Identification of *Salicornia* Populations: Comparison between Morphological Characterization and RAPD Fingerprinting

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Abstract: In Japan, there are two taxa of the genus *Salicornia* plants; *S. europaea* L. distributed in Hokkaido and *S. herbacea* L. distributed on the coast of Inland Sea of Seto. To estimate the polymorphism of the *Salicornia* plants, we statistically analyzed the morphological features and random amplified polymorphic DNA (RAPD) of five groups from three populations found at Lake Tofutsu and Lake Notori in Hokkaido and Okayama Prefecture on the coast of Inland Sea of Seto. The morphological features, such as plant length, segment number, length and number of branches, and incidence of the secondary branches showed variations among locations. The morphological plasticity of *Salicornia* plants was also observed at different plant densities. Thereby these features were difficult to use for identifying the populations. On the other hand, the genotype based on the RAPD markers implied five groups: two groups from the Notori population, two groups from the Tofutsu population and one group from the Okayama population. Additionally the Notori and Tofutsu populations were identified as genotypically related, and different from the Okayama population. The RAPD method, which is one of the simplest and fastest molecular techniques, was found useful for identifying the type of *Salicornia* plant.

**Key words**: DNA fingerprinting, Genotype, Glasswort, Halophyte, Phenotype.

*Salicornia* plants are annual halophytes, which are widespread in salt marshes on the Eurasia Continent, North America, Middle East and South Africa. The plants can grow in the presence of high concentrations of sodium salt, since they may accumulate large amount of salts in the tissues during their growth period (Flowers et al., 1977; Momonoki et al., 1996). Recently, direct seawater irrigation has been attempted using halophytes in desert environments. *Salicornia* plants have emerged as a potential candidate for seawater crops, since the seeds are available for oil production and the plants can be eaten by some animals and fishes for livestock (Glenn et al., 1991; Belal and Al-Dosari, 1999; Noaman and El-Haddad, 2000). Furthermore, *Salicornia* plants are also used as a model plant for comparing salt resistance in many crops.

The first finding of *Salicornia* plants in Japan was around Lake Akkeshi in Hokkaido in northern Japan in 1891. These plants were identified as *Salicornia herbacea* L., and were also found along the Inland Sea of Seto in southwest Japan in 1913 (Makino, 1913). On the other hand, *Salicornia europaea* L. is distributed broadly in Europe and the northern part of the Eurasia Continent. Therefore the scientific name *Salicornia europaea* L. was adapted for the *Salicornia* plants distributed in Hokkaido Island in the mid-1950s. Then, two species of *Salicornia* plants in Japan have been described: *S. herbacea* L. on the coast of Inland Sea of Seto and *S. europaea* L. in Hokkaido Island.

Since the annual grasses generally tend to exhibit plasticity in phenotype (Niwa, 2002), classification of the *Salicornia* plants based on phenotype has been considered difficult. Since the summer of 1998, a new group of *Salicornia* plants appeared unexpectedly on the accumulated sand in an open space near Lake Tofutsu, located in eastern Hokkaido, habitats of many native *S. europaea* L.. The accumulated sand was brought from a depth of one meter from the bottom of Lake Tofutsu. The plants probably derived from buried seeds in the lakebed. However, their morphology was very different from the endemic populations growing naturally in this area.

The random amplified polymorphic DNA (RAPD) method (Williams et al., 1990) has been successfully used for differentiatin of bacteria species (Campbell et al., 2000) and as a tool for epidemiological and taxonomic studies. It also facilitates the characterization of DNA polymorphisms in plants (Crockett et al., 2000; Xie et al., 2001), insects (Beeman and Brown, 1999) and animals (Oliver et al., 1999). In order to develop a strategy for identifying *Salicornia* plants, we analyzed two groups from the Lake Tofutsu population (including newly found group), two groups from the Lake Notori population and one group from the Okayama population on the coast of Inland Sea of Seto using RAPD techniques. To assess the specific limit of RAPD, we compared the genotypic classification based on the RAPD patterns with morphological classification based on plant
length, segment number, length and number of branches and incidence of the secondary branches.

**Materials and Methods**

1. **Plant materials**
   The newly found group of *Salicornia* plants was named “Tofutsu-large” as illustrated in Fig. 1-A-a. All plant materials except for the Okayama population were collected in mid-August in 2000 from around Lake Notori (44° 04' N, 144° 12' E) and Lake Tofutsu (43° 56' N, 144° 20' E) in Hokkaido as shown in Fig. 1-B. Each of the Notori and Tofutsu populations was classified into

![Image A](image1.png)

![Image B](image2.png)

![Image C](image3.png)

**Fig. 1.** *Salicornia* plants used in this study. A a, Plants collected from Lake Notori and Lake Tofutsu; A b, Plant collected from Okayama Prefecture. B, The collection sites of *Salicornia* plants used in this study. C, Schematic diagram of *Salicornia* plants. The numbers and length of the primary and secondary branches were measured at the apical, middle and basal parts.
two groups of 'large' and 'ordinary'. Twenty to sixty plants were collected at random from each group by the quadrant method using an area of three square meters for the Tofutsu large group and a square meter for other groups. These plants were analyzed based on morphological features and DNA polymorphisms. To harvest seeds, we collected about 300 plants each, from the groups of Tofutsu-large and Notori-ordinary at the beginning of October in 2000 at the same places a described above. After that, the plants were dried in air for about 2 months and stored at 4°C in the dark.

Ten plant materials from the Okayama population, which equates with the Okayama group, were collected at random from a place in Okayama prefecture (34'26' N, 133'49' E) (Fig. 1-B) by the quadrant method using an area of five square meters in mid-October in 2000. Based on collection sites and phenotype of plants in each group, the provisional groups of the plants were defined as Fig. 1-A-a and -b. To estimate the plant morphology, we used ten to sixty plants from each group. For RAPD analysis, ten plants from each group were used.

2. Morphological comparison among populations

Comparison and characterization of plants from each population were made by measuring plant length, segment number, length of the primary branches, number of the primary and secondary branches and incidence of the secondary branches in the apical, middle and basal, parts of the plant portions as shown in Fig. 1-C. A data matrix was created using actual measurement values of each plant as multivariate data, including plant length, segment number, length of the primary branches, number of the primary and secondary branches and incidence of the secondary branches. To characterize the morphology of each plant, we performed the principal component (PC) analysis based on the data matrix. The dissimilarity matrix was computed from the data matrix to create the cluster dendrogram for all plants, and hierarchic cluster analysis was carried out based on the generated matrix by the method of Ward's linkage (Ward, 1963). All statistical analyses were performed using JMP 4J (SAS Institute Inc. Cary, NC, USA).

3. Cultivation of Salicornia plants

The seeds were harvested from about 300 plants each from the Notori-ordinary group, and Tofutsu-large group, at the beginning of October in 2000 and stored at 4°C in the dark. The plants were cultivated in a glasshouse at Tokyo University of Agriculture, Okhotsk campus. On April 16, 2001, the branches and stems with seeds mixed with the powder of branches and stems (about 150 g) were sown onto a plastic box (90 × 40 × 30 cm) filled with river sand. After 2 weeks, seedlings were transplanted into Wagner's pot (1/5000a), which was filled with washed river sand. The planting density was 10- or 200-plants/pot, with five replications for each plant group. The plants were grown in the solution of Shimose et al. with some modification (1987). Four weeks after sowing, when the plants had rooted, the salt treatment in the solution containing 1% NaCl started. Plant length, length and the number of the primary and secondary branches, were measured 12 weeks later.

4. Preparation of genomic DNA for polymerase chain reaction (PCR)

Genomic DNA of each Salicornia plant was extracted from the fine meal of the stems by benzyl chloride method (Zhu et al., 1993) using an ISOPLANT DNA extraction kit (Nippon Gene, Osaka, Japan) according to the manufacturer's instructions. The DNA was further purified by phenol/chloroform/isoamylalcohol extraction. To remove contaminating small fragments of DNA and RNA, we separated the DNA preparations by agarose gel electrophoresis (AGE) and extracted high molecular weight DNA from the gels using Easy Trap 2 DNA extraction kit (TaKaRa, Ohtsu, Japan).

5. RAPD-PCR

For the RAPD-PCR, sixty DNA dodecamers (DNA Oligomer (12) Set A-1–A-5; Nippon Gene) were used as random primers. The RAPD-PCR was performed in 25 μL of reaction mixture containing 5 ng of template DNA, 1 μM of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 1.25 units of Gene Taq polymerase (Nippon Gene). The PCR was carried out for 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 30°C for 1 min, and extension at 72°C for 1 min, using Gene-Amp automated thermal cycler (Model 9700, PE Applied Biosystems, Foster City, CA, USA). The RAPD fragments were separated by AGE and visualized by UV illumination after ethidium bromide staining and the presence or absence of each RAPD marker was observed. The bands which commonly appeared in all ten samples in each group, were defined as positive markers. On the other hand, the bands whose presence or absence varied with the plant individual in the same group, were not used as markers. The RAPD markers were scored as 1 for positive marker and as 0 for negative marker. To presume the genetic distance among each group, we compared the similarity matrix by the Nei and Li method (Nei and Li, 1979) using the scores, and created the phylogenetic tree by the unweighted pair group method for arithmetic averages (UPGMA). The calculation of the matrix and construction of the tree were performed with FreeTree software (available at http://www.natur.cuni.cz/~fllegr/programs/) (Hampel et al., 2001).

Results

1. Morphological characterization of Salicornia plants

As shown in Table 1, the plant length and segment
number were the highest in the Okayama population among all the populations. Both were approximately 3 times higher than the Notori-large and Tofutsu-large groups. The primary branches were longest in the Okayama population among all populations, and were over 100 mm length at the basal and middle parts (Table 2). The Okayama population was characterized by long stems.

Significant differences were found among the Notori and Tofutsu populations, in their morphologies (Tables 1 and 2). Especially, the Tofutsu-large group exhibited a high incidence of secondary branches. The Notori-large group had hardly any the secondary branches. Incident rates of the secondary branches at the basal, middle and apical parts of the plant in the Notori-large population were 58.1, 12.7 and 0%, respectively. By contrast, those in the Tofutsu-large were 96.9, 97.5 and 55.4%, respectively, which displayed wide spread of the branches just like a Christmas-tree shape.

To classify the Salicornia plants into phenotypic groups, we computed the dissimilarity matrix with actual morphological measurement values of each plant as multivariate data, and performed hierarchical cluster analysis with Ward’s linkage (Ward, 1963). From the results (Fig. 2), we clustered the plants predominantly into three groups, I, II and III. Group I was mainly composed of the Okayama population. Group II consisted of the newly found Tofutsu-large group, and group III consisted of the remaining groups of Tofutsu and Notori populations. Moreover, group III could be roughly separated into two subgroups consisting of the Notori-large group, and Notori-ordinary and Tofutsu-ordinary groups. The variations of Salicornia plant morphologies were examined by PC analysis. The first PC accounted for 57.14% of total variation and the second component represented 18.18%. Since the cumulative contribution percentage of the first two PCs was 75.32%, the two-dimensional graph clearly separates the Salicornia plants into three groups: newly found group, Okayama plants and remaining group as shown in Fig. 3.

2. Effect of planting density on Salicornia morphology

The Notori-ordinary and Tofutsu-large groups were planted at two densities, 10 and 200 plants/pot. As shown in Table 3, plant length at 10-plants/pot was 1.2 - 1.3 times higher than that at 200-plants/pot, and the difference was significant. The length of the primary branches tended to be larger at the low plant density except for the apical part of the plants in the Tofutsu-large group. The number of the primary branches in plants/pot exceeds those at 200 plants/pot in all parts of the plants. Consequently the plant lengths, the length and the number of the primary branches were highest in the low-density stand.

3. RAPD fingerprinting of Salicornia plants

By adapting RAPD-PCR using sixty oligonucleotides for the Salicornia plants, 192 RAPD markers were obtained. Of the 192 RAPD markers, 140 markers
appeared in every preparation, while 52 markers showed unstable exhibition depending on the preparation. Fig. 4 summarizes the RAPD markers that showed polymorphisms. Seven specific RAPD markers for identifying both of the Notori-ordinary and Notori-large groups were detected. A25-4 marker band did not appear specifically in the Notori population. All of these 8 markers were available to define the Notori species. As specific markers for Tofutsu and Okayama, one (A31-3) and nine bands respectively, were obtained. Inversely, as null markers for Tofutsu and Okayama, one (A25-2) and 13 bands, respectively, were obtained. In total, two markers for Tofutsu and 22 markers for Okayama were available to distinguish from each other. Additionally, specific markers were available: one for the Notori-ordinary group, 10 for the Notori-large group, one for the Tofutsu-ordinary group and one for the Tofutsu-large group.

Using the similarity matrix calculated from the positive and negative pattern of RAPD markers for \textit{Salicornia} plants, a phylogenetic tree was created by the UPGMA using the FreeTree software and the robustness of the tree topology was assessed by bootstrap analysis with repetitions value of 5,000. As shown in Fig. 5, the Okayama population was separated as a distinct group from the Hokkaido populations with a bootstrap value of 100%. Furthermore, the Hokkaido populations were divided into the Notori and Tofutsu populations, with a bootstrap value of 95%. Furthermore, the Notori and Tofutsu populations were divided into the ordinary and large types, but the bootstrap values were only 76 and 80%, respectively.

\textbf{Discussion}

\textit{Salicornia} plants in Japan have been identified mainly as two species. The plants distributed in Hokkaido Island have been conventionally recognized as \textit{S. europaea} \textit{L.}, and those on the coast of Inland Sea of Seto as \textit{S. herbacea} \textit{L.}. On the other hand, in 1998, new group of \textit{Salicornia} plants appeared on the accumulated sand brought from the lakebed of Lake Tofutsu. The \textit{Salicornia} plants in the newly found group exhibited typical morphology with a high incidence rates of the secondary branches, showing a Christmas-tree shape. Initially, we attempted to classify of the plant populations based on the morphological features. The plant materials were collected from two endemic populations in the neighborhood, Lake Notori and Lake Tofutsu, and additionally the specimen plants were obtained from Okayama Prefecture on the coast of Inland Sea of Seto. Furthermore, the Notori and Tofutsu populations were each classified according to plant size into two groups of large (including newly found plants) and ordinary. The Okayama population equates with the Okayama group.

According to the cluster and PC analyses by using the morphological measurements, the five groups from three populations were classified into three groups one consist-
Fig. 3. Scatter diagram of the first two principal components for plant morphology.

Table 3. Effect of planting density on plant length, length and number of the primary branches in Salicornia plants.

| Population (Group) | Notori-ordinary | Tofutsu-large |
|--------------------|-----------------|--------------|
| Plants/pot         | 200             | 10           | 200         | 10  |
| Plant length (mm)  | 161.1±6.0       | 203.2±12.8   | 150.6±7.2   | 197.7±5.3 |
| Length of the Apical primary branches (mm) | 8.5±2.8       | 25.2±3.8     | 20.7±2.2    | 14.4±1.5 |
| Middle             | 10.8±1.1        | 49.5±2.9     | 27.9±1.6    | 45.5±2.2 |
| Basal              | 20.6±1.6        | 105.1±4.8    | 33.1±1.6    | 94.8±3.5 |
| No. of the Apical primary branches | 0.8±0.2       | 5.2±0.9      | 2.6±0.5     | 7.5±0.8 |
| Middle             | 5.6±0.6         | 11.3±0.9     | 6.4±0.5     | 11.9±0.8 |
| Basal              | 6.5±0.6         | 11.4±1.3     | 2.6±0.5     | 7.5±0.8 |

Values are shown as means± standard errors.

Band Primer

Notori-ordinary-large

Okayama

Tofutsu-ordinary-large

* newly found population

Fig. 4. RAPD markers that showed polymorphism of the Salicornia plants. Each column corresponds to plant materials and each row to marker bands. The black and white boxes indicate the presence and absence of markers, respectively.

ing of the Okayama group, one consisting of the Tofutsu-large (newly found) group, and third group consisting of the Tofutsu-ordinary, Notori-large and Notori-ordinary groups. The Salicornia plants tend to have phenotypic variation depending on the environmental conditions such as temperature, quality of soil, concentration of salt, and density of population (Ungar et al., 1979; Ellison, 1987; Boorman et al., 2001). Thereby Ball (1964) suggested that the specific limits of the classification of the Salicornia plants based on the mor-
phological features, especially based on those of the dried Salicornia plants are obscure. To prove the relevance between genotype and phenotype in the Salicornia plants, we analyzed the genetic variability by RAPD fingerprinting.

The genotype based on the RAPD markers implied that the Notori-ordinary and Notori-large groups were closely related with each other. The Tofutsu-ordinary group was different from the Notori-ordinary group indicating that the Tofutsu population was derived from an ancestry different from that of the Notori population. Furthermore, the RAPD revealed that in the Tofutsu population, the newly found large group was closely related with the ordinary group. On the other hand, the Okayama population was quite different from all other populations.

Consequently, the Salicornia populations used in this study were divided into the following three genetic groups: group 1, consisting of the Notori-large and Notori-ordinary groups; group 2, consisting of the Tofutsu-ordinary and newly found Tofutsu-large groups; and group 3, consisting of the Okayama population. Thus, the discrepancy was observed between phenotypic and genotypic classifications of the Salicornia populations. In the experimental cultivation, the Salicornia plant grown from identical population seeds exhibited significant diversity in their morphology at a different planting density. Moreover, the size and shape of the Salicornia flower also showed wide diversity depending on the salt concentration in the soil (data not shown). Consequently the classification of Salicornia plants based on the morphology was also obscure in this study as suggested by Ball (1964).

On the other hand, the Salicornia populations were certainly classified into interzonal groups depending on the UPGMA phylogenetic tree with high robustness of 95–100% bootstrap values. Use of molecular markers is considered to be best for analysis of genetic diversity since there is no effect of stage of development, environment or management practices (Choudhury et al., 2001) and is available for even dead plants when the genomic DNA is extractable. Furthermore, among the available molecular marker systems, the RAPD technique is the fastest and simplest (Choudhury et al., 2001). Altogether, RAPD fingerprinting may be a reliable strategy for identification of the type of plant populations.

Why did the newly found Salicornia plants exhibit superior growth with high incidence rates of the secondary branches? According to Ellison (1987), the branching of individual Salicornia plants is decreased probably due to reduced light availability in dense stands. Furthermore, the growth of Salicornia plants is affected by both interspecific and intraspecific competition (Ungar et al., 1979).

In the newly found Salicornia group in the Tofutsu population, the density of the plants was sparse compared with other populations, because the habitat was open place without any competitive plants. In fact the results of cultivation under the experimental condition with a different planting density showed that the plant vigor of the Salicornia tended to increase in a sparse condition. Consequently, it was speculated that the seeds could be recovered for germination at optimum environmental conditions after digging them out from a lakebed, and then the plants grew superiorly.

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*In Japanese*