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Effect of environmental gradient in coastal vegetation on communities of arbuscular mycorrhizal fungi associated with *Ixeris repens* (Asteraceae)

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Abstract The community structure of arbuscular mycorrhizal (AM) fungi associated with *Ixeris repens* was studied in coastal vegetation near the Tottori sand dunes in Japan. *I. repens* produces roots from a subterranean stem growing near the soil surface which provides an opportunity to examine the effects of an environmental gradient related to distance from the sea on AM fungal communities at a regular soil depth. Based on partial sequences of the nuclear large subunit ribosomal RNA gene, AM fungi in root samples were divided into 17 phylotypes. Among these, five AM fungal phylotypes in *Glomus* and *Diversispora* were dominant near the seaward forefront of the vegetation. Redundancy analysis of the AM fungal community showed significant relationships between the distribution of phylotypes and environmental variables such as distance from the sea, water-soluble sodium in soil, and some coexisting plant species. These results suggest that environmental gradients in the coastal vegetation can be determinants of the AM fungal community.

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Introduction

Coastlands are a stressful environment with low fertility, high salinity, intermittent drought, changeable temperature, unstable sandy substrate, etc. Plant species found in this environment, known as coastal plants, are specifically adapted to the extreme environmental conditions. Usually, a transition of the coastal plant species is found corresponding to the environmental gradient with distance from the sea, the vegetation closest to the seaside experiencing the most stressful conditions. Similar to coastal plants, some arbuscular mycorrhizal (AM) fungi may be specifically adapted to the coastal environment. For example, isolates of the AM fungi *Glomus geosporum* and *Glomus mosseae* from a salt marsh showed significantly higher spore germination ability compared to non-marsh spore isolates under increased levels of salinity (Carvalho et al. 2004). Many studies have reported AM fungal spores collected from coastal sand dunes (see review by Sridhar and Beena 2001). Also, Koske (1975) reported that the density of AM fungal spores was greater in stabilized dunes than that in foredunes in New South Wales, Australia. Applying molecular methods, Yamato et al. (2008) examined AM fungal communities associated with *Ipomoea pes-capre* within 10 m from the seaward forefront of coastal vegetation in Okinawa, Japan, and found dominance of some closely related AM fungi.

However, extensive studies are lacking on the effect of environmental gradients on AM fungal communities in coastal vegetations. In the present study, we examined the

AM fungal community associated with *Ixeris repens* (L.) A. Gray (Asteraceae), a coastal plant distributed in East Asia (Kitamura et al. 2002), in order to determine whether specific coastal AM fungi are found and whether changing environmental gradients alter the AM fungal communities with increasing distance from the sea. *I. repens* grows in a wide stretch of coastal vegetation near the Tottori sand dunes in Tottori Prefecture, Japan, which was selected as the study site. This vegetation is not fragmented by any roads, and the environmental gradients are uninterrupted. A mycorrhizal association was reported in roots of *I. repens* by Funatsu et al. (2005), but the causal AM fungi were not identified. This plant produces roots from a subterranean stem growing approximately 1–3 cm below the soil surface, and the roots grow from the base of leaves, enabling easy identification of root locations from above ground. Consequently, the effects of an environmental gradient related to distance from the sea on AM fungal communities can be examined in root samples taken at a regular soil depth.

Materials and methods

Sampling

The study site (35°32′30″ N, 134°12′30″ E) was located in the Arid Land Research Center at Tottori University in Tottori Prefecture, Japan. The mean annual temperature and the total annual precipitation recorded at the nearest meteorological station (Tottori) were 14.9 °C and 1,914.0 mm, respectively (averages of 1981–2010). A flat sand beach extends for approximately 100 m from the shoreline and then transitions to sand dunes. The edge of the vegetation was located about 30 m from the shoreline. *I. repens* and *Calystegia soldanella* (L.) R. Br. (Convolvulaceae) were widely distributed, and four other plant species were common: *Wedelia prostrata* (Hook. & Arn.) Hemsl. (Asteraceae), *Carex kobomugi* Ohwi (Cyperaceae), *Ischaemum anthephoroides* (Steud.) Miq. (Poaceae), and *Zoysia macrostachya* Franch. & Savat. (Poaceae).

A sampling plot of 20×50 m was established in the vegetation on June 9, 2010, with the 20-m long side facing the sea. The long axis (50 m) of the study plot was almost flat with less than 2 m difference in altitude between the two extremes. In the study plot, 37 sampling points were randomly chosen (Table S1). At each sampling point, a sampling area of 20×20 cm was established, and surface soil was removed from around a leaf of *I. repens* to expose the subterranean stem. To collect the roots, 100 ml of a soil core sample, 5 cm in depth, was taken from under the leaf. In each sampling area, three soil cores were collected and mixed to form one sample. For almost all samples, only roots of *I. repens* were collected. All plant species found in a 1.0×1.0-m area surrounding the sampling areas were recorded.

Soil chemical analysis

Soil pH (soil to water, 1:2.5, *v*/*v*) and available phosphate (Truog-P; Truog 1930) were measured for all soil samples. Water-soluble Na-ion concentrations (soil Na) were analyzed by atomic absorption spectrophotometer Z-2310 (Hitachi High-Technologies, Tokyo, Japan) after extraction (soil to water, 1:5, *v*/*v*).

Root colonization by AM fungi

All roots collected from sampling sites were cut into small segments (approximately 1 cm), and a small subsample (approximately 20 mg fresh weight) was used for determination of mycorrhizal colonization levels. The roots were cleared with 10 % KOH and stained with 0.05 % trypan blue. Colonization levels were determined using the gridline intersection method (Brundrett et al. 1996), based on three times of analyses at least 100 intersections per sample.

Molecular analysis

After sampling for root colonization analyses, remaining roots (24.2–190 mg fresh weight) were used for the molecular identification of AM fungi. Total DNA was extracted from each root sample using the DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. Partial sequences (approximately 750 bp) of nuclear large subunit ribosomal RNA gene (LSU rDNA) were amplified by polymerase chain reactions (PCR) from the extracted DNA using Takara Ex Taq™ Hot Start Version (Takara Bio, Otsu, Japan) using the universal forward primer LR1 (van Tuinen et al. 1998) and the fungal-specific reverse primer FLR2 (Trouvelot et al. 1999). The PCR reaction mixture contained 1.0 μl extracted DNA, 0.75 units Taq polymerase, 0.25 μM each primer, 200 μM each dNTP, and 3.0 μl manufacturer's PCR buffer, in a total volume of 30 μl. The PCR program (PC-818S Program Temp Control System; Astec, Fukuoka, Japan) was: 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min, then 72 °C for 5 min. When direct PCR was unsuccessful, nested PCR was applied. First round PCR was performed with the primers LR1 and NDL22 (van Tuinen et al. 1998) using the same program with 30 cycles, and second round of PCR was performed using 1.0 μl of a 1:100 dilution of the first PCR product with the primers LR1 and FLR2 using the same program. It was confirmed that the primers LR1 and FLR2 show complete match with most arbitrarily selected AM fungi in the four orders Archaeosporales, Diversisporales, Glomerales, and Paraglomerales (Table S2).

PCR products were cloned using the pGEM-T Easy Vector System I (Promega, Madison, WI, USA). For each PCR product, at least 16 clones were randomly chosen, and plasmid DNA was extracted using MagExtractor-Plasmid (Toyobo, Tokyo, Japan). The DNA inserts were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit using T7 and SP6 promoter primers on a 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). Sequences were subjected to BLAST searches (Altschul et al. 1997), and those not identified as Glomeromycota fungi were excluded from further analyses.

Multiple sequence alignment was performed using ClustalX 2.0.12 (Larkin et al. 2007). Neighbor joining analyses (Saitou and Nei 1987) were performed for the aligned data sets using ClustalX with bootstrap analysis of 1,000 replications (Felsenstein 1985). AM fungal phylotypes were defined based on sequence similarities computed by Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI). The rarefaction curve was computed for each sample by plotting the number of AM fungal phylotypes detected against the number of sequences using the Analytic Rarefaction 1.3 software (Hooland 2003). For each sample, additional clones were sequenced until the rarefaction curve tended to plateau.

Representative DNA sequences were selected for each AM fungal phylotype, according to the tree topology and deposited in the DNA Data Bank of Japan database with accession numbers AB670043–AB670115. The selected sequences were subjected to BLAST searches, and similar sequences were downloaded from GenBank. For the set of sequenced and downloaded data, multiple sequence alignments were carried out as described above. For phylogenetic analysis, the maximum likelihood (ML) method was applied with PhyML 3.0 (Guindon et al. 2010). The best fit ML tree was inferred under the GTR model. To check statistical support for the tree topology obtained, bootstrap analysis was performed with 1,000 replications. The tree obtained in the analysis was drawn using Treeview software (Page 1996).

Statistical analysis

Correlations between distance from the shoreline and number of plant species around the sampling point, and between number of plant species and AM fungal phylotypes, were examined using Pearson's coefficient test. In addition, correlations among the environmental variables (distance from the shoreline, soil Na, soil pH, available soil P) and AM fungal colonization levels were examined.

Multivariate analyses were applied using CANOCO 4.5 (ter Braak and Smilauer 2002) in order to infer relationships between the AM fungal community and the environmental factors. In the data in the response variables table (distribution of AM fungal phylotypes), the presence or absence of each phylotype in each sample was scored by "1" or "0." For the explanatory (environmental) variables data, distances from the shoreline, soil Na, and soil pH were used as quantitative variables. Coexisting plant species were used as nominal variables when plant species were found at more than three sampling points.

Detrended correspondence analysis (DCA) was first applied to the response variables data to estimate heterogeneity in species turnover units throughout the length of the community composition gradients. After confirming the length of composition gradients on the first DCA axis, principal component analysis (PCA) was applied to infer relationships between AM fungal distribution and environmental variables. PCA was performed by scaling interspecies correlations, with division by standard deviation and centering per species. The resulting diagram was visualized using CanoDraw.

To explain the effects of environmental variables, redundancy analysis (RDA) was applied by scaling interspecies correlations, with division by standard deviation and centering per species. Monte Carlo permutation tests with unrestricted 999 permutations were then performed for manually selected environmental variables.

Results

Vegetation analysis

Among the plant species recorded in the 1.0-m^2 area surrounding the sampling point (Table S1), *I. repens* and *C. soldanella* were dominantly found within 20 m of the seaward forefront of the vegetation, which suggests that these plants have a high tolerance for salinity. Meanwhile, in the inland portion of the plots, *W. prostrata*, *C. kobomugi*, and *I. anthephoroides* were dominant, together with *I. repens*. The number of plant species found around each sampling point (1 m^2) increased significantly with the distance from shoreline (R=0.664, P<0.001).

Soil chemical analysis and AM colonization

The chemical properties of the examined soils are shown in Table S1. Soil pH ranged from 5.9 to 7.6, with an average of 6.9. No correlation was found between distance from the shoreline and soil pH. Available soil P ranged from 3.8 to 6.3 mg kg^{-1}, with an average of 4.9 mg kg^{-1}, indicating low soil fertility. A positive correlation was found between the distance from the shoreline and the available soil P (R=0.541, P<0.001). Water-soluble soil Na levels ranged from 8.1 to 25.8 mg kg^{-1}, with an average of 13.7 mg kg^{-1}. Soil Na was negatively correlated with distance from the shoreline (R=−0.669, P<0.001). AM colonization levels were variable (0–52.8 %), with an average of 17.4 %. No correlation was found with any environmental variable.

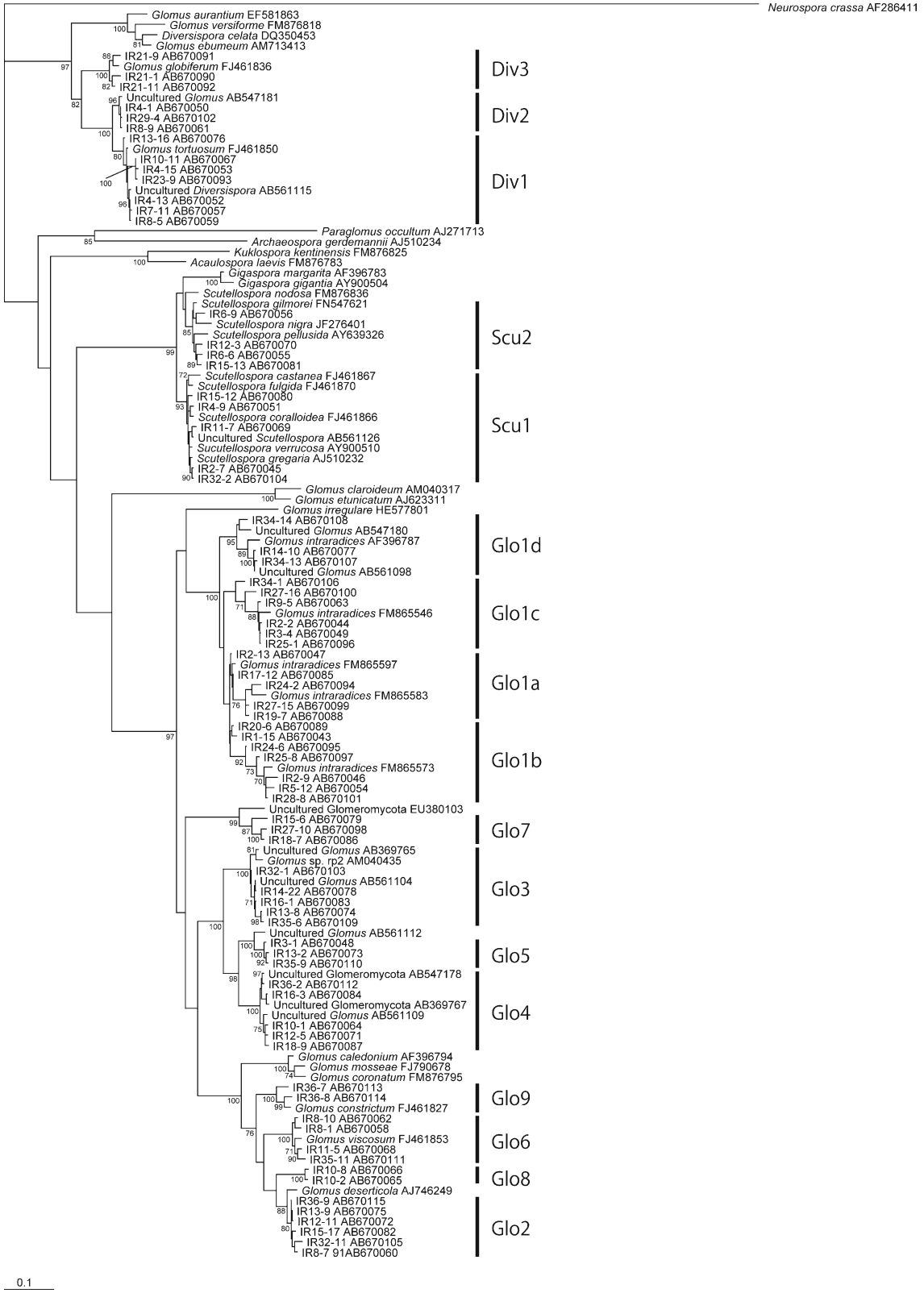

Fig. 1 Maximum likelihood phylogenetic tree based on partial large subunit sequences of the nuclear ribosomal RNA gene (LSU rDNA) of Glomeromycota obtained from *I. repens* in a coastal vegetation in Tottori Pref. and the GenBank database. The tree is rooted to *Neurospora crassa* (AF286411) in Ascomycota. The sequence numbers relate to sample number (IR1–IR37) and the clone numbers. The phylotypes (Glo1a-d, Glo2-9, Scu1, Scu2, Div1-3) are shown. Bootstrap values are shown where they exceed 70 % (1,000 replications). The scale is shown so that

Molecular analysis

A total of 597 partial sequences of LSU rDNA of AM fungi were obtained from 31 samples of *I. repens* roots (Table S3), of which 19 samples were analyzed by one-step PCR and 12 samples with nested PCR. The ML analysis resulted in one ML tree (ln L=−13,124.01212; Fig. 1). The sequences obtained were divided into 17 phylotypes, where each phylotype had a sequence similarity score of more than 95 %. Twelve of the phylotypes grouped in the genus *Glomus* (Glo1a–1d and Glo2–9), two in *Scutellospora* (Scu1 and Scu2), and three in *Diversispora* (Div1–3). Sequences of *Glomus intraradices* were found in the four phylotypes Glo1a–1d and were thus defined as subclades of Glo1. One sequence corresponded to *Glomus irregulare* (HE577801), an AM fungal species recently separated from *G. intraradices* (Błaszkowski and Czerniawska 2008; Stockinger et al. 2009), and was not included in the Glo1 clade.

Glo1a was the most frequent AM fungal phylotype detected in the analysis of *I. repens* roots. A total of 158 sequences of this phylotype were obtained from 17 samples (Table S3). All Glo1c sequences were obtained with Glo1a sequences in these same samples. Div1 and Div2 sequences also occurred together in 10 of the samples. Div1 formed a clade with *Glomus tortuosum* (FJ461850). Div3, found in only one root sample of *I. repens* (IR30), was closely related to Div1 and Div2 and formed a clade with *Glomus globiferum* (FJ461836). Div3 fungi were also detected in *I. repens* roots at 0.3 m from the seaward forefront of the vegetation. Glo2, Glo6, Glo8, and Glo9 phylotypes were somewhat related to one another in occurrence. Glo2 was closely related to *Glomus deserticola*, Glo6 to *Glomus viscosum*, and Glo9 to *Glomus constrictum*. Glo3, Glo4, and Glo5 formed a clade in which no previously identified AM fungi were included. *Scutellospora* species were also detected with a division into Scu1 and Scu2. Scu1 was found in eight samples, and Scu2 was found in six samples. Scu1 was closely related to *Scutellospora collaroidea*, *Scutellospora verrucosa*, and *Scutellospora gregaria*, whereas Scu2 was closely related to *Scutellospora gilmorei*, *Scutellospora nigra*, and *Scutellospora pellucida*.

AM fungal community composition and environmental variables

The number of AM fungal phylotypes detected in *I. repens* root samples significantly increased with distance from the shoreline (R=0.602, P<0.001). Five phylotypes, Glo1a, Glo1b, Glo1c, Div1, and Div2, were dominant within 11.5 m of the seaward forefront of the vegetation (Fig. 2). A weak but positive relationship was also found between the number of plant species and the number of AM fungal phylotypes (R=0.459, P<0.01).

Multivariate analyses were applied to AM fungal phylotypes that occurred in at least three samples. The length of the community composition gradients of the first axis was computed to be 3.64 from DCA. Based on this result, PCA was applied as the linear ordination method (Fig. 3). The eigenvalues of the first and second axes were 0.426 and 0.159, respectively. The cumulative percentage variance of species data showed that the first two PCA axes explain 58.5 % of the variability in species data. The resulting ordination diagram (Fig. 3) indicates that the soil Na gradient is related to distance from the shoreline. Glo1 occurred preferentially in a higher soil Na range, whereas Glo3, Scu1, and Scu2 preferred lower soil Na. Glo2 and Glo6 were more frequent at relatively higher pH.

Analysis of coexisting plant species showed that *C. soldanella* was more common in the higher soil Na range near the seaward forefront of the vegetation, whereas *W. prostrata* preferred the lower soil Na range. The Monte Carlo permutation tests on RDA showed that distance from the shoreline, soil Na, and presence of coexisting plants (*W. prostrata* and *C. soldanella*) had significant relationships with the distribution of the AM fungal phylotypes (Table 1).

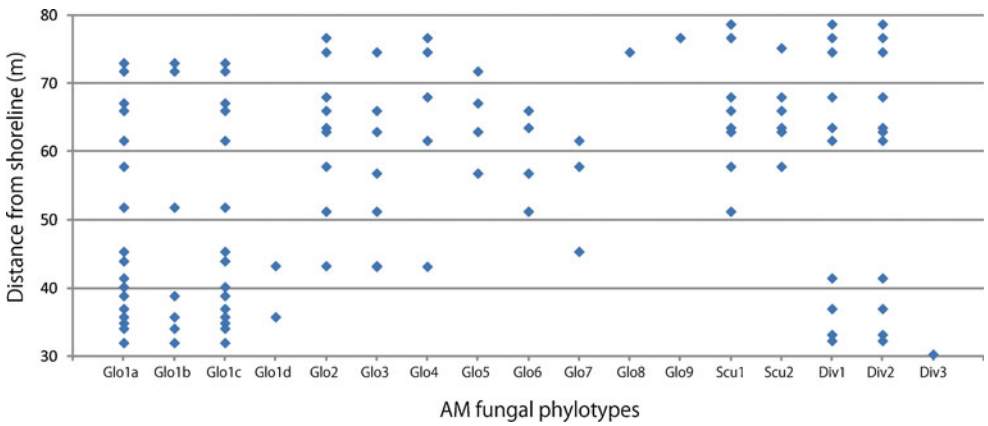

Fig. 2 Distribution of the AM fungal phylotypes in relation to distances from the shoreline

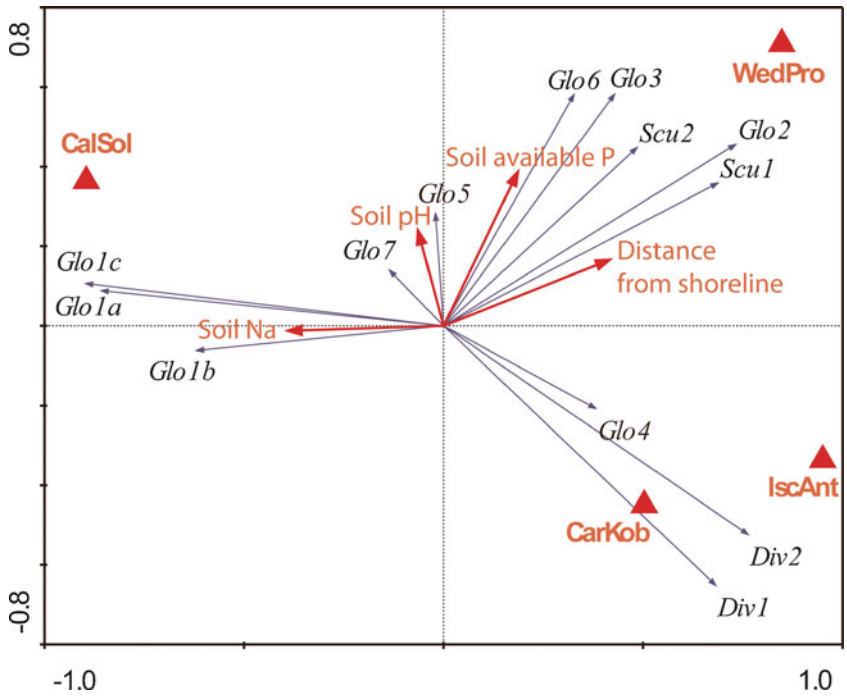

Fig. 3 Diagram of a PCA of arbuscular mycorrhizal fungal communities with environmental variables, distance from shoreline, soil Na, soil pH, soil available P, and coexisting plant species in the examined coastal vegetation in Tottori Pref. The eigenvalues of the first and second PCA axes were 0.426 and 0.159, respectively. *CalSol*, *C. soldanella*; *CarKob*, *C. kobomugi*; *IscAnt*, *I. anthephoroides*; *WedPro*, *W. prostrata*

Discussion

Because plant species can be determinants of AM fungal diversity (Sanders 2002), this study focused on a single plant species to examine the effects of environmental variables on AM fungal community composition along a coastline gradient. Some coastal plants grow deep roots, to more than 1 m (Abe and Katsuya 1995). This can be problematic for sampling because depth can also be a determinant of AM fungal distribution (Abe and Katsuya 1995). To overcome this, *I. repens* was selected as the plant species in the coastland site studied. The fact that this plant is widely distributed in the coastal vegetation and that its roots can be collected at a consistent soil depth made it appropriate to examine the effects of environmental gradients on the AM fungal community.

Mycorrhizal colonization of the examined *I. repens* roots was variable, and environmental variables showed a very limited relationship to colonization levels. Forty percent of the root samples were colonized by AM fungi to less than 10 %. This observation may indicate that AM fungal inoculum is sparsely and unequally distributed in the coastland soil environment. Roots of *I. repens* are produced from many sites on one subterranean stem, which may compensate for the lower levels of AM colonization in some parts.

Molecular analysis of the AM fungi associated with *I. repens* roots revealed 17 phylotypes across the coastland study site. Monte Carlo permutation tests on RDA showed that distance from the shoreline and soil Na had significant relationships with phylotype distribution, which suggests that environmental gradients in the coastland affect the AM fungal community. The distribution of two coexisting plant species, *W. prostrata* and *C. soldanella*, also had significant relationships with phylotype distribution; these plants may share AM fungal communities with the roots of *I. repens*. Similar to coastal plant species specifically adapted to the stressful environment, the *Glomus* and *Scutellospora* phylotypes identified in the present study site may be adapted to the coastal environment.

From the phylogenetic analysis, Glo1 was closely related to *G. intraradices* (Schenck and Smith 1982). This *Glomus* species is known as a generalist found in various environments, including some harsh conditions with high aridity, salinity, acidity, etc. (Öpik et al. 2006; An et al. 2008; Yamato et al. 2008, 2009), suggesting that it is highly tolerant of various stressful environmental factors. Glo2 was closely related to *G. deserticola* (Trappe et al. 1984), an AM fungal

Table 1 Result of Monte Carlo permutation tests (999 permutations) on RDA for the relationships with environmental variables including coexisting plant species on arbuscular mycorrhizal fungal community

	F value	*P* value
Distance from shoreline	2.80	0.023
Soil Na	2.69	0.029
Soil P	1.76	0.116
Soil pH	0.86	0.499
W. prostrata	3.26	0.024
C. soldanella	3.17	0.021
C. kobomugi	1.38	0.201
I. anthephoroides	0.62	0.737

species known as one of the most common AM fungi in arid and coastal environments (Trappe et al. 1984; Beena et al. 2001). Glo3 formed a clade with an uncultured *Glomus* (AB561104), while Glo4 and Glo5 were closely related to the sequences AB561109 and AB561112 of another uncultured *Glomus* previously identified from coastal vegetation in Ishikari, Hokkaido, Japan (A. Kawahara, personal communication). Glo6 formed a clade with *G. viscosum* (Walker et al. 1995), and Glo9 formed a clade with *G. constrictum* (FJ461827); both fungal species having been reported in arid environments (Requena et al. 1996; Ferrol et al. 2004; Dandan and Zhiwei 2007). Div1 formed a clade with *G. tortuosum* (Schenck and Smith 1982), which has been isolated from a coastal dune plant, *Elymus mollis* Trin. in Ibaraki Prefecture, Japan (Abe et al. 1994). The sequence AB561115 of an uncultured *Diversispora* detected from coastal vegetation in Ishikari, Hokkaido, Japan (A. Kawahara, personal communication) was also included in this clade. Div3 formed a clade with *G. globiferum* (Koske and Walker 1986a), which has been reported from a coastal sand dune in Florida (Sylvia 1986). The *Glomus* species phylogenetically characterized as Diversisporales have not been formally renamed because of the few morphological characteristics that distinguish them from other *Glomus* species (Redecker et al. 2007; Schüßler and Walker 2010).

Scutellospora phylotypes detected in the coastland study site were separated into two clades, Scu1 and Scu2. However, many *Scutellospora* species were included in the clades, suggesting that the LSU region is not appropriate for the species-level distinction of *Scutellospora* fungi. *Scutellospora* species have previously been reported in sand dune ecosystems (Koske and Walker 1986b; Koske and Gemma 1995). Although spores of *Gigaspora* species have been reported from sand dunes (Koske and Halvorson 1981; Beena et al. 2000), no *Gigaspora* sequences were found in this study.

In conclusion, the present observations indicate that some AM fungi may be specifically adapted to the coastal environment. Previous studies have shown that the AM symbiosis can reduce the concentration of Na in shoots of plants growing in saline conditions (Dixon et al. 1993; Sharifi et al. 2007; Zuccarini and Okurowska 2008; Yamato et al. 2008), which may be important in allowing coastal plants to grow in saline conditions. Further studies are required to determine the role of the functional diversity of AM fungi in coastal environments for improvement of plant resistance to saline conditions.

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