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MOLECULAR GENOTYPING OF RUMEN FUNGI BASED ON RFLP ANALYSIS

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A B S T R A C T

Eight polycentric strains of the genera *Orpinomyces* and five monocentric strains of the genera *Neocallimastix* were examined for the restriction fragment length polymorphisms. EcoRI-digested nuclear DNA of isolates of rumen fungi and DNA-DNA hybridization with probe RS2 revealed several distinct cleavage profiles. Results are ambiguous and interpretation is difficult with respect to the high genetic variability of rumen fungi isolates.

Key words: microbiology / anaerobic fungi / molecular genetics / RFLP / classification / rumen

MOLEKULARNA GENOTIPIZACIJA VAMPNIH GLIV NA OSNOVI ANALIZE RFLP

I Z V L E Č E K

Proučevali smo polimorfizme restrikcijskih profilov osmih policentričnih sevov rodu *Orpinomyces* in petih monocentričnih sevov rodu *Neocallimastix*. Na podlagi z EcoRI razrezane jedrne DNK in DNK : DNK hibridizacij s sondo RS2 smo odkrili več različnih restrikcijskih profilov izolatov vampnih gliv. Rezultati so dvoumni in interpretacija težka glede na visoko genetsko variabilnost glivnih izolatov iz vampa.

Ključne besede: mikrobiologija / anaerobne glive / molekularna genetika / RFLP / razvrščanje / vamp

INTRODUCTION

The anaerobic fungi represent a special group of microorganisms inhabiting digestive tract of herbivorous animal. The intensive study of rumen fungi have been induced by their capacity to produce highly effective fibre-digesting enzymes. Anaerobic fungi penetrate and preferentially colonize tissues traditionally regarded as resistant to degradation, such as sclerenchyma and vascular tissues.

Ultrastructure studies have shown the absence of mitochondria. In place of mitochondria rumen fungi possess hydrogenosomes capable of coupling the metabolism of hexose to cellular energy formation with the concomitant production of H 2. Unlike mitochondria hydrogenosomes do not contain DNA. No extrachromosomal DNA is present in rumen fungi. Genomic DNA of rumen fungi is unique with respect to the base composition. G+C content is very low as 15 – 20%. These data indicate that anaerobic fungi have the most A+T rich genomes of any organisms identified so far.

All rumen fungi have been assigned to the family of the Neocallimasticaceae of the class Chytridiomycetes. The phylogenetic relationship between anaerobic fungi and aerobic chytrids has been recently proved by sequence analysis of 18s rRNA. Up to now in the family Neocallimasticaceae six genera have been described: Neocallimastix, Piromyces, Caecomyces (monocentric fungi), and Orpinomyces, Anaeromyces, Ruminomyces (polycentric fungi). These genera can be partially characterized on the basis of thallus morphology and ultrastructure of zoospores. The main problem of species identification is an extensive morphological variation of isolates depending on environmental conditions. Molecular techniques offer new approach to systematics. In this study we attempted to determine the feasibility of using restriction fragment length polymorphism (RFLP) analysis to distinguish among isolates of anaerobic rumen fungi.

MATERIAL AND METHODS

Fungal Cultures and Growth Conditions

8 polycentric fungi of genus Orpinomyces and 5 monocentric fungi of genus Neocallimastix used in this study were determined by classical taxonomic methods. The origins of the cultures appear in Table 1. Strains Cx and RE1 were kindly provided by C. Orpin (Rowett Research Institute). Other strains of rumen fungi were isolated in our laboratory according to the method of Joblin (1981).

All rumen fungi were cultured anaerobically at 38^{o} C on medium M10 (Caldwell and Bryant, 1966) enriched with 20% (v/v) rumen fluid. Cellobiose (4g/l) was used as a carbon source. Mycelia were harvested after 72 h of cultivation.

Isolation and Purification of Fungal DNA

Nuclear DNA was isolated by method of Brownlee (1989). Mycelium was ground to a fine powder in liquid nitrogen and suspend in extraction buffer (2% Triton X-100, 1% SDS, 0.25M NaCl, 0.1M Tris-HCl, 0.1M EDTA). DNA was purified using phenol/chloroform (24:1) and precipitated by isopropanol.

DNA Digestion, Electrophoresis and Hybridization

1ug of genomic DNA was digested with EcoRI enzyme and fragments were separated on 0.7% agarose gel (Maniatis *et al.*,1982). Digested DNA was transferred from the gel to nylon membrane and non-radioactively hybridized at 60 ^{o} C with RS2 repetitive element .

RESULTS AND DISCUSSION

Restriction fragment length polymorphism was applied to eight polycentric and five monocentric rumen fungi. Strains used in this study were isolated from different animals living in different territories (Tab.1) and identified using light microscopy. Nuclear DNAs from 13 fungal isolates were first characterized by digestion of total DNA with enzyme EcoRI. The EcoRI restriction revealed three different cleavage profiles (Fig.1a). Digestion of DNA of polycentric fungi generated two distinct patterns. Profile I for Orpinomyces fungi Zu2, LG2 and ALP created by bands 3.9, 3.4 and 1.7 kb differs from profile II for Orpinomyces fungi K1, Zu1, LG1 and DK15 (3.9, 2.5 and 1.7 kb) only in one band. Cleavage profile III for monocentric Neocallimastix strains created by fragments 1.8, 3.7 and 4.1 kb has no common band with

polycentric strains. Exclamation marks in Fig.1a indicate two monocentric (BV2 and PJ1/8) and one polycentric (A4) rumen fungi with distinctly different restriction fragment patterns.

Table 1. List of studied strains of rumen fungi

Isolate	Source	Origin	Animal	Genus
A4	Rumen	Kazachstan	Camel	*Orpinomyces*
K1	Rumen	CZ	Cow	*Orpinomyces*
Zu1	Faeces	CZ	Bison	*Orpinomyces*
Zu2	Faeces	CZ	Bison	*Orpinomyces*
LG1	Faeces	CZ	Llama	*Orpinomyces*
LG2	Faeces	CZ	Llama	*Orpinomyces*
ALP	Faeces	CZ	Llama	*Orpinomyces*
DK15	Faeces	CZ	-	*Orpinomyces*
Cx	Rumen	Scotland	-	*Neocallimastix*
RE1	Rumen	Scotland	-	*Neocallimastix*
PJ1/8	Faeces	CZ		*Neocallimastix*
BV2	Faeces	CZ	Buffalo	*Neocalimastix*
DK5	Faeces	CZ	-	*Neocallimastix*

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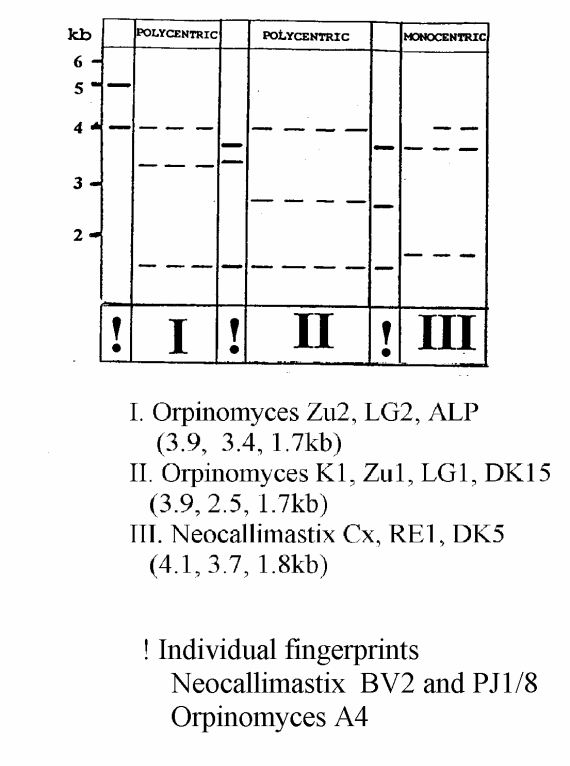

Figure 1a. EcoRI digestion of nuclear DNAs

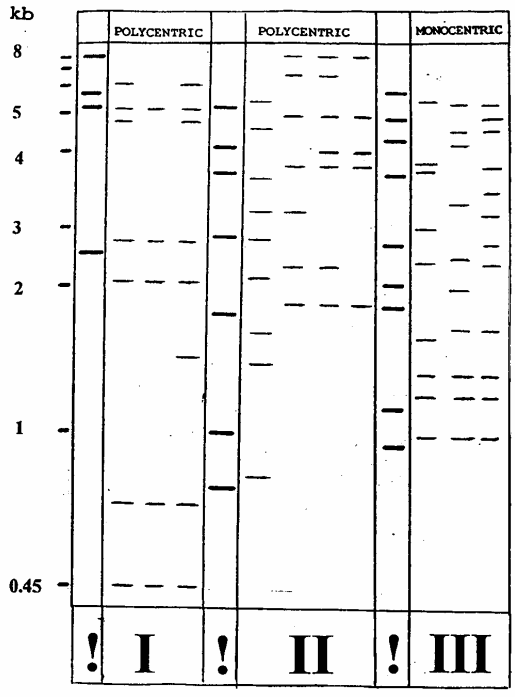

Figure 1b. Hybridization with probe RS2

EcoRI digested DNA samples of 13 rumen fungi were further characterized by hybridization with repetitive element RS2 (Fig.1b). This DNA-DNA hybridization experiment proved high similarity among isolates in group I and group II (with exception of strain DK15 that exhibits

different fingerprint). The isolates of group III were less homogenous and strains BV2, PJ1/8 and A4 exhibited again the individual hybridization patterns.

Experiments based on the restriction fragment length polymorphism revealed extensive genetic variability. Our results are ambiguous. Their interpretation is complicated by three different cleavage profiles for Orpinomyces strains (profile I, profile II and strain A4). The study have not found RFLP analysis useful for distinguishing among genera or species of rumen fungi. However, if we admit the possibility that there are more than six genera of rumen fungi, RFPL analysis of large amount of different strains could bring some improvement to the classification of anaerobic rumen fungi.

So far no methods relying on analysis of DNA have been successfully used to establish new taxonomic classification of rumen fungi. The most reliable method to detect genetic variation between fungal species is analysis of rDNA that contains highly conserved DNA sequences as well as more variable regions. Sequence analysis of ITS1 spacer (Brookman *et al*., 1997) seems a promising tool for comparing a variety of rumen fungal isolates.

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