Genetic Diversity of Symbiotic Green Algae of *Paramecium bursaria* Syngens Originating from Distant Geographical Locations

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1. Introduction

The unicellular ciliate *Paramecium bursaria* (Peniculida, Oligohymenophorea) is a host of endosymbiotic algal species. The mutualistic symbiosis exhibited by *P. bursaria* suppresses the genetic change of the inhabitant and ensures a nutritionally stable environment. Doebeli and Knowlton [1] reported that the rate of nucleotide substitutions was lower in symbiotic algae than in free-living relatives and their corresponding inhabitants since their co-evolution from an ancient association. *Paramecium* spp. usually comprise several sexually separated sibling groups, termed “syngens”, which are morphologically indistinguishable. Currently, *P. bursaria* strains have been assigned to five syngens (R1 to R5), which may correspond to some syngens described by Bomford [2,3]. Each syngen in Bomford’s collection (which was lost) had specific geographical distributions. Based on some similarities between syngens from the “old” and “new” collections, it has been suggested that syngen R1 is widespread in Europe; syngen R2 is widespread in Europe, extending eastwards to Siberia and Australia; syngen R4 is fairly widespread in the USA; and syngen R3 is present in Russia, Japan, China and the USA; finally, syngen R5 is represented by only four strains from two locations in western Europe [4].
Symbiotic algae isolated from different Paramecium bursaria syngens are represented by Chlorella-like species belonging to two genetically distinct “European” and “American” populations [5]. Gaponova et al. [6] confirmed the existence of two groups of symbionts based on the analysis of rDNA PCR products of two different lengths, which corresponded to the southern (three introns) or northern (single intron) group. Phylogenetic analyses based on the 28S rDNA gene, ITS 1, 5.8S rDNA and ITS 2 sequences suggested the existence of five different endosymbionts: Chlorella vulgaris, Chlorella variabilis, Micractinium conductrix comb. nov., Choricystis minor (Choricystis parasitica comb. nov.) and Coccomyxa simplex. Pröschold et al. [7] have confirmed the occurrence of two endosymbiont groups and found that Micractinium conductrix and Chlorella vulgaris belonged to the “European” population. Hoshina and Imamura [8] have found that Chlorella vulgaris is a symbiont of Paramecium bursaria strain. Chlorella variabilis represents the “American” population and has been found in Paramecium bursaria strains (CCAP211/84, 211/109 and 211/110) collected in the USA [7]. Algal symbionts of all P. bursaria strains of two different origins form one clade, but are split into two distinct lineages.

An evolutionary scenario for P. bursaria with respect to algal acquisition and subsequent switching assumes the coexistence of both species belonging to the “American” and “European” endosymbiont groups in one cell of ancestral P. bursaria. This sympatric relationship led to a continuous intron transmission. During evolution, the host “chose” one of the endosymbionts, and later “European” algae may have diverged into a lineage with a weakened host–algal partnership, in which accidental switching of the algae occurred twice [9,10].

Hoshina and Imamura [8] and Gaponova et al. [6] have shown that P. bursaria can contain different endosymbionts, depending on their origin. Nakahara et al. [11] identified an additional endosymbiont, Choricystis minor, in a strain from Florida (USA). Pröschold et al. [7] studied 17 strains of endosymbionts isolated from various hosts and different geographical locations. Phylogenetic analyses revealed that they were polyphyletic. The most studied ciliate, P. bursaria, harbors endosymbionts representing at least five different species: Coccomyxa sp., Choricystis minor, Micractinium conductrix, Chlorella vulgaris and Chlorella variabilis. C. vulgaris, C. variabilis and Micractinium conductrix are obligate endosymbionts of P. bursaria [7]. M. tetrahymenae forms a symbiotic association with Tetrahymena utriculariae only under anoxic or microaerobic conditions. Phylogenetic analyses using complex evolutionary models based on secondary structure have demonstrated that this endosymbiont represents a new species of Micractinium, which belongs to the so-called Chlorella clade (Trebuoxiophyceae) [12].

In the present study, we investigated 43 strains of algal symbionts isolated from P. bursaria strains belonging to five syngens. The strains were collected in remote geographical locations. Twenty sequences of symbionts were available in GenBank (28S rDNA and ITS1-5.8S rDNA-ITS2 fragment). The strains of Coccomyxa chodatii, Stigeoclonium tenue, Stigeoclonium variabile, Parachlorella kessleri and Actinastrium hantzschii were used as outgroups. Three loci: a fragment of the ITS1-5.8S rDNA-ITS2 region and a fragment 28S rDNA, as well as chloroplast genes encoding ribosomal protein L36 (rpl36) and translation initiation factor IF-1 (infA) were applied to study phylogenetic relationships of symbiotic algae. The selected ribosomal primers were specific to symbiotic cells, which did not allow the simultaneous amplification of P. bursaria rDNA fragments. The 28S rDNA is characterized by higher variability than the 18S rDNA [8]. The ITS1-5.8S rDNA-ITS2 region is highly variable among the sequences of different species, while it is relatively conserved among the sequences of the same species of algae. Furthermore, this fragment is most commonly available in GenBank, which facilitates comparative analysis. The 3′rpl36-5′infA gene fragment has been selected due to the presence of an intergenic region, which is suspected to have more potential substitution sites than the gene-coding regions.

The main aim of the study was to determine the molecular phylogenetic relationships among green algal endosymbionts of P. bursaria in order to explore the history of the symbiosis events. We tried to answer whether endosymbiosis of a green algae in the host P.
Plants 2021, 10, 609

*bursaria* took place prior to the diversification of the host lineage into the various syngens or if endosymbionts are incorporated over and over again. In the latter case we assess whether endosymbionts are host-specific or if there is no relationship between host syngens and endosymbiont lineage.

2. Results

2.1. Syngen Identification

Identification of *Paramecium bursaria* syngens was performed by mating the studied strain with standard strains representing all mating types of each sygen. The number of symbiotic strains of algal species identified in each of the five *P. bursaria* syngens is presented in Table 1.

Table 1. The number of symbiotic strains of particular algal species identified in five syngens of *Paramecium bursaria*.

| Endosymbiont Species       | Syngen of *Paramecium bursaria* |
|---------------------------|---------------------------------|
|                           | R1     | R2     | R3     | R4     | R5     |
| Chlorella vulgaris        | 2      | 10     | 4      | 1      | 1      |
| Chlorella variabilis      | 1      | 4      | 2      | 1      | 1      |
| Chlorella sorokiniana     | 0      | 3      | 1      | 0      | 0      |
| Micractinium conductrix   | 3      | 7      | 0      | 0      | 0      |

2.2. Geographical Distribution of Paramecium Bursaria Symbionts

*P. bursaria* syngens and their geographical distribution are shown in Figure 1 and Table 2. Syngen R1 from central Asia (Tajikistan) harbored *C. vulgaris* strain but those from Europe (Wien) contained *C. variabilis*. Endosymbiotic *Micractinium conductrix* was isolated from the syngen originating from north-eastern Europe (St. Petersburg, Tver). Syngen R2 of *P. bursaria* was collected most frequently, and 10 endosymbionts from central Asia (Altai, Lake Baikal), eastern Europe (Astrakhan), eastern Europe (Tver, Yaroslavl, Kaliningrad), and Scotland (Europe) were assigned to *C. vulgaris*. Four strains from eastern Europe (Astrakhan), Far East (Kamchatka) and from Germany (Europe) belonged to *C. variabilis*. Two strains from Kamchatka and one from central Asia (Lake Baikal) were assigned to *C. sorokiniana*. Seven strains of *Micractinium conductrix* from Asia and Europe were found in this syngen. Green endosymbionts from syngen R3 sampled in Japan and Far East (Khabarovsk) belonged to the *C. vulgaris* clade, but *C. variabilis* (Khanka Nature Reserve) and *C. sorokiniana* strains were also found in China. One strain of *C. variabilis* was isolated in Europe (Italy). Strains isolated from syngen R4 of *P. bursaria* originating from the USA were assigned to *C. vulgaris* and *C. variabilis*. Endosymbionts isolated from syngen R5 originating from eastern Europe (Astrakhan) were assigned to *C. vulgaris*, while the strain isolated from the same *P. bursaria* syngen sampled in north-eastern Europe (St. Petersburg) was *C. variabilis*.

2.3. Molecular Results

Results of the analysis of ITS1-5.8S-rDNA-ITS2, 28S rDNA and 3′rpl36-5′infA chloroplast gene fragments revealed similarity of the isolated strains to the species described as *Chlorella vulgaris*, *Chlorella variabilis*, *Chlorella sorokiniana* and *Micractinium conductrix*. Phylogenetic inference showed that these strains belonged to four distinct clades, thus the endosymbionts were polyphyletic.

2.3.1. Analysis of the ITS1-5.8S rDNA-ITS2 Fragment

Results of the analysis of the ITS1-5.8S rDNA-ITS2 fragments (543 bp) of 37 endosymbionts revealed the existence of 29 haplotypes in the studied dataset. The value of the interspecific haplotype diversity was Hd = 0.987 and the nucleotide diversity was \( \pi = 0.16040 \). Nucleotide frequencies were as follows: A = 20.5%, T = 22.6%, C = 30.1% and G = 26.8%.
Figure 1. Geographical distribution of *Paramecium bursaria* symbionts with numbers corresponding to those in Table 2.
Table 2. Strains of symbiotic algae studied in the current survey.

| No. | Algal (Endosymbiont) Species | Algal (Endosymbiont) Strain | Paramaecium bursaria (Host) Strain | Taxonomic Designation of the Host | Origin of the Host | GenBank Accession Number | References |
|-----|-----------------------------|-----------------------------|-----------------------------------|---------------------------------|-------------------|--------------------------|------------|
|     | Chlorella vulgaris           | CVG-SHT-56                  | SHT-56                            |                                | Tajikistan        | KX639563                  | KX639603   |
| 1.  | Chlorella vulgaris           | CVG-TR54-4                  | TR54-4                            |                                | Tver, Russia      | KX639564                  | KX639536   |
| 2.  | Chlorella vulgaris           | CVG-RA2-1                   | RA2-1                             |                                | Altai Forelands, Russia | KX639562  | KX639562 | This study |
| 3.  | Chlorella vulgaris           | CVG-MitR                    | MitR                              |                                | Japan             | KX639561                  | KX639563   |
| 4.  | Chlorella vulgaris           | CVG-JR-16                   | JR-16                             |                                | Japan             | KX639560                  | KX639560   |
| 5.  | Chlorella vulgaris           | CVG-HK319-12                | HK319-12                          |                                | Khabanovsk, Russia | KM203671                  | KM203663   |
| 6.  | Chlorella vulgaris           | CVG-Bya129-5                | Bya129-5                          |                                | Yaroslavl, Russia  | KM203559                  | KM203598   |
| 7.  | Chlorella vulgaris           | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203557                  | KM203596   |
| 8.  | Chlorella vulgaris           | CVG-BRR178-9                | BRR178-9                          |                                | Lake Baikal, Russia| KM203556                  | KM203595   |
| 9.  | Chlorella vulgaris           | CVG-AZ21-3                  | AZ21-3                            |                                | Astrakhan Nature Reserve, Russia | KX639555    | KX639594 | This study |
| 10. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KX639554    | KX639593 | This study |
| 11. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203670    | KM203662 |
| 12. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203673    | KM203661 |
| 13. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203674    | KM203661 |
| 14. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203675    | KM203661 |
| 15. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203676    | KM203661 |
| 16. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203677    | KM203661 |
| 17. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203678    | KM203661 |
| 18. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203679    | KM203661 |
| 19. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203680    | KM203661 |
| 20. | Chlorella vulgaris           | CVG-AZ10-1                  | AZ10-1                            |                                | Astrakhan Nature Reserve, Russia | KM203681    | KM203661 |
| 21. | Chlorella variabilis         | CVA-AZ8-2                   | AZ8-2                             |                                | Yaroslavl, Russia  | KM203544                  | KM203544   |
| 22. | Chlorella variabilis         | CVA-IP                      | IP                                |                                | Pisa, Italy       | KM203549                  | KM203549   |
| 23. | Chlorella variabilis         | CVA-AR10-3                  | AR10-3                            |                                | Ardmore, USA      | KM203667                  | KM203658   |
| 24. | Chlorella variabilis         | CVA-GB15-2                  | GB15-2                            |                                | Boston, USA       | KM203673                  | KM203661   |
| 25. | Chlorella variabilis         | CVG-ARZ1-4                  | ARZ1-4                            |                                | Botanical Garden in St. Petersburg, Russia | KM203669    | KM203659  |
| 26. | Chlorella variabilis         | CVG-ZK-126                  | ZK-126                            |                                | Kaliningrad, Russia| KM203672                  | KM203660   |
| 27. | Chlorella variabilis         | CVG-BRR15-3                 | BRR15-3                           |                                | Lake Baikal, Russia| KM203558                  | KM203597   |
| 28. | Chlorella variabilis         | CVG-BRR178-9                | BRR178-9                          |                                | Lake Baikal, Russia| KM203556                  | KM203595   |
| 29. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203555                  | KM203590   |
| 30. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203554                  | KM203585   |
| 31. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203553                  | KM203581   |
| 32. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203552                  | KM203583   |
| 33. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203551                  | KM203584   |
| 34. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203550                  | KM203585   |
| 35. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203549                  | KM203586   |
| 36. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203548                  | KM203587   |
| 37. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203547                  | KM203588   |
| 38. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203546                  | KM203589   |
| 39. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203545                  | KM203590   |
| 40. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203544                  | KM203591   |
| 41. | Chlorella variabilis         | CVG-BRR180-10               | BRR180-10                         |                                | Lake Baikal, Russia| KM203543                  | KM203592   |
| No. | Algal (Endosymbiont) Species | Algal (Endosymbiont) Strain | *Paramecium bursaria* (Host) Strain | Taxonomic Designation of the Host | Origin of the Host | GenBank Accession Number | References |
|-----|-----------------------------|-----------------------------|-------------------------------------|----------------------------------|------------------|-------------------------|------------|
| 42  | *Micractinium conductrix*   | MC-TR54-1                    | TR54-1                              | R1                               | Tver, Russia     | KX639572, KX639579      | This study |
| 43  | *Micractinium conductrix*   | MC-SKB9-1                    | SRB9-1                              | R2                               | River Danube, Serbia | KX639571, KX639578, KX639539 | This study |
| 44  | *Micractinium conductrix*   | MC-TOSI-7                    | TOSI-7                              | R2                               | Togliatti, Russia | KM203676, KM203665, nd | [33]       |
| 45  | *Micractinium inermum*      | NLP-F014                     | nd                                  | nd                               | nd               | KF597304.1, nd, nd     | Unpublished data |
| 46  | *Chlorella sorokiniana*     | UTEX 1665                    | nd                                  | nd                               | nd               | KJ676113.1, nd, nd     | [14]       |
| 47  | *Micractinium sp.*          | KNUA029                      | nd                                  | nd                               | nd               | KM243321.1, nd, nd     | [25]       |
| 48  | *Micractinium reisseri*     | (endosymbiont)               | nd                                  | nd                               | nd               | nd                      | nd         |
| 49  | *Micractinium sp.*          | MCVWW15                      | nd                                  | nd                               | nd               | nd                      | nd         |
| 50  | *Micractinium sp.*          | MCVWW4                       | nd                                  | nd                               | nd               | nd                      | nd         |
| 51  | *Micractinium sp.*          | MCVWW5                       | nd                                  | nd                               | nd               | nd                      | nd         |
| 52  | *Micractinium sp.*          | MCVWW10                      | nd                                  | nd                               | nd               | nd                      | nd         |
| 53  | *Micractinium sp.*          | KNUA032                      | nd                                  | nd                               | nd               | nd                      | nd         |
| 54  | *Micractinium reisseri*     | (endosymbiont)               | nd                                  | nd                               | nd               | nd                      | nd         |
| 55  | *Micractinium reisseri*     | EdL_CII_MAF                  | nd                                  | nd                               | nd               | nd                      | nd         |
| 56  | *Chlorella sp.*             | SAG 13.81                    | nd                                  | nd                               | nd               | nd                      | nd         |
| 57  | *Chlorella sp.*             | IFRPD                        | nd                                  | nd                               | nd               | nd                      | [1]        |
| 58  | *Chlorella sorokiniana*     | KLL-2018                     | nd                                  | nd                               | nd               | nd                      | nd         |
| 59  | *Chlorella sorokiniana*     | KL219                        | nd                                  | nd                               | nd               | nd                      | nd         |
| 60  | *Chlorella variabilis*      | CCAP 211/84                  | nd                                  | nd                               | nd               | nd                      | nd         |
| 61  | *Chlorella variabilis*      | SAG 211-6                    | nd                                  | nd                               | nd               | nd                      | nd         |
| 62  | *Chlorella variabilis*      | EdL_CII_3NB                  | nd                                  | nd                               | nd               | nd                      | nd         |
| 63  | *Chlorella vulgaris*        | DRL3                         | nd                                  | nd                               | nd               | nd                      | nd         |
| 64  | *Coscompha chodatii*        | SAG 216-2                    | nd                                  | nd                               | nd               | nd                      | nd         |
| 65  | *Stigeoclonium tenue*       | CCAP 477/11A                 | nd                                  | nd                               | nd               | nd                      | nd         |
| 66  | *Stigeoclonium variabile*   | CCAP 477/13                  | nd                                  | nd                               | nd               | nd                      | nd         |
| 67  | *Parachlorella koscheri*    | SAG 211-11g                  | nd                                  | nd                               | nd               | nd                      | nd         |
| 68  | *Actinastrum hantzschii*    | SAG 2015                     | nd                                  | nd                               | nd               | nd                      | nd         |
The haplotype network of the ITS1-5.8S rDNA-ITS2 fragment was constructed for the inference and visualization of genetic relationships between green endosymbionts of *P. bursaria* (Figure 2). Four haplogroups were identified for the rDNA fragment in the studied strains, i.e., *C. vulgaris*, *C. variabilis*, *C. sorokiniana* and *M. conductrix*. The clade of *C. vulgaris* was composed of 12 haplotypes; one of them comprised two strains isolated from *P. bursaria* syngen R2: CVG-BBR-180-10 and CVG-BL15-3 sampled from the Baikal Lake (central Asia). The clade of *C. variabilis* included six haplotypes. Three strains: CCAP 211/84, SAG 211-6 and Edl_CI2_3NB from GenBank formed a common haplotype. The remaining strains represented single haplotypes.

The clade of *C. sorokiniana* was composed of two unique haplotypes. The first one consisted of two *Chlorella* sp. strains, CB4 and IFRPD, and the second one of *Chlorella sorokiniana* KLL-G018 and KU219 from GenBank.

The following clade, *Micractinium*, included nine haplotypes and seven of them represented unique haplotypes; two of them were composed of two strains: *Micractinium* sp., MCWW5 and MCWW10 from GenBank, and the second haplotype: *Micractinium reisseri* EDL_CI1_MAF from GenBank and SW1-ZK1 from Germany. There were 88 to 112 differences between *C. variabilis* and *C. sorokiniana*, 81 to 128 between *C. vulgaris* and *C. variabilis*, 72 to 100 between *C. variabilis* and *Micractinium*, 149 to 192 between *Micractinium* and *C. vulgaris* and 168 to 204 differences between *C. vulgaris* and *C. sorokiniana*. Intraspecific variation among haplotypes was the result of several substitutions (Table 2, Figure 2).
2.3.2. Analysis of the 28S rDNA Fragment

Results of the analysis of 28S rDNA fragments (555 bp) of 43 symbionts isolated from different *P. bursaria* strains showed the presence of 29 haplotypes. The value of the interspecific haplotype diversity was $H_d = 0.908$ and the nucleotide diversity was $\pi = 0.03165$. Nucleotide frequencies were as follows: $A = 26.7\%$, $T = 18.7\%$, $C = 23.8\%$ and $G = 30.8\%$.

The haplotype network of the 28S rDNA fragment grouped the strains into four clades: *C. vulgaris*, *C. variabilis*, *C. sorokiniana* and *Micractinium*. The clade of *C. variabilis* was composed of 10 unique haplotypes with 2 to 9 substitutions between them (Figure 3).

![Haplotype network constructed for 43 symbiotic algae of *P. bursaria* strains based on sequence comparison of the 28S rDNA gene fragment, (a) with strain abbreviations, (b) geographical origin of *P. bursaria* strains and syngens. The size of the dots is proportional to haplotype frequency. Median vectors that represent hypothetical intermediates or un-sampled haplotypes are shown as black dots. Hatch marks on individual branches represent nucleotide substitutions between individual haplotypes (corresponding number was assigned for more than 10). Haplotypes marked as “no locality data” were acquired from GenBank.](image-url)
The *C. vulgaris* clade consisted of five unique haplotypes. One of them included 14 strains: CVG-Bya129-5 (Yaroslavl) and CVG-TR54-4 (Tver) from eastern Europe, CVG-SHT56 (Tajikistan) from central Asia, CVG-RA2-1 (Altai) and CVG-BBR178-9, CVG-BBR180-10 (Baikal Lake) from central Asia, CVG-AZ10-1, CVG-AZ20-1, CVG-AZ21-3, (Astrakhan) from eastern Europe, CVG-HKV19-12 (Khabarovsk) from the Far East, CVG-JR-16, CVG-MitR and CVG-Yad1-g from Japan, CVG-AB2-51 (Boston) from USA. The second haplotype was composed of two strains: CVG-AZ7-14 (Astrakhan) from eastern Europe and CVG-Ard7 (Ardmore) from USA. The other haplotypes were represented by the following single strains: CVG-Bl15-3, CVG-KZ-126 and CVG-Gb15-2. The *C. variabilis* clade was composed of 10 single strains.

The *Micractinium* clade was composed of 10 haplotypes. One of them included four strains from Europe: MC-PMP1-3-1, (St. Petersburg, north-eastern Europe), MC-SRB9-1 (Serbia, southern Europe), MC-TOS1-7 (Togliattii, south-eastern Europe) and SW1-ZK (Germany, western Europe). The other nine corresponded to single strains: MC-4231-1, MC-Vm-14, MC-Rn88-4, MC-Ms-1, MC-Gb7-2, MC-1142-2, MC-TR54-1, NLP-F014 and KNUA029.

The last clade consisted of *C. sorokiniana* representatives, and included four haplotypes. One haplotype was formed by two strains from the Far East origin: CS-11231-2 and CS-1135-2 (Kamchatka) and the other two represented single strains: CS-Bbr51-1 and CS-Cs-2.

Interspecific variability was higher when *C. vulgaris* to *Micractinium* or *C. variabilis* to *Micractinium* were compared (28–58 differences). There was a low number of substitutions between *C. vulgaris* and *C. variabilis* (1–20 differences) (Table 2, Figure 3).

2.3.3. Analysis of the rpl36-infA Genes Fragment

Results of the rpl36-infA gene fragment (267 bp) analysis in symbionts isolated from 43 *P. bursaria* strains showed the presence of 36 haplotypes. The value of the interspecific haplotype diversity was $H_d = 0.984$, and the nucleotide diversity was $\pi = 0.07886$. Nucleotide frequencies were as follows: $A = 29.6\%$, $T = 36.0\%$, $C = 18.5\%$ and $G = 15.9\%$.

The haplotype network of chloroplast gene fragments grouped the strains into four clades: *C. vulgaris*, *C. variabilis*, *C. sorokiniana* and *M. conductrix* (Figure 4). The *C. vulgaris* clade included 17 haplotypes; one haplotype was represented by three strains. Two strains from Europe: CVG-GB15-2 (Scotland), CVG-KZ-126 (Kaliningrad) isolated from *P. bursaria* syngen R2, and one strain from central Asia: CVG-SHT-56 (Tajikistan) from syngen R1. The remaining haplotypes consisted of single strains.

The clade of *C. variabilis* consisted of nine haplotypes and eight of them included single strains. Strain CVA-B5-7 (St. Petersburg, north-eastern Europe) from syngen R5 and strain CVA-AZ20-4 (Astrakhan, eastern Europe) from syngen R2 belonged to the ninth haplotype.

The *C. sorokiniana* clade was composed of four unique haplotypes corresponding to single strains.

The *M. conductrix* clade included six haplotypes, five of them represented single strains and one haplotype was composed of the five following strains: MC-PMP1-3-1 and MC-Ms-1 (St. Petersburg, north-eastern Europe), isolated from syngen R1, MC-SRB9-1 (Serbia, southern Europe), MC-TOS1-7 (Togliattii, south-eastern Europe), and MC-Vm-14 (Valaam, northern Europe) isolated from syngen R2.

There were 18 to 43 substitutions between *C. vulgaris* and *C. variabilis*, 19 to 49 substitutions between *C. vulgaris* and *C. sorokiniana*, 41 to 51 between *C. sorokiniana* and *M. conductrix*, and 35 to 57 substitutions between *M. conductrix* and *C. variabilis* (Table 2, Figure 4).
3. Discussion

*Paramecium bursaria* is an archetypical outbreeder, which presumably means that its effective population size is large. *P. bursaria* is divided into five syngens which are characterized by a specific geographical distribution. Nyberg [22] concluded that *P. bursaria* syngens, as extreme outbreeders, should be globally distributed, but Bomford [2] and Greczek-Stachura et al. [4] postulated that most sibling species were restricted to certain geographical regions, and thus adapted to specific conditions. Based on the comparison of syngens from Bomford’s collection and new syngen annotations, it is known that syngens R3 and R4 have been found in the United States [23], and syngen R3 has been reported later in China [24]. According to the study by Hoshina et al. [25], *P. bursaria* strains from Japan were also classified as syngen R3. Two syngens, R1 and R2, are only of Eurasian origin, and have been recorded at various locations from Great Britain to central Siberia; in addition, two strains of syngen 2 have been found in one locality in Australia. Syngen R3 strains have been isolated in far-eastern Russia and south-eastern Siberia (but never western Siberia), China, Japan, and the USA. Recently, this syngen has been reported in Europe, namely in Austria and in Italy (although the strain from Pisa was collected in a botanical garden, where it could have been brought along with some tropical plants). Syngen 4 strains are restricted to the USA. Strains belonging to syngen 5 have been found in the Volga delta, known for its great migration routes of waterfowl that are suspected transmitters of paramecia [4,26]. The current investigation of different syngens of *P. bursaria* collected in Europe, Asia and North America confirmed the previous knowledge about
their biogeography. *P. bursaria* syngen R1 has been found in central Asia and north-eastern Europe. Strains of syngen R2 have been found in Asia and Europe. Syngen R3 was sampled in Japan, Far East and China. Strains of syngen R4 originate from the USA and syngen R5 strains are derived from eastern Europe and north-eastern Europe (Figure 1, Table 2).

The existence of syngens is the result of the process of speciation. The key question regarding evolution is: what are the driving forces behind initial speciation of *Paramecium bursaria*? Geographic isolation is often the main speciation factor, but its significance in protists is uncertain as there is still disagreement over their distribution—whether it is cosmopolitan or endemic.

If *P. bursaria* syngens are hosting the same species of endosymbiotic algae, they can be sympatric or other speciation mechanisms may play a leading role. Therefore, in our opinion, identification of species of endosymbiotic algae can explain a possible process of co-evolution. In the present study, we have identified four species of endosymbiotic algae, i.e., *C. vulgaris*, *C. variabilis*, *C. sorokiniana* and *M. conductrix*. Spanner et al. [27], based on ITS-2 sequencing, identified *Chlorella variabilis* and *Micractinium conductrix* in *Paramecium bursaria* cells. The two above endosymbionts have been identified in strains belonging to syngens R1 and R2 of *P. bursaria*, which originated from Europe. Moreover, we have found *C. vulgaris* and *C. variabilis* in all five syngens of *P. bursaria*, *M. conductrix* was present in syngen R1 and R2, and *C. sorokiniana* in syngen R2 and R3 (Table 1). Gaponova et al. [6] have also found *M. conductrix* in *P. bursaria* isolates collected in North Karelia (Russia). Overall, it seems that *M. conductrix* occurs only in Europe, whereas *C. variabilis* is distributed worldwide. Hoshina et al. [5,10] established the geographical distribution of *Micractinium* sp. in the regions of England, Germany, Austria and northern Karelia, which was consistent with the results obtained by Luo et al. [17,28]. Strains belonging to the American group derived from USA, Japan, China and southern Australia carried symbiotic algae classified as *Chlorella vulgaris* and *Chlorella variabilis* [7]. Hoshina and Imamura [9] identified the strains from Kaliningrad as *C. vulgaris*, similar to our findings i.e., the strain isolated from syngen R2. Pröschold et al. [7] have suggested that *C. variabilis* is characteristic of the American but not the European group; however, according to our results, the strains from St. Petersburg and Valaam as well as strains from central Europe (Pisa, River Danube in Serbia) have been assigned to *C. variabilis* and *M. conductrix*.

Our findings suggest that there is no correlation between *P. bursaria* syngen and the species of symbiont, as was previously argued by Weis [29]. Similarly, Reisser et al. [30] stated that *P. bursaria* strains of American or European origin formed a stable symbiosis with symbionts of both groups. Then, Meier and Wiessner [31] demonstrated that *P. bursaria* could eliminate symbionts and subsequently be reinfected by new symbionts. Summerer et al. [32] mixed two aposymbiotic *P. bursaria* strains with symbiotic and free-living *Chlorella* strains. Symbioses were formed with endosymbiotic *Chlorella*, with the exception of those from *H. viridis* and free-living algae. Similarly, in the current survey we demonstrated that there is no strong relationship between species of symbionts and the geographical distribution of their host, *P. bursaria*. This may be explained by the ancestral aposymbiotic ciliate *P. bursaria* possibly having acquired different species of green algae and later diverging into a lineage with a host-algal partnership where accidental algal change may have occurred. Summerer et al. [33] analyzed nuclear 18S rDNA, the ITS1 region and chloroplast 16S rDNA from algal symbionts of *P. bursaria* strains originating from two lakes in Austria. These strains formed a clade with two distinct lineages, suggesting the existence of a biogeographic pattern. Genetic differences between symbiotic algae are 10 times higher than between free-living algae. This suggests that multiple symbiotic origins are more likely than the divergence of one symbiotic species to different symbiotic algae existing currently [25]. The endosymbiotic lifestyle has evolved many times in green algae, as evidenced by the presence of numerous haplotypes of endosymbiotic algae in the haplotype network based on the nuclear ITS1-5.8S rDNA-ITS2 fragment, 28S rDNA fragment and 3′rpl36-5′infA gene sequences. Endosymbionts of the Chlorellaceae species, which also
serve as specific hosts for large dsDNA viruses known as chloroviruses, do not cluster together, providing strong evidence for independent transitions to endosymbiosis [34].

Therefore, we suppose that the speciation of \textit{P. bursaria} syngens was an earlier evolutionarily event than the establishment of symbiosis, as evidenced by the diversity of symbionts and their lack of specificity.

4. Materials and Methods

4.1. Strain Cultivation and Strain Crosses

\textit{Paramecium bursaria} strains were cultivated on a lettuce medium according to Sonneborn [35], fed \textit{Klebsiella pneumoniae} (SMC) and stored at 18 °C (12L/12D). We investigated 43 symbiotic strains isolated from \textit{P. bursaria} cells derived from different geographical locations. We also analyzed 20 sequences of symbiotic algae available in GenBank and strains of \textit{Coccomyxa chodatii}, \textit{Stigeoclonium tenue}, \textit{Stigeoclonium variabile}, \textit{Parachlorella kessleri} and \textit{Actinastrum hantzschii} as outgroups (Table 2).

Identification of \textit{P. bursaria} syngens was performed by mating reaction of a studied strain with standard strains representing all mating types of each syngen. The studied strains were assigned to a certain syngen based on the occurrence of strong clumping at the beginning of the mating reaction, the presence of mating couples and survival of F\textsubscript{1} progeny.

4.2. Molecular Methods

Symbiotic DNA was extracted using the GeneJET Plant Genomic DNA Purification Kit (ThermoScientific) according to the protocol. Dense \textit{P. bursaria} culture (1.5 mL) was harvested from a liquid culture by centrifugation. Then, the pellet was sonicated on ice for 10 s at 40 W. Subsequently, the standard extraction protocol was followed. The ITS1-5.8S rDNA-ITS2 fragment was amplified using the following primers pairs: ITS1 [32]/ITS2R (primer designed for the present study, Table 3) and ITS1F/ITS2R (primers designed for the present study, Table 3) according to the protocol with the following parameters: initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 2 min, extension at 72 °C for 3 min and a final extension at 72 °C for 5 min.

Table 3. Primers used in the present study.

| DNA Fragment | Primer | Sequence 5′-3′ | References |
|--------------|--------|---------------|------------|
| ITS1-5.8S rDNA-ITS2 | ITS1 | TCCGTAGGTTGAACCTGCGG | [33] |
| | ITS1F | AATCTATCGAATCCACTTTGGTAAC | Designed in the present study |
| | ITS2R | CTGCTAGGTCTCCAGCAAAG | Designed in the present study |
| 28S rDNA fragment | HLR0F | GGCAAGACTACCGCTGAA | [8] |
| | HLR4R | TTCAGACGGGCGCAGT | [8] |
| 3′rpl36-5′infA genes | UCP2F | CTTTGWCKTTGTTATGTTTGG | [36] |
| | UCP2R | GCTCATGTYTCHGGBAAATWCG | [36] |

The fragment of a 28S rDNA was amplified by polymerase chain reaction (PCR) using the HLR0F/HLR4R primer pair [8,37] (Table 3), according to the protocol described by Hoshina et al. [38]. The fragment of 3′rpl36-5′infA genes was amplified using the UCP2F and UCP2R primer set (Table 3), according to Provan et al. [36]. After amplification, PCR products were separated by electrophoresis in 1% agarose gel for 1 h at 95 V and then gel-purified using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). Sequencing reaction was performed in both directions using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, USA). Sequencing products were precipitated using Ex Terminator (A&A Biotechnology, Gdynia, Poland).
4.3. Data Analyzes

Sequences were examined and corrected using Chromas Lite (Technylesium), and aligned using BioEdit [39]. The analysis of haplotype diversity (Hd) and nucleotide diversity (π) was carried out using DnaSP v5.10.01 [39]. The analysis of nucleotide frequencies and identification of the best nucleotide substitution models for maximum likelihood tree reconstruction (T92 + G for three loci) were conducted using Mega v5.1. Haplotype networks were constructed using the Median Joining method implemented in the Network 4.6.1.3 software [40,41].

5. Conclusions

The ITS1-5.8S rDNA-ITS2 fragment is the most appropriate molecular marker to identify and resolve evolutionary relationship between symbionts of *Paramecium bursaria*. We assigned symbiotic algae of *P. bursaria* to four species: *Chlorella vulgaris*, *Chlorella variabilis*, *Chlorella sorokiniana* and *Micractinium conductrix*. The division of *P. bursaria* endosymbionts into the American and European groups and the correlation between *P. bursaria* syngen and a symbiotic species has not been confirmed. No strong relationships have been found between symbiotic species and geographical distribution of their host *P. bursaria*.

Molecular markers: ITS1-5.8S rDNA-ITS2, 28S rDNA fragments and 3′*rpl36*-5′*infA* gene fragments are useful molecular tools for distinguishing closely related taxa of *P. bursaria* symbionts. The ITS1-5.8S rDNA-ITS2 fragment is the most appropriate due to its high interspecific and low intraspecific variability. Additionally, the application of two independent genome fragments (nuclear and chloroplast) increases the reliability of the results.

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