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Full Length Research Paper

Identification and nitrogen fixation effects of symbiotic *Frankia* isolated from *Casuarina* spp. in Zhejiang, China

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Fourteen symbiotic isolates were obtained from root nodules of *Casuarina equisetifolia* and *Casuarina cunninghamiana* in Zhejiang, China. All isolates exhibited typical *Frankia* morphological characteristics, including filamentous hyphae, vesicles, and multilocular sporangia borne terminally or in an intercalary position. Combined with 16S rDNA sequence alignment results, all isolates were identified as *Frankia* spp. 4 isolates belonged to the physical Group A, 7 to Group B, and 3 to Group AB. The strains demonstrated varied nitrogenase activities, with the ZCN192 strain being the highest (2.897 μmol·mg^{-1}h^{-1}) and ZCN199 the lowest (0.056 μmol·mg^{-1}h^{-1}). After *in vivo* inoculation, all strains significantly increased seedling height, basal diameter, and dry biomass of *Casuarina* spp. Generally, strains with higher nitrogenase activities exhibited more effective nitrogen fixation *in vivo.*

Keywords: *Casuarina* spp., *Frankia*, identification, nitrogen fixation effect.

INTRODUCTION

Coastal shelter forests are crucial components of the ecological construction and natural disaster prevention system of China. Zhejiang Province, located in the southeastern coast of China, has been vigorously constructing coastal shelter forests since 1991. However, the shelter forests are still considered of insufficient quantity and quality that could scarcely combat tsunamis or severe storm tides. Poor site conditions, and weak soil enzyme and microbial activities are the primary factors inhibiting the rapid growth and sustainable ecological protection features of coastal shelter forests. Therefore, choosing tree species with root systems that have nitrogen-fixing bacteria and can fertilize soil through nitrogen fixation, improve soil structure, increase soil biodiversity, and promote the virtuous cycle of the soil ecosystem will help expedite the construction efforts of Zhejiang Province. Consequently, the quality of coastal shelter forests will be improved and their ecological protection features will be stabilized.

Casuarina spp. is a typical symbiotic tree species that are essential not only to the construction of shelter forests but also to that of timber forests (Baker and Mullin, 1992; Lechevalier, 1994). Especially in the leading edge of sandy coastal zones, *Casuarina* spp. have unique advantages and play important roles. Such roles include defending against wind and retaining sand, preventing wave erosion, as well as recovering coastal ecosystems. Since its introduction into China in the 1950s, *Casuarina* spp. has become significant shelter forest tree species along the coastline of Zhejiang Province because of their exceptional characteristics (Wang et al., 1992; Zhong et al., 2005). However, planting *Casuarina* spp. for successive years leads to sand shortage and soil acidification. *Frankia* is a bacterial species isolated from *Casuarina* spp. that plays an important role in such processes as symbiotic nitrogen fixation, soil fertilization, plant growth, and soil ecosystem restoration (Meesters et al., 1985; Rönkkö et al., 1993). Research has characterized the symbiotic *Frankia* from red bayberry in Zhejiang Province (He et al., 2003; Tan et al., 2008), whereas systematic data on *Frankia* strains from *Casuarina* spp. in this location remain limited. Given the wide range of *Frankia* host plants and their genotypic diversity, we sampled root nodules from *Casuarina* plants found in the coastal areas of Zhejiang Province, isolated

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Table 1. Experimental strains of *Frankia* spp. and nodulation effects on roots of *Casuarina* spp..

Bacterial strain	Location	Host plant	Number of nodulated / inoculated strain	Average number of nodule (nodules/strain)
ZCN1	Cangnan District, Wenzhou	*C. equisetifolia*	7/15	6.3
ZCN7	Cangnan District, Wenzhou	*C. equisetifolia*	14/15	10.5
ZCN13	Cangnan District, Wenzhou	*C. equisetifolia*	12/15	6.9
ZCN19	Oujiang District, Wenzhou	*C. equisetifolia*	15/15	11.0
ZCN23	Oujiang District, Wenzhou	*C. equisetifolia*	8/15	6.3
ZCN55	Beicang District, Ningbo	*C. cunninghamiana*	15/15	6.8
ZCN67	Beicang District, Ningbo	*C. cunninghamiana*	11/15	8.7
ZCN90	Zhenhai District, Ningbo	*C. cunninghamiana*	9/15	8.1
ZCN101	Putuo District, Zhoushan	*C. equisetifolia*	9/15	8.8
ZCN113	Putuo District, Zhoushan	*C. equisetifolia*	14/15	6.7
ZCN157	Dinghai District, Zhoushan	*C. equisetifolia*	13/15	7.1
ZCN192	Shujiang District, Taizhou	*C. equisetifolia*	11/15	11.4
ZCN199	Shujiang District, Taizhou	*C. equisetifolia*	11/15	6.5
ZCN201	Shujiang District, Taizhou	*C. equisetifolia*	7/15	6.5
Y5	Qintian, Lishui	*Myrica sp.*	3/15	4.7

and identified *Frankia* strains, as well as analyzed their nitrogen fixation effects. Our aim was to provide a theoretical basis for developing such strains for wider applications.

MATERIALS AND METHODS

Isolation of *Frankia*

To increase the probability of finding diversity, root nodules were sampled from 2007 to 2009 according to different geographical and ecological factors as well as host tree species. Seven typical sampling sites in four major coastal cities and counties were selected. A total of 218 root nodules were collected from two types of nodulated plants, *Casuarina equisetifolia* and *Casuarina cunninghamiana.*

Frankia strains were isolated based on a previously described method with some modifications (Lalonde et al., 1981). After wrapping with double-layered gauze, the fresh nodules were thoroughly cleaned with tap water and disinfected for 10 to 20 min in 30% (w/v) hydrogen peroxide (H_2O_2). The nodules were rinsed with sterile distilled water before being dissected into thin slices and transferred to test tubes containing anazotic benzyl aminopurines (BAP) (He et al., 2003). All slices from one individual nodule were incubated in one tube in the dark at a constant temperature of 28°C for 1 to 3 months. The isolated strains were microscopically identified by the presence of characteristic *Frankia* morphological structures, including hyphae, vesicles, sporangia, and spores. The strains were then cultured and reserved in a new test tube. A *Frankia* strain originally isolated from *Myrica* sp. was used as the reference strain.

Inoculation tests

Nodulation was induced many times from March to June 2008 and from April to July 2009. *C. equisetifolia* and *C. cunninghamiana* seeds were soaked in water at 40°C for 3 days and sterilized with 2% sodium hypochlorite. The resulting seeds were germinated on trays of sterile potting mix (1:2 peat: vermiculite with 1.25 g of $CaCO_3$ to bring the pH to 6.0). After 4 weeks, seedlings were transplanted into 9 ml free-draining plastic pots (1 seedling per pot) containing 120 g of the potting mix described above with a nitrogen-free nutrient formulation, consisting of 0.3 g superphosphate, 0.13 g KC1, and 0.3 g $MgSO_4$.

The strains were grown at 28°C in BAP medium for about 2 months. Cells were collected by centrifugation (5000 rpm, 4°C), washed and resuspended in BAP, homogenized by repetitive passages through a 0.7 mm needle, and used as inoculum at 2 εg/ml protein of culture medium. About 5 ml of the bacterial suspension was subsequently extracted and injected into the soil around the root system. Each strain inoculated 15 replicate original host seedlings once every month. After three months, nodulation was observed and the number of root nodules was recorded.

A total of 37 isolates were obtained from 218 root nodules. Among them, 14 strains exhibited invariable infection to the original host plant. Compared with the reference strain Y5, the strains achieved a higher nodulation rate and produced even more nodules. Detailed information on the experimental strains is listed in Table 1.

Identification of *Frankia*

Morphological characteristics

The experimental strains were cultured in anazotic BAP liquid medium for 4 weeks. A droplet of suspension was aseptically absorbed, placed onto a clean glass slide, and directly observed under a microscope for its mycelium, vesicles and sporangia. The detailed structural features of the strains were characterized by scanning electron microscopy (SEM). Hyphae were fixed in 4% glutaraldehyde and 1% osmic acid, dehydrated with ethanol, placed in isoamyl acetate overnight and vacuum dried. Sections were mounted on stubs covered with 25 nm of gold, and observed under a PHILIPS XL-30E SEM.

Physiological groups

The physiological type of the strains was classified according to

Lechevalier et al. (1983).

16S rDNA sequencing analysis

DNA extraction was performed as described by Clawson et al. (1998). The 16S rDNA sequences were amplified using the 27 f 5Nj AGA GTT TGA TCA TGG CTC AG-3Njand 1492 r 5NJTACG GTT ACC TTG TTA CGA CTT-3Njuniversal primers (synthesized by Shanghai Bioengineering Co., Ltd). Amplification was carried out using Shanghai Bioengineering polymerase chain reaction (PCR) kits under the following PCR conditions: pre-denaturation at 95°C for 45 s, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 2 min, followed by a final extension at 72°C for 7 min. The amplified products were detected using 0.8% agarose electrophoresis. Sequence analysis was completed using MEGA5.02 software, and the measured 16S r DNA sequences were compared online via the NCBI database of the U.S. National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/blast). Homologous analysis was also performed, with relevant information obtained from GenBank. A neighbor-joining phylogenetic tree for closely related strains was also constructed using the MEGA5.02_software.

Nitrogen fixation effect of *Frankia*

Acetylene reduction methods reported by Tjepkema and Torrey (1983) and Baker and Danell (1986) were adopted to evaluate the effects of *in vitro* nitrogen fixation. The tested 14 strains were cultivated in static anazotic BAP culture medium at 28 to 30°C for 15 days. About 6 ml of each bacterial suspension was transferred into a 10 ml vial under sterile conditions. The vial was obturated after 1 ml of acetylene gas was injected into the mixture, which was gently shaken at 28°C (20 rpm). After 8 days, acetylene (C_2H_2) reduction, as a measure of nitrogenase activity was sampled via gas chromatography (GC) using a SHIMADZU GC 9A/9AM system. Mycoprotein production was measured according to the Bradford (1976) method. The process was repeated three times for each strain, with the sterile culture solution and the reference strain Y5 as the negative and positive controls, respectively.

The same method used for root nodule inoculation was adopted to study the *in vivo* nitrogen fixation effects of the strains. Experiments were conducted in a conservatory using two tree species (*C. equisetifolia* and *C. cunninghamiana*) for which the seedling height was 15 cm. Each strain was correspondingly inoculated into 3 young original host plants. Inoculation was processed once a month and stopped after a successive 3-month cycle. Nodulation was observed after 5 months and the plants were pulled out of the culture pan. The height, basal diameter, and dry weight of the plants were measured. Their increasing rates (R) were calculated as (V ï C) / C, where V is the measured value of inoculated plants and C is the value of the control. The control was used for injecting the sterile culture solution. The procedures were repeated three times at 3-month intervals.

Statistical analysis

One-factor ANOVA was performed to assess significant differences in nitrogenase activity (N) as well as the increase rates in the plant height (R1), basal diameter (R2), and dry weight (R3) of the tested isolates. Multivariate correlation analysis was performed to recognize the significant contributor of R1, R2, R3, and their interaction (independent variables) with the dependent variable N using a stepwise procedure. All statistical analyses were conducted using the DPS software (Tang and Feng, 2007).

RESULTS

Identification of strains

Morphological characteristics

The experimental strains grew slowly, and hyphal growth was observed after 4 weeks of static culture in non-nitrogen BAP growth medium. Much of the mycelium settled to the bottom of the pots, whereas a small amount attached to the pot walls. The colonies were mostly cotton-like and granular. Optical microscopy using the bacterial suspension revealed that the experimental strains generated typical hyphae, vesicles, and multi-locular sporangia of *Frankia* (Table 2). The ultra-microstructure of bacterial strain ZCN192 is shown in Figure 1.

Physiological group classification

The morphological characteristics and physiological groupings of the strains are listed in Table 2. In the presence of Tween-80, the experimental strains differed in their growth response. ZCN55 isolated from *C. cunninghamiana* as well as ZCN13, ZCN23, and ZCN201 isolated from *C. equisetifolia* were categorized into physiological Group A. Seven strains demonstrated better growth after Tween-80 was added, including ZCN67 and ZCN90 from *C. cunninghamiana*, and ZCN1, ZCN7, ZCN19, ZCN113, and ZCN157 from *C. equisetifolia*. They were classified into physiological Group B. Physiological Group AB was composed of three strains whose mycelium growth was not significantly affected in the presence or absence of Tween-80. These preliminary experimental results clearly indicated that more B-type strains were obtained from among the tested bacteria.

16S rDNA sequencing analysis

Polymerase chain reaction (PCR) demonstrated that the reference strain Y5 and the 14 experimental strains all yielded clear single bands with a sequence length of approximately 1.5 kb. A homologous comparison with GenBank strains (for example, strain AcoN24d, FCg07, AVN17s, Cea5.1, Ea1-2, Sn4-3, FrCth, whose Genbank number is L40610.1, AY502037.1, L40613.1, U72718.1, L40618.1, AJ408874.1, AF050759.1, respectively) confirmed that the experimental and *Frankia* strains shared the highest homology, with a level of similarity greater than 97%. Phylogenetic analysis using closely related strains and the DNASTAR software revealed that variation at the DNA sequence level was sufficient to distinguish between different strains and groups under the *Frankia* genus. Morphological characteristics and the

Table 2. Morphological characteristics and physiological groups of *Frankia* strains.

Bacterial strain	Water-soluble pigment	Generation of cytocysts	Generation of vesicles	Characteristics of liquid culture	Characteristics of solid culture	Physiological group
ZCN1	—	++	+	Whitish granules	Yellowish brown, convexity, white halo	B
ZCN7	—	+	++	Whitish floccus	Grey yellow, convexity, white halo	B
ZCN13	—	++	+	Whitish granules	Yellowish brown, convexity, white halo	A
ZCN19	—	++	+	Whitish floccus	Grey yellow, convexity, white halo	B
ZCN23	—	+++	++	Whitish granules	Yellowish brown, convexity, white halo	A
ZCN55	—	+	+	Whitish floccus	Grey yellow, convexity, white halo	A
ZCN67	—	++	+	Whitish granules	Yellowish brown, convexity, white halo	B
ZCN90	—	++	+	Whitish floccus	Grey yellow, convexity, white halo	B
ZCN101	—	++	+	Whitish granules	Yellowish brown, convexity, white halo	AB
ZCN113	—	+	+++	Whitish granules	Yellowish brown, convexity, white halo	B
ZCN157	—	++	++	Whitish granules	Yellowish brown, convexity, white halo	B
ZCN192	—	+++	+++	Whitish granules	Grey yellow, convexity, white halo	AB
ZCN199	—	+	+	Whitish granules	Grey yellow, convexity, white halo	AB
ZCN201	—	++	++	Whitish granules	Yellowish brown, convexity, white halo	A
Y5	—	+	++	Pale red, floc particles	Reddish-brown, radial pattern	B

ñ+,ò indicates not produced; ñ+,ò minor; ñ++,ò moderate; ñ+++,ò excessive. A, the presence of Tween-80 inhibited the glucose utilization of the strain. B, Tween-80 was conducive to glucose utilization. AB, Tween-80 did not affect glucose utilization.

characterization of strains into physiological groups appeared to have little correlation when mapped into the 16S rDNA phylogeny (as presented in Figure 2). On the other hand, the strains obtained from the same host plant and distributed in the same region had the propensity to cluster. For example, ZCN55, ZCN67, and ZCN90 from *C. cunninghamiana* in Ningbo comprised a monophyletic clade within the phylogeny, as did ZCN1, ZCN7, and ZCN19 from *C. equisetifolia* in Wenzhou, and *Frankia* sp. AVN17s (obtained from GenBank, accession number

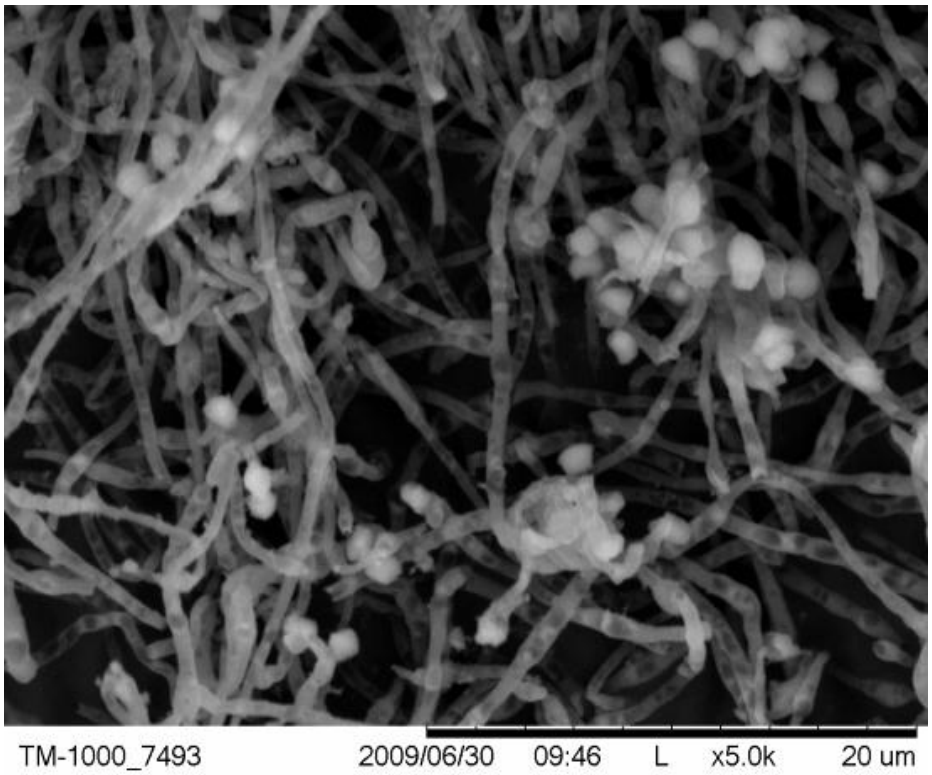

Figure 1. Sporangia of strain ZCN192 observed under scanning electron microscopy.

L40613). Baker and Schwintzer (1990) confirmed that actinomycete *Frankia* is able to fix atmospheric nitrogen and associate symbiotically within root nodules of non-legume, actinorhizal plants belonging to eight families of dicotyledons. In this present study, the reference strain Y5 from *Myrica* sp. appeared nested within the phylogeny of Casuarina-inhabiting *Frankia* spp. This testified that *Frankia* possess a wide host range and would confirm that host-switching of *Frankia* spp. into other host plants occurred during the evolution of the genus.

Nitrogen fixation effects of strains

The results of acetylene reduction showed that all experimental strains exhibited nitrogenase activity *in vitro*. However, the enzymatic activities of the strains greatly varied ($F_{14, 120} = 146.66$, $P = 0.0001$). The nitrogenase activities of strains from the same host plant showed great differences, as shown in Figure 3: Plant height ($F_{14,120} = 100.08$, $P = 0.0001$), diameter ($F_{14,120} = 67.00$, $P = 0.0001$), and dry weight ($F_{14,120} = 516.69$, $P = 0.0001$). The strains with much more vesicles also had the propensity of achieving a higher enzymatic activity compared with those with fewer ones. For example, the number of vesicles for strain ZCN192 was much greater than that of strain ZCN199, and the acetylene reduction activity levels of the strains were 2.897 and 0.056 εmol·mg-1·h-1, respectively. This result conformed to the findings of Zhang et al. (2006).

Nitrogen fixation effects *in vivo* demonstrated that the experimental strains, including the reference strain Y5, improved the height, basal diameter, and dry weight of young seedlings of *Casuarina* spp. at varying extents compared with the control. Apparent nitrogen fixation discrepancies among the strains were also observed.

By multivariate correlation analysis, all independent variables measured were found to be significantly related to the *in vitro* nitrogenase activity of strain (N). We used the following linear equation:

$$N = 0.06 + 3.62R3 - 6.47R1R3 + 3.17R2R3 \quad (R2 = 0.821;\ F_{3, 11} = 16.85;\ P = 0.0002).$$

The most influential factor was R3. Variables R1 and R2 were only influential upon interaction with R3. These variables made an overall contribution of 82.1% to the variation in the *in vitro* nitrogenase activity.

DISCUSSION

A total of 37 symbiotic strains of *Frankia* spp. were obtained by the slicing method from the root nodule of *Casuarina* spp. collected from four major coastal areas in Zhejiang Province, China. Cultural characteristics combined with morphological features confirmed that the

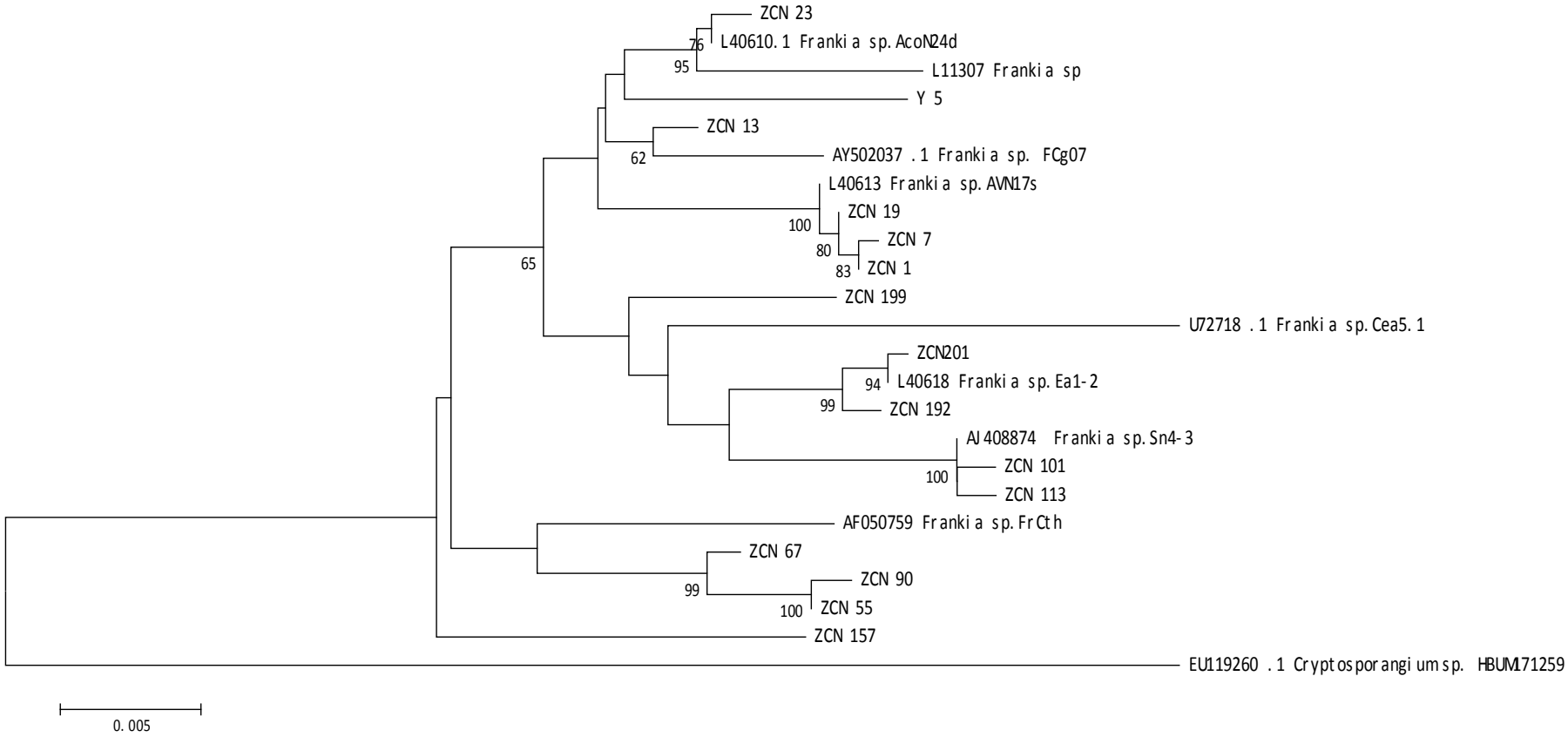

Figure 2. Neighbor-joining phylogenetic tree of the experimental strains of *Frankia* spp. from *Casuarina* spp., Zhejiang Province, China, with additional sequences from GenBank.

isolates were all *Frankia* strains. However, repeated inoculation tests revealed that only 14 strains steadily produced nodules. The results were congruent with a previous report that *Frankia* widely exists in the environment, and that some are saprobiotic soil inhabitants (Wolters et al., 1999; Jeong and Myrold, 2001), whereas others were originally symbiotic but have lost their infectivity as a result of being isolated (Nazaret et al., 1989). Reddell and Bowen (1986) have testified that although *Frankia* possesses a wide range of host plants and symbiotically nodulated into a variety of woody plants, the same strain may have different affinities for different host plants. The current study used the *Frankia* strain Y5 isolated from red bayberry (the original host plant) to contrast evaluation. The results reveal that the strains isolated from *Casuarina* spp. had higher nodulation rates and more nodule numbers, possibly due to the preference of the original host (that is, the strain isolated from red bayberry infects red bayberry more easily compared with *Frankia* isolated from *Casuarina* spp.). Further investigations are needed to confirm this.

Morphological features have always been the primary basis for identifying *Frankia*. The present

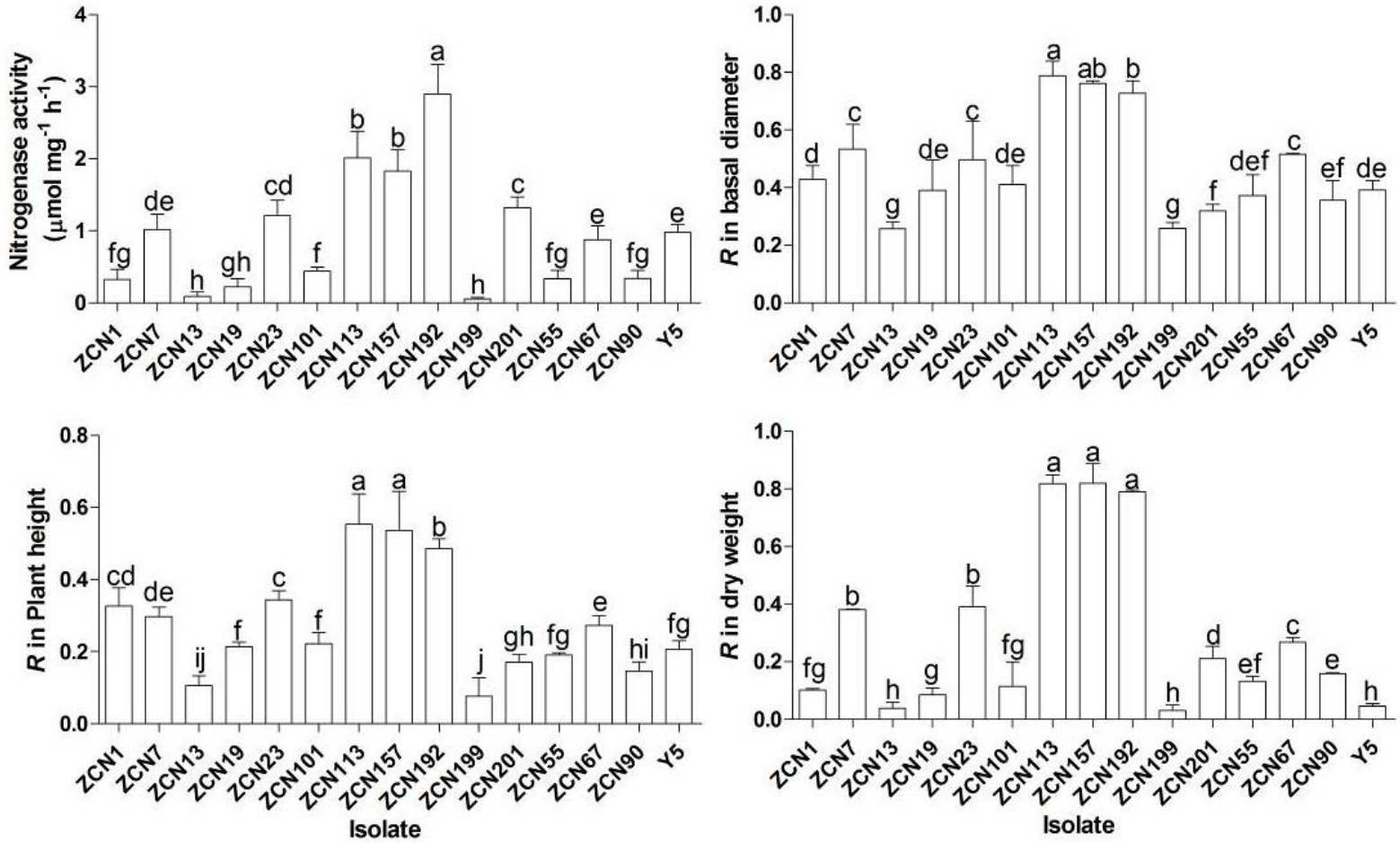

Figure 3. Nitrogenase activities *in vitro* and nitrogen fixation effects *in vivo*. Significant differences are denoted by lowercase letters ($P < 0.05$).

study observed various strains under microscopy and found that the experimental strains exhibited typical structures of *Frankia*, including branching hyphae, sporangia, and vesicles. Universal bacterial primers were selected to amplify the 16S rDNA sequences of all the strains, and sequences were compared to confirm their taxonomy. The results show that the level of genetic similarity between the 14 experimental strains and other published *Frankia* was greater than 97%, thereby confirming them as *Frankia* spp. Previous studies on *Frankia* strains from different host plants and different regions have indicated that there is a variety of genotypes within the *Frankia* groups (Clawson et al., 2003; Gtari et al., 2007; Mirza etal., 2009). This was confirmed in this present study, even within a single genus in the localised region of Zhejiang Province.

The 14 strains in the current study significantly differed in their *in vitro* nitrogenase activities, and quantity of vesicles. These correlations signified that vesicles are essential to *Frankia* nitrogen fixation (Meesters et al., 1987; Berry et al., 1991; Harriott et al., 1991). Among the experimental *Frankia* isolates, strains ZCN113, ZCN157, and ZCN192 expressed the highest levels of nitrogenase activity and were the most effective in increasing plant growth parameters such as plant height, basal diameter and dry weight biomass. However, the specific performances of strains in their environments may also be determined by their colonization and survival capability, as well as other biological and environmental factors. For instance, Rajendran and Devaraj (2003) have found via field inoculation studies that the biomass of *C. equisetifolia* inoculated with *Frankia* is higher than that of the non-inoculated control. The biomass of *Casuarina* spp. with *Frankia*, phosphate bacteria, and arbuscular mycorrhizas is also higher than that of species separately inoculated with *Frankia*. This finding is likely caused by the inhibitory effect of limited soil phosphorus on the growth of *Frankia*, whereas arbuscular mycorrhizas can provide the phosphorus necessary for the nitrogen fixation and nodulation of *Frankia*. These data clearly indicate that the environmental applications of *Frankia* merit further investigation.

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