

Genetic Diversity of *Nostoc* Microsymbionts from *Gunnera tinctoria* Revealed by PCR-STRR Fingerprinting

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ABSTRACT

The cyanobacteria belonging to the genus *Nostoc* fix atmospheric nitrogen, both as free-living organisms and in symbiotic associations with a wide range of hosts, including bryophytes, gymnosperms (cycads), the small water fern *Azolla* (Pteridophyte), the angiosperm genus *Gunnera*, and fungi (lichens). The *Gunnera*–*Nostoc* symbiosis is the only one that involves a flowering plant. In Chile, 12 species of *Gunnera* have been described with a broad distribution in the temperate region. We examined the genetic diversity of *Nostoc* symbionts from three populations of *Gunnera tinctoria* from Abtao, Chiloé Island, southern Chile, and microsymbionts from other two species of *Gunnera* from southern Chile, using PCR amplification of STRR (short tandemly repeated repetitive) sequences of the *Nostoc* infected tissue. To our knowledge, this is the first report of PCR fingerprinting obtained directly from symbiotic tissue of *Gunnera*. Genetic analyses revealed that *Nostoc* symbionts exhibit important genetic diversity among host plants, both within and between *Gunnera* populations. It was also found that only one *Nostoc* strain, or closely related strains, established symbiosis with an individual plant host.

Introduction

Cyanobacteria (blue-green algae) are a group of prokaryotic organisms exhibiting the general characteristics of Gram-negative bacteria. They are unique among the prokaryotes because of their capacity to carry out oxygenic photosynthesis. Furthermore, some cyanobacteria also have the

capacity to fix atmospheric nitrogen. These qualities place cyanobacteria among the most successful and widespread group among the prokaryotes, occupying a wide range of terrestrial and aquatic environments [14]. The cyanobacteria belonging to the genus *Nostoc* fix atmospheric nitrogen, both as free-living microorganisms and in symbioses with higher plants and fungi. Successful symbiotic associations are established with a diversity of hosts including bryophytes, gymnosperms (cycads), the small water fern *Azolla* (Pteridophytes), the angiosperm genus

Gunnera (Gunneraceae), and fungi (lichens) [2, 14, 19, 25]. Although the taxonomic identity of the host and the structures in which the microsymbiont is housed vary greatly, the systematic and genetic diversity of *Nostoc* symbionts is largely unknown [18].

The symbiosis between *Nostoc* and plant species in the genus *Gunnera* is the only association between *Nostoc* and a flowering plant. The association is facultative; the two partners can be isolated and cultivated independently and the symbiosis may be easily reconstituted [2]. The *Nostoc*–*Gunnera* symbiosis exhibits unique features compared to other cyanobacterial–plant symbioses. The cyanobacterium infects specialized gland organs located in the stems and rhizome of the host plant. Once it has invaded the interior of the gland, the cyanobacterium also enters the *Gunnera* cells; here it begins to form heterocysts (specialized nitrogen fixing cells) at the highest frequency recorded in any cyanobacterial population [2, 14]. The cyanobacteria comprise only a very small fraction of the host plant biomass, but their presence can be shown to be highly beneficial, making the host plants autotrophic with respect to nitrogen [19].

Gunnera occurs naturally in Central and Southern Africa, Madagascar, New Zealand, Tasmania, Indonesia, the Philippines, Hawaii, Mexico, Central and South America, and the Juan Fernandez Islands. Hence, all extant *Gunnera* species are largely restricted to the Southern Hemisphere and the Pacific Ocean [2]. These plants often inhabit areas with heavy rainfall. In Chile, 12 species of *Gunnera* have been described with a broad distribution in the temperate region, but the number of species could be lower because of synonymy [11].

For a long time, microbial ecology has largely depended on the use of conventional microbiological techniques such as isolation and characterization of pure cultures. Most microorganisms, including *Nostoc*, are still recalcitrant to growth in culture; therefore, the traditional techniques may not reflect the real diversity present in *Nostoc* populations in the wild. The development of methods for amplification of microsymbiont DNA from symbiotic tissue by PCR (polymerase chain reaction), avoiding the isolation step, permits an estimation of the genetic diversity and relatedness of microsymbionts. It also provides a means to conduct molecular ecological studies of *Nostoc* to address issues such as competition, distribution, and host specificity. Previous attempts to obtain a DNA fingerprint directly from symbiotic tissue failed mainly due to inhibition of PCR [17]. Families of repeated sequences

have been described in cyanobacteria, among them three types of STRR (short tandemly repeated Repetitive) sequences which are specific genomic DNA components of the heterocystous strains [13]. Consensus sequences of STRR1, STRR2, and STRR3 are CCCCA(A/G)T, TT(G/T)GTCA, and CAACAGT, respectively. In addition, longer sequences (about 37 bp), denominated LTRR (long tandemly repeated repetitive), have also been identified in *Anabaena* strain PCC 7120 [21]. The conserved status of these repetitive sequences makes them methodologically important tools for diversity studies among related microorganisms [19, 21].

The aims of this work were (i) to assess the genetic diversity of *Nostoc* microsymbionts present in the rhizome of different plants of *Gunnera tinctoria* from three different populations in the locality of Abtao, Chiloé Island (42° S; 30' W), southern Chile, and (ii) to compare the diversity of *Nostoc* microsymbionts found in the populations of *Gunnera tinctoria* with that of *Nostoc* microsymbionts inhabiting other *Gunnera* species from Chile and Juan Fernandez Island. Thus, we will examine genetic differentiation of microsymbionts among different individuals, populations, and species of their *Gunnera* hosts.

Materials and Methods

Sampling Procedure

Tissue samples of *Gunnera* rhizomes were collected in February and September 2000 from the localities and taxa shown in Table 1. Rhizome fragments were conserved at 4°C in polyethylene bags to conserve humidity. Samples of *Gunnera tinctoria* were collected from 10 different plants at sites 1, 2, and 3 in the Abtao sector of Chiloé Island. In the field, plants grew in the vicinity of the road cuts at different elevations (200–400 m) over a distance of 5 km. Each collection site was considered a different *Gunnera* population, as they were separated from one another by >1 km. Samples of *Gunnera tinctoria* and *G. magellanica* collected from the locality of Aytui, Chiloé Island, *G. magellanica* from Tierra del Fuego, and *G. masafuerae* and *G. peltata* from Juan Fernandez Island (Alejandro Selkirk Island) (populations 4, 5, 6, 7, and 8, respectively) were included in the analysis in order to compare with the diversity of microsymbionts associated with *Gunnera tinctoria* growing outside of the Abtao sector, and to assess the *Nostoc* genotypes associated with other *Gunnera* species. Three different sections of infected tissue in each rhizome were used to isolate *Nostoc* DNA. Each tissue portion was treated individually, separated from the rest of the rhizome (approx. 500 mg of infected tissue), and stored at –20°C prior to DNA extraction. *Gunnera* leaves were also collected to be used as negative controls in the PCR-STRR1 amplification.

Table 1. Samples of microsymbionts (*Nostoc*) obtained from different species and populations of *Gunnera* from Chile grouped by locality of origin

Samples	Subsample number	Host plant species	Locality ^a
Gt-01	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-02	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-03	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-04	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-05	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-06	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-07	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-08	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-09	1	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-10	1	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (1)
Gt-11	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-12	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-13	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-14	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-15	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-16	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-17	1	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-18	1	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (2)
Gt-19	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-20	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-21	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-22	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-23	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-24	3	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-25	1	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-26	1	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-27	1	<i>Gunnera tinctoria</i>	Abtao, Chiloé Island (3)
Gt-28	1	<i>Gunnera tinctoria</i>	Aytui, Chiloé Island (4)
Gt-29	8	<i>Gunnera tinctoria</i>	Aytui, Chiloé Island (4)
Gt-30	1	<i>Gunnera tinctoria</i>	Aytui, Chiloé Island (4)
Gm-01	1	<i>Gunnera magellanica</i>	Aytui, Chiloé Island (5)
Gm-02	1	<i>Gunnera magellanica</i>	Aytui, Chiloé Island (5)
Gm-03	1	<i>Gunnera magellanica</i>	Tierra del Fuego (6)
Gm-04	1	<i>Gunnera magellanica</i>	Tierra del Fuego (6)
Gm-05	1	<i>Gunnera magellanica</i>	Tierra del Fuego (6)
Gm-06	1	<i>Gunnera magellanica</i>	Tierra del Fuego (6)
Gp-01	8	<i>Gunnera peltata</i>	Alejandro Selkirk Island (7)
Gmf-01	5	<i>Gunnera masafuerae</i>	Alejandro Selkirk Island (8)

^a The number in parenthesis represents each *Gunnera* population.

DNA Isolation

DNA isolation was carried out by the CTAB method [4]. Samples of infected plant tissue were rinsed with sterile distilled water and then gently homogenized to liberate the bacterial cells. Debris was decanted and the cyanobacterial cell pellets were collected from the supernatant by centrifugation (10 min, 7000 ×g) and were rinsed twice with sterile TENP buffer (50 mM Tris, 20 mM EDTA disodium salt pH 8.0, 100 mM NaCl and 1% PVPP w/v). The cell pellets were ground in 500 µL of TEN-CPP buffer (100 mM Tris 100, 20 mM EDTA disodium salt pH 9.5, 1.4 M NaCl, 2% w/v CTAB, 0.5% w/v PVP, 0.5% w/v PVPP), using Eppendorf mini-grinders, and proteinase K was added to give a final concentration of 100 µg/mL. The solution was incubated at 65°C for 60 min, then subjected to three successive thermal shocks (−70°C for 20 min; boiling water for 5 min). Debris was sedimented by centrifugation (10 min, 5000 rpm). The supernatant was ex-

tracted with an equal volume of chloroform–octanol (24:1), then twice with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) followed by one extraction with an equal volume of chloroform–isoamyl alcohol (24:1). Two volumes of ice-cold absolute ethanol and 0.1 volume of 3 M sodium acetate were added, and the total genomic DNA was precipitated overnight (−20°C) and pelleted by centrifugation at 10,000 rpm at room temperature for 10 min. The pellet was rinsed once with 70% ethanol and dried. Finally, the DNA was resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and then incubated with DNase-free RNase (50 µg/mL) at 37°C for 1 h. The integrity and concentration of the isolated genomic DNA were determined by 0.8% agarose gel electrophoresis (ultrapure; Gibco BRL) against the λ *Hind*III molecular weight DNA marker.

DNA was also isolated from *Gunnera* leaves by the method of Doyle and Doyle [8] and their concentration was determined in the same manner as cyanobacterial DNA.

PCR-STRR Amplification

For PCR-STRR amplification the STRR1A primer, 5'-CCARTCCCCARTCCCC-3' [19], was used. All PCR reactions were carried out in 25 μ L volume containing 15 ng/ μ L of template DNA, 0.4 μ M of primer, 1.5 mM MgCl₂, 80 μ M of each dNTPs, 10 μ g/mL of BSA, and 1.5 U of *Taq* polymerase in reaction buffer. Thermal cycles were as follows: one cycle at 95°C for 6 min; 30 cycles of 94°C for 1 min; 56°C for 1 min, and 65°C for 5 min; one cycle at 65°C for 16 min; and a final holding step at 4°C. Following amplification, 10- μ L aliquots of the PCR products were resolved by agarose gel electrophoresis at 60 V for 60 min in 1.5% (wt/vol) agarose (ultrapure; Gibco BRL) in TAE (40 mM Tris-acetate, 1 mM EDTA).

As negative controls for the PCR-STRR amplifications, we used DNA extracted from *G. tinctoria* and from the following bacteria: *Escherichia coli*, *Frankia*, and *Rhizobium* spp. *E. coli* was grown in LB medium [15] at 37°C with shaking. BAP medium [16] was used for cultivation of *Frankia* and yeast extract-mannitol medium (YEM) [24] for *Rhizobium* spp. Both cultures were incubated at 28°C without shaking.

Data Analyses

The size of the PCR products was determined by the Kodak Digital Science ID program. Fingerprints generated from different microsymbionts were compared and all bands were scored. The presence or absence of particular DNA fragments was converted into binary data and the Jaccard distance index was used to obtain the similarity matrix. The tree was constructed using the UPGMA (unweighted pair-group method using arithmetic averages) program in PHYLIP [9].

Results

DNA from 96 cyanobacterial microsymbionts was isolated from 38 individual plants of *Gunnera*, from four different species, distributed in six different localities in southern Chile and Juan Fernandez Islands (Table 1). A high yield per sample (~8–10 ng/mg symbiotic tissue) of a high molecular weight DNA (approx. 23 kb) was obtained directly from the *Gunnera* rhizome using the extraction protocol described above (data not shown). We estimate that PCR-STRR profiles generated from each infected tissue sample, including bundles of symbiotic cells (gland), are likely to represent those of the microsymbiotic genome. This conclusion is supported because, of all three bacterial species included as controls, only a minor PCR product was obtained for *Rhizobium* and because DNA samples from plant leaves were not amplified with this primer (data not shown). The PCR-STRR amplifications of

microsymbiont DNA using the STRR1A primer yielded multiple distinct DNA bands ranging in size from approximately 250 to 3500 bp. These distinct banding patterns represent a unique DNA fingerprint for each sample.

Nostoc Genetic Diversity within a Single Host Plant

Three to eight symbiotic tissue samples were analyzed for each individual plant of *G. tinctoria*, as well as for the species *G. peltata* and *G. masafuerae*. All DNA fingerprints obtained from different samples of symbiotic tissue derived from a single plant were virtually identical, indicating that each plant was infected by a genetically unique *Nostoc* strain or by closely related strains (Fig. 1).

Nostoc Genetic Diversity within and between *Gunnera tinctoria* Populations

In order to assess the cyanobacterial diversity within narrow geographic areas, we collected tissue samples from three populations of *G. tinctoria* growing in Abtao, Chiloé Island, and determined the DNA fingerprint directly from symbiotic tissue. A total of 17 genetically different microsymbionts were identified from the infected rhizome tissue of 27 plants of *G. tinctoria* (samples Gt-01 to Gt-27) representing three populations from Abtao, Chiloé Island (Figs. 2 and 4). A similar degree of variability among in-

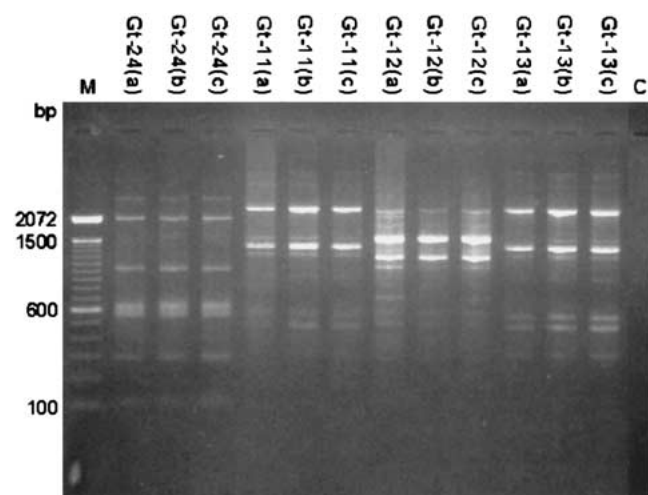


Fig. 1. STRR1-PCR fingerprint patterns of *Nostoc* microsymbionts obtained from genomic isolates from symbiotic tissue of different plants of *Gunnera tinctoria*. The letters a, b, and c represent different sections of symbiotic tissue within a single plant. Lane C, control with no template DNA. Lane M, molecular weight standard (100 bp ladder).

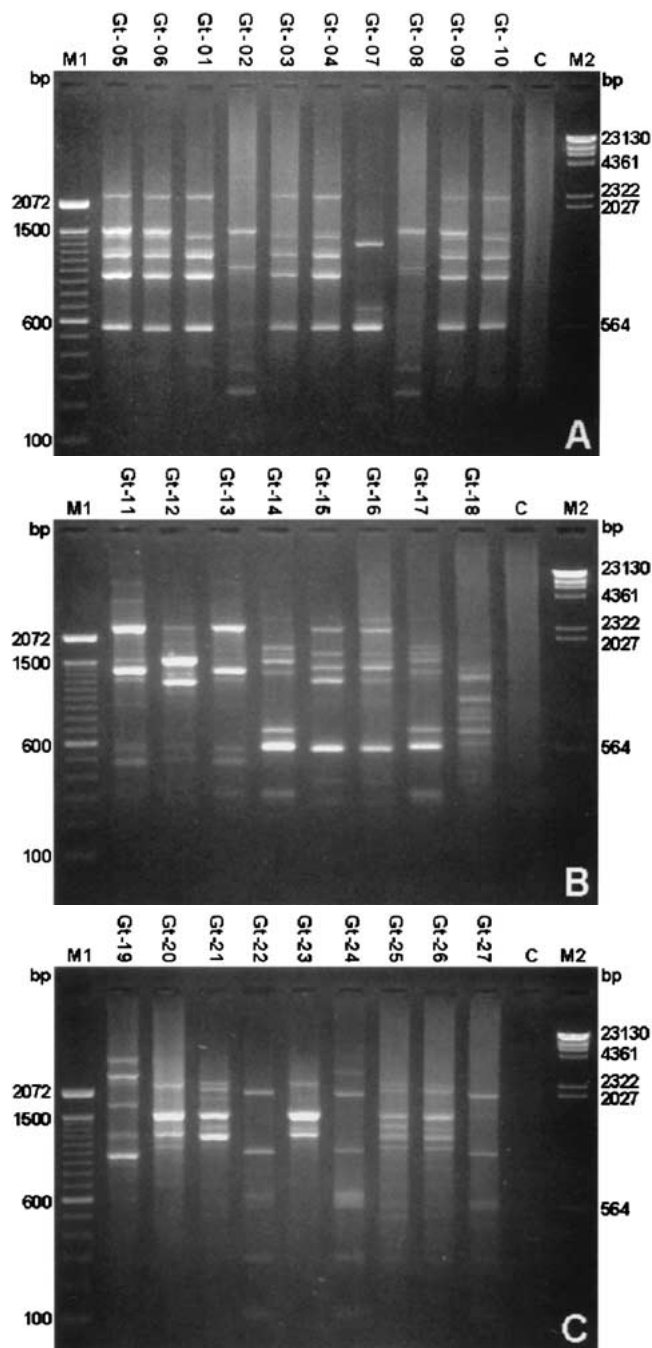


Fig. 2. STRR1-PCR fingerprint patterns of *Nostoc* microsymbionts inhabiting plant tissue of three populations *Gunnera tinctoria* in Abtao, Chiloé Island. Panels A, B, and C represent populations 1, 2, and 3, respectively. Lane C in each panel represents the control without DNA template. Lanes M are molecular weight standards. M1, 100 bp ladder, M2, λ HindIII.

dividuals within populations was found, since the 17 different STRR profiles identified were distributed as follows: five in population 1 and six each in populations 2 and 3 (Table 2). Although five STRR profiles were found in

population 1, the STRR patterns P1 and P2 are exhibited by most of the microsymbionts. There were no shared *Nostoc* DNA fingerprints among the three populations from Abtao. Nevertheless, two samples of symbiotic tissue from population 3 shared identical STRR patterns with a microsymbiont obtained from a *Gunnera tinctoria* plant collected in Aytui, a locality distant 50 km from Abtao. Accordingly, *Nostoc* microsymbionts exhibited a high genetic diversity both within and between populations even from geographically close localities.

Nostoc Genetic Diversity within and between Host Plant Species

STRR-PCR markers from *Nostoc* inhabiting plants of the species *G. magellanica* growing in Chiloé Island and in Tierra del Fuego showed different fingerprints from *G. tinctoria*, even when the two species were growing in the same geographic locality (Fig. 3) and despite the fact that some bands were shared. Different DNA patterns were obtained from *G. peltata* and *G. masafueræ* collected from Alejandro Selkirk Island (33° 50'S 80° 00'W) (Fig. 3).

Clustering of DNA profiles revealed that all profiles were about 50% similar at the host-plant interspecific level. However, in some cases microsymbionts from different species of *Gunnera* formed a single group (i.e., Gt-11, Gt-13 and Gm-01) (Fig. 4). These results indicate a certain degree of genetic heterogeneity among cyanobacterial symbionts associated with the same *Gunnera* species, as well as strong genetic resemblance among isolates from different *Gunnera* species. UPGMA clustering of DNA fingerprints revealed at least five main groups of microsymbionts; in three of them we can find microsymbionts from different localities and/or from different host plant species (Fig. 4).

Discussion

Although the function of STRR and LTRR sequences is still unknown, it has been suggested that STRR sequences might be the target of specific DNA-binding proteins responsible for chromosome condensation and/or might be involved in the control of chromosome distribution or replication during heterocyst differentiation [10, 12]. However, the conserved status of these repetitive sequences makes them methodologically important tools for diversity studies among related microorganisms [19, 21]. Rasmussen and Svenning [19] used primers correspond-

Table 2. Occurrence of cyanobacterial STRR-DNA patterns in each population and species of *Gunnera* studied (see Table 1)^a

Patterns	Geographic location							
	<i>Gunnera tinctoria</i>			<i>Gunnera magellanica</i>		<i>Gunnera peltata</i>		<i>Gunnera masafuerae</i>
	Abtao	Aytui	Aytui	Aytui	Tierra del Fuego	Alejandro Selkirk	Alejandro Selkirk	
1	2	3	4	5	6	7	8	
P1	+	-	-	-	-	-	-	-
P2	+	-	-	-	-	-	-	-
P3	-	+	-	-	-	-	-	-
P4	-	+	-	-	-	-	-	-
P5	-	+	-	-	-	-	-	-
P6	-	+	-	-	-	-	-	-
P7	-	-	-	-	+	-	-	-
P8	-	-	-	-	+	-	-	-
P9	+	-	-	-	-	-	-	-
P10	-	-	-	+	-	-	-	-
P11	-	+	-	-	-	-	-	-
P12	-	-	-	+	-	-	-	-
P13	-	-	-	-	-	-	-	-
P14	+	-	-	-	-	-	-	-
P15	-	-	+	-	-	-	-	-
P16	-	-	+	-	-	-	-	-
P17	-	-	+	-	-	-	-	-
P18	-	-	-	-	-	+	-	-
P19	-	+	-	-	-	-	-	-
P20	-	-	+	-	-	-	-	-
P21	-	-	+	+	-	-	-	-
P22	-	-	+	-	-	-	-	-
P23	-	-	-	-	-	+	-	-
P24	-	-	-	-	-	+	-	-
P25	-	-	-	-	-	+	-	-
P26	-	-	-	-	-	-	+	-
P27	-	-	-	-	-	-	-	+

^a The presence (+) and absence (-) of STRR DNA patterns are shown for each geographic locality. The number of *Nostoc* samples that shared identical patterns in each locality is indicated within parentheses.

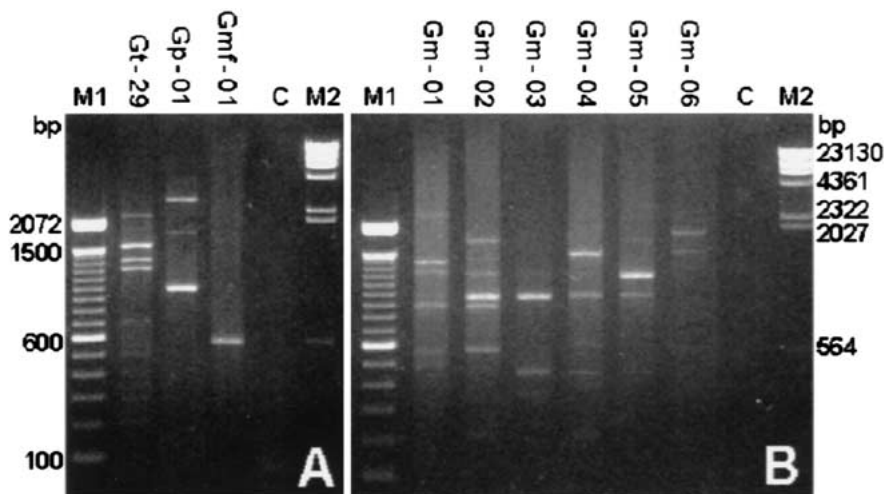


Fig. 3. STRR1-PCR fingerprint patterns of *Nostoc* microsymbionts extracted from different *Gunnera* species. (A) *G. tinctoria*, *G. peltata*, and *G. masafuerae*, (B) *G. magellanica* from three different localities (Table 1). Lane C, control with no template DNA, lanes M, molecular weight standards. M1, 100 bp ladder M2, λ *Hind*III.

ing to the STRR and LTRR sequences in PCR, resulting in a method which generates specific fingerprints for individual cyanobacterial isolates. Therefore, the PCR-STRR molecular markers are useful for studying the genetic diversity of cyanobiont populations in the wild, either free-living or symbiotic.

Nostoc Genetic Diversity within a Single Host Plant

All samples obtained from one individual host plant exhibited the same STRR-DNA fingerprint, indicating that the microbial symbiont population colonizing a single rhizome contained a reduced genetic variability. This result suggests that a mechanism selecting for a genetically restricted microbial partner could be operating in the establishment of the *Nostoc*-*Gunnera* symbiosis in wild populations. The infection of the stem gland cells depends on the presence of the *Nostoc* hormogonia in the exudate from the plants' apices. *Gunnera* possesses a unique gland on its stem that interacts with cyanobacteria [14]; therefore it is likely that a single compatible strain enters the rhizome tissue during early bacterial infection. Once inside the plant tissue, new symbiotic *Nostoc* colonies should arise from the first colonies and become disseminated throughout the plant rhizome as it grows. For *Anthoceros*/*Nostoc* symbiosis, Meeks and Elhai [14] proposed a similar mechanism for new infections that is dependent on a low number of hormogonia produced by preexisting *Nostoc* colonies. In addition, experimental inoculation assays have demonstrated that only one strain appears to be able to develop an effective symbiosis with the host plant, although several strains have been described in the outermost mucus of the *Gunnera* rhizome [3]. Accordingly,

only strains that were able to differentiate hormogonia (motile cell) were infective, even when other *Nostoc* strains were placed on plant apices close to newly formed glands. In contrast to these and our results, Nilsson et al. [17], using PCR-STRR as molecular marker, found that from eight isolates obtained in culture from the same *Gunnera* plant, three yielded different DNA fingerprints. The disagreement with our result could be attributed to their use of a *Nostoc* culture compared to the symbiotic tissue used in our work. Their method, based on an isolation process from the symbiotic tissue slice, could most likely purify additional strains present in the vicinity of the *Gunnera* gland, instead of the true microsymbiont. In other cases, surface contaminants have also been isolated from symbiotic tissue as putative symbionts [26].

On the other hand, Costa et al. [7], using molecular genetic tools without culture, showed that in *Nostoc*-Bryophyte symbiosis the same tRNA^{Leu} (UAA) intron sequence was obtained from all analyzed symbiotic samples within a single bryophyte thallus. The authors suggested that the development of individual symbiotic colonies seems to become stabilized at an early stage of the infection process, and new *Nostoc* strains do not seem to establish symbiosis. A similar specificity can be seen using identical methodological approaches in some lichens where only one *Nostoc* strain colonizes the lichen thallus [18].

The presence of one or more than one microsymbiont strain associated with a single host could be the result of the infection mode used in the symbiotic relationships. For example, in the symbioses of *Frankia*-actinorhizal plants and *Rhizobium*-leguminous plants there are several possible sites to establish an effective symbiosis, because the

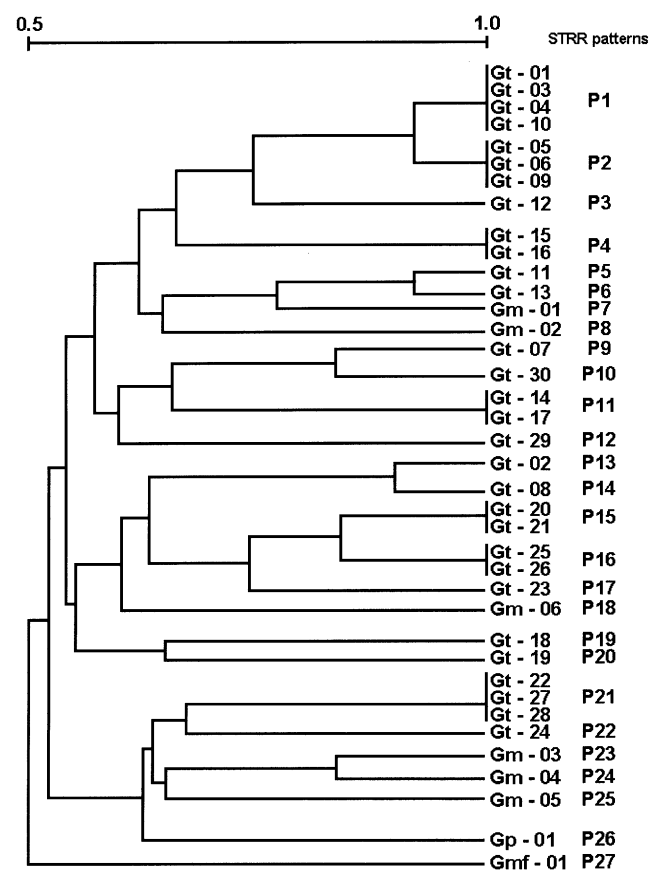


Fig. 4. Dendrogram of relatedness of STRR-PCR fingerprinting for *Nostoc* microsymbionts determined by cluster and the un-weighted pair-group method with arithmetic averages (UPGMA). The geographical origin and host plants of the microsymbionts studied are shown in Table 1.

infective bacteria can attach to different root hairs and multiple symbiotic organs (root nodules) are evolved by the plants to house the microsymbiont. In fact, in the actinorhizal symbiosis the root nodules usually contain more than one *Frankia* strain as different microsymbionts have been found colonizing specific lobes of a single nodule [1, 6, 20]. In the case of the *Rhizobium*-legume symbiosis, although a plant with multiple nodules was generally nodulated by more than one strain, a single nodule was often occupied by a single strain [22, 23]. In this manner, cyanobacteria such as *Nostoc* seem to be passive partners in all associations with plants, in contrast to *Frankia* and *Rhizobium*, which actively induce root nodulation by a multistage process that integrates both symbiotic partners. An extreme case has been described in the vesicomid clams of the genus *Calyptonia*, where it was determined that the microsymbiont partial 16S rRNA sequences amplified from ovaries of the clam were identical to the symbiont

sequences recovered from somatic tissue. This could be the consequence of the mechanism of symbiont transmission, which is inherited transovarially through the eggs [5].

Nostoc Genetic Diversity within and between *Gunnera tinctoria* Populations

Previous studies showed that 27 *Nostoc* isolates from six different *Gunnera* species growing in New Zealand were grouped into six DNA fingerprints. Similar results were obtained with 10 strains isolated from two Chilean *Gunnera* species (*G. tinctoria*, *G. magellanica*), which exhibited four different DNA patterns [17]. In addition, one DNA pattern was shared by several strains isolated from *Gunnera* from New Zealand, Chile, and a botanical garden in Sweden. These data suggest a moderate genetic diversity of *Nostoc* strains even though they were isolated from different *Gunnera* species growing in a broad geographic area [17].

In contrast, our results showed that *Nostoc* symbionts from three *Gunnera tinctoria* populations growing in the Abtao, Chiloé Island, exhibited 17 different DNA fingerprints among 27 host plants, indicating a higher genetic diversity (Figs. 2 and 4) than that obtained from cyanobacterial cultures studied by Nilsson et al. [17]. One explanation of this finding could be that the range of potential associations is greater in some environments because the symbiosis depends on many factors, among them the infective capacity of bacterial populations present in the soil, their interactions with the host plant, and local edaphic conditions. On the other hand, because our results were obtained from symbiotic tissue (noncultured) they may reflect better the actual genetic diversity of cyanobacterial microsymbionts in the host plant populations, since this approach eliminates the limitations associated with purification and culture of microorganisms.

Nostoc Genetic Diversity within and between Host-Plant Species

Our results are similar to those obtained by Rasmussen and Svenning [19], who found genetic heterogeneity among cyanobacterial isolates from the same *Gunnera* species, as well as genetic similarity among isolates from different *Gunnera* species.

Early work in reconstitution experiments indicated that several *Nostoc* strains were compatible with *Gunnera* species different from the original host-plant species,

suggesting that the specificity of the *Nostoc/Gunnera* symbiosis could not be particularly high [2, 14]. Zimmerman and Bergman [27] indicated that the main selective factor in the establishment of the symbiotic association is the availability of compatible and infective *Nostoc* strains in the soil, rather than the *Gunnera* species. The cluster analysis (UPGMA) showed that the microsymbionts did not group in relation to the host plant or the sites sampled (Fig. 4). These findings are in agreement with results from reconstitution experiments described above.

Further studies using STRR-PCR and with a more extensive sampling of symbiotic tissue from several *Gunnera* species should reveal a clearer picture of the cyanobacterial biodiversity present in natural populations.

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