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 halophytic 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 cladosporoides*. 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 plants¹⁻³. Some dematiaceous fungi that produce dark mycelia and spores have also been isolated during studies of fungal endophytes⁴⁻⁶. For example, dematiaceous fungi such as *Alternaria* and *Cladosporium* are known to be facultative endophytes of terrestrial plants⁷. 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 published⁸⁻¹⁷. Further studies on fungal communities on halophytes are required to examine their biodiversity and functional roles.

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*Salicornia europaea* L. (Chenopodiaceae) is a halophytic succulent plant widely distributed in salt marshes and seashores in temperate regions of the Northern Hemisphere¹⁸,¹⁹. 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 Notoro 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 Kitaame Ships (trade ships between Hokkaido and Setouchi regions in the 19th century)²⁰. Recently, molecular analyses have revealed that the two populations of *S. europaea* have different origins²⁰,²¹. Hoshino et al.²⁰ 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 Notoro (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 Notoro 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

<table>
<thead>
<tr>
<th>Region</th>
<th>Temp. (°C)</th>
<th>Prec. (mm)</th>
<th>Temp. (°C)</th>
<th>Prec. (mm)</th>
<th>Temp. (°C)</th>
<th>Prec. (mm)</th>
<th>Average in 1981–2010</th>
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<tr>
<td></td>
<td>1997</td>
<td>1998</td>
<td>2006</td>
<td></td>
<td></td>
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<tr>
<td>Hokkaido (Tokoro)</td>
<td>6.2</td>
<td>5.9</td>
<td>6.5</td>
<td>6.0</td>
<td>700</td>
<td></td>
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<tr>
<td>Setouchi (Takamatsu)</td>
<td>16.5</td>
<td>17.5</td>
<td>16.5</td>
<td>16.3</td>
<td>1082</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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 Notoro and 40 from Lake Saroma, including plants that turned red), July 1998 (20 plants each from Lake Notoro and Lake Saroma), and August 1997 (20 plants each from Lake Notoro 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'-GGAAGTAAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGTTATTGATATGC-3')22. 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), ITS3 (5'-GCACTGGAAGAACCGGAGC-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/blastclust) 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 X23, 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-TATCAATAAGCGGAGGAAAG-3') and NL4 (5'-GGTCGGTGTTCAGACGG-3')23.

Data analyses

Isolation frequency (IF) and colonization rate (CR) of fungi were calculated using the following formula: IF = Nt/Ni × 100 and CR = Nc/Nt × 100, where Nt is the number of plants from which a given fungal species was isolated, Nc is the total number of plants from which fungi were isolated in a sample and Ni 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 software25, based on data of all species. Sample-based rarefaction and extrapolation (for which the Bernouilli 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% (Nt/Ni = 219/230) in total; 94% (Nt/Ni = 141/150) in Hokkaido and 97.5% (Nt/Ni = 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 corneal sea-water agar plate (arrows). Bar = 5 cm.
Table 2. Species richness and diversity indices of endophytic fungi in each examination

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>No. of samples</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>15</td>
<td>5</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>No. of plants</td>
<td>70</td>
<td>40</td>
<td>40</td>
<td>150</td>
<td>50</td>
<td>30</td>
<td>80</td>
</tr>
<tr>
<td>Colonization rate (%)</td>
<td>100.0</td>
<td>85.0</td>
<td>92.5</td>
<td>94.0</td>
<td>96.0</td>
<td>100.0</td>
<td>97.5</td>
</tr>
<tr>
<td>No. of isolates</td>
<td>178</td>
<td>40</td>
<td>63</td>
<td>281</td>
<td>105</td>
<td>71</td>
<td>176</td>
</tr>
<tr>
<td>No. of species observed</td>
<td>12</td>
<td>9</td>
<td>7</td>
<td>18</td>
<td>12</td>
<td>7</td>
<td>15</td>
</tr>
</tbody>
</table>

Species richness

- **Chao-2**
  - Hokkaido – 2006: 12.21
  - Hokkaido – 1998: 12.75
  - Hokkaido – 1997: 13.00
  - Hokkaido – total: 25.62
  - Setouchi – 1998: 16.00
  - Setouchi – 1997: 7.22
  - Setouchi – total: 25.71

- **Jackknife**
  - Hokkaido – 2006: 13.71
  - Hokkaido – 1997: 10.00
  - Hokkaido – total: 24.53
  - Setouchi – 1998: 16.00
  - Setouchi – 1997: 8.33
  - Setouchi – total: 21.12

- **Bootstrap**
  - Hokkaido – 2006: 13.00
  - Hokkaido – 1998: 11.02
  - Hokkaido – 1997: 8.33
  - Hokkaido – total: 20.91
  - Setouchi – 1998: 13.73
  - Setouchi – 1997: 7.66
  - Setouchi – total: 17.63

Diversity

- **Shannon index (H')**
  - Hokkaido – 2006: 1.93
  - Hokkaido – 1998: 1.80
  - Hokkaido – 1997: 1.44
  - Hokkaido – total: 1.97
  - Setouchi – 1998: 1.84
  - Setouchi – 1997: 1.57
  - Setouchi – total: 1.90

- **Fisher's alpha (F)**
  - Hokkaido – 2006: 1.72
  - Hokkaido – 1998: 1.63
  - Hokkaido – 1997: 1.10
  - Hokkaido – total: 2.57
  - Setouchi – 1998: 1.89
  - Setouchi – 1997: 1.07
  - Setouchi – total: 2.25

- **Simpson’s index (D)**
  - Hokkaido – 2006: 5.56
  - Hokkaido – 1998: 4.87
  - Hokkaido – 1997: 3.36
  - Hokkaido – total: 5.14
  - Setouchi – 1998: 4.97
  - Setouchi – 1997: 4.14
  - Setouchi – total: 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 μm.

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 Notoro, 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*

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Strain no.</th>
<th>Accession no. (ITS/28S D1D2)</th>
<th>BLAST search result based on ITS (upper) and 28S D1D2 (lower) (strain no. of BRCs)</th>
<th>Query cover (%)/identity (%) (ITS (upper), 28S (lower))</th>
<th>Isolation frequency (%)</th>
</tr>
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<tr>
<td></td>
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<tr>
<td>Pleospora sp.</td>
<td>IOC-1348</td>
<td>AB975284/AB975310</td>
<td><em>Stenophialium solani</em> AF204448</td>
<td>100/98, 98/100</td>
<td>60.0, 27.5, 67.5, 46.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Pleospora herbarum</em> var. <em>herbarum</em> AF382385 (CBS 191.86)</td>
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<td>56.7</td>
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<td>Alternaria alternate</td>
<td>IOC-1351</td>
<td>AB975286/AB975312</td>
<td><em>Alternaria alternata</em> KJ533950</td>
<td>100/100</td>
<td>70.0, 30.0, 47.5, 44.0</td>
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<td><em>Alternaria alternata</em> AB132875 (IFM 53880)</td>
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<td>86.7</td>
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<tr>
<td>Alternaria phragmospora (syn.</td>
<td>IOC-1496</td>
<td>AB975297/AB975323</td>
<td><em>Embellisia phragmospora</em> FI53731-</td>
<td>100/99, 97/100</td>
<td>5.7, 10.0, 20.3, 0.0</td>
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<tr>
<td><em>Embellisia phragmospora</em></td>
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<td></td>
<td><em>Alternaria phragmospora</em> KF147565</td>
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<td>40.0</td>
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<td>Cladosporium cladosporioides</td>
<td>IOC-1350</td>
<td>AB975285/AB975311</td>
<td><em>Cladosporium cladosporioides</em> K5986320</td>
<td>100/100</td>
<td>2.9, 5.0, 0, 64.0</td>
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<td><em>Cladosporium cladosporioides</em> KCS5410 (ATCC 11275)</td>
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<td>30.0</td>
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<td>Sarocladium strictum</td>
<td>IOC-1404</td>
<td>AB975290/AB975316</td>
<td><em>Sarocladium strictum</em> KC11519</td>
<td>100/100</td>
<td>22.9, 0, 0, 0</td>
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<td><em>Sarocladium strictum</em> AY138483 (CBS 346.7T)</td>
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<td>Phoma sp.</td>
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<td>AB975294/AB975320</td>
<td><em>Phoma</em> sp. AY153965</td>
<td>100/100, 99/100</td>
<td>1.4, 0, 0, 2.0</td>
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<td><em>Peyronellaceae graminea</em> J938876 (DAOM 214575)</td>
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<td>Hypocephales sp.</td>
<td>IOC-1534</td>
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<td><em>Hypocephales</em> sp. HQ649990</td>
<td>100/98, 99/96</td>
<td>0, 0, 2.5, 0</td>
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<td><em>Acremonium implicatum</em> x <em>rain</em> HQ232046 (CBS 243.59)</td>
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<td><em>Paradenophyllia arenariae</em> DQ411539 (CBS 181.58)</td>
<td>97/100, 100/100</td>
<td>8.6, 5.0, 10.3, 0</td>
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<td><em>Paradenophyllia arenariae</em> EU84587</td>
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<td>Phoma betae</td>
<td>IOC-1493</td>
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<td><em>Phoma betae</em> EU59452</td>
<td>99/99, 98/99</td>
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<td><em>Phoma betae</em> EU574178 (CBS 109410)</td>
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<td>AB975288/AB975314</td>
<td><em>Fusarium sporotrichoides</em> K866346</td>
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<td>11.4, 0.0, 0.3, 0</td>
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<td><em>Emericithopsis pallida</em> EU045572</td>
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<td><em>Fusarium cereals</em> DQ45969 (NRRL 25805)</td>
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<td>Fusariumavenaceum</td>
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<td><em>Gibberella avenuea</em> AY147285</td>
<td>100/100, 100/100</td>
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<td>(Telomospor: <em>Gibberella avenuea</em>)</td>
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<td><em>Fusarium avenuea</em> JN938913 (DAOM 215454)</td>
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<th>Fungus</th>
<th>Strain no.</th>
<th>Accession no. (ITS/28S D1D2)</th>
<th>BLAST search result based on ITS (upper) and 28S D1D2 (lower) (strain no. of BRCs)</th>
<th>Query cover (%)</th>
<th>Isolation frequency (%)</th>
<th>Hokkaido (n = 70)</th>
<th>Seto Inland Sea (n = 50)</th>
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</table>
| *Epicoccum nigrum*        | IOC-14:9   | AB975291/AB975317            | *Epicoccum nigrum* K867291
**Epicoccum sp.** JF73674 | 100/100       | 2.9                      | 0.0               | 0                       | 0               | €                       |
| *Alternaria sp.*          | IOC-15:46  | AB975300/AB975326            | *Alternaria sp.* QJ77551
**Leuca eureka** DQ678044 (DAOM 195275) | 100/96       | 0                       | 2.5               | 0                       | 0               | €                       |
| *Leuca sy.*               | IOC-15:49  | AB975301/AB975327            | *Leuca infectioria* JX454532
**Chalastospora ochlavata** FJ839651 (CBS 124120) | 100/98       | 0                       | 2.5               | 0                       | 0               | €                       |
| *Tolypocladium cylindrosporum* | IOC-15:56  | AB975302/AB975328 | *Tolypocladium cylindrosporum* FJ411410
**Tolypocladium cylindrosporum** AF245301 | 100/100       | 0                       | 2.5               | 0                       | 0               | €                       |
| *Nigrospora oryzae*       | IOC-14:24  | AB975292/AB975318            | *Nigrospora oryzae* EU436680
**Nigrospora sp.** AB808205 | 100/100       | 1.4                      | 0                 | 0                       | 0               | €                       |
| *Fusarium incarnatum*     | IOC-15:68  | AB975304/AB975330            | *Fusarium incarnatum* KJ572780
**Fusarium incarnatum** ABY33745 | 100/100       | 0                       | 0                 | 12.0                     | 16.7             | €                       |
| *Colletorichum goeiosporioides* | IOC-15:83  | AB975306/AB975332 | *Colletorichum goeiosporioides* JX080148 (CBS 273.51)
**Glomerella cingulata** DQ266195 | 100/100       | 0                       | 0                 | 22.0                     | €               |                       |
| *Colletorichum boninense* | IOC-15:66  | AB975303/AB975329            | *Colletorichum boninense* SE819619
**Colletorichum boninense** JQ286161 | 100/100       | 0                       | 0                 | 6.0                       | €               |                       |
| *Diaporthe sp.*           | IOC-15:88  | AB975308/AB975334            | *Diarothe phaseolorum* AF001017
**Diarothe ceratotumiae** JQ444140 (CBS 131306) | 100/98       | 0                       | 0                 | 6.0                       | €               |                       |
| *Ascochya caurina*        | IOC-14:95  | AB975296/AB975322            | *Phoma betae* JX292134
**Pleospora calvata** EU754131 (CBS 233.25)
**Pleospora calvata** EU754131 (CBS 233.25) | 96/99        | 2.0                      | 0                 | 0                       | €               |                       |
| *Cochliobolus sp.*        | IOC-15:82  | AB975305/AB975331            | *Cochliobolus sp.* JX960591
**Cochliobolus geniculatus** JN941528 (NBRC 7407) | 100/99        | 0                       | 0                 | 2.0                       | €               |                       |
| *Phoma macrostoma*        | IOC-15:85  | AB975307/AB975333            | *Phoma macrostoma* DQ474092 (DAOM 17540)
**Ascochya sp.** AE808053 | 100/100       | 0                       | 0                 | 2.0                       | €               |                       |
| *Pleosporales sp.*        | IOC-15:91  | AB975309/AB975335            | *Pleospora sp.* HQ649967
**Birnur rzezianzidea** AY016356 (CBS 167.79) | 96/99        | 2.0                      | 0                 | 0                       | €               |                       |

1ATCC, American Type Culture Collection (USA); CBS, Centraalbureau voor Schimmel cultures, Filamentous funghi and Yeast Collection (The Netherlands); DAOM, National Mycological Herbarum (Canada); IFM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University (Japan); NHC, NBRC Culture Collection (Japan); NRR., Agricultural Research Service Culture Collection (USA).
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 (Freisen.) 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., Emericellopsis sp., Epicoccum nigrum Link, Alternaria sp., Lewia sp., Tolyposcladium cylindrosporum W. Gams and Nigrospora oryzae (Berk. & Broome) Petch from Hokkaido; Fusarium incarnatum (Desm.) Sacc., Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., C. boninense Moriwa, Toy. Sato & Tsukib., Diaporthe sp., Ascochyta caudina (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. They discussed that cultures of five species, including *P. herbarum* could not be
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distinguished using four loci (rDNA ITS regions, the protein encoding GPD and EF-1 alpha genes and the intergenic spacer between vmad and vpsA). Thus, further studies are required for precise identification of the Pleospora spp.

Petri and Fisher[13] reported three Pleospora species, namely, P. biortlingii 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., was isolated at the highest frequency, followed by Stagonospora sp. 1. Crabtree and Gessner[25] 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.[8] 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[12]. 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[15,16]. 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)[8]. Unidentified species of Alternaria and its teleomorph Levia were also isolated from the Hokkaido samples, albeit at a lower frequency.

In addition to the above-mentioned Pleosporales fungi, Cladosorium cladosporioides (Capnodiales) was isolated at a relatively high frequency. Another species of the genus, C. herbarum (Pers.) Link was frequently isolated from S. europaea in Canada[8]. Such dematiaceous fungi isolated from aerial tissues of various terrestrial plants are generally thought to be epiphytes or facultative endophytes[7,28]. 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[29] 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[8,12,13,15,16,26]. These fungi are known to produce high levels of pigment[30], which suggests that such pigment may protect the fungi inhabiting plants growing...
in high-salinity environments. Kogej et al.31 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 attack32. Dark-pigmented mycelia produced by *Camarosporium roumeguerii* Sac. are thought to absorb more UV radiation than white mycelia.7, English and Gerhardt33 reported that spores with dark-coloured walls showed greater resistance to UV radiation than those with light-coloured walls. Bell and Wheeler32 and Taborda et al.34 reported that melanin enhanced fungal resistance against UV radiation, wave desiccation and extreme temperatures. Suryanarayanan et al.35 suggested that melanin in the hyphae of *Phyllosticta capitalesis* 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.8,9 Thus, compounds such as melanin are likely to influence the survival rate of fungi that reside in the outer and/or inner environment of halophilic 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*10,12,13 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 speciality 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.

References


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