were identified based on the criteria of Yoshida (1983), were collected within both its native and introduced ranges (Fig. 4.1, Table 4.1), either by snorkeling or sampling during low tide. Specimens were collected 1 – 2 m apart to

Table 4.1 Collection details of *S. muticum* populations. Acronyms for the collection sites are also given. All the ITS2 and Rubisco spacer sequences obtained are identical. Refer to Fig. 4.1 for the number of TrnW_I spacer haplotype sequences and the location of these collection sites.

Localities	Acronym	Date of collection	Collectors	No. of ITS2 sequences	No. of Rbc spacer sequences
1. Tateyama, Chiba Prefecture, Japan (139°51'E, 34°59'N)	JpTY	24 Jan 07	D. Fujita	15	15
2. Yaizu, Shizuoka Prefecture, Japan (138°19'E, 34°51'N)	JpYZ	30 Jan 08	K. Matsuyama-Serisawa	7	7
3. Awajishi, Hyogo Prefecture, Japan (135°1'E, 34°34'N)	JpAJ	8 Apr 07	C.C. Cheang and P. O. Ang	11	13
4. Oojima, Yamaguchi Prefecture, Japan (132°26'E, 33°57'N)	JpOJ	3 Apr 07	C.C. Cheang and P. O. Ang	9	13
5. Jangheung, Chollanam-Do, South Korea (126°56'E, 34°27'N)	KrJH	3 May 07	H. G. Choi	6	8
6. Beidaihe, Hebei Province, China (119°31'E, 39°49'N)	CnBDH	10 May 05	C.C. Cheang and S. C. C. Suen	5	5
7. Rungcheng, and Weihai, Shandong Province, China (122°29'E, 37°09'N and 122°7'E, 37°30'N)	CnRC and CnWH	20 Jun 07 and 26 Jun 07	D. Duan	5	4
8. Qingdao, Shandong Province, China (120°21'E, 36°2'N)	CnQD	13 Jun 04	C.C. Cheang and P. O. Ang	5	5
9. Newcastle Island, Nanaimo, BC, Canada (123°56'W 49°10'N)	CanBC	20 May 07	K. Marr	6	6
10. Corona del Mar, CA, USA (117°52'W, 33°35'N)	USACA	24 Apr 07	J. Smith	10	13
11. Bergen, Norway (5°18'E, 60°23'N)	NorBe	4 Jul 04	K. Sjotun	5	8
12. Connemara, Ireland (9°56'W, 53°32'N)	IrCo	11 Dec 03	A. Critchley	5	5
13. Pembroke, UK (4°56'W 51°42'N)	UKPem	03 Dec 03	A. Critchley	0	0
14. Helgoland, Germany (7°52'E, 54°10'N)	GeHel	30 Jan 07	M. Mollis	8	10
15. Saint-Vaast-la-Hougue, France (1°16'W, 49°35'N)	FrSVLH	21 Jan 04	P. O. Ang	4	4
16. Rue du Caro, Brest, France and Perharidy, Roscoff, France (4°29'W, 48°33'N and 4°1'W, 48°43'N)	FrBr and FrRo	10 Apr 05	P. O. Ang	10	17
17. Pormenande, El Franco, Spain (6°49'W, 43°33'N)	SpPor	May 07	C. Fernandez	6	8

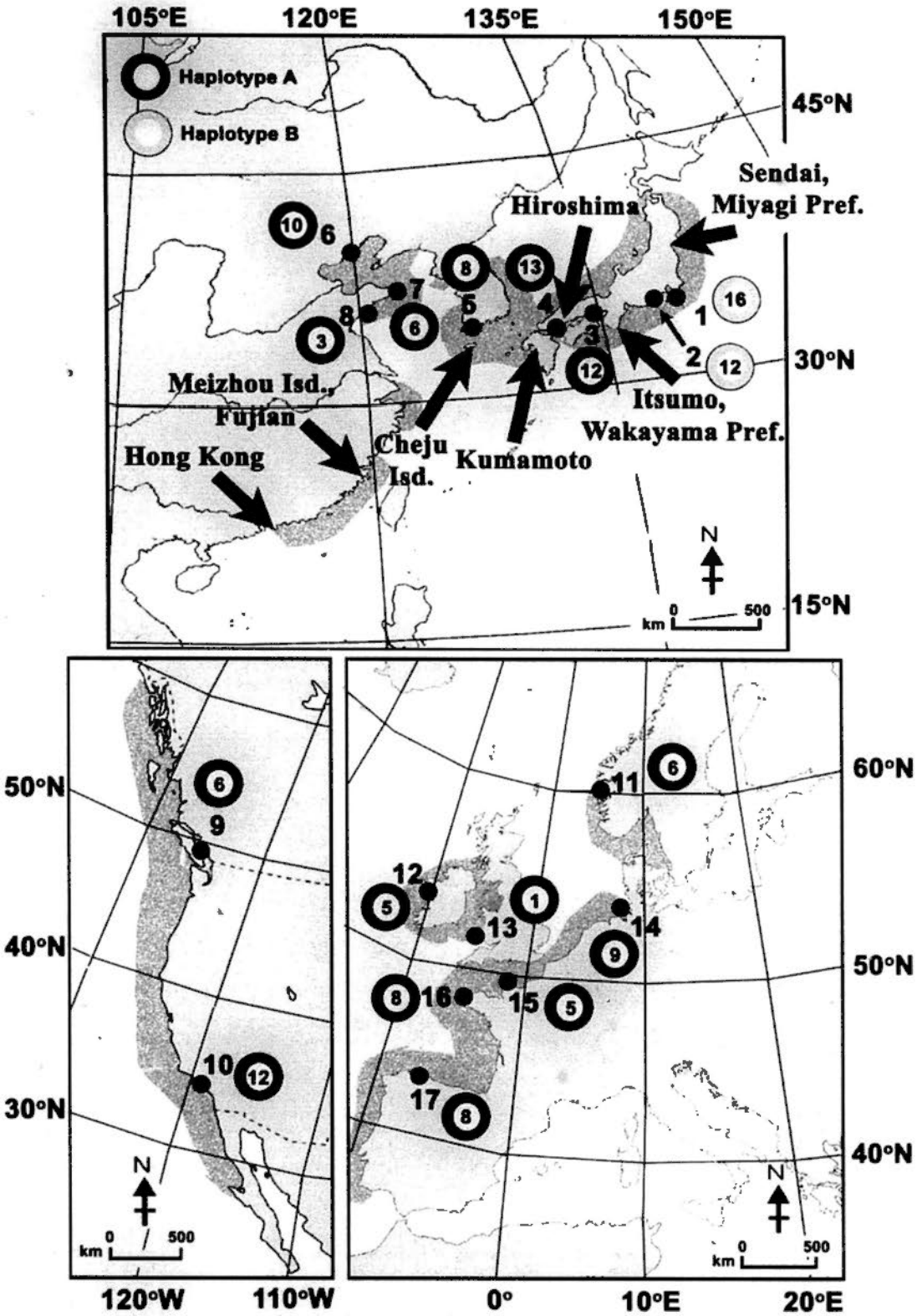

Figure 4.1 Distribution (shaded area) of *S. muticum* in its native range, the northwestern Pacific, as well as in the introduced range, the northeastern Pacific and the northeastern Atlantic. Sampling sites are indicated in numbers, and the number of specimens sequenced for TrnW_I spacer is shown inside the donut chart. Frequency of various haplotypes was indicated in the donut charts for each population. Refer to Table 4.1 for details of the sampling information.

avoid collecting individuals from the same mother plant, as *Sargassum* germlings are known to have a short dispersal distance (Kendrick & Walker 1995). Two to three vouchers haphazardly selected from each population were air dried and deposited in the Marine Science Laboratory Herberium, the Chinese University of Hong Kong.

About 1 g of samples from the new branches without epiphytes were cleaned with brushes and then preserved either in 95% ethanol or silica gel until molecular study was carried out. Genomic DNA of specimens was extracted by modified cetyltrimethylammonium bromide (CTAB) method (Protocol two of Ho *et al.* 1995). The extracted genomic DNA was then purified by GENECLEAN II Kit (BIO 101 Inc., CA, USA), and the manufacturer's instructions were followed (Yoshida *et al.* 2000).

4.2.2 Polymerase chain reaction (PCR) and direct sequencing

Spacer between the Rubisco large and small subunit in plastid DNA, spacer between TrnW and TrnI in mitochondrial DNA and ITS2 region in the nuclear genome, in which ITS2 region was the least variable and TrnW_I spacer the most, have been shown to effectively differentiate populations of *S. hemiphyllum* (Chapter 3). These markers were applied in this study (Table 4.2), using PCR profile as follows: initial 3 min at 96°C, 32 cycles of 40 s at 96°C, 30 s at 55°C 50 s at 72°C, final 3 min at 72°C. PCR products were subjected to commercial sequencing (Macrogen Inc., Seoul, South Korea). Twenty haphazardly selected specimens were sequenced twice in order to verify the results. Sequences obtained, together with the available sequences of *S. muticum* from GenBank, were visually edited and aligned

Table 4.2 The primers and PCR profile used for the three genetic markers: nuclear ITS2, plastid Rubisco spacer and mitochondrial TrnW_I spacer.

Markers	Forward/ Backward	Sequences
ITS2	F	ITS2FC2[1]: 5'-TTGTCGGGGAGGAGGAGG-3'
	R	25BR2-Reverse[2]: 5'-TCCTCCGCTTAGTATATGCTTA-3'
Rubisco spacer	F	RBCLF1[3]: 5'-GACCTTTAAAAGCAGCTTTAGAT-3'
	R	M1R[4]: 5'-CCCCATAGTTCCCTAATACGCATT-3'
TrnW_I spacer	F	TrnW-I-FC[5]: 5'-GTTCAAGTCCCTCTCTTTCTGT-3'
	R	TrnW-I-R[6]: 5'-CCTACATTGTTAGCTTCATGAGAA-3'

[1] modified from the sequence (GenBank Accession number: AY150006) of *Sargassum hemiphyllum* (Oak *et al.* 2002)
[2] based on Yoshida *et al.* (2000).
[3] modified from the sequence (GenBank Accession number: AF292068) of *Sargassum muticum* (Phillips *et al.* 2005)
[4] based on Cheang (2003).
[5] modified from the sequence (GenBank Accession number: AY494079) of *Fucus vesiculosus* (Oudot-Le Secq *et al.* 2006)
[6] based on Voisin *et al.* (2005).

using the software MEGA ver. 4 (Tamura *et al.* 2007). New sequences obtained were deposited in GenBank.

Heteroplasmy of the mitochondrial markers in *Fucus serratus* Linnaeus has been documented (Coyer *et al.* 2004). The problem of heteroplasmy could hinder the applicability of the mitochondrial marker in phylogenetic reconstruction or inference of demographic history (Posada & Crandall 2002). This problem was not significant in the present study, since all the sequences demonstrate clear single peak in their chromatograms (data not shown).

4.2.3 Statistical treatments

Neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) trees were generated by PAUP 4.0 Beta (Swofford 2000), and the Bayesian inference (BI) the program MrBayes v.3.12 (Ronquist & Huelsenbeck 2003), in order to analyze the phylogenetic relationships among populations. Akaike information criterion implemented in Modeltest ver. 3.7 (Posada & Crandall 1998) was applied, *a priori*, to estimate the appropriate substitution model to be used in the NJ, ML and BI methods. TVM model was found to be the optimal model for ITS2, and HKY model for both Rubisco spacer and TrnW_I spacer. For NJ, MP and ML methods, significance of the branching was assessed by 1000 bootstrapping replicates, in each of which 100 random taxa were added and the tree was heuristically searched by tree bisection-reconnection branch swapping method (Felsenstein 1985). For BI, two independent Markov-chain-Monte-Carlo searches with random starting points were conducted for each data set until the divergence between two runs became small and stationary. Trees were sampled every 100 cycles

in at least 1,500,000 generations with the burn-in value set to the later third-forth of the sampling trees, in which consensus was reached for the two parallel runs. *Posteriori* possibility was then calculated from the sampled trees to illustrate the statistical confidence for the BI tree. *Sargassum thunbergii* (Mertens ex Roth) Kuntze, *S. pallidum* (Turner) C. Agardh and *S. miyabei* were designated as outgroups, since they were all phylogenetically closely related to *S. muticum* (Stiger *et al.* 2003, Phillips 2005). The number of haplotypes was recorded for each sampling locality. The computer software TCS ver.1.13 (Clement *et al.* 2000) was utilized to generate the TCS haplotype network.

Time of divergence among the populations was estimated based on the molecular clock calibrated in Chapter Three. Based on the grouping of the introduced and native populations, the haplotype and nucleotide diversities were calculated. Both Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) neutrality tests were carried out to evaluate if the markers utilized are selectively neutral. This could be assessed by the significant negative values to reveal whether the populations have undergone recent range expansion (Chiang *et al.* 2004). The mismatch distribution analysis was also conducted to detect any history of sudden range expansion. This analysis is a chi-square based statistics testing whether the observed distribution pattern of the pair-wise difference among all pairs of haplotypes is significantly different from the expected one that is generated based on the sudden expansion model (Roger & Harpending 1992). All calculations of diversity indices, the neutrality and mismatch distribution tests were carried out using ARLEQUIN ver.3.0 (Excoffier *et al.* 2005)

4.3 Results

Although the taxonomic status of *Sargassum muticum* and *S. miyabei* in the older literatures is confusing due to the different applications of the name *S. kjellmanianum* (cf. Tseng & Chang 1954), reference to and citation of *S. muticum* and *S. miyabei* appear readily distinguishable in the most updated records and checklists for the NW Pacific (e.g. Yoshida 1998, Tseng & Lu 2000). Tseng and Lu (2000) reported the northern limit of *S. muticum* in Asia to be the Kurile Islands of Russia. However, this was not supported by Yoshida (1998) wherein this species was not recorded in Hokkaido of Japan nor in the Kurile Islands. The occurrence of *S. muticum* in this region, thus, remains questionable. The northern limit of the distribution of *S. muticum* is likely to be, by consensus, the northern Honshu of Japan (41°N) (Fig. 4.1). The southern limit, again based on Tseng and Lu (2000), is around the Guangdong Province of southern China (23°N). Critchley (1983a) doubted if the records in southern China such as Fujian and Guangdong Provinces were based on misidentified materials. Attempts to collect *S. muticum* in Fujian and Guangdong Provinces (including Hong Kong) were not successful. Some materials from Meizhou Island, Fujian Province (Fig. 4.1) localities, where previous records of *S. muticum* had been reported, resembled *S. muticum* morphologically. But sequences obtained from their ITS2 region, when blasted against those in the GenBank, indicate that they are unlikely to be *S. muticum* (data not shown). Materials from southern China identified as *S. muticum* in CK Tseng Herbarium in Qingdao, China would need to be rechecked to confirm the southern distribution range of *S. muticum*.

In its introduced range, *S. muticum* is distributed from South-east Alaska (55°-59°N) (Lindstrom 1977) to the Mexican coast (28° N) (Espinoza 1990) in

Pacific coast of North America (Fig. 4.1). In contrast, the latitudinal range of its distribution in the European coast started further north, ranging from Norway (62° N) (Rueness 1989) to Northern Spain (43°N) (Fernández 1990). More recently, its southern distribution range has been reported to reach the southern shore of Portugal near Faro (36°N) (R. Santos, personal communication). This species was also reported in the Mediterranean coast of Italy (45° N) (Curiel *et al.* 1998).

Among the seven populations from its native range in northwest Pacific and nine populations from the introduced areas in North America and Europe, a total of 117, 141, and 140 sequences from ITS2, Rubisco spacer and TrnW_I spacer regions respectively were obtained (Table 4.1). The aligned sizes of ITS2, Rubico spacer and TrnW_I spacer regions were 580 bp, 297 bp and 177 bp respectively. Based on all these markers, highly homogenous genetic population structure was detected (Fig. 4.2). All *S. muticum* sequences from various populations formed a single cluster in the NJ and MP trees of ITS2 and Rubisco spacer (Fig. 4.2A and B), while two clades, one of which consisting of all the specimens from Tateyama and Yaizu of Japan, could be recognized based on the TrnW_I spacer (Fig. 4.2C).

The ITS2 sequences of NrDNA, the most conserved marker among the three markers used (Chapter 3), were shown to be identical in all samples from the different localities, both within the native and the introduced ranges (Fig. 4.3A). This sequence is identical to the sequences obtained from GenBank (AB043503 and AB043772-4), which were based on specimens of *S. muticum* from Chiba (the prefecture of JpTY), Miyagi, Hokkaido in Japan and Cheju Island, South Korea as well as the sequence of *S. miyabei* from Hokkaido, Japan (AB043502) (Fig. 4.3A).

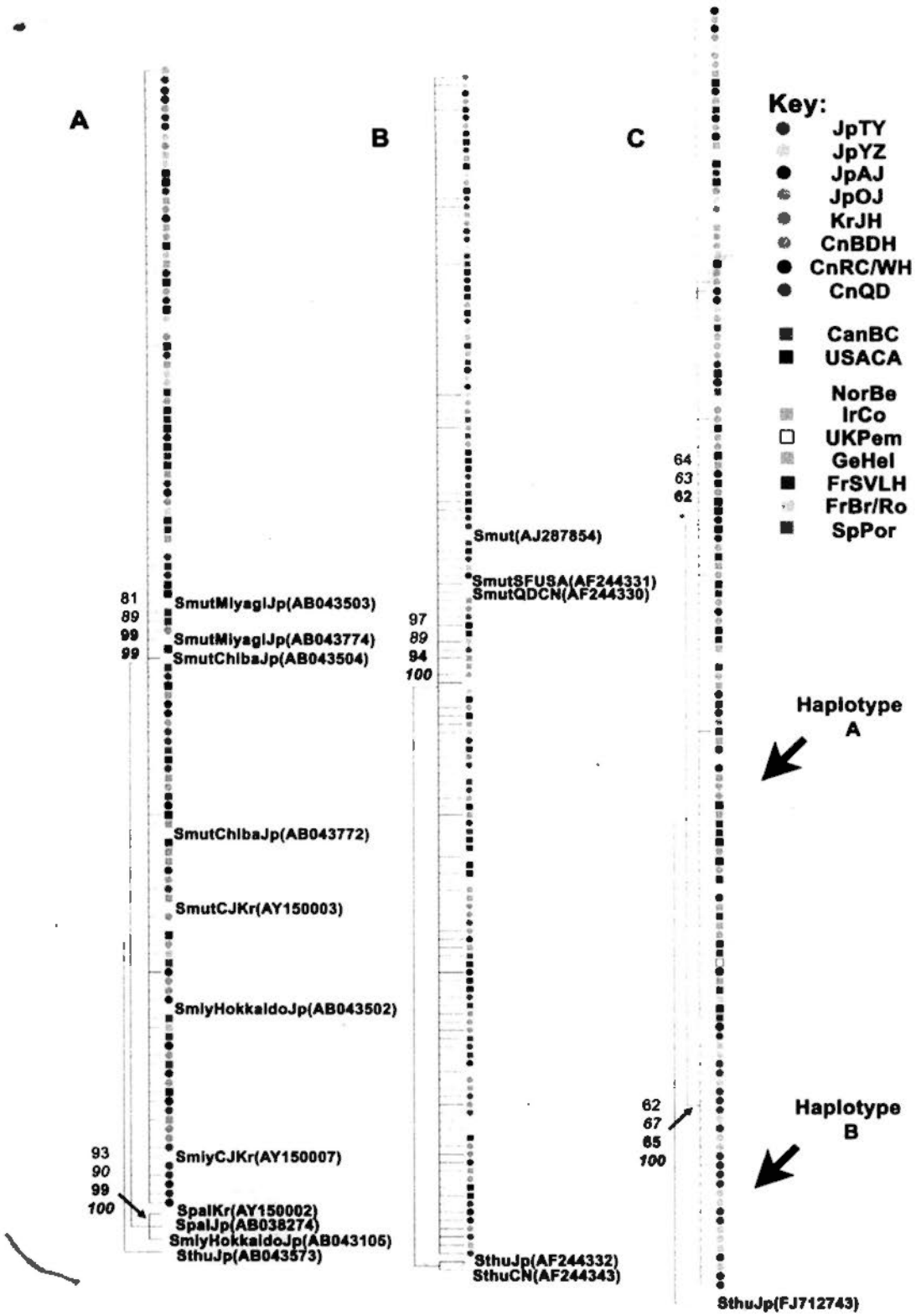

Figure 4.2 Neighbor-joining (NJ) tree illustrating the relationship among different populations of *S. muticum* based on (A) ITS2, (B) Rubisco spacer, and (C) TrnW_I spacer. The same topology of the trees was obtained for the maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) approaches. Bootstrap values (1000 replicates for NJ, MP and 100 for ML) for NJ (regular), MP (italic) and ML (bold) methods and the posteriori probabilities of BI (bold and italic) method are indicated at the nodes. Available sequences from GenBank are included in the trees for ITS2 (*S. muticum*: AY150003, AB043503-4, AB043772-4; *S. miyabei*: AB043105, AB043502, AY150007; *S. pallidum*: AB038274, AY150002; *S. thunbergii*: AB043573), Rubisco spacer (*S. muticum*: AF244330-1, AF292068, AJ287854; *S. thunbergii*: AF244332, AF 244343) and TrnW_I spacer (*S. thunbergii*: FJ712742).

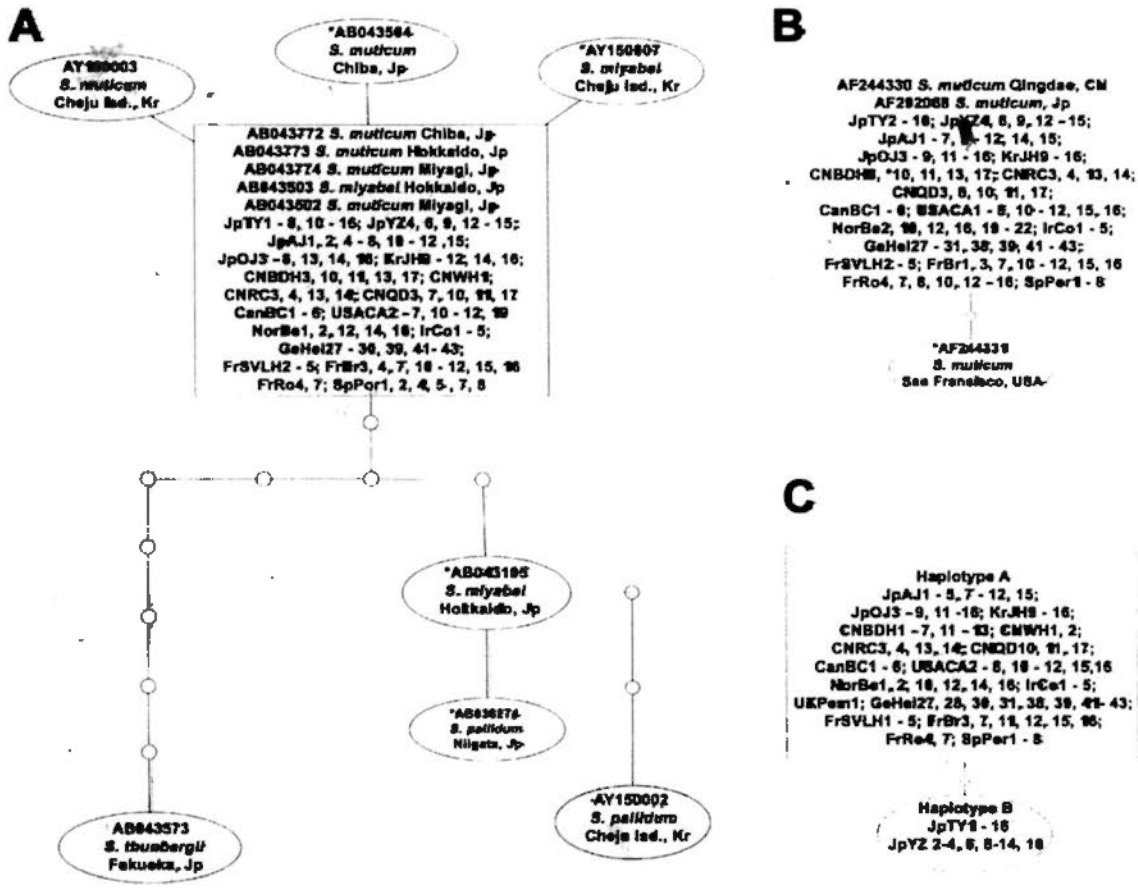

Figure 4.3 The TCS networks based on the markers A). ITS2, B). Rubisco spacer and C). TrnI_W spacer. Sampling localities of the haplotype are shown inside the rectangles (ancestral) and the ellipses (derived), while each node represents a missing haplotype possessing one nucleotide change. Sequences of *S. thunbergii* (AF244332-3) were not shown, as they were not linked to the network. Sequence of *S. muticum* of Rubisco spacer (AJ287854) was incomplete, hence was also excluded from the networks. Refer to Table 4.1 for the code for the localities. The ambiguous sequences with multiple peaks were labeled with asterisk.

Three other sequences (AY150003, AB043504 *of S. muticum* from Cheju Is. and Chiba respectively; AY150007 of *S. miyabei* from Cheju Is.), which were clustered within the clade of *S. muticum* (Fig. 4.2A), showed a one base pair difference from the above sequences of all the present *S. muticum* specimens (Fig. 4.3A). Sequences of other out-group species, such as *S. pallidum*, differed from those of *S. muticum* by at least four base pairs in ITS2 (Fig. 4.3A). Sequences downloaded from GenBank (AB043504, AY150007, AB043105, AB038274) being ambiguous sequences with at least one multiple peak have to be noted (Fig. 4.3A).

All the sequences of Rubisco spacer obtained in this study were identical with those reported for *S. muticum* from Qingdao, China and an unknown site in Japan (AF244330 and AF292068) (Fig. 4.3B). All specimens from the introduced areas shared identical haplotype with those from the native populations (Fig. 4.3B). Another sequence of *S. muticum* (AF244331) from San Francisco, USA, which is ambiguous, was different from the main haplotype of *S. muticum* by two base pair (Fig. 4.3B).

As the most variable marker among the three markers studied, two haplotypes of TrnW_I spacer were obtained in this study. Haplotype A differs from haplotype B by two base pairs (Fig. 4.3C). These two haplotypes appear reciprocally monophyletic (Fig. 4.1). Haplotype A was the most dominant haplotype (GenBank accession: FJ712727), which could be found in samples of *S. muticum* from all the introduced areas plus almost all those from its native range (Fig. 4.1). Haplotype B (FJ712728) was demonstrated in all specimens from Tateyama and Yaizu, Japan but not from any other localities (Fig. 4.1). The divergence time for the two lineages

(haplotypes) was estimated to be 0.29 Myr - 0.48 Myr, within the middle Pleistocene.

As there was only one haplotype detected in the ITS2 and Rubisco spacer regions, neither the diversity indices, the neutrality test nor the mismatch distribution analysis could be carried out for these two markers. In the case of TrnW_I spacer, only native populations could be analyzed since none of the nucleotide substitution was recorded in samples from the introduced range. Within the native populations of *S. muticum*, the haplotype diversity (0.3652 ± 0.0550) was high compared to the nucleotide diversity (0.0041 ± 0.0035). Both Tajima's D test and Fu's F_S test of neutrality tests yielded positive values with $p > 0.05$ (Table 4.3), indicating that the spacer region investigated is selectively neutral and the recent range expansion was not likely within the native range of *S. muticum*. For the mismatch distribution analysis, the probability of the sum of squared deviation (SSD) was not small enough to reject the null hypothesis that the observed pairwise difference among haplotypes was the same as the expected difference generated by the model of the sudden range expansion (Table 4.3). A bimodal mismatch distribution was obtained, however, rather than a typical unimodal distribution of a suddenly expanded population (Fig. 4.4).

Table 4.3 Summary of genetic diversity, neutrality test and mismatch distribution analysis for the native populations of *S. muticum* examined

	Native populations
Genetic diversity	
h ± SD	0.3652 ± 0.0550
π ± SD	0.004127 ± 0.0035
Neutrality test	
Sample size	80
Tajima's D test	1.25, p=0.88 (N.S.)
Fu's F_{ST} test	3.13, p=0.90 (N.S.)
Mismatch distribution analysis	
Sum of Squared Deviation	0.13, p=0.073 (N.S.)
Raggedness index	0.67, p=0.196 (N.S.)

C.I. = confidence interval; N.S.= non-significant, *h:* haplotype diversity; SD: standard deviation; π: nucleotide diversity

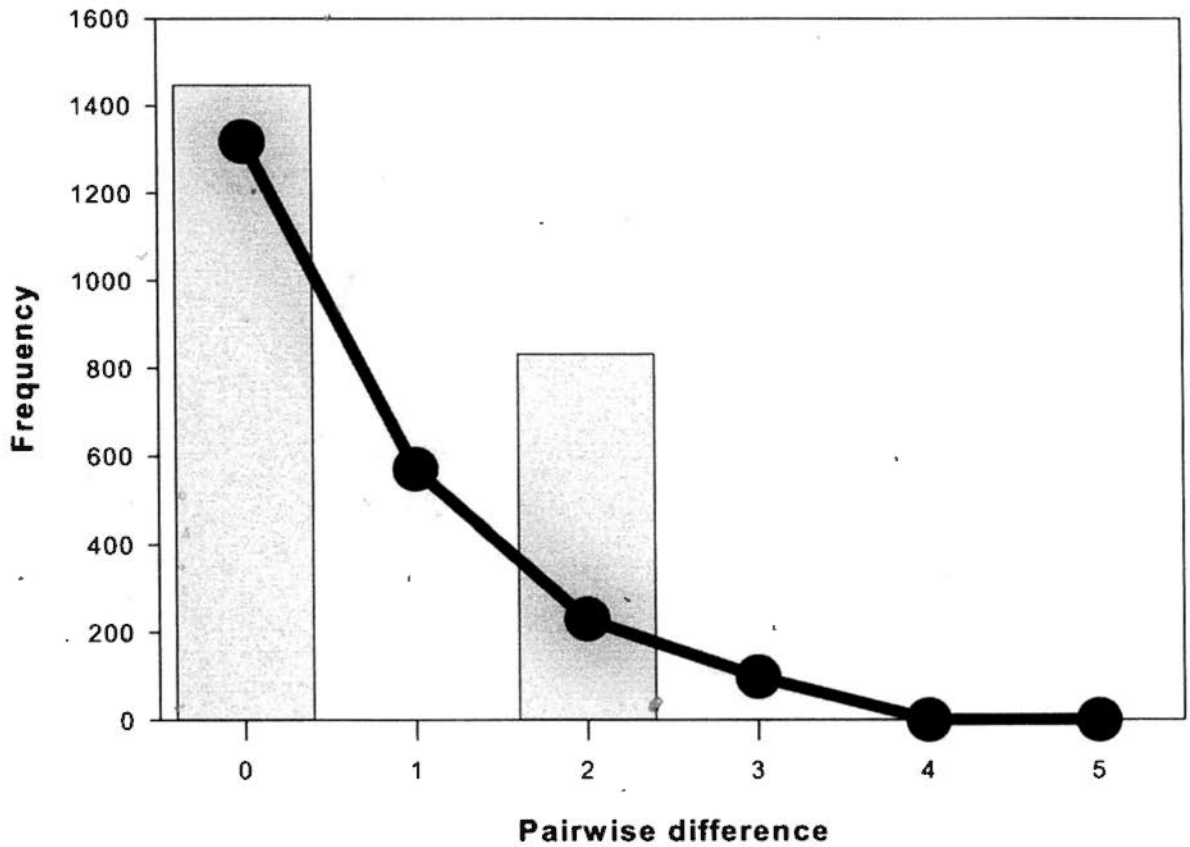

Figure 4.4 Observed (bars) and expected (line) mismatch distributions of the native populations of *S. muticum* under a model of sudden demographic expansion for the TrnW_I spacer. Refer to Table 4.3 for details of the parameters revealed by this mismatch distribution analysis.

4.4 Discussion

4.4.1 Confusion between *S. muticum and S. miyabei.*

Although *S. miyabei* is phylogenetically the most closely related species to *S. muticum* (Stiger *et al.* 2003), it has not been examined in more details by other researchers (Critchley 1983a). The inclusion of this species in the analysis of ITS2 sequences reflected that there were misidentifications of this species, in which at least one GenBank sequence AB043502 (Hokkaido) was identical with the sequences of *S. muticum*. Another sequence of *S. miyabei*, AY150007 (Cheju Is.) that possess one base pair difference, may also belong to *S. muticum*. Misidentification is highly likely especially when the reproductive organs were not yet developed. Scagel (1956) raised a similar concern with regards to the misidentification of *S. hemiphyllum* as *S. muticum* and suggested the use of diagnostic feature of holdfast to address this question. The holdfast of *S. hemiphyllum* is ramifying and that of *S. muticum*, discoid in shape. This same diagnostic feature may also be used to differentiate between *S. muticum* and *S. miyabei* in which the holdfasts of *S. miyabei* have short (<1 cm) creeping extensions (Yoshida 1978, Yoshida *et al.* 2000). Compared to the different histological characters of *S. muticum* identified by Fensholt (1955), this morphological character is operationally more convenient in field identification. This would have contributed to more successful discrimination between materials belonging to *S. muticum* and *S. miyabei* in the records in NW Pacific (Yoshida 1998, Tseng & Lu 2000). Based on the phylogenetic data using three markers carried out in this study, all the materials collected from the introduced range of *S. muticum* were truly *S. muticum*, and *S. miyabei* was absent.

4.4.2 Intraspecific variation and population structure of *S. muticum*

The intraspecific variation of *S. muticum* exhibited in the studies of Stiger *et al.* (2003) and Phillips *et al.* (2005) may not reflect the variation occurred among the populations, since the ITS2 sequence of Chiba (AB043504) and the Rubisco spacer sequence of San Francisco (AF244331) are ambiguous sequences. These ambiguous sequences could attribute to factors like the heterozygoticity or the intragenomic variant of the gene family (Dover 1982). These two sequences should be excluded from the interpretation of the genetic variation at the population level (Harris & Crandall 2000).

The ITS2 sequence of Cheju Isd., South Korea (Oak *et al.* 2002), which is an unambiguous sequence, possess one base-pair difference from the other *S. muticum* sequences. It is geographically close to the sampling locality KrJH of this study (Fig. 4.1). However, no such sequence was obtained in any localities in this study (especially in KrJH), suggesting that this sequence may be in minority. The intraspecific variation revealed based on markers of ITS2 and Rubisco, thus, appears extremely low.

The finding of two genetically distinct haplotypes of TrnW_I spacer, haplotypes A and B found in the Seto inland sea and the eastern Honshu in Japan respectively, is concordant with the observation by Yoshida (1983) that there are two forms of *S. muticum* in Japan. One is the lectotype specimen of *S. kjellmanianum* forma *muticus* Yendo, collected from Itsumo, Wakayama Prefecture (Fig. 4.1) and possesses 3 cm long basal leaves densely covering the young shoot; while the other

form, with longer (up to 5 cm) and more sparsely emerged basal leaf compared with the typical form, was collected in Seto Inland Sea (Yoshida 1983). In this study, the specimens with haplotype B may correspond to the typical *S. muticum*, while haplotype A could be associated with the non-typical *S. muticum* with larger leaves. The two morphologically different forms originally described as *S. kjellmanianum* (*S. miyabei*) and its forma *muticus* (*S. muticum*) by Okamura (1928), though they were suggested to be conspecific to *S. muticum* by Yoshida (in Critchley 1983a), may correspond to specimens belonging to the two lineages revealed in this study. The morphological difference described by both Yoshida and Okamura, however, was not observed in all the voucher specimens of both haplotypes examined in the present study, the basal leaves of which are all smaller than 2 cm in length (data not shown). While this may be due to differences in the growing stage of the present specimens examined, the fact that these two recently diverged cryptic lineages could still be sharing an undifferentiated phenotype could not be ruled out.

Populations of *S. muticum* found along the coast of northern China possess identical haplotype for the three markers with those from central Japan. This suggests that *S. muticum* found in these two regions are conspecific. This finding is consistent with the postulation of Yoshida (1978) that *S. muticum* f. *longifolium*, which is restricted to the Gulf of Bohai and Yellow Sea of China (Tseng & Chang 1954), is probably conspecific to *S. muticum*.

4.4.3 The past demography of *S. muticum* in NW Pacific

Based on the TrnW_I spacer, there was unlikely a recent demographic expansion of the populations in the native range of *S. muticum*, as shown by the

neutrality tests (Table 4.3). For mismatch distribution analysis, the *p*-value of SSD appears marginal (p=0.073), and the raggedness index (r=0.67), though not significant, was high compared to the studies on other organisms (e.g. r=0.008-0.095 for barnacle, Tsang *et al.* 2008, r=0.0004-0.001 for anemonefish, Timm & Kochzius 2008). In addition, there was a bimodal mismatch distribution rather a typical unimodal one, again suggesting no sudden demographic expansion of the native *S. muticum*. Following the scheme proposed by Zink (2002) in classifying the evolutionary history of the organisms, lineages that show reciprocal monophyly probably possess a history of isolation. As the populations with the two haplotypes of TrnW_I spacer appear reciprocally monophyletic with a slight variation (2/177 bp), it is reasonable to postulate that the lineages with these two haplotypes diverged recently (during the middle Pleistocene). This divergence could be related to the sea level fluctuation during the Quaternary period, as proposed for the closely related species *S. hemiphyllum* (Cheang *et al.* 2008). The separation of the populations due to the formation of ancient refugia in NW Pacific has been postulated to lead to the divergence of *S. hemiphyllum* (Cheang *et al.* 2008). The area of the refugium for *S. muticum*, however, remains unclear. Considering the present distribution of the two lineages covering the region from Northern China, South Korea to the Honshu of Japan, we can deduce that the possible area of refugium for haplotypes A could be the Sea of Japan basin and/or the East China Sea basin. These areas were hypothesized to be isolated during the middle Pleistocene (Ota 1998), and were involved in the lineage differentiation of various marine lives such as the fish *Chelon haematocheilus* [Temminck & Schlegel, 1845] (Liu *et al.* 2007), the Dall's porpoise *Phocoenoides dalli* [True, 1885] (Hayano *et al.* 2003) and *S. hemiphyllum* (Chapter 3). The ancestor of haplotype B may, in contrast, may have survived through the

glacial period on the Pacific side of the Honshu, Japan, the water body of which is thought to be disconnected from the Sea of Japan (Ono 1990 in Hayano *et al.* 2003).

4.4.4 Genetic diversity of *S. muticum* compared to other invasive species

In contrast to other invasive marine seaweeds (e.g. McIvor *et al.* 2001, Voisin *et al.* 2005) in which cryptic diversity has frequently been discovered, *S. muticum* exhibits no cryptic diversity in the introduced populations. All specimens from the introduced range were identical in terms of the sequences of the three markers from various genomes. Whether the founder effect is significant in the introduced populations remains unknown, since the genetic variability of the native populations was extremely low compared to other well known invasive macroalgae such as *Codium fragile* (Suringar) Hariot (Provan *et al.* 2008) and *Undaria pinnatifida* (Harvey) Suringar (Uwai 2006a,b). Introduced *S. muticum* exhibits only one haplotype for all the three markers used in this study, while the number of haplotypes found in the introduced regions of various other invasive macroalgae ranges from 1 to 20 (Table 4.4), depending on the variability of the markers examined. No concrete evidence of multiple introductions was found in *S. muticum*, unlike that, for example, demonstrated in *Undaria pinnatifida* (Voisin *et al.* 2005). Moreover, *S. muticum* does not exhibit hybridization, which occasionally occurs in other invasive species such as *Caulerpa racemosa* (Forsskål) J. Agardh and *C. taxifolia* (M. Vahl) C. Agardh in their introduced range (Verlaque *et al.* 2003, Meusnier *et al.* 2004).

Table 4.4 Summary of the genetic variability of the invasive marine macroalgae within their native (N) and introduced (I) ranges as reported in the literature. Inside the bracket are the numbers of sequences over the numbers of populations considered for each species.

Invasive Species	Nuclear marker				
	ITS1, ITS1-ITS2	Intron, 18S	LSU D1-D3 domain	Rpl16-rps3 spacer	psbJ-psbL spacer
Chlorophyta					
Caulerpa racemosa (Forsskål) J. Agardh	N: 12 (17/ 7P); I: 16 (26/ 11P)	N: 7 (9/ 5P); I: 11 (13/ 8P)			
Caulerpa taxifolia (M. Vahl) C. Agardh	*N: 5 (132/ 26P); I: 2 (33/ 5P)				
Codium fragile ssp. *tomentosoides* (van Goor) P.C. Silva				N: 1 (32/ 8P); I: 1 (55/ 15P)	N: 1 (32/ 8P) : I: 1 (55/ 15P)
Rhodophyta					
Asparagopsis armata Harvey			N: 1 (1/ 1P); I: 9 (33 /19P)		
Grateloupia turuturu Yamada					
Polysiphonia harveyi J. Bailey					
Phaeophyceae					
Undaria pinnatifida (Harvey) Suringar					
Undaria pinnatifida					
Sargassum muticum	N: 1 (63/9P); I: 1 (54/9P)				

*** Indelotype instead of haplotype was used in the original article**

Plastid marker			Mitochondrial marker			Other markers or evidence	First record of year of introduction	Haplotype newly hybridized in introduced area?	Reference
trnG-5S spacer	RbcL	Rbc L-S spacer	Cox2-3 spacer	Cox3	TrnW-I spacer				
						Morpho-logical data	1926	Yes	Verlaque *et al.* 2003
							1984	Yes	Meusnier *et al.* 2004
N: 2 (32/ 8P); I: 1 (55/ 15P)						plastid micro-satellite data	~1900	No	Provan *et al.* 2005
		N: 1 (1 /1P); I: 1 (23 /8P)	N: 1 (3 /2P); I: 12 (22/ 10P)				1920's	Unknown	Andreakis *et al.* 2007
	N: 2 (4/ 3P); I: 2 (6/ 4P)						1970's	No	Gavio & Fredericq. 2002
	N: 4 (4/ 4P); I: 2 (18/ 18P)						1908	No	McIvor *et al.* 2001
					**N: 8 (118/ 5P); I: 20 (475/ 16P)		1971	No	Voisin *et al.* 2005
				N: 9 (106/ 21P)			NA	NA	Uwai *et al.* 2006b
		N: 1 (70 /9P); I: 1 (71 /9P)			N: 2 (79/9P); I: 1(69/9P)		1944 to USA; 1973 to Europe	No	This study

** the result was based on concatenated sequences of trnI_W spacer and atp8_trnS spacer

Within their native range, one to 12 haplotypes were found in eight other invasive seaweed species reported in the literature (Table 4.4). The majority of these studies, however, examined only a few native populations. In contrast, among the seven populations in the native range of *S. muticum* that we examined, only one haplotype in ITS2 and Rubisco spacer, and two haplotypes in TrnW_I spacer regions were found, indicating a low genetic variability. The only comparable example of invasive species with such low genetic variability is *Codium fragile* subspecies *tomentosoides* (van Goor) P.C. Silva, which possesses only one haplotype in two markers (Table 4.4).

4.4.5 Eco-physiological tolerances and range expansion of *S. muticum*

The eurythermal and euryhaline characteristics of *S. muticum* are evident especially in the introduced region (Norton 1977b, Hales *et al.* 1989, Steen 2003). In contrast to the other invasive species in which the eurythermal ability may be an attribute of cryptic genetic variability (e.g. McIvor *et al.* 2001), *S. muticum* appears to be a highly homogenous species that possesses such tolerance. The genus *Sargassum* was believed to have originated in the tropical region (Phillips 1995). It is suggested (see Chapter 3) that the ancestors of subgenus *Bactrophycus* of genus *Sargassum*, to which *S. muticum* belongs, radiated probably during the late Pliocene to Pleistocene in the Sea of Japan, which was influenced by the cold and anoxic surface water with low salinity (Oba *et al.* 1991). *Sargassum muticum* may have, thus, developed the ability to tolerate a wider range of temperature and salinity. This potential evolutionary history may account for its eurythermal and euryhaline abilities that eventually offered an eco-physiological advantage in its range

expansion in the introduced area. It would not matter if the introduction of *S. muticum* in Europe was saturated (e.g. Critchley 1983b) or not (e.g. Karlsson & Loo 1999), the difference in the latitudinal range of this species along the three continental coasts (Fig. 4.1) might suggest that photoperiod, which is directly correlated with the latitude where the species is found, would not be a critical factor in affecting the distribution of *S. muticum*. Photoperiodicity, however, has been shown to affect the production of the erect thallus of this species (Hwang & Dring 2002).

4.4.6 Reconstructing the possible chronology of introduction

The suspected vector for the introduction of *S. muticum* was the Pacific oyster, *Crassostrea gigas* (Critchley & Dijkema 1984, Goulletquer *et al.* 2002). According to Barrett (1963), the natural spats of *C. gigas* in Japan were allowed to settle on empty shells of oyster and other mollusk that were mounted on wires and rafts and suspended in the middle of water body during July to September. In order to "harden" the shell of oysters and reduce the mortality of oyster during shipment, the wires were transferred to the intertidal zone where the spats were exposed and submerged regularly following the tidal cycle starting from September. These shells with at least 10 oyster spats each were packed and shipped to US in about January or February (Barrett 1963). As *S. muticum* becomes reproductive during winter to early summer in Japan (Yoshida 1983), the most probable period for the attachment of *S. muticum* on the shells of the spats or the mollusks could be when they were exposed in the intertidal area and about to be packed in January or February. These tiny germlings could remain barely visible during the packing and shipping period, which may make the introduction of *S. muticum* possible even though there was standard

cleaning process before shipment (Quayle 1964). Until now, there was no direct observation of *S. muticum* growing on the shell of the Pacific oyster (there is, however, for another commercial oyster *Ostrea edulis* [Linnaeus, 1758], Critchley & Dijkema 1984). Mineur *et al.* (2007), however, were able to rear *S. muticum* from adult *C. gigas* after the treatments of a series of testing conditions as well as the usual cleaning practice of commercial exportation.

In the American Pacific Northwest, the seeds of *Crassostrea gigas* were first imported into Puget Sound, Washington, USA and British Columbia, Canada as early as 1902 and 1912 respectively (Scagel 1956). Scagel (1956) suggested that the populations of *S. muticum* had been established before World War II. He, however, did not pinpoint any source location of introduction in Japan. One potential location, deduced based on literatures, is Matsushima Bay, Miyagi Prefecture (Fig. 4.1) in the northeastern Honshu of Japan (Barrett 1963). This area and its vicinity, which were also evident to be the source locality for the introduction of the gastropod *Batillaria attramentaria* [Sowerby II, 1855] in the American Pacific Northwest associated with the import of Pacific oyster (Miura *et al.* 2006), is the centre of the seed-producing industry of *C. gigas* (Barrett 1963). The other potential source location is the Kumamoto Prefecture of western Kyushu (Fig. 4.1), from where the seed of another variety of the Pacific oyster (currently believed to be a distinct species, *C. sikamea* [Amemiya 1928], Banks *et al.* 1994) were shipped to California and Washington, USA starting from 1946 (Barrett 1963, Pijanka 2006). However, the transported amount of this variety, which was regarded as superior in both form and flavor compared to the Miyagi's oyster, was small as they mainly served for the experimental plantings in 1950's (Barrett 1963).

Among these two localities mentioned by Barrett (1963), the present data show that Miyagi is less likely to be the source location because the haplotype B of TrnW_I spacer (typical *S. muticum*) found in Tateyama and Yaizu, which is geographically near Miyagi, was different from the haplotype A (non-typical *S. mutiucm*) found in all American populations. Should Miyagi be the source location, it is unlikely that no haplotype B would have been recorded in the Amercian populations. In contrast, Kumamoto, which is situated between the sampling sites of Oojima, Japan and Jangheung, Korea of this study (Fig. 4.1), was more likely to be the source as the haplotype of all the specimens from Oojima and Jangheung is identical to that of the North American specimens (Fig. 4.3). However, the record of first occurrence of *S. muticum*, as *Cystophyllum geminatum* (C. Agardh) J. Agardh, in US during 1944 (Scagel 1956) seems to predate the first shipment of the Kumamoto oyster in 1946 (Pijanka 2006). It remains unclear whether the chronological records of *S. muticum* appearance in US are accurate or the information about the time of first shipment of Kumamoto oyster is reliable.

It is worth noting that, other than the Miyagi Prefecture, Hiroshima situated in the Seto Inland Sea (Fig. 4.1) is another centre of oyster seed-producing industry starting from 1950's (Thomson 1952). It was here where the imported *C. gigas* in Australia originated (Thomson 1952). Among the literature stating the source of the exportation of Pacific oysters to American Pacific Northwest (c.f. Scagel 1956, Barrett 1963, Quayle 1964, Grizel & Héral 1991), Hiroshima was not mentioned as the source except for two shipments during 1902-1903 and 1907-1908 (Steele 1964). These imported oysters, however, failed to establish an industry due to the high mortality of the spats during shipment (Steele 1964). Since the American and Seto

inland sea populations shared the same TrnW_I spacer haplotype, the introduction of *S. muticum* associated with the Pacific oyster from Hiroshima could not be completely ruled out.

In Europe, the time of introduction of *C. gigas* appears coincident with the first occurrence of *S. muticum* in southern England (Farnham *et al.* 1973). The first area of introduction was suspected to be northwestern France as a result of uncontrolled transport of *C. gigas* from British Colombia, Canada by air during 1970-1975 (Farnham *et al.* 1973, Grizel & Héral 1991), though oyster spats were also introduced from Sendai, Miyagi Prefecture (Fig. 4.1) of northeastern Honshu of Japan during 1971-1977 (Grizel & Héral 1991). The oyster industry was, thereafter, self-sustaining using broodstocks in Europe (Grizel & Héral 1991, Mineur 2007). Compared to Sendai, British Columbia is more likely to be the source location of introduction in Europe, since haplotype A (of non-typical *S. muticum*) found in European populations was in common with those in North American populations, while no haplotype B found in Tateyama was detected in the introduced European populations. Besides, Yoshida (1983) found that specimens in Zeeland district of the Netherlands were morphologically similar to those in Seto Inland Sea population and did not represent the typical form of the lectotypes found in Wakayama, Japan. This observation was also supported by the present findings that the haplotype of the European populations were all the same as those of non-typical *S. muticum* in Oojima in the Seto Inland Sea, rather than those in Tateyama and Yaizu which most likely represent the typical *S. muticum*.

Based on the results of this present study, it would not be possible to infer whether the range expansions of *S. muticum* in North America and Europe are the results of single or multiple event(s) of introduction. The elucidation of the finer introductory routes of *S. muticum* awaits the use of more variable genetic markers, such as the other more variable mitochondrial marker and nuclear microsatellite loci. This study, nevertheless, reveals two recently diverged lineages in the native range of *S. muticum*, and indicates that the lineage originated in central and western Japan, South Korea and the northern China dominate all the introduced population. By incorporating information from the literature, this study helps to elucidate the potential source area of the invasive *S. muticum* in its native range.

Chapter Five: Comparative Phylogeography of *Sargassum* spp. in NW Pacific

5.1 Introduction

Phylogeography, first coined by Avise *et al.* (1987), provides the link between systematics and population genetics in enhancing our understanding of recent evolutionary history of living organisms (Avise *et al.* 1987). Phylogeographic studies on many different species in both the terrestrial and marine realms have since been carried out (reviewed by Avise 2000). Information about the congruence (or incongruence) of evolutionary histories among different taxa inhabiting the same area or region allows us to deduce any past (vicariant) event in the earth history which had governed the evolutionary history of the biota of the said region (Arbogast & Kenagy 2001, Zink 2002). This comparative phylogeographic approach (Avise 2000) has revealed the existence of vicariant barriers in various regions (e.g. Avise 1992, Riddle *et al.* 2000, Hoffmann & Baker 2003, Barber *et al.* 2006, Soltis *et al.* 2006). For example, by compiling the phylogeographic information on several maritime species such as the American oyster (Reeb & Avise 1990), the horseshoe crab (Saunders *et al.* 1986) and the killifishes (Duggins *et al.* 1995), a consistent genetic divergence was demonstrated between the populations from the Atlantic side

and the Gulf of Mexico side of the Florida Key in US (Avise 2000). The Florida Key, thus, was proposed to have played a key role in the divergence of these species (Avise, 2000). Moreover, recently identified ancient marine refugia in northern Europe were hypothesized to have played a role in shaping the genetic structure of several macroalgal species (Provan *et al.* 2005). The high haplotype and nucleotide diversities of a European red algal species *Palmaria palmata* (Linnaeus) Kuntze found in the English Channel and in SW Ireland suggest that these two places could be the refugia for this species during the glacial period. The species recolonized the other European coast as far west as Iceland and also the North America thereafter (Provan *et al.* 2005). Phylogeographic studies on the brown alga *Fucus serratus* Linnaeus, using microsatellites (Coyer *et al.* 2003) and mitochondrial marker (Coyer *et al.* 2007), revealed a high genetic diversity in the area of Brittany, France, located near the England Channel and SW Ireland. Both pieces of evidence point to the same conclusion that the England Channel and SW Ireland were probably two of the ancient refugia for seaweeds in Europe.

Other than investigating the congruence of evolutionary histories of organisms belonging to different taxonomic groups found in the same area, study on some closely related species could also provide important insight on not just the common

response to the geological vicariant event, but also the idiosyncratic ecological responses underlying the evolutionary history of these organisms (Rocha *et al.* 2002). For example, Lourie *et al.* (2005) revealed two general patterns of genetic break in Southeast Asia among four species of seahorse belonging to the same genus *Hippocampus*. The difference in the break pattern was suggested to be related to individual ecological responses of different taxa towards a common geological event (e.g. the Pleistocene fragmentation of marine basins) (Lourie *et al.* 2005).

Unlike the above mentioned cases in East Atlantic, European and Tropical Indo-west Pacific waters, only very limited number of comparative phylogeographic works has been done in NW Pacific. One of these on the intertidal snail (genus *Cerithidea*) was carried out in Ryukyu, Japan to verify the existence of any analog to the two well known terrestrial genetic breaks, Tokara and Kerama gaps, in the marine fauna (Kojima *et al.* 2006). Although some degrees of genetic divergence within these three *Cerithidea* species were verified, this study covered only a relatively small area within the NW Pacific and thus cannot provide more insight on the evolutionary history of other marine biota in the whole region. More recently, phylogeographic studies on other groups of marine lives, such as seaweed (Cheang *et al.* 2008), fish (Liu *et al.* 2006, 2007) and barnacles (Tsang *et al.* 2008), have been

carried out covering a larger regional scale in NW Pacific. While the differentiation of populations of various marine species has been shown to be related to the sea level fluctuation during the Quaternary period (e.g. Lourie *et al.* 2005, Liu *et al.* 2006, Timm & Kochzius 2008), the frequent alternation between the formation and disappearance of marine refugia during this period led to a complex evolutionary history as well as a complex genetic structure among many of these species (e.g. Hoarau *et al.* 2007). Inconsistencies do exist in the patterns of genetic structure examined among closely related species (e.g. *Sargassum hemiphyllum* vs. *S. muticum*, see Chapters 3 and 4), making it difficult to interpret the implications of these genetic data with respect to the evolutionary history of these species.

Besides the Tokara and Kerama gaps in Ryukyu, which were believed to be important in contributing to the genetic break among populations of some marine species in NW Pacific (Kojima *et al.* 2006), two other refugia were considered to be potentially critical. One is the East China Sea basin, which remained an almost enclosed marine region in the glacial period, surrounded by the continental Chinese coast and the Ryukyu Island chain (Ota 1998). Further south is the South China Sea basin, which was disconnected from the East China Sea basin by the land bridge formed in the Taiwan Strait. These two basins were hypothesized to be the refugia for

the two varieties of the brown alga *Sargassum hemiphyllum* (Chapter 3). A clear divergence between the two varieties, allopatrically distributed in the Japan-Korea region and the southern China region, was exhibited by the PCR-RFLP data of Rubisco spacer (Cheang *et al.* 2008). The divergence of the two varieties was believed to be maintained by the large volume of freshwater discharge from the Yellow and Yangtze Rivers in China (Cheang *et al.* 2008), with a genetic break located between the regions from Zhejiang Province, China to Cheju Island, Korea. In contrast, the phylogeographic work on another *Sargassum* species, *S. muticum* (Chapter 4), reveals no genetic structure among its populations in NW Pacific, based on ITS2 and Rubisco spacer, and a slight genetic structure based on the sequencing data of TrnW_I spacer. This apparent contradictory finding on these two closely related species (both belong to the same subgenus *Bactophycus*) might suggest their differential responses to the past geological event(s) due to some species-specific biological properties.

To gain a better understanding of how geological event(s) could affect closely related species, such as those belonging to the genus *Sargassum* in NW Pacific, the genetic structures of additional species of *Sargassum* were examined. Four species of *Sargassum*, all belonging to subgenus *Bactrophycus*, were selected for this purpose.

These species, *Sargassum hemiphyllum*, *S. fusiforme*, *S. muticum* and *S. thunbergii*, are not only four of the top ten most widely distributed *Sargassum* species in NW Pacific (Chapter 2), they also exhibit two distinct distribution patterns (Fig. 5.1). *Sargassum hemiphyllum* and *S. fusiforme* are discontinuously distributed. They are not found inside the highly blackish Bohai region along the Chinese coast (Fig. 5.1A, B). In contrast, both *S. muticum* and *S. thunbergii* are distributed continuously along the Chinese coast. They are found abundantly inside the Bohai Gulf (Fig. 5.1C, D). The region of Bohai Gulf, together with the coastal regions along the Yellow Sea to East China Sea, constitutes the area heavily affected by the freshwater outflow from the two big rivers of China, Yangtze and Yellow Rivers (Cheang 2003). The associated environmental conditions, such as low salinity (Steen 2004) and heavy sedimentation (Umar *et al.* 1998), do not favor the growth of most *Sargassum* species that require oceanic condition and a hard substratum. This sea region with its adverse condition for *Sargassum* was postulated to provide a dispersal barrier in maintaining the genetic differentiation among some species of *Sargassum* (Cheang *et al.* 2008).

By investigating the genetic population structures of these four closely related *Sargassum* species in NW Pacific with different geographical distribution patterns,

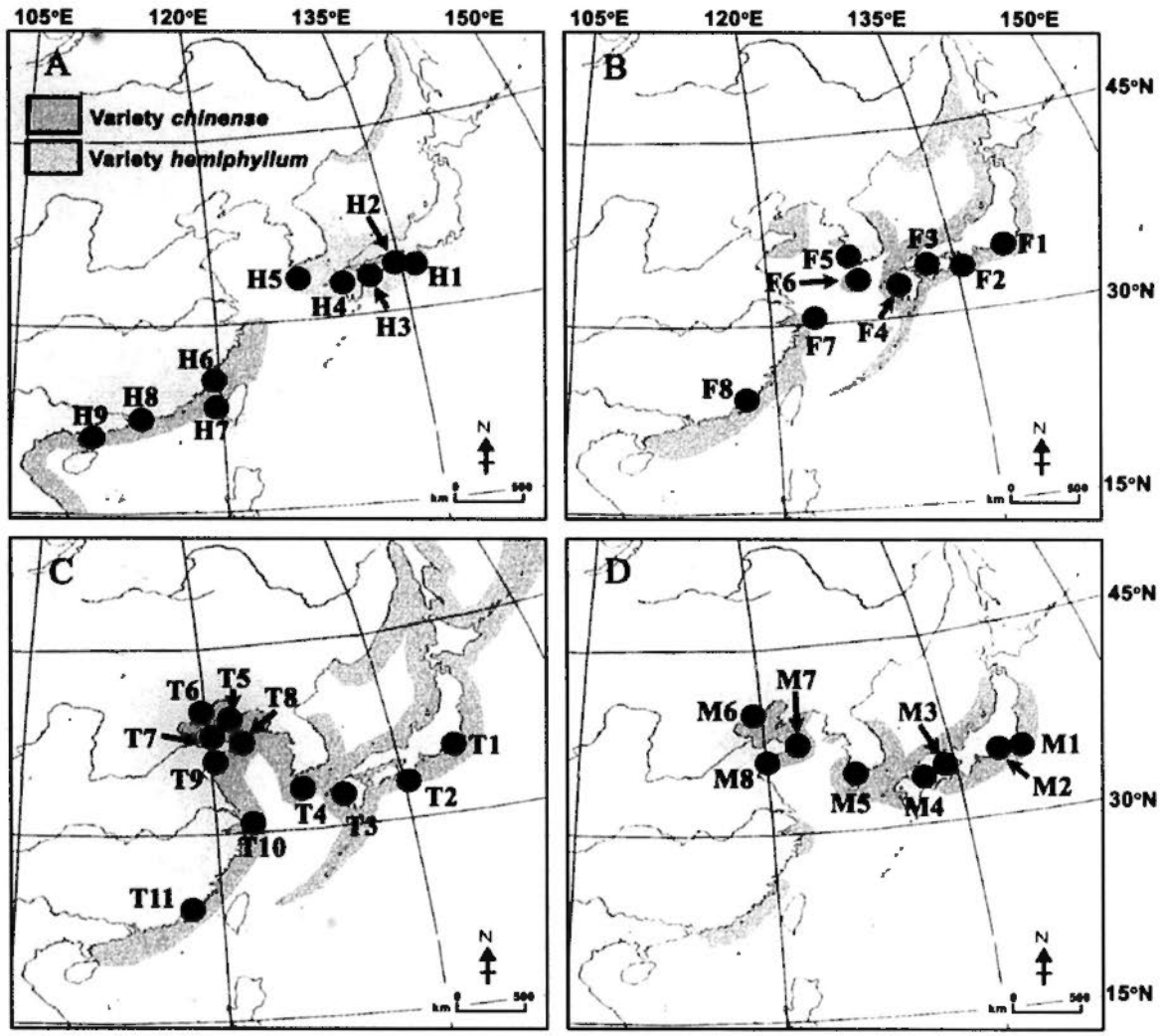

Figure 5.1 The distribution (shaded areas) of *Sargassum hemiphyllum* (A), *S. fusiforme* (B), *S. thunbergii* (C) and *S. muticum* (D) in NW Pacific. Graphs A and B refer to species with discontinuous distribution which does not extend to Bohai Gulf and East China Sea, while graphs C and D refer to those with continuous distribution. Distribution data were compiled based on the literature records (Chapter 2). Refer to Table 5.1 for details of the sampling localities indicated.

we might be able to shade light on the effect of the rivers, or its associated adverse condition for *Sargassum*, on the evolutionary history of *Sargassum* species in NW Pacific. Both ecological vs. evolutionary processes involved in shaping the genetic population structures of these species may be deciphered using this comparative phylogeographic approach.

5.2 Materials and Methods

5.2.1 Specimen sampling, DNA extraction, PCR amplification and direct sequencing

The distribution pattern of the four *Sargassum* species was examined based on the literature records (Chapter 2). As much as possible, samples were collected throughout the whole recorded range of the species by snorkeling or sampling during low tide (Fig. 5.1, Table 5.1). However, logistic constraints often prevented collection to be made in some specific sites. Nonetheless, the location and number of sampling sites for each species should be representative of its distribution range. Specimens were collected 1 – 2 m apart to avoid collecting individuals from the same mother plant, as *Sargassum* germlings are known to have a short dispersal distance (Kendrick & Walker 1995). Two to three vouchers haphazardly selected from each population were air dried and deposited at the Marine Science Laboratory,

Table 5.1 Collection details of the samples used in this study. Letters in ( ) refer to the grouping used in AMOVA: N: northern, C: central, S: southern regions. For details of these regions, refer to descriptions given under the Materials and Methods.

Localities	Acronym (N/C/S)	Date of collection	Collectors
Sargassum hemiphyllum			
H1. Seto Marine Biological Laboratory of Kyoto University, Wakayama Prefecture, Japan (135°20'E, 33°41'N)	JpWY (N)	22 Mar 01	M. Sato
H2. Shishikui, Tokushima Prefecture, Japan (134°18'E, 33°33'N)	JpTS (N)	7 Apr 07	C.C. Cheang & P. O. Ang
H3. Sukumo, Kochi Prefecture, Japan (132°42'E, 32°54'N)	JpSM (N)	6 Apr 07	C.C. Cheang & P. O. Ang
H4. Kawatana, Nagasaki Prefecture, Japan (129°52'E, 33°03'N)	JpNS (N)	19 Apr 07	N. Murase
H5. Sungsan, Cheju Island, South Korea (126°56'E, 33°27'N)	KrCJ (N)	14 Feb 01	J. H. Kim
H6. Hoatian, Pin Tan, Fujian Province, China (119°47'E, 25°40'N)	CnFJ (S)	25 May 01	Y. Zhang
H7. Kwangyinting, Penghu, Taiwan (119°33'E, 23°34'N)	TwPH (S)	24 Mar 02	C.C. Cheang & P. O. Ang
H8. Sai Kung, Hong Kong, China (114°16'E, 22°22'N)	HkSK (S)	26 Feb 02	F. F. Yeung
H9. Naozhoudao, Zhanjiang, Guangdong Province, China (110°37'E, 20°55'N)	CnZJ (S)	14 May 01	C.C. Cheang & P. O. Ang
Sargassum fusiforme			
F1. Izu Shirahama, Shizuoka Prefecture, Japan (138°21'E, 34°41'N)	JpSO (N)	16 Jun 07	K. Matsuyama-Serisawa
F2. Shirahama, Wakayama Prefecture, Japan (135°21'E, 33°41'N)	JpWY (N)	9 Apr 07	C. C. Cheang & P. O. Ang
F3. Oojima, Yamaguchi Prefecture, Japan (132°26'E, 33°57'N)	JpOJ (N)	3 Apr 07	C.C. Cheang & P. O. Ang

F4. Kawatana, Nagasaki Prefecture, Japan (129°52'E, 33°03'N)	JpNS (N)	19 Apr 07	N. Murase
F5. Sungsan, Cheju Island, South Korea (126°56'E, 33°27'N)	KrCJ (N)	14 May 06	P. O. Ang
F6. Yeonggwang, Jeollanam-do, South Korea (126°23'E, 35°20'N)	KrYG (N)	26 Jun 07	H. G. Choi
F7.Shen Shan, Zhejiang, Province, China (122°47'E, 30°43'N)	CnSS (S)	30 May 07	H. L. Fung & Y. M. Tsang
F8.Meizhou, Fujian Province, China (119°07'E, 25°05'N)	CnFJ (S)	31 May 07	P. O. Ang
F9. NanO, Shantou, Guangdong Province, China (117°06'E, 23°24'N)	CnST (S)	28 Apr 07	Y. H. Lam & P. O. Ang
Sargassum thunbergii			
T1. Aburatsubo, Kanagawa Prefecture, Japan (139°36'E, 35°9'N)	JpKG (N)	22 May 05	M. Honda
T2. Shirahama, Wakayama Prefecture, Japan (135°21'E, 33°41'N)	JpWY (N)	9 Apr 07	C. C. Cheang & P. O. Ang
T3. Kawatana, Nagasaki Prefecture, Japan (129°52'E, 33°03'N)	JpNS (N)	19 Apr 07	N. Murase
T4. Sungsan, Cheju Island, South Korea (126°56'E, 33°27'N)	KrCJ (N)	21 Oct 06	P. O. Ang
T5. Dalian, Liaoning Province, China (121°41'E, 38°51'N))	CnDL (C)	16 Jun 04	C. C. Cheang & R. Luan
T6. Beidaihe, Hebei Province, China (119°31'E, 39°49'N)	CnBDH (C)	10 May 05	C.C. Cheang & S. C. C. Suen
T7. Yantai, Shandong Province, China (121°25'E, 37°35'N)	CnYT (C)	16 Jun 04	D. Duan
T8. Qingdao, Shangdong Province, China (120°20'E, 36°02'N)	CnQD (C)	13 Jun 04	C. C. Cheang & P. O. Ang
T9. Shen Shan, Zhejiang, Province, China (122°47'E, 30°43'N)	CnSS (S)	30 May 07	H. L. Fung & Y. M. Tsang

T10. Meizhou, Fujian Province, China (119°07'E, 25°05'N)	CnFJ (S)	31 May 07	P. O. Ang
Sargassum muticum			
M1. Tateyama, Chiba Prefecture, Japan (139°51'E, 34°59'N)	JpTY (N)	24 Jan 07	D. Fujita
M2. Yaizu, Shizuoka Prefecture, Japan (138°19'E, 34°51'N)	JpSO (N)	30 Jan 08	K. Matsuyama-Serisawa
M3. Awajishi, Hyogo Prefecture, Japan (135°1'E, 34°34'N)	JpAJ (N)	8 Apr 07	C.C. Cheang & P. O. Ang
M4. Oojima, Yamaguchi Prefecture, Japan (132°26'E, 33°57'N)	JpOJ (N)	3 Apr 07	C.C. Cheang & P. O. Ang
M5. Jangheung, Jeollanam -Do, South Korea (126°56'E, 34°27'N)	KrJH (N)	3 May 07	H. G. Choi
M6. Beidaihe, Hebei Province, China (119°31'E, 39°49'N)	CnBDH (C)	10 May 05	C.C. Cheang & S. C. C. Suen
M7. Rungcheng & Weihai, Shandong Province, China (122°29'E, 37°09'N & 122°7'E, 37°30'N)	CnRC & CnWH (C)	20 Jun 07 & 26 Jun 07	D. Duan
M8. Qingdao, Shandong Province, China (120°21'E, 36°2'N)	CnQD (C)	13 Jun 04	C.C. Cheang & P. O. Ang

the Chinese University of Hong Kong.

Detailed procedure of the treatment of specimens and DNA extraction followed those described in Chapter Three. Briefly, about 1 g of the new branches without epiphytes was cleaned with brushes and subsequently preserved in 95% ethanol or silica gel until molecular study was carried out (Cheang 2003). Genomic DNA of these specimens was extracted by modified cetyltrimethylammonium bromide (CTAB) method (Protocol two of Ho *et al.* 1995). The extracted genomic DNA was further purified by GENECLEAN II Kit (BIO 101 Inc., CA, USA), following the manufacturer's instructions (Yoshida *et al.* 2000).

The spacer between the Rubisco large and small subunits in plastid DNA, spacer between the TrnW and TrnI in mitochondrial DNA and ITS2 region in the nuclear genome of all the specimens from the four species were amplified by PCR (profile: initial 3 min at 96°C, 32 cycles of 40 s at 96°C, 30 s at 55°C 50 s at 72°C, final 3 min at 72°C) by the thermocycler (Mastercycler Gradient, Eppendorf®, Hamburg, Germany). The primer sets used are listed in Table 5.2. Both the cocktail for amplifications and the subsequent procedure, up to the point of commercial automated sequencing (Macrogen Inc., Seoul, South Korea), were identical with

Table 5.2 List of primers used for the three genetic markers: nuclear ITS2, plastid Rubisco spacer and mitochondrial TrnW_I spacer. Letters inside ( ) represent the species in which the primer(s) were applied. (H: *S. hemiphyllum*, F: *S. fusiforme*, M: *S. muticum*, T: *S. thunbergii*)

Markers	Forward/ Backward	Sequences
ITS2	F	ITS2FC2*: 5'-TTGTCGGGGAGGAGGAGG-3' **(H, F, M, T)**
	R	25BR2-Reverse[1]: 5'-TCCTCCGCTTAGTATATGCTTA-3' **(H, F, M, T)**
Rubisco spacer	F	M1F[2]: 5'-GACCTTTAAAAGCAGCTTTAGAT-3' **(H, F)**
		RBCLF1[3]: 5'-GACCTTTAAAAGCAGCTTTAGAT-3' **(M, T)**
	R	M1R[2]: 5'-CCCCATAGTTCCCTAATACGCATT-3' **(H, F, M, T)**
TrnW_I spacer	F	TrnW-I-F[4]: 5'-GGGGTTCAAATCCCTCTCTT-3' **(H, F, T)**
		TrnW-I-FC[5]: 5'-GTTCAAGTCCCTCTCTTTCTGT-3' **(M)**
	R	TrnW-I-R[4]: 5'-CCTACATTGTTAGCTTCATGAGAA-3' **(H, M)**
		TrnW-I-RC1[5]: 5'-GTTCAAGTCCCTCTCTTTCTGT-3' **(F, T)**

* modified from the sequence (GenBank Accession number: AY150006) of *Sargassum hemiphyllum* (Oak *et al.* 2002)

[1] based on Yoshida *et al.* (2000).

[2] based on Cheang (2003).

[3] modified from the sequence (GenBank Accession number: AF292068) of *Sargassum muticum* (Phillips *et al.* 2005)

[4] based on Voisin *et al.* (2005).

[5] modified from the sequence (GenBank Accession number: AY494079) of *Fucus vesiculosus* (Secq *et al.* 2006)

those described in Chapter Three. The sequences obtained, together with those of the four species available from GenBank, were visually edited and aligned using the software MEGA ver. 4 (Tamura *et al.* 2007). *Sargassum horneri*, which belongs to the same subgenus (*Bactrophycus*) as the four species and was found to be the most closely related to the four species based on ITS2 (Stiger *et al.* 2003), serves as the outgroup for ITS2 tree. No sequence of species under the subgenus *Bactrophycus* was available in GenBank for Rubisco spacer, so the tree was tentatively rooted as in the case of ITS2. An unknown *Sargassum* sp. from the GenBank (EU169849) serves as outgroup in TrnW_I spacer tree since it is the only available TrnW_I sequence of *Sargassum* sp. New sequences obtained in this study, if any, were deposited to the GenBank.

The heteroplasmy of the mitochondrial markers in *Fucus serratus* Linnaeus has been documented (Coyer *et al.* 2004). The problem of heteroplasmy could hinder the applicability of the mitochondrial marker in phylogenetic reconstruction or inference of demographic history (Posada & Crandall 2002). This problem was not significant in the present study, since all the sequences demonstrate clear single peak in their chromatograms (data not shown).

5.2.2 Statistical treatments

Neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) trees were generated by the software package PAUP 4.0 Beta (Swofford 2000), and Bayesian inference (BI), by MrBayes v.3.12 (Ronquist & Huelsenbeck 2003), to analyze the phylogenetic relationships among the cytoplasmic haplotypes and nuclear genotypes of all the four species. Same haplotype/genotypes shared by different localities were treated as different units according to their sampling localities so that the population structure could be visualized in the tree. The detailed settings for the NJ, MP and BI approaches were the same as those described in Chapter Three. Akaike information criterion implemented in Modeltest ver. 3.7 (Posada & Crandall 1998) was used, *a priori*, to estimate the appropriate model to be applied in the NJ, ML and BI methods. The optimal models found for the four species among the three markers were TVM+ I (ITS2), GTR (Rubisco spacer) and HKY (TrnW_I spacer) for *S. hemiphyllum*; HKY+I (ITS2, Rubisco spacer) and TrN+I (TrnW_I spacer) for *S. fusiforme*; K81UF (ITS2), TIM+I (Rubisco spacer) and HKY (TrnW_I spacer) for *S. thunbergii*, and TVM + I (ITS2), TVM (Rubisco spacer) and HKY (TrnW_I spacer) for *S. muticum*.

The number of haplotypes was recorded for each sampling locality. The computer software TCS ver.1.13 (Clement *et al.* 2000) was utilized to generate the TCS network, which demonstrated the relationship among haplotypes of the four species. The occurrence and frequency of the haplotypes obtained for the four species were mapped according to their sampling localities, so that any concordant distribution pattern of the haplotypes across the four species could be visualized.

Parameters of the population structure, neutrality tests, sudden range expansion and the analysis of molecular variance (AMOVA) were compared across the four species. Basic parameters of the populations, such as sequence divergence, nucleotide diversity and haplotype diversity (Nei 1987) were calculated and compared. Both Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) neutrality tests were carried out to test if the markers utilized were selectively neutral and significant negative values could tell whether the populations underwent recent range expansion (Chiang *et al.* 2004). The mismatch distribution analysis was also conducted for the detection of any history of sudden range expansion (Roger & Harpending 1992). This is essentially a Chi-square test testing on the deviation of the observed frequency distribution of pairwise nucleotide difference from the expected unimodal distribution derived from the sudden range expansion model. The raggedness index implemented

in this test was able to quantify the shape of the frequency distribution curve so as to indicate whether the distribution was unimodal (i.e. with sudden range expansion).

AMOVAs were carried out to test if the Φ_{ST} was statistically significant. In order to test whether the genetic population structure of the *Sargassum* species are related to the discharge of the two rivers in China, the populations were grouped with respect to their location in areas influenced by either the Yangtze or the Yellow Rivers, namely the northern, central and southern populations. The northern population included all the Japanese and Korean populations; and the southern one, all populations in southern China south of Zhejiang Province, China (Table 5.1). The central populations referred to those from the region of Bohai and Yellow Sea, which are heavily influenced by the two rivers (Fig. 5.1). Comparison among the Φ_{ST} of the four species revealed whether there was consistent differentiation due to the presence of the rivers across the four species. All these calculations were done using the software Arlequin ver. 3.1 (Excoffier *et al.* 2005).

5.3 Results

5.3.1 Sequence data and diversity

A total of 62, 142 and 93 sequences of *S. hemiphyllum*, 26, 49 and 58 sequences of *S. fusiforme*, 45, 85 and 102 sequences of *S. thunbergii*, and 56, 70 and 79 sequences of *S. muticum* were obtained for ITS2, Rubisco and TrnW_I spacers respectively (Table 5.3). Variations in sizes were observed in ITS2 sequences of *S. hemiphyllum* and *S. fusiforme*. The aligned length ranged from 504 to 506 and 500 to 506 respectively (Table 5.3), yielding two genotypes in *S. hemiphyllum* and six genotypes in *S. fusiforme*. The greater variation in length in *S. fusiforme* and *S. hemiphyllum* could be attributed to the existence of relatively more insertion-deletion sites in the sequences together with various base substitutions (Table 5.3), as compared to those in *S. thunbergii* and *S. muticum*. The latter possessed a single genotype of ITS2 sequences (497 bp). The aligned length of Rubisco sequences was 263 bp, except that of *S. fusiforme* which was four base pairs longer. The number of polymorphic sites in *S. hemiphyllum* (5 bp), however, was higher than that in *S. fusiforme* (2 bp), although both species possessed three different haplotypes. Both *S. thunbergii* and *S. muticum* exhibited single type of sequences in Rubisco spacer without any base-pair substitutions. The length of TrnW_I spacer sequences ranged from 165 bp (*S. fusiforme*) to 159 bp (*S. thunbergii*), with *S. hemiphyllum* and *S.*

Table 5.3 Basic information about the sequences and the genetic diversities of the four *Sargassum* species studied, based on ITS2, Rubisco and TrnW_I spacers.

Parameters[1]	*S. hemiphyllum*	*S. fusiforme*	*S. thunbergii*	*S. muticum*
ITS2				
(aligned sizes)	504,6bp	500,2,4,6bp	497bp	497bp
n / n_{Pop} / n_h	62/9/2	26/6/6	45/11/1	56/8/1
s (T_S/T_V/Indel)	5 (0/3/2)	13 (2/5/6)	0 (0/0/0)	0 (0/0/0)
h ± SD	0.42 ± 0.049	0.88 ± 0.044	0.00 ± 0.00	0.00 ± 0.00
π ± SD	0.0037 ± 0.0023	0.060 ± 0.030	0.00 ± 0.00	0.00 ± 0.00
Rbc spacer				
(aligned sizes)	263bp	267bp	263bp	263bp
n / n_{Pop} / n_h	142/9/3	49/9/3	85/10/1	70/8/1
s (T_S/T_V/Indel)	5 (0/5/0)	2 (1/1/0)	0 (0/0/0)	0 (0/0/0)
h ± SD	0.65 ± 0.015	0.32 ± 0.078	0.00 ± 0.00	0.00 ± 0.00
π ± SD	0.0099 ± 0.0058	0.0012 ± 0.0014	0.00 ± 0.00	0.00 ± 0.00
TrnW_I spacer				
(aligned sizes)	160bp	165bp	159bp	160bp
n / n_{Pop} / n_h	93/8/4	58/9/4	102/9/2	79/8/2
s (T_S/T_V/Indel)	9 (2,7,0)	3 (2,1,0)	1 (0,1,0)	2 (1,1,0)
h ± SD	0.75 ± 0.013	0.25 ± 0.074	0.058 ± 0.032	0.37 ± 0.055
π ± SD	0.026 ± 0.014	0.0024 ± 0.0023	0.00036 ± 0.0009	0.0041 ± 0.0035

[1] n: No. of sequences; n_{Pop}: no. of populations, n_h: no. of haplotypes; *s*: no. of polymorphic sites; T_I: no. of transition site; T_V: no. of transversion site; Indel: no. of insertion/deletion site; *h*: haplotype diversity; SD: standard deviation; π: nucleotide diversity

muticum demonstrating the same size (160 bp). *Sargassum hemiphyllum* showed the most number of polymorphic sites (9 bp), followed by *S. fusiforme* (3 bp) and *S. muticum* (2 bp). *Sargassum thunbergii* demonstrated the least number of variable sites (1 bp) in the marker TrnW_I spacer. Both *S. hemiphyllum* and *S. fusiforme* exhibited four different haplotypes, while *S. thunbergii* and *S. muticum* each possessed only two different haplotypes.

No haplotype diversity nor nucleotide diversity were revealed in *S. thunbergii* and *S. muticum* based on ITS2 and Rubisco spacer due to the lack of sequence variation. In contrast, *S. hemiphyllum* and *S. fusiforme* demonstrated higher genetic diversities, especially in haplotype diversities (Table 5.3). For the TrnW_I spacer, *S. hemiphyllum* was the most genetically diverse, while *S. thunbergii* the least. The diversity indices of *S. fusiforme* are lower than those of *S. muticum* for this marker (Table 5.3).

5.3.2 Phylogenetic relationship among haplotypes and their distribution in the four species

The phylogenetic relationships among the four species examined are essentially consistent based on all the three markers investigated (Figs. 5.2 to 5.4). *Sargassum*

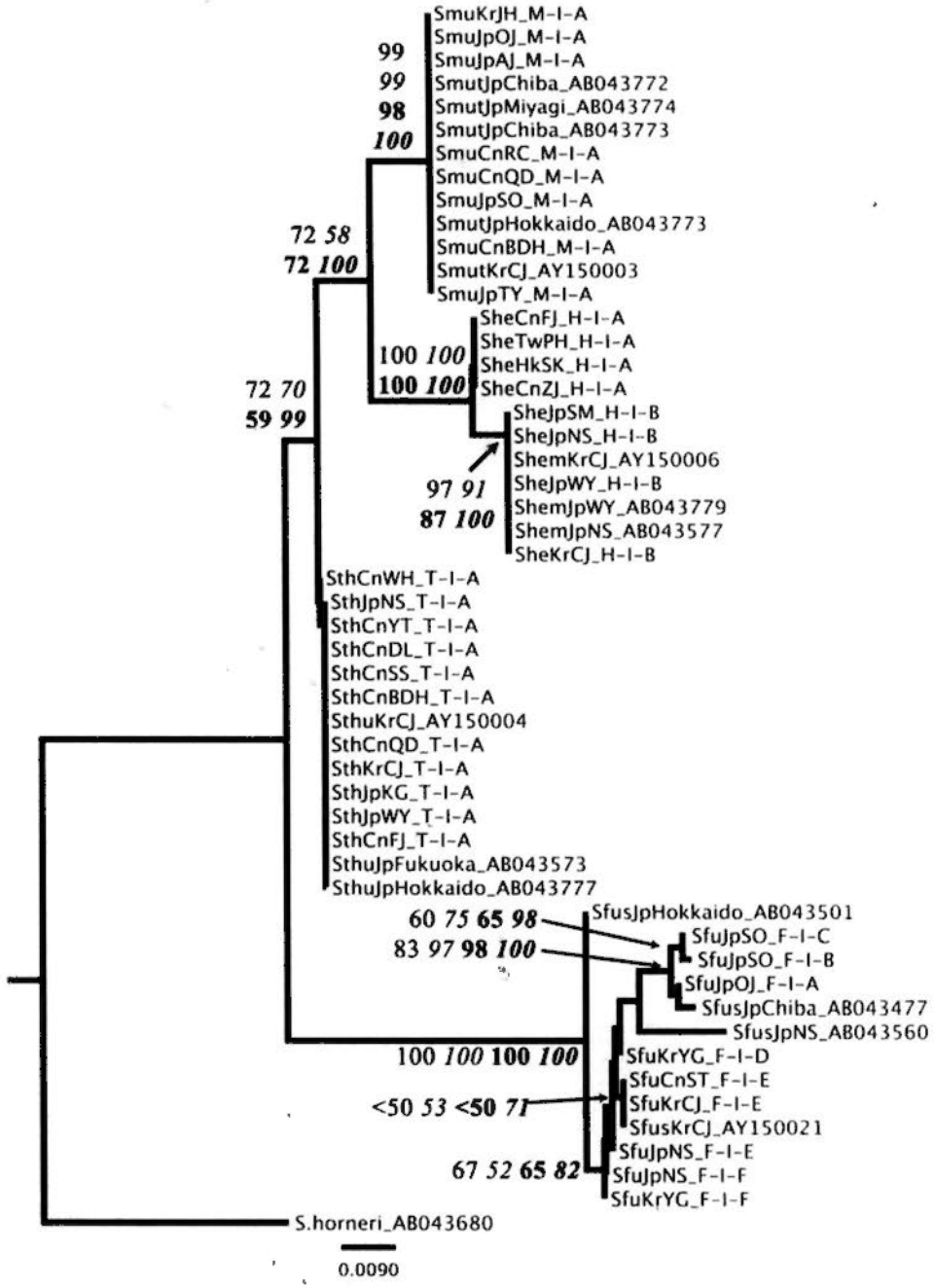

Figure 5.2 Neighbor-joining (NJ) tree illustrating the relationship among different haplotypes of *S. hemiphyllum*, *S. fusiforme*, *S. thunbergii* and *S. muticum* based on ITS2. The same topology of the trees was obtained for the maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) approaches. Refer to Fig. 5.5 for the intraspecific relationship among haplotypes. Bootstrap values (1000 replicates for NJ, MP and 100 for ML) for NJ (regular), MP (italic) and ML (bold) methods and the posteriori probabilities of BI (bold and italic) method are indicated at the nodes. Available sequences from GenBank were included in the analyses (*S. hemiphyllum*: AB043577, AB0435779, AY150006 from Nagasaki, Wakayama of Japan and Cheju Island of Korea respectively; *S. fusiforme*: AB043477, AB043501, AB043560, AY150021 from Chiba, Hokkaido, Nagasaki of Japan and Cheju Island of Korea respectively; *S. thunbergii*: AB043777, AB043573, AY150003 from Hokkaido, Fukuoka of Japan and Cheju Island of Korea respectively; *S. muticum*: AB043503, AB043772-4, AY150003 from Miyagi, Chiba, Hokkaido, Miyagi of Japan and Cheju Island of Korea respectively), while *S. horneri* (AB043680) serves as outgroup.

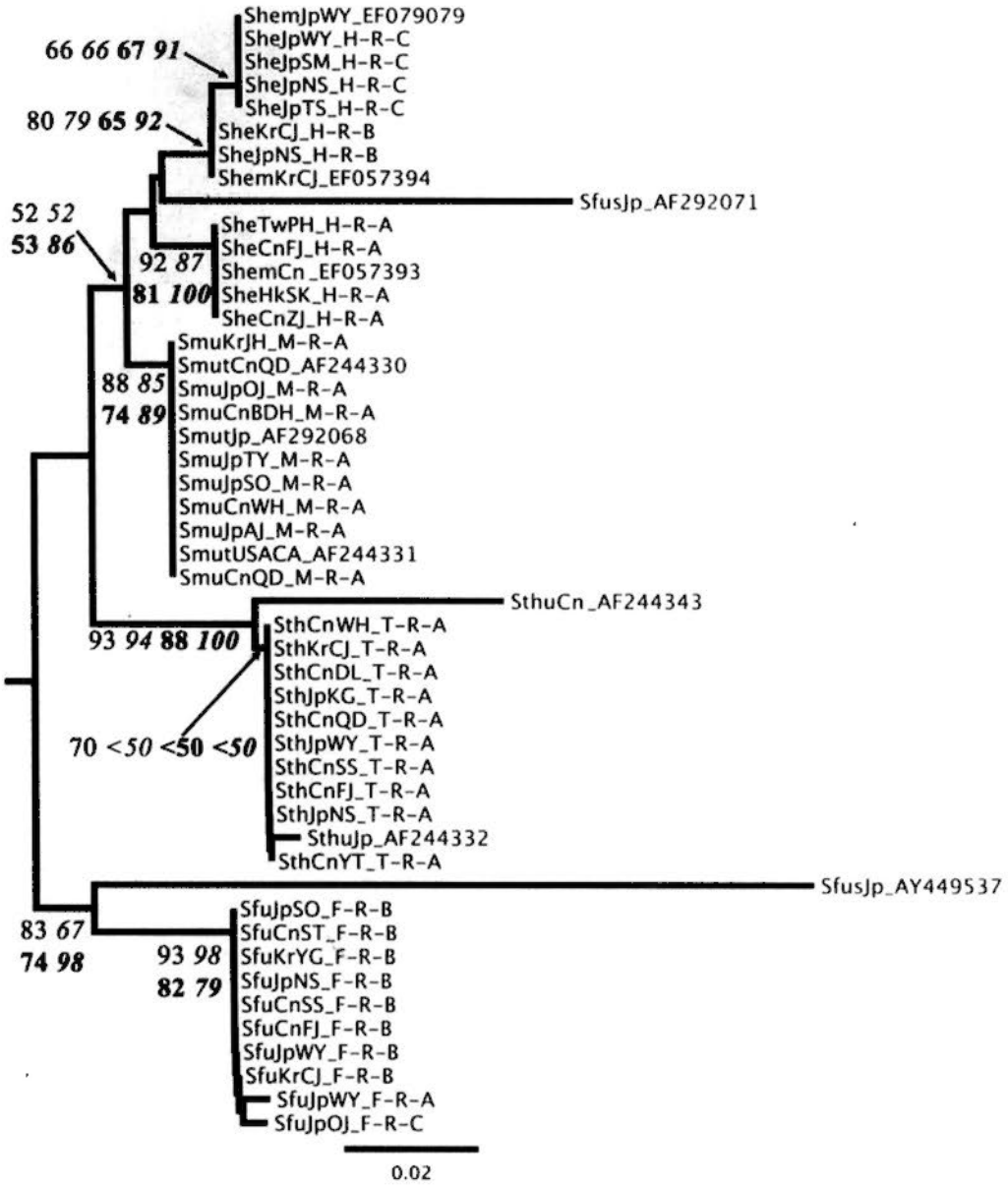

Figure 5.3 Neighbor-joining (NJ) tree illustrating the relationship among different haplotypes of *S. hemiphyllum*, *S. fusiforme*, *S. thunbergii* and *S. muticum* based on Rubisco spacer. The same topology of the trees was obtained for the maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) approaches. Refer to Fig. 5.6 for the intraspecific relationship among haplotypes. Bootstrap values (1000 replicates for NJ, MP and 100 for ML) for NJ (regular), MP (italic) and ML (bold) methods and the posteriori probabilities of BI (bold and italic) method are indicated at the nodes. Available sequences from GenBank were added into the trees (*S. hemiphyllum*: EF079079, EF057393-4 from Wakayama of Japan, Cheju Island of Korea and Fujian of China respectively; *S. fusiforme*: AF292071 and AY449537 both from Japan; *S. thunbergii*: AF244332 and AF244343 from Japan and China respectively; *S. muticum*: AF244330-1 and AF292068 from Qingdao of China, California of USA and Japan respectively). No suitable outgroup sequence was found in GenBank, so the tree was tentatively rooted as in the case of ITS2 (Fig. 5.2).

Figure 5.4 Neighbor-joining (NJ) tree illustrating the relationship among different haplotypes of *S. hemiphyllum*, *S. fusiforme*, *S. thunbergii* and *S. muticum* based on TrnW_I spacer. The same topology of the trees was obtained for the maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) approaches. Refer to Fig. 5.7 for the intraspecific relationship among haplotypes. Bootstrap values (1000 replicates for NJ, MP and 100 for ML) for NJ (regular), MP (italic) and ML (bold) methods and the posteriori probabilities of BI (bold and italic) method are indicated at the nodes. An unknown *Sargassum* sp. from the GenBank (EU169849) serves as outgroup since it is the only available TrnW_I sequence of *Sargassum* sp.

fusiforme was the most basal species with *S. thunbergii* being the sister group to the clades of *S. muticum* and *S. hemiphyllum*.

With respect to ITS2, no intraspecific structure was revealed among the populations of both *S. thunbergii* and *S. muticum* (Fig. 5.2). Only one haplotype was found in these two species (*S. thunbergii*: T-I-A, *S. muticum*: M-I-A), which was distributed throughout their entire range (Fig. 5.5). In comparison, genetic population structure was revealed in both *S. hemiphyllum* and *S. fusiforme* (Fig. 5.2). The genetic divergence of the two varieties of *S. hemiphyllum* (genotypes H-I-A and H-I-B) contributed to the intraspecific structure of this species. Genotype H-I-A found in Japanese and Korean populations was different from Genotype H-I-B occurring along the Chinese coast by five nucleotide changes (Fig. 5.5). Comparable to *S. hemiphyllum*, *S. fusiforme* also possessed two main lineages that corresponded to the eastern Japanese (JpOJ and JpSO) and the southwestern Japanese-Korean-Chinese (KrCJ, KrYG, JpNS and CNST) populations (Figs. 5.2 and 5.3). The lineage of JpOJ and JpSO appeared to be a distinct group (confidence levels ranged from 83 to 100 depending on the tree constructing methods), which consisted of the genotypes F-I-A in JpOJ, F-I-B and F-I-C in JpSO and a GenBank sequence (AB043477) from Chiba of eastern Japan (Stiger *et al.* 2003). Though

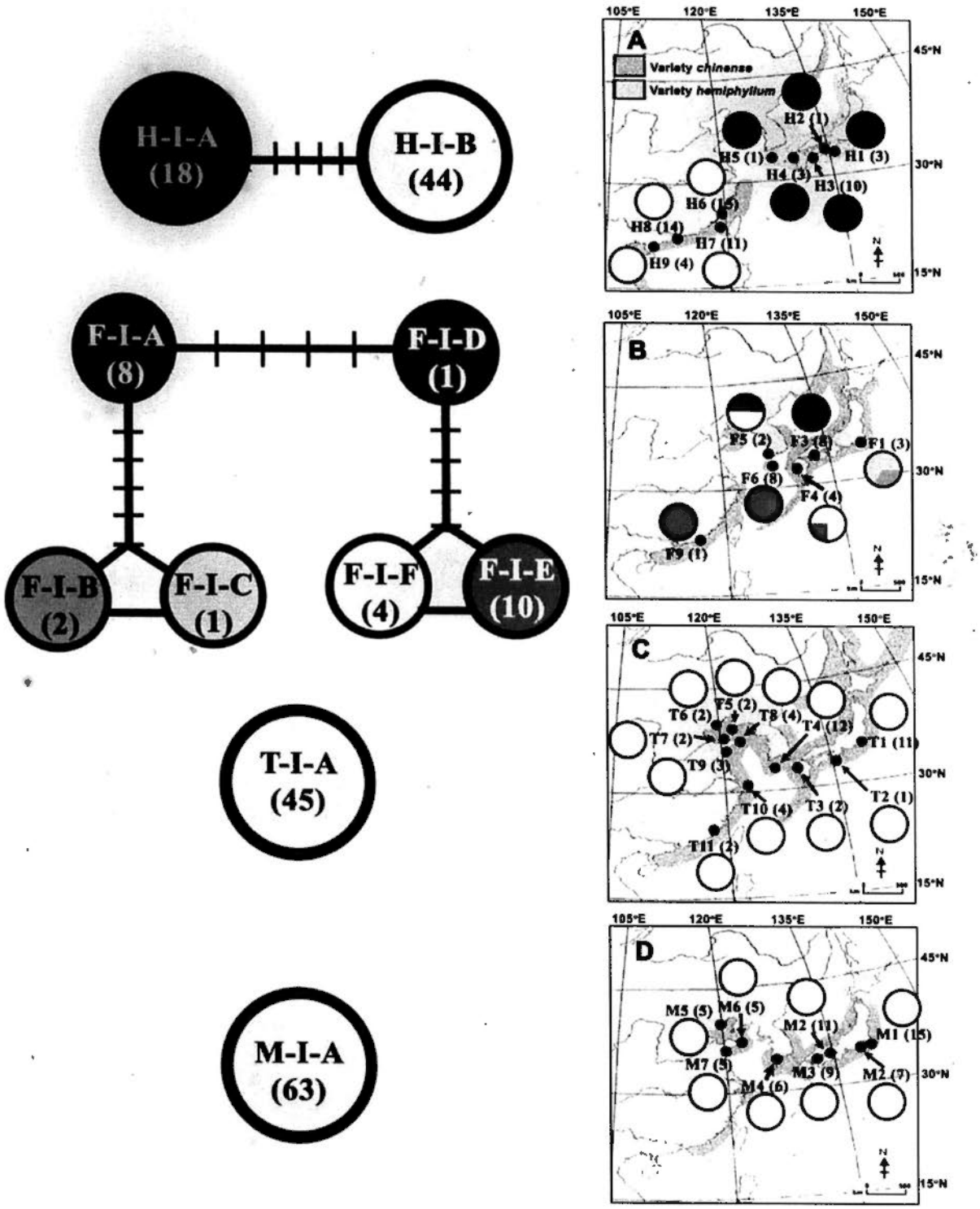

Figure 5.5 The TCS haplotype networks and the haplotype distribution of A) *Sargassum hemiphyllum*, B) *S. fusiforme*, C) *S. thunbergii* and D) *S. muticum* in NW Pacific, based on ITS2 marker.

being within the same lineage, genotype F-I-A differed from both F-I-B and F-I-C by six base pairs (Fig. 5.3). The southwestern Japanese-Korean-Chinese lineage, which included genotypes F-I-D, F-I-E and F-I-F, plus the GenBank sequences from Nagasaki of western Japan, exhibited at least five bp differences with the eastern Japan lineage of *S. fusiforme* (Fig. 5.5). Genotype F-I-E appeared to be the major genotype (10 sequences) in this lineage that was distributed in KrCJ and CnST (Fig. 5.5). There was a rather large nucleotide difference (5 steps) between genotypes F-I-D (KrYG) and F-I-E (KrCJ, CnST), F-I-F (KrYG). A weakly supported subclade, which included genotype E from KrCJ and CnST as well as another GenBank sequence from Cheju Island (AY50021, Oak *et al.* 2002), could be recognized inside this lineage (Fig. 5.2).

The single haplotypes of both *S. muticum* and *S. thunbergii* revealed based on Rubisco spacer led to a homogenous intraspecific clade (Fig. 5.3). These haplotypes occurred in the entire distribution range of the two species (Fig. 5.6). In contrast, the two varieties of *S. hemiphyllum* could be distinguished, forming two distinct intraspecific lineages. The sequences from China (haplotype H-R-A, Fig. 5.6) formed a distinct lineage (Fig 5.3), whereas the sequences from eastern Japan, such as those from JpWY, JpTS, JpSM and JpNS (haplotype H-R-C), formed a subgroup

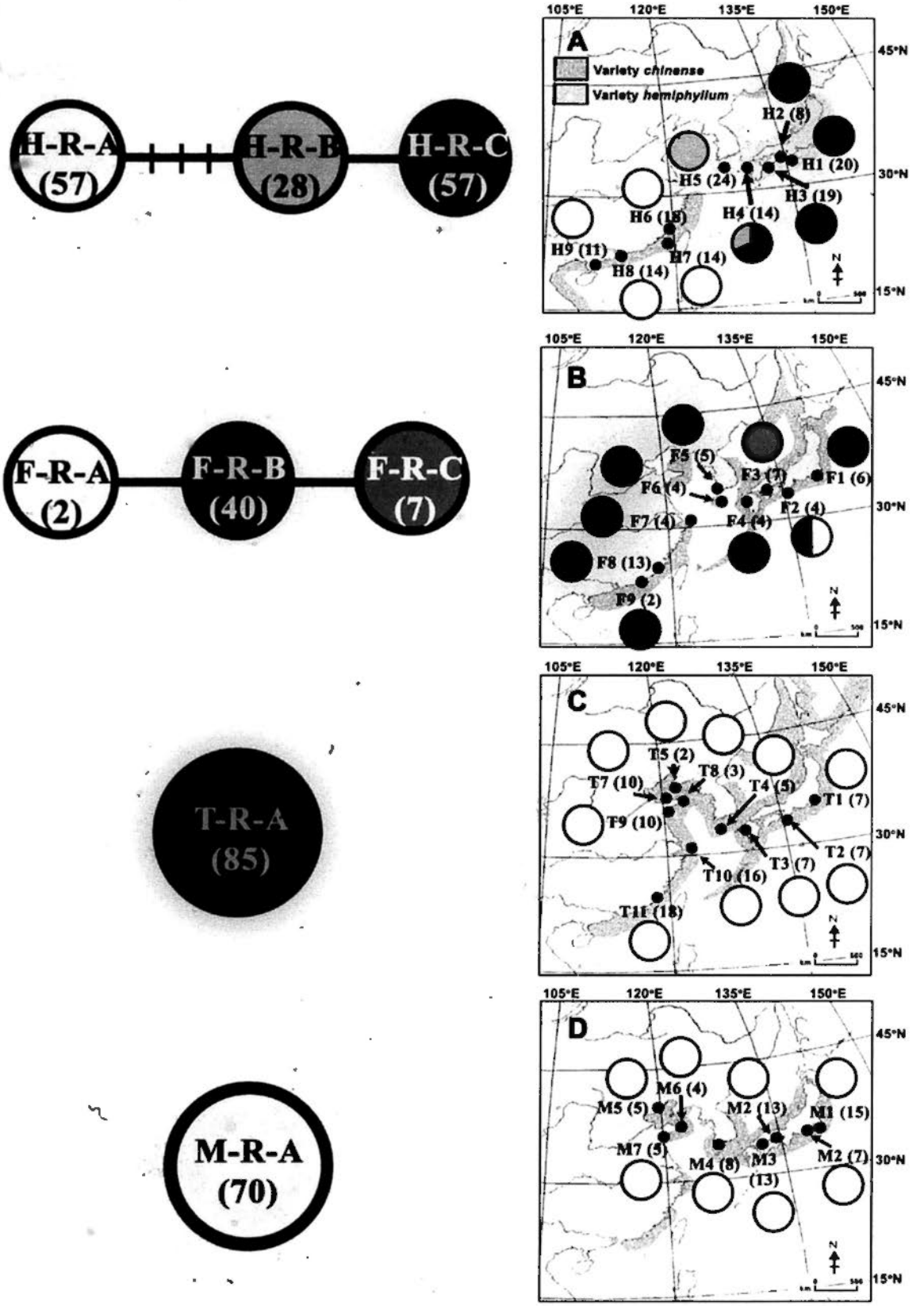

Figure 5.6 The TCS haplotype networks and the haplotype distribution of A) *Sargassum hemiphyllum*, B) *S. fusiforme*, C) *S. thunbergii* and D) *S. muticum* in NW Pacific, based on Rubisco spacer marker.

within the var. *hemiphyllum* lineage. This latter lineage also included the sequences from KrCJ and JpNS (haplotype H-R-B, Fig. 5.6). The smallest difference between these two lineages was five bp (Fig. 5.6). Sequences of *S. fusiforme*, in which only a single clade could be recognized, are more homogenous than those of *S. hemiphyllum*, (Fig. 5.3). This clade consisted of three haplotypes (F-R-A to F-R-C), and is different from those of *S. muticum* and *S. thunbergii* in being constituted only by one haplotype. The two lineages of *S. fusiforme* revealed based on ITS2, the eastern Japanese (JpOJ and JpSO) and the southwestern Japanese-Korean-Chinese (KrCJ, KrYG, JpNS and CNST) lineages, could not be identified based on Rubisco spacer. Instead, haplotype F-R-B appeared to be the single major and widely distributed haplotype. Sequences from JpOJ (haplotype F-R-C) were distinct and differed from those of haplotype F-R-B in other locations by one change, while a minor lineage F-R-A was found to be admixed with haplotype F-R-B in JpWY (Fig. 5.6).

In terms of TrnW_I spacer, both *S. muticum* and *S. thunbergii* demonstrated weak intraspecific structure, the subclade within which was supported by relatively low bootstrap values (Fig. 5.4). Each of the species possessed two haplotypes, which differed from one another by a single change (T-T-A and T-T-B, M-T-A and M-T-B,

Fig. 5.7). The major haplotypes, T-T-A and M-T-A, were distributed almost throughout the entire range of both species, except in eastern Japan (JpSO and JpTY for *S. muticum*, JpKG for *S. thunbergii*). Haplotype T-T-B of *S. thunbergii* was admixed with the major haplotype T-T-A in JpKG (Fig. 5.7). In contrast, there were two well-separated lineages for *S. hemiphyllum*, both of which consisted of subclades with relatively strong support (Fig. 5.4). The separation of these two lineages of *S. hemiphyllum* was so distinct that, instead of grouping with each other, one of the lineages appeared as the sister group of *S. muticum*, but with a low confidence value for all the tree constructing methods (Fig. 5.4). The TCS network of the haplotypes corresponding to these two lineages could not be linked as well (Fig. 5.7). The haplotypes H-T-A and H-T-B of the var. *chinense* lineage differed from each other by one nucleotide. They were mainly distributed along the Chinese coast, but some of them were also found in JpWY. The two haplotypes of var. *hemiphyllum*, haplotypes H-T-C and H-T-D, also differed from each other by a single bp. They were found entirely in Korea and Japan, in which H-T-C was more localized in Korea and eastern Japan and H-T-D in JpSM and JpWY (Fig. 5.7). In contrast to the data of Rubisco spacer, the differentiation of the eastern Japanese (JpOJ and JpSO) and the southwestern Japanese-Korean-Chinese (KrCJ, KrYG, JpNS and CNST) populations of *S. fusiforme* observed in the analysis of ITS2 region was also evident in the tree of

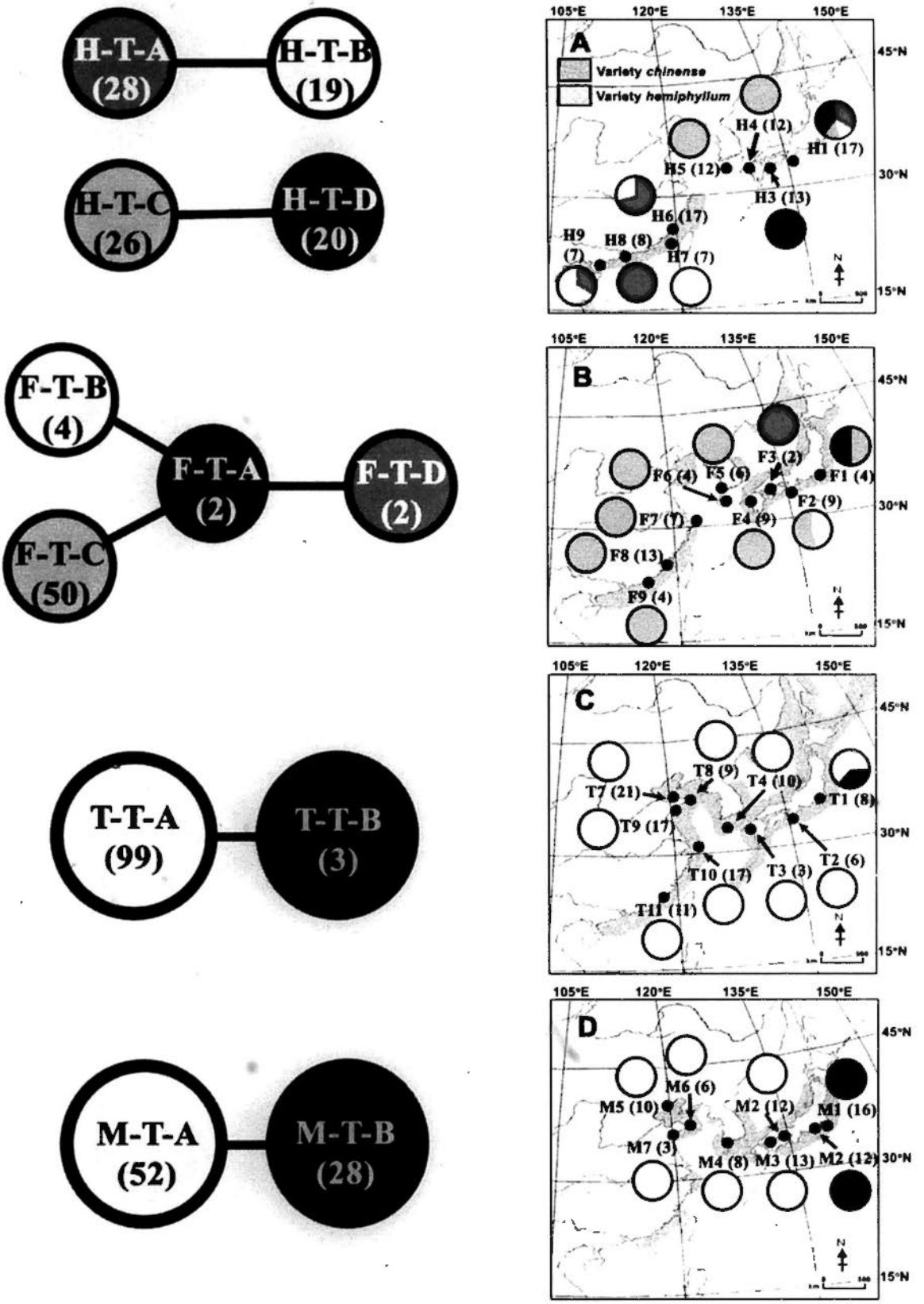

Figure 5.7 The TCS haplotype networks and the haplotype distribution of A) *Sargassum hemiphyllum*, B) *S. fusiforme*, C) *S. thunbergii* and D) *S. muticum* in NW Pacific, based on TrnW_I spacer marker.

TrnW_I spacer, but with a lower confidence ranging from 63 to 92 depending on the tree construction methods (Fig. 5.4). Haplotypes F-T-A and F-T-D, with two bp difference, corresponded to the eastern Japanese lineage of *S. fusiforme* (Fig. 5.4). They were found in JpOJ and JpSO only (Fig. 5.7). Haplotype F-T-C was the dominant haplotype in China, Korea and Japan. It was found admixed with F-T-A in JpSO and F-T-B in JpWY (Fig. 5.7).

5.3.3 Population structures and sudden range expansion

No Φ_{ST} was calculated for *S. thunbergii* and *S. muticum* due to the lack of sequence variation in ITS2 and Rubisco spacer (Table 5.4). The Φ_{ST} of *S. hemiphyllum* and *S. fusiforme*, in contrast, are high for the two markers, ranging from 0.990 to 1.000 (*S. hemiphyllum*) and 0.853 to 0.919 (*S. fusiforme*). The Φ_{ST} values of TrnW_I spacer are generally smaller than those of ITS2 and Rubisco spacer in both species (0.862 and 0.523, respectively). *Sargassum thunbergii* demonstrated a lower Φ_{ST} (0.324), and yet *S. muticum* exhibited a higher Φ_{ST} (1.000) in marker TrnW_I spacer.

The "among-groups" partition of variance in AMOVA, which indicates the percentage of variance explained by the grouping of the northern, central and

Table 5.4 The overall Φ_{ST} among the populations, and the results of neutrality tests (Tajima's D and Fu's F_{ST}) and the mismatch distribution analysis, of the four *Sargassum* species studied based on ITS2, Rubisco and TrnW_I spacers.

Parameters[1]	*S. hemiphyllum*	*S. fusiforme*	*S. thunbergii*	*S. muticum*
ITS2				
Φ_{ST} (all populations)	1.000**	0.919**	N.A.	N.A.
Tajima's D/	1.91 (N.S.)/	0.70659 (N.S.)/	0.00 (N.S)/	0.00 (N.S)/
Fu's F_{ST}	7.88 (N.S.)	-0.10396 (N.S.)	N.A.	N.A.
SSD / R	0.24* /	0.17*/	N.A.	N.A.
	0.69 (N.S.)	0.33**		
Rbc spacer				
Φ_{ST} (all populations)	0.990**	0.853**	N.A.	N.A.
Tajima's D/	3.38 (N.S.)/	-0.47 (N.S.)/	0.00 (N.S)/	0.00 (N.S)/
Fu's F_{ST}	8.22 (N.S.)	-0.46 (N.S.)	N.A.	N.A.
SSD / R	0.12 (N.S.)/	0.0033 (N.S.)/	N.A.	N.A.
	0.22 (N.S.)	0.23 (N.S.)		
TrnW_I spacer				
Φ_{ST} (all populations)	0.862**	0.523**	0.324**	1.000**
Tajima's D/	3.50 (N.S.)/	-0.65 (N.S.)/	-0.80 (N.S.)/	1.25 (N.S.)/
Fu's F_{ST}	10.17 (N.S.)	-1.05 (N.S.)	-1.06 (N.S.)	3.13 (N.S.)
SSD / R	0.11 (N.S.)/	0.033 (N.S.)/	0.00001 (N.S.)/	0.13 (N.S.)/
	0.13 (N.S.)	0.51 (N.S.)	0.79 (N.S.)	0.67 (N.S.)

[1] Φ_{ST} (all populations); SSD: Sum of Squared Deviation; R: Raggedness index; C.I.: confidence interval; N.A.: not applicable.

* $p<0.05$; **: $p < 0.01$; N.S.: non-significant,

southern populations with respect to the areas influenced by Yangtze and Yellow Rivers, was highest (74.05% - 100%) in *S. hemiphyllum* for all the three markers examined (Table 5.5). This variance of "among group" level in the other three species for the three markers is very low compared to that in *S. hemiphyllum* (Table 5.5). The high negative values in *S. fusiforme* for all the markers revealed that the variance obtained for this species is unlikely to be explained by the grouping patterns with respect to areas influenced by the two rivers (Table 5.5).

None of the two neutrality indices of all the species and were statistically significant (Table 5.4). *Sargassum fusiforme* showed almost all negative values of both Tajima's D and Fu's F_{ST} tests across the three markers investigated (Table 5.4), indicating that this species may have undergone sudden range expansion in its evolutionary history. A sudden range expansion may also occur in *S. thunbergii*, which exhibited negative value in the two neutrality tests, though the values of the tests could not be calculated based on ITS2 and Rubisco spacer markers. In contrast, neither *S. hemiphyllum* nor *S. muticum* revealed a negative value in both the neutrality tests (Fig. 5.4).

Table 5.5 Summary of the partitions of variance in AMOVA of the four species of *Sargassum* investigated, based on the three markers studied.

AMOVA	*S. hemiphyllum*	*S. fusiforme*	*S. thunbergii*	*S. muticum*
ITS2				
Among groups	100.00	-58.74	N.A.[1]	N.A.
Among populations within groups	0.00	150.66	N.A.	N.A.
Within populations	0.00	8.08	N.A	N.A
Rbc spacer				
Among groups	92.92	-20.18	N.A.	N.A.
Among populations within groups	6.17	105.48	N.A.	N.A.
Within populations	0.91	14.71	N.A.	N.A.
TrnW_I spacer				
Among groups	74.05	-2.86	-3.62	10.18
Among populations within groups	12.17	55.12	36.05	89.82
Within populations	13.77	47.74	67.57	0.00

[1] N.A.: Not applicable.

With regard to the mismatch distribution analysis, the sum of squared deviation (SSD) was statistically significant only for *S. hemiphyllum* and *S. fusiforme* in ITS2 (Table 5.4), reflecting that the frequency distribution of the pairwise nucleotide difference of these two species did not follow the model of sudden range expansion. In contrast, all the species possessed non-significant SSD for both Rubisco and TrnW_I spacers. Only *S. fusiforme* demonstrated a statistically significant raggedness index based on ITS2 sequences, which also indicated that the distribution curve of this species is not unimodal.

5.4 Discussion

5.4.1 Genetic differentiation among the four *Sargassum* species

Based on the markers used in this study, the pattern of genetic population differentiation among the four species studied appears to match the two types of geographical distribution patterns observed. *Sargassum muticum* and *S. thunbergii*, which are found inside the Bohai Gulf, Yellow Sea and its vicinity, possess no (ITS2, Fig. 5.2) or little (TrnW_I spacer, Fig. 5.4) genetic divergence among populations. In contrast, *S. hemiphyllum* and *S. fusiforme*, which are discontinuously distributed and do not occur in Bohai region, demonstrate deep intraspecific divergence in all the three markers (Figs. 5.2-5.4). The latter two species exhibit more haplotypes than the

former two with regard to the three markers studied (Table 5.3). While the distributions of the haplotypes of both *S. hemiphyllum* and *S. fusiforme* are relatively more localized, the major haplotypes of *S. muticum* and *S. thunbergii* are widely spread (Figs. 5.5-5.7).

The patterns of differentiation between *S. hemiphyllum* and *S. fusiforme*, however, were not consistent with each other (Figs. 5.5 - 5.7). *Sargassum hemiphyllum* possesses distinct genetic population differentiation that corresponds to the divergence of the northerly and southerly distributed varieties, with outflow from the two major rivers in eastern China serving potentially as a barrier (Fig. 5.5, refer to discussion in Chapter 3 for other details). The genetic break between the two major lineages of *S. fusiforme*, based on ITS2 sequences, appeared to be located in between eastern and southwestern Japan (Fig. 5.5). Moreover, this genetic break is not consistent among the three markers investigated. The sequences of Rubisco spacer marker from Seto Inland Sea population (JpOJ), but not including those from eastern Japan (JpSO), are distinct from those of the other populations (Fig. 5.6). On the other hand, the differentiation of eastern and southwestern Japanese populations (with Korean and Chinese populations) was barely observable based on TrnW_I spacer, with an admixture of haplotype F-T-C (dominant haplotype in southwestern

Japanese populations) with F-T-A in JpSO (Fig. 5.7). The genetic grouping of these populations could not be explained in terms of their biogeographic pattern of distribution with respect to the two rivers; in contrast to the case for *S. hemiphyllum* (Table 5.5).

No unequivocal conclusion could be drawn on whether sudden range expansion occurred in the four species due to inconsistency in various results obtained between neutrality tests and mismatch distribution analyses among different markers. No negative values were obtained in both neutrality tests throughout the three markers investigated for *S. hemiphyllum*, suggesting that this species might not have experienced range expansion recently (Table 5.4). This finding, however, can only be partially supported by the mismatch distribution analysis, in which the pairwise nucleotide differences followed a unimodal distribution curve in Rubisco and TrnW_I spacer but not in ITS2 (Table 5.4). The high negative values of the neutrality tests based on the three markers in *S. fusiforme* indicate that this species had likely undergone sudden range expansion (Table 5.4). However, this result is again not completely consistent with the mismatch distribution analysis as pairwise nucleotide differences followed a unimodal distribution curve only in analyses based on Rubisco and TrnW_I spacers (Table 5.4). On the other hand, *S. thunbergii* exhibits a

more consistent result showing a sudden range expansion based on both neutrality tests and mismatch distribution analysis, but it should be noted that the results are based only on the TrnW_I spacer data (Tables 5.4). For *S. muticum*, both neutrality tests yield positive values with $p > 0.05$ (Table 5.4), indicating that the spacer region investigated is selectively neutral and any recent range expansion was not likely. An inconsistent result, however, was obtained for the mismatch distribution analysis. The null hypothesis that the observed pairwise difference among haplotypes follows the model of the sudden range expansion was not rejected (Table 5.4). More specimens may be needed to verify the above findings on sudden range expansion in all the four species, and more variable markers may also be sought, in particular for *S. thunbergii* and *S. muticum*, so that more conclusive findings could be reached.

5.4.2 Glacial refugia and the intraspecific population differentiations

Whether the populations of a species are genetically differentiated depends on the balance between the differentiating force, such as mutation, genetic drift and selection, and homogenizing factors like the gene flow (Slatkin 1987). The allopatric speciation took place in various glacial refugia (e.g. Provan *et al.* 2005), for example, was driven by the diminished homogenizing factors (block of gene flow). Thus, the

diversity of the lineages (haplotypes) among populations of a species could reflect the fragmentation these populations experienced in their evolutionary history.

As mentioned in Chapter Three, the divergence of the two varieties of *S. hemiphyllum* was postulated to be related to the glacial refugia formed in East and South China Seas. These refugia harbored the ancestors of *S. hemiphyllum* and, because of their isolation during the last glacial periods, led to allopatric divergence of the species into two varieties. While these two varieties of *S. hemiphyllum* possess distinct sequences of ITS2 and Rubisco spacer regions, such pattern with distinct sequences among populations was not observed for both *S. thunbergii* and *S. muticum*. This suggests that no similar divergence occurred for these two species throughout their evolutionary histories. With reference to the most variable marker TrnW_I spacer, though there are divergences in both *S. muticum* and *S. thunbergii*, such divergences are shallow and involved only one base pair when compared with those in *S. hemiphyllum* and *S. fusiforme* (Figs. 5.2-5.4). The populations of *S. muticum* and *S. thunbergii* probably did not experience the same vicariant event and/or did not respond to the vicariant event in a similar way as *S. hemiphyllum* (Fig. 5.4).

Although *S. thunbergii* and *S. muticum* might not experience the same vicariant event as *S. hemiphyllum*, whether their ancestors survived through the glacial refugia in East and South China basins, as *S. hemiphyllum* postulated to be, remains unclear. Given their distribution range occurring mainly in Japan, Korea and northern China, the East China basin, instead of South China basin, is more likely to be involved in their evolutionary histories. The role of another possible refugium in the Sea of Japan, which was postulated to be important to the divergence of the subgenus *Bactrophycus* during the Pliocene to Pleistocene (Chapter 2), could not be ruled out. The divergence of the two main lineages of *S. fusiforme* may also be related to the glacial refugia in the Sea of Japan and the East China Sea. The eastern Japanese lineage may have evolved from the descendants that survived the Sea of Japan refugium, and the southwestern Japanese-Korean-Chinese lineage, the East China Sea refugium.

The presence of the four *Sargassum* spp. of subgenus *Bactrophycus* in various glacial refugia demonstrated in this study may suggest a different extent of the southward expansion among *Sargassum* spp. during glacial period, from the Sea of Japan where the *Bactrophycus* was suspected to radiate (Chapter 2). The deep divergence between the varieties of *S. hemiphyllum* and *S. fusiforme* may indicate an

early colonization of the southward-migrated ancestor throughout the glacial period, whereas the relatively shallow divergence in *S. thunbergii* and *S. muticum* restricted in Japanese populations may suggest a late differentiation of populations in the northern NW Pacific during Pleistocene.

5.4.3 Contemporary environment and the intraspecific population differentiations

The diversity of haplotypes might provide some hint on how the lineages of a species were differentiated in the past, whereas the present day distribution of haplotypes may reflect how these differentiated lineages were maintained and how the differentiated populations are connected by gene flow. The contemporary environment and the ecological demography of the organisms will undoubtedly affect this homogenizing force and, thus contribute to shaping the pattern of distribution of present genetic populations. Numerous factors are believed to help maintain population differentiation (Slatkin 1987, Lande 1988). For example, dispersal among different lineages in heterogeneous environments may be mediated through different regimes of ocean currents (Muss *et al.* 2001, Tsang *et al.* 2008). Different life histories among lineages may evolve due to environmental fluctuation (Kalisz *et al.* 2001). Different rates of local extinction/colonization may be

experienced by different lineages because of environmental change (McCauley 1991), like the presence of large river that led to adverse condition for maritime species (Lessios *et al.* 2001).

Salinity, although demonstrated to play a less important role in determining the biogeography of the genus *Sargassum* (Chapter 2), may be important in maintaining the phylogeographic pattern of some *Sargassum* species. The consistent allopatric distribution of the two *S. hemiphyllum* varieties with the genetic break occurring in the Yangtze and Yellow Rivers affected area may suggest the role of salinity in maintaining the differentiation of this species. On the other hand, for *S. fusiforme* that also possesses distinct genetic population differentiation, the river-associated environmental constraints do not seem to contribute to the maintenance of its intraspecific differentiation (Table 5.5). The major haplotypes of this species, such as F-R-B and F-T-C, are widely distributed not just in the Chinese coast but also along the Korean and southwestern Japanese coasts. Some of these dominant haplotypes were also found as far as JpSO, which is thought to be the region where the other lineage (eastern Japanese) inhabits. This strongly suggests that the maintenance of the population structure of this species is not related to the river discharge in China. Reduced salinity or other river-associated factors such as sedimentation did not stop

S. muticum and *S. thunbergii* from invading into the Gulf of Bohai. This may indicate that these two species possess stronger tolerance to brackish water compared to *S. hemiphyllum*. No genetic differentiation occurred in these two species along the Yangtze and Yellow Rivers affected area. This suggests that the physiochemical tolerance of these two species to fluctuating salinity is an essential trait aiding these species in expanding their range into the brackish waters.

Other factors such as the oceanic current regime may also affect the present genetic population structure of *Sargassum* species, as shown in other marine organisms in NW Pacific (Muss *et al.* 2001, Tsang *et al.* 2008). Two fruiting types of *Sargassum horneri*, namely autumn and spring fruiting types, were recognized in the Seto Inland Sea of Japan (Yoshida *et al.* 2001). Although these two types of *S. horneri* reproduce in different seasons, they could be crossed to produce offspring (Uchida & Arima 1993), indicating that these two types are conspecific. The admixture of the two types of *S. horneri* in Seto Inland Sea may suggest that it is, in fact, the admixture of two recently differentiated cryptic lineages. These two lineages may probably be the descendants of populations isolated separately in the Sea of Japan and the southern Japan during the last glacial maximum. The existence of these cryptic lineages awaits verification by other phylogeographic works. Nevertheless,

the genetic population structure of the four *Sargassum* species investigated in this study did not show a similar admixture of different lineages in the localities of the Seto Inland Sea (such as JpOJ, JpAJ and JpTS). Instead, the region of southern Honshu of Japan (such as JpWY and JpSO) remains the admixing region for at least *S. hemiphyllum* and *S. fusiforme* based on various markers.

Some of the haplotypes (e.g. H-T-A, H-T-B in *S. hemiphyllum*; F-R-B, F-T-D in *S. fusiforme*) in JpWY/JpSO are commonly found in the Chinese coast, suggesting that there might be an introgression of the southwestern Japanese and/or Chinese populations of these two species back to the eastern Japanese population. Direct evidence of transport of the drifting *S. horneri*, albeit with low frequency, was documented from the Zhejiang Province (the mouth of Yangtze River) to the eastern Kyushu of Japan (Komatsu *et al.* 2007). This transport is likely to be mediated by the combined effect of the coastal current (most probably the Yangtze Ring Haline Front due to the freshwater discharge from the Yangtze River, Park & Chu 2006) and the Kuroshio Current. Both *S. hemiphyllum* and *S. fusiforme* were found in the drifting *Sargassum* assemblage right off the locality, i.e. Wakayama of Japan (Komatsu *et al.* 2007), where admixture of haplotypes of *S. hemiphyllum* was detected. There is probably an unidirectional transport of drifting algal thalli from China to Japan

(Chapter 3), which may be mediated by the coastal currents and the Kuroshio Current during winter to late summer from the Chinese coast to Japan (Sekine & Kutsuwada 1994). This season corresponds to or is right after the reproductive period of *S. hemiphyllum* (February to May, Ang 2006) and *S. fusiforme* (April to June, Zou *et al.* 2006) in southern China. Similar situation was revealed in other marine taxa such as the intertidal barnacle *Tetraclita japonica* [Pilsbry, 1916]. Putative hybrids of which could be found in the southern Pacific side of Honshu, Japan (Tsang *et al.* 2008).

While *S. muticum* recorded in southern China may be a misidentification (Chapter 4), the occurrence of *S. thunbergii* and *S. fusiforme* in southern China may be the result of a recent range expansion (as indicated partly in neutrality and mismatch distribution tests) from north (i.e. Korea and Japan) to south (i.e. Taiwan and southern China) following the direction of the coastal current peaked in fall and winter (Liu *et al.* 2003). The successful recruitment of these two species in Hong Kong (the southern limit of the species) was different from year to year (Ang unpub. data), suggesting that the recruitment process is highly dynamic among years. This may be supported by evidence found in a cold-water copepod *Calanus sinicus* [Brodsky, 1962], the occurrence of which in Taiwan and Hong Kong are highly

correlated with the abovementioned coastal current (Hwang & Wong 2005). The occasional occurrence of *S. fusiforme* and *S. thunbergi* may be a result of the annual variation of the coastal currents flowing from the north to the south along the Chinese coasts.

To conclude, the present study elucidates contrasting patterns of genetic population structures among four closely related *Sargassum* species with continuous vs. discontinuous distribution patterns with regards to their presence or absence in areas heavily affected by the Yangtze and Yellow Rivers in China. The haplotype diversity of these species appears to be related to their evolutionary history, depending on whether they were harbored by various glacial refugia in NW Pacific or not. The distribution patterns of the haplotypes are largely determined by the contemporary environmental factors. While the seawater temperature *per se* probably does not contribute much to the present distribution pattern of the haplotypes in the four *Sargassum* species investigated, reduced salinity and other adverse environmental stresses associated with the river discharge in China may affect the genetic population structure of *S. hemiphyllum*, but not the other three species. The maintenance of the population structure of *S. fusiforme*, the other species possessing discontinuous distribution, appears not to be correlated with the effects of the two

rivers. Instead, such population structure may have been influenced by the oceanic and coastal currents. Extremely low genetic population differentiation is found in *S. muticum* and *S. thunbergii*, the distribution of which are continuous and extends into the rivers affected Bohai Gulf region. The reason behind this low genetic population differentiation, however, remains unclear. Their occurrences in the Bohai region may be attributed to their inherited physiochemical trait in tolerating reduced salinities. Physiological experiments would be needed to confirm these hypotheses.

Chapter Six: Preliminary study on the effects of reduced salinity on the growth and survival of the germlings and vegetative branches of *S. hemiphyllum* var. *chinense*

6.1 Introduction:

As a group of economically important marine organisms, seaweeds have been attracting interest from many researchers to study their cultivation under controlled conditions (e.g. Drew 1949, Deysher & Dean 1986). Artificial cultivation of seaweeds has focused on the completion of their life cycle (Drew 1949) and, since then, the improvement of the method of cultivation (e.g. Westermeier *et al.* 2006). Many researchers took advantage of the advancement of cultivation techniques to address various ecological questions, such as verifying the causes of biological invasion (Norton 1977b, Hales & Fletcher 1989, 1990).

Different environmental factors, e.g. seawater temperature and salinity, were shown to affect the survival, growth and reproduction of seaweeds (Hales & Fletcher 1989, 1990, Steen 2004). The individual or the synergistic effects of these factors are evident (Norton 1977b, Deysher & Dean 1986). In contrast to the sea surface temperature which has been considered to be the prime factor affecting algal biogeography (Lüning 1990), salinity is believed to act on a localized scale (Chapter

2). The hyposaline environment was also hypothesized to induce genetic population differentiation in algae over an evolutionary time scale (Cheang *et al.* 2008).

Environmental factors may affect *Sargassum* species differentially in their different growing stages. Norton (1977b) investigated the effect of temperature and salinity on *Sargassum muticum* and found that the performances of the germlings and the vegetative branches were different under different conditions. Other studies on the cultivation of *Sargassum* species, however, focus mainly on the effect of environmental factors on the germlings (e.g. Hales & Fletcher 1989, Steen 2004, Zhao *et al.* 2008b). The germling stage is suspected to be the most vulnerable period in the life cycle of the algae that will be influenced by adverse environmental constraint(s).

Ang (2006) found that *S. hemiphyllum* var. *chinense* in Hong Kong underwent the slowest growing stage during summer and then a rapid growth in autumn to winter. The rapid growth period was suggested to be initiated by the decrease in seawater temperature only. De Wreede (1978) pointed out that not just the low temperature but also high salinity will favor the growth and initiate the reproduction of *Sargassum* species in Hawaii. While salinity was shown to be an important factor in affecting the growth of other *Sargassum* species (Hales & Fletcher 1989, Steen 2004), Ang (2006) did not consider the role of the salinity in the phenology of *S. hemiphyllum*.

In the biogeography of *Sargassum* in NW Pacific, the reduced salinity condition in Yellow and Yangtze Rivers associated area was postulated to

discriminate the growth of *S. hemiphyllum*, and thus block the gene flow between the two varieties of this species, var. *chinense* and var. *hemiphyllum* (Cheang *et al.* 2008). This hypothesis can be verified by conducting laboratory culture of the species under different salinity conditions. The present study, thus, aimed at investigating the effect of salinity on the growth of the vegetative shoots as well as the germlings of *S. hemiphyllum* var. *chinense* under laboratory condition. The data were then compared with the field salinity data in order to understand how this factor could affect the phenology of this species. Results from the present study should provide direct evidence on whether reduced salinity adversely affects the growth of *S. hemiphyllum* and thus serves as a selective force in blocking the gene flow between the two varieties of this species, as postulated earlier.

6.2 Materials and Methods

This study consisted of tests on the growth and survival of vegetative branches and the newly developed germlings of *Sargassum hemiphyllum* under different salinities. Specimens of *S. hemiphyllum* were collected by snorkeling in Tung Ping Chau and Lung Ha Wan, Hong Kong and transported to the laboratory. Prior to the test on their salinity tolerance, all specimens were acclimated for two days in 0.45 μm-filtered (Millipore membrane filters, Billerica, MA) natural seawater (33 ppt) under the following conditions: photoperiod of 12h:12h (light: dark), irradiance of 35 μmol photon m^{-2} s^{-2} and temperature of 17°C for both germlings and branches. Both the acclimation and the salinity tests were carried out in a low temperature incubator (Model 2015, Sheldon Manufacturing Inc., Oregon, USA) with cool white fluorescent lamps.

6.2.1 Growth and survival of germlings

This part of the experiment was carried out from 17th May to 26th June in 2008. Fertile branches with mature receptacles were excised from different reproductive specimens and cultured in twenty 90 mm Petri dishes, each containing 50 mL of natural seawater (33 ppt). Since *S. hemiphyllum* is dioecious, separate branches with male and female receptacles were cultured in the same dish. After one day, the oocytes were released and fertilized. The zygotes eventually detached from the receptacles and sank to the bottom of the dish. Germlings were haphazardly collected from the fertilization Petri dishes and transferred to the treatment Petri dishes, each of which eventually contained 30 - 60 germlings. These dishes contained water in one of the seven salinity levels, 0 ppt, 5 ppt, 10 ppt, 15 ppt, 20 ppt, 25 ppt and 30 ppt, each with five replicates. Natural seawater at 33 ppt was used as a control. To enable the germlings to adapt to lower salinities gradually, the germlings were treated with stepwise reduction of salinity level, 5 ppt at every 1.5 h interval, until the desired salinity level was reached (adapted from Norton 1977b). Germlings in all the treatment dishes were cultured in the same enriched seawater IMR 1/2 medium (Eppley *et al.* 1967). The culture media were renewed every week till the end of the experiment in 40 days. In each treatment dish, the longest length of the pigmented portion of 10 haphazardly selected germlings was measured to the nearest 0.05 mm every 2 to 3 days under the dissecting microscope. The morphology of the germlings was also observed at the same time. The number of live germlings was counted in each dish, and germlings that lost their pigment completely were considered as dead.

6.2.2 Growth and survival of vegetative branch

This part of the experiment was conducted from 24th Mar to 4th May in 2004. New secondary shoots with length of around 5 cm were collected from separate *Sargassum* individuals and kept in 250 ml conical flasks, each with 150 ml seawater culture medium. Salinity levels of the water at 0 ppt, 10 ppt, 20 ppt, and 30 ppt, were adjusted by adding respective proportions of double distilled water with natural seawater. Natural seawater at 33 ppt was used as a control. Flasks with each level of salinity were prepared in triplicates, each with one branch. All treatments were cultured for 40 days, during which the culture media were renewed every week. The net wet weight, to the nearest 0.01 g, of each branch was monitored every two to three days using an electronic balance (Model GF-800, A&D Engineering, Inc., San Jose, USA). Branches that lost their pigment completely were noted as a sign of mortality.

6.2.3 Statistical analysis

For both the germlings and the branches, the relative growth rate (RGR) was calculated using the following equation (Hunt 1982):

$$\mathbf{RGR = (In\ W_2 - In\ W_1)/(t_2 - t_1)}$$

where W_1 and W_2 referred to the wet weight of branches (or the length of germlings) respectively at times t_1 and t_2. The same branch was measured repeatedly in the same sample; the equation was therefore used directly to calculate the RGR of each branch. For growth of the germlings however, ln W was an average of the 10 germlings measured so RGR was calculated for each Petri dish (Hoffmann & Poorter 2002). Furthermore, the survivorship of the germlings was calculated by dividing the number of live germlings with that counted in the previous measurement. Two-way

analysis of variance (ANOVA) was used to evaluate any significant changes in the RGR (calculated from the mean length of germlings) and the survivorship of germlings, with salinity levels and culture duration as the factors. Two-way ANOVA with one repeated-measures factor (culture duration) and one between-subjects factor (salinity levels) was used instead to evaluate any significant difference among RGRs (calculated from the mean weight of branches) of branches cultured under different salinity levels, since the branches measured were the same throughout the experiment. For both tests, homogeneity of sample variance was tested using Levene's test of homogeneity and data were transformed if parametric assumptions cannot be met (Zar 1999). Student-Newman-Keuls <SNK> test was used as a post hoc test to reveal the homogenous subsets of the tested parameter among treatments, in case a significant difference among samples was detected. All statistical tests were carried out using SPSS for Windows ver. 11.5.0 (SPSS Inc., Chicago, USA). The experimental results showing sensitivity of the germling growth and survivorship to salinity levels were evaluated with respect to the field salinity data monitored monthly in the sampling site since March, 2002, using a portable multimeter (Model 85, YSI Inc., Yellow springs, USA). How salinity change could have affected the phenology of *S. hemiphyllum* was then projected.

6.3 Results

6.3.1 Survivorship of the germlings

The survivorship of the germlings was significantly different under different salinities and over time (Table 6.1). No interacting effect between salinities and time (duration) was detected. All germlings cultured under different salinities demonstrated a type three survivorship curve (Pearl 1928), in which high mortality

Table 6.1 Results of the two-way analysis of variance (ANOVA) on the factors, salinities and culture duration, affecting the RGR and survivorship of germlings; and two-way ANOVA on the effect of culture duration as a repeated-measures factor and salinity levels as a between-subjects factor on the RGR of vegetative branches. Homogeneity subsets identified by post hoc SNK's test are indicated in Figs. 6.1, 6.2 and 6.5 for the effect of salinities on RGR of branches, survivorship and RGR of germlings respectively.

Factor	Sum of Squares	df	F	Sig.
Germlings				
Survivorship (Ln transformed)				
Duration (D)	26.085	16	16.030	<0.001
Salinity (S)	40.693	6	66.687	<0.001
D x S	7.589	82	0.910	0.694
Within Treatment	42.714	420		
Total	296.018	525		
RGR				
Duration (D)	0.922	16	23.311	<0.001
Salinity (S)	0.078	5	6.318	<0.001
D x S	0.463	80	2.341	<0.001
Within Treatment	1.008	408		
Total	3.287	510		
Branches				
RGR				
Duration (D)*	0.035	23	1.363	0.130
Within Treatment for D*	0.258	230		
Salinity (S)	0.045	4	6.951	0.006
Within Treatment for S	0.016	10		
D x S*	0.082	92	0.799	0.893

*: sphericity of data assumed

occurred early in the developing stage. There were different degrees of survival for germlings under different salinities, whereas only all those cultured in 0 ppt died in day 6 (Fig. 6.1). Germlings grown under 10 ppt shrank to as small as only half of their original size (Fig. 6.2) while those under 15 ppt attained the highest survivorship of 80% at the end of the experiment, followed by those cultured in 20 ppt and 10 ppt. High salinities at 25 ppt and 30 ppt were the worst conditions for the survival of germlings, with 60% and 70% mortality exhibited respectively in day 40 under these salinities.

6.3.2 Growth of the germlings

Both the culturing duration and salinity were demonstrated to be statistically significant factors affecting the RGR of *Sargassum* germlings (Table 6.1). The cross effect of these two factors was also significant. Based on the observed increase in the length of the germlings (Fig. 6.2), it can be deduced that the effect of culturing duration on RGR of germlings increased with the rise in the levels of salinity. There was no negative growth of the germlings recorded under all the salinity levels, while all the germlings cultured in 0 ppt salinity eventually died (Fig. 6.2). The sizes of the germling elongated at different rates, depending on the level of salinity they were in. At the end of the experiment at Day 40, their size differences ranged from 2 (5 ppt) to 7 times (25 ppt) of their original size at Day 0 (Fig. 6.2). The increase in the length of the germlings cultured at 30 ppt was the lowest, with the optimal growth rate being recorded in those cultured under 25 ppt (Fig. 6.2). The lowest lethal limit for the germling was within the range of 0 and 5 ppt.

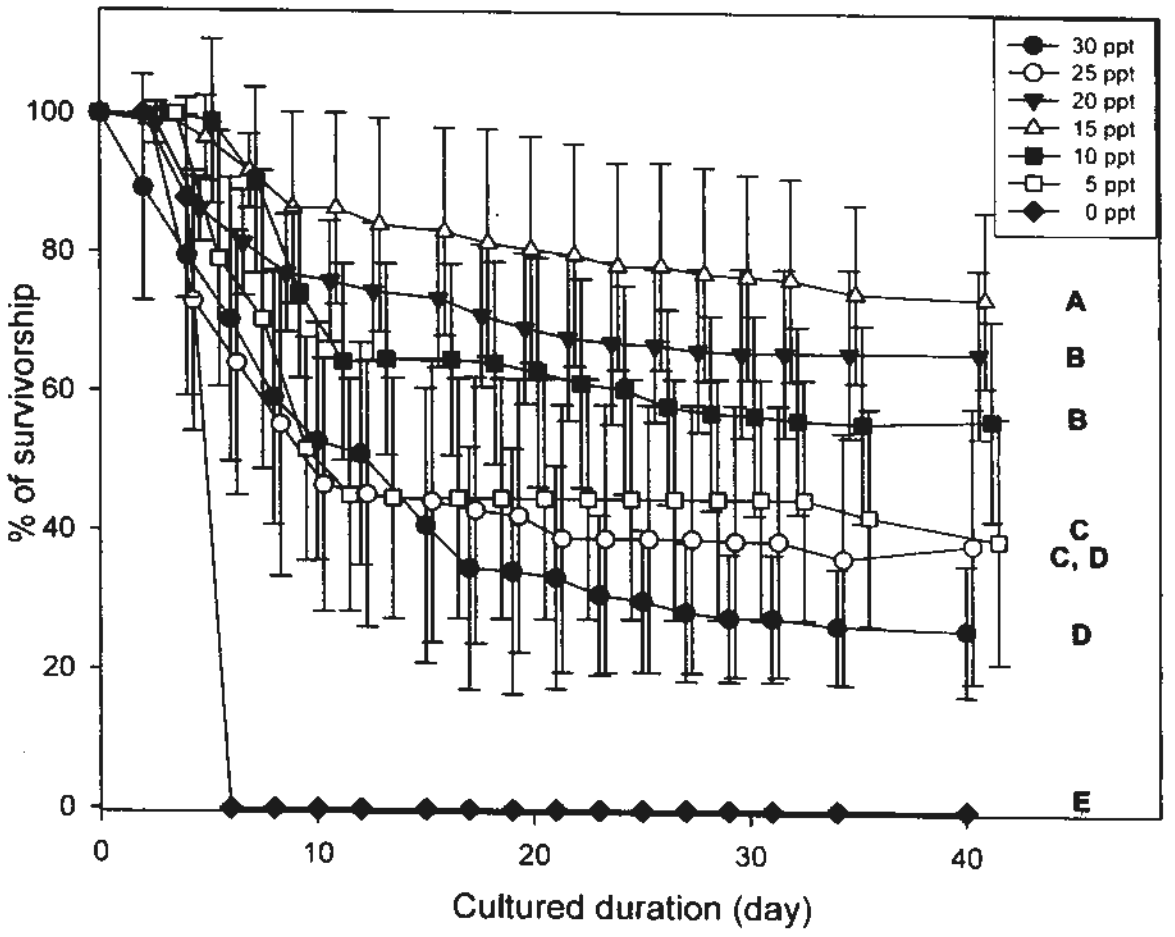

Figure 6.1 The mean survivorship (% ± SD) of germlings grown under different salinities. Survivorship was expressed as a percentage of the number of germlings remained alive at time t over the initial number of germlings at time 0 (Day 0). Results of post-hoc test for the factor salinity are also shown, with survivorship patterns which are not statistically significantly different under different salinity levels being indicated with the same letters.

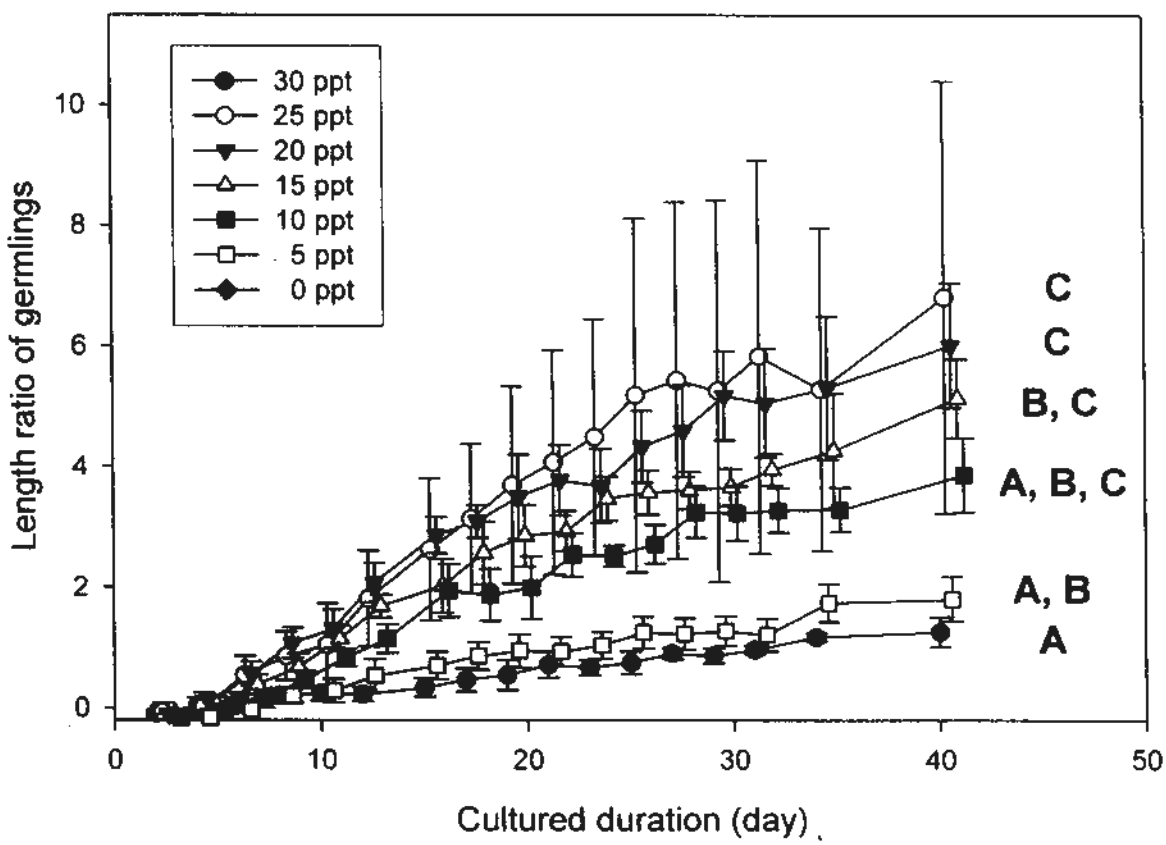

Figure 6.2 The effect of salinity on the growth of the germlings of *Sargassum hemiphyllum*. Growth was expressed as changes in the mean ratio (±SD) of germling length at time t over the initial length at time 0 (Day 0). Results of post-hoc test for the factor salinity are also shown, with growth patterns which are not statistically significantly different under different salinities being indicated with the same letters.

6.3.3 Morphological development of the germlings

Before the onset of the experiment, zygotes (of about 150 μm in diameter) in 2-cell (Fig. 6.3D), 3-cell (Fig. 6.3E) and 4-cell stages (Fig. 6.3F, G) were found emerging and adhering on the surface of the mature receptacles (Fig. 6.3A-C) being cultivated. Further developed multicellular germlings (Fig. 6.3H, I) became detached from the receptacle and anchored on the bottom of the Petri dish (Fig. 6.3J). Differential morphological development of the germlings grown under different salinities could be observed. Throughout the first 10 days of the experiment, active growth of "rhizoid" was observed in almost all the germlings, irrespective of the salinities of the culture medium, except those under 0 ppt (Fig. 6.4). Thereafter, the pigmented portion of the germlings increased conspicuously in length (Fig. 6.4) under higher salinity levels (i.e. 10 ppt or higher), with initial bifurcation developing at the apical part (e.g. at Day 10, 25 ppt, Fig. 6.4). Uniserial filaments developed in the distal part of the germlings cultured between 10 ppt and 25 ppt starting from day 10 (Fig. 6.4). Blades were obviously recognizable after day 19 (Fig. 6.4). At the end of the experiment at Day 40, many germlings demonstrated a sympodial growth (Fig. 6.4), during which the main growing axis was replaced by the growth of lateral branches or "leaves". Germlings reared under lower salinity levels (e.g. 5 ppt or 10 ppt), however, demonstrated a deficiency in rhizoidal development when compared to those cultured under higher salinities levels (Fig. 6.4). Their blades also did not develop as well as those under higher salinity conditions (Fig. 6.4). Cryptostomata were observed occasionally on the blades of the germlings cultured under salinity greater than 10 ppt starting from day 27.

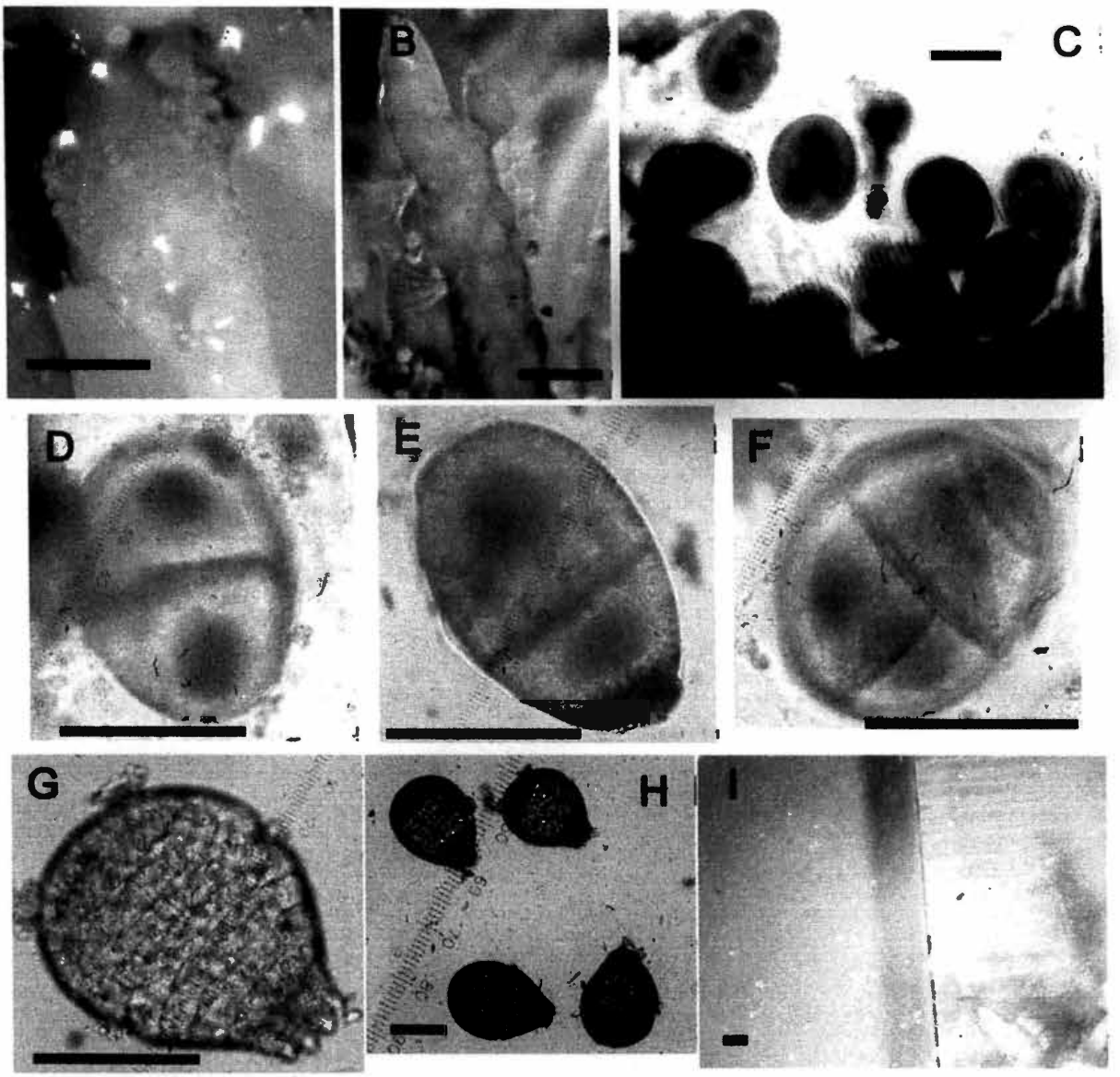

Figure 6.3A-I Photographs of receptacles with (A) emerging zygotes and (B), zygotes being released. (C) Various stages of zygotes adhering on receptacle surface, (D) two-cell stage, (E) three-cell stage with basal cell, (F) four-cell stage and (G, H) multiple-cell stage of zygotes, and (I) germlings attached on Petri dish. Scale bar = 1 mm (A, B, I), 100 μm (C-H)

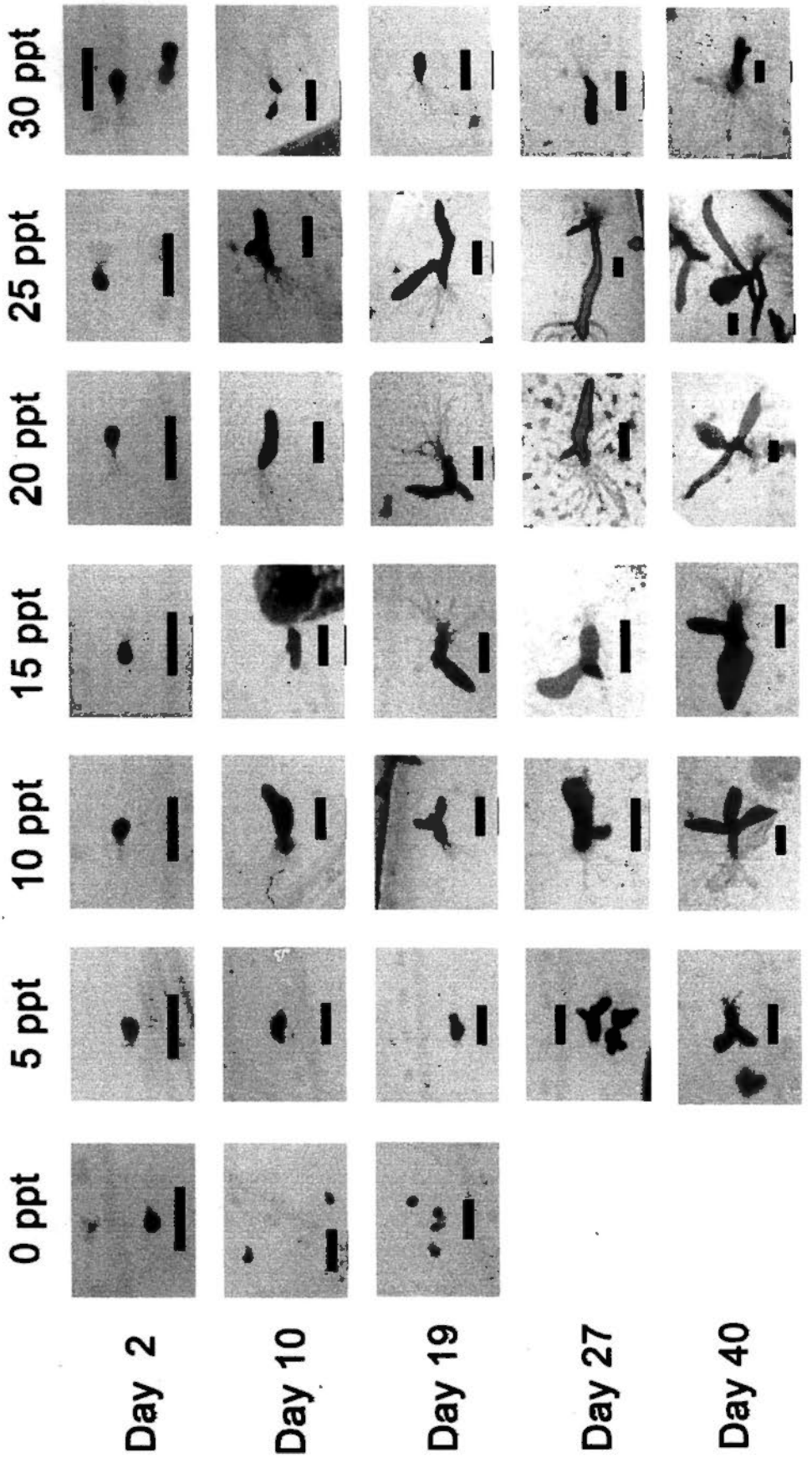

Figure 6.4 Morphological changes of the germlings of *S. hemiphyllum* grown under different salinity levels over time. All germlings died about Day 19 under 0 ppt. Scale bar = 500 μm

6.3.4 Growth and survival of vegetative branches

The vegetative branches of *Sargassum hemiphyllum* grown in 0 ppt of water appeared pale and were dead within 2 days of the experiment. The lethal limit of reduced salinity, thus, lies below 10 ppt (Fig. 6.5). All other branches survived through the end of the experiment under different salinity conditions. Branches responded significantly differently to different levels of salinity conditions (Fig. 6.5, Table 6.1). The growth rate of the branches increased with the rise in salinity level, with those under 33 ppt (control) sustaining the maximum growth. These branches attained 1.17 ± 0.51 (S.D.) times of their original weight at the end of the experiment. The RGR did not significantly change over time (Table 6.1), indicating a steady growth rate during the experimental period. No additive effect was revealed between the factors, salinity levels and the culturing duration, on the RGR of vegetative branches (Table 6.1).

6.3.5 The salinity change in the natural environment from 2002 to 2008

Salinity fluctuated within the range of 24 ppt to 35 ppt over the six-year period from 2002 to 2008. It varied between 31 and 35 ppt during winter and between 24 and 31 ppt during wet summer season (Fig. 6.6). This general pattern was repeated each year with a slight variation in the lowest level of salinity recorded in summer.

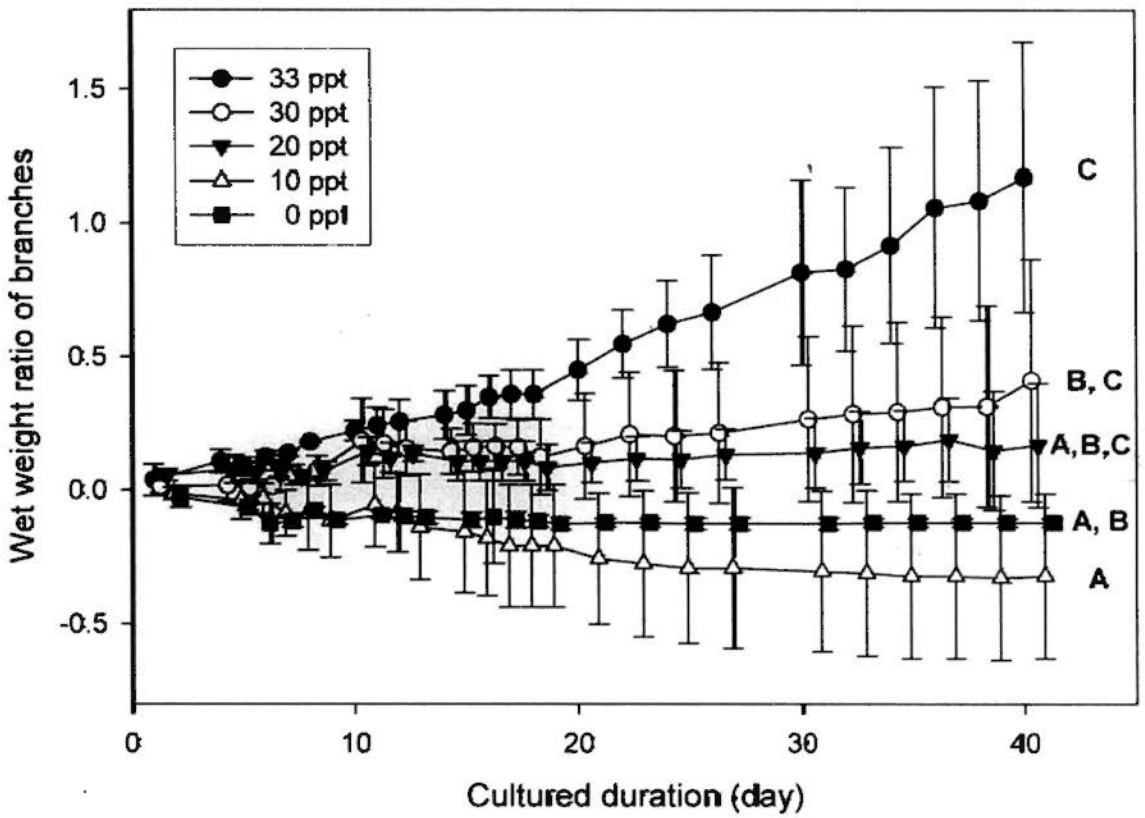

Figure 6.5 The effect of salinity on the growth of vegetative branches of *Sargassum hemiphyllum*. Growth was expressed as changes in the mean ratio (±SD) of branch wet weight at time t over the initial weight at time 0 (Day 0). Results of post-hoc test for the factor salinity are also shown, with growth patterns which are not statistically significantly different under different salinities being indicated with the same letters.

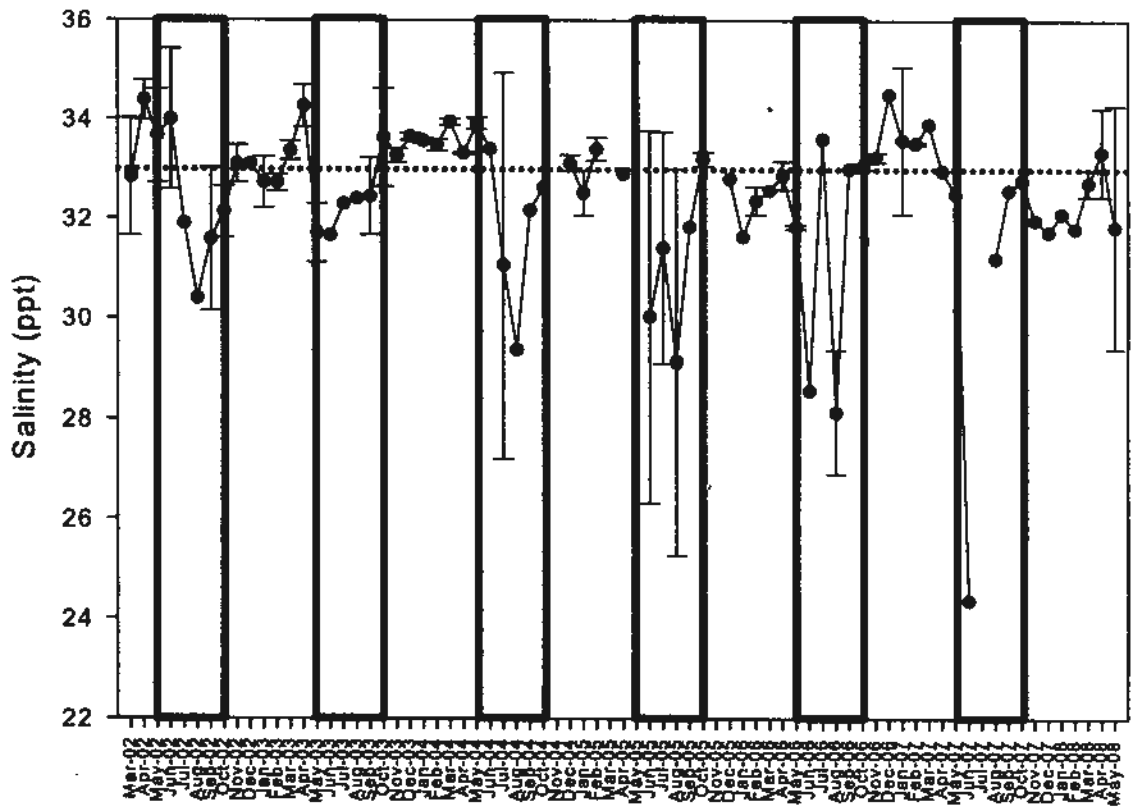

Figure 6.6 The salinity level (± S.D.) in Lung Lok Shui, Tung Ping Chau Marine Park, Hong Kong from Mar 2002 to May 2008. Dotted line refers to the optimal growing salinity for vegetative branches determined in this study, while the rectangle refers to the slow growing period of *S. hemiphyllum* var. *chinense* (Ang 2006).

6.4 Discussion

6.4.1 The morphological development of zygote

The size of the zygote of *S. hemiphyllum* var. *chinense* (~ 150 μm) is slightly larger than that of *S. hemiphyllum* var. *hemiphyllum* (125 μm from Inoh 1930). It is comparable to those of other *Sargassum* species that ranged from 100 to 300 μm in diameter (Norton 1977b, Honda & Okuda 1990, Yoshida *et al.* 1999, Hwang *et al.* 2006, Zhao *et al.* 2008b). The observed patterns of morphological development agree well with those reported in other *Sargassum* species such as *S. muticum* (Norton 1977b), *S. micracanthum* (Honda & Okuda 1990) and *S. thunbergii* (Zhao *et al.* 2008b). After the first equatorial division, the zygotes of these species developed the basal cell (Norton 1977b) (Fig. 6.3E), which soon differentiated into the rhizoid. The appearance of the multicellular germlings with basal rhizoids (Fig. 6.3H) is almost identical in all *Sargassum* species studied (Inoh 1930, 1932). Similar to *S. hemiphyllum*, development of rhizoid was observed prior to the elongation of the main body (Fig. 6.4) in species like *S. micracanthum* (Honda & Okuda 1990) and *S. thunbergii* (Zhao *et al.* 2008b). Furthermore, the germlings of *S. hemiphyllum* var. *chinense* demonstrated a sympodial growth (Fig. 6.4), like that in other *Sargassum* species (Norton 1977b, Arai & Miura 1991).

6.4.2 The effect of reduced salinity on the growth and survival of the germlings

The germlings could not survive in freshwater (0 ppt), while positive growth was recorded at salinity as low as 5 ppt or higher. This lower lethal limit of 0-5 ppt resembles that in the closely related species *S. muticum*, which ranges from 3.4 to 6.8 ppt (Hales & Fletcher 1989). Both of these species belong to the subgenus

Bactrophycus. In contrast, this limit is lower than that between 10 and 15 ppt for the juveniles of *S. ringgoldianum*, a member of subgenus *Sargassum* (Arai & Miura 1991). Steen (2004) also found that the germlings of *S. muticum* could not survive at 0 ppt even though they were allowed to recover at 30 ppt after one week of exposure. The survival of the germlings was decreased to 40% after a week of exposure to 5 ppt medium in their first week of development (Steen 2004). This value is comparable to ~40% survivorship recorded for *S. hemiphyllum* var. *chinense* under 5 ppt salinity level in the present study (Fig. 6.1). Comparing with the 70%- 100% survivals of the germlings of *S. muticum* in salinities between 10 ppt and 30 ppt when the recovery was allowed (Steen 2004), the highest survivorship (~80%) of var. *chinense* occurred in 15 ppt and the survivorship in other salinity levels (10 ppt, 20 ppt, 25 ppt and 30 ppt) ranged between 30% to 70% (Fig. 6.1).

The optimal growth of germlings of *S. muticum* was recorded between 30 ppt to 35 ppt by different authors (Steen 2004, Hales & Fletcher 1989, Norton 1977b), whereas the optimal growth was recorded at 30 ppt in *S. ringgoldianum* (Arai & Miura 1991). All the germling of *S. muticum* and *S. ringgoldianum* showed gradual decrease in length with the decrease in salinity (Norton 1977b, Hales & Fletcher 1989, Arai & Miura 1991, Steen 2004). The RGR of germlings cultured at low salinities was shown to be different from the controlled treatment as well (Steen 2004). Similar gradual suppression of growth in the germlings cultured at lower salinity levels was also observed in the present study. However, the optimal growth of the germlings of *S. hemiphyllum* var. *chinense* at 25 ppt was relatively lower. Some epiphytic growth of diatoms on the seaweed in the culture medium after day 14 was observed and this may have contributed to the slower growth of the

germlings under 30 ppt. Nonetheless, the presence of different optimal growth conditions among the germlings of different *Sargassum* species could not be ruled out.

Other than the survivorship and growth of germlings, reduced salinity also affected the development of rhizoid. The length of the rhizoid of species like *S. echinocarpum, S. horneri* and *S. muticum* decreases with a reduction in salinity (De Wreede 1978, Ogawa 1986). Some of the individuals of *S. echinocarpum* and *S. obtusifolium* demonstrated zero growth of the rhizoid at 15 ppt (De Wreede 1978), while no germination of rhizoid in *S. fusiforme, S. horneri* and *S. muticum* occurred at salinities lower than 6.5 ppt, 16.3 ppt and 12.9 ppt respectively (Ogawa 1986, 1994, Ogawa *et al.* 1996). The range of the salinity that the rhizoid of *S. hemiphyllum* var. *chinense* was unable to develop was between 5 ppt and 10 ppt, which is comparable with those of the closely related species such as *S. muticum* (Ogawa 1994). Although the germlings of var. *chinense* cultured at 5 ppt or 10 ppt survived throughout the experimental period, the poor development of rhizoid may make it difficult for them to attach, hence to grow into larger thalli under natural environment with strong waves.

6.4.3 The effect of reduced salinity on the growth and survival of the vegetative branches

Similar to the germlings, the vegetative branches of *S. hemiphyllum* could not survive at 0 ppt, and the lethal salinity limit lies between 0 ppt and 10 ppt. This limit also seems to be comparable to that for *S. muticum* (Norton 1977b), in which the growth of the detached branches was recorded to increase with salinity from 9 ppt

within the temperature ranges of 10 to 25°C. The lethal limit for the receptacles of *S. muticum* was also shown to lie between 3.4 ppt and 8.5 ppt in another study (Hales & Fletcher 1990).

The salinity (27 ppt) for the optimal growth of *S. muticum* (Norton 1977b), however, appears to be different from that (33 ppt) of *S. hemiphyllum* var. *chinense* in the present study. Similar optimal salinity level at 23.8 ppt (Hales & Fletcher 1990) was exhibited for the receptacles of *S. muticum*. It could be suggested that these two species likely perform differentially in the field environment with different fluctuating salinity conditions.

6.4.4 The possible effect of low salinity on the natural population of *S. hemiphyllum*

Sargassum hemiphyllum var. *chinense* grows slowly in summer and rapidly in autumn and winter (Ang 2006). While lowering of the temperature in autumn has been suggested as a cue to rapid growth for this species, the findings of this study provide another possible explanation on the cause of the slow growth in summer. Salinity fluctuation during summer is a common phenomenon in the coastal subtropical region of southern China like Hong Kong, as this is also the SE monsoon season characterized with heavy precipitation. Salinity in the sampling site in Tung Ping Chau reached a level as low as 24 ppt over this period (Fig. 6.6). The slow growth of *Sargassum* during summer may thus be due to the suboptimal condition for the growth of the vegetative branches at salinity level lower than 33 ppt (Fig. 6.6). The level of salinity increases to 31 ppt or above during autumn and winter, a level that is suitable for *S. hemiphyllum*, hence the higher growth rate recorded

during this period (Ang 2006). De Wreede (1978) found out that the condition (salinity: 30 – 35 ppt and temperature: 24°C) of maximum growth in three Hawaiian *Sargassum* species coincided with that in the natural environment when *Sargassum* became reproductive during winter and early spring. This observation appears to be applicable in the present case of *S. hemiphyllum* as well, at least with respect to the salinity level in the natural environment.

Autumn in Hong Kong is characterized by lowering of seawater temperature and shortening of photoperiod. These other environmental factors may affect the growth rate of *S. hemiphyllum* synergistically with increase in salinity. The tolerance of *Sargassum* spp. to hyposaline condition has been shown to change with temperature (Norton 1977b, De Wreede 1978). The integrative effect of different environmental factors with the salinity in affecting the growth and survival of both vegetative branches and germlings of *S. hemiphyllum* would thus need to be verified in further studies to obtain a better picture of the effect of environmental conditions in affecting the structure of the coastal community in subtropical areas.

6.4.5 The tolerance of reduced salinity of *S. hemiphyllum* var. *chinense* and its distribution

The maximum growth of *S. hemiphyllum* var. *chinense* under 33 ppt salinity and the inability for its germlings to develop rhizoid under low salinity level (5 ppt, 10 ppt) clearly indicate that this species is more suitable to survive in a truly marine coastal environment rather than in a brackish shore. The southern limit of distribution of *S. hemiphyllum* var. *chinense* is reported to be in northern Vietnam. The Pearl River estuary in southern China appears not to be a barrier to its southern

spread. In contrast, the northern limit of this variety in the Zhejiang province of China is immediately south of the mouth of the Yangtze River. The huge hyposaline environment of the Yangtze River and Yellow River estuaries appears to create a dispersal barrier for this variety to expand further northward, especially given the presence of prevailing southward flowing coastal currents in this region (Hwang & Wong 2005).

Whether there is ecotypic differentiation of the two varieties of *S. hemiphyllum* with regards to their tolerance to hyposaline environment remains to be verified by similar experimental evaluation of salinity tolerance of specimens of var. *hemiphyllum*. The absence of var. *hemiphyllum* in the southern Chinese coast as well as the distinct genetic difference between the two varieties (Cheang *et al.* 2008) suggest that var. *hemiphyllum* may also be incapable of crossing this extensive hyposaline barrier created by the Yangtze and the Yellow Rivers. This hyposaline coastal area of Bohai and Yellow Sea may be a significant factor in maintaining the intraspecific population differentiation of *S. hemiphyllum*, by effectively blocking the gene flow between its two varieties.

In summary, the condition of reduced salinity affects both the growth and survivorship of *S. hemiphyllum* var. *chinense*. The maximum growth rate of its vegetative branches in 33 ppt salinity appears to coincide with the environmental condition associated with its fast growing stage as well as its reproductive period. On the other hand, the suboptimal growth of its branches and germlings under lower salinity level demonstrated in the laboratory appears to be reflected in its inactive slow growth period associated with low and fluctuating salinity condition in summer.

Salinity may be a significant environmental factor in maintaining its intraspecific population differentiation, by long blocking the gene flow between its two varieties. The synergistic effect of reduced salinity with other environmental factors, such as temperature, would need to be further assessed to obtain a more comprehensive understanding of the role of salinity in affecting the distribution of *Sargassum hemiphyllum* in NW Pacific.

Chapter Seven: Conclusion and Perspective

7.1 Conclusion

7.1.1 The distribution of *Sargassum* spp. in NW Pacific and the potential governing environmental factor

A total of 151 species of *Sargassum* were recorded in NW Pacific. Based on their presence or absence among the 47 Operational Geographical Units (OGUs) defined (Chapter 2), three main biogeographic clades of *Sargassum* were recognized in this region. The highest species richness was found in southern China and southern Japan. The degrees of compositional difference of *Sargassum* species among OGUs were most positively correlated with the combined effect of the mean lowest sea surface temperature (SST) in winter, latitude (which reflected photoperiodicity) and mean annual salinity, compared to lower correlation with geographical distance and Euclidean distance among OGUs, generated by differences in environmental parameters among them. The SST was believed to be the most critical factor in structuring *Sargassum* biogeographic patterns with the SST in the coldest month having the most profound effect. There was a transition zone of occurrence between the species under subgenus *Bactrophycus* and those under subgenus *Sargassum*. The *Bactrophycus* spp. are likely to be more recently radiated

especially in north NW Pacific and possessed variable eurythermal ability. In contrast, species under subgenus *Sargassum* are more primitive and originated in the Tropics.

7.1.2 Phylogeographic studies of *Sargassum* spp. in population level

The present distribution pattern of *Sargassum* in the NW Pacific has been synthesized (Chapter 2) and a hypothetical scenario to account for the pattern was suggested. However, the effect of different environmental factors on the biogeography of *Sargassum* spp. in a finer scale, i.e. the population level, remains unclear. The studies of phylogeography of *Sargassum hemiphyllum* and *S. muticum* in Chapters Three and Four respectively demonstrate two different phylogeographic patterns represented by these two species, both of which are under subgenus *Bactrophycus* and phylogenetically closely related (Stiger *et al.* 2003).

Two allopatrically distributed varieties of *S. hemiphyllum*, var. *chinense* and var. *hemiphyllum*, were found to be genetically distinct in terms of the ITS2 and Rubisco spacer. The genetic break between these two varieties, with var. *chinense* distributed in southern Chinese coast and var. *hemiphyllum* in Japan and Korea, is situated in the

region including Bohai, Yellow Sea and East China Sea. This region is heavily influenced by the Yangtze and Yellow Rivers in China. An introgression of the mitochondrial genome from var. *chinense* to var. *hemiphyllum* is evident based on the mitochondrial marker TrnW_I spacer. This introgression is likely to be mediated by the Kurushio current. The hybridization between the two varieties may still be ongoing since the concerted evolution of ITS2 is not yet saturated in the Korean population, which is geographically in-between the distribution of the two varieties.

In contrast, *S. muticum* exhibited no variation in ITS2 and Rubisco spacer sequences, regardless of the native or introduced populations examined. A fixed one-nucleotide difference in the TrnW_I spacer occurs between the population of eastern Japan and the rest of the other populations. This finding aids in revealing the western and central Japan (Seto Inland Sea) as the most likely source localities of the introduced *S. muticum* populations in both the eastern Pacific and eastern Atlantic coasts. The intense Pacific oyster farming industry and exportation of oysters from these regions in Japan to establish other farming industries in North America and Europe was believed to account for the introduction.

7.1.3 Comparison of the phylogeographic studies of the four *Sargassum* spp.

To investigate the effect of river outflow on shaping the genetic population structure of *Sargassum* spp., a comparative phylogeographic study was carried out in Chapter Five on four closely related *Sargassum* species which show two different distribution patterns. *Sargassum hemiphyllum* and *S. fusiforme* are not found in Bohai and Yellow Sea, which are heavily influenced by the Yangtze and Yellow Rivers in China, and demonstrate a discontinuous distribution pattern. *Sargassum thunbergii* and *S. muticum*, in contrast, occur in the rivers affected region and exhibit a continuous distribution pattern along the coast of China.

A contrasting pattern of genetic population structure was obtained, based on the nuclear ITS2, plastid Rubisco spacer and mitochondrial TrnW_I spacer, between the species showing these two distribution patterns. No or little population differentiation, depending on the markers, is revealed for the species with a continuous distribution. The discontinuously distributed species, however, exhibit a deep genetic divergence among populations based on various genetic markers. Two distinct lineages corresponding to the two varieties inhabiting the northern and southern regions of the Bohai and Yellow region is revealed in *S. hemiphyllum*, as shown in more details in

Chapter Three. Two main lineages are also present in *S. fusiforme* based on ITS2 and TrnW_I sequences, but the genetic break between the two lineages is situated in the region between eastern and southwestern Japan, i.e., in a location different from that separating the two *S. hemiphyllum* varieties. The two *Sargassum* species with discontinuous distribution exhibit more haplotypes (e.g. four in TrnW_I spacer) than species with continuous distribution (two in TrnW_I spacer). This is likely related to whether they survive through the glacial refugia and how fragmented their ancient populations were. Glacial refugia in the Sea of Japan or the East China Sea may harbor the ancestors of *S. thunbergii* and *S. muticum*, while the two main lineages of *S. fusiforme* might survive in both refugia.

The current distribution of the haplotypes was largely determined by the contemporary environmental factors. While the presence of large rivers was postulated to maintain the genetic population differentiation in *S. hemiphyllum* (Cheang *et al.* 2008, also Chapter 3), the present study has shown that reduced salinity and other adverse environmental stresses associated with the occurrence of the two rivers in China affect only the genetic population structure of *S. hemiphyllum*, but not that of *S. fusiforme* which is also discontinuously distributed. The maintenance of the population structure of *S. fusiforme* appears not to be correlated

with the effect of the two rivers. The oceanic current is believed to contribute to the present genetic population structure to certain extant, since there are introgressions in the eastern Japanese population of *S. hemiphyllum* and *S. fusiforme*. Shallow differentiations were detected in *S. muticum* and *S. thunbergii*. Both of these species are continuously distributed and extend into coastal areas heavily affected by the rivers. This differentiation may be due to more recent vicariant events unlikely to be similar to the ones experienced by *S. hemiphyllum* and *S. fusiforme*. The occurrence of *S. muticum* and *S. thunbergii* in the Bohai region may be attributed to their inherited physiochemical trait in being able to tolerate brackish water.

7.1.4 The tolerance of *S. hemiphyllum* v. *chinense* to reduced salinity

In order to directly verify the effect of low salinity on *S. hemiphyllum* var. *chinense*, which was absent from the brackish water in Bohai and Yellow sea, the tolerance of both germlings and vegetative branches to reduced salinity was tested in Chapter Six. The growth rate of the branches rose with the increase in salinity level till 33 ppt. The lethal limit of vegetative branches to reduced salinity lies between 0 ppt and 10 ppt. Germlings cultured in 15 ppt attained the highest survivorship. The optimal growth of the germlings occurred at 25 ppt, while the lowest lethal limit was

within the range of 0 ppt and 5 ppt. Germlings reared under low salinity were deficient in rhizoid development, making them highly unlikely to grow into large thallus in the natural environment exposed to strong waves.

The maximum growth of *S. hemiphyllum* var. *chinense* under salinity 33 ppt and the inability to develope rhizoid under low salinity level (5 ppt, 10 ppt) indicate that this species is suitable to survive in an oceanic coastal area rather than in an estuarine coast. The salinity level of maximum vegetative growth coincides with the environmental condition for fast growing stage as well as the reproductive period of this variety in Hong Kong, whereas the suboptimal growth of branches and germlings under the lower salinity levels resembles the inactive period over its life history.

The northern limit of the distribution range of variety *chinense* of *S. hemiphyllum* may be determined by the tolerance of this variety to hyposaline condition. Salinity may be a significant environmental factor in maintaining the intraspecific population differentiation of *S. hemiphyllum*, by blocking the northward dispersal of variety *chinense*, as well as, possibility stopping the southward transport of variety *hemiphyllum*. The lethal limits of both vegetative branches and germlings

of *S. h.* var. *chinense* are comparable to those of *S. muticum*, which occurs in the estuarine Bohai region. The optimal growth of branches of *S. muticum* occurred under salinity level of 27 ppt, in contrast to the optimal salinity level of *S. hemiphyllum* at 33 ppt. This could have explained the absence of *S. hemiphyllum* in brackish water and support the suggestion that river discharge serves as a barrier for the exchange of genetic materials among its populations.

7.2 Future prospective

7.2.1 The analytical biogeographic analysis

Although some practical problems remain to be addressed (Lewis 1990), the rich literature documenting algal distribution throughout the world provides a ready and comprehensive dataset for analytical biogeography. Continuing efforts to compile and analyze these datasets will undoubtedly enable us to obtain a more reliable picture of global phycogeography than the conventional models based on the projection of particular algal groups.

The study in Chapter Two illustrates the usefulness of multivariate clustering and related techniques, e.g. "BEST", "LINKTREE", in identifying potential environmental factors structuring algal assemblages. Besides identifying the prime

factor (winLSST in this study), this approach enabled other minor factors (e.g. salinity) to be determined as well, and seems clearly superior to conventional approaches that are unable to provide such information. The multivariate clustering analysis together with analyses like "BEST" and "LINKTREE" should serve as useful tools to elucidate the structuring forces of other algal assemblages as well as other ecological communities.

The data on the abundance of each *Sargassum* species also offer another useful information for characterizing the OGUs. Although two studies have considered the phenology of *Sargassum* in NW Pacific (Yoshida *et al.* 2001, Ang 2006), the available information is inadequate to yield a complete picture for this region. Phenological studies on *Sargassum* from different localities are necessary to better understand the underlying structuring force(s) behind their biogeographical distribution, not just in the NW Pacific but elsewhere in the world.

7.2.2 The phylogeographic studies on *Sargassum* spp.

Although some molecular phylogenetic studies on *Sargassum* (Stiger *et al.* 2000, 2003, Oak *et al.* 2002, Phillips *et al.* 2005) have been conducted, a robust taxonomic scheme of *Sargassum* reflecting its phylogeny has not yet been achieved. It is

necessary to obtain more molecular data in order to improve the quality of the available biogeographic information, for instance to clarify the status of the morphological variants which may have been misidentified as new species.

Besides, the elucidation of the ecological demography among populations by molecular markers also provides insight(s) on the factor governing the biogeography of *Sargassum* in this region. This, however, requires relatively variable genetic markers. Engel *et al.* (2008) compared eight markers in the mitochondria genome, together with Rubisco spacer in plastid genome, for their performance in analyzing the phylogeny and the phylogeographic pattern in nine laminarialean and three fucoid species of brown algae, in which no Sargassoceous species were included. Some markers, e.g. rps3_rps19 and TrnW_I spacers, demonstrated intraspecific variation and are proposed to be good in the phylogeographic studies. The studies in Chapter Two, Three and Four have, indeed, proven the applicability of the mitochondrial marker (TrnW_I spacer) in the phylogeographic study in some *Sargassum* species. For the *Sargassum* spp. with little or even no genetic population differentiation based on mitochondrial markers, other more variable markers such as microsatellite (van der Strate *et al.* 2002) could be used to demonstrate the demography of these species. For instance, the source of introduction of the invasive

S. muticum from its native range would be better resolved if more variable markers are used. The contrasting patterns of population differentiations among the four *Sargassum* species investigated illustrate that, though being phylogenetically closely related, *Sargassum* species could experience different evolutionary histories and/or respond differently to the same geological history. A more comprehensive picture of how the populations of these *Sargassum* spp. differentiated is sought, and yet this could be achieved only when more phylogeographic works of *Sargassum* are done in NW Pacific. The different patterns of population differentiation revealed in *Sargassum* undoubtedly help to shed lights on the understanding of the different evolutionary histories existed in the marine flora of this region.

7.2.3 The potential effect of elevated SST on *Sargassum* spp.

The importance of SST in structuring *Sargassum* biogeography revealed in Chapter Two should alert researchers to the potential effect of elevated SST due to global warming on the current and future distribution of *Sargassum*. The potential is always there for a range expansion or range shift of *Sargassum* spp., which are important components of the marine coastal environment. Should these occur, a possible consequence would be a change in the whole coastal community or ecosystem structure with a potential shift in the composition and diversity of other

associated organisms. It is therefore essential to monitor the range distribution and phenology of *Sargassum* and other macroalgae on a long-term basis, to enable the effect of global warming on changes in the structure of coastal communities to be more effectively assessed.

All in all, the brown macroalgal genus *Sargassum* is an ecologically important component of the marine ecosystem in NW Pacific region. The elucidation of the biogeography of *Sargassum* spp. and how the biogeographic pattern was formed and/or sustained are certainly providing insights for a better understanding of the biogeography of the marine flora in NW Pacific, which is one of the crucial components in understanding the past history of this planet.

References:

Abbott, I. A. 1985-1999. *Taxonomy of Economic Seaweeds: With Reference to Pacific Species*. California Sea Grant College, La Jolla, USA.

Adey, W. H. and Steneck, R. S. 2001. Thermogeography over time creates biogeographic regions: a temperature/space/time-integrated model and an abundance-weighted test for benthic marine algae. *Journal of Phycology* **37**:677-98.

Ajisaka, T., Nang, H. Q., Dinh, N. H., Lu, B., Chiang, Y. M. and Yoshida T. 1997. *Sargassum hemiphyllum* (Turner) C. Agardh var. *chinense* J. Agardh from Vietnam, the Chinese mainland, and Taiwan (Fucales, Phaeophyta). *In:* Abbott, I. A. [Ed.] *Taxonomy of Economic Seaweeds with Reference to Some Pacific Species. Vol. 6.* . California Sea Grant College Program, University of California, La Jolla, USA, pp. 37-50.

Alverson, A. J. and Kolnick, L. 2005. Intragenomic nucleotide polymorphism among small subunit (18s) rDNA paralogs in the diatom genus *Skeletonema* (Bacillariophyta). *Journal of Phycology* **41**:1248-57.

Anderson, E. 1953. Introgressive hybridization. *Biological Reviews* **28**:280-307.

Andreakis, N., Procaccini, G., Maggs, C. and Kooistra, W. H. C. F. 2007. Phylogeography of the invasive seaweed *Asparagopsis* (Bonnemaisoniales, Rhodophyta) reveals cryptic diversity. *Molecular Ecology* **16**:2285-99.

Andrew, N. L. and Viejo, R. M. 1998. Ecological limits to the invasion of *Sargassum muticum* in northern Spain. *Aquatic Botany* **60**:251-63.

Ang, P. O. 2006. Phenology of *Sargassum* spp. in Tung Ping Chau Marine Park, Hong Kong SAR, China. *Journal of Applied Phycology* **18**:629-36.

Arai, A. and Miura, A. 1991. Effects of salinity and light intensity on the growth of brown algae, *Sargassum ringgoldianum*. *Suisanzoshoku* **39**:315-19.

Arbogast, B. S. and Kenagy, G. J. 2001. Comparative phylogeography as an integrative approach to historical biogeography. *Journal of Biogeography* **28**:819-25.

Avise, J. C. 1992. Molecular population structure and the biogeographic history of a regional fauna : a case history with lessons for conservation biology. *Oikos* **63**:62-76.

Avise, J. C. 2000. *Phylogeography: the History and Formation of Species*. Harvard University Press, Cambridge, USA, 447 pp.

Avise, J. C. 2004. *Molecular Markers, Natural History, and Evolution.* Sinauer Associates, Inc. , Sunderland, USA, pp. 684 pp.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A. and Saunders, N. C. 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics* **18**:489-522.

Ayala, F. J. 1997. Vagaries of the molecular clock. *Proceedings of the National Academy of Sciences of the United States of America* **94**:7776-83.

Baba, M. 2007. Effects of temperature and irradiance on germling growth in eight Sargassaceous species. *Report of Marine Ecology Research Institute* **10**:9-20.

Bäck, S., Collins, J. C. and Russell, G. 1992. Effects of salinity on growth of Baltic and Atlantic *Fucus vesiculosus*. *British Phycological Journal* **27**:39-47.

Báez, J., C., Real, R., Vargas, J. M. and Flores-Moya, A. 2004. A biogeographical analysis of the genera Audouinella (Rhodophyta), Cystoseira (Phaeophyceae) and Cladophora (Chlorophyta) in the western Mediterranean Sea and Adriatic Sea. *Phycologia* **43**:404-15.

Banks, M. A., McGoldrick, D. J., Borgeson, W. and Hedgecock, D. 1994. Gametic incompatibility and genetic divergence of Pacific and Kumamoto oysters, *Crassostrea gigas* and *C. sikamea*. *Marine Biology* **121**:127-35.

Barber, P. H., Erdmann, M. V. and Palumbi, S. R. 2006. Comparative phylogeography of three codistributed stomatopods: origins and timing of regional lineage diversification in the coral triangle. *Evolution* **60**:1825-39.

Barrett, E. M. 1963. The California oyster industry. *In: The Resources Agency of California Department of Fish and Game Fish Bulletin 123.* pp. 1-103.

Benzie, J. A. H., Ballment, E. and Edyvane, K. 2000. Allozymes as genetic identification markers of *Sargassum* spp. (Phaeophyta) from the Great Barrier Reef, Australia. *Botanica Marina* **43**:169-70.

Bermingham, E. and Martin, A. P. 1998. Comparative mtDNA phylogeography of neotropical freshwater fishes: testing shared history to infer the evolutionary landscape of lower Central America. *Molecular Ecology* **7**:499-517.

Bolton, J. J. 1994. Global seaweed diversity: Patterns and anomalies. *Botanica Marina* **37**:241-45.

Bolton, J. J., Leliaert, F., Clerck, O., Anderson, R. J., Stegenga, H., Engledow, H. E. and Coppejans, E. 2004. Where is the western limit of the tropical Indian Ocean seaweed flora? An analysis of intertidal seaweed biogeography on the east coast

of South Africa. *Marine Biology* **144**:51-59.

Boudouresque, C. F. and Verlaque, M. 2002. Biological pollution in the Mediterranean Sea: invasive versus introduced macrophytes. *Marine Pollution Bulletin* **44**:32-38.

Bray, R. T. and Curtis, J. T. 1957. An ordination of the upland forest communities of Southern Wisconsin. *Ecological Monographs* **27**:325-49.

Breeman, A. M. 1988. Relative importance of temperature and other factors in determining geographic boundaries of seaweeds: Experimental and phenological evidence. *Helgoland Marine Research* **42**:199-241.

Breeman, A. M., Oh, Y. S., Hwang, M. S. and Van den Hoek, C. 2002. Evolution of temperature responses in the *Cladophora vagabunda* complex and the *C. albida/sericea* complex (Chlorophyta). *European Journal of Phycology* **37**:45-58.

Breeman, A. M. and Pakker, H. 1994. Temperature ecotypes in seaweeds: Adaptive significance and biogeographic implications. *Botanica Marina* **37**:171-80.

Briggs, J. C. 1974. *Marine Zoogeography*. McGraw-Hill, New York, USA, 475 pp.

Briggs, J. C. 1995. *Global Biogeography*. Elsevier Science B. V., Amsterdam, Netherlands, 452 pp.

Carballo, J. L., Olabarria, C. and Osuna, T. G. 2002. Analysis of four macroalgal assemblages along the Pacific Mexican Coast during and after the 1997–98 El Niño. *Ecosystems* **5**:749-60.

Cheang, C. C. 2003. *Genetic and morphological variation among populations of* Sargassum hemiphyllum *(Phaeophyta)*. Master Thesis, The Chinese University of Hong Kong, 136 pp.

Cheang, C. C., Chu, K. H. and Ang, P. O. 2008. Morphological and genetic variation in the populations of *Sargassum hemiphyllum* (Phaeophyceae) in northwestern Pacific. *Journal of Phycology* **44**:855-65.

Chen, C. S. 1986. *The structure and function of* Sargassum *community in northern Taiwan*. Doctoral Dissertation, National Taiwan University, 127 pp.

Chiang, T. Y., Chiang , Y. C., Chen, Y. J., Chou, C. H., Havanond, S., Hong, T. N. and Huang, S. 2001. Phylogeography of *Kandelia candel* in East Asiatic mangroves based on nucleotide variation of chloroplast and mitochondrial DNAs. *Molecular Ecology* **10**:2697-710.

Chiang, Y.-C., Schaal, B. A., Ge, X. J. and Chiang, T.-Y. 2004. Range expansion leading to departures from neutrality in the nonsymbiotic hemoglobin gene and the cpDNA *trn*L-*trn*F intergenic spacer in *Trema dielsiana* (Ulmaceae). *Molecular Phylogenetics and Evolution* **31**:929-42.

Chiang, Y. M. 1960. Marine algae of northern Taiwan (Cyanophyta, Chlorophyta, Phaeophyta). *Taiwania* **7**:51-75.

Choi, T. S. and Kim, K. Y. 2004. Spatial pattern of intertidal macroalgal assemblages associated with tidal levels. *Hydrobiologia* **512**:49-56.

Chu, K. H., Li, C. P. and Ho, H. Y. 2001. The first internal transcribed spacer (ITS-1) of ribosomal DNA as a molecular marker for phylogenetic and population analyses in Crustacea. *Marine Biotechnology* **3**:355-61.

Clarke, K. R. and Ainsworth, M. 1993. A method of linking multivariate community structure to environmental variables. *Marine Ecology Progress Series* **92**:205-19.

Clarke, K. R. and Gorley, R. N. 2006. *PRIMER v6: User manual/tutorial.* PRIMER-E, Plymouth, UK, 192 pp.

Clarke, K. R., Somerfield, P. J. and Chapman, M. G. 2006. On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray-Curtis coefficient for denuded assemblages. *Journal of Experimental Marine Biology and Ecology* **330**:55-80.

Clement, M., Posada, D. and Crandall, K. A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**:1657-59.

Comes, H. P. and Abbott, R. J. 2001. Molecular phylogeny, reticulation, and lineage sorting in Mediterranean *Senecio* sect. *Senecio* (Asteraceae). *Evolution* **55**:1943-62.

Coyer, J. A., Hoarau, G., Stam, W. T. and Olsen, J. L. 2004. Geographically specific heteroplasmy of mitochondrial DNA in the seaweed, *Fucus serratus* (Heterokontophyta: Phaeophyceae, Fucales). *Molecular Ecology* **13**:1323-26.

Coyer, J. A., Hoarau, G., Stam, W. T. and Olsen, J. L. 2007. Hybridization and introgression in a mixed population of the intertidal seaweeds *Fucus evanescens* and *F. serratus*. *Journal of Evolutionary Biology* **20**:2322-33.

Coyer, J. A., Peters, A. F., Stam, W. T. and Olsen, J. L. 2003. Post-ice age recolonization and differentiation of *Fucus serratus* L. (Phaeophyceae; Fucaceae) populations in Northern Europe. *Molecular Ecology* **12**:1817-29.

Coyer, J. A., Smith, G. J. and Andersen, R. A. 2001. Evolution of *Macrocystis* spp. (Phaeophyceae) as determined by ITS1 and ITS2 sequences. *Journal of Phycology* **37**:574-85.

Crisci, J. V. 2001. The voice of historical biogeography. *Journal of Biogeography* **28**:157-68.

Critchley, A. T. 1983a. *Sargassum muticum*: A taxonomic history including world-wide and western Pacific distributions. *Journal of Marine Biological Association of the United Kingdom* **63**:617-25.

Critchley, A. T. 1983b. The establishment and increase of *Sargassum muticum* (Yendo) Fensholt populations within the Solent area of southern Britain. II. An investigation of the increase in canopy cover of the alga at low water. *Botanica Marina* **26**:539-45.

Critchley, A. T., De Visscher, P. R. M. and Nienhuis, P. H. 1990. Canopy characteristics of the brown alga *Sargassum muticum* (Fucales, Phaeophyta) in Lake Grevlingen, southwest Netherlands. *Hydrobiologia* **204/205**:211-17.

Critchley, A. T. and Dijkema, R. 1984. On the presence of the introduced brown alga *Sargassum muticum*, attached to commercially imported *Ostrea edulis* in the S.W. Netherlands. *Botanica marina* **27**:211-16.

Critchley, A. T., Farnham, W. F. and Morrell, S. L. 1983. A chronology of the new European sites of attachment for the invasive brown alga, *Sargassum muticum*, 1973-1981. *Journal of the Marine Biological Association of the United Kingdom* **63**:799-811.

Cunningham, E. M., Guiry, M. D. and Breeman, A. M. 1993. Environmental regulation of development, life history and biogeography of *Helminthora stackhousei* (Rhodophyta) by daylength and temperature. *Journal of Experimental Marine Biology and Ecology* **171**:1-21.

Curiel, D., Bellemo, G., Marzocchi, M., Scattolin, M. and Parisi, G. 1998. Distribution of introduced Japanese macroalgae *Undaria pinnatifida*, *Sargassum muticum* (Phaeophyta) and *Antithamnion pectinatum* (Rhodophyta) in the Lagoon of Venice. *Hydrobiologia* **385**:17-22.

De Wreede, R. E. 1978. Growth in varying culture conditions of embryos of three Hawaiian species of *Sargassum* (Phaeophyta, Sargassaceae). *Phycologia* **17**:23-31.

Deysher, L. and Norton, T. A. 1982. Dispersal and colonization in *Sargassum muticum* (Yendo) Fensholt. *Journal of Experimental Marine Biology and Ecology* **56**:179-95.

Deysher, L. E. and Dean, T. A. 1986. Interactive effects of light and temperature on sporophyte production in the giant kelp *Macrocystis pyrifera*. *Marine Biology* **93**:17-20.

Dover, G. 1982. Molecular drive: a cohesive mode of species evolution. *Nature* **299**:111-17.

Drew, K. M. 1949. Conchocelis-phase in the life-history of *Porphyra umbilicalis* (L.) Kütz. *Nature* **164**:748-49.

Duggins, C. J., Karlin, A., Mousseau, T. and Relyea, K. 1995. Analysis of a hybrid zone in Fundulus majalis in a northeastern Florida ecotone. *Heredity* **74**:117-28.

Durand, C., Manuel, M., Boudouresque, C. F., Meinesz, A., Verlaque, M. and Le Parco, Y. 2002. Molecular data suggest a hybrid origin for the invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta) in the Mediterranean Sea. *Journal of Evolutionary Biology* **15**:122-33.

Engel, C. R., Billard, E., Voisin, M. and Viard, F. 2008. Conservation and polymorphism of mitochondrial intergenic sequences in brown algae (Phaeophyceae). *European Journal of Phycology* **43**:195-205.

Engle, V. D. and Summers, J. K. 1999. Latitudinal gradients in benthic community composition in Western Atlantic estuaries. *Journal of Biogeography* **26**:1007-23.

Eppley, R. W., Holmes, R. W. and Strickland, J. D. H. 1967. Sinking rates of marine phytoplankton measured with fluorometer. *Journal of Experimental Marine Biology and Ecology* **1**:191-208.

Espinoza, J. 1990. The southern limit of *Sargassum muticum* (Yendo) Fensholt (Phaeophyta, Fucales) in the Mexican Pacific. *Botanica Marina* **33**:193-96.

Excoffier, L., Laval, G. and S., S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**:47-50.

Famà, P., Olsen, J. L., Stam, W. T. and Procaccini, G. 2000. High levels of intra- and inter-individual polymorphism in the rDNA ITS1 of *Caulerpa racemosa* (Chlorophyta). *European Journal of Phycology* **35**:349-56.

Farnham, W. F., Fletcher, R. L. and Irvine, L. M. 1973. Attached *Sargassum* found in Britain. *Nature* **243**:231-32.

Fautin, D. G. 2007. The coastal and marine environmental database, Biogeoinformatics of Hexacorallia. http://hercules.kgs.ku.edu/hexacoral/envirodata/locationcart/loccartmap.cfm

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-91.

Fensholt, D. E. 1955. An emendation of the genus *Cystophyllum* (Fucales). *American Journal of Botany* **42**:305-22.

Fernández, C. 1999. Ecology of *Sargassum muticum* (Phaeophyta) on the North Coast of Spain: IV. Sequence of Colonization on a Shore. *Botanica Marina* **42**:553-62.

Freeland, J. R. 2005. *Molecular Ecology*. John Wiley and Sons, Chichester, UK, 400 pp.

Freshwater, D. W., Fredericq, S. and Bailey, J. C. 1999. Characteristics and utility of nuclear-encoded large-subunit ribosomal gene sequences in phylogenetic studies of red algae. *Phycological Research* **47**:33-38.

Fu, Y. X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**:915-25.

Galysheva, Y. A. 2004. Subtidal macrobenthos communities of Vostok Bay (Sea of Japan) under conditions of anthropogenic impact. *Russian Journal of Marine Biology* **30**:363-70.

Garbary, D. 1987. A critique of traditional approaches to seaweed distribution in light of the development of vicariance biogeography. *Helgoland Marine Research* **41**:235-44.

Gavio, B. and Fredericq, S. 2002. *Grateloupia turuturu* (Halymeniaceae, Rhodophyta) is the correct name of the non-native species in the Atlantic known as *Grateloupia doryphora*. *European Journal of Phycology* **37**:349-59.

Geller, J. B. 1996. Molecular approaches to the study of marine biological invasions. *In*: Ferraris, J. D. and Palumbi, S. R. [Eds.] *Molecular Zoology*. Wiley, New York, pp. 119-32.

Geller, J. B., Walton, E. D., Grosholz, E. D. and Ruiz, G. M. 1997. Cryptic invasions of the crab *Carcinus* detected by molecular phylogeography. *Molecular Ecology* **6**:901-06.

Goulletquer, P., Bachelet, G., Sauriau, P. G. and Noel, P. 2002. Open Atlantic coast of Europe- A century of introduced species into French waters. *In*: Leppäkoski, E., Gollasch, S. and Olenin, S. [Eds.] *Invasive Aquatic Species of Europe. Distribution, Impacts and Management*. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 276-90.

Grizel, H. and Héral, M. 1991. Introduction into France of the Japanese oyster (*Crassostrea gigas*). *ICES Journal of Marine Science* **47**:399-403.

Guiry, M. D. and Guiry, G. M. 2008. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org

Gurgel, C. F. D., Fredericq, S. and Norris, J. N. 2004. Phylogeography of *Gracilaria tikvahiae* (Gracilariaceae, Rhodophyta): A study of genetic discontinuity in a continuously distributed species based on molecular evidence. *Journal of Phycology* **40**:748-58.

Gusmão, J., Lazoski, C. and Solé-Cava, A. M. 2000. A new species of *Penaeus* (Crustacea: Penaeidae) revealed by allozyme and cytochrome oxidase I analyses. *Marine Biology* **137**:435-46.

Hales, J. M. and Fletcher, R. L. 1989. Studies on the recently introduced brown alga *Sargassum muticum* (Yendo) Fensholt. IV. The effect of temperature, irradiance and salinity on germling growth. *Botanica Marina* **32**:167-76.

Hales, J. M. and Fletcher, R. L. 1990. Studies on the recently introduced brown alga *Sargassum muticum* (Yendo) Fensholt. V. Receptacle initiation and growth and gamete release in laboratory culture. *Botanica Marina* **33**:241-49.

Hanisak, M. D. and Samuel, M. A. 1987. Growth rates in culture of several species of *Sargassum* from Florida, USA. *Hydrobiologia* **151-152**:399-404.

Haraguchi, H., Murase, N., Mizukami, Y., Noda, M., Yoshida, G. and Terawaki, T. 2005. The optimal and maximum critical temperatures of nine species of the *Sargassaceae* in the coastal waters of Yamaguchi Prefecture, Japan. *Japanese Journal of Phycology* **53**:7-13.

Harries, D. B., Cook, E., Donnan, D. W., Mair, J. M., Harrow, S. and Wilson, J. R. 2007a. The establishment of the invasive alga *Sargassum muticum* on the west coast of Scotland: Rapid northwards spread and identification of potential new areas for colonization. *Aquatic Invasions* **2**:367-77.

Harries, D. B., Harrow, S., Wilson, J. R., Mair, J. M. and Donnan, D. W. 2007b. The establishment of the invasive alga *Sargassum muticum* on the west coast of Scotland: a preliminary assessment of community effects. *Journal of the Marine Biological Association of the United Kingdom* **87**:1057-67.

Harris, D. J. and Crandall, K. A. 2000. Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): Implications for phylogenetic and microsatellite studies. *Molecular Biology and Evolution* **17**:284-91.

Hayano, A., Amano, M. and Miyazaki, N. 2003. Phylogeography and population structure of the Dall's porpoise, *Phocoenoides dalli*, in Japanese waters revealed by mitochondrial DNA. *Genes and Genetic Systems* **78**:81-91.

Hillis-Colinvaux, L. 1986. Deep water populations of *Halimeda* in the economy of an atoll. *Bulletin of Marine Science* **38**:155-69.

Ho, C.-L., Phang, S.-M. and Pang, T. 1995. Molecular characterisation of *Sargassum polycystum* and *S. siliquosum* (Phaeophyta) by polymerase chain reaction (PCR) using random amplified polymorphic DNA (RAPD) primers. *Journal of Applied Phycology* **7**:33-41.

Hoarau, G., Coyer, J. A., Veldsink, J. H., Stam, W. T. and Olsen, J. L. 2007. Glacial refugia and recolonization pathways in the brown seaweed *Fucus serratus*. *Molecular Ecology* **16**:3606-16.

Hoffmann, F. G. and Baker, R. J. 2003. Comparative phylogeography of short-tailed bats (*Carollia*: Phyllostomidae). *Molecular Ecology* **12**:3403-14.

Hoffmann, W. A. and Poorter, H. 2002. Avoiding bias in calculations of relative growth rate. *Annals of Botany* **90**:37-42.

Honda, M. and Okuda, T. 1990. Egg liberation, germling development and .seasonal changes in photosynthetic rates of autumnal *Sargassum mieracanthum*. *Japanese Journal of Phycology* **38**:263-68.

Huang, S. F. 1990. The marine algal flora of Hsiao-Liuchiu island. *Botanical Bulletin of Academia Sinica* **31**:245-55.

Huang, S. F. 2002. Temporal and spatial variation of benthic marine algae in northeastern Taiwan. *Journal of the National Taiwan Museum* **55**:9-20.

Hunt, R. 1982. *The Functional Approach to Plant Growth Analysis*. Edward Arnold, London, UK, 248 pp.

Hwang, E., Park, C. and Baek, J. 2006. Artificial seed production and cultivation of the edible brown alga, *Sargassum fulvellum* (Turner) C. Agardh: Developing a new species for seaweed cultivation in Korea. *Journal of Applied Phycology* **18**:251-57.

Hwang, E. K. and Dring, M. J. 2002. Quantitative photoperiodic control of erect thallus production in *Sargassum muticum*. *Botanica Marina* **45**:471-75.

Hwang, J.-S. and Wong, C. K. 2005. The China Coastal Current as a driving force for transporting *Calanus sinicus* (Copepoda: Calanoida) from its population centers to waters off Taiwan and Hong Kong during the winter northeast monsoon period. *Journal of Plankton Research* **27**:205-10.

Hwang, R. L., Tsai, C. C. and Lee, T. M. 2004. Assessment of temperature and nutrient limitation on seasonal dynamics among species of *Sargassum* from a coral reef in southern Taiwan. *Journal of Phycology* **40**:463-73.

Inoh, S. 1930. Embryological studies on *Sargassum*. *Science Reports of the Tohoku Imperial University, Series 4 (Biology)* **5**:421-38.

Inoh, S. 1932. Embryological Studies on *Sargassum* and *Cystohpyllum*. *Journal of the Faculty of Science, Hokkaido University. Series 5, Botany* **1**:125-33.

Inoue, K., Odo, S., Noda, T., Nakao, S., Takeyama, S., Yamaha, E., Yamazaki, F. and Harayama, S. 1997. A possible hybrid zone in the *Mytilus edulis* complex in Japan revealed by PCR markers. *Marine Biology* **128**:91-95.

Ishizuka, A. and Tanaka, J. 2004. Seaweed and seagrass flora at the Tenjin-jima Island in the west coast of Miura Peninsula, Kanagawa Pref. *Science Report of the Yokosuka City Museum* **51**:53-66.

Kafanov, A. I., Ivanova, M. B. and Koltypin, M. V. 2004. On the knowledge of the intertidal zone of Russian Far Eastern Seas. *Russian Journal of Marine Biology* **30, Suppl. 1**:S56-S67.

Kalisz, S., Nason, J. D., Hanzawa, F. M. and Tonsor, S. J. 2001. Spatial population genetic structure in *Trillium grandiflorum*: the roles of dispersal, mating, history, and selection. *Evolution* **55**:1560-68.

Kang, J. W. 1966. On the geographical distribution of marine algae in Korea. *Bulletin of Pusan Fisheries College* **7**:1-125.

Karlsson, J. and Loo, L. O. 1999. On the distribution and the continuous expansion of the Japanese seaweed - *Sargassum muticum* - in Sweden. *Botanica Marina* **42**:285-94.

Kashenko, N. V. 1999. Benthic communities of macrophytic algae in Vostok Bay, the Sea of Japan. *Russian Journal of Marine Biology* **25**:360-64.

Kawai, T. 1997. Distribution of large algae at Suttsu Bay, western Hokkaido, Japan. *Scientific Reports of the Hokkaido Fisheries Experiment Station* **51**:77-82.

Kendrick, G. A. and Walker, D. I. 1995. Dispersal of propagules of *Sargassum* spp. (Sargassaceae: Phaeophyta): Observations of local patterns of dispersal and consequences for recruitment and population structure. *Journal of Experimental Marine Biology and Ecology* **192**:273-88.

Khfaji, A. K. and Norton, T. A. 1979. The effects of salinity on the distribution of *Fucus ceranoides*. *Estuarine and Coastal Marine Science* **8**:433-39.

Kim, B. J., Lee, H. J., Yum, S., Lee, H. A., Bhang, Y. J., Park, S. R., Kim, H. J. and Kim, J. H. 2004. A short-term response of macroalgae to potential competitor removal in a mid-intertidal habitat in Korea. *Hydrobiologia* **512**:57-62.

Kim, K. Y., Choi, T. S., Huh, S. H. and Garbary, D. J. 1998. Seasonality and community structure of subtidal benthic algae from Daedo Island, southern Korea. *Botanica Marina* **41**:357-65.

Kim, M. K. and Kim, K. T. 2000. Studies on the seaweeds in the Islands of Ullungdo and Dokdo: I. Decrease of algal species compositions and changes of marine algal flora *Algae* **15**:119-24.

Kim, M. K., Shin, J. K. and Cha, K. H. 2004. Variation of species composition of benthic algae and whitening in the coast of Dokdo Island during summer. *Algae* **19**:69-78.

Kim, Y. H., Ahn, J. K., Lee, J. I. and Eum, H. M. 2004. Effects of heated effluents on the intertidal macroalgal community near Uljin, the east coast of Korea. *Algae* **19**:257-70.

Kim, Y. H., Nam, K. W. and Sohn, C. H. 1997. Intertidal benthic marine algae at Chumunjin on the east coast of Korea: Flora, distribution and community structure. *Algae* **12**:117-30.

Kim, Y. H. and Park, S. H. 1997. Succession pattern of intertidal benthic algal communitites in Cheju Island. *Algae* **12**:23-30.

Kim, Y. H., Yoon, H. J. and Yoo, J. S. 1995. Species composition and biomass of marine algal community on the mid-western coast of Korea. *Journal of Plant Biology* **38**:389-98.

Kitayama, T. 1996. Brown algae from Onagawa Bay, Miyagi Pref., Japan. *Memoris of the National Science Museum of Tokyo* **29**:223-30.

Kitayama, T. 1998. Brown algae from Fukue Island, Nagasaki Pref., Japan. *Memoris of the National Science Museum of Tokyo* **31**:97-106.

Kitayama, T., Dai, H. P. and Han, J. X. 1995. Brown algae from Zhoushan Islands, Zhejiang Province, China. *Bulletin of the National Science Museum, Tokyo, series B* **21**:169-78.

Klochkova, N. G. 1998. An annotated bibliography of marine macroalgae on northwest coast of the Bering Sea and the Southeast Kamchatka: the first revision of flora. *Algae* **13**:375-418.

Koh, C. H. and Shin, H. C. 1990. Growth and size distribution of some large brown algae in Ohori, east coast of Korea. *Hydrobiologia* **204-205**:225-31.

Kojima, S., Kamimura, S., Iijima, A., Kimura, T., Kurozumi, T. and Furota, T. 2006. Molecular phylogeny and population structure of tideland snails in the genus *Cerithidea* around Japan. *Marine Biology* **149**:525-35.

Komatsu, T., Tatsukawa, K., Filippi, J. B., Sagawa, T., Matsunaga, D., Mikami, A., Ishida, K., Ajisaka, T., Tanaka, K., Aoki, M., Wang, W.-D., Liu, H.-F., Zhang, S.-D., Zhou, M.-D. and Sugimoto, T. 2007. Distribution of drifting seaweeds in eastern East China Sea. *Journal of Marine Systems* **67**:245-52.

Konishi, Y. and Hayashida, F. 2004. Vegetation of benthic marine algae in Suruga Bay, Central Japan. *Journal of the School of Marine Science and Technology* **1**:15-27.

Kurihara, A. and Iima, M. 1999. On growth and maturation of *Sargassum thunbergii* from southern part of Nagasaki Prefecture, Japan. *Japanese Journal of Phycology* **47**:179-86.

Kusumo, H. T. and Druehl, L. D. 2000. Variability over space and time in the genetic structure of the winged kelp *Alaria marginata*. *Marine Biology* **136**:397-409.

LaJeunesse, T. C. and Pinzón, J. H. 2007. Screening intragenomic rDNA for dominant variants can provide a consistent retrieval of evolutionarily persistent ITS (rDNA) sequences. *Molecular Phylogenetics and Evolution* **45**:417-22.

Lande, R. 1988. Genetics and demography in biological conservation. *Science* **241**:1455-60.

Le Provost, C., Bennett, A. F. and Cartwright, D. E. 1995. Ocean tides for and from TOPEX/POSEIDON. *Science* **267**:639-42.

Lee, I. K. and Kim, Y. H. 1999. Biodiversity and distribution of marine benthic organisms and used of algal resources in the coastal zone of Korea and Japan I. Benthic Marine Algae in the East Coast of Korea. *Algae* **14**:91-110.

Lee, J. W., Oh, B. G. and Lee, H. B. 1997. Marine algal flora and community of Padori area in the Taean Peninsula, the west coast of Korea. *Algae* **12**:131-38.

Lee, J. W., Oh, B. G. and Lee, H. B. 2000. Marine benthic algal community at Padori, west coast of Korea. *Algae* **15**:111-17.

Lee, S. Y., Lee, J. W. and Lee, H. B. 1997. Marine benthic algal flora of Yongil Bay and its adjacent areas, the eastern coast of Korea. *Algae* **12**:303-11.

Lee, W. J., Yoon, H. S. and Boo, S. M. 1997. Marine algal community of Gyonggiman Islets on the west sea of Korea. *Algae* **12**:139-44.

Lessios, H. A., Kessing, B. D. and Pearse, J. S. 2001. Population structure and speciation in tropical seas: Global phylogeography of the sea urchin *Diadema*. *Evolution* **55**:955-75.

Lewis, J. E. 1990. Evaluating taxonomic databases for biogeographic use. *Bulletin of Marine Science* **47**:1990.

Lincoln, J. M. and Schlanger, S. O. 1991. Atoll stratigraphy as a record of sea level change: Problems and prospects. *Journal of Geophysical Research* **96**:6727-52.

Lindstrom, S. C. 2001. The Bering Strait connection: dispersal and speciation in boreal macroalgae. *Journal of Biogeography* **28**:243-51.

Lindstrom, S. C. 1977. *An Annotated Bibliography of the Benthic Marine Algae of Alaska.* Alaska Department of Fish and Game, Juneau, Alaska, 172 pp.

Lindstrom, S. C. 1987. Possible sister groups and phylogenetic relationships among selected North Pacific and North Atlantic red algae. *Helgoland Marine Research* **41**:245-60.

Liu, J.-X., Gao, T.-X., Wu, S.-F. and Zhang, Y.-P. 2007. Pleistocene isolation in the Northwestern Pacific marginal seas and limited dispersal in a marine fish, *Chelon haematocheilus* (Temminck and Schlegel, 1845). *Molecular Ecology* **16**:275-88.

Liu, J.-X., Gao, T.-X., Yokogawa, K. and Zhang, Y.-P. 2006. Differential population structuring and demographic history of two closely related fish species, Japanese sea bass (*Lateolabrax japonicus*) and spotted sea bass (*Lateolabrax maculatus*) in Northwestern Pacific. *Molecular Phylogenetics and Evolution* **39**:799-811.

Liu, K.-K., Peng, T.-H., Shaw, P.-T. and Shiah, F.-K. 2003. Circulation and biogeochemical processes in the East China Sea and the vicinity of Taiwan: an overview and a brief synthesis. *Deep Sea Research Part II: Topical Studies in Oceanography* **50**:1055-64.

Loughnane, C. and Stengel, D. B. 2002. Attached *Sargassum muticum* (Yendo) Fensholt found on the west coast of Ireland. *Irish Naturalists' Journal* **27**:70-72.

Lourie, S. A., Green, D. M. and Vincent, A. C. J. 2005. Dispersal, habitat differences, and comparative phylogeography of Southeast Asian seahorses (Syngnathidae: *Hippocampus*). *Molecular Ecology* **14**:1073-94.

Lu, B. R. and Tseng, C. K. 2004. Studies on four new species of the malacocarpic *Sargassum* (Sargassaceae, Heterokontophyta) in China. *Hydrobiologia* **512**:193-99.

Lüning, K. 1980. Control of algal life-history by daylength and temperature. *In*: Price, J. H., Irvine, D. E. G. & Farnham, W. F. [Eds.] *The Shore Environment* Vol. 2. *Ecosystems.* Systematics Association Special Volume 17b. Academic Press, London, UK, pp. 915-43

Lüning, K. 1986. New frond formation in *Laminaria hyperborea* (Phaeophyta): a photoperiodic response. *British Phycological Journal* **21**:269-73.

Lüning, K. 1990. *Seaweeds: Their Environment, Biogeography, and Ecophysiology.* John Wiley and Sons, Inc., New York, USA, 527 pp.

Lüning, K. and Dring, M. J. 1979. Continuous underwater light measurement near Helgoland (North Sea) and its significance for characteristic light limits in the sublittoral region. *Helgoland Marine Research* **32**:403-24.

McCauley, D. E. 1991. Genetic consequences of local population extinction and recolonization. *Trends in Ecology and Evolution* **6**:5-8.

McCourt, R. M., Casanova, M. T., Karol, K. G. and Feist, M. 1999. Monophyly of genera and species of Characeae based on rbcL sequences, with special reference to Australian and European *Lychnothamnus barbatus* (Characeae: Charophyceae). *Australian Journal of Botany* **47**:361-69.

McIvor, L., Maggs, C. A., Provan, J. and Stanhope, M. J. 2001. rbcL sequences reveal multiple cryptic introductions of the Japanese red alga *Polysiphonia harveyi*. *Molecular Ecology* **10**:911-19.

Meusnier, I., Valero, M., Olsen, J. and Stam, W. 2004. Analysis of rDNA ITS1 indels in *Caulerpa taxifolia* (Chlorophyta) supports a derived, incipient species status for the invasive strain. *European Journal of Phycology* **39**:83-92.

Michanek, G. 1979. Phytogeographic provinces and seaweed distribution. *Botanica Marina* **22**:375-91.

Middlebrook, R., Hoegh-Guldberg, O. and Leggat, W. 2008. The effect of thermal history on the susceptibility of reef-building corals to thermal stress. *Journal of Experimental Biology* **211**:1050-56.

Miller, K. G., Kominz, M. A., Browning, J. V., Wright, J. D., Mountain, G. S., Katz, M. E., Sugarman, P. J., Cramer, B. S., Christie-Blick, N. and Pekar, S. F. 2005. The Phanerozoic record of global sea-level change. *Science* **310**:1293-98.

Mineur, F., Belsher, T., Johnson, M. P., Maggs, C. A. and Verlaque, M. 2007. Experimental assessment of oyster transfers as a vector for macroalgal introductions. *Biological Conservation* **137**:237-47.

Miura, O., Torchin, M. E., Kuris, A. M., Hechinger, R. F. and Chiba, S. 2006. Introduced cryptic species of parasites exhibit different invasion pathways. *Proceedings of the National Academy of Sciences of the United States of America* **103**:19818-23.

Mosisch, T. D. 1993. Effects of salinity on the distribution of *Caloglossa leprieurii* (Rhodophyta) in the Brisbane river, Australia. *Journal of Phycology* **29**:147-53.

Muirhead, J. R., Gray, D. K., Kelly, D. W., Ellis, S. M., Heath, D. D. and Macisaac, H. J. 2008. Identifying the source of species invasions: sampling intensity vs. genetic diversity. *Molecular Ecology* **17**:1020-35.

Murase, N. O., Kito, H. I., Mizukami, Y. U. and Maegawa, M. I. 2000. Productivity of a *Sargassum macrocarpum* (Fucales, Phaeophyta) population in Fukawa Bay, Sea of Japan. *Fisheries Science* **66**:270-77.

Muss, A., Robertson, D. R., Stepien, C. A., Wirtz, P. and Bowen, B. W. 2001. Phylogeography of *Ophioblennius*: the role of ocean currents and geography in reef fish evolution. *Evolution* **55**:561-72.

Myers, A. A. and Giller, P. S. 1988. *Analytical Biogeography. An Integrated Approach to the Study of Animal and Plant Distributions*. Chapman and Hall, London, UK, 578 pp.

Nam, K. W., Kim, Y. S., Kim, Y. H. and Sohn, C. H. 1996. Benthic marine algae in the east coast of Korea: Flora, distribution and community structure. *Journal of the Korean Fisheries Society* **29**:727-43.

Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York, USA, 256 pp.

Ng, K. Y. 2009. *The study of epifaunal assemblage of Sargassum bed in Hong Kong SAR, China*. Master Thesis, The Chinese University of Hong Kong.

Nguyễn, H. D., Huynh, Q. N., Trần, N. B. and Nguyễn, V. T. 1993. Phaeophyta. *Marine algae of north Vietnam*. Nhà xuất ban Khoa học và kỹ thuật, Hà Nôi (Ha Noi), Vietnam, pp. 107-80.

Nichols, S. A. and Barnes, P. A. G. 2005. A molecular phylogeny and historical biogeography of the marine sponge genus *Placospongia* (Phylum Porifera) indicate low dispersal capabilities and widespread crypsis. *Journal of Experimental Marine Biology and Ecology* **323**:1-15.

Norton, T. A. 1976. Why is *Sargassum muticum* so invasive? *British Phycological Journal* **11**:197–98.

Norton, T. A. 1977a. The growth and development of *Sargassum muticum* (Yendo) Fensholt. *Journal of Experimental Marine Biology and Ecology* **26**:41-53.

Norton, T. A. 1977b. Ecological experiments with *Sargassum muticum*. *Journal of the Marine Biological Association of the United Kingdom* **57**:33-43.

Norton, T. A. 1992. Dispersal by macroalgae. *European Journal of Phycology* **27**:293-301.

Oak, J. H., Suh, Y. and Lee, I. K. 2002. Phylogenetic relationships of *Sargassum* subgenus *Bactrophycus* (Sargassaceae, Phaeophyceae) inferred from rDNA ITS sequences. *Algae* **17**:235-48.

Oba, T., Kato, M., Kitazato, H., Koizumi, I., Omura, A., Sakai, T. and Takayama, T. 1991. Paleoenvironmental changes in the Japan Sea during the last 85,000 years. *Paleoceanography* **6**:499-518.

Oba, T. and Murayama, M. 2004. Sea-surface temperature and salinity changes in the northwest Pacific since the Last Glacial Maximum. *Journal of Quaternary Science* **19**:335-46.

Ogawa, H. 1986. Combined effects of temperature and salinity on the early development of marine algae. II. Rhizoid development of *Sargassum horneri* (Turner) C. Agardh. *Japanese Journal of Phycology* **34**:137-41.

Ogawa, H. 1994. Effects of temperature and salinity on the rhizoid development of *Sargassum muticum*. *Suisanzoshoku* **42**:25-31.

Ogawa, H., Kanaya, N. and Kiuchi, E. 1995. Effects of temperature and salinity on the rhizoid development of *Sargassum yezoense*. *Suisanzoshoku* **43**:445-48.

Ogawa, H., Kanaya, N. and Kiuchi, E. 1996. Effects of temperature and salinity on the rhizoid development of brown alga, *Hizikia fusiformis*. *Suisanzoshoku* **44**:407-11.

Ohno, M. 1984. Observation on the floating seaweeds of near-shore waters of southern Japan. *Hydrobiologia* **116-117**:408-12.

Ohta, M. and Ninomiya, S. 1990. Relationship between distribution of *Sargassum* species and water movement. *Japanese Journal of Phycology* **38**:179-85.

Okamura, K. 1928. *Icones of Japanese Algae*. Kazamashobo, Tokyo, Japan, 202 pp.

Ono, Y. 1990. The northern landbridge of Japan. *Quaternary Research* **29**:183-92.

Orfanidis, S., Venekamp, L. A. H. and Breeman, A. M. 1999. Ecophysiological adaptations of two Mediterranean red algae in relation to distribution. *European Journal of Phycology* **34**:469-76.

Ornellas, A. B. and Coutinho, R. 1998. Spatial and temporal patterns of distribution and abundance of a tropical fish assemblage in a seasonal *Sargassum* bed, Cabo Frio Island, Brazil. *Journal of Fish Biology* **53**:198-208.

Ota, H. 1998. Geographic patterns of endemism and speciation in amphibians and reptiles of the Ryukyu Archipelago, Japan, with special reference to their

paleogeographical implications. *Population Ecology* **40**:189-204.

Pakker, H., van Reine, W. F. P. h. and Breeman, A. M. 1994. Temperature responses and evolution of thermal traits in *Cladophoropsis membranacea* (Siphonocladales, Chlorophyta). *Journal of Phycology* **30**:777-83.

Palumbi, S. R. 1994. Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics* **25**:547-72.

Palumbi, S. R. 1997. Molecular biogeography of the Pacific. *Coral Reefs* **16**:S47-S52.

Pamilo, P. and Neí, M. 1988. Relationships between gene trees and species trees. *Molecular Biology and Evolution* **5**:568-83.

Park, S. and Chu, P. 2006. Thermal and haline fronts in the Yellow/East China Seas: Surface and subsurface seasonality comparison. *Journal of Oceanography* **62**:617-38.

Pearl, R. 1928. *The Rate of Living.* Alfred A. Knopf., New York, 185 pp.

Peters, A. F. and Breeman, A. M. 1993. Temperature tolerance and latitudinal range of brown algae from temperate Pacific South America. *Marine Biology* **115**:143-50.

Phillips, N. 1995. Biogeography of *Sargassum* (Phaeophyta) in the Pacific basin. *In*: Abbott, I. A. [Ed.] *Taxonomy of Economic Seaweeds Volume 5.* California Sea Grant College Program, La Jolla, USA, pp. 107-45.

Phillips, N. and Fredericq, S. 2000. Biogeographic and phylogenetic investigations of the pan-tropical genus *Sargassum* (Fucales, Phaeophyceae) with respect to Gulf of Mexico species. *Gulf of Mexico Science* **18**:77-87.

Phillips, N. E., Smith, C. M. and Morden, C. W. 2005. Testing systematic concepts of *Sargassum* (Fucales, Phaeophyceae) using portions of the rbcLS operon. *Phycological Research* **53**:1-10.

Pijanka, R. 2006. History on the half-shell- The Kumamoto oyster flourishes on North American menus. *Pacific Rim Magazine Online.*

Porzecanski, A. L. and Cracraft, J. 2005. Cladistic analysis of distributions and endemism (CADE): using raw distributions of birds to unravel the biogeography of the South American aridlands. *Journal of Biogeography* **32**:261-75.

Posada, D. and Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**:817-18.

Posada, D. and Crandall, K. A. 2002. The effect of recombination on the accuracy of phylogeny estimation. *Journal of Molecular Evolution* **54**:396-402.

Proches, S. 2005. The world's biogeographical regions: cluster analyses based on bat distributions. *Journal of Biogeography* **32**:607-14.

Provan, J., Booth, D., Todd, N. P., Beatty, G. E. and Maggs, C. A. 2008. Tracking biological invasions in space and time: elucidating the invasive history of the green alga *Codium fragile* using old DNA. *Diversity and Distributions* **14**:343-54.

Provan, J. I. M., Murphy, S. and Maggs, C. A. 2005. Tracking the invasive history of the green alga *Codium fragile* ssp. *tomentosoides*. *Molecular Ecology* **14**:189-94.

Quayle, D. B. 1964. Distribution of introduced marine mollusca in British Columbia waters. *Journal of Fisheries Research Board of Canada* **21**:1155-81.

Reeb, C. A. and Avise, J. C. 1990. A genetic discontinuity in a continuously distributed species: mitochondrial DNA in the American oyster, *Crassostrea virginica*. *Genetics* **124**:397-406.

Riddle, B. R., Hafner, D. J., Alexander, L. F. and Jaeger, J. R. 2000. Cryptic vicariance in the historical assembly of a Baja California Peninsular Desert biota. *Proceedings of the National Academy of Sciences of the United States of America* **97**:14438-43.

Rieseberg, L. H. and Soltis, D. E. 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants* **5**:65-84.

Rocha, L. A., Bass, A. L., Ross Robertson, D. and Bowen, B. W. 2002. Adult habitat preferences, larval dispersal, and the comparative phylogeography of three Atlantic surgeonfishes (Teleostei: Acanthuridae). *Molecular Ecology* **11**:243-51.

Rogers, A. R. and Harpending, H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* **9**:552-69.

Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**:1572-74.

Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145**:1219-28.

Rueness, J. 1989. *Sargassum muticum* and other introduced Japanese macroalgae: Biological pollution of European coasts. *Marine Pollution Bulletin* **20**:173-76.

Ruiz, G. M., Carlton, J. T., Grosholz, E. D. and Hines, A. H. 1997. Global invasions of marine and estuarine habitats by non-indigenous species: Mechanisms, extent, and consequences. *American Zoologist* **37**:621-32.

Russell, G. 1987a. Salinity and seaweed vegetation. *In*: Crawford, R. M. M. [Ed.] *Plant Life in Aquatic and Amphibious Habitats*. Blackwell Scientific Publications, Oxford, Boston Mass., U.S.A, pp. 35-52.

Russell, G. 1987b. Spatial and environmental components of evolutionary change: interactive effects of salinity and temperature on *Fucus vesiculosus* as an example. *Helgoland Marine Research* **41**:371-76.

Sakai, A. K., Allendorf, F. W., Holt, J. S., Lodge, D. M., Molofsky, J., With, K. A., Baughman, S., Cabin, R. J., Cohen, J. E., Ellstrand, N. C., McCauley, D. E., O'Neil, P., Parker, I. M., Thompson, J. N. and Weller, S. G. 2001. The population biology of invasive species. *Annual Review of Ecology and Systematics* **32**:305.

Sarich, V. M. and Wilson, A. C. 1973. Generation time and genomic evolution in primates. *Science* **179**:1144-47.

Sasaki, H., Flores-Moya, A., Henry, E. C., Muller, D. G. and Kawai, H. 2001. Molecular phylogeny of Phyllariaceae, Halosiphonaceae and Tilopteridales (Phaeophyceae). *Phycologia* **40**:123-34.

Sato, M. and Wada, K. 2000. Resource utilization for decorating in three intertidal majid crabs (Brachyura: Majidae). *Marine Biology* **137**:705-14.

Saunders, N. C., Kessler, L. G. and Avise, J. C. 1986. Genetic variation and geographic differentiation in mitochondrial DNA of the horseshoe crab, *Limulus polyphemus*. *Genetics* **112**:613-27.

Scagel, R. F. 1956. Introduction of a Japanese alga, *Sargassum muticum* into the northeast Pacific. *Fisheries Research Papers. Department of Fisheries, State of Washington* **1**:49-58.

Schelly, R., Salzburger, W., Koblmüller, S., Duftner, N. and Sturmbauer, C. 2006. Phylogenetic relationships of the lamprologine cichlid genus *Lepidiolamprologus* (Teleostei: Perciformes) based on mitochondrial and nuclear sequences, suggesting introgressive hybridization. *Molecular Phylogenetics and Evolution* **38**:426-38.

Schils, T. and Wilson, S. C. 2006. Temperature threshold as a biogeographic barrier in northern Indian Ocean macroalgae. *Journal of Phycology* **42**:749-56.

Schonbeck, M. W. and Norton, T. A. 1978. Factors controlling the upper limits of fucoid algae on the shore. *Journal of Experimental Marine Biology and Ecology* **31**:303-13.

Schroth, W., Jarms, G., Streit, B. and Schierwater, B. 2002. Speciation and phylogeography in the cosmopolitan marine moon jelly, *Aurelia* sp. *BMC*

Evolutionary Biology **2**:1-10.

Secq, M.-P., Goër, S., Stam, W. and Olsen, J. 2006. Complete mitochondrial genomes of the three brown algae (Heterokonta: Phaeophyceae) *Dictyota dichotoma*, *Fucus vesiculosus* and *Desmarestia viridis*. *Current Genetics* **49**:47-58.

Sekine, Y. and Kutsuwada, K. 1994. Seasonal variation in volume transport of the Kuroshio south of Japan. *Journal of Physical Oceanography* **24**:261-72.

Senjyu, T., Enomoto, H., Matsuno, T. and Matsui, S. 2006. Interannual salinity variations in the Tsushima Strait and its relation to the Changjiang discharge. *Journal of Oceanography* **62**:681-92.

Serrão, E. A., Alice, L. A. and Brawley, S. H. 1999. Evolution of the Fucaceae (Phaeophyceae) inferred from nrDNA-ITS. *Journal of Phycology* **35**:382-94.

Setchell, W. A. 1931a. Hong Kong Seaweeds, Part I. *The Hong Kong Naturalist: a Quarterly Illustrated Journal Principally for Hong Kong and South China* **2**:39-60.

Setchell, W. A. 1931b. Hong Kong Seaweeds, Part II Sargassaceae. *The Hong Kong Naturalist: a Quarterly Illustrated Journal Principally for Hong Kong and South China* **2**:237-53.

Setchell, W. A. 1933. Hong Kong Seaweeds, III, Sargassaceae, Plates 3 - 20. *The Hong Kong Naturalist: a Quarterly Illustrated Journal Principally for Hong Kong and South China* **Supp. 2**:33-49.

Setchell, W. A. 1936. Hong Kong Seaweeds, V. *Hong Kong Naturalist* **Supp. 5**:1-20.

Shen, X. Q., Fan, W. and Cui, X. S. 2004. Study on the relationship of fishing ground distribution of *Ommastrephes bartrami* and water temperature in the Northwest Pacific Ocean. *Marine Fisheries Research*:11-15.

Shen, Y. F. and Fan, K. C. 1950. Marine algae of Formosa. *Taiwania* **1**:317-45.

Shi, G. R. 1993. Multivariate data analysis in palaeoecology and palaeobiogeography - a review. *Palaeogeography, Palaeoclimatology, Palaeoecology* **103**:199-234.

Shimabukuro, H., Terada, R., Sotobayashi, J., Nishihara, G. N. and Noro, T. 2007. Phenology of *Sargassum duplicatum* (Fucales, Phaeophyceae) from the southern coast of Satsuma peninsula, Kagoshima, Japan. *Bulletin of the Japanese Society of Scientific Fisheries* **73**:454-60.

Silva, P. C. 1992. Geographic patterns of diversity in benthic marine algae. *Pacific Science* **46**:429-37.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. *Science* **236**:787-92.

Soltis, D. E. and Kuzoff, R. K. 1995. Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). *Evolution* **49**:727-42.

Soltis, D. E., Morris, A. B., McLachla, J. S. N., Manos, P. S. and Soltis, P. S. 2006. Comparative phylogeography of unglaciated eastern North America. *Molecular Ecology* **15**:4261-93.

Southward, A. J., Hawkins, S. J. and Burrows, M. T. 1995. Seventy years' observations of changes in distribution and abundance of zooplankton and intertidal organisms in the western English Channel in relation to rising sea temperature. *Journal of Thermal Biology* **20**:127-55.

Stæhr, P. A., Pedersen, M. F., Thomsen, M. S., Wernberg, T. and Krause-Jensen, D. 2000. Invasion of *Sargassum muticum* in Limfjorden (Denmark) and its possible impact on the indigenous macroalgal community. *Marine Ecology Progress Series* **207**:79-88.

Steele, E. N. 1964. *The Immigrant Oyster (*Ostrea gigas*) Now Known as the Pacific Oyster*. Pacific Coast Oyster Growers Association, Inc, Olympia, USA, 179.

Steen, H. 2003. Intraspecific competition in *Sargassum muticum* (Phaeophyceae) germlings under various density, nutrient and temperature regimes. *Botanica Marina* **46**:36-43.

Steen, H. 2004. Effects of reduced salinity on reproduction and germling development in *Sargassum muticum* (Phaeophyceae, Fucales). *European Journal of Phycology* **39**:293-99.

Steen, H. and Rueness, J. 2004. Comparison of survival and growth in germlings of six fucoid species (Fucales, Phaeophyceae) at two different temperature and nutrient levels. *Sarsia* **89**:175 - 83.

Stephenson, T. A. 1948. The constitution of the intertidal fauna and flora of South Africa. *Annals of the Natal Museum* **11**:207-324.

Stiger, V., Horiguchi, T., Yoshida, T., Coleman, A. W. and Masuda, M. 2000. Phylogenetic relationships of *Sargassum* (Sargassaceae, Phaeophyceae) with reference to a taxonomic revision of the section Phyllocystae based on ITS-2 nrDNA sequences. *Phycological Research* **48**:251-60.

Stiger, V., Horiguchi, T., Yoshida, T., Coleman, A. W. and Masuda, M. 2003. Phylogenetic relationships within the genus *Sargassum* (Fucales, Phaeophyceae), inferred from ITS-2 nrDNA, with an emphasis on the taxonomic subdivision of the genus. *Phycological Research* **51**:1-10.

Strømgren, T. 1994. Apical elongation of fucoid algae exposed to low salinities. *Aquatic Botany* **49**:67-74.

Sündermann, J. and Feng, S. 2004. Analysis and modelling of the Bohai sea ecosystem--a joint German-Chinese study. *Journal of Marine Systems* **44**:127-40.

Sunnucks, P. 2000. Efficient genetic markers for population biology. *Trends in Ecology and Evolution* **15**:199-203.

Swofford, D. L. 2000. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Ver. 4.0b10. 4.0b10 ed. Sinauer Associates, Sunderland, USA.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**:585-95.

Taki, K. and Ogishima, T. 1997. Distribution of some developmental stages and growth of *Euphausia pacifica* Hansen in the northwestern Pacific (Japan) on the basis of norpac net samples. *Bulletin of Tohoku National Fisheries Research Institute* **59**:95-117.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) Software version 4.0. *Molecular Biology and Evolution* **24**:1596-99.

Taylor, R. B. 1998. Short-term dynamics of a seaweed epifaunal assemblage. *Journal of Experimental Marine Biology and Ecology* **227**:67-82.

Terry, L. A. and Moss, B. L. 1980. The effect of photoperiod on receptacle initiation in *Ascophyllum nodosum* (L.) Le Jol. *European Journal of Phycology* **15**:291 - 301.

Thomson, J. M. 1952. The acclimatization and growth of the Pacific oyster (*Gryphaea gigas*) in Australia. *Australian Journal of Marine and Freshwater Research* **3**:64-73.

Timm, J. and Kochzius, M. 2008. Geological history and oceanography of the Indo-Malay Archipelago shape the genetic population structure in the false clown anemonefish (*Amphiprion ocellaris*). *Molecular Ecology* **17**:3999-4014.

Tokida, J. and Masaki, T. 1959. A list of marine algae collected in the vicinity of Oshoro Marine Biological Station, at Oshoro, Hokkaido, Japan. *Bulletin of the Faculty of Fisheries Hokkaido University* **10**:173-95.

Tokuda, H., Kawashima, S., Ohno, M. and Ogawa, H. 1994. *A Photographic Guide to Seaweeds of Japan.* Midori Shobo Co. Ltd., Tokyo, Japan, 194 pp.

Tomanek, L. and Somero, G. N. 1999. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from

different thermal habitats: implications for limits of thermotolerance and biogeography. *Journal of Experimental Biology* **202**:2925-36.

Tsai, C. C., Wong, S. L., Chang, J. S., Hwang, R. L., Dai, C. F., Yu, Y. C., Shyu, Y. T., Sheu, F. and Lee, T. M. 2004. Macroalgal assemblage structure on a coral reef in Nanwan Bay in southern Taiwan. *Botanica Marina* **47**:439-53.

Tsang, L. M., Chan, B. K. K., Ma, K. Y. and Chu, K. H. 2008. Genetic differentiation, hybridization and adaptive divergence in two subspecies of the acorn barnacle *Tetraclita japonica* in the northwestern Pacific. *Molecular Ecology* **17**:4151-63.

Tseng, C. K. 1983. *Common Seaweeds of China.* Science Press, Beijing, China, 316 pp.

Tseng, C. K. 1985. *Sargassum* sect. *Phyllocystae* sect. *nov.*, a new section of *Sargassum* subgenus *Bactrophycus*. *In*: Abbott, I. A. [Ed.] *Taxonomy of Economic Seaweeds, Volume 1.* California Sea Grant College Program, La Jolla, USA, pp. 15.

Tseng, C. K. 1996. Species of Hong Kong *Sargassum* and their biogeography. *In*: Morton, B. [Ed.] *The Marine Biology of the South China Sea III.* Hong Kong University Press, Hong Kong, China, pp. 3-10.

Tseng, C. K. and Chang, C. F. 1954. Studies on Chinese *Sargassums*, II. *Sargassums kjellmanianum* Yendo. *Acta Botanica Sinica* **3**:353-66.

Tseng, C. K. and Lu, B. R. 2000. *Flora Algarum Marinarum Sinicarum, Tomus III Phaeophyta, No. II Fucales.* Science Press, Beijing, China, 238 pp.

Uchida, T. and Arima, S. 1993. Crossing experiments between autumn- and spring-fruiting types of *Sargassum horneri* (Phaeophyta). *Bulletin of the Japanese Society of Scientific Fisheries* **59**:1685-88.

Umar, M. J., McCook, L. J. and Price, I. R. 1998. Effects of sediment deposition on the seaweed *Sargassum* on a fringing coral reef. *Coral Reefs* **17**:169-77.

Umezaki, I. 1984a. Ecological studies of *Sargassum horneri* (Turner) C. Agardh in Obama Bay, Japan Sea. *Bulletin of the Japanese Society of Scientific Fisheries* **50**:1193-200.

Umezaki, I. 1984b. Ecological studies of *Sargassum hemiphyllum* C. Agardh in Obama Bay, Japan Sea. *Bulletin of the Japanese Society of Scientific Fisheries* **50**:1677-83.

Uwai, S., Nelson, W., Neill, K., Wang, W. D., Aguilar-Rosas, L. E., Boo, S. M., Kitayama, T. and Kawai, H. 2006b. Genetic diversity in *Undaria pinnatifida* (Laminariales, Phaeophyceae) deduced from mitochondria genes - origins and succession of introduced populations. *Phycologia* **45**:687-95.

Uwai, S., Yotsukura, N., Serisawa, Y., Muraoka, D., Hiraoka, M. and Kogame, K. 2006a. Intraspecific genetic diversity of *Undaria pinnatifida* in Japan, based on the mitochondrial cox3 gene and the ITS1 of nrDNA. *Hydrobiologia* **553**:345-56.

Valeria Ruggiero, M. and Procaccini, G. 2004. The rDNA ITS region in the Lessepsian marine angiosperm *Halophila stipulacea* (Forssk.) Aschers. (Hydrocharitaceae): Intragenomic variability and putative pseudogenic sequences. *Journal of Molecular Evolution* **58**:115-21.

van den Hoek, C. 1975. Phytogeographic provinces along the coasts of the northern Atlantic Ocean. *Phycologia* **14**:317-30.

van den Hoek, C. 1982. The distribution of benthic marine algae in relation to the temperature regulation of their life histories. *Biological Journal of the Linnean Society* **18**:81-144.

van den Hoek, C. 1984. World-wide latitudinal and longitudinal seaweed distribution patterns and their possible causes, as illustrated by the distribution of Rhodophytan genera. *Helgoland Marine Research* **38**:227-57.

van der Strate, H. J., van de Zande, L., Stam, W. T. and Olsen, J. L. 2002. The contribution of haploids, diploids and clones to fine-scale population structure in the seaweed *Cladophoropsis membranacea* (Chlorophyta). *Molecular Ecology* **11**:329-45.

van Oppen, M. J. H., Diekmann, O. E., Wiencke, C., Stam, W. T. and Olsen, J. L. 1994. Tracking dispersal routes: phylogeography of the Arctic-Antarctic disjunct seaweed *Acrosiphonia arcta* (Chlorophyta). *Journal of Phycology* **30**:67-80.

van Oppen, M. J. H., Klerk, H., Graaf, M., Stam, W. T. and Olsenz, J. L. 1996. Assessing the limits of random amplified polymorphic DNAs (RAPDs) in seaweed biogeography. *Journal of Phycology* **32**:433-44.

Verlaque, M., Durand, C., Huisman, J., Boudouresque, C. F. and Le Parco, Y. 2003. On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). *European Journal of Phycology* **38**:325-39.

Veron, J. 2000. *Corals of the World.* Australian Institute of Marine Science Press, Townsville, Australia, 1382 pp.

Voisin, M., Engel, C. R. and Viard, F. 2005. Differential shuffling of native genetic diversity across introduced regions in a brown alga: Aquaculture vs. maritime traffic effects. *Proceedings of the National Academy of Sciences of the United States of America* **102**:5432-37.

Voris, H. K. 2000. Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and time durations. *Journal of Biogeography* **27**:1153-67.

Wang, W. D. 2003. Investigation and study on *Sargassum* and *Hizikia* in Zhejiang Province. *Journal of Shanghai Fisheries University* **12**:227-32.

Wang, W. L. and Chiang, Y. M. 2001. The marine macroalgae of Lu Tao (Green Island), Taiwan. *Taiwania* **46**:49-61.

Warwick, R. M., Clarke, K. R. and Suharsono 1990. A statistical analysis of coral community responses to the 1982–83 El Niño in the Thousand Islands, Indonesia. *Coral Reefs* **8**:171-79.

Westermeier, R., Patiño, D., Ines Piel, M., Maier, I. and Mueller, D. G. 2006. A new approach to kelp mariculture in Chile: production of free-floating sporophyte seedlings from gametophyte cultures of *Lessonia trabeculata* and *Macrocystis pyrifera*. *Aquaculture Research* **37**:164-71.

Wilkinson, M., Telfer, T. and Grundy, S. 1995. Geographical variation in the distributions of macroalgae in estuaries. *Aquatic Ecology* **29**:359-68.

Williamson, M. and Fitter, A. 1996. The varying success of invaders. *Ecology* **77**:1661-66.

Willis, B. L., van Oppen, M. J. H., Miller, D. J., Vollmer, S. V. and Ayre, D. J. 2006. The role of hybridization in the evolution of reef corals. *Annual Review of Ecology, Evolution, and Systematics* **37**:489-517.

Womersley, H. B. S. 1954. Australian species of *Sargassum*, subgenus *Phyllotrichia*. *Australian Journal of Botany* **2**:337-54.

Wright, S. 1943. Isolation by distance. *Genetics* **28**:114-38.

Xu, X. and Oda, M. 1999. Surface-water evolution of the eastern East China Sea during the last 36,000 years. *Marine Geology* **156**:285-304.

Yang, H. N. 1994. Marine algal flora of Pengchia Yu and its special place in the marine phytogeography of Taiwan. *Botanica Marina* **37**:429-32.

Yang, H. N. 1995. *Taiwanese taxa of* Sargassum *(Sargassaceae, Fucales, Phaeophyta)*. Doctoral Dissertation, Taiwan National University, 212 pp.

Yendo, K. 1907. The Fucacaeae of Japan. *Journal of College of Science, Imperial University of Tokyo* **21**:1-174.

Yokogawa, K. 1997. Morphological and genetic differences between Japanese and Chinese red ark shell *Scapharca broughtonii*. *Fisheries Science* **63**:332-37.

Yokoyama, H., Ishihi, Y., Toyokawa, M., Yamamoto, S. and Ajisaka, T. 1999. Community structure on sargassoceous beds in Gokasho Bay II. Seasonal growth, maturation periods of sargassoceous species, and annual net production of seaweeds. *Bulletin of National Research Institute of Aquaculture* **28**:27-37.

Yoo, J. S. 2003a. Biodiversity and community structure of marine benthic organisms in the rocky shore of Dongbaekseom, Busan. *Algae* **18**:225-32.

Yoo, J. S. 2003b. Community dynamics of benthic marine algae in the intertidal and subtidal rocky shore of Samyang, Jejudo Island. *Algae* **18**:301-09.

Yoo, J. S. 2003c. Structural characteristics of benthic algal community in the subtidal zone of Yeongil Inner and Oter Bay. *Algae* **18**:365-69.

Yoshida, G., Murase, N. and Terawaki, T. 1999. Comparisons of germling growth abilities under various culture conditions among two *Sargassum horneri* populations and *S. filicinum* in Hiroshima Bay. *Bulletin of Fisheries and Environment of Inland Sea* **1**:45-54.

Yoshida, G., Yoshikawa, K. and Terawaki, T. 2001. Growth and maturation of two populations of *Sargassum horneri* (Fucales, Phaeophyta) in Hiroshima Bay, the Seto Inland Sea. *Fisheries Science* **67**:1023-29.

Yoshida, T. 1978. Lectotypification of *Sargassum kjellmanianum* and *S. miyabei* (Phaeophyta, Sargassaceae). *Japanese Journal of Phycology* **26**:121-24.

Yoshida, T. 1983. Japanese species of *Sargassum* subgenus *Bactrophycus* (Phaeophyta, Fucales). *Journal of faculty of science, Hokkaido Imperial University, Series V (Botany)* **13**:99-246.

Yoshida, T. 1998. *Marine Algae of Japan*. Uchida Rokakuho Publishing Co., Ltd., Tokyo, Japan, 1222 pp.

Yoshida, T., Stiger, V. and Horiguchi, T. 2000. *Sargassum boreale* sp. nov. (Fucales, Phaeophyceae) from Hokkaido, Japan. *Phycological Research* **48**:125-31.

Yotsukura, N., Denboh, T., Motomura, T., Horiguchi, T., Coleman, A. W. and Ichimura, T. 1999. Little divergence in ribosomal DNA internal transcribed spacer -1 and -2 sequences among non-digitate species of *Laminaria* (Phaeophyceae) from Hokkaido, Japan. *Phycological Research* **47**:71-80.

Zar, J. H. 1999. *Biostatistical Analysis*. Prentice Hall, Upper Saddle River, USA, 929 pp.

Zhang, J., Cai, Z. and Huang, L. 2006. Population genetic structure of crimson snapper *Lutjanus erythropterus* in East Asia, revealed by analysis of the mitochondrial control region. *ICES Journal of Marine Science* **63**:693-704.

Zhao, F., Liu, F., Liu, J., Ang, P. and Duan, D. 2008a. Genetic structure analysis of natural *Sargassum muticum* (Fucales, Phaeophyta) populations using RAPD and ISSR markers. *Journal of Applied Phycology* **20**:191-98.

Zhao, Z., Zhao, F., Yao, J., Lu, J., Ang, P. and Duan, D. 2008b. Early development of germlings of *Sargassum thunbergii* (Fucales, Phaeophyta) under laboratory conditions. *Journal of Applied Phycology* **20**:925-31.

Zhou, S. Q., Guo, F., Wu, L. S. and Li, R. G. 2001. The study on the ecology of the benthic community in intertidal zone, Fujian islands. *Acta Oceanologica Sinica* **23**:104-09.

Zink, R. M. 2002. Methods in comparative phylogeography, and their application to studying evolution in the North American aridlands. *Integrative and Comparative Biology* **42**:953-59.

Zou, D., Gao, K. and Ruan, Z. 2006. Seasonal pattern of reproduction of *Hizikia fusiformis* (Sargassaceae, Phaeophyta) from Nanao Island, Shantou, China. *Journal of Applied Phycology* **18**:195-201.

Zuccarello, G. C., West, J. and Rueness, J. 2002. Phylogeography of the cosmopolitan red alga *Caulacanthus ustulatus* (Caulacanthaceae, Gigartinales). *Phycological Research* **50**:163-72.

Appendix 2.1 List of all the *Sargassum* species/species complexes and the OGUs compiled in this study for NW Pacific.

Species/ species complexes
Subgeus *Bactrophycus*
S. ammophilum Yoshida et T. Konno
S. araii Yoshida
S. autumnale Yoshida
S. boreale Yoshida et Horiguchi
S. confusum C. Agardh
S. coreanum J. Agardh/ *S. ringgoldianum* Harvey subsp *coreanum*
S. filicinum Harvey
S. fulvellum (Turner) C. Agardh/ *S. enerve* C. Agardh
S. fusiforme (Harvey) Setchell/ *Hizikia fusiformis* (Harvey) Okamura
S. giganteifolium Yamada
S. hemiphyllum (Turn.) v. *chinense* J. Agardh
S. hemiphyllum v. *hemiphyllum* (Turner) C. Agardh
S. herklotsii Setchell
S. horneri (Turn.) C. Agardh
S. macrocarpum C. Agardh
S. micracanthum (Kützing) Endlicher
S. microceratium (Turner) C. Agardh
S. miyabei Yendo/ *S. kjellmanianum* Yendo
S. muticum (Yendo) Fensholt
S. nigrifolioides Tseng et Lu
S. nigrifolium Yendo
S. nipponicum Yendo
S. okamurae Yoshida et. T. Konno
S. ringgoldianum Harvey/ *S. ringgoldianum* Harvey Subsp *ringgoldianum*
S. rostratum J. Agardh
S. sagamianum Yendo
S. segii Yoshida/ *S. racemosum* Yamada & Segi
S. serratifolium (C. Agardh) C. Agardh
S. siliquastrum (Turner) C. Agardh/ *S. tortile* (C. Agardh) C. Agardh
S. spathulophyllum J. Tanaka et Murakami
S. tenuifolium Yamada
S. thunbergii (Mertens ex Roth) Kuntze
S. trichophyllum (Kutzing) O. Kuntze

S. wakayamaense Yoshida

S. yamadae Yoshida et Konno

S. yamamotoi Yoshida

S. yezoense (Yamada) Yoshida et. T. Konno

Subgenus *Sargassum*

S. agaviforme Tseng et Lu

S. alternato-pinnatum Yamada

S. amabile Yamada

S. angustifolium (Turn.) C. Agardh

S. aquifolium (Turner) C. Agardh

S. assimile Harvey

S. baccularia (Mertens) C. Agardh

S. beihaiense Tseng et Lu

S. biserrula J. Agardh

S. biserrulioides Tseng et Lu

S. bulbiferum Yoshida

S. capilliforme Tseng et Lu

S. capitatum Tseng et Lu

S. carpophyllum J. Agardh

S. carpophyllum v. *compressum* Grunow

S. cervicorne Greville

S. cinctum J. Agardh

S. cinereum J. Agardh

S. crassifolium J. Agardh

S. crispifolium Yamada

S. cristaefolium C. Agardh/ *S. berberifolium* J. Agardh/ *S. duplicatum* Bory de Saint-Vincent

S. duplicatum (J. Agardh) J. Agardh

S. cymosum C. Agardh

S. cystophyllum Montagne v. *parcespinosa* Grunow

S. distichum Sonder/ *S. aemulum* Sonder in Linn.

S. dotyi Trono

S. erumpens Tseng et Lu

S. euryphyllum (Grunow) Tseng et Lu

S. feldmannii Pham

S. frutescens Tseng et Lu

S. fruticulosum Tseng et Lu

S. fujianense Tseng et Lu

S. fuliginosoides Tseng et Lu

S. fuscifolium Tseng et Lu

S. gemmiphorum Tseng et Lu

S. glaucescens J. Agardh

S. gracillimum Reinbold.

S. graminifolium (Turner) J. Agardh

S. granuliferum C. Agardh

S. guangdongii Tseng et Lu

S. hainanense Tseng et Lu

S. henslowianum C. Agardh

S. heterocystum (Kuetzing) Montagne

S. ilicifolioides Tseng et Lu

S. ilicifolium (Turner) C. Agardh v. *conduplicatum* (J. Ag.) Grunow

S. ilicifolium (Turner) C. Agardh/ *S. sandei* Reinbold

S. incanum Grunow

S. integerrimum Tseng et Lu

S. intermedium Tseng et Lu

S. kashiwajimanum Yendo

S. kasyotense Yamada

S. kuetzingii Setchell

S. kushimotense Yendo

S. leizhouense Tseng et Lu

S. longicaulis Tseng et Lu

S. longifructum Tseng et Lu

S. mcclurei f. *duplicatum* A. Zin et H.D. Nguyen

S. mcclurei Setchell

S. megalocystum Tseng et Lu

S. myriocystum J. Agardh

S. naozhouense Tseng et Lu

S. odontocarpum Sonder/ *S. coriifolium* J. Agardh

S. oligocystum Montagne/ *S. binderi* Sonder ex J. Agardh

S. paniculatum J. Agardh

S. parvifolioides Tseng et Lu

S. parvifolium (Turn.) C. Agardh

S. patens C. Agardh

S. patens C. Agardh v. *phylizophylla* (Kuetz.) Yendo

S. patens C. Agardh v. *rodgersianum* (Harvey) Grunow

S. patens v. *typicum* Setch.

S. piluliferum (Turner) C. Agardh

S. pinnatifidum Harvey

S. plagiophyllum C. Agardh

S. polycystum C. Agardh

S. polycystum v. *linearifolium* H.N.Yang & Y.M. Chiang

S. polyporum Montagne

S. primitivum Tseng et Lu

S. pseudolanceolatum Tseng et Lu

S. pumilum Tseng et Lu

S. qingdaoense Tseng et Lu

S. qinzhouense Tseng et Lu

S. qionghaiense Tseng et Lu

S. rhizophorum Tseng et Lu

S. salicifolioides Yamada/ *S. hyugaense* Yamada

S. sanyaense Tseng et Lu

S. shandongense Tseng, C. F. Zhang et Lu

S. shangchuanii Tseng et Lu

S. siliculosoides Tseng et Lu

S. siliquosum J. Agardh

S. silvae Tseng et Lu

S. spinifex C. Agardh

S. squarrosum Greville

S. subdroserifolium Tseng et Lu

S. subspathulatum Grunow

S. sullivanii Trono

S. swartzii (Turn.) C. Agardh/ *S. acutifolium* Greville

S. symphyorhizoideum Tseng et Lu

S. telephifolium (Turner) C. Agardh

S. tenerrimum J. Agardh

S. tenue J. Agardh

S. tenuifolioides Tseng et Lu

S. tosaense Yendo

S. vachellianum Greville

S. vietnamense A. Zin. et H.D. Nguyen

S. virgatum C. Agardh

S. weizhouense Tseng et Lu

S. wenchangense Tseng et Lu

S. wightii Greville

S. xishaense Tseng et Lu

S. yendoi Okamura et. Yamada/ *S. henslowianum* v. *condensatum* Yamada

S. yingehaiense Tseng et Lu

S. yongxingense Tseng et Lu

Unknown subgenus

S. kwangyangenses Kang

OGU

1: Kuril Islands (North), Russia

2: Kuril Islands (South), Russia

3: Southern Hokkaido, Japan

4: Northern Honshu 1, Pacific side, Japan

5: Northern Honshu 2, Pacific side, Japan

6: Izu Islands, Pacific side, Japan

7: Eastern Honshu 1, Pacific side, Japan

8: Seto Inland Sea, Japan

9: Southern Shikioku, Japan

10: Eastern Kyushu, Japan

11: Ryukyu Islands, Japan

12: Western Kyushu, Japan

13: Western Honshu 1, Sea of Japan side, Japan

14: Western Honshu 2, Sea of Japan side, Japan

15: Western Hokkaido, Japan

16: Northern Hokkaido, Japan

17: Sakhalin South, Russia

18: Russian coast of Vladivostok Sea

19: N. Korea North East (Shimpo & Genzan)

20: Korea, 1 (East)

21: Dokdo & Ullundo Islands, South Korea

22: Korea, 2 (East)

23: Korea, 3 (South)

24: Cheju Island, South Korea

25: Korea, 4 (South)

26: Korea, 5 (West)

27: Korea, 6 (West)

28: Korea, 7 (West)

29: China mainland, Lioudong East

30: China mainland, Lioudong west

31: China mainland, Shandong North and East

32: China mainland, Shandong South

33: China mainland, Jiangsu

34: China mainland, Zhejiang North

35: China mainland, Zhejiang South

36: China mainland, Fujian North

37: Taiwan Penghu (west)

38: Taiwan North

39: Taiwan East

40: Taiwan Ludao/ Landao (southeast)

41: Taiwan South

42: China mainland, Fujian South

43: China mainland, Guangdong East (Including Hong Kong and Macau)

44: China mainland, Guangdong West

45: China mainland, Hainan

46: China mainland, Guangxi

47: Vietnam, North