

# Assemblages of endophytic fungi on *Salicornia europaea* disjunctively distributed in Japan: towards clarification of the ubiquity of fungal endophytes on halophytes and their ecological roles

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**We studied assemblages of endophytic fungi on the halophilic plant *Salicornia europaea* in Japan. This plant is disjunctively distributed in the eastern Hokkaido and the Seto Inland Sea (Setouchi) regions. Various dematiaceous fungi that form dark spores and/or mycelia (and the related ascigerous stages) were common in *S. europaea*. Among them, *Pleospora* sp. (anamorph: *Stemphylium*) and *Alternaria alternata* were the major endophytes found in the plant, followed by *Alternaria phragmospora* and *Cladosporium cladosporioides*. Fungal assemblages from Hokkaido were similar among years, as were assemblages collected in completely segregated regions in Japan, Hokkaido and Setouchi. While such dematiaceous fungi are generally known to be epiphytes living on the surface of terrestrial vascular plants, they may ubiquitously inhabit halophytes such as *Salicornia*.**

**Keywords:** Dematiaceous fungi, endophytic fungi, halophytes, *Salicornia europaea*.

## Introduction

VARIOUS endophytic fungi have been found in living tissues of terrestrial vascular plants<sup>1–3</sup>. Some dematiaceous fungi that produce dark mycelia and spores have also been isolated during studies of fungal endophytes<sup>4–6</sup>. For example, dematiaceous fungi such as *Alternaria* and *Cladosporium* are known to be facultative endophytes of terrestrial plants<sup>7</sup>. In contrast, several reports of isolation of dematiaceous fungi (including those in the teleomorphic stages) from chenopodiaceous and other halophytes growing in salt marshes, mangrove forests and desert areas have been published<sup>8–17</sup>. Further studies on fungal communities on halophytes are required to examine their biodiversity and functional roles.

*Salicornia europaea* L. (Chenopodiaceae) is a halophytic succulent plant widely distributed in salt marshes and seashores in temperate regions of the Northern Hemisphere<sup>18,19</sup>. In Japan, *S. europaea* is discontinuously distributed in eastern Hokkaido and the Seto Inland Sea (Setouchi) regions. The plant is found in salt marshes along Lake Noto and Lake Saroma (brackish lakes), and other salt marshes located in eastern Hokkaido and the sites of salt farms along the coast of Setouchi (Okayama, Kagawa and Ehime Prefectures). Populations of *S. europaea* in the Setouchi region are thought to be artificially introduced from Hokkaido by Kitamae Ships (trader ships between Hokkaido and Setouchi regions in the 19th century)<sup>20</sup>. Recently, molecular analyses have revealed that the two populations of *S. europaea* have different origins<sup>20,21</sup>. Hoshino *et al.*<sup>20</sup> found that the population in Setouchi was identical to that in Korea, and they did not consider that *S. europaea* in the Setouchi region was derived from Hokkaido.

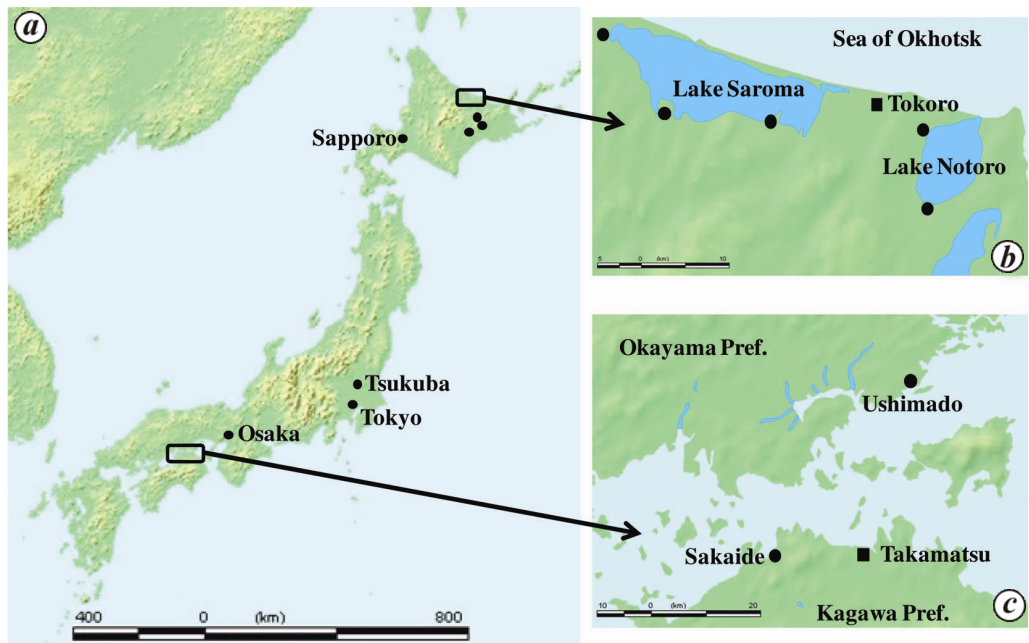
In this study, we investigated assemblages of endophytic fungi inhabiting living tissues of aerial components of *S. europaea* collected in Hokkaido in different years, and compared them with plants collected in Setouchi to examine the ubiquity of endophytic fungal communities.

## Materials and methods

### Sampling

The aerial components of healthy *S. europaea* were collected at the lakeshore of Lake Noto (44.03°N, 144.09°E) and Lake Saroma (44.08°N, 143.48°E) located in eastern Hokkaido (Figure 1). The two lakes were adjacent and connected to the Sea of Okhotsk at some points, and contained brackish water. Another region investigated was the seacoast of Setouchi, namely Ushimado town in Okayama Prefecture (34.38°N, 134.09°E) and Sakaide

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**Figure 1.** Distribution of *Salicornia europaea* and sampling sites. **a**, Distribution of *S. europaea* in Japan (black dots and squares); **b**, Sampling sites at Lake Noto and Lake Saroma (black dots); **c**, Sampling sites adjacent to the Seto Inland Sea (Setouchi). Black squares in **b** and **c** show locations of observation stations of the Japan Meteorological Agency.

**Table 1.** Daily mean temperature (Temp.) and annual total precipitation (Prec.) of each region<sup>a</sup>

Region	Year							
	1997		1998		2006		Average in 1981–2010	
	Temp. (°C)	Prec. (mm)	Temp. (°C)	Prec. (mm)	Temp. (°C)	Prec. (mm)	Temp. (°C)	Prec. (mm)
Hokkaido (Tokoro <sup>b</sup> )	6.2	555	5.9	816	6.5	953	6.0	700
Setouchi (Takamatsu <sup>b</sup> )	16.5	1096	17.5	1442	16.5 <sup>c</sup>	1212 <sup>c</sup>	16.3	1082

<sup>a</sup>Data are derived from Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>). <sup>b</sup>Observation stations are near sampling sites in Hokkaido and Seto Inland Sea respectively. <sup>c</sup>Reference data.

city in Kagawa Prefecture (34.23°N, 133.55°E; Figure 1). The two regions were 1400 km apart. Atmospheric data of these regions are provided in Table 1. Sampling in Hokkaido was conducted in September 2006 (30 plants from Lake Noto and 40 from Lake Saroma, including plants that turned red), July 1998 (20 plants each from Lake Noto and Lake Saroma), and August 1997 (20 plants each from Lake Noto and Lake Saroma). Sampling in the Setouchi region was conducted in October 1998 (20 plants from Ushimado and 30 from Sakaide) and November 1997 (10 plants from Ushimado and 20 from Sakaide). Aerial components of symptom-free plants were placed in a paper bag and then in a plastic bag to prevent over-drying and kept under ca. 4°C before processing (2–3 days after collection).

### Fungal isolation

Plant material was immersed in 70% ethanol solution for 1 min and sodium hypochlorite solution (1% available

chlorine) for 2 min, then rinsed in sterile distilled water and blotted dry in sterile paper towels for 3 h. After sterilization and drying, the main stems of the plant were divided into three segments and placed on the surface of cornmeal seawater agar (CMSWA: commercial cornmeal agar (Nissui Co Ltd, Tokyo) dissolved in 15 ppt salinity seawater (S. Jamarin; Jamarin Lab, Osaka) (Figure 2). The plates were incubated at 17°C for several months. Mycelia growing from plant segments and spores that formed on and around the segment were isolated and cultured on potato dextrose agar (PDA; Nissui Co Ltd, Tokyo).

### Identification

We performed morphological observation and sequence analysis of nuclear ribosomal DNA to identify the isolated fungi. Based on morphological observations, subcultures were incubated on PDA plates at 25°C. Fungal materials were mounted in one drop of lactophenol

solution on glass slides for observation with light microscopy.

For sequence analysis, the mycelia of isolates incubated for 2 weeks at 25°C on PDA plates were harvested and placed in 2 ml plastic tubes for nuclear DNA extraction. DNA was extracted using the Nucleon PhytoPure DNA extraction kit (GE Healthcare UK Ltd, Buckinghamshire, England) or DNeasy Plant Mini Kit (QIAGEN, Tokyo) according to the manufacturer's instructions. The ITS regions of rDNA were amplified by polymerase chain reaction (PCR) using TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan) or Blend Taq Plus (TOYOBO, Fukui, Japan) as a single fragment with the standard primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')<sup>22</sup>. Amplification of the desired fragment was performed with a GenAmp PCR System 7000 thermal cycler (Applied Biosystems, CA, USA) as follows: 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension for 2 min at 72°C, followed by an incubation for 5 min at 72°C and soaking at 4°C. Amplified DNA was sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) in a thermal cycler as follows: 25 cycles of 15 sec at 96°C and 4 min at 55°C, followed by a 4°C soak. Nucleotide sequences were determined in both directions using the primers ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'), ITS3 (5'-GCATCGATGAAGAACGGAGC-3'), ITS4 and ITS5 (ref. 22). Sequences were analysed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

ITS sequence data (length: 522–589 bp) were analysed using BLASTClust (<http://toolkit.tuebingen.mpg.de/blast-clust>) to generate a non-redundant sequence cluster, and some representatives of the ITS sequences of each cluster were applied to BLAST search to identify similar sequences in DNA databases. After performing clustering analysis with Clustal X<sup>23</sup>, species were identified using morphological and molecular analyses.

In addition to analyses using ITS sequences, the 5'-end of the 28S rDNA (including D1 and D2; length: 561–571 bp) was examined in the BLAST search. The 28S

rDNA regions were amplified and sequenced as described above, but using the universal primers NL1 (5'-GCA-TATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3')<sup>24</sup>.

### Data analyses

Isolation frequency (IF) and colonization rate (CR) of fungi were calculated using the following formula:  $IF = N_i/N_t \times 100$  and  $CR = N_c/N_t \times 100$ , where  $N_i$  is the number of plants from which a given fungal species was isolated,  $N_c$  is the total number of plants from which fungi were isolated in a sample and  $N_t$  is the total number of plants examined for isolation.

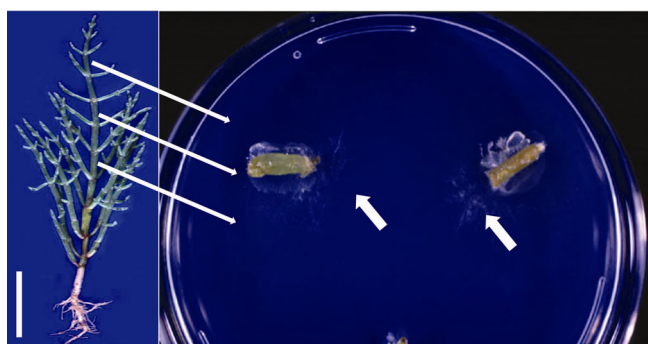
Analysis of species richness was conducted using Chao-2, Jackknife and Bootstrap (estimators of the species number). These and the following analyses were made using the EstimateS ver. 9.1 software<sup>25</sup>, based on data of all species. Sample-based rarefaction and extrapolation (for which the Bernoulli product model was used with EstimateS) of fungi in both the Hokkaido and Setouchi regions were calculated. Similarity indices (Jaccard, Sorensen, Bray–Curtis and Morisita–Horn indices) and diversity indices (Shannon index ( $H'$ ), Fisher's alpha ( $F$ ) and Simpson's index ( $D$ )) were calculated to compare fungal assemblages between samples obtained in different regions and in different years.

Fungi originating from plant samples on agar plates were observed microscopically, and 457 isolates were temporarily cultured to record their occurrence and calculate the IF. Of these isolates, 146 representative strains were selected and subcultured for identification based on morphological and molecular analyses.

### Results

A total of 457 fungi were isolated from 230 *S. europaea* plants. CRs of fungi were 95.2% ( $N_c/N_t = 219/230$ ) in total; 94% ( $N_c/N_t = 141/150$ ) in Hokkaido and 97.5% ( $N_c/N_t = 78/80$ ) in Setouchi. Detailed results are provided in Table 2. CRs of each sample ranged from 85% to 100%.

Of the 457 isolates, 146 selected isolates were subjected to morphological and DNA sequence analyses. Based on BLASTClust analysis, the 146 ITS sequences of the selected isolates were grouped into 28 clusters under a 98% identity threshold (sequence length covered: 70%), being more significant than the 95% identity threshold by which 17 groups were recognized and morphologically different species clustered in the same group, e.g. *Alternaria alternata* (Fr.) Keissl. and *Alternaria phragmospora* Emden. Among the 28 clusters, 2 were significantly associated with other monophyletic groups based on Clustal X; namely *Pleospora* sp. and *Hypocreales* sp. Thus, we recognized 26 taxa from *S. europaea* of Japan based on DNA sequence and morphological analyses (Table 3).



**Figure 2.** (Left) A plant of *S. europaea* and three parts of the aerial component examined to isolate fungi. (Right) Fungi growing on a cornmeal sea-water agar plate (arrows). Bar = 5 cm.

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Table 2. Species richness and diversity indices of endophytic fungi in each examination

Sampling	Hokkaido – 2006	Hokkaido – 1998	Hokkaido – 1997	Hokkaido – total	Setouchi – 1998	Setouchi – 1997	Setouchi – total
No. of samples	7	4	4	15	5	3	8
No. of plants	70	40	40	150	50	30	80
Colonization rate (%)	100.0	85.0	92.5	94.0	96.0	100.0	97.5
No. of isolates	178	40	63	281	105	71	176
No. of species observed	12	9	7	18	12	7	15
Species richness							
Chao-2	12.21	12.75	13.00	25.62	16.00	7.22	25.71
Jackknife	13.71	13.50	10.00	24.53	16.00	8.33	21.12
Bootstrap	13.00	11.02	8.33	20.91	13.73	7.66	17.63
Diversity							
Shannon index ( <i>H'</i> )	1.93	1.80	1.44	1.97	1.84	1.57	1.90
Fisher's alpha ( <i>F</i> )	1.72	1.63	1.10	2.57	1.89	1.07	2.25
Simpson's index ( <i>D</i> )	5.56	4.87	3.36	5.14	4.97	4.14	5.16

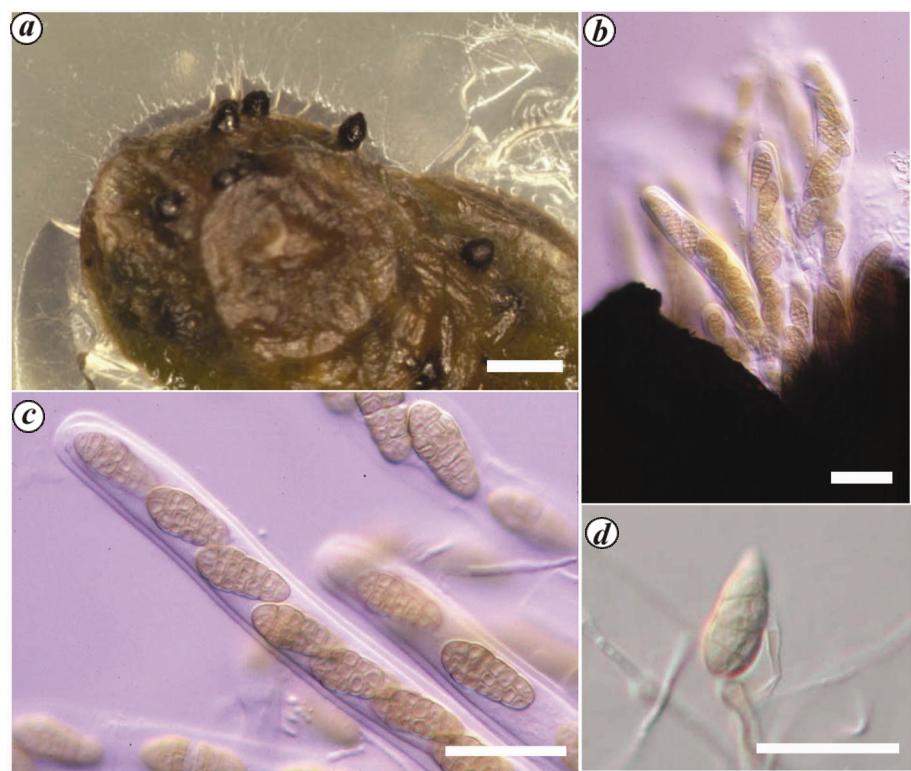


Figure 3. *Pleospora* sp. **a**, Ascomata produced on a plant segment cultured on CMSWA. **b**, Asci coming from an ascoma. **c**, Ascospores inside asci. **d**, Conidium of *Stemphylium* anamorph. Bar: (**a**) = 1 cm; (**b**)–(**d**) = 50  $\mu$ m.

Endophytic fungi isolated from *S. europaea* and IFs of each fungus are shown in Table 3. *Pleospora* sp. (Figure 3) was isolated at a high frequency from plants collected in all regions and sites and in each sampling year. IFs of *Pleospora* sp. from Hokkaido in each year were over 60%, except for that in 1998 (Table 3). IFs of the fungus from the two lake areas in Hokkaido were as follows (data not shown in Table 3): 76.7% in 2006, 55% in 1998

and 80% in 1997 at Lake Noto, and 47.5% in 2006, 0% in 1998 and 55% in 1997 at Lake Saroma. In the Setouchi region, IFs of *Pleospora* sp. were also high; 35% in 1998 and 100% in 1997 at Ushimado, and 53.3% in 1998 and 35% in 1997 at Sakaide (data not shown in Table 3).

*Alternaria alternata* was isolated at a high frequency from plants collected in both regions. The IF of *Alternaria alternata* ranged from 30% to 70% in plants from

**Table 3.** Isolation frequencies of endophytic fungi from *Salicornia europaea*

Fungus	Strain no.	Accession no. (ITS/28S D1D2)	BLAST search result based on ITS (upper) and 28S D1D2 (lower) (strain no. of BRCs) <sup>a</sup>	Query cover (%)/ identity (%) [ITS (upper), 28S (lower)]	Isolation frequency (%)				
					Hokkaido			Seto Inland Sea	
					2006 (n = 70)	1998 (n = 40)	1997 (n = 40)	1998 (n = 50)	1997 (n = 30)
<i>Pleospora</i> sp.	IOC-1348	AB975284/AB975310	<i>Stemphylium solani</i> AF203448 <i>Pleospora herbarum</i> var. herbarum AF382386 (CBS 191.86)	100/98 98/100	60.0	27.5	67.5	46.0	56.7
<i>Alternaria alternata</i>	IOC-1351	AB975286/AB975312	<i>Alternaria alternata</i> KJ538950 <i>Alternaria alternata</i> AB192875 (IFM 53880)	100/100 100/100	70.0	30.0	47.5	44.0	86.7
<i>Alternaria phragmospora</i> (syn. <i>Embellisia phragmospora</i> )	IOC-1496	AB975297/AB975323	<i>Embellisia phragmospora</i> FJ357314 <i>Alternaria phragmospora</i> KF417565	100/99 97/100	35.7	10.0	20.0	0.0	40.0
<i>Cladosporium cladosporioides</i>	IOC-1350	AB975285/AB975311	<i>Cladosporium cladosporioides</i> KJ596320 <i>Cladosporium cladosporioides</i> KC585410 (ATCC 11275)	100/100 100/100	32.9	5.0	0	64.0	30.0
<i>Sarocladium strictum</i>	IOC-1404	AB975290/AB975316	<i>Sarocladium strictum</i> KC311519 <i>Sarocladium strictum</i> AY138483 (CBS 346.7T)	100/100 100/100	22.9	0	0	0	3.3
<i>Phoma</i> sp.	IOC-1465	AB975294/AB975320	<i>Phoma</i> sp. AY513965 <i>Peyronellaea glomerata</i> JN938876 (DAOM 214575)	100/100 99/100	1.4	0	0	2.0	0
<i>Hypocrea</i> sp.	IOC-1504	AB975299/AB975325	<i>Hypocrea</i> sp. HQ649990 <i>Acremonium implicatum</i> strain HQ232046 (CBS 243.59)	100/98 100/96	0	0	2.5	0	3.3
<i>Paradendryphiella</i> sp.	IOC-1364	AB975287/AB975313	<i>Paradendryphiella arenariae</i> DQ411539 (CBS 181.58) <i>Paradendryphiella arenariae</i> EU848587	97/100 100/100	8.6	5.0	10.0	0	0
<i>Phoma betae</i>	IOC-1493	AB975295/AB975321	<i>Phoma betae</i> EU594572 <i>Phoma betae</i> EU754178 (CBS 109410)	99/99 98/99	0	15.0	5.0	0	0
<i>Fusarium sporotrichioides</i>	IOC-1365	AB975288/AB975314	<i>Fusarium sporotrichioides</i> KC866346 <i>Fusarium sporotrichioides</i> JN938901	99/100 99/99	11.4	0.0	0.0	0	0
<i>Emericellopsis</i> sp.	IOC-1503	AB975298/AB975324	<i>Emericellopsis pallida</i> EU045572 <i>Emericellopsis maritima</i> F1176861	100/99 100/99	0	0.0	5.0	0	0
<i>Fusarium cerealis</i>	IOC-1375	AB975289/AB975315	<i>Fusarium cerealis</i> DQ459869 (NRRL25805) <i>Fusarium cerealis</i> DQ459869 (NRRL25805)	100/100 100/100	4.3	0.0	0	0	0
<i>Fusarium avenaceum</i> (Teleomorph: <i>Gibberella avenacea</i> )	IOC-1461	AB975293/AB975319	<i>Gibberella avenacea</i> AY147285 <i>Fusarium avenaceum</i> JN938913 (DAOM 215454)	100/100 100/100	2.9	0.0	0	0	0

(Contd)



Table 3. (Contd)

Fungus	Strain no.	Accession no. (ITS/28S D1D2)	BLAST search result based on ITS (upper) and 28S D1D2 (lower) (strain no. of BRCs) <sup>a</sup>	Query cover (%)/ identity (%) [ITS (upper), 28S (lower)]	Isolation frequency (%)				
					Hokkaido		Seto Inland Sea		
					2006 (n = 70)	1998 (n = 40)	1997 (n = 40)	1998 (n = 50)	1997 (n = 30)
<i>Epicoecium nigrum</i>	IOC-1419	AB975291/AB975317	<i>Epicoecium nigrum</i> KC867291 <i>Epicoecium</i> sp. JF773674	100/100 100/100	2.9	0.0	0	0	0
<i>Alternaria</i> sp.	IOC-1546	AB975300/AB975326	<i>Alternaria</i> sp. JQ775551 <i>Lewia aurea</i> DQ678044 (DAOM 195275)	100/96 100/99	0	2.5	0	0	0
<i>Lewia</i> sp.	IOC-1549	AB975301/AB975327	<i>Lewia infectoria</i> JX454532 <i>Chalastospora obclavata</i> F1839651 (CBS 124120)	100/98 100/99	0	2.5	0	0	0
<i>Tolypocladium cylindrosporum</i>	IOC-1556	AB975302/AB975328	<i>Tolypocladium cylindrosporum</i> FJ411410 <i>Tolypocladium cylindrosporum</i> AF245301	100/100 100/100	0	2.5	0	0	0
<i>Nigrospora oryzae</i>	IOC-1424	AB975292/AB975318	<i>Nigrospora oryzae</i> EU436680 <i>Nigrospora</i> sp. AB808205	100/100 100/100	1.4	0	0	0	0
<i>Fusarium incarnatum</i>	IOC-1568	AB975304/AB975330	<i>Fusarium incarnatum</i> KJ572780 <i>Fusarium incarnatum</i> AY633745	100/100 100/100	0	0	0	12.0	16.7
<i>Colletotrichum gloeosporioides</i>	IOC-1583	AB975306/AB975332	<i>Colletotrichum gloeosporioides</i> JX010148 (CBS 273.51) <i>Glomerella cingulata</i> DQ286193	100/100 100/100	0	0	0	22.0	0
<i>Colletotrichum boninense</i>	IOC-1566	AB975303/AB975329	<i>Colletotrichum boninense</i> KF819619 <i>Colletotrichum boninense</i> DQ286161	100/100 100/100	0	0	0	6.0	0
<i>Diaporthe</i> sp.	IOC-1588	AB975308/AB975334	<i>Diaporthe phaseolorum</i> AF001017 <i>Diaporthe ceratocarpiae</i> JQ044440 (CBS 131306)	100/98 100/99	0	0	0	6.0	0
<i>Ascochyta caulina</i> (Teleomorph: <i>Pleospora calvenscens</i> )	IOC-1495	AB975296/AB975322	<i>Phoma betae</i> JX292134 <i>Pleospora calvenscens</i> EU754131 (CBS 246.79)	96/99 98/100	0	0	0	2.0	0
<i>Cochliobolus</i> sp.	IOC-1582	AB975305/AB975331	<i>Cochliobolus</i> sp. JX960591 <i>Cochliobolus geniculatus</i> JN941528 (NBRC 7407)	100/99 100/100	0	0	0	2.0	0
<i>Phoma macrostoma</i>	IOC-1585	AB975307/AB975333	<i>Phoma macrostoma</i> DQ474092 (DAOM 175940) <i>Ascochyta</i> sp. AB808053	100/100 100/99	0	0	0	2.0	0
<i>Pleosporales</i> sp.	IOC-1591	AB975309/AB975335	<i>Pleosporales</i> sp. HQ649967 <i>Bimuria novae-zelandiae</i> AY016356 (CBS 107.79)	96/99 100/97	0	0	0	2.0	0

<sup>a</sup>ATCC, American Type Culture Collection (USA); CBS, Centraalbureau voor Schimmelfungi and Yeast Collection (The Netherlands); DAOM, National Mycological Herbarium (Canada); IFM, Research Center for Pathogenic Fungi and Microbial Toxins, Chiba University (Japan); NBRC, NBRC Culture Collection (Japan); NRRL, Agricultural Research Service Culture Collection (USA).

**Table 4.** Similarities of fungal assemblages among different samplings and regions by Morishita–Horn (left) and Bray–Curtis (right) indices

Sample	Setouchi–total	Hokkaido–1998	Hokkaido–1997	Setouchi–1998	Setouchi–1997
Hokkaido–total	0.891/0.685				
Hokkaido–2006		0.881/0.438	0.855/0.665	0.781/0.534	0.942/0.798
Hokkaido–1998			0.910/0.600	0.680/0.403	0.888/0.430
Hokkaido–1997				0.630/0.489	0.872/0.643
Setouchi–1998					0.776/0.590

Hokkaido and from 44% to 86.7% in plants from Setouchi (Table 3). IFs of the fungus in each area (data not shown in Table 3) were 56.7% in 2006, 10% in 1998 and 50% in 1997 at Lake Notoro; 80% in 2006, 50% in 1998 and 45% in 1997 at Lake Saroma; 55% in 1998 and 60% in 1997 at Ushimado, and 36.7% in 1998 and 100% in 1997 at Sakaide.

Another species of *Alternaria*, *A. phragmospora*, was isolated with an IF of 10% to 40% in these regions, although the fungus was not isolated from plants collected in Setouchi in 1998.

In addition to the aforementioned fungi that are phylogenetically similar, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries was isolated at relatively high frequencies. IFs ranged from 5% to 64%, although the fungus was not found in plants collected in Hokkaido in 1997. Other fungi isolated from both the Hokkaido and Setouchi regions included *Sarocladium strictum* (W. Gams) Summerb., *Phoma* sp. and *Hypocreales* sp.

The following fungi were isolated from either Hokkaido or Setouchi: *Paradendryphiella* sp., *Phoma betae* A.B. Frank, *Fusarium sporotrichioides* Sherb., *F. cerealis* (Cooke) Sacc., *F. avenaceum* (Fr.) Sacc., *Emericlesporium* sp., *Epicoccum nigrum* Link, *Alternaria* sp., *Lewia* sp., *Tolypocladium cylindrosporum* W. Gams and *Nigrospora oryzae* (Berk. & Broome) Petch from Hokkaido; *Fusarium incarnatum* (Desm.) Sacc., *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *C. boninense* Moriwaki, Toy. Sato & Tsukib., *Diaporthe* sp., *Ascochyta caulina* (P. Karst.) Aa & Kesteren, *Cochliobolus* sp., *Phoma macrostoma* Mont. and *Pleosporales* sp. from Setouchi. A total of seven species were common to both regions, and 11 and 8 species were found only in Hokkaido and Setouchi respectively.

Ecological analyses are shown in Tables 2 and 4, and Figure 4. Overall, species richness and diversity were not significantly different between samplings for different years in Hokkaido, while the observed species number and each index of the 1997 sample tended to be lower than those of the 1998 and 2006 samples (Table 2). The similarity indices of Morisita–Horn among three samples in Hokkaido were >0.8 and those of Bray–Curtis were >0.6, excluding between samples in 2006 and 1998 (0.438; Table 4; other indices are not shown).

For the Setouchi samples, the observed species number and each index of the 1997 sample were lower than those

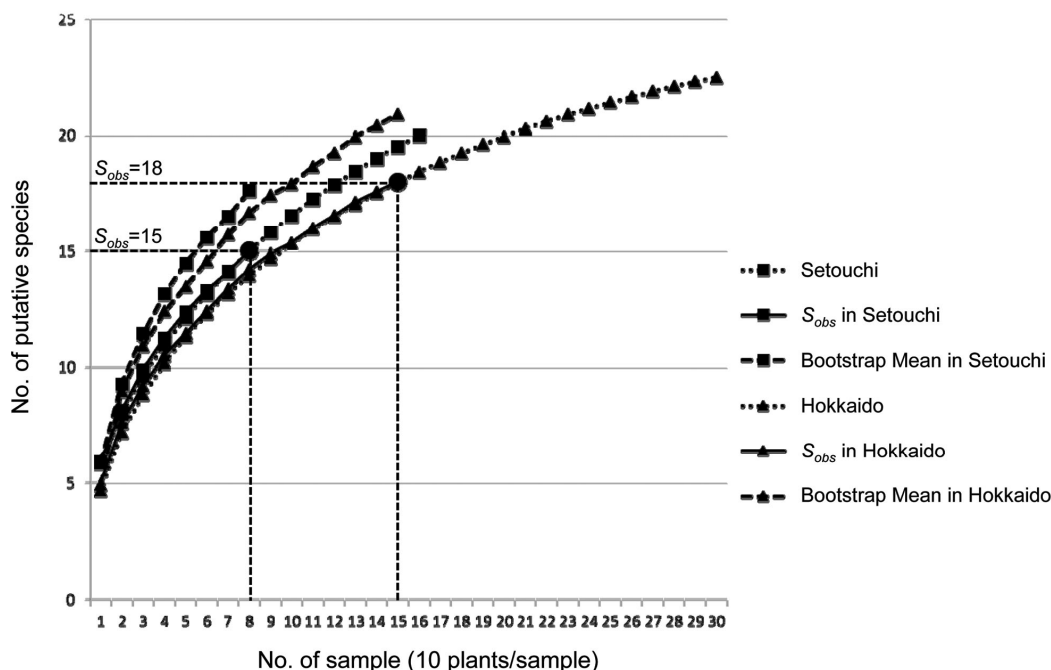
of the 1998 sample (Table 2). The similarity indices of Morisita–Horn and Bray–Curtis between two samples in Setouchi were 0.776 and 0.590 respectively (Table 4).

Indices of species richness and diversity were similar in the Hokkaido and Setouchi populations (Table 2). The similarity indices of Morisita–Horn and Bray–Curtis between the two populations were 0.891 and 0.685 respectively (Table 4). Sample-based rarefaction and extrapolation from two sampling tests of *S. europaea* from Hokkaido and Setouchi demonstrated that species richness of the two populations did not differ significantly, while both were non-asymptotic (Figure 4). Bootstrap estimates of richness of the two populations did not exceed the 95% confidence interval (data not shown) around observed species richness.

## Discussion

CRs and IFs of fungal endophytes showed that *S. europaea* harbours diverse endophytic fungi. Several species were present at a high frequency (Tables 2 and 3), with relatively high consistency among samplings at various times (decades apart) conducted in Hokkaido, and between the geographically and climatically different regions of Hokkaido and Setouchi (Figure 1; Tables 1 and 2). While analysis of species richness using sample-based rarefaction and extrapolation (Figure 4) demonstrated that more samples are required to produce an accurate interpretation of endophytic fungal community diversity, species richness and diversity did not differ significantly between the two regions. IFs of some fungi differed between samples in the same region, potentially reflecting small-scale year-to-year variation, seasonal factors, or small-scale structure in communities within sites. For example, IFs of *C. cladosporioides* and *S. strictum* in Hokkaido, and *A. alternata* and *A. phragmospora* in Setouchi were higher in samples obtained in the later months, potentially revealing a seasonal pattern that can be explored in future work.

*Pleospora* sp. in this study (Figure 3) was morphologically similar to *P. herbarum* (Pers.) Rabenh. However, ITS and 28S sequence analyses could not be used for species identification of this fungus, as reported by Inderbitzin *et al.*<sup>26</sup>. They discussed that cultures of five species, including *P. herbarum* could not be



**Figure 4.** Sample-based rarefaction and extrapolation from two reference samplings of *S. europaea* from Hokkaido and the Seto Inland Sea (Setouchi).

distinguished using four loci (rDNA ITS regions, the protein encoding GPD and EF-1 alpha genes and the intergenic spacer between *vmaA* and *vpsA*). Thus, further studies are required for precise identification of the *Pleospora* spp.

Petrini and Fisher<sup>13</sup> reported three *Pleospora* species, namely, *P. biorlingii* Byford, *P. herbarum* Rabenh. and *P. salicorniae* Jaap, from *Salicornia perennis* growing on the seacoast in England. *P. salicorniae*, which is synonymous with *Decorospora gaudefroyi* (Pat.) Inderb., Kohlm. & Volkm.-Kohlm.<sup>26</sup>, was isolated at the highest frequency, followed by *Stagonospora* sp. 1. Crabtree and Gessner<sup>27</sup> also reported *D. gaudefroyi* (as *Pleospora gaudefroyi* Pat.) from *Salicornia* sp. Although the species may differ, morphologically similar fungi that form pigmented spores are likely predominant on *Salicornia*.

Booth *et al.*<sup>8</sup> reported fungal assemblages on *S. europaea* in southern Manitoba and Saskatchewan, Canada. In their study, *Alternaria* species were isolated at a high frequency from plants, but samples were processed using a washing method. Similar results were found regarding the fungal assemblages of *Salicornia rubra* in an inland salt marsh in Manitoba<sup>12</sup>. In the study, other halophytes were also examined; *Alternaria* species were isolated from halophytes, including *S. rubra*, at a high frequency, as well as from *S. europaea*. Furthermore, *Alternaria* species were major endophytes on chenopodiaceous plants growing in desert areas in China<sup>15,16</sup>. Moreover, *Paradendryphiella arenariae* (Nicot) Woudenberg & Crous, which was found in the Hokkaido samples, was

also reported in *S. europaea* in Canada (as *Dendryphiella arenariae* Nicot)<sup>8</sup>. Unidentified species of *Alternaria* and its teleomorph *Lewia* were also isolated from the Hokkaido samples, albeit at a lower frequency.

In addition to the above-mentioned Pleosporales fungi, *Cladosporium cladosporioides* (Capnoidiales) was isolated at a relatively high frequency. Another species of the genus, *C. herbarum* (Pers.) Link was frequently isolated from *S. europaea* in Canada<sup>8</sup>. Such dematiaceous fungi isolated from aerial tissues of various terrestrial plants are generally thought to be epiphytes or facultative endophytes<sup>7,28</sup>. However, the present study revealed that some fungi could penetrate fresh tissues of *S. europaea*. These fungi are thought to be an important endophyte of halophilic chenopodiaceous plants.

Momonoki and Kamimura<sup>29</sup> reported that during the growth period of wild *S. europaea* found around Lake Notoro, the pH and osmotic pressure of the plants increased from 7.6 to 8.8, and from 650 to 2000–2600 mOsm/kg (1 mOsm/kg = 17.02 mm Hg) respectively. The internal environments of halophytes such as *S. europaea* are likely stressful to microbes, including fungi.

Pigmented spore- and/or mycelia-forming fungi, such as *Pleospora* spp. (anamorph: *Stemphylium* spp.), *Alternaria* spp., *Cladosporium* spp. and *Camarosporium* spp. are frequently isolated from halophytes in salt marsh and desert areas<sup>8,12,13,15,16,26</sup>. These fungi are known to produce high levels of pigment<sup>30</sup>, which suggests that such pigment may protect the fungi inhabiting plants growing



in high-salinity environments. Kogej *et al.*<sup>31</sup> hypothesized that melanization of the halophilic black yeast, *Hortaea werneckii* (Horta) Nishim. & Miyaji can decrease the permeability of its cell wall to its major compatible solute, glycerol. This may allow the species to tolerate a wider range of salt concentrations. It is also known that melanin in fungi enhances penetration into host plants with pigmented appressoria and functions as a defence system against microbial attack<sup>32</sup>. Dark-pigmented mycelia produced by *Camarosporium roumeguerii* Sacc. are thought to absorb more UV radiation than white mycelia<sup>27</sup>. English and Gerhardt<sup>33</sup> reported that spores with dark-coloured walls showed greater resistance to UV radiation than those with light-coloured walls. Bell and Wheeler<sup>32</sup>, and Taborda *et al.*<sup>34</sup> reported that melanin enhanced fungal resistance against UV radiation, radio waves, desiccation and extreme temperatures. Suryanarayanan *et al.*<sup>35</sup> suggested that melanin in the hyphae of *Phyllosticta capitalensis* Henn. was responsible for the success of this fungus as a cosmopolitan endophyte that could survive in stressful environments. It is pertinent to mention here that melanization of hyphae protects marine fungi against osmotic shock<sup>36</sup>. Thus, compounds such as melanin are likely to influence the survival rate of fungi that reside in the outer and/or inner environment of halophytic plants growing under high salt concentration and UV radiation.

Regarding fungi other than the above-mentioned dematiaceous species, many fungal genera found in this study have been reported from *Salicornia*<sup>8,12,13</sup>, although their IFs were not high, except for some fungi that were isolated from either Hokkaido or Setouchi: *F. sporotrichioides*, *F. incarnatum* and *C. gloeosporioides*. Endophytic fungi of *S. europaea* appear to reflect those of other chenopodiaceous plants. In order to clarify specialty and stability of the endophyte ecology on halophytes, further studies on tolerance of endophytes against both UV and high salinity (high osmotic pressure) conditions are required.

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