Folia biologica (Kraków), vol. **55** (2007), No 1-2

Review

Molecular Phylogenetics of Representative *Paramecium* Species

Agnieszka MACIEJEWSKA

Accepted September 20, 2006

MACIEJEWSKA A. 2007. Molecular phylogenetics of representative *Paramecium* species. Folia biol. (Kraków) **55**: 1-8.

The genus *Paramecium* has been known to science for 250 years and contains some of the most widely studied species of ciliates. At present, the basic research object for phylogenetic studies is the genome of various paramecia. One of the most widely used markers are genes coding for various rRNA's. Comparative analyses of sequences coding rRNA were applied for resolving the systematic position of some paramecia species and also for the establishment of an accurate taxonomy of *Paramecium.* Paramecia were also model organisms for their systematic group in more general studies in a comparative analysis among ciliates, fungi, plants and multicellular animals, illustrating the evolutionary relationships between Archaebacteria and Eucaryota. A new, revolutionary genealogy proposed the shifting of presumptively advanced groups towards more primitive ones, and traditionally primitive forms were located closer to highly specialized taxa, but rRNA analysis did not unambiguously resolve associations within the studied groups. Because of the aforementioned concerns, the number of molecular markers used for alternative studies is growing, such as genes coding proteins from the Hsp family or histone proteins. Other promising candidate markers may be hemoglobin genes or genes coding á-tubulins. In case of comparative analyses of nucleotide sequences, the outcome of the research usually depends upon a subjective choice of DNA. One of the directions of research in molecular phylogenetics include indirect methods that allow for an estimation of entire genomes, for example RAPD-PCR-fingerprinting.

Key words: *Paramecium*, molecular phylogenetics, molecular markers, rRNA, hsp70, histone genes, hemoglobin genes, α-tubulin genes, RAPD.

Agnieszka MACIEJEWSKA, Department of Genetics, Szczecin University, Piastów 40B, 71-065 Szczecin, Poland.
E-mail: maciejew@univ.szczecin.pl

In protozoology, paramecia are the most often used model organisms in studies assessing behavioural responses to environmental conditions, their reactions and reproduction, and particularly in investigations concerning sexual processes in populations and their life histories and those of clonal units. The ease, speed and low cost of paramecia culturing has made them a common model species in population genetic and breeding system studies, as well in work concerning cell biology, physiology and biochemistry.

Additionally, paramecia show a high species diversity. At present, many more species are known than what had been previously estimated (SONNEBORN 1957). Sibling species, common in ciliates, seem to be particularly important in evolutionary studies. Sibling species are considered real biological species that possess complete reproductive barriers coupled with a minimal amount or total lack of morphological differentiation. Sibling species are not limited to ciliates, indeed, they have been detected in a number of organisms. Sibling species are quite similar at the morphological level because of their recent separation and are therefore useful in investigations dealing with the processes of evolution. They originate through the actions of selection, genetic drift and isolation, processes that induce genome differentiation. Reproductive isolation, a key element of speciation, is a byproduct of these processes. The structure and functions of *Paramecium*, and particularly the life history promoting a high evolutionary tempo, permits investigations at a variety of cellular organizational levels, making these organisms especially valuable in evolutionary and phylogenetic studies.

Taxonomic species of *Paramecium* are similar in cell shape but are morphologically differentiated and show many important biological differ-

ences (the morphology and number of contractile vacuoles and micronuclei, mating type, nuclear type and its reorganization during conjugation, occurrence of endocytobionts). All of these traits allow for many morphometric, karyological, cyto- logical and physiological analyses (FOKIN *et al.* 2001).

The largest diversity is seen at the molecular level in *Paramecium*. The genome of paramecia, and all other organisms, accumulates mutations, therefore it is possible to look at the number of differences between nucleotide sequences and establish the age of the most recent common ancestor. By comparative analysis of genomes, the field of molecular phylogenetics allows the determination of DNA sequence similarity and a description of the evolutionary relationships among species. The data needed for these comparisons are gathered through the examination of polymorphic loci in organisms. At present, the basic research object for phylogenetic studies is the genome of various paramecia. Protein sequences can also be used, although nucleotide sequences are applied more often because of their stronger phylogenetic signal. Nucleotide sequences of homologous genes harbor more information than amino acid sequences because synonymous mutations change only DNA sequences. Additionally, further information can be derived from variation in noncoding regions. Contemporary PCR methods and sequencing also greatly facilitate the collection of data.

The recent development of molecular genetic methods has greatly increased the number of papers attempting to resolve relationships between organisms. Paramecia have been the objects of variation and evolutionary studies for quite a long quite, so it is obvious that they also should be subjected to various molecular phylogenetic analyses. Below a short review of phylogenetic analyses of paramecia based on molecular markers is presented.

Analysis of rRNA sequences and genes coding rRNA molecules

One of the most widely used markers are genes coding for various rRNA's. Ribosomal RNA is recognized as one of the best molecules for these analyses because of its ubiquity and identical function in all cells. The most useful rRNAs are from the small ribosomal subunits (SSU rRNA), mainly because of the sequence length and an appropriate amount of variable sites for comparative analyses. Most of the regions coding rRNA can be easily isolated from the genomes of different organisms (MCCARROL *et al.* 1983). These genes are quite conservative, facilitating the analysis of divergent evolutionary lineages. In the late 1960's WOESE argued that variation in SSU rRNA genes can be used to demonstrate the relationships among diverse forms of life, i.e. they can be used as a universal molecular chronometer (WOESE 1998). Genes coding the smaller rRNA molecules (5S and 5.8S), included in the larger ribosomal subunit (LSU rRNA), are used to a lesser extent because of their small size and low information content, making them more difficult to analyse statistically (STACKEBRANDT & WOESE 1981).

STRÜDER-KYPKE *et al.* (2000a, 2000b) applied the rRNA of small ribosomal subunits (SSU rRNA) to decipher relationships among *Paramecium* and Peniculia. On the basis of observations and morphometry, the genus *Paramecium* was divided into two groups, the "Bursaria" group resembling a footprint and the "Aurelia" group with more elongated cells (WOODRUFF 1921). In order to verify the systematic placement of species in each of these groups in Oligohymenophorea, comparative analyses of the above mentioned rDNA were undertaken. At first the following species were included: *P. bursaria*, *P. calkinsi*, *P. dubosqui*, *P. jenningsi*, *P. nephridiatum*, *P. primaurelia* and *P. polycaryum*. Dendrograms were constructed illustrating the phylogenetic relationships among *Paramecium* species using the neighbor-joining technique and the Kimura 2-parameter model (KIMURA 1980), maximum likelihood, and maximum parsimony. All methodological approaches recovered a monophyletic *Paramecium* lineage among Oligohymenophorea. This clade consisted of a separate, basal branch with *P. bursaria*, while the remaining "bursaria" species were not monophyletic. On the other hand, species from the "Aurelia" group formed a compact monophyletic group (STRÜDER-KYPKE *et al.* 2000a).

In subsequent studies using the same markers, the number of species was increased to include *P. caudatum*, *P. multimicronucleatum*, *P. putrinum* and *P. woodruffi* in order to resolve the relationships among *Paramecium* and also in the subclass Peniculia. *P. bursaria* was revealed as a separate lineage and the monophyly of the „Aurelia" subgroup, including *P. caudatum* and *P. multimicronucleatum,* was confirmed. Detailed analyses amongst the *Paramecium* clade revealed further branches including a *P. putrinum* and *P. dubosqui* branch, and another containing *P. woodruffi*, *P. nephridiatum*, *P. polycaryum* and *P.calkinsi*. The subclass Peniculia was divided into two orders, Urocentrida and Peniculida, the latter of which was further divided into two suborders, Frontoninа and Peniculina (STRÜDER-KYPKE *et al.* 2000b; MIAO *et al.* 2004).

Comparative analyses of sequences coding the 18S rRNA (SSU rRNA) gene were applied for resolving the systematic position of *P. schewiakoffi* and also for the establishment of an accurate taxonomy of *Paramecium* (FOKIN *et al.* 2004). The

sequenced fragment was compared with earlier sequences (STRÜDER-KYPKE *et al.* 2000a, 2000b). The neighbor-joining technique, maximum likelihood and maximum parsimony were used. To determine the robustness of the phylogenetic trees, analyses were performed with application of filter sets including only those positions that are conserved in at least 50% of all eukaryotic and paramecia sequences present in the database. *P. schewikoffi* was concluded to be the sister species of *P. jenningsi* in the "Aurelia" group. The phylogenetic tree was in accordance with earlier studies using the same molecular marker. On the basis of the molecular and morphological results, it was shown that *Paramecium* can be divided into 4 groups, treated as subgenuses. *P. bursaria* forms the first group, *P. putrinum* and *P. dubosqui* the second, while the „woodruffi"group comprises *P. woodruffi*, *P. nephridiatum*, *P. calkinsi* and *P. polycaryum*, and finally the Aurelia group with *P. aurelia*, *P. jenningsi*, *P schewiakoffi*, *P. caudatum* and *P. multimicronucelatum*.

Genes coding LSU rRNA's were also applied to phylogenetic studies concerning ciliates. 23S rRNA (D2 region) was used for analyses with *Colpoda*, *Paramecium* and *Tetrahymena*, representing two classes, the Colpodea and Oligohymenophorea (NANNEY *et al.* 1998). Dendrograms were constructed using parsimony with many additional factors and revealed considerable molecular divergence between strains that could not be differentiated by morphology. It was hypothesized that in all protists a high level of molecular variation is masked by morphological conservatism linked with limitations of ancestral cytological conditions.

Paramecia were also model organisms for their systematic group in more general studies. rDNA coding the small ribosomal subunit in *P. tetraurelia* was used in a comparative study among ciliates, fungi, plants and multicellular animals (SOGIN & ELWOOD 1986). Similarity indices and algorithms incorporating graphical transformations were applied to dendrogram construction. The results showed that ciliates diverged from eukaryotes as a dissipitated group during a radiation that encompassed fungi, plants and animals. This early divergence of ciliates and eukaryotes may be the cause of the morphological differentiation among contemporary protists.

In 1998 SOGIN and SILBERMAN reviewed phylogenetic studies of ciliates and other organisms based on various rRNA's of the small ribosomal subunit (the so-called 16S-like rRNA). *P. tetraurelia* was one of the most often incorporated species (SOGIN & SILBERMAN 1998). The deeply branched phylogenetic tree of eukaryotes obtained using SSU rRNA's revealed larger sequence variation in Eucaryota than in Archea and Bacteria. The results of studies using genes coding rRNA confirmed the early origin of protist evolutionary lineages preceding the great radiation of other eukaryotes.

The same *Paramecium* species was also used in analyses of 5S rRNA sequences, illustrating the evolutionary relationships between Archaebacteria and Eucaryota (NANNEY *et al.* 1991). The goal of this study was to find the origin of the lineage leading to eukaryote cells. The 5S rRNA molecule was chosen because of its ubiquity in many cells and high conservatism that allows testing relationships between taxa with a long evolutionary history. It was also assumed that the short sequence length will not compromise the results because of the correction schemes used in the computer program. Dendrogram construction was based mostly on parsimony taking into account numerous additional variables, checking the validity and correcting the topology of the tree. The analysis showed that eukaryotes diverged probably at the time of origin of halobacteria and methane bacteria. The identification of the last common ancestor of all eukaryotes proved to be difficult because the earliest representative organisms show high divergence amongst each other and to present-day species.

A full appreciation of phylogenetic relationships among eukaryotes based on analyses of rRNA is not complete without the study of PHILIPPE and GERMOT (2000). These authors used maximum likelihood in concatenated LSU and SSU rRNA sequences in 32 species, including *P. tetraurelia*. The taxa were chosen for high biodiveristy. The topology of the obtained tree was very similar to those from SSU rRNA. The order in which particular evolutionary lineages appeared was for the most part congruent with other studies. The small inconsistencies were interpreted as artifacts associated with the long-branch attraction phenomenon (PHILIPPE & GERMOT 2000).

Analysis of sequences coding Hsp70

A promising candidate gene seems to be the gene coding proteins from the Hsp70 family which occur as orthologs in almost all organisms. This marker, as rRNA markers, confirms the monophyly of morphological groups, which is advantageous compared to other protein molecules. Another convenience is the availability of closer related outgroups than in the case of rRNA, which lowers the risk of long-branch attraction.

Sequences of Hsp70 were applied to phylogenetic analysis of various strains of *P. tetraurelia* and *Euplotes aediculatus* compared to orthologs

of other eukaryotes (BUDIN & PHLIPPE 1998). Various Hsp70 genes located in different cell regions were used (cytosol, endoplasmatic reticulum, mitochondria). The sequences were compared using neighbor-joining, maximum likelihood and maximum parsimony. An analysis of concatenated sequences using cytosolic, endoplasmatic and mitochondrial sequences was also applied. The dendrograms revealed a deep divergence between Hypotricha and Oligohymenophorea, which confirmed the rRNA analyses (WRIGHT & LYNN 1997). On the other hand, a unique feature of the Hsp70 study was low stability of trees due to low bootstrap values, which seems to confirm the hypothesis of a great radiation at the base of the eukaryote clade making it difficult to precisely identify the order in which taxa appeared.

It is also worth mentioning the possibility of applying Hsp70 genes to more detailed analyses. PRZYBOŚ *et al.* (2003a) used the cytosolic Hsp70 gene for confirming the identity of a new strain of *P. quadecaurelia* from Namibia. A comparative analysis of this sequence based on maximum similarity produced a dendrogram confirming classical interstrain crossings that classified the new African strain as belonging to *P. qudecaurelia*.

Analysis of sequences coding histone proteins

Other recently proposed markers are genes for the histone proteins (mainly H3 and H4). They occur in the vast majority of organisms but are considered highly conservative which precludes their role in phylogenetic studies, especially in distantly related organisms. However, in ciliates these markers show considerable variation unique to the living world. Sequence variation among ciliates may be higher than that between plants and animals (BENDER *et al.* 1992; HECKER 1993). The causes of this variation remain to be discovered. One hypothesis concerns the atypical organization of the macronucleus in which mini- or subchromosome fragments appear in place of chromosomes. These fragments do not need the effective transcriptional control reserved to chromosomes, which may have released the histones from selective pressure and caused the accumulation of substitutions and insertions. Additionally, the macronucleus does not divide mitotically, which may have changed the selection regime on conservative domains of histones involved in mitosis in other organisms.

The large sequence variation in ciliate histones and independent evolution of these proteins relative to other macromolecules shows their potential applicability to cross-check analyses. BERNHARD and SCHLEGEL (1998) performed a comparative analysis of fragments coding H4 and H3 and an intergenic DNA fragment between them, additionally including their amino acid sequences. Their neighbor-joining and parsimony analyses of phylogenetic relationships of ciliates, including *P. tetraurelia* and *P. bursaria,* were congruent to rDNA and morphological groupings, arguing for the correctness of the phylogeny (BAROIN-TOURANCHEAU *et al.* 1992; BERNHARD *et al.* 1995; HIRT *et al.* 1995; HAMMERSCHMIDT *et al.* 1996).

New contributions have applied the H4 protein for resolving relationships among eukaryotes. KATZ *et al.* (2004) sequenced this gene in 13 ciliate species and compared them to analogous fragments in other eukaryotes, including *Paramecium*. Their neighbor-joining and maximum similarity diagrams confirmed the large variation seen in ciliates as compared to other eukaryotes, probably as a result of functional constraints and adaptive evolution of this protein due to nuclear dualism.

Analysis of sequences coding hemoglobin genes

Other promising candidate markers may be hemoglobin genes present in the genomes of all *Paramecium* representatives which show a level of variation adequate for phylogenetic analysis. YAMAUCHI *et al.* (1995) conducted a study of various strains of *P. multimicronucleatum*, *P. triaurelia*, *P. jenningsi* and *P. caudatum* as a reference species. Maximum similarity was used to show the sister relationship between *P. triaurelia* and *P. jenningsi* and to formulate an hypothesis on the origin of sibling species of *P. aurelia* from *P. jenningsi*. Comparative sequence analysis revealed a single intron in the same position in all analysed hemoglobin genes (YAMAUCHI *et al.* 1992, 1993).

In conjunction with the hemoglobin genes, amino acid sequences of hemoglobin proteins were also analysed in *Paramecium.* HIRAI *et al.* (1992) revealed the inter- and intra strain variation between *P. caudatum* strains in the proteins Hb10 and bHb. Unfortunately these proteins are not adequate molecular markers for differentiating syngens of *P. caudatum* because of the wide distribution of identical forms which produces similar profiles in liquid chromatography for particular syngens and strains.

Analysis of sequences coding α-tubulin genes

Genes coding α-tubulins have been applied to a different area of research. These genes form a family that came into being through duplications. Comparisons of sequences allows for the tracking of duplicated copies in various organisms, which gives valuable information on variation in rate of

evolution through the level of substitution and also produces phylogenetic hypotheses. ISRAEL *et al.* (2002) conducted a study in the following ciliates: *P. tetraurelia*, *Euplotes crassus*, *Halteria grandinella*, *Stylonychia lemnae* and *Chilidonella uncinata.* It was shown that the rate of evolution is different in the particular organisms, while the topology of the dendrograms differed from those using other molecular and morphological markers. This may be due to an uneven rate of evolution in the studied species, gene conversion or asymmetrical duplication of the genes.

Phylogenetic analysis using the RAPD technique

Comparative analyses of nucleotide sequences are quite popular among phylogenetic researchers and evolutionary biologists. However, they do not always give straightforward answers, while many questions remain to be resolved. Additionally in case of these methods, the outcome of the research usually depends upon a subjective choice of DNA sequence that is not always applicable to phylogenetic studies, which may produce false results.

One of the directions of research in molecular phylogenetics includes indirect methods that allow for an estimation of divergence of entire genomes. Direct sequencing methods are difficult because of the high cost involved and limited computational capacity of most computers, while indirect methods give information about entire genome variation using simple, quick and relatively low cost procedures.

One of the best contemporary indirect methods is RAPD-PCR-fingerprinting (random amplified polymorphic DNA). Variation on the level of DNA sequences occurs in species or strains, and can be observed as different band patterns on agarose gels. These patterns can be used for illustrating the relationships and for identification of strains, species or individuals, and through analogy to dactyloscopic methods, they are often referred to as genomic fingerprints. Band patterns can be subjected to mathematical analysis using various similarity indices, such as the Nei and Li index (NEI & LI 1979) and the Jaccard index (SOKAL & MICHENER 1958). Despite the mathematical similarity, mean values from both indices are quite similar and can be used in neighbor-joining diagrams.

This method was used for the first time in *Paramecium* for the identification of sibling species by STOECK & SCHMIDT (1998) in the *P. aurelia* complex. Morphometric indices could not be used because these species are practically identical. Electrophoresis of isoenzymes and electrokaryotyping gave satisfying results only in some cases (RAUTIAN & POTHEKIN 2002). However, RAPD-PCR-fingerprinting is quick, highly dependable and allows for simultaneous comparisons of many strains. Another asset is the fact that cultures are not needed, because even a single cell suffices for analysis. In the previously mentioned study, the authors explored the structure of a complex composed of 15 sibling species (ct. PRZYBOŚ 1986a). The species of this group are genetically isolated and have different gene pools. The cells may conjugate freely within species, but interspecific crosses do not occur. These are real biological species, geographically isolated with different ecological requirements and variable life cycles. Depending on the amount of inbreeding in species of the *P. aurelia* complex, the strains within species are more or less isolated and show a variable number of surviving clones in interstrain crosses (PRZYBOŚ 2001; PRZYBOŚ & FOKIN 1999, 2000). Molecular confirmation of the separateness of the species was shown by the characteristic RAPD-PCR-fingerprinting pattern obtained with the Ro 460-04 primer (STOECK *et al.* 1998, 2000a).

Apart from identification of European species of the *P. aurelia* complex, RAPD has been used for differentiating between *P. multimicronucleatum* and *P. caudatum* (STEOCK *et al.* 2000b), and brackish species such as *P. nephridiatum*, *P. calkinsi*, *P. duboscqui*, and *P. woodruffi* (FOKIN *et al.* 1999). This method has also been used for intraspecific studies in the *P. aurelia* complex and *P. jenningsi* (PRZYBOŚ *et al.* 1999, 2003b; SKOTARCZAK *et al.* 2004a, 2004b).

The initiation of studies of variation within *P. jenningsi* (PRZYBOŚ 1975, 1978, 1980, 1986b) had two main causes. One important factor was the finding of FOKIN (1997) of new localities of this species in Japan (Honshu, Yamaguchi), which expanded the known range of this species. Second, it was shown that other *Paramecium* species are morphologically and biochemically conservative, while variable at the molecular level (STRÜDER-KYPKE *et al.* 2000a, FOKIN *et al.* 2001). This suggested the occurrence of genetic polymorphism that did not involve polymorphism at other cellular levels.

Both discoveries prompted studies of natural variation within *P. jenningsi* from Japan, India, Madagascar, Uganda and Florida, using RAPD-PCR-fingerprinting with Ro 460-04 (PRZYBOŚ *et al.* 1999). The pattern observed was different from other *Paramecium* species. The Nei and Li index was used to calculate the similarity among strains of *P. jenningsi*. Three genotypic groups were acknowledged within this species:

– an eastern group from India and Madagascar

– a western group from Uganda and Florida

– a Japanese group.

It was shown that the genotype of *P. jenningsi* changes with increasing latitude from tropical areas, which are thought to be the areas of origin. The genotype of *P. jenningsi* also changes with the type of inbreeding.

The discovery of new localities of *P. jenningsi* in Japan, China and Saudi Arabia prompted further phylogenetic investigations. Interstrain and intrastrain crosses were performed in addition to RAPD-PCR-fingerprinting with primer Ro 460-04. This study revealed greater genetic variation than before and identified two reproductively isolated syngens from India, Saudi Arabia, and China, and also from Japan (PRZYBOŚ *et al.* 2003b).

Subsequent studies involved more detailed analyses of continental and Japanese strains. RAPD-PCR-fingerprinting was performed with 11 primers (Ro 460-01 to Ro 460-10 and Ro 360-04), with considerable variation that permitted the construction of characteristic band patterns for each strain. The phylogenetic analysis, incorporating earlier results of crosses showing the reproductive isolation between Japanese and continental strains, concluded that two sibling species occur within *P. jenningsi* (SKOTARCZAK *et al.* 2004a, 2004b).

The RAPD method was also used for identification of *P. schewiakoffi* (FOKIN *et al.* 2004). The band pattern characteristic for *P. schewiakoffi* was distinct from *P. jenningsi*. Phylogenetic analysis illustrated the close relationships between *P. schewiakoffi* and Japanese *P. jenningsi* strains that were proposed to be one of two sibling species within the *P. jenningsi* complex.

The review of molecular phylogenetic studies incorporating *Paramecium* species presented here gave a summary of the avenues of research in this area. It is incomplete because of the many ambiguities and unanswered questions that remain. The higher level sytematics and taxonomy of ciliates, and studies at the more detailed specific level, are constantly being revised as progress in methodology, especially molecular techniques, is attained.

At the present time many broad scale studies are being conducted in many scientific institutions concerning the phylogeny of various taxa, even distantly related ones. New molecular markers are being sought and tested against a phylogenetic framework. Sequencing studies are also being conducted in order to obtain the entire genome of *Paramecium*, e.g. the group of Polish researchers from the Warsaw laboratory for sequencing and oligonucleotide construction in the Institute of Biochemistry and Biophysics that managed to sequence the largest somatic chromosome of *Paramecium* (ZAGULSKI *et al.* 2004). These kinds of discoveries give new perspectives for planning and conducting experiments that may answer pertinent evolutionary and phylogenetic questions.

References

BAROIN-TOURANCHEAU A., DELGADO P., PERASSO R., ADOUTTE A. 1992. A broad molecular phylogeny of ciliates: identyfication of major evolutionary trends and radiations within phylum. Proc. Natl. Acad. Sci. USA **89**: 9764-9768.

BENDER K., BETSCHART B., SCHALLER J., KAMPFER U., HECKER H. 1992. Sequence differences between histones of procyclic *Trypanosoma brucei* and higher eukaryotes. Parasitology **105**: 97-104.

BERGER J. D. 2001. Riding the ciliate cell cycle – a thirty-five-year prospective. J. Eukaryot. Microbiol. **48**: 505-518.

BERNHARD D., LEIPE D., SOGIN M. L., SCHLEGEl M. 1995. Phylogenetic relationships of the Nassulida within the phylum Ciliophora inferred from the complete small subunit rRNA gene sequences of *Furgasonia blochmanni*, *Obertrumia georgiana* and *Pseudomicrothorax dibius*. J. Euk. Microbiol. **42**: 126-131.

BERNHARD D., SCHLEGEL M. 1998. Evolution of histone H4 and H3 genes in different Ciliate lineages. J. Mol. Evol. **46**: 344-354.

BRICHEUX G., BRUGEROLLE G. 1997. Molecular cloning of actin genes in *Trichomonas vaginalis* and phylogeny interferred from actin sequences. FEMS Microbiol. Lett. **53**: 205-213.

BRINKMANN H., PHILIPPE H. 1999. Archaea sister group of Bacteria? Indications from tree reconstruction artifacts in ancient phylogenies. Mol. Biol. Evol. **16**: 817-825.

BUDIN K., PHILIPPE H. 1998. New insights into the phylogeny of Eukaryotes based on Ciliate Hsp70 sequences. Mol. Biol. Evol. **15**: 943-956.

FOKIN S. I. 1997. Morphological diversity of the micronuclei in *Paramecium*. Arch. Protistenkd. **148**: 375-387.

FOKIN S. I., PRZYBOŚ E., CHIVILEV M. 2001. Nuclear reorganization variety in *Paramecium* (Ciliophora: Peniculida) and its possible evolution. Acta Protozool. **40**: 249-261.

FOKIN S. I., PRZYBOŚ E., CHIVILEV M., BEIER C. L., HORN M., SKOTARCZAK B., WODECKA B., FUJISHIMA M. 2004. Morphological and molecular investigations of *Paramecium schewiakoffi* sp. nov. (Ciliophora, Oligohymenophorea) and current status of distribution and taxonomy of *Paramecium* species. Europ. J. Protistol. **40**: 225-243.

FOKIN S. I., STOECK T., SCHMIDT J. 1999. *Paramecium duboscqui* Chatton, Brachon, 1933. Distribution, ecology and taxonomy. Europ. J. Protistol. **35**: 161-167.

GAJADHAR A. A., MARQUARDT W. C., HALL R., GUNDERSON J., ARIZTIA-CARMONA E. V., SOGIN M. L. 1991. Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Cryptheconidium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates and ciliates. Mol. Biochem. Parasitol. **45**: 147-154.

GALTIER N., GOUY M. 1995. Inferring phylogenies from DNA sequences of unequal base compositions. Proc. Natl. Acad. Sci. USA **92**: 11317-11321.

GALTIER N., GOUY M. 1998. Inferring pattern and process: maximum likelihood implementation of a nonhomogeneous model of DNA sequence evolution for phylogenetic analysis. Mol. Biol. Evol. **15**: 871-879.

GRIBALDO S., PHILIPPE H. 2002. Ancient phylogenetic relatinships. Theor. Popul. Biol. **61**: 391-408.

HAMMERSCHMIDT B., SCHLEGEL M., LYNN D. H., LEIPE D. D., SOGIN M. L., RAIKOV I. B. 1996. Insights into the evolution of nuclear dualism in the ciliates revealed by phylogenetic analysis of rRNA sequences. J. Euk. Microbiol. **43**: 225-230.

HECKER H. 1993. Man and sea urchin – more closely related than African and American trypanosomes. Parasitol. Today **9**: 57-58.

HIRAI I, USUKI I, OCHIAI T. 1992. Inter- and intrasyngenic variations of the hemoglobin components in *Paramecium caudatum*. J. Protozool. **39**: 636-639.

HIRT R. P., DYAL P. L., WILKINSON M., FINLAY B. J., ROBERTS D. M., EMBLEY T. M. 1995. Phylogenetic relationships among karyorelictids and heterotrichs inferred from small subunit rRNA sequences: resolution at the base of the ciliate tree. Mol. Phylogent. Evol. **4**: 77-87.

ISRAEL R. L., KOSAKOVSKY POND S. L., MUSE S. V., KATZ L. A. 2002. Evolution of duplicated alpha-tubulin genes in ciliates. Int. J. Org. Evol. **56**: 1110-1122.

KAMAISHI T., HASHIMOTO T., NAKAMURA Y., MASUDA Y., NAKAMURA F., OKAMOTO K., SHIMIZU M., HASEGAWA M. 1996. Complete nucleotide sequences of the genes encoding translation elongation factors 1á and 2 from a microsporidian parasite, *Glugea plecoglossi*: implications for the deepest branching of eukaryotes. J. Biochem. **120**: 1095-1103.

KATZ L. A., BORNSTEIN J. G., LASEK-NESSELQUIST E., MUSE S. V. 2004. Dramatic diversity of Ciliate histone H4 genes revealed by comparisonsof patterns of substitutions and paralog divergences among Eukaryotes. Mol. Biol. Evol. **21**: 555-562.

KEELING P. J., DOOLITTLE W. F. 1996. Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. Mol. Biol. Evol. **13**: 318-338.

KIMURA M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**: 111-120.

MCCARROL R., OLSEN G. J., STHAL Y. D., WOESE C. R. SOGIN M. L. 1983. Nucleotide sequence of Dictyostelium dictyostelium small-subunit ribosomal ribonucleic acid inferred from the gene sequence: evolutionary implications. Biochemistry **22**: 5858-5868.

MIAO W., FEN W-S., YU Y-H., ZHANG X-Y., SHEN Y-F. 2004. Phylogenetic relationships of yhe subclass Peritrichia (Oligohymenophorea, Ciloiphora) inferred from Small-Subunit rRNA gene sequences. J. Eukaryot. Microbiol. **51**: 180-186.

MOREIRA D., LE GUYADER H., PHILIPPE H. 1999. Unusually high evolutionary rate of the elongation factor 1á genes from the Ciliophora and its impact on the phylogeny of Eukaryotes Mol. Biol. Evol. **16**: 234-245.

NANNEY D. L. 1989. What can molecules tell us about evolution. Boll. Zool. **56**: 205-221.

NANNEY D. L., MOBLEY R. M., PREPARATA R. M., MEYER E. B., SIMON E. M. 1991. Eukaryotic origins: string analysis of 5S ribosomal sequences from some relevant organisms. J. Mol. Evol. **32**: 316-327.

NANNEY D. L., PARK C., PREPARATA R., SIMON E. M. 1998. Comparison of sequence differences in a variable 23S rRNA domain among sets of cryptic species of ciliated protozoa. J. Eukaryot. Microbiol. **45**: 91-100.

NEI M., LI W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**: 5269-5273.

PHILIPPE H., GERMOT A. 2000. Phylogeny of Eukaryotes based on ribosomal RNA: long-branch attraction and models of sequence evolution. Mol. Biol. Evol. **17**: 830-834.

PHILIPPE H., LAURENT J. 1998. How good are deep phylogenetic trees? Curr. Opin. Genet. Devel. **8**: 616-623.

PRZYBOŚ E. 1975. Genetic studies of *Paramecium jenningsi* strains (Diller & Earl, 1958). Folia biol. (Kraków) **23**: 425-471.

PRZYBOŚ E. 1978. Cytological and karyological studies of *Paramecium jenningsi*. Folia biol. (Kraków) **26**: 25-29.

PRZYBOŚ E. 1980. The African strain of *Paramecium jenningsi*. Cytological and karyological investigations. Folia biol. (Kraków) **28**: 391-397.

PRZYBOŚ E. 1986a. Species structure in Ciliates. Folia biol. (Kraków) **34**: 103-132.

PRZYBOŚ E. 1986b. Chromosomes in *Paramecium jenningsi* (Diller Earl, 1958): A serial section study. Folia biol. (Kraków) **34**: 133-160.

PRZYBOŚ E. 2001. The *Paramecium aurelia* species complex in Ukraine. Folia biol. (Kraków) **49**: 229-233.

PRZYBOŚ E., FOKIN S. I. 1999. Investigations on the species of the *Paramecium aurelia* complex (Ciliophora, Protista) in Israel. Israel J. Zool. **45**: 295-298.

PRZYBOŚ E., FOKIN S. I. 2000. Data on the occurence of species of the *Paramecium aurelia* complex world-wide. Protistology **1**: 179-184.

PRZYBOŚ E., FOKIN S. I., STOECK T., SCHMIDT J. 1999. Occurrence and ecology of *Paramecium jenningsi* strains. Folia biol. (Kraków) **47**: 53-59.

PRZYBOŚ E., HORI M., FOKIN S. I. 2003a. Strains of *Paramecium quadecaurelia* from Namibia, Africa; genetic and molecular studies. Acta Protozool. **42**: 357-360.

PRZYBOŚ E., SKOTARCZAK. B., WODECKA B. 2003b. Phylogenetic relationships of *Paramecium jenningsi* strains (classical analysis and RAPD studies). Folia biol. (Kraków) **51**: 130-145.

RAUTIAN M. S., POTHEKIN A. A. 2002. Electrokaryotypes of macronuclei of several *Paramecium* species. J. Eukaryot. Microbiol. **49**: 296-304.

ROGER A. J., SANDBLOM O., DOOLITTLE R. F., PHILIPPE H. 1999. An evaluation of elongation factor 1á as a phylogenetic marker for Eukaryotes. Mol. Biol. Evol. **16**: 218-233.

ROGER A. J., SMITH M. W., DOOLITTLE R. F., DOOLITLE W. F. 1996. Evidence for the Heterolobosea from phylogenetic analysis of genes encoding glyceraldehyde-3-phosphate dehydrogenase. J. Euk. Microbiol. **43**: 475-485.

SCHLEGEL M. 1991. Protist evolution and phylogeny as discerned from small subunit ribosomal RNA sequence comparisons. Eur. J. Protistol. **27**: 207-219.

SKOTARCZAK B., PRZYBOŚ E., WODECKA B., MACIEJEWSKA A. 2004a. Sibling species within *Paramecium jenningsi* revealed by RAPD. Acta Protozool. **43**: 29-35.

SKOTARCZAK B., PRZYBOŚ E., WODECKA B., MACIEJEWSKA A. 2004b. Random amplified polymorphic DNA fingerprinting as a marker for *Paramecium jenningsi* strains. Folia biol. (Kraków) **52**: 117-124.

SOGIN M. L., ELWOOD H. J. 1986. Primary structure of the *Paramecium tetraurelia* small-subunit rRNA coding region: phylogenetic relationships within the Ciliophora. J. Mol. Evol. **23**: 53-60.

SOGIN M. L., SILBERMAN J. D. 1998. Evolution of the Protists and protistan parasites from the perspective of molecular systematics. Int. J. Parasitol. **28**: 11-20.

SOKAL R. R., MICHENER C. E. 1958. A statistical method for evaluating systematic relationships. Univ. Kans. Sci. Bull. **38**: 1409-1438.

SONNEBORN T. M. 1957. Breeding systems, reproductive methods, and species problems in Protozoa. (In: The Species Problem. E. Mayr ed. AAAS, Washington): 155-324.

STACKEBRANDT E., WOESE C. R. 1981. Evolution of prokaryotes. (In: Molecular and Cellular Aspects of Microbial Evolution. M. J. Carlile, J. F. Collins, B. E. B. Moseley eds. Cambridge University Press, Cambridge, England): 1-31.

STOECK T., PRZYBOŚ E., KUSCH J., SCHMIDT J. 2000a. Intraspecies differentiation and level of inbreeding of diffrent sibling species of the *Paramecium aurelia* complex. Acta Protozool. **39**: 15-22.

STOECK T., PRZYBOŚ E., SCHMIDT J. 1998. A combination of genetics with inter- and intra-strain crosses and RAPD-fingerprints reveals diffrent population structures within the *Paramecium aurelia* species complex. Europ. J. Protistol. **34**: 348-355.

STOECK T., SCHMIDT J. 1998. Fast and accurate identification of european species of the *Paramecium aurelia* complex by RAPD-fingerprints. Microb. Ecol. **35**: 311-317.

STOECK T., WELTER H., SEITZ-BENDER D., KUSCH J., SCHMIDT J. 2000b. ARDRA and RAPD-fingerprinting reject the sibling species concept for the ciliate *Paramecium caudatum* (Ciliophora, Protoctista). Zool. Scr. **29**: 75-82.

STRÜDER-KYPKE M. C., WRIGHT A. G., FOKIN S. I., LYNN D. H. 2000a. Phylogenetic reletionships of the genus *Paramecium* inferred from Small Subunit rRNA gene sequences. Mol. Phylogenet. Evol. **14**: 122-130.

STRÜDER-KYPKE M. C., WRIGHT A. G., FOKIN S. I., LYNN D. H. 2000b. Phylogenetic reletionships of the subclass Peniculia (Oligohymenophorea, Ciliophora) inferred from Small Subunit rRNA gene sequences. J. Eykaryot. Microbiol. **47**: 419-429.

WOESE C. 1998. The universal ancestor. Proc. Natl. Acad. Sci. USA **95**: 6854-6859.

WOODRUFF L. L. 1921. The structure, life history and intergenic relationships of *Paramecium calkinsi*, sp. nov. Biol. Bull. **41**: 171-180.

WRIGHT A.-D. G., LYNN D. H. 1997. Monophyly of the trichostome ciliates (phylum Ciliophora: class Litostomatea) tested using new 18S rRNA sequences from the vestibuliferids, *Isotricha intestinalis* and *Dasytricha ruminantium*, and the haptorian, *Didinium nasutum*. Eur. J. Protistol. **33**: 305-315.

YAMAUCHI K., OCHIAI T., USUKI I. 1992. The unique structure of the *Paramecium caudatum* hemoglobin gene: the presence of one intron in the middle of the coding region. Biochim. Biophys. Acta **1171**: 81-87.

YAMAUCHI K., TADA H., OCHIAI T., USUKI I. 1993. Structure of the *Paramecium caudatum* gene encoding the B-type of the major hemoglobin component. Gene **126**: 243-246.

YAMAUCHI K., TADA H., USUKI I. 1995. Structure and evolution of *Paramecium* hemoglobin genes. Biochim. Biophys. Acta **1264**: 53-62.

ZAGULSKI M., NOWAK J. K., LE MOUEL A., NOWACKI M., MIGDALSKI A., GROMADKA R., NOEL B., BLANC I., DESSEN P., WINCKER P., KELLER A. M., COHEN J., MEYER E., SPERLING L. 2004. High coding density on the largest *Paramecium tetraurelia* somatic chromosome. Curr. Biol. **14**: 1397-1404.