

Doctoral Thesis

Microbiomics and phylogeography of bacteria
associated with *Umbilicaria* and related
rock tripe lichens

(Summary)

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(イワタケ類地衣類に共在する微生物のマイクロバイオーーム解析および系統地理学的研究)

Lichens (地衣類) are one of the toughest organisms on the Earth and are living in most diverse habitats on terrestrial biosphere. They look as single organisms but are composites of filamentous fungi and photosynthetic organisms (algae and/or cyanobacteria), namely mycobionts and photobionts, respectively, in a symbiotic association. Mycobionts provide filamented protection to photobionts from environmental stresses such as UV radiation and desiccation, while in return photobionts provide photosynthetically produced organic nutrients to heterotrophic mycobionts. They benefit each other, and thus the symbiotic association has often been regarded as a good example of mutualistic symbiosis. In addition to the fungal and algal, both eukaryotic partners and photosynthetic cyanobacteria, possible involvement of non-photosynthetic prokaryotic agents, i.e., bacteria, in the symbiosis has arisen as a recent topic in microbial ecology as well as in lichenology. This doctoral study and the doctoral thesis aimed at phylogenetic characterizations (or microbiomics) of lichen-associated bacteria, as well as of lichen-forming fungi and algae, as a possible third component of the lichen symbiosis and biogeographic (or phylogeographic) profiling of lichen samples collected from a global-wide, namely Pole-to-Pole, range of lichen habitats. This doctoral thesis consists of five chapters: Chapter 1, Introduction; Chapter 2, Materials and Methods; Chapter 3, Results and Discussion; Chapter 4, General Discussion; and, Chapter 5, Conclusions and Future Prospect, as explained below.

Chapter 1 Introduction give general biological characteristics of lichens whose bulk (visible) bodies are composed of about 17,000-20,000 species of lichen-forming fungi; and, the lichen-forming fungi comprises of approximately 20% of the total fungal species. The largest and almost entirely lichen-forming fungal taxonomical class is the Lecanoromycetes with about 14,200 known lichen species. Although lichen is a composite of fungi, algae and/or cyanobacteria, the scientific name for lichen is the same as that of the fungi (mycobiont). About 90% of lichens have less diverse green algal photobionts, of which algal genera *Trebouxia* and *Trentepohlia*, along with cyanobacterial genus *Nostoc* are the major photobionts. Generally, lichens are known to be bipartite (two-membered) of which, lichens that have green algal photobionts comprised of about 85%, approximately 10% of

lichens have cyanobacterial photobionts and about 3% comprises of both photobionts, hence forming tripartite (three-membered) lichens. This chapter also introduced rock tripe, the fungal genus *Umbilicaria* which belongs to the family Umbilicariaceae, in the order Umbilicariales within the sub-class Umbilicariomycetidae of the Fungi kingdom. The world-wide distributed *Umbilicaria*, which is the target specimen used in this study are highly distributed at the intermediate latitudes of both the Northern and Southern Hemispheres and decreases in diversity around equator and the bipolar regions. Then, the possibility of bacteria as a third agent of lichen-formers is explained in this chapter, with a speculation that lichen-associated bacteria may play significant roles such as N₂ fixers, phosphate-solubilization, organic materials degraders and antibiotic producers. If there would be lichen-symbiotic bacteria, they would always be associated with lichens despite the geographic variety of the host lichens. Search for bacterial phylotypes that always co-exist with lichens was therefore validated. On the other hand, such phylotypic search on the lichens collected all over the world would reveal geographic patterns in co-existing bacterial phylotypes (or microbiomes), leading to a hypothesis of phylotypic biogeography, or phylogeography.

Chapter 2 Materials and Methods describes the lichen samples collected from many places of the world as well as environmental settings of the sampling site, ranging from Arctic and sub-Arctic cryospheres, a glacier on the top of a 5000 m-mountain on the Equator in Uganda, the top of the table mountains in Guiana, a high arid region in South Africa, and to Antarctic ice-free fellfields. From these various sites, only the “rock tripe” lichens (岩茸) were selectively collected because they are easy to identify and easy to sample by cutting at the single attachments to rocks. The representative rock tripe genus is *Umbilicaria*, whose name came from “umbilical” after their single attachments to rocks. Total >40 rock tripe lichen samples (out of >100 specimens) were used and analyzed. The methods used in this study were mainly DNA sequencings (conventional Sanger-sequencing and next-generation MiSeq-sequencing) of four target genomic sequences (18S rRNA gene, eukaryotic ITS region, 16S rRNA gene, and V3-V4 region of 16S rRNA gene) and bio-informatic treatments and analyses of the obtained sequences. Phylotypes were generated by grouping similar sequences (having 97% or higher similarities) and compared for phylotype compositions (microbiomics) and site-to-site variations (phylogenetics) by cluster analyses to draw phylogenetic trees, dendrograms and heatmaps.

Chapter 3 reports the results of DNA sequencings of the rock tripe lichen samples, which are ~4,000,000 sequences, phylotypes, microbiomic and phylogeographic features. With the massive

sequences, this chapter is comprised mainly of three parts. The first part provides a introductory representation of phylotypes of a typical rock tripe lichen-forming mycobionts (fungi) and photobionts (algae and/or cyanobacteria) as exemplified with Antarctic *Umbilicaria*, which revealed unexpected cryptic diversities in the phylotypes of algae-derived chloroplasts and in non-typical (non-*Nostoc*) cyanobacterial associates in lichens. In contrast, the phylotypes of eukaryotic photobionts were all affiliated with only two green algal genera of *Trebouxia* and *Coccomyxa*, compared with the prokaryotic photobionts (cyanobacteria) diversity. The second part continues with the Antarctic *Umbilicaria* but targets at bacterial phylotypes that was dominated by the phylotypes affiliated with the genus *Mucilaginibacter* of the phylum Bacteroidetes. And the third unveiled the massive bacterial phylotypes associated with rock tripe lichens from worldwide sites and revealed their microbiomic and phylogeographic features in detail.

Chapter 4 General Discussion proposes rather challenging hypothesis about flexibility or plasticity in the fungal-algal and algal-chloroplast partnerships. The multiple and plastic “polygamous” partnership hypothesis challenges the traditional “monogamous” view of lichens consisting of one-fungi and one-alga. Moreover, the cryptic diversity of chloroplasts in the “one-alga” leads to a new and original idea of adaptation via chloroplast selection. This point was included in the article manuscript submitted to *Microorganisms*. In addition, biogeographic (or phylogeographic) consideration on the lichen microbiomes leads to the idea of a biogeographic boundary between Antarctic and non-Antarctic sites. Bacterial phylotypes associated with the Antarctic lichens were commonly predominated by *Mucilaginibacter*, a genus of the phylum Bacteroidetes, while bacterial phylotypes associated with the non-Antarctic lichens were dominated by Proteobacteria. Dendrograms demonstrated clear separation of Antarctic clusters from those of other sites and ambiguous-but-visible, site-specific clusters for other sites.

Chapter 5 Conclusions and Future Prospects clarify the biological and ecological meanings of the new findings in the thesis as highlighted as follows: 1) Cryptic diversities in chloroplasts and cyanobacteria in lichens were revealed by the next-generation massive parallel sequencing by the MiSeq high throughput platform as well as the conventional Sanger sequencing; 2) Bacterial microbiomes that are associated with rock tripe lichens inhabiting various locations on the Earth were characterized for comparative analyses; and 3) Biogeographic boundary, such as the Wallace Line in animal biogeography, was first proposed for bacterial biogeography or phylogeography between Antarctica and non-Antarctica localities. More findings on cryptic diversities in bacterial phylotypes

and candidate lichen-symbiotic bacteria are focused as future prospect, particularly in terms of biotechnological applications to production of bioactive and/or antibiotic substances. Unexpected dominance of *Microcoleus* in the cyanobacterial phylotypes associated with the Antarctic *Umbilicaria* may also add previously unknown significance of the genus in lichen biology and ecology and thus contribute to future prospect. In addition, the importance of continued field surveys and samplings, particularly in the central Asia, Australia and South America, is added to the future prospect to increase resolution of bacterial biogeography or phylogeography.

The first part of Chapter 3 and part of Chapter 4 were collectively bound as an article published in the academic journal *Microorganisms*. Other parts of the thesis are also being bound as separate article manuscripts to be submitted to corresponding academic journals, as well as reported as technical reports and book chapters. And, the thesis and derived publications may help younger generations in my local islands, as well as globally in every community, widening their views and rowing to vast external world.