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Actin evolution in ciliates (Protist, Alveolata) is characterized by high diversity and three duplication events ☆

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ABSTRACT

Ciliates possess two distinct nuclear genomes and unique genomic features, including highly fragmented chromosomes and extensive chromosomal rearrangements. Recent transcriptomic surveys have revealed that ciliates have several multi-copy genes providing an ideal template to study gene family evolution. Nonetheless, this process remains little studied in ciliated protozoa and consequently, the evolutionary patterns that govern it are not well understood. In this study, we focused on obtaining fine-scale information relative to ciliate species divergence for the first time. A total of 230 actin gene sequences were derived from this study, among which 217 were from four closely related *Pseudokeronopsis* species and 13 from other hypotrichous ciliates. Our investigation shows that: (1) At least three duplication events occurred in ciliates: diversification of three actin genes (Actin I, II, III) happened after the divergence of ciliate classes but before that of subclasses. And several recent and genus-specific duplications were followed within Actin I (*Sterkiella*, *Oxytricha*, *Uroleptus*, etc.), Actin II (*Sterkiella*), respectively. (2) Within the genus *Pseudokeronopsis*, Actin I gene duplication events happened after *P. carnea* and *P. erythrina* diverged. In contrast, in the morphologically similar species *P. flava* and *P. rubra*, the duplication event preceded diversification of the two species. The Actin II gene duplication events preceded divergence of the genus *Pseudokeronopsis*. (3) Phylogenetic analyses revealed that actin is suitable for resolving ciliate classes, but may not be used to infer lower taxon relationships.

1. Introduction

Actin is a ubiquitous protein in eukaryotic cells, participating in many essential cellular processes, such as cytoskeletal structure, maintenance of cell shape, cell motility, cell division, endocytosis and intracellular transport (Kaksonen et al., 2006; Sehring et al., 2007; Zheng et al., 2009). In animals, plants and many protozoans, multigene families typically encode for actin (Bhattacharya et al., 2000; Carlini et al., 2000). The number and organization of the actin gene family among different organisms varies. Vertebrates and arthropods contain up to six different actin genes (Hooper and Thuma, 2005; Lahr et al., 2011), while plants possess dozens of them (McCurdy et al., 2001). In microbial eukaryotes such as amoebae (Lahr et al., 2011), red algae (Tanifuji and Archibald, 2010), and dinoflagellates (Bachvaroff and Place, 2008), evolutionary patterns of actin genes are variable and large families are often present. In ciliates, 25 highly divergent genes have been found in the *Paramecium tetraurelia* somatic genome (Sehring et al., 2007).

Ciliates are microbial eukaryotes with two types of nuclei: a germline micronucleus (MIC) and a somatic macronucleus (MAC). Chromosomal fragmentation as well as gene duplication influenced by both Mendelian inheritance and epigenetic phenomena occur during the formation of the macronucleus (Kim et al., 2011; Robinson and Katz, 2007), which results in extremely high copy numbers of genes (Jahn and Klobutcher, 2002). And recent transcriptomic surveys (Gentekaki et al., 2014; Feng et al., 2015) have revealed that ciliates have genes with paralogs spanning the eukaryotic tree or specific groups, and might be an ideal template to study gene family evolution. The actin gene family of the phylum Ciliophora is highly divergent with the number of actin genes in the somatic genome varying not only among the different ciliate lineages but also within the same lineage (Kim et al., 2004; Pérez-Romero et al., 1999; Zufall et al., 2006). For example, the somatic genome of *Sterkiella nova* (Croft et al., 2003; Dizick and Prescott, 1999; Dubois and Prescott, 1995; Mitcham and Prescott, 1994) and *S. histriomuscorum* (Dubois and Prescott, 1997; Swart et al., 2013) contains three and two actin genes respectively, all of which

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show high divergence even within the same species (Dizick and Prescott, 1999). Though the high diversity of actin genes of ciliates is known for a long time, we still know little about their diversification and evolution since only a few species have been studied. To our knowledge, there has been no investigation focusing on duplication of the actin gene family for morphologically closely related ciliate species, which will reveal fine-scale evolutionary patterns.

Here, we focus on hypotrichous species, a group of spirotrichean ciliates possessing highly processed macronuclear genomes (Zufall et al., 2006). We examined the number and variation of actin genes in four closely related species of *Pseudokeronopsis* and eight additional species within the subclass Hypotrichia. Our aim was to survey duplication events of the actin gene family in Hypotrichia and assess the evolutionary patterns that underline them. We also examined whether actin is an appropriate phylogenetic marker for resolving the evolutionary relationships within Hypotrichia.

2. Material and methods

2.1. Sample collection and cultures

Ciliate populations used in this study are listed in Supplementary material, Table S1. All populations were cultured at room temperature in artificial seawater and a rice grain was used to enrich bacterial growth (Chen et al., 2015; Zhang et al., 2014; Zhao et al., 2015). Three clonal lines of *Pseudokeronopsis rubra* p4 were generated by isolating single cell, respectively. In total eight populations of *Pseudokeronopsis* were used in this study: four for *P. rubra*, two for *P. flava* and one for each of *P. erythrina* and *P. carnea*.

For morphological identifications, we employed live microscopy and staining methods as described in previous investigation (Liu et al., 2015; Pan et al., 2015). We provided SSU rDNA sequences for all eight *Pseudokeronopsis* populations used in this study. All populations of the same species share the same SSU rDNA sequences, while sequences of different species are distinct from each other. And these new sequences also match those for same species in GenBank (Supplementary material, Fig. S1a).

2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, but the dosage of each reagent was modified to only 1/4 of the suggested volume (Chen et al., 2015).

The following primers were used in amplification—ACTII: ACTIIF 5′-AACTGGGAYGAYATGGARAAGAT-3′ and ACTIIR 5′-ATCCA CATYTGYTGGAANGT-3′ (Zufall et al., 2006); ACTI-1: ACTI-1F 5′-T CAGGARTGKTYAARGCYGG-3′ and ACTI-1R 5′-GAYRCAYTTTCKRT GRACGAT-3′(Pérez-Romero et al., 1999); ACTI-2: ACTI-2F 5′-AACT GGGAYGAYATGGARAAGAT-3′ and ACTI-2R 5′-ATCCACATKSHGGC GAAGGT-3′ (revised from Tekle et al. (2007)) (Supplementary material, Fig. S1b). The amplified fragment size of ACTI-1, ACTI-2 and ACTII was 1048, 799 and 796 bp (excluding primer sequence) predicted to encode amino acid sequences of 349, 266 and 265 sites, respectively. In addition, because only one sequence of hypotrichous species (*Sterkiella nova*) was annotated as Actin III and this sequence fell into the Actin I clade in our phylogenetic trees, we did not design primers to amplify Actin III of hypotrichous species. Consequently, we selected two pairs of actin primers (ACTII and ACTI-2) to amplify actin gene of seven populations of other hypotrichous genera (Supplementary material, Table S1). In order to explore whether genetic difference were present among different clonal lines in a population, we chose ACTII to amplify the actin gene of three clonal lines of *P. rubra* p4, all of which were classified into same paralog. Therefore, we performed no more experiments detecting inter-clonal genetic divergences.

In order to reduce the impact of PCR-mediated recombination and improve accuracy, we minimized the initial template concentration and the number of cycles during our preliminary experiments. Additionally, we used Thermo ScientificTM Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Espoo, Finland), for which mismatch is only 4.4×10^{-7}. The amplification conditions were initial denaturation at 98 °C for 3 min; followed by 30 cycles of denaturation at 98 °C for 15 s, annealing at 54 °C for 15 s, and extension at 72 °C for 90 s; and final extension at 72 °C for 10 min. Reactions were run in total volume of 50 μl, using concentrations of reagents per manufacturers recommendations (1X Phusion HF buffer, 0.2 mM each deoxyribonucleotide triphosphate, 0.5 μM each primer and 0.02 U/μl polymerase). The final concentrations of template DNA were 0.5–1 ng/μl. For *P. flava* p1, *P. flava* p2, and *P. rubra* p1, ACTII was used to amplify actin gene with three parallel PCR replications with the same templates and amplification conditions respectively. Since sequences of different PCR replications for each population were assigned into the same paralog, no more primers or populations were selected for further experiment. This result indicates that genetic divergences within a population are not PCR-mediated.

The PCR products were visualized with agarose gel electrophoresis and purified using Universal DNA purification kit (TIANGEN, Beijing, China). Purified PCR products were cloned with the pEASY-Blunt Cloning Vector and Trans 1-T1 Phage Resistant Chemically Competent Cells (TransGen, Beijing, China). All plasmids containing inserts were purified using a QIAGEN plasmid mini kit (Qiagen, Hilden, Germany). A total of 230 positive colonies were sequenced with M13F and M13R sequencing primers on an ABI 3730 sequencer at the Beijing Genomics Institute (BGI) in Shenzhen, China. In order to obtain different actin genes from each population, 5–10 clones from each population-primer-specific PCR cloning reaction were sequenced.

2.3. Chimera check, genetic distances and phylogenetic analysis

We used the USEARCH method implemented in Qiime 1.8 (Caporaso et al., 2010) to perform de novo chimera check, and no chimera was detected in our new sequences. Subsequently, all sequences were used in downstream analyses. Paralogs defined with 99% nucleotide sequence similarity were selected with Qiime 1.8 (Caporaso et al., 2010). And then only one representative sequence of each paralog was used to construct phylogenetic trees.

Genetic distances within each population and between populations were calculated with MEGA 5.0 (Tamura et al., 2011). Mean pairwise nucleotide distances were calculated using the Kimura 2-parameter correction model (Kimura, 1980), and mean pairwise amino acid distances were calculated using the Poisson correction parameter (Nei and Kumar, 2000).

To explore the relationship among Actin I, II and III of hypotrichous species, as well as their relationship to other ciliate groups, phylogenetic trees were constructed with 30 hypotrichous nucleotide sequences along with an additional 25 sequences from representative species of other ciliate lineages (Figs. 1 and 2). We selected two populations of *Cyanidioschyzon merolae*, a species of Rhodophyta as the outgroup taxa. Multiple sequence alignments of all the actin nucleotide sequences and actin amino acid sequences were constructed with CLUSTAL W implemented in BioEdit 7.0.9.1 (Hall, 1999), and then manually modified. A nucleotide Maximum Likelihood (ML) analysis was constructed with the RAxML online program (http://www.phylo.org/). Searches for the best tree were conducted starting from 1000 random trees, and bootstrapping was incorporated with 1000 replicates (Miller et al., 2010) under the GTR + I + G model of nucleotide substitution