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Unravelling the Evolutionary Patterns of Genus *Frontonia*: An Integrative Approach with Morphological and Molecular Data

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Simple Summary: Ciliates, particularly the genus *Frontonia*, have been studied to understand their evolutionary history, but challenges remain. This study investigated the evolutionary patterns of *Frontonia* using genetic and morphological data. Molecular analysis of the SSU rRNA gene revealed four major phylogenetic groups within *Frontonia*, suggesting its paraphyly. The common ancestor existed approximately 420 million years ago, with distinct groups emerging during the Mesozoic era. Diversification analysis showed higher extinction rates than speciation rates within the genus. Morphological traits, including habitat adaptations, were examined through ancestral state reconstructions, revealing a complex evolutionary history. Habitat transitions were not directly linked to morphological traits such as contractile vacuoles, emphasizing the role of genetic diversity and environmental adaptation. These findings provide valuable insights into the interplay between evolution, extinction, and morphology in ciliates, advancing our understanding of biodiversity and evolutionary biology.

Abstract: Ciliates of the genus *Frontonia* have been extensively studied to resolve their phylogenetic and evolutionary history, but challenges remain. This study used molecular analyses of SSU rRNA genes, phylogenetic tree reconstruction, molecular dating, and diversification analysis, together with ancestral state reconstruction of morphological traits and habitat preferences. Data included newly sequenced Korean species, GenBank records and published morphological information. Phylogenetic trees revealed paraphyly within *Frontonia*, identifying four groups that emerged in the Mesozoic era: Group I (~172 mya), Group II (~83 mya), Group III (~115 mya), and Group IV (~190 mya), with a common ancestor dating to ~420 mya in the Palaeozoic era. Diversification analysis revealed higher extinction rates (0.826 and 0.613 species/year) than speciation rates (0.011 and 0.016 species/year). Morphological evolution showed habitat adaptation and plasticity, with habitat transitions unrelated to contractile vacuolar traits. The SSU rRNA gene polymorphism likely contributed to the paraphyletic state of *Frontonia*. These results highlight the complex evolutionary patterns of the genus, shaped by genetic diversity, morphology, and environmental constrains.

Keywords: *Frontonia*; evolutionary history; morphological evolution; diversification analysis; SSU rRNA gene; molecular dating; ancestral state reconstruction

1. Introduction

The research on ciliates, with a particular focus on the genus *Frontonia,* has indeed advanced significantly due to the integration of molecular and morphological studies. The genus *Frontonia,* established nearly two centuries ago, represents a diverse group of ciliate species [1–7]. While various research efforts have contributed to the understanding of their morphological traits, the phylogenetic relationships and evolutionary history of many ciliate lineages, including *Frontonia,* remain unresolved [8]. Molecular analyses, particularly of the SSU rRNA gene, have provided crucial insights into the phylogenetic relationships within *Frontonia.* These analyses have revealed the paraphyletic nature of the genus, with several distinct clades identified. Furthermore, certain species consistently cluster with different genera in the SSU rRNA gene phylogenetic trees, suggesting a complex evolutionary history [8–12].

However, the molecular analysis of the SSU rRNA gene has been the primary tool used to construct phylogenetic trees within *Frontonia,* with limited integration of morphological data beyond species descriptions. To address this gap, a multifaceted approach is proposed in the current study. This approach involves the integration of molecular data, in particular the SSU rRNA gene, for molecular clock analysis. At the same time, morphological data will be used to reconstruct ancestral states, allowing for a more comprehensive understanding of the phylogenetic relationships and evolutionary history of *Frontonia*. By combining molecular and morphological data, the study aims to elucidate the evolutionary patterns, phylogenetic relationships, and divergence time of the genus *Frontonia*. These integrated approaches will provide deeper insights into the diversification patterns and evolutionary processes of the lineages in *Frontonia.*

2. Materials and Methods

Sample Collection and Morphological Study

Newly sequenced species of *Frontonia* were collected from different locations in South Korea. Detailed information on the collection sites can be found in Table 1. The *Frontonia* species were cultivated in petri dishes using water collected from their respective habitats. Initial in vivo cell observations were made using both bright field and differential interference contrast microscopy techniques. Subsequent detailed examinations were performed using a compound microscope, with magnifications ranging from 100x to 1000x for both live cells and stained samples. The protargol-impregnated method was employed to visualize the structure of the buccal area, the oral apparatus and the ciliary pattern.

Table 1. *Frontonia* species from Korea and their accession number for SSU rRNA gene sequence.

Taxon	**Collection site**	**accession no.**
Frontonia cf. *acuminata* pop1	Gwangju, Korea	PV016905
Frontonia cf. *acuminata* pop2	Ulsan, Korea	PV016906
Frontonia cf. *acuminata* pop3	Gunsan, Korea	PV016907
Frontonia cf. *acuminata* pop4	Ulsan, Korea	PV016908
Frontonia cf. *atra* pop1	Daejon, Korea	PV016909
Frontonia cf. *atra* pop2	Gunsan, Korea	PV016910
Frontonia cf. *atra* pop3	Masan, Korea	PV016911
Frontonia cf. *atra* pop4	Chungcheongbukdo, Korea	PV016912
Frontonia cf. *atra* pop5	Gyeongsangnamdo, Korea	PV016913
Frontonia cf. *atra* pop6	Ulsan, Korea	PV016914
Frontonia cf. *atra* pop7	Gyeongju, Korea	PV016917
Frontonia cf. *leucas*	Gwangju, Korea	PV016904

Frontonia sp. 1	Ulsan, Korea	PV016915
Frontonia sp. 2	Ulsan, Korea	PV016916
Frontonia sp. 3	Gyeongju, Korea	PV016918

DNA Extraction, Amplification, and Sequencing

The genomic DNA extraction was carried out using the RED Extract-N-Amp Tissue PCR Kit from Sigma (St. Louis, MO, USA) according to the manufacturer's instructions. The polymerase chain reaction (PCR) was performed using the forward primer EUKA (5'- GAC CGT CCT AGT TGG TC-3') [13] or 82F (5'-CTC GGT AAG CGT CAA AG-3') [14] and the reverse primers D1, D2 rev2 (5'-GAC TGC ACG TTT AGC TAG CA-3') or D1, D2 rev4 (5'- GTG CCT GGT TCY TCA GAT TG -3'). PCR amplifications were performed using the TaKaRa ExTaq DNA polymerase kit from TaKaRa Bio-medicals (Otsu, Japan) following a specific protocol: an initial denaturation cycle at 94°C for 2 minutes, followed by 37 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 40 seconds, and extension at 72°C for 4 minutes. A final extension step was then carried out at 72°C for 10 minutes [15].

Alignment and Phylogenetic Analysis

In this study, 71 sequences from the species of the genus *Frontonia* obtained from NCBI and newly sequenced in Korea (*Table A1*), 20 sequences from related genera (*Paranassula, Paramecium, Apofrontonia, Stokesia, Disematosoma, Marituja, Lembadion, Urocentrum,* and *Tetrahymena*) were selected as reference sequences and two species from the genus *Tetrahymena* were selected as outgroup (*T. pyriformis* & *T. rostrata*) (Figure 1*)*. The alignment for this data set was performed by using MAFFT version 7.0 [16]. Subsequent refinement and masking of the alignments were carried out using G-blocks version 0.91b (Castresana, 2000). To determine the best evolutionary models, jModeltest version 2.1.7 [18,19] was employed. Further analysis included maximum likelihood (ML) using PhyML 3.0 software with 1000 non-parametric bootstrap replicates [20]. In addition, Bayesian Inference (BI) analysis was performed using MrBayes version 3.1 [21].

Molecular Dating and Diversification Analysis

The analysis used 33 sequences from representative species of the genus *Frontonia*, selected on the basis of the availability of both molecular and morphological information (*Suppl. Table 1*). In addition, 18 sequences from species of related genera were included in the analysis. Divergence times were estimated using a Bayesian framework that implemented in BEAST ver. 2.4.5 [22,23]. The software BEAUti ver.2.4.5 [23] was used to generate the BEAST software input XML file with the following settings; (i) calibrated Yule model, (ii) GTR+I(=0.389)+Γ(=0.412), (iii) four gamma categories for substitution rate heterogeneity, (iv) strict molecular clock, (v) clock rate prior assuming a normal distribution with a mean of 3.88×10^{-4} nucleotide substitutions per site per one million years and a 95% credibility interval ranging from 1.24×10^{-4} to 9.14×10^{-4} (Rataj and Vďačný, 2018), and (vi) Yule birth rate set with shape parameter set to 0.001 and scale parameter set to 1000 (gamma shape parameter).

For the estimation of the divergence time, calibration nodes from the genera *Paramecium* and *Tetrahymena* were used. These nodes were based on microfossil evidence from *Paramecium triassicum* and *Tetrahymena rostrata*-like species found on an amber Triassic slab during the Upper Triassic period, approximately 220-230 million years ago (Mya) [24]. The mean divergence time for these calibration nodes was estimated to be 225 Mya. Markov Chain Monte Carlo (MCMC) analyses started with a random seed and ran for 600,0000 generations, with trees and all other parameters were saved every 10,000th iteration. The quality of the MCMC analysis was assessed using Tracer version 1.6 [25] to ensure convergence and adequate burn-in. The final maximum credibility tree was generated using TreeAnnotator ver. 1.8.1[22] after discarding the first 10% of sampled trees.

The analysis of speciation rate versus extinction rate in the genus *Frontonia* was performed using two software programs: Divbayes and SubT [26]. Divbayes used data containing information on the time of ancestral divergence and the corresponding number of species. On the other hand, SubT used 95% height data for each branch obtained from a chronogram generated by the BEAST software, together with species number data used in the analysis, including predicted data for species numbers where actual data were not available (*Table A2*). These analyses were conducted to explore patterns and dynamics of species diversification through time.

Reconstruction of Ancestral Morphologies

For the prediction of ancestral states, 33 species of the genus *Frontonia* and *Apofrontonia dohrni* were selected as representative taxa. The selection was based on the availability and credibility of their molecular and morphological data, as shown in Supplementary Table 2. To perform the ancestral state reconstruction, 12 important and significant characters were selected within the genus *Frontonia.* These characters included the number of ciliary rows in peroral membranes (PM), the number vestibular kineties (VK), the number of postoral kineties (PK), the number of ciliary rows in peniculi 1-3, the structure of P 3 (shortened and linearity with P1 and P2), the habitat, the number of contractile vacuoles (CV), the number of contractile vacuolar pores (CVP) and the presence of contractile canals (CC). The character matrix and ancestral state reconstruction were performed using the parsimony model in Mesquite software ver. 3.70 [27]. The reconstruction of ancestral states was based on the topology of the best likelihood tree obtained from the RAxML analysis performed on CIPRES [28].

3. Results

3.1. Phylogenetic Analyses

The phylogenetic analyses based on SSU rRNA gene sequences have provided insights into the evolutionary relationships within the genus *Frontonia,* revealing the existence of four main groups: Group I: This group includes species such as *F. canadensis, F. subtropica, F. salmastra, F. sinica, F. magna, F. mengi, F. tchibisovae,* and *F. lynni.* Both Bayesian Inference (BI) and Maximum Likelihood (ML) analyses strongly support this group, with node support values of 1.00/100%. Group II: Comprising species such as *F. paramagna, F. leucas, F. vesiculosa, F. vernalis, F. shii, F. paravernalis, F. leucas, F. angusta,* and *F.* cf. *leucas,* Group II also shows robust node support values of 1.00/100% for both BI and ML. Group III: This group includes species such as *F. didieri, F. ocularis, F. elegans, F. pusilla,* and *F. anatolica,* with slightly lower node support values of 0.98/85% for BI and ML compared to Groups I and II. Group IV: Including species such as *F. terricola, F.* cf. *acuminata, F. acuminata, F. atra, F. apoacuminata, F. minuta,* and *F.* cf. *atra,* Group IV shows moderate node support (BI/ML, 1.00/88%) (Figure 1).

Furthermore, Group III shows a close relationship with the genus *Apofrontonia*, albeit with lower node support (BI/ML, 0.81/60%). Group IV forms a cluster with the genera *Stokesia, Marituja,* and *Disematostom*a, with strong node support (BI/ML, 1.00/100%). These phylogenetic relationships shed light on the evolutionary dynamics and diversification patterns within the genus *Frontonia* highlighting both strong and moderate support for the identified groups and their relationships with related taxa (Figure 1).

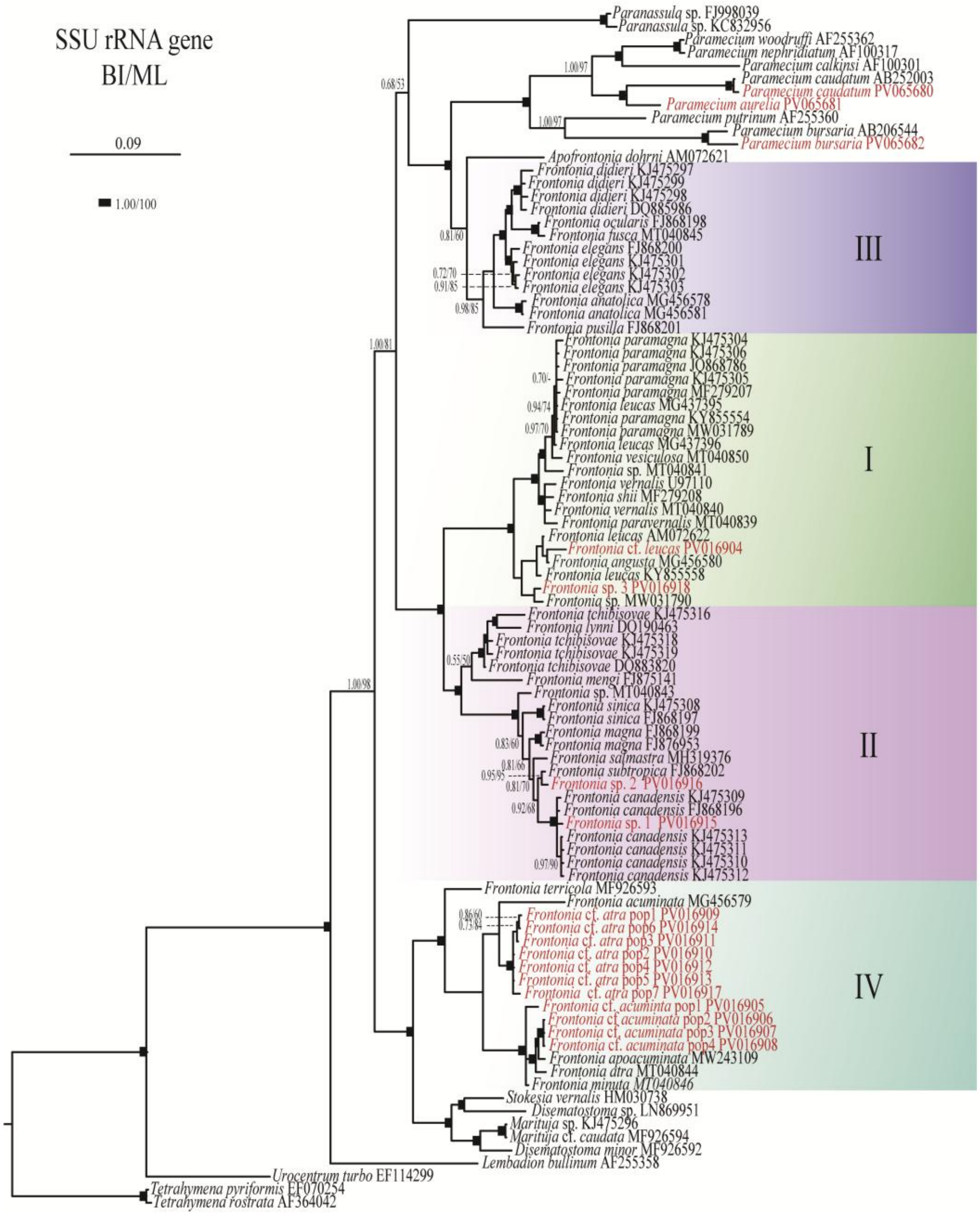

Figure 1. Phylogenetic tree generated from Bayesian Inference (BI) using Mr Bayes ver. 3.1 software and Maximum Likelihood (ML) from PHYML ver. 3.0 based on the SSU rRNA (top) and SSU-ITS-LSU rRNA (bottom) gene sequences of the genus *Frontonia* and related genera. The scale bar indicates the number of base changes per 1000 nucleotide positions in BI analysis. Node support is represented as follows: BI posterior probability/ ML bootstrap. The newly sequenced species of the genus *Frontonia* are shown in red.

3.2. Estimation of Divergence Times and Diversification Using the SSU rRNA Gene

The divergence time within the genus *Frontonia* can be estimated using two methods: relaxed clock and strict clock. Relaxed clock is commonly used at higher taxonomic levels such as family or order, while strict clock is favored for intraspecific level analyses where low rates of variation between branches are expected [29–32]. In the case of the genus *Frontonia*, the strict clock approach is more suitable for estimating divergence time due to the limited data set within the genus level, and the minimal variation between branches.

The analyses of divergence time in the genus *Frontonia* performed in this study show that Peniculia is estimated to have originated approximately 750 million years ago (Mya), confirming the previous findings of Rataj and Vďačný (2018) (Figure 2). Using the strict clock approach, the SSU rRNA gene showed a mean clock rate of 2.36 x 10e-4 per year. Node calibrations for the *Tetrahymena* clade and *Paramecium* clades indicate their emergence at 223 Mya and 226 Mya, respectively. The common ancestor of the genus *Frontonia* appeared around 420 Mya and serves as an ancestral point for all members of the Penicullida. Notably, the emergence of three clades within the genus *Frontonia* occurred relatively recently: Group I around 172 Mya, Group II around 83 Mya, Group III around 115 Mya, and Group IV around 190 Mya (Figure 2).

The results obtained from Divbayes and SubT for speciation and extinction indicate a higher extinction rate compared to the speciation rate with genus *Frontonia*: 0.826 species/year over speciation rate: 0.011 species/year (DivBayes 1.1) and extinction rate: 0.613 species/year over speciation rate: 0.016 species/year (SubT1.1).

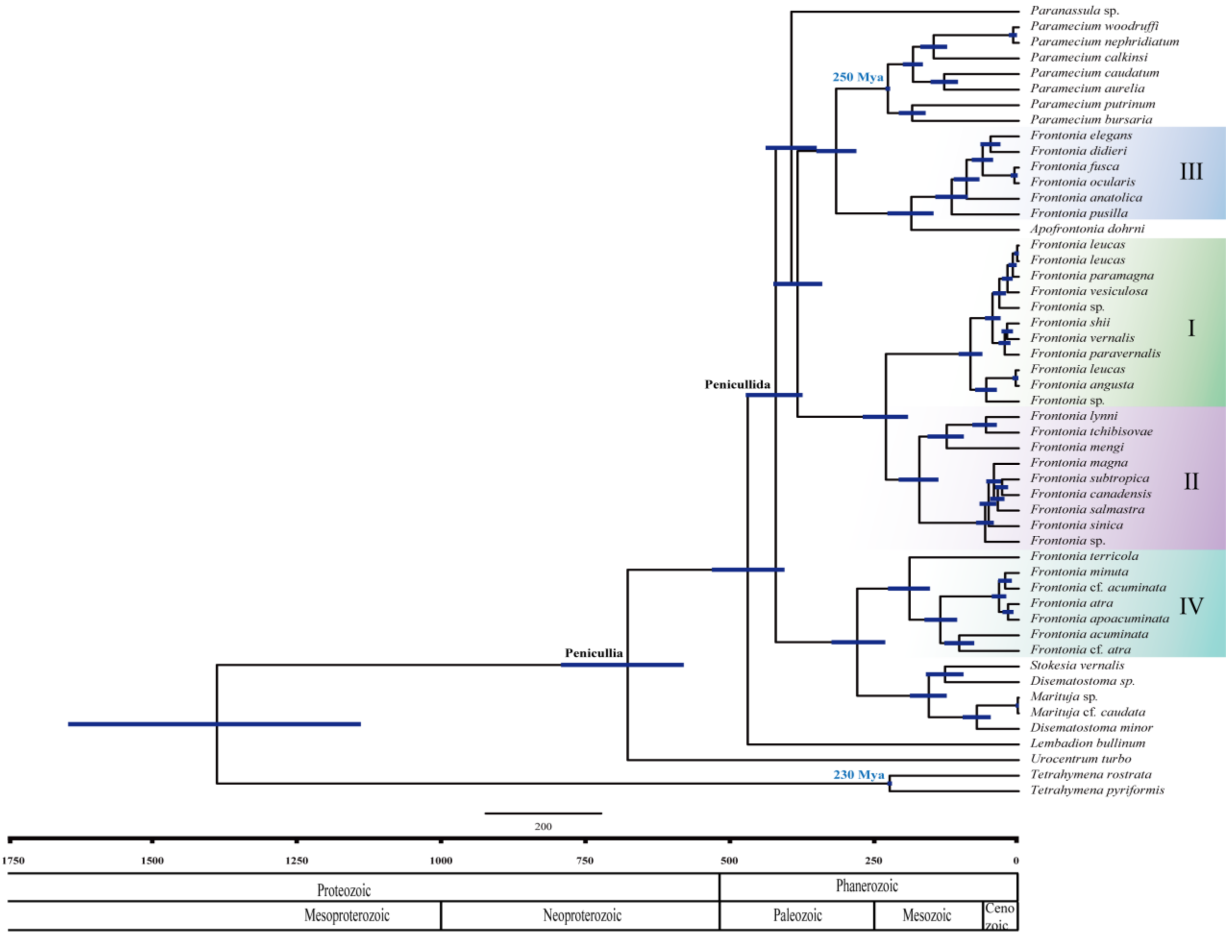

Figure 2. Maximum credibility tree showing posterior means of divergence times of the genus *Frontonia* and related genera obtained using Bayesian relaxed molecular dating in BEAST. The 95% credibility intervals are shown as bars for all nodes. The horizontal axis represents the time scale in millions of years.

3.3. Reconstruction of Ancestral Character State

The analysis of ancestral states in the genus *Frontonia* reveals intriguing evolutionary patterns across several morphological characters;

- **Vestibular kineties (VK)**: Group III retains the ancestral state of three rows of VK, whereas Group I and II show increases to four and five rows, respectively. Notably, *F. canadensis* shows transitions from three to five and back to three rows, consistent with the ancestral state of the genus *Frontonia*. Group IV undergoes significant changes, expanding to four, five, and even six rows (Figure 3A).

- **Postoral kineties (PK)**: Group I shows various increases in PK number (six to eight rows), Group II retains the ancestral state PK number except for *F. magna*, Group III decrease PK number except for *F, anatolica*, whereas Group IV shows both decreasing and increasing tendencies, with some species reaching up to six rows (Figure 3B).
- **Peroral membrane (PM)**: Groups II and III show an increases in PM ciliary rows, although species such as *F. sinica*, *F. salmastra*, and *F. lynni* revert from two rows to one (Figure 3C).
- **Peniculi 1 and 2 (P1 and P2):** Group I shows a marked increase from four to five rows, while other groups, with a few exceptions, retain the ancestral state (Figure 3D-E).
- **Peniculi 3 evolution**: Groups I and II show an increases in ciliary rows from three to four or five, whereas *F. mengi* and *F. sinica* uniquely decrease to two rows. Group IV members retain the ancestral state with several species increasing to four or five rows, while Group III decreases from the ancestral three ciliary rows to two. Most *Frontonia* species retain the ancestral linear structure of peniculi 3, with shortening of peniculi 3 considered an ancestral character (Figure 3F-H).

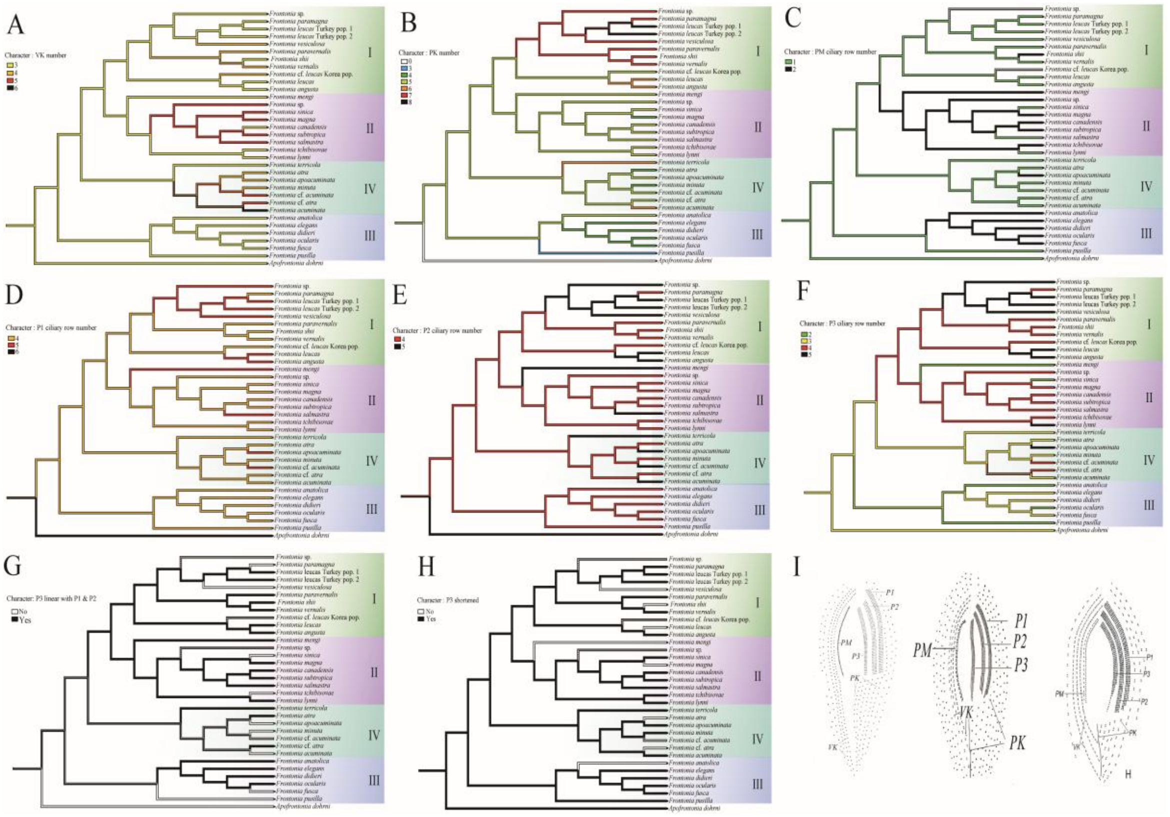

Figure 3. Ancestral state reconstruction of morphological characteristics; **A)** vestibular kinety (VK) number, **B)** postoral kinety (PK) number, **C)** peroral membrane (PM) row number, **D)** peniculi 1 (P1) row number, **E)** peniculi 2 (P2) row number, **F)** peniculi 3 (P3) row number, **G)** linearity of peniculi 3 (P3) in relation to peniculi 1 (P1) and peniculi 2 (P2), **H)** length of peniculi 3 (P3) in relation to peniculi 1 (P1) and peniculi 2 (P2) (shortened), **I)** *F. shii* [33] (right) as an example of species in which P3 has the same structure as P1 and P2, *F. paramagna* [33] (middle) as an example of species in which P3 does not have the same structure as P1 and P2, and *F. canadensis* [34] (left) as an example of species in which P3 is shortened compared to P1 and P2. *0: no, 1: yes.

- **Habitat adaptation**: The genus *Frontonia* originates from brackish habitats before adapting to freshwater environments. Group III remains in the brackish environment and adapts to marine habitats for several species, while Group I and IV retain a freshwater adaptation, except for *F. acuminata*, which adapts to a marine environment. Groups II evolves from freshwater to marine and to brackish habitats (Figure 4).

- **Contractile vacuole (CV)**: The ancestral analysis suggests that the genus originally possessed a single CV, a character often retained. Group III, excluding some species, increases from one to two CVs, while unique species in Group IV show up to 10 CVs per individual (Figure 4).
- **Contractile vacuolar pores (CVP) and collecting canals (CC)**: The evolution of CVPs and CCs is not closely correlated with the evolution of CVs (Figure 4).

These results shed light on the evolutionary history and adaptive strategies of *Frontonia* species, providing valuable insights into morphological evolution and habitat preferences.

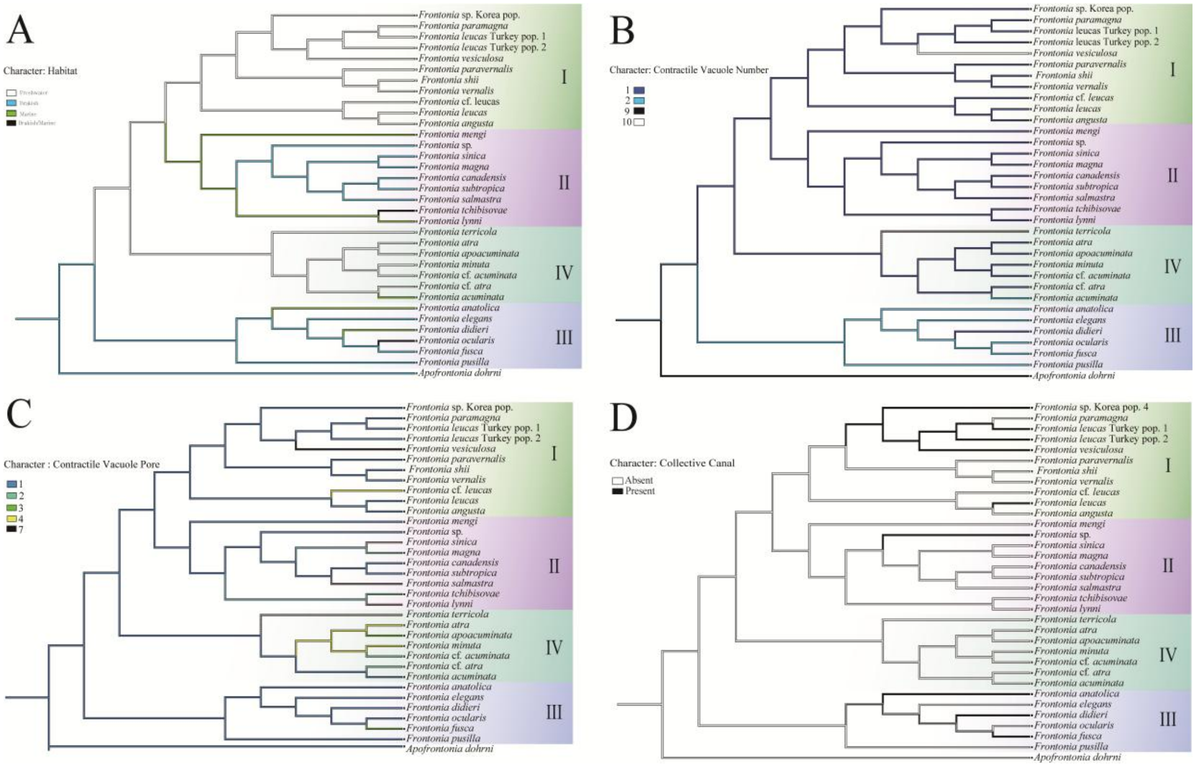

Figure 4. Reconstruction of ancestral state of morphological characteristics; A) habitat, B) number of contractile vacuole (CV), C) number of contractile vacuolar pore (CVP) and D) presence of contractile canal (CC).

4. Discussion

4.1. Evolutionary Patterns of Morphological Characters in the Genus Frontonia

The ancestral state reconstruction analysis provides valuable insights into the evolutionary patterns and morphological adaptations within the genus *Frontonia.* By examining the ancestral characters of *Frontonia* species, we can better understand the evolutionary trajectory of their oral apparatus and its adaptation to different habitats.

The results suggest that the common ancestor of *Frontonia* probably inhabited brackish waters and possessed specific characteristics related to its oral apparatus. This ancestor had a single contractile vacuole (CV), a single contractile vacuolar pore (CVP) and no contractile canals (CC). In addition, the ancestral species had consistent number of vestibular kineties (VK), postoral kineties (PK), and peroral membrane (PM) ciliary rows, as well as distinct structures in peniculi 1, 2, and 3. *Frontonia canadensis*, while closely resembling the ancestral state, shows evolutionary changes such as variations in the number of ciliary rows in peniculi 3 (P3) and changes in the structure of preoral membrane (PM). These changes highlight the dynamic nature of the morphological evolution of *Frontonia* [34].

The morphological characteristics of the oral apparatus, including the number of VK, PK, PM ciliary rows, and the structure of peniculi 1-3, serve as crucial markers for distinguishing *Frontonia* species. Reconstructing ancestral state using these characters reveals distinct evolutionary patterns for each character. While some characters, such as the number of VKs, show predictable evolutionary trends, others, such as the number of PM ciliary row, show reversals, highlighting the complexity of morphological evolution in *Frontonia.*

Habitat plays an important role in shaping morphological charcteristics of ciliates such as Frontonia [35–37]. However, our analysis indicates that there is no direct correlation between habitat type and the number of contractile vacuoles, pores, and canals in Frontonia species. This suggests that other factors, perhaps related to physiological adaptations, also influence the evolution of these traits. The observed stability of certain morphological characters across different habitats is consistent with the neutral morphological theory, suggesting that certain characters may be favored in different ecological contexts [38].

4.2. Evolutionary History of Genus Frontonia

The evolutionary history of the genus *Frontonia* provides insights into its emergence and divergence over millions of years, and sheds light on the environmental factors and evolutionary processes that have shaped its diversity. The common ancestor of the four groups within genus *Frontonia* and members of Penicullida emerged and diverged approximately 420 million years ago during the Palaeozoic era (Figure 2) [39]. Each group or clade within *Frontonia* originated at different times in geological time. Groups I and II shared a common ancestor at the beginning of the Mesozoic era about 230 million years ago, with Group I diverging about 172 million years ago and Group II diverging about 83 million years ago. Group III, morphologically close to the genus *Apofrontonia,* shared a common ancestor at the beginning of the Mesozoic era, about 185 million years ago, while Group III itself diverged about 115 million years ago. Group IV appeared about 190 million years ago, making it one of the oldest groups within *Frontonia.* Group I is the youngest, emerging at the end of the Mesozoic era.

The ancestor of the genus *Frontonia* probably diverged during the Cambrian explosion, a period of significant biological diversification. While species numbers increased during the Cambrian period, environmental changes, possibly driven by glacial cycles, led to mass extinctions in the Paleozoic era following the Cambrian explosion. Although fossil evidence for ciliates is scarce, it is plausible that the harsh environmental conditions limited the survival and development of the ancestral *Frontonia* populations, resulting in long branches without speciation during the Palaeozoic era [39–41].

Diversification tests suggest a higher extinction rate than the speciation rate in *Frontonia*, indicating the challenges faced by ancestral populations in maintaining their existence over geological time scales. Surviving *Frontonia* species persisted into the early Mesozoic era, during which time they diverged, coinciding with a warmer Earth and potentially more favorable environmental conditions [42,43].

The current paraphyly within the genus *Frontonia* may be due to SSU rRNA gene polymorphism in its ancestor, contributing to the high genetic diversity observed in the past. Harsh environmental conditions during the Palaeozoic era probably subjected ancestral *Frontonia* populations to natural selection, resulting in the decline of many populations. However, surviving populations retained variations in their SSU rRNA gene, potentially leading to divergent evolutionary trajectories. Over time, during the Mesozoic era, these divergent populations underwent speciation, possibly in response to changing environmental conditions, as supported by recent studies [8].

5. Conclusions

This study provides a comprehensive investigation of the phylogenetic, evolutionary and diversification dynamics within the genus *Frontonia* by integrating molecular data from the SSU rRNA gene with analyses of morphological characters. Reconstructions of ancestral states have provided valuable insights into the unique evolutionary patterns of each morphological character within *Frontonia.* In particular, habitat transitions do not show a direct correlation with the number of contractile vacuoles, contractile vacuole pores, and contractile canals, suggesting the influence of complex factors on these characters. The historical perspective of the genus *Frontonia* traces its origins back to the Cambrian explosion, revealing survival challenges during the Paleozoic era and subsequent diversification throughout the Mesozoic era. The observed paraphyly within *Frontonia* is attributed to SSU rRNA gene polymorphism in its ancestor, reflecting high genetic diversity and adaptation to changing environmental conditions over time.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table A1: *Datasheet Genus Frontonia members in this Study;* Table A.2. Datasheet that input into speciation rate analysis software.

Author Contributions: Conceptualization, R.K.W. and M.K.S.; methodology, R.K.W. and R.A.; software, R.K.W..; validation, R.K.W. and M.K.S.; formal analysis, R.K.W.; investigation, R.K.W. and R.A.; resources, M.K.S.; data curation, R.K.W.; writing—original draft preparation, R.K.W.; writing—review and editing, R.K.W., M.K.S., and R.A.; visualization, R.K.W.; supervision, M.K.S.; project administration, M.K.S.; funding acquisition, M.K.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Appendix A.1

Table A1. *Datasheet Genus Frontonia members in this Study.*

Species	Accession No.	Origin	Molecular information (Source by publication based on SSU rRNA gene)	Morphological information
	SSU			
Frontonia anatolica	MG456578	Turkey	Kizildang & Yildiz, 2019	Kizildang & Yildiz, 2019, Yildiz & Senler, 2013
Frontonia anatolica	MG456581	Turkey		
Frontonia acuminata	MG456579	Turkey	Kizildang & Yildiz, 2019	Kizildang & Yildiz, 2019, Foissner, et al., 1994, Kahl, 1931, Pernard, 1922
Frontonia angusta	MG456580	Turkey	Kizildang & Yildiz, 2019	Kizildang & Yildiz, 2019, Foissner, et al.,

				2002, Kahl, 1931, Foissner, et al., 1994
Frontonia canadensis	KJ475313	Shenzen, China	Zhao et al., 2016	Pan, X., et al., 2013, Roque. and de Puytorac, 1972.
Frontonia canadensis	KJ475311	Huizhou, China		
Frontonia canadensis	KJ475309	Huizhou, China		
Frontonia canadensis	KJ475310	Nansha, China		
Frontonia canadensis	KJ475312	Hongkang, China		
Frontonia canadensis	FJ868196	Nansha, China	Fan et al., 2013	
Frontonia cf. *acuminata*	PV016905	Gwangju, Korea	present study	present study
Frontonia cf. *acuminata*	PV016906	Ulsan, Korea		
Frontonia cf. *acuminata*	PV016907	Gunsan,Korea		
Frontonia cf. *acuminata*	PV016908	Ulsan, Korea		
Frontonia cf. *atra*	PV016909	Daejon, Korea		
Frontonia cf. *atra*	PV016910	Gunsan, Korea		
Frontonia cf. *atra*	PV016911	Masan, Korea		
Frontonia cf. *atra*	PV016912	Chungcheongbukdo, Korea		
Frontonia cf. *atra*	PV016913	Gyeongsangnamdo, Korea		
Frontonia cf. *atra*	PV016914	Ulsan, Korea		
Frontonia cf. *atra*	PV016917	Gyeongju, korea		
Frontonia atra	MT040844	Serchio river, Italy	Serra et al., 2021	Serra et al., 2021, Dragesco & Dragesco-Kerneis, 1986, Foissner, et.al., 1994
		Gangneung-si		Omar and Jung, 2021
Frontonia didieri	KJ475297	Qingdao, China	Zhao et al., 2016	Long et al., 2008
Frontonia didieri	KJ475299	Qingdao, China		
Frontonia didieri	KJ475298	Shenzen, China		
Frontonia didieri	DQ885986	Qingdao, China	Long et al., 2008	

Frontonia ocularis	FJ868198	Guangzhou, China	Pan et al., 2013	Pan et al., 2013
		Gangneung-si		Jung, 2021
Frontonia elegans	FJ868200	Guangzhou, China	Fan et al., 2013	Fan et al., 2013
Frontonia elegans	KJ475301	Huizhou, China	Zhao et al., 2016	
Frontonia elegans	KJ475302	Huizhou, China		
Frontonia elegans	KJ475303	Huizhou, China		
Frontonia cf. *leucas*	PV016904	Gwangju, Korea	present study	present study
Frontonia leucas	MG437395	Turkey	Yildiz & Kizildang , 2017	Yildiz & Kizildang, 2019
Frontonia leucas	MG437396			
Frontonia leucas	AM072622	Italy	Fokin et al., 2006	Serra et al., 2021, Dragesco & Dragesco-Kerneis, 1986, Foissner, et.al., 1994, Kahl, 1931
Frontonia leucas	KY855558	Andhra Pradesh, India	Serra et al., 2021	
Frontonia lynni	DQ190463	Qingdao, China	Long et al., 2008	Long et al., 2005
Frontonia magna	FJ868199	Zhuhai, China	Zhao et al., 2016	Fan et al., 2011
Frontonia magna	FJ876953	Shenzen, China	Fan et al., 2011	
Frontonia mengi	FJ875141	Qingdao, China	Fan et al., 2011	Fan et al., 2011
Frontonia paramagna	KJ475304	Guangzhou, China	Zhao et al., 2016	
Frontonia paramagna	KJ475306	Sichuan, China	Zhao et al., 2016	
Frontonia paramagna	JQ868786	Harbin, China	Chen et al., 2014	Chen et al., 2014
Frontonia paramagna	KJ475305	Qingdao, China	Zhao et al., 2016	
Frontonia paramagna	MF279207	Harbin, China	Cai et al., 2018	Cai et al., 2018
Frontonia paramagna	KY855554	Andhra Pradesh, India	Serra et al., 2021	Serra et al., 2021
Frontonia paramagna	MW031789	Heilojiang, China	Sun et al.,2021	Sun et al.,2021
Frontonia shii	MF279208	Harbin, China	Cai et al., 2018	Cai et al., 2018
Frontonia sinica	KJ475308	Qingdao, China	Zhao et al., 2016	Fan et al., 2013
Frontonia sinica	FJ868197	Shenzen, China	Fan et al., 2013	
Frontonia subtropica	FJ868202	Shenzen, China	Fan et al., 2013	Pan et al., 2013
Frontonia tchibisovae	KJ475318	Qingdao, China	Zhao et al., 2016	Long et al., 2008

Frontonia tchibisovae	KJ475316	Qingdao, China		
Frontonia tchibisovae	KJ475319	Qingdao, China		
Frontonia tchibisovae	DQ883820	Yantai, China	Long et al., 2008	
Frontonia terricola	MF926593	Guangzhou, China	Xu et al., 2018	Xu et al., 2018, Foissner, 1987, Foissner, et al., 2002
Frontonia pusilla	FJ868201	Guangzhou, China	Fan et al., 2013	Fan et al., 2013
Frontonia vernalis	U97110	UK	Hirt et al., 1997	
Frontonia vernalis	MT040840	Tusacany, Italy	Serra et al., 2021	Serra et al., 2021
Frontonia paravernalis	MT040839	Tusacany, Italy	Serra et al., 2021	Serra et al., 2021
Frontonia salmastra	MH319376	Tusacany, Italy	Fokin et al., 2019	Fokin et al., 2019, Dragesco & Dragesco-Kerneis, 1986
Frontonia vesiculosa	MT040850	Andhra Pradesh, India	Serra et al., 2021	Serra et al., 2021, Dragesco & Dragesco-Kerneis, 1986, Kahl, 1932
Frontonia minuta	MT040846	Serchio river, Italy	Serra et al., 2021	Serra et al., 2021, Dragesco & Dragesco-Kerneis, 1986
Frontonia fusca	MT040845	Serchio river, Italy	Serra et al., 2021	Serra et al., 2021, Fokin, 2008, Kahl, 1932
Frontonia sp.	MT040843	India	Serra et al., 2021	Serra et al., 2021
Frontonia sp.	MT040841	Serchio river, Italy	Serra et al., 2021	Serra et al., 2021

Appendix A.2

Table A2. Datasheet that input into speciation rate analysis software.

DivBAyes 1.1		SubT 1.1	
Estimated taxa[a]	**Diversification date**	**Data of taxa[b]**	**Subtitution taxa[c]**
69 species	450 Mya	33 species	36 species

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