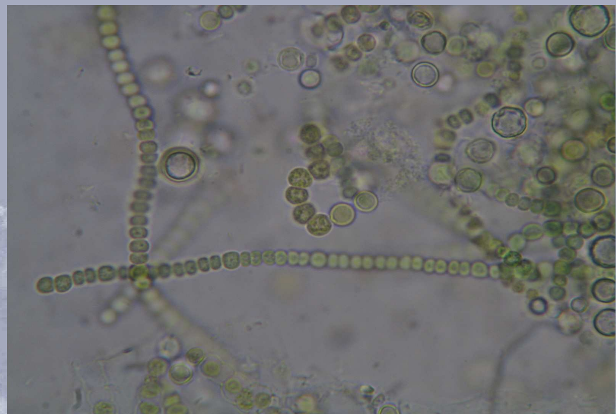
A COLLECTION OF POLAR CYANOBACTERIA FOR THE EXPLORATION OF DIVERSITY AND BIOTECHNOLOGICAL APPLICATIONS

A. Wilmotte[1], K. Waleron[2], M. Waleron[2], Z. Namsaraev[1], P. Simon[1], R. Fernandez[1], A. Taton[1], V. Storms[3], W.F. Vincent[4], R.W. Castenholz[5], F. Marinelli[6]

1 - University of Liege, Centre for Protein Engineering, Liege, Belgium, 2 - University of Gdansk, Department of Biotechnology, Gdansk, Poland, 3 - University of Ghent, Laboratory of Microbiology, Ghent, Belgium, 4 - Université Laval, Department of Biology, Quebec, Canada, 5 - University of Oregon, Department of Biology, Eugene, USA, 6 - University of Insuburia, DBSM, Varese, Italy

2006: BCCM (Belgian Coordinated Collections of Microorganisms) stimulation project on the elaboration of culture collections of microorganisms that were not yet available in the current consortium: **a collection of polar cyanobacteria is being built.**



- A way to preserve and further characterize strains isolated during projects

- But ‘New duties’ for Quality Assurance purposes

OUTLINE

1. **Antarctic strains, their origin, isolation procedure, morphological diversity, genotypic diversity,**
2. **Arctic strains, their origin**
3. **Siberian strains, their origin**
4. **Characterisations for the collection of polar cyanobacteria**
5. **First dedicated screening of about 50 Antarctic strains for bioactive compounds**

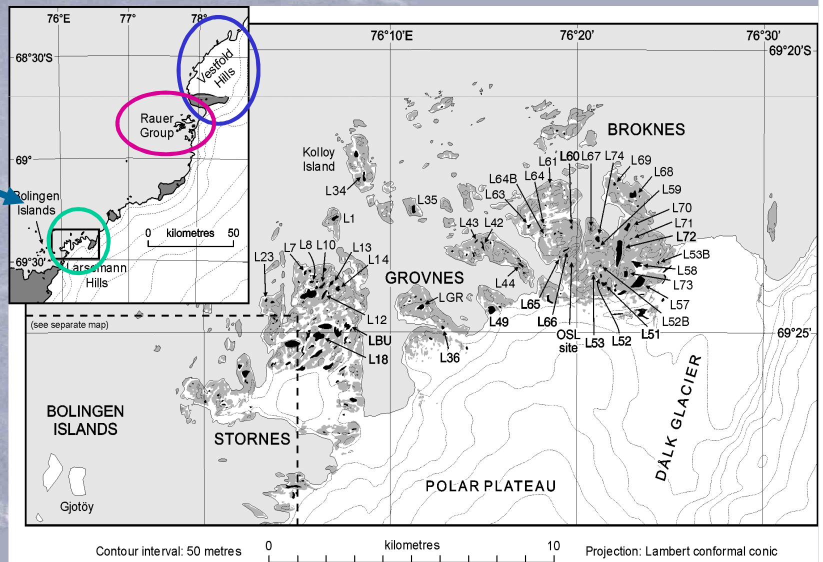
1. Microbial mats in Antarctic lakes

South Victoria Land

Lake Fryxell, Dry Valleys

Larsemann Hills:

Lakes Reid, Firelight, Progress, Heart



Rauer Islands

Vestfold Hills

EC project MICROMAT, LTER Dry Valleys, AAD

Examples of Antarctic strains isolation & culture

✓ **Several isolation techniques, 17 culture media, 3 incubation temperatures**

➔ **59 strains from 23 lakes**

- **34 strains at 22℃**
- **23 strains at 12℃**
- **2 strains at 5℃**

→ ***Correlated to lower growth rates at low temperatures***

✓ **Morphological description → 12 taxa**

- **Oscillatoriales: 43 strains**
- **Nostocales: 15 strains**
- **Chroococcales: 1 strain**

→ ***In agreement with the dominance of Oscillatoriales in microbial mats***

✓ **16S rRNA gene sequences (56 strains)**

Morphological diversity

Oscillatoriales

S40 *Phormidium murrayi* **(Ace lake)**

S12 *Pseudophormidium sp* ***(lake Gentner)***

S26 *Phormidium priestleyi* **(lake Broknes)**

S32 *Leptolyngbya antarctica* **(lake Gentner)**

Morphological diversity

Nostocales

S16 *Calothrix sp* (lake Broknes)

S38 Coleodesmium sp (lake Broknes)

S08 *Nostoc sp.* (lake Progress)

S60 *Petalonema sp.* (lake Gentner)

GENOTYPIC DIVERSITY Antarctic strains

OTU (Operational Taxonomic Unit): group of 16S rRNA gene sequences with more than 97.5% of sequence similarity for positions of *E. coli*: 405-780

→ 21 OTUs were obtained

Each OTU might correspond to more than one species, following the bacterial genospecies definition, but is surely **distinct** from other OTUs at the **specific level** (Stackebrandt & Goebel, 1994)

→ **Underestimation of the diversity !**

Examples of Antarctic strains isolation & culture

✓ Several isolation techniques, 17 culture media, 3 incubation temperatures

➔ 59 strains from 23 lakes

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✓ Morphological description → 12 taxa

- Oscillatoriales: 43 strains
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→ In agreement with the dominance of Oscillatoriales in microbial mats

✓ 16S rRNA gene sequences (56 strains) → 21 OTUs

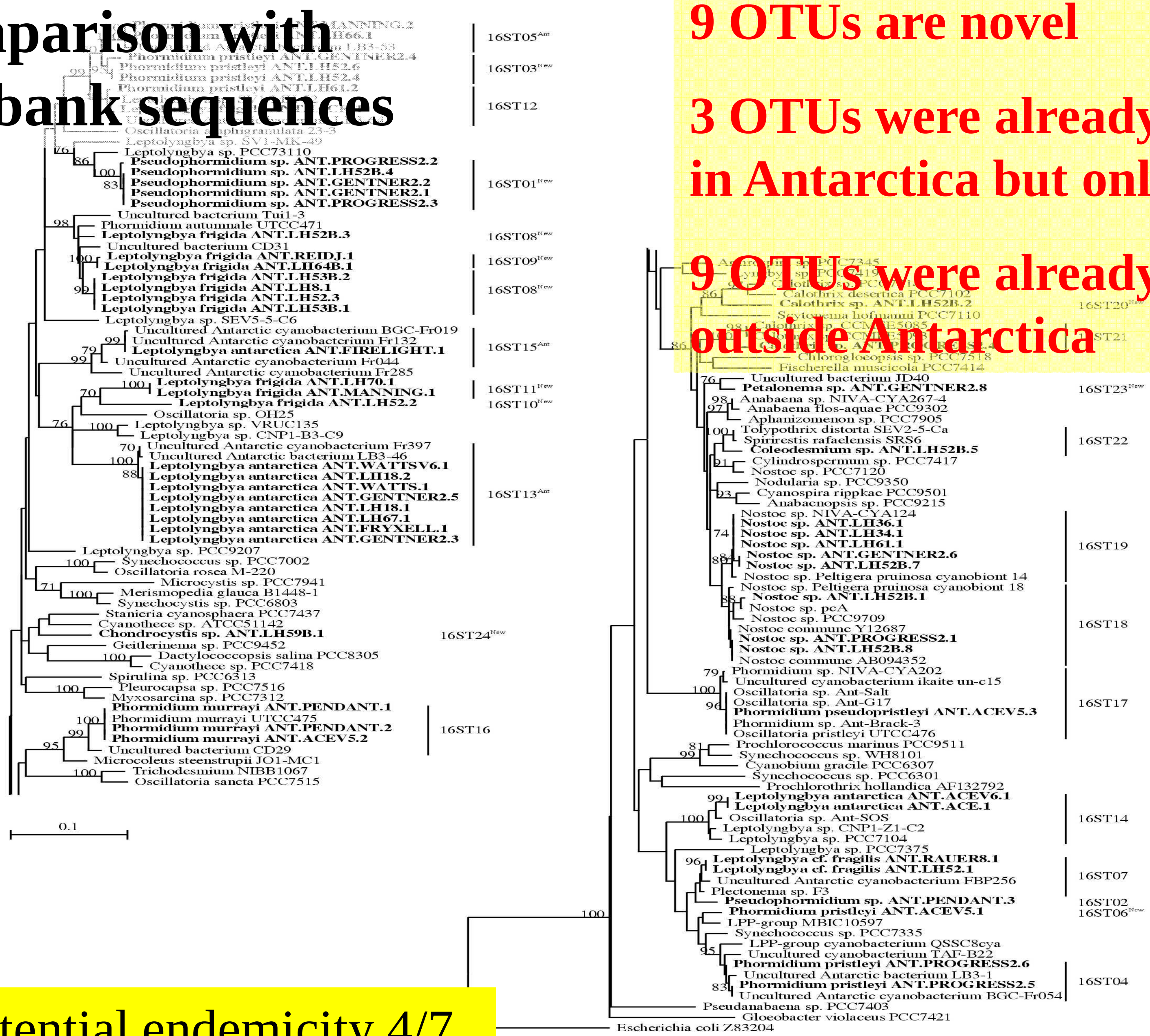
→ Molecular diversity > morphological diversity

Comparison with Genbank sequences

9 OTUs are novel

3 OTUs were already found in Antarctica but only there

9 OTUs were already found outside Antarctica

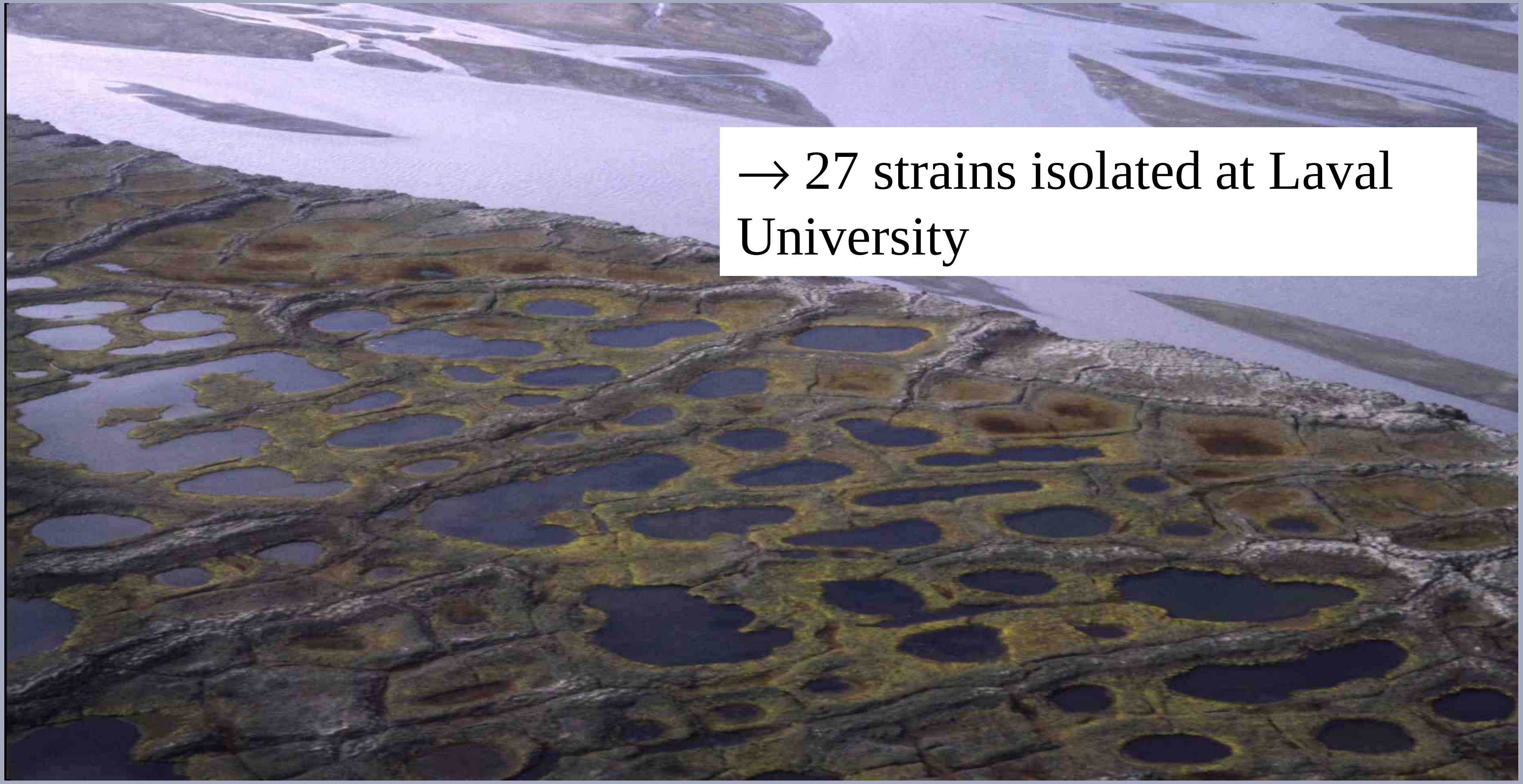


→ Potential endemicity 4/7

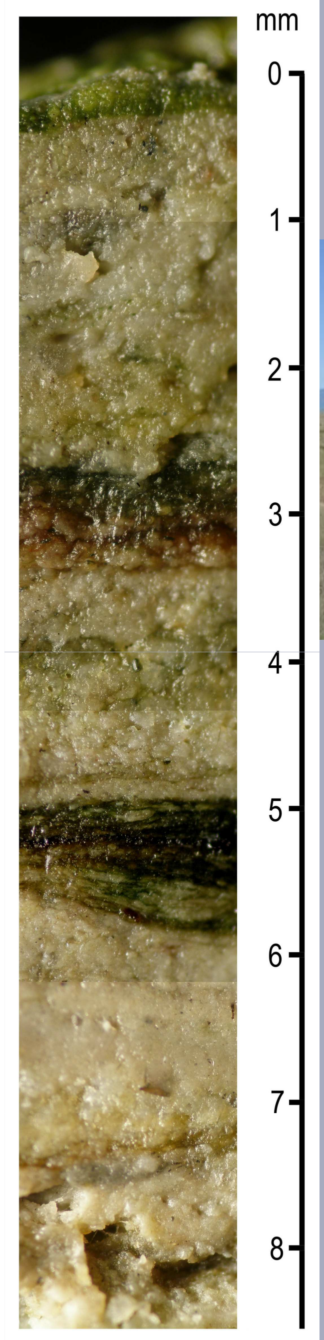
Taton et al. 2006 J. Phycology

2. Microbial mats and water in Arctic lakes

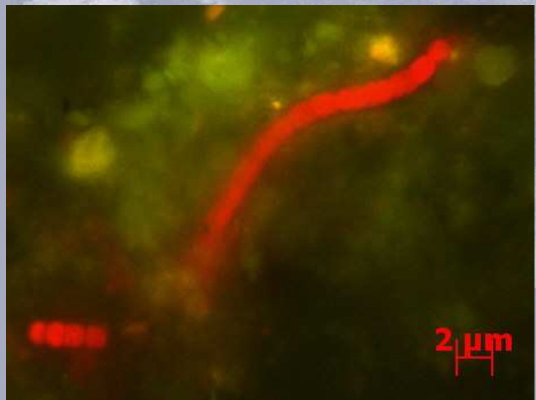
Tundra & thermokarst lakes, Bylot Island, Nunavut, Canada



→ 27 strains isolated at Laval University



3. Siberian hypersaline lakes



Benthic microbial mats in soda lakes subjected to temperatures from –40° to +40°C and unstable water regimes

→ 20 strains isolated at the Winogradsky Institute, RAS

4. Characterisations for the collection of polar cyanobacteria

Tests for viability, purity and authenticity:

-16S rRNA gene

-ITS spacer between the 16S and 23S rRNA gene

-Fingerprints by variants of HIP (Highly Iterated Palindrome)

- GCGATCGC overrepresented in most cyanobacteria (Robinson et al. 1995)
- Smith et al. (1999): use of variants with 2 additional bases to obtain genomic fingerprints to distinguish closely related strains

Distance tree based on the 16S rRNA

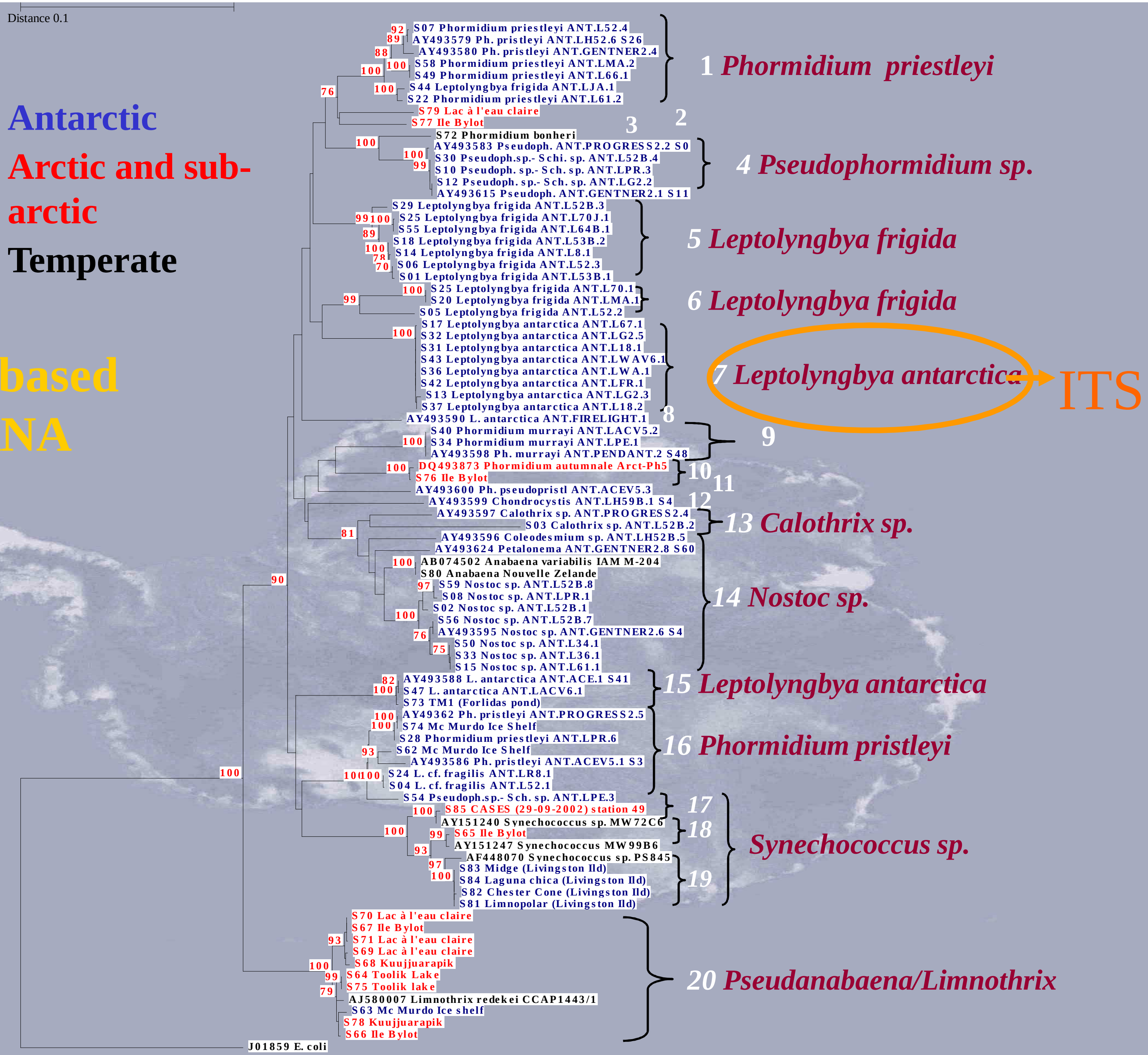
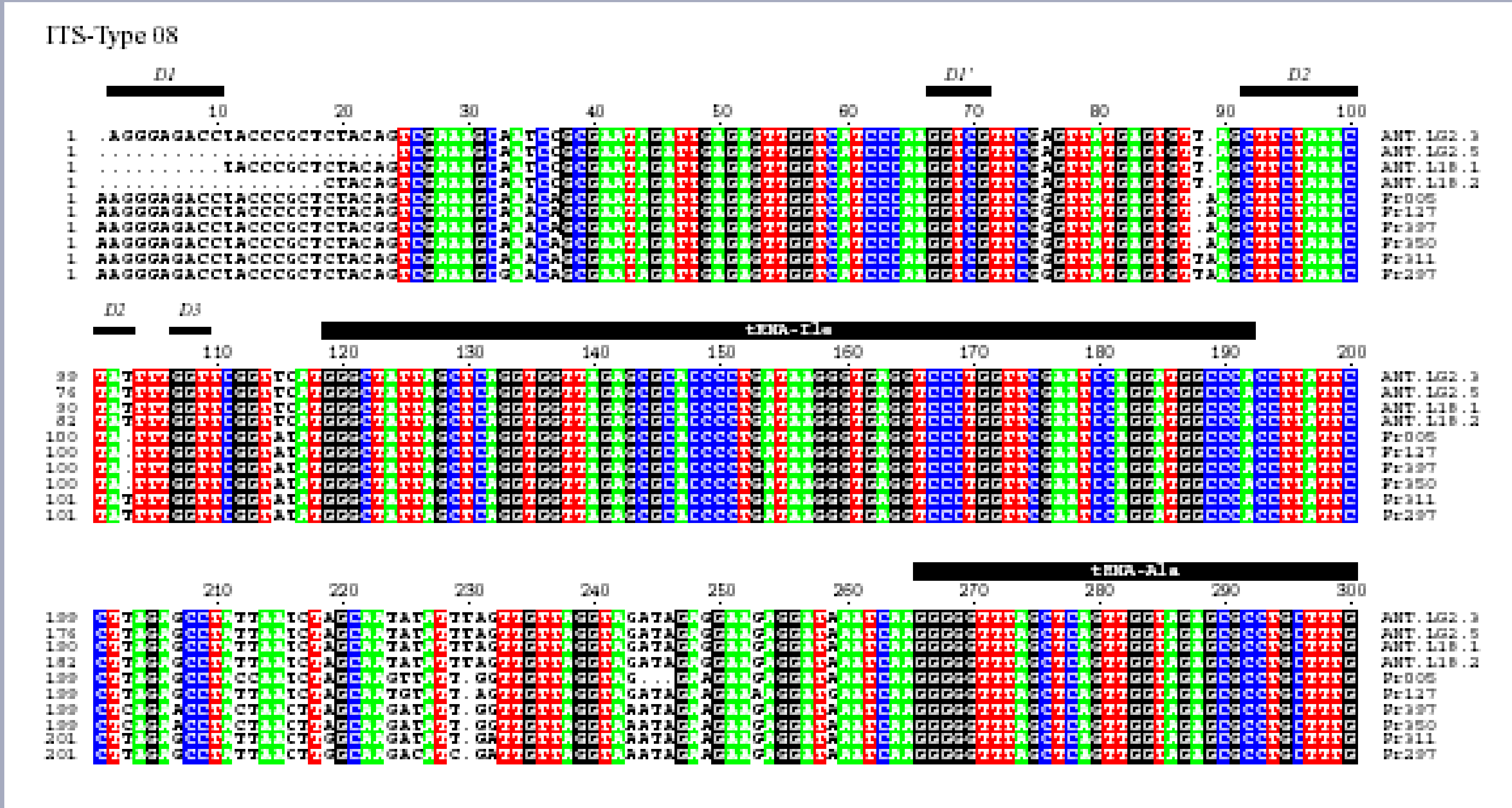


Fig. 1. Neighbor-joining tree based on 1061 16S rRNA positions, with a Jukes-Cantor correction for multiple mutations. The most similar sequence in Genbank was extracted by Sequence Match of RDPII. Indels were not taken into account. Bootstrap values higher than 70 % are indicated besides the nodes.

ITS sequences group 7

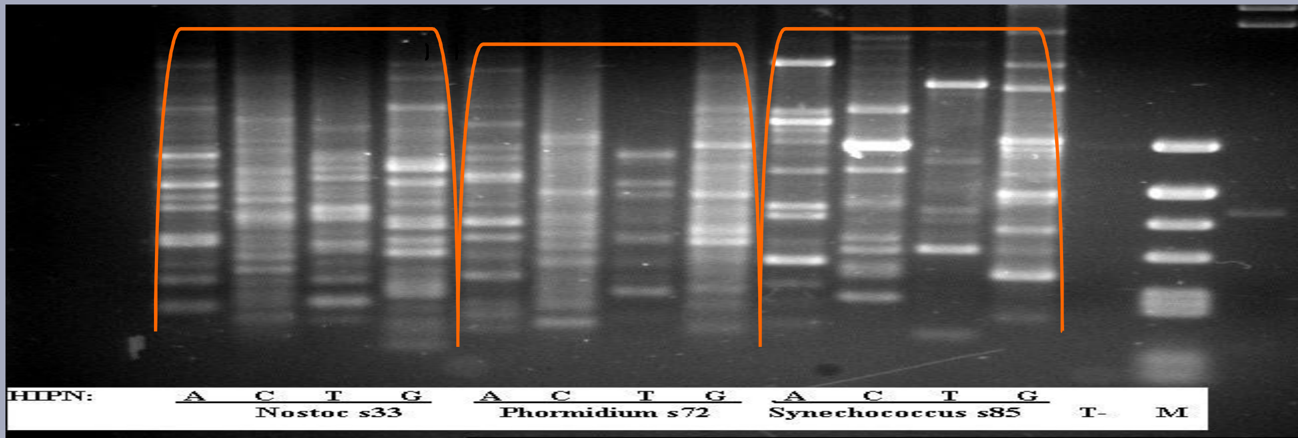


4 sequences of 2 Prydz Bay lakes and 6 sequences from Lake Fryxell

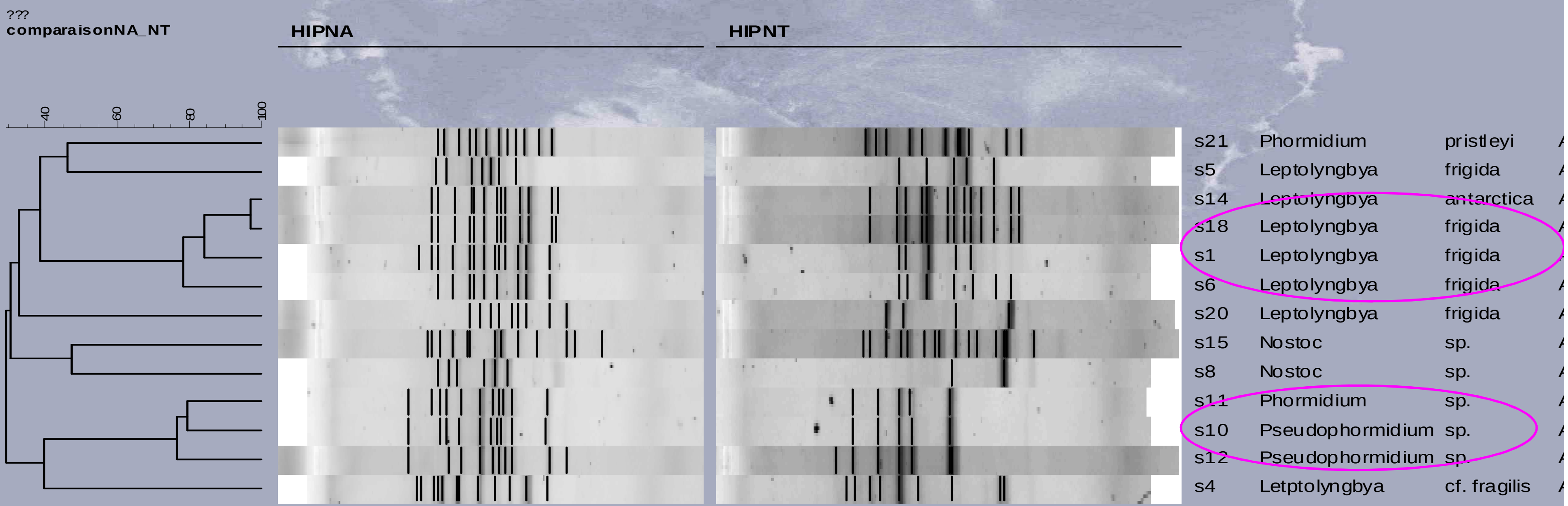
→ 11% polymorphic positions

Genomic fingerprints with HIP variants

1. Different variants: HIP-NA, HIP-NC, HIP-NT, HIP-NC



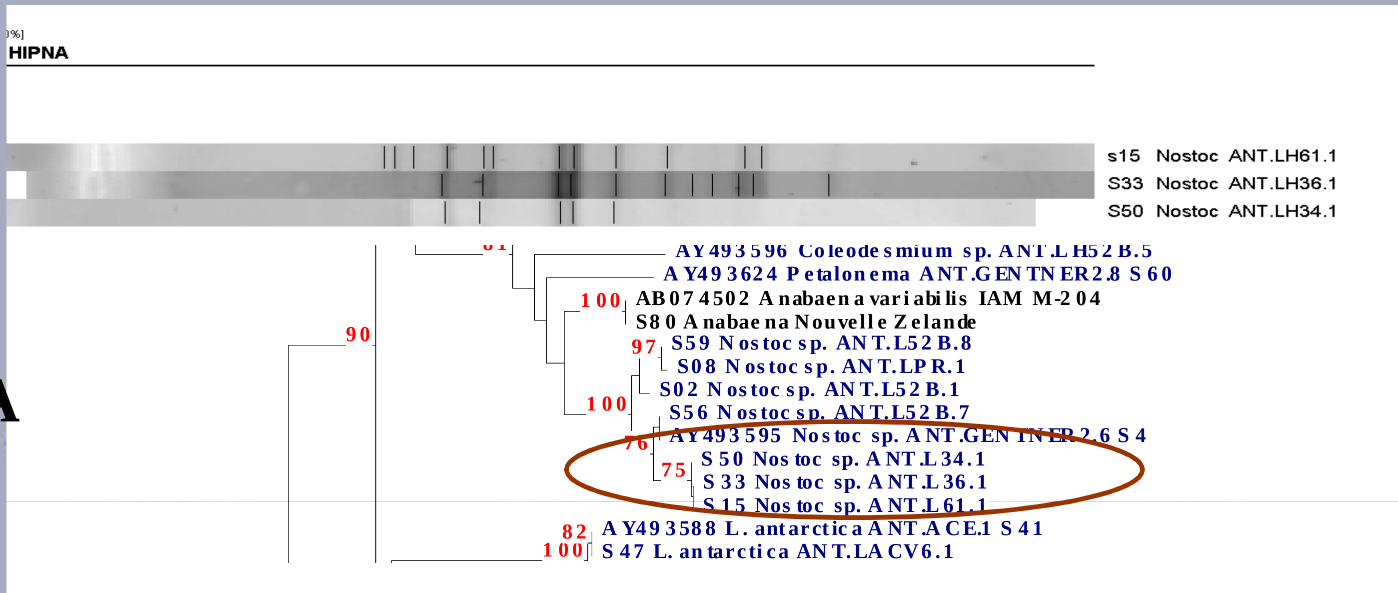
2. Fingerprints analyzed with GelComparII by cluster analysis

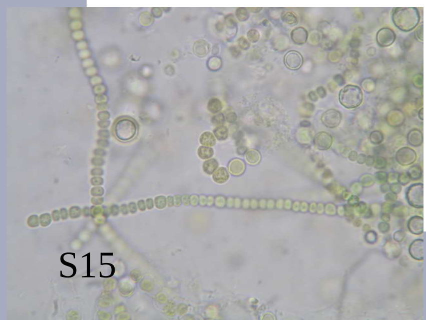


Nostoc strains S15, S33, S50

HIPNA

16S rRNA



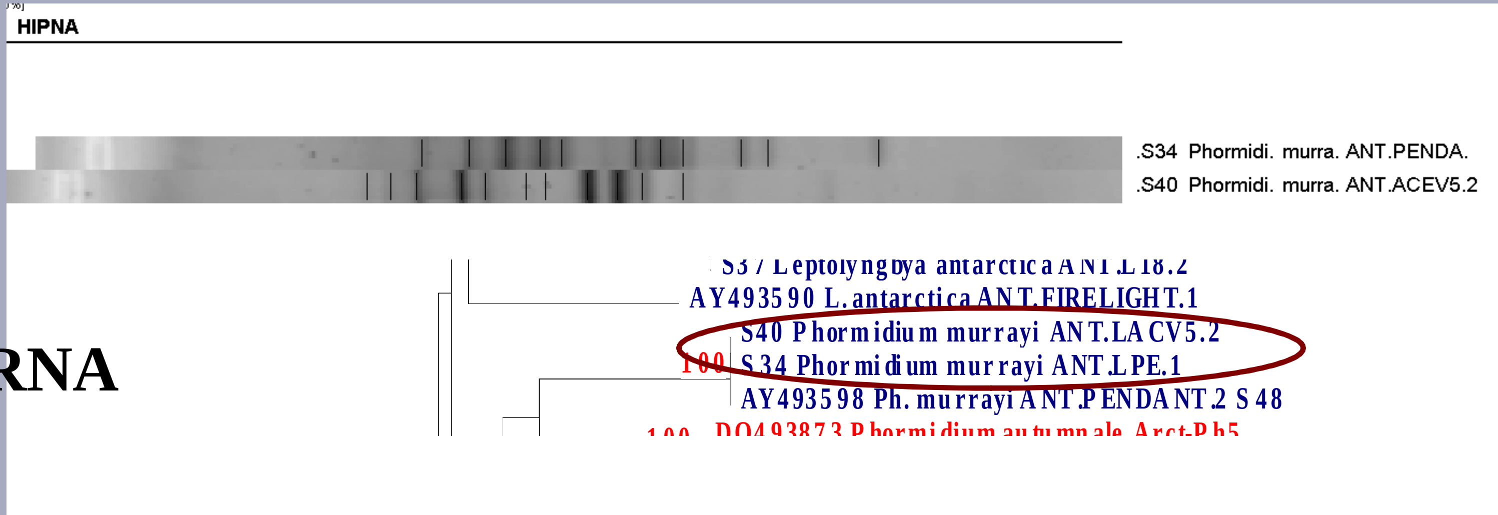


HIPNA differentiates *Nostoc* strains with identical 16S rRNA sequences

Strains *Phormidium murrayi* S34-S40

HIPNA

16S rRNA

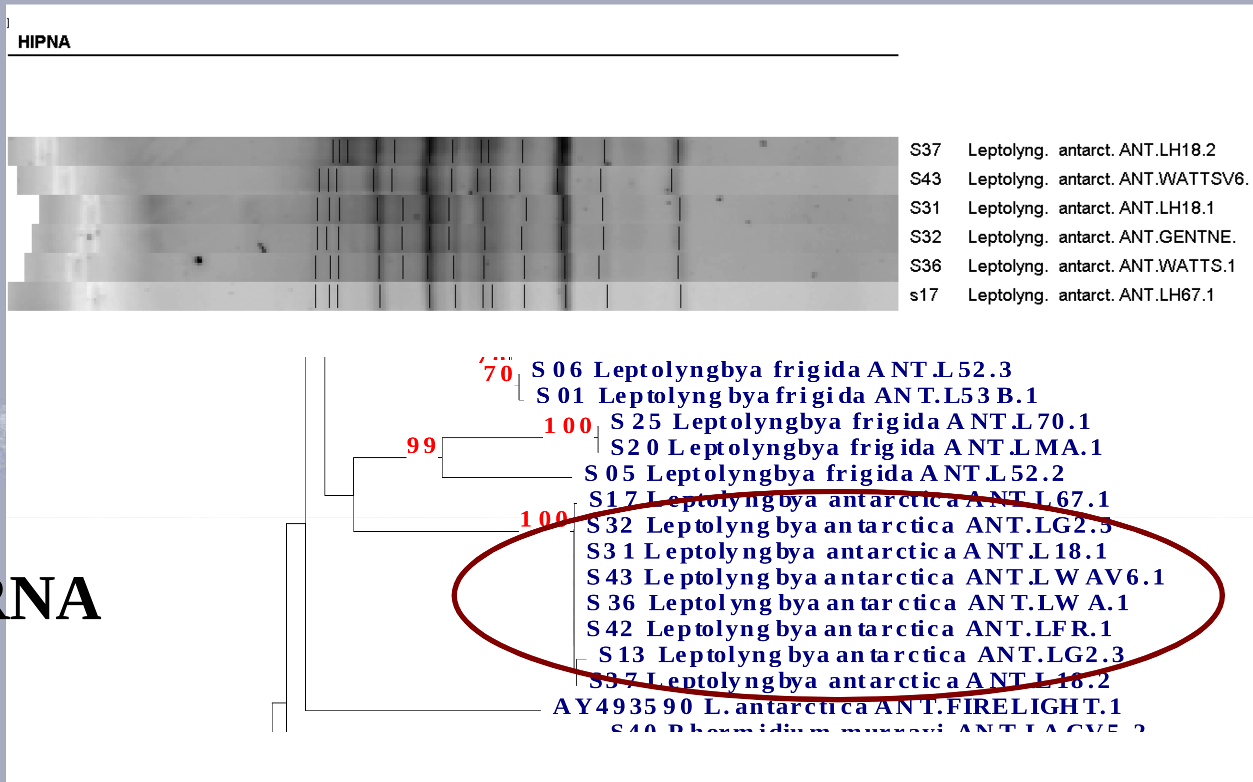


HIPNA differentiates *P. murrayi* strains with identical 16S rRNA and ITS sequences

Leptolyngbya antarctica S17-S31-S32-S36-S37-S43

HIPNA

16S rRNA



HIPNA does <u>not</u> seem to differentiate well the 6 strains with identical 16S rRNA and ITS sequences

→ Need to test new HIP variants or another strategy (MLST…)

On-going activities

Characterization of the strains with (novel) techniques having **different levels of resolution**

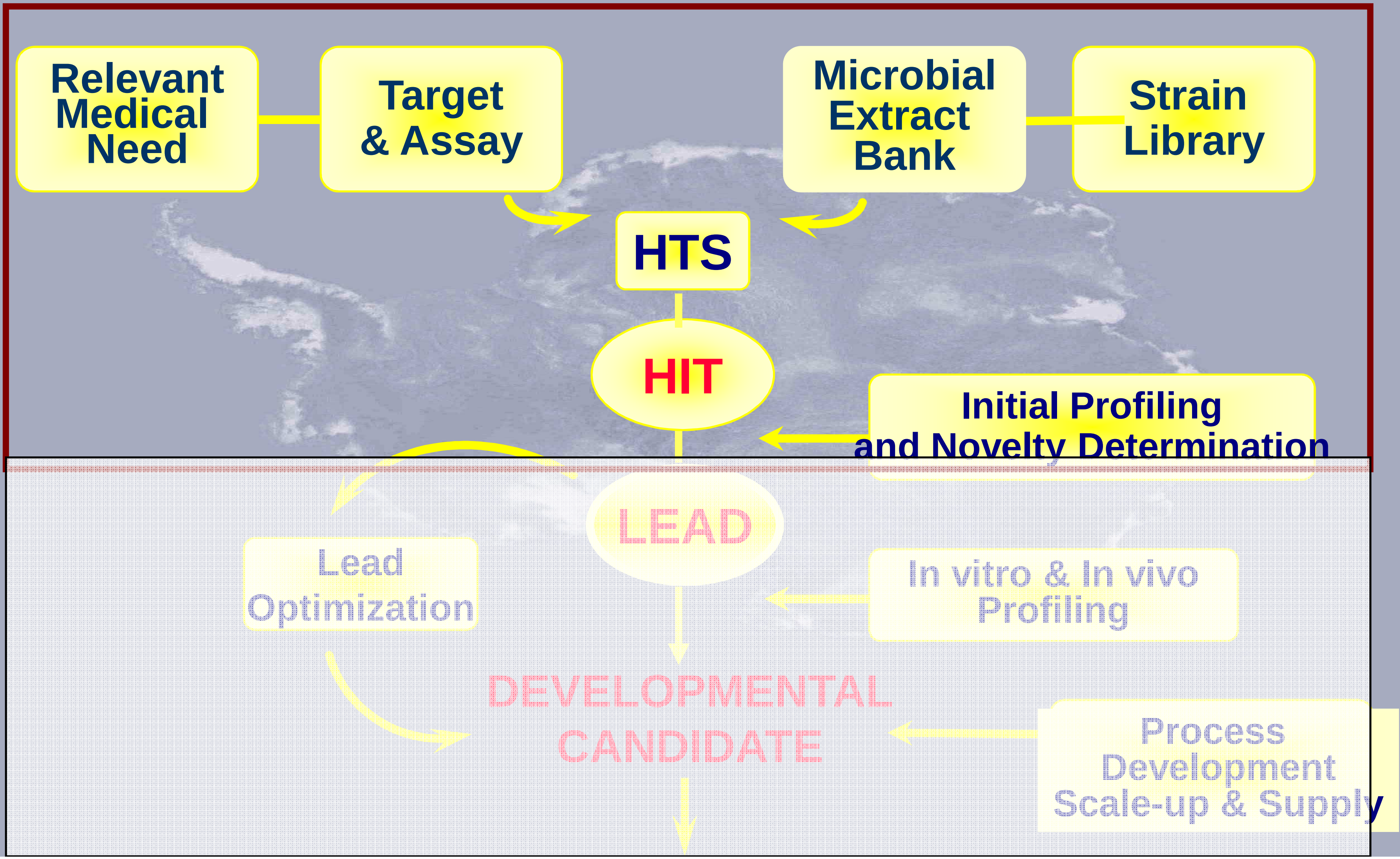
→ Additional data on the diversity and biogeography of polar cyanobacteria

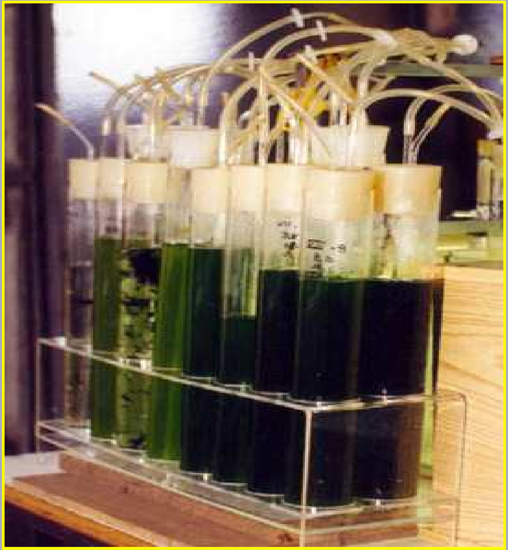
Possibility to test **cryopreservation** :

- Antarctic strains: 83 % viable after 6 months (DMSO)
- Arctic strains more problematic

5. Screening of the Antarctic strains for bioactive compounds

Identification of Novel Compounds
Integrated Approach





1 liter culture of 51 antarctic cyanobacterial strains and extract preparation

(Mario Tredici & Natascia Biondi, Università di Firenze)

Nostoc sp.

Microbial activity profile of 126 extracts from 48 strains & selection of two hits for further characterization

(Vicuron Pharmaceuticals (Biosearch Italia))

Biondi et al. 2008 J. Appl. Microbiol.

Mass cultivation conditions

•Slow-growing (biomass productivity <60 mg L^{-1} d^{-1})

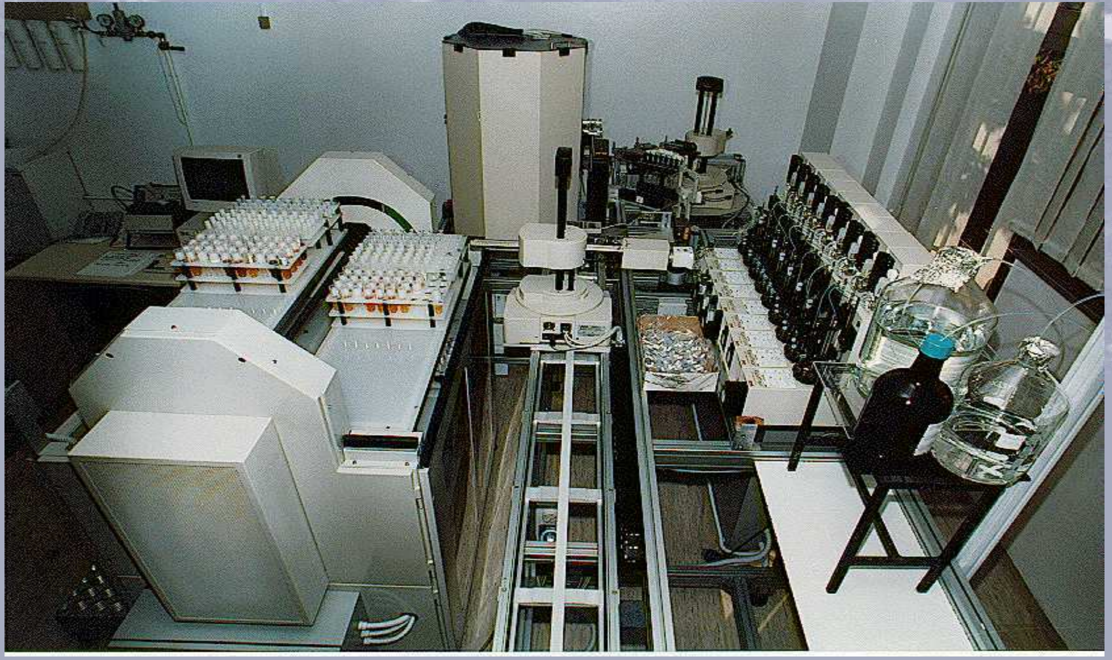
•Optimal growth at 20-22°C

Inability to adapt to high light intensity and to air bubbling cultivation limited their biomass productivity more than temperature, as they were almost all psychrotolerant, rather than psychrophilic

Biondi et al. 2008 J. Appl. Microbiol.

For 48 isolates, enough biomass produced for screenings.

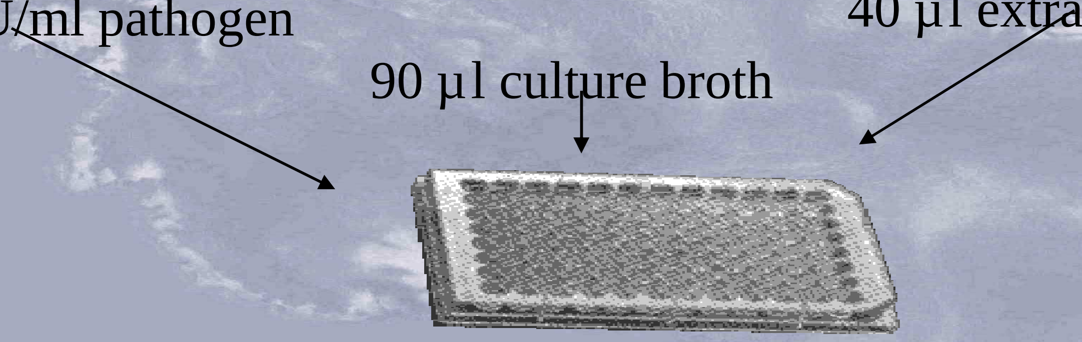
126 chemically diverse extracts (ethyl acetate or methanol from biomass or thawing water) screened by a combination of HT antimicrobial assays and cytotoxicity tests.



Antimicrobial assays:

Microtiter assay in liquid, with measure of optical density at 620 nm to detect pathogen growth inhibition

Active strains: more than 80% of pathogen growth inhibition in comparison to controls (100%)



O.D. reading after 18-24 hours for *S. aureus*, *E. coli* and *C. albicans*, and after 48 hours for *A. fumigatus*, *C. neoformans*

+ Confirmation by broth micro-dilution assays (Gaspari et al. 2005)

Cytotoxicity to HeLa cells:

Incorporation of ^{3}H-thymidine in cell cultures

Cytotoxic strains: more than 40% of inhibition relative to controls (100%) after 24H incubation

Cyanobacterial screenings

17 cyanos produced antimicrobial activities (35%)
25 were cytotoxic (50%)

29 % versus *S.aureus*
20% versus *C.neoformans*
2% versus A.fumigatus

No activities against *E.coli* and *C.albicans*

Bioactivities were not in coincidence with the phylogenetic relationship, but rather specific to certain strains
i.e
Identical strains (rRNA) isolated from the same lakes or from different lakes may produce different patterns of bioactivity

Hits selected, tested in endpoint and fractionated by LC-MS

16rRNA OTU	Morphotype	Identification	Hits	Activity on *S.aureus*	Activity on *A.fumigatus*	Activity on *C.neoformans*	Cytotoxicity
16ST03 New	OS-II	*Phormidium pristleyi*	ANT. LH52.4	0	512	1024	0
16ST03 New	OS-II	*Phormidium pristleyi*	ANT. LH52.6	8	512	512	160
16ST01 New	OS-I	*Pseudophor-midium* sp.	ANT. PROGRESS 2.2	64	0	512	640
16ST13 Ant	OS-V	*Leptolyngbya antarctica*	ANT. GENTNER 2.3	64	0	0	640
16ST19	NO-I	*Nostoc* sp.	ANT. LH34.1	8	0	0	0

Microbiological activity: the highest dilution which inhibits 80%of the pathogen growth

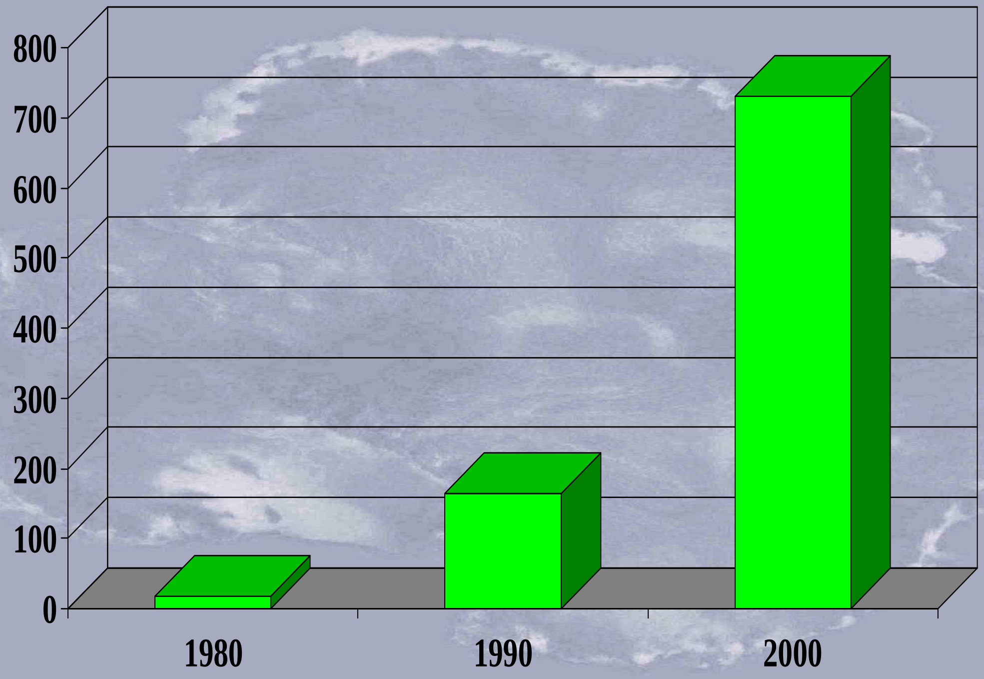
Cytotoxicity: the highest dilution which inhibits 40% of HeLa cell thymidine uptake

HPLC fractionation + LC-MS of active fractions of the two hits (antifungal activity) showed very similar chromatographic profiles (→ duplicates?), whereas the fraction active against *A. fumigatus* was separated from the fraction exhibiting cytotoxicity.

For the 3 other interesting strains, LC-MS showed that the fraction active against *S. aureus* eluted at similar retention times, suggesting that the three strains produced similar novel antibacterial compounds (Luc Jacquet, personal communication).

Cyanobacteria = Promising microbial group but somewhat difficult to cultivate

New secondary metabolites from cyanobacteria



VICURON database, Lazzarini et al. (2000) *Antonie van Leeuwenhoek* 78:399-405

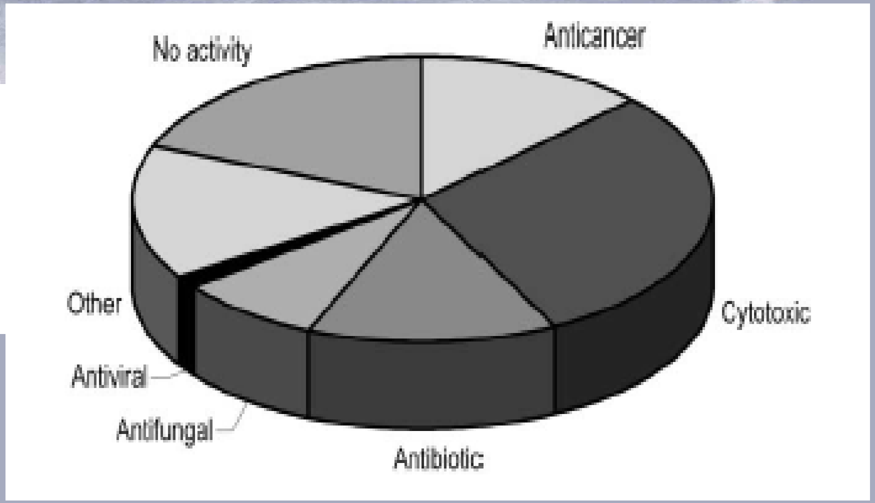
Examples of bioactivities in the 5 cyanobacterial orders

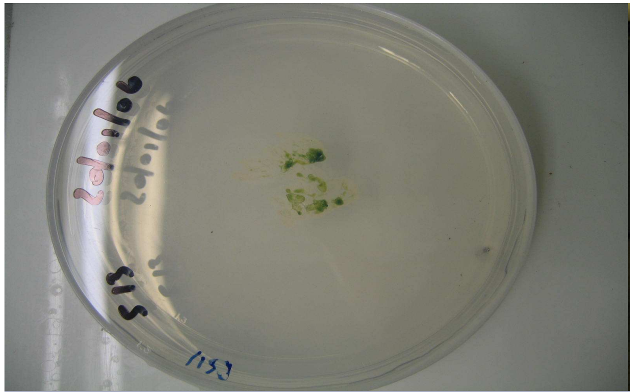
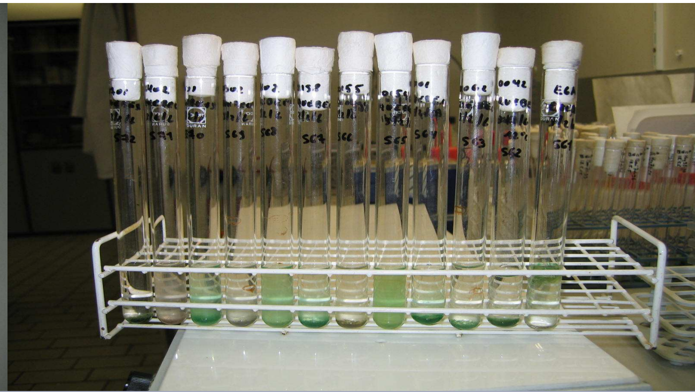
9350 A. M. Burja et al. / Tetrahedron 57 (2001) 9347–9377

Table 1. (*continued*)

Order	Compounds	Activities
Chroococcales (11)	36	Enzyme inhibitor, cytotoxic, cell-differentiation, tumor promoter, endotoxic, hepatotoxic (6)
Pleurocapsales (1)	2	Antifungal, no activity (2)
Oscillatoriales (15)	197	Antialgal, anticancer, anti-HIV, antifeedant, antifungal, anti-inflammatory, antimicrobial, antimitotic, antiproliferative, antiviral, brine shrimp toxicity, cytotoxic, cytoskeleton disruption, herbicidal, hepatotoxin, icthyotoxic, immunosuppressive, molluscidal, neurotoxic, no activity, PBDu binding, tumor promoter, protein kinase activator, skin irritant, sunscreen pigment, toxin (26)
Nostocales (41)	126	Anticancer, antifungal, antimalarial, anti-HIV, cardioactive, hepatotoxic, antimicrobial, antimitotic, anti-inflammatory, antiviral, cytotoxic, enzyme inhibitor, toxin, neurotoxin, pigment, no activity (16)
Stigonematales (6)	16	Antifungal, antibiotic, anticancer, antimitotic, cytotoxic, herbicidal, no activity (7)

Biological activities of 424 compounds from marine cyanobacteria (2001)



→ Renewed interest for biotechnological exploitation of cyanobacteria, with a steadily increasing number of cyanobacteria found to produce a variety of novel and biologically active compounds

→ Current discussion at ATCM level concerning the exploitation of Antarctic genetic ressources and how this fits with the Antarctic Treaty and the Madrid Protocol

Thanks for your attention !

New Belgian Antarctic Station in Dronning Maud Land !