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Paramecium Diversity and a New Member of the *Paramecium aurelia* Species Complex Described from Mexico

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Abstract: *Paramecium* (Ciliophora) is an ideal model organism to study the biogeography of protists. However, many regions of the world, such as Central America, are still neglected in understanding *Paramecium* diversity. We combined morphological and molecular approaches to identify paramecia isolated from more than 130 samples collected from different waterbodies in several states of Mexico. We found representatives of six *Paramecium* morphospecies, including the rare species *Paramecium jenningsi*, and *Paramecium putrinum*, which is the first report of this species in tropical regions. We also retrieved five species of the *Paramecium aurelia* complex, and describe one new member of the complex, *Paramecium quindecaurelia* n. sp., which appears to be a sister species of *Paramecium biaurelia*. We discuss criteria currently applied for differentiating between sibling species in *Paramecium*. Additionally, we detected diverse bacterial symbionts in some of the collected ciliates.

Keywords: biogeography; ciliates; *Paramecium quindecaurelia*; cytochrome C oxidase subunit I gene; sibling species; species concept in protists; bacterial symbionts

1. Introduction

Paramecium is one of the most studied genera of ciliates. Currently, at least fourteen morphological species of *Paramecium* are recognized as valid, and several more require reinvestigation [1–3]. Most of the morphological species include a number of genetically isolated groups, referred to as syngens. In some cases, syngens have been elevated to full species (the *P. aurelia* species complex, [4]) or could be described at least as genetic species due to the well-proved absolute reproductive barrier separating them, such as syngens in *P. bursaria* [5]. Additionally, several cryptic species of *Paramecium* have been reported [3], yet they are disputed as true species due to the failure to establish their laboratory culturing and to collect more specimens in nature. Finally, some contested *Paramecium* species were documented only once from specific localities and were not subjected to thorough morphological, physiological or molecular description [1,6,7].

Most of the *Paramecium* morphospecies have recognizable morphological traits, allowing easy and fast identification of new representatives isolated from nature [1]. Numerous molecular phylogenetic analyses of inter- and intraspecific diversity of *Paramecium* have been performed, and molecular barcoding of different genes has allowed researchers to discriminate between morphospecies when morphological features were blurred [8,9], or even between sibling species or syngens within morphological species [5,10–12]. While the 18S rRNA gene has proven to be too conservative

to disclose the intraspecies relationships in *Paramecium*, the cytochrome C oxidase subunit I (COI) gene is routinely used as a barcoding sequence in *Paramecium* molecular phylogenetic analysis [3,9,13,14]. This gene is sufficiently divergent and permits the inference of reliable genus level tree configuration and existence of intraspecific groups within each *Paramecium* morphospecies [14–16].

Extensive sampling, even in well-studied territories, allows researchers to find new *Paramecium* species or to confirm and validate species previously described [2,3,17,18]. Moreover, as many geographic regions remain poorly studied for *Paramecium* species occurrence, it is very possible that knowledge of the diversity of this genus is incomplete. Central America has not been surveyed systematically for *Paramecium* diversity. In this study, we provide new data on *Paramecium* occurrence and distribution in Mexico, including description of a new species of the *P. aurelia* complex found in two remote localities. Additionally, we report the presence of bacterial symbionts in some collected *Paramecium* strains since their occurrence has been considered as a possible criterion for *Paramecium* species [19].

2. Materials and Methods

2.1. Sampling and Maintenance of Paramecium Strains

About 130 freshwater samples were taken from more than 40 different waterbodies (natural and artificial lakes and ponds, canals, streams, drains, wetlands, and even a water reservoir in a roof-top garden) in several localities of seven states of Mexico: Ciudad de México, Estado de México, Hidalgo, Querétaro, Veracruz, Quintana Roo, and Yucatán in January–March 2019. The volume of each water sample collected was 10–30 mL. Samples from the same waterbody were taken at a distance of at least 20 m from each other and, thus, were considered as representing separate populations of ciliates. The samples were quickly transported to the laboratory and screened for paramecia within 24 h after sampling. Ciliates were detected under a Nikon SMZ 800 (Nikon Corporation, Tokyo, Japan) stereomicroscope. Then, all samples were kept on rice grains for 10–14 days and monitored every three days for previously unnoticed paramecia to show up. Several cells from each "positive" sample were isolated separately into depression slides; when possible, we isolated up to 10 cells from each sample, aiming to represent paramecia of different sizes and cell shapes. The ciliates introduced in culture were maintained on lettuce medium bacterized the day before use with *Enterobacter cloacae*, and supplemented with 0.8 mg/L of β-sitosterol (Merck, Darmstadt, Germany), as described earlier [19]. All currently alive strains used in the study are available upon request from RC CCM collection (World Data Centre for Microorganisms, RN 1171), Saint Petersburg State University, Saint Petersburg, Russia.

2.2. DIC Microscopy and Stainings

Live cells observations were made with differential interference contrast (DIC) microscopy with a Nikon Labophot-2 microscope equipped with a Nikon Digital Sight DS2Mv (Nikon Corporation) camera. We observed the cytological features important for quick species identification in *Paramecium*, namely cell size and shape, size, number and structure of micronuclei, structure of contractile vacuoles, and presence of algal symbionts [1]. Several staining techniques were employed, including the Feulgen procedure in De Lamater protocol, Harris hematoxylin, silver nitrate impregnation after Champy's fixation, silver carbonate and protargol [20]. Morphometric measurements were taken from stained cells.

2.3. Molecular Identification of Paramecium Strains and Bacterial Symbionts

Paramecium strains isolated from all samples and attributed to different morphospecies were subjected to sequencing of the mitochondrial COI gene. Additionally, in order to reconstruct a complete molecular phylogenetic tree of the *P. aurelia* species complex, COI gene sequences were obtained for the *P. primaurelia* strains Ir 4-2 (Russia) and FT11 (Pakistan), *P. pentaurelia* strains NR-2 (USA) and Nr1-9 (Russia), *P. septaurelia* strains 227 and 38 (USA). The total cell DNA was extracted

from 100 to 200 cells of each strain using the GenElute Mammalian Genomic DNA Purification Kit (Sigma, Germany), according to the protocol "Genomic DNA from tissue". The PCR was performed using Encyclo Taq polymerase (Evrogen, Russia). The 767 bp-long partial COI gene sequences were amplified using the primers F388 and R1184, which are suitable for the majority of *Paramecium* species as described by Strüder-Kypke et al. [9]. For *P. primaurelia*, *P. triaurelia*, *P. pentaurelia* and *P. septaurelia*, the primers COI-long F (GATAAGGCTTGAGATGGCATACCCAGGAAG), and COI-longR (CAAAACCCATGTAAGCCATAACGTAGACAG) were designed, and 35 cycles of PCR were performed with an annealing temperature of 60 °C. Additionally, the partial sequences were obtained for the mitochondrial cytochrome C oxidase subunit II (COII) gene of some strains of *P. biaurelia* and presumably new species of the *P. aurelia* complex (see below; GenBank accession numbers MT318927–MT318930). In all cases, the same primers were used for PCR and sequencing. The partial 16S rRNA gene sequence for bacterial symbionts inhabiting *P. putrinum* strain K8 was amplified by PCR using the primers 16S alfa F19 and 16S alfa R1517, and sequenced with the primer 16S F343 [21]. All oligonucleotides were synthesized by Eurofins DNA (Germany). The PCR products were directly purified and sequenced at the Core Facility Center "Molecular and Cell Technologies" (St Petersburg State University, Saint Petersburg, Russia).

2.4. Molecular Phylogenetic Analysis

The COI gene sequences obtained in this study (GenBank accession numbers MT078136–MT078152, MT318931–MT318935) were aligned with COI gene sequences manually selected from GenBank or retrieved from ParameciumDB [22] for the strains with sequenced mitochondrial genomes [23]. Manual selection of entries was performed, since many COI gene sequences in GenBank are either too short, incorrectly assigned to a species or simply missing for some species in GenBank. The longer sequences were trimmed manually to obtain the 767 bp-long COI gene fragment, and the incomplete COI gene sequences from Genbank were chosen so that all sequences in the final set were at least 600 bp long. MUSCLE algorithm was used for alignment (online multiple alignment program [24]). Phylogenetic trees were constructed to infer the relationships of all isolated *Paramecium* strains using Phylogeny.fr [25,26]. The trees were computed by the bootstrapping procedure (500 bootstraps) and approximate likelihood ratio test method PhyML 3.1/3.0 aLRT [27]. The Maximum Likelihood analysis was performed with the HKY model.

2.5. Mating Tests

For the strains of the presumably new species of the *P. aurelia* complex, preparation of cell lines for mating tests and studies of autogamy were carried out by method of daily re-isolations [28]. The sexually reactive cultures were mixed with each other and also with *P. biaurelia* tester strains IST, Rieff, and Ts from the RC CCM collection. Strains Rieff and IST belong to the same mating type compatible with the Ts strain mating type. The conjugation was observed only between +18 °C and +21 °C. The conjugating couples were picked with the Pasteur pipette, then exconjugant cells were isolated into separate microaquariums, and F1 clones were established. The fragment of the nuclear *mtB* gene involved in mating type control [29] was amplified for F1 clones in 32 cycles of PCR using the primers bi-mtBF (GCACACCCTCTTAAAATAAGT) and bi-mtBR (AAATCTCGCAAACAACTACTG) with an annealing temperature of 55 °C. This fragment was sequenced using the same primers to confirm the heterozygosity of F1 clones (i.e., to confirm the exchange of pronuclei in conjugation), as allelic single nucleotide polymorphisms were visible on chromatograms as double peaks (data not shown). F2 progeny were obtained after autogamy of F1 clones, and survival rates of the F2 generation were counted. The F2 clones were considered as viable if they completed more than 6 divisions after autogamy, since old macronuclei remain functional during the first 5–6 vegetative divisions in *Paramecium* [19], and confer otherwise inviable cells to survive during that period.

3. Results

3.1. Diversity of Paramecium and Its Bacterial Symbionts Revealed by Extensive Sampling in Several Regions of Mexico

From all sampled Mexican localities, paramecia were found in 19 waterbodies. Strains from 30 populations of six morphospecies, namely representatives of the *P. aurelia* species complex, *P. jenningsi*, *P. caudatum*, *P. multimicronucleatum*, *P. bursaria*, and *P. putrinum* were introduced in the laboratory cultures. The most frequently recorded species was *P. multimicronucleatum* (15 populations from 9 waterbodies). Six populations from different waterbodies contained representatives of the *P. aurelia* species complex, while other morphospecies were relatively rare (Table 1). It was not uncommon to find several *Paramecium* species in the same community. The greatest diversity was detected in the lakes, ponds, and streams of the Cantera Oriente reserve (Mexico City), where we isolated a number of strains of *P. caudatum*, *P. multimicronucleatum*, *P. bursaria*, and *P. putrinum.* In several populations, some *Paramecium* specimens were inhabited by bacterial endosymbionts (Table 1, Figure 1). Most of these symbiotic bacteria still have to be identified and are currently being studied. Among the most interesting findings were presumably *Trichorickettsia* sp. abundantly present in host cytoplasm in several *P. putrinum* strains from Cantera Oriente (Figure 1A), as well as unknown cytoplasmic (Figure 1C,D) and intranuclear (Figure 1E,F) symbionts in different strains of *P. multimicronucleatum*. Tiny bacteria able to produce R-bodies resembling *Caedibacter* sp. or *Caedimonas* sp. [30] were found in cytoplasm of *P. tetraurelia* cells from Xochimilco Lake (Mexico City), while other cytoplasmic symbionts were detected in the *P. octaurelia* strain from Cenote Azul (Quintana Roo).

Table 1. List of all *Paramecium* strains isolated from natural populations in Mexico in the current study.

Morphological Species	Sibling Species/ Intraspecific Group	Strain Index	Waterbody	Origin	State	Coordinates	Bacterial Symbionts
The *Paramecium aurelia* species complex	*P. primaurelia*	CH	Water supply pond	Temozon, near Cenote Hubiku	Yucatán	20°49′05″ N/ 88°10′25″ W	ND [1]
	P. triaurelia	Chp3-1	Lake	Mexico City, Chapultepec lake	Ciudad de México	19°25′23″ N/ 99°11′07″ W	ND
	P. tetraurelia	X38	Lake	Mexico City, Xochimilco lake	Ciudad de México	19°16′46″ N/ 99°06′09″ W	Cytoplasmic, R-body-producing
	P. octaurelia	CA1	Cenote	Cenote Azul, Bacalar	Quintana Roo	18°38′51″ N/ 88°24′45″ W	Cytoplasmic
	P. quindecaurelia n. sp.	A65	Pond	Amealco	Querétaro	20°11′22″ N/ 100°08′28″ W	ND
		D88	Drain	Mexico City, Los Dinamos	Ciudad de México	19°16′02″ N/ 99°17′31″ W	ND
P. jenningsi	Syngen [3]	DK	Roof garden	Mexico City	Ciudad de México	19°25′36″N/ 99°09′35″W	ND
P. caudatum	NA [2]	K1-1	Pond	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	ND
		K5-2	Pond	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	ND
		V-1	Stream	Santuario Bosque de Niebla	Veracruz	19°30′47″ N/ 96°56′49″ W	ND
P. multimicronucleatum	Clade I	Chp5-3	Lake	Mexico City, Chapultepec lake	Ciudad de México	19°25′23″ N/ 99°11′07″ W	Intranuclear, in macronucleus [3]
		Chp3-4	Lake	Mexico City, Chapultepec lake	Ciudad de México	19°25′23″ N/ 99°11′07″ W	Intranuclear, in macronucleus
		E59	Lake	Endho lake	Hidalgo	20°08′25″ N/ 99°21′41″ W	ND

Table 1. *Cont.*

Morphological Species	Sibling Species/ Intraspecific Group	Strain Index	Waterbody	Origin	State	Coordinates	Bacterial Symbionts
	Clade II	K4-2	Pond	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	ND
		Chp10-2	Lake	Mexico City, Chapultepec lake	Ciudad de México	19°25′23″ N/ 99°11′07″ W	ND
		R49	Lake	Requena lake	Hidalgo	19°56′31″ N/ 99°19′54″ W	ND
		R51	Lake	Requena lake	Hidalgo	19°56′31″ N/ 99°19′54″ W	Intranuclear, in macronucleus
		R53	Lake	Requena lake	Hidalgo	19°56′31″ N/ 99°19′54″ W	ND
		R58	Lake	Requena lake	Hidalgo	19°56′31″ N/ 99°19′54″ W	ND
		L72	Wetlands	Lerma	Estado de México	19°15′35″ N/ 99°29′29″ W	ND
		SMM80-11	Lake	San Miguel Almaya	Estado de México	19°12′53″ N/ 99°26′18″ W	Cytoplasmic
		SK6	Wetlands	Laguna Chunyaxché, Sian Ka'an	Quintana Roo	20°04′15″ N/ 87°34′24″ W	ND
		LB2	Lagoon	Bacalar	Quintana Roo	18°41′46″ N/ 88°22′34″ W	ND
	Clade III	T42	Canal	Mexico City, Tláhuac	Ciudad de México	19°15′59″ N/ 99°00′31″ W	Cytoplasmic
		SMM81	Lake	San Miguel Almaya	Estado de México	19°12′53″ N/ 99°26′18″ W	ND
P. putrinum	NA	K6	Lake	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	Cytoplasmic [4]
		K8	Lake	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	Cytoplasmic
		K11-3	Stream	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	Cytoplasmic
P. bursaria	Syngen R3	K11-4	Stream	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	ND
		K15-1	Lake	Mexico City, Cantera Oriente	Ciudad de México	19°19′05″ N/ 99°10′22″ W	ND
		A66	Lake	Amealco	Querétaro	20°11′22″ N/ 100°08′28″ W	ND

[1] ND—not detected; [2] NA—non-applicable; [3] Similar in ChP5-3 and ChP3-4 strains; [4] Similar in K6, K8 and K11-3 strains.

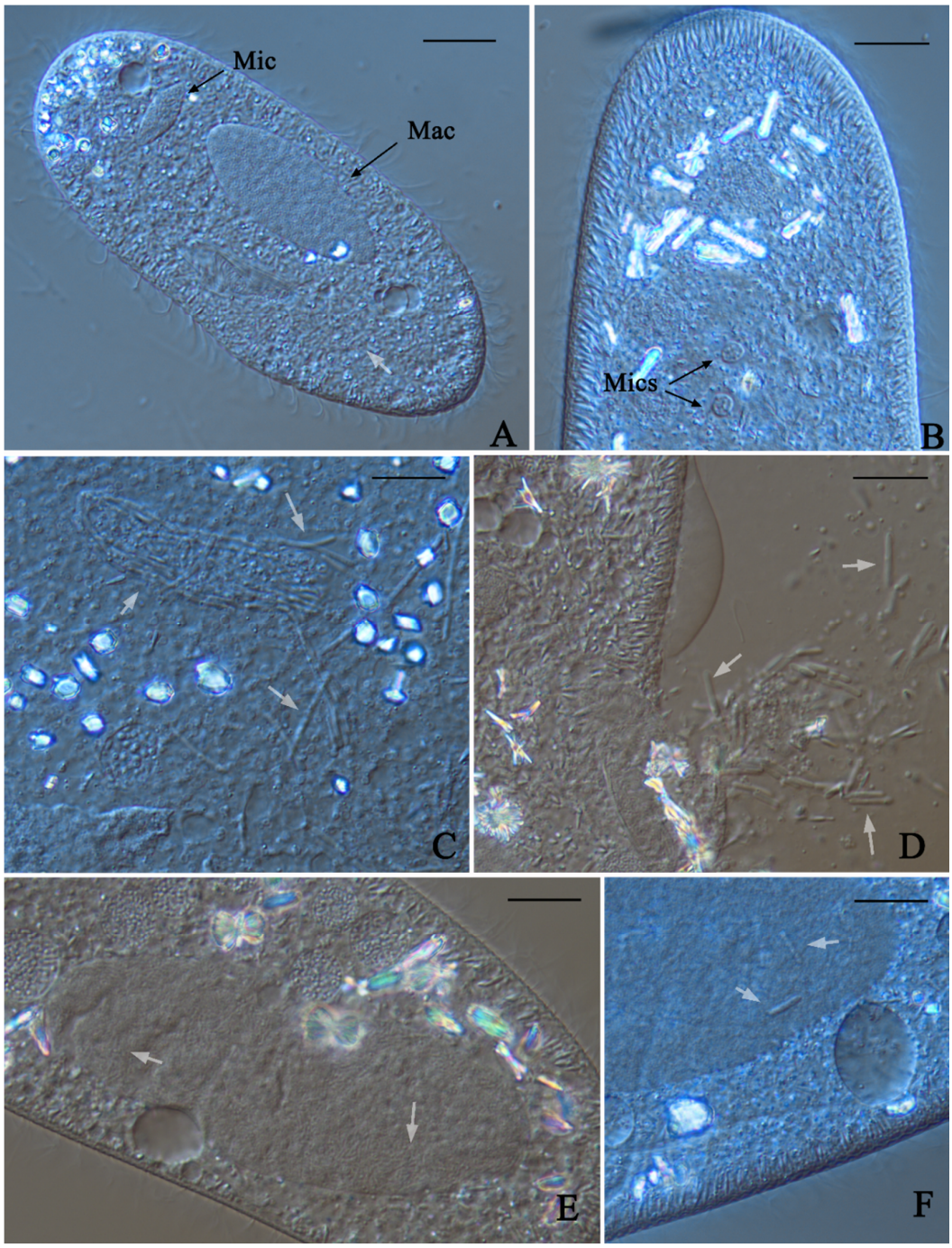

Figure 1. Diversity of *Paramecium* and its bacterial symbionts discovered in Mexico. (**A**) *Paramecium putrinum* cell (strain K8) with abundant bacteria in cytoplasm; (**B**) *Paramecium jenningsi* cell (strain DK) with two species-characteristic micronuclei; (**C**) Cytoplasmic bacteria in squashed cell of *Paramecium multimicronucleatum* (strain SMM80-11); (**D**) Cytoplasmic bacteria in squashed cell of *Paramecium multimicronucleatum* (strain T42); (**E**) Congregations of bacteria in the macronucleus of *Paramecium multimicronucleatum* cell (strain ChP5-3); (**F**) Bacteria in the macronucleus of *Paramecium multimicronucleatum* cell (strain R51). Symbiotic bacteria are marked with the grey arrows. Mac = macronucleus, Mic = micronucleus. Scale bars: 6 μm (**A**), 10 μm (**B–F**).

3.2. *Phylogenetic Analysis of the Collected Strains*

The strain attribution to certain morphospecies by DIC microscopy of the living cells was confirmed by COI gene sequencing and further positioning of a strain within the *Paramecium* phylogenetic tree.

Molecular characterization by COI and COII genes sequencing is the fastest and most reliable way to discriminate between different species of the *P. aurelia* complex [9,10]. All sibling species form separate branches on trees inferred from these molecular markers [10,31], and COI and COII gene barcodes make it possible to identify each species of the *P. aurelia* complex, eliminating the need for laborious round-robin mating tests with representatives of all species of the complex. COI gene sequencing can also reveal different haplotypes that cluster into intraspecific groups within *P. multimicronucleatum* [16,32] and into reproductively isolated syngens in *P. bursaria* [5]. However, the haplotypes revealed within *P. caudatum* do not form pronounced and well-supported branches [3,14]. Thus, analysis of COI gene sequences of Mexican *Paramecium* strains (Figure 2) showed that *P. bursaria* isolated from the lakes in Cantera Oriente (Mexico City) and Amealco (Querétaro) belonged to syngen R3, which is known to be widespread in Far East Russia, China, Japan, and South America [5]. *Paramecium multimicronucleatum* strains sorted into three branches within this morphospecies cluster. *Paramecium jenningsi* from Mexico City grouped together with strains of the species found in Asia and Africa (Figure 3). Finally, we succeeded in recovering four known species of the *P. aurelia* complex: *P. primaurelia*, *P. triaurelia*, *P. tetraurelia*, and *P. octaurelia*. Strains from two populations (Dinamos, Mexico City and Amealco, Querétaro) clustered in a separate branch as a sister species for *P. biaurelia* (Figure 3). This suggested to us that we may have discovered a novel member of the complex, so, these strains were thoroughly studied in order to figure out if they actually represented a new species of the *P. aurelia* complex.

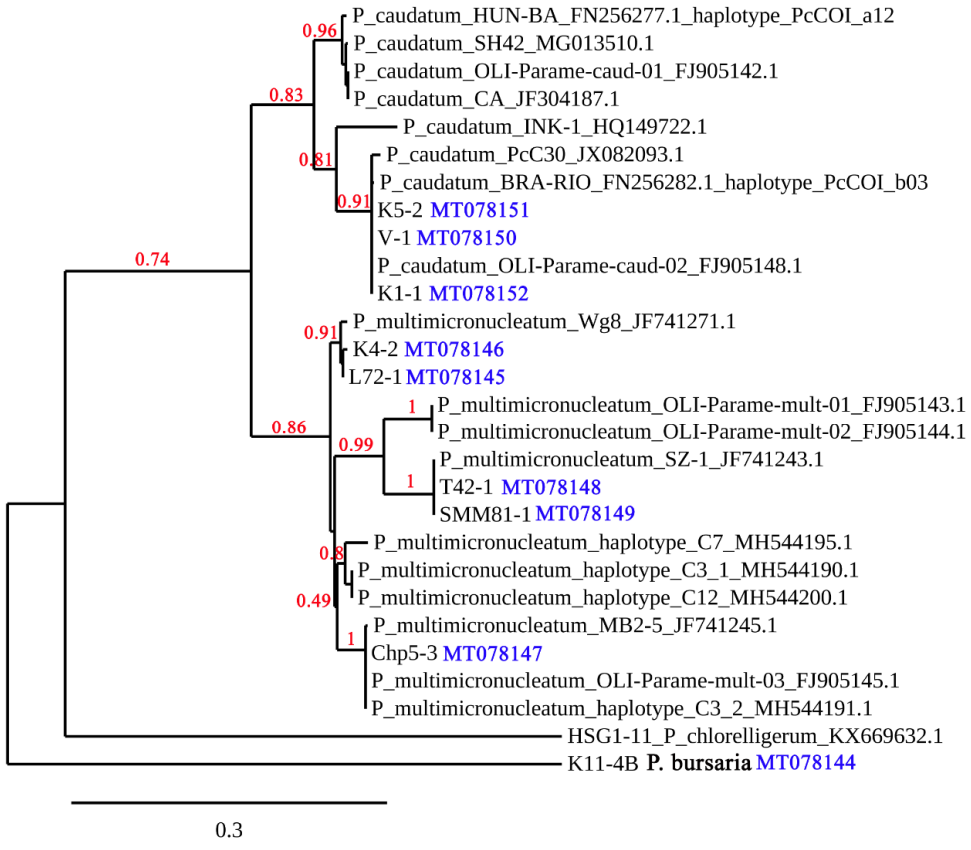

Figure 2. Phylogenetic position of *Paramecium caudatum* and *Paramecium multimicronucleatum* strains collected in this study on the mitochondrial COI gene tree. The sequences of *P. chlorelligerum* and *P. bursaria* are included as outgroups. Groups I, II and III within *P. multimicronucleatum* (see Table 1) are indicated. The tree was computed by the bootstrapping procedure (500 bootstraps) and approximate likelihood ratio test method PhyML 3.1/3.0 aLRT. Numbers at nodes represent posterior probabilities higher than 0.4. The scale bar represents the branch length, corresponding to 0.3 substitution per site. The COI gene sequence accession numbers of the strains collected in this study are shown in blue.

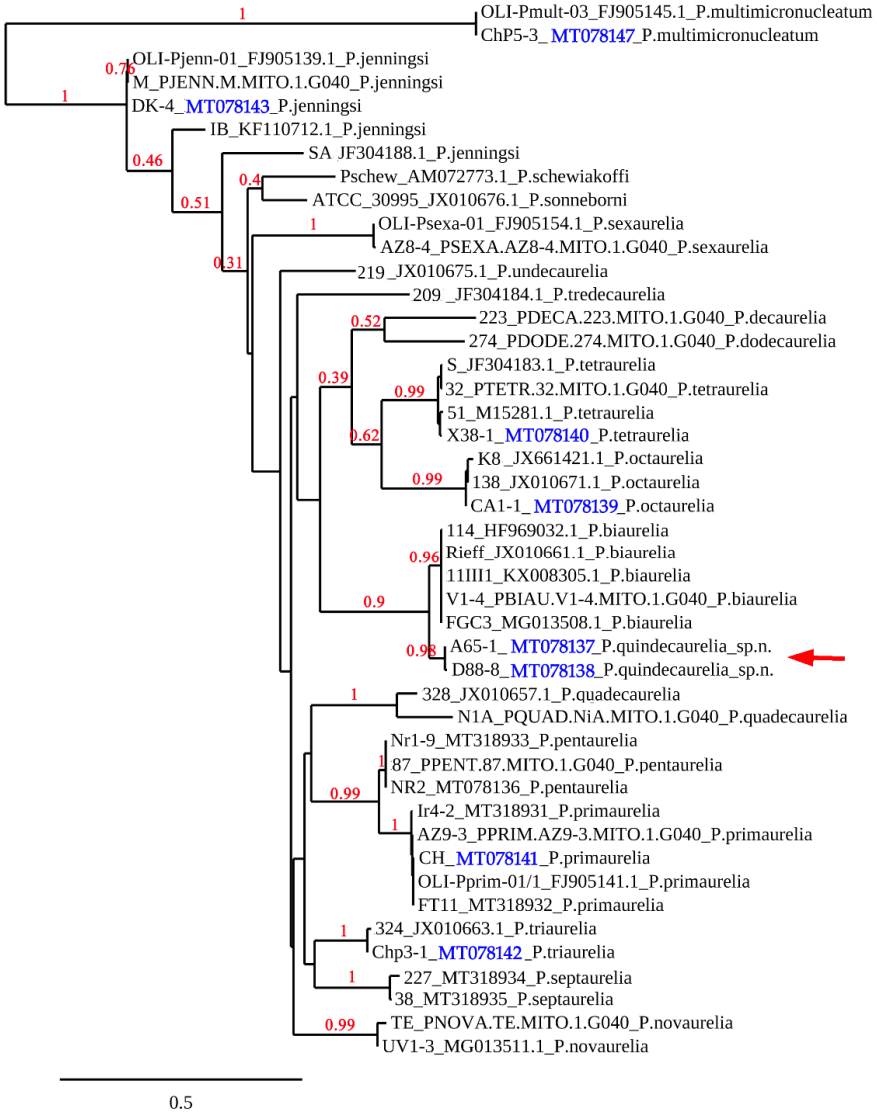

Figure 3. The phylogenetic tree of the *Paramecium aurelia* species complex inferred from mitochondrial COI gene sequences. *Paramecium multimicronucleatum* was used as an outgroup. The red arrow indicates position of *P. quindecaurelia* n. sp. The red brace shows the pair of sister species *P. biaurelia* and *P. quindecaurelia* n. sp.; the green brace shows the pair of sister species *P. primaurelia* and *P. pentaurelia*; the blue brace shows the pair of sister species *P. tetraurelia* and *P. octaurelia*. The tree was computed by the bootstrapping procedure (500 bootstraps) and approximate likelihood ratio test method PhyML 3.1/3.0 aLRT. Numbers at nodes represent posterior probabilities higher than 0.3. The scale bar represents the branch length, corresponding to 0.5 substitution per site. The COI gene sequence accession numbers of the strains collected in this study are shown in blue.

3.3. New Species of the Paramecium Aurelia Complex, P. Quindecaurelia n. sp.

The ciliates were collected from a small lake near the highway in Amealco, Querétaro (population A65) and from the lake drain in Los Dinamos, the National Park in the mountains on Mexico City territory (population D88). Several cells from both populations were isolated, and the obtained strains were introduced into laboratory cultures. They can be maintained in a variety of temperatures but demonstrated the best growth rate, three divisions per day, at 21–23 °C. The ciliates look like typical representatives of the *P. aurelia* complex (Figure 4). The cells are rather large, and maximum body length of 80 fixed specimens ranged from 104 to 168 µm with a mean length of 125 µm (16.9 µm standard error). Maximum body width of 80 specimens ranged from 19 to 39 µm with a mean width of

28 µm (8.0 µm standard error). The average kineties number was 55. The length of the infundibulum ranged from 15 to 36 µm with an average of 26 µm. All cells had one roundish macronucleus that was longer than wide (Figure 4I), as well as two micronuclei of the vesicular type (Figure 4G,H) typical for *P. aurelia* [1,4]. The average length of the macronucleus was 32 µm, and average width was 12 µm. Two contractile vacuoles were present per cell, and each vacuole had one pore and six or seven canals in different cells (Figure 4E,F). The macronucleus is fragmented into 35–40 pieces during autogamy, and two macronuclear anlagen are formed in the cell (Figure 4J). All morphometric data confirm that these strains are very similar to *P. biaurelia* ([33], Supplementary Table S1).

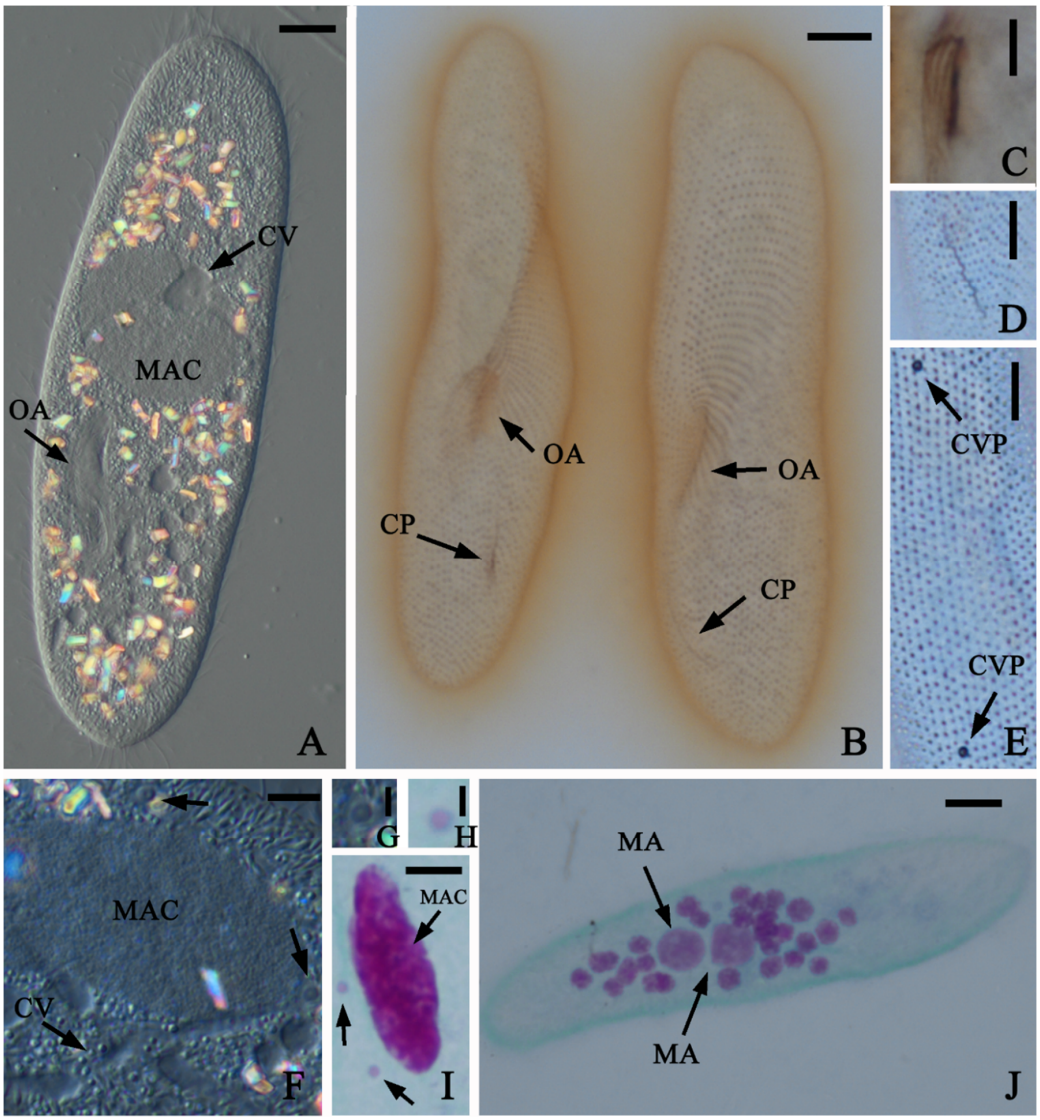

Figure 4. Morphological features of *Paramecium quindecaurelia* n. sp. (**A**) DIC live micrograph of a specimen; OA—oral aperture, CV—contractile vacuole with 6 or 7 canals; MAC—macronucleus. Silver nitrate impregnated cells: (**B**) Ventro-lateral and dorso-lateral cell projections. CP—cytoproct; (**C**) Buccal overture with buccal ciliature; (**D**) Cytoproct region; (**E**) One pore (CVP) per one contractile vacuole is characteristic. (**F**) DIC live micrograph showing the contractile vacuole (CV), the macronucleus (MAC) and two micronuclei (marked by the arrows). Micronuclei have typical vesicular shape shown by DIC (**G**) and on Feulgen stained cell (**H**,**I**). (**J**) Feulgen stained specimen in autogamy: two new macronuclear anlagen (MA) surrounded by numerous fragments of old macronucleus. Scale bars: 8 µm (**A**,**B**,**J**), 4 µm (**C**,**D**,**E**,**F**), 3 µm (**G**,**H**,**I**).

The COI gene sequencing was performed to assign A65 and D88 strains to a certain species of the *P. aurelia* complex. The strains from the same populations had identical sequences (Table 2), while between A65 and D88 strains the sequence was 99.3% similar (5 single nucleotide polymorphisms (SNPs) per 760 bp-long sequence). The best match in GenBank for both strains was KX008305.1, belonging to *P. biaurelia*. This sequence was 94.9% similar to A65 (39 SNPs per 760 bp-long sequence) and 94.5% similar to D88 (42 SNPs per 760 bp-long sequence). In the *P. aurelia* phylogenetic tree inferred from the COI gene sequences, strains A65 and D88 formed a separate branch sister to *P. biaurelia* (Figure 3, red brace), mirroring another pair of closely related species, *P. primaurelia* and *P. pentaurelia* (Figure 3, green brace). The COI gene sequence similarity between and within the latter two species is of the same range (Table 3).

The first cells were entering autogamy only after 20 divisions in daily re-isolations cycle, while synchronous autogamy of the culture was observed after 25–27 vegetative divisions. Two macronuclear anlagen were formed after the sexual process, also typical for the *P. aurelia* species. We never observed selfing (intrastrain conjugation) in A65 and D88 strains, thus, the mating type determination is not stochastic as in some species of the complex [31]. Furthermore, we never obtained conjugation by mixing mature cells of different strains from both populations, so, presumably, all isolated strains were of the same mating type. In closely related *P. biaurelia*, the mating types are inherited maternally [19,29], and it is very likely that cytoplasmic inheritance is characteristic for these strains also.

Since we were unable to obtain conjugation between A65 and D88 strains, we also tested the possibility of conjugation between these strains and tester strains of *P. biaurelia*. Several conjugating couples were observed in crosses between IST and D88 and between Rieff and D88 strains after incubation of slightly starved reactive cells at + 18 °C, while only single couples were observed in mating tests of A65 with Rieff and IST strains. No conjugation was observed between Ts strain and either A65 or D88. In the control mating tests between *P. biaurelia* strains, conjugation was well pronounced at the same conditions. None of the few exconjugants in crosses utilizing A65 survived.

Exconjugant cells were isolated and F1 clones were established for the crosses IST × D88 and Rieff × D88. All F1 clones fit well and grew like healthy paramecia. F2 progeny were obtained by autogamy from all F1 clones. Survival rate of F2 clones was 3% (2 of 60) in the IST × D88 cross, and 8% (5 of 60) in the Rieff × D88 cross. At the same time, survival rate of F2 clones obtained in the IST intrastrain cross was 95%, and in the cross of *P. biaurelia* strains Rieff × Ts, it was 77%. Moreover, during the following month of maintenance, no F2 clones from the IST × D88 cross survived, and only two F2 clones remained viable from the Rieff × D88 cross. This rate of survival is very low and could be due to a technical error of the experiments. For example, some cells could have remained vegetative and were occasionally selected from autogamous culture of F1 heterozygous clones or some F1 cells might regenerated old macronuclei in autogamy.

Table 2. COI gene sequence identity values for sister species *P. quindecaurelia* n. sp. and *P. biaurelia*.

	A65-1 ***P. quindecaurelia*** **(this study)**	**D88-8** ***P. quindecaurelia*** **(this study)**	**114** ***P. biaurelia*** **(HF969032.1)**	**V1-4** ***P. biaurelia*** **(PBIAU.V1-4.MITO.1.G040)**	**11III1** ***P. biaurelia*** **(KX008305.1)**	**FGC3** ***P. biaurelia*** **(MG013508.1)**
A65-1 ***P. quindecaurelia*** (this study)	100	99.3 (755/760)	94.6 (671/709)	95.0 (722/760)	94.9 (721/760)	94.8 (651/687)
D88-8 ***P. quindecaurelia*** (this study)	99.3 (755/760)	100	94.5 (670/709)	94.6 (719/760)	94.5 (718/760)	94.6 (650/687)
114 ***P. biaurelia*** (HF969032.1)	94.6 (671/709)	94.5 (670/709)	100	99.9 (708/709)	99.7 (707/709)	99.9 (686/687)
V1-4 ***P. biaurelia*** (PBIAU.V1-4.MITO.1.G040)	95.0 (722/760)	94.6 (719/760)	99.9 (708/709)	100	99.9 (756/757)	100% (687/687)
11III1 ***P. biaurelia*** (KX008305.1)	94.9 (721/760)	94.5 (718/760)	99.7 (707/709)	99.9 (756/757)	100	99.9 (686/687)
FGC3 ***P. biaurelia*** (MG013508.1)	94.8 (651/687)	94.6 (650/687)	99.9 (686/687)	100% (687/687)	99.9 (686/687)	100

Table 3. COI gene sequence identity values for sister species *P. pentaurelia* and *P. primaurelia*.

	87/5 ***P. pentaurelia*** **(PPENT.87.MITO.1.G040)**	**NR-2/5** ***P. pentaurelia*** **(This Study)**	**Nr1-9/5** ***P. pentaurelia*** **(This Study)**	**AZ9-3/1** ***P. primaurelia*** **(PPRIM.AZ9-3.MITO.1.G040)**	**OLI-prim01** ***P. primaurelia*** **FJ905141.1**	**IR4-2** ***P. primaurelia*** **(This Study)**	**FT11** ***P. primaurelia*** **(This Study)**	**CH** ***P. primaurelia*** **(This Study)**
87/5 ***P. pentaurelia*** (PPENT.87.MITO.1.G040)	100	99.9 (652/653)	100 (767/767)	94.3 (712/755)	94.2 (711/755)	94.4 (724/767)	94.3 (723/767)	94.1 (722/767)
NR-2/5 ***P. pentaurelia*** (this study)	99.9 (652/653)	100	99.9 (652/653)	94.3 (616/653)	94.2 (615/653)	94.2 (615/653)	94.2 (615/653)	94.0 (614/653)
Nr1-9/5 ***P. pentaurelia*** (this study)	100 (767/767)	99.9 (652/653)	100	94.3 (712/755)	94.2 (711/755)	94.4 (724/767)	94.3 (723/767)	94.1 (722/767)
AZ9-3/1 ***P. primaurelia*** (PPRIM.AZ9-3.MITO.1.G040)	94.3 (712/755)	94.3. (616/653)	94.3 (712/755)	100	99.9 (759/760)	99.5 (751/755)	99.9 (754/755)	99.8 (753/755)
OLI-prim01 ***P. primaurelia*** FJ905141.1	94.2 (711/755)	94.2 (615/653)	94.2 (711/755)	99.9 (759/760)	100	99.4 (750/755)	100 (755/755)	99.9 (754/755)
IR4-2 ***P. primaurelia*** (this study)	94.4 (724/767)	94.2 (615/653)	94.4 (724/767)	99.5 (751/755)	99.4 (750/755)	100	99.4 (762/767)	99.2 (761/767)
FT11 ***P. primaurelia*** (this study)	94.3 (723/767)	94.2 (615/653)	94.3 (723/767)	99.9 (754/755)	100 (755/755)	99.4 (762/767)	100	99.9 (766/767)
CH ***P. primaurelia*** (this study)	94.1 (722/767)	94.0 (614/653)	94.1 (722/767)	99.8 (753/755)	99.9 (754/755)	99.2 (761/767)	99.9 (766/767)	100

4. Discussion

4.1. Overview of Paramecium Diversity Revealed in Mexico

Screening of samples collected by us in Mexico showed unsurprisingly that *Paramecium* is widely represented in fresh waterbodies in this part of the world. The territory of Mexico, and in general, of Central America, remains unexplored by ciliatologists compared to Europe or some parts of the USA. Several *Paramecium* species, which we found in the current studies, were reported for the first time from these vast territories. To compensate the lack of knowledge about the *Paramecium* biogeography of Central America, we summarized the retrospective records of *Paramecium* according to [34–47]; all *Paramecium* records available from Mexico as well as Central and South America are shown in Table 4.

Besides the most important discovery of a new member of the *P. aurelia* species complex, it is worth noting several new facts on *Paramecium* biogeography. First is the occurrence of *P. putrinum* in the waterbodies of Mexico City. This species is not very frequent but can be considered common for temperate climate zones [1,11]. To our best knowledge, it has never been documented from tropical climates, but our observation is the southern-most collection of *P. putrinum* and may represent the southern extent of the species range. The same concerns *P. triaurelia* collected in Chapultepec Lake, Mexico City, as this species was not previously isolated as far south or in a tropical environment [4]. Record of *P. jenningsi* is notable, as this species is rare, and, unlike *P. putrinum*, usually inhabits waterbodies of tropical or subtropical zones [48]. This is only the third finding of *P. jenningsi* in the region, after Panama and Florida, USA [49]. *Paramecium jenningsi* strain DK from Mexico belonged to the same genotypic group as *P. jenningsi* strains collected in Japan and Madagascar [50]. Interestingly, *P. bursaria* strains found in two localities in Mexico belonged to syngen R3, common in Japan, China and also known from South America [5]. As for *P. caudatum*, all Mexican strains appeared to be related to each other and grouped in molecular phylogenetic trees with strains from Brazil, China and the European part of Russia (Figure 2), thus, confirming that the genotypes within these species do not show any special geographical pattern [3,14,32]. *Paramecium multimicronucleatum* strains found in Mexico belonged to three branches within this morphospecies. All branches include strains from all over the world.

Table 4. All records of *Paramecium* species in Mexico and Latin America.

Species/Mexican States	CdM	Chi	EdM	Gro	Hgo	Mor	Oax	Pue	Qro	Q.R.	Sin	Ver	Yuc	CA *	SA *
P. aurelia	X		X	X		X	X	X	**X**	X	X	X	**X**	X	X
P. primaurelia													**X**	X	X
P. biaurelia															X
P. triaurelia	**X**														
P. tetraurelia	**X**										X			X	X
P. sexaurelia														X	X
P. octaurelia										**X**				X	
P. tredecaurelia						X *								X	
P. quadecaurelia															X
P. quindecaurelia	**X**								**X**						
P. bursaria	**X**		X				X		**X**					X	X
P. calkinsi									X			X			
P. caudatum	**X**	X	X			X	X	X	X			**X**	X	X	X
P. duboscqui															X
P. jenningsi	**X**													X	
P. multimicronucleatum	**X**		**X**		**X**	X		X		**X**		X		X	X
P. polycaryum								X							
P. putrinum	**X**		X		X										

CdM = Ciudad de México, Chi = Chiapas, EdM = Estado de México, Gro = Guerrero, Hgo = Hidalgo, Mor = Morelos, Oax = Oaxaca, Pue = Puebla, Qro = Querétaro, Q.R. = Quintana Roo, Sin = Sinaloa, Ver = Veracruz, Yuc = Yucatán, CA = Central America, SA = South America; records of this study are highlighted in red. * according to [1,4,51].

4.2. Paramecium Quindecaurelia n. sp. and a Species Concept in Paramecium

Since Sonneborn [4] gave species rank to fourteen syngens of the *P. aurelia* complex, only one more member of this complex has been discovered: *P. sonneborni* [52]. Actually, it would be amazing if there were no more currently unknown sibling species of this complex in nature. Here, we report the sixteenth species of the *P. aurelia* complex, suggesting the name *Paramecium quindecaurelia* n. sp., as numerical tradition was interrupted with the description of *P. sonneborni*.

Morphologically, all species of the *P. aurelia* complex are indistinguishable [4], except *P. sonneborni*, which has a unique micronuclei morphology [52]. Morphometric characteristics do not show any significant variation among the sibling species of the complex [33]. The number of kineties is not considered important to differentiate between the *P. aurelia* species, while features of oral cortex are very conservative in *Paramecium* and cannot be used as a species characteristic within this genus [53]. Thus, morphological analysis of *P. quindecaurelia* n. sp., expectedly, revealed that it has no discriminating features and in particular, is extremely similar to its closest relative, *P. biaurelia*.

We reconstructed the complete phylogenetic tree for the *P. aurelia* species complex inferred from the COI gene sequence (Figure 3). *Paramecium quindecaurelia* n. sp. branches in the same cluster with *P. biaurelia*, but the COI gene identity between these two species does not exceed 95.0%, while within each of these species, it is not less than 99.3% (Table 2). The comparison of COII gene sequences of *P. quindecaurelia* n. sp. and *P. biaurelia* strains gives very similar values: 94.8–95.1% between two species and 99.6–99.9% within each species (Supplementary Table S2). The phylogenetic distance between *P. quindecaurelia* n. sp. and *P. biaurelia* inferred from COI gene sequence analysis is of the same range as between the most closely related sister species *P. primaurelia* and *P. pentaurelia* (Figure 3, Table 3) where identity of the COI gene sequences is 94.0–94.4%. According to Sonneborn's data [19], *P. primaurelia* and *P. pentaurelia* were genetically isolated from each other but had identical isozyme patterns while all other species of the *P. aurelia* complex were characterized by unique zymograms. The only additional differences between these two species were unstable mating type O in *P. pentaurelia*, while in the clonal life of *P. primaurelia* the mating types never changed [19], and "a very weak and unreliable mating reaction, not leading to conjugation, occurred between type E of *P. pentaurelia* and type O of *P. septaurelia*", but the same was not true for *P. primaurelia* [4]. Thus, *P. primaurelia* and *P. pentaurelia* are also very similar physiologically to each other.

It is not surprising that *P. quindecaurelia* n. sp. is able to mate with *P. biaurelia*, and that *P. primaurelia* can conjugate with *P. pentaurelia* [19]. However, existence of the pronounced reproductive barrier between *P. quindecaurelia* n. sp. and *P. biaurelia*, despite the isolation is not absolute, further confirms that we found a novel member of the *P. aurelia* complex. The survival of F2 progeny in crosses between *P. biaurelia* and *P. quindecaurelia* n. sp. is slightly greater than zero. It is assumed that the reproductive species criterion is reliable but not absolute, as in many zoological species interspecific hybrids are known, and sometimes they also are considered as separate species (for example, water frogs from the *Pelophylax esculentus* complex—[54]). The F1 interspecies hybrids of *P. biaurelia* and *P. quindecaurelia* n. sp. cross are perfectly viable, but lethality in the F2 generation is very high, so the hybrids can be considered effectively sterile. Unfortunately, there are no data in the literature on F1 and F2 survival in crosses between *P. primaurelia* and *P. pentaurelia*. In the third pair of closely related species, *P. tetraurelia* and *P. octaurelia* (Figure 3), F1 hybrids had problems with survival and growth, and F2 post-autogamous progeny never survived [55]. Still, their COI gene sequence similarity is just 83.9%, and the phylogenetic distance between the latter two species is bigger than between *P. primaurelia* and *P. pentaurelia* or between *P. biaurelia* and *P. quindecaurelia* n. sp. (see Figure 3 and Tables 2 and 3). Different molecular markers have different resolution in *Paramecium*. For example, comparison of 18S rRNA gene provides good overview of the whole genus phylogeny [8,15], while it becomes useless if applied to the *P. aurelia* complex [8]. Mitochondrial COI and COII genes are rather conserved within *P. aurelia* sibling species (polymorphism in these genes among strains of the same species does not exceed 1–2%), while even the closest sibling species differ at least in 5% of these gene sequences ([9,13] and this work). At the same time, in related to *Paramecium* genus *Tetrahymena*, the divergence for

more than 1% in the *COI* gene sequence is considered reliable interspecific difference [56]. Obviously, only molecular phylogenomic analysis would allow us to understand the diagnostic level of molecular divergence between closely related species of ciliates.

In our opinion, at present, our data are sufficient to claim that *P. quindecaurelia* n. sp. is a separate species and not just a divergent group in *P. biaurelia*. Interestingly, *P. biaurelia*, the most common species of the *P. aurelia* complex in cold and moderate climate zones, at least in Europe [57], is not known from tropical environments [4,58], which may shelter its twin species, *P. quindecaurelia* n. sp.

4.3. Paramecium Quindecaurelia n. sp. Taxonomic Summary

Diagnosis. Classical species of the *Paramecium aurelia* complex, sister species of *P. biaurelia*.

Type locality. Los Dinamos National Park, Mexico City (19°16′02″ N/99°17′31″ W).

Type slides. Several holotype and paratype slides have been deposited in the collection of microscopical slides of the Department of Invertebrate Zoology, Saint Petersburg State University, Russia.

Type culture. The type strain D88-8 and other strains of the species are maintained in the RC CCM culture collection (World Data Centre for Microorganisms, RN 1171) of Saint Petersburg State University, Saint Petersburg, Russia.

Sequence availability. The nucleotide sequence of the mitochondrial cytochrome c oxidase I gene of the type strain was deposited in the NCBI GenBank database under accession number MT078138, the sequence of the mitochondrial cytochrome c oxidase II gene under accession number MT318928.

Zoobank Registration LSID: http://zoobank.org/urn:lsid:zoobank.org:act:B5A24294-3165-40DA-A425-3AD2D47EB8E7.

Further remarks. The strains of this species are able to conjugate with *P. biaurelia* strains. No endosymbionts have been detected in the species so far.

5. Conclusions

In this work, we confirmed that sampling in poorly studied regions, such as Central America, may reveal broad diversity of *Paramecium*, making it possible also to find new species. We found representatives of six *Paramecium* morphological species, and the collected strains belonged to different groups within some of these species (as in the case of the *P. aurelia* species complex and *P. multimicronucleatum*). Numerous molecular phylogenetic data demonstrate that each *Paramecium* morphospecies includes a number of intraspecific groups, which in some cases are known to correspond to syngens [5,11]. Genomic analyses showed that the *P. aurelia* complex emerged as a result of three whole genome duplications followed by species radiation [59], and speciation has been actively going on in this group [60,61]. It is known that the survival rate of F2 progeny in crosses of strains belonging to the same species, for example, in *P. sexaurelia* [62], can vary significantly. Inability to form conjugating couples seems to be one of the most limiting components of the reproductive barrier between sibling species of the *P. aurelia* complex. If couples can be formed, the possibility that a small proportion of the progeny from interspecies crosses may survive cannot be completely ruled out. Comparative genomics can potentially elucidate if all sibling species of the *P. aurelia* complex are absolutely isolated from each other. Pairs of twin species, like *P. primaurelia* / *P. pentaurelia* and *P. biaurelia* / *P. quindecaurelia* n. sp. may serve as the best models to study genetic isolation and gene flow between the *P. aurelia* species.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/12/5/197/s1, Table S1: Morphometric characteristics of *P. quindecaurelia* n.sp. and *P. biaurelia*., Table S2: COII gene sequence identity values for sister species *P. quindecaurelia* sp.n. and *P. biaurelia*.

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