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## Rapid Identification of Rumen Protozoa by Restriction Analysis of Amplified 18S rRNA Gene

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**Summary.** A rapid method has been developed for molecular identification of rumen ciliates without the need for cultivation. Total DNA was isolated from single protozoal cells by the Chelex method and nearly complete protozoal 18S rRNA genes were amplified and subjected to restriction fragment length polymorphism analysis. On the basis of restriction patterns generated a molecular key was elaborated allowing identification of protozoa solely by a molecular technique without prior knowledge of morphology. No differences were observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species. The ARDREA analysis described here provides a rapid and convenient way for identification and diversity studies of rumen protozoa.

**Key words:** 18S rRNA, ARDREA, ciliate, identification, protozoa, rumen.

**Abbreviations:** ARDREA - Amplified Ribosomal DNA Restriction Analysis, PCR - Polymerase Chain Reaction.

### INTRODUCTION

Rumen protozoa represent a substantial part of the rumen microbial population contributing up to 50 % of the total microbial biomass (Williams and Coleman 1992). However, due to highly specific growth requirement and their complex morphology our understanding

of the role of protozoa in rumen fermentation is still limited. Ciliate protozoa in the rumen are classified on the basis of the micro- and macronucleus and the presence and morphology of exterior spines and lobes or internal skeletal plates as well as the shape and size of cells (Dogiel 1927, Ogimoto and Imai 1981, Williams and Coleman 1992). Based on such morphological characteristics a large number of genera and species have been described, but it is not clear to what extent these represent true species. Furthermore, morphological classification and identification of protozoa is made more complex as many of the morphological traits rapidly

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change or completely disappear under *in vitro* conditions (Williams and Coleman 1992). Molecular methods based on DNA analysis and fingerprinting provide a rational alternative to the classic morphology. In recent years the PCR amplification and sequencing of small subunit (SSU) rRNA genes from rumen bacteria and fungi have revolutionised studies on rumen microbial ecology. However, relatively few studies have applied this methodology to study the rumen protozoa (Hori and Osawa 1987, Lee and Kugrens 1992, Wright *et al.* 1997, Hirt *et al.* 1998), primarily due to the demanding growth requirement of rumen protozoa. The aim of this study was to develop a PCR fingerprinting method, which is independent of cultivation, for rapid identification of predominant rumen protozoa.

## MATERIALS AND METHODS

**Collection of protozoa.** Twenty protozoal species were included in the study. The cultures came from monofaunated sheep obtained during the ERCULE project and species included (country of original place of isolation is shown in parentheses) *Dasytricha ruminantium* (France), *Diplodinium dentatum* (Poland), *Diploplastron affine* (Poland), *Enoploplastron trilorcatum* (Poland), *Entodinium bursa* (Slovakia), *Ent. caudatum* (Scotland and Slovakia), *Ent. furca monolobum* (Slovakia), *Ent. nanellum* (Slovakia), *Ent. simplex* (Poland), *Epidinium ecaudatum* (Poland), *Eudiplodinium maggii* (France and Poland), *Isotricha intestinalis* (Poland), *I. prostoma* (France and Slovakia), *Metadinium medium* (Poland), *Ophryoscolex caudatus* (Poland), *Ophr. purkynjei* (Poland), *Ostracodinium gracile* (Poland), *Ostr. dentatum* (Poland), *Polyplastron multivesiculatum* (France and Poland). For study of protozoal variability *Ophr. caudatus* (forma *tricornatus*) cells were directly picked out from fresh rumen fluid of goat and sheep from Slovakia. The morphological variants of *Dipl. dentatum* were picked from an *in vitro* culture kept in Kielanowski Institute of Animal Physiology and Nutrition of Jablonna. *Entodinium caudatum* type and forma *dubardii* cells were from an *in vitro* culture kept in Institute of Animal Physiology, Kosice, Slovakia.

**DNA isolation and analysis.** A single protozoal cell was picked either from rumen fluid or from *in vitro* culture under the microscope, washed twice in drop of sterile water and put into 50 µl of 5% Chelex-100 (BioRad, California, USA) in water. Pre-incubated proteinase K (Merck, Germany) was then added to the reaction mixture to a final concentration of 20 µg/ml. After proteinase treatment (55°C for 30 min) DNA was released from cell by heating the sample at 98°C for 5 min. After rapid cooling to 0°C and centrifugation (3000g for 5 min) DNA containing supernatant was directly used for PCR amplification. All isolation and manipulation steps were done under aerobic conditions.

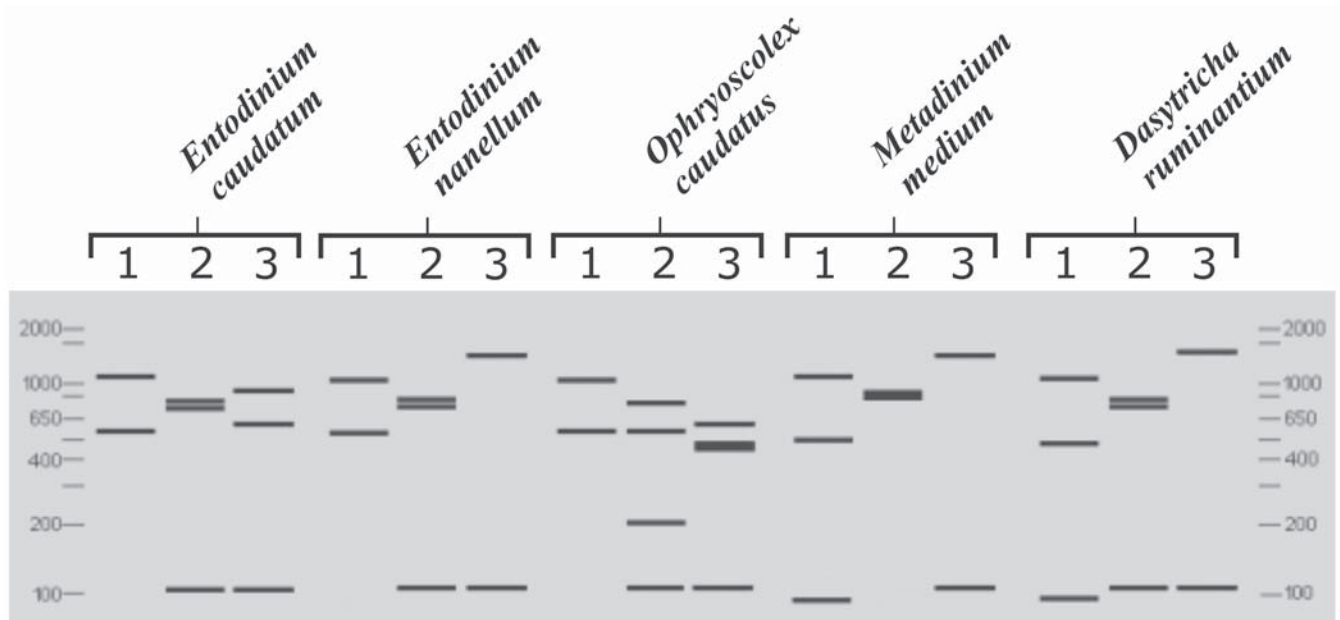
**Polymerase chain reaction.** Five µl of isolated DNA was amplified using a Techne Thermal Cycler Progene in a 50 µl reaction mix containing 0.04 mM of each deoxynucleosidetriphosphate, 20 pmol of each primer, PCR reaction buffer (Perkin Elmer), and 0.5 U of

AmpliQaq DNA polymerase (Perkin Elmer). An initial denaturation step at 95°C for 5 min was followed by 35 cycles of (94°C for 1 min, 52°C for 1 min and 72°C for 1 min), and a final incubation at 72°C for 10 min. Primers based on conserved regions in eukaryotic 18S rDNA genes were used in the PCR amplification: EukFor (5'-AATATGGTTGATCCTGCCAGT-3' and EukRev (5'-TGATCCTTCTGCAGGTTTCACCTAC-3'). Quality and quantity of amplified DNA was determined by electrophoresis in 1% agarose gel (Maniatis *et al.* 1982).

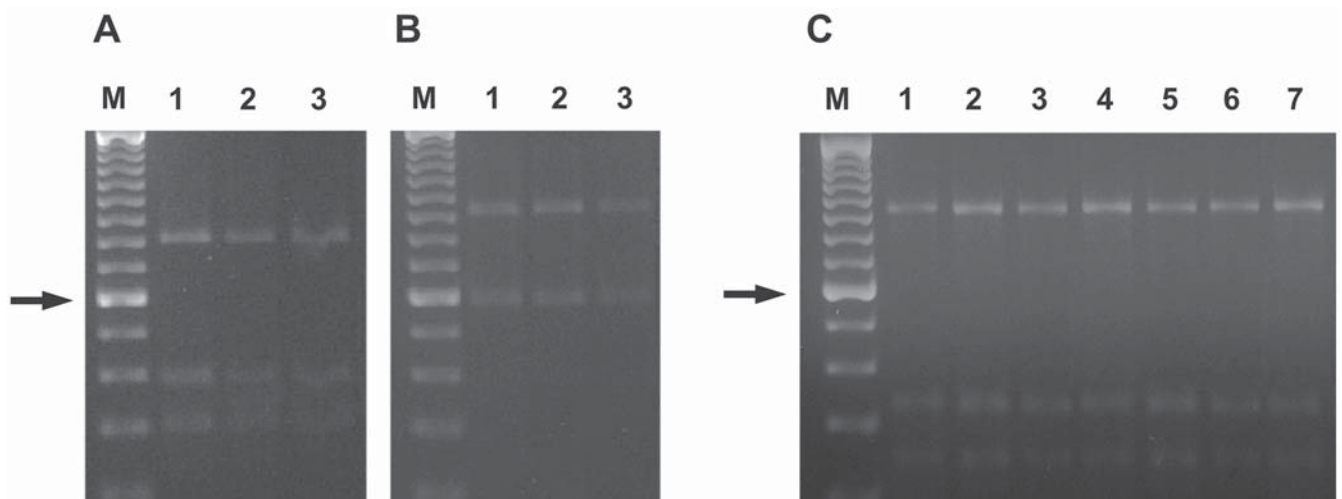
Amplified DNA (about 0.5 µg per reaction) was digested by 18 restriction endonucleases - *AccI*, *AseI*, *AvaII*, *BanI*, *BstUI*, *CfoI*, *EcoRV*, *HaeIII*, *HindIII*, *MaeII*, *MluI*, *MspI*, *NruI*, *SacII*, *SpeI*, *StuI*, *StyI* and *XmaI* (New England Biolabs and Gibco BRL), according to manufacturer's instructions for 1 h. Digestion products were separated by horizontal 2% agarose gel electrophoresis in Tris-acetate buffer (Maniatis *et al.* 1982).

## RESULTS

A rapid method has been developed for molecular identification of rumen protozoa without the need for cultivation. Total DNA was isolated from single protozoal cells by the Chelex method and used as a target for PCR amplification using primers directed to the 18S rDNA gene. The nearly complete SSU rDNA gene was obtained by PCR amplification from all tested samples. Amplified DNA was then subjected to cleavage by several (18) restriction endonucleases recognizing tetra- or hexa-nucleotide sequences and fragments generated were resolved by agarose gel electrophoresis. Specific DNA fingerprints were obtained after agarose gel electrophoresis (Fig. 1). While for example all tested species produced identical banding patterns after cleavage by *BanI* and *CfoI* restriction endonucleases, substantial variability was observed after the cleavage by *AccI* (four different profiles) or *AseI* endonuclease (five different profiles). The highest discriminatory power was observed for *MspI* and *BstUI* endonuclease (six profiles). The restriction fragment length polymorphism (RFLP) analysis of amplified 18S rDNA genes was found to clearly discriminate between all species studied, with DNA from each species giving unique sets of patterns. The fingerprints were recorded and species were grouped into similarity groups. The data obtained from DNA cleavage were then correlated with data obtained by computer-aided analysis of available 18S rDNA sequences from GenBank and a molecular key was designed. Identification starts by *AvaII* cleavage and by using six selected endonucleases any from 20 protozoal species be can unambiguously identified (Table 1).



**Fig. 1.** Schematic representation of ARDREA profiles of selected protozoal species obtained by *Ava*II (lane 1), *Ase*I (lane 2) and *Msp*I (lane 3) restriction endonucleases.



**Figs 2A-C.** Geographic and morphological invariability of ARDREA profiles of *Ophryoscolex caudatus* f. *tricoronatus* strains isolated from Slovak sheep - lane 1, Slovak goat - lane 2 and Polish sheep - lane 3 obtained by *Bst*UI (part **A**) and *Msp*I (part **B**) restriction endonuclease. Part **C** - ARDREA profiles of *Entodinium caudatum* type strains (lanes 1-3) were compared to those of f. *dubardii* (lanes 4-7) obtained by *Bst*UI restriction endonuclease. Lane M - 100 bp DNA ladder (Gibco BRL, California USA), standard of molecular weight. The arrow indicates marker band of 600 bp.

No differences were observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species. Analysis of animal-to-animal variability did not

reveal any variability in *Ophr. caudatus* f. *tricoronatus* (Figs 2A, B). No differences were found in morphologically different *Dipl. dentatum* strains (data not shown) and similarly no differences were found in several

**Table 1.** Key for RFLP analysis of rumen protozoa.

1. Digestion with <i>AvaII</i> :		
a) (fragments 1080, 550)		2
b) (fragments 1080, 460, 90)		10
2. Digestion with <i>NruI</i>		
a) (no cleavage, fragment 1650)	<i>Entodinium</i> sp.	3
b) (fragments 1100, 450)		7
3. Digestion with <i>MspI</i>		
a) (fragments 1530, 100)		4
b) (fragments 920, 610, 100)		5
4. Digestion with <i>AccI</i>		
a) (fragments 910, 620)	<i>Ent. nannellum</i>	
b) (no cleavage, fragment 1650)	<i>Ent. bursa</i>	
5. Digestion with <i>EcoRV</i>		
a) (fragments 910, 620)	<i>Ent. caudatum</i>	
b) (no cleavage, fragment 1650)		6
6. Digestion with <i>AccI</i>		
a) (fragments 910, 620)	<i>Ent. simplex</i>	
b) (no cleavage, fragment 1650)	<i>Ent. furca monolobum</i>	
7. Digestion with <i>AccI</i>		
a) (fragments 750, 600, 300)	<i>Ophryoscolex</i> sp.	8
b) (no cleavage, fragment 1650)	<i>Epidinium</i> sp.	9
8. Digestion with <i>MspI</i>		
a) (fragments 920, 610, 100)	<i>Ophr. purkynjei</i>	
b) (fragments 610, 480, 400, 100)	<i>Ophr. caudatum</i>	
9. Digestion with <i>MspI</i>		
a) (fragments 1330, 200, 100)	<i>Epid. caudatum</i>	
b) (fragments 920, 610, 100)	<i>Epid. ecaudatum</i>	
10. Digestion with <i>StyI</i>		
a) (fragments 800, 530, 300)		11
b) (fragments 800, 530, 150)		12
11. Digestion with <i>AccI</i>		
a) (fragments 750, 600, 300)	<i>Enoploplastron trilorlicatum</i>	
b) (no cleavage, fragment 1650)	<i>Isotricha intestinalis</i>	
12. Digestion with <i>MspI</i>		
a) (fragments 1530, 100)		13
b) (more fragments)		16
13. Digestion with <i>NruI</i>		
a) (fragments 1100, 450)		14
b) (no cleavage, fragment 1650)		15
14. Digestion with <i>AccI</i>		
a) (fragments 1000, 650)	<i>Metadinium medium</i>	
b) (fragments 1350, 300)	<i>P. multivesiculatum</i>	
c) (no cleavage, fragment 1650)	<i>Diploplastron affine</i>	
15. Digestion with <i>AccI</i>		
a) (fragments 1350, 300)	<i>Dasytricha ruminantium</i>	
b) (no cleavage, fragment 1650)	<i>Isotricha prostoma</i>	
16. Digestion with <i>NruI</i>		
a) (fragments 1100, 450)		17
b) (no cleavage, fragment 1650)	<i>Ostracodinium dentatum</i>	
17. Digestion with <i>AseI</i>		
a) (fragments 840, 800)	<i>Eudiplodinium maggii</i>	
b) (no cleavage, fragment 1650)		18
18. Digestion with <i>AccI</i>		
a) (fragments 1350, 300)	<i>Ostracodinium gracile</i>	
b) (no cleavage, fragment 1650)	<i>Diplodinium dentatum</i>	

morphotypes of *Ent. caudatum* (Fig. 2C), indicating that morphological variability observed is not due to genetic heterogeneity.

## DISCUSSION

The rumen ciliates are the most abundant protozoa in the rumen and are involved in host metabolism and digestion of plant material. By classical morphological criteria more than 250 species of ciliates have been described which live in the rumen of various feral and domesticated ruminants (Williams and Coleman 1992). Identification of protozoa by these criteria is extremely tedious and requires extensive special knowledge and skills. Moreover, the validity of classical identification is questioned, since many of the "species" described exhibit a substantial morphological plasticity (Dehority 1994). Introduction of modern molecular methods based on DNA analysis and fingerprints, especially the methods targeted at ribosomal RNA operon provide exact insight into similarity studies of micro-organisms. Recent research using molecular characterisation has suggested that the protozoal diversity within the rumen is even greater than that first anticipated, but despite recent progress with molecular ecological studies (Karnati *et al.* 2003), the level of diversity present between individuals remains unclear.

While there are numerous examples of application of molecular methods for identification of bacteria (e.g. Blanc *et al.* 1997), only a few papers deal with identification of protozoa (Yang *et al.* 2002). The primary objective of the present work was to develop a rapid method for identification of predominant rumen protozoa. Restriction analysis of amplified ribosomal DNA method described here was found to be able to clearly discriminate between all strains studied. Use of the Chelex method enabled isolation of DNA from single protozoal cell thus reducing possible contamination by foreign DNA from feeds or fungi. On the basis of DNA fingerprints obtained a molecular key was designed. While all 18 restriction endonucleases provided some discrimination between strains, the key was optimized in order to minimize the number of cleavage steps and endonucleases used. While small *Entodinia* could be identified in four steps, up to six restriction endonucleases cleavage steps had to be used to unequivocally identify *Ostr. dentatum* and *Diplod. dentatum* species. RFLP patterns were found to be stable and reproducible.

In addition, to providing a simple method for discrimination of morphologically similar species, the RFLP technique also demonstrated that new morphologically different variants (e.g. Dehority 1994) are not necessarily new species. No differences were observed between identical protozoal species originating from different animals or geographic locations, or between morphological variants of the same species, indicating limited intra-species variability of studied protozoa. These data are in correlation with a previous report on very limited intra-species sequence variation among eight isolates of the rumen ciliate *I. prostoma* (Wright 1999).

In conclusion, the methodology and molecular key described here provides a rapid and convenient way for identification of rumen protozoa. Furthermore, it enables the examination of the diversity of rumen protozoa without requiring specialist knowledge regarding the morphological characteristics of the ciliates being studied. The technique may be performed in even a basic molecular laboratory and by a researcher unskilled in the identification of rumen protozoa by traditional means.

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