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ORIGINAL ARTICLE

Worldwide sampling reveals low genetic variability in populations of the freshwater ciliate *Paramecium biaurelia* (*P. aurelia* species complex, Ciliophora, Protozoa)

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Abstract
Species (or cryptic species) identification in microbial eukaryotes often requires a combined morphological and molecular approach, and if possible, mating reaction tests that confirm, for example, that distant populations are in fact one species. We used *P. biaurelia* (one of the 15 cryptic species of the *P. aurelia* complex) collected worldwide from 92 sampling points over 62 years and analyzed with the three above mentioned approaches as a model for testing protistan biogeography hypotheses. Our results indicated that despite the large distance between them, most of the studied populations of *P. biaurelia* do not differ from each other (rDNA fragment), or differ only slightly (*COI* mtDNA fragment). These results could suggest that in the past, the predecessors of the present *P. biaurelia* population experienced a bottleneck event, and that its current distribution is the result of recent dispersal by natural or anthropogenic factors. Another possible explanation for the low level of genetic diversity despite the huge distances between the collecting sites could be a slow rate of mutation of the studied DNA fragments, as has been found in some other species of the *P. aurelia* complex. COI haplotypes determined from samples obtained during field research conducted in 2015–2016 in 28 locations/374 sampling points in southern Poland were shared with other, often distant *P. biaurelia* populations. In the Kraków area, we found 5 of the 11 currently known COI *P. biaurelia* haplotypes. In 5 of 7 reservoirs from which *P. biaurelia* was obtained, two different COI haplotypes were identified.

Keywords Ciliates · *Paramecium aurelia* complex · Microbial biogeography and distribution · Haplotype variability · Field research

Introduction

Although members of genera like *Paramecium* or *Tetrahymena* have become objects of research in many fields of life sciences (Kodama and Fujishima 2014; Picariello et al. 2014; Tomazic et al. 2014; Benbouzid et al. 2015), data from populations of particular species living in natural habitats remain scarce (Katz and Turkewitz 2013). Therefore estimating and evaluating the species composition, biodiversity and distribution of a variety of ecosystems is crucial for generating hypotheses on the course of microevolution, ecology and potential protection of ciliates and other microbial eukaryotes. As other free-living protists, ciliates play a key ecological role in various types of environment (Weisse 2006; Pawlowski et al. 2012). However, in many cases determining the boundaries between eukaryotic microbial species is difficult due to the lack of a strict species definition (Boenigk et al. 2012), scarce data on natural populations (Heger et al. 2014) as well as existence of cryptic species (Nanney and McCoy 1976; Gentekaki and Lynn 2010; McManus et al. 2010; Katz et al. 2011).

In the genus *Paramecium*, strain crosses and molecular analyses have revealed the occurrence of cryptic differentiation (for example Sonneborn 1975; Greczek-Stachura et al. 2012; Tarcz et al. 2014; Przyboś and Tarcz 2016) in the majority of the 19 valid morphospecies (Fokin 2010/2011;

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Krenek et al. 2015). One of these is *Paramecium aurelia*, which is in fact a complex of 15 sibling species (Sonneborn 1975; Aufderheide et al. 1983) that are morphologically indistinguishable but sexually isolated. However reciprocal relationships within the *P. aurelia* species complex are still not clear because in some strains inconsistencies have been found between DNA fragment comparisons and mating test classifications (Catania et al. 2009), which may be the result of incomplete lineage sorting or hybridization-introgression processes (Tarcz et al. 2013). Although the latter issue is rare in microbial eukaryotes and rather concerns parasitic species (Messenger et al. 2012), interspecies mating reactions between some species of the *P. aurelia* complex have been reported (Sonneborn 1975). In order to avoid misidentification during species delimitation, a combination of different (molecular, morphological and physiological) approaches should be applied (Duff et al. 2008; Caron 2013; Agatha and Strüder-Kypke 2014). Thus, in *P. aurelia* complex for proper cryptic species designation different approaches are used: comparing cell morphology features, characteristics of the nuclear apparatus, testing genetic isolation by strain crosses, and analysis of DNA fragment variability. Until now two DNA regions have most frequently been used in *P. aurelia* studies: ribosomal (ITS1-5.8S-ITS2-5'LSU rDNA), and mitochondrial (a part of *COI* gene) regions (Tarcz et al. 2013). Moreover these regions have been proposed as a DNA barcoding tool for other ciliates (Strüder-Kypke and Lynn 2010; Stoeck et al. 2014b), and have been used as a markers for population studies in particular ciliates (Gentekaki and Lynn 2009; Zufall et al. 2012) as well as in several *Paramecium* species (Snoke et al. 2006; Zhao et al. 2013).

Faunistic data on the *P. aurelia* complex (Sonneborn 1975; Przyboś and Surmacz 2010) can be used to recognize the existence of cryptic species with either a narrow (for example *P. undecaurelia*) or wide range of occurrence (for example *P. biaurelia*). The latter seem to be ideal objects of research in the fields of wide microbial biogeography and population genetics. *Paramecium biaurelia*, according to Sonneborn (1975), is found in Europe, Asia, Australia and New Zealand, North and South America and is generally common in cold to moderate climates and never found in the tropics. The cell length of *P. biaurelia* is about 133 μm (Fokin and Chivilev 2000) and its characteristic feature, which distinguishes it from the other *P. aurelia* species, is that it carries out mating reactions below 21 °C and between 9 PM and 7 AM (Sonneborn 1975). Importantly, there is no evidence for gene flow between *P. biaurelia* and other *P. aurelia* species (Sonneborn 1975). A preliminary molecular survey of 12 strains originating from distant sampling sites (Tarcz et al. 2013) showed that *P. biaurelia* forms a well separated clade, distinct form other species of the complex and reveals very low genetic variability.

Due to the sparse data on the spatial variability of other free-living, freshwater microbial eukaryotes it could be supposed that more accurate sampling of distant as well as local populations may reveal previously hidden genetic variation in *P. biaurelia*.

Materials and methods

Strain maintenance

Most strains used in the present study are part of the strain collection of the Department of Experimental Zoology, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences. Ciliates were collected between 1954 and 2016 and identified as *P. biaurelia* according to the Sonneborn method (1970) by researchers working on *Paramecium* (E. Przyboś and others (see supplementary Table S1), sent by collaborators, or donated (mainly standard strains) by Prof. T. M. Sonneborn (Department of Biology, Indiana University, Bloomington, USA) and Prof. G. H. Beale (Institute of Genetics, Edinburgh University, Scotland, UK)). Strains were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes* according to Sonneborn's method (1950, 1970) and supplemented with 0.8 mg/ml ß sitosterol (Merck, Darmstadt, Germany). For phylogenetic analysis, we used 139 strains of *P. biaurelia* originating from Europe, Asia, Australia, and North and South America (Fig. 1).

Molecular techniques

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 that the cell culture was centrifuged for 20 min at 13,200 rpm. The supernatant was then removed and the remaining cells were suspended in lysis buffers and proteinase K. Fragments of rDNA (1062 bp) and *COI* (638 bp) genes were amplified, sequenced and analyzed. The rDNA fragment was amplified with an ITS1 universal eukaryotic primer (5′-TCCGTAGGTGAACCTGCGG-3′ (White et al. 1990)) and a 3pLSU primer (5′-CAAG ACGGGTCAGTAGAAGCC-3′ using the protocol previously described by (Tarcz et al. 2012)). The *COI* fragment of mitochondrial DNA was amplified with the primer pair F388dT (5′-TGTAAAACGACGGCCAGTGG wkCbAAAGATGTwGC-3′) and R1184dT (5′-CAGG AAACAGCTATGACTAdACyTCAGGGTG ACCrAAAAATCA-3′) using the protocol previously described by (Strüder-Kypke and Lynn 2010). The composition of the reaction mixtures, PCR programs, and purification

Fig. 1 The origin ($N = 92$) of *Paramecium biaurelia* strains used in present studies

procedure were presented in detail in Tarcz et al. (2012). Cycle sequencing was done in both directions with BigDye Terminator v3.1 chemistry (Applied Biosystems, USA). The primers used in the PCR reactions were again used for sequencing the rDNA region and the primer pair M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-CAGG AAACAGCTATGAC-3′) (Strüder-Kypke and Lynn 2010) was used for sequencing the *COI* fragment. The details of the sequencing procedure were as in Tarcz et al. (2012). The sequences of both studied fragments are available in the NCBI GenBank database (see supplementary Table S1).

Data analysis

Sequences were examined using Chromas Lite (Technelysium, Australia) to evaluate and correct chromatograms. Alignments of the studied sequences were performed using BioEdit software (Hall 1999) and checked manually. All the obtained sequences were unambiguous and were used in the later analyses. Phylograms were constructed for the studied fragments in Mega v6.0 (Tamura et al. 2013), using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). All positions containing gaps and missing data were eliminated. NJ analysis was performed in Mega 6.0 by bootstrapping with 1000 replicates. MP analysis was evaluated with a min-min heuristic parameter (at level 2) and bootstrapping with 1000 replicates. Mega 6.0 identified the T92 model for the ITS1-5.8S-ITS2-5′ end of LSU rDNA and for *COI* mtDNA as the best nucleotide substitution model for maximum likelihood tree reconstruction. Bayesian inference (BI) was performed in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003); the analysis was run for 5,000,000 generations and trees were sampled every 100 generations. All trees for BI analysis were visualized in TreeView 1.6.6 (Page 1996). Analysis of haplotype diversity (Hd) and its sampling variance (SD), nucleotide diversity (π), and polymorphic sites was done in DnaSP v5.10.01 (Librado and Rozas 2009). Haplotype networks, which presented the distribution and relationships among haplotypes of *P. biaurelia* strains, were reconstructed with the Median Joining method (Bandelt et al. 1999), as implemented in PopART v. 1.7 (Leigh and Bryant 2015).

Results

Variability of the studied DNA fragments within the *Paramecium biaurelia* strains

In the present study, we sampled 123 strains (from the ISEA PAS collection) in terms of both analyzed DNA fragments (12 of them were studied previously in Tarcz et al. 2013). Moreover for the molecular analysis we included an extra 2 (in the case of rDNA fragment) and 16 (in the case of *COI* mtDNA fragment) sequences from GenBank. Altogether 92 sampling points were tested, with most of them from the following ecozones: the Western Palearctic (67), Central Palearctic (10), Nearctic (8),

Eastern Palearctic (4), Australasia (2) and finally one location in the Neotropic (Supplementary Table S1).

Studies on the ITS1-5.5S-ITS2-5'LSU rDNA fragment showed that all ($N = 125$) studied *P. biaurelia* strains were almost identical—only three haplotypes (Pa2RD_01, Pa2RD_02 and Pa2RD_03) were identified (Table S1). Intraspecific haplotype diversity among the studied *P. biaurelia* strains ($N = 125$) was Hd = 0.032 (0.00048), and nucleotide diversity was $\pi = 0.00003$. The mean divergence over all *P. biaurelia* ($N = 125$) sequence pairs was 0.000 (ranging from 0.000 to 0.001), and only 2 variable positions (0 parsimony informative) were detected in the ITS1-5.5S-ITS2-5'LSU rDNA fragment (1062 bp).

In the *COI* mtDNA dataset intraspecific haplotype diversity among the studied *P. biaurelia* strains ($N = 139$) was Hd = 0.626 (0.00144), which indicates that the studied DNA fragment has rather low variability. Nucleotide diversity amounted to $\pi = 0.00248$. The mean divergence over all *P. biaurelia* ($N = 139$) sequence pairs was 0.002 (ranging from 0.000 to 0.016) and only 18 variable positions (7 parsimony informative) were detected in the *COI* fragment (638 bp). A total of 11 *COI* haplotypes were found in the studied dataset (Table S1). Notably, 6 of the 11 were private haplotypes (Pa2COI_01, Pa2COI_07, Pa2COI_08, Pa2COI_09, Pa2COI_10, Pa2COI_11) and the remaining 5 (Pa2COI_02, Pa2COI_03, Pa2COI_04, Pa2COI_05, Pa2COI_06) were shared between 133 of 139 strains of *P. biaurelia*. The most frequent haplotype was Pa2COI_03, observed in 79 isolates, followed by Pa2COI_02 in 28 *P. biaurelia* strains, Pa2COI_04 in 15 strains, Pa2COI_06 in 7 strains, and finally Pa2COI_05, which was characteristic for 4 strains from one location (Table S1). For better understanding and to preserve continuity between previous and future *Paramecium* studies, we decided to number the individual haplotypes according to the formula first used by Barth et al. (2006).

Inter- and intraspecific relationships of *P. biaurelia* species based on the comparison of ITS1-5.8S-ITS2-5'LSU rDNA and *COI* mtDNA sequences

Based on the obtained rDNA and *COI* sequences and the constructed tree we found that *P. aurelia*, *P. jenningsi* complexes together with *P. schewiakoffi* form a monophyletic clade distant from *P. caudatum* and *P. multimicronucleatum*, which were used as an outgroup in the present study (Figs. 2 and 3).

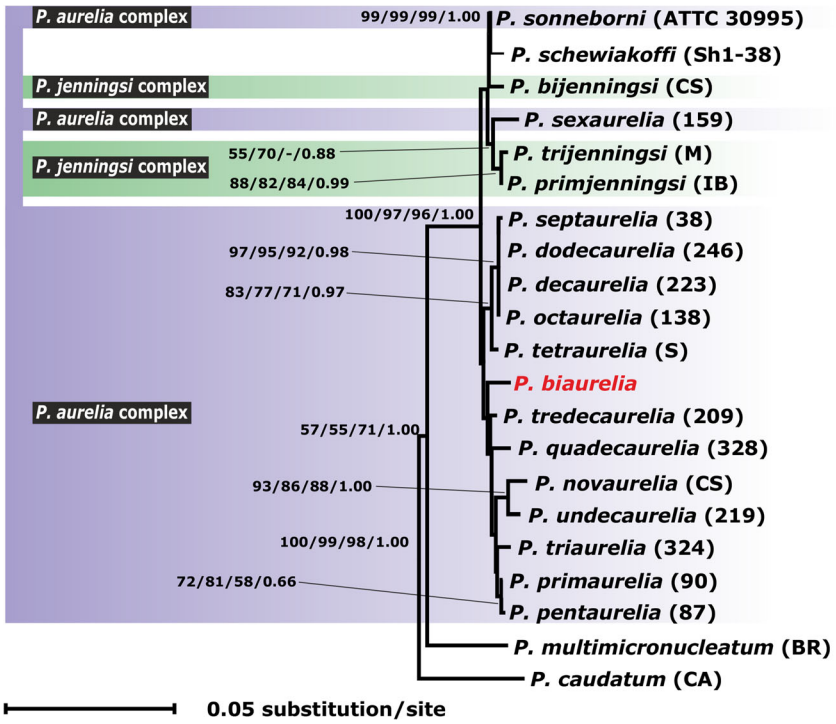

Fig. 2 Phylogenetic tree constructed for *Paramecium aurelia* complex, *P. jenningsi* complex and *P. schewiakoffi* (two species: *P. caudatum* and *P. multimicronucleatum* were used as an outgroup). The tree was constructed on the basis of a comparison of sequences from the ribosomal ITS1-5.8S-ITS2-5'LSU fragment using the maximum likelihood method. Bootstrap values for neighbor joining, maximum parsimony, maximum likelihood, and posterior probabilities for Bayesian inference are presented. Bootstrap values smaller than 50% (posterior probabilities < 0.50) are not shown. Dashes represent no bootstrap or posterior value at a given node. All positions containing gaps and missing data were eliminated. Phylogenetic analyses were conducted using MEGA 6.0 (NJ/ML) and MrBayes 3.1.2 (BI). The analysis involved 145 nucleotide sequences. There was a total of 1062 positions in the final dataset

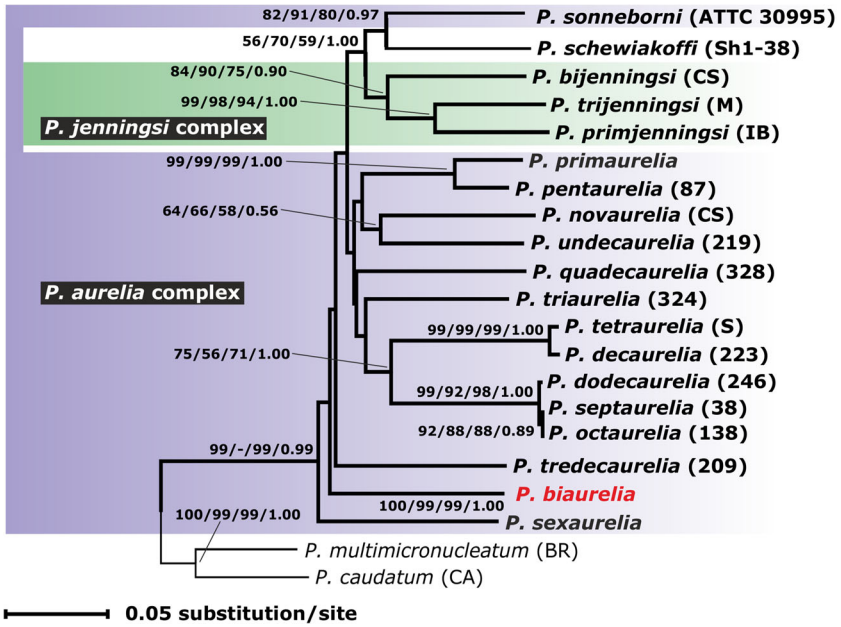

Fig. 3 Phylogenetic tree constructed for *Paramecium aurelia* complex, *P. jenningsi* complex and *P. schewiakoffi* (two species: *P. caudatum* and *P. multimicronucleatum* were used as an outgroup). The tree was constructed on the basis of a comparison of sequences from the mitochondrial *COI* fragment using the maximum likelihood method. Bootstrap values for neighbor joining, maximum parsimony, maximum likelihood, and posterior probabilities for Bayesian inference are presented. Bootstrap values smaller than 50% (posterior probabilities < 0.50) are not shown. Dashes represent no bootstrap or posterior value at a given node. All positions containing gaps and missing data were eliminated. Phylogenetic analyses were conducted using MEGA 6.0 (NJ/ML) and MrBayes 3.1.2 (BI). The analysis involved 159 nucleotide sequences. There was a total of 638 positions in the final dataset

Similarly, *P. biaurelia* forms a well-defined monophyletic clade within the *P. aurelia* complex and the other species of the subgenus *Paramecium* (Figs. 2 and 3). In the case of ribosomal data, *P. biaurelia* is situated close to *P. tredecaurelia* and *P. quadecaurelia* (Fig. 2). The intraspecific relationships of *P. biaurelia* rDNA haplotypes (Figs. 4a and 5a) indicate the existence of one dominating haplotype Pa2RD_01 ($N = 123$) and two private haplotypes Pa2RD_02, Pa2RD_03, which are different from Pa2RD_01 by one nucleotide substitution.

In turn, the *COI* dataset revealed that *P. biaurelia* is rather distant from the other *P. aurelia* species and is situated between the *P. sexaurelia* and *P. tredecaurelia* branches (Fig. 3). The intraspecific relationships of *P. biaurelia COI* haplotypes (Figs. 4b and 5b) showed the existence of a dominating haplotype located in the central part of the network—Pa2COI_03 ($N = 79$). Most *P. biaurelia* COI haplotypes are directly connected with Pa2COI_03 and differ from it by one to three substitutions. It is worth noting that there are only 10 nucleotide substitutions between the most distant haplotypes (Pa2COI_10 and Pa2COI_11) (Figs. 4b and 5b).

The biogeographical pattern within the studied *P. biaurelia* strains

The most frequent haplotype, Pa2RD_01 was observed in 123 isolates (Palearctic, Nearctic, Australasia ecozones), while haplotypes Pa2RD_02 and Pa2RD_03 were characteristic for MbIII.3 (Münster, Botanical Garden) and US_Bl 11III1 (Indiana, Bloomington, Miller Showers Park) strains, respectively (Fig. 4a, Table S1). The COI fragment median-joining network showed that haplotype Pa2COI_03 ($N = 79$) was found in the Palearctic, Nearctic and Australasia ecozones (Fig. 4b, Table S1). The second most common haplotype (Pa2COI_02; $N = 28$) was from Europe and the Central Palearctic. The remaining haplotypes with broader geographical range (Pa2COI_04; $N = 15$) were identified from four sampling points in the Eastern and Western Palearctic, Nearctic and Australasia, as well as (PaCOI_06; $N = 7$) from the Western Palearctic and Nearctic realms. Finally there were seven rare haplotypes with restricted distributions: Pa2COI_05 (four sampling points in Boston, USA), Pa2COI_01 (one sampling point in Scotland, Great Britain), Pa2COI_07, Pa2COI_08, Pa2COI_10 (three different sampling points in Poland), Pa2COI_09 from North Carolina, USA and Pa2COI_11 from Chile. The constructed COI (similarly as rDNA) network revealed no median vectors or reticulate patterns between the obtained haplotypes. Despite intercontinental sampling, most (8 of 11 haplotypes) of the identified *P. biaurelia* COI haplotypes were only present in strains originating from Europe (except Pa2COI_05, 09, 11), and (7 of 11 haplotypes) Poland (Fig. 4b, Table S1).

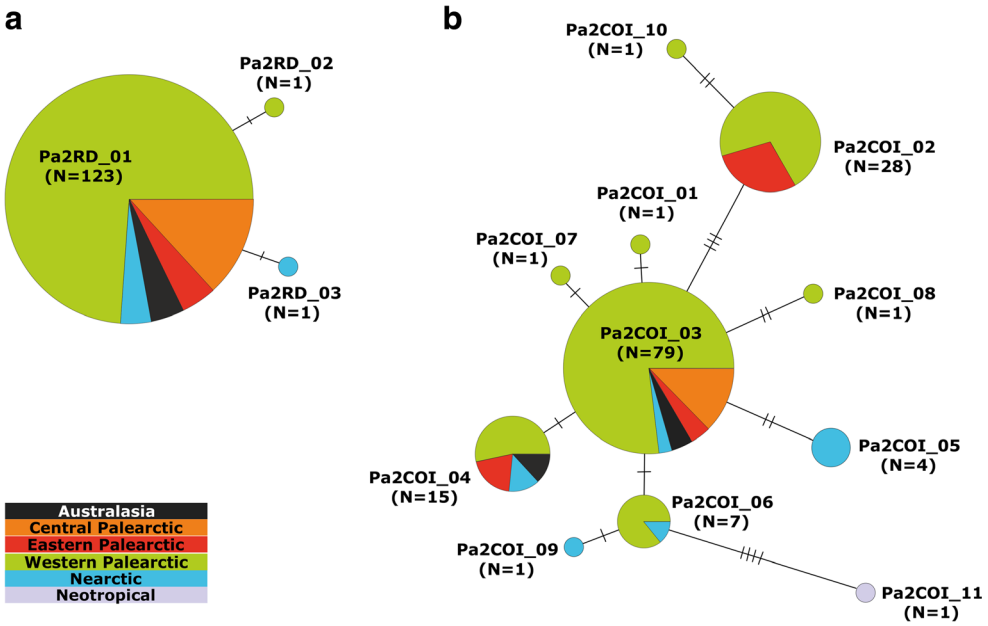

Fig. 4 Haplotype network of *Paramecium biaurelia* constructed using the 123 sequences of ribosomal ITS1-5.8S-ITS2-5'LSU fragments (**a**) and 139 of mitochondrial *COI* fragments (**b**). The network presents reciprocal relationships between, and the origin of *P. biaurelia* haplotypes identified in current study. Black dashes on particular branches represent nucleotide substitutions between particular haplotypes. Analyses were conducted using the Median Joining method in PopART software v. 1.7

Analysis of *P. biaurelia* natural population variability

In order to more accurately analyze molecular variability of *P. biaurelia* populations, we conducted field research (seasons 2015–2016) by sampling 49 water reservoirs in 28 locations (Southern Poland, mainly Kraków), which finally gave 177 sampling points altogether. Some reservoirs were sampled 2–6 times, which consequently led to 374 examined sampling points; *P. biaurelia* was detected in 3.74% ($N = 14$) of them (Table S2). In the studied area (within the administrative boundaries of Kraków, and one sampling point in Pieskowa Skała about 20 km northwest of Kraków), based on genetic crosses (the biological species concept) and *COI* mtDNA analysis (the phylogenetic species concept), we identified 4 out of the 11 known *P. biaurelia COI* haplotypes (no differences were detected in the rDNA fragment). All identified haplotypes were shared with the other, often distant *P. biaurelia* populations. A comparison of the obtained *COI* data with the other studied *P. biaurelia* strains showed that 5 of the 11 known haplotypes of the global *COI* variability were detected within the Kraków area (Fig. 5b, Table S2). In 5 of the 7 reservoirs in which *P. biaurelia* was identified, two different COI haplotypes were detected (at different sampling points) (Fig. 6, Table S2).

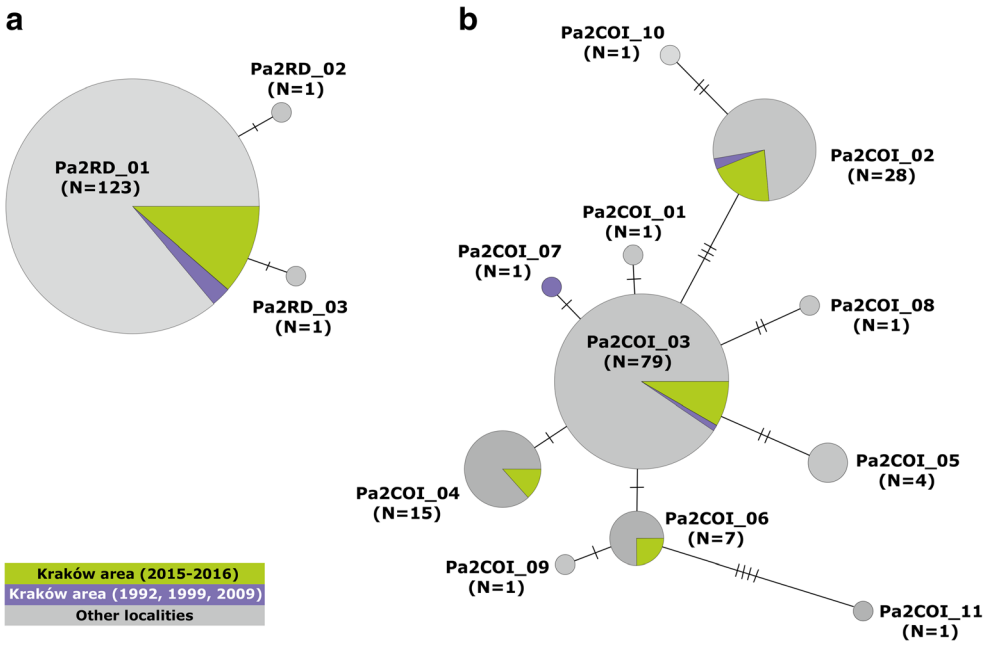

Fig. 5 Haplotype network of *Paramecium biaurelia* constructed using the 123 sequences of ribosomal ITS1-5.8S-ITS2-5'LSU fragments (**a**) and 139 of mitochondrial *COI* fragments (**b**). The network presents a comparison of haplotypes obtained in the Kraków area vs. the other localities, where molecular data for *P. biaurelia* is available. Black dashes on particular branches represent nucleotide substitutions between particular haplotypes. Analyses were conducted using the Median Joining method in PopART software v. 1.7

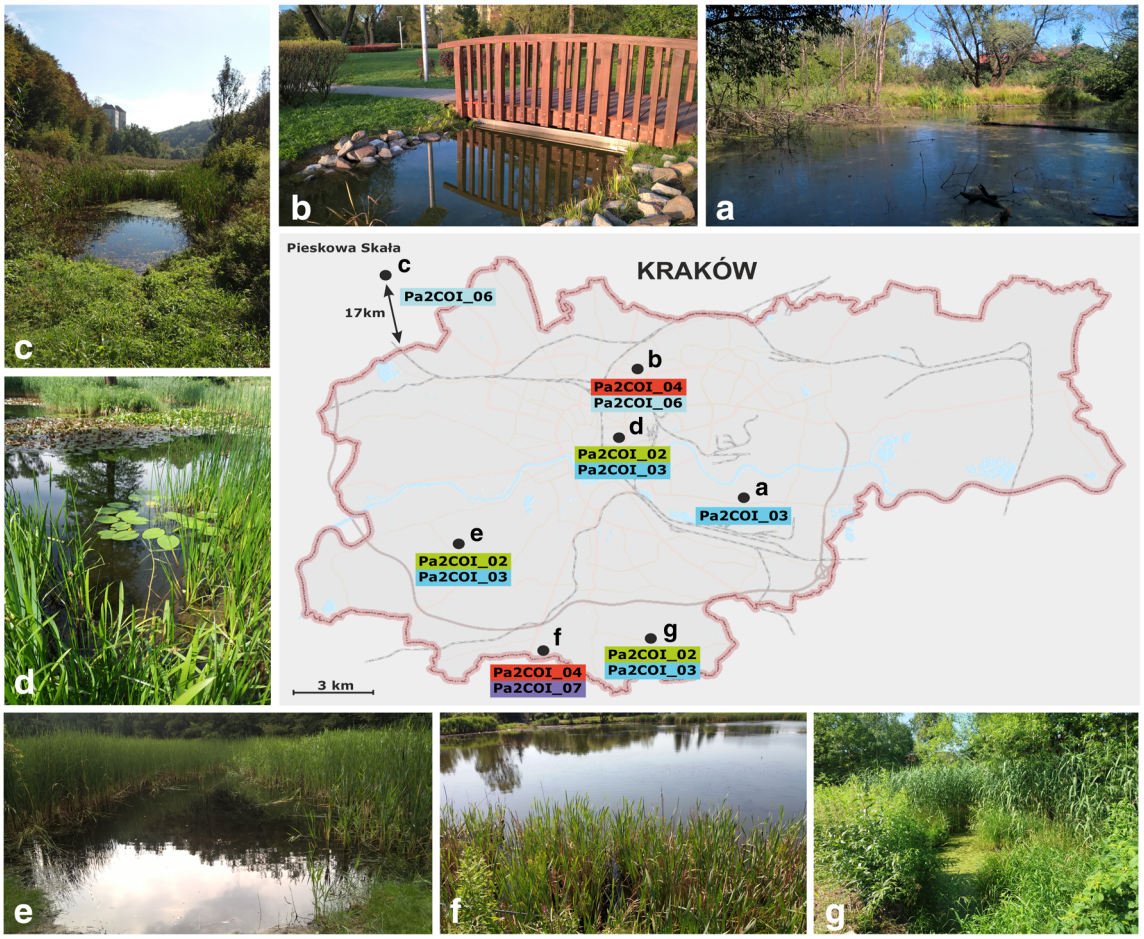

Fig. 6 Map of sampling sites of *Paramecium biaurelia* strains collected during field research in the Kraków area. **a** Kraków, "At the brickyard" pond, 1 sampling point. **b** Kraków, Zaczarowana Dorożka Park (pond), 2 sampling points. **c** Pieskowa Skała (pond), 1 sampling point. **d** Kraków, Botanical Garden (3 ponds), 6 sampling points. **e** Kraków, Królówka (pond), 2 sampling points. **f** Kraków, Opatkowice (pond), 2 sampling points. **g** Kraków, Rajsko (pond), 2 sampling points

The occurrence of different COI haplotypes in one sampling point or even in one water sample has been observed before at locations outside the Kraków area—for example in a small river near Moscow three COI haplotypes were identified (Table S1).

Discussion

Paramecium biaurelia as a good model for testing microbial biogeography

Despite ciliates from the *Paramecium* genus being one of the most characteristic microorganisms of aquatic ecosystems, known since the seventeenth century thanks to Leeuwenhoek's observations, their biogeography and distribution remain poorly understood compared with animals or plants (Azovsky et al. 2016). This problem is similar among most other protists. Currently two different models describe the observed distribution patterns of microbial eukaryotes—the "ubiquity model" (UM) (Fenchel and Finlay 2004; Finlay et al. 2006) and the "moderate endemicity model" (MEM) (Foissner 2006; Foissner et al. 2008). At present, despite intensive debate over these hypotheses neither has been fully confirmed. Reasons for this are twofold: firstly, the difficulty of defining a species in protistology (Caron 2013), which is crucial for shaping protist biogeography (Finlay 1998; Dijoux et al. 2014), and secondly—an under-sampling of particular species, which precludes determining whether they have wide or narrow ranges (Foissner 2006; Caron et al. 2009; Fokin 2010/2011).

Recent developments in molecular analyses have given rise to new possibilities not only for testing the UM and MEM biogeography models (Darling et al. 2007; Watts et al. 2011), but also for more easily designating protist species (Chantangsi et al. 2007; Gentekaki and Lynn 2010), testing their phylogenetic relationships (Kosakyan et al. 2013; Lahr et al. 2013) or even assessing the biodiversity of entire ecosystems (Sogin et al. 2006; Stoeck et al. 2014a; Lara et al. 2015; Pawlowski 2015). The latter issue, based on next generation sequencing technology allows for the most accurate

examination of protistan diversity (Mahe et al. 2015) through the detection of species or molecular operational taxonomic units (MOTU) composition. However, for the obtained data to be useful for identifying new species, they must be matched with previously described taxons by comparing them with sequences deposited in databases like GenBank or BOLD (Thomsen and Willerslev 2015). Moreover, there exists the potential for the accidental sequencing of paralogs of study genes (West et al. 2014) as well as of orthologs from environmental samples (Thomsen and Willerslev 2015), which would make it almost impossible to study the biogeographical occurrence of some protist species. An alternative to the above and proposed in the current study may be the collection of samples of particular, well-defined species from as many sampling points as possible. Species (or cryptic species) identification in microbial eukaryotes often requires at least a morpho-molecular approach (Boenigk et al. 2012), and if possible, mating reaction tests that confirm distant populations to be in fact one species. Thus, we suppose that *P. biaurelia* collected worldwide from 92 sampling points over 62 years and then analyzed with the three above presented approaches seems to be an appropriate model for testing protistan biogeography hypotheses.

The reasons for the wide distribution of *Paramecium biaurelia*

The occurrence of several species from the *P. aurelia* complex may be restricted by temperature barriers, as *P. quadecaurelia* is restricted to the tropical zone (Przyboś et al. 2013), and *P. sexaurelia*, *P. octaurelia* as well as *P. sonneborni* to the warm zone (Przyboś et al. 2014, 2015; Przyboś and Prajer 2015). On the other hand, *Paramecium biaurelia* is one of the species of the *P. aurelia* complex that is characterized by wide distribution in areas with colder climates. Moreover, many years of sampling (since 1959) of different regions of Poland have revealed that it is a common species, recorded in 88 habitats (40.36%) among 218 studied (Przyboś et al. 2011). *P. biaurelia's* frequent occurrence could be explained by fact that it is capable of invading marginal habitats, and adapts to circumstances that enforce inbreeding, such as intraclonal mating. Gill and Hairston (1972) found that *P. biaurelia* inhabits nearly all parts of two investigated woodland seeps at very low density over the whole year, in contrast to other species that occurred only at various locations at different times. Furthermore, this species is less sensitive to low temperatures (Hairston 1958), low food levels (Hairston and Kellermann 1965) and bacterial endosymbionts (Beale and Preer 2008) that may be toxic to other species of the complex. Therefore, the role of competition between species from the same ecological niche may be an important factor shaping the observed diversity of occurrence of particular *P. aurelia* species (Hairston 1958; Hairston and Kellermann 1965). It has been shown that *P. biaurelia* can exert a dominating or inhibitory effect on other paramecia (*P. triaurelia*), probably caused by the presence of killer strains in this species (*P. biaurelia*) (Beale and Preer 2008). In fact this species is characterized by the greatest variety of endosymbionts among the whole *P. aurelia* complex (Sonneborn 1974). The above characters lead to the conclusion that *P. biaurelia* is a species with superior fitness for inhabiting new, unpromising ecological conditions and could be called a pioneer species. In summary, a high adaptive capacity for new environmental conditions (Gill and Hairston 1972) as well as a vast population size (Finlay 2002) may be key factors for the wide distribution of *P. biaurelia*. In contrast, small population size causes restricted gene flow and restricted distribution in the other flagship ciliate—*Tetrahymena thermophila* (Zufall et al. 2012).

Explanation of the observed low genetic variation of *P. biaurelia*

The comparison of COI sequences from 139 *P. biaurelia* strains (Table S1) revealed the existence of 11 haplotypes of which only 4 were frequent in the studied dataset and observed on different continents whereas 6 of them appeared only once in the studied dataset and finally one haplotype has been identified in 4 strains in one location (Fig. 4b). Moreover, the number of nucleotide differences between particular haplotypes did not exceed four (usually only one or two) substitutions.

An explanation of the obtained results may be that the *P. biaurelia* population (predecessors of the present population) went through a bottleneck in the past, and its current distribution is the result of recent dispersal by natural or anthropogenic factors. This hypothesis is supported by fact that the *P. biaurelia* effective population size is smaller than that of the other widespread *Paramecium* species: *P. primaurelia* and *P. tetraurelia* (Snoke et al. 2006). At present, the dispersal of the *P. aurelia* complex (among them *P. biaurelia*) as well as other paramecia is still not completely solved despite several suggestions and attempts at explanation. It is worth noting that the existence of cysts has never been reported in the *Paramecium* genus (Landis 1988; Gutierrez et al. 1988; Beale and Preer 2008). Therefore the passive dispersal of paramecia is possible, with some drops of water on migrating birds, even over long non-stop routes (Hedenström 2010), mammals over further distances (Sonneborn 1975; Coleman 2005) or by insects over shorter distances (Razowski 1996). Furthermore, Foissner (2006) suggested an important role for human activities in the dispersal of microeukaryotes. However, the bottleneck explanation for low molecular

variability contradicts the suggestion that *P. biaurelia* is a pioneer species.

In our opinion extraordinary genome stability is a more likely explanation for the observed low molecular diversity in *P. biaurelia*, and has been reported previously in *P. tetraurelia* (Sung et al. 2012). This hypothesis could be supported by the fact that *P. biaurelia* diverged from the same lineage as *P. tetraurelia* (McGrath et al. 2014) and therefore similarly to *P. tetraurelia* could be characterized by a low mutational rate (Sung et al. 2012) that led to low molecular diversity between strains belonging to the same species. Moreover, it has been supposed that whole genome duplication (WGD) has seemingly not influenced phenotypic innovations in some *P. aurelia* species (McGrath et al. 2014). In contrast, in other taxa WGD events have played an important role in increasing their complexity and molecular diversity, for example in land plants (Van de Peer et al. 2009) or yeast (Thomson et al. 2005).

Sampling the neighborhood—can almost the entire global diversity of *P. biaurelia* be observed in a nearby pond?

As was mentioned above, under-sampling (in all ecozones as well as particular ecosystems/ecological niches) negatively affects biogeographical studies of protists species (Foissner 2006; Fokin 2010/2011), especially in the case of freshwater habitats (Filker et al. 2016). Moreover, it has been shown that detailed sampling of particular species can reveal previously hidden molecular variability. This happened recently both in the model algae species of the genus *Klebsormidium*, where 66% of *rbcL* genotypes from nearctic and palaearctic colonies were identified for the first time (Ryšánek et al. 2015), and in the ciliate *Tetrahymena thermophila*, which is restricted to east coast of the USA (Zufall et al. 2012). Similarly, in five *Paramecium* species (7 populations from the Qingdao neighborhood) as many as 90 *COI* haplotypes were recently identified (Zhao et al. 2013). Studies carried out recently (2015) and previously (1992, 1999) in ponds of the botanical garden in Kraków revealed two different *P. biaurelia* COI haplotypes (Pa2COI_02 and Pa2COI_03) (Przyboś et al. 2016). However, it is worth noting that in the case of the botanical gardens, the observed molecular variability could have been connected with new populations (not only microorganisms but also aquatic invertebrates) having been transported to the water bodies with tropical plants (Kolicka et al. 2015).

The present *P. biaurelia* study revealed that 6 of 11 COI haplotypes occurred only once in the analyzed dataset ($N = 139$), and three of them (Pa2COI_07, Pa2COI_08, Pa2COI_10) were obtained from Poland (Table S1). Due to the observed high incidence in the various ecosystems tested to date (Przyboś et al. 2011) and the ability to invade new habitats (Gill and Hairston 1972), perhaps more accurate sampling of small areas (i.e., one or several ponds) will reveal previously hidden genetic variation of the studied microorganism.

Generally, during the recent sampling (2015–2016) of 28 locations in southern Poland (374 sampling points), we confirmed that *P. biaurelia* is one of the most common species from the *P. aurelia* complex (24% among eight *P. aurelia* species identified) in the studied reservoirs. Interestingly, no new COI haplotypes were revealed, and haplotypes Pa2COI_02, Pa2COI_03 dominated within the studied area, which in turn is in concordance with worldwide *P. biaurelia* analysis (Figs. 5b and 6). Further to the above, in the Kraków area we detected the Pa2COI_04, Pa2COI_06 haplotypes. Therefore it may be concluded that every COI haplotype in the studied dataset ($N = 139$) that appeared in more than one location was observed in the Kraków area (Figs. 5b and 6, Table S1), confirming the global ubiquity of frequent haplotypes and local endemism of private haplotypes as in Ryšánek et al. (2015). On the other hand, the less frequent haplotypes in current dataset (Pa2COI_05, Pa2COI_09, Pa2COI_11) may turn out to be widespread in the Nearctic and Neotropical ecozones (Fig. 4) after careful sampling of these areas. The observed distinctiveness of *P. biaurelia* collected in both Americas (haplotypes Pa2COI_05, Pa2COI_09, Pa2COI_11) is in concordance with a previous analysis of genomic data (883 genes) of 11 strains that suggested a USA-Europe separation (Johri et al. 2017). However, our results, similarly to (Pawlowski et al. 2007), showed that despite isolation by distance, gene flow is still possible between populations from different ecozones.

Another finding of the present sampling of the Kraków area is that in most of the reservoirs in which *P. biaurelia* was detected, two different COI haplotypes were identified (Fig. 6, Table S2). The most plausible explanation of this finding is independent colonization of the reservoirs by different *P. biaurelia* populations, as was observed in *Coleps* sp. (Barth et al. 2008).

We are aware that the majority of the studied *P. biaurelia* COI variability is based on strains from the Palearctic ecozone. However, we expect that more accurate sampling of reservoirs in Australasia, the Southern part of the Ethiopian and Neotropical ecozones as well as the Nearctic ecozone where *P. biaurelia* is very common (Hairston 1958) will bring new data on the distribution of this microbial eukaryote.

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