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1 **The presence of free D-aspartate in marine macroalgae is restricted to the**  
2 **Sargassaceae family**

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14 *Abbreviations:* Asp, aspartate; OPA, *o*-phthalaldehyde ; NAC, *N*-acetyl-L-cysteine; HPLC,  
15 high-performance liquid chromatography.

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17       **The presence of D-aspartate (D-Asp), a biologically rare amino acid, was evaluated in**  
18       **38 species of marine macroalgae (seaweeds). Despite the ubiquitous presence of free**  
19       **L-Asp, free D-Asp was detected in only 5 species belonging to the Sargassaceae family of**  
20       **class Phaeophyceae (brown algae) but not in any species of the phyla Chlorophyta**  
21       **(green algae) and Rhodophyta (red algae). All other members of Phaeophyceae,**  
22       **including 3 species classified into the section *Teretia* of Sargassaceae did not contain**  
23       **D-Asp. These results indicate that the presence of free D-Asp in marine macroalgae is**  
24       **restricted only to the Sargassaceae family, excluding the species in the section *Teretia*.**

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26       **Key words:** D-amino acid; D-aspartate; macroalga (seaweed); Sargassaceae; *Sargassum*

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45 In the past four decades, various D-amino acids have been found in animals and plants.<sup>1, 2)</sup>  
46 Within aquatic animals, D-aspartate (D-Asp) and D-alanine have been identified in  
47 cephalopods,<sup>3, 4)</sup> crustaceans,<sup>5)</sup> and mollusks.<sup>6)</sup> Recently, it was reported that D-Asp and  
48 aspartate racemase [EC 5.1.1.13] are involved in anaerobic energy metabolism in the bivalve  
49 mollusk *Anadara broughtonii* (formerly *Scapharca broughtonii*).<sup>7, 8)</sup> Although little attention  
50 has been paid to the natural occurrence and physiological role of D-Asp in macroalgae, study  
51 on the presence of D-amino acids in macroalgae commenced following the observation of  
52 high levels of free D-Asp in the marine macroalga *Sargassum fusiforme*.<sup>9)</sup> *S. fusiforme*  
53 germinates in late summer and grows from autumn to spring in the sea near Iwate Prefecture,  
54 northern part of the largest island of Japan, showing rapid growth particularly in early spring.  
55 The D-Asp content also increases during this early growth period. These findings suggest that  
56 D-Asp plays an important role in the early growth stages of *S. fusiforme*,<sup>9)</sup> but the exact  
57 biological role of D-Asp in this alga is yet to be clarified. To gain deeper insight into the  
58 biological role of D-Asp in *S. fusiforme*, we prepared a polyclonal antibody against D-Asp and  
59 confirmed that D-Asp is present in the intracellular structure of *S. fusiforme*.<sup>10)</sup> These results  
60 exclude the possibility that D-Asp is derived from attached or symbiotic organisms, such as  
61 marine bacteria.

62 Nagahisa et al.<sup>11)</sup> reported the presence of free D-Asp in some marine macroalgae in Japan.  
63 They found that D-Asp was present at high levels, with concentrations proportional to those of  
64 L-Asp, in *Costaria costata*, *S. fusiforme*, and *S. yezoense* that belong to Phaeophyceae (brown  
65 algae). However, D-Asp was not found in many other species of macroalgae, and even if it  
66 was detected, it was present only in trace amounts. *Sargassum fulvellum*, *S. fusiforme*, and *S.*  
67 *yezoense* thrive in the same intertidal zones in the sea. Although *S. fusiforme* and *S. yezoense*  
68 are reported to have high levels of D-Asp, its content in *S. fulvellum* is very low. Thus, the  
69 question arises as to why the amount of D-Asp largely differs in marine macroalgae belonging  
70 to the same genus, *Sargassum*. In the present study, the distribution of D-Asp in many marine  
71 macroalgae, particularly those belonging to the family Sargassaceae, was examined to clarify  
72 the seaweeds that contain D-Asp.

73 This is the first report presenting a taxonomical view of the presence of free D-Asp by  
74 relating it to the classification of marine macroalgae.

75

## 76 **Materials and Methods**

77 *Materials.* Marine macroalgae were collected from the coast of Iwate and Kanagawa  
78 Prefecture, Japan, in 2013 and 2014 as shown in Table 1. Algal extracts were prepared from 5  
79 species of Chlorophyta (green algae), 15 species of Rhodophyta (red algae), and 18 species of  
80 Phaeophyceae. All chemicals, including D- and L-Asp, *o*-phthalaldehyde (OPA), and  
81 *N*-acetyl-L-cysteine (NAC), were purchased from Wako Pure Chemicals (Osaka, Japan), and  
82 were of analytical-grade purity. Milli-Q water for the experiments was obtained by purifying  
83 distilled water using a Simplicity UV (Merck Millipore, Billerica, MA, USA) system.

84

85 *Preparation of algal extract.* A part of the thalli of each sampled alga (about 10 g wet  
86 weight) was rinsed with distilled water to remove attached foreign materials, such as small  
87 organisms. The algae were dried by wrapping the thalli tightly with paper towel and a portion  
88 of the thallus was homogenized with 10 volumes of 80% (v/v) ethanol using a polytron  
89 homogenizer (PT-K, Kinematica, Lucerne, Switzerland) followed by centrifugation at 50,000  
90  $\times g$  at 4 °C for 15 min. The resultant precipitate was extracted with the same volume of  
91 ethanol solution for maximal extraction. The supernatants (ethanolic extracts) were combined  
92 and evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in a  
93 small amount of Milli-Q water and then rinsed with a sufficient amount of diethyl ether to  
94 remove pigments and fatty materials. The aqueous solution layer was transferred to an  
95 eggplant-shaped flask and evaporated to dryness. The obtained residue was dissolved in 50  
96 mL of Milli-Q water and stored at -20 °C until further analysis with high-performance liquid  
97 chromatography (HPLC).

98

99 *Derivatization of amino acids with OPA/NAC.* Each sample extract was passed through a  
100 0.20  $\mu\text{m}$  filter. For derivatization of amino acids, 10  $\mu\text{L}$  of the sample solution was mixed with

101 70  $\mu$ L of a saturated sodium borate solution and 20  $\mu$ L of a mixture of 10 mg OPA and 10 mg  
102 NAC in 1 mL of methanol. After allowing the mixture to react for 1 min at room temperature,  
103 the reaction mixture was injected directly into the HPLC system.

104

105 *Determination of D- and L-aspartate.* Chromatography was performed as described by  
106 Nimura and Kinoshita<sup>12)</sup> with some alterations.<sup>13)</sup> Chromatographic analysis was performed  
107 with a JASCO (Tokyo, Japan) HPLC system consisting of a PU-2089 quaternary gradient  
108 pump with a degasser, a CO-2065 column oven, an AS-2057 autosampler with a cooling  
109 system, an FP-2020 fluorescence detector, and a ChromNAV data processor. The analytical  
110 column was a reversed-phase ODS-80T<sub>S</sub> (4.6  $\times$  250 mm) (Tosoh, Tokyo, Japan) with a guard  
111 column (3.2  $\times$  15 mm) packed with the same resin. Elution was performed with a mixture of  
112 solvent A (50 mM sodium acetate buffer at pH 5.6) and solvent B (methanol:solvent A =  
113 80:20) at 40 °C; the flow rate was set at 1.0 mL min<sup>-1</sup>. For fluorometric detection of eluted  
114 OPA/NAC derivatives, the excitation and emission wavelengths were set to 350 and 450 nm,  
115 respectively. The elution gradient was set as follows: 0–20 min, 0–20% solvent B in solvent  
116 A; 20–35 min, 20% solvent B in solvent A.

117

## 118 **Results**

### 119 *Distribution of free D- and L-aspartate in marine macroalgae*

120 Typical HPLC chromatograms of the converted D- and L-enantiomers of standard amino  
121 acids, an extract of *S. fusiforme* with the presence of D-Asp, and an extract of *S. thunbergii*  
122 with no D-Asp are shown in Fig. 1. Retention times of both D- and L-Asp derivatives fully  
123 corresponded with each peak obtained for extracts of *S. fusiforme* (Fig. 1; peak number 1 and  
124 2).

125 Although L-Asp was detected in all species examined, D-Asp was not detected in species  
126 belonging to Chlorophyta, Rhodophyta, and most species of Phaeophyceae. However, D-Asp  
127 was present in some species of Phaeophyceae belonging to the genus *Sargassum* and  
128 *Stephanocystis* of Sargassaceae (Table 1).

129 Figure 2 shows a concentration of D- and L-Asp in Sargassaceae. No traces of D-Asp were  
130 detected in *Sargassum confusum*, *S. hemiphyllum*, and *S. thunbergii*. However, *S. fusiforme*, *S.*  
131 *horneri*, *S. siliquastrum*, and *S. patens* contained D-Asp in significant concentrations (0.41,  
132 0.41, 5.13, and 7.70  $\mu\text{mol} \cdot \text{g}^{-1}$  wet weight, respectively). *Stephanocystis hakodatensis* also  
133 contained 0.83  $\mu\text{mol} \cdot \text{g}^{-1}$  wet weight of D-Asp. In addition, the percent content of  $[\text{D}/(\text{D} + \text{L})]$   
134  $\times 100$  (%) was generally high in *Sargassum fusiforme*, *S. siliquastrum*, and *S. patens* (45.6,  
135 45.8, and 51.4 %, respectively), whereas that in *S. horneri* and *S. hakodatensis* was 25.2 and  
136 28.4%, respectively.

137

### 138 **Discussion**

139 The presence of free D-Asp in marine macroalgae was confirmed using pre-column HPLC  
140 amino acid analysis. D-Asp was detected only in 5 distinguishable species out of the total of  
141 38 species examined (13% of the species). The macroalgae containing D-Asp all belonged to  
142 the Sargassaceae family, indicating that free D-Asp is specifically presents in the family  
143 Sargassaceae of the class Phaeophyceae, although *S. confusum*, *S. hemiphyllum*, and *S.*  
144 *thunbergii*, which also belong to Sargassaceae, contained no D-Asp (Table 1).

145 Generally, Sargassaceae exist at similar locations i.e., on rocks in the lower intertidal zone  
146 to the infralittoral zone; Their growth season is also recognized as the duration from  
147 autumn to spring in Japan. Therefore, we presumed that the presence of D-Asp is related to  
148 algae's taxonomic ranks rather than their habitat. In biological classification, taxonomic ranks  
149 comprise species, genus, family, order, class, phylum (division), and kingdom. However,  
150 taxonomic ranks in botany include the secondary ranks, such as "section" between genus and  
151 species. Thus, the knowledge about detailed classification of Phaeophyceae should be  
152 considered to gain deeper insight into the diverse distribution of D-Asp in Sargassaceae.  
153 According to detailed taxonomic ranks, it was found that D-Asp was detected in the entire  
154 family Sargassaceae, except the section *Teretia* (Fig. 3). Therefore, the presence of free D-Asp  
155 in macroalgae is taxonomically restricted to certain sections in Sargassaceae.

156 The taxonomical position of *S. fusiforme* has been discussed for a long time.<sup>14)</sup> At present, *S.*

157 *fusiforme* belongs to the section *Hizikia* in the genus *Sargassum*.<sup>14)</sup> The genus of *Sargassum* in  
158 to subgenus *Bactrophycus* is mainly based on the morphology of the basal part supplemented  
159 by the morphology of receptacles. In the present study, we reported high accumulation of  
160 D-Asp in *S. fusiforme* from the section *Hizikia*, but its absence in species belonging to the  
161 section *Teretia*. Although the section *Hizikia* is positioned near the section *Teretia*  
162 morphologically, the presence of D-Asp is unique.

163 Nagahisa et al.<sup>11)</sup> reported a very low content of D-Asp in *S. fulvellum* from the section  
164 *Teretia* (0.0012  $\mu\text{mol} \cdot \text{g}^{-1}$  wet weight) compared to that in *S. yezoense* (0.0485  $\mu\text{mol} \cdot \text{g}^{-1}$  wet  
165 weight) from the section *Halochloa*, whereas *C. costata* contained 0.066  $\mu\text{mol} \cdot \text{g}^{-1}$  wet weight  
166 of D-Asp; these results were not corroborated in the present study (Table 1). This difference in  
167 D-Asp content may have resulted from different protocols implemented during the preparation  
168 of the samples before analyses. Namely, in the present study, thalli were carefully cleaned  
169 from any attached organisms and the identification of macroalgae species was verified.

170 It is known that the content of amino acids in marine macroalgae changes with seasons  
171 and/or locations. Thus, D-Asp is present in *S. fusiforme* throughout the year although its level  
172 vary with time.<sup>9)</sup> Similarly, the presence of D-Asp in *S. fusiforme* was confirmed in thalli of  
173 samples collected at both Iwate and Kanagawa (data not shown), suggesting that the presence  
174 of D-Asp in *S. fusiforme* is independent of season and/or location. It is speculated that the  
175 D-Asp is present in other marine macroalgae of the family Sargassaceae, except the section  
176 *Teretia*, regardless of the season and/or location, although more detailed studies are necessary.

177 The available information on the biological role and metabolic pathway of D-Asp in marine  
178 organisms is limited. It was reported that D-Asp and aspartate racemase are involved in  
179 anaerobic energy metabolism in the bivalve mollusk, *A. broughtonii*.<sup>7, 8)</sup> In contrast, the  
180 biological role and metabolic pathway of D-Asp in marine macroalgae remain unclear.

181 Funakoshi et al.<sup>15)</sup> reported the presence of D-amino acid transaminase [EC 2.6.1.21], which  
182 catalyzes transamination between D-Asp and other D-amino acids, in the terrestrial plant,  
183 *Arabidopsis thaliana*. Recently, Ito et al.<sup>16)</sup> reported that mammalian enzyme serine racemase,  
184 the primary enzyme responsible for brain D-serine production also catalyzes Asp racemization.



185 Unfortunately, aspartate racemase, serine racemase, and/or D-amino acid transaminase activity  
186 remain to be detected in any macroalgae through biochemical analyses.<sup>2)</sup> Moreover, there is  
187 no genome information about species belonging to the Sargassaceae. Thus, it is necessary to  
188 identify and characterize enzymes involved in the metabolism of D-Asp in Sargassaceae  
189 through molecular and biochemical testing, which provides novel insights into  
190 lineage-specific presence, biosynthetic pathways, and physiological functions of D-Asp in  
191 marine brown macroalgae.

192

### 193 **Author Contribution**

194 T. Yokoyama performed the experimental design and experiments. Experimental  
195 interpretation of data was conducted by all authors. T. Yokoyama wrote the paper, and the  
196 other authors commented on the manuscript.

197

### 198 **Disclosure statement**

199 No potential conflict of interest was reported by the authors.

200

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204

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207 Kitasato University for their assistance in this study.

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268 **Figure Legends**

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270 **Fig. 1.** Typical HPLC chromatograms of (A) the OPA/NAC derivatives of D- and  
271 L-enantiomers of standard amino acids, (B) an extract of the macroalgae *Sargassum fusiforme*  
272 and (C) *Sargassum thunbergii*.

273 A 10 µL aliquot of derivatized sample solution was injected into HPLC. Each peak of (A)  
274 standard amino acids represents 10 pmol.

275 1. D-Asp, 2. L-Asp, 3. L-Asn, 4. D-Asn, 5. D-Ser, 6. L-Ser, 7. L-Glu, 8. D-Glu, 9. L-Gln, 10.  
276 D-Gln, 11. D, L-His, 12. D-Thr, 13. Gly, 14. L-Thr.

277

278 **Fig. 2.** Concentrations of aspartate in macroalgae belonging to the family Sargassaceae.

279 N.D.: not detected.

280

281 **Fig. 3.** Convincing interpretation for the presence of free D-aspartate in Sargassaceae.

282 Infrageneric classification is based on the algae database. The data on *Sargassum fulvellum*  
283 (quite low) and *S. yezoense* (\*) are based on the report by Nagahisa et al.<sup>11)</sup>

284

285 **Table 1.** Distribution of free D- and L-aspartate in marine macroalgae.

286 N.D.: not detected.

287

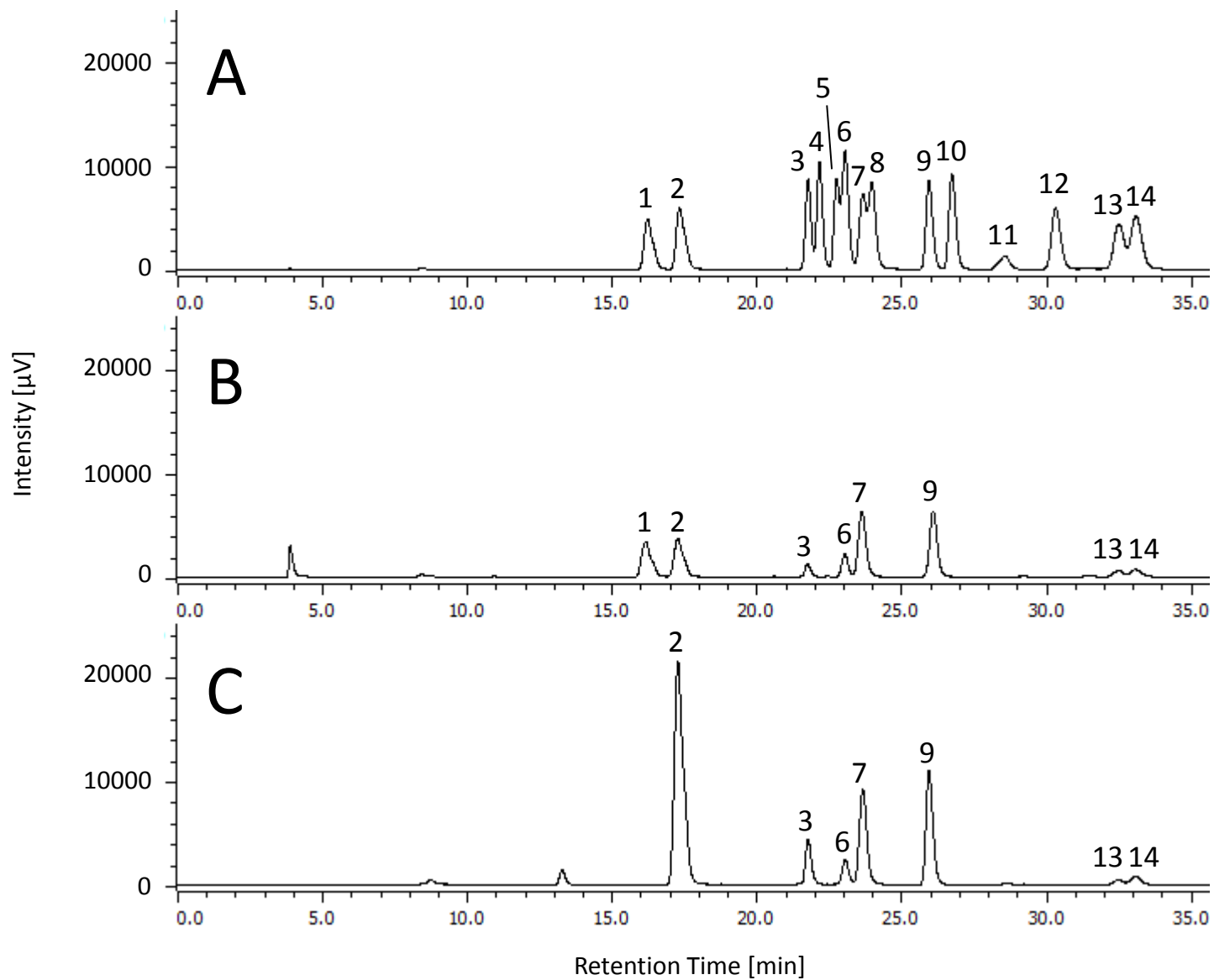


Fig.1. Yokoyama *et al.*

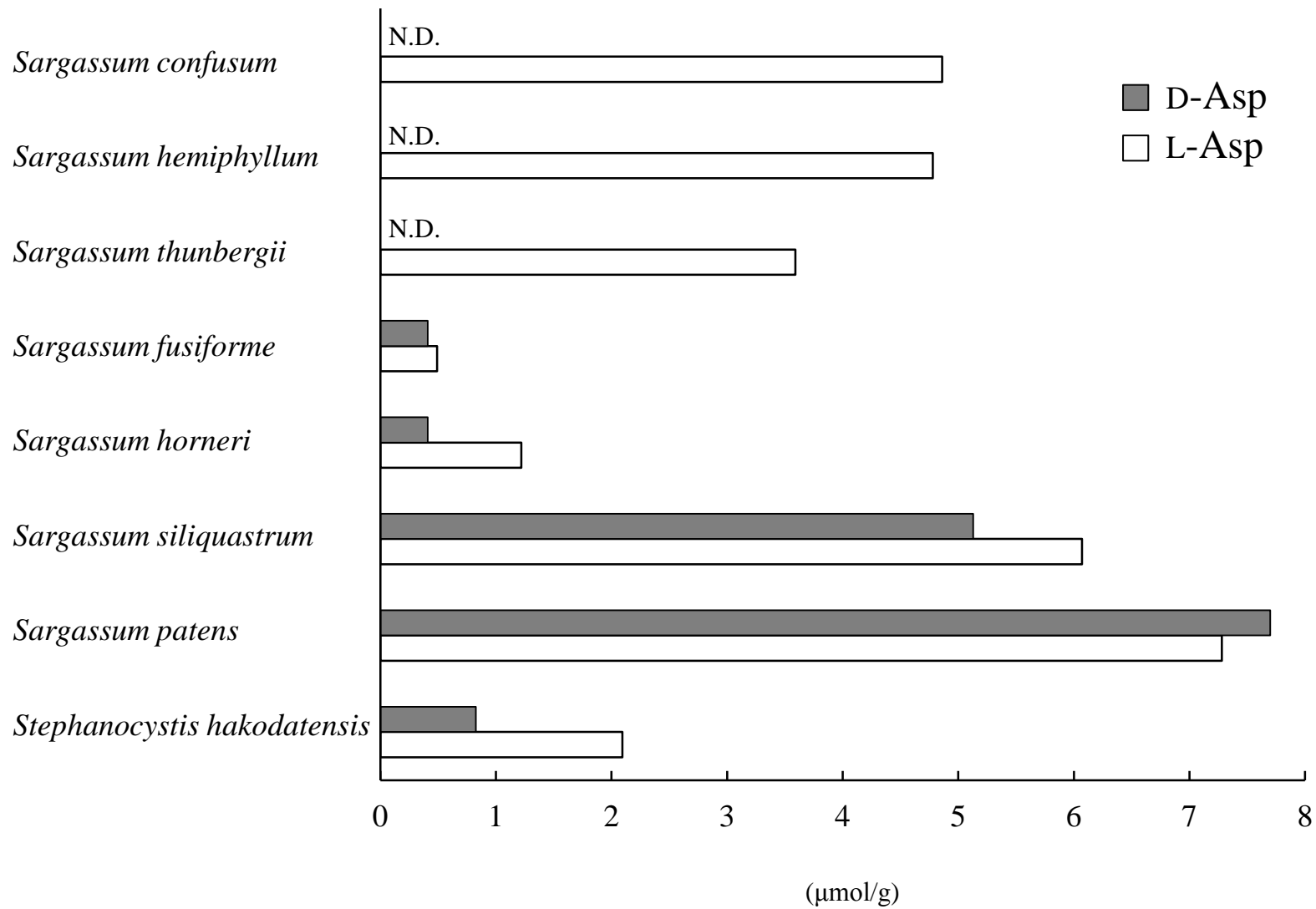


Fig.2. Yokoyama *et al.*

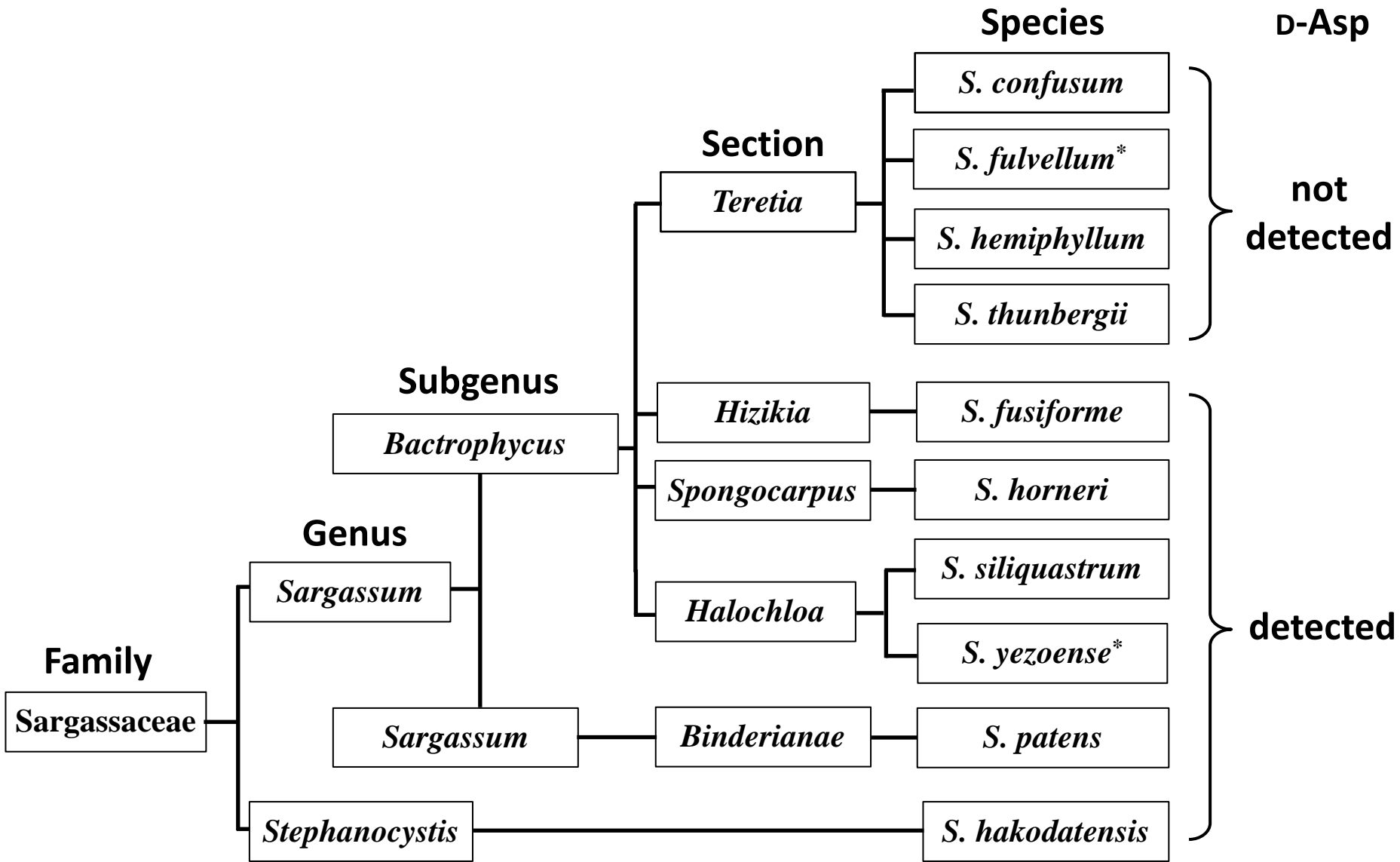


Fig.3. Yokoyama *et al.*

Species	D-Asp ( $\mu\text{mol/g}$ )	L-Asp ( $\mu\text{mol/g}$ )	D/(D+L) $\times$ 100 (%)	Sampling date	Sampling area
<b>CHLOROPHYTA</b>					
<i>Cladophora opaca</i>	N.D.	5.69	—	20130610	Iwate
<i>Codium fragile</i>	N.D.	2.40	—	20131217	Iwate
<i>Ulva australis</i>	N.D.	0.18	—	20130610	Iwate
<i>Ulva intestinalis</i>	N.D.	0.28	—	20131104	Kanagawa
<i>Ulva linza</i>	N.D.	1.81	—	20131202	Kanagawa
<b>RHODOPHYTA</b>					
<i>Ahnfeltiopsis paradoxa</i>	N.D.	1.13	—	20130610	Iwate
<i>Chondria crassicaulis</i>	N.D.	2.19	—	20130610	Iwate
<i>Chondrophyucus undulatus</i>	N.D.	17.11	—	20131202	Kanagawa
<i>Chondrus ocellatus</i>	N.D.	1.00	—	20130610	Iwate
<i>Chondrus yendoii</i>	N.D.	0.26	—	20130610	Iwate
<i>Gloiopeltis furcata</i>	N.D.	0.25	—	20130610	Iwate
<i>Grateloupia lanceolata</i>	N.D.	0.89	—	20140614	Iwate
<i>Grateloupia sparsa</i>	N.D.	0.83	—	20131217	Iwate
<i>Hypnea asiatica</i>	N.D.	22.35	—	20131217	Iwate
<i>Hypnea japonica</i>	N.D.	0.25	—	20131202	Kanagawa
<i>Lomentaria hakodatensis</i>	N.D.	0.56	—	20130610	Iwate
<i>Neodilsea yendoana</i>	N.D.	0.69	—	20140614	Iwate
<i>Polysiphonia senticulosa</i>	N.D.	1.83	—	20130610	Iwate
<i>Pyropia yezoensis</i>	N.D.	1.39	—	20140614	Iwate
<i>Schizymenia dubyi</i>	N.D.	1.67	—	20131217	Iwate
<b>PHAEOPHYCEAE</b>					
<i>Analipus japonicus</i>	N.D.	1.73	—	20130610	Iwate
<i>Alaria crassifolia</i>	N.D.	1.48	—	20140614	Iwate
<i>Costaria costata</i>	N.D.	0.89	—	20140614	Iwate
<i>Eisenia bicyclis</i>	N.D.	1.16	—	20131104	Kanagawa
<i>Ishige okamurae</i>	N.D.	1.79	—	20131104	Kanagawa
<i>Petalonia binghamiae</i>	N.D.	4.28	—	20131202	Kanagawa
<i>Saccharina japonica</i>	N.D.	69.70	—	20131111	Iwate
<i>Saccharina japonica var. religiosa</i>	N.D.	1.08	—	20140614	Iwate
<i>Sargassum confusum</i>	N.D.	4.86	—	20130610	Iwate
<i>Sargassum fusiforme</i>	0.41	0.49	45.6	20130610	Iwate
<i>Sargassum hemiphyllum</i>	N.D.	4.78	—	20131104	Kanagawa
<i>Sargassum horneri</i>	0.41	1.22	25.2	20130610	Iwate
<i>Sargassum patens</i>	7.70	7.28	51.4	20131104	Kanagawa
<i>Sargassum siliquastrum</i>	5.13	6.07	45.8	20131219	Iwate
<i>Sargassum thunbergii</i>	N.D.	3.59	—	20140614	Iwate
<i>Scytosiphon lomentaria</i>	N.D.	0.55	—	20130610	Iwate
<i>Stephanocystis hakodatensis</i>	0.83	2.09	28.4	20140614	Iwate
<i>Undaria pinnatifida</i>	N.D.	2.25	—	20140614	Iwate

Table 1. Yokoyama *et al.*