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The *Gunnera* Symbiosis: DNA Restriction Fragment Length Polymorphism and Protein Comparisons of *Nostoc* Symbionts

William J. Zimmerman[1]* and Birgitta Bergman[2]

[1]Department of Agronomy & Soils, Washington State University, Pullman, Washington 99164-6420, USA and [2]Department of Physiological Botany, University of Uppsala, Box 540, S-751 21 Uppsala, Sweden

Abstract. Cyanobacteria separated from symbiosis with several species of the angiosperm *Gunnera* were comparatively characterized and correlated with the locales and taxonomy of their host plants. All were identified as strains of *Nostoc.* Protein profiles and DNA restriction fragment length polymorphisms (from hybridizations with heterologous *nif*H and *gln*A probes) determined that three of the four cyanobacteria from *Gunnera* grown at one site in Sweden, each from a different host species, were very similar or identical. Plants of one species, *G. manicata,* grown in a second location at the site were infected with a different cyanobiont. Among five isolates from two species of *Gunnera,* collected in the same locale in New Zealand, three subgroups were documented. Isolates from three different *Gunnera* species grown in separate locations in the United States were each uniquely different. None of the cyanobacteria differed in the molecular weights of their glutamine synthetase and Fe-nitrogenase proteins. The diversity and accessibility of compatible *Nostoc* populations present in the soil micro-environment, not a critical selective factor required by *Gunnera,* were concluded to be a major determinant in symbiont selection.

Introduction

Gunnera L. is one of only a few genera of higher plants, together with five genera of liverworts, which form symbioses with cyanobacteria. It is the only angiosperm known to live in such a mutualistic association. *Gunnera* is a genus of approximately fifty species in its own monotypic family (Gunneraceae). These herbaceous plant taxa, which vary considerably in size, occur predominantly at higher elevations in the tropics but are cultivated as ornamentals elsewhere (Fig. 1). The *Gunnera-Nostoc* symbiosis, first described by von Reinke [37], is facultative in that neither partner needs the other for survival [3, 5, 37].

* Corresponding author. *Present address:* Department of Natural Sciences, University of Michigan, Dearborn, MI 48128, USA.

Fig. 1. *Gunnera manicata* cultivated in an outdoor flat in Uppsala, Sweden (August 1988).

The *Nostoc* cyanobiont initiates the symbiosis by populating the external mucilage at the stem apex of *Gunnera* and later migrating to develop heterotrophically inside glandular cells of the plant [3, 27]. The parameters for successful establishment of symbiosis by a cyanobacterium are the abilities to infect the stem apex, form motile hormogonia, maintain consistent growth and nitrogen fixation, and penetrate host cell walls [4]. Recognition mechanisms between the symbiotic partners have been presumed to include lectins [33].

This diazotroph is able to satisfy the total nitrogenous needs of both partners during early stages of development [29]. However, there is no apparent correlation between the development of the cyanobiont and the host [35]. *Nostoc* cells eventually become intracellular by penetrating cell walls, but not the protoplasm, of *Gunnera* [7]. All cyanobacterial cells penetrate into host tissue [21, 28]. This compares to only 50% penetration into host cells of some cycads, mentioned by Duckett et al. [7], or none in the cases of other cycads, liverworts, and the aquatic fern *Azolla* [30].

Gunnera cyanobionts are relatively easily isolated and cultured [5, 27], as they are from various cycads and liverworts. Symbiont-free *Gunnera* seedlings may even be successfully infected with different *Nostoc* species [4] as can *Anthoceros, Blasia,* or cycads [8, 13–15, 26]. Such a reconstitution is severely limited in *Azolla* [17]. The primary cyanobiont of *Azolla* is apparently not culturable [12, 19], although minor symbiotic strains have been grown independently [39].

In *Gunnera,* the mode of infection by cyanobacteria, the successful infection by different strains in controlled experiments, and the lack of retention of the symbiosis throughout the life cycle suggest that the process of symbiotic association is not greatly regulated. This would belie early reports that the cyanobiont is of only one species, *N. punctiforme* [16, 38], which infers a greater selective role by the host.

This study characterizes and compares cyanobacteria isolated from *Gunnera* plants grown in soil environments of Sweden and New Zealand (and to a lesser extent, the United States). The intent is to elucidate the potential diversity of the cyanobionts and to relate their presence to *Gunnera* species and habitats.

Table 1. *Nostoc* strains isolated from *Gunnera*

Cyanobacterial strain code	Host species	Region of *Gunnera* cultivation
SW-Gtc	*G. tinctoria*	Uppsala, Sweden
SW-Gnz	*Gunnera* sp.	Uppsala, Sweden
SW-Gmn1	*G. manicata*	Uppsala, Sweden
SW-Gmn2	*G. manicata*	Uppsala, Sweden
NZ-7901[a]	*G. arenaria*[b]	New Zealand
NZ-8001	*G. chilensis*[b]	New Zealand
NZ-8002	*G. chilensis*[b]	New Zealand
NZ-8005	*G. chilensis*[b]	New Zealand
NZ-NG[c]	*G. arenaria*	New Zealand
US-H	*G. kaalensis*	U.S.A. (Hawaii)
US-K	*G. killipiana*	U.S.A. (Berkeley, CA)
US-R	*Gunnera* sp.	U.S.A. (Coos Bay, OR)

[a] The NZ strains bear the numerical code of the collection of J. Meeks (University of California, Davis, USA)
[b] Probable host species (W. B. Silvester, personal communication)
[c] "NG" is the "*Nostoc* ex *Gunnera*" isolate [28]

Materials and Methods

Cyanobiont Origins, Isolation, and Culture

Twelve cyanobacterial isolates from species of *Gunnera* were selected for this investigation (Table 1). All were maintained on agar and in liquid culture with nitrogen-free BG-11_0 medium [25]. Five accessions from *Gunnera* native to New Zealand, isolated by W. B. Silvester, were obtained from the collection of J. Meeks (University of California, Davis, USA). One cyanobiont from *G. kaalensis* in Hawaii, isolated by E. Towata, was provided by B. Rosen (University of Tampa, FL, USA).

The remaining six accessions were acquired by direct extraction from *Gunnera* plants. The four cyanobacteria isolated in Sweden came from *Gunnera* species indigenous to South America. The plants were grown from seeds planted in a standard soil mixture of potting soil and peat moss. Three were cultivated within a greenhouse while one, Gmn2, was growing in an outdoor flat at the time of cyanobacterial extraction. Stalk sections from mature plants of two *Gunnera* species exotic to the USA were acquired from California (Dept. Botany, University of California, Berkeley) and Oregon.

Stem pieces of *Gunnera* were first washed in sterile water and then in 70% ethanol. Using flame-sterilized scalpels and dissecting needles, cortical plant tissue was removed and thin sections of plant tissue from areas of cyanobacterial glands were sliced, rinsed in 70% ethanol, and again in sterile water. These sections were either placed into Petri plates containing BG-11_0 medium and 1.5% bacto-agar or first teased in a drop of water to free cyanobacterial cells, which were then transferred onto the plates.

To remove contaminants from the cyanobacteria, individual motile hormogonia were picked from young colonies growing on agar plates and used to inoculate fresh agar plates amended with 20–40 mM sodium arsenate [34]. This procedure was repeated and sometimes combined with a sonication step so that single cells could be spread over agar plates. After this procedure was completed, hormogonia were used to initiate liquid cultures in 50 ml Erlenmeyer flasks containing BG-11_0.

Cyanobacteria from these flasks were taken to grow 700–800 ml of culture aseptically in aerated

one-liter flasks. The air flow was supplemented with 1.0% CO_2, sterilized with a 0.2 μm nylon filter, and hydrated through a water trap before reaching each culture. Light was provided by cool-white fluorescent lamps (approx. 100 μmol m^{-2} sec^{-1}) and the temperature was kept at 25°C. The cyanobacteria were harvested by centrifugation at 10 to 14 days after inoculation. The cell biomass was washed in TE (10 mM Tris : HCl, pH 8.0/1 mM EDTA, pH 8.0), centrifuged again, and stored at −20°C until thawed for DNA extraction.

Protein Extraction, Electrophoresis, and Western Blots

Total soluble proteins were extracted by transferring samples from the 50 ml Erlenmeyer flasks into sterile conical polyethylene tubes. The cells were centrifuged to obtain 1 ml of packed biomass, washed with TE, and centrifuged again. The contents were transferred to glass vials and 0.5% (v/v) 2-mercaptoethanol was included. Cells were ruptured on ice by sonication and stored at −20°C until use.

Electrophoresis and protein staining were conducted with a Pharmacia PhastSystem (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden) using the manufacturer's recommended protocols. Pairs of mini-gels (8–25% T, 2% C) with protein samples denatured with sodium dodecyl sulfate (SDS) were subjected simultaneously to electrophoresis—one for staining of total protein profiles and the other for use in Western blots of specific proteins.

The immunostaining procedure was the one utilized by Bio-Rad (Richmond, CA, USA). Two rabbit sera containing monospecific antibodies against glutamine synthetase (GS) purified from *Anabaena* PCC 7120 [2, 23] or against the Fe-protein of nitrogenase [1, 6] purified from *Rhodospirillum rubrum* were used with Western blots. The GS antiserum was provided by R. Haselkorn (University of Chicago, Chicago, IL, USA) and the nitrogenase antiserum by S. Nordlund (University of Stockholm, Stockholm, Sweden).

DNA Extraction and Purification

Thawed cyanobacterial biomass (0.5–1.0 g fresh weight) was refrozen in liquid nitrogen, ground into powder with a mortar and pestle, resuspended inside sterile polypropylene tubes with an extraction buffer, and incubated for 1 hour at 37°C. The extraction buffer consisted of 3 ml of 25 mM Tris : HCl (pH 8.0)/50 mM EDTA (pH 8.0), 1.7 ml of 30% sucrose, and 1 ml of lysozyme (50 mg ml^{-1} stock).

SDS was then added to each tube to 1% v/v (10% stock). The contents were mixed carefully by inversion and 25 units (78 units mg^{-1}) of predigested RNase A (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden) were added. The tubes were incubated 1–2 hours at 37°C.

The DNA phase was extracted initially with one or more treatments with an equal volume of Tris-equilibrated phenol (molecular biology grade). This was followed by repeated extractions in 1:1 phenol : chloroform/isoamyl alcohol (24/1), and chloroform/isoamyl alcohol. DNA was precipitated from the aqueous phase with 2.5 M ammonium acetate and 96% ethanol. After centrifugation, the pellet was washed in 70% ethanol and resuspended in 4 ml TE. The contents were ultracentrifuged in a CsCl isopycnic gradient (with 0.5 mg ml^{-1} ethidium bromide) in a VTi65 rotor at 50,000 rpm for 12–18 hours at 20°C.

After side-puncture removal of the DNA band from each tube under ultraviolet light, the ethidium bromide was extracted with multiple changes of isopropanol saturated with 20× SSC (3 M NaCl, 0.34 M sodium citrate, pH 7.0), and the CsCl removed by dialysis in TE. The DNA was then pipetted into sterile microfuge tubes, precipitated, and rinsed as before, then resuspended into TE. The DNA purities were estimated by measurement of the optical densities at 260 nm and 280 nm.

The continued presence of residual polysaccharides was later removed using a hexadecyltrimethylammonium bromide (CTAB) precipitation. This DNA purification technique subsequently

eliminated the need for CsCl and ultracentrifugation in the final cyanobacterial harvests. Following the suspension of DNA in TE, NaCl was added to 0.7 M (from 5 M stock) and then CTAB to 1% (from a 10% CTAB/0.7 M NaCl stock). Tubes were incubated at 65°C for 10–20 min, and the aqueous phase extracted twice with chloroform/isoamyl alcohol. The DNA was precipitated after the last extraction with 96% ethanol, pelleted, and resuspended as previously described into 300 µl TE.

DNA hybridization

The two restriction enzymes *Eco*RI and *Hin*dIII were used to digest the cyanobacterial genomic DNA according to the manufacturers' recommendations (New England Biolabs, Inc., Beverly, MA, USA; Bethesda Research Laboratories, Bethesda, MD, USA). The DNA was fractionated by electrophoresis in 0.7% (w/v) agarose and transferred either to nitrocellulose or to Zeta Probe nylon membranes (both from Bio-Rad Laboratories, Richmond, CA, USA) by neutral and alkaline capillary blot procedures, respectively.

The hybridization probes were taken from subclones of pAn154.3 and pAn503 containing *nif*H and *gln*A genes, respectively, from *Anabaena* PCC 7120. The *nif*H insert was a 1.8 kb *Hin*dIII fragment containing 0.9 kb *nif*H, 0.15 kb of *nif*D, and 0.75 kb of non-*nif* DNA [20]. The *gln*A insert was a 1.4 kb *Eco*RI fragment containing 0.94 kb of coding sequence and 0.46 kb of 3′-flanking sequence of the *gln*A gene [11, 36]. Both probes were oligolabelled with (α^{32}P)dCTP according to strict protocols [9, 10]. Hybridization was carried out in 6× SSPE at 60°C. Autoradiograms of x-ray film were exposed from 2–36 hours at −70°C. Membranes were reused after stripping the hybridized probes with either boiling water (nitrocellulose) or 0.4 N NaOH (nylon).

Results

Three of the four Swedish accessions from greenhouse-cultivated *Gunnera* plants were similar in their colonial morphology, growing as loose aggregates on agar and in stationary liquid culture. The exception was the Gmn2 isolate, taken from a plant grown outdoors, with its tendency to form more definitive colonies. The New Zealand accessions were also similar, except for the varied cell shapes and sizes of the NG isolate. These cyanobacteria were taken from *Gunnera* plants grown at only one general locale. None of the three isolates from plants grown in the United States resembled each other, and their sites were widely separated. All of the cyanobacteria were identified as *Nostoc* strains by their cell morphology and the presence of hormogonia.

Protein profiles confirmed the similarities within each of the two major groups (Fig. 2). The exceptions were once again the Gmn2 and NG isolates, where the sizes of the majority of the soluble proteins differed from those of the other cyanobacteria in their respective groups. The NG isolate also exhibited a protein doublet of bands at 23 and 25 kDa (the other cyanobacteria possessed two bands at 20 and 25 kDa) that correlated with those observed in *Anabaena* strains rather than in *Nostoc* (W. J. Zimmerman, unpublished data). The banding patterns of the U.S. accessions were distinct from these and from each other (profiles not shown). However, the protein subunits of glutamine synthetase (MW 50 kDa) and of the Fe-protein of nitrogenase (MW approx. 36 kDa), examples shown in Fig. 2C, did not differ in any of the isolates from the known

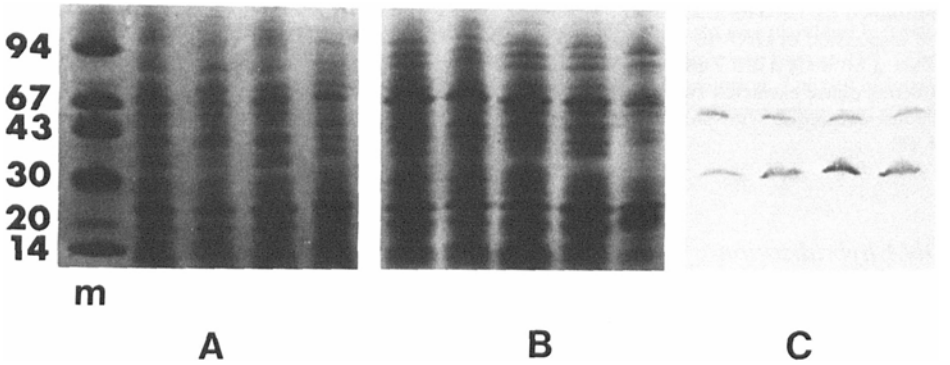

Fig. 2. Total soluble protein profiles (SDS-PAGE) and immunoblots of cyanobacterial cell-free extracts. A) Swedish group: Gtc, Gnz, Gmnl, Gmn2 (left to right); B) New Zealand group: 7901, 8001, 8002, 8005, NG (left to right); C) The glutamine synthetase protein at 50 kDa and the Fe-nitrogenase protein at about 36 kDa as identified by Western blots (Swedish isolates in the order given in 2A; composite picture of two separate membranes). m = molecular weight markers from 14.4–94 kDa.

molecular masses of those proteins for free-living cyanobacteria [22, 31, 32] or symbiotic cyanobacteria [2, 6].

Likewise, these same correlations were observed for the cyanobacteria in their hybridization patterns with *nif*H and *gln*A (Table 2; Figs. 3, 4). The limited data obtained from the DNA restriction fragment length polymorphisms (RFLPs) of the U.S. cyanobacteria were sufficient to reconfirm their dissimilarities with each other and the strains from Sweden and New Zealand.

The RFLPs from *nif*H hybridizations for the Swedish cyanobacteria were generally more complex than those with the cyanobacteria from New Zealand plants. The three identical Swedish isolates, Gtc-Gnz-Gmn1, yielded more restriction fragment bands (four) with the *Hin*dIII digest than did the other accessions; the *nif*H gene is known to be multi-copy [24]. The radioactive signal of the smallest DNA fragment (1.3 kb) was much less intense than the others and judged to be a minor band, that is, the result of hybridization to a very short similar DNA sequence. This band number exceeded that from other free-living and symbiotic *Nostoc* and *Anabaena* strains, but gave three, instead of four bands, with the *Eco*RI/*Hin*dIII double digest [19]. The lack of a documented fourth band may be attributed to either the presence of a co-migrating band or a small fragment (<500 bp) which had migrated off the gels.

The restriction fragment patterns of 7901 and 8001 were identical for both probes, as were those of 8002 and 8005 of the New Zealand isolates. The *nif*H hybridizations with the *Eco*RI DNA digests of 8002 and 8005 also showed that they both possessed the same weakly hybridizing minor band (observable in Fig. 4 from an over-exposed autoradiogram). The high molecular-weight band in this same hybridization, after checking with repeated digestions and probing from different DNA stocks and by sequential probing with the *gln*A insert, was verified not to be a product of a partial digest. The hybridized fingerprints of mixed DNA from distinct isolates (8005 + 7901; 8005 + 8001; 8002 + 7901) were also able to be accurately interpreted.

Table 2. Tabulated sizes (kb) of genomic DNA fragments from *Nostoc* strains, following single and double digestions with the restriction enzymes *Eco*RI and *Hin*dIII and hybridizations with *gln*A and *nif*H probes (as described in Materials and Methods)

	*gln*A		
Strain	*Eco*RI	*Hin*dIII	*Hin*dIII/ *Eco*RI
SW-Gtc	1.80, 0.76	3.4	—[a]
SW-Gnz	1.80, 0.76	3.4	1.10, 0.76
SW-Gmn1	1.80, 0.76	3.4	1.10, 0.76
SW-Gmn2	1.85, 0.76	5.0	—
NZ-7901	3.8, 0.76	4.5	2.5, 0.76
NZ-8001	3.8, 0.76	4.5	—
NZ-8002	0.87, 0.76	3.8	0.87, 0.76
NZ-8005	0.87, 0.76	3.8	0.87, 0.76
NZ-NG	3.0	—	—
US-H	1.60, 0.87	6.5	—
US-K	1.65, 0.76	4.6	—
US-R	—	2.6	—
	*nif*H		
Strain	*Eco*RI	*Hin*dIII	*Hin*dIII/ *Eco*RI
SW-Gtc	11, 4.7, 3.3	7.4, 2.3, 1.9, (1.3)[b]	—
SW-Gnz	11, 4.7, 3.3	7.4, 2.3, 1.9, (1.3)	5.6, 1.35, 1.15
SW-Gmn1	11, 4.7, 3.3	7.4, 2.3, 1.9, (1.3)	5.6, 1.35, 1.15
SW-Gmn2	4.4, 2.9, 1.10	4.6, 1.9, 1.5	—
NZ-7901	4.4, 1.20	4.4, 3.3	2.5, 1.30
NZ-8001	4.4, 1.20	—	—
NZ-8002	>20, (4.4)	4.5	3.9
NZ-8005	>20, (4.4)	4.5	3.9
NZ-NG	10, 6.6, 4.2	—	—
US-H	—	—	—
US-K	4.4, 1.15	4.8, 3.6	—
US-R	—	5.9, 5.0, 3.6	—

[a] No hybridization conducted
[b] Parentheses indicate a minor band

Discussion

The molecular results in this study characterized the similarity among the *Nostoc* isolates from *Gunnera* plants cultivated in Sweden, among those from plants in New Zealand, and the differences betwen these groups and the cyanobacteria from the USA. The New Zealand cyanobacteria, containing two identical pairs of *Nostoc,* are not well documented and have been in extended culture. However, the fact that three of the freshly cultured Swedish isolates are identical implies that those from New Zealand should not be dismissed as clonal cultures.

Only one genus of cyanobiont is generally identified with a host genus in any symbiosis; the few reports of other endophytic genera are supposedly due to incorrect identification or unusual exceptions [33]. *Nostoc* has been the only cyanobacterial genus found in symbiosis with *Gunnera.* This is also true in

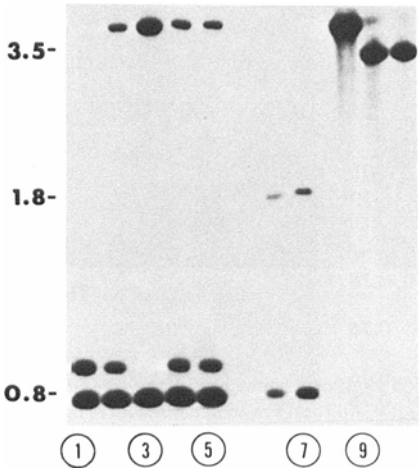

Fig. 3. Representative Southern blot analyses with *gln*A hybridizations of total cyanobacterial DNA from isolates of the three major subgroups from New Zealand and Sweden. From left to right, lanes 1–7: 8002, 8005 + 7901, 7901, 8005 + 8001 (twice), Gtc, Gmnl (*Eco*RI digestion); lanes 8–10: 8002, Gnz, Gmnl (*Hin*dIII digestion).

several other plant symbioses, and perhaps even so with *Azolla* and its symbiotic "*Anabaena azollae*" [19]. *Anabaena,* the closest relative to *Nostoc,* and other Nostoccalean genera were never capable of re-establishing a symbiosis.

Although plant hosts of cyanobacterial symbioses may be selective for *Nostoc,* a number of species or strains of that genus are apparently successful in becoming cyanobionts. Investigations involving the artificial reconstitution of the symbiosis in liverworts found that, as with *Gunnera* [4], only *Nostoc* strains were successful and that they could be naturally free-living or originally symbiotic [8, 26]. This genus-specific, species-flexible symbiont selection was also documented in cycads by Grobbelaar et al. [15] where 30 species of *Encephalartos* and one of *Stangeria* were examined and found to contain *Nostoc* (six species) in all but one case.

Grilli Caiola [14] likewise observed only *Nostoc* in 12 species of four cycad genera. However, those cyanobionts were subsequently categorized into three major types according to host range with the conclusion that a single species of cyanobacterium is found for each cycad genus or group. That investigation somewhat paralleled ours in that cyanobacteria were isolated from plants growing in several different locales, although no geographic correlation was made. On the other hand, we interpreted our results quite differently. Although one *Nostoc* strain may have infected three disparate host species in Sweden, and another *Nostoc* was found in two *Gunnera* species in New Zealand, five different symbiotic cyanobacteria from those two sites were isolated altogether. There was no selective cyanobacterial host range to be inferred from our observations.

Current research with cyanobacteria has shown the ability of DNA RFLP techniques to accurately fingerprint morphologically similar cyanobacterial strains [12, 18, 19]. This capacity to distinguish *Nostoc* taxa from one another has proved in *Azolla* [19] and in cycads [18] that multiple cyanobionts may exist in symbiosis with a plant. This is probably also true for *Gunnera,* although not definitively evident in our study. Our restrictive isolation technique (single-hormogonium transfers) and serial subculturing presumably resulted in the selection of only one cyanobacterial strain per *Gunnera* plant. However, the

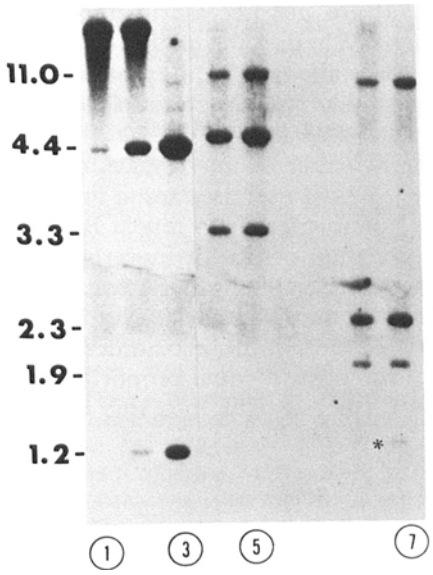

Fig. 4. Representative Southern blot analyses with *nif*H hybridizations of total cyanobacterial DNA from isolates of the three major subgroups from New Zealand and Sweden. From left to right, lanes 1–5: 8005, 8002 + 7901, 7901, Gnz, Gmnl (*Eco*RI digestion); lanes 6–7: Gnz, Gmnl (*Hin*dIII digestion; * refers to faint bands).

multiple bands observed from the *nif*H hybridizations for Gtc-Gnz-Gmnl might conceivably indicate the presence of a second Swedish strain in those plants.

Nevertheless, our combined evidence illustrating the diversity of the *Gunnera* cyanobionts denies the possibility of their being only varieties of the single species, *Nostoc punctiforme*. The selection of the different cyanobionts by *Gunnera* species seems to be primarily influenced by the accessibility of compatible cyanobacteria, not species-specific host recognition. This point is shown in *G. manicata*, grown in the same soil but either in a greenhouse or an outdoor flat, which formed associations with different cyanobacteria.

The diversity of microbial populations established in different soil substrates may therefore be a selective force in successful cyanobacterial symbiosis. This would even include the sand cultures used to grow *Gunnera* by Silvester [27]. The variation in the geographic habitats of *Gunnera* taxa would be a factor in selection only indirectly, i.e., if a given cyanobacterial composition of a particular soil or area is unique. The *Gunnera* association apparently is more analogous to that in cycads than to the severely regulated *Azolla* symbiosis.

The only experiments proving the capability of several *Nostoc* strains to infect *Gunnera* were performed under controlled experimental conditions using surface-sterilized seeds germinated in sterile vermiculite [4]. Our observations with cyanobacteria taken from plants grown in a "natural" environment—relatively uncontrolled light and temperature, nonsterile, nitrogen-containing soil, the presence of various cyanobacterial populations subject to selection pressures—corroborate those results; our initial characterization demonstrates the potential diversity of infectable *Nostoc* strains.

We also isolated two free-living *Nostoc* isolates, dominant in the Swedish soils, which did not resemble the Swedish cyanobionts morphologically when cultured nor in their protein profiles. This indicates that strains meeting the

infection criteria of Bonnett and Silvester [4] are not necessarily the most abundant taxa in a habitat containing *Gunnera.* Whether the choice of one or more cyanobionts by *Gunnera* plants at the same site is related exclusively to the diversity and relative abundance of compatible cyanobacterial species in that soil is still uncertain.

The next investigative step is to determine whether *Gunnera* takes up any compatible cyanobacterial strain or whether the host exercises some measure of selectivity beyond mere recognition. One possible test is to repeat Bonnett and Silvester's experiments using a mixed inoculum of two or more isolates, known to successfully infect *Gunnera,* which are separable by DNA restriction fragment length polymorphisms (such as those demonstrated here in Figs. 3 and 4) or isoenzyme profiles. An examination of the symbiotic colonies in the mature plant to find if the two strains infect the host in equal proportions or if one is preferentially chosen will provide further insight into this unique association.

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