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Phylogeographic analysis of the genus *Ulva* (Ulvales, Chlorophyta), including bloom sample in Qingdao, China

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Abstract — In June 2008, news agencies worldwide reported a 'green tide' phenomenon that was threatening the upcoming Olympic sailing events in Qingdao, China. This was caused by the green macroalgal genus *Ulva*. Previous work showed that the species involved in this phenomenon belongs to a complex of tubular species called the LPP complex clade (i.e. including the species *U. linza* Linnaeus, *U. prolifera* O.F. Müller and *U. procera* (K. Ahlner) Hayden et al.). To understand the relationship between the bloom formed in open water and the attached populations of *Ulva* found on the shorelines surrounding Qingdao, we performed some phylogenetic analyses based on the Internal Transcribed Spacer (ITS) region of the nuclear rDNA, on a larger set of samples. Due to a lack of resolution of this marker at the intraspecific level, we performed some new analyses based on the spacer region between tandemly repeated 5S rDNA. Samples were divided into two groups: the *U. linza* group, which includes unbranched foliose thalli collected from marine environment; and the *U. prolifera* group, which includes unbranched folious and well-branched tubular thalli from both seas (including Qingdao samples) and rivers. The bloom samples and a part of the attached samples had identical sequences for the 5S rDNA spacer region. ML tree and statistical parsimony network (SPN) tree indicated that they were closely related with populations from the Sea of Japan. Other attached samples of the Qingdao area possessed identical sequence to samples widely distributed on the Pacific coast of Japan, from Okinawa to the Kanto area.

Key words: Japan, Phylogeography, Qingdao, River, Taxonomy, *Ulva*

Introduction

The green macroalgal genus *Ulva* (including *Enteromorpha*) is one of the most common seaweeds found on seashores from all over the world (Shimada et al. 2003, van den Hoek et al. 1995). The genus *Ulva* is very common in marine environment but can also be found in brackish & freshwater (Canter-Lund and Lund 1995, Ichihara et al. 2009a, b, Martins et al. 1999, McAvoy and Klug 2005, Reed and Russell 1979; Shimada et al. 2007). For example, *U. prolifera* O.F. Müller is growing from the estuary to 6–7 km upstream of the Shimanto River, Kochi Prefecture, Japan (Ohno and Takahashi 1988). Fishermans harvest *U. prolifera* from the Shimanto River in winter to spring, and dry it for commercial purposes, and Japanese use it for Miso-soup, Tenpura, and so on (Ohno 2004). Due to an increasing demand, harvesting is also complemented by land-based cultivation in tanks. The aquaculture of *U. prolifera* is conducted in Kochi Prefecture using deep seawater (Hiraoka and Oka 2008).

However, *Ulva* species can also have negative effects on human activities. Extensive biomass of free-floating thalli can accumulate on shallow beaches under particular circumstances. This phenomenon, involving mainly the genus *Ulva*, is called 'green tide' (Fletcher 1996, Ohno 1999). In June 2008, news agencies worldwide reported a vast algal bloom in Qingdao, China. An army of more than 10,000 of recruits have been deployed during one week to remove the algal bloom. Qingdao city reported that the total mass of the bloom was about 10,000 wet weight ton (Liu et al. 2009).

From the gross morphology, it made no doubt that the bloom species belonged to the genus *Ulva*, and molecular phylogenetic analysis of ITS (Internal Transcribed Spacer) region of nrDNA indicated that the Qingdao bloom samples were included in the clade comprising *U. linza* L., *U. prolifera* and *U. procera* (K. Ahlner) Hayden et al. (called LPP

complex clade) (Leliaert et al. 2009), although they did not make an accurate identification of the bloom specimens at the species level.

Liu et al. (2009) mentioned the possibility of non-local sources for the Qingdao bloom from satellite images data. According to these observations, on the 15th of May 2008, some small green patches covering a total surface of about 80 km^2 were observed near the coasts of Yancheng and Lianyungang, Jiangsu province. Soon, it grew rapidly and moved into the middle of the Yellow Sea, towards to Qingdao. Finally huge quantities of biomass stranded and formed the famous green tide on the 28th of June along the coasts of Qingdao. Liu et al. (2009) emitted the hypothesis that the bloom was originating from *U. prolifera* populations growing on the aquaculture nets of the nori farms (producing *Porphyra yezoensis* Ueda) in the Jiangsu province, China.

The present study aims to understand the relationship between the population of *Ulva* forming the bloom, and the attached populations of *Ulva* found in the surroundings of Qingdao. For this purpose, we use two markers: the nuclear encoded ITS region, traditionally used *Ulva* species, and a more sensitive marker, the spacer region between tandemly repeated 5S rDNA, especially developed for the LPP clade (Shimada et al. 2008).

Materials and Methods

The bloom samples were collected from Qingdao (2008/Jul/6), and were transported alive to the Usa Marine Biological Institute, Kochi University, Japan for culture studies. Unialgal cultures were established from zoids. Male gametophyte (M2) and female gametophyte (F1) were isolated from a thallus and used in the present molecular phylogenetic study. Attached dried thalli were collected from rocks in the intertidal zone in Qingdao (C883 and C884:2009/May/5, C885 and C886:2009/Jul/20). Voucher herbarium specimens are deposited in the Herbarium of the Graduate School of Science, Hokkaido University, Sapporo (SAP 108022-108024).

Total DNA was extracted from the six samples mentioned above by using the Dneasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) according to the specifications of the manufacturer. The regions selected for PCR amplification and automated sequencing were the nuclear-encoded ITS region including the 5.8 S rDNA and the 5 S rDNA spacer region (Shimada et al. 2008). The newly determined sequences were added to the alignment files used in the previous report (Shimada et al. 2008), and phylogenetic analyses were performed using the maximum likelihood (ML) algorithm available in the computer program PAUP* 4.0 b10 (Swofford, 2002). Identical sequences were excluded from the alignment. The program MODELTEST version 3.7 (Posada and Crandall, 1998) was used to find the model of sequence evolution that best fit each dataset by a hierarchical likelihood ratio test (α=0.01). When the best sequence evolution model was determined, ML tree searches were performed using the estimated model parameters with the following options: starting tree option=obtained by stepwise addition, and branch swapping algorithm=TBR. Bootstrap values based on 100 re-samplings in ML, 2000 re-samplings in maximum parsimony (MP) and neighbor-joining (NJ) of the dataset were calculated (TBR, full heuristic search option) (Felsenstein, 1985).

A statistical parsimony network (SPN) tree for the 32 haplotypes of the 5S rDNA spacer region was inferred using TCS v. 1.18 (Clement et al., 2000). Each gap was treated as 5th state in the construction of a SPN tree.

Results and Discussions

The six samples collected from Qingdao, including bloom and attached thalli, all possessed identical sequence of ITS rDNA. The phylogenetic tree obtained with the ML method is presented in Fig. 1. Likelihood settings from the best-fit model (GTR+I+G) were selected by a hierarchical likelihood ratio test in the program MODELTEST version 3.7: assumed nucleotide frequencies A=0.14400, C=0.37800, G=0.32330, and T=0.15470; substitution-rate matrix with AC=1.226900, AG=2.180000, AT=2.801000, CG=1.401000, CT=3.666100 and GT=1; proportion of invariable sites=0.3781; gamma distribution with shape parameter=0.5223. Based on these settings, a heuristic search was performed with the TBR branch swapping option (-ln L=4599.99070) after 10369 rearrangements (Fig. 1). The six Qingdao samples were included in the LPP complex clade containing *U. linza, U. prolifera* and *U. procera*. It received 82 % bootstrap support in ML, 94 % in MP and 100 % in NJ analyses. There were only 0–7 substitutions (0–1.149 %) in the LPP complex. The phylogenetic position of the 6 samples was the same as for the population studied by Leliaert et al. (2009). However, the relationships of all the different populations/species belonging to the LPP clade is not well resolved using the ITS marker.

The unrooted phylogenetic tree obtained with ML analysis of 5S rDNA spacer region (389 bp) is presented in Fig. 2. For the ML method, likelihood settings from the best-fit model (HKY85+G) were selected by a hierarchical likelihood ratio test in the program MODELTEST version 3.7: assumed nucleotide frequencies A=0.16540, C=0.42130, G=0.23030, and T=0.18300; gamma distribution with shape parameter=0.7038. Based on these settings, a heuristic search was performed with the TBR branch swapping option (-ln L=1186.7596) after 68569 rearrangements (Fig. 2). The LPP complex were divided into two groupings: the *U. linza* group including only marine littoral representatives and the

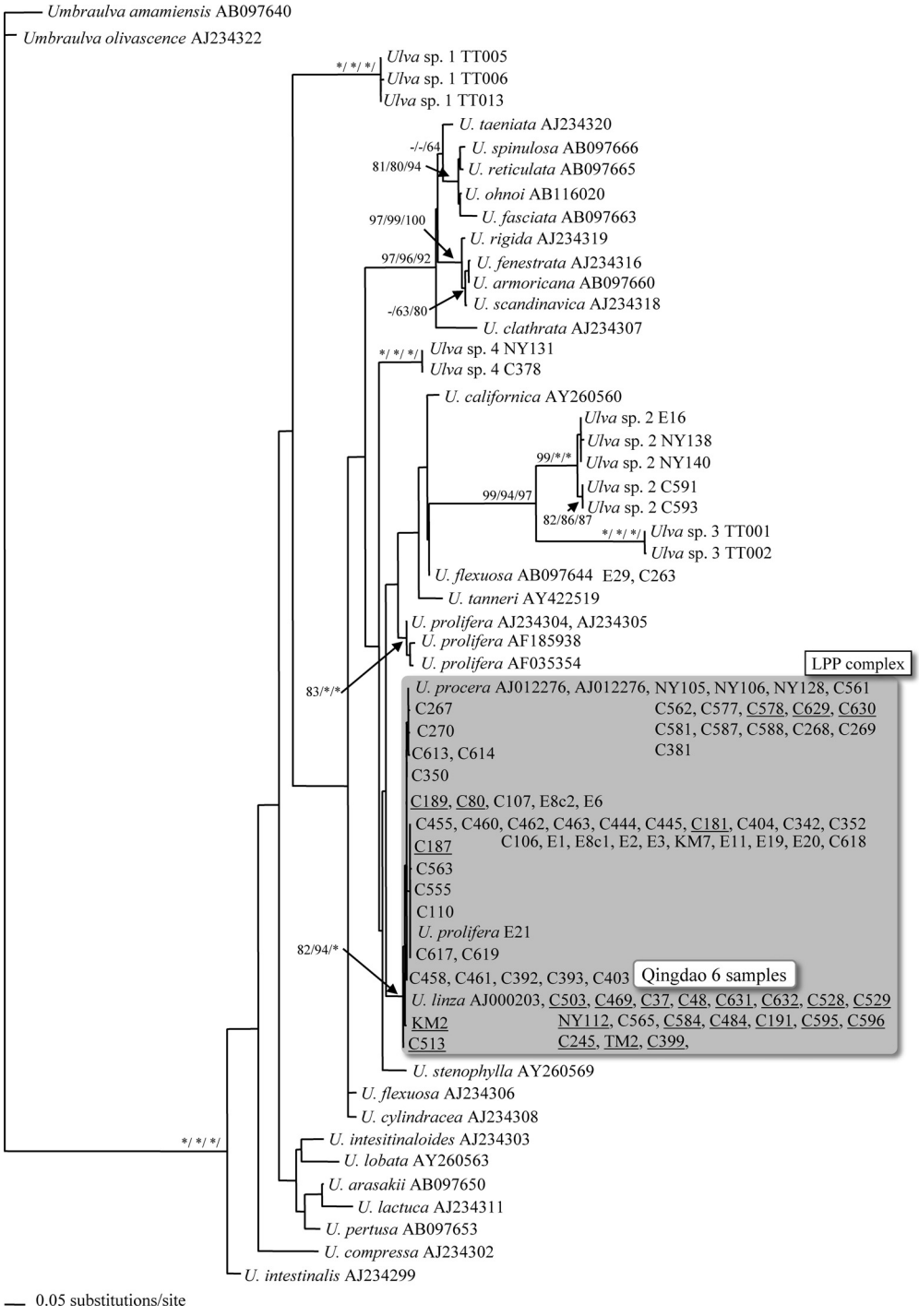

Fig. 1. Phylogenetic tree of the maximum likelihood (ML) analysis inferred from the nuclear encoded ITS regions including 5.8 S rDNA of *Ulva*. *Umbraulva amamiensis* and *Umbraulva olivascens* were used as outgroups. Numerals at internal nodes are bootstrap values >50% for 100 replicates in ML, 2000 replicates in maximum parsimony (MP) and neighbor-joining (NJ) analyses (ML/MP/NL). The underlined sample numbers were collected from seashores. * denotes 100% bootstrap values.

U. prolifera group composed of a mixture of both marine (10 samples) and all river representatives. There are 124 bp indels between two groups. These two groupings were supported by 100 % bootstrap values in all analyses. The ten marine samples contained in the *U. prolifera* group did not form a monophyletic clade. All six samples collected from Qingdao were contained in the *U. prolifera* group. The two bloom samples (F1, M2) as well as two attached samples (C885, C886) were all identical (haplotype QB). There are 54 bp indels between haplotype QB and other haplotype of the *U. prolifera* group. The haplotype QB was closely related to the haplotype S collected from Toyama Pref., the Sea of Japan. Other two attached Qingdao samples (C883, C884) were identical to the haplotype A distributed from Okinawa to Kanto area, the Pa-

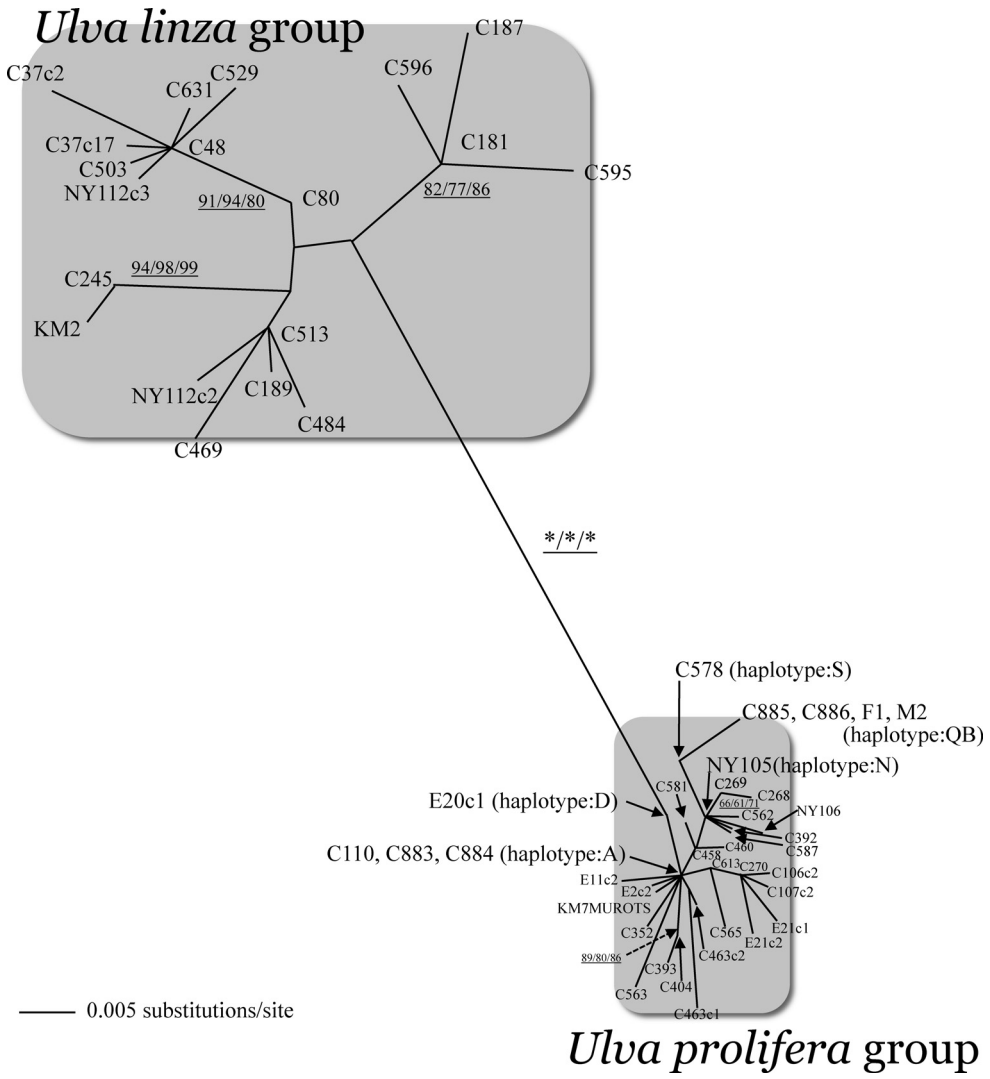

Fig. 2. Unrooted ML tree of the 5 S rDNA spacer region of the LPP complex. Numerals at internal nodes are bootstrap values >50% for 100 replicates in ML, 2000 replicates in maximum parsimony (MP) and neighbor-joining (NJ) analyses (ML/MP/NL). * denotes 100% bootstrap values.

cific coast of Japan. The *U. linza* group and the *U. prolifera* group were clearly separated, and we did not find any hybrid samples that possessed the *U. linza* group type sequence and the *U. prolifera* group type sequence of the 5S rDNA spacer region. This result indicated that the two entities restrict gene flow and might be independent species. Thus, Qingdao bloom can be identified as *Ulva prolifera*.

The *U. linza* group includes only unbranched foliose thalli collected from the seas, and the *U. prolifera* group includes unbranched folios thalli and well-branched tubular thalli from the seas and rivers. *Ulva prolifera* commonly possesses well-branched tubular thalli (Koeman 1982), but its gross morphology varies in different environmental conditions (Shimada et. al 2001). In the culture experiments, the zoids from unbranched folios *U. prolifera* (similar to *U. linza*, but identified as *U. prolifera* by 5S rDNA spacer region data) grew to well-branched tubular thalli (Shimada unpubl.). Pang et al. (2009) tracked the algal origin of the *Ulva* bloom in Qingdao by a combination of molecular (ITS rDNA and *rbc*L data set), morphological and physiological analyses. However, these DNA markers are too conservative to identify the different population and to track their origin. In addition, field-materials are shown to harbor a high phenotypical variation. For example, gross morphology varies with wave action (Mshigeni and Kajumulo 1979), branching is affected by salinity (Blomster et al. 1998), thallus thickness varies through the season and is thus probably related to age (Blidding 1968, Phillips 1988). Cell size is known to vary under different salinity (Koeman and van den Hoek 1981), the shape of basal cells is variable with the age, the ploidy and/or the sex of individual plants (Coat et al. 1998). The presence or absence of the marginal teeth is influenced by wave action (Philips 1988), and the number of pyrenoids is also thought to be variable under the nutritious condition such as micro-

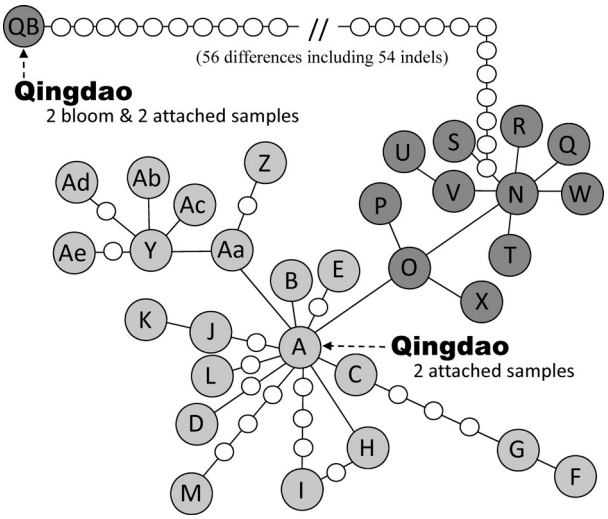

Fig. 3. SPN tree of the *U. prolifera* group in the LPP complex. Haplotype number is correlated in the previous report (Fig. 4, Shimada et al. 2008).

scopic green algae (Kapraum 1970, Tanner 1986, Phillips 1988, Malta et al. 1999). Therefore, it is better to use 5S rDNA spacer region as DNA marker for species/population identification of the LPP complex, or to execute culture or cross experiments to accede the morphological or biological species concept.

The SPN tree of the *U. prolifera* group is presented in Fig. 3. Haplotype number is correlated in the previous report (Fig. 4, Shimada et al. 2008). Haplotype A connected with 11 other lineages. One lineage (dark gray circles in Fig. 3) comprised 12 haplotypes including Qingdao bloom haplotype QB which connected to the haplotype S collected from Toyama Pref., the Sea of Japan and the haplotype N extended from the Pacific coast of the Tohoku region to the Sea of Japan. This result indicated that the Qingdao bloom haplotype QB might be derived from the lineage of the Sea of Japan. We had not found out this mutant haplotype QB in other locations. We need more samples in Korean and Chinese coasts to understand detailed root of this blooms.

In conclusion, two types of *U. prolifera* coexist in Qingdao: one is widely distributed and grows in winter to spring; and the other is only distributed in Qingdao, grows in summer and caused the bloom. The present bloom of *U. prolifera* may have been derived from the Sea of Japan phylogenetically, and the original population of the bloom might exist in the coast of the Yellow Sea. The occurrence of further blooms should be monitored in order to understand the pattern of green tides in the Yellow Sea, as well as their possible occurrence in surrounding areas.

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