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Sampling hidden microbial eukaryotic biodiversity in the tropics: new insights from the *Paramecium aurelia* complex (Ciliophora, Protozoa)

Sebastian Tarcz, Marta Surmacz and Ewa Przyboś

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Ciliates, including the genus *Paramecium*, are among the most thoroughly researched groups of free-living microbial eukaryotes. However, our knowledge of their biodiversity appears to be restricted. Therefore, more data is required for tropical regions, to generate a more accurate picture of the distribution of the cryptic *Paramecium* species. In the current paper, recent data on the tropical biodiversity of the *Paramecium aurelia* species complex is presented. We believe that the COI mtDNA fragment allows for an evaluation of the geographic variation of particular cryptic species within the *Paramecium aurelia* complex, while also being sufficient for species identification. The obtained data indicates that the examined tropical populations may be very variable (with more than 50% previously unknown COI haplotypes discovered). Consequently, it is reasonable to assume that tropical environments reveal a high biodiversity of *Paramecium* ciliates.

Key words: biogeography, COI haplotypes, ecozones, freshwater ciliates, haplotype networks.

Sebastian Tarcz✉, Marta Surmacz, Ewa Przyboś, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków 31-016, Sławkowska 17, Poland.
E-mail: tarcz@isez.pan.krakow.pl

Although ciliates are probably the most intensively studied group of free-living microbial eukaryotes and occupy different ecological niches, where they are a very significant component of food webs, the understanding of their biodiversity seems to be limited (Liu *et al.* 2022, Lynn 2008). The causes of this problem could be the complex structure of the ciliate species (Caron 2013; Nanney & McCoy 1976) or its under-sampling in many ecosystems (Foissner et al. 2008, Fokin 2010/2011). Moreover, the poor understanding of the group's biodiversity is also observed within the 'pets' of ciliatologists (Foissner 2006), including the *Paramecium* genus studied herein, whose species are model organisms in many studies concerning genetics, evolution, physiology and biochemistry. This has been confirmed by the recently described new morphospecies (Krenek *et al*. 2015, Melekhin *et al*. 2022, Paiva *et al*. 2016), as well as by cryptic species (Greczek-Stachura *et al*. 2021, Potekhin & Mayén-Estrada 2020, Przyboś & Tarcz 2016) which have been reported in the majority of the over 20 valid *Paramecium* species. One of these is *Paramecium aurelia*, which is a complex of sixteen cryptic species (Aufderheide *et al.* 1983, Potekhin & Mayén-Estrada 2020, Sonneborn 1975) that are morphologically indistinguishable but sexually isolated. Up-to-date faunistic data on the *P. aurelia* complex indicates the existence of species with both broad (Tarcz *et al*. 2018) and narrow ranges of occurrence (Przyboś *et al*. 2014). However, the most common

currently known sampling localities of the *Paramecium aurelia* complex are in Palearctic and Nearctic ecozones (Przyboś & Surmacz 2010). Therefore, to obtain a more complete picture presenting the distribution of cryptic *P. aurelia* species, greater amounts of data are needed in relation to tropical areas (Fokin 2010/2011) because, for example, it may turn out that cryptic species known only from a few 'tropical' sites are common in these climate zones.

Based on the current knowledge, populations of some cosmopolitan species of the *P. aurelia* complex have been found in the tropics, which were isolated from both colder and warmer ecosystems, such as *P. primaurelia* and *P. tetraurelia* (Tarcz *et al.* 2013), as well as species restricted to or found mainly in the tropics including *P. sexaurelia*, *P. tredecaurelia*, *P. quadecaurelia*, *P. sonneborni* and *P. quindecaurelia* (Potekhin & Mayén-Estrada 2020, Przyboś *et al.* 2013a, Przyboś *et al.* 2013b, Tarcz *et al.* 2013, Przyboś *et al.* 2014).

To properly delimit a ciliates species, a combination of different (molecular, morphological and physiological) approaches should be employed (Caron 2013, Stoeck *et al.* 2014). However, the application of molecular techniques, such as DNA barcoding (Hebert *et al.* 2003; Pawlowski *et al.* 2012) or high-throughput sequencing (HTS) technologies (Reuter *et al.* 2015), has given rise to new possibilities for the easier species detection of these microbial eukaryotes (Gentekaki & Lynn 2010). In the case of the *P. aurelia* complex as well as the other *Paramecium* species, there are many studies (Greczek-Stachura *et al.* 2021, Przyboś & Tarcz 2019, Tarcz *et al.* 2018) which indicate that species identification is possible based only on the haplotypes of the COI gene fragment. Despite some pitfalls (Rataj & Vďačný 2021, Obert *et al.* 2022), this region has been successfully used as a DNA barcoding tool for other ciliates (Strüder-Kypke & Lynn 2010), and has been used as a marker for ciliates population studies (Gentekaki & Lynn 2009) as well as in several *Paramecium* species (Snoke *et al.* 2006, Zhao *et al.* 2013).

In the current paper, we present new data concerning the tropical biodiversity of the *Paramecium aurelia* species complex. We suggest that the COI fragment is sufficient for *Paramecium* species identification, but also provides an opportunity to assess the spatial variability of particular cryptic species within the *Paramecium aurelia* complex.

Material and Methods

Material

Paramecium strains studied in the present paper representing the *P. aurelia* species complex are listed in Table S1 (SM.01). Newly identified strains were collected in the Dominican Republic (Neotropical realm), Madagascar and Namibia (Afrotropical realm) (Fig. 1).

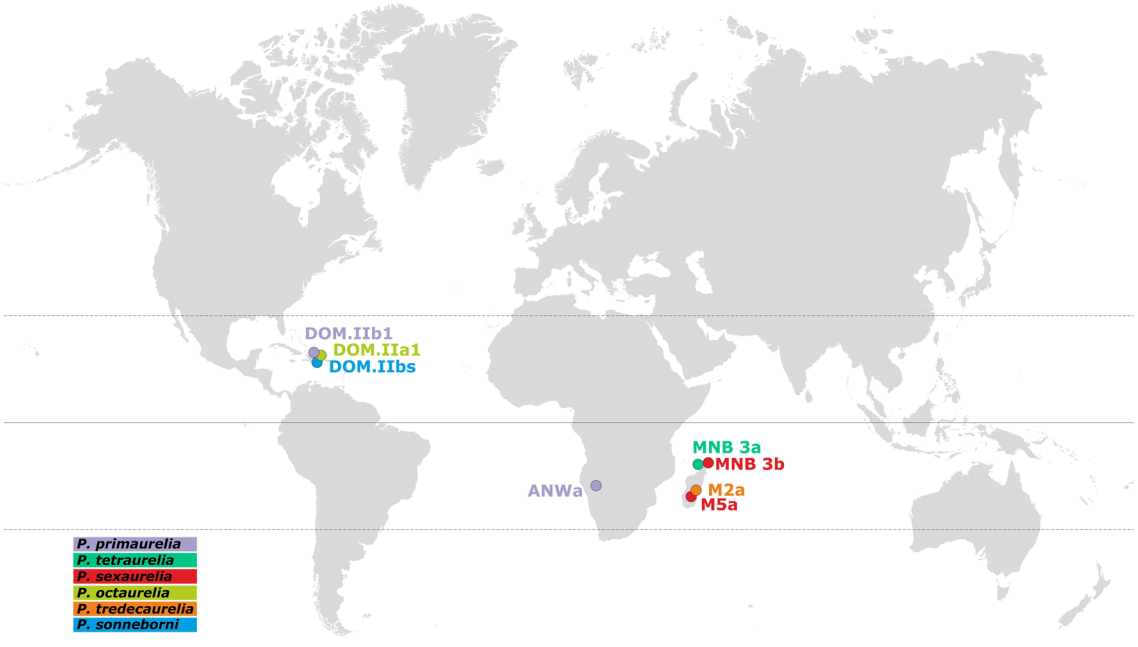

Fig. 1. Geographic distribution of newly identified strains of the *Paramecium aurelia* complex. The Equator is marked with a solid line, while the Tropics of the Cancer and Capricorn with dashed lines.

Methods

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

Sonneborn's methods (1950, 1970) for the cultivation and identification of strains were used. Paramecia were cultured at 27°C in a medium made of dried lettuce in distilled water, then 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 based on a strong conjugation between the studied strain and the reference strain of the species (Przyboś & Tarcz 2019).

The following standard strains were used:

Strain 90 of *P. primaurelia* from Pennsylvania, USA;

Strain S of *P. tetraurelia* from Sydney, Australia;

Strain 159 of *P. sexaurelia* from Puerto Rico;

Strain 138 of *P. octaurelia* from Florida, USA;

Strain TaB of *P. tredecaurelia* from Bangkok, Thailand;

Strain ATCC 30995 of *P. sonneborni* from Texas, USA.

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.

Molecular techniques

The genomic DNA of *Paramecium* was isolated (approx. 1000 cells were used for the DNA extraction) from vegetative cells at the end of the exponential phase using the NucleoSpin Tissue Kit (Macherey-Nagel, Germany), according to the manufacturer's instructions for DNA isolation from human or animal tissue and cultured cells. The only modification was a cell culture centrifugation for 20 min at 13,200 rpm. Then, the supernatant was removed and the remaining cells were resuspended in a lysis buffer and proteinase K. The proteinase K buffer step consisted of two parts: pre-lyse sample incubation at 56°C for 3h; and lyse sample incubation at 70°C for 10 min. Details of the protocol are available at https://www.mn-net.com/media/pdf/5b/d0/d9/Instruction-NucleoSpin-Tissue.pdf. Both the quantity and purity of the extracted DNA were evaluated using a NanoDrop-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Fragments of the COI gene were amplified, sequenced and analysed. The COI fragment of mitochondrial DNA was amplified using a pair of primers: forward F388dT (5'-TGTAAAACGACGGCCAGTGGwkCbAAAGATGTwGC-3') and reverse R1184dT (5'-CAGGAAACAGCTATGACTAdACyTCAGGGTGACCrAAAAATCA-3'), according to a protocol previously described in Strüder-Kypke and Lynn (2010). The amplification cycles were as follows: 4 min at 94°C; followed by 5 cycles of 94°C for 45 s, 45°C for 1:15 min and 72°C for 1:30 min; 30 cycles of 94°C for 45 s, 55°C for 1:15 min and 72°C for 1:30 min; and a final extension at 72°C for 8 min. The PCR amplification was carried out in a final volume of 40 µl containing 30 ng DNA, 1.5 U Taq polymerase (EURx, Poland), 0.8 µl of 20 µM each primer, 10 x PCR buffer, and 0.8 µl of 10 mM dNTPs. To assess the quality of the amplification, the PCR products were electrophoresed in 1% agarose gel for 30 min at 85 V with a DNA molecular weight marker (MassRuler Low Range DNA Ladder, Thermo Fisher Scientific, USA).

To purify the PCR products, 5 µl of each product were mixed with 2 µl of Exo-BAP Mix (EURx, Poland), and were subsequently incubated at 37°C for 15 min, followed by another 15 min at 80°C. Cycle sequencing was performed in both directions using the BigDye Terminator v3.1 chemistry (Applied Biosystems, USA). The forward M13F (5'-TGTAAAAC-GACGGCCAGT-3') and reverse M13R (5'-CAGGAAACAGCTATGAC-3') primers (Messing 1983, Strüder-Kypke & Lynn 2010) were used for sequencing the COI fragment. Details of the sequencing procedure are derived from (Tarcz *et al.* 2012, 2014). The studied COI sequences are available in the NCBI GenBank database (see Supplementary Table S1).

The sequences were evaluated using Chromas Lite v2.1.1 (Technelysium, Australia). An alignment of the studied COI mtDNA fragment was constructed using BioEdit v7.2.5 software (Hall 1999) and was checked manually. All sequences obtained were unambiguous and were used for further analyses. The mean uncorrected p-distances were calculated using Mega v6.0 (Tamura *et al.* 2013). Neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using the Mega v6.0 program, by bootstrapping with 1000 replicates. All positions containing gaps and missing data were eliminated. The MP analysis was evaluated with the min-min heuristic parameter (at level 2) and bootstrapping with 1000 replicates. An HKY+G+I model for mtDNA (G = 0.758, I = 0.198) has been identified as the best nucleotide substitution model for a maximum likelihood tree reconstruction using Mega v6.0 software. The Bayesian inference (BI) was performed using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003); the analysis was run for 5,000,000 generations with the GTR+G+I model, and the trees were sampled every 100 gen-

erations. All trees for the BI analysis were visualised using TreeView v1.6.6 (Page 1996).

The number of haplotypes (h) and intraspecific haplotype diversity (Hd), as well as the nucleotide diversity (π), were determined with DnaSP v5.10.01 (Librado & Rozas 2009). The haplotype network, representing the distribution and relationships among the haplotypes of *Paramecium aurelia* strains, was reconstructed using the Median Joining method (Leigh & Bryant 2015) implemented in PopART v1.7 software (Bandelt *et al.* 1999).

Results and Discussion

Cryptic species of *Paramecium aurelia* complex from tropical environments

The distribution of microbial eukaryotes, including ciliate genera such as *Paramecium*, *Euplotes* and *Tetrahymena,* is currently explained by two complementary concepts: 'Everything is everywhere but the environment selects' (Fenchel & Finlay 2004); and the moderate endemic concept (Foissner 2017, Foissner *et al*. 2008). The information obtained on individual species, i.e. the number and distribution of the sampling sites, significantly influences the verification of the above hypotheses.

In the case of the genus *Paramecium*, the well-studied Palearctic contrasts with the poorly studied tropical regions (Fokin *et al*. 2004) where, during occasional sampling, new data has been obtained every time (Przyboś & Tarcz 2018, Przyboś *et al*. 2013a,b, 2014, 2017). Unfortunately, to date, no studies on the seasonal variation of the *Paramecium* species have been carried out in the tropics. This type of monitoring of one or two-three water bodies would help to better understand the biogeography of ciliates, not only by analysing their spatial but also their seasonal variability (Lu *et al*. 2019, Przyboś *et al*. 2011, 2016).

In the material studied here from the Dominican Republic (Neotropical realm), Madagascar and Namibia (Afrotropical realm), an analysis of the COI fragments as well as strain crosses revealed the occurrence of six of the sixteen currently known *P. aurelia* species: *P. primaurelia*, *P. tetraurelia*, *P. sexaurelia*, *P. octaurelia*, *P. tredecaurelia* and *P. sonneborni* (Fig. 1, Tab. S2 - SM.02). Almost all of them appeared in monophyletic clusters representing particular *P. aurelia* species (Fig 2), except *P. octaurelia* (see below). The occurrence of *Paramecium* specimens has been reported for the first time in a Dominican territory. In the two sampling points, three cryptic species of the *Paramecium aurelia* complex were identified: *P. primaurelia*, *P. octaurelia* and *P. sonneborni*. It is worth noting that this is the third locality of *P. sonneborni* to be established overall, as it has previously been found in the USA (Texas) and Cyprus (Aufderheide *et al*. 1983, Przyboś *et al*. 2014). In turn, *P. primaurelia*, a cosmopolitan species, has been identified worldwide in both tropical and colder climates (Tarcz *et al*. 2013). In the current study, apart from in the Dominican Republic, *P. primaurelia* was detected in Namibia (Afrotropical realm), where previously one stand of *P. quadecaurelia* has been identified (AN1-1, Vindhoek) (Przyboś *et al*. 2003). The last species currently found in the Dominican Republic, *P. octaurelia*, has a relatively wide distribution, but mainly in areas with a moderately warm and tropical climate (Przyboś & Prajer 2015, Przyboś & Rautian 2017, Sonneborn 1975). Similarly, as in previous studies (Przyboś & Tarcz 2018), some strains classified as *P. octaurelia* by mating tests appeared in both the *P. tetraurelia* as well as in the *P. octaurelia* clades of the COI tree (Fig. 2). This discrepancy between the mating tests and the molecular results has previously been identified in the *P. aurelia* complex (Catania *et al*. 2009), and was explained as caused by incomplete line sorting or a hybridisation/introgression event (Tarcz *et al*. 2013). It is worthwhile to emphasise that the Neotropical realm is known for its high biodiversity of flora and fauna (Ceballos & García 1995), which seems to be promising in relation to microeukaryote biodiversity studies. For example, a new cryptic species of the *P. aurelia* complex (Potekhin & Mayén-Estrada 2020) has recently been identified in the southern part of Mexico. Similar recent discoveries have also been made regarding another genus of ciliates, *Loxodes* (Méndez-Sánchez *et al.* 2022). Although the last of the areas studied herein,– Madagascar, is one of the foremost biodiversity hotspots (Ralimanana *et al*. 2022), it is still a poorly studied region with regard to ciliates diversity. So far, representatives of just two morphological species of *Paramecium,* i.e. *Paramecium aurelia* (Przyboś & Tarcz 2018, Przyboś et al. 2013b) and *Paramecium jenningsi* (Przyboś & Tarcz 2019), have been found in Madagascar. In the current study, we identified representatives of *P. tetraurelia* and *P. sexaurelia* for the first time in Madagascar, as well as a second (or third – see below) locality of *P. tredecaurelia*.

Overall, tropical realms have a good potential for discovering new *Paramecium* species (Fokin 2010/2011) and for increasing our knowledge of the molecular variability of known species (see below). However, some of the tropical *Paramecium* spe-

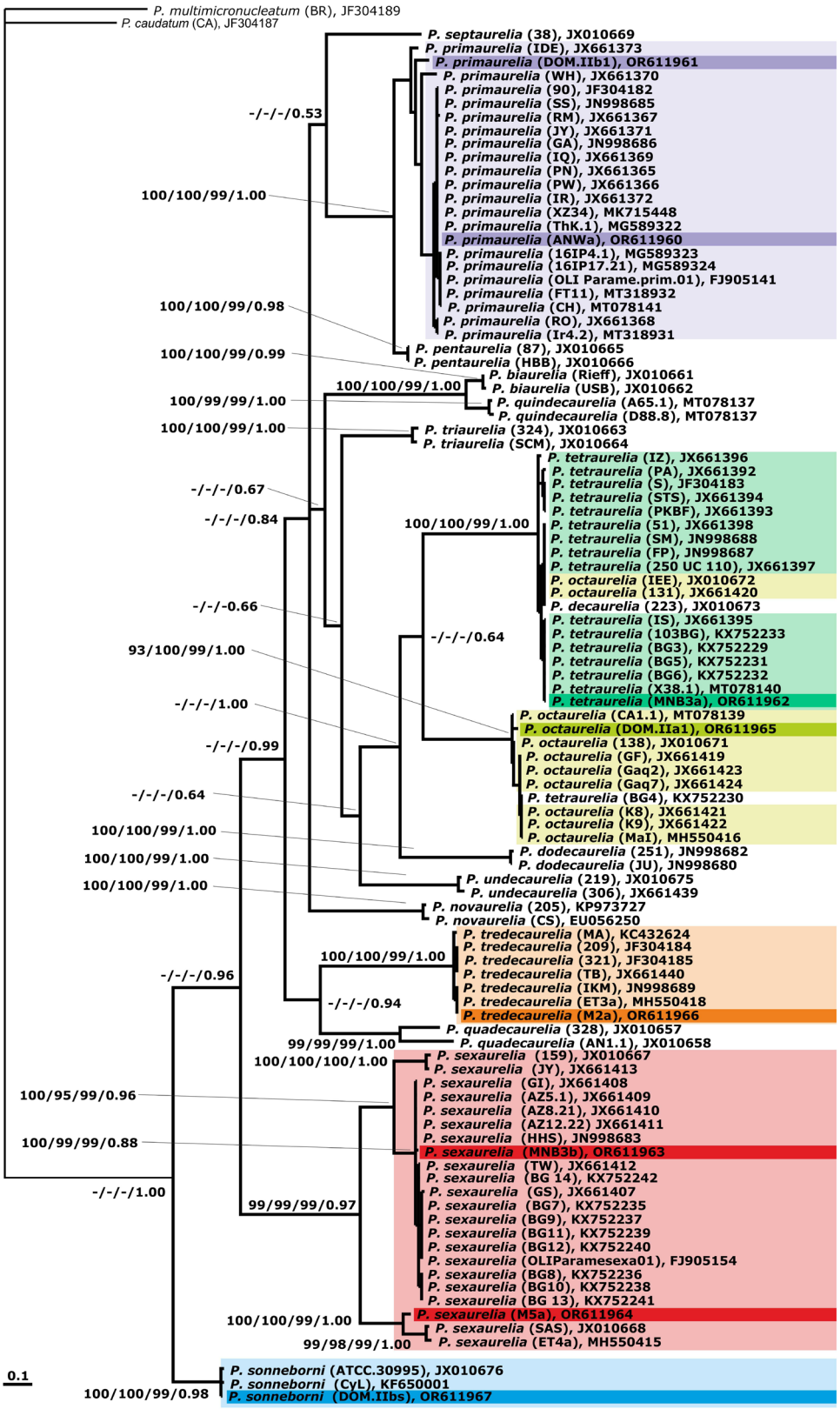

Fig. 2. Phylogenetic tree constructed for 100 *Paramecium aurelia* strains (*Paramecium caudatum* and *Paramecium multimicronucleatum* species were used as an outgroup). All strains are listed in Table S1. The tree was built on the basis of the mitochondrial COI fragment using the Bayesian inference (BI). Bootstrap values for neighbor joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and posterior probabilities for Bayesian inference (BI) are presented. Bootstrap values lower 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 v6.0 (NJ/MP/ML) and MrBayes 3.1.2 (BI). The analysis involved 102 nucleotide sequences. There were a total of 638 positions in the final dataset.

cies (i.e. *P. africanum*, *P. jankowskii*, *P. ugandae*, *P. wihtermanii*) are known only from data in the literature, so their proper affiliation cannot be verified due to a lack of living strains (cf Krenek *et al*. 2015). In such cases, there is no possibility to analyse a standardised DNA fragment (e.g. the COI DNA barcode for ciliates), which would not only confirm the species affiliation, but would also allow for an assessment of the haplotype variability.

Tropical biodiversity of the *P. aurelia* species complex: new insights from the COI haplotype variability

The application of a COI DNA fragment analysis allows not only for ciliate species or cryptic species identification, but also an intraspecific biodiversity assessment (Rajter *et al*. 2022, Strüder-Kypke & Lynn 2010). In the *Paramecium* genus, COI DNA fragments were successfully used for the first time almost twenty years ago (Barth *et al*. 2006). Currently, the method is widely used and has allowed for the clear discrimination of cryptic species in the *P. aurelia*, *P. bursaria* and *P. jenningsi* complexes (Greczek-Stachura *et al*. 2021, Przyboś & Tarcz 2016, Tarcz *et al*. 2013), as well as intraspecific variability assessments (Tarcz *et al*. 2018) or an identification of the hidden biodiversity of known species (Przyboś *et al*. 2016).

Eight COI haplotypes were found in the currently studied material (six species of the *P. aurelia* complex), with five of them identified for the first time (Figs 3, 4). This indicates that sampling tropical water bodies and the finding of 'hidden' molecular variability may change our understanding of ciliate biodiversity. Moreover, all collections of the water samples were made 'by the way' of the other activities (e.g. holidays, excursions or attending conferences) and not during professional protozoological research.

Haplotypes distribution of the newly identified *Paramecium aurelia* strains

Paramecium primaurelia. A cosmopolitan species known from all zoogeographic realms, except the Australasian ecozone (Przyboś & Surmacz 2010, Tarcz *et al*. 2013). An up-to-date COI fragment analysis revealed the occurrence of nine haplotypes, including haplotype Pa1COI_09 identified in specimens from the water sample collected in the Dominican Republic (Fig. 3). The second currently identified *P. primaurelia* population, from Namibia, is characterised by haplotype Pa1COI_01 with a global range of occurrence (Fig 3, Table S1). Most of the *P. primaurelia* populations studied so far are characterised by the haplotype Pa1COI_01. The other haplotypes are mainly characteristic of isolated populations from the tropics: Pa1COI_03 and Pa1COI_06 from the Indomalayan realm; Pa1COI_08 and Pa1COI_09 from the Neotropical realm. It is also worth noting that these haplotypes are genetically distant from other *P. primaurelia* haplotypes. In summary, *P. primaurelia* is a species with a wide range of occurrences, but also with a high variability of COI haplotypes (intraspecific *p*-distances 0.000-0.041). Moreover, new haplotypes not found elsewhere are being identified in tropical populations (Fig. 4), which suggests the potential undiscovered biodiversity of this species.

Paramecium tetraurelia. A species with a worldwide occurrence pattern (Tarcz *et al*. 2013); however, with low intraspecific variability (intraspecific *p*-distances 0.000-0.014 without strains, which appeared in the *P. octaurelia* clade) in comparison, for example, to *P. primaurelia* (Fig. 3). Two dominant COI haplotypes are present on several ecozones: Pa4COI_02 in the Palearctic and Nearctic; and Pa4COI_04 in the Palearctic, Afrotropical and Neotropical, including the currently identified strain from Madagascar.

Paramecium sexaurelia has the highest level of genetic variability within the *Paramecium aurelia* complex (intraspecific *p*-distances 0.000-0.108). The number of nucleotide substitutions between different haplotypes of *P. sexaurelia* is equal to, or even higher than, those found between the particular species of the *Paramecium aurelia* complex (Fig. 3). It is supposed that the occurrence of such high variability may be related to the fact that *P. sexaurelia* may have been the first species to diverge from among the current species of the *P. aurelia* complex, and was dispersed globally before the continents split (McGrath *et al*. 2014). The COI haplotypes from Madagascar (Pa6COI_10 and Pa6COI_11) were identified for the first time. Moreover, they are very distant from each other. Similarly, the haplotypes Pa6COI_01 (Puerto Rico) and Pa6COI_09 (Ethiopia) are also distant from the other *P. sexaurelia* haplotypes. Despite such interspecific differences (Fig. 3), *P. sexaurelia* forms a monophyletic clade on the tree (Fig. 2). It is noteworthy that, in previous studies of tropical *P. sexaurelia* populations, several new COI haplotypes were also found (Przyboś & Tarcz 2018, Przyboś *et al*. 2016).

Paramecium octaurelia is widespread throughout tropical and subtropical areas (Przyboś & Tarcz 2018, Sonneborn 1975), including its discovery for the first time in a Neotropical locality (Dominica). However, despite the considerable

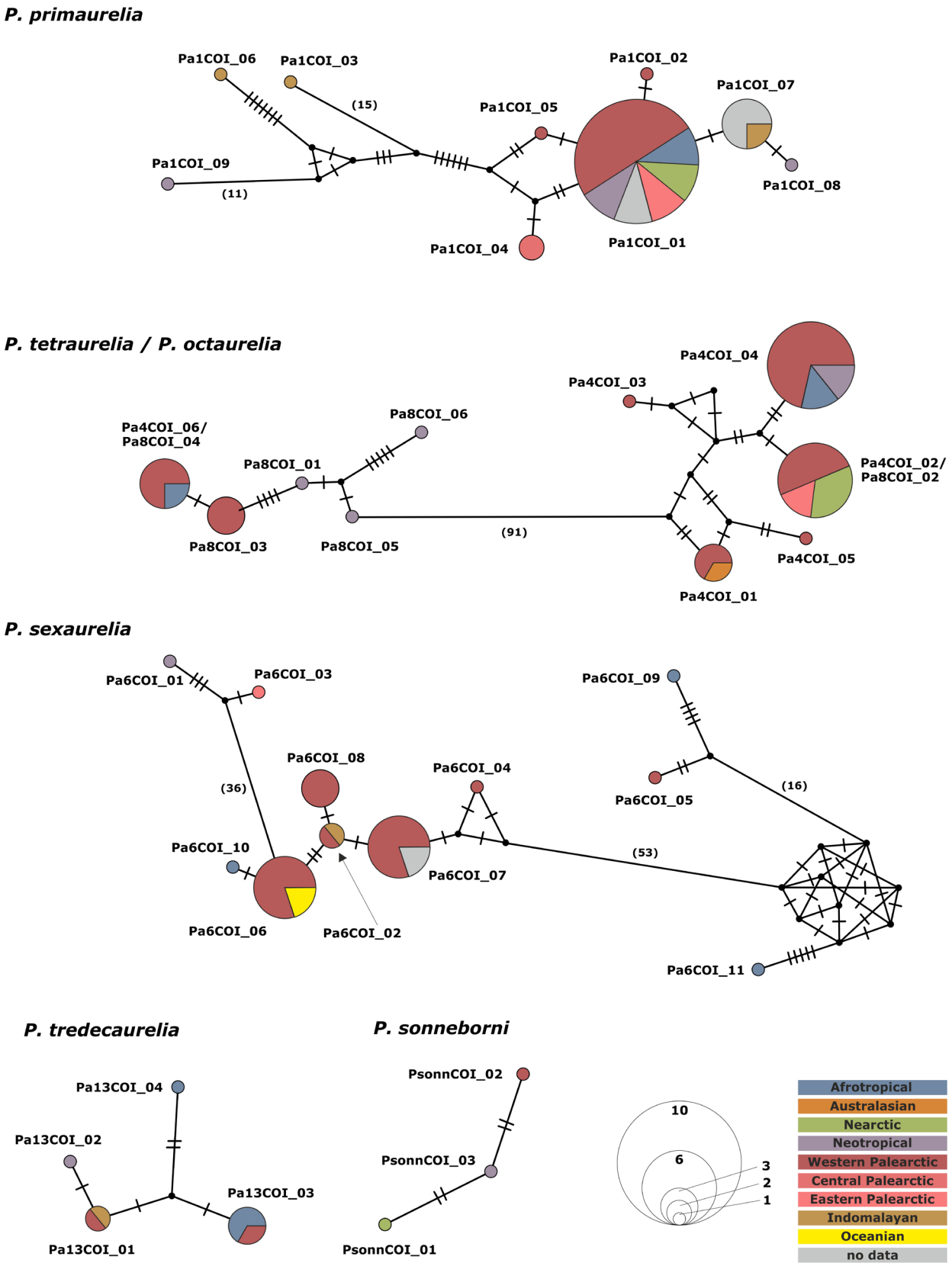

Fig. 3. Haplotype network of the *Paramecium aurelia* complex constructed using 100 mitochondrial COI gene sequences. All strains are listed in Table S1. The network presents interrelationships between *P. aurelia* COI haplotypes concerning their geographical origin. The different colors indicate the corresponding zoogeographical regions. Hatch marks on individual branches represent nucleotide substitutions (the corresponding number is provided for more than 10 substitutions). Analyses were conducted using the median joining method in PopART software v. 1.7.

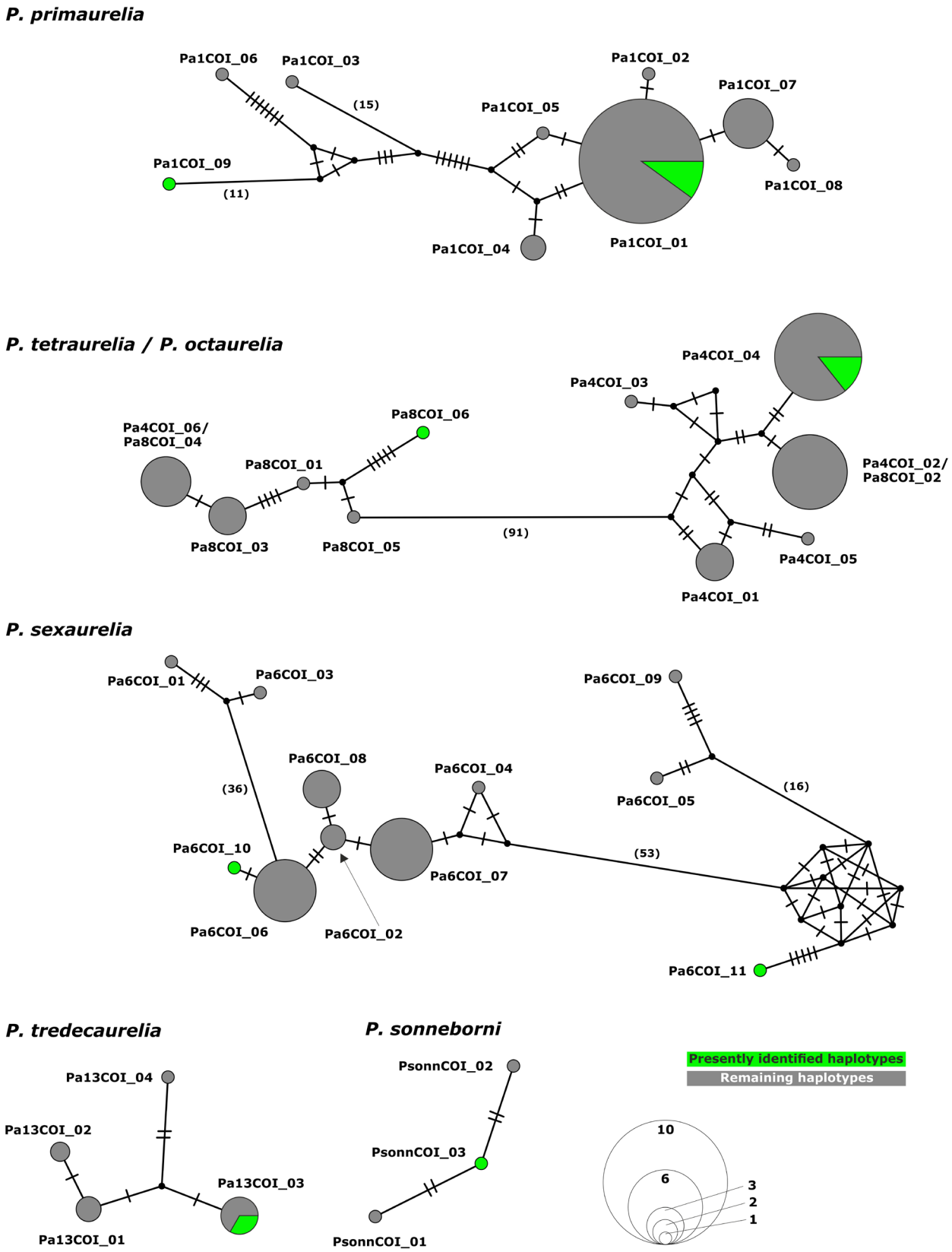

Fig. 4. Haplotype network of the *Paramecium aurelia* complex constructed using 100 mitochondrial COI gene sequences. All strains are listed in Table S1. The network presents a comparison of haplotypes obtained in the current study (light green) vs. the other localities (grey), where molecular data for particular *P. aurelia* species were available. Hatch marks on individual branches represent nucleotide substitutions (the corresponding number is provided for more than 10 substitutions). Analyses were conducted using the median joining method in PopART software v. 1.7.

intercontinental distances between the sampling sites, *P. octaurelia* presents a relatively low variability (intraspecific *p*-distances 0.000-0.017 without strains, which appeared in the *P. tetraurelia* clade) in the COI fragment (Fig. 3). In the current study, we identified the new COI haplotype Pa8COI_06, which is the most distant from the other *P. octaurelia* haplotypes. Moreover, the neotropical haplotypes (Pa8COI_01, Pa8COI_05, Pa8COI_06) present a higher COI variability than the others (from Afrotropical and Palearctic ecozones) and are grouped by themselves on the haplotype network according to their geographical origin (Fig. 3). The current results may suggest that *P. octaurelia* is a common species in the Neotropics.

Paramecium tredecaurelia. In the current survey, we identified the seventh (or eighth – see below) stand of *Paramecium tredecaurelia*, and the second (or third – see below) in Madagascar (Table S1). With only seven strains previously documented, it is an uncommon species in the *P. aurelia* complex, given that the first strains (from France, Madagascar and Mexico) of *P. tredecaurelia* were described over sixty years ago (Rafalko & Sonneborn 1959). As was mentioned above, there are three Madagascar *P. tredecaurelia* stands (Przyboś & Tarcz 2018, Rafalko & Sonneborn 1959, present study), but we only have molecular data for two of them. Strain 328, described by Rafalko & Sonneborn in 1959, probably doesn't exist. It is also worth noting that this species, although rarely observed, has a wide distribution range in warm climates (Mexico, France, Israel, Ethiopia, Madagascar and Thailand). Moreover, three of the eight *P. tredecaurelia* sampling points were situated in Madagascar, which could indicate that it is quite a characteristic species for this island. According to the intraspecific variability, only four COI haplotypes have been identified to date, and half of them were found in Madagascar (Pa13COI_03 and Pa13COI_04). In addition, the current study confirms the previous hypothesis that *P. tredecaurelia* seems to be a non-polymorphic species (Przyboś *et al*. 2013b) (intraspecific *p*-distances 0.000-0.005).

Paramecium sonneborni. In the current study, we identified not only the third locality, but also the third COI haplotype (PsonnCOI_03). Although the first *P. sonneborni* strain has been found in the Nearctic ecozone (Aufderheide *et al*. 1983), it is close to the Neotropics. Therefore, the studies to date indicate that *P. sonneborni* might be characteristic of the neotropical realm, and has a low intraspecific variability (intraspecific *p*-distances 0.000-0.006).

Conclusions

In the current paper, new data on tropical localities of *the P. aurelia* complex has been presented, including the first data on its occurrence in the Dominican Republic (Neotropical realm). The obtained results suggest a potentially high variability in the tropical regions studied (more than 50% of the identified COI haplotypes were new). It is worth noting that the *Paramecium* sampling to date has been done only 'by the way' and not as a planned protozoological study. Each of the identified *Paramecium aurelia* species presents a different biogeographical variation: some are restricted to the tropics only, while some have a wide distribution. Some cosmopolitan species are also characterised by 'tropical' COI haplotypes that do not occur anywhere else. Therefore, it can be supposed that there is a potentially high biodiversity of *Paramecium* ciliates hidden in tropical areas.

Acknowledgements

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Author Contributions

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

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary Materials to this article can be found online at: http://www.isez.pan.krakow.pl/en/folia-biologica.html

Supplementary files:

Table SM.01. A list of currently studied *Paramecium* strains.

Table SM.02. p-distance matrix of the studied COI mtDNA fragments.

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