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# *Paramecium tredecaurelia*: A Unique Non-Polymorphic Species of the *P. aurelia* spp. Complex (Oligohymenophorea, Ciliophora)

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**Abstract.** New stands of *Paramecium tredecaurelia*, a rare species of the *P. aurelia* spp. complex, were identified in Thailand and Madagascar on the basis of mating reactions and molecular markers (rDNA and mtDNA). Analysis of DNA fragments showed that all *P. tredecaurelia* strains, the recently recorded ones and the ones known previously from France, Mexico, and Israel, form a monophyletic and well-defined clade in the *P. aurelia* species trees. All of these strains, collected from different localities around the world, represent identical or nearly identical haplotypes in terms of all the studied DNA fragments. Given the huge distances between particular collection sites, such a low level of variability of the studied sequences may result from a slow rate of evolution in *P. tredecaurelia*.

**Key words:** *P. aurelia* species complex, intraspecific polymorphism, rDNA (ITS1-5.8S- ITS2-5'LSU rDNA), cytochrome c oxidase subunit I gene (*COI*), cytochrome b gene (*CytB*).

**Abbreviations:** c.m.t. – complementary mating types.

## INTRODUCTION

Among the 15 known species of the *Paramecium aurelia* complex (Sonneborn 1975, Aufderheide *et al*. 1983), some are cosmopolitan (*P. primaurelia*, *P. biaurelia*, *P. tetraurelia* and *P. sexaurelia*), while others, such as *P. quadecaurelia*, have been found only in a few localities (Sonneborn 1975, Przyboś *et al*. 2003, Przyboś *et al*. 2013).

*Paramecium tredecaurelia* is also a rare species with only four strains known until recently, that is, from Paris, France; Benenitra, Madagascar; Cuernavaca Valley, Mexico (Rafalko and Sonneborn 1959, Sonneborn 1975), and Kiryat Motzkin, Israel (Przyboś *et al*. 2002). This species does not conjugate or even enter into any mating reactions with any other species of the complex. It is also characterized by a synclonally inherited (Mendelian) mating type system, unique among the species of the complex (Sonneborn 1975). This means that two mating types are determined by different alleles at the

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same locus and the homozygote does not undergo any change of mating type at autogamy. The reference specimens of the species come from strain 209 from France, genetically restricted to mating type O (odd) and from strain 321 from Mexico, genetically restricted to mating type E (even) (Sonneborn 1974). This is also the only species that requires high temperature to mate, as cultures should be grown at 27–31°C. As concerns isozyme patterns (Tait 1970, Allen *et al*. 1973), the species is characterized by two cathodal bands for mitochondrial isocitrate dehydrogenase and two anodal bands for cytoplasmic isocitrate dehydrogenase. No other species has such a combination of two patterns as that shown by *P*. *tredecaurelia*. Symbionts were not found in that species.

Recently, new stands of this species were recorded in Bangkok, Thailand and St. Luce, Madagascar, and are presented in this paper. The strains were identified at first by molecular markers and then by mating reactions. Three genome fragments: one ribosomal (ITS1-5.8S-ITS2-5'LSU rDNA) and two mitochondrial (cytochrome c oxidase subunit I and cytochrome b gene fragments, designated *COI* and *CytB*, respectively) were employed to assist in identification and characterization. We chose them as markers because each of these genome fragment has likely evolved at a different rate, and this in turn leads to different gene trees (Bull *et al*. 1993). This is especially important in the case of a rapid and recent speciation process (Bull *et al*. 1993), which was postulated in the species of the *Paramecium aurelia* complex (Barth *et al*. 2008, Przyboś *et al*. 2012b). Furthermore, the proposed DNA fragments were successfully applied for molecular comparison of other species of the genus *Paramecium*: *P. dodecaurelia* (Przyboś *et al*. 2012b), *P. novaurelia* (Tarcz 2012), *P. calkinsi* (Przyboś *et al*. 2012a), *P. multimicronucleatum* (Tarcz *et al*. 2012), and *P. bursaria* (Greczek-Stachura *et al*. 2012). Besides the above-mentioned markers, the small subunit rRNA gene was used for the characterization of the recently redescribed species *P*. *chlorelligerum* Kahl 1935 (Kreutz *et al*. 2012). Similarly, nuclear genes encoding the H4 histone subunit (Przyboś *et al*. 2006) and the hsp70 protein (Hori *et al*. 2006, Przyboś *et al*. 2003) were applied for species identification and comparison of evolutionary distances among syngens and sibling species of *Paramecium*, respectively.

The aim of our present study was to characterize molecularly and genetically the newly recorded strains of *P. tredecaurelia* as well as intraspecific relationships within this species. It is worth noting that our study does not only involve a comparison of DNA fragments (the phylogenetic species concept), but also classical genetic crosses (the biological species concept). In particular, in closely related microbial eukaryotes, the assessment of species boundaries requires "more than one line of evidence" (Boenigk *et al*. 2012). This is in concordance with former proposals (Schlegel and Meisterfeld 2003, Duff *et al*. 2008), which suggested the application of combined morphological-molecular approaches to the identification of planktonic protists.

## MATERIAL AND METHODS

### Identification of species of the *P. aurelia* spp. complex by mating reactions

New strains originating from Bangkok, Thailand and St. Luce, Madagascar, as well as other strains of *P. tredecaurelia* known previously from Paris, France; Cuernavaca, Mexico; and Kiryat Motzkin, Israel (Table 1), were used in the present study. Data concerning other species of the *P. aurelia* complex are given in Table 2.

Paramecia were cultured in a lettuce medium inoculated with *Enterobacter aerogenes* and supplemented with 0.8 mg/ml β-sitosterol (Merck, Darmstadt, Germany). They were identified according to Sonneborn's methods (1950, 1970) by mating reactions. The new strains from Thailand and Madagascar, mature for conjugation, were mated with reactive complementary mating types (c.m.t.) of the reference (standard) strains of several species of the *P. aurelia* complex. The following standard strains were used: *P. primaurelia*, strain 90 (Pennsylvania, USA); *P. tetraurelia*, strain from Sydney, Australia; *P. pentaurelia*, strain 87 (Pennsylvania, USA); *P. sexaurelia*, strain 159 (Puerto Rico); *P. octaurelia*, strain 138 (Florida, USA); *P. dodecaurelia*, strain 246 (Mississippi, USA); and *P. tredecaurelia*, strain 209 (Paris, France) and strain 321 (Cuernavaca, Mexico).

Species determination was based on the occurrence of 85 to 95% initial agglutination of paramecia, followed by the presence of tight conjugating pairs formed by paramecia of the strain from Thailand or Madagascar and the reference strain of *P. tredecaurelia* from France, Paris (strain 209). To make sure that no intra-strain conjugation (selfing) occurred within the c.m.t., controls of non-crossed c.m.t. were also cultured and observed. A temperature of 29°C was optimal for the conjugation of this species.

The $F_1$ generation of hybrids from inter-strain crosses of *P. tredecaurelia* (strains from Thailand or Madagascar, both restricted to the even mating type × strain 209 from France, restricted to the odd mating type; strain 209 from France, odd mating type × strain 321 from Mexico, even mating type) was obtained by conjugation, and $F_2$ by autogamy by the daily isolation lines method (according to Sonneborn 1950, 1970). The occurrence of autogamy was examined in preparations stained by acetocarmine.

The survival of clones in both generations was estimated from a total of 100 clones. Clones were considered survivors after un-

**Table 1.** Studied *Paramecium tredecaurelia* strains.

| No. | Strain designation | Strain geographical origin | Collector's name | Reference | GenBank accession numbers | | |
|---|---|---|---|---|---|---|---|
| | | | | | ITS1-5.8S-ITS2-5'LSU rDNA | *COI* mtDNA | *CytB* mtDNA |
| 1. | 209* | France, Paris | G. Beale | Rafalko and Sonneborn 1959 | JF304165 | JF304184 | HM001352 |
| 2. | 321** | Mexico, Cuernavaca, Taxco Valley | T. M. Sonneborn | Rafalko and Sonneborn 1959 | JF304166 | JF304185 | KC432625 |
| 3. | IKM** | Israel, Kiryat Motzkin | E. Przyboś 2001 | Przyboś *et al*. 2002 | JN998647 | JN998689 | KC432626 |
| 4. | MA** | Madagascar, St. Luce, Ambandrika Lake | M. Barresi 2011 | Present paper | KC432623 | KC432624 | KC432628 |
| 5. | TaB** | Thailand, Bangkok | T. Fokina 2010 | Present paper | JX661364 | JX661440 | KC432627 |

* – Strain 209 restricted to odd mating type (XXV according Sonneborn 1975).
** – Strains 321, IKM, MA, TaB restricted to even mating type (XXVI Sonneborn 1975).
Strain from Madagascar, Benenitra (designated 238, restricted to even mating type) reported by Rafalko and Sonneborn (1959) was not available for our studies.

dergoing 6–7 fissions during 72 hours following the separation of conjugation partners or post-autogamous caryonids (the two products of the first fission of each autogamous *Paramecium*). The procedures were carried out following Chen (1956). The percentage of surviving hybrid clones in crosses was compared in $F_1$ and $F_2$ (Table 3) because *Paramecium* species were identified not only on the basis of their capacity to conjugate with the reference specimens, but also to produce viable recombinant $F_2$ clones (Sonneborn 1975).

All the reference (standard) strains of particular species of the *P. aurelia* complex were obtained from the laboratories of Prof. T. M. Sonneborn (Department of Biology, Indiana University, USA) or Prof. G. H. Beale (Institute of Animal Genetics, Edinburgh University, Great Britain). They are kept at the Department of Experimental Zoology, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Cracow, Poland.

## Molecular methods

All strains used for DNA isolation were homozygous, as they passed autogamy previously. *Paramecium* genomic DNA was isolated from vegetative cells at the end of the exponential phase (approx. 1000 cells were used for DNA extraction) using a NucleoSpin Tissue Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions for DNA isolation from cell cultures. The only modification was centrifugation of the cell culture for 20 min. at 13,200 rpm. The supernatant was removed and the remaining cells were suspended in lysis buffers and proteinase K.

Fragments of rDNA, *COI*, and *CytB* genes were sequenced and analyzed. First, rDNA fragments were amplified with ITS1 and ITS4 universal eukaryotic primers (White *et al*. 1990) and ITS3zg and 3pLSU primers developed with OligoAnalyzer 3.1 (http://www.eu.idtdna.com/analyzer/applications/oligoanalyzer) (Table 4). F388dT and R1184dT primers (Table 4) and the protocol previously described by Strüder-Kypke and Lynn (2010) were used for the amplification of the *COI* fragment of mitochondrial DNA. In some cases, when the above *COI* pair of primers did not yield a well-defined product, the internal primer CoxH10176 (Barth *et al*. 2006) was used instead of R1184dT. To amplify the *CytB* gene fragment, the primer pair CytBF/PaCytR and the protocol previously described by Barth *et al*. (2008) were used.

PCR amplification for all analyzed DNA fragments was carried out in a final volume of 40 µL containing 30 ng of DNA, 1.5 U Taq-Polymerase (EURx, Poland), 0.8 µL of 20 µM of each primer, 10 × PCR buffer, and 0.8 µL of 10 mM dNTPs.

In order to assess the quality of the amplification, PCR products were electrophoresed in 1% agarose gel for 45 min. at 85 V with a DNA molecular weight marker (Mass Ruler Low Range DNA Ladder, Fermentas, Lithuania). NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) was used for purifying PCR products. In some PCR products, additional sub-bands were obtained apart from the main band. In these cases, 30 µL of each PCR product was separated on 1.8% agarose gel (100 V/60 min.) with a DNA molecular weight marker (Mass Ruler Low Range DNA Ladder, Fermentas, Lithuania). Then the band representing the examined fragment was cut out and purified.

Sequencing was done in both directions with the application of BigDye Terminator v3.1 chemistry (Applied Biosystems, USA). The primers used in PCR reactions were applied for sequencing the rDNA region, and the primer pair M13F/M13R was used for se-

**Table 2.** Other strains of *Paramecium aurelia* species used in this studies. Strains of *P. multimicronucleatum* were used as an out-group.

| No. | Species | Strain designation | Strain geographical origin | GenBank accession numbers | | |
|---|---|---|---|---|---|---|
| | | | | ITS1-5.8S-ITS2-5'LSU rDNA | *COI* mtDNA | *CytB* mtDNA |
| 1. | *P. primaurelia* | 90 (SO-90*) | USA, Pennsylvania | JF304163 | JF304182 | AM949780* |
| 2. | *P. primaurelia* | SS | Spain, Andalusia, Sevilla | JN998643 | JN998685 | N/A |
| 3. | *P. primaurelia* | PR-08* | Russia, Astrakhan Nature Reserve | N/A | N/A | AM949779* |
| 4. | *P. biaurelia* | Rieff | Great Britain, Scotland, Rieff | JX010640 | JX010661 | N/A |
| 5. | *P. biaurelia* | UB2 | USA, Boston | JX010641 | JX010662 | N/A |
| 6. | *P. biaurelia* | PR-34* | Russia, Irkutsk | N/A | N/A | AM949784* |
| 7. | *P. biaurelia* | DB-05* | Germany, Saidenbach Reservoir | N/A | N/A | AM949785* |
| 8. | *P. triaurelia* | 324 (SO-324*) | USA, Florida | JX010642 | JX010663 | AM949778* |
| 9. | *P. triaurelia* | SCM | Spain, Castile | JX010643 | JX010664 | N/A |
| 10. | *P. triaurelia* | HH-05* | Germany, Hannover | N/A | N/A | AM949777* |
| 11. | *P. tetraurelia* | S (PR-92*) | Australia, Sydney | JF304164 | JF304183 | AM949771* |
| 12. | *P. tetraurelia* | FP | France, Paris | JN998645 | JN998687 | N/A |
| 13. | *P. tetraurelia* | SO-51* | USA, Indiana | N/A | N/A | AM949770* |
| 14. | *P. pentaurelia* | 87 (SO-87*) | USA, Pennsylvania | JX010644 | JX010665 | AM949782* |
| 15. | *P. pentaurelia* | HBB | Hungary, Balatonfüzfo | JX010645 | JX010666 | N/A |
| 16. | *P. pentaurelia* | NA-05* | Italy, Naples | N/A | N/A | AM949781* |
| 17. | *P. sexaurelia* | 159 (SO-159*) | Puerto Rico | JX010646 | JX010667 | AM949765* |
| 18. | *P. sexaurelia* | SAS | Spain, Andalusia, Seville | JX010647 | JX010668 | N/A |
| 19. | *P. sexaurelia* | FO-128* | Japan, Yamaguchi | N/A | N/A | AM949764* |
| 20. | *P. septaurelia* | 38 (SO-38*) | USA, Florida | JX010648 | JX010669 | AM949766* |
| 21. | *P. septaurelia* | AZ24-4 | Russia, Astrakhan Nature Reserve | JX010649 | JX010670 | N/A |
| 22. | *P. septaurelia* | PO-162* | Russia, Astrakhan Nature Reserve | N/A | N/A | AM949768* |
| 23. | *P. octaurelia* | 138 (SO-168*) | USA, Florida | JX010650 | JX010671 | AM949767* |
| 24. | *P. octaurelia* | IEA (PR-169*) | Israel, Ein Effek | JX010651 | JX010672 | AM949772* |
| 25. | *P. novaurelia* | CS | Czech Republic, Ceske Skalnice | JX010652 | EU056250 | N/A |
| 26. | *P. novaurelia* | UG (PR-175*) | Ukraine, Gorgany Mts. | JX010653 | EU056263 | AM949776* |
| 27. | *P. novaurelia* | ED-05* | UK, Edinburgh | N/A | N/A | AM949775* |
| 28. | *P. decaurelia* | 223 (SO-223*) | USA, Florida | JX010654 | JX010673 | AM949769* |
| 29. | *P. decaurelia* | JH | Japan, Honshu Island | JX010655 | JX010674 | N/A |
| 30. | *P. undecaurelia* | 219 (SO-219*) | USA, Texas | JX010656 | JX010675 | AM949783* |
| 31. | *P. dodecaurelia* | 246 (SO-246*) | USA, Mississipi | JN998639 | JN998681 | AM949763* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 32. | *P. dodecaurelia* | GM | Germany, Münster | JN998615 | JN998657 | N/A |
| 33. | *P. dodecaurelia* | SK-199* | USA, Hawaii | N/A | N/A | AM949762* |
| 34. | *P. quadecaurelia* | 328 (SO-328)* | Australia, Emily Gap near Alice Springs | JX010635 | JX010657 | AM949773* |
| 35. | *P. quadecaurelia* | AN1-1 (DO-207)* | Namibia, Vindhoek, Pond in park | JX010636 | JX010658 | AM949774* |
| 36. | *P. sonneborni* | ATTC 30995 (AU-208*) | USA, Texas | JF304167 | JX010676 | AM949786* |
| 37. | *P. multimicronucleatum* | AB9-20 | USA, Boston | JF741241 | JF741273 | N/A |
| 38. | *P. multimicronucleatum* | BR | USA, Louisiana, Baton Rouge | JF304172 | JF304189 | N/A |
| 39. | *P. multimicronucleatum* | GMA-2* | Germany, Martinfeld | N/A | N/A | AM949757* |
| 40. | *P. multimicronucleatum* | ISN-11* | Italy, Naples | N/A | N/A | AM949756* |

* – Designations and GenBank numbers as in (Barth *et al*. 2008)

quencing the *COI* fragment (Table 4). The sequencing reaction was carried out in a final volume of 10 μL containing 3 μL of template, 1 μL of BigDye (1/4 of the standard reaction), 1 μL of sequencing buffer, and 1 μL of 5 μM primer. Sequencing products were precipitated using Ex Terminator (A & A Biotechnology, Poland) and separated on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, USA). The sequences are available in the NCBI GenBank database (see Tables 1 and 2).

### Data analysis

Sequences were examined using Chromas Lite (Technelysium, Australia) to evaluate and correct the chromatograms. The alignment of the studied sequences was performed using ClustalW (Thompson *et al*. 1994) within the BioEdit software (Hall 1999) and checked manually. All of the obtained sequences were unambiguous and were used for analysis. Phylograms were constructed for the studied fragments with Mega v5.0 (Tamura *et al*. 2011), using neighbor-joining (NJ) (Saitou and Nei 1987), maximum parsimony (MP) (Nei and Kumar 2000), and maximum likelihood (ML) (Felsenstein 1981). All positions containing gaps and missing data were eliminated. NJ analysis was performed using Mega v5.0 program, by bootstrapping with 1000 replicates (Felsenstein 1985). MP analysis was evaluated with the min-mini heuristic parameter (at level 2) and bootstrapping with 1000 replicates. Bayesian inference (BI) was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003); analysis was run for 5,000,000 generations and trees were sampled every 100 generations. All trees for BI analysis were visualized with TreeView 1.6.6 (Page 1996). Analysis of uncorrected p-distance estimation for NJ analysis, and identification of substitution models (T92 + G + I for rDNA and *COI* mtDNA fragments or HKY + G + I for *CytB* mtDNA fragments) for ML and BI analysis were done with Mega v5.0 (Tamura *et al*. 2004, 2011).

## RESULTS AND DISCUSSION

New stands of *P. tredecaurelia* were found in Bangkok, Thailand (the first discovery of the species in Eastern Asia) and St. Luce, Madagascar. Previously, *P. tredecaurelia* had been recorded in Madagascar (Rafalko and Sonneborn 1959), but in a different locality (Benenitra). However, that strain was not available for our study. Currently, the species is known from isolated localities in America, Europe, Africa, and Asia.

The viability of the offspring of the hybrid exconjugant clones of inter-strain crosses (strains from: Thailand × France; Madagascar × France; France × Mexico) observed in $F_1$ was high (88–94%), but in the $F_2$ generation it was low (21–26%) (Table 3). Similar results were obtained previously, when the strain from Israel was crossed with the strain from France: 90% hybrid clones survived in $F_1$, but only 26% in $F_2$ (Przyboś *et al*. 2002). Rafalko and Sonneborn (1959) also reported very high mortality in the $F_2$ generation of crosses

**Table 3.** Percentage of surviving clones of *Paramecium tredecaurelia* inter-strain hybrids.

| No. | Strain designation / origin | Percentage of surviving clones | | Reference |
|---|---|---|---|---|
| | | $F_1$ obtained by conjugation | $F_2$ obtained by autogamy | |
| 1. | 209 × 321 (France x Mexico) | 88 | 21 | Present paper |
| 2. | TaB × 209 (Thailand x France) | 94 | 22 | Present paper |
| 3. | MA × 209 (Madagascar x France) | 91 | 22–25 | Present paper |
| 4. | IKM × 209 (Israel x France) | 90 | 26 | Przyboś *et al*. 2002 |

**Table 4.** Primers used for amplification and sequencing of studied DNA fragments.

| DNA fragment | Primer | Sequence 5'–3' | References |
|---|---|---|---|
| ITS1-5.8S-ITS2 | ITS1 | TCCGTAGGTGAACCTGCGG | White *et al*. (1990) |
| ITS1-5.8S-ITS2 | ITS4 | TCCTCCGCTTATTGATATGC | White *et al*. (1990) |
| LSU rDNA | ITS3zg | CryAwCGATGAAGAACGCAGCC | Tarcz *et al*. (2012) |
| LSU rDNA | 3pLSU | CAAGACGGGTCAGTAGAAGCC | Tarcz *et al*. (2012) |
| *CytB* mtDNA | CytF | GGWACMATGCTRGCTTTYAG | Barth *et al*. (2008) |
| *CytB* mtDNA | PaCytR | GGYCTAAAATATCAATGRGGTGC | Barth *et al*. (2008) |
| *COI* mtDNA | F388dT* | **TGTAAAACGACGGCCAGT**GGwkCbAAAGATGTwGC | Strüder-Kypke and Lynn (2010) |
| *COI* mtDNA | R1184dT* | **CAGGAAACAGCTATGAC**TAdACyTCAGGGTGACCrAAAAATCA | Strüder-Kypke and Lynn (2010) |
| *COI* mtDNA | CoxH10176 | GAAGTTTGTCAGTGTCTATCC | Barth *et al*. (2006) |
| sequencing primer | M13F | TGTAAAACGACGGCCAGT | Strüder-Kypke and Lynn (2010) |
| sequencing primer | M13R | CAGGAAACAGCTATGAC | Strüder-Kypke and Lynn (2010) |

* – primers used for amplification of *COI* fragment are composed of two parts – first one is a degenerate primer (Forward or Reverse), specific to amplified *COI* sequence and the second is a sequencing primer M13 (Forward or Reverse) (bold).

of strain 238 from Benenitra, Madagascar with strain 209 from Paris, France, and of strain 321 from Mexico with strain 209 from France. Similar results were obtained in the present study and in our previous studies (Przyboś *et al*. 2002, 2007). Autogamy was observed in Madagascar and Thailand strains after 12–16 fissions (the growth rate of the cultures was 4 fissions per day) in daily isolation lines cultivated at 27°C, according to Sonneborn's method (1970). It is noteworthy that the majority of known *P. tredecaurelia* strains are restricted to the even mating type. These are strains from Thailand, Madagascar (both localities), Israel, and Mexico. Only the strain from France is characterized by and restricted to the odd mating type.

In the present analyses, based on a comparison of rDNA and mtDNA (*COI* and *CytB*) fragments, the average genetic distance (uncorrected p-distance) between the studied strains of *P. tredecaurelia* is 0.000/0.003/0.004 (rDNA/*COI*/*CytB*). This means that the studied strains are identical in the compared ribosomal fragments and differ in 5 (*COI*) and 6 (*CytB*) nucleotide positions. Variation among all of the studied *Paramecium* strains is presented in Tables S1–S3 (supplementary material). Previous studies revealed similar, very low variability in *P. tredecaurelia* strains (Przyboś *et al*. 2007, Tarcz *et al*. 2006). For example, in RAPD fingerprint analyses, strains from France and Israel showed a 92% similarity of band patterns, which differed only by the presence of only one band at about 1000 bp in the strain from Israel (Przyboś *et al*. 2007). The alignment of both rDNA gene fragments containing 3'SSU rDNA-ITS1 and 5'LSU rDNA revealed only one polymorphic nucleotide position which differentiated the strains from Israel and France (Tarcz *et al*. 2006).

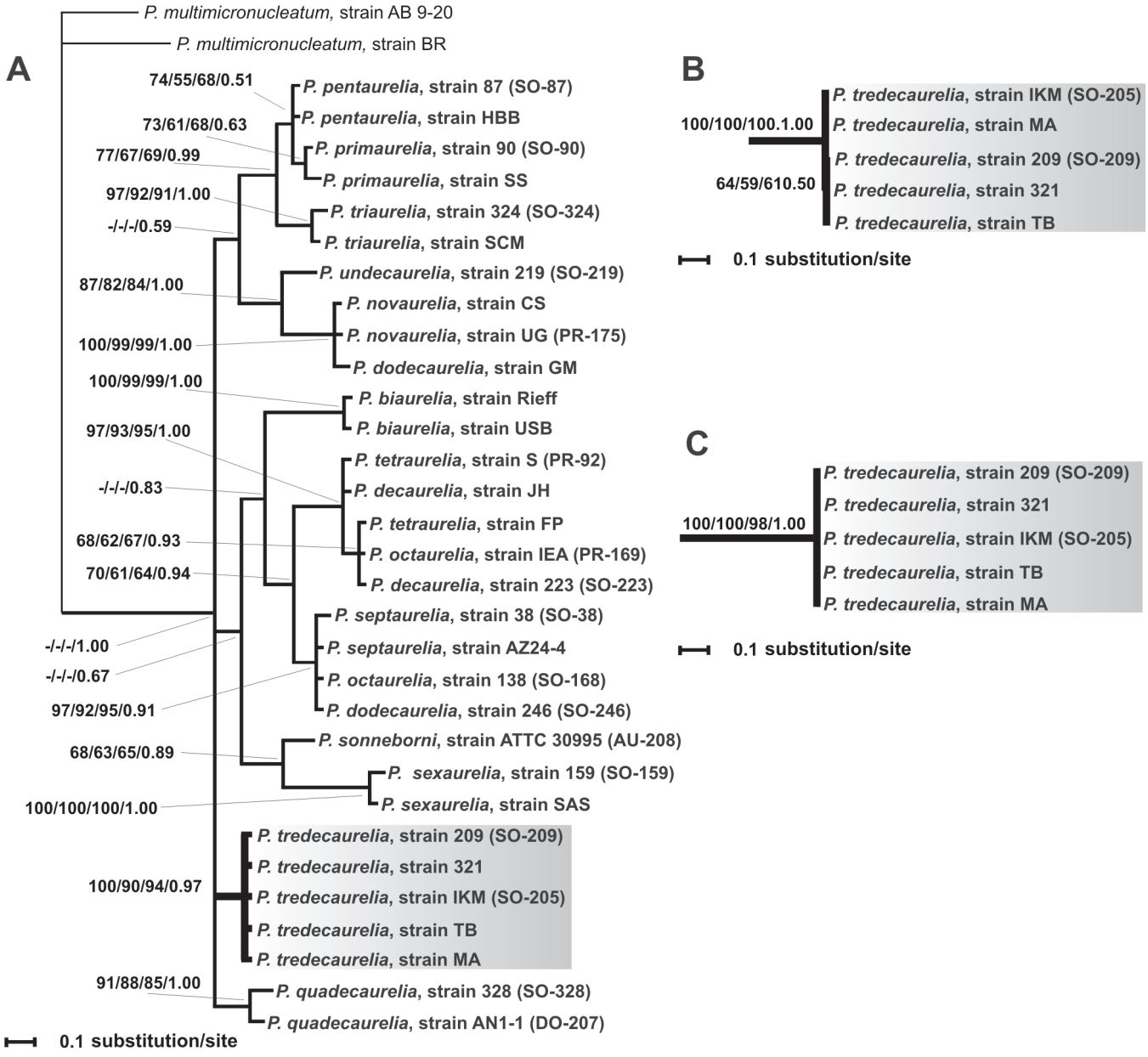


**Fig. 1.** Phylogram constructed for 31 strains of the *P. aurelia* species complex (including the 5 studied strains of *P. tredecaurelia*) and two strains of *P. multimicronucleatum* used as an outgroup. The trees were constructed on the basis of a comparison of sequences from the ITS1-5.8S-ITS2-5'LSU rDNA fragment (**A**), *COI* (**B**), and *CytB* (**C**) using the Bayesian inference method. Bootstrap values for neighbor joining, maximum parsimony analysis, maximum likelihood, and posterior probabilities for Bayesian inference are shown. Bootstrap values smaller 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 5.0 (NJ/MP/ML) and MrBayes 3.1.2 (BI).

All *P. tredecaurelia* strains, the new ones recorded recently from Madagascar and Thailand and the ones known previously from France, Mexico, and Israel, differ only minimally based on the studied DNA fragments and form a separate and well-supported clade in all the constructed trees (Fig. 1A, B, C). Each tree reveals differences in the mutual relationships of *P. tredecaurelia* representatives. Thus, it is difficult to hypothesize about

the place of origin of *P. tredecaurelia* and the direction in which it spread. Based on over 60 years of sampling for the *P. aurelia* species complex, it can be concluded that *P. tredecaurelia* is an extremely rare member of the complex as only 6 strains have been found to date (Rafalko and Sonneborn 1959, Przyboś *et al*. 2002). We are aware that the tropics and generally the southern hemisphere are poorly studied with regards to the occurrence of the *P. aurelia* species complex and future investigations may possibly reveal new stands of *P. tredecaurelia*. The present results suggest that in the past the *P. tredecaurelia* population (predecessors of the present population) probably went through a bottleneck, and its current distribution is the result of a recent dispersal by natural or anthropogenic factors. Such a low level of variability of the studied sequences despite the huge distances between particular localities of the strains may have also been caused by a slow rate of *P. tredecaurelia* evolution. Catania *et al*. (2009) claimed that the above phenomenon can be explained by strong selection against nucleotide substitutions at silent sites, which plays a significant role in sharing very similar or even identical haplotypes in distant populations.

Similar examples of low molecular variability have also been described in some ciliates and other protists. Weisse *et al*. (2008) found that the SSU rDNA and ITS sequences of clones of the freshwater spirotrich ciliate *Meseres corlissi* originating from distant areas (Austria and Australia) were identical, and the clones differed only slightly in morphology and temperature response. According to the authors, these features were typical of rare species. Also, different morphotypes of tintinnids (ciliates) were found to have identical sequences at the ITS locus by Snoeyenbos-West *et al*. (2002) (cited in Foissner *et al*. 2008). In their paper devoted to protist distribution, Bass and Boenigk (2011) presented a study by Logares *et al*. (2007), which showed that two dinoflagellate morphospecies *Scrippsiella hangoei* and *Peridinium aciculiferum* reveal morphological differences, but have identical ITS, 5.8S, ITS2 and partial LSU rDNA sequences.

In contrast to the monophyletic nature of the *P. tredecaurelia* cluster, other species of the *P. aurelia* complex exhibit different levels of intraspecific polymorphism. This was previously revealed by molecular methods based on PCR fingerprinting (Stoeck *et al*. 1998, 2000; Przyboś *et al*. 2007) as well as sequencing gene fragments in several species, e.g., in *P. pentaurelia* (Przyboś *et al*. 2011), *P. sexaurelia* (Przyboś *et al*. 2010), *P. octaurelia* (Przyboś *et al*. 2009). Among the species of the complex, *P. dodecaurelia* shows a high level of intraspecific variation. It seems to be a polyphyletic species with several haplotypes similar or even shared with other members of the *P. aurelia* species complex (Przyboś *et al*. 2012b). Differentiation of strains was also observed in *P. quadecaurelia* (Przyboś *et al*. 2013), another rare species of the *P. aurelia* complex, known only from four localities. Trees based on genome fragments similar to those studied in the present paper showed that *P. quadecaurelia* strains form a monophyletic but differentiated clade. The genetic differentiation among *P. quadecaurelia* strains was equal to or even greater than the distances between some other *P. aurelia* species. These two rare species of the *P. aurelia* complex, i.e., *P. quadecaurelia* and the presently studied *P. tredecaurelia,* also differ in the manner of their mating type inheritance system. The former possesses a caryonidal system, while the latter has a synclonal one (Sonneborn 1975). However, there is no scientific evidence for a clear connection between the system of mating type inheritance and the rate of speciation within species.

The present results also reveal a notable discordance between the lack of (rDNA) or very low (mtDNA) sequence variability in *P. tredecaurelia* strains and the low viability of $F_2$ inter-strain hybrids. Currently, it is difficult to determine whether this is due to the gradual appearance of a reproductive barrier between the studied strains or rather the result of certain characteristics of strain 209 from France: this strain was the only representative of the mating type O (odd) used in all the genetic crosses (Table 3). Future sampling may lead to finding other strains with an odd mating type, which would allow us to test whether the low viability of the $F_2$ generation observed in the present study is a characteristic feature of *P. tredecaurelia*.

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