POLAR RESEARCH, 2017
VOL. 36, 1374123
https://doi.org/10.1080/17518369.2017.1374123

RESEARCH ARTICLE

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Patterns of fungal–algal symbiont association in *Usnea aurantiaco-atra* reveal the succession of lichen–moss communities in Fildes Peninsula, Antarctica

Shunan Cao [a], Fang Peng [b], Hongyuan Zheng [c], Feng Wang [c], Chuanpeng Liu [d] & Qiming Zhou [d]

[a]Key Laboratory for Polar Science, State Ocean Administration, Polar Research Institute of China, Shanghai, China; [b]China Center for Type Culture Collection, College of Life Sciences, Wuhan University, Wuhan, China; [c]College of Environmental Science and Engineering, Tongji University, Shanghai, China; [d]School of Life Science and Technology, Harbin Institute of Technology, Harbin, China

ABSTRACT

Usnea aurantiaco-atra is the most widespread flora in Fildes Peninsula. There are two growth types of *U. aurantiaco-atra*: the erect form on rocks and the prostrate form associated with mosses. Phylogenetic analysis showed that individuals of the two growth forms share genotypes. Moreover, haploid disequilibrium testing indicated no significant genetic difference for the two growth forms when fungal and algal internal transcribed spacer rDNA were treated as two alleles of one lichen individual. The two growth forms of *U. aurantiaco-atra* appear to reflect different stages of lichen–moss community succession. A mode is proposed for demonstrating the occurrence of this succession.

KEYWORDS

Haplotype; ITS rDNA; linkage disequilibrium; mycobiont; populations; reproduction

ABBREVIATIONS

ITS: internal transcribed spacer; PCR: polymerase chain reaction

Lichens are pioneer organisms in harsh environments and may dominate the terrestrial vegetation. This is especially true in Antarctica, where lichens are the major contributors to biomass and diversity (Domaschke et al. 2012). Lichens are composed of fungal and algal symbionts that contribute to the thallus biomass and form the typical morphological characters. A relatively stable relationship exists between the fungal and algal partners over time. Lichens are formed when a fungal spore germinates and the hyphae encounter the correct algal partner. The hyphae grow and cover the algal cells, eventually resulting in formation of typical lichen structures and completing the lichenization process. This process is observed in lichens with sexual reproduction, such as those with apothecia. Lichens can also reproduce by vegetative growth and this is common in lichens with soredia, isidia or thallus fragments. Sexual reproduction is an opportunity for a new algal partner to be introduced into the original fungal partner. A horizontal transmission of photobiont can then occur (Nelsen & Gargas 2008; Dal Grande et al. 2012). In vegetative reproduction, both fungal and algal partners are transmitted vertically from the parental thallus to the new individual and the offspring genotype is unchanged from that of the lichen parent.

Linkage disequilibrium, a measure of the non-random association of alleles at different loci, is useful for studying the population genetic diversity differences in lichens or fungi that disperse via sexual and asexual reproduction (Walser et al. 2004; De Fine Licht et al. 2006; Molina-Montenegro et al. 2013). Selected gene markers often include ITS rDNA, β-tublin, EF-1α, group I intron in small subunit rDNA (Cassie & Piercey-Normore 2008; O'Brien et al. 2009). Higher genetic diversity is typically observed in populations with sexual reproduction (Molina-Montenegro et al. 2013). For the co-dispersion of mutualistic organisms, Werth and Scheidegger (2012) suggested that linkage disequilibrium could provide compelling evidence. Significant linkage disequilibrium has been observed between fungal and algal loci indicated mutual lichen thalli propagated by clonality, which indicated that the fungal and algal partners in mutual lichen thalli were vertically transmitted.

The Fildes Peninsula is located at the northern tip of Antarctica. It has a mean yearly temperature <0°C, sunshine duration < 600 h, and yearly rainfall/snow < 600 mm (Yang et al. 2013). It is an ideal natural area to study flora succession resulting from glacial retreat, ice melt and rising sea levels. Conditions around this island present a transition from a glacial to a pedogenic geosystem. Only one vascular plant – *Deschampsia antarctica* Desv. – has been observed on the island but at least 120 lichen species have been reported (http://www.aari.aq/KGI/Vegetation/lst_lichens.html). The marshy grassland area here is a unique feature of the Antarctic terrestrial continent and the plant community is primarily composed of lichens, especially *Usnea arurantiaco-atra* (Jacq.) Bory, and mosses.

CONTACT Qiming Zhou genbank@vip.sina.com School of Life Science and Technology, Harbin Institute of Technology, 2 Yikuang Street, Nangang Distinct, Harbin150080, China

Supplemental data for this article can be accessed here.

Usnea aurantiaco-atra (formerly *U. fasciata* Torr. or *Neuropogon aurantiaco-ater* [Jacq.] I.M. Lamb in older literature) is the most conspicuous lichen in Fildes Peninsula. There are two common growth types of this lichen. The erect type grows on rocks and often harbours apothecia. The prostrate type is attached to moss tufts and lacks apothecia. *Usnea antarctica* Du Rietz is another widespread species in this region; it grows erect and seems to reproduce via the vegetative structures soredia. Recent studies, using molecular data, demonstrated that *U. antarctica* should be included in *U. aurantiaco-atra* (Seymour et al. 2007). Hence, *U. antarctica* is treated as a erect growth type of *U. aurantiaco-atra* in this study. The coexistence of different growth forms in the same location make *U. aurantiaco-atra* a good candidate for exploring transformation and evolution of the growth forms.

In the current study, *U. aurantiaco-atra* with different reproduction and growth forms were collected around Fildes Peninsula and the ITS rDNA of both fungal and algal partners were sequenced. The relationship between the erect and prostrate types of *U. aurantiaco-atra* was studied using haplotype and linkage disequilibrium analysis based on ITS rDNA data. The ITS rDNA of *U. aurantiaco-atra* fungal and algal symbionts were set as alleles and the linkage disequilibrium of these alleles was investigated. We assumed that if linkage disequilibrium (between fungal ITS and algal ITS regions) was not observed within the prostrate forms with strictly vegetative reproduction nor within the erect sexual reproduction populations, there should be an evolutionary succession where the erect *U. aurantiaco-atra* was replaced by the prostrate form coinciding with the appearance of moss. Based on these analyses, a possible lichen–moss communities succession is detailed.

Material and methods

A total of 132 *U. aurantiaco-atra* individuals were collected from 12 sites around Fildes Peninsula, Antarctica (Fig. 1; Supplementary Table S1).

The total DNA was exacted using a modified cetyl trimethylammonium bromide method (Cao et al. 2015). The primer pairs used are listed in Supplementary Table S2. The sequences retrieved from GenBank are listed in Supplementary Table S3. A 50 µL PCR reaction system was used which consisted of 5 µL amplification buffer (containing 25 mmol L^{-1} of $MgCl_2$), 1.25 units of Taq DNA polymerase (TaKaRa Biotechnology Co. Ltd.), 4 µL 2.5 mmol L^{-1} of each dNTP, 2 µL10 µmol L^{-1} of each primer, 6 µL of diluted template DNA and 33 µL H_2O. The PCR amplification conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 2–4 min. These cycles were followed by a final extension at 72°C for 10 min. An ABI3730XL Sequencer was used and double-stranded PCR products were sequenced.

The SEQMAN program within Lasergene version 7.1 software package (DNASTAR Inc.) was used to check and assemble the double-directional sequence

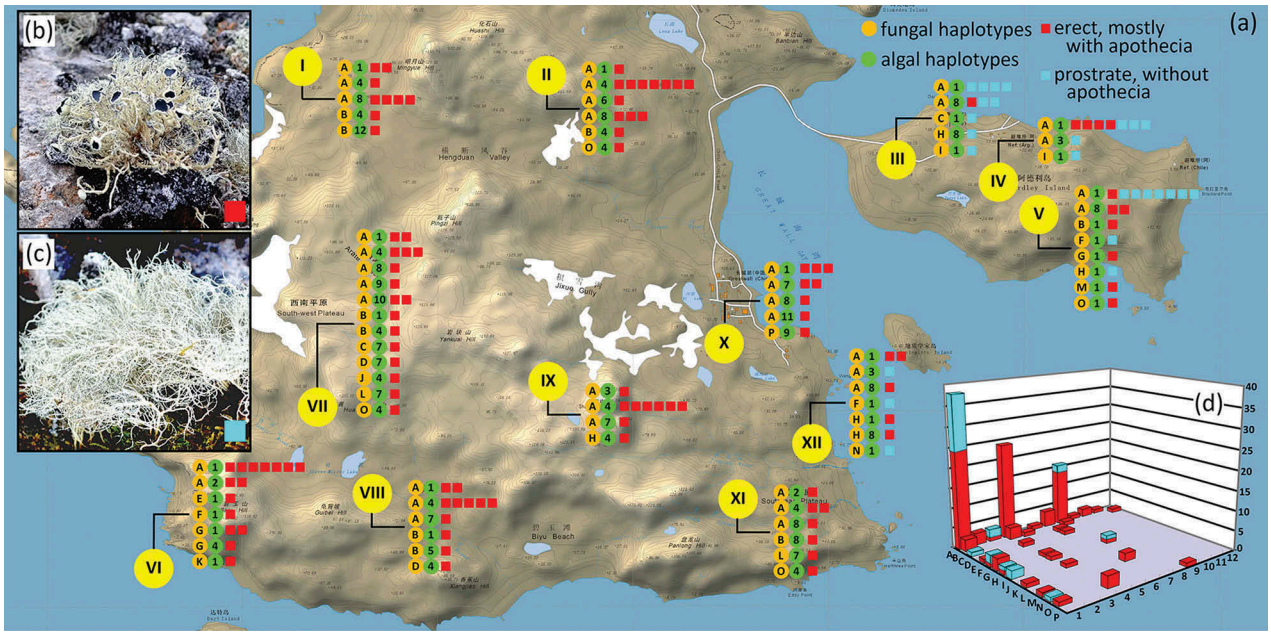

Figure 1. Distribution of *Usnea aurantiaco-atra* symbionts (fungal and algal) genotypes and sampling sites. (a) Map of the Fildes Peninsula. Twelve sampling sites are marked with Roman numerals from I to XII. The fungal ITS rDNA haplotypes are represented by capital letters, A – P. The algal ITS rDNA haplotypes are represented by Arabic numerals, 1 – 12. Most erect individuals had apothecia whereas prostrate individuals lacked them. (b) Typical *U. aurantiaco-atra* with apothecia, erectly growing on rocks. (c) Typical prostrate *U. aurantiaco-atra*, growing with moss. (d) Fungal–algal genotypes distribution displayed as a column diagram.

data. The small subunit and large subunit regions of rDNA were trimmed off. Preliminary alignment of the fungal ITS rDNA sequences together with those retrieved from GenBank was performed using ClustalW algorithm within MEGA 5 and then adjusted manually (Tamura et al. 2011). The same operation was performed for the algal ITS rDNA sequences. The fungal and algal ITS haplotype networks were calculated using TCS 2.1 (Clement et al. 2000), respectively (Supplementary Fig. S1).

The linkage disequilibrium of ITS rDNA between fungal and algal symbionts was calculated using GenAlEx 6.502 (Peakall & Smouse 2012). Nucleotide diversity, genetic diversity and molecular diversity indexes were calculated using Arlequin 3.5 (Excoffier & Lischer 2010).

Results and discussion

The fungal and algal ITS sequences for all 132 samples were submitted to GenBank (Supplementary Table S1). Fungal ITS rDNA sequences clustered into one branch as *U. aurantiaco-atra* supported with 99 bootstraps (Supplementary Fig. S1a). Meanwhile, all the algal ITS rDNA assigned as *Trebouxia jamesii* (Supplementary Fig. S1d).

Both the phylogenetic analyses and haplotype network results revealed 16 haplotypes for the fungal symbiont (marked from "A" to "P," Supplementary Fig. S1b). A total of 14 haplotypes were identified from the erect growth types, 10 of which were unique (haplotypes B, D, E, G, J, K, L, M, O and P). Six haplotypes were identified from the prostrate growth types, two of which were unique (haplotypes I and N). Four fungal ITS haplotypes were shared by both growth types (haplotypes A, C, F and H) (Fig. 1a, d). Both the phylogenetic analyses and haplotype network results showed 12 haplotypes for the algal symbiont (marked from "1" to "12," Supplementary Fig. S1d). All the 12 algal ITS haplotypes have been identified from the erect growth types, and three haplotypes were identified from the prostrate growth types (haplotypes 1, 3 and 8) (Fig. 1a, d).

The most widespread *U. aurantiaco-atra* genotype was "A1" ("A" is the fungal haplotype and "1" is the algal haplotype). Thirty-eight of 132 individuals possessed genotype A1, and 24 of them appeared within the erect populations. A total of 14 individuals were prostrate forms without apothecia (Fig. 1a, d), Supplementary Table S1). *Usnea aurantiaco-atra* individuals with two distinct growth types not only share the same algal partner *T. jamesii* but also share the same genotypes. For example, individuals III-02 and III-08 share the same genotype "A8", but the former has an erect growth type and the latter has a prostrate growth type (Fig. 1a, d), Supplementary Table S1).

Arlequin results showed that there was only minor molecular diversity difference between two *U. aurantiaco-atra* growth forms. For the fungal ITS rDNA (498 bp after alignment), prostrate *U. aurantiaco-atra* showed relatively higher nucleotide diversity (0.0021 ± 0.0016), genetic diversity (0.5200 ± 0.1143) and molecular diversity indexes (1.022) than those with erect growth type (0.00160 ± 0.0013; 0.4918 ± 0.0590; 0.787, respectively). For the algal ITS rDNA (663 bp after alignment), *U. aurantiaco-atra* with prostrate growth had relatively lower nucleotide diversity (0.0023 ± 0.0016), genetic diversity (0.3415 ± 0.1104) and molecular diversity indexes (1.523) than individuals with erect growth (0.0023 ± 0.0016; 0.7585 ± 0.0238; 1.548, respectively). For the fungal—algal ITS rDNA (1161 bp after alignment), *U. aurantiaco-atra* with prostrate growth had a relatively higher nucleotide diversity (0.0022 ± 0.0014), molecular diversity index (2.545), and relatively lower genetic diversity (0.7077 ± 0.0946) than individuals with erect growth (0.0020 ± 0.0012; 0.8803 ± 0.019; 2.335, respectively).

The ITS rDNA from fungal and algal partners were treated as one allele to estimate the combination pattern of the symbionts, and to test the difference of haploid disequilibrium between the erect and prostrate types (Table 1). There was no significant difference between the erect type ($p = 0.649$) and prostrate type ($p = 0.704$). This result indicated the fungal and algal symbionts within the two *U. aurantiaco-atra* growth types were randomly matched. Haploid disequilibrium was not observed in the prostrate individuals lacking apothecia.

Discussion

Although there are two distinct growth types of *U. aurantiaco-atra*, the lichen-forming fungi and its algal partner were identified as one species, respectively, based on ITS rDNA analyses (Supplementary Fig. S1). Molecular diversity indicated only minor differences between these two growth types (with and without apothecia).

The ITS rDNA of *U. aurantiaco-atra* fungal and algal symbionts were set as alleles and the linkage disequilibrium of these alleles was investigated. We supposed that the fungal and algal partners were transmitted vertically (clonally) if significant linkage

Table 1. Haploid disequilibrium test of *Usnea aurantiaco-atra* symbionts (haploid disequilibrium, 999 randomizations).

Growth type	No. samples	Ve[a]	Vo[b]	Vo/Ve[c]	No. permutations	P (Vr ≥ Vo)[d]
Erect	106	0.437	0.447	1.024	999	0.649 (ns)
Prostrate	26	0.471	0.448	0.951	999	0.704 (ns)

[a]The expected variance. [b]The observed variance. [c]The index of linkage disequilibrium. [d]Vr calculated for each random sample as the variance of the randomized data set and the probability of observing a Vr value as extreme as that measured for the original data (Vo).

disequilibrium of the alleles was observed. When *U. aurantiaco-atra* propagated by sexual reproduction, the linkage disequilibrium could not be observed within offspring because of the recombination of fungi and algae during lichenization. There are two distinct growth types in *U. aurantiaco-atra*, and reproduction within these two growth forms was obviously different. Individuals with erect growth may propagate with apothecia (sexual reproductive structure) or soredia, but individuals with prostrate growth lack such structures and probably undergo dispersal via thallus fragments. The linkage disequilibrium values of the assumed allele (ITS rDNA of fungal and algal partner) of these two *U. aurantiaco-atra* populations were similar. These data suggested an explanation of the evolutionary succession that may have occurred in the Fildes Peninsula.

Since the fungal partner of the sampled lichen thalli was confirmed to be one identical species (*U. aurantiaco-atra*), the offspring thalli of *U. aurantiaco-atra* with sexual reproduction produced by ascospore-lichenization and the offspring thalli of *U. aurantiaco-atra* with vegetative reproduction produced clonally (fungal and algal partners co-dispersal) could be distinguished from their population structures and the linkage disequilibrium values. Linkage disequilibrium (between fungal ITS and algal ITS regions) was not observed within the populations with sexual reproduction. In contrast, an obvious linkage disequilibrium was observed within the prostrate forms that reproduce vegetatively.

The value of linkage disequilibrium within the prostrate population was similar to that of the erect populations. This suggests that these two populations had a similar "sexual ancestor." Combining the observations from the local micro-environment, we propose an evolutionary succession model of lichen–moss communities (Fig. 2). Initially only *U. aurantiaco-atra* can grow on gravel (Fig. 2a, d); after soil appears following the growth of *U. aurantiaco-atra*, moss colonizes the area (Fig. 2b, d); The environmental factors are changed by the development of mosses and humidity increases within the moss vegetation. As a direct result, *U. aurantiaco-atra* detaches from the gravel because the root portion of lichen thalli may decay in high humidity micro-environment (Fig. 2c, d). The prostrate growth form of *U. aurantiaco-atra* degrades as a result of moss competition (Kappen & Redon 1987). Unattached *U. aurantiaco-atra* must grow in direct contact with mosses or else winds would blow the thalli into the sea. As a result, a long and curly plant morphology evolves (Fig. 2d). The prostrate thalli never bear fruiting bodies and they are less productive than erect form individuals (Kappen 1985). This suggests that the prostrate individuals are actually "the degraded" or "root rotten-thalli unattached" status of those with erect form, which explains why a similar linkage disequilibrium value was observed within both populations.

We consider the ITS rDNA of the fungus and its algal partner as a pair of alleles. The prostrate individuals of *U. aurantiaco-atra* were expected to exhibit a strict linkage for ITS rDNA of the two partners if they originated from sterile ones by vegetative reproduction such as lichen fragment dispersal. Such a linkage would not be observed in erect *U. aurantiaco-atra* because most individuals were considered to have derived from the joint union of a parent fungal spore and its photobiont; a random match occurred when these parent fungal spore met a compatible algal partner. We could also conclude that the reproduction of the lichen populations experiences the evolution of horizontal transmission if no linkage occurs between the fungal and algal partners.

Molina-Montenegro et al. (Molina-Montenegro et al. 2013) reported the nurse effect of *U. antarctica*

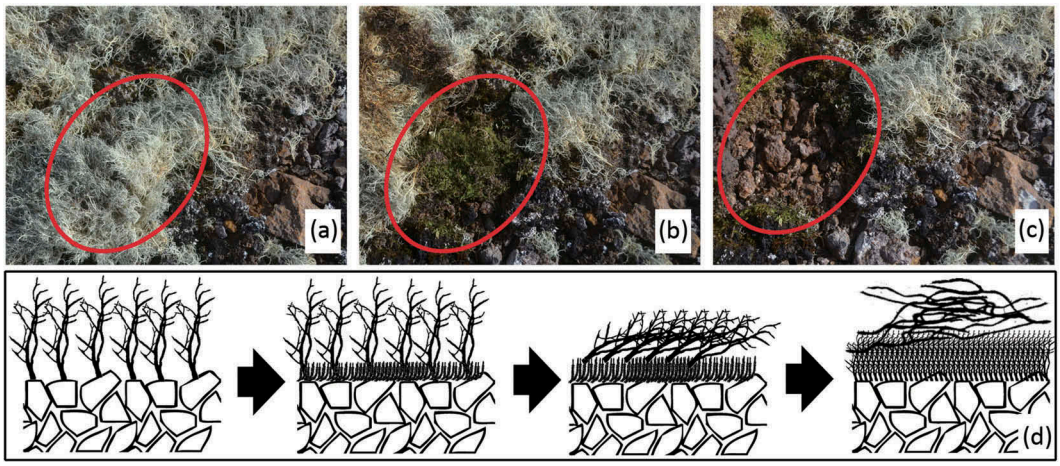

Figure 2. Photographs of (a) tangled prostrate *Usnea aurantiaco-atra*, (b) moss beneath *U. aurantiaco-atra* (tangled prostrate removed), and (c) gravel beneath moss (moss removed); and (d) diagram illustrating the succession of lichen–moss communities.

in which cushions (formed by prostrate *Usnea*) ameliorate the extreme conditions of Antarctic islands by increasing temperature, soil moisture and nutrient availability, decreasing radiation, and water loss from evaporation. We noted moss cushions under the tangled prostrate *U. aurantiaco-atra* in most circumstances, and the lowest substrates were gravels (Fig. 2a–c). Hence, a succession model of lichen–moss communities with different stages is presented (Fig. 2d). Erect *U. aurantiaco-atra* is ancestral and derived from lichenized fungal spores and compatible algal partners. The prostrate type is a degraded type of the erect type, and the diffusion method for prostrate individuals without apothecia is not vertical transmission, as revealed by the linkage disequilibrium analysis. A loss of vitality for prostrate type individuals would be expected because they are degraded without roots attached to gravel and they have to adapt to a different micro-environment and bryophyte competition. The growth rate and the potential net photosynthesis of prostrate *U. aurantiaco-atra* are much lower than in the erect form (Elisabeth Tschermak-Woess 1988; Li et al. 2014).

Conclusion

The haploid disequilibrium test results indicated no linkage disequilibrium in the prostrate population of *U. aurantiaco-atra* and there was no significant difference between the erect and the prostrate growth types. Since linkage disequilibrium can be viewed as an indicator of reproduction mode in lichens, this result indicates that the prostrate individuals were not clonally derived. *Usnea aurantiaco-atra* without apothecia should be derived from sexual reproduction, similar to those with apothecia.

A succession model of Antarctica lichen–moss is proposed to explain the differentiation of the two *U. aurantiaco-atra* phenotypes. The micro-environment has an important influence on lichens that have phenotypic plasticity. This succession model is helpful for understanding lichen evolution in the harsh Antarctic environment.

Acknowledgements

We are indebted to the anonymous reviewers for valuable suggestions that improved this manuscript. We are grateful to the Chinese Arctic and Antarctic Administration for helping in carrying out the project at the Great Wall Station. Our research was facilitated by the Polar Samples resource-sharing platform (http://birds.chinare.org.cn/) and the Polar Science data-sharing platform (http://www.chinare.org.cn) maintained by the Polar Research Institute of China and the Chinese National Arctic & Antarctic Data Center.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by the International cooperation programme of the Chinese National Arctic Research Expedition (IC201514), Chinese Polar Environment Comprehensive Investigation and Assessment Programmes (no. CHINARE2016-02-01) and National Infrastructure of Natural Resources for Science and Technology Program of China (no. NIMR-2017-8); State Oceanic Administration [IC201514]; State Oceanic Administration [2012GW03003].

ORCID

Shunan Cao http://orcid.org/0000-0002-3264-851X
Qiming Zhou http://orcid.org/0000-0002-2892-6329

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