J. Eukaryot. Microbiol., 54(1), 2007 pp. 83–85

DOI: 10.1111/j.1550-7408.2006.00149.x

Reverse Arrangement of rRNA Subunits in the Microsporidium *Glugoides intestinalis*

DOMINIK REFARDT[1] and LAURENCE MOUTON

Zoologisches Institut der Universität Basel, Evolutionsbiologie, Vesalgasse 1, CH-4051 Basel, Switzerland

ABSTRACT. The rRNA gene cluster of microsporidia is typically arranged in the order small subunit-internal transcribed spacer-large subunit, which conforms with the general arrangement of these genes in nearly all organisms. We found a rearrangement of the cluster in the microsporidium *Glugoides intestinalis*, where the large subunit precedes the small subunit. Such a rearrangement has already been reported for several species in the microsporidian genus *Nosema*, and we provide evidence that the arrangement reported here is a second, independent event.

MICROSPORIDIA are highly specialized fungi and obligate intracellular parasites of eukaryotes. Possibly due to their parasitic life-style, they have a highly compacted genome, which has a remarkably conserved organization, despite a high rate of evolution (Katinka et al. 2001; Slamovits et al. 2004). Compaction has also affected the rRNA gene cluster, which, in microsporidia, typically consists of the small subunit (SSU), an internal transcribed spacer (ITS), and the two parts of the large subunit (5.8S and 28S) fused together.

Recently, a rearranged rRNA gene cluster has been found in the microsporidium *Nosema bombycis*: the large subunit (LSU) precedes the SSU (Huang et al. 2004). Subsequently, the same arrangement was found in *N. spodopterae* (Tsai, Huang, and Wang 2005) and *N. antheraeae* (Wang et al. 2006). The genus *Nosema* is currently an assemblage of different unrelated genera, and it has been suggested by Tsai et al. (2005) that this reverse arrangement may be used as a diagnostic feature for the ''true'' *Nosema* species (sensu Baker et al. 1994).

The microsporidium *Glugoides intestinalis* is only distantly related to the genus *Nosema* (Larsson et al. 1996; Refardt et al. 2002). Attempts to amplify a presumed ITS 3′ of the SSU using primers in both SSU and LSU have failed and it has been noted that the non-coding DNA 3′ of the SSU is unusually long for an ITS (Refardt et al. 2002). Upon the publication by Huang et al. (2004), we suspected that a similar arrangement might be present in *G. intestinalis*, which proved to be the case. We provide evidence that this rearrangement happened independently of the rearrangement in the *Nosema* species.

MATERIALS AND METHODS

Glugoides intestinalis infects gut epithelial cells of the small freshwater crustacean *Daphnia magna* (Larsson et al. 1996). Infected hosts were found at two localities: host clone DK-35-9 came from Munich, Germany and harbours *G. intestinalis* isolate Gi1. Its SSU sequence has already been published together with 753 bp of non-coding DNA 3′ of the SSU (Refardt et al. 2002; GenBank Accession no. AF394525); and host clones CN-2-1 and CN-2-2 came from the Nesyt Pond, southeastern Czech Republic, and harbour parasite isolates Gi2 and Gi3, respectively.

Total DNA from single infected hosts was extracted as described in Refardt and Ebert (2006). DNA amplification was performed in a 20-μl reaction vol. containing 1 μl DNA extract, 0.5 U HotStarTaq DNA polymerase (Qiagen, Hildesheim, Germany), 1 × PCR buffer, 200 μM dNTPs, and 0.5 μM of the primers LSU1fwd (5′-CCCGTCTTGAAACACGGACCAAG GAG-3′) and SSUrev (5′-GGCTCCTCTATTCCTATCCA-3′) (Fig. 1). The PCR profile included an initial denaturation step (95 °C, 15 min) followed by 40 amplification cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 4 min). Amplified products were visualized on a 1.3% agarose gel with ethidium bromide staining. A single band with a length of ~ 2.8 kb was isolated (GenElute gel extraction kit, Sigma, Buchs, Switzerland) and cloned (TOPO TA cloning kit, Invitrogen, Groningen, the Netherlands). Colonies were selected by blue/white-screening, plasmids were purified (GenElute plasmid miniprep kit, Sigma), and digested with *Eco*RI to check whether they contained the desired insert. Sequencing was done on both strands by automated means by Microsynth (Balgach, Switzerland). The internal primers LSU2-fwd (5′-AGAAGGAGGTGATACGAGCC-3′), LSU3fwd (5′-GGTAAGCTTCGAGGGGAAGG-3′), SSUfwd (5′-CAGCAGG TTGATTCTGCCTG-3′), LSU1rev (5′-ACAAAGGATGAACC CGTCTC-3′), LSU2rev (5′-TCTATCTCACTAGAGTCAAGC-3′), and LSU3rev (5′-TGATACTGCCTCAAATCCTCGC-3′) were used for complete sequencing (Fig. 1). Sequences of the three isolates were published in GenBank (Accession nos. DQ680156, DQ680157, DQ680158).

RESULTS AND DISCUSSION

The ribosomal gene cluster of the three isolates of *G. intestinalis* analyzed included 2,776 bp of the LSU, a spacer region, and the SSU. At the 3′-end there is a 424-bp overlap with an already published sequence of isolate Gi1, to which our sequences are completely identical. Taken together, the LSU has been partially sequenced (2,048 bp) and has a G/C content of 46%; the spacer sequence has a length of 235 bp and a G/C content of 36%; the SSU has a length of 1,260 bp and a G/C content of 46%; and the following non-coding DNA has a length of 753 bp and a G/C content of 38% (Fig. 1).

The typical gene order for microsporidia is SSU–ITS–LSU and conforms with the general arrangement of the rRNA operon where the SSU precedes the LSU from the 5′ to 3′ direction. The rRNA genes of *G. intestinalis* have been rearranged in the order LSU-spacer-SSU (Fig. 1). It remains to be shown whether the spacer region is transcribed.

A similar rearrangement of the rRNA genes has occurred in several *Nosema* species that belong to the so called ''true'' *Nosema* (Baker et al. 1994; Huang et al. 2004; Tsai et al. 2005; Wang et al. 2006). The question therefore arises, whether these rearrangements are two independent events or whether they share a common ancestry. A subtree of the phylum microsporidia taken

Corresponding Author: D. Refardt, School of Biological Sciences, University of Auckland, 3A Symonds Street, Auckland, New Zealand—Telephone number: +64-9-3737599; FAX number: +64-9-3737416; e-mail: dominik.refardt@gmail.com

[1]Present address: Evolutionary Genetics and Microbial Ecology Laboratory, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand.

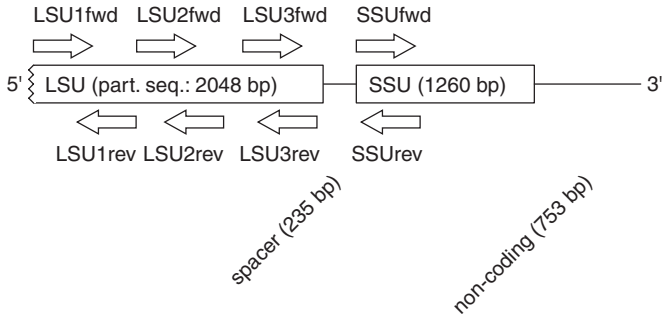

Fig. **1.** Schematic diagram of the rRNA gene cluster of the microsporidium *Glugoides intestinalis*. Gene domains are boxed. Details of the primers are given in the text.

from a phylogenetic analysis by Vossbrinck and Debrunner-Vossbrinck (2005) shows the microsporidia with rearranged rRNA genes highlighted and those set in bold for which the normal arrangement has been confirmed by sequencing (i.e. sequences in GenBank contained at least parts of both 16S and 23S; the normal arrangement of the rRNA gene cluster of *Endoreticulatus* has been kindly confirmed by Huang, W. F., pers. commun.) (Fig. 2). The tree topology suggests further that the rearrangement is confined to *G. intestinalis*, as it occurs neither in the genus *Endoreticulatus*, to which *G. intestinalis* is basal, nor in the genera *Nucleospora* and *Enterocytozoon*, both basal to *Glugoides* (Fig. 2). It remains to be shown whether the rearrangement in *Nosema* is exhibited by all ''true'' *Nosema* species or whether it occurred only in a subgroup of this clade.

Tandemly arranged rRNA operons occur in many eukaryotes and have been reported from the microsporidium *N. apis* (Gatehouse and Malone 1998). Therefore the question may arise whether two adjacent repeats of subunits were sequenced and a rearrangement was erroneously inferred. If this were true, the noncoding region 3′ of the SSU would be the real ITS followed by the LSU. However, several attempts to amplify the region 3′ of the SSU using conserved primers situated in the SSU and LSU failed (data not shown). These primers have been used successfully with a broad range of species in the phylum microsporidia (Canning et al. 2002; Refardt et al. 2002). Furthermore, the 235-bp length of the spacer region between LSU and SSU in *G. intestinalis* is only slightly longer than those reported for the three *Nosema* species in which the rearrangement has also been observed (*N. antheraeae*: 192 bp; *N. bombycis*: 179 bp; *N. spodopterae*: 185 bp). In contrast, the length of the repeat unit in *N. apis* has a length of approximately 18 kb.

The fact that this rearrangement occurred twice in the phylum Microsporidia is intriguing. Unusual arrangements for rRNA genes have been reported in plastid genomes of several protists (de Koning and Keeling 2006 and references therein). Yet, these rearrangements differ in that they either caused a separation of the two subunits or the subunits are still adjacent but the rRNAs are encoded on opposite strands of the DNA.

An analysis of ribosomal DNA regions in an isolate of *N. bombycis* revealed several copies of the 16S subunit, some of which were fragmented, yet still transcribed (Iiyama et al. 2004). A transposon-like element was found upstream of one of these copies, suggesting that mobile genetic elements may play a role in the duplication of rRNA genes. Indeed there is evidence that long-terminal repeat retrotransposon elements are associated with genome size variation and syntenic discontinuities in *N. bombycis* (Xu et al. 2006). However, it remains to be explained why those

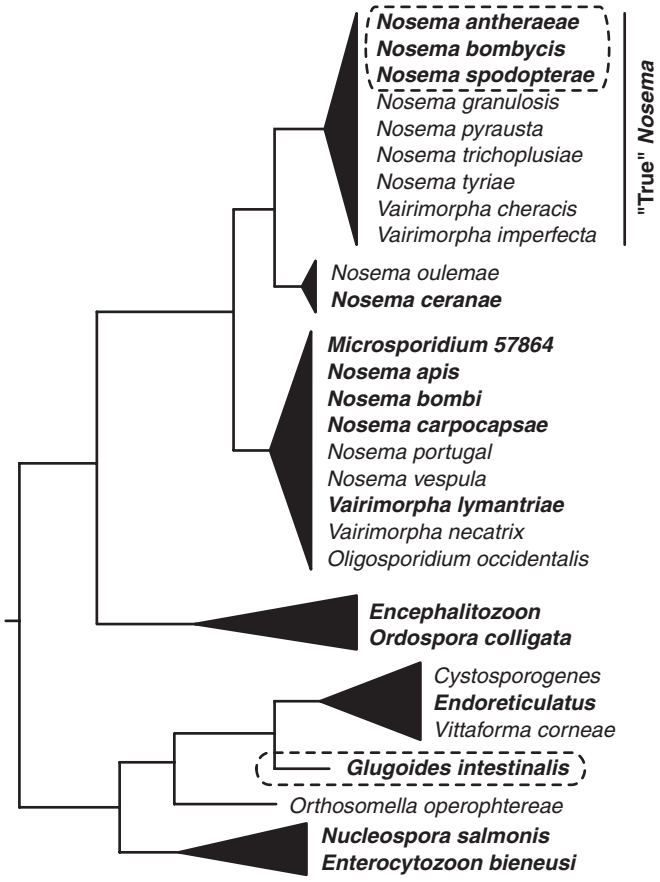

Fig. **2.** Small subunit rRNA phylogeny showing the relationship of *Glugoides intestinalis* to those *Nosema* species in which a rearrangement of the rRNA subunits has been documented (encircled species names). The tree is redrawn from Vossbrinck and Debrunner-Vossbrinck (2005) and has been simplified by collapsing certain clades. Genus and species names set in bold indicate sequences where the canonical arrangement of the rRNA genes has been verified by sequencing (see text for details).

rearranged rRNA clusters were functional and fixed twice in a microsporidian species.

We suggest the following: first, compaction of the rRNA in microsporidia may have caused the loss of processing sites, which then facilitated the processibility of a rearranged cluster. Second, if clusters are tandemly repeated, these repeats may now allow the transcription of an additional overlapping functional unit whose LSU stems from the first and whose SSU stems from the second repeat. If this leads to an overall increase in the rate of ribosome production, it may be favoured by natural selection as an alternative to repeat duplication (Weider et al. 2005). The subsequent loss of flanking units would then leave the organism with a rearranged rRNA. There is currently an increasing interest in the evolution of microsporidian genomes and insights gained from these studies may help to elucidate the plausibility of this scenario and shed light on the evolutionary origin of rearranged rRNA in microsporidia.

ACKNOWLEDGMENTS

We thank D. Ebert for supporting this research under a grant from the Swiss National Science Foundation. We also thank S. Lass and A. Petrusek for the provision of parasite isolates.

LITERATURE CITED

Baker, M. D., Vossbrinck, C. R., Maddox, J. V. & Undeen, A. H. 1994. Phylogenetic relationships among *Vairimorpha* and *Nosema* species (Microspora) based on ribosomal RNA sequence data. *J. Invertebr. Pathol.*, **64**:100–106.

Canning, E. U., Refardt, D., Vossbrinck, C. R., Okamura, B. & Curry, A. 2002. New diplokaryotic microsporidia (Phylum Microsporidia) from freshwater bryozoans (Bryozoa, Phylactolaemata). *Eur. J. Protistol.*, **38**:247–265.

Gatehouse, H. S. & Malone, L. A. 1998. The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. *J. Invertebr. Pathol.*, **71**:97–105.

Huang, W. F., Tsai, S. J., Lo, C. F., Soichi, Y. & Wang, C. H. 2004. The novel organization and complete sequence of the ribosomal RNA gene of *Nosema bombycis*. *Fung. Genet. Biol.*, **41**:473–481.

Iiyama, K., Chieda, Y., Yasunaga-Aoki, C., Hayasaka, S. & Shimizu, S. 2004. Analyses of the ribosomal DNA region in *Nosema bombycis* NIS 001. *J. Eukaryot. Microbiol.*, **51**:598–604.

Katinka, M. D., Duprat, S., Cornillot, E., Metenier, G., Thomarat, F., Prensier, G., Barbe, V., Peyretaillade, E., Brottier, P., Wincker, P., Delbac, F., El Alaoui, H., Peyret, P., Saurin, W., Gouy, M., Weissenbach, J. & Vivares, C. P. 2001. Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. *Nature*, **414**: 450–453.

de Koning, A. P. & Keeling, P. J. 2006. The complete plastid genome sequence of the parasitic green alga *Helicosporidium* sp. is highly reduced and structured. *BMC Biol.*, **4**:12.

Larsson, J. I. R., Ebert, D., Vávra, J. & Voronin, V. N. 1996. Redescription of *Pleistophora intestinalis* Chatton, 1907, a microsporidian parasite of *Daphnia magna* and *Daphnia pulex*, with establisment of the new genus *Glugoides* (Microspora, Glugeidae). *Eur. J. Protistol.*, **32**: 251–261.

Refardt, D. & Ebert, D. 2006. Quantitative PCR to detect, discriminate and quantify intracellular parasites in their host: an example from three microsporidians in *Daphnia*. *Parasitology*, **133**:11–18.

Refardt, D., Canning, E. U., Mathis, A., Cheney, S. A., Lafranchi-Tristem, N. J. & Ebert, D. 2002. Small subunit ribosomal DNA phylogeny of microsporidia that infect *Daphnia* (Crustacea: Cladocera). *Parasitology*, **124**:381–389.

Slamovits, C. H., Fast, N. M., Law, S. J. & Keeling, P. J. 2004. Genome compaction and stability in microsporidian intracellular parasites. *Curr. Biol.*, **14**:891–896.

Tsai, S. J., Huang, W. & Wang, C. H. 2005. Complete sequence and gene organization of the *Nosema spodopterae* rRNA gene. *J. Eukaryot. Microbiol.*, **52**:52–54.

Vossbrinck, C. R. & Debrunner-Vossbrinck, B. A. 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitol.*, **52**:131–142.

Wang, L. L., Chen, K. P., Zhang, Z., Yao, Q., Gao, G. T. & Zhao, Y. 2006. Phylogenetic analysis of *Nosema antheraeae* (Microsporidia) isolated from Chinese oak silkworm, *Antheraea pernyi*. *J. Eukaryot. Microbiol.*, **53**:310.

Weider, L. J., Elser, J. J., Crease, T. J., Mateos, M., Cotner, J. B. & Markow, T. A. 2005. The functional significance of ribosomal (r)DNA variation: impacts on the evolutionary ecology of organisms. *Annu. Rev. Ecol. Evol. Syst.*, **36**:219–242.

Xu, J., Pan, G., Fang, L., Li, J., Tian, X., Li, T., Zhou, Z. & Xiang, Z. 2006. The varying microsporidian genome: existence of long-terminal repeat retrotransposon in domesticated silkworm parasite *Nosema bombycis*. *Int. J. Parasitol.*, **36**:1049–1056.

Received: 06/09/06, 08/26/06; accepted: 08/27/06