

Intraspecific Genetic Divergence of *Paramecium bursaria* and Re-construction of the Paramecian Phylogenetic Tree

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Summary. *Paramecium bursaria*, a species belonging to the genus *Paramecium*, is quite unique in that the species intracellularly maintains several hundreds of symbiotic algae. This species has been regarded as firstly branched-off species within this genus, and reported its higher genetic variation. We determined rDNA 5' half regions of 13 strains of *P. bursaria*, and investigated their intraspecific genetic divergences with comparisons of 18S rDNA and internal transcribed spacer 2 (ITS2). The 18S rDNA divergence among the strains of *P. bursaria* reached 1.61% which definitely greater than that of *P. caudatum*, *P. multimicronucleatum* and *P. aurelia* complex, and also greater than some interspecific relations (e.g. between *P. jenningsi* and *P. schewiakoffi*). These results seemed to be caused by the strains used for the experiments; we used large number (14) of *P. bursaria* strains and small number of others (four *P. caudatum*, three *P. multimicronucleatum* and three *P. aurelia* complex strains). Comparison of the ITS2 region using 10 *P. bursaria*, 13 *P. caudatum*, 11 *P. multimicronucleatum* and 18 *P. aurelia* complex strains showed inverted results: *P. multimicronucleatum* and *P. aurelia* complex diverged greater than *P. bursaria* in the quantitative (number of nucleotide substitutions) as well as the qualitative sense (presence/absence of [hemi-] compensatory base change). Increasing the divergent sequence number occasionally changed the topology of the phylogenetic tree. We re-evaluated the phylogenetic relationships of *Paramecium* spp. as inferred from 18S rDNA with our 20 added paramecian sequences. The trees indicated that paramecia divided into four lineages: i) *P. bursaria*, ii) *P. duboscqui* and *P. putrinum*, iii) *P. calkinsi*, *P. jenningsi*, *P. nephridiatum*, *P. polycaryum*, *P. woodruffi*, and *P. aurelia* complex, and iv) *P. caudatum*, *P. multimicronucleatum* and *P. schewiakoffi*; in strict correspondence with the latest subgeneric concept of „*Chloroparamecium*”, „*Helianter*”, „*Cypriostomum*” and „*Paramecium*”, respectively. „*Chloroparamecium*” and „*Helianter*” of which there are similar characteristics were claded, as were „*Cypriostomum*” and „*Paramecium*”.

Key words: CBC, „*Chloroparamecium*”, „*Cypriostomum*”, „*Helianter*”, ITS2, „*Paramecium*”, 18S rDNA, phylogeny.

Abbreviations: CBC - compensatory base change, ITS - internal transcribed spacer, ML - maximum likelihood, MP - most parsimonious, NJ - neighbor joining, OTU - operational taxonomic unit.

INTRODUCTION

The common freshwater ciliate, *Paramecium* spp. has been well and widely studied for two hundred

years. Early microscopic technology caused insufficient descriptions, and undeveloped methods concerning species identification resulted in many dubious species (Wichterman 1953, 1986). With the development of modern techniques, e.g., electron microscopy and staining techniques (Fokin 1986, 1997; Shi *et al.* 1997; Fokin and Chivilev 1999; Fokin *et al.* 1999), the species concepts have been reorganized. Consequently, 17 paramecian species (as the *P. aurelia* complex con-

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verted into one species) are now recognized as valid (Fokin *et al.* 2004).

Among *Paramecium* spp., morphology and other characteristics are diverse, which have brought subgeneric concepts into this genus (Woodruff 1921; Jankowski 1969, 1972). The latest concept based upon morphometric, biological and molecular differences divides the above 17 species into four subgenera: „*Chloroparamecium*”, „*Helianter*”, „*Cypriostomum*” and „*Paramecium*” (Fokin *et al.* 2004). „*Chloroparamecium*” is composed of only one species *P. bursaria*, and prior studies (Strüder-Kypke *et al.* 2000a, b; Fokin *et al.* 2004) have suggested this species diverged first in the genus *Paramecium*.

If *P. bursaria* is regarded as an “old species”, the intraspecific genetic divergence is possibly larger. In fact, Stoeck *et al.* (1998) reported higher genetic diversities than in other paramecian species. The highest 18S rDNA pairwise distance seen in *P. bursaria* reached 1.57% (gaps were included, Hoshina *et al.* 2005); e.g. the recently described species *P. schewiakoffi* (Fokin *et al.* 2004; GenBank accession no., AJ548821) differed by 0.73% from *P. jenningsi* (AF100311) and by 1.11% from *P. tetraurelia* (X03772).

The sibling species concept seen in *Paramecium* spp. has been investigated with various methods e.g. with isozymes (Allen and Gibson 1971) and random amplified polymorphic DNA (RAPD, Stoeck *et al.* 1998, 2000; Stoeck and Schmidt 1998). However, it is problematic to consider their phylogeny or distance. Coleman (2000, 2005) proposed the utility of internal transcribed spacer 2 (ITS2) sequence comparison for such species or non-species affair. Although this region is a non gene-coding region, it might be limited to primary and secondary sequence variation in the potential gene exchange group. She also showed the applicability of this region for paramecian species, and indicated the linkage between sequence variation and gene exchange. Although the precise cause of above genetic distances in *P. bursaria* are as yet not well known, in this study we compared their ITS2 sequences as well as the 18S rDNA sequences.

We have collected *P. bursaria* strains from studies relating their symbionts (Hoshina *et al.* 2004, 2005) and the other comparative paramecian stocks, and have used 18S rDNA sequence comparison. As a result, we obtained 20 operational taxonomic units (OTUs) of 18S rDNA sequences from *Paramecium* spp. (Hoshina *et al.* 2005 and this study), which more than doubles the number of available sequences compared to other phy-

logenetic assessments based on this sequence (Fokin *et al.* 2004). It is well known that increases in the numbers of sequences frequently changes the phylogenetic tree topology (e.g. Graur and Li 2000, Nei and Kumar 2000). Consequently, we attempted to re-construct the paramecian phylogenetic tree.

MATERIALS AND METHODS

Paramecium bursaria, *P. caudatum*, *P. multimicronucleatum* and *P. tetraurelia* stocks (Table 1) were maintained in aka-endoumame medium (Tsukii *et al.* 1995) or in accordance with CCAP (Culture Collection of algae and Protozoa, UK) instructions, at 25°C with cool white light illumination (16 L: 8 D, 100 µmol photons m⁻² s⁻¹).

For DNA extraction, we used the modified CTAB method (outlined in Hoshina *et al.* 2004), or the DNeasy plant mini kit (Qiagen, GmbH, Düsseldorf, Germany) according to manufacturer’s directions. A part of the total DNA used for the following analyses were those obtained by Hoshina *et al.* (2005).

DNA amplification and sequencing primers for ribosomal DNAs are described in Hoshina *et al.* (2005). For ITS amplification, we used the alternative primer sets of *Paramecium*500F (Hoshina *et al.* 2005)/ITS4 (White *et al.* 1990) or *Paramecium*730F (5’-GTGTTTCAGGCAGGTTTTCG-3’, forward direction)/ITS4. The PCR products were once sequenced directly. Low-quality electrophorograms were observed in the sequences of ITS regions of *P. bursaria* CCAP 1660/12 and 1660/13, which were cloned using the TArget Clone kit (Toyobo, Osaka, Japan) according to manufacturer’s recommended protocols.

We compared all available (published by Strüder-Kypke *et al.* 2000a, b; Coleman 2005; Barth *et al.* 2006) and our (Table 1) sequences of *P. bursaria*, *P. caudatum*, *P. multimicronucleatum* and *P. aurelia* complex. 18S rDNA sequences (14 strains of *P. bursaria*, four *P. caudatum*, three *P. multimicronucleatum* and three *P. aurelia* complex) were manually aligned using Clustal X version 1.81 (Thompson *et al.* 1997). For ITS2 comparison, once we made secondary structural models for every sample, these were referred to that of *P. tetraurelia* (Coleman 2005; a brief diagram is shown in Fig. 1). Helix constructions were predicted by mfold version 3.2 (Zuker 2003) and ITS2 sequences (10 *P. bursaria*, 13 *P. caudatum*, 11 *P. multimicronucleatum* and 18 *P. aurelia* complex) were aligned by considering the above secondary structure. Unrooted distance trees based on each 18S rDNA and ITS2 were constructed (Fig. 2) by Neighbor Joining (uncorrected “p” distance) in PAUP version 4.0b10 (Swofford 2000).

For the re-constructing of the paramecian phylogenetic tree, we employed all available paramecian sequences (published by Strüder-Kypke *et al.* 2000a, b; Fokin *et al.* 2004) with 15 other ciliates (see Fig. 3). Once we aligned them using Clustal X, they were compared to available 18S rRNA structures (Comparative RNA Web Site, Cannone *et al.* 2002), manually adjusted, and ambiguous regions lying at hairpin loops, internal loops or bulges were removed. Phylogenetic trees based on this 18S rDNA dataset were constructed using PAUP. For maximum likelihood (ML) analyses, optimal likelihood settings for evolutionary models were determined by Modeltest version 3.6 (Posada and Crandall 1998), which resulted in a model and respective

parameters or proportions for each dataset. With those settings, a heuristic search was performed (nearest-neighbor interchange algorithm, starting tree obtained via neighbor joining). Maximum parsimony (MP) analysis was performed using a heuristic search with the bisection-reconnection option and random sequence addition with 10 replications; gaps were treated as a fifth base. Neighbor Joining (NJ) analysis of Saito and Nei was also performed. The bootstrap probabilities were calculated by all ML (100 replicates), MP (1000) and NJ (1000) analyses.

RESULTS

Sequences and genetic intraspecies diversity of *P. bursaria*

The variation seen in multiple copies of a single locus has been reported in a paramecium (Preer *et al.* 1999). However, most of our electrophorograms from direct sequencing showed definite single peaks. If different sequences were present, their copy number in the genome seems proportionately below our detection level. It probably needs tens or hundreds of subcloning procedure to detect such minor variation, and truthfully, the quest for all minor variation is never ending without

whole genome analysis. Indeed, recent intraspecific studies of paramecia using rDNA cistron also chose

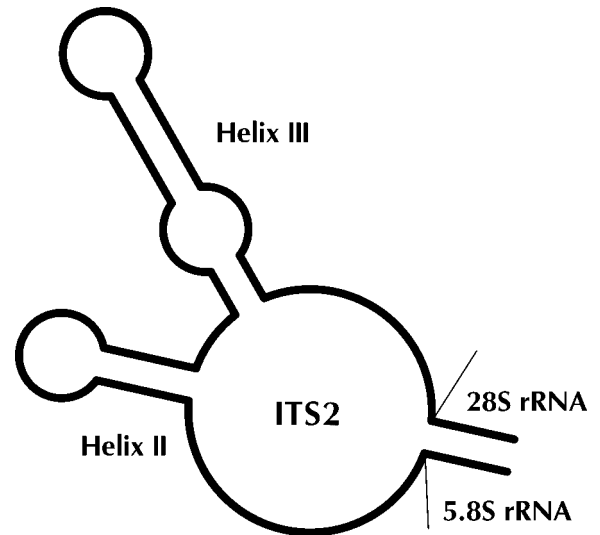


Fig. 1. Secondary structural diagram of paramecian ITS2 modified from Coleman, 2005. ITS2 usually forms a four-fingered hand structure in eukaryotic organisms (Coleman 2003); helices I and IV occasionally evolve rapidly, and in paramecia, these are absent (see Coleman 2005).

Table 1. Paramecian strains of which DNA sequence were obtained in this study.

Strains	syngen/mating	Collection site	Accession	
			18S rDNA	ITS
<i>P. bursaria</i> OK1	1/I	Aich, Japan	AB206537 ^a	AB252010
<i>P. bursaria</i> So13	1/I	Nagano, Japan	AB206538 ^a	AB252011
<i>P. bursaria</i> F36	1/II	^b	AB206539 ^a	AB252012
<i>P. bursaria</i> KM2	1/I	Shimane, Japan	AB206540 ^a	
<i>P. bursaria</i> Dd1	1/II	Ibaraki, Japan	AB206541 ^a	
<i>P. bursaria</i> Bnd1	1/III	Hiroshima, Japan	AB206542 ^a	
<i>P. bursaria</i> Cs2	1/II	Shanghai, China	AB206543 ^a	AB252013
<i>P. bursaria</i> MRBG1	?	Melbourne, Australia	AB219526 ^a	AB252014
<i>P. bursaria</i> PB-SW1	?	Schwarzwald, Germany	AB206544 ^a	AB252015
<i>P. bursaria</i> CCAP1660/10	2/V	?	AB252000	AB252016
<i>P. bursaria</i> CCAP1660/11	2	Cambridge, UK	AB206545 ^a	AB252017
<i>P. bursaria</i> CCAP1660/12	?	Cambridge, UK	AB252001	AB252018-24
<i>P. bursaria</i> CCAP1660/13	?	Cambridge, UK	AB252002	AB252025-38
<i>P. caudatum</i> Isn4	12/XXIII	Iwate, Japan	AB252003	AB252039
<i>P. caudatum</i> KNZ2	3/VI	Kanazawa, Japan	AB252004	AB252040
<i>P. caudatum</i> Yhm	?	Yamaguchi, Japan	AB252005	AB252041
<i>P. multimicronucleatum</i> TH105	2/IV	?	AB252006	AB252042
<i>P. multimicronucleatum</i> YM25	cycler ^c	Ishinomaki, Japan	AB252007	AB252043
<i>P. tetraurelia</i> 51	4/VII	USA	AB252008	AB252044
<i>P. tetraurelia</i> hrd	4/VIII	?	AB252009	AB252045

^a Accessions obtained in Hoshina *et al.* (2005); ^b One of F1 crossbreed between T151 (1/IV, Ibaraki, Japan) and Mts4 (1/I, Nagano, Japan); ^c Janiform entity of 2/III and 2/IV, see Barnett (1966).

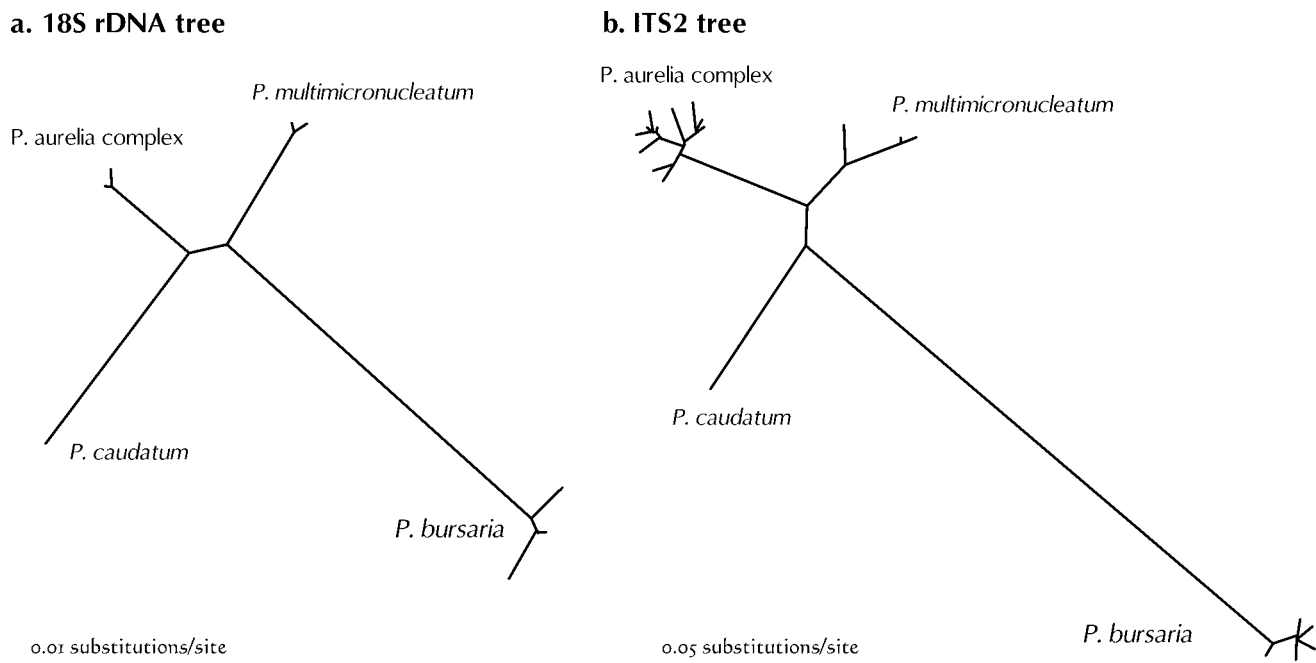


Fig. 2. Unrooted phylograms showing genetic divergences among *Paramecium bursaria*, *P. caudatum*, *P. multimicronucleatum* and *P. aurelia* complex: a, based on 18S rDNA; b, based on 163 aligned nucleotides (gaps included) of ITS2.

direct sequencing method (Coleman 2005, Barth *et al.* 2006). Thus, we quit subsequent clonings for the PCR products of which electrophorograms have undoubted peaks. The visible double peaks or dubious electrophorograms were seen only in spacer regions of two strains of *P. bursaria*, which were defined by a subcloning method. Seven clones detected a single nucleotide polymorphism (SNP, C/T mixtures) and 19 nucleotide insertion/deletions in ITS1 of CCAP 1660/12; and in 14 clones a C/T variation was detected in the 5.8S rDNA and two C/T and two A/G variations in the ITS2 region of CCAP 1660/13. The positions of SNPs corresponded to those of the double peaks seen when sequenced directly.

Four types of 18S rDNA sequences were found in *P. bursaria* (Fig. 4a): A) CCAP 1660/12 and 1660/13; B) 1660/10 and a Canadian strain (Strüder-Kypke *et al.* 2000a, AF100314); C) PB-SW1 and 1660/11; D) OK1, So13, F36, KM2, Dd1, Bnd1, Cs2, MRBG1. The intraspecific distance reached 23 substitutions + 4 insertion/deletions between genetic group of C and D. Oppositely, there were only two transitions between A and B. The unrooted 18S rDNA tree (Fig. 2a) revealed big intraspecific distances of *Paramecium bursaria* com-

pared to the other three species; five substitutions were seen in *P. multimicronucleatum* and in *P. aurelia* complex, and no divergence in *P. caudatum*.

Six different ITS2 sequences were recognized when 14 clones of CCAP 1660/13 were regarded as independent OTUs (Fig. 4b): i) CCAP 1660/10, 1660/11, 1660/12 and 1660/13 clones 01-03, 05-07 and 11-14; ii) PB-SW1 and CCAP 1660/13 clone 08; iii) CCAP 1660/13 clone 04; iv) CCAP 1660/13 clone 09; v) CCAP 1660/13 clone 10; vi) OK1, So13, F36, Cs2 and MRBG1. There were only one or two transitions among the genetic groups i through v, and the group vi differed two or more nucleotides + one insertion/deletions from the others. The ITS2 tree (Fig. 2b) showed the reverse result to that of the 18S rDNA. Up to 6 substitutions were seen both in *P. multimicronucleatum* and in *P. aurelia* complex, yet 3 substitutions + 1 insertion/deletion was found among *P. bursaria* strains as a maximum (group i vs. groups iii-vi). No divergence was seen in *P. caudatum*.

Depending on species (-complex), there were several varieties such as helix length and bulges, although the general structure of ITS2 was common to all four species. For intraspecific comparison, while most of the

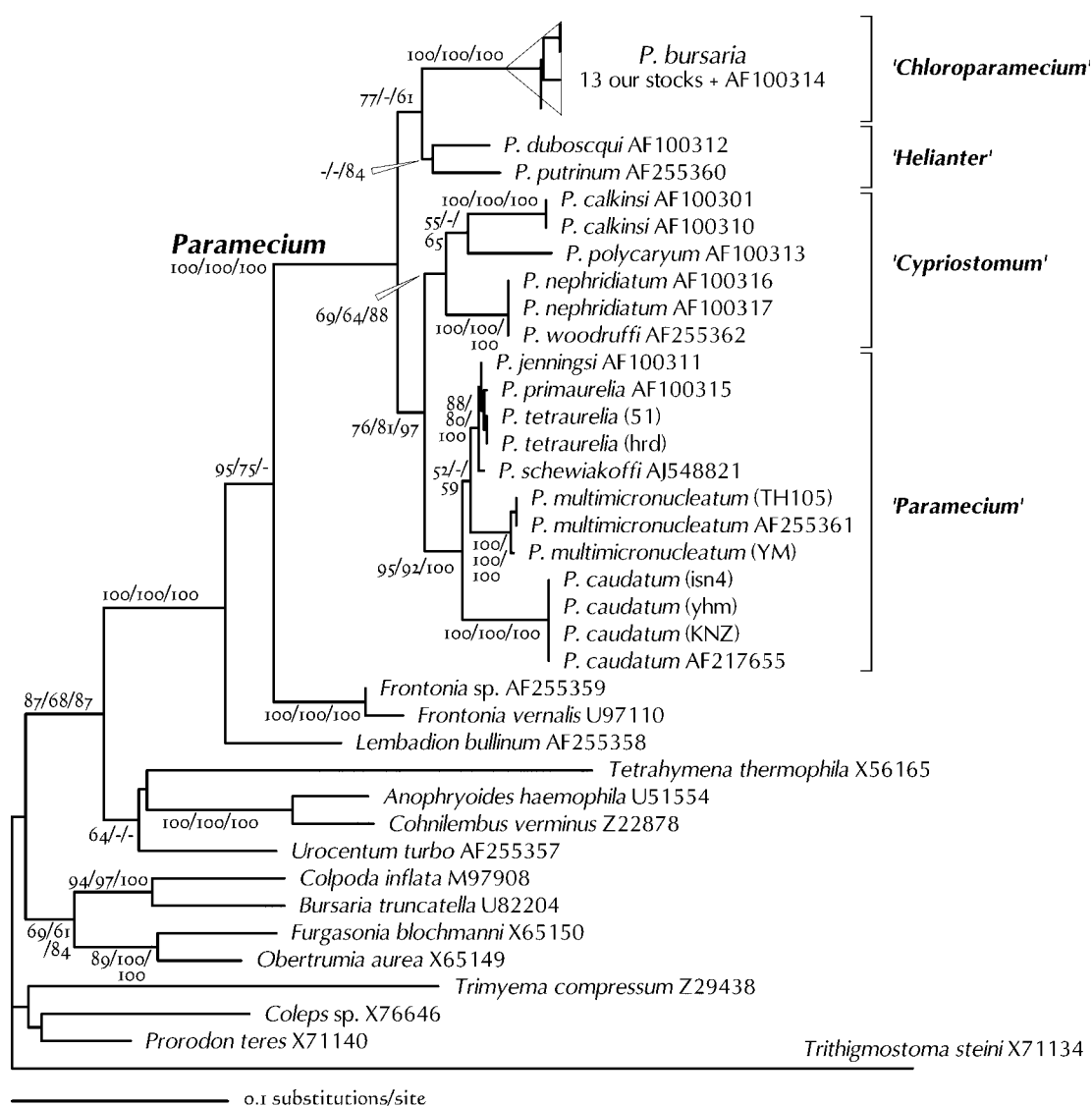


Fig. 3. Phylogenetic tree of paramecia constructed from an analysis of 18S rRNA gene sequences. Tree topology was obtained by ML analysis. Numerals at each nodes are bootstrap probabilities of ML (100 replicates) / MP (1000 replicates) / NJ (1000 replicates) analyses; only values above 50% support are shown.

nucleotide substitutions or insertion/deletions were seen at single loops (including bulges), two compensatory nucleotide substitutions without structural changes were also seen. One was hemi-CBC (compensatory change on only one side of a helix pairing: U-A/U-G) at helix III (Fig. 1) in *P. aurelia* complex, which was reported by Coleman (2005). The other was CBC (compensatory base change: U-A/C-G) found at the tip of helix II (Fig. 1) of *P. multimicronucleatum*. Neither CBC nor hemi-CBC was found in species of *P. bursaria*.

Paramecian phylogeny

Phylogenetic analyses of the 34 paramecian 18S rDNA sequences were performed with 15 other ciliates. Once we tried multiple alignment with the out group taxa as employed by Strüder-Kypke *et al.* (2000b). Although they instructed on gap-handlings for length polymorphic region Helix E10_1 among them, such length polymorphic regions were abundant when compared to the outermost spirotrich taxa. We therefore employed

evolutionary event, when a species diverges into two or more species, requires a long time for completion. Consequently, any species is just in the process of speciation. When we look at organisms around us, we can occasionally recognize plural different units within a species. In protists, it is well known that sexually isolated groups are present in a morphologically identical taxon (Finlay and Esteban 2001, Coleman 2002). In the case of *Paramecium* spp., such sexually isolated groups are called syngens. It is a fact that an entity of paramecia (hitherto *P. aurelia*) was re-described as 14 independent species with the findings of a separated character for each syngen (Sonneborn 1975). Later, *P. sonnebornii* was added to this group as 15th species (Aufderheide *et al.* 1983). As each of the other paramecia remains inclusive of several syngens within a species, the above separation has created active debate over its pros and cons, and is now evolving into “what is a species?” All of the species derived from hitherto *P. aurelia* are generally collectively called *P. aurelia* complex (Allen *et al.* 1983).

To fill a gap between morphological species and biological (producing fertile offspring) species, Coleman (2000) proposed the Z clade concept which indicates the zygote formation group, regardless of whether they produce fertile offspring or not. Furthermore, to assess delimits of Z clades, the utility of a region of ribosomal DNA cistron, the internal transcribed spacer 2 (ITS2), has been advocated (Fabry *et al.* 1999; Coleman 2000, 2005; Coleman and Vacquier 2002; Young and Coleman 2004). ITSs organize certain secondary structures, which helps splicing for 18S (SSU), 5.8S and LSU rRNA. Therefore, ITSs are not mere spacers but may be regarded as genes. Compensatory Base Change (CBC) is a phenomenon when one side base of a pair changes, the opposite side base also changes, compensating to conserve that base pair in a double strand helix. Only one side change also remaining a structure called hemi-CBC. Both are used to establish the confidential folding pattern for rRNAs (Gutell *et al.* 1994). Although the ITS2 sequence has nothing directly to do with whether two organisms can mate or not, when there is a CBC in ITS2 there is no sexual compatibility (Coleman 2000, Coleman and Vacquier 2002, Behnke *et al.* 2004). There was a hemi-CBC (indicating incompatibility next to CBC) in *P. aurelia* complex (Coleman 2005), and we here found a CBC intraspecies of *P. multimicronucleatum*. However, neither CBC nor hemi-CBC was found among our 10 strains of *P. bursaria*. It is a fact that intraspecific divergence of

P. bursaria looked higher than those of the other species by 18S rDNA comparison (Fig. 2a), but this is different with ITS2 (Fig. 2b). Definitely, ITS2 divergence of *P. bursaria* was lower in quality and quantity than that of *P. aurelia* complex and of *P. multimicronucleatum*. The principal factor of the above reversion might be the number of strains (see Materials and methods) and syngens; for instance, we could have used only two (or more) *P. bursaria* syngens, but compared them with 13 syngens of *P. aurelia* complex for ITS2 analysis (most of *P. multimicronucleatum* sequence were obtained in Barth *et al.* (2006), of which syngens have not been made public). Although CBC indicates different biological species, the converse is not always true (Coleman 2005). To assess the degree of *P. bursaria* speciation, all six syngens should be analyzed.

We detected five different sequence variants in CCAP 1660/13 ITS2 region. We handled them as independent OTUs for the comparisons, although it might be inappropriate procedure. As a matter of course, it is not certain the other strains have same sequence copies within the genome. The genetic groups of which based on 18S rDNA and ITS2 were not congruent (see Fig. 4). 18S rDNA group C can be taken as a simple example; PB-SW1 belongs to ITS2 group ii with one of 1660/13 (group A) cloning products, and 1660/11 belongs to group i with those 18S group A (1660/12 and 1660/13 clones) and B (1660/10). The other hand, the member of 18S group D congruently belonged to ITS2 group vi. The genetic group D = vi is also characterized by that they commonly possess “American” type symbionts (Hoshina *et al.* 2005). From the geographical viewpoint, although sampling is meager, group D distributes to Asia and Oceania whereas groups A, B and C distribute to Europe and Northern America (Table 1). We defined four genetic groups intraspecies of *P. bursaria* based on 18S rDNA, however, it might be regarded that the greater autonomy of group D.

Tree topologies can differ according to outgroup selections, multiple alignment, increasing OTUs and tree construction models (Graur and Li 2000, Nei and Kumar 2000). Although the number of paramecian species was not changed from the previous analysis (Fokin *et al.* 2004), we increased genetic diversity of paramecian materials. For example, although only one *P. bursaria* sequence has ever been published, we sprinkled several new sequences of *P. bursaria*, of which the diversity is equivalent to some interspecific diversities (mentioned above). After the first paramecian analysis (Strüder-Kypke *et al.* 2000a), the organisms

phylogenetically surrounding the peniculines were stable, offering a choice of the closer outermost group to construct a phylogenetic tree. Closer outgroups resulted in more accurate alignment. In addition, we relentlessly removed doubtful regions which corresponded to loops or bulges in SSU rRNA foldings.

The accumulation of such tiny differences generated the unique topology. The point with the greatest difference is the first division of *Paramecium* spp. The topologies conducted by Strüder-Kypke *et al.* 2000a, b; Fokin *et al.* 2004 commonly showed that *P. bursaria* diverged at first at the genus and the monophyly of the rest of the species were moderately to highly supported. In our analyses, however, the paramecia branched into two, the clade of *P. bursaria*, *P. duboscqui* and *P. putrinum* and the clade constructed of *P. calkinsi*, *P. polycaryum*, *P. nephridiatum*, *P. woodruffi*; *P. jenningsi*, *P. aurelia* complex, *P. schewiakoffi*, *P. multimicronucleatum* and *P. caudatum* (Fig. 3). Although Fokin *et al.* (2004) acknowledged that *P. duboscqui* and *P. putrinum* were secondary branching species, they pointed out the similarities to *P. bursaria*, for example by the multiple mating-type system and nuclear reorganization process. This monophyly was supported up to 77% obtained by ML analysis, and six MP and NJ trees also showed the same topology.

Although there are negative aspects to some lower bootstraps (except NJ analysis) or some shorter internodes, the members of the paramecia can be perceived as four phylogenetic groups, namely, *P. bursaria*; *P. duboscqui* and *P. putrinum*; *P. calkinsi*, *P. polycaryum*, *P. nephridiatum*, and *P. woodruffi*; *P. jenningsi*, *P. aurelia* complex, *P. schewiakoffi*, *P. multimicronucleatum* and *P. caudatum*. This separation is consistent with the subgeneric concept of Fokin *et al.* (2004) emphasizing morphometric analysis. Each corresponds to „*Chloroparamecium*”, „*Helianter*”, „*Cypriostomum*” and „*Paramecium*”, respectively (see Fig. 3). All topologies obtained from ML, MP and NJ analyses are congruent even at the most unreliable branch, indicating „*Helianter*” (*P. duboscqui* and *P. putrinum*) and NJ analysis gives strong bootstrap support. Therefore, we generally support the subgeneric classification of Fokin *et al.* (2004) from the phylogenetic view point.

The same as with published trees (Strüder-Kypke *et al.* 2000b, Fokin *et al.* 2004), *Paramecium* spp.

constructed a robust clade with *Frontonia* and *Lembadion* spp. (Subclass Peniculia, see Strüder-Kypke *et al.* 2000b) which share the short helix E10_1 in the 18S rDNA (Strüder-Kypke *et al.* 2000b) and the morphological characteristics of nematodesmata (Didier 1970). In this clade, *Paramecium* spp. were derivatively divided in ML and MP analyses; incongruently, *Frontonia* and *Lembadion* were claded as a sister to *Paramecium* in NJ analysis. Referring to their cell shape, the alternative foot shape or cigar shape which was the first dividing concept for paramecia (Woodruff 1921) resulted in cigar shaped species aggregated to a clade of the subgenus „*Paramecium*” (Fig. 3). The other three subgenera have a foot shaped body in common. *Frontonia* and *Lembadion* also appear to be foot shaped. Consequently, it can be said that the foot shape is the plesiomorphic character for the genus *Paramecium* and the cigar shape is the synapomorphic one for the subgenus „*Paramecium*”. Can we say that „*Chloroparamecium*” is the lineage acquiring algal-holding ability? When we glance around the Peniculia, there is another green ciliate, *Frontonia vernalis* Ehrenberg, which is a typical algae-hold host (Berninger *et al.* 1986). If the algal-holding ability was obtained at the lineage to „*Chloroparamecium*”, the same ability must have been independently gained at the lineage to *Frontonia vernalis*. It is also possible that the ancestor of Peniculia (because the relationship between *Frontonia* and *Lembadion* was unresolved, as mentioned above) had already gained this ability. This needs at least five evolutionary instances of one gain and four independent losses: the lineages to (i) *Lembadion*, (ii) heterotrophic *Frontonia*, (iii) „*Helianter*”, (iv) the ancestor of „*Cypriostomum*” and „*Paramecium*”. Derived characters are usually equally weighted in cladistics, but it is an accepted norm in evolution that the loss of a character is easier than the gain of a character. With the example of eyes in arthropods, it is occasionally taken as a central role of evolution that eyes are lost over 30 times as often as they are gained (Oakley and Cunningham 2002). Another example is the trophically diverse group, the dinoflagellates, although it is not clear when they got plastid-control ability. At least eight independent instances of plastid loss (with three of replacement) have been shown (Saldarriaga *et al.* 2001). The evolutionary facts will be settled once the gene permitting algal control has been elucidated.

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