Research paper

Using a PCR-DGGE Method to Analyze Diazotrophic Diversity in Soil and Root Nodules of *Casuarina* in the Sihu Area

Tsung-Po Chang,[1] Chun-Hsiung Hung,[2] Yu-Ting Wu,[3] Lei-Chen Lin[4,5]

【Summary】

Casuarina spp. are major windbreak species in coastal forests of Taiwan. With great ability for rapid growth and adaptability to adverse environmental conditions, they enhance soil stabilization and provide protection and rehabilitation for coastal areas. Many factors may limit the reproduction of *Casuarina* in coastal areas such as infertile soil, drought, salt stress, litterfall, and weeds. In addition, nitrogen is generally considered one of the major nutrients in plant growth, and *Casuarina* plants can obtain nitrogen sources through association with *Frankia* via root nodules. In order to understand the interaction between diverse diazotrophic diversity and different test sites of *Casuarina* spp. determine whether it is the diversity level of diazotrophic on healthy and on declining *Casuarina* spp., or the number of nitrogen-fixing bacteria in the nodules of *Casuarina* spp., direct amplification of the *nifH* gene for a polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis with the primer pair polF-GC/polR was applied in this study. The results demonstrated that there were 2 groups of nitrogen-fixing bacteria collected from these root nodule samples that appeared on the PCR-DGGE profiles. Whether *Casuarina* stands are on the decline or not, a high diversity of diazotrophic community has been observed among all test sites. Therefore, this study shows that the declining *Casuarina* is not related to the diazotophic diversity.

Key words: *Casuarina equisetifolia*, denaturing gradient gel electrophoresis (DGGE), diazotrophic diversity, *Frankia*, *nifH* gene, root nodule.

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研究報告

利用PCR-DGGE技術分析四湖地區木麻黃林分土壤及根瘤之固氮微生物多樣性

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摘 要

木麻黃是臺灣沿海地區主要之防風樹種，其具有生長快速及廣泛的逆境適應能力，可提供土壤穩定、沿海保護及棲地復育之功能。在臺灣西部沿海木麻黃防風林遭遇到許多天然更新之限制因子，例如：土壤貧瘠、乾旱、鹽分逆境、枯落物及雜草競爭。然而，氮元素通常是植物生長主要的限制因子之一，木麻黃植物可以透過與固氮菌*Frankia*共生形成根瘤，藉此，獲得氮源。為了瞭解不同木麻黃林分與固氮微生物多樣性之交互關係，健康的木麻黃林分與衰敗的林分相較，是否具有較高的固氮微生物多樣性，以及木麻黃根瘤內具有多少株固氮微生物。本研究藉由引子polF-GC/polR直接擴增*nifH*固氮基因，並進行變性梯度膠體電泳分析，以了解土壤中固氮微生物之多樣性。研究結果顯示木麻黃根瘤中應具有2群團之固氮微生物，而不同木麻黃林分土壤中具有高度的固氮微生物群落多樣性，無論林分衰敗與否。因此，本研究證實木麻黃林分之衰敗與固氮微生物多樣性無直接的關係。

關鍵詞：木賊葉木麻黃、變性梯度膠體電泳、固氮菌多樣性、*Frankia*、*NifH*基因、根瘤。

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INTRODUCTION

Most coastal sandy soils lack organic matter and are deficient in nutrients for plant growth (Chen et al. 2008). Nitrogen is an essential element for plant growth in the forms of amino acids, proteins, nucleic acids, and other nitrogen-containing components (Mengel and Pilbeam 1992). Although 80% of the atmosphere is nitrogen (N_2), N_2 is difficult to be directly used by plants. Nitrogen-fixing microbes are able to convert nitrogen gas from the atmosphere into ammonium (NH_4^+) in a process of biological nitrogen fixation possibly located in root nodules and induced by symbiotic, associative, and free-living soil bacteria including 2 major bacterial groups: *Rhizobia* and *Frankia* which can form nodules to fix nitrogen symbiotically within vascular plants (Franche et al. 2009). *Frankia* can form root nodules in almost all genera of the Casuarinaceae, including *Casuarina* plants (Benson and Clawson 2000).

Casuarina spp. are the most important coastal forests, which grow rapidly with broad adaptability for stress tolerance and provide soil stabilization, coastal protection, and rehabilitation (Liao 2011, Sayed 2011, Deng et al. 2013). *Casuarina* stands can only sustain 20~30 yr of growth and decline thereafter (Sheu 2006), and it is difficult for them to naturally regenerate on the west coast of Taiwan (Liao 2011). Recently, considerable natural generating saplings were discovered in the Sihu area since 2010 (Deng et al. 2013). However, symbiosis with *Frankia* is an important factor for the survival of *Casuarina* plants under various conditions. Infor-

mation on *Frankia* populations in different stand conditions is scant. Therefore, the study of *Frankia* is very important for improving *Casuarina* plant growth and survival under different conditions (Sayed 2011).

The polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique based on the *nifH* gene has been one of the common molecular tools to study diazotrophic assemblages in different ecosystems (Zehr et al. 2003, Mårtensson et al. 2009). We used the PCR-DGGE technique to explore the diversity of *Frankia*-associated endophytic nitrogen fixers in root nodules of *Casuarina*, and the diazotrophic community in *Casuarina* stands at different sites.

MATERIALS AND METHODS

Sample collection

Four sites with dead, declining, healthy, and regenerating *Casuarina* stands were selected in Sihu Township, Yunlin County, Taiwan (23°40′35″N, 120°9′40″E) in June 2013 (Fig. 1). Soils were randomly sampled thrice from the surface soil (0~15 cm in depth) in a quadrate of approximately 15×15 cm at each site. Approximately 5~7 ml of each replicate was homogenized into 1 composite sample. Root nodules were collected from *Casuraina* trees at the sites of healthy growth stand; we did not find nodules at other sites. All of the samples were kept at 4℃ to preserve them.

Soil was collected from 4 sites of windbreak forests at Sihu of western Taiwan based on growth situations of *Casuarina* stands, including dead trees (Fig. 2A), decline in growth under water stress (Fig. 2B), healthy growth (Fig. 2C), and natural regeneration (Fig. 2D).

DNA extraction and the PCR

DNAs from soil samples and root nodules were extracted using a NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) and the

Fig. 1. Location of the sampling sites in the Sihu area.

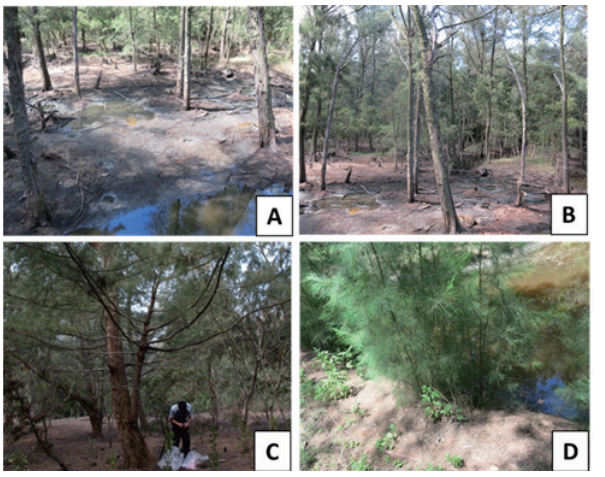

Fig. 2. Four test samples were collected from *Casuarina equisetifolia* in the Sihu area. A, Site of dead trees; B, site of a declining area; C, site of healthy trees; D, site of a regeneration area.

CTAB method (Möller et al. 1992), respectively. These DNAs were used in the PCR.

The polF/polR primers were used to amplify the *nifH* gene in all samples (Poly et al. 2001). To do the DGGE analysis, a GC clamp was added to the 5' end of the polF primer (Ferris et al. 1996). The PCR contained 2×PCR master mix (Promega, Madison, WI, USA), 10 ng of a DNA template, and 2 μM of each primer. Amplification was performed in an iCycle™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR products were checked on a 1.5% agarose gel and then stored at 4℃ until the DGGE analysis.

DGGE analysis

PCR products were separated on an acrylamide-bisacrylamide gel with a 30~70% denaturing gradient in 1×TAE at 60℃ and 80 V for 12 h with a Dcode system (BioRad). Gels were stained with ethidium bromide (EtBr) and visualized by a UV transilluminator (G: Box, Syngene, Frederick, MD, USA). PCR-DGGE band profiles were analyzed and compared using Sorensen pairwise similarity coefficients (Cs), which were determined by: $Cs = (2j/(a+b)) \times 100$, where a is the number of bands in lane 1, b is the number of bands in lane 2, and j is the number of common bands (Gillan et al. 1998). Clustering of groups was performed by the unweighted pair group method using arithmetic averages (UPGMA) with MVSP software (Kovach 1999).

Sequencing

The bands of root nodule samples were excised from the DGGE gels, and extracted by repeat transfer from room temperature to -80℃ three times. The extracted DNAs were re-amplified using the primers polF and polR without the GC-clamp. Sequencing was performed using the ABI 3730XL system (Genomics BioSci & Tech Co., New Taipei City, Taiwan).

Sequence alignment and analysis

The nucleotide sequences of successful sequencing were aligned using Clustal W running in Bioedit (vers. 7.1.3.0) (Hall 1999). A sequence blast was performed with a basic local alignment search tool (BLAST) from the GenBank database (National Center for Biotechnology Information, NCBI). The strains

were assigned to different groups according to the identification of taxa.

For the phylogenetic analysis, the Neighbor-joining method (Saitou and Nei 1987) was constructed with 1000 bootstrap replicates (Felsenstein 1985) using MEGA 5.0 (Tamura et al. 2011).

RESULTS

Morphology of Casuarina root nodules

Root nodules were only observed at the site with a healthy *Casuarina* stand. Most nodules grew on the litter horizon and were a brown color. The morphological characteristics of nodules were described by Athar and Mahmood (2001), the structure of the nodules was a coralloid shape. In this study, we classified 2 types of nodules according to the shape. Type 1 of nodules had short root lobes (Fig. 3A), and type 2 had long root lobes (Fig. 3B).

Diversity of Casuarina-associated nitrogen-fixing bacteria

Amplification of the *nifH* gene was successfully performed in all samples (4 soil samples and 1 root nodule sample) using the polF-GC/polR primers (Fig. 4A). There was no length diversity within the samples. About 380 bp of PCR products was amplified. The strains of nitrogen-fixing bacteria appeared in the PCR-DGGE profile in both soil samples and root nodules (Fig. 4B).

The DGGE profiles were compared to one another using the Sorensen pairwise similarity coefficients (Cs) and a sketch map (Fig. 4C) of diazotrophic communities from 4 growth situation sites and 1 root nodule sample. There were many diazotrophs in the soil, even though there was no vegetation on the ground in the dead area. The highest diazotrophic diversity was found in the soil sample of the dead tree area (27 bands) than in the declining area (22 bands), healthy tree area (19 bands), regeneration area (16 bands), and nodule sample. The lowest diazotrophic diversity was present in the root nodule sample.

The result of the UPGMA clustering showed that similarities among samples were not very high (Cs value > 52.2%) (Fig. 5). The dead tree sample was grouped with healthy trees (Cs value = 52.2%). The declining sample was grouped with the group of dead and healthy trees (Cs value = 33.4%). The regeneration area was grouped with the root nodule sample (Cs value = 24.0%).

Endophytes of *Casuarina* root nodule

In the root nodule pattern, the PCR-DGGE bands of strains were assigned to groups a and b (Fig. 4B). These represented 2 groups of nitrogen-fixing bacteria found in the root nodules. The blast against the NCBI database is shown in Table 1. The sequence of strain a1 had the best hit to unidentified nitrogen-fixing bacteria (FJ008366) with 81% identity. Sequences of

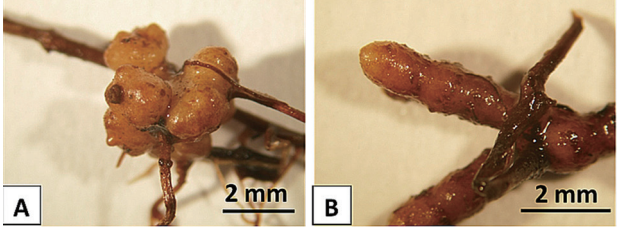

Fig. 3. Different morphologies of root nodules collected from healthy *Casuarina equisetifolia*. A, Short root lobes of a nodule; B, long root lobes of a nodule.

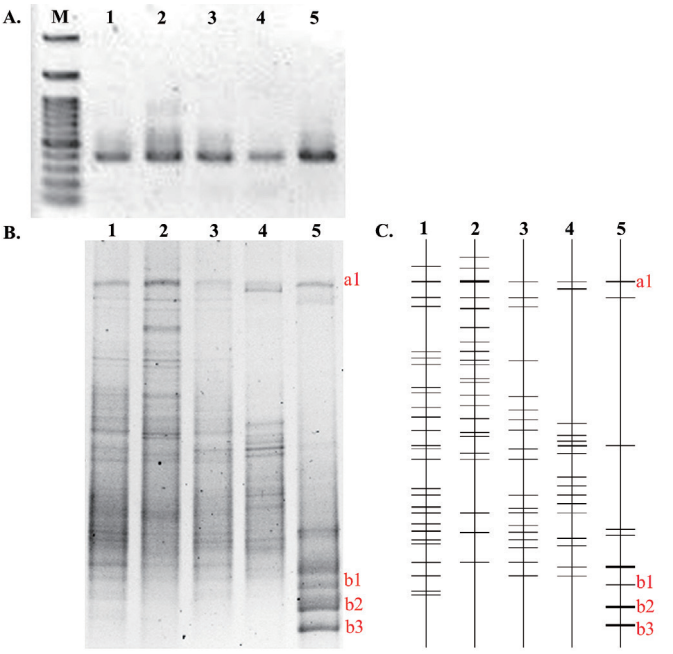

Fig. 4. Electropherograms of PCR products amplified from the *nifH* gene from different sites. A. Using primer polF-GC/polR, we could successfully amplify all samples of about 380 bp of PCR products. B. The DGGE analysis showed high diazotrophic diversity, even in the dead tree area. In the root nodule pattern, constituents of diazotrophs differed in the various soil samples. There were 2 groups of strains within nodules: a1 strain which belonged to unidentified nitrogen-fixing bacteria; and b1~3 strains which were identified to the *Frankia* group. C. Sketch map of DGGE profiles (1: dead tree area, 2: declining growth area, 3: healthy tree area, 4: regeneration area, and 5: sample of root nodule of *Casuarina equisetifolia* from healthy trees. The numbers denote nitrogen-fixing bacterial strains).

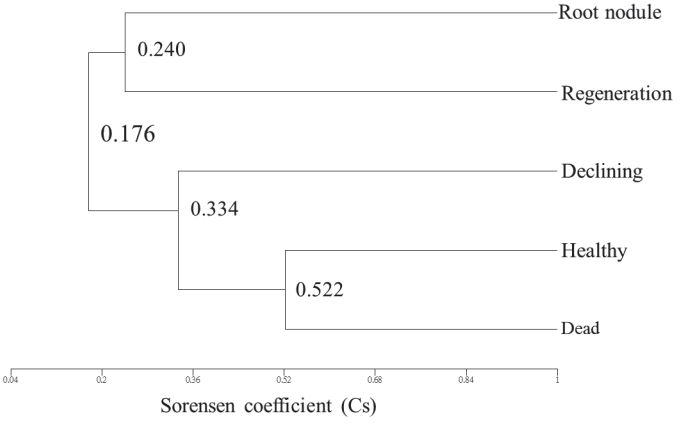

Fig. 5. UPGMA clustering of DGGE bands according to the Sorensen coefficient (Cs). Dead: dead tree areas; declining: declining growth area; healthy: healthy tree area; regeneration: regeneration area; and root nodule: root nodule of *Casuarina equisetifolia* from healthy trees.

strains b1, b2, and b3 were the same, and all sequences of group b hit *Frankia* (EU862918) with 99% identity. Unfortunately, the remaining strains were not successfully sequenced.

The phylogenetic analysis showed that strain a1 was belonged to an unknown group. In the unknown group, the uncultured nitrogen-fixing bacteria formed a unique group distinct from the *Frankia* group and legume group. Strains b1, b2, and b3 belonged to the *Frankia* group (Fig. 6).

DISCUSSION

The morphology of *Casuarina* root nodules

Healthy *Casuarina* trees are generally associated with abundant nodules with an ovate and rod-like shape (Athar and Mahmood 2001). Different morphologies may be caused by an association with the strain diversity of symbiotic bacteria in root nodules. There are many nitrogen-fixing bacteria in root nodules of *Casuarina*.

Using *nifH* PCR-DGGE to analyze the diazotrophic diversity

The *nifH* gene is a commonly used marker to investigate diazotrophic diversity in different environments and ecosystems (Zehr et al. 2003, Mårtensson et al. 2009). PCR-DGGE has the advantages of understanding diversity of bacteria without strain isolation and can study non-culturable populations (Franche et al. 2009, Mårtensson et al.

Table 1. The *nifH* gene sequences from root nodule sample BLAST of the NCBI database

Code*	Accession no.	BLAST closest match	Overlap (%)	Identities (%)
a1	FJ008366	Uncultured soil bacteria	91	218/270 (81)
b1	EU862918	*Frankia* sp. CcI3	99	289/293 (99)
b2	EU862918	*Frankia* sp. CcI3	99	289/293 (99)
b3	EU862918	*Frankia* sp. CcI3	99	289/293 (99)

* Denotes nitrogen-fixing bacterial strains from the DGGE analysis.

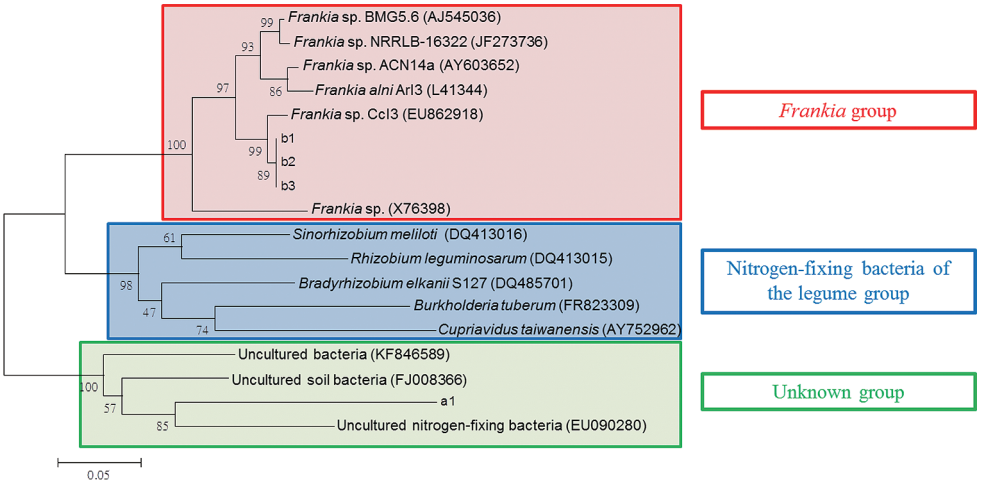

Fig. 6. Neighbor-joining tree of the partial *nifH* gene sequences from different nitrogen-fixing bacterial groups. Sequences a1, b1, b2, and b3 were from the DGGE analysis of this study.

2009). In Parker's (1957) study, there were many non-symbiotic nitrogen-fixing bacteria in the soil. In this study, some strains from the soil were not present in root nodules of *Casuarina*; this indicates that there are many nitrogen-fixing bacteria which were not symbiotic with *Casuarina*. We found abundant strain diversity of nitrogen-fixing bacteria from different growth situations of *Casuarina* stands, even the site with dead trees. This result suggests that there are many nitrogen-fixing bacteria in soils from different growth situations of *Casuarina* stands in the Sihu area, and declining *Casuarina* is not related to diazotophic diversity.

A quick way to understand the dominant strains of test samples is to use the PCR-DGGE profile analysis. But there are some defects in this technique, as we could not totally know the constituents of strains from the samples. In the soil patterns, the PCR-DGGE profile showed abundant diversity of diazotrophs, but the abundant bands were too dense to cut the gels for DNA sequencing. In this study, we could not figure out the diversity or constituents of diazotrophic strains among different growth situations of *Casuarina* stands. In future study, we should establish a DNA library of diazotrophic strains, to clarify whether there are specific strains in distinct stands.

Endophytes of *Casuarina* root nodules

We know the *Casuarina* trees are symbiotic with *Frankia* strains (Benson and Clawson 2000, Sayed 2011). In the pattern of root nodules from DGGE, strain a1 was a hit to unidentified nitrogen-fixing bacteria. Non-*Frankia* nitrogen-fixing bacteria have been reported from nodules of *C. equisetifolia* (Valdés et al. 2005). However, as many strains of *Frankia* were also present in root nodules from *Casuarina* trees, the result may show that more than 1 *Frankia* strain is present in actinorhizal root nodules, similar to observations by Normand and Lalonde (1982).

According to Echbab et al.'s (2007) study, *Frankia* strains can successfully occur with *Casuarina* in axenic conditions. In this study, strains b1, b2, and b3 were absent from soils of all stands, even in the healthy growth stand. This result may demonstrate that *Frankia* belongs to symbiotic bacteria, and is not the dominant diazotroph in soil, but is often found in nodules of host plants. However, the phylogenetic analysis showed that strain a1 was distinct from the *Frankia* group and legume group. This result demonstrates that there are non-*Frankia* nitrogen-fixing bacteria in nodules, as in Valdés et al.'s (2005) study.

We found 2 groups of nitrogen-fixing bacteria in root nodules using *nifH* gene sequences. Unfortunately, this method cannot detect non-nitrogen-fixing bacteria. In a future study, we will try to isolate the endophyte of root nodules of *Casuarina*, and use other gene regions (like 16S rDNA) to identify bacteria that are symbiotic with Casuarina trees.

CONCLUSIONS

A quick way to understand the richness of strains in sample is to use a PCR-DGGE analysis. There are many diazotrophs in soils from different growth situation stands, even is an area with dead trees. A declining *Casuarina* stand was not related to the diazotophic diversity. In this study, we were able to categorize root nodules of *C. equisetifolia* into 2 morphological types, and 2 groups of bacteria were present in the root nodules, unidentified nitrogen-fixing bacteria and *Frankia*.

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