

NOTE

Genomic Characterization of *Neoparamoeba pemaquidensis* (Amoebozoa) and Its Kinetoplastid Endosymbiont[∇]

Goro Tanifuji,¹† Eunsoo Kim,¹† Naoko T. Onodera,¹† Rebecca Gibeault,¹
Marlena Dlutek,¹ Richard J. Cawthorn,² Ivan Fiala,³ Julius Lukeš,³
Spencer J. Greenwood,⁴ and John M. Archibald^{1*}

Canadian Institute for Advanced Research, Integrated Microbial Biodiversity Program, Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4R2¹; Department of Pathology and Microbiology and AVC Lobster Science Centre, University of Prince Edward Island, Charlottetown, PE, Canada C1A 4P3²; Biology Centre, Institute of Parasitology, Czech Academy of Sciences and Faculty of Sciences, University of South Bohemia, 37005 České Budějovice (Budweis), Czech Republic³; and Department of Biomedical Sciences and AVC Lobster Science Centre, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada C1A 4P3⁴

Received 10 March 2011/Accepted 31 May 2011

We have performed a genomic characterization of a kinetoplastid protist living within the amoebozoan *Neoparamoeba pemaquidensis*. The genome of this “*Ichthyobodo*-related organism” was found to be unexpectedly large, with at least 11 chromosomes between 1.0 and 3.5 Mbp and a total genome size of at least 25 Mbp.

Kinetoplastids are an important group of eukaryotic microbes named by virtue of their shared possession of a conspicuous mass of DNA—the “kinetoplast”—inside the mitochondrion (21, 31). They are remarkable for their bizarre suite of biochemical features (e.g., spliced leader [SL] *trans* splicing and mitochondrial RNA editing) (1, 5, 21) and are best known as pathogens: the so-called “trityps” are responsible for mass mortality and morbidity in humans and other animals (1, 14, 31). The kinetoplastids also contain a variety of other parasites, such as the fish pathogen *Cryptobia*, as well as many free-living groups (e.g., *Bodo*) (31). Phylogenetic studies suggest that within kinetoplastids, parasitic lineages have evolved from free-living ones on at least four occasions (31). Despite many genomics-enabled advances for the trityp pathogens (e.g., see references 2, 14, and 18), a comprehensive evolutionary framework for understanding the biology of these important pathogens is currently lacking.

The kinetoplastid endosymbiont of *Neoparamoeba pemaquidensis*, an amoebozoan that causes disease in fish (e.g., Atlantic salmon) and invertebrates such as lobster (20, 27), represents an apparent example of “recent” adaptation to intracellularity. We refer to this enigmatic endosymbiont as the “*Ichthyobodo*-related organism” (IRO) based on 18S ribosomal DNA (rDNA) analyses showing its close affinity to *Ichthyobodo* (6, 10–12). In this study we characterized genes from *N. pemaquidensis* and its kinetoplastid endosymbiont and

carried out the first investigation of the chromosomes of both organisms using pulsed-field gel electrophoresis (PFGE) and Southern blotting.

N. pemaquidensis strains CCAP 1560/4 and 1560/5 and *Neoparamoeba branchiphila* AFSM3/II were cultured as described previously (13). Using standard transmission electron microscopy (TEM) and 4',6-diamidino-2-phenylindole (DAPI) staining protocols, *N. pemaquidensis* strain 1560/4 was mostly found to possess 1 (and only occasionally 2) oval or round IRO per cell, each 4 to 6 μm in diameter. The IRO was typically very closely associated with the host cell nucleus, with their surfaces often appearing to be in direct contact (Fig. 1A and C). This is consistent with previous reports based on studies of multiple strains of *Neoparamoeba* (e.g., see references 10, 12, 13, and 17). Within the IRO, a single mitochondrion was found to occupy more than half of the endosymbiont cell volume, with its distinctive kinetoplast DNA (kDNA) appearing as a complex fibrillar structure in TEM micrographs (Fig. 1A and B). The close proximity of the IRO to the amoeba host nucleus was also apparent under DAPI staining (Fig. 1C). The IRO nucleus stained weakly with DAPI relative to the amoeba nucleus, suggesting that the host and endosymbiont nuclei differ greatly in terms of their DNA content (Fig. 1C).

In order to gain insight into the evolutionary origins of *Neoparamoeba* and the IRO, we amplified six protein genes from both the host and its endosymbiont using PCR and/or reverse transcriptase PCR (RT-PCR) (alpha-tubulin [αTub], beta-tubulin [βTub], elongation factor 2 [EF2], heat shock protein 90 [hsp90], an RNA polymerase I subunit [Rpa1], and an RNA polymerase II subunit [Rpb1]; primer sequences are available upon request). PCR products were cloned into suitable vectors, and several clones per amplicon were sequenced on a CEQ8000 capillary DNA sequencer (Beckman Coulter,

* Corresponding author. Mailing address: Department of Biochemistry & Molecular Biology, Dalhousie University, Sir Charles Tupper Medical Building, 5850 College Street, Halifax, Nova Scotia B3H 4R2, Canada. Phone: (902) 494-2536. Fax: (902) 494-1355. E-mail: john.archibald@dal.ca.

† These authors contributed equally to this work.

∇ Published ahead of print on 10 June 2011.

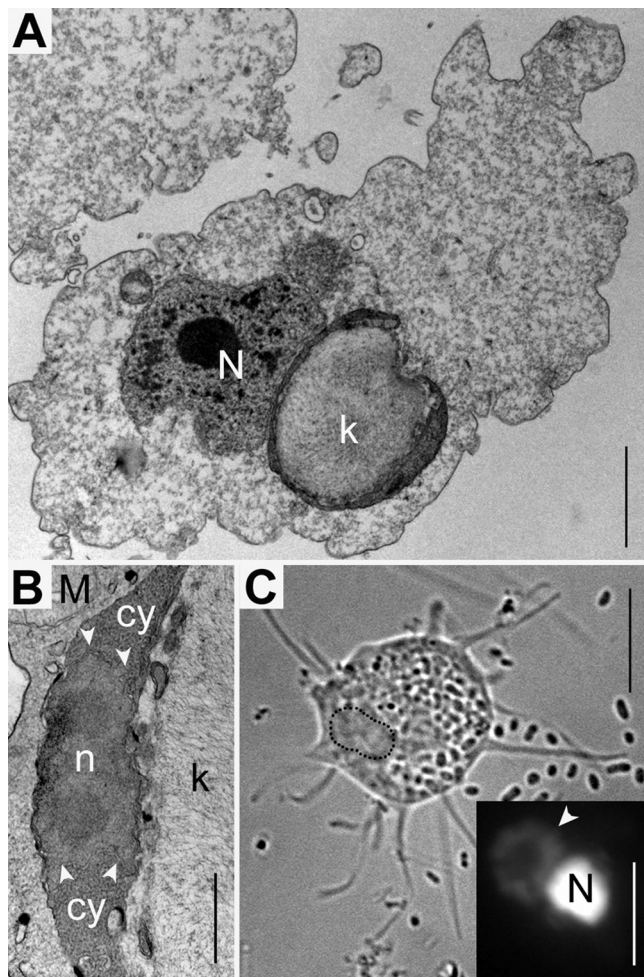


FIG. 1. Morphological observation using TEM and DAPI staining of *Neoparamoeba pemaquidensis* CCAP1560/4 and its kinetoplastid endosymbiont (i.e., the IRO). (A) Cross section of the host amoeba cell, showing its nucleus (N) and the IRO with its distinctive kinetoplast DNA (k). Scale bar = 2 μ m. (B) Close-up view of the IRO nucleus (n) with its double membrane envelope (arrowheads). Two nucleoli are visible. The cytoplasm of the IRO (cy) is rich with ribosomes. Scale bar = 500 nm. (C) Differential interference contrast (DIC) image showing an *N. pemaquidensis* cell with extended pseudopods. Bacteria are apparent outside and inside the cell. Scale bar = 10 μ m. The inset box shows an enlarged DAPI-stained view of the region outlined by the dotted line. The position of the IRO relative to the amoeba nucleus (N) is indicated by an arrowhead. Scale bar for inset box = 5 μ m.

Inc., Fullerton, CA). Protein alignments were analyzed individually (data not shown) and as a single concatenate. Trees inferred from the concatenated data set showed the IRO branching robustly with the tritryps *Leishmania* and *Trypanosoma*, to the exclusion of two other euglenozoans, *Diplonema* and *Euglena* (Fig. 2). We also amplified and sequenced the SL RNA gene from the IRO genome and confirmed the presence of an SL mini-exon addition to IRO alpha- and beta-tubulin mRNAs by RT-PCR and 5' rapid amplification of cDNA ends (RACE)-PCR (data not shown). Additional RACE-PCR experiments showed that the host-derived alpha-tubulin mRNA does not undergo mini-exon addition. These results suggest

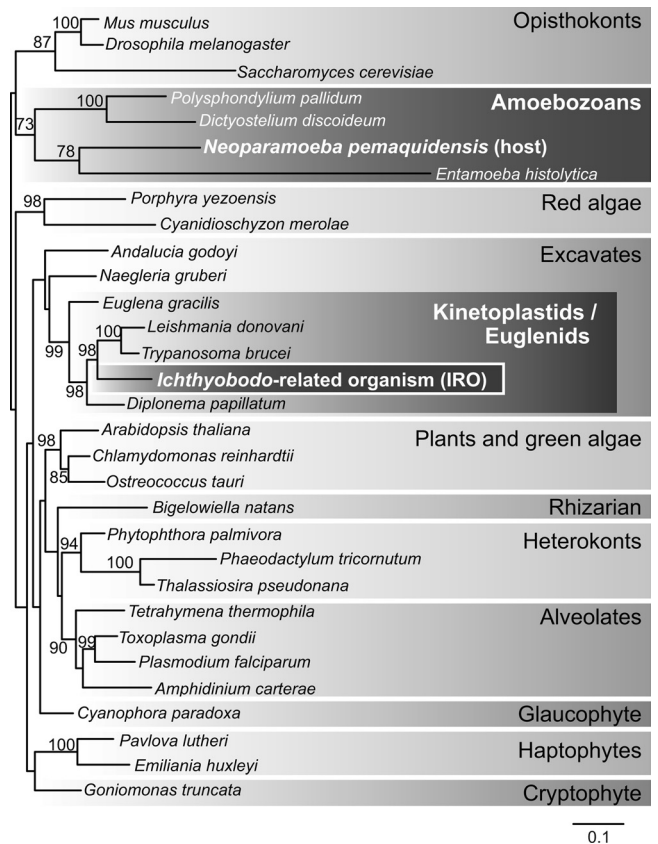


FIG. 2. Phylogeny of *Neoparamoeba pemaquidensis* CCAP1560/4 and its *Ichthyobodo*-related organism (IRO). The phylogenetic tree shown was constructed using maximum likelihood (ML) from a concatenated data set including the alpha-tubulin, beta-tubulin, elongation factor 2, and heat shock protein 90 proteins (Rpb1 and Rpa1 sequences were excluded due to the partial nature of the *N. pemaquidensis* and IRO sequences). ML analysis was performed using the RAxML software program, ver. 7.0.4 (33), with the WAG+Gamma+I model, and bootstrap analysis was based on 1,000 replicates with the CAT model. The amino acid alignment consisted of 31 operational taxonomic units (OTUs) and 942 unambiguously aligned positions. The tree was rooted arbitrarily. Bootstrap support values are shown if > 70%. The scale bar indicates an inferred number of amino acid substitutions per site.

that SL *trans* splicing takes place in the kinetoplastid endosymbiont but not in its host. No spliceosomal introns were found in any of the IRO protein genes sequenced herein, which are among the first to be determined, so it remains to be seen whether both *cis* and *trans* splicing occurs in these organisms, as has been shown for euglenoids and other kinetoplastids (3).

Consideration of the *Neoparamoeba* host- and IRO-derived sequences revealed interesting differences in base composition. The G+C content of the IRO genes was 51.0 to 65.5% for the protein coding genes sequenced in our study, and the previously sequenced ribosomal intergenic spacer regions (ITS1 and -2) showed 68.1% G+C when considered together. In stark contrast, sequences derived from the *Neoparamoeba* host were 49.8 to 55.2% G+C in protein-coding genes and were much lower overall in G+C content, especially in noncoding regions, such as the internal transcribed spacer (ITS) (21.5%). Although low G+C content is often seen in endosymbiont and

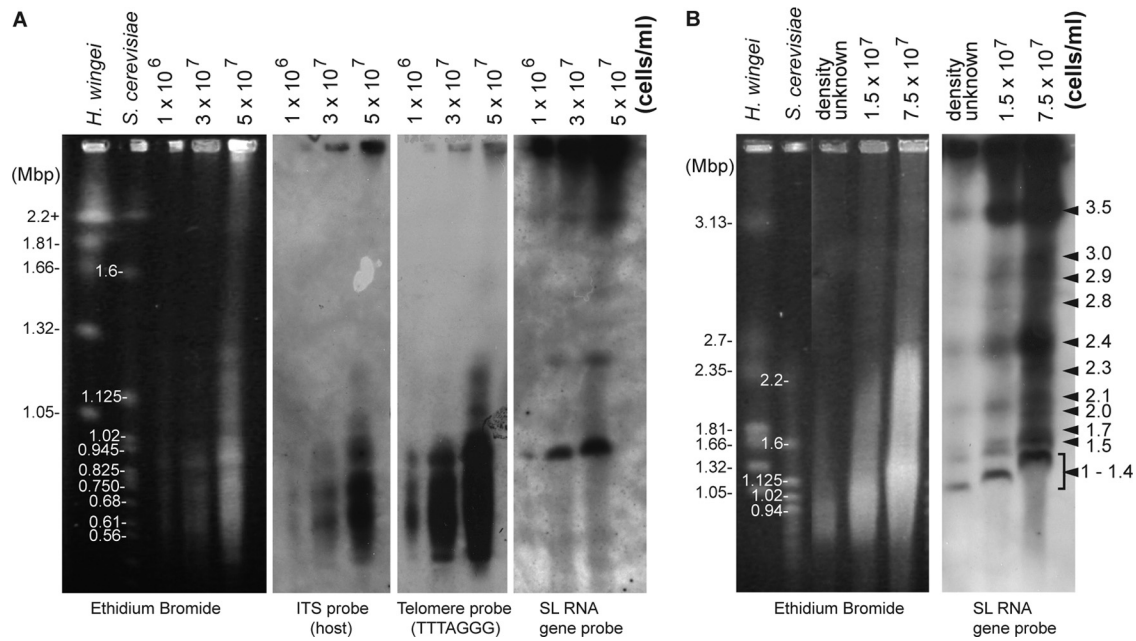


FIG. 3. Karyotype analysis of *Neoparamoeba pemaquidensis* CCAP1560/4 and its IRO. Ethidium bromide-stained pulsed-field gels are shown alongside Southern blots using probes designed against the *N. pemaquidensis* internal transcribed spacer (ITS) sequence and telomeric repeat (TTTAGGG), as well as the IRO spliced-leader (SL) RNA gene. Southern hybridizations were performed using the Roche DIG System (Roche Diagnostics, Mannheim, Germany). *Hansenula wingei* and *Saccharomyces cerevisiae* were used as DNA size markers. Cell densities of DNA plugs of *Neoparamoeba pemaquidensis* CCAP1560/4 are shown, as are the chromosome sizes of the markers. (A) Pulsed-field gel run with 1% agarose, $0.5 \times$ Tris-borate-EDTA (TBE), 14°C , a run time of 24 h at 6 V/cm, 120° angle, and a 60- to 120-s pulse time. (B) A 1% agarose pulsed-field gel, $0.5 \times$ TBE, 14°C , 48-h running time, 3 V/cm, 106° angle, and 500-s pulse time.

organellar genomes, the *N. pemaquidensis* host and IRO genomes exhibit the opposite pattern.

In addition to changes in base composition, adaptation to a life of intracellularity often leads to a reduction in genome size. This phenomenon is commonly seen in plastids and mitochondria (15, 32), bacterial endosymbionts, such as *Buchnera* (25, 26, 30), and eukaryotic intracellular pathogens, such as microsporidia (7, 8, 35). A strong signal for coevolution of *Neoparamoeba*-IRO pairs is clear from analyses of rDNA sequences (6, 11), and given that *Neoparamoeba* is invariably found with at least one IRO residing within it, the relationship between the two is very likely obligate (6). How big is the IRO nuclear genome, and where does this enigmatic organism reside along the continuum between transient endosymbiont and organelle? To address this question, we used pulsed-field gel electrophoresis (PFGE) to investigate the structure of the host and endosymbiont genomes of *N. pemaquidensis*. PFGE plugs were prepared using the general procedure described by Tanifuji et al. (34), with *N. pemaquidensis* prey bacteria being removed by filtration with a $3\text{-}\mu\text{m}$ pore polycarbonate membrane (Sterlitech, Kent, WA) prior to plug formation. A strong “smear” of similarly sized chromosomes in the range of ~ 0.45 to 1 Mbp was apparent under a variety of different PFGE conditions and ethidium bromide stainings and with different PFGE plug concentrations (Fig. 3 A and B). In order to differentially target host and IRO chromosomes, nonradioactive Southern hybridization analyses were performed using digoxigenin (DIG)-labeled probes against the predicted telomeric repeat of the *N. pemaquidensis* host [(TTTAGGG)_n], the host rDNA ITS, and the SL RNA gene of the IRO. Southern

hybridizations using host ITS and telomere probes detected strong signals between 0.45 and 1.2 Mbp, a pattern that was also seen with a host 28S rDNA probe (data not shown). These results suggest that the host genome contains many chromosomes in the 0.45- to 1.2-Mbp range.

Chromosomes of the *N. pemaquidensis* IRO were detected in Southern hybridization analyses using an SL RNA gene probe. Euglenoids and dinoflagellates are known to possess SL RNA genes in high copy number (2, 18, 28, 36), and our results suggest that the same is true for the *N. pemaquidensis* IRO. To our surprise, hybridizations using the SL RNA gene probe against membranes derived from multiple independent PFGE runs and using various plug cell concentrations revealed a total of 11 distinct hybridizing bands between ~ 1.0 and 3.5 Mbp; particularly strong signals were detected at ~ 1.0 , 2.4, and 3.5 Mbp (Fig. 3). Taking into account the fact that the chromosomal band migration distance was found to vary depending on PFGE plug cell density (see Fig. 3B; this is often the case in PFGE), these 11 bands total ~ 25 Mbp. We consider this to be a tentative absolute lower bound for the size of the *N. pemaquidensis* IRO nuclear genome. This estimate must be considered in the context of several important caveats, including (i) the likelihood of comigrating chromosomes in our PFGE analyses, (ii) interchromosomal variation in the SL RNA gene copy number (and thus variation in hybridization intensity), and (iii) the possibility that not all IRO chromosomes contain an SL RNA gene.

Across the breadth of eukaryotic diversity, nuclear genome size varies tremendously (16), but even free-living organisms, such as the red-alga *Cyanidioschyzon merolae* and the green

alga *Ostreococcus tauri*, can have very “small” genomes (16.5 Mbp and 12.6 Mbp, respectively) (9, 22). Trypanosomatid haploid genomes are in the range of 35 to 55 Mbp (14), and based on the preliminary data presented herein, it is possible that the IRO genome is similar in size. Our base composition analyses are also significant. A reduction in G+C content is also a hallmark feature of many (but not all) reduced genomes (15, 23, 24, 26, 29, 32), but our newly determined IRO protein gene sequences exhibit a G+C content between 55% and 65%, similar to that seen in other kinetoplastid taxa, including the free-living bodonid *Bodo saltans* (19). In fact, the rDNA ITS of the *N. pemaquidensis* IRO is 68.1% G+C over 188 bp, more G+C rich than the ITS of the prokinetoplastid *Ichthyobodo necator* (40.8% G+C) and the metakinetoplastids *Bodo caudatus* (49.2% G+C), *Trypanosoma brucei*, and *Leishmania major* (33.6 to 49.0%) (2, 4, 18). In sum, while the *N. pemaquidensis* IRO nuclear genome is very likely not “large,” there is currently no evidence to suggest that it has undergone significant reductive evolution in response to what is clearly a life of obligate intracellularity. In concert with investigation of the *Neoparamoeba* nuclear genome, determining the sequence of the IRO genome will provide a framework for better understanding *Neoparamoeba* pathogenesis and the nature of this interesting host-endosymbiont relationship.

Nucleotide sequence accession numbers. Genes of *Neoparamoeba* and the IRO, encoding the following proteins, were sequenced: alpha-tubulin, beta-tubulin, elongation factor 2, heat shock protein 90, an RNA polymerase I subunit, and an RNA polymerase II subunit; GenBank accession numbers are JF262536 to -42, JF262544 to -53, JF419568 to -72, FJ706693 to -98, and JF706718. Sequence of the SL RNA gene from the IRO genome has been deposited in GenBank under accession numbers JF262543 and JF441171.

This work was supported by an operating grant (ROP85016) from the Canadian Institutes of Health Research (CIHR) regional Partnership Program, together with the Nova Scotia Health Research Foundation (NSHRF). G.T. and E.K. are supported by the Tula Foundation. J.M.A. holds a CIHR New Investigator Award, and J.M.A. and J.L. are Fellows of the Canadian Institute for Advanced Research, Program in Integrated Microbial Biodiversity. J.L. is supported by the Czech Ministry of Education and the Praemium Academiae award.

We thank Mary Ann Trevors for assistance with TEM preparation.

REFERENCES

- Barrett, M. P., et al. 2003. The trypanosomiasis. *Lancet* **362**:1469–1480.
- Berriman, M., et al. 2005. The genome of the African trypanosome *Trypanosoma brucei*. *Science* **309**:416–422.
- Breckenridge, D. G., Y. Watanabe, S. J. Greenwood, M. W. Gray, and M. N. Schnare. 1999. U1 small nuclear RNA and spliceosomal introns in *Euglena gracilis*. *Proc. Natl. Acad. Sci. U. S. A.* **96**:852–856.
- Callahan, H. A., R. W. Litaker, and E. J. Noga. 2002. Molecular taxonomy of the suborder Bodonina (order Kinetoplastida), including the important fish parasite, *Ichthyobodo necator*. *J. Eukaryot. Microbiol.* **49**:119–128.
- Campbell, D. A., N. R. Sturm, and M. C. Yu. 2000. Transcription of the kinetoplastid spliced leader RNA gene. *Parasitol. Today*. **16**:78–82.
- Caraguel, C. G. B., et al. 2007. Microheterogeneity and coevolution: an examination of rDNA sequence characteristics in *Neoparamoeba pemaquidensis* and its prokinetoplastid endosymbiont. *J. Eukaryot. Microbiol.* **54**:418–426.
- Cornman, R. S., et al. 2009. Genomic analyses of the microsporidian *Nosema ceranae*, an emergent pathogen of honey bees. *Plos. Pathog.* **5**:e1000466.
- Corradi, N., J. F. Pombert, L. Farinelli, E. S. Didier, and P. J. Keeling. 2010. The complete sequence of the smallest known nuclear genome from the microsporidian *Encephalitozoon intestinalis*. *Nat. Commun.* **1**:77. doi: 10.1038/ncomms1082.
- Derelle, E., et al. 2006. Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. *Proc. Natl. Acad. Sci. U. S. A.* **103**:11647–11652.
- Dyková, I., I. Fiala, J. Lom, and J. Lukeš. 2003. *Perkinsiella amoebae*-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*. *Eur. J. Protistol.* **39**:37–52.
- Dyková, I., I. Fiala, and H. Pecková. 2008. *Neoparamoeba* spp. and their eukaryotic endosymbionts similar to *Perkinsiella amoebae* (Hollande, 1980): coevolution demonstrated by SSU rRNA gene phylogenies. *Eur. J. Protistol.* **44**:269–277.
- Dyková, I., A. Figueras, and Z. Peric. 2000. *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Dis. Aquat. Organ.* **43**:217–223.
- Dyková, I., et al. 2005. *Neoparamoeba branchiphila* n. sp., and related species of the genus *Neoparamoeba* Page, 1987: morphological and molecular characterization of selected strains. *J. Fish Dis.* **28**:49–64.
- El-Sayed, N. M., et al. 2005. Comparative genomics of trypanosomatid parasitic protozoa. *Science* **309**:404–409.
- Gray, M. W., B. F. Lang, and G. Burger. 2004. Mitochondria of protists. *Annu. Rev. Genet.* **38**:477–524.
- Gregory, T. R. 2005. The C-value enigma in plants and animals: a review of parallels and an appeal for partnership. *Ann. Bot. (Lond.)* **95**:133–146.
- Hollande, A. 1980. Identification du parasite (Nebenkernel) de Janickina pigmentifera a un symbiote (*Perkinsiella amoebae* nov gen-nov sp.) apparente aux flagelles kinetoplastidies. *Protistologica* **16**:613–625.
- Ivens, A. C., et al. 2005. The genome of the kinetoplastid parasite, *Leishmania major*. *Science* **309**:436–442.
- Jackson, A. P., M. A. Quail, and M. Berriman. 2008. Insights into the genome sequence of a free-living kinetoplastid: *Bodo saltans* (Kinetoplastida: Euglenozoa). *BMC Genomics* **9**:594.
- Lee, L. E. J., et al. 2006. High yield and rapid growth of *Neoparamoeba pemaquidensis* in co-culture with a rainbow trout gill-derived cell line RTgill-W1. *J. Fish Dis.* **29**:467–480.
- Lukeš, J., et al. 2002. Kinetoplast DNA network: evolution of an improbable structure. *Eukaryot. Cell* **1**:495–502.
- Matsuzaki, M., O. Misumi, T. Shin, et al. 2004. Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. *Nature* **428**:653–657.
- McCutcheon, J. P., B. R. McDonald, and N. A. Moran. 2009. Origin of an alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. *Plos. Genet.* **5**:e1000565.
- Moore, C. E., and J. M. Archibald. 2009. Nucleomorph genomes. *Annu. Rev. Genet.* **43**:251–264.
- Moran, N. A., J. P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**:165–190.
- Moran, N. A., H. J. McLaughlin, and R. Sorek. 2009. The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. *Science* **323**:379–382.
- Mullen, T. E., et al. 2004. Paramoebiasis associated with mass mortality of American lobster *Homarus americanus* in Long Island Sound, USA. *J. Aquat. Anim. Health* **16**:29–38.
- Murthy, V. K., K. M. Dibbern, and D. A. Campbell. 1992. PCR amplification of mini-exon genes differentiates *Trypanosoma cruzi* from *Trypanosoma rangeli*. *Mol. Cell Probe* **6**:237–243.
- Nakabachi, A., et al. 2006. The 160-kilobase genome of the bacterial endosymbiont *Carsonella*. *Science* **314**:267.
- Shigenobu, S., H. Watanabe, M. Hattori, Y. Sakaki, and H. Ishikawa. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407**:81–86.
- Simpson, A. G. B., J. R. Stevens, and J. Lukeš. 2006. The evolution and diversity of kinetoplastid flagellates. *Trends Parasitol.* **22**:168–174.
- Smith, D. R. 2009. Unparalleled GC content in the plastid DNA of *Selaginella*. *Plant Mol. Biol.* **71**:627–639.
- Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**:2688–2690.
- Tanifuji, G., M. Erata, K. Ishida, N. Onodera, and Y. Hara. 2006. Diversity of secondary endosymbiont-derived actin-coding genes in cryptomonads and their evolutionary implications. *J. Plant Res.* **119**:205–215.
- Texier, C., C. Vidau, B. Vignes, H. El Alaoui, and F. Delbac. 2010. Microsporidia: a model for minimal parasite-host interactions. *Curr. Opin. Microbiol.* **13**:443–449.
- Zhang, H., et al. 2007. Spliced leader RNA trans-splicing in dinoflagellates. *Proc. Natl. Acad. Sci. U. S. A.* **104**:4618–4623.