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Ribosomal DNA sequences reveal gregarine pathogens (Apicomplexa: Gregarinia) in mites and other arachnids (Arachnida)

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Abstract

Ribosomal RNA genes are widely applied in phylogenetic studies due to the ubiquitous presence and relative conservation of many regions of their nucleotide sequences. Many specific PCR primers have been developed for amplification of the rDNA cluster fragments from particular taxa. However, the use of universal primers hybridising to the conserved rDNA regions enables discovering sequences of eukaryote endosymbiont or pathogen origin in the analysed DNA sample. This approach makes it possible to detect gregarines (Protozoa: Apicomplexa) that are obligate parasites of digestive tracts and body cavities in invertebrate animals. Here we report new uncultured gregarine clones detected by rDNA sequencing in one harvestmen, *Rilaena triangularis* (Opiliones: Phalangioidea), and two astigmatid feather mite species, *Proctophyllodes stylifer* and *P. ateri* (Acari: Analgoidea). To our knowledge, this is the first record of parasitic Astigmata (cohort Psoroptidia) infected with gregarines. Comparison of the amplified rDNA fragments with sequences deposited in GenBank revealed that analysed sequences corresponded to 18S rDNA from the genera *Gregarina* (85 and 90 % identity with *G. chortiocetes* for sequences found in *P. ateri* and harvestman, and *Syncystis* (87 % identity with *S. mirabilis* for sequences found in *P. stylifer*). The differences observed between nucleotide sequences of the gregarine 18S rDNA fragments indicated that the gregarine isolates from which the sequences were derived belonged to presumably three different species.

Keywords: rDNA, endoparasite, *Rilaena triangularis*, *Proctophyllodes ateri*, *P. stylifer*

Zusammenfassung

Kodierende DNA-Sequenzen der ribosomalen RNA-Gene werden in phylogenetischen Studien wegen ihres ubiquitären Vorkommens und zahlreicher relativ konservativer Regionen in ihren Nukleotid-Sequenzen regelmäßig angewandt. Es sind viele spezifische PCR-Primer zur Amplifikaktion einer Reihe von rRNA-Fragmenten aus verschiedenen Taxa entwickelt worden. Die Anwendung der universalen Primer, die mit konservativen Regionen der rDNA in eukaryotischer Kern-DNA hybridisieren, ermöglicht jedoch auch die Aufdeckung zusätzlicher Sequenzen der eukaryotischen Endosymbionten oder Pathogene, die aus den analysierten DNA Proben stammen.

Die hier beschriebene Methode erlaubt den Nachweis der Gregarinen (Protozoa: Apicomplexa), die obligate Endoparasiten des Darms und der Körperhöhlen wirbelloser Tiere sind. Wir stellen hier neue, durch rDNA-Sequenzierung entdeckte und bisher nicht kultivierte Klone von Gregarinen vor: eine im Weberknecht *Rilaena triangularis* (Opiliones: Phalangioidea) und zwei in den Federmilben *Proctophyllodes stylifer* und *P. ateri* (Acari: Astigmata, Analgoidea). Nach dem vorliegenden Wissen ist dies der erste Nachweis parasitischer Astigmata (Kohorte Psoroptidia), die mit Gregarinen infiziert waren. Der Vergleich der amplifizierten rDNA-Fragmente mit in GenBank deponierten Sequenzen hat gezeigt, dass die analysierten Sequenzen mit der 18S rDNA aus den Gattungen *Gregarina* (85 Identität mit *G. chortiocetes* für Sequenzen aus *P. ateri* und 90 % aus dem Weberknecht) und *Syncistis* (87 % Identität mit *S. mirabilis* aus *P. stylifer*) übereinstimmen. Die Unterschiede zwischen den Nukleotid-Sequenzen dieser 18S rDNA-Fragmente zeigen, dass die analysierten Gregarinen-Isolate zu wenigstens drei verschiedenen Arten gehören

1. Introduction

The gene coding for the small subunit of ribosomal RNA (SSU RNA) is the most intensively sequenced marker for phylogenetic studies in all groups of organisms, including mites. Newly obtained sequence data can be quickly and easily compared with all published sequences of this marker deposited in GenBank (NCBI) database. Numerous specific primers for PCR amplification of 18S rRNA gene from particular taxa have been developed (see: Ribosomal DNA Primer Database, http://bioinformatics.psb.ugent.be/webtools/rRNA /primers). On the other hand, the use of universal primers that can amplify fragments of rDNA sequences of any eukaryote species in polymerase chain reaction (PCR) makes it possible not only to amplify rDNA from the studied specimens, but also from pathogens infecting them. Unfortunately, heterogenic copies of amplified 18S rRNA gene fragments have to be cloned in plasmids and then individually sequenced (Valles & Pereira 2003), which makes the studies more laborious and expensive. We propose to overcome this problem by amplifying a fragment of rDNA comprising a part of 18S rRNA gene, both internal transcribed spacers (ITS1 and ITS2), and 5.8S rRNA. Because ITS sequences evolve much more rapidly than rRNA genes and accumulate more insertion-deletion (indel) mutations, PCR amplification of this rDNA region from different species produces amplicons that differ in length. These amplicons could be easily separated by agarose gel electrophoresis and directly sequenced. A sequence of the part of 18S rRNA gene could be compared with sequences deposited in GenBank for species identification or taxon delineation.

Gregarines (Alveolata: Apicomplexa) are obligatory protozoan parasites of invertebrate animals, mainly of terrestrial arthropods. Most species of gregarines are found in the host gut, although a transition from intestinal parasitism to infecting the body cavity and internal organs is observed in some families (Simdyanov & Kuvardina 2006). Numerous cases of gregarine occurrence have been reported in Opiliones (Cokendolpher 1993). However, there are only a few cases of protozoan infections reported from mites, mainly from oribatids (Poinar & Poinar 1988, van der Geest et al. 2000) and water mites (Issi & Lipa 1968).

Here we describe a simple and rapid method for the detection and identification of eukaryotic pathogens in arachnids. Three new uncultured gregarine clones have been discovered by this approach: one in harvestmen, *Rilaena triangularis* (Opiliones: Phalangioidea), and two in astigmatid feather mite species, *Proctophyllodes stylifer* and *P. ateri* (Acari: Analgoidea).

2. Materials and methods

2.1. Animal material

Harvestmen material of *Rilaena triangularis* (Opiliones, Phalangidae) was sampled in the city forest Las Marceliński in Poznań, Poland, 5 June 2005, coll. J. Dabert, voucher UAMRil-01. Both *Proctophyllodes* species were collected during trapping of autumn migratory birds in Kopań near Darłowo, Poland, 20 October 2002, coll. P. Solarczyk. Mite material of *Proctophyllodes ateri* (Acari, Proctophyllodidae) and *P. stylifer* was extracted from feathers of *Parus ater* (Passeriformes; Paridae) and *Parus caeruleus.* Samples were preserved in 96 % ethyl alcohol and stored at 4° C until DNA extraction.

2.2. DNA extraction, amplification, and sequencing

Whole genomic DNA was extracted using DNAeasy Tissue Kit (Qiagen) following a standard manufacturer's protocol. Total genomic DNA was extracted from 15 whole specimens of feather mites, and a fragment of the harvestman's leg. Amplified rDNA fragments encompassed the 3'-end of the 18S rRNA gene starting from an universal helix 36, internal transcribed spacers ITS1 and ITS2, the gene coding for the 5.8S rRNA, and a part of the 28S rRNA. The 18S rRNA helix numbering system is after Wuyts et al. (2000). For PCR amplification the following primers were used: developed in this study forward primer f1300 [5'-TGCATGGCCGTTCTTAGTTG-3'] and reverse primer ITS2-28S [5'-ATATGCTTAAATTCAGGGGG-3'] (Navajas et al. 1998). Amplification was performed on a MiniCycler MJ Research using a program: initial step with 3 min at 96° C followed by 35 steps with 10 sec at 95 °C, 10 sec at 50 °C and 2 min at 72 °C, and with final elongation 5 min at 72 °C. Reaction mix contained 5 µl of DNA template, 0.1 mM dNTP (Fermentas), 1.5 mM $MgCl_2$, 1x reaction buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08 % Nonidet P40), 0.25 µM each primer and 1.25 U Taq polymerase HiFi (Novazym) in a total volume of 25 µl. Amplification products were separated by electrophoresis on 1.5 % agarose gel, purified using QIAquick Gel Extractinion Kit (Qiagen), and sequenced on genetic analyser ABI Prism 3130XL (Applied Biosystems) using the BigDye Terminator v3.1 chemistry of the same manufacturer. Contigs were aligned and manually assembled in ChromasPro v. 1.32 (Technelysium Pty Ltd.) and GeneDoc v. 2.7.000 (Nicholas & Nicholas 1997). A BLASTn (Altschul et al. 1997) search was used to infer the taxonomic affiliation of these sequences. A 198-bp fragment, encompassing the V7 and V8 areas of 18S rRNA gene, was chosen for comparison of the gregarine-like sequences with corresponding sequences from the GenBank database. For phylogenetic analysis we use 38 sequences in total, including: 3 gregarine-like sequences from this study, 26 sequences designated as Gregarinia, and 4 uncultured gregarine clones isolated from arthropods (an ingroup), 4 other apicomplexan species (a close outgroup), and a representative of alveolate Dinophyceae (a distant Alveolate outgroup). Sequence alignment is available from the authors on request. The phylogenetic tree was reconstructed by Maximum Likelihood method implemented in on-line version of PhyML software (Guindon et al. 2005). Appropriate model of sequence evolution (GTR + G = 0.930 + I = 0.265) was selected by software Modeltest 3.7 (Posada & Crandall 1998, Posada & Buckley 2004). Tree visualisation was prepared by tree editing tools in MEGA 4 software (Tamura et al. 2007). DNA sequences were deposited in GenBank database under Accession Nos. EU156377–EU156379.

3. Results

Amplification of the rDNA fragment with primers f1300 and ITS2-28S from the genomic DNA isolated from *Rilaena triangularis* revealed two PCR products that differed significantly in length (Fig. 1). Comparison of the nucleotide sequence of resulted amplicons with sequences deposited in GenBank showed that the 1.9 kb fragment was most similar to the *Phalangium opilio* 18S ribosomal RNA gene (Acc. AF124937) revealing 449/450 (99 %) identity. The sequence of the 1 kb product (Acc. EU156377) was most similar to the *Gregarina chortiocetes* 18S ribosomal RNA gene [Acc. L31841; 426/473 (90 %) identity].

Using the same primer pair for amplification of rDNA from feather mites of the genus *Proctophyllodes* (Acari: Analgoidea) two other 18S rDNA sequences were discovered that belonged to protozoan of the phylum Apicomplexa (Cavalier-Smith 1993). An additional fragment of 18S rRNA gene (UAMPs05-2, Acc. EU156378) that was amplified from total genomic DNA isolated from *P. ateri* showed similarity to undefined uncultured eukaryote clone LEMD003, [AF372797; 148/167 (88 %) identity]. However, from identified organisms, the sequence UAMPs05-2 was homologous to gregarine 18S rDNA sequences: *G. caledia* [L31799; 145/169 (85 %) identity] and *G. chortiocetes* [144/168 (85 %) identity]. Furthermore, the sequence UAMPs04-2 found in *P. stylifer* (Acc. EU156379) corresponded to that reported for *Syncystis mirabilis* (Apicomplexa: Gregarinia) [DQ176427; 302/345 (87 %) identity].

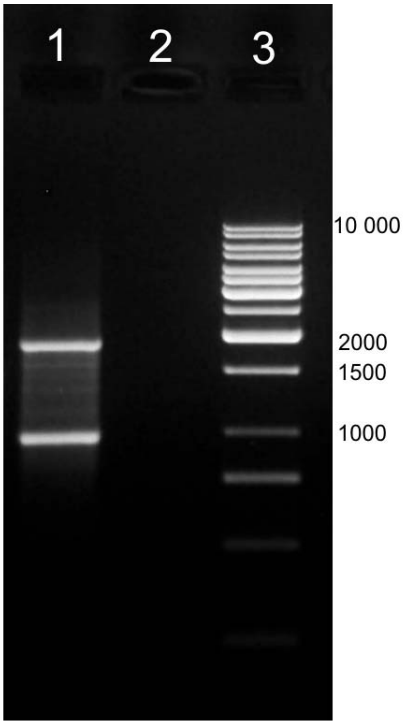

Fig. 1 Results of PCR amplification of the rDNA fragment with primers f1300 and ITS2-28S using DNA isolated from *Rilaena triangularis* as a template. Lanes: 1: two PCR amplification products, the upper band represents the harvestman, and the lower one a protozoan parasite; 2: PCR blank; 3: 1-kb ladder (Fermentas). Numbers on side indicate sizes (base pairs) of marker bands.

Sequence comparison of the gregarine-like 18S rRNA gene fragments isolated from harvestman and two feather mite species showed that they differed clearly (Fig. 2). Among 198-bp, 69 sites were variable and only four of which were indels. Most substitutions were observed in the variable areas V7 and V8, however the isolate UAMPs04-2 from *P. stylifer* differed from two others in conserved helices 34, 36, and 38. Sequence identity in the compared 18S rRNA gene fragment ranged from 68 to 76 %.

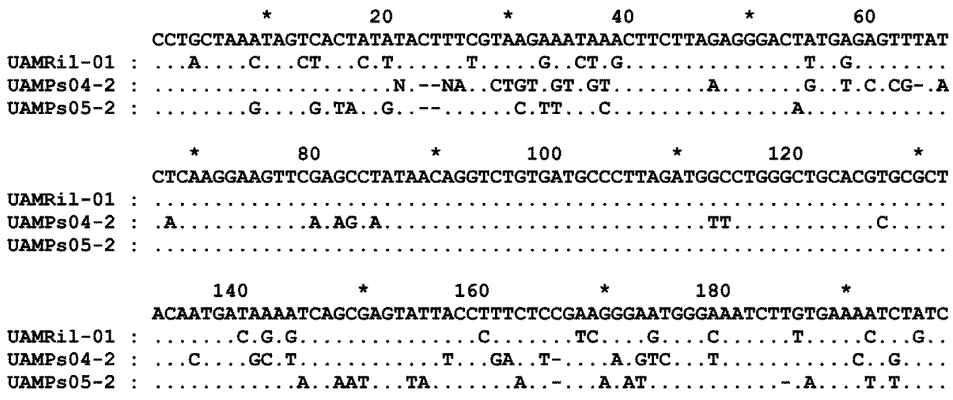

Fig. 2 Sequence alignment of the gregarine-like 18S rRNA gene fragments isolated from *Rilaena triangularis* (UAMRil-01), *Proctophyllodes stylifer* (UAMPs04-2), and *P. ateri* (UAMPs05 2).

To reveal the phylogenetic position of the isolated gregarine-like sequences, corresponding 18S rDNA sequences that have been annotated to Gregarinia in the GenBank database were used. The ML analysis proved that all three isolates are deeply nested in frames of two clades in Gregarinia tree (Fig. 3). Isolates UAMPs05-2 and UAMRil-01 are closely related and belong most probably to *Gregarina* the genus. The third isolate UAMPs04-2 is situated basal to *Synsystis-Monocystis* clade in distant part of the tree.

4. Discussion

This work describes a simple and rapid method for the detection and identification of eukaryotic pathogens or endosymbiots in arachnids. This simple assay could be added to the standard procedures commonly used to isolate rDNA sequences for molecular phylogenetic studies. An 18S rRNA gene-based PCR test employs conserved primers useful for simultaneous amplification of nuclear rDNA fragments from both host and pathogen genomes. Infested hosts result in amplification of PCR products having distinct sizes, which is mainly caused by variability in the length of the ITS sequences. The amplicons can be easily separated by size from each other by simple agarose gel electrophoresis and directly

sequenced. Unlike other PCR-based methods for the detection of pathogens, which depend on taxon-specific primers (Sparagano et al. 1999, Valles & Pereira 2003, Adelson et al. 2004) the proposed approach is able to detect taxa for which no sequence data are available.

There is very little information available concerning the eukaryotic pathogens or endosymbiots of Arachnida, especially Acari. This is, to our knowledge, the second report of Astigmata infected with gregarines (Loo et al. 2004) and the first record for parasitic mites of cohort Psoroptidia. Taking this into consideration, the proposed method seems to be a promising approach to study protozoan diversity by providing a rapid and easy screening of host organisms for which traditional microscopic techniques could be ineffective.

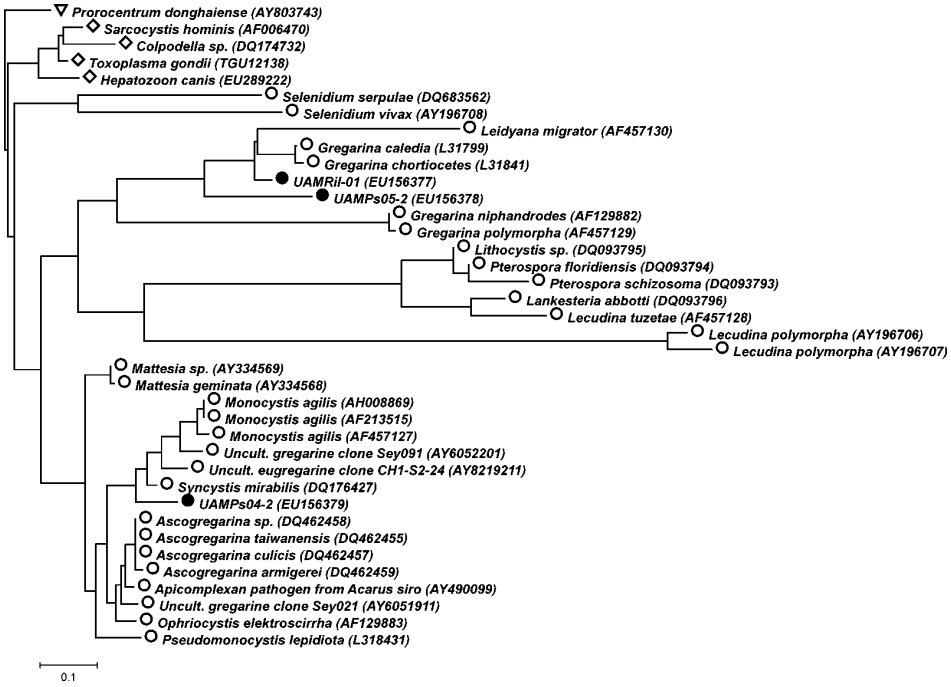

Fig. 3 Phylogenetic position of the isolated gregarine-like sequences. Maximum likelihood tree inferred from nucleotide sequences of 18S rRNA gene fragments of the isolate from harvestman (UAMRil01), two isolates from feather mites (UAMPs04-2, UAMPs05-2) (black circle), and corresponding sequences from the GenBank database annotated to Gregarinia (circle). Representatives of other Apicomplexa (diamond) and alveolate Dinophyceae (triangle) were used as outgroups. GenBank accession numbers are in brackets.

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