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New Stands of the *Paramecium aurelia* spp. Complex (Protista, Oligohymenophorea) in Ethiopia, Madagascar, Taiwan, and Romania

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Knowledge about the occurrence and distribution of species from the *Paramecium aurelia* complex is important for understanding their evolution. However, sampling of paramecia in the southern hemisphere, and, in general, in the tropics was usually done only occasionally. Therefore, biogeographical information from areas such as Africa, South America and Australia may bring new, interesting data. In the current survey, based on mating test results as well as COI mtDNA analysis we showed the existence of new stands of *P. sexaurelia*, *P. octaurelia*, *P. novaurelia*, *P. tredecaurelia,* and *P. quadecaurelia*. In the case of Ethiopia and Taiwan these are the first data in terms of occurrence of the *P. aurelia* complex.

Key words: Ciliates; *Paramecium aurelia* species complex; biogeography, *COI* mtDNA, molecular analysis.

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Ciliates occupy different ecological niches in which they play an important role as trophic links in food webs. Some of them, such as a bacterivorous organisms, can also clean water by reducing bacteria populations (LYNN 2008). More generally, according to CORLISS (2004), protists are important members of the biological world. However, their geographical diversity (biogeography) has been under discussion for many years. One model supports cosmopolitan (FENCHEL & FINLAY 2004) and the second a moderately endemic distribution (FOISSNER *et al*. 2008; FOISSNER 2017).

Among ciliates within the *Paramecium* genus, there are a few flagship species being the subjects of many studies (ALTERMATT *et al*. 2014) concerning several general biological problems (cf BEALE & PREER 2008), including biodiversity (e.g. SONNEBORN 1975; GRECZEK-STACHURA *et al*. 2012; PRZYBOŚ & TARCZ 2016). Studies of the latter issue in connection with taxonomy may bring a more complete understanding of the role of ciliate communities in ecosystems (CLAMP & LYNN 2017).

One of the "flagship" models for biodiversity studies is *Paramecium aurelia* – a complex of 15 sibling species (SONNEBORN 1975; AUFDERHEIDE *et al*. 1983). Some of them are cosmopolitan (*P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, and *P. sexaurelia*), whereas the occurrence of others is limited by temperature barriers (SONNEBORN 1975). Restriction to the warm zone has been revealed in some species such as *P. quadecaurelia* (PRZYBOŚ *et al*. 2013b), or *P. sonneborni* (PRZYBOŚ *et al*. 2014, 2015).

Some geographical regions have been sampled more extensively, such as Europe, the USA in North America, and several localities in Asia, as e.g. Japan, Asiatic Russia, partly India and Israel (PRZYBOŚ & FOKIN 2000; PRZYBOŚ & SURMACZ 2010). Generally, the northern hemisphere is much better explored than the southern one which still has "white spots" in Africa, South America, and Australia (FOKIN 2010/2011).

Here we present new data on the geographical distribution of five members of the *Paramecium aurelia* species obtained from Ethiopia, Taiwan (locations never studied before), Madagascar (sampled occasionally), and Romania.

Material and Methods

Material

Paramecia strains studied in the present paper representing the *P. aurelia* species complex are listed in Table 1. They were collected in: Asia, Taiwan; Africa, Ethiopia and Madagascar; Europe, Romania (Table 1, Fig. 1). In Taiwan, samples were collected in Wufeng, district of Taizhong town, from a stream near a Buddhist graveyard. Taiwan is situated in the monsoon tropical climatic zone.

In Ethiopia, samples of water were collected in Lalibela (altitude about 2630 m a.s.l.) from a cistern with papyrus, situated near one of the rock-hewn churches from the XII-XIII century. Ethiopia has a moderate warm climate as the majority of the country is situated at high altitude (FLIS 1967).

A sample of water was collected in Madagascar, Isalo National Park from a mountain stream in a shaded ravine. The park is situated on a plateau, 820 to 1240 m a.s.l., built from sandstone forma-

Table 1

New stands of species of the *Paramecium aurelia* complex

Species	Clone index	Collection place	Coordinates	Collector and date of collection	GenBank acc number
P. sexaurelia	Et-4,a	Ethiopia, Lalibela, cistern	12°01′45.660′′N / 39°02′25.512′′E	A. Bielecka, November, 2017	MH550415
P. octaurelia	MaI	Madagascar, Isalo National Park, stream	22°32′22.8′′ S / 45°22′52.4′′E	Ł. Przybyłowicz, April, 2018	MH550416
P. novaurelia	RB	Romania, Bran, pond	45°30′23.94′′N / 25°22′7.04′′E	E. Przyboś, June, 2017	MH550417
P. tredecaurelia	Et-3,a	Ethiopia, Lalibela, cistern	12°01′45.660′′N / 39°02′25.512′′E	A. Bielecka, November, 2017	MH550418
P. quadecaurelia	Tai	Taiwan, Taizhong, Wufeng, pond	24°03′60.00′′N / 120°41′59.99′′E	P. Wojtal, September, 2017	MH550420

Fig. 1. The origin of studied currently *Paramecium aurelia* species.

tions with deep canyons. The island has a tropical climate with seasonal rainfall (RATAJSKI 1967). It belongs to the afro-tropical region, its fauna is characterized by diverse ecosystems and unique wildlife thanks to the origin of the island from the breakup of the Gondwana supercontinent and following the split from the Indian peninsula.

In Romania, material was collected from a pond near Bran castle ("Dracula castle"), Transylvania.

Methods

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

SONNEBORN's methods (1950, 1970) of cultivation and identification of strains were used. Paramecia were cultured at 27°C in a medium made of dried lettuce in distilled water, inoculated with *Enterobacter aerogenes* and supplemented with 0.8 mg/ml β-sitosterol (Merck, Darmstadt, Germany). New strains were identified as particular species of the *P. aurelia* complex on the basis of strong conjugation between the studied strain and the reference strain of the species.

The following standard strains were used:

strain 159 of *P. sexaurelia* from Puerto Rico,

strain 138 of *P. octaurelia* from Florida, USA,

strain 205 of *P. novaurelia* from Edinburgh, Scotland, UK,

strain TaB of *P. tredecaurelia* from Thailand, Bangkok,

strain An1-1 of *P. quadecaurelia* from Africa, Namibia,

strain 328 of *P. quadecaurelia* from Australia.

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

Survival of inter-strain hybrids

In the inter-strain crosses, the F_1 generation was obtained by conjugation and F_2 by autogamy (using the method of daily isolation lines). Inter-strain crosses were carried out between recently identified strains from Taiwan, Ethiopia, Madagascar, and Romania representing particular species of the *P. aurelia* complex and the standard strains of the species (Table 2). The occurrence of the desired stage of autogamy (specimens at the stage of two macronuclear anlagen, i.e., after finishing the reorganization of the nuclear apparatus) was examined on preparations stained with aceto-carmine. The type of macronuclear anlagen is also an important feature for taxonomic diagnosis. Survival of clones in both generations was estimated as percentages. 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. A detailed description of procedures can be found in PRZYBOŚ *et al*. (2013b).

Molecular techniques

Paramecium genomic DNA was isolated from vegetative cells at the end of the exponential phase (approximately 1000 cells were used for DNA extraction) using a Genomic Micro AX Tissue Gravity (A&A Biotechnology, Poland), according to the manufacturer's instructions for DNA isolation from cell cultures. Both the quantity and purity of the extracted DNA were evaluated with a NanoDrop-2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The *COI* fragment (about 800 bp) of mitochondrial DNA was amplified with the primer pair forward F388dT (5'-TGTAAAACGACGGCCAGTGGwkCbAA AGATGTwGC-3') and reverse R1184dT (5'-CAGGAAACAGCTATGACTAdACyTCAGGG TGACCrAAAAATCA-3') using the protocol previously described by STRÜDER-KYPKE & LYNN (2010). For all analyzed DNA fragments,

Table 2

Percentage of surviving hybrid clones in inter-strain crosses of the *Paramecium aurelia* spp. complex

Species	Crossed strains: studied x standard of particular species	Percentage of surviving hybrid clones in:	
		F1 (obtained by conjugation)	F2 (obtained by autogamy)
P. sexaurelia	Et-4,a x 159	76	50
P. octaurelia	MaI x 138	98	82
P. novaurelia	RB x 205	97	87
P. tredecaurelia	Et-3,a x TaB	98	15.7
P. quadecaurelia	Tai x AN1-1	82	60

PCR amplification was carried out in a final volume of 40 µl containing 30 ng of DNA, 1.5 U Taq-Polymerase (EURx, Poland), 0.8 µl of 20 µM of each primer, 10 × PCR buffer, and 0.8 µl of 10 mM dNTPs. In order to assess the quality of amplification, PCR products were electrophoresed in a 1% agarose gel for 30 min at 85 V with a DNA molecular weight marker (Mass Ruler Low Range DNA Ladder, Thermo Fisher Scientific, USA).

For purifying PCR products, 5 µl of each were mixed with 2 µl of Exo-BAP Mix (EURx, Poland), and then incubated at 37°C for 15 min and afterwards at 80°C for another 15 min. Cycle sequencing was done in both directions with BigDye Terminator v3.1 chemistry (Applied Biosystems, USA). The primer pair forward M13F (5'-TGTAAAACGACGGCCAGT-3') and reverse M13R (5'-CAGGAAACAGCTATGAC-3') (MESSING 1983; STRÜDER-KYPKE & LYNN 2010) was used for sequencing the *COI* fragment. Details of the sequencing procedure can be found in TARCZ *et al*. (2012). The sequences of the studied *COI* mtDNA fragment are available in the NCBI GenBank database (Table 1).

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 for analyses. Phylograms were constructed for the studied fragments by means of MEGA v6.0 (TAMURA *et al*. 2013), using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) by bootstrapping with 1000 replicates. All positions containing gaps and missing data were eliminated. 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). MEGA 6.0 (TAMURA *et al.* 2013) identified HKY+G+I for *COI* mtDNA as the best nucleotide substitution models for maximum likelihood tree reconstruction.

Results and Discussion

Based on mating tests (Table 2), as well as *COI* mtDNA analysis (Fig. 2) we showed the presence of *P. sexaurelia*, *P. octaurelia*, *P. novaurelia*, *P. tredecaurelia*, and *P. quadecaurelia* in the studied localities (Table 1, Fig. 1).

For the first time the areas of Ethiopia and Taiwan were examined for the occurrence of *Paramecium*, in particular the *P. aurelia* complex. Worth noting is the presence of rare species of the complex, i.e. *P. tredecaurelia* in Ethiopia and *P. quadecaurelia* in Taiwan. However, as only a few samples of water with plankton were collected, the occurrence of other species of the complex in these regions cannot be excluded.

Paramecium sexaurelia

Paramecium sexaurelia is a wide-ranging species, appearing in tropical to moderate temperature zones (SONNEBORN 1975), however, rather preferring warm zone (PRZYBOŚ & PRAJER 2015). The species was previously found in North (USA) and Central (Puerto Rico) Americas, Africa (Kenia, Mozambique), Asia (India, Thailand, Indonesia, Japan, China), and Europe (Spain, Germany, Poland, Greece, Croatia, Russia), (SONNEBORN 1975; PRZYBOŚ & SURMACZ 2010; PRZYBOŚ & PRAJER 2015).

The present study revealed new stands in Africa in Ethiopia, Lalibela (Table 1, Fig. 1). According to studies based on molecular analyses (CATANIA *et al*. 2009; TARCZ *et al*. 2013; MCGRATH *et al*. 2014; LYNCH 2015) *P. sexaurelia* seems be an old species, distant on phylogenetic trees from other species of the *P. aurelia* complex. *P. sexaurelia* presents a high level of inbreeding (SONNEBORN 1957; KOŚCIUSZKO & PRAJER 1988; PRZYBOŚ *et al*. 2010) among species of the *P. aurelia* complex (generally characterized as inbreeders) (SONNEBORN 1975; LANDIS 1986), as well as a high intraspecific variability revealed by RAPD fingerprints (PRZYBOŚ *et al*. 2007b,c), *cytb* (PRZYBOŚ *et al*. 2010), and *hsp70* gene comparison (HORI *et al*. 2006).

The present results confirm intra-specific differentiation of *P. sexaurelia* as the percentage of surviving hybrid clones (50%) observed in F_2 of inter-strain cross of the Ethiopian strain with the *P. sexaurelia* standard strain 159 from Puerto Rico. Similarly, 62% of hybrids survived in the F_2 generation in a cross of the Mozambique strain with the Puerto Rican strain (PRZYBOŚ & PRAJER 2015); as well as a rather low percentage (60 and 63%) of surviving clones in a cross of strains from Poland, Kraków, Botanical Garden with the standard strain of the species (PRZYBOŚ *et al*. 2016). In this case *P. sexaurelia* was recorded for the first time in Poland, and as a species limited to tropical regions, it might have been transported to the water bodies with some tropical plants. An even lower (40% and 11%) percentage of surviving hybrid clones than in the present study was observed previously in crosses of Spanish

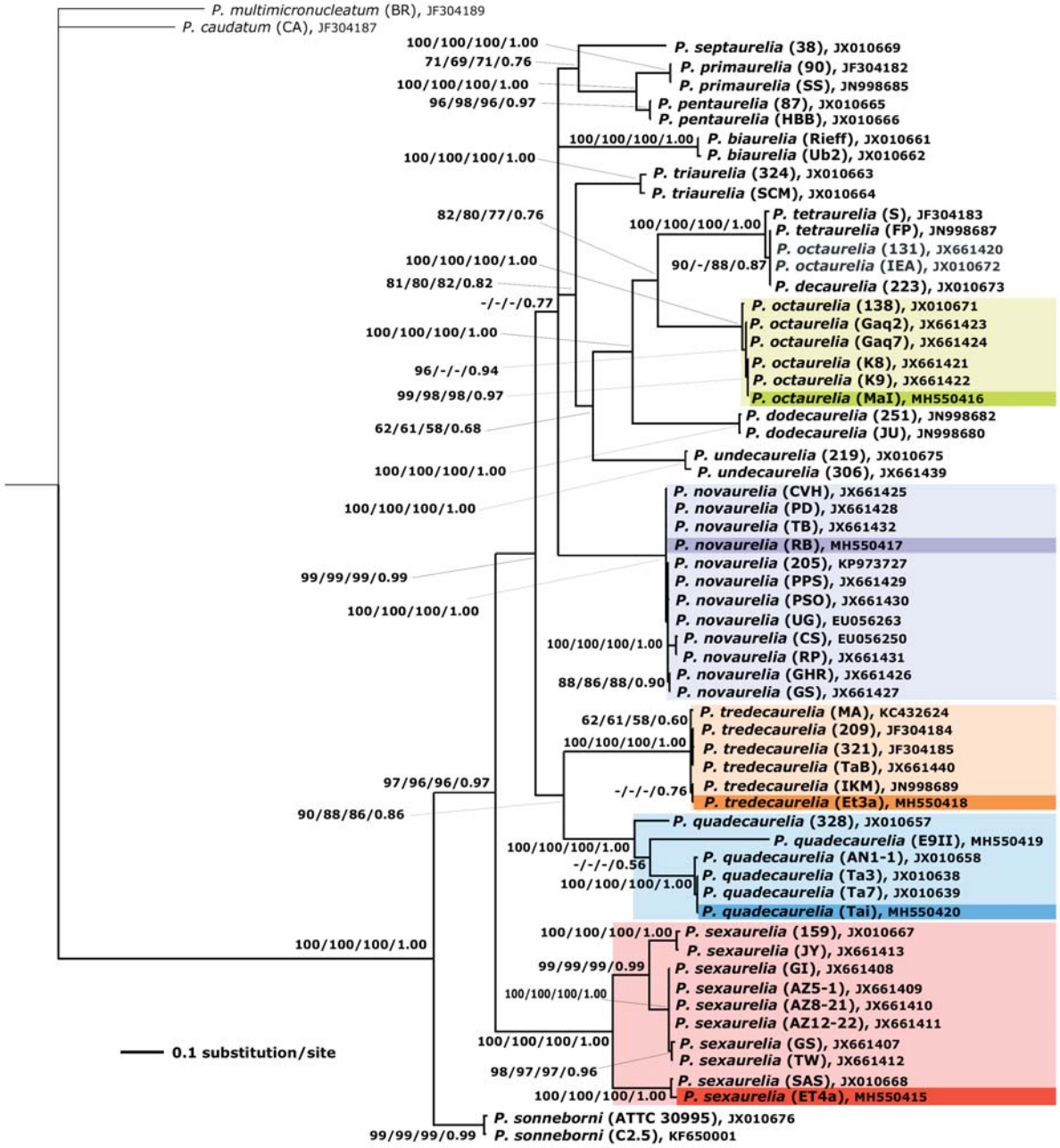

Fig. 2. A phylogenetic tree constructed for 60 strains of *Paramecium aurelia* complex (two species: *P. caudatum* and *P. multimicronucleatum* were used as an outgroup). The tree was constructed on the basis of a comparison of sequences from the mitochondrial *COI* fragment using the Bayesian inference 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. The strains highlighted by a darker color are the newly acquired ones. In the parentheses are the strain names, and GenBank accession numbers are in smaller font. All positions containing gaps and missing data were eliminated. Phylogenetic analyses were conducted using MEGA 6.0 (NJ/MP/ML) and MrBayes 3.1.2 (BI). The analysis involved 62 nucleotide sequences. There was a total of 638 positions in the final dataset.

strains from Seville with the standard strain (PRZYBOŚ 1990). The low percentage of surviving hybrid clones observed in the present study and previous ones may be caused by high genetic divergence between strains originating from remote habitats (Fig. 2).

Paramecium octaurelia

Paramecium octaurelia is common in tropical and subtropical Americas (SONNEBORN 1975), and is also found in Germany, Austria, Russia, Czech Republic, Cyprus, Georgia, Israel and the

Republic of South Africa (cf PRZYBOŚ & PRAJER 2015). Recently, it was also noted on Guam (PRZYBOŚ & RAUTIAN 2017). At present, *P. octaurelia* was identified in a water sample collected from Isalo National Park, Madagascar (Table 1, Fig. 1). Survival of inter-stain hybrids (MaI, Madagadscar, Isalo x 138, Florida, USA) was high, 98% and 82%, respectively in F_1 and F_2 generations (Table 2). *P. octaurelia* can mate with *P. tetraurelia*, however, in spite of strong mating reaction (up to 40% of mating pairs), their offspring is nonviable (SONNEBORN 1975). The latter issue can be seen on the COI tree (Fig. 2), where strains classified by mating tests as *P. octaurelia* appear in two clades. The first one is monophyletic, and contains six *P. octaurelia* strains (including the currently studied MaI strain), whereas the second one is paraphyletic and consists of *P. tetraurelia*, *P. octaurelia* and *P. decaurelia* (Fig. 2). Such discrepancies between results of mating tests and molecular studies has been noted previously in the *P. aurelia* complex (CATANIA *et al*. 2009; TARCZ *et al*. 2013).

Paramecium novaurelia

A new stand of *Paramecium novaurelia* was recorded in Romania, Transylvania, near Bran castle (Table 1, Fig. 1), based on mating reaction with the standard strain of the species (strain 205 from Scotland) as well as *COI* mtDNA analysis (Fig. 2). The survival of inter-strain hybrids of both strains in F_1 and F_2 generations (Table 2) was high, 97 and 87% respectively, in spite of the geographical distance between the crossed strains originating from Scotland and Romania. The species was already recorded in Romania in Petresti de Jos, Cluj region (PRZYBOŚ & PRAJER 2015), and also in other localities near Cluj, Bucharest, and Constanta (PRZYBOŚ 1968).

P. novaurelia is characterized by a wide temperature tolerance, and is thought to be the most common *P. aurelia* species in Europe (PRZYBOŚ & SURMACZ 2010), known from Sweden, Finland, Scotland, Germany, France, Spain, Poland, Czech Republic, Hungary, Bulgaria, Romania, Ukraine, Russia. However, it was also recorded from the Asiatic part of Turkey (PRZYBOŚ 1998), USA, Boston (PRZYBOŚ *et al*. 2007a), Asiatic part of Russia, Yakutia (RAUTIAN *et al*. 2015) and Altay (PRZYBOŚ & RAUTIAN 2017).

Intra-specific polymorphism observed by RAPD analysis and a comparison of two loci (STOECK *et al*. 2000; PRZYBOŚ *et al*. 2006; TARCZ 2013) might be connected with the degree of inbreeding characteristic for the species (SONNEBORN 1957; LANDIS 1986), and therefore *P. novaurelia* should be considered as a moderate inbreeder (STOECK *et al*. 2000; HORI *et al*. 2006). As a moderate inbreeder *P. novaurelia* may have a wider adaptive possibility and a potentially broader range of occurrence.

Paramecium tredecaurelia

A new stand of *Paramecium tredecaurelia* was recorded in Lalibela, Ethiopia (Table 1, Fig. 1). It is a rare species of the *P. aurelia* complex, with only a few (six) strains known until recently. Strains from Paris, France (strain 209); Curnavaca, Mexico (strain 321) and Benenitra, Madagascar (strain 238) were described as *P. tredecaurelia* by RAFALKO and SONNEBORN in 1959. Later, strain IKM was recorded in Israel (PRZYBOŚ *et al*. 2002), strain MA again in St. Luce, Madagascar, and strain TaB in Bangkok, Thailand (PRZYBOŚ *et al*. 2013a). *P. tredecaurelia* seems be limited to the warm temperature zone, known from isolated localities in America, Europe, Africa and Asia.

The species is completely sexually isolated from other species of the *P. aurelia* complex, and is characterized by a Mendelian (synclonally inherited) mating type system (SONNEBORN 1975), unique among the species of the complex. RAFALKO and SONNEBORN (1959) found that strain 209 was restricted to mating type XXV (odd) and strains 328 and 321 to mating type XXVI (even). PRZYBOŚ *et al*. (2013a) observed that other strains except strain 209 were also restricted to an even mating type. This species requires a high temperature (29-31°C) for conjugation (SONNEBORN 1975). Autogamy was observed in the recently recorded strain from Ethiopia (Et-3,a) starved for 16-20 fissions (clonal age) in daily isolated lines (according to SONNEBORN 1975) cultivated at 29°C in a medium enabling 4 fissions daily. The Ethiopian strain (Et-3,a) represents the odd mating type.

Noteworthy is fact that despite a lack of or a very low molecular variability within *P. tredecaurelia* (PRZYBOŚ *et al*. 2007b; TARCZ *et al*. 2006; PRZYBOŚ *et al*. 2013a), inter-strain hybrids yield high mortality in the F_2 generation (RAFALKO & SONNEBORN 1959; PRZYBOŚ *et al*. 2007b; PRZYBOŚ *et al*. 2002; PRZYBOŚ *et al*. 2013a). Similarly, in the current survey low hybrid survival in F_2 was also revealed (Table 2), whereas all *P. tredecaurelia* strains formed a compact, monophyletic clade in the species tree of the *P. aurelia* complex (Fig. 2).

Currently it is difficult to explain the above phenomenon, and in our opinion including more *P. tredecaurelia* strains from remote localities may resolve this issue. In the other species of the complex – *P. biaurelia* a low variability of over 120 worldwide sampled strains has been explained by a bottleneck event or by a slow rate of mutation of the studied DNA fragments (TARCZ *et al*. 2018).

Paramecium quadecaurelia

The fifth stand of *P. quadecaurelia* was recorded in Taizhong, Taiwan (Table 1, Fig. 1) based on conjugation with the strain representing this species from Namibia (AN1-1), and a comparison of the *COI* mtDNA fragment (Fig. 2). The species was previously known only from Australia (strain 328, Emily Gap near Alice Springs) (SONNEBORN 1975), Namibia (AN1-1, Vindhoek) (PRZYBOŚ *et al*. 2003), Ecuador (E9II, Loyola) and Thailand (two clones from one sample – Ta3, Ta7, Samui island) (PRZYBOŚ *et al*. 2013b). It seems that the range of *P. quadecaurelia* similarly as *P. tredecaurelia* is limited to the warm zone (SONNEBORN 1975; PRZYBOŚ & PRAJER 2015).

In the inter-strain cross of the Taiwan strain (Tai) with the African strain (AN1-1), 82% of hybrids in F_1 and 60% in F_2 generations survived (Table 2). Previously (PRZYBOŚ *et al*. 2013b), a higher percentage of surviving hybrid clones in F_1 (90-100%) and F_2 (57-96%) generations was observed. However, unlike the case of *P. tredecaurelia,* variation in the *COI* fragment within *P. quadecaurelia* was equal or even greater than between for example *P. primaurelia* and *P. pentaurelia* (Fig. 2). Probably, according to PRZYBOŚ *et al*. (2013b), strains of *P. quadecaurelia* in the future might be separated into different, reproductively isolated lines.

Inbreeding, a general characteristic for species of the *P. aurelia* (SONNEBORN 1957) complex, seems to be an important factor causing reduction of intra-specific hybrid survival, especially in species being characterized by a high degree of inbreeding, as *P. sexaurelia* (STOECK *et al*. 1998). Particular strains of this species originating from distant localities are more genetically isolated (dependent on autogamy to renew their clonal life) than strains in the other species of the complex such as *P. novaurelia,* a moderate inbreeder (see results of the present paper).

Biogeographical aspects of the current findings

The dispersal of paramecia is a very important aspect influencing their biogeography, and is especially intriguing in the case of rare species of the *P. aurelia* complex. As cysts are unknown in paramecia (LANDIS 1986; GUTIERREZ *et al*. 1998; BEALE & PREER 2008) they can only be transported by animals or human activities with some drops of water. Although sampling of paramecia in the southern hemisphere has been done occasionally (PRZYBOŚ & RAUTIAN 2017), and biogeographical data from Africa, South America, and Australia are very limited, every time they bring new, interesting data. For example, sampling in Guam (PRZYBOŚ & RAUTIAN 2017), Taiwan and Ethiopia (this study) done for the first time, revealed the occurrence of rare species of the *P. aurelia* complex. Sampling in Madagascar done once again also showed the presence of another rare species (*P. octaurelia*) on that island.

Under-sampling of the southern hemisphere may cause limitation in our understanding of the ranges of particular species or even existence of new species, such as the finding of *Paramecium schewiakoffi* in Shanghai, China (FOKIN *et al*. 2004), or the spirotrich ciliate *Rubrioxytricha guamensis* on Guam (KUMAR *et al*. 2017). We suppose that future studies may bring new data concerning the occurrence of rare species of the *P. aurelia* complex, improving our understanding of the ways of their dispersal and evolution.

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Author Contributions

Research concept and design: E.P.; Collection and/or assembly of data: E.P., S.T.; Data analysis and interpretation: E.P., S.T.; Writing the article: E.P.; Critical revision of the article: S.T. ; Final approval of article: S.T.

Conflict of Interest

The authors declare no conflict of interest.

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