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Species Diversity and Seasonal Changes of Dominant *Ulva* Species (Ulvales, Ulvophyceae) in Mikawa Bay, Japan, Deduced from ITS2 rDNA Region Sequences

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Frequent occurrences of green tides caused by *Ulva* species (Ulvales, Ulvophyceae) associated with eutrophication along enclosed coasts are currently causing environmental problems in coastal ecosystems. In addition, increasing intercontinental introductions of coastal marine organisms, including *Ulva*, are also a serious issue. However, due to the considerable morphological plasticity of this genus, the taxonomy of *Ulva* species based on morphological studies is problematic. Therefore, in order to elucidate the species diversity and seasonal changes of the dominant *Ulva* species in Mikawa Bay, central Honshu, Japan, we made seasonal collections of *Ulva* species at seven localities, and identified the dominant species using the ITS2 rDNA region sequences. We identified the following nine taxa as common *Ulva* species in the area: 1) *Ulva pertusa* Kjellman; 2) *U. ohnoi* Hiraoka et Shimada; 3) *U. linza* L.; 4) *U. californica* Wille; 5) *U. flexuosa* Wulfen; 6) *U. fasciata* Delile; 7) *U. compressa* L.; 8) *U. armoricana* Dion *et al.*; 9) *U. scandinavica* Bliding. Among the species, *U. pertusa* was most common and dominant from spring to summer, and *U. ohnoi* from autumn to winter. *Ulva californica* and *U. scandinavica* have not been reported before from Japan.

Key Words: ITS2, non-indigenous species, trans-ocean introduction, *Ulva* species

INTRODUCTION

Ulva and *Enteromorpha* (Ulvales, Ulvophyceae) species are among the most common inhabitants of intertidal ecosystems. The genus-level taxonomy of *Ulva* and *Enteromorpha* has been extensively revised based on molecular phylogenetic studies. Thus while *Ulva* and *Enteromorpha* were distinguished based on differences in thallus anatomy, the indepedence of the genus *Enteromorpha* was questioned based on molecular phylogenetic analyses (Tan *et al.* 1999; Shimada *et al.* 2003), and subsequently *Enteromorpha* was reduced to synonymy with *Ulva* (Hayden *et al.* 2003). In Japan, 18 species of *Ulva* are currently recognized (Yoshida *et al.* 2005). The species level taxonomy of *Ulva* species is difficult because of their simple and remarkably plastic morphology (Blomster 2000). One result of these difficulties is that 95 *Ulva* and 133 *Enteromorpha* species have been described worldwide (Index Nominum Algarum 2007), and 125 names are flagged as currently used names by the AlgaeBASE database (Guiry 2007).

On highly eutrophic enclosed coasts, (e.g. Tokyo Bay, Mikawa Bay and the Seto Inland Sea including Osaka Bay in Japan), *Ulva* species grow abundantly to form 'green tides', and occasionally cause various environmental problems. These include mass stranding of the plants creating a nuisance due to the rotten smell, and deterioration of tidal flat communities (Ohno 1999). There are high risk of frequent long-distance introductions of non-indigenous *Ulva* species, because they are one of the commonest ship-fouling species (Blomster *et al.* 2000), and have been identified in ballast waters (Flagella *et al.* 2007; Kawai *et al.* unpublished data).

Along the coast of Gamagori City in Mikawa Bay, frequent mass stranding of *Ulva* species has become a significant environmental issue. However, the taxonomy of these *Ulva* species and their phenologies have not been

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clarified. In the present study, in order to identify the dominant species of *Ulva* causing mass strandings, we carried out seasonal collections of dominant species in seven localities in Mikawa Bay and identified the species using ITS2 rDNA (internal transcribed spacer between 5.8S and 26S rDNA) region sequences as a molecular marker.

MATERIALS AND METHODS

Specimens of *Ulva* were collected at the following localities and brought back to the laboratory: Takagi, Nizaki, Tahara, Takeshima, Ebisu, Isshiki, and Kowa, in Mikawa Bay, Aichi Prefecture, Japan (Fig. 1). If there were large populations composed of a single species, judging from the gross morphology, the most typical specimens were sampled. If there were no large populations, specimens were randomly collected. In the laboratory, two (or three) representative specimens in each collection were selected based on their gross morphology. If the collection from a locality was composed of only one species, judging from the morphology, two specimens were randomly selected from the collection. If the collection included more than one species, two or three specimens representing the most dominant two taxa were selected (Table 1). A part of the selected specimens (roughly 40 x 40 mm) was cut off and quickly dried in silica gel. The rest of the specimen was pressed to keep as voucher specimens. Silica gel-dried specimens as well as the voucher specimens analyzed in the present study are deposited in the herbarium of the Kobe University Research Center for Inland Seas.

Approximately 100 mg (wet weight) of algal tissue ground in liquid nitrogen was used for genomic DNA extractions, which were performed using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the ITS2 region was carried out using a PC-708-02 thermal cycler (Astec, Fukuoka, Japan) and a TaKaRa Ex Taq (Takara Shuzo, Shiga, Japan) reaction kit (total reaction volume of 30 μL was composed of 3.0 μL 10x Ex Taq Buffer, 2.4 μL dNTP mixture, 1.5 μl DMSO, 0.5 μL of each primer, 0.2 μL TaKaRa Ex Taq and 1.0 μL DNA solution). We used the pair of primers for amplification of the ITS2 region: Forward 5'-CTCTCAACAACGGATATCT-3'; Reverse 5'-TGATATGCTTAAGTTCAGC-3'. The profile of PCR conditions was as follows: initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, extension at 68°C for 60 s. PCR products were directly sequenced using a BigDye Terminator Cycle Sequencing Reaction Kit v.3.1 (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequences of 26 species (28 samples) of the Ulvaceae were downloaded from GenBank and included in the alignment: *Ulvaria fusca* Ruprecht (AB097637), *Umbraulva japonica* (Holmes) Bae et Lee (AB097638), *Um. amamiensis* (Tanaka) Bae et Lee (AB097640), *Um. olivascens* (Dangeard) Bae et Lee (AJ234322), *Ulva intestinalis* L. (AJ234299), *U. compressa* L. (AJ234302), *U. intestinaloides* (Koeman et Hoek) Hayden *et al.* (AJ234303), *U. arasakii* Chihara (AB097650), *U. lactuca* L. (AJ234311), *U. pertusa* Kjellman (AB097653), *U. flexuosa* Wulfen (AJ234306, AB097645, AF099719), *U. cylindracea* (Herbarium name by Blomster) (AJ234308), *U. prolifera* O.F. Müller (AF035354), *U. lobata* (Kützing) Harvey (AY260563), *U. tanneri* Hayden *et* Waaland (AY422519), *U. californica* Wille (AY260560), *U. stenophylla* Setchell et Gardner (AY260569), *U. linza* L. (AJ000203), *U. procera* (Ahlner) Hayden *et al.* (AY260558), *U. clathrata* (Roth) C. Agardh (AJ234307), *U. taeniata* (Setchell) Setchell et Gardner (AJ234320), *U. fasciata* Delile (AB097663), *U. ohnoi* Hiraoka *et* Shimada (AB116034), *U. scandinavica* Bliding (AJ234318), *U. armoricana* Dion *et al.* (AB097660), and *U. rigida* C. Agardh (AJ234319). The ITS2 sequences (296 bp) were aligned using the mFOLD program (Zuker

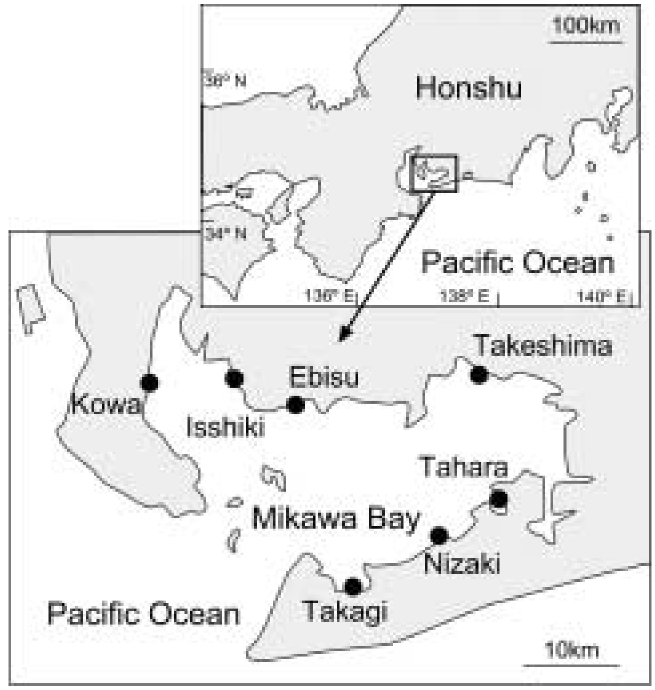


Fig. 1. Collection sites of *Ulva/Enteromorpha* specimens in Mikawa Bay, Aichi Pref., Japan.

1989) with regard to their secondary structure. The alignment is available from the first author upon request. *Ulvaria fusca*, *Umbraulva japonica*, *Um. amamiensis* and *Um. olivascens* were designated as outgroups in this analysis because these species represent a sister group to a monophyletic group composed of *Ulva* in a previous study (Shimada *et al.* 2003).

Phylogenetic trees were inferred by the maximum likelihood (ML) and maximum parsimony (MP) methods. The ML analysis was implemented with *PAUP* 4.0 b10* (Swofford 2002). Identical sequences were excluded from the alignment. We used the JC model (Jukes and Cantor 1969) in the ML analysis following Takahashi and Nei (2000). Bootstrap analysis (Felsenstein 1985) was based on 100 re-samplings of the dataset (TBR, full heuristic search option). Parsimony analysis was performed with *PAUP**. All sites were treated as unordered and equally weighted. The heuristic search option with random addition of sequences (2000 replicates) and tree-bisection-reconnection branch swapping algorithm (TBR) was used for tree searching. Bootstrap analysis was based on 2000 re-samplings of the dataset (10 random additions, TBR, full heuristic search).

RESULTS AND DISCUSSION

The phylogenetic tree obtained from the ML analysis (-*ln* L = 2051.62787) is shown in Fig. 2. Based on JC model settings, the heuristic search was performed with the TBR branch swapping option. In the MP analysis, 288 most-parsimonious trees (311 steps) were obtained (not shown). The topologies of the ML and MP trees were almost congruent, except for the clades whose bootstrap values were low.

Based on the phylogenetic tree as well as the comparisons with the published sequence data of related taxa, nine species are deduced as major components of the dominant populations in this area: 1) *Ulva pertusa*, 2) *U. ohnoi*, 3) *U. linza*, 4) *U. californica*, 5) *U. flexuosa*, 6) *U. fasciata*, 7) *U. compressa*, 8) *U. armoricana*, and 9) *U. scandinavica*. The most common species through the area and seasons were *U. pertusa* and *U. ohnoi*, followed by *U. linza*, *U. californica* and *U. flexuosa*.

There was a clear seasonal pattern in the occurrence of the two dominant species: *Ulva pertusa* dominated during spring to early summer, whereas *U. ohnoi* dominated during autumn to early winter (Table 1). The phenology of both species in this area was similar to previous reports of the species from other localities. Floating populations (thalli not attached on substrates) of *Ulva pertusa* in Kanagawa Prefecture, Pacific coast of central Japan, showed their maximum biomass during April to June (spring to early summer in the area), and the population almost disappeared before October (Ohno 1999). Similarly, Kim *et al.* (2004) reported the occurrence of a unimodal seasonal pattern for the population of *U. pertusa* at Jege, Namhaegun, on the south coast of Korea, in which biomass peaked in May and dropped significantly from June to September. On the other hand, the population of *U. ohnoi* (as *Ulva* sp.) in Kochi Prefecture, Pacific coast of Shikoku Island, attained its maximum biomass in mid-August, and then markedly decreased in November (Ohno 1988). Therefore, although sampling was not sufficient for statistical validation in the present study, we consider *U. pertusa* and *U. ohnoi* to be responsible for causing ‘green tides’ in Mikawa Bay area, and the dominant species may be different between spring and late summer to autumn.

Ulva scandinavica, originally described from Sweden (Bliding 1968), has not been reported previously from Japan, and it is considered to be a non-indigenous species in the region. The ITS2 rDNA sequence of this species was identical with that of the species collected from England, U.K. (AJ234318) and the Netherlands (AB097659). *U. scandinavica* was described as an asexual species having larger cells than that of the sexual species, *U. rigida* (Bliding 1968). Although the occurrence of *U. armoricana* was also indicated by the present analyses, the taxonomic status of this entity is problematic. *U. armoricana* is most closely related to *U. scandinavica* both in morphology and the ITS2 sequence data (Coat *et al.* 1998; Dion *et al.* 1998). Morphologically *U. armoricana* differs from *U. scandinavica* only in having longitudinal ribs containing closely packed bundles of rhizoids except in the youngest thalli. In ITS2 sequences, there are only three base substitutions and one gap between *U. scandinavica* and *U. armoricana*. In fact it was very difficult to distinguish morphologically the two specimens identified by ITS2 sequences from Mikawa Bay (MK-81, *U. scandinavica* and MK-78, *U. armoricana*). The original distributional ranges of *U. armoricana* and *U. scandinavica* are considered to be similar: the Adriatic (Battelli and Tan 1998), France (Dion *et al.* 1998), Sweden (Bliding 1968), and Spain (Diaz-Tapia and Bárbara 2005). Further study is therefore required in both Europe and Japan to evaluate the taxonomy of these species.

Ulva californica has not been reported previously from Japan, and is also considered to be a non-indigenous

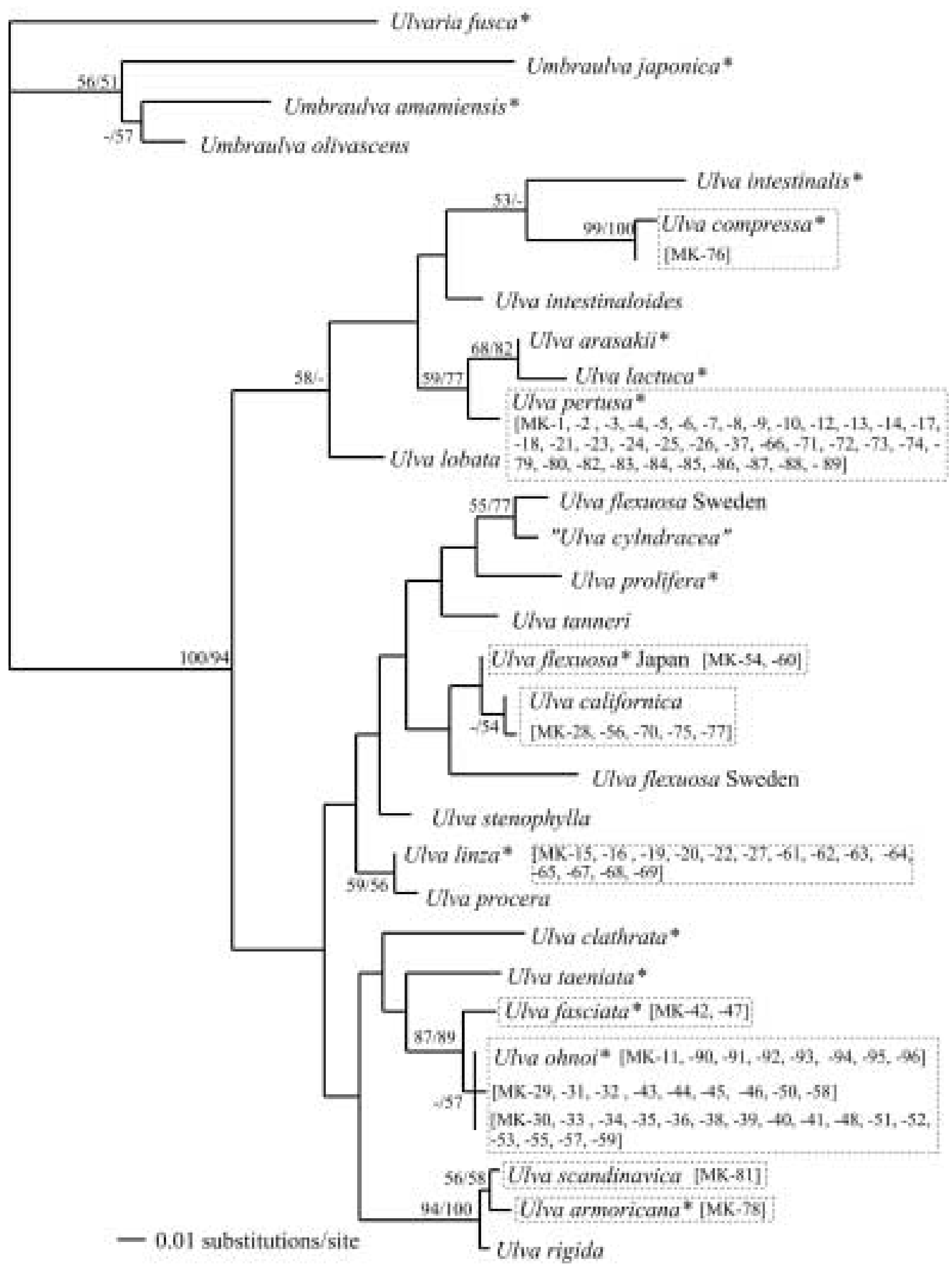


Fig. 2. Phylogenetic tree of *Ulva* inferred from ML analysis of ITS2 rDNA sequences (296 bp). *Ulvaria fusca*, *Umbraulva japonica*, *U. amamiensis* and *U. olivascens* were used as outgroups. Only values above 50% bootstrap support (ML / MP) are shown. "–" means < 50%. Taxa with * have been reported in Japan (Yoshida *et al.* 2005).

species. It was originally described from California, Pacific North America (Collins *et al.* 1899). Tanner (1986) investigated the morphological variations of *U. californica* and its related taxa, and suggested *U. angusta* Setchell & Gardner and *U. scagelii* Chihara to be synonyms of *U. californica*. Consistent with this notion, *U. californica* shows a considerable range of morphological diversities, from a reniform thallus up to 15-20 mm tall (Collins 1909; Abbott and Hollenberg 1976) to a reniform, linear or oblanceolate thallus attaining 300 mm (Tanner 1986). Japanese materials were linear, oblanceolate to oblong, 170-210 mm tall and 25-130 mm wide, and they had only

Table 1. Collection sites and sequence data used for molecular phylogenetic analysis, including their database accession numbers

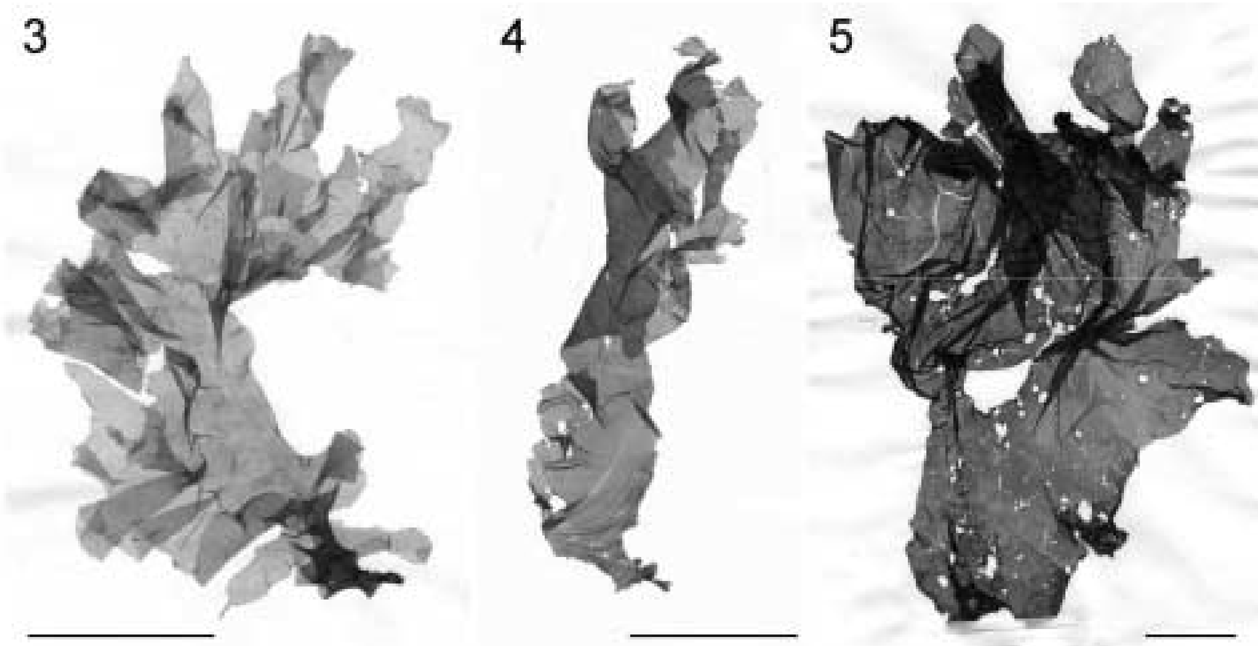
Specimen code	Species	Collection sites in Mikawa Bay, Japan	Collection date	Accession no. for ITS
MK-1	*Ulva pertusa*	Takagi	16 June 2004	AB280825
MK-2	*Ulva pertusa*	Takagi	16 June 2004	AB280826
MK-3	*Ulva pertusa*	Nizaki	16 June 2004	AB280827
MK-4	*Ulva pertusa*	Nizaki	16 June 2004	AB280828
MK-5	*Ulva pertusa*	Tahara	16 June 2004	AB280829
MK-6	*Ulva pertusa*	Tahara	16 June 2004	AB280830
MK-7	*Ulva pertusa*	Takeshima	16 June 2004	AB280831
MK-8	*Ulva pertusa*	Takeshima	16 June 2004	AB280832
MK-9	*Ulva pertusa*	Ebisu	16 June 2004	AB280833
MK-10	*Ulva pertusa*	Ebisu	16 June 2004	AB280834
MK-11	*Ulva ohnoi*	Isshiki	16 June 2004	AB280884
MK-12	*Ulva pertusa*	Isshiki	16 June 2004	AB280835
MK-13	*Ulva pertusa*	Kowa	16 June 2004	AB280836
MK-14	*Ulva pertusa*	Kowa	16 June 2004	AB280837
MK-15	*Ulva linza*	Takagi	12 Mar. 2004	AB280868
MK-16	*Ulva linza*	Takagi	12 Mar. 2004	AB280869
MK-17	*Ulva pertusa*	Nizaki	12 Mar. 2004	AB280838
MK-18	*Ulva pertusa*	Nizaki	12 Mar. 2004	AB280839
MK-19	*Ulva linza*	Tahara	12 Mar. 2004	AB280870
MK-20	*Ulva linza*	Tahara	12 Mar. 2004	AB280871
MK-21	*Ulva pertusa*	Takeshima	12 Mar. 2004	AB280840
MK-22	*Ulva linza*	Takeshima	12 Mar. 2004	AB280872
MK-23	*Ulva pertusa*	Ebisu	12 Mar. 2004	AB280841
MK-24	*Ulva pertusa*	Ebisu	12 Mar. 2004	AB280842
MK-25	*Ulva pertusa*	Isshiki	12 Mar. 2004	AB280843
MK-26	*Ulva pertusa*	Isshiki	12 Mar. 2004	AB280844
MK-27	*Ulva linza*	Kowa	12 Mar. 2004	AB280873
MK-28	*Ulva californica*	Kowa	12 Mar. 2004	AB280863
MK-29	*Ulva ohnoi*	Takagi	12 Nov. 2003	AB280892
MK-30	*Ulva ohnoi*	Takagi	12 Nov. 2003	AB280902
MK-31	*Ulva ohnoi*	Takagi	12 Nov. 2003	AB280893
MK-32	*Ulva ohnoi*	Nizaki	12 Nov. 2003	AB280894
MK-33	*Ulva ohnoi*	Nizaki	12 Nov. 2003	AB280895
MK-34	*Ulva ohnoi*	Nizaki	12 Nov. 2003	AB280903
MK-35	*Ulva ohnoi*	Tahara	12 Nov. 2003	AB280904
MK-36	*Ulva ohnoi*	Tahara	12 Nov. 2003	AB280905
MK-37	*Ulva pertusa*	Tahara	12 Nov. 2003	AB280845
MK-38	*Ulva ohnoi*	Takeshima	12 Nov. 2003	AB280906
MK-39	*Ulva ohnoi*	Takeshima	12 Nov. 2003	AB280907
MK-40	*Ulva ohnoi*	Takeshima	12 Nov. 2003	AB280908
MK-41	*Ulva ohnoi*	Ebisu	12 Nov. 2003	AB280909
MK-42	*Ulva fasciata*	Ebisu	12 Nov. 2003	AB280882
MK-43	*Ulva ohnoi*	Ebisu	12 Nov. 2003	AB280896
MK-44	*Ulva ohnoi*	Isshiki	12 Nov. 2003	AB280897
MK-45	*Ulva ohnoi*	Isshiki	12 Nov. 2003	AB280898
MK-46	*Ulva ohnoi*	Isshiki	12 Nov. 2003	AB280899
MK-47	*Ulva fasciata*	Kowa	12 Nov. 2003	AB280883
MK-48	*Ulva ohnoi*	Kowa	12 Nov. 2003	AB280910
MK-50	*Ulva ohnoi*	Takagi	12 Nov. 2004	AB280900
MK-51	*Ulva ohnoi*	Takagi	12 Nov. 2004	AB280911
MK-52	*Ulva ohnoi*	Takeshima	12 Nov. 2004	AB280912
MK-53	*Ulva ohnoi*	Takeshima	12 Nov. 2004	AB280913
MK-54	*Ulva flexuosa*	Takeshima	12 Nov. 2004	AB280861

Table 1. (continued)

Specimen code	Species	Collection sites in Mikawa Bay, Japan	Collection date	Accession no. for ITS
MK-55	*Ulva ohnoi*	Ebisu	12 Nov. 2004	AB280914
MK-56	*Ulva californica*	Ebisu	12 Nov. 2004	AB280864
MK-57	*Ulva ohnoi*	Isshiki	12 Nov. 2004	AB280915
MK-58	*Ulva ohnoi*	Isshiki	12 Nov. 2004	AB280901
MK-59	*Ulva ohnoi*	Kowa	12 Nov. 2004	AB280916
MK-60	*Ulva flexuosa*	Kowa	12 Nov. 2004	AB280862
MK-61	*Ulva linza*	Takagi	13 April 2005	AB280874
MK-62	*Ulva linza*	Takagi	13 April 2005	AB280875
MK-63	*Ulva linza*	Nizaki	13 April 2005	AB280876
MK-64	*Ulva linza*	Nizaki	13 April 2005	AB280877
MK-65	*Ulva linza*	Tahara	13 April 2005	AB280878
MK-66	*Ulva pertusa*	Takeshima	13 April 2005	AB280846
MK-67	*Ulva linza*	Takeshima	13 April 2005	AB280879
MK-68	*Ulva linza*	Ebisu	13 April 2005	AB280880
MK-69	*Ulva linza*	Isshiki	13 April 2005	AB280881
MK-70	*Ulva californica*	Isshiki	13 April 2005	AB280865
MK-71	*Ulva pertusa*	Kowa	13 April 2005	AB280847
MK-72	*Ulva pertusa*	Kowa	13 April 2005	AB280848
MK-73	*Ulva pertusa*	Takagi	8 June 2005	AB280849
MK-74	*Ulva pertusa*	Takagi	8 June 2005	AB280850
MK-75	*Ulva californica*	Nizaki	8 June 2005	AB280866
MK-76	*Ulva compressa*	Nizaki	8 June 2005	AB280824
MK-77	*Ulva californica*	Tahara	8 June 2005	AB280867
MK-78	*Ulva armoroicana*	Tahara	8 June 2005	AB280918
MK-79	*Ulva pertusa*	Tahara	8 June 2005	AB280851
MK-80	*Ulva pertusa*	Takeshima	8 June 2005	AB280852
MK-81	*Ulva scandinavica*	Takeshima	8 June 2005	AB280917
MK-82	*Ulva pertusa*	Ebisu	8 June 2005	AB280853
MK-83	*Ulva pertusa*	Isshiki	8 June 2005	AB280854
MK-84	*Ulva pertusa*	Isshiki	8 June 2005	AB280855
MK-85	*Ulva pertusa*	Kowa	8 June 2005	AB280856
MK-86	*Ulva pertusa*	Takagi	20 Sept. 2005	AB280857
MK-87	*Ulva pertusa*	Takagi	20 Sept. 2005	AB280858
MK-88	*Ulva pertusa*	Nizaki	20 Sept. 2005	AB280859
MK-89	*Ulva pertusa*	Nizaki	20 Sept. 2005	AB280860
MK-90	*Ulva ohnoi*	Tahara	20 Sept. 2005	AB280885
MK-91	*Ulva ohnoi*	Tahara	20 Sept. 2005	AB280886
MK-92	*Ulva ohnoi*	Takeshima	20 Sept. 2005	AB280887
MK-93	*Ulva ohnoi*	Takeshima	20 Sept. 2005	AB280888
MK-94	*Ulva ohnoi*	Isshiki	20 Sept. 2005	AB280889
MK-95	*Ulva ohnoi*	Kowa	20 Sept. 2005	AB280890
MK-96	*Ulva ohnoi*	Kowa	20 Sept. 2005	AB280891

one base substitution when compared to *U. californica* collected from La Jolla, California (AY260560). Until the late 1990s, *Ulva californica* was reported only from the Pacific coast of North America: Alaska (Scagel *et al.* 1989), British Columbia (Scagel *et al.* 1989), Washington (Scagel *et al.* 1989), Oregon (Hansen 1997) and California (Scagel *et al.* 1989). However, recently John *et al.* (2004) reported the species from western Africa using morphological criteria, and Hayden and Waaland (2004) from Europe based on molecular data. Therefore, considering our new report of its occurrence in the western Pacific, we conclude that *U. californica* is expanding its geographical distribution by trans-ocean introductions.

The occurrence of green tides associated with the eutrophication of coasts has become a common environmental problem in extensive areas of temperate coasts worldwide (Sfriso 1994; Fletcher 1996; Martins *et al.* 2001; Kim *et al.* 2004). Many studies have been done on the



Figs 3-5. Probable non-indigenous species of *Ulva* newly found in Mikawa Bay, Aichi Pref. Japan. Figs 3, 4. *U. californica* (3, MK-28; 4, MK-70). 5. *U. scandinavica* (MK-81). Scale bars, 50 mm.

dynamics of the dominant populations, based on identifications of *Ulva/Enteromopha* species using gross thallus morphology, but these identifications may not be very reliable because of the remarkable morphological plasticity as well as the occurrence of cryptic invasive species. We have found that the application of molecular techniques to the identification of these taxa is very effective, and can become a powerful tool for ecological studies of green tide species.

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