UNIVERSITY OF ICELAND

Alternative nitrogen fixation in lichens

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Introduction

Lichens are a prime example for symbiosis, formed by a fungal partner (mycobiont) and a photosynthetic partner (photobiont) like green algae or cyanobacteria (Figure 1). Growing on almost all surfaces wherever it is natural or man-made, lichen has become a very successful organism. Lichens caught special attention as a sensitive environmental indicator for air pollution.

Figure 1 *Peltigera membranacea* as an example for lichen (Snow, 2010)

Most of the *peltigera* lichens are so-called cyanolichens which means that the photobiotic partners are cyanobacteria. That has a huge meaning for the lichen since cyanobacteria are not only providing nutrients through photosynthesis but additionally are able to fixate nitrogen from the atmosphere. A few cells of Nostoc develop a different phenotype forming so called heterocysts (Figure 2). With a low oxygen level there are perfect to sustain oxygen sensitive nitrogenases. Heteocysts occur with a rate of one to around 20 normal cells. Due to their low oxygen permeability through their thick cell walls, no photosystem II activity occurs. This leads to a division of work between the bacteria cells: normal cells provide nutrition from photosynthesis and heterocysts provide in exchange fixated nitrogen (Sakurai and Masukawa, 2006). Thereby the fungus benefits from both.

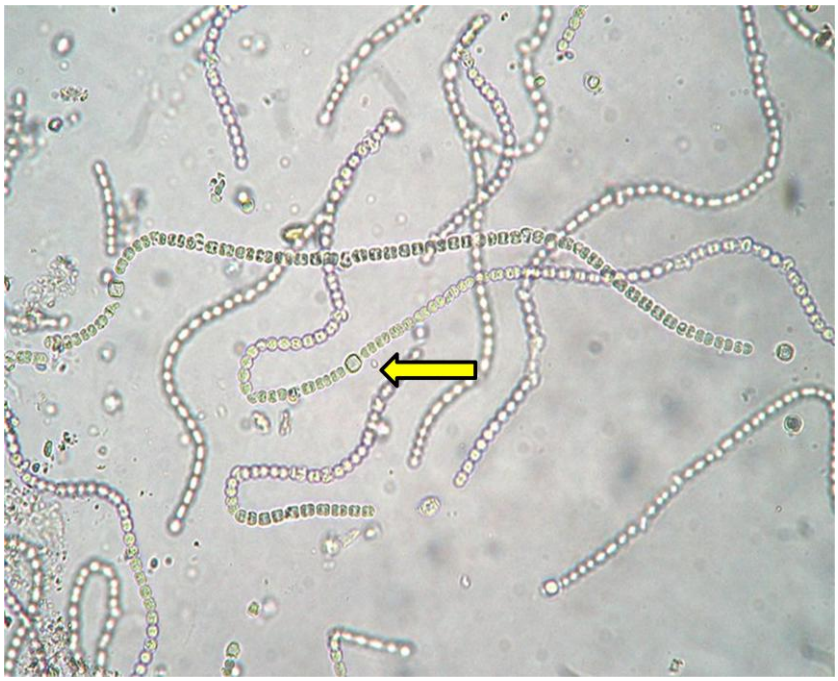

Figure 2 *Nostoc commune* as an example of cyanobateria, note the marked heterocyst with different phenotype responsible for nitrogen fixation (Peters)

Since water and sunlight are normally not limited, the availability of nitrogen becomes an important factor not only for microbiotic but also for higher organisms. Thus in cyanobacteria three different classes of nitrogenase have been found. Mo-nitrogenase, V-nitrogenase and Fe-nitrogenase. The best known nitrogenase is the Mo-dependent one, which shows also the highest efficiency. Only under Mo-limited environment other nitrogenases are expressed, if present (Meeks et al., 2001; Bothe et al., 2010). The alternative V-nitrogenase was now found in cyanolichens, which highlights once again the importance of lichens for the ecosystem, this time as a source for bound nitrogen.

Other than the regular molybdenum and iron containing protein (MoFe protein), the nitrogenase contains vanadium and iron. This nitrogenase is not produced by a simple replacement of molybdenum in the enzyme but is a distinct molecule, expressed by a gene cluster called *vnf*. Still both are very similar to each other (see Eady, 2003).

In this project the *vnf* gene cluster, responsible for the Vn-nitrogenase, was screened over different locations all over Iceland. The *vnf* occurence could be in relation to the Mo-concentration, which is mostly influenced through volcanic activity and normally the limiting factor for the regular nitrogenase.

Materials and Methods

Processing of lichens

The collected samples were cleaned by removing dirt and any traces of other macroorganisms under a stereo microscope using brushes and tweezers. A part of the lichen was stored uncleaned as a reference. Cleaned lichens were first dried at room temperature and then stored at -20°C for extraction and inoculation.

DNA extraction

To extract the DNA of the lichen around 1 cm^2 of the lichen thallus was cut off and DNA was isolated after a modified method of Sinnemann et al., 2000 (procedure in appendix)

PCR

To amplify specific DNA fragments to confirm or deny the existence of genes in a DNA sample Polymerase chain reactions (PCRs) were used (standard protocol and thermocycler program in appendix).

Results were applied on an 1% agaraose gel with a 1 kb ladder.

Primers

Primers were synthesized by microsynth.

gene	primer name	sequence
vnfN	vnfN3Fa	GTTTGTCGTTGGGACTTGC
vnfN	vnfN7Ra	CTATCCTCTATCCCAATCGTTAGCAA
rbcL	CX	GGGGCAGGTAAGAAAGGGTTTCGTA
rbcL	CW	CGTAGCTTCCGGTGGTATCCACGT

Table 1 List of primers used in this project

Cell culture of cyanobacteria

Cyanobacteria were cultivated on BG-11_0 media (composition in appendix) under artificial light (12h/day) at room temperature. Repeated subcultering ended with pure colonies of cyanobacteria of the lichen.

Freezing of cell cultures

Cells were scrapped off the petridish and transfered to 1.5 ml liquid BG-11_0. After 2 days of incubation at 21°C 70 µl of DMSO were added and the tubes were frozen at -80°C for future usage.

Sequencing

To confirm the PCR a part of the PCR products were sequenced (see appendix).

Firstly, PCR products were cleaned of extra nucleotides in an Exo Sap reaction. Purified PCR products were then used as templates for sequencing using Big Dye. Finally sequencing reaction products were precipitated and the DNA pellet was dissolved in HiDi and sequenced by a 3500XL Genetic Analyzer (Applied Biosciences).

Results

PCRs showed that out of 87 lichen samples 60 were positive on the primers for *vnfN*, which is a part of the gene cluster responsible for the V based nitrogenase (Figure 3)

The locations of these samples are spread over Iceland (Figure 4). While the east shows a high number of occurrence of the V-Nitrogenase, the west of Iceland show no occurrence of it. The north and especially south and southeast, where a lot of samples were available, showed both, lichen with and without the additional V-Nitrogenase (Table with location and sample numbers in appendix).

Next to the single samples two lichen populations (BK-01 and GU1) were tested with the *vnfN*-primers. Both populations showed a high frequency of the alternative nitrogenase. BK-01 population had 15 positive lichen out of 18 and GU1 even 30 positive out of 30 (Figure 4).

Additionally, isolated cyanobacteria of lichens were tested in a colony PCR. Only 4 out of 13 of the available cultures were positive. Strangely, one sample which was positive in lichen overall isolation, showed up negative in the colony PCR.

Furthermore, a part of the PCR products (samples: 82, 114, 146 and 148) were sequenced to confirm the PCR (see appendix). The succesfull sequenced products showed, as expected, high coverage (96-98%) to the vanadium nitrogenase associated proteins of *Anabaena variabilis* in the ncbi database. Admittedly, the sequences are only reliable to about half of their size due to the long product size (about 1kb), a sequence run with the opposing primer are missing.

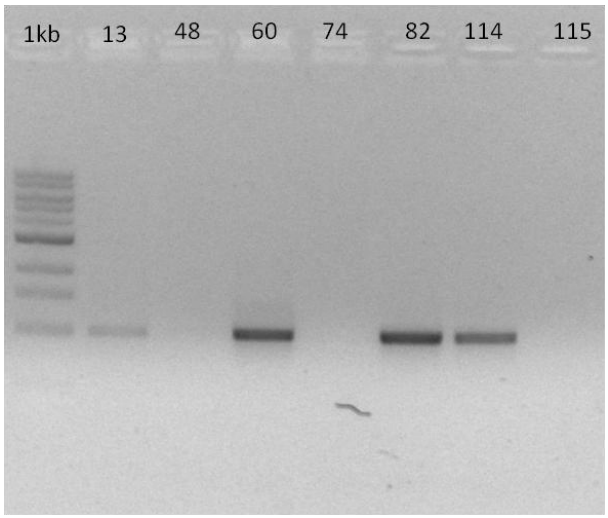

Figure 3 Example of PCR screening for vnfN, part of the gene cluster responsible for V-Nitrogenase. Sample 13, 60, 82 and 114 are showing bands of the right size, slightly above 1000 bp.

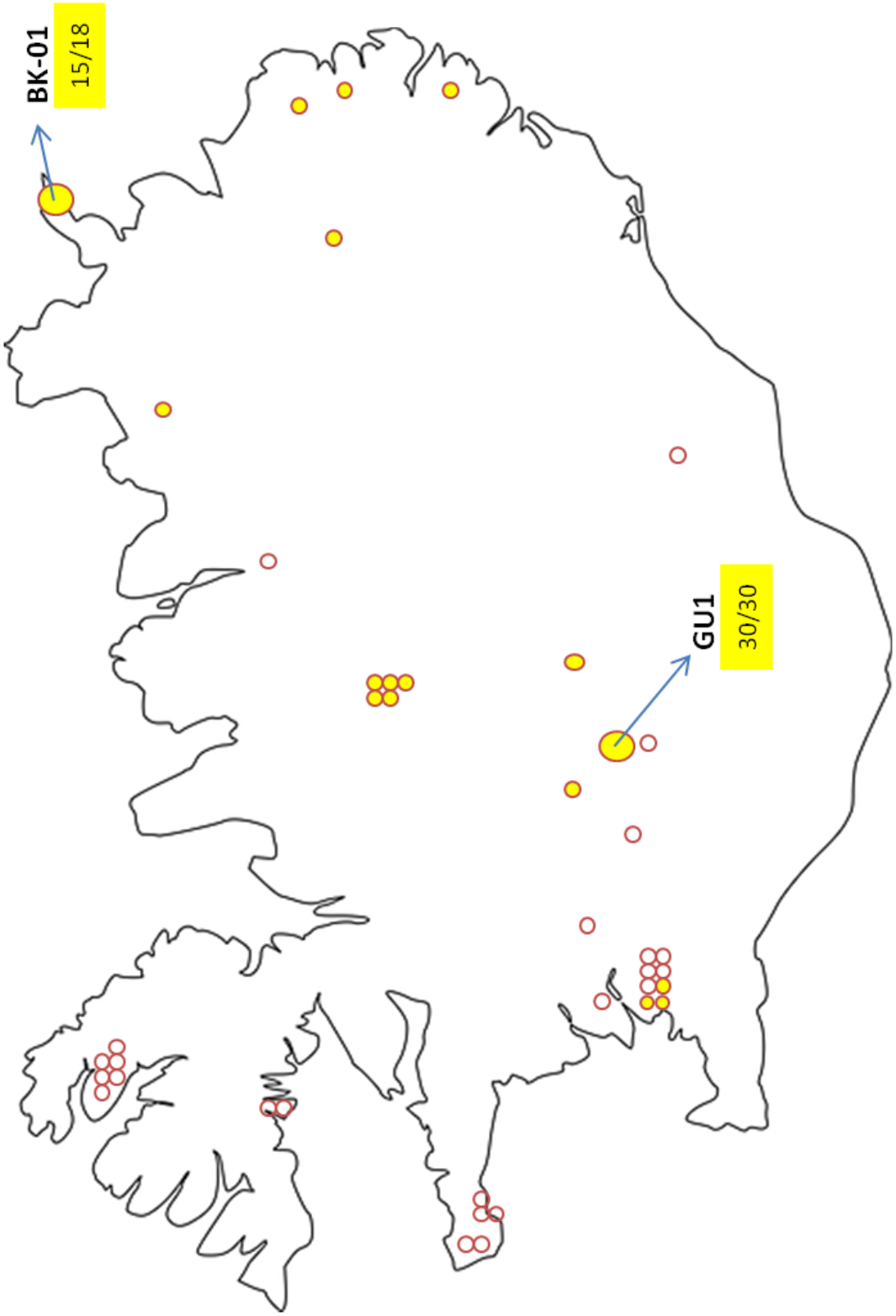

Figure 4 Distribution of vnfN over Iceland. Yellow marked spots are positive for vnfN, empty negative.

(Note that the colony PCRs are not included through contradictory results.)

Discussion

The PCRs show us that an additional, distinct nitrogenase is available in a part of the lichens. This gives the lichen the possibility to switch nitrogenase depending on the environmental conditions making it more adaptable.

If we compare the resulting map (Figure 4) to the distribution of molybdenum in Iceland (Oskarsdottir et al., 2011), the starting assumption, that the V-Nitrogenase distribution correlates with available amount of molybdenum, cannot be seen yet. Interestingly, there seem to be some kind of disequilibrium in the distribution of *vnf*.

For further investigation the *vnf* screening has to be continued, which is now with established primers easily possible. It would be interesting if a distinct is visible under a larger scale of samples. Also fascinating would be the question, if this alternative enzyme occurs more or less often dependent on the species of the cyanobacteria.

Literature

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Appendix

Used Samples

sample number	positive for vnfN	location
BK-01 (18samples)	15/18	N66° 10' 42.75" W15° 13' 57.83"
GU1 (30 samples)	30/30	N64° 15' 41.21"W20° 13' 17.64"
13	X	Keldur
25		Akureyri-Starri
28		Laugauatushellis
29	X	Bruarhlod
48		Kaldakvisl
60	X	Hahver
74		Keldur
82	X*	Keldur
107		Keldur
111		Keldur
113		Keldur
114	X*	Keldur
115		Keldur
139		Bláskógar
145		Skaftafell
146	X*	Keldurhverfi
148	X*	Stórurð
154	X	Loðmundarfjörður
162		Hrafnholar
220		Dalsheiði
222		Hvalhaushöfði
224		Hvalhaushöfði
225		Dalsheiði
226		Dalsheiði

227		Dalsheiði
228		Dalsheiði
229		Dalsheiði
252		Arnarstapi
253		Arnarstapi
254		Arnarstapi
255		Snæfellsnes
257		Djúpalónssandur
267	X	N65° 13'13,5" W19° 32'13,9"
268	X	N65° 13'13,5" W19° 32'13,9"
269	X	N65° 13'13,5" W19° 32'13,9"
270	X	N65° 13'13,5" W19° 32'13,9"
271	X	N65° 13'13,5" W19° 32'13,9"
303	X	Djúpivogur
307	X	Kálfell

Table 2 Used Sample for vnfN screening and the location. Where no location was known, the gps coordinates are indicated. * indicates sequencing

DNA-Isolation

- 0,02 g lichen grinded du powder in bead beater in ~10 min or with mortar and pestle together with liquid Nitrogen
- Added 0.25 ml of lysis buffer and mixed
- Incubated at 65°C for 20 min
- Centrifuged at 14000 rpm for 4 min
- Transfered the supernatant to new tube, discarded old tube
- Added 0,086 ml of 10,5M ammonium acetate and mixed
- Incubated on ice for 20 min
- Centrifuged at 14000 rpm for 4 min
- Again transered the supernatant to new tube, discarded old one
- Added 0.128 ml Isopropanol
- Mixed and incubated on ice for 20 min
- Centrifuged at 14000 rpm for 4 min
- Discarded supernatant
- Added 100 µl RNAse A mix (1ml TE + 4µl RNAse A) and 200 µl TE buffer
- Incubated at 50°C for 20 min while tapping to mix
- Added equal volume of phenol:chloroform:isoamzl alcohol (25:24:1)
- Vortexed briefly
- Centrifuged at 14000 rpm for 4 min
- Transfered upper phase into new tube
- Added equal volume of saturated chloroform
- Vortexed briefly
- Centrifuged at 14000 rpm for 4 min
- Transfered upper phase into new tube
- Added 1/10 volume of 3M sodium acetate (ph 7) and 2 volume of 95% EtOH
- Centrifuged at 14000 rpm for 4 min
- Discarded supernatant
- Added ~500 µl of 70% EtOH
- Centrifuged at 14000 rpm for 4 min
- Discarded supernatent
- Air dry pellet
- Disscolved pellet in ~30 µl of TE buffer

Standard PCR

	1x	Final
10x Thermo Pol Buffer	1,5 µl	1x
25 mM $MgCl_2$	1,5 µl	2,5 mM
dNTPs (each 2mM)	1,5 µl	0,2 mM
10 µM Forward Primer	0,36 µl	0,2 µM
10µM Reverse Primer	0,36 µl	0,2 µM
Taq Polymerase	0,12 µl	20U/ml
Template DNA	X	~90 ng
dd H_2O	up to 15 µl	

Depending on availability and reaction Eppendorf Mastermix or One Taq Master buffer were used after producer instructions to produce more stable results.

Standard program of thermocycler

T [°C]	t [min]
94	2:00
94	0:20
52-58 (depending on primers)	0:20
72	~1:00 /kb of product
72	10:00
12	∞

In case of of colonie PCR the same procedure was used except that the denaturation step was extended to 5 minutes.

BG-11$_0$

A) Stock solutions for BG-11:

Stock 1:

Na_2MG EDTA	0.1g/liter
Ferric ammonium citrate	0.6g/liter
Citric acid . $1H_2O$	0.6g/liter
$CaCl_2$. $2H_2O$	3.6g/liter

→ autoclave

Stock 2:

$MgSO_4$. $7H_2O$	7.5g/liter

→ autoclave

Stock 3:

K_2HPO_4 . $3H_2O$	4.0g/liter
or K_2HPO_4	3.05g/liter

→ autoclave

Stock 5 (Microelements):

H_3BO_3	2.86g/liter
$MnCl_2$ $4H_2O$	1.81g/liter
$ZnSO_4$ $7H_2O$	0.222g/liter
$CuSO_4$ $5H_2O$	0.079g/liter
$COCl_2$ $6H_2O$	0.050g/liter
$NaMoO_4$ $2H_2O$	0.391g/liter
or MoO_4 (85%)	0.018g/liter

B) For basic BG11 medium combine the following stock solutions:

Stock Solution	Per Liter of medium
Stock 1	10 ml
Stock 2	10 ml
Stock 3	10 ml
Na_2CO_3	0.02g
Stock 5	1.0 ml

Combine stocks 1-4 and adjust pH to 7.5 (use 1.0N HCl). Aliquot into flasks, add 1% noble agar for solid media and autoclave. After autoclaving add Stock 5.

Sequencing

Firstly, the products were cleaned off from any traces of the PCR reaction by an ExoSap reaction:

Exo Sap	1X
ddH2O	3,7
Anartic Phosphatase buffer	1
Antartic phosphatase 0.2X5U/µl =1U	0,2
Exo I 0.1X20U/µl =2U	0,1
PCR product	5
Total	10

Exo Sap Cycle
38°C - 35min
80°C - 20min
12°C - infinity

After that the Big Dye needed for the base detection was added:

Sequencing Rxn	
ddH2O	5,25
5X Sequencing buffer	2,75
Big Dye (v3.1)	0,5
Primer (1.6pmol/µl)	1,5
Exo Sap Treated PCR product	5
Total	15
To dispense	8,5

Master mix (Minus Primer & PCR product)	8,5
Primer (1.6pmol/µl)	1,5
Exo Sap Treated PCR product	5
Total	15

Sequencing Cycle	
96°C	10sec
96°C	10sec
55°C	5sec
60°C	2min
12°C	7min
12°C	infinity

Finally the samples were washed and precipitated:

For 24 samples	
Material	Quantity (µl)
Nuclease free water	1125
3M CH3COONa	125
Glycogen	6,25
Total	1256,25

Mix together 50µl of mix and 15µl of sample. Then add 125µl of 96% EtOH (ice cold)
Spin at 10, 500 rpm for 15 mins at 4°C
Take the liqiud off with suction. Note: DNA is bound to the walls at the bottom of the tube
Wash with 250µl of 70% EtOH. Spin at 10,500rpm for 5-6 min at 4°C. Take the liquid off with suction. Repeat this step
Air dry the samples in a dark place until dry. (Can be stored at 4°C)
Add 12µl of Hi-Di to the samples.
Vortex, heat the samples at 92°C for 2mins, place on ice, Briefly spin, load 10µl of the sample into the sequencing tray.

Sequencing results (Fasta formate)

Sample 82

GKTKTGGGKKAKKKKGGATCATGCCTTCATAGCCAAATATGGCTTATCAACATAAGTTTCTCTGTTAGTG
GGGTAGGCCCAAAGCAATTTTTATTCCTAGTTCCTCTGCCATGTAAATTCCCATGCCGAGCCAATCACAG
CATCAACATCAAACTCTTCTAAAGTCTGTTTTACCTGATAGCCATCTGCCSAAAATATTACGCGGGAAGT
AAGACCGAGGCTGTGCAGTTCTCGCTCCAAAATTGAACGAGAATCAGGCATCGCCGAGCGAAATAATAAG
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CATTTCTTCGCCTTGTTTGATGATTGGCTCTACTTTTTCATCTATCTTAAAATGTGCCGCCAATGCCTTC
AACCACCGAGTAGTGTTGTTTACACCTATTGGTAAAGGTATATCATCAAGGATCAAAGGGATGTCATGGG
TCTGTTGCATTTTCCGAGCAAACTTATACCCAACATCATGACTGAGAACAATATTGGCGGTTGCCTCACA
GCTTGCTCTAGTTCTTCAAAGGAGGTATTATGAGAGAAGACCGTCTGTACTTTAATCCTTAAAGATTTTA
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TACAATCCTTGGCTTCCTACCCCTTCTCTTAGTCTGCCTTTTTTTGATAAAAGGAAGTAAAGCCTCCAAA
CCCATCTCCAAGCCATCGKAARSAWTTGCCCCRAAAMCCCACCARCCATTAAAAGGGAAACGAGTTTGGG
CTTTTGATATCTGGCTGAAGCTGGGTTGCACAATCCCCGCAATATCTTCMCCAATGAATGTCTGCCGSGC
AAGTCCMAACGAMCAAACAAATG

Sample 114

GGCATGTTTTTTGATMTGCCTTCATAGCCAATATGGCTTATCAACATAAGTTTCTCTGTTAGTGGGGTAA
AACATCAAAAGCAATTTTTATTCCTAGTTCCTCTGCCATGTWTTTTTCCCATGCCGAGCCAATCACAGCA
TCAACATCAAACTCTTCTAAAGTCTGTTTTACCTGATAGCCATCTGCCGAAAATATTACGCGGGAAGTAA
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ACGGCAATTCTACAGTTACGATATCGGGGAATAATCATCAATGCTCGTTTGCGAAGTGTATCTACAACCA
TTTCTTCGCCTTGTTTGATGATTGGCTCTACTTTTTCATCTATCTTGAAATGTGCCGCCAATGCCTTCAA
CCACTGAGTAGTGTTGTTTACACCTATTGGTAAAGGTATATCATCAAGGATCAAAGGGAGTCATGGGTCT
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AATCCTTGGCTTCCTACCCCTTCTCTTAGTCTGCCTTTTTTTGATAAAAGGAAGTAAAGCCTCCAAACCC
ATCTCCAAGCCATCGTAAGCATTGCCCCGAAACCCACCAGCCATTAAAGGAACGAGTTTGGCTTTGATAT
CTGGCTGAAGCTGGTTGCACAATCCCGCAATATCTTCACCAATAATGTCTGCCGCGCAAGTCCMAACGAC
AAAC

Sample 146

GTCCCAGTAGACATCTKGTTGGTCTGCTTCTAGCCAAAATATGGCTTATCAACATAAGTTTCTCTGTTAG
TGGGGTGGACCCAAAAGCAATTTTTATTCCTAGTTCCTCTGCCATGTAAAARKCMMRKGCCGAGCCAATC
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AAGTAAGACCGAGGCTGTGCAGTTCTCGCTCCAAAATTGAACGAGAATCAGGCATCGCCGAGCGAAATAA
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CAAACCCATCTCCAAGCCATCGTAAGCATTGCCCCGAAAMCCCACCAGCCATTAAAGGAACGAGTTTGGC
TTTGATATCTGGCTGAAGCTGGGTTGCACAATCCCGCAATATCTTCCMCMAATAAATGTCTGCCGSSGCA
AGTCCCAACGAMAAAACCATT

Sample 148

TTCCMAAAAGSACATATTTTTTTGCTGCCTTCTGCCAAATATGGCTTAYCAACATAAGTTTCTCTGTTAG
TGGGGTAAAASGTCAAAAGCAATTTTTATTCCTAGTTCCTCTGCCATGTAAAAATCCCATGCCGAGCCAA
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TGATATTTACAATCCTTTGGCTTCCTACCCCCTTCTCTTAGYCTGCCTTTTTTTGATAAAAGGAAAGTAA
AAGCCTCCAAMCCTATCTCCMAGCCATCATAA