NGS for ecologists: DNA metabarcoding

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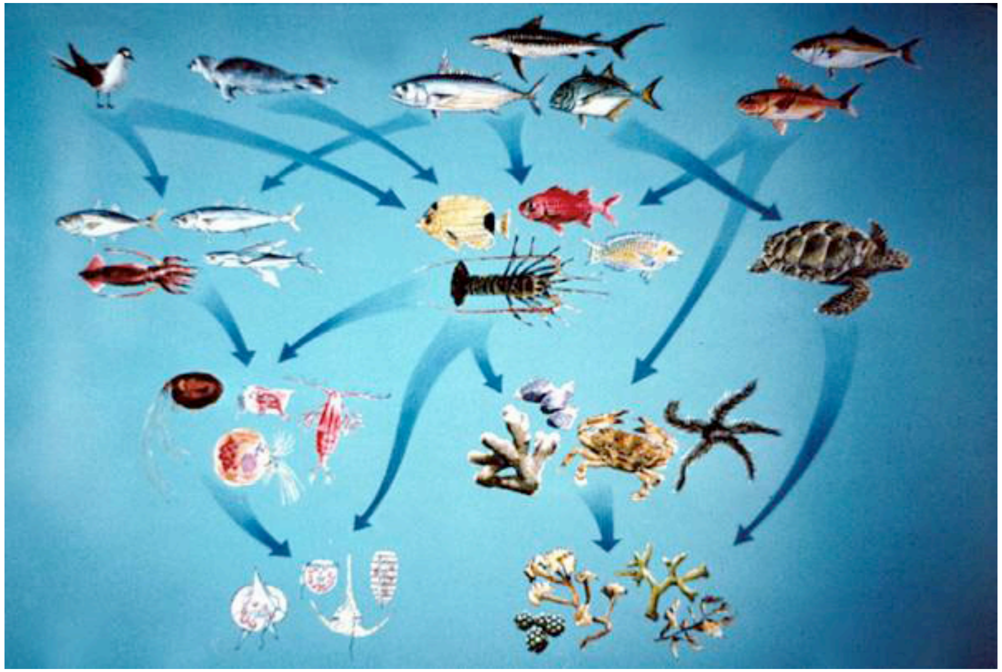
LECA
Laboratoire d'Ecologie Alpine

What determine patterns of species diversity?

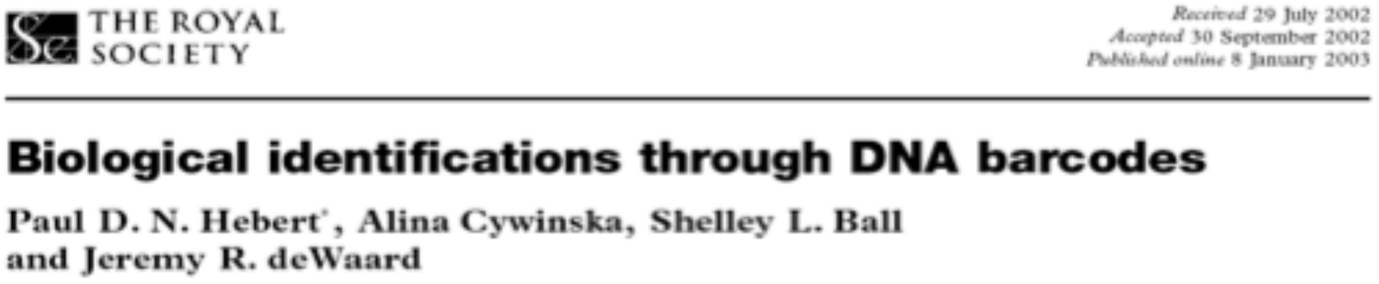
Foodwebs:
niche partitioning,
energy flows in
ecosystems …



Barcoding : an old concept …

Kangethe, E.K.*, et al.* **1982**
Identification of the species origin of fresh meat using an enzyme-linked immunosorbent-assay procedure. *Meat Science* 7, 229-240

… and recent developments



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Biological identifications through DNA barcodes

**Paul D. N. Hebert[*], Alina Cywinska, Shelley L. Ball
and Jeremy R. deWaard**

Different approaches of DNA Barcoding

Sensu stricto

« short DNA sequence from a standardized and agreed-upon position in the genome as a molecular diagnostic for species-level identification »

CONSORTIUM FOR THE BARCODE OF LIFE

Sensu lato

« DNA-based taxon identification using diverse techniques »

Review

Cell PRESS

DNA barcoding for ecologists

Alice Valentini[1,2], François Pompanon[1] and Pierre Taberlet[1]

DNA metabarcoding

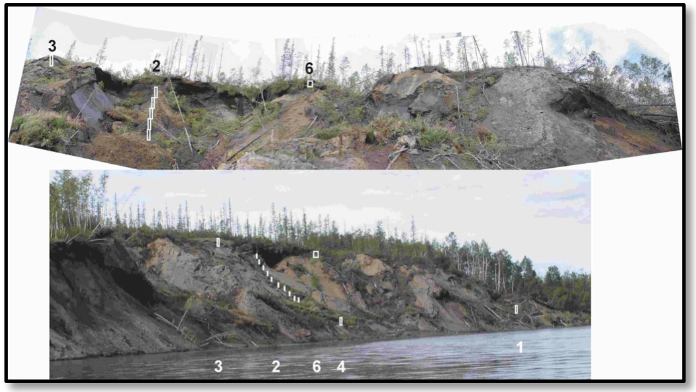
Combination of the barcoding concept and Next generation sequencing

→ High throughput plant & animal identification

DNA metabarcoding

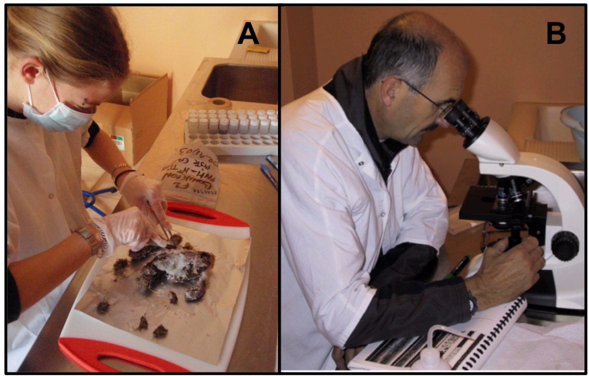
An alternative to traditional methods

Too many species …



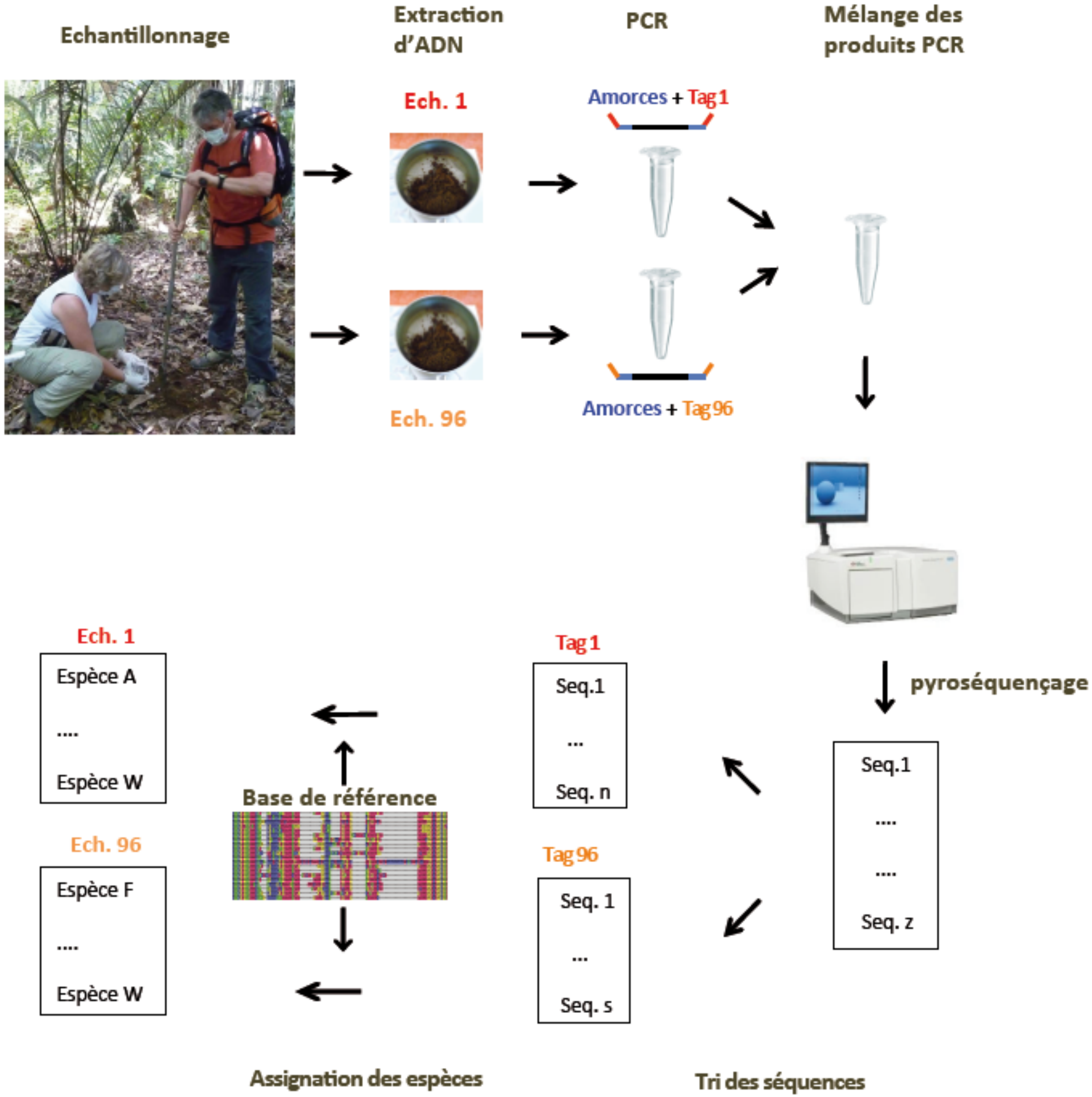
Too old ecosystems …

Too secretive species …



Too many time…

The metabarcoding approach



Echantillonnage

Extraction d'ADN

PCR

Mélange des produits PCR

Ech. 1

Amorces + Tag1

Ech. 96

Amorces + Tag96

pyroséquençage

Seq.1
....
....
Seq. z

Tag1
Seq.1
...
Seq. n

Tag96
Seq. 1
...
Seq. s

Base de référence

Ech. 1
Espèce A
....
Espèce W

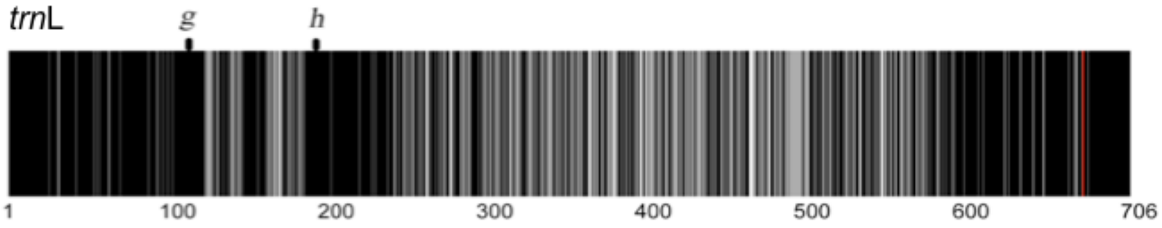
Ech. 96
Espèce F
....
Espèce W

Assignation des espèces

Tri des séquences

Constraints of working with degraded DNA

A barcode should be		taxonomy	ecology
Discriminant	Low variation within species & high variation between species	✓✓	✓
Standardized	One region for as many taxa as possible	✓✓	✓
Phylogenetically informative	Assigning unknown species to a taxon (genus, family, etc.)	✓✓	✓
Robust	Highly conserved priming sites, and highly reliable DNA amplifications	✓	✓✓
Short	Allowing amplification from as many substrates as possible (even degraded)	✓	✓✓

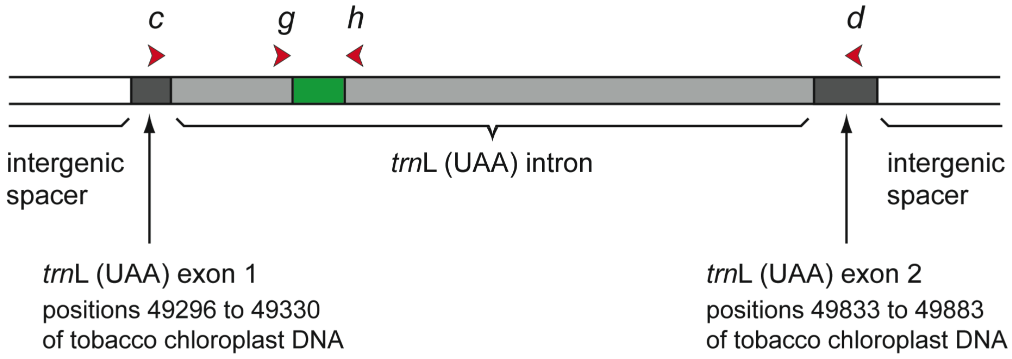


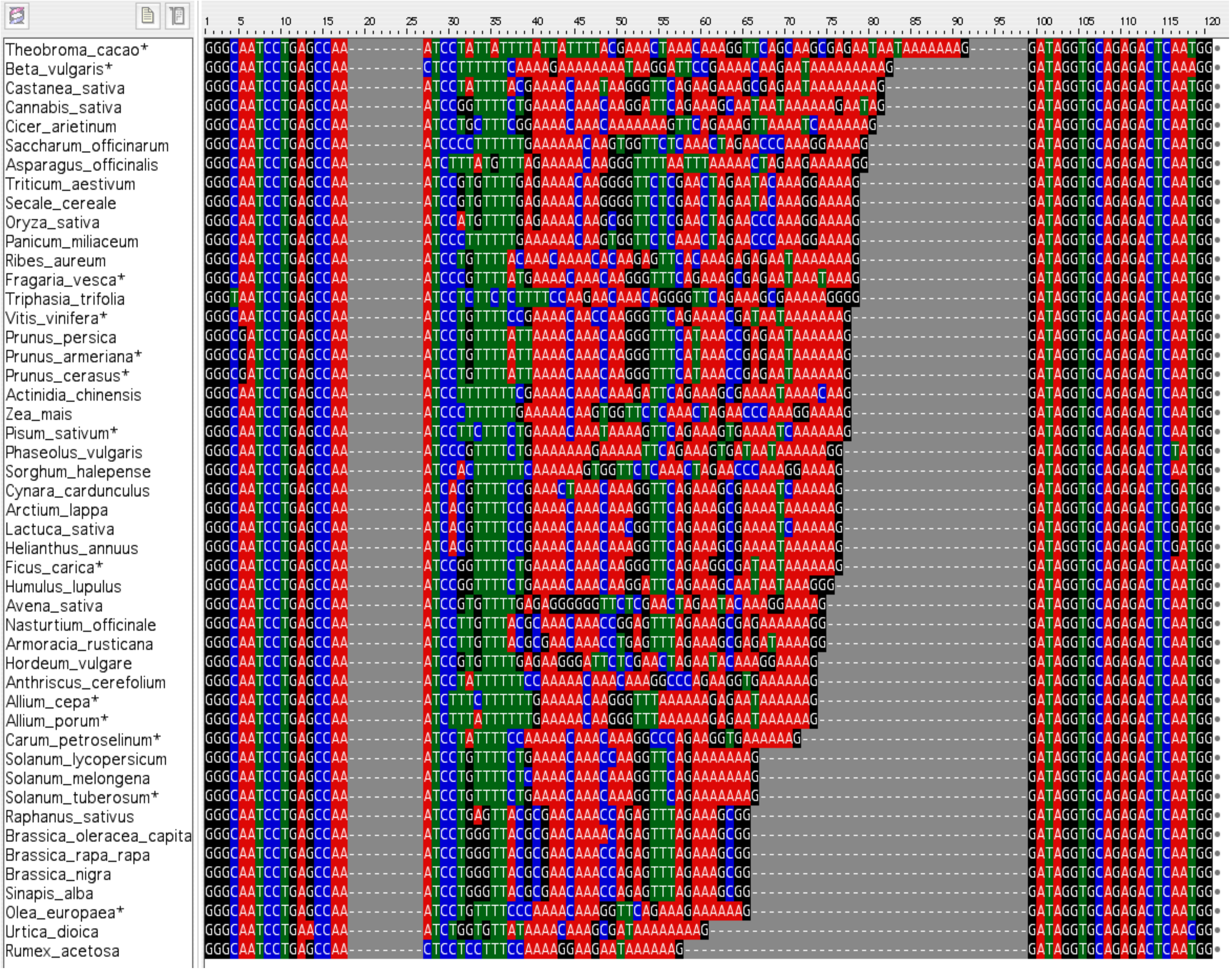
sampling

DNA amplification

- Using universal primers amplifying a very short DNA fragment (< 100 bp)
- Possibility of using many primer pairs targeting different taxonomic groups

The chloroplast *trn*L(UAA) intron





```
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Beta_vulgaris*            GGGCAATCCTGAGCCAA---------CTCCTTTTTTCAAAAGAAAAAAAATAAGGATTCCGAAAACAAGAATAAAAAAAAAG----------------GATAGGTGCAGAGACTCAAAGG
Castanea_sativa           GGGCAATCCTGAGCCAA---------ATCCTATTTTACGAAAACAAATAAGGGTTCAGAAGAAAGCGAGAATAAAAAAAAG-----------------GATAGGTGCAGAGACTCAATGG
Cannabis_sativa           GGGCAATCCTGAGCCAA---------ATCCGGTTTTCTGAAAACAAACAAGGATTCAGAAAGCAATAATAAAAAAGAATAG-----------------GATAGGTGCAGAGACTCAATGG
Cicer_arietinum           GGGCAATCCTGAGCCAA---------ATCCTGCTTTCGGAAAACAAACAAAAAAAGTTCAGAAAGTTAAAATCAAAAAAG------------------GATAGGTGCAGAGACTCAATGG
Saccharum_officinarum     GGGCAATCCTGAGCCAA---------ATCCCCTTTTTTGAAAAAACAAGTGGTTCTCAAACTAGAACCCAAAGGAAAAG-------------------GATAGGTGCAGAGACTCAATGG
Asparagus_officinalis     GGGCAATCCTGAGCCAA---------ATCTTTATGTTTAGAAAAACAAGGGTTTTAATTTAAAAACTAGAAGAAAAAGG-------------------GATAGGTGCAGAGACTCAATGG
Triticum_aestivum         GGGCAATCCTGAGCCAA---------ATCCGTGTTTTGAGAAAACAAGGGGTTCTCGAACTAGAATACAAAGGAAAAG--------------------GATAGGTGCAGAGACTCAATGG
Secale_cereale            GGGCAATCCTGAGCCAA---------ATCCGTGTTTTGAGAAAACAAGGGGTTCTCGAACTAGAATACAAAGGAAAAG--------------------GATAGGTGCAGAGACTCAATGG
Oryza_sativa              GGGCAATCCTGAGCCAA---------ATCCATGTTTTGAGAAAACAAGCGGTTCTCGAACTAGAACCCAAAGGAAAAG--------------------GATAGGTGCAGAGACTCAATGG
Panicum_miliaceum         GGGCAATCCTGAGCCAA---------ATCCCTTTTTTGAAAAAACAAGTGGTTCTCAAACTAGAACCCAAAGGAAAAG--------------------GATAGGTGCAGAGACTCAATGG
Ribes_aureum              GGGCAATCCTGAGCCAA---------ATCCTGTTTTACAAACAAAACACAAGAGTTCACAAAGAGAGAATAAAAAAAG--------------------GATAGGTGCAGAGACTCAATGG
Fragaria_vesca*           GGGCAATCCTGAGCCAA---------ATCCCGTTTTATGAAAACAAACAAGGGTTTCAGAAAGCGAGAATAAATAAAG--------------------GATAGGTGCAGAGACTCAATGG
Triphasia_trifolia        GGGTAATCCTGAGCCAA---------ATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTTCAGAAAGCGAAAAAGGGG--------------------GATAGGTGCAGAGACTCAATGG
Vitis_vinifera*           GGGCAATCCTGAGCCAA---------ATCCTGTTTTCCGAAAACAACCAAGGGTTCAGAAAACGATAATAAAAAAAG---------------------GATAGGTGCAGAGACTCAATGG
Prunus_persica            GGGCGATCCTGAGCCAA---------ATCCTGTTTTATTAAAACAAACAAGGGTTTCATAAACCGAGAATAAAAAAG---------------------GATAGGTGCAGAGACTCAATGG
Prunus_armeriana*         GGGCGATCCTGAGCCAA---------ATCCTGTTTTATTAAAACAAACAAGGGTTTCATAAACCGAGAATAAAAAAG---------------------GATAGGTGCAGAGACTCAATGG
Prunus_cerasus*           GGGCGATCCTGAGCCAA---------ATCCTGTTTTATTAAAACAAACAAGGGTTTCATAAACCGAGAATAAAAAAG---------------------GATAGGTGCAGAGACTCAATGG
Actinidia_chinensis       GGGCAATCCTGAGCCAA---------ATCCTTTTTTTCGAAAACAAACAAAGATTCAGAAAGCGAAAATAAAACAAG---------------------GATAGGTGCAGAGACTCAATGG
Zea_mais                  GGGCAATCCTGAGCCAA---------ATCCCTTTTTTGAAAAACAAGTGGTTCTCAAACTAGAACCCAAAGGAAAAG---------------------GATAGGTGCAGAGACTCAATGG
Pisum_sativum*            GGGCAATCCTGAGCCAA---------ATCCTTCTTTCTGAAAACAAATAAAAGTTCAGAAAGTGAAAATCAAAAAAG---------------------GATAGGTGCAGAGACTCAATGG
Phaseolus_vulgaris        GGGCAATCCTGAGCCAA---------ATCCCGTTTTCTGAAAAAAAGAAAAATTCAGAAAGTGATAATAAAAAAGG----------------------GATAGGTGCAGAGACTCTATGG
Sorghum_halepense         GGGCAATCCTGAGCCAA---------ATCCACTTTTTTCAAAAAAGTGGTTCTCAAACTAGAACCCAAAGGAAAAG----------------------GATAGGTGCAGAGACTCAATGG
Cynara_cardunculus        GGGCAATCCTGAGCCAA---------ATCACGTTTTCCGAAACTAAACAAAGGTTCAGAAAGCGAAAATCAAAAAG----------------------GATAGGTGCAGAGACTCGATGG
Arctium_lappa             GGGCAATCCTGAGCCAA---------ATCACGTTTTCCGAAAACAAACAAAGGTTCAGAAAGCGAAAATAAAAAAG----------------------GATAGGTGCAGAGACTCGATGG
Lactuca_sativa            GGGCAATCCTGAGCCAA---------ATCACGTTTTCCGAAAACAAACAACGGTTCAGAAAGCGAAAATCAAAAAG----------------------GATAGGTGCAGAGACTCGATGG
Helianthus_annuus         GGGCAATCCTGAGCCAA---------ATCACGTTTTCCGAAAACAAACAAAGGTTCAGAAAGCGAAAATAAAAAAG----------------------GATAGGTGCAGAGACTCGATGG
Ficus_carica*             GGGCAATCCTGAGCCAA---------ATCCGGTTTTCTGAAAACAAACAAGGGTTCAGAAGGCGATAATAAAAAAG----------------------GATAGGTGCAGAGACTCAATGG
Humulus_lupulus           GGGCAATCCTGAGCCAA---------ATCCGGTTTTCTGAAAACAAACAAGGATTCAGAAAGCAATAATAAAGGG-----------------------GATAGGTGCAGAGACTCAATGG
Avena_sativa              GGGCAATCCTGAGCCAA---------ATCCGTGTTTTGAGAGGGGGGTTCTCGAACTAGAATACAAAGGAAAAG------------------------GATAGGTGCAGAGACTCAATGG
Nasturtium_officinale     GGGCAATCCTGAGCCAA---------ATCCTTGTTTACGCAAACAAACCGGAGTTTAGAAAGCGAGAAAAAAGG------------------------GATAGGTGCAGAGACTCAATGG
Armoracia_rusticana       GGGCAATCCTGAGCCAA---------ATCCTTGTTTACGCGAACAAACCTGAGTTTAGAAAGCGAGATAAAAGG------------------------GATAGGTGCAGAGACTCAATGG
Hordeum_vulgare           GGGCAATCCTGAGCCAA---------ATCCGTGTTTTGAGAAGGGATTCTCGAACTAGAATACAAAGGAAAAG-------------------------GATAGGTGCAGAGACTCAATGG
Anthriscus_cerefolium     GGGCAATCCTGAGCCAA---------ATCCTATTTTTTCCAAAAACAAACAAAGGCCCAGAAGGTGAAAAAAG-------------------------GATAGGTGCAGAGACTCAATGG
Allium_cepa*              GGGCAATCCTGAGCCAA---------ATCTTTCTTTTTTGAAAAACAAGGGTTTAAAAAAGAGAATAAAAAAG-------------------------GATAGGTGCAGAGACTCAATGG
Allium_porum*             GGGCAATCCTGAGCCAA---------ATCTTTATTTTTTGAAAAACAAGGGTTTAAAAAAGAGAATAAAAAAG-------------------------GATAGGTGCAGAGACTCAATGG
Carum_petroselinum*       GGGCAATCCTGAGCCAA---------ATCCTATTTTCCAAAAACAAACAAAGGCCCAGAAGGTGAAAAAAG---------------------------GATAGGTGCAGAGACTCAATGG
Solanum_lycopersicum      GGGCAATCCTGAGCCAA---------ATCCTGTTTTCTGAAAACAAACCAAGGTTCAGAAAAAAAG--------------------------------GATAGGTGCAGAGACTCAATGG
Solanum_melongena         GGGCAATCCTGAGCCAA---------ATCCTGTTTTCTCAAAACAAACAAAGGTTCAGAAAAAAAG--------------------------------GATAGGTGCAGAGACTCAATGG
Solanum_tuberosum*        GGGCAATCCTGAGCCAA---------ATCCTGTTTTCTGAAAACAAACAAAGGTTCAGAAAAAAAG--------------------------------GATAGGTGCAGAGACTCAATGG
Raphanus_sativus          GGGCAATCCTGAGCCAA---------ATCCTGAGTTACGCGAACAAACCAGAGTTTAGAAAGCGG---------------------------------GATAGGTGCAGAGACTCAATGG
Brassica_oleracea_capita  GGGCAATCCTGAGCCAA---------ATCCTGGGTTACGCGAACAAAACAGAGTTTAGAAAGCGG---------------------------------GATAGGTGCAGAGACTCAATGG
Brassica_rapa_rapa        GGGCAATCCTGAGCCAA---------ATCCTGGGTTACGCGAACAAACCAGAGTTTAGAAAGCGG---------------------------------GATAGGTGCAGAGACTCAATGG
Brassica_nigra            GGGCAATCCTGAGCCAA---------ATCCTGGGTTACGCGAACAAACCAGAGTTTAGAAAGCGG---------------------------------GATAGGTGCAGAGACTCAATGG
Sinapis_alba              GGGCAATCCTGAGCCAA---------ATCCTGGGTTACGCGAACAAACCAGAGTTTAGAAAGCGG---------------------------------GATAGGTGCAGAGACTCAATGG
Olea_europaea*            GGGCAATCCTGAGCCAA---------ATCCTGTTTTCCCAAAACAAAGGTTCAGAAAGAAAAAAG---------------------------------GATAGGTGCAGAGACTCAATGG
Urtica_dioica             GGGCAATCCTGAACCAA---------ATCTGGTGTTATAAAACAAAGCGATAAAAAAAAG--------------------------------------GATAGGTGCAGAGACTCAACGG
Rumex_acetosa             GGGCAATCCTGAGCCAA---------CTCCTCCTTTCCAAAAGGAAGAATAAAAAAG-----------------------------------------GATAGGTGCAGAGACTCAATGG
```
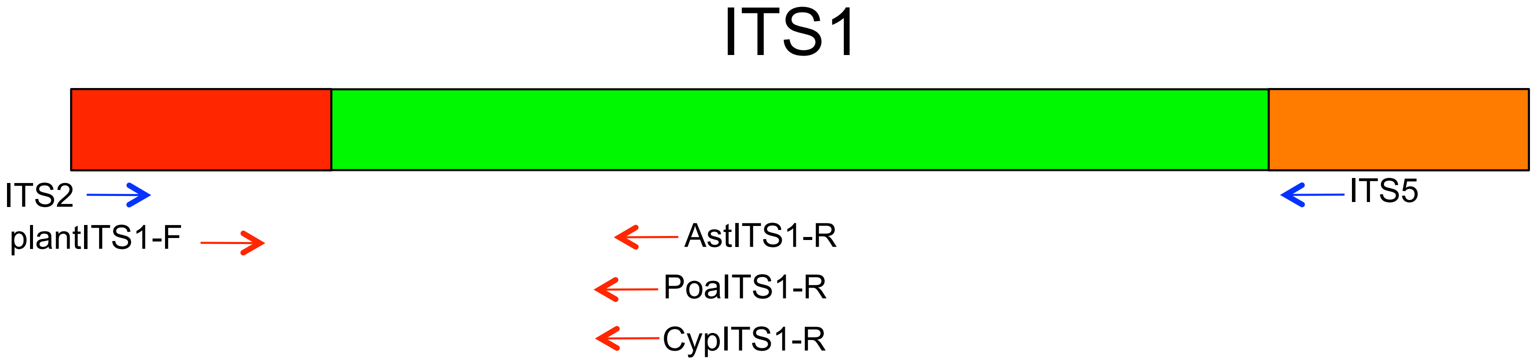

New ITS barcode primers for Asteraceae, Poaceae, and Cyperaceae

- Asteraceae
  Length: < 90bp; 68% Asteraceae with a maximum of 2 mismatches
- Poaceae
  Length: 54bp- 88bp; 98.5% Poaceae with a maximum of 2 mismatches
- Cyperaceae
  - Length: 46bp - 78bp; 93% Cyperaceae with a maximum of 2 mismatches



sequencing

400 bp fragments
10^6 reads /run

GS FLX Roche Diagnostic®

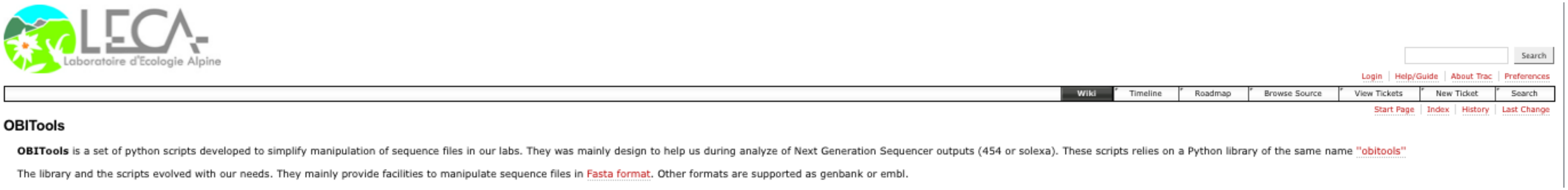
100 bp fragments
(2x100 paired-ends)

2 10^9 reads / run

HighSeq 2000 Illumina®

taxonomic assignment

- Reference database (extracted from public databases / built on purpose)
- Difficult with very short barcodes (identification of families, genera, or species)
- Possibility to work with a low resolution or without taxonomic assignment (Ecological clades / MOTUs)



OBITools

OBITools is a set of python scripts developed to simplify manipulation of sequence files in our labs. They was mainly design to help us during analyze of Next Generation Sequencer outputs (454 or solexa). These scripts relies on a Python library of the same name "obitools"

The library and the scripts evolved with our needs. They mainly provide facilities to manipulate sequence files in Fasta format. Other formats are supported as genbank or embl.

DNA BARCODING

New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the *trn*L approach

ALICE VALENTINI,*† CHRISTIAN MIQUEL,* MUHAMMAD ALI NAWAZ,‡§ EVA BELLEMAIN,*
ERIC COISSAC,* FRANÇOIS POMPANON,* LUDOVIC GIELLY,* CORINNE CRUAUD,¶
GIUSEPPE NASCETTI,† PATRICK WINCKER,¶ JON E. SWENSON‡** and PIERRE TABERLET*

Mollusks

Deroceras reticulatum

Arion rufus

Helix aspera

Insects

Gonfophocerippus rufus

Chorthippus biguttulus

Birds

Tetrao urogallus aquitanicus

Tetrao urogallus major

Mammals

Ursus arctos

Marmota caudata

Valentini *et al.* (2009) *Molecular Ecology Resources*, **9**, 51-60.

Marmot and bear diets

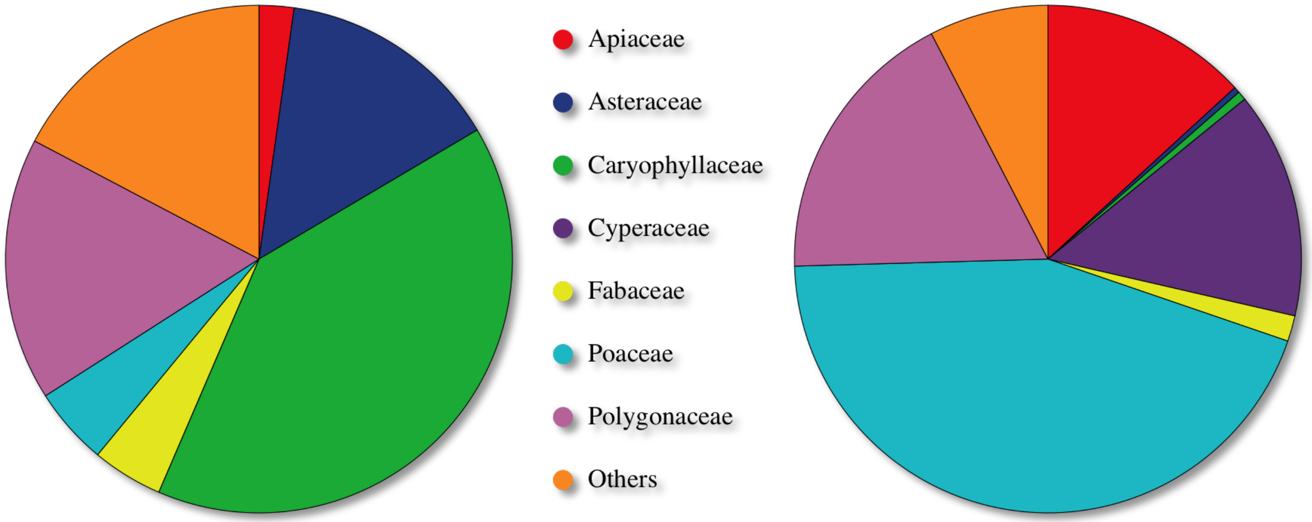
Deosai National Park, Pakistan

© Usman Ghani

Golden marmot

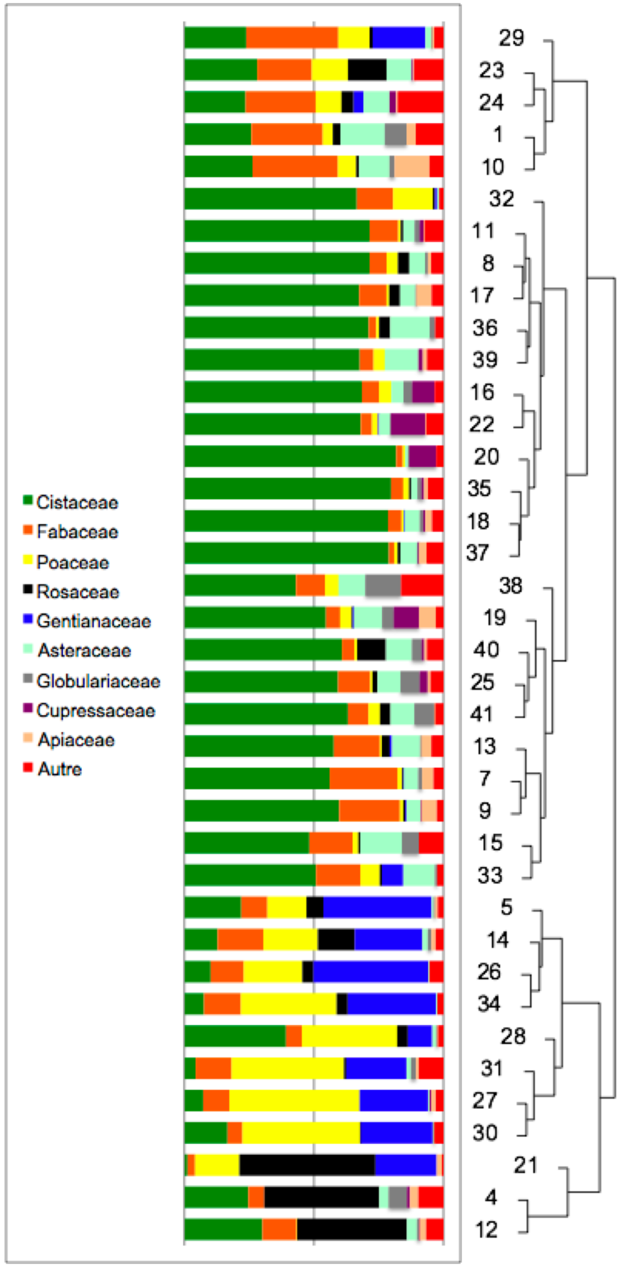
© Usman Ghani

Brown bear

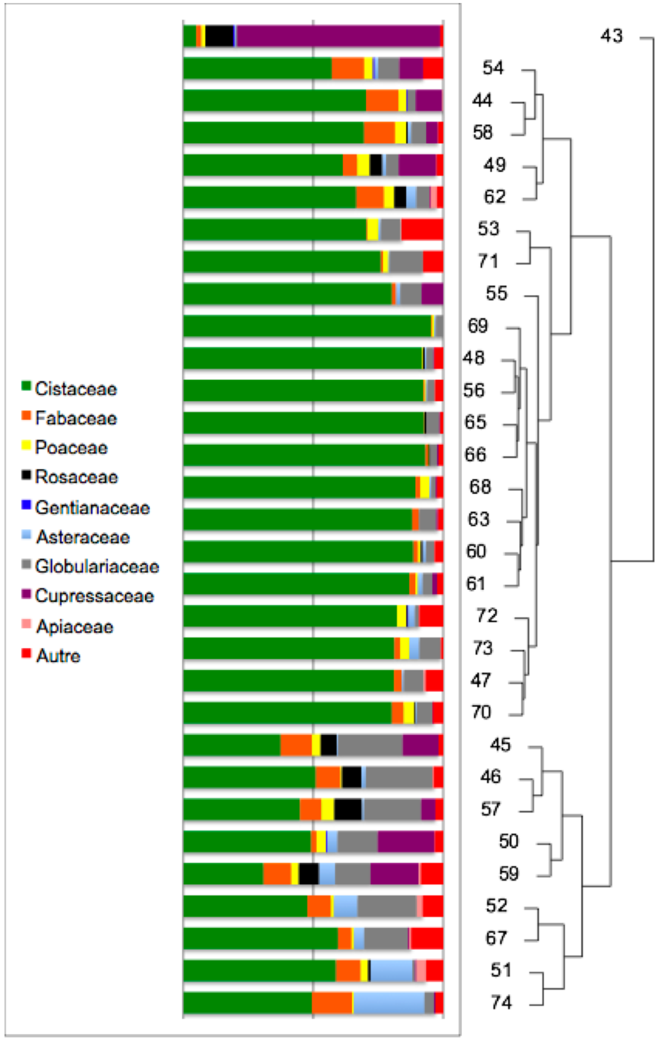


Bauges massif, France

Chamois diet

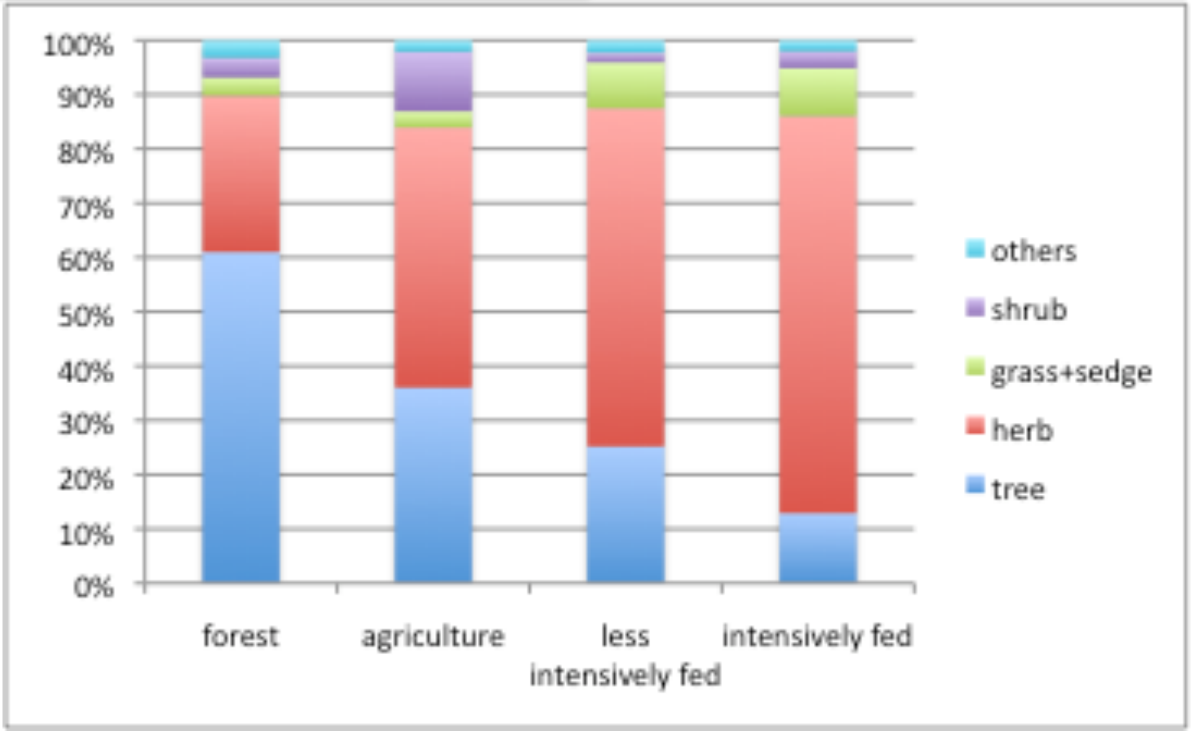


October



November

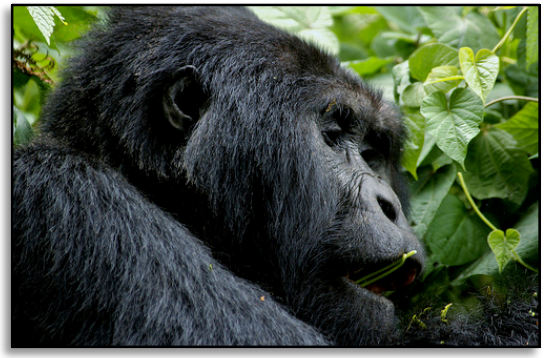
Rayé *et al.* (2011) *Ecological Research*, DOI 10.1007/s11284-010-0780-5.

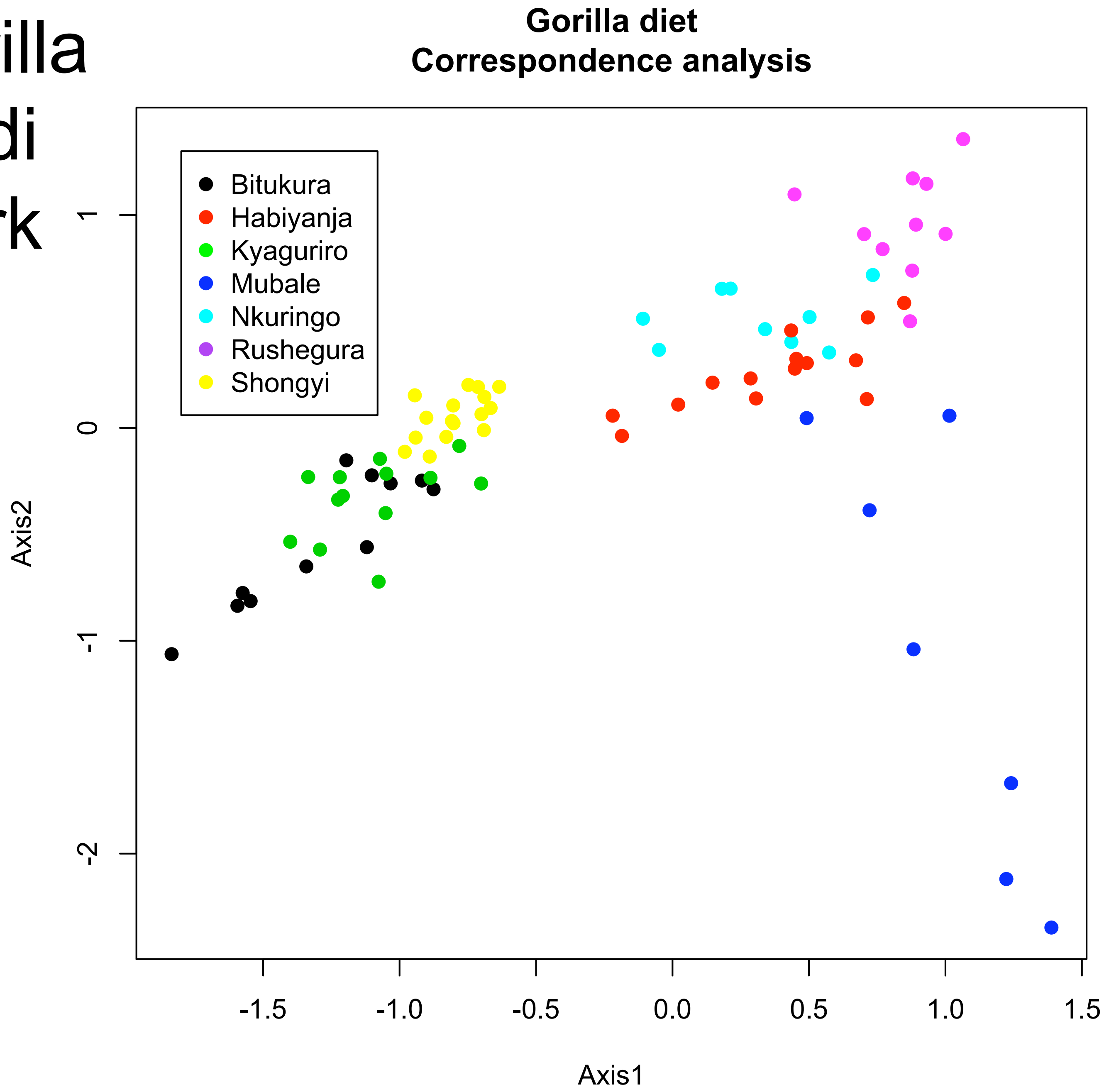


Bison diet in 4 management schemes

Kowalczyk *et al.* (2011) *Forest Ecology and Management*, **261**, 821-828.

Mountain Gorilla diet in Bwindi National Park (Uganda)





Collaboration with Juliette Ogubi, Innocent Rwego, and Charles Masembe
Makerere University, Kampala, Uganda

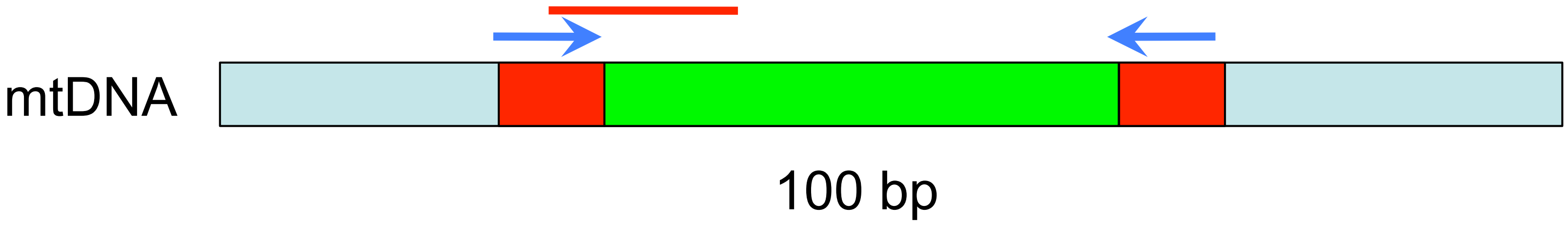
Diet analysis of carnivores

- Technically more difficult
- How to amplify the prey DNA, without amplifying host DNA when using universal primers?

→ using a blocking primer for host DNA during the amplification

Experimental protocol

- Sampling in the field
- DNA extraction
- DNA amplification
  - with mtDNA universal primers → ←
  - with and without blocking primer —



- DNA sequencing (NGS)

Snow leopard diet

Influence of the blocking primer during DNA amplification

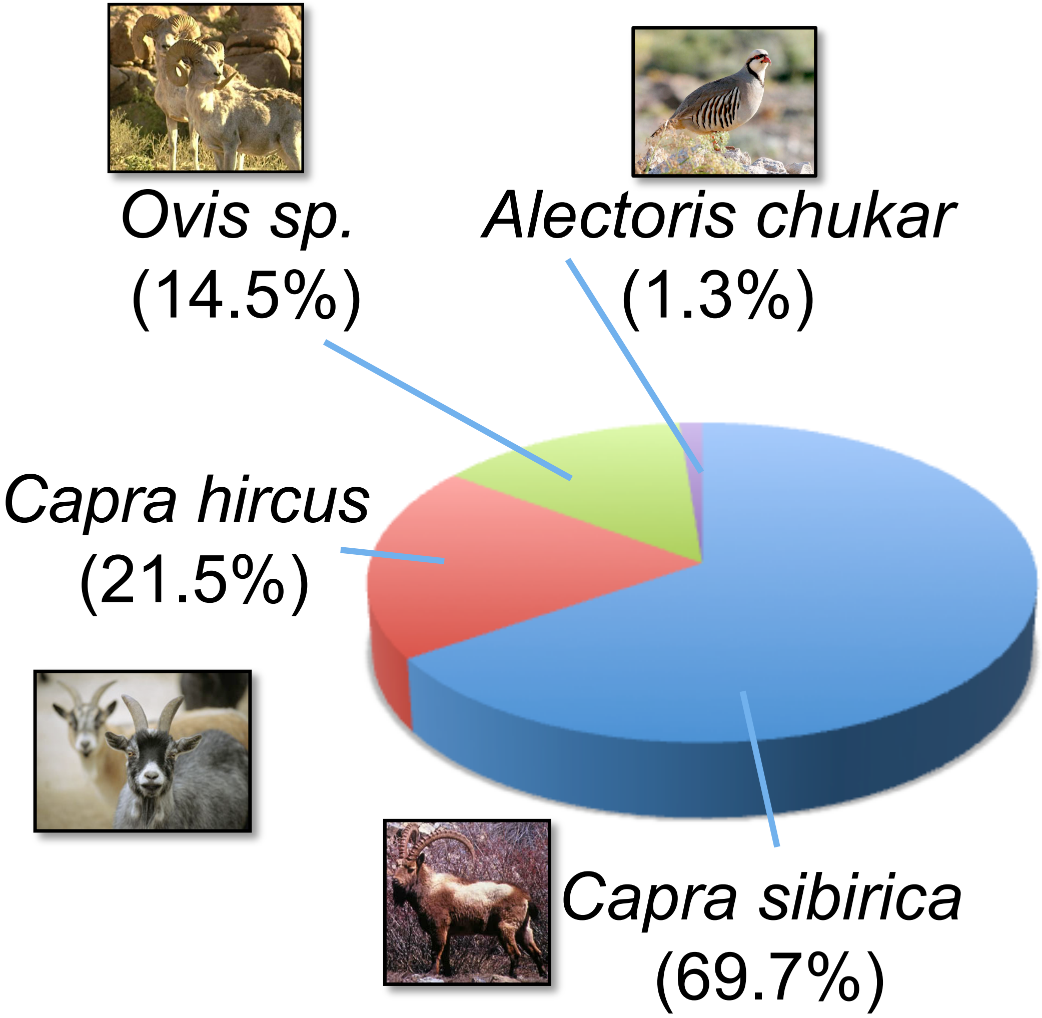
	feces 1		feces 2		feces 3	
Uncia uncia	74%	1%	97%	43%	100%	100%
Capra sibirica	26%	99%	0%	0%	0%	0%
Capra hircus	0%	0%	0%	0%	0%	0%
Ovis sp.	0%	0%	3%	57%	0%	0%

forward primer + reverse primer

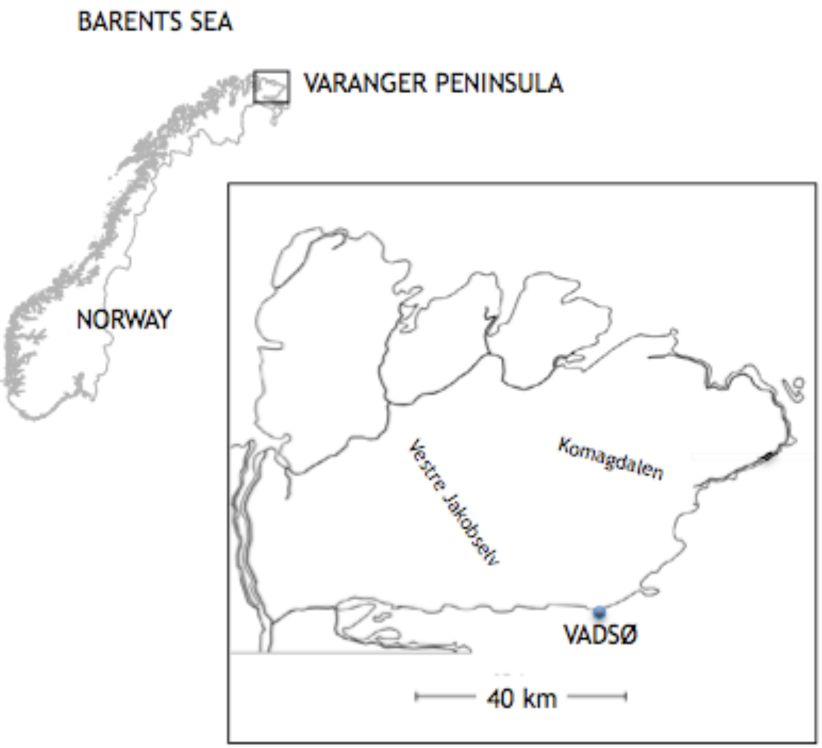
forward primer + reverse primer + blocking primer

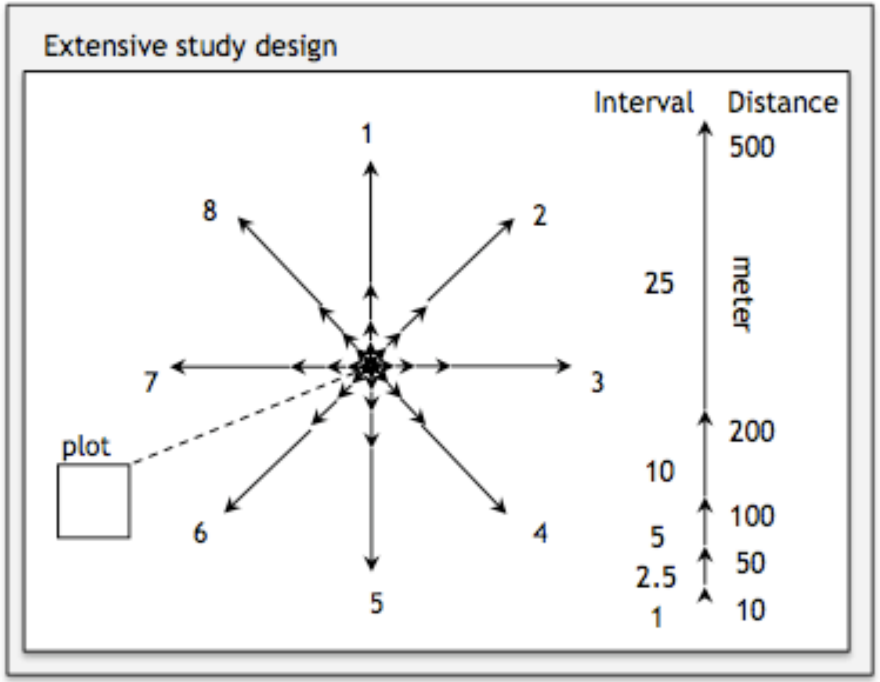
Snow leopard diet in Mongolia

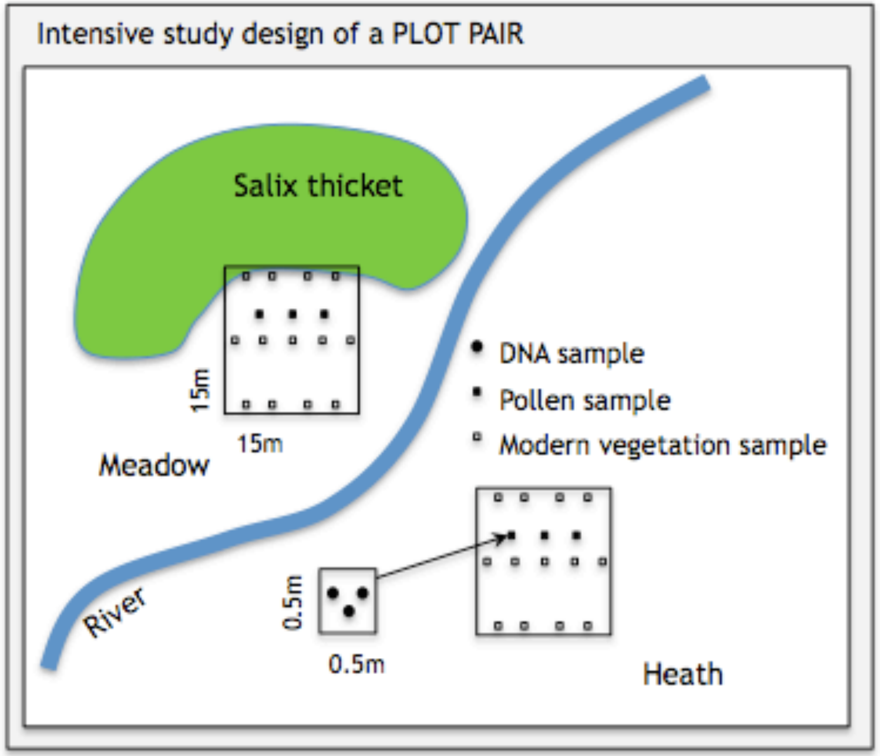
70 faeces analyzed



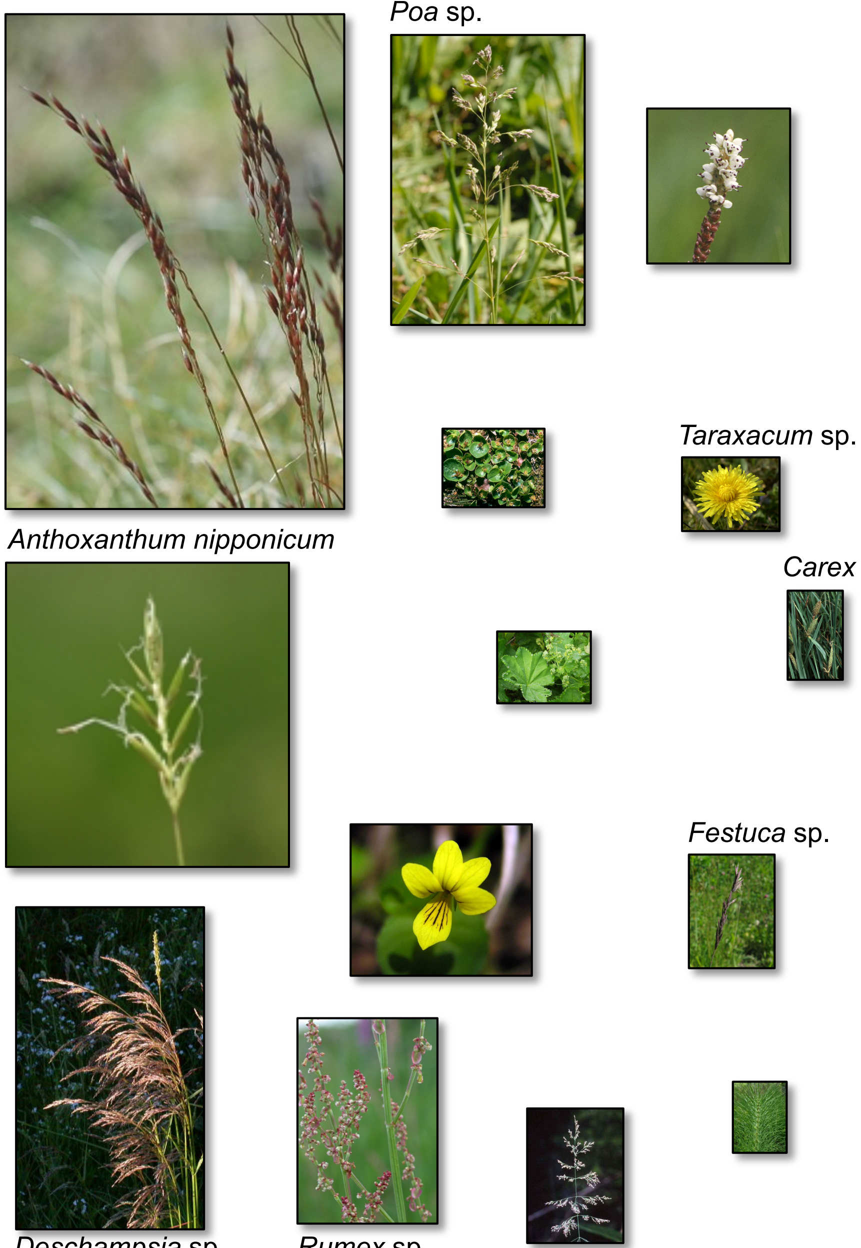
Calibration of soil DNA with aboveground species at Varanger, Northern Norway




Extensive study design


Intensive study design of a PLOT PAIR

Above ground analysis



Avenella flexuosa

Poa sp.

Taraxacum sp.

Anthoxanthum nipponicum

Carex sp.

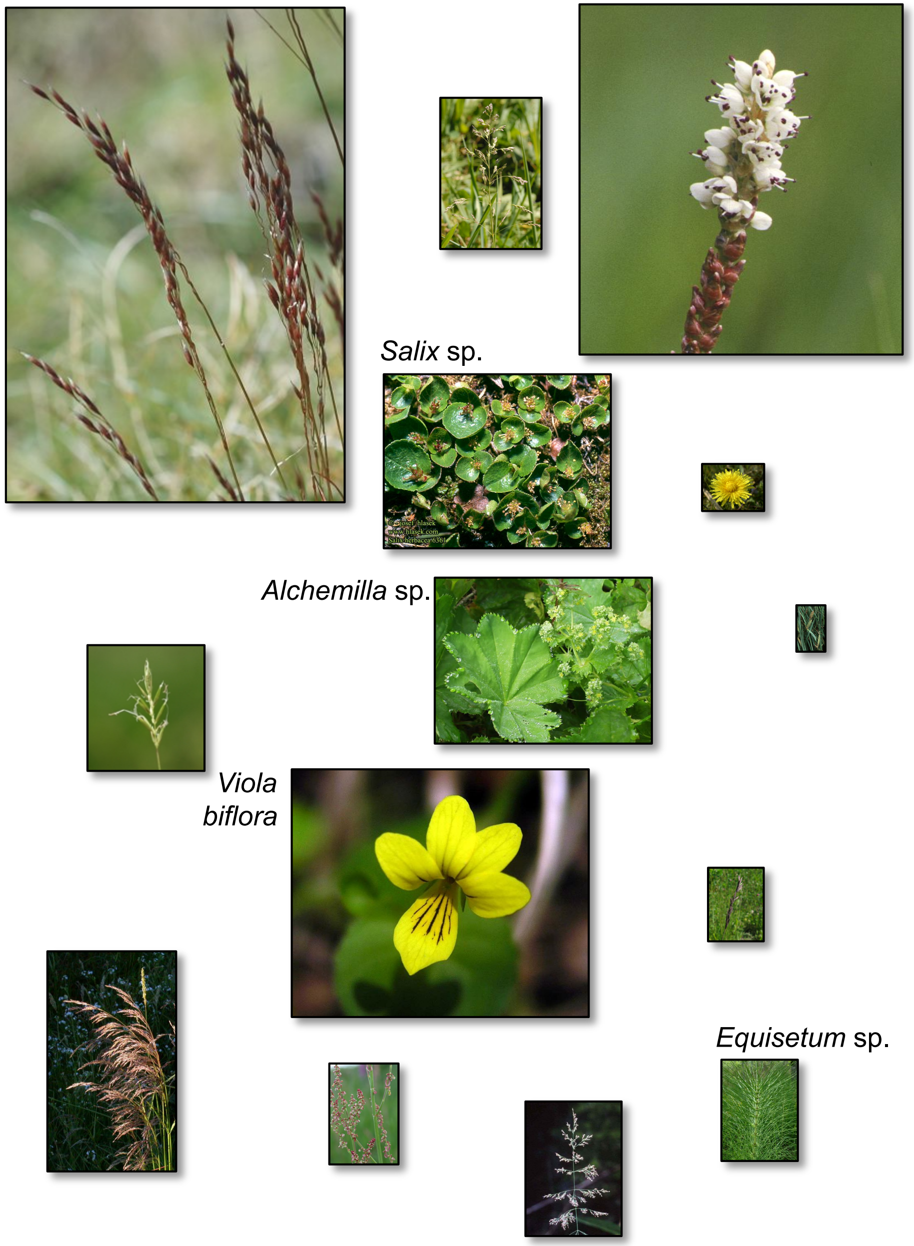
Festuca sp.

Deschampsia sp.

Rumex sp.

Calamagrostris sp.

DNA-based soil analysis



Bistorta vivipara

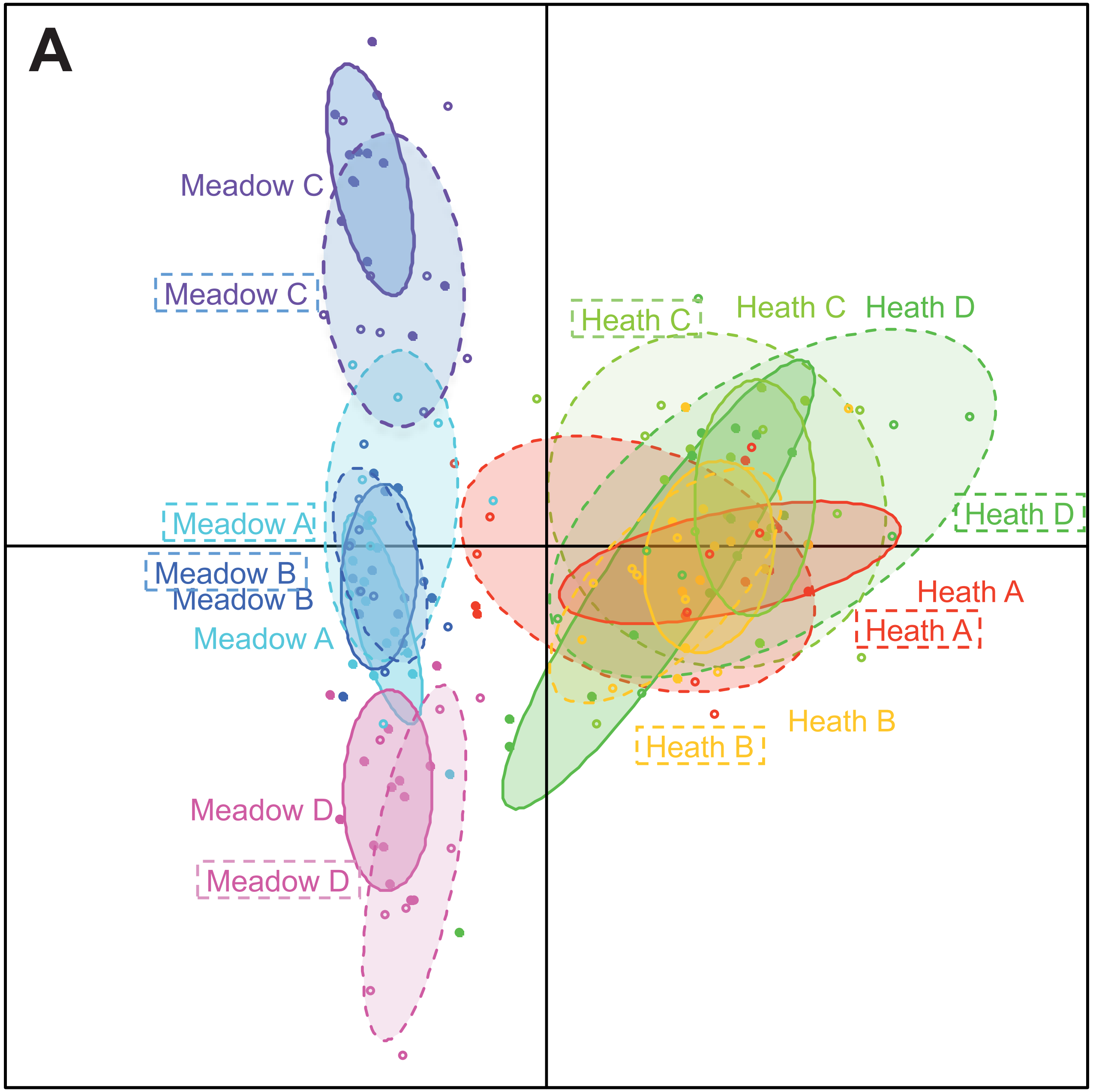
Salix sp.

Alchemilla sp.

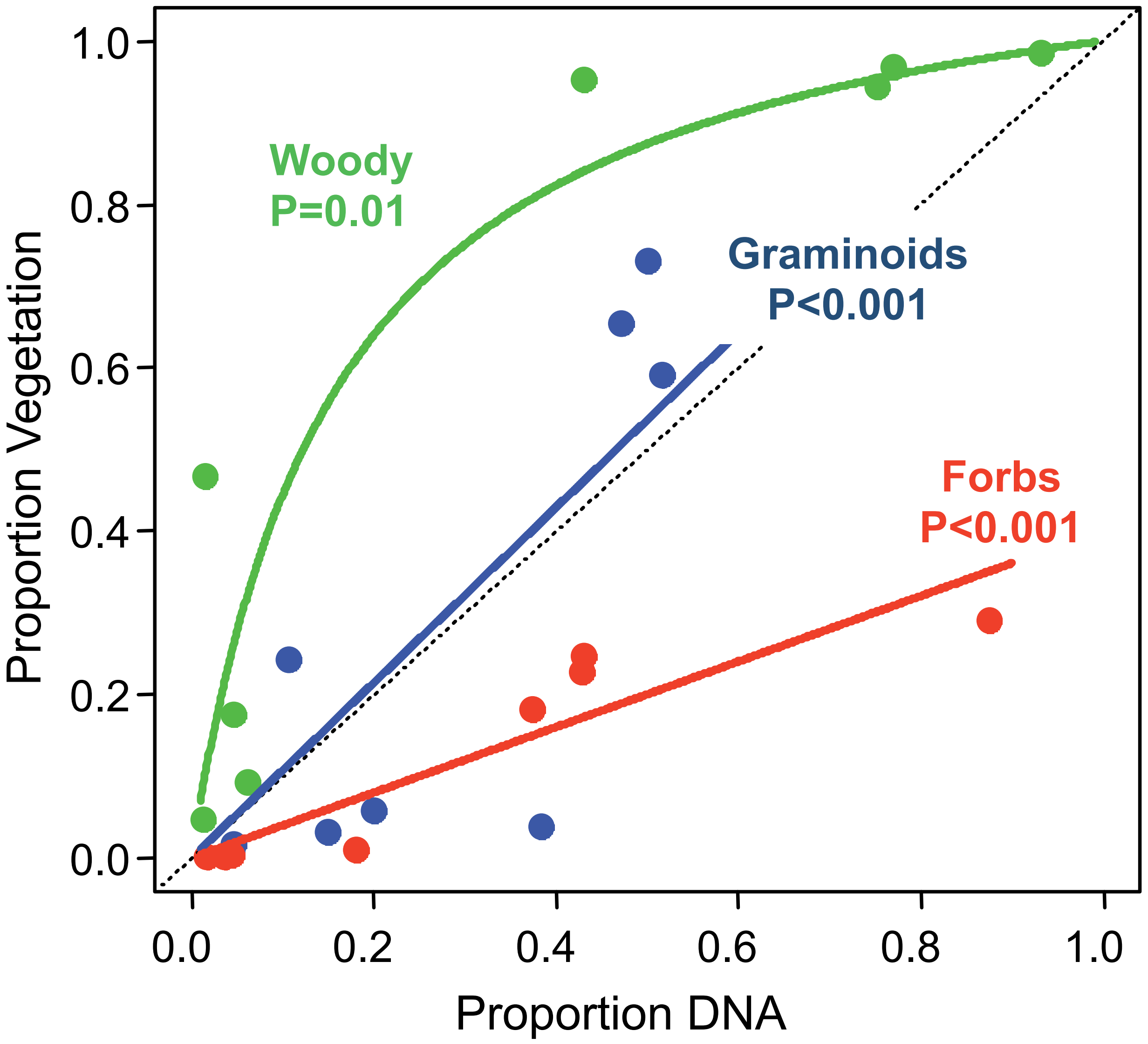
Viola biflora

Equisetum sp.

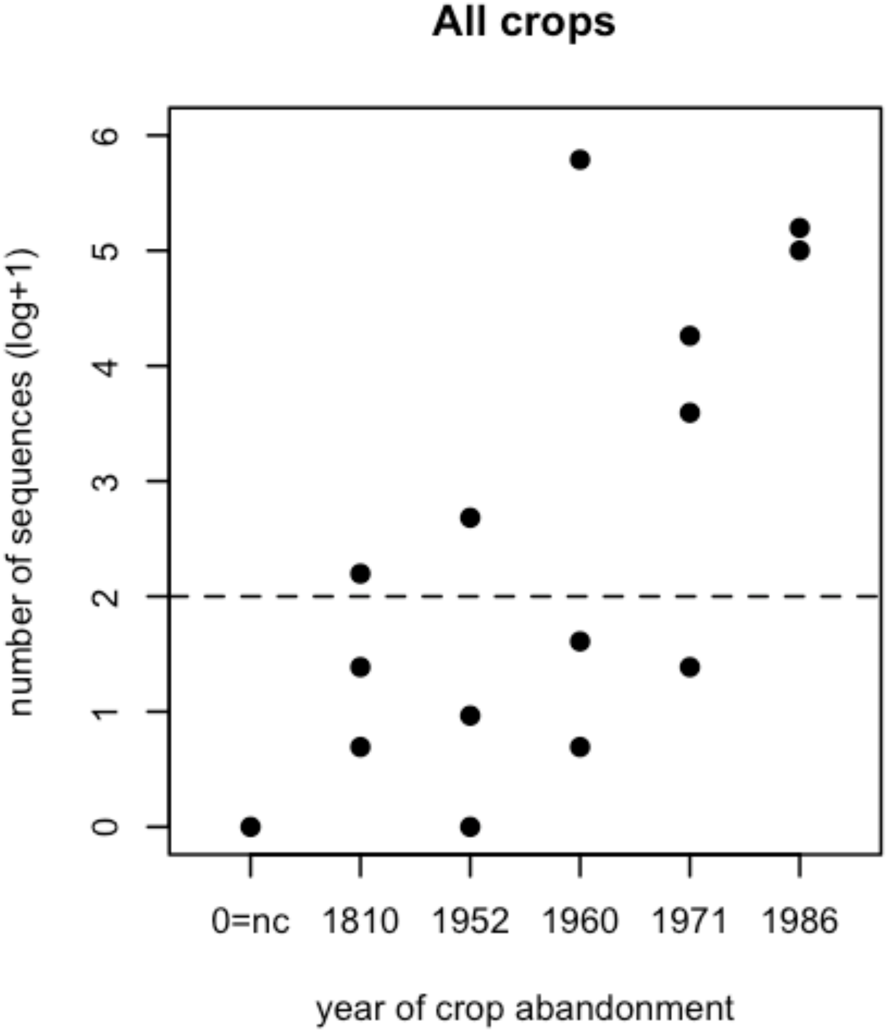
DNA in soil mirrors above ground plant diversity



DNA-based soil analysis: functionnal groups



How long does a DNA molecule persist in soil?



DNA-based soil analysis: broad applicability

C Sites	No of core soil samples	No of plots	Sequencing platform	No of sequences analyzed	No of families identified
Boreal (Varanger Fjord, Norway)	72	8	Roche 454 FLX	176 283	33
Temperate (French Alps, France)	8	1	Illumina GA IIx	396 054	5
Tropical (Nouragues Field Station, French Guiana)	49	1	Illumina GA IIx	1 636 455	34

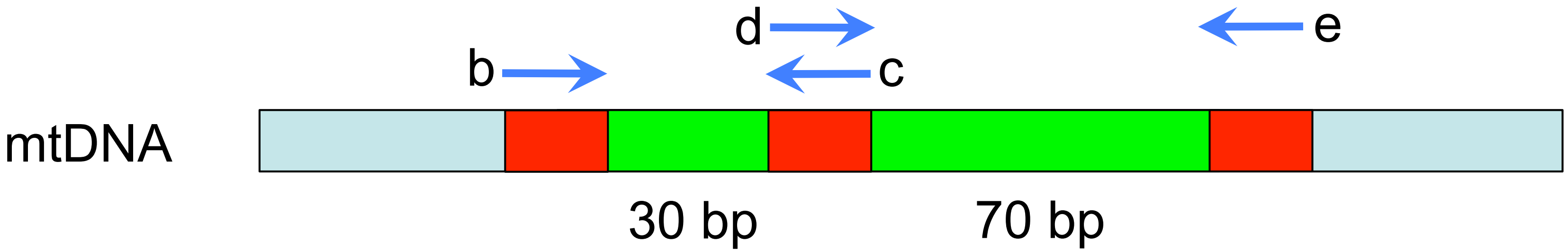
Permafrost analysis

Nb Seq	Family	Genus	Species
937			*Bistorta vivipara*
482		*Equisetum*	*arvense / fluviatile / sylvaticum*
94	Salicaceae	3	*Salix sp. / Chosenia arbutifolia / Populus balsamifera*
60			*Armeria scabra*
55			*Thymus oxyodontus*
43			*Lagotis glauca*
37	Asteraceae	3	Asteraceae 4
35			*Avenella flexuosa*
27		*Aconogonon*	*alaskanum / ocreatum / tripterospermum*
26		*Rumex*	5
19	Asteraceae	2	*Packera* sp. / *Senecio* sp.
19	Poaceae		Poaceae 2
16		*Ranunculus*	*acris / subborealis / turneri*
15		*Festuca*	9
13			*Hulteniella integrifolia*
11			*Saxifraga hirculus*
9			*Trientalis europaea*
8	Asteraceae	4	Asteraceae 2
7		*Valeriana*	*capitata / sambucifolia / transjenisensis*
6			*Myosotis alpestris*
6	Asteraceae	5	Asteraceae 3
6		*Empetrum*	*sibiricum / subholarcticum*
5			*Anthoxanthum nipponicum*
5			*Crepis chrysantha*
4		*Saxifraga*	5
3		*Papaver*	19
3	Poaceae	2	*Elymus* sp. / *Leymus* sp.
3			*Trollius europaeus*

A 22,960 years old flora

Earthworms from soil DNA

- Eight soil samples collected per plot
- Universal short barcodes for earthworms
- Reference database built using samples identified with the standardized COI barcoding approach



Earthworms from soil DNA: results

		Chartreuse		Grenoble	
Species	**Barcode**	**Plot 1**	**Plot 2**	**Plot 1**	**Plot 2**
Aporrectodea icterica	catcttaatgaagactaaaacttcactaaa	836954	649677	834031	1359355
Aporrectodea longa	tattttaacaaaaacccaaaaattttcaataaa	2	6	244463	271829
Aporrectodea sp	cattttaataaaaattataaattttactaaa	0	0	236024	236678
Octolasion cyaneum	cattttaatagaagcttactattctaataaa	468462	3823	0	2
Unidentified Oligochaeta	tattttaataaaatagtaaattttactaaa	334804	96337	0	1
Unidentified Oligochaeta	tattataaatcaattaataattgagcata	0	372828	0	0
Lumbricus terrestris	aatttaaataaatataaaaaatttactaaa	0	0	174286	143682
Octolasion tyrtaeum	cattttaatagaaaaataatatcctaataaa	306476	0	0	2
Unidentified Oligochaeta	tatcacaatatttatacaataaatattatg	183116	68615	0	0
Unidentified Oligochaeta	tatttttcttatactttagtaaacaaaaa	96924	42148	0	0
Lumbricus castaneus	aatttaaataaatataaaaaaatttactaaa	0	0	56	131001
Aporrectodea longa	tattttaacaaaacccaaaaattttcaataaa	2469	105312	159	145
Allobophora chlorotica	cattttaataaagatataaactttactaaa	0	0	51953	43196
Unidentified Oligochaeta	tattttatttacctaaaacagtaacaaaa	0	0	62901	0
Unidentified Oligochaeta	tatttttcttatactttagtaaataaaaa	592	61802	0	0
Unidentified Oligochaeta	taccttaacaaatattattatttcgaag	30571	0	0	0
Aporrectodea caliginosa	tattttaataaaaaaatataaatttttaataa	0	23005	0	0

DNA metabarcoding

- Works extremely well for diet analysis using feces as a source of DNA
  - For herbivores
  - For carnivores
  - Can be adjusted for other type of diet
- Works well for plant biodiversity using soil samples
  - In the Arctic
  - In the temperate region
  - In the tropical region
- Need to be further adjusted for animal biodiversity using soil samples
  - Optimistic for animals with high biomass (earthworms, etc.)
  - Might be problematic for animals with low biomass

Number of occurrences	P6 loop (chloroplast *trn*L (UAA) intron) sequences	Identification
3103	ATCCGGTTCATGGAGAC-AATAGTTT-CTT-CTTTTATTCTCCTAAGATA-GGAAGGG	*Picea*
45	.................-........-...-....-..............-.......	*Picea* variant
42	.................-........-...-...................-......-	*Picea* variant
13	..........................-...-...................-......A	*Picea* variant
9	.................-........-...T...................-.......	*Picea* variant
9	.................-........-...-.......C...........-.......	*Picea* variant
6	.................-........-..C-...................-.......	*Picea* variant
6	.................-........-...-...C...............-.......	*Picea* variant
6	.................-........-...C....-..............-.......	*Picea* variant
5	............A....-........-...-...................-.......	*Picea* variant
5	.................-........-...-........T..........-.......	*Picea* variant
5	.................T........-...-...................-.......	*Picea* variant
5	.................-.G......-...-...................-.......	*Picea* variant
5	.................-........-...-...................A.......	*Picea* variant
5	.................-........T...-...................-.......	*Picea* variant
5	.................A.T......-...-...................-.......	*Picea* variant
4	.................-........-...-...................-....A..	*Picea* variant
4	-................-........-...-...................-.......	*Picea* variant
4	................T-........-...-...................-.......	*Picea* variant
4	.................-........-...-...................G.......	*Picea* variant
4	......C..........-........-...-...................-.......	*Picea* variant
4	.................-........-...-....-..............-......-	*Picea* variant
4	.................-........-...-...................-...G...	*Picea* variant
4	..............A..-........-...-...................-.......	*Picea* variant
236	ATCCGGTTCATAGAGAAAAGGGTTTCTCTCCTTCTCCTAAGGAAAGG	*Abies*
4	..................-............................	*Abies* variant

P6 loop (chloroplast *trn*L (UAA) intron) sequences obtained after high throughput pyrosequencing for a bird faeces sample (*Tetrao urogallus major*)

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Thank you for your attention