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Intraspecific differentiation of *Paramecium novaurelia* strains (Ciliophora, Protozoa) inferred from phylogenetic analysis of ribosomal and mitochondrial DNA variation

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Abstract

Paramecium novaurelia Beale and Schneller, 1954, was first found in Scotland and is known to occur mainly in Europe, where it is the most common species of the *P. aurelia* complex. In recent years, two non-European localities have been described: Turkey and the United States of America. This article presents the analysis of intraspecific variability among 25 strains of *P. novaurelia* with the application of ribosomal and mitochondrial loci (ITS1-5.8S-ITS2, 5′ large subunit rDNA (5′LSU rDNA) and cytochrome c oxidase subunit 1 (*COI*) mtDNA). The mean distance observed for all of the studied *P. novaurelia* sequence pairs was $p = 0.008/0.016/0.092$ (ITS1-5.8S-ITS2/5′LSU rDNA/*COI*). Phylogenetic trees (NJ/MP/BI) based on a comparison of all of the analysed sequences show that the studied strains of *P. novaurelia* form a distinct clade, separate from the *P. caudatum* outgroup, and are divided into two clusters (A and B) and two branches (C and D). The occurrence of substantial genetic differentiation within *P. novaurelia*, confirmed by the analysed DNA fragments, indicates a rapid evolution of particular species within the *Paramecium* genus.

Keywords: *Paramecium novaurelia*; Sibling species; *Paramecium aurelia* species complex; rDNA; *COI* mtDNA; Molecular phylogeny

Paramecium species live mainly in freshwater (lakes, ponds, rivers, marshes, streams and lakes (Sonneborn 1975)) or brackish water (Fokin and Chivilev 1999) at temperatures ranging from about 3 °C to 28 °C (Landis 1988). The *P. aurelia* species complex Müller, 1773, is composed of 15 genetic species; 14 of them were named by Sonneborn (Sonneborn 1975). The fifteenth (*P. sonneborni*) was described separately (Aufderheide et al. 1983). Despite the fact that particular species of the complex are morphologically indistinguishable, they have isolated gene pools. Moreover, particular species differ according to geographical distribution, temperature requirements, culture conditions necessary for conjugation and system of mating type inheritance (Stoeck et al. 2000a). One of the members of the *P. aurelia* complex is *P. novaurelia* Beale and Schneller, 1954, which was found in Scotland for the first time and is known to occur mainly in Europe, where it is the most common species of the *P. aurelia* complex (Przyboś 2005; Sonneborn 1975).

Information on intra-specific molecular diversity within the species *P. novaurelia* is rather scarce. The first genetic comparison showed that *P. novaurelia* can be distinguished from other *P. aurelia* species by a characteristic random amplification of polymorphic DNA (RAPD) pattern (Stoeck and Schmidt 1998). Further detailed analysis revealed that the same RAPD pattern occurred in 14 *P. novaurelia* strains, but the additional band patterns observed during the analysis

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presented four 'genotypes' mostly correlated with geographical origin (Stoeck et al. 2000a). RAPD fingerprints were later used by Przyboś et al. (2006) in order to analyse the differentiation of the newly identified strains of *P. novaurelia* from Russia and other European strains, which showed correlations between geographic and genetic distances. Several types of band patterns within *P. novaurelia* were also observed by Przyboś et al. (2007a), who compared strains from Asia, Europe, and North America, and identified two main groups of strains. However, due to the restricted number of characters that can be analysed, RAPD data are inferior to sequence data. Thus, the interpretation of RAPD results is insufficient for solving phylogenetic problems (Stoeck et al. 2000b). Sequencing and comparing homologous DNA fragments is more promising, but in previous studies, only a few *P. novaurelia* strains were used. For example, two *P. novaurelia* strains from Germany were used for the analysis of an ITS1-5.8S-ITS2 ribosomal DNA (rDNA) variation within the complex of *P. aurelia* (Coleman 2005). Similarly, two *P. novaurelia* strains were used in an investigation of relationships between strains in the genus *Paramecium* with the *hsp70* gene (Hori et al. 2006).

Until now, the most common DNA sequences used in phylogenetic studies across the genus *Paramecium* have been rDNA fragments and part of the cytochrome c oxidase subunit 1 mitochondrial DNA (*COI* mtDNA) gene. Ribosomal ITS1-5.8-ITS2 sequences have been used in molecular studies of *P. bursaria* (Greczek-Stachura et al. 2010; Hoshina et al. 2006), of the *P. aurelia* species complex (Coleman 2005), and of *P. caudatum* and *P. multimicronucleatum* (Barth et al. 2006). The mitochondrial part of *COI* mtDNA, which is characterised by much greater variability, was examined in two species for the first time: *P. caudatum* and *P. multimicronucleatum* (Barth et al. 2006). Previous analyses showed that the *COI* fragment was differentiated, while the ribosomal fragments were identical. This was observed not only in the genus *Paramecium* (Barth et al. 2006; Przyboś et al. 2012), but also in other ciliate genera, for example, *Tetrahymena* (Lynn and Strüder-Kypke 2006). The *COI* fragment was also used to analyse relationships in nine *P. novaurelia* strains (Przyboś et al. 2007a), revealing two clusters and one separate branch. The obtained tree topology was not exactly in contradiction to the geographical origin of the studied strains.

This article presents the first study concerning genetic relationships within 25 strains of one member of the *P. aurelia* complex, originating from distant localities, using ribosomal and mitochondrial loci. It is an expansion of previous work (Przyboś et al. 2007a) and targets the relationships between genetic profile and geographical locality.

In the current study, instead of the 3′ small subunit (SSU) rDNA fragment (there were no differences among the studied *P. novaurelia* and *P. caudatum* sequences, Przyboś et al. 2007a), the variable ITS2 region was analysed. Sequencing a larger number of strains and more variable regions of the ribosomal DNA enables a more exact analysis of the relationships within particular members of the *P. aurelia* species complex and may provide new information about the diversity and evolution of eukaryotic microorganisms such as *Paramecium*.

Material and Methods

Material

The study examined strains of *P. novaurelia* from different geographical regions (Table 1). They were kept in the collection of the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Cracow. *Paramecia* were cultivated on lettuce medium inoculated with *Enterobacter aerogenes*, according to Sonneborn (1970).

Molecular methods

Paramecium genomic DNA was isolated from vegetative cells at the end of the exponential phase (approx. 1000 cells were used for DNA extraction) using a NucleoSpin Tissue Kit (Macherey-Nagel, Germany), according to the manufacturer's instructions for DNA isolation from cell cultures. The only modification was centrifugation of the cell culture for 20 min at 13 200 rpm. Then, the supernatant was removed and the remaining cells were suspended in lysis buffers and proteinase K.

The primers used for polymerase chain reaction (PCR) reactions are listed in Table 2. PCR amplification of both ribosomal fragments (ITS1-5.8S-ITS2 and 5′ large subunit rDNA (5′LSU rDNA)) was carried out in a final volume of 40 μl containing: 30 ng of DNA, 1.5 U Taq-Polymerase (Qiagen™, Germany), 0.8 μl 20 mM of each primer, 10× PCR buffer and 0.8 μl of 10 mM deoxy nucleotide triphosphates (dNTPs) in a TPersonal Thermocycler™ (Biometra GmbH, Göttingen, Germany) or Mastercycler-ep™ (Eppendorf GmbH, Germany). The amplification protocol consisted of initial denaturation at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s and extension at 72 °C for 60 s, with the final extension at 72°C for 5 min. Amplification of the *COI* gene fragment was carried out in the same volume as in the case of ribosomal fragments (see above). The protocol followed Barth et al. (2006).

After amplification, PCR products were electrophoresed in 1% agarose gel for 45 min at 85 V with a DNA molecular-weight marker (Mass Ruler Low Range DNA Ladder, Fermentas, Vilnius, Lithuania). NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) was used for purifying PCR reactions. In some PCR reactions, additional sub-bands were obtained apart from the expected band. In these cases, 30 μl of each PCR product was separated on 1.8% agarose gel (100 V/60 min) with a DNA molecular-weight marker (Mass Ruler Low Range DNA Ladder, Fermentas, Lithuania). Then, the band representing the examined fragment was cut out and purified.