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# The DNA based characterization of the diet from digested samples: a reliability study in ruminants

**V. Niderkorn[1,*], A. Farruggia[1], M. De Barba[2], D. Rioux[2], R. Baumont[1] and F. Pompanon[2]**

[1]INRA, UMR1213 Herbivores, F-63122 Saint-Genès Champanelle (France)
[2]LECA, Université Grenoble Alpes, UMR CNRS 5553, F-38041 Grenoble (France)
*e-mail: vincent.niderkorn@clermont.inra.fr

**Abstract.** Characterizing the diet of ruminants at pasture in diversified grasslands is a difficult step that could be realized by using the DNA-based methods recently developed for assessing the composition of environmental samples (i.e. metabarcoding approach). In this study, we performed two experiments to assess the performance of this method. The diet was inferred from rumen and faecal samples after DNA extraction, subsequent amplification of a short fragment of chloroplast DNA with universal primers used for plant DNA identification (*trn*L approach), and sequencing of the PCR products using HighSeq Illumina system. The first experiment compared the results obtained from replicated extractions and PCRs performed on faecal samples from cattle with a diversified diet, and showed a high reproducibility of the method. The second experiment was designed to assess the semi-quantitative aspects of this approach. Five diets were allocated to five sheep fed *ad libitum* according to a 5 × 5 Latin square design. The diets were mixtures of green fodder differing by their white clover: ryegrass ratios (i.e., 0:100, 25:75, 50:50, 75:25 and 100:0). The analysis of rumen contents allowed a reliable estimation of the proportion of each species ingested, while the information was much less accurate from faecal samples. Technical and analytical solutions are currently tested in order to improve the quantitative information obtained from faecal samples.

**Keywords.** Ruminants – Diet analysis – DNA metabarcoding – Chloroplast *trn*L intron – Sequencing.

***Caractérisation du régime alimentaire des ruminants à partir de l'analyse des produits de la digestion par DNA metabarcoding***

***Résumé.*** *La caractérisation du régime alimentaire des ruminants au pâturage au sein de prairies diversifiées est un verrou méthodologique qui pourrait en partie être levé à l'aide de méthodes basées sur l'analyse de l'ADN présent dans des échantillons environnementaux (métabarcoding). Pour évaluer la fiabilité de cette technique, nous avons mené deux expérimentations au cours desquelles l'ADN issu d'échantillons de contenu du rumen et de fèces a été extrait. Un fragment court d'ADN chloroplastique a été amplifié en utilisant des amorces universelles (approche trnL) et les produits PCR ont été séquencés en utilisant un système HighSeq Illumina. La première expérimentation a consisté à comparer les résultats obtenus à partir d'extractions et de PCRs répétées sur des fèces de bovins alimentés avec un régime diversifié et a permis de valider la reproductibilité de la méthode. La deuxième expérimentation a été conçue pour évaluer les aspects semi-quantitatifs de cette approche. Cinq moutons ont été alimentés* ad libitum *selon un carré latin 5 × 5 avec cinq mélanges binaires de ray-grass anglais et de trèfle blanc en proportions variables (0:100, 25:75, 50:50, 75:25 et 100:0). Les analyses de contenu du rumen ont permis de quantifier de manière fiable les proportions ingérées de chaque espèce, tandis que l'information issue de l'analyse des fèces était beaucoup moins précise. Des solutions techniques et analytiques sont actuellement examinées pour améliorer la quantification à partir des échantillons fécaux.*

***Mots-clés.*** Ruminants – Analyse du régime alimentaire – ADN metabarcoding – Intron chloroplastique *trn*L – Séquençage.

## I – Introduction

Characterizing the diet of grazing ruminants in complex environment is an important methodological gap for the research on the animal-plants relationships in diversified grasslands (permanent or multispecies temporary grasslands). The current methods are based on the direct observation of the foraging behaviour (Holechek *et al.*, 1982), the microhistological techniques (Alipayo *et al.*, 1992), the extraction and the analysis of long chain alkanes presents in plant cuticular wax (Dove and Mayes, 1996), or the spectral methods (NIRS, near infrared reflectance spectroscopy) (Keli *et al.*, 2008). These methods are time-consuming, not very accurate, or not well adapted to diversified swards.

The recent development of a molecular technique allowing the identification of plant species in complex or degraded matrices (DNA metabarcoding) could allow a methodological innovation in this field (Pegard *et al.*, 2009). This technique is based on the analysis of very short residual fragments of plant DNA in digestive residues using a couple of universal primers targeting a variable area of intron of gene chloroplastic *trn*L (UAA) of the plants (Valentini *et al.*, 2009). DNA metabarcoding has recently been tested to assess qualitatively the composition of the diet of cattle in grasslands with contrasted botanic diversity (Farruggia *et al.*, 2012). Twice as many taxons were found in the faeces from animals grazing on species rich grassland compared to faeces from animals grazing on species poor grassland.

The objective of this work was to test the reproducibility and the potential of the DNA metabarcoding technique to characterize quantitatively the diet of ruminants fed multispecies forages.

## II – Materials and methods

***Experiment 1:*** Faecal samples were taken on five dairy cows grazing a species-rich grassland. From each of the five samples, three subsamples were prepared on which the DNA was extracted with the DNeasy Blood and Tissue Kit (QIAgen) following the manufacturer's instructions. Each DNA extracts were amplified in triplicate with the *trn*L gh primers (g primer, GGGCAATCCTGAGCCAA; h primer, CCATTGAGTCTCTGCACCTATC; Taberlet *et al.*, 2007). Paired-end sequencing (100 nucleotides on each extremity of the DNA fragments) from all the PCR products (n = 45) was carried out at the French National Sequencing Centre (CEA Genoscope) on a high-throughput sequencing technology (Illumina HiSeq 2000 system).

***Experiment 2:*** To test the semi-quantitative approach of the *trn*L approach for estimating the diet of herbivores, an experiment on sheep was conducted. During the period of May–July 2011, pure plots of white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) were used to test five binary mixtures differing by their clover:ryegrass ratios of 0:100, 25:75, 50:50, 75:25 and 100:0. The five diets were allocated to five 1-year-old male Texel sheep fed *ad libitum* according to a 5 × 5 Latin square design. For each sheep and each diet, one rumen sample and one faecal sample were collected on two successive days (n = 50). The collection started 13 days after the beginning of the diet in order to prevent an effect from the previous diet. The DNA analysis was performed following the procedure used in Experiment 1. The sequences produced were assigned to a species using programs from the OBITools software package (avalaible at http://metabarcoding.org/obitools). The relative number of sequences obtained for each plant species in the PCR products was compared to the actual relative proportion of these species in the diet.

## III – Results and discussion

In Experiment 1, each replicate has been first characterized by a set of sequences called MOTUS (Molecular taxonomic unit) without species identification. A principal component analysis (PCA) was

carried out on these MOTUS to test the reproducibility of the method (Fig. 1). The three axis explained 53%, 14% and 11% of the total variance, respectively. Axis 1 and 2 highlighted one deviant PCR product. Axis 2 and 3 of the PCA showed a good discrimination of the MOTUS on the basis of cows but not on the basis of extractions and PCR. Hierarchical ANOVA performed on the factorial coordinates of the PCA confirmed that there was no effect of extraction or PCRs. The observed deviant PCR product could result from the production of errors or the amplification of very minor species in the first cycles of the PCR, leading to a non-representative final product (Coissac *et al*., 2012). Taking these results together, it can be recommended to perform one extraction per sample and three PCRs per extraction, which makes it possible to eliminate the deviant PCRs detected by the PCA. The same analysis has been made after assignment of taxa to generated sequences. The reproducibility of the results was better because this step also took into account the possibility of errors on the assigned sequences when comparing at the reference index.

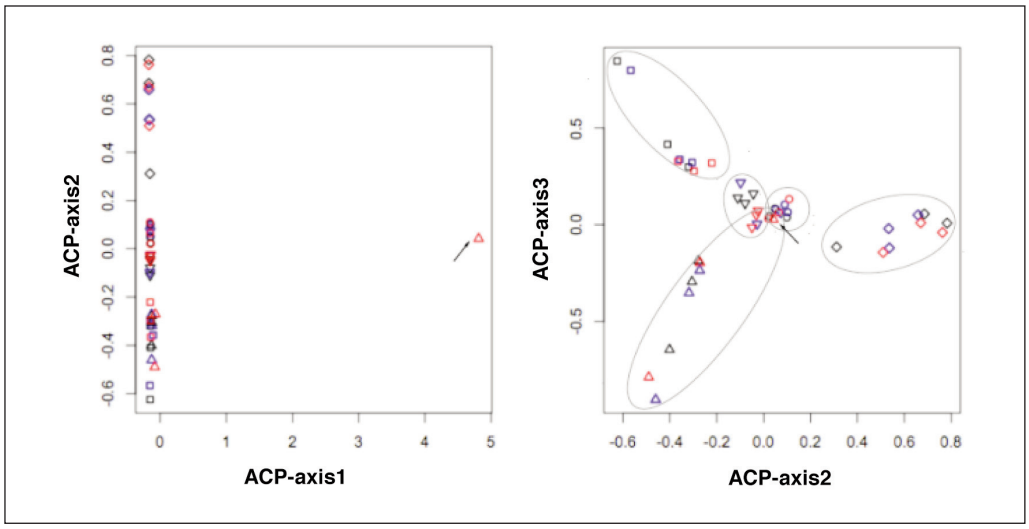


**Fig. 1. Principal Component Analysis based on the sequences (MOTUS, Molecular Taxonomic Units) generated with *trn*L approach. Each type of marker represents a cow, each colour corresponds to one extraction and each point is a PCR. Each ellipse corresponds to one of the five cows.**

In Experiment 2, the results obtained from rumen contents showed a very good determination of the proportions of ryegrass and white clover in the diet (Fig. 2). The Pearson correlation coefficient between the actual fraction of ryegrass in the diet and the proportion estimated using the DNA metabarcoding approach was highly significant ($r = 0.87$, $P<0.001$).

The semi-quantification was less accurate when the DNA metabarcoding analyses were carried out on faeces, since the proportions of ryegrass in the diet were underestimated (Fig. 2). This could be explained by a lower recovery of the ryegrass DNA in faeces in comparison with rumen contents due to a differential in the post-rumen digestibility. A bias could occur due to the method of DNA extraction used, which tends to support the recovery of the intracellular DNA.

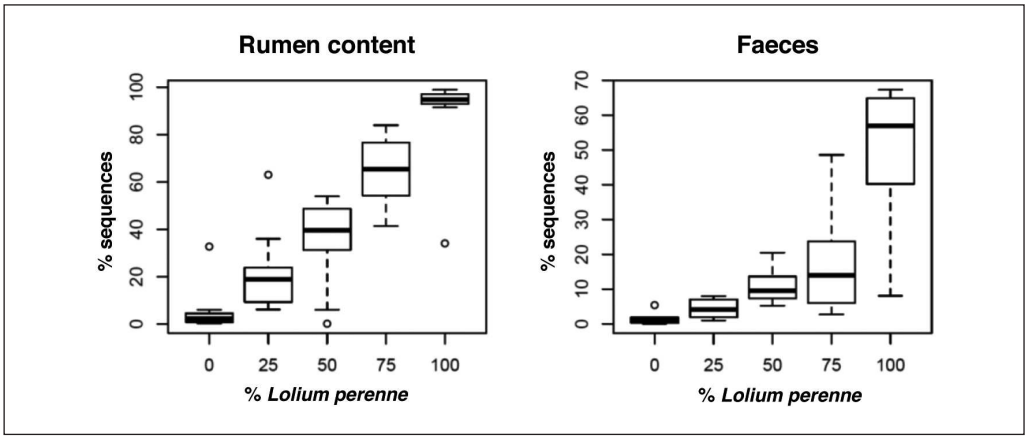


**Fig. 2. Percentages of ryegrass (*Lolium perenne*) sequences found in the PCR products generated from rumen contents and faeces from sheep fed ryegrass-white clover mixtures in various proportions.**

## IV – Conclusions

Overall, the DNA metabarcoding appears reproducible to characterize the diet of ruminants, although deviant PCRs can be observed. One extraction per sample and three PCRs per extraction can be recommended. Very promising results were obtained from rumen contents which validates the possibility of determining the proportions of species by DNA metabarcoding for simple mixtures. The comprehension of the biases observed between plant species and the kind of digestive residues (rumen or faeces) should make it possible to improve the use of this methodology, in particular by improving the method of DNA extraction.

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