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Through a narrow lens: exploring the molecular biodiversity of *Paramecium sexaurelia* – tropical freshwater ciliate populations from the Palm House of the Jagiellonian University Botanical Garden, Kraków

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Original article

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The biodiversity of protists, which are key players in many ecosystems, remains understudied, particularly in tropical regions. The ciliate *Paramecium sexaurelia*, a cryptic species within the *Paramecium aurelia* complex, is typically restricted to warm climates. In this study, we examine the genetic variability of *P. sexaurelia* populations collected over three years (2016-2018) from water bodies in the palm houses of the Jagiellonian University Botanical Garden in Kraków. These artificial palm-house environments, which contain tropical plants, may serve as reservoirs for microbial eukaryotes native to warm climates, thereby providing a unique opportunity to study protist diversity outside their native regions. Our molecular analysis revealed a considerable amount of genetic diversity within these populations, as we detected 13 distinct COI haplotypes (Pa6COI_02, 07, 14-24). While two haplotypes (Pa6COI_02 and 07) matched previously known sequences, the remaining eleven haplotypes (Pa6COI_14-24) were novel to this study, demonstrating the unexplored genetic richness of *P. sexaurelia,* even in artificial habitats. Given the high genetic diversity and widespread distribution of the species, these results provide valuable insights into its population structure in controlled environments. The presence of *P. sexaurelia* in a temperate-climate palm house suggests possible plant-mediated introductions, raising intriguing questions about the dispersal and persistence of tropical protists beyond their native ranges. These findings highlight the often-overlooked role of botanical gardens in preserving microbial and eukaryotic diversity, while underscoring the value of such artificial habitats as natural laboratories for studying the biodiversity of tropical protists in non-native settings.

Key words: artificial habitats, COI haplotypes, greenhouses, microbial eukaryotes, seasonal variability, urban biodiversity hotspots.

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Protists, representing the paraphyletic assemblage of eukaryotic microorganisms once classified within Whittaker's (1969) five-kingdom system, play a crucial role in the functioning of many ecosystems (Perrin & Dorrell 2024; Singer *et al*. 2021). Despite their important ecological roles as primary producers, predators, decomposers and parasites (Massana *et al*. 2015), microbial eukaryotes are still largely underestimated in biodiversity assessments (Burki *et al*. 2021; Del Campo *et al*. 2014). This is particularly true in tropical regions (Lentendu *et al*. 2019), which are considered to be biodiversity hotspots (Myers *et al*. 2000).

The limited understanding of microbial eukaryote biodiversity stems from multiple interconnected factors. These include the small size of the organisms and the fact that some taxa may actually be comprised of complexes of cryptic species (Caron *et al*. 2009),

as well as the difficulty of culturing many species (Stepanauskas *et al*. 2012), the intrinsic complexity of their taxonomy and evolutionary relationships (Adl *et al*. 2019), insufficient reference databases (Gelis *et al*. 2024), and technical challenges in relation to molecular methodologies (Forster *et al*. 2019) or data accessibility (Paupério *et al*. 2023). Additionally, biased sampling of the environments (Lehtiniemi *et al*. 2022), particularly the undersampling of tropical areas (Tarcz *et al*. 2023), have further compounded these limitations. Each of these factors creates a bottleneck in our ability to fully catalogue and recognise the vast diversity of microbial eukaryotes, underscoring the need for continued methodological advancements and interdisciplinary research in order to comprehensively understand protists' role in the biosphere (Lukeš *et al*. 2024).

Addressing these challenges requires an integrative approach combining advanced molecular techniques, improved culturing methods, better curation of the reference data and comprehensive environmental sampling (Clamp & Lynn 2017). However, despite the considerable research potential of freshwater protist communities (Downing *et al*. 2006), field collection efforts are often hindered by natural factors, geopolitical instability and financial constraints, which collectively render comprehensive surveys of many water bodies nearly impossible (Tarcz 2024).

Beyond planned expeditions and scientific collaborations (Asănică *et al*. 2024), additional avenues for obtaining valuable protist biodiversity data include data sharing from high-throughput sequencing projects (Berry *et al*. 2021), citizen science initiatives (Chandler *et al*. 2017) and capacity-building efforts, such as training programmes for field biologists (Rieder *et al*. 2024).

An interesting yet often overlooked approach involves sampling water bodies within the palm houses and greenhouses of botanical gardens, which often function as miniature tropical islands of biodiversity in urban areas with colder climates (Kolicka *et al*. 2015; Komala & Przyboś 2001). These artificial tropical environments, particularly those housing Bromeliaceae plants with their water-filled phytotelmata, provide habitats for diverse organisms, including protists (Dunthorn *et al*. 2012; Durán-Ramírez *et al*. 2015; Kolicka *et al*. 2016). Representatives of the flagship ciliate genus *Paramecium* have been documented in both phytotelmata (Buosi *et al*. 2014) and palm house ponds (Komala & Przyboś 2001; Przyboś *et al*. 2016), demonstrating the potential of these artificial habitats for protist research.

One protist species previously recorded in palm house ponds is the ciliate *Paramecium sexaurelia* (Przyboś *et al*. 2016), one of sixteen cryptic species within the *Paramecium aurelia* complex commonly found in tropical regions (Tarcz *et al*. 2023). Its worldwide distribution, though restricted to warm climates (Figure 1), may have originated before the continental separation, allowing it to achieve

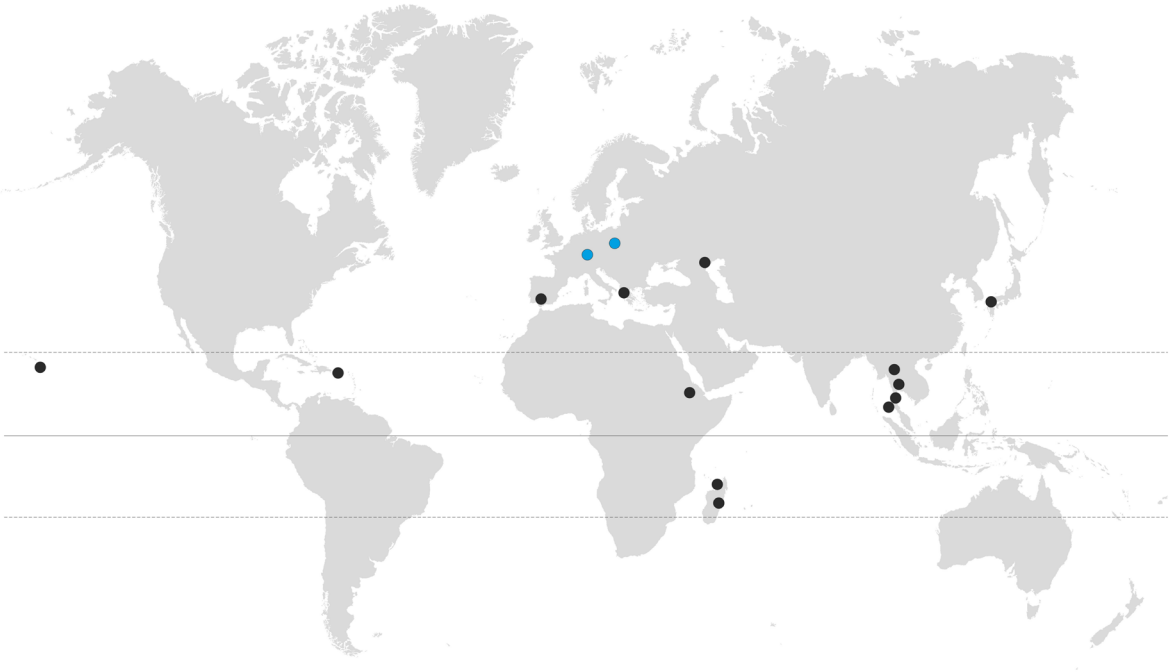

Fig. 1. Geographic distribution of the studied strains of the *Paramecium sexaurelia* complex. The Equator is marked with a solid line, while the Tropics of Cancer and Capricorn are marked with dashed lines. Black circles indicate locations of natural water bodies, while blue circles indicate locations of water bodies in botanical gardens located in Stuttgart and Kraków.

a global range without an extensive recent migration (Johri *et al*. 2017). However, *P. sexaurelia* has not been documented as naturally occurring in temperate climates, including Poland, despite more than 60 years of intensive faunistic research beginning in the 1960 s (Przyboś & Surmacz 2010). Therefore, its presence in palm house water bodies likely resulted from an introduction via tropical plant transport (Przyboś *et al.* 2016). Importantly, *P. sexaurelia* exhibits a high level of genetic variability compared to other species in the *P. aurelia* complex (Przyboś *et al*. 2010; Tarcz *et al*. 2023), making it a suitable model for studying tropical microbial eukaryote biodiversity in artificial 'tropical islands' within cooler climates.

The current survey employs DNA barcode mitochondrial COI gene sequencing with the aim to assess both the spatial and temporal genetic variation of *Paramecium sexaurelia* populations collected over three years (2016-2018) from two water bodies located in the palm houses of the Jagiellonian University Botanical Garden in Krakow. Given the occurrence of *P. sexaurelia* in various regions and its potential genetic diversity, examining the population variability in these reservoirs, which house plants from tropical regions, may provide insights into the genetic diversity of the species under different environmental conditions. Such research may provide a foundation for future studies targeting specific geographic regions.

Materials and Methods

Material

The *Paramecium* strains studied in the present paper, representing the *P. aurelia* species complex, are listed in Supplementary Materials: Tables S1 and S2. Newly identified strains were collected from two palm-house water bodies in the Botanical Garden of the Jagiellonian University, Kraków. The Botanical Garden, established in 1783, is situated east of the Old Town and covers an area of 9.6 hectares. It contains three ponds and two greenhouses (palm houses). The garden has been described in detail in Przyboś *et al.* (2016).

Methods

Methods of collecting samples and establishing strains

The material for this study was collected over three vegetation seasons (spring, summer and autumn) between 2016 and 2018, from two artificial water bodies located within the palm houses of the Jagiellonian University Botanical Garden in Kraków. In each water body, water samples (50 ml) containing plankton and plant debris were collected from four sampling points (designated 1-4) that were evenly distributed along the shoreline (for details, see Przyboś *et al.* (2016)). Samples were taken from the surface layer near the edge of the water body.

This sampling approach allowed for an analysis of the species microdistribution at each sampling point, reflecting natural species associations at specific locations and times, as well as seasonal dynamics and dominance patterns in the studied habitats. *Paramecium* cells found in the freshly collected material were isolated, either directly or after supplementation with a small amount of fresh culture medium. In samples where representatives of the *Paramecium aurelia* species complex were detected, up to ten clonal strains per sample were established for the purpose of species identification. The strains are listed in Table S1 (SM.01).

Identification of established strains of *P. sexaurelia*

Sonneborn's methods (1950, 1970) of cultivating and identifying strains were used. Paramecia were cultured at 27°C in a medium of dried lettuce in distilled water, then inoculated with *Enterobacter aerogenes* and supplemented with 0.8 mg/ml β-sitosterol (Merck, Darmstadt, Germany). They were identified initially by eye under a stereo microscope Nikon SMZ800 as members of the *P. aurelia* species complex. New strains were then identified as *Paramecium sexaurelia* based on a strong conjugation between the studied strain and the reference strain (strain 159 from Puerto Rico) of the species (Sonneborn 1975). The standard strain belongs to the collection of *P. aurelia* spp. of the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków, Poland.

Molecular techniques

Genomic DNA of the *Paramecium* was isolated (approximately 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. The supernatant was then removed and the remaining cells were resuspended in lysis buffer and proteinase K. The proteinase K buffer

step consisted of two parts: pre-lysis sample incubation at 56°C for 3 h; and lysis sample incubation at 70°C for 10 min. Protocol details 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'), with a protocol previously described in Strüder-Kypke & 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 min 15 s, 72°C for 1 min 30 s and 30 cycles of 94°C for 45 s, 55°C for 1 min 15 s, 72°C for 1 min 30 s, 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 × 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 was mixed with 2 µl of Exo-BAP Mix (EURx, Poland), and 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'-TGTAAAACGACGGCCAGT-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). The studied COI sequences are available in the NCBI GenBank database (see SM.01. Table S1).

Data analyses

The sequences were evaluated using Chromas Lite v2.1.1 (Technelysium, Australia). The alignment of the studied COI mtDNA fragment was constructed using BioEdit v7.2.5 software (Hall 1999) and was checked manually. All the sequences obtained were unambiguous and were used for further analyses. The mean uncorrected p-distances were calculated using MEGA v12 (Kumar *et al*. 2024). Neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using MEGA v12 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 = 2.336, I = 0.535) was identified as the best nucleotide substitution model for the maximum likelihood tree reconstruction using MEGA v12 software. 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 generations. 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 haplotypes of the *Paramecium sexaurelia* strains, was reconstructed using the Median-Joining method (Bandelt *et al.* 1999) implemented in PopART v1.7 software (Leigh & Bryant 2015).

Results

Spatial and temporal dynamics of COI haplotype diversity in *Paramecium sexaurelia* strains isolated from the Kraków Botanical Garden

An intensive, multi-year sampling campaign was conducted in the Jagiellonian University Botanical Garden in Kraków, Poland, to characterise the genetic diversity of *Paramecium sexaurelia*.

Spatially, the haplotype composition differed markedly between the water bodies. At the Palm-House I waterbody, spanning 2016-2018, we identified 9 haplotypes (Pa6COI_02, 07, 14, 16, 19, 21, 22, 23, 24), while the Palm-House II waterbody yielded 7 haplotypes (Pa6COI_07, 15, 17, 18, 19, 20, 21). Only three haplotypes (Pa6COI_07, 19, 21) were shared by both sites; the others were unique to one habitat, with Pa6COI_02, 14, 16, 22-24 found only in Palm-House I and Pa6COI_15, 17, 18, 20 exclusive to Palm-House II.

Temporal patterns also emerged across the study period. In 2016 we recovered haplotypes 07, 14, 15; while in 2017 we found 02, 07, 16-21; and in 2018 we observed haplotypes 02, 07, 19, 21-24. Several haplotypes, notably Pa6COI_07, 19, and 21, persisted

Fig. 2. Phylogenetic tree constructed for 83 *Paramecium aurelia* strains (*Paramecium caudatum* and *Paramecium multimicronucleatum* species were used as outgroups). All the strains are listed in Tables S1-S2 (SM.01., SM.02.) The tree was built based on the mitochondrial COI fragment using the maximum likelihood (ML). Bootstrap values for neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and posterior probabilities for the Bayesian inference (BI) are presented. Bootstrap values lower than 50% (posterior probabilities <0.50) are not shown. Dashes represent no bootstrap or posterior probability value at a given node. All positions containing gaps and missing data were eliminated. The phylogenetic analyses were conducted using MEGA v12 (NJ/MP/ML) and MrBayes 3.1.2 (BI). The analysis involved 85 nucleotide sequences. There was a total of 641 positions included in the final dataset.

across multiple years, whereas others appeared only in a single year; for example, Pa6COI_14 and 15 were detected only in 2016, while Pa6COI_22-24 appeared only in 2018.

In summary, the multi-year sampling effort in the Kraków Botanical Garden revealed a stable, dominant resident population (Pa6COI_07) coexisting with a surprisingly large number of rare haplotypes. It also confirmed the presence of a stable, non-native haplotype (Pa6COI_02), previously identified in Thailand (see SM.01 Table S1), highlighting the Botanical Garden's role as a 'melting pot' for microbial diversity.

Intraspecific COI diversity and phylogenetic structure of *Paramecium sexaurelia*

Based on 55 COI sequences representing 24 COI haplotypes, including 11 newly identified in this study, *P. sexaurelia* exhibits substantial intraspecific genetic diversity. The haplotype diversity (Hd) is high (0.8781), and it seems to be high on a global scale. Pairwise p-distances ranged from 0.000 to a maximum of 0.143, observed between strains A126_ISEA.PAS (Pa6COI_01) and A567_ISEA.PAS (Pa6COI_09). These findings confirm a broad range of mitochondrial COI divergence within the species (SM.03. Table S3).

The phylogenetic analyses placed all *P. sexaurelia* strains within a single, well-supported monophyletic clade of the *P. aurelia* species complex, with high bootstrap values supporting the base of the clade (Figure 3). However, the internal relationships among haplotypes remained largely unresolved, with many nodes receiving low statistical support. Despite this, the species can be subdivided into at least three genetically distinct lineages (designated here as A, B and C), corresponding to major haplotype clusters observed in both the phylogenetic tree and haplotype network (Figs 2-4).

Haplotype network structure and biogeographic patterns in *Paramecium sexaurelia*

The haplotype network of *P. sexaurelia* reveals 24 distinct COI haplotypes, reflecting a high level of intraspecific genetic diversity and a complex evolutionary structure. Three major genetic groups (A, B, C) emerged from the network analysis, which is consistent with previous phylogenetic findings. All the COI sequences obtained in the current study, as well as those remaining from the Botanical Garden, belonged to haplogroup A (Figure 4). The Pa6COI_07 haplotype is comprised of 18 sequences primarily from the Kraków Botanical Garden, along with representatives from Thailand (HH2.1). This group occupies a central and a highly connected position

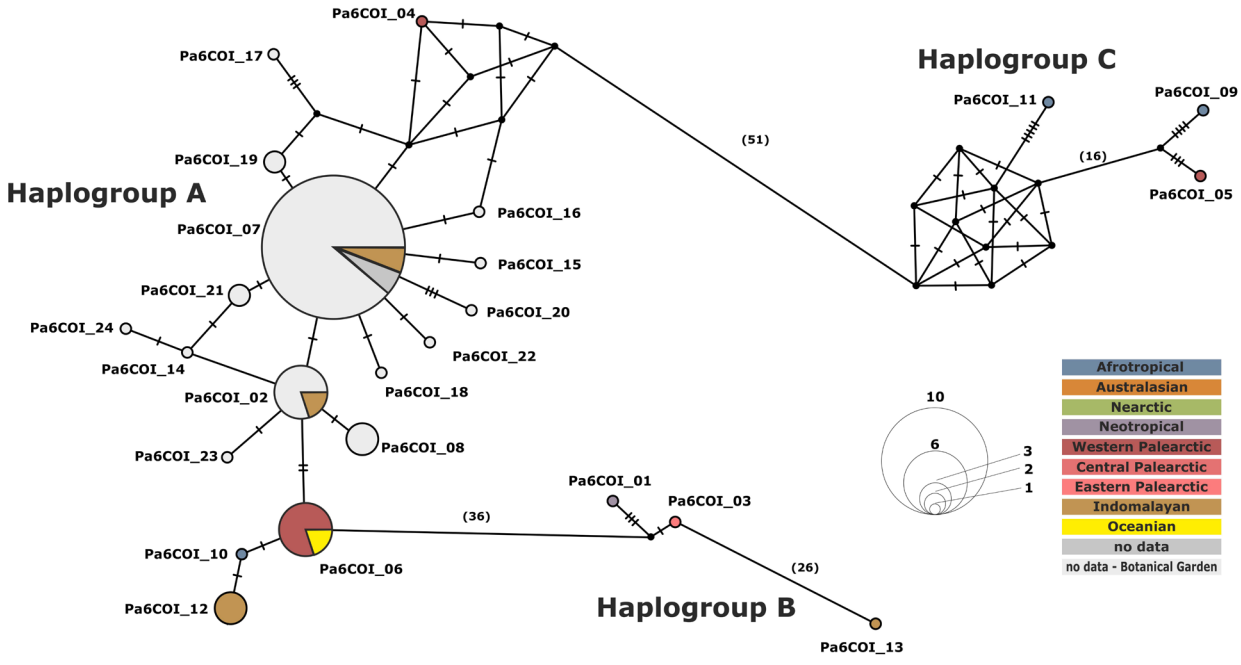

Fig. 3. Haplotype network of the *Paramecium sexaurelia* constructed using 55 mitochondrial COI gene sequences. All the strains are listed in Table S1. The network presents interrelationships between *P. sexaurelia* COI haplotypes concerning their geographical origin. The different colours indicate the corresponding zoogeographical regions. Hatch marks on individual branches represent nucleotide substitutions (the corresponding number is provided for more than 10 substitutions). The analyses were conducted using the median joining method in PopART software v. 1.7.

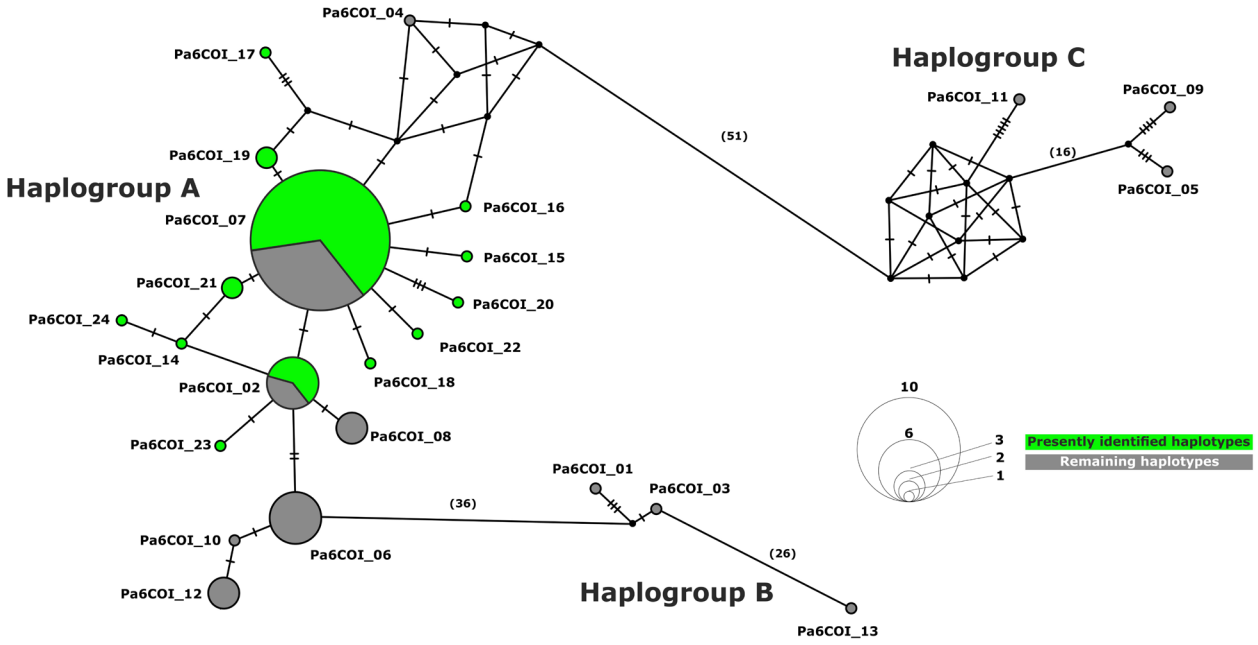

Fig. 4. Haplotype network of the *Paramecium sexaurelia* constructed using 55 mitochondrial COI gene sequences. All the strains are listed in Table S1. The network presents a comparison of haplotypes obtained in the current study (light green) vs the other localities (grey), where molecular data for particular *P. sexaurelia* strains were available. Hatch marks on individual branches represent nucleotide substitutions (the corresponding number is provided for more than 10 substitutions). The analyses were conducted using the median joining method in PopART software v. 1.7.

within the network, suggesting it may represent either an ancestral lineage or a particularly successful evolutionary variant with a broad geographic distribution. The centrality of this group implies its potential role as a hub for subsequent diversification events within haplogroup A. The Pa6COI_02 haplotype forms a distinct cluster that includes strains from both Thailand and the Botanical Garden Kraków (BG14, A713, A723, A724), positioned at a considerable genetic distance from Pa6COI_07. This separation by multiple mutational steps supports its interpretation as a divergent lineage that has undergone independent evolutionary trajectories. In contrast, the Pa6COI_06, 10, 12 cluster occupies a more peripheral position and is comprised of haplotypes from geographically diverse regions, including Greece, Germany, Madagascar, Russia, Spain, Thailand and Hawaii. This group maintains a clear genetic separation from Pa6COI_07 while displaying internal connectivity among its constituent members, suggesting a distinct evolutionary history with a subsequent geographic dispersal (Figure 3).

A particularly noteworthy feature of the network is the presence of eleven newly described haplotypes (Pa6COI_14 - Pa6COI_24) identified in this study from the Kraków Botanical Garden. These novel variants are dispersed throughout the network structure and are often connected to central haplotypes such as Pa6COI_02 or Pa6COI_07 by single mutational steps.

The geographic distribution patterns reveal both widespread and geographically restricted haplotypes. Conversely, certain haplotypes show a pronounced geographic restriction, exemplified by Pa6COI_09-11 from the Afrotropical region and Pa6COI_14-24 from the Kraków Botanical Garden. This geographic structuring suggests that the *P. sexaurelia* diversity reflects a complex interplay of ancient dispersal events, regionally persistent lineages and ongoing local evolutionary processes.

Discussion

Paramecium sexaurelia: an example of a microeukaryote gateway to tropical biodiversity

Genetic diversity serves as a fundamental driver of the adaptation of species to environmental change. Its erosion poses critical threats to an ecosystem's resilience and functionality. Recent studies have documented substantial reductions in diversity across multiple taxa (Shaw *et al.* 2025). These findings raise serious concerns about the stability of eco-

logical networks. While biodiversity loss has been extensively examined in well-characterised groups such as plants, animals and fungi, microbial eukaryotes, particularly protists, are experiencing equally alarming levels of decline. These declines remain poorly documented due to insufficient taxonomic resolution, limited sampling efforts and the persistent Linnean Shortfall – the discrepancy between described and actual species. This problem is compounded by the Taxonomic Impediment, which reflects shortages in taxonomic expertise and resources (de Araujo *et al.* 2018; Emerson 2025; Wiens 2023).

Protists play pivotal roles in nutrient cycling, food web dynamics and ecosystem regulation. They remain paradoxically among the least understood eukaryotic groups, despite their ecological importance. Although they have a relatively low number of formally described species, molecular surveys have consistently revealed extensive cryptic diversity (Stern *et al.* 2018). This suggests that their true richness may rival that of better-known multicellular lineages. To help mitigate these limitations, researchers have increasingly advocated for spatially explicit approaches. These have focused on biodiversity hotspots, which are geographic regions harbouring exceptional species richness while simultaneously facing imminent threats (Myers *et al.* 2000). Many of these hotspots strikingly coincide with areas of high protist diversity, particularly in tropical ecosystems globally recognised as epicentres of microbial eukaryotic richness (Bass & Cavalier-Smith 2004; Foissner 2006).

Environmental DNA (eDNA) metabarcoding has emerged as a transformative methodology for assessing microbial diversity. This approach, which operates in a non-invasive and high-throughput manner, is proving to be especially powerful in tropical regions where logistical and taxonomic challenges hinder traditional sampling techniques. The technique enables the detection of hundreds to thousands of operational taxonomic units from single soil or water samples. It reveals orders of magnitude of greater diversity than conventional morphological studies (Bik *et al.* 2012; Burki *et al.* 2021; de Vargas *et al.* 2015; Mahé *et al.* 2017; Pawlowski *et al.* 2016).

With the growing accumulation of eDNA and metagenomic datasets, the challenge has shifted from data generation to data interpretation (Karlicki *et al.* 2022). This transition is opening new avenues for artificial intelligence applications that are increasingly being recognised as key tools for addressing biodiversity shortfalls. AI offers a promise for automating species identification, modelling species distributions and predicting ecosystem responses. Machine learning algorithms can process massive image-based or sequence-based datasets (Miller *et al.* 2025; Pollock *et al.* 2025; Reynolds *et al.* 2025). However, a major limitation remains: most current AI classifiers are trained on temperate-region datasets. They fail to generalise effectively to the hyperdiverse and often unique communities found in tropical ecosystems. This creates an urgent need for region-specific training datasets and algorithms that are optimised for tropical protist diversity.

A promising solution involves leveraging well-studied model organisms that are both widely distributed and genetically diverse. The species studied here, *Paramecium sexaurelia*, represents such a candidate as a well-characterised ciliate species within the *P. aurelia* complex that has long served as a model in microbial eukaryotic genetics. This organism provides insights into cryptic speciation, genome rearrangement, cytoplasmic inheritance and endosymbiosis (Van Houten 2023). It exhibits a high degree of intraspecific genetic variability and wide geographic distribution in the tropics that largely overlaps with Myers' biodiversity hotspots (Figure 1) (Przyboś & Prajer 2015; Tarcz *et al.* 2023). Given its distinctive phylogeographic profile (Figure 3) and established model organism status, *P. sexaurelia* is uniquely positioned to serve as a benchmark species for developing and validating eDNA and AI-based biodiversity assessment tools. Its presence in tropical regions enhances its value as an ecological indicator that is capable of reflecting changes in the microbial community structure and ecosystem health. Furthermore, its well-characterised genomic data makes it ideal for the future training of AI models in species identification and predictive ecological modelling. This integration could ultimately bridge the gap between data-rich technological advances and data-poor biodiversity regions, thereby advancing our understanding and preservation of global biodiversity before it disappears unnoticed.

Artifcial ponds in greenhouses as potential biodiversity reservoirs for microeukaryotes

Understanding tropical protist biodiversity presents significant challenges, particularly when broad environmental DNA (eDNA) surveys may generate overwhelming amounts of data that obscure the resolution of individual, often cryptic species (Tarcz 2024). A more practical approach focuses on well-characterised model taxa that can serve as biodiversity indicators. Studies of the *Paramecium aurelia* complex have successfully employed specific marker genes in order to track species distributions and

haplotype diversity across different environments (Barth *et al.* 2006; Coleman 2005; Przyboś & Tarcz 2019).

The consistent discovery of novel genetic diversity across multiple sampling efforts underscores the remarkable cryptic variation within tropical protistan populations. Previous studies on *P. sexaurelia* have demonstrated that a COI mtDNA gene analysis of tropical localities routinely yields new haplotypes with each sampling campaign (Melekhin *et al.* 2024; Przyboś & Tarcz 2018; Tarcz *et al.* 2023), a pattern that was also clearly evident in the initial greenhouse study performed at the Jagiellonian University Botanical Garden (Przyboś *et al.* 2016). This tropical richness contrasts dramatically with temperate environments, where natural Kraków pond sampling at Opatkowice revealed a limited level of diversity within the *P. aurelia* complex, detecting only a few species with minimal COI haplotype variation (Przyboś *et al.* 2011).

The artificial tropical greenhouse ponds at the Jagiellonian University Botanical Garden harboured a substantially higher amount of diversity, with multiple *P. aurelia* species and extensive haplotype variation. During our comprehensive three-year survey (2016-2018) of these greenhouse systems, we isolated 28 *P. sexaurelia* strains representing 13 COI haplotypes, with 11 of them being newly identified, further supporting the pattern that every (or almost every) sampling effort in tropical or tropical-like environments may reveal previously unknown levels of genetic diversity. These findings are aligned with previous population genomic studies demonstrating that *P. sexaurelia* possesses significantly higher genome-wide polymorphism compared to other members of the *P. aurelia* complex (Johri *et al.* 2017), while reinforcing the exceptional genetic potential of tropical protistan lineages and the importance of continued systematic sampling in these biodiversity hotspots. The emergence of new haplotypes each season observed in the current survey may suggest multiple introduction events, as was previously observed in the case of *Coleps* (Barth *et al.* 2008). Based on the current research findings, botanical gardens are emerging as invaluable windows into the hidden diversity of tropical protistan communities, offering critical insights that can guide strategic biodiversity surveys in natural habitats. The comprehensive three-year study of *Paramecium sexaurelia* populations in the Jagiellonian University Botanical Garden's greenhouse systems exemplifies this potential, revealing a remarkable amount of genetic diversity that would be difficult to capture through traditional field sampling alone.

The spatial and temporal patterns observed in these greenhouse ponds provide crucial insights into the biogeographical processes that shape protistan diversity in natural tropical ecosystems. Despite their physical proximity, each water body maintained unique haplotype compositions, with Pond I containing 10 haplotypes and Pond II harbouring only 7. This suggests that fine-scale habitat differences can maintain genetically distinct populations. This pattern of genetic differentiation between neighbouring artificial habitats mirrors what might be expected in natural tropical water bodies, where environmental heterogeneity could drive even greater diversification. Therefore, the greenhouse study provides both a methodological framework and a compelling rationale for expanded biodiversity surveys in the world's tropical regions, where the true extent of protistan diversity awaits discovery.

Conclusions

The current survey demonstrates that *Paramecium sexaurelia* serves as an exceptional model organism for understanding tropical protist biodiversity patterns. The comprehensive analysis of artificial greenhouse ponds at the Jagiellonian University Botanical Garden revealed a remarkable amount of genetic diversity, with 28 strains representing 13 COI haplotypes, 11 of which were newly identified.

These findings underscore the urgent need for region-specific biodiversity assessment tools, particularly as current AI-based classification systems remain biased toward temperate datasets. *P. sexaurelia*'s well-characterised genetics, wide tropical distribution and overlap with Myers' biodiversity hotspots position it as an ideal benchmark species for developing eDNA and machine learning applications that are tailored to tropical ecosystems.

These results emphasise that even small, human-created habitats can maintain significant protist diversity, while offering accessible windows into tropical biodiversity patterns that would otherwise remain hidden in remote natural habitats.

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Author Contributions

Research concept and design: S.T. Collection and/or assembly of data: S.T., K.D., M.H., M.S., E.P.; Data analysis and interpretation: S.T. K.D., M.H., M.S., 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 Materials

Supplementary Materials to this article can be found online at:

http://www.isez.pan.krakow.pl/en/folia-biologica.html

Supplementary files:

SM.01. Table S1. A list of currently studied *Paramecium sexaurelia* strains.

SM.02. Table S2. A list of currently studied members of the *Paramecium aurelia* complex and two other *Paramecium* morphospecies used as an outgroup in the tree reconstruction.

SM.03. Table S3. Estimates of evolutionary divergence between the *Paramecium* sequences.

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