ISSN 0015-5497, e-ISSN 1734-9168
Folia Biologica (Kraków), vol. 64 (2016), No 4
doi:10.3409/fb64_4.253

Seasonal Variability of the *Paramecium aurelia* Complex in the Botanical Garden of the Jagiellonian University, Kraków – in the Light of Species Composition and *COI* Haplotype Variation*

Ewa PRZYBOŚ, Marta SURMACZ, and Sebastian TARCZ

Accepted September 15, 2016 *Published December 2016*

PRZYBOŚ E., SURMACZ M., TARCZ S. 2016. Seasonal variability of the *Paramecium aurelia* complex in the Botanical Garden of the Jagiellonian University, Kraków – in the light of species composition and *COI* haplotype variation. Folia Biologica (Kraków) **64**: 253-265.

The temporal occurrence of some *Paramecium aurelia* species is still an intriguing problem as cysts were never reported to exist in the *Paramecium* genus. A sequence of species occurrence was studied (by strain crosses and molecular identification) in five water-bodies of the Jagiellonian University Botanical Garden in Kraków in different sampling sites and different seasons of the year. In the current study 20 *P. aurelia* strains were isolated from collected water samples and identified as *P. biaurelia*, *P. tetraurelia, P. sexaurelia* (the first record in Poland), *P. novaurelia* (the first record in the Botanical Garden). Generally only one species was found in the particular water body in a single sampling point in a given season – an exception was observed in the case of some strains of *P. tetraurelia* and *P. sexaurelia*. The latter species were mostly isolated from two water bodies situated in the Palm Houses (higher temperature preference) and *P. biaurelia* with *P. novaurelia* from water bodies located outside (lower temperature preference). Sequencing of the *COI* mtDNA fragment revealed 9 haplotypes in the studied area which were characteristic for particular species. The most variable species was *P. sexaurelia* – 8 strains studied and 3 haplotypes identified. In contrast, *P. novaurelia* has only one haplotype for 6 strains collected in different seasons. The present study supports the hypothesis that botanical garden water bodies may be a hot-spot for microbial eukaryotic species such as *Paramecium*.

Key words: *Paramecium aurelia* spp., botanical garden, temporal occurrence, seasonal variability, *COI* mtDNA, molecular analysis.

Ewa PRZYBOŚ, Marta SURMACZ, Sebastian TARCZ, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków 31-016, Sławkowska 17, Poland.
E-mail: przybos@isez.pan.krakow.pl

The genus *Paramecium* (Protista, Ciliophora, Oligohymenophorea) and within it especially the *Paramecium aurelia* species complex represents a popular model organism applied in different kinds of biological studies. The complex is composed of 15 freshwater species known world-wide (SONNEBORN 1975; AUFDERHEIDE *et al*. 1983), some species are cosmopolitan, others were recorded only in a few or single habitats (SONNEBORN 1975; PRZYBOŚ & FOKIN 2000; PRZYBOŚ & SURMACZ 2010; FOKIN 2010/2011). However, not all parts of the world were studied sufficiently – for example the southern hemisphere is in need of investigation. In North America, only the USA was studied in greater detail, and the majority of species (12 among 15 known) of the complex were recorded there (SONNEBORN 1975; AUFDERHEIDE *et al*. 1983).

Europe has also been studied intensively as 13 of 15 known species of the complex were found there as well as much information on the frequency of species occurrence (PRZYBOŚ & SURMACZ 2010; PRZYBOŚ *et al*. 2014; RAUTIAN *et al*. 2014; PRZYBOŚ & PRAJER 2015). *P. undecaurelia* and *P. quadecaurelia* are the only species not yet recorded in Europe.

The territory of Poland was sampled since 1959 (KOMALA & KOŚCIUSZKO 1959), and the presence of *P. primaurelia, P. biaurelia, P. triaurelia, P. tetraurelia, P. pentaurelia, P. novaurelia,* and *P. dodecaurelia* was recorded (cf. PRZYBOŚ *et al*. 2011) in 218 studied habitats. The most frequent among these species was *P. novaurelia* (found in

*The molecular studies were supported by a grant from the National Science Centre, Poland, no. UMO-2014/15/B/NZ8/00258.

113 habitats), followed by *P. biaurelia* (recorded in 88 habitats), and *P. primaurelia* (in 56 habitats). *P. tetraurelia* was less frequent (found in 20 habitats, among them 10 were in the Tatras, later eliminated by pollution, KOMALA & PRZYBOŚ 1984). Other species (*P. triaurelia, P. pentaurelia*, and *P. dodecaurelia*) were found only in two habitats per species.

The problem of temporal occurrence and distribution of species of the *P. aurelia* complex, despite numerous investigations (HAIRSTON 1958; LANDIS 1986; PRZYBOŚ & KOMALA 1993; PRZYBOŚ & FOKIN 2000; PRZYBOŚ *et al*. 2011), still seems unsolved. Moreover, an intriguing issue stimulating further research is the fact that cysts (as a way of passive dispersal) were never reported in the *Paramecium* genus (LANDIS 1986; GUTIERREZ *et al*. 1988; BEALE & PREER 2008). It is suggested that the only manner of transport is with drops of water on migrating birds or mammals for longer distances (SONNEBORN 1975; COLEMAN 2005), or by insects such as water beetles for shorter distances (RAZOWSKI 1996). In turn, FOISSNER (2006) emphasized that human activities play an important role in dispersal of microorganisms. Moreover, the application of phylogenetic, ecophysiological and environmental data showed that physical environmental parameters (temperature, salinity) are important in shaping the genetic structure of microbial populations (LOWE 2015).

Although many microbial biogeography surveys are focused on global biodiversity (LE BESCOT *et al*. 2016; ROSSI *et al*. 2016), analysis of the variability in a small area significantly complements knowledge on this subject (GENTEKAKI & LYNN 2009; PRZYBOŚ *et al*. 2011). An example of the latter is the current study of *P. aurelia* species occurrence in five water-bodies of the Jagiellonian University Botanical Garden in Kraków. We decided to carry out this analysis as a complex project, testing different sampling sites of particular water-bodies and different seasons of the year, because previous reports KOMALA & PRZYBOŚ 1999; 2000; 2001a,b, PRZYBOŚ & KOMALA 2001) concern the presence of particular species of the *P. aurelia* complex in some water-bodies of the garden, but without systematic investigation. The aim of the present study was to register the *P. aurelia* species (by strain crosses and molecular identification) in the studied water-bodies comparing their appearance in different seasons. Moreover, application of the *COI* mtDNA fragment allowed for an assessment of haplotype diversity between and within identified species obtained from different water-bodies, sampling points and seasons.

Material and Methods

Material

Species of the *P. aurelia* complex as well as other *Paramecium* morpho – species, established from water samples collected in the Botanical Garden of the Jagiellonian University, Kraków are presented in Table 1a.

Table 1a

Characteristics of the studied water-bodies of the Jagiellonian University Botanical Garden in Kraków

Season	Kind of water body	Characteristics of water during sampling		Ambient temp.(°C)	*Paramecium* species present in the samples		
		Temp. (°C)	pH		*P. aurelia* spp.	*P. bursaria*	*P. caudatum*
Spring, 16 April 2015	Pond I	15.2	6.8-7.2	22	+	+	+
	Pond II	15.2	6.8-7.2	22		+	+
	Pond III	14.6	6.4	20	+		
	Palm-house I	23	6.8	30	+		
	Palm-house II	26	7.6	26	+		
Summer, 31 August 2015	Pond I	21	7.2	23.4	+		+
	Pond II	21.5	6.0	24. 4		+	+
	Pond III	20	5.8	23. 8			
	Palm-house I	25.1	6.4	25	+		
	Palm-house II	27.8	5.4	25	+		
Autumn, 19 October 2015	Pond I	8	6.4	9	+		+
	Pond II	10	6.5	16			+
	Pond III	9	5.5	11.9	+	+	+
	Palm-house I	20	6.5	22	+		
	Palm-house II	25	6.5	21	+		

The Botanical Garden founded in 1783 is located east of the Old Town and occupies 9.6 ha. It houses a plant collection from around the world, trees, shrubs, other plants growing outside and in greenhouses. In the garden, there are three ponds, one natural (designated by us as Pond I) with an island situated in the middle of it with the tree *Taxodium distychum* growing on the island, two artificial (designated by us as Pond II and Pond III), and two greenhouses (palm-houses) (Figs 1& 2). One green-

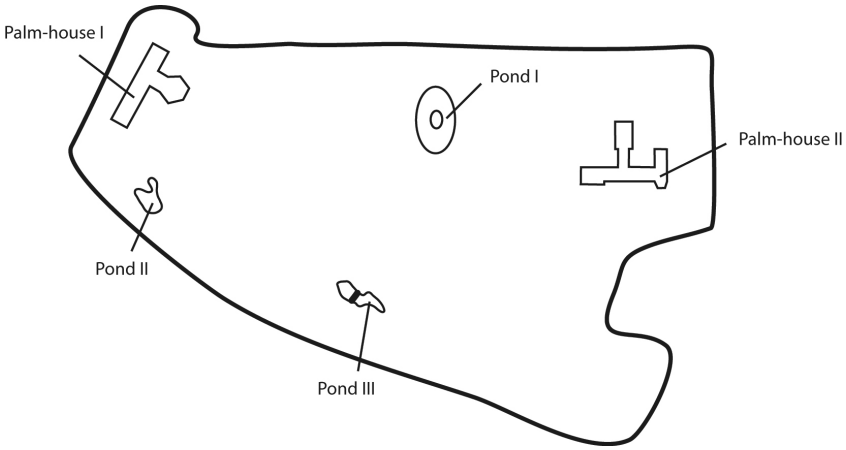

Fig. 1. Sketch map of the Botanical Garden of the Jagiellonian University in Kraków with the marked sampled water – bodies: pond I, pond II, pond III, palm-house I, and palm-house II.

Fig. 2. Photographs of the studied water – bodies. a. pond I, b. pond II, c. pond III, d. Palm-House I, e. Palm-House II.

house (designated by us as palm-house II) is the oldest one in the garden and was especially built for the cultivation in the concrete pond (9 x 7 m) of the Nymphaceae water plants *Victoria regia* (from the Amazon River basin) and *Victoria cruziana* (from Parana, Argentina), it is named the "Victoria" green-house. Tropical and subtropical plants from Africa, South America, and south-eastern Asia grow in the pond and on its banks. The second green-house (designated by us palm-house I) was built mainly for the cultivation of palms but also contains a pond with water plants from Africa, southern and eastern Asia, and Polynesia.

Methods

Methods of collecting samples and establishing strains

The material for investigations was collected in three seasons of 2015, i.e. spring, summer, and autumn from three ponds (one natural and two artificial) and from ponds (reservoirs) situated within two palm-houses. The water samples (2×25 ml) with plankton and plant remnants were collected from four sampling points (s.p., designated 1-4) situated along each of the studied water-bodies, and at a similar distance from each other (Fig. 3). Samples were taken from the surface of water near the edge. During the sampling, the pH and water temperature were measured as well as ambient temperature (Table 1a).

The applied method of sample collection permitted the study of the "microdistribution" of species at a given sampling point. This should reflect the relations between species existing in nature in a particular time and place, seasonality of appearance, and dominance in the particular water bodies.

Paramecia found in the samples (collected in the field) were isolated directly and after the addition of a small quantity of fresh culture medium to the samples. From each sample in which paramecia

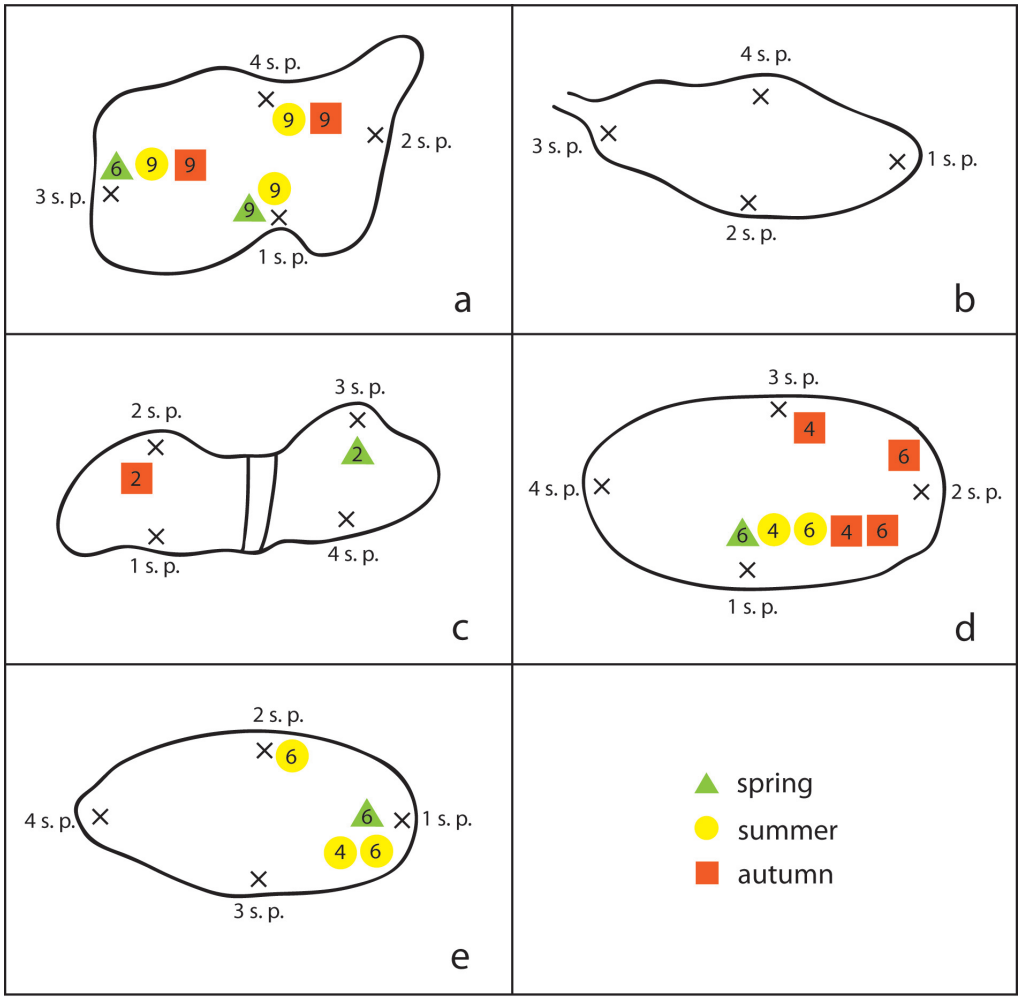

Fig. 3. Occurrence of the *P. aurelia* spp. in the studied water-bodies (a. pond I, b. pond II, c. pond III, d. Palm-House I, e. Palm-House II), and their sampling points (designated by 'x' and numerals) in different seasons. Numbers in green triangles – refer to species revealed in spring collection, in yellow rings – to species from summer collection, in red squares – to species from autumn collection. Number 2 refers to *P. biaurelia*, number 4 to *P. tetraurelia*, number 6 to *P. sexaurelia*, number 9 to *P. novaurelia*.

belonging to the *P. aurelia* spp. complex were found directly after water collection, up to 10 clones were established for species identification. The studied strains of the *P. aurelia* spp. complex are presented in Table 1b.

Other *Paramecium* species, *P. caudatum* and *P. bursaria*, were also identified (Table 1a) in the collected water samples based on an analysis of the type of their micronuclei (VIVIER 1974) on slides stained by acetocarmine (SONNEBORN 1950) as well as the shape of cells.

Identification of established strains of the *P. aurelia* spp.

SONNEBORN's methods (1950, 1970) of cultivation, induction of conjugation, autogamy, and strain crosses were applied. Paramecia were cultivated at 27°C in the lettuce medium prepared from powdered, baked lettuce and distilled water and inoculated with *Enterobacter aerogenes* supplemented with 0.8 mμ/ml β-sitisterol. The established strains were identified as particular species of the *P. aurelia* complex (by mating reaction) on the basis of strong conjugation between reactive complementary mating types of the studied strains and the mating types of a standard (reference) strain of particular species of the *P. aurelia* complex. The following standard strains were used: strain Rieff, Scotland, Great Britain – *P. biaurelia*, strain 51, Indiana, USA – *P. tetraurelia*, strain 159, Puerto Rico – *P. sexaurelia*, strain 138, Florida, USA – *P. octaurelia*, strain 205, Edinburgh, Scotland, Great Britain – *P. novaurelia*.

The standard strains belong to the collection of strains of the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków, Poland.

Survival of inter-strain hybrids

In intra- and inter-strain crosses the F1 generation was obtained by conjugation and F2 by autogamy (using the method of daily isolated

Table 1b

The number of identified clones of the *P. aurelia* species complex (in brackets directly selected and after enrichment) in the studied water-bodies in particular sampling points and different seasons

Season	Kind of water body	Sampling point	Species of the *P. aurelia*			
			P. biaurelia	*P. tetraurelia*	*P. sexaurelia*	*P. novaurelia*
Spring	Pond I	1				1(I)
		3			1(I)	
	Pond III	3	10(I) 2(II)			
	Palm-house I	1			10(I) 2(II)	
	Palm-house II	1			10(I) 2(II)	
Summer	Pond I	1				2(I)
		3				9(I) 2(II)
		4				5(I) 2(II)
	Palm-house I	1		2(I)	8(I) 2(II)	
	Palm-house II	1		5(I)	5(I)	
		2			8(I) 2(II)	
Autumn	Pond I	3				4(I)
		4				2(I)
	Pond III	2	10(I)			
	Palm-house I	1		6(I)	4(I)	
		2			10(I)	
		3		10(I)		

I – clones established directly after water collection
II – clones established after medium was added into collected water samples

lines). The occurrence of the desired stage of autogamy (specimens at the stage of two macronuclear anlagen) was examined on preparations stained with aceto-carmine.

The survival of hybrids in F1 and F2 generations in inter-strain crosses between the studied strains and the standard strain of the particular species was investigated and estimated as percentages (Table 2). According to CHEN (1956), clones can be considered as surviving after passing 6-7 fissions during 72 hours after separation of partners of conjugation or post-autogamous caryonids.

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). A fragment of the *COI* gene (638 bp) was amplified, sequenced and analyzed.

To amplify a fragment of the mitochondrial *COI* gene, the following primer pair designed for ciliates (STRÜDER-KYPKE & LYNN 2010) was used: F388dT, 5'-TGTAAAACGACGGCCAGTGG wkCbAAAGATGTwGC 3'; and R1184dT, 5'-CAGGAAACAGCTATGACTAdACyTCAG GGTGACCrAAAAATCA-3'. If the above *COI* pair of primers did not yield a well-defined product, another reverse primer CoxH10176 5'-GAAGTTTGTCAGTGTCTATCC -3' (BARTH *et al*. 2006) was used instead of R1184dT. Cycle sequencing was done in both directions with the application of BigDye Terminator v3.1 chemistry (Applied Biosystems, USA) using the primer pair M13F/M13R (STRÜDER-KYPKE & LYNN 2010) and a primer used in PCR reactions (CoxH10176). The details of the amplification, purification as well as sequencing procedures were previously presented in TARCZ *et al*. (2013). The sequences are available from the NCBI GenBank database (see Table 3 for accession numbers).

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

Table 2

Survival (as percentage) of the inter-strain hybrids in species of the *Paramecium aurelia* complex

Season	Kind of water-body		Species of the *P. aurelia* complex		Percentage of surviving clones in hybrid generations	
		Sampling point		Crossed strains (studied x standard of particular species)	F1	F2
Spring	Pond I	3	*P. sexaurelia*	PI,3,1 x 159 (6)	92	82
		1	*P. novaurelia*	PI,1,1 x 205 (9)	90	87
	Pond III	3	*P. biaurelia*	PIII,3,2 x Rieff (2)	85	98
	Palm-house I	1	*P. sexaurelia*	PhI,11 x 159 (6)	95	78
	Palm-house II	1	*P. sexaurelia*	PhII,1,1 x 159 (6)	98	90
Summer	Pond I	3	*P. novaurelia*	PI,3,7 x 205 (9)	90	93
		1	*P. novaurelia*	PI, 1,1 x 205(9)	100	97
		4	*P. novaurelia*	PI,4,1 x 205 (9)	100	87
	Palm-house I	1	*P. sexaurelia*	PhI,1 1 x 159 (6)	96	80
		1	*P. tetraurelia*	PhI, 1, 3 X 51 (4)	96	80
	Palm-house II	2	*P. sexaurelia*	PhII,2,2 x 159 (6)	96	98
		1	*P. tetraurelia*	PhII,1, 1 x 51(4)	90	97
		1	*P. sexaurelia*	PhII, 1,10 x 159(6)	80	97
Autumn	Pond I	3	*P. novaurelia*	PI,3, 1 x 205 (9)	100	96
		4	*P.novaurelia*	PI, 4,1 x 205(9)	100	86
	Pond III	2	*P. biaurelia*	PIII, 2, 1 x Rieff (2)	88	88
	Palm-house I	1	*P. tetraurelia*	PhI, 1, 1 x 51 (4)	90	64
			P. sexaurelia	PhI, 1,8 x 159(6)	80	63
		2	*P. sexaurelia*	PhI ,2, 1 x 159 (6)	95	60
		3	*P. tetraurelia*	PhI, 3,2 x 51 (4)	90	70

Table 3

Species of the *P. aurelia* complex recorded in the water bodies of the Botanical Garden in particular years of the studies

Species	Strain index	Water body	Years and seasons of sampling	GenBank acc number	*COI* haplotype
P. biaurelia	nd	Pond I	1960, autumn	nd	nd
	76BG	Pond II	1992, autumn	KX752225	Pa2COI_1
	77BG	Pond I	1999, autumn	KX752226	Pa2COI_2
	BG1	Pond III	2015, spring	KX752227	Pa2COI_2
	BG2		2015, autumn	KX752228	Pa2COI_2
P. tetraurelia	BG3	Palm-house I	2015, summer	KX752229	Pa4COI_1
	BG4	Palm-house II		KX752230	Pa4COI_2
	BG5	Palm-house I	2015, autumn	KX752231	Pa4COI_1
	BG6			KX752232	Pa4COI_1
	103BG		2000, summer	KX752233	Pa4COI_1
	104BG	Pond II	1998, autumn	KX752234	Pa4COI_3
P. sexaurelia	BG7	Pond I	2015, spring	KX752235	Pa6COI_1
	BG8	Palm-house I		KX752236	Pa6COI_2
	BG9	Palm-house II		KX752237	Pa6COI_1
	BG10	Palm-house I	2015, summer	KX752238	Pa6COI_2
	BG11	Palm-house II		KX752239	Pa6COI_1
	BG12			KX752240	Pa6COI_1
	BG13	Palm-house I	2015, autumn	KX752241	Pa6COI_2
	BG14			KX752242	Pa6COI_3
P. novaurelia	BG15	Pond I	2015, spring	KX752243	Pa9COI_1
	BG16		2015, summer	KX752244	Pa9COI_1
	BG17			KX752245	Pa9COI_1
	BG18			KX752246	Pa9COI_1
	BG19		2015, autumn	KX752247	Pa9COI_1
	BG20			KX752248	Pa9COI_1

used for analyses. Phylograms were constructed for the studied fragments by means of Mega v6.0 (TAMURA *et al*. 2013), using neighbor-joining (NJ) and maximum likelihood (ML). All positions containing gaps and missing data were eliminated. NJ analysis was performed using the Mega v6.0 program by bootstrapping with 1000 replicates. Bayesian inference (BI) was performed with MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003); analysis was run for 5,000,000 generations and trees were sampled every 100 generations. All trees for BI analysis were visualized with TreeView 1.6.6 (PAGE 1996). Analysis of haplotype diversity (Hd) and its sampling variance (SD), nucleotide diversity (π), and polymorphic sites was done with DnaSP v5.10.01 (LIBRADO & ROZAS 2009). Mega 6.0 (TAMURA *et al.* 2013) identified the HKY+G+I for *COI* mtDNA (G = 0.68, I=0.48) as the best nucleotide substitution models for maximum likelihood tree reconstruction. A haplotype network of the *COI* mtDNA fragment, which presented the distribution and relationships among haplotypes of *P. aurelia* strains, was done by using the median-joining method as implemented in the program Network 4.6.1.1 (http://www.fluxus-engineering.com/, BANDELT *et al*. 1999).

Results and Discussion

Species of *Paramecium* identified in the Botanical Garden of the Jagiellonian University

During three-seasonal (spring-summer-autumn) sampling in the studied water - bodies of the Botanical Garden, several species of the *P. aurelia* complex were identified, i.e., *P. biaurelia* (2 strains), *P. tetraurelia* (4 strains), *P. sexaurelia* (8 strains), and *P. novaurelia* (6 strains) (Table 1b). Identification was based on mating tests and a high (percentage) survival of inter-strain hybrids (Table 2) obtained by crosses between the studied strain and the standard (reference) strain of the particular species. Furthermore, molecular analysis of the *COI* mtDNA fragment of the studied strains was performed, allowing a comparison of relationships

between identified strains of four species as well as for studying their relationships with all representatives of the *P. aurelia* complex (Figs 4, 5). Other *Paramecium* morpho-species, *P. bursaria* (4 strains) and *P. caudatum* (7 strains) were also found in the studied water-bodies (Table 1a).

Spatial and seasonal variability of collected *P. aurelia* strains

Often, only one species was found in the particular water body in a single sampling point (s.p.) and in different seasons (Fig. 3, Table 1b). However, in natural pond I two species (*P. sexaurelia* and *P. novaurelia*) were recorded, each in different s.p. in spring (Fig. 3, Table 1b). *P. novaurelia* appeared in three s.p. in this pond in summer, and in two s.p. in autumn. In turn, in water collected in summer in palm-houses I and II, two species (*P. tetraurelia* and *P. sexaurelia*) were identified in the same s.p., and also in palm-house I in autumn. *P. sexaurelia* was also found in the other s.p. of palm-house II in summer, and in two other s.p. of palm-house I in autumn (Fig. 3, Table 1b).

P. sexaurelia was recorded in both studied palm-houses (I and II) in spring and summer, as well as in palm-house I in autumn (Fig. 3, Table 1b). Identification of *P. sexaurelia* in three water-bodies of the Botanical Garden is a noteworthy finding because this is its first record in Poland. The occurrence of this species is rather limited by a temperature barrier (SONNEBORN 1975) and usually to the tropical regions (PRZYBOŚ & PRAJER 2015). We suppose that the species might have been transported to the water-bodies of the Botanical Garden with some tropical plants (in the case of palm-houses) and, may have been transferred later to pond I.

In the present study *P. sexaurelia* was the most frequent species (8 to 20 identified strains) in the water-bodies of the Garden. Similarly, this species was found in the Botanical Garden and Zoo in Stuttgart, Germany (PRZYBOŚ & FOKIN 1997), and may be transferred with water borne plants or animals.

P. tetraurelia, beside the mentioned occurrence in the palm-houses in summer together with *P. sexaurelia*, was also found in autumn in palm-house I in two s.p. Its occurrence in the Botanical Garden water-bodies is limited to palm-houses because of a preference for higher temperatures.

P. biaurelia was recorded only in pond III in spring and in autumn, in different s.p. It was the only *Paramecium* species collected from this water-body. According to HAIRSTON (1958) *P. biaurelia* has probably an inhibitory effect on the other species caused by the presence of killing symbionts.

Finally, *P. novaurelia* was identified for the first time in the Botanical Garden in pond I in all studied seasons, i.e. in spring in one s.p., in summer in three s.p., and in autumn in two s.p. (Table 1b). Although it is supposed to be the most frequent species of the *P. aurelia* complex in Poland (cf PRZYBOŚ *et al*. 2011), it was never found during sampling done in 1960, 1992, 1997,1999 and 2000.

The seasonal occurrence of different *Paramecium* species in particular ecosystems (water-bodies) or even micro-niches (sampling points) seems to be a characteristic feature of paramecia communities (PRZYBOŚ *et al*. 1967; REHMAN *et al*. 2007; PRZYBOŚ *et al*. 2011). It is supposed that the species occupied different niches with various chemical and biological conditions suitable for their requirements (LANDIS 1986). Such "environmental heterogeneity" (LANDIS 1986) allows for coexistence of different species (FENCHEL 1987).

Studies carried out in the Poznań Palm house (KOLICKA *et al*. 2015) concerning aquatic invertebrates (Rotifera) showed that palm houses were "a convenient habitat for a prevalence of native and introduced" species. Specific environmental conditions of water-bodies (reservoirs, ponds) within palm-houses (greenhouses) with high and constant ambient and water temperature and raised humidity, may favor rapid adaptation of tropical species (as *P. sexaurelia*) transferred with tropical water plants. According to JABLONKA & RAZ (2009), adaptation to the new habitat may be rapid due to the selection of epigenetic variants.

Current results in the light of previous sampling of the Botanical Garden and biogeography features of identified *P. aurelia* species

Among species of the *P. aurelia* complex recorded in the water bodies of the Botanical Garden in Kraków, *P. biaurelia*, *P. tetraurelia*, and *P. sexaurelia* are considered as cosmopolitan species (SONNEBORN 1975). However in Poland, *P. sexaurelia* was never found before and *P. tetraurelia* seems to be rather rare (cf Table 5 in PRZYBOŚ *et al*. 2011), and might be eliminated by environment pollution (KOMALA & PRZYBOŚ 1984).

P. biaurelia and *P. tetraurelia* were recorded in particular water bodies of the Botanical Garden during previous studies (KOMALA & PRZYBOŚ 1999, 2000, 2001a,b; PRZYBOŚ & KOMALA 2001). Sometimes, the presence of these species was only temporal or recorded only once during single studies (Table 3).

P. tetraurelia was found (KOMALA & PRZYBOŚ 2001b) in palm-house (in the present paper desig-

nated as palm-house I) reservoir in three s.p., water samples were collected in September 2000. *P. tetraurelia* and *P. sexaurelia* were also recorded in both palm-houses in summer, and in palm-house I in autumn.

P. biaurelia was identified in an artificial pond (pond II), (KOMALA & PRZYBOŚ 1999, sampling being carried out in 1992). The pond was rebuilt and refilled in 1997, and *P. tetraurelia* was found in samples collected in October 1998 (KOMALA & PRZYBOŚ 2000). During the current sampling *Paramecium* sp. were not recorded there.

P. biaurelia was identified also in natural pond I, in water samples collected in November 1999 (PRZYBOŚ & KOMALA 2000). It is remarkable that the pond was completely rebuilt and refilled in 1999, the previous sampling done in 1992 did not reveal the occurrence of any species of the *P. aurelia* complex there (KOMALA & PRZYBOŚ 1999). According to the KOMALA (1960, unpublished) this species was also found in this pond. At present, *P. sexaurelia* was recorded in spring, and *P. novaurelia* in spring, summer, and autumn in the pond, in autumn more dispersed, and three sampling points were found in summer. In contrast, *P. sexaurelia* was no longer present in the summer and autumn water samples.

P. biaurelia was also found at present in pond III in spring, and autumn, however, sampling carried out in May 2000 did not reveal the presence of the *P. aurelia* spp. (KOMALA & PRZYBOŚ 2001).

Haplotype diversity of studied *P. aurelia* species

Current molecular analyses, based on environmental high-throughput sequencing (envHTS) of small subunit rRNA (SSUrRNA) gene may provide new data on ciliate communities (SONG *et al*. 2015, ROSSI *et al*. 2016). However, analysis of intraspecific variability of particular ciliate species (for example from the *Paramecium* genus) requires more variable mtDNA fragments (BARTH *et al.* 2006, 2008; PRZYBOŚ *et al.* 2011).

Here, sequences of the genes encoding cytochrome c oxidase subunit I (638 bp) were obtained from 20 *P. aurelia* strains (identified as *P. biaurelia*, *P. tetraurelia, P. sexaurelia* and *P. novaurelia*) collected in the Botanical Garden in 2015. Moreover, in the current study we have the opportunity to compare molecular data from two other strains of *P. tetraurelia* collected in 1998 (identified in pond II) and 2000 (identified in palm-house I) (Table 3). Similarly as in the case of *P. tetraurelia,* we could compare *COI* sequences form two *P. biaurelia* strains collected in 1992 (pond II) and in 1999 (pond I) (Table 3).

Altogether we identified 9 *COI* haplotypes which were characterized by haplotype diversity Hd=0.8804 (SD=0.035) and nucleotide diversity π= 0.5081. The mean divergence of all obtained from this area *P. aurelia* (N=24) sequence pairs was 0.002 (ranging from 0.000 to 0.349). In turn, in particular studied species the mean intraspecific variability was: *P. biaurelia* (0.002), *P. tetraurelia* (0.065), *P. sexaurelia* (0.002) and *P. novaurelia* (only one haplotype observed). In the studied *COI* fragment, we found 214 variable positions (200 parsimony informative) among the *P. aurelia* strains.

The constructed phylogenetic tree (Fig. 4) showed that the studied strains identified in the Botanical Garden form monophyletic groups with the other representatives of particular *P. aurelia* species except one strain GB4 of *P. tetraurelia*. Although genetic crosses were conducted before and repeated after molecular analysis, a discordance between these two approaches was revealed (Fig. 4, Table 2). This phenomenon was observed previously in the *P. aurelia* complex and explained as incomplete lineage sorting (TARCZ *et al*. 2013).

Analysis of the *COI* network (Fig. 5) revealed the existence of three haplogroups which correspond to *P. biaurelia* (2 haplotypes – Pa2COI_1, Pa2COI_2), *P. sexaurelia* (3 haplotypes – Pa6COI_1, Pa6COI_2, Pa6COI_3) and 3 from 4 strains of *P. tetraurelia* (2 haplotypes – Pa4COI_1, Pa4COI_3). Additionally, one strain of *P. tetraurelia* appears as a distant haplotype (Pa4COI_2), and finally the most common (among studied strains) haplotype (Pa9COI_1) was characteristic for 6 strains of *P. novaurelia*.

Three different *COI* haplotypes of *P. sexaurelia* (Fig. 5) were characteristic for particular waterbodies. All strains established from the palm-house I (BG8, BG10, BG13) are identical (haplotype Pa6COI_2) although they were established in different seasons (spring, summer, and autumn) but from the same s.p. (1). However, strain (BG14) also from palm-house I (established from Autumn collection, 2 s.p.) is slightly different (haplotype Pa6COI_3) from other strains originating from this reservoir. Strains from palm-house II (BG9, BG11, BG12) are characterized by a third haplotype (haplotype Pa6COI_3) despite being established in different seasons and s.p. similarly as strain BG7 originated from pond I. In our opinion, the observed haplotype richness might be connected with introduction of three different *P. sexaurelia* strains with plants originating from diverse locations. For example the strain BG14 has an identical haplotype (haplotype Pa6COI_3) to strain TW from Taiwan (Fig. 4).

It is worth noting that within 9 identified *P. aurelia* haplotypes only 3 were identified for the first

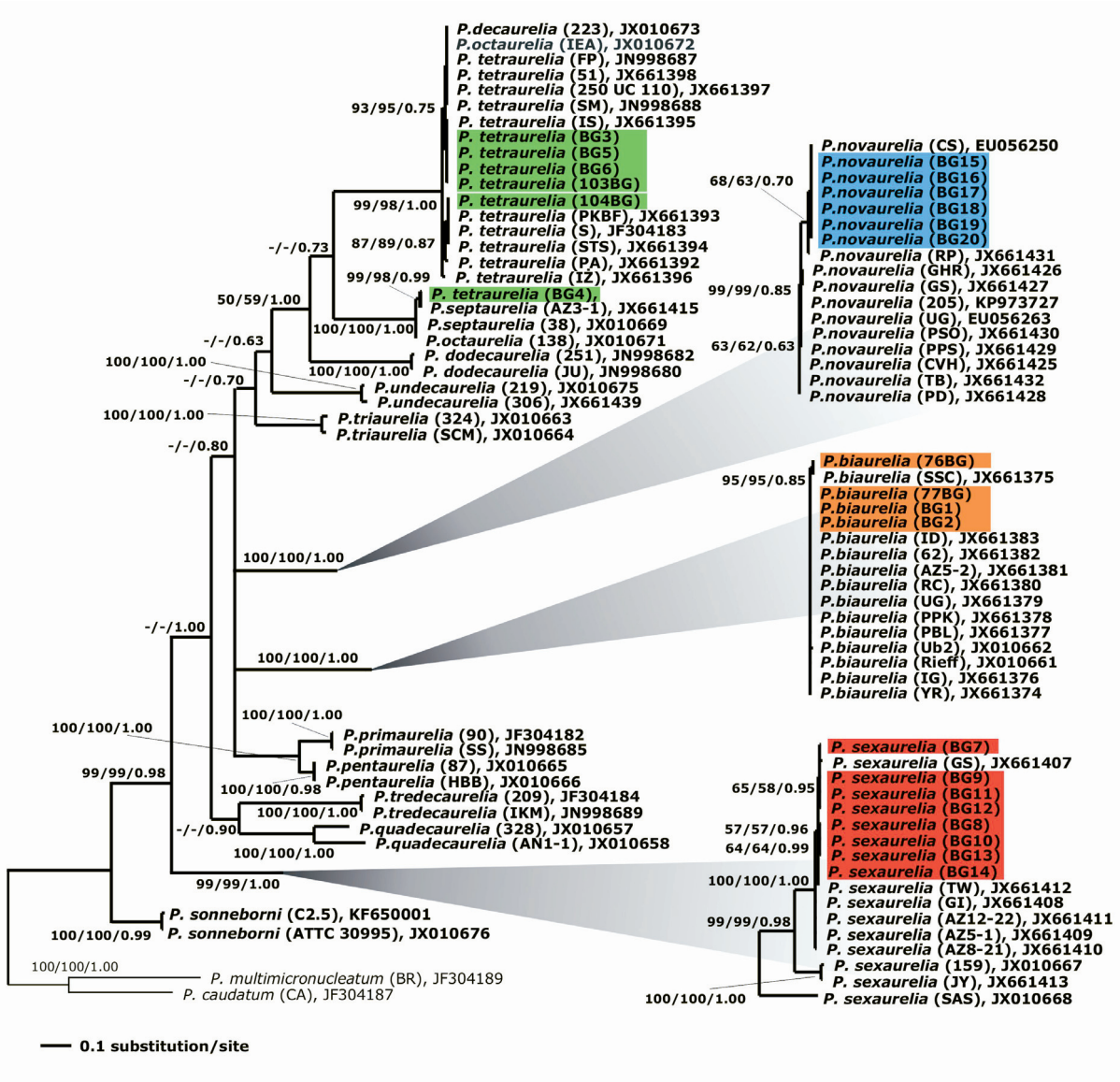

Fig. 4. Phylogenetic tree constructed for 87 strains of *Paramecium aurelia* complex (two species: *P. caudatum* and *P. multimicronucleatum* were used as an outgroup). The tree was constructed on the basis of a comparison of sequences from the mitochondrial *COI* fragment using the Maximum Likelihood method. Bootstrap values for neighbor joining, maximum likelihood, and posterior probabilities for Bayesian inference are presented. Bootstrap values smaller than 50% (posterior probabilities .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 89 nucleotide sequences. There was a total of 638 positions in the final dataset.

time – two from *P. sexaurelia* (Pa6COI_1, Pa6COI_2) and one from *P. tetraurelia* (Pa4COI_2). The remaining 6 haplotypes were revealed in earlier studies (TARCZ *et al*., 2013) in both close and distant sampling points (Fig. 5).

Generally, during the current survey we did not observe intraspecific variability in one water body. Exceptions concern *P. sexaurelia*, but here two different haplotypes were identified in two different sampling points in one water body in one season. A second exception concerns *P. biaurelia* and *P. tetraurelia* from which different haplotypes were revealed over a longer interval (15-23 years). The observed spatial (between different water bodies) and seasonal (between different years) haplotype diversity was observed previously in other studies (BARTH *et al*. 2006, 2008; PRZYBOŚ *et al*. 2011).

In our opinion studies of Botanical Garden water bodies allow not only to study species composition and population variability of *P. aurelia* species in term of space and time, but reveal some ecological features of these closely related taxa.

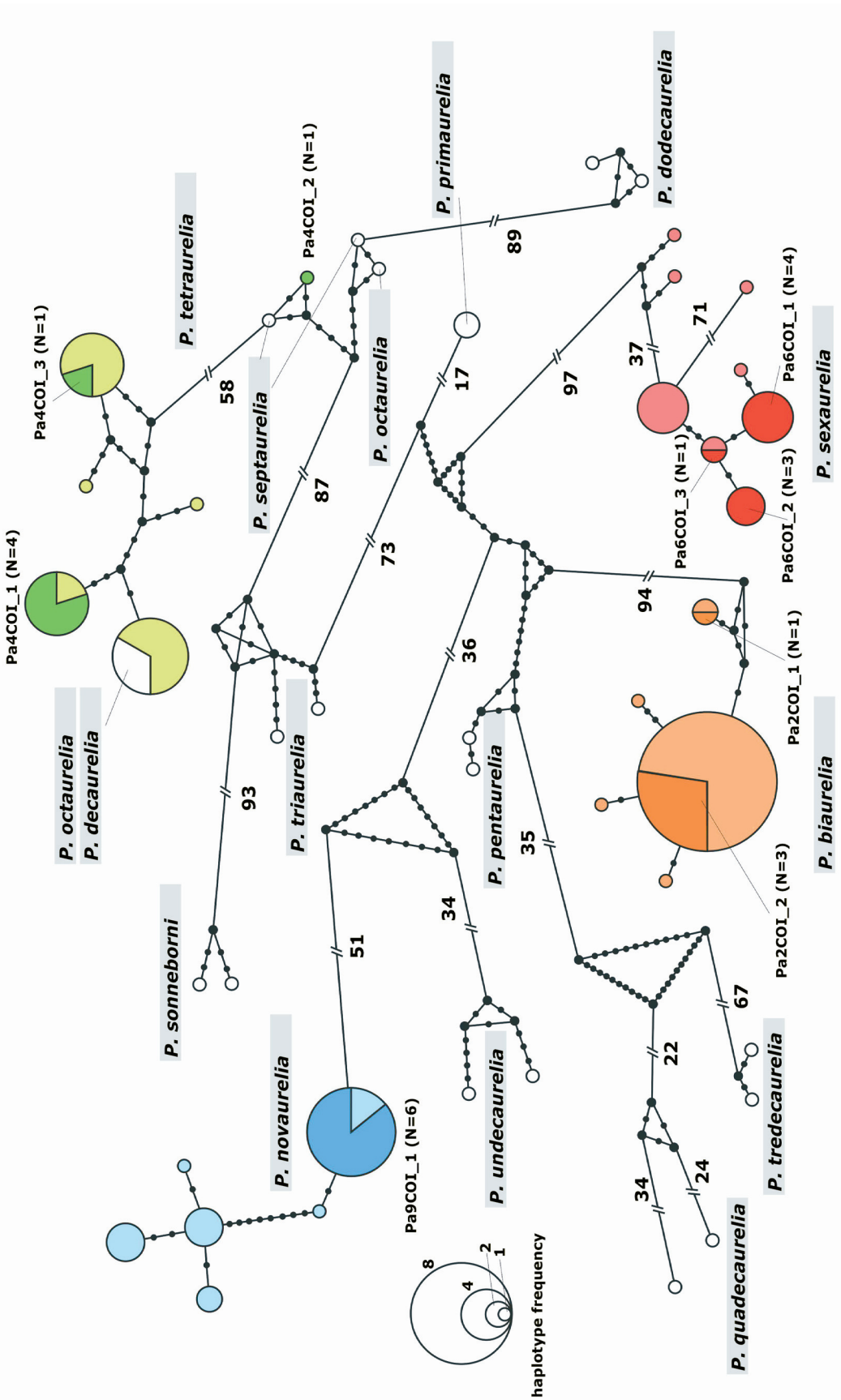

Fig. 5. Haplotype network of *Paramecium aurelia* species constructed using the 87 sequences from mitochondrial *COI* fragments. Haplotypes identified in Botanical Garden (Kraków) are marked in dark orange (*P. biaurelia*), dark green (*P. tetraurelia*), dark red (*P. sexaurelia*) dark blue (*P. novaurelia*) in contrast to light orange, green, red and blue colors which represents other *COI* haplotypes of particular species obtained from GenBank. Haplotypes characteristic for the remaining *P. aurelia* species are marked white. The median vectors that represent hypothetical intermediates or unsampled haplotypes, are shown in black circles. Black dots on particular branches represent nucleotide substitutions between particular haplotypes (in the case of over 10 a corresponding number was given). Analyses were conducted using the Median Joining method in Network 4.6.1.1.

Acknowledgements

The authors are very grateful to Professor dr hab. Józef R. MITKA, head of the Botanical Garden of the Jagiellonian University, Kraków for permission to carry out the studies in the garden.

References

AUFDERHEIDE K.J., DAGGETT P.-M., NERAD T. A. 1983. *Paramecium sonneborni* n. sp., a new member of the *Paramecium aurelia* species -complex. J. Protozool. **30**: 128-131.

BANDELT H.J., FORSTER P., RÖTHL A. 1999. Median-Joining Networks for Inferring Intraspecific Phylogenies. Mol. Biol. Evol. **16**: 37-48.

BARTH D., KRENER S., FOKIN S.I., BERENDONK T.U. 2006. Intraspecific genetic variation in *Paramecium* revealed by mitochondrial cytochrome c oxidase I sequences. J. Eukaryot. Microbiol. **53**: 20-25.

BARTH D., TISCHNER K., BERGER H., SCHLEGEL M., BERENDONK T. U. 2008. High mitochondrial diversity of *Coleps s*p. (Ciliophora:Prostomatida). Environm. Microbiol. **10**: 626-634.

BEALE G.H., PREER J.R.Jr 2008. *Paramecium*. Genetics and Epigenetics. CRC Press, Taylor & Francis Group, Boca Raton (FL), London, New York. Pp. xxi +191.

CHEN T.T. 1956. Varieties and mating types in *Paramecium bursaria* . II. Variety and mating types found in China. J. Exp. Zool. **132**: 255-268.

COLEMAN A. W. 2005. *Paramecium aurelia* revisited. J. Eukaryot. Microbiol. **52**: 68-77.

FENCHEL T. 1987. Ecology of Protozoa. The biology of free-living phagotrophic protists. Springer Verlag. Berlin. 197 pages.

FOISSNER W. 2006. Biogeography and dispersal of micro-organisms: a review emphasizing protists. Acta Protozool. **45**: 111- 136.

FOKIN S.I. 2010/2011. *Paramecium* genus: biodiversity, some morphological features and the key to the main morphospecies discrimination. Protistology **6**: 227-235.

GENTEKAKI E., LYNN D.H. 2009. High-level genetic diversity but no population structure inferred from nuclear and mitochondrial markers of the peritrichous ciliate *Carchesium polypinum* in the Grand River basin (North America). Appl Environ Microbiol **75**: 3187-3195.

GUTIERREZ J. C., MARTIN-GONZALES A., CALLEJAS S. 1988. Nuclear changes , macronuclear chromatin reorganization and DNA modifications during ciliate encystment. Europ. J. Protistol. **34**: 97-103.

HAIRSTON N.G. 1958. Observations on the ecology of *Paramecium*, with comments on the species problem. Evolution **12**: 440-450.

HALL T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis programm for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41**: 95-98.

JABLONKA E.V.A., RAZ G.A.L. 2009. Transgenerational epigenetic inheritance prevalence, mechanisms , and implications for the study of heredity and evolution. Q. Rev. Biol. **84**: 131-171.

KOLICKA M., DZIUBA M.K., ZAWIERUCHA K., KUCZYŃSKA-KIPPEN N., KOTWICKI L. 2015. Palm house – biodiversity hotspot or risk of invasion? Aquatic invertebrates: the special case of Monogononta (Rotifera) under greenhouse conditions. Biologia **70**: 94-103 (Section Zoology).

KOMALA Z., KOŚCIUSZKO H. 1959. First report on the geographical distribution of different varieties of *Paramecium aurelia* in Poland. Folia Biol. (Kraków) **7**: 83-88.

KOMALA Z., PRZYBOŚ E. 1984. Distribution of the *Paramecium aurelia* species complex (Protozoa, Ciliophora) in the Carpathian chain of Poland. Zoologica Scripta **13**: 161-163.

KOMALA Z. , PRZYBOŚ E. 1999. *Paramecium biaurelia* and other accompanying zooplankton in the ponds of the Botanical Garden in Kraków. Folia Biol. (Kraków) **47**: 51-52.

KOMALA Z. , PRZYBOŚ E. 2000. Other investigations on the zooplankton of water bodies in the Botanical Garden of the Jagiellonian University in Kraków. Folia Biol. (Kraków) **48**: 49-51.

KOMALA Z., PRZYBOŚ E. 2001a. Zooplankton in the artificial pond of the Botanical Garden of the Jagiellonian University in Kraków. Folia Biol. Kraków **49**: 99-101.

KOMALA Z., PRZYBOŚ E. 2001b. Zooplankton in the ponds with tropical plants in greenhouses of the Botanical Garden of the Jagiellonian University in Kraków. Folia Biol. Kraków **49**: 225-228.

LANDIS W.G. 1986. The interplay among ecology, breeding systems, and genetics in the *Paramecium aurelia* and *Paramecium bursaria* complexes. (In: Progress in Protistology, J.O. CORLISS and D. J.PATTERSON eds . Vol. 1 Biopress, Bristol): 287-307.

LE BESCOT N., MAHÉ F., AUDIC S., DIMIER C., GARET M.J., POULAIN J., WINCKER P., VARGAS C., SIANO R. 2016. Global patterns of pelagic dinoflagellate diversity across protist size classes unveiled by metabarcoding. Environmental Microbiology **18**: 609-626.

LIBRADO P., ROZAS J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics **25**: 1451-1452.

LOWE C. 2015. Beyond everything is everywhere – the burgeoning field of landscape genetics and its application for understanding Protist biogeography. VII European Congress of Protistology – International Society of Protistologists Joint Meeting , 5-10 September 2015, Seville, Spain, abstracts, p. 157.

PAGE R.D.M. 1996. TreeView: An application to display phylogenetic tress on personal computers. Bioinformatics **12**: 357-358.

PRZYBOŚ E., FOKIN S. I. 1997. Species of the *Paramecium aurelia* complex Sonneborn in Germany. Archiv Protistenk. **148**: 167-172.

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

PRZYBOŚ E., KOMALA Z. 1993. The *Paramecium aurelia* species complex of Poland. Folia Biol. (Kraków) **41**: 11-16.

PRZYBOŚ E., KOMALA Z. 2001. *Paramecium aurelia* species complex in a natural but newly reconstructed pond of the Botanical Garden of the Jagiellonian University in Kraków. Folia Biol. (Kraków) **48**: 149-150.

PRZYBOŚ E., KOŚCIUSZKO H., KOMALA Z.1967. The occurrence of *Paramecium aurelia* syngens in a natural water reservoir in different seasons of the year. Folia Biologica (Kraków) **15**: 399-404.

PRZYBOŚ E., PRAJER M. 2015. New stands of species of the *Paramecium aurelia* complex; is the occurrence of particular species limited by temperature barriers? Folia Biol. (Kraków) **63**: 215-220.

PRZYBOŚ E., SURMACZ M. 2010. New, world-wide data on the distribution of species of the *Paramecium aurelia* complex (Ciliophora, Protozoa). Folia biol. (Kraków) **58**: 185-188.

PRZYBOŚ E., TARCZ S., GRECZEK-STACHURA M., SURMACZ M. 2011. Seasonal and spatial variability of species occurrence

of the *Paramecium aurelia* complex in a single natural pond (Protista, Ciliophora). Hydrobiologia **663**: 233-244.

PRZYBOŚ E., TARCZ S., RAUTIAN M., LEBEDEVA N. 2014. The first European stand of *Paramecium sonneborni* (*P. aurelia* complex), a species known only from North America (Taxas, USA). Europ. J. Protistol. **50**: 236-247.

RAUTIAN M., PRZYBOŚ E., SURMACZ M., LEBEDEVA M. 2014. New stands of species of the *Paramecium aurelia* complex in Africa and Europe. Folia Biol. (Kraków) **62**: 361-366.

RAZOWSKI J. 1996. Dictionary of Insect Morphology. Wydawnictwo Naukowe PWN, Warszawa-Kraków, p. 140. (In Polish).

REHMAN A., SHAKOORI F. R., SHAKOORI A. R. 2007. Seasonal variation in protozoan population in tannery effluents. Pakistan J. Zool. **39**: 69-76.

RONQUIST F., HUELSENBECK J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.

ROSSI A., BOSCARO V., CARDUCCI D., SERRA V., MODEO L., VERNI F., FOKIN S. I., PETRONI G. 2016. Ciliate communities and hidden biodiversity in freshwater biotopes of the Pistoia province (Tuscany, Italy). Europ.J. Protistol. **53**: 11-19.

SONG W., LI J., LIU W., AL-RASHEID K.A.S., HU X., LIN X. 2015. Taxonomy and molecular phylogeny of four *Strombidium* species, including description of *S. pseudostylifer* sp. nov. (Ciliophora, Oligotrichia). System. Biodivers. **13**: 76-92.

SONNEBORN T.M. 1950. Methods In the general biology and genetics of *Paramecium*. J. Exp. Zool. **113**: 87-148.

SONNEBORN T.M. 1970. Methods in *Paramecium* research. In: Prescott D.M. ed. Methods in Cell Physiology, vol.4. Academic Press, New York, London: 241-339.

SONNEBORN T.M. 1975. The *Paramecium aurelia* complex of fourteen sibling species. Trans. Amer. Micros. Soc. **94**: 155-178.

STRÜDER-KYPKE M.C., LYNN D.L. 2010. Comparative analysis of the mitochondrial cytochrome c oxidase subunit I (COI) gene in ciliates (Alveolata, Ciliophpra) and evaluation of its suitability as a biodiversity marker. Syst. Biodiv. **8**: 131-148.

TAMURA K., STECHER G., PETERSON D., FILIPSKI A. KUMAR S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. **30**: 2725-2729.

TARCZ S., PRZYBOŚ E., SURMACZ M. 2013. An assessment of haplotype variation in ribosomal and mitochondrial DNA fragments suggests incomplete lineage sorting in some species of the *Paramecium aurelia* complex (Ciliophora, Protozoa). Mol. Phylogenet. Evol. **67**: 255-265.

VIVIER E. 1974. Morphology, taxanomy and general biology of the genus *Paramecium*. In: Wagtendonk W.I. ed.. *Paramecium*. A Current Survey. Elsevier, Amsterdam: 1-89.