Evolutionary patterns of the SSU rRNA (V4 region) secondary structure in genus *Euplotes* (Ciliophora, Spirotrichea): insights into cryptic species and primitive traits

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ABSTRACT

The genus *Euplotes*, a group of ciliated protists, has attracted attention as a model organism due to its widespread distribution and ease of cultivation. This study examines the evolutionary patterns of the SSU rRNA secondary structure within this genus, aiming to elucidate its role in supporting evolutionary relationships and uncovering cryptic species. By predicting the secondary structure of SSU rRNA and applying the CBC (Compensatory Base Change) concept analysis, we examined 69 species of the genus *Euplotes*, with 57 SSU rRNA gene sequences retrieved from GenBank and 12 newly sequenced specimens from South Korea. Our analysis revealed significant variations in the V4 region secondary structure, particularly in helix E23_8, across different clades of *Euplotes*. Reconstruction of the ancestral state indicated a transition from a simpler (Type I) to a more complex (Type II) secondary structure, with several species showing a reversal to Type I especially species in clade VI, suggesting of reverse evolution. In addition, our study identified cryptic species within *Euplotes* based on differences in the secondary structure of the V4 region, particularly evident in clade VI, where CBC analysis highlighted differences in *E. minuta* compared to *E. vannus* and *E. crassus*. These results highlight the utility of molecular data in refining species boundaries and evolutionary patterns within the genus *Euplotes*.

Subjects Biodiversity, Bioinformatics, Evolutionary Studies, Microbiology, Molecular Biology
Keywords *Euplotes*, V4 region, SSU rRNA, Secondary structure, Evolutionary pattern, Cryptic species, Primitive character

Submitted 16 April 2024
Accepted 20 December 2024
Published 23 January 2025

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Academic editor
Joseph Gillespie

Additional Information and Declarations can be found on page 10

DOI **10.7717/peerj.18852**

INTRODUCTION

Genus *Euplotes* is a remarkably diverse genus of ciliates, comprising more than 100 species (*Bisby et al., 2010*). This genus is notable for its cosmopolitan distribution and ease of cultivation in the laboratory, as it is commonly found in a wide variety of environment. *Euplotes* serves as a valuable model organism alongside *Paramecium* and *Tetrahymena*

(*Aury et al., 2006*; *Greider & Blackburn, 1989*; *Kruger et al., 1982*; *Sonneborn, 1975*). *Euplotes* has been extensively utilized in studies investigate the adaptation of single-celled organisms to extreme conditions such as cold environments (*La Terza et al., 2001*; *Marziale et al., 2008*; *Mozzicafreddo et al., 2021*), as well as research in mating on processes and pheromone studies (*Di Giuseppe et al., 2011*; *Gong et al., 2022*; *Pedrini et al., 2022*), the symbiotic relationships between symbionts and hosts (*Boscaro et al., 2019a*, *2019b*; *Vannini et al., 2017*), and the geographical distribution of unicellular organisms (*Syberg-Olsen et al., 2016*). Given the importance of *Euplotes*'s as a model organism, numerous studies have focused on species delimitation and the exploration of new species across diverse environments and biogeographies (*Abraham et al., 2021*; *Han, Pan & Jiang, 2022*; *Lian et al., 2020*; *Syberg-Olsen et al., 2016*; *Valbonesi et al., 2021*; *Zhao et al., 2018*). In the process of delimitation within the genus *Euplotes* several cryptic species emerge due to their indistinguishable morphological character (*Wang et al., 2021*; *Zhao et al., 2018*). Molecular data have mainly played a supporting role in the phylogenetic studies of *Euplotes* and in the attempt to reveal cryptic species within genus *Euplotes* (*Zhao et al., 2018*). However, it should be noted that molecular data can play a more significant role than previously discussed. For example, in the study of the genus *Euplotes*, molecular data, such as SSU rRNA secondary structure, can serve as a key feature to identify new species (*Abraham et al., 2021*).

The SSU rRNA secondary structure, which serving as a molecular character, exhibits specific evolutionary patterns that have proven valuable in phylogenetic studies of different taxa (*Marin et al., 2003*; *Rusin et al., 2001*; *Telford, Wise & Gowri-Shankar, 2005*; *Voronova & Chelomina, 2020*). This is evident in phylogenetic studies of ciliates (*Du, Zhao & Tang, 2018*; *Gao, Katz & Song, 2013*; *Gao et al., 2014*; *Li et al., 2008*; *Wang et al., 2015*; *Zhang et al., 2014*; *Zhang et al., 2015*). Components of the RNA secondary structure, including stems, inner loops, hairpin loops, and bulges, are features that can support traditional cladistics and contribute to our understanding of the universal tree of life by examine the evolutionary patterns inherent in these molecular characters (*Caetano-Anollés, 2002*).

The secondary structure of SSU rRNA contains nine variable regions (V1–V9) that hold phylogenetic information. Among these, the V4 region is one of the most studied in ciliates due to its superior ability to resolve phylogenetic relationships compared to the V9 region (*Dunthorn et al., 2012*, *2014*; *Santoferrara, McManus & Alder, 2013*). The V4 region has been widely used to differentiate closely related ciliate taxa, such as scuticociliates (*Gao et al., 2014*), litostomes (*Strüder-Kypke et al., 2006*), and haptorians (*Zhang, Simpson & Song, 2012*). Furthermore, the V4 region is valuable for estimating the diversity of different protist taxa due to its high mutation rate (*Stoeck et al., 2010*, *2014*; *Santoferrara, McManus & Alder, 2013*; *Dunthorn et al., 2014*).

In addition to elucidating taxonomic relationships and evolutionary history, the secondary structure of SSU rRNA can also be used to distinguish between different species. This is achieved through compensatory base change (CBC) analysis on the helices of the secondary structure, which detects nucleotide base changes on both sides of the paired bases. An experimental study by *Coleman & Vacquier (2002)* demonstrated a correlation

between CBCs and sexual compatibility between species. The study found that taxa differing by CBCs, even by just a single CBC in conserved pairing positions, showed differences in sexual compatibility.

Therefore, this study aims (1) to observe the evolutionary pattern of the SSU rRNA secondary structure of V4 region in the genus *Euplotes* as a speciose and model organism group and its usability in supporting the phylogenetic and evolutionary of the genus *Euplotes*, and (2) to reveal cryptic species within the genus *Euplotes* through CBC analysis of the secondary structure of the V4 region.

MATERIALS AND METHODS

Sample collection and morphological study

Twelve species of *Euplotes* were collected from various locations in South Korea (Table 1). These specimens were cultured in Petri dishes containing water from their respective habitats. The cells were initially observed in their live state using a stereomicroscope (Nikon SMZ800, Nishioi, Shinagawa-ku, Tokyo) to assess their typical shape, movement, and behavior. For more detailed analysis, a differential interference contrast (DIC) microscope (Axio Imager A.1, Carl Zeiss, Oberkochen, Germany) was used, allowing for magnification between 100× and 1,000× for both live and stained samples.

DNA extraction, amplification, and sequencing

Genomic DNA extraction was performed using the RED Extract-N-Amp Tissue PCR Kit from Sigma (St. Louis, MO, USA), according to the manufacturer's instructions. For polymerase chain reaction (PCR), the forward primer EUK A (5′-GAC CGT CCT AGT TGG TC-3′) and the reverse primer EUK B (5′-CTT GGA CGY CTT CCT AGT-3′) were used, as described by *Medlin et al. (1988)*. PCR amplification was performed using the TaKaRa ExTaq DNA polymerase kit from TaKaRa Bio-medicals (Otsu, Japan) according to this specific protocol: an initial denaturation at 94 °C for 2 min, followed by 37 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 40 s, and extension at 72 °C for 4 min. This was followed by a final extension at 72 °C for 10 min (*Kim et al., 2011*). Sequencing was performed with bidirectional sequencing using the primers used in the PCR reaction (EUK A and EUK B).

Predicting the secondary structure of the V4 region of SSU-rRNA and CBC (compensatory base change) concept analysis

To predict the secondary structure of the SSU-rRNA, we performed an alignment of 69 SSU rRNA gene sequences from the species of genus *Euplotes* retrieved from GenBank (NCBI) and together with newly sequenced species from Korea (Table 1), related genera members (*Certesia quadrinucleata, Aspidisca fusca, Euplotidium Rosati, Diophrys scutum, Uronychia xinjiangensis*, and *Discocephalus pararotatorius*) selected as an outgroup. The alignment was performed using SSU-ALIGN and based on the alignment results from SSU-ALIGN, it was determined that the V4 region is a longer variable region (163–261 bp) (Table S1) and has more mutations compared to other variable regions (Fig. S1). From the SSU-ALIGN results, we isolated the V4 region of the SSU rRNA gene (*Nawrocki, 2009*). To

Table 1 Characterization of SSU rRNA gene sequences of *Euplotes* species from Korea.

Taxon	Collection site	SSU rRNA gene			Clade
		GenBank entry	Length (nt)	GC (%)	
Euplotes cf. *inkystans*	Dokdo, Korea	PP648189	1,794	45.2	V
Euplotes cf. *mutabilis*	Dokdo, Korea	PP648190	1,836	44	VI
Euplotes crenosus	Dokdo, Korea	PP648191	1,972	42	VI
Euplotes neopolitanus	Dokdo, Korea	PP648192	1,874	42	IV
Euplotes trisulcatus	Busan, Korea	PP648193	1,750	45.3	V
Euplotes gracilis	Ulsan, Korea	PP648194	1,803	44	V
Euplotes muscorum oligomembrana n.subsp.	Ulsan, Korea	PP648195	1,814	43	V
Euplotes paramuscicola n.sp.	Ulsan, Korea	PP648196	1,807	46	V
Euplotes vannus pop.1	Pohang, Korea	PP648197	1,840	43	VI
Euplotes vannus pop.2	Pohang, Korea	PP648198	1,846	43	VI
Euplotes n. sp.	Dokdo, Korea	PP648199	1,807	45	V
Euplotes sp.	Dokdo, Korea	PP648200	1,797	45	V

generate consensus secondary structure of the V4 region of genus *Euplotes* member, we used RNAalifold software (*Hofacker, 2008*), the consensus secondary structure of V4 region used as a reference for predicting the secondary structure of the V4 region for each species. The prediction of the secondary structure for each species was achieved by using MFOLD, which calculates the minimum energy (*Zuker, 2003*). To guide the construction of the secondary structure using MFOLD, we used the consensus secondary structure and several criteria. First, we closed bilateral bulges (internal loops) present in published models if they could consistently form G:C pairs with non-canonical pairing bases in the stem. Second, we did not retain paired structures if multiple non-canonical base pairings occurred, instead of canonical (G:C or A:U) or wobble (G:U) base pairs. Final, in cases where multiple structures were predicted, we selected the structure with either the minimum free energy or the best compatibility with similar sequences (*Řeháková et al., 2014*; *Voigt, Erpenbeck & Wörheide, 2008*). The final secondary structure of the hypervariable region of SSU-rRNA was visualized using RNAviz software (*De Rijk, Wuyts & De Wachter, 2003*).

For the compensatory base change (CBC) analysis, we used 4SALE (*Seibel et al., 2006*) to detect CBCs between sequence-structure pairs within the alignment. The CBC analysis was applied to members of clade VI within the genus *Euplotes* (*E. minuta, E.* cf. *mutabilis, E. crenosus, E. japonicum, E. cristatus, E. crassus*, and *E. vannus*) (Fig. S2).

Reconstruction of ancestral state

For the ancestral state analysis of the V4 (SSU-rRNA) secondary structure, we used representative species from the genus *Euplotes* and related genera (*Certesia quadrinucleata, Aspidisca fusca, Euplotidium rosati, Diophrys scutum, Uronychia xinjiangensis*, and *Discocephalus pararotatorius*) (Table S1). The presence or absence of

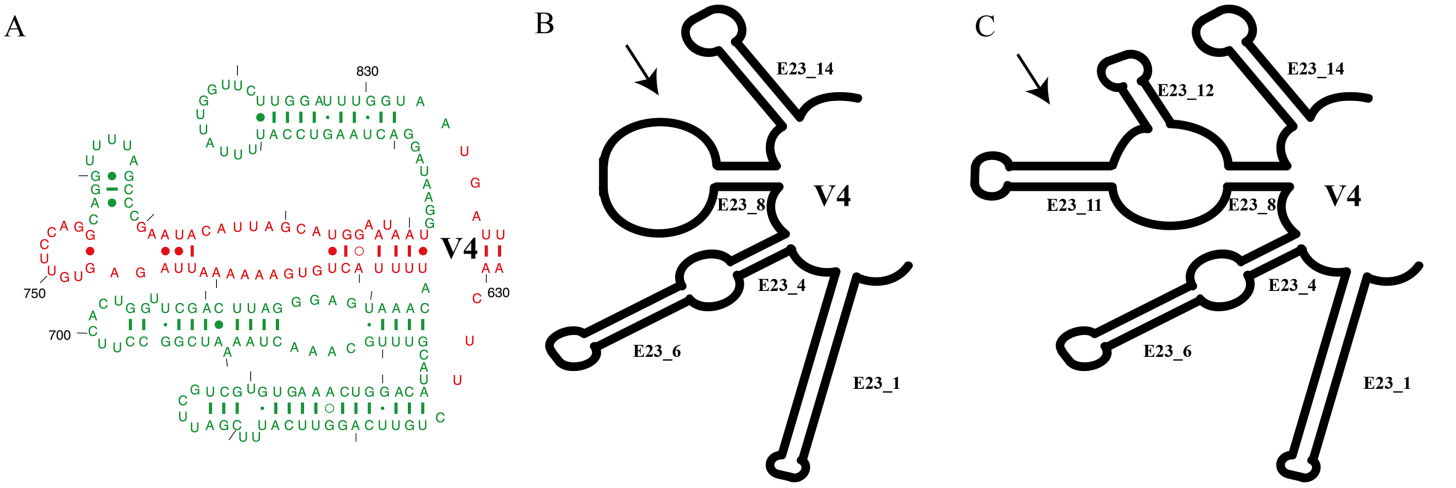

Figure 1 Summary of V4 secondary structure on *Euplotes* and related genera. (A) Helices of V4 secondary structure marked in red, representing helices commonly found in Prokaryotes (Bacteria), and in green mark, representing extended helices in Eukaryotes (*Lee & Gutell, 2012*), (B) V4 secondary structure type I in genus *Euplotes* members, featuring one hairpin loop at the end of E23_8, (C) V4 secondary structure type II in genus *Euplotes* members, with two extra helices (E23_11 & E23_12). Full-size DOI: 10.7717/peerj.18852/fig-1

two additional helices on the extension helix E23_8 (Type I *vs*. Type II) (Fig. 1) was mapped onto the best-likelihood tree generated by RAxML analysis on the CIPRES platform (*Miller, Pfeiffer & Schwartz, 2011*). The character matrix and subsequent ancestral state reconstruction were performed using the parsimony model in Mesquite software (version 3.70) (*Maddison & Maddison, 2021*) (Table S1).

RESULTS

Significant characters of SSU rRNA secondary structure

In this study, we focused on the secondary structures of the V4 region. This choice was prompted by the observation of a significant number of mutations in this region, as indicated by the results of SSU-Align and primary sequence alignment. The consensus secondary structure predicted for the V4 region by the RNAalifold software identified four major helices (Fig. 1). In general, the secondary structure of each member of the genus *Euplotes* consists of four helices, with variations observed specifically in helix E23_8. These variations include two additional helices or hairpin loops at the end of the helix, highlighting the structural diversity within the genus *Euplotes* in the V4 region (Fig. 1).

Each clade depicted in the phylogenetic tree (Fig. S2) has a distinct secondary structure pattern. Members of clade I display a single large hairpin loop composed of 21 nucleotides in helix E23_8 (Fig. 2). This structural feature is also shared by the members of clade II, but smaller in size, consisting of 13 nucleotides (Fig. 2). Clade III is divided into two groups based on the characteristics of the V4 secondary structure. The first group include species with a hairpin loop consisting of five nucleotides (Fig. 2). Members of this group include *E. curdsi, E. dominicanus, E. estuarianus, E. nobili, E. raikovi*, and *E. shii* (Fig. 3). The second group is characterized by a long helix in E23_8 and the presence of two additional helices (E23_11 & E23_12) (Fig. 2). This group includes *E. bergeri, E. elegans, E. qatarensis*, and *E. wuhanensis* (Fig. 3). All members of clade IV share a V4 secondary structure

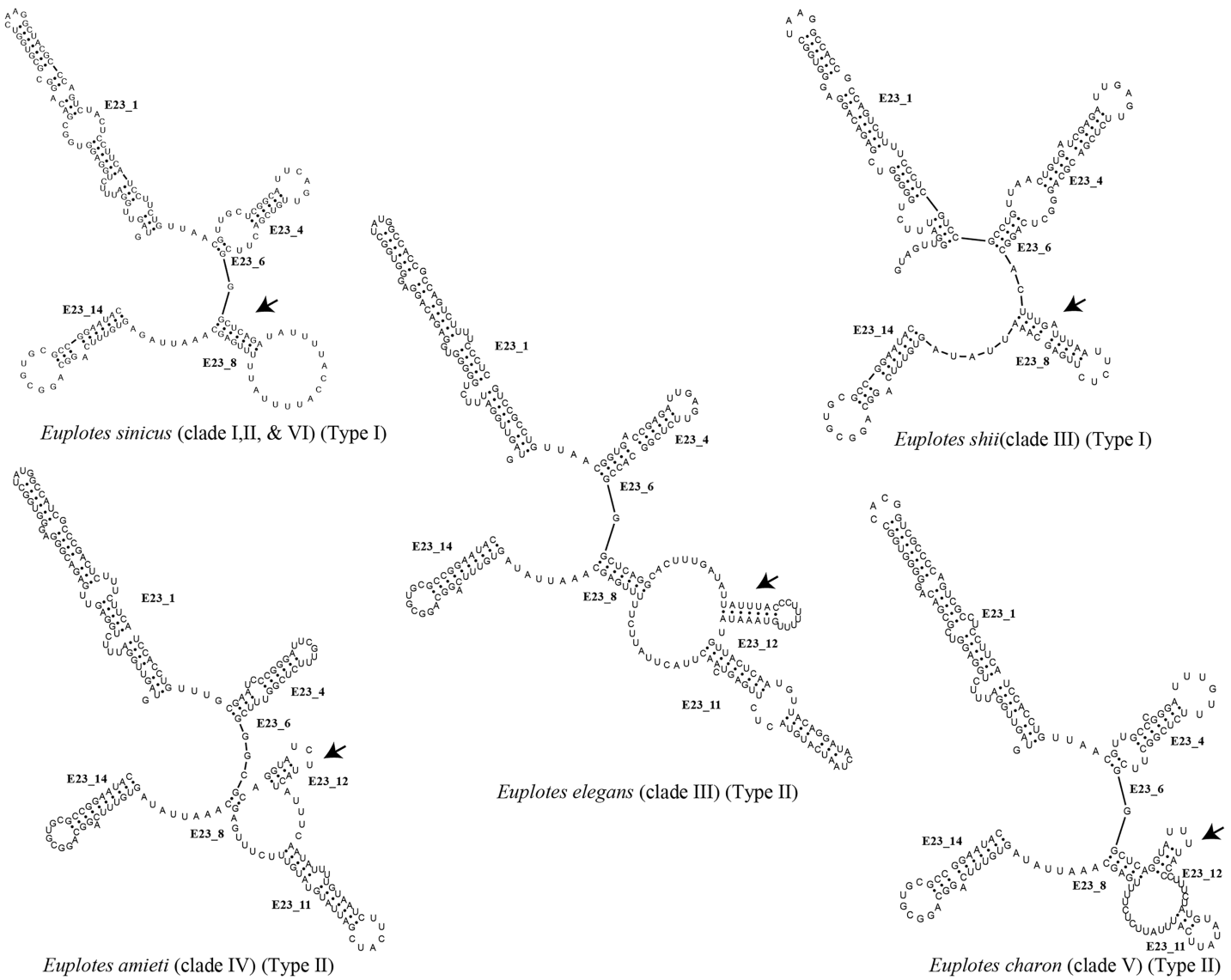

Figure 2 **Several species of the genus *Euplotes* and their V4 secondary structure (E23_8, E23_11, and E23_12).**
Full-size DOI: 10.7717/peerj.18852/fig-2

characterized by the presence of two additional helices (E23_11 & E23_12), with the helix E23_8 shorter compared to the second group of clade III (Fig. 2). All members of clade V have two additional helices, except for three species that have a single hairpin loop at the end of helix E23_8 (Fig. 3). These three species are *E. trisulcatus* (hairpin loop consisting of 32 nucleotides), *E. trisulcatus* (hairpin consisting of 17 nucleotides) and *E. shini* (hairpin loop consisting of 29 nucleotides) (Fig. 2). In clade VI, members share a common pattern of a single large hairpin loop composed of 25–29 nucleotides. Interestingly, *E. minuta*, a member of clade VI, deviates from this pattern and show two additional helices (E23_11 & E23_12) (Fig. 3 and Fig. S2).

In addition to secondary structure, we applied compensatory base change (CBC) analysis to the members of clade VI, focusing specifically on the genus *Euplotes*

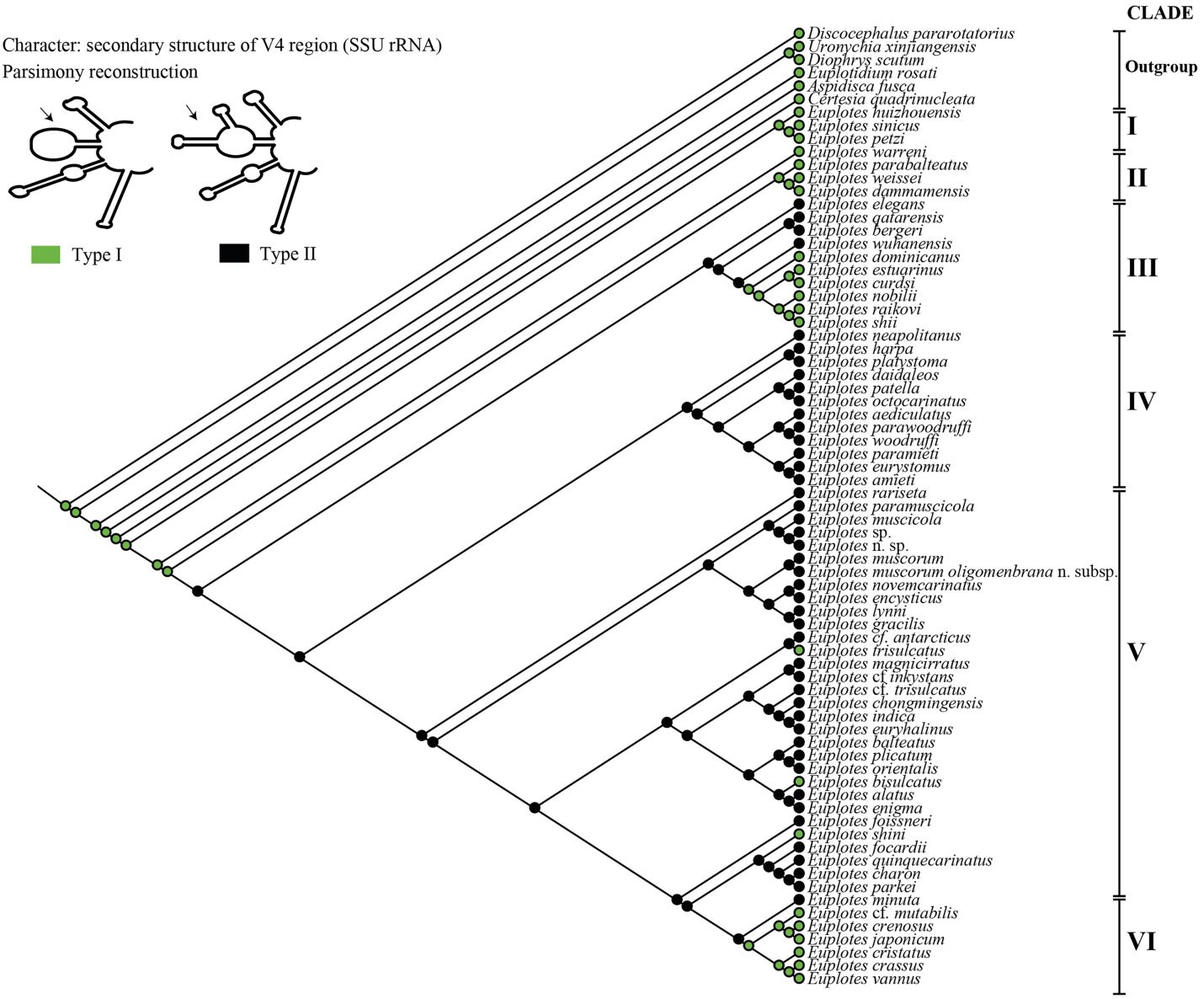

Figure 3 **Ancestral state analysis of V4 in the genus *Euplotes*, with V4 secondary structure "Type I" and "Type II" as characters.**
Full-size DOI: 10.7717/peerj.18852/fig-3

vannus-minuta-crassus complex. The CBC shows that *E. minuta* has one CBC compared to other members of clade VI (Fig. 4).

Ancestral state of V4 secondary structure within genus *Euplotes*

The ancestral state was analyzed using the V4 secondary structure within the genus *Euplotes* to discern the evolutionary pattern of molecular characters, with particular focus on helix E23_8 (hairpin loop *vs.* two additional helices) (Fig. 1). The V4 secondary structure type I represents a molecular feature inherited from the ancestor of the genus *Euplotes*, and it is retained in members of clade I and II (Fig. 3). The ancestral state analysis shows that Type II of the V4 secondary structure, indicating the addition of two helices, is

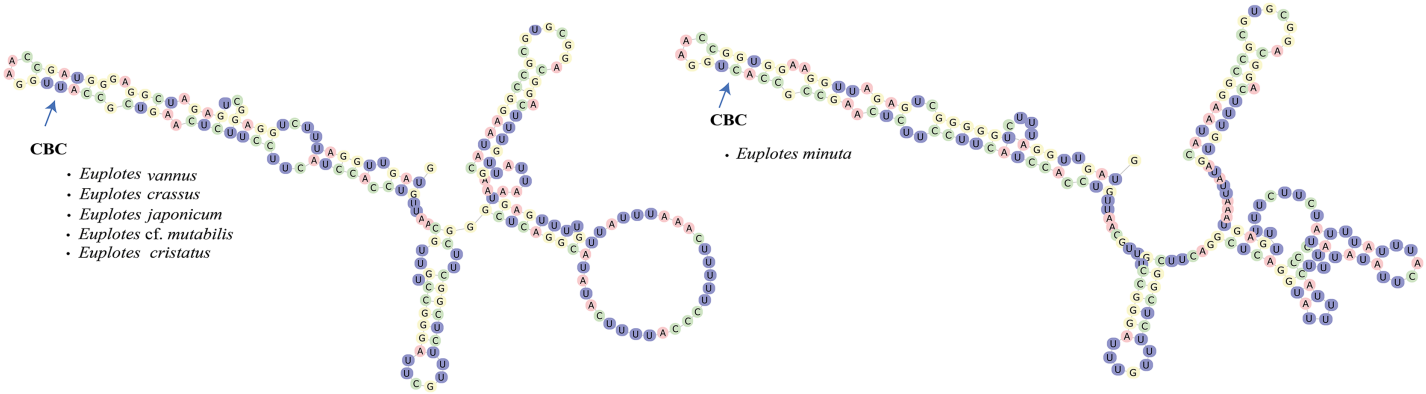

Figure 4 ***Euplotes minuta*** **shows one compensatory base change (CBC) compared to another member of Clade VI.**
Full-size DOI: 10.7717/peerj.18852/fig-4

evolve feature compared to Type I of the V4 secondary structure (Fig. 3). Type II V4 secondary structure is commonly observed in almost all members of clade III to V with the exceptions of a few species that have Type I of the V4 secondary structure as their molecular character. These species include *E. dominicanus, E. estuarinus, E. curdsi, E. nobilii*, and *E. shii* in clade III, and *E. trisulcatus, E. bisulcatus* and *E. shini* in clade V (Fig. 3).

An interesting observation arises in the members of clade VI, where all members except *E. minuta* have Type I of the V4 secondary structure as their molecular character. Clade VI appears to represent a relatively recent clade in the phylogenetic tree of the genus, and these results suggest a remark reappearance of primitive characters reappearing in the most advanced species within this clade (Fig. 3).

DISCUSSION

Reverse evolutionary pattern of the V4 secondary structure within the genus *Euplotes*

The Type I of V4 secondary structure is regarded as simpler compared to Type II, as it shares structural similarities to the V4 secondary structure found in prokaryotes. In prokaryotes, the E23 (V4) region typically lacks an additional helix, indicating a primitive feature compared to eukaryotes (*Lee & Gutell, 2012*). This primitive feature is observed in members of the genus *Euplotes* and outgroup genera used as the earliest divergence group at the base of the phylogenetic tree, as supported by molecular clock analysis (Fig. S3). The primitive character of the V4 secondary structure was also observed in some later diverging species, particularly in Group VI (Fig. 3 & Fig. S1).

The secondary structure of V4 in the common ancestor of the genus *Euplotes* is characterized by Type I, which later evolved into the more complex Type II structure (Fig. 3). Interestingly, in certain evolved species there was a reduction in the E23_11 and E23_12 helices, resulting in a reversion to the Type I V4 secondary structure (Fig. 3). This pattern is suggestive of reverse evolution, where a character state changes to resemble the

ancestral state, involving reversals and regressions that reflect evolutionary patterns of reversion to earlier or simplified forms after initially becoming more complex (*Porter & Crandall, 2003*; *Teotónio & Rose, 2001*).

The structural variations observed in the V4 secondary structure result from deletions or insertions in the V4 SSU rRNA region (Fig. S4). This pattern suggests that the occurrence of insertions or deletions in this region implies its lack of conservation and limited relevance to ribosome function, and thus the high degree of evolutionary change in this region is unlikely to have a significant impact on ribosomal function (*Wuyts, Van de Peer & De Wachter, 2001*). Although this region may not directly affect ribosome function, it is important because of the exceptionally high mutation rate within the SSU rRNA gene sequence. This region is likely to play a critical role in maintaining the free energy level to support the conservation of the SSU rRNA secondary structure. This study shows that the Type II of the V4 secondary structure has a more favorable free energy profile when compared to Type I (Table S1). The reverse evolution of the V4 secondary structure is not driven by the energy favorability, but is likely due to deletions that occur within this specific region.

The evolutionary pattern of V4 secondary structure supports the primitive nature of the basal clade in the genus *Euplotes*

The genus *Euplotes* shows distinct groupings, with a basal group (clade I) containing species such as *E. huizhouensis, E. petzi*, and *E. sinicus*, and subsequent divergent groups (clades II to VI) (Fig. 3). The evolutionary pattern of the V4 secondary structure shows that clade I, as a basal group, has primitive or ancestral characteristics compared to the later evolved groups. This pattern extends beyond molecular characters, morphological characters also follow a similar trend. Specifically, the basal group (clade I) displays a distinctive double-pattern argyrome character, representing the ancestral state of the genus *Euplotes*. In addition, these species display 10 fronto-ventral cirri (FVC), another character considered primitive or plesiomorphic in the genus *Euplotes*. In summary, molecular evidence from the V4 secondary structure supports the idea that species in the basal group (clade I) of the genus *Euplotes* are characterized by primitive traits compared to species in later diverging groups (clades II to VI), as reflected in their morphological characters (*Syberg-Olsen et al., 2016*; *Zhao et al., 2018*).

Cryptic species within the genus Euplotes in clade VI are revealed by the CBC concept in the V4 secondary structure

In this study, the CBC concept is applied to the members within clade VI, that potentially contain cryptic species. The presence of cryptic species makes it difficult to distinguish the species by morphological characters. By examining the secondary structure of V4, it is clear that *E. minuta* has a different structure compared to other members of clade VI. The CBC shows changes in nucleotide bonds in *E. minuta* compared to *E. crassus, E. vannus, E.* cf. *mutabilis*, and *E. japonicum*. To date, cryptic species have been identified among *E. minuta, E. crassus*, and *E. vannus*, which can be distinguished mainly by cell size alone

(*Dini, 1984*; *Valbonesi, Ortenzi & Luporini, 1988*). However, the presence of CBC in *E. minuta* allows us to reasonably conclude that *E. minuta* represents a separate species from *E. crassus* and *E. vannus*. Furthermore, the CBC concept in *E. crassus* and *E. vannus* is consistent with evidence that both species are capable of mating under laboratory conditions (*Valbonesi, Ortenzi & Luporini, 1988*). This mating compatibility results from their morphological and chemical compatibility, in particular their pheromones. Considering both mating compatibility and the CBC concept, it is plausible that these two species are more appropriately classified as a single species.

CONCLUSIONS

The study of the V4 secondary structure within the genus *Euplotes* provides valuable insights into the evolutionary dynamics of this group. The observed pattern of reverse evolution, in which the V4 structure reverts from the more complex Type II to the simpler Type I, suggests a reversion to ancestral features. Furthermore, the application of CBC analysis within Clade VI reveals the presence of cryptic species, providing a more nuanced understanding of species differentiation within *Euplotes*. The CBC analysis not only supports the distinct classification of species such as *E. minuta* but also raises the possibility that *E. crassus* and *E. vannus* may share such close genetic similarities that they could potentially be considered as a single species. In conclusion, this research highlights the evolutionary complexity within the genus *Euplotes* and demonstrates the effectiveness of molecular tools such as V4 secondary structure analysis and CBC in elucidating species relationships and evolutionary history. These findings contribute to a deeper understanding of the processes driving diversity within the genus.

ACKNOWLEDGEMENTS

We thank MD Abu Taher (University of Ulsan, University of British Columbia) for providing sample (*Euplotes gracilis, Euplotes vannus* pop1 and pop2) for analysis in this article.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the NRF Korea grant funded by the Ministry of Education of the Republic of Korea (2021R1I1A2048744). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Ministry of Education of the Republic of Korea: 2021R1I1A2048744.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Ratih Kusuma Wardani conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Ragib Ahsan performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Mann Kyoon Shin conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The newly sequenced species are available at GenBank: PP648189 to PP648201.

Data Availability

The following information was supplied regarding data availability:

The data is available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.18852#supplemental-information.

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