· Article ·
doi: 10.13679/j.advps.2015.4.00274

Advances in Polar Science
December 2015 Vol. 26 No. 4: 274-281

The various substrates of *Usnea aurantiaco-atra* and its algal sources in the Fildes Peninsula, Antarctica

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Received 4 July 2015; accepted 9 September 2015

Abstract The lichen species *Usnea aurantiaco-atra* (Jacq.) Bory is the most dominant vegetation on the Fildes Peninsula, Antarctica. Most individuals grow on rocks, and some are found with mosses. During the 27th and 28th Chinese National Antarctic Research expeditions of the Great Wall Station, *U. aurantiaco-atra* was observed growing on the lichen thallus of *Umbilicaria antarctica* Frey & I.M. Lamb, or on wood, which indicated that *Usnea aurantiaco-atra* could grow on various substrates. The diversities of the symbionts in *U. aurantiaco-atra* collected in the Fildes Peninsula were investigated using ITS rDNA sequences. The results showed that the sequences from mycobionts of *U. aurantiaco-atra* growing on various substrates did not exhibit significant differences. All photobionts in this lichen species were the green algae *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner. The identical sequences from the photobionts of both *Umbilicaria antarctica* and *Usnea aurantiaco-atra* indicated there was an algae pool in this area and different mycobionts could obtain their algal partners from this pool. The variety of substrates for *U. aurantiaco-atra* suggested its photobiont could be obtained from a mature lichen thallus by vegetative propagation; from other lichen thalli (e.g. *Umbilicaria antarctica*); or from the surroundings. This study will promote understanding of the distribution of photobionts and the process of lichenization.

Keywords **lichen, molecular systematics, phylogeny, photobiont pool, substratum preference**

Citation: Cao S N, Zheng H Y, Liu C P, et al. The various substrates of *Usnea aurantiaco-atra* and its algal sources in the Fildes Peninsula, Antarctica. Adv Polar Sci, 2015, 26: 274-281, doi: 10.13679/j.advps.2015.4.00274

1 Introduction

Lichen is a typical symbiotic association, comprising the lichenized fungus (mycobiont) and its photosynthetic partner (alga or cyanobacterium, photobiont). In this symbiosis, the photobiont provides carbon sources, such as polybasic alcohol (green algae) or glucose (cyanobacteria) by photosynthesis activity[1–3], and the mycobiont protects its photosynthetic partner from strong radiation and desiccation by enveloping the algae cells. In the lichen thallus, the sexual reproduction of the photobiont is inhibited[4] or suppressed[5]. However, the mycobiont has various ways of reproducing, such as by vegetative propagation (soredia or segment of the thallus), by a sexual procedure (the ascospores), or by an asexual method (the conidiospores). The ascospores of fungi must meet and recognize their compatible photobiont partner before they form a stabilized relationship developing into lichen thalli. This process is known as "lichenization". The sources of lichenized algae have attracted considerable attention because free photobionts are very rare in nature[6].

About 17500 lichenized fungi have been identified[7],

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but only 200 photosynthetic partners have been reported based on morphological studies (100 green algae and 100 cyanobacteria)[8]. This means that many different lichens must harbor the same photobiont. Conversely, some lichenized fungi may incorporate different algae as their photobionts[9-10], and it is believed that the mycobionts are able to adapt to various environments in this way[11-12].

There are two flowering plants, 104 mosses and about 427 lichens in Antarctica[13]. In the Fildes Peninsula, where the Great Wall Station is located, about 120 lichens have been reported (http://www.aari.aq/kgi/Vegetation/lst_lichens.html), of which the most dominant species is the fruticose lichen *Usnea aurantiaco-atra* (Jacq.) Bory. Most individuals of this species grow on rock, have erect and strong thalli, and apothecia; the minority grow with mosses, have thin and flattened thalli, and without apothecia. *Umbilicaria antarctica* Frey & I. M. Lamb, whose diameter could reach 20 cm, was the most abundant foliose lichen in this area. Lichen substrates provide the micro-environment for their survival and have some specificity[14]. For example, all species in the genus *Umbilicaria* Hoffm. were found to grow on rocks except *U. yunnana* (Nyl.) Hue; besides, wood is rarely also the substrate for some *Umbilicaria* lichens under harsh environmental conditions[15-16].

Antarctica is an ideal area for the study of the recognition and association between mycobionts and photobionts. During the 27th and 28th Chinese National Antarctic Research expeditions (CHINARE), a few *Usnea aurantiaco-atra* individuals were found growing on the lichen *Umbilicaria antarctica* and on wood. This provides a new insight in understanding the lichenization process.

ITS rDNA is one of the most widely used molecular markers in taxonomy, systematics and phylogenetics[17–19], and has been used as a DNA barcodes marker[20]. ITS rDNA was used to identify and analyze the phylogenetics of symbionts from *Umbilicaria antarctica* and *Usnea aurantiaco-atra* in Fildes Peninsula, Antarctica. Our study clarified the algal source in lichenization, and revealed the ecological function of the substratum preference.

2 Materials and Methods

2.1 Materials

2.1.1 Sampling

During the 27th and 28th CHINAREs, seven typical *Usnea aurantiaco-atra* individuals (two growing on rock, two on wood, two with moss and one on *Umbilicaria antarctica*) and seven *Umbilicaria antarctica* individuals (Table 1) were collected in the Fildes Peninsula and Ardley Island, Antarctica. Fragments of thalli (200–500 mg) from 280 (on rock) and 104 (with moss) individuals of *Usnea aurantiaco-atra* were gathered for molecular phylogenetic analysis. The sampling sites were marked using Google Earth 7.1.2.2041 (Google Inc., USA) (Figure 1).

2.2 Methods

2.2.1 Morphology

Typical individuals of *Usnea aurantiaco-atra* (with well-grown thalli and clear features such as apothecia or soredia) and the special individuals (growing on other substrates such as woods or other lichens), together with *Umbilicaria antarctica* individuals, were photographed and collected. The morphological features were investigated using a SMZ–168 stereo zoom microscope (Motic China Group Co., LTD.) in the Scientific Research Building of the Great Wall Station.

Table 1 Samples used in this study

Sample ID	Species	BIRDS No.	Substrates	GenBank Accession No.	
				Mycobiont	Photobiont
AG017	*Umbilicaria antarctica*	2131C0001ASBM200010	Rock	KR053307	KR053350
AG019	*Umbilicaria antarctica*	2131C0001ASBM000082	Rock	KR053308	KR053351
AG023	*Umbilicaria antarctica*	2131C0001ASBM000083	Rock	KR053309	KR053352
AG024	*Umbilicaria antarctica*	2131C0001ASBM000085	Rock	KR053310	KR053348
AG028	*Umbilicaria antarctica*	2131C0001ASBM000081	Rock	KR053311	KR053349
AG035	*Umbilicaria antarctica*	2131C0001ASBM200009	Rock	KR053312	KR053353
AG041	*Umbilicaria antarctica*	2131C0001ASBM200013	Rock	KR053313	KR053354
AG235	*Usnea aurantiaco-atra*	2131C0001ASBM000068	Woods	KR053314	KR053358
AG236	*Usnea aurantiaco-atra*	2131C0001ASBM000069	Woods	KR053315	KR053359
AG247	*Usnea aurantiaco-atra*	2131C0001ASBM000070	Moss	KR053316	KR053355
AG249	*Usnea aurantiaco-atra*	2131C0001ASBM000071	Moss	KR053317	KR053356
AG251	*Usnea aurantiaco-atra*	2131C0001ASBM000072	Rock	KR053318	KR053357
AG282	*Usnea aurantiaco-atra*	2131C0001ASBM000074	*Umbilicaria*	KR053319	KR053360
AG297	*Usnea aurantiaco-atra*	2131C0001ASBM000073	Rock	KR053320	KR053361

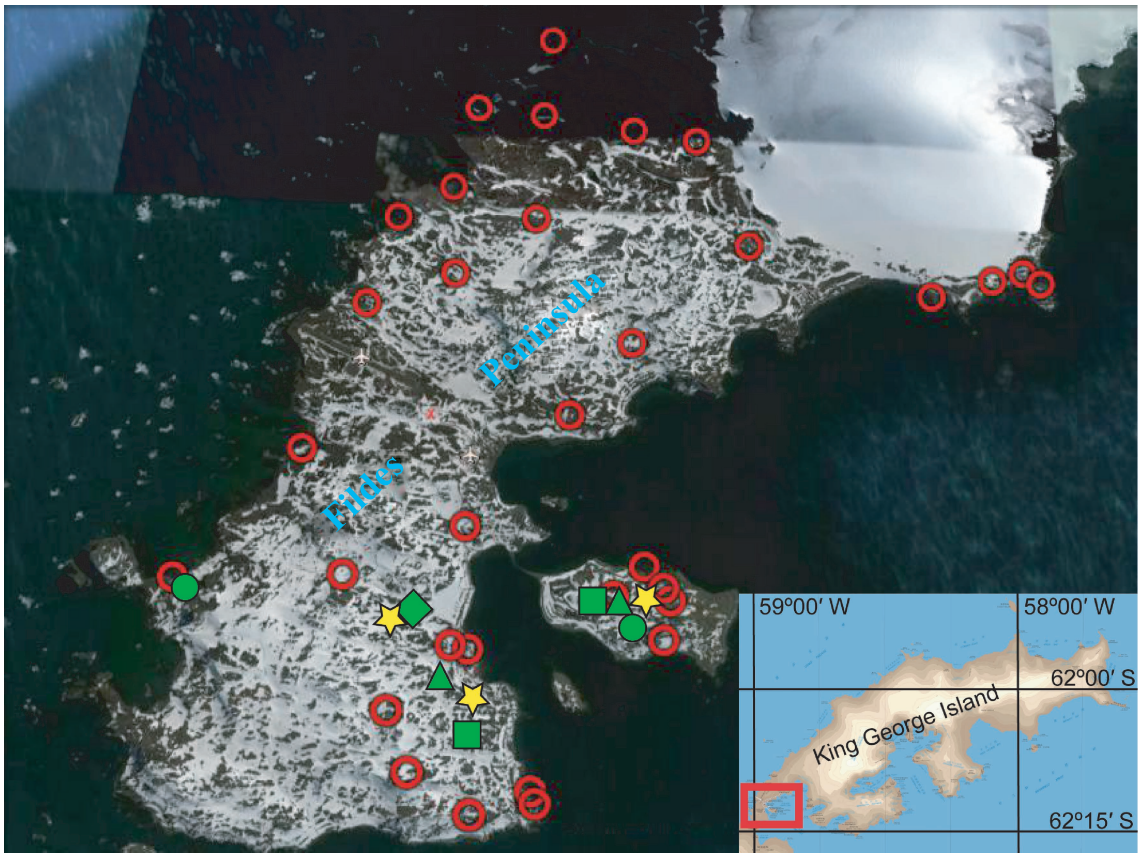

Figure 1 Map of sampling locations. ○: *Usnea aurantiaco-atra* thalli fragments; ● : *U. aurantiaco-atra* on rock; ■ : *U. aurantiaco-atra* on moss; ▲: *U. aurantiaco-atra* on wood; ◆ : *U. aurantiaco-atra* on thallus of *Umbilicaria antarctica*; ✦: *Umbilicaria antarctica*.

2.2.2 Extraction of total DNA

Total DNA was extracted using a modified CTAB Method[21] from the 14 morphologically inspected samples (seven *Umbilicaria antarctica* and seven *Usnea aurantiaco-atra*) and 384 *U. aurantiaco-atra* collected with a small amount of their thalli

2.2.3 ITS rDNA Amplification

The primer pairs ITS5 (5′–GGAAGTAAAAGTCGTAACAA GG–3′)/ITS4 (5′–TCCTCCGCTTAT TGATATGC–3′)[22] and nrSSU–1780–5′(5′–CTGCGGAAGGATCATTGATTC –3′) /nr LSU–0012–3′ (5′–AGTTCAGCGGGTGGTCTTG –3′)[4] were used to amplify ITS rDNA regions from the mycobiont and photobiont, respectively. PCR reactions were performed in a 50 μL reaction volume (100 ng of DNA template, 200 nM of each primer, 400 nMdNTP, 1×buffer, 1 U of rTaq) as follows: an initial denaturation at 95°C for 5 min, followed by 33 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 2 min, and completed with an extra extension at 72°C for 10 min.

2.2.4 Gel electrophoresis

The PCR product of each sample was detected in 1.2% agarose gel electrophoresis with DL2000 DNA Marker (Takara Biotechnology Co., Ltd., Dalian, China) as the marker.

2.2.5 Sequencing of the PCR product

PCR products were purified using E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek Inc., USA). The products for those listed in Table 1 were bi-directionally sequenced using ABI3730XL. The PCR products from those samples collected in small amounts were digested with Sau3AI and detected by electrophoresis. Based on the electrophoresis results, products from 27 mycobionts and 24 photobionts were selected arbitrarily and sequenced.

2.2.6 Alignment and phylogenetic analysis

Sequences were assembled with Lasergene SeqMan Pro (DNASTAR, Inc., USA) and corrected manually, and then aligned using ClustalW in Mega 5.10[23-24]. The phylogenetic analysis was executed with Mega 5.10 software, and the Kimura-2 parameter was selected as the nucleotide substitution model. The maximum likelihood (ML) method was used to construct the phylogenetic tree and the reliability of the inferred tree was tested by 1000 bootstrap replications[25].

3 Results

3.1 Morphology and attachment

Usnea aurantiaco-atra individuals growing on rock (Figure 2a), with moss (Figures 2b, 2c), on wood (Figure 2d) or on *Umbilicaria antarctica* (Figure 2e–2f) were inspected, and specimens were stored in the Resource-sharing Platform of Polar Samples (BIRDS) (Table 1). In general, apothecia were observed on the thalli of those attached to rocks (Figure 2a). Rare apothecia could be observed from those associated with mosses, both those growing on spalls beneath mosses (Figure 2b), and the others with moss but without any attachment (Figure 2c). Specially, there were no apothecia on those *Usnea aurantiaco-atra* individuals growing on wood (Figure 2d) or on the lichen *Umbilicaria antarctica* (Figure 2e–2f).

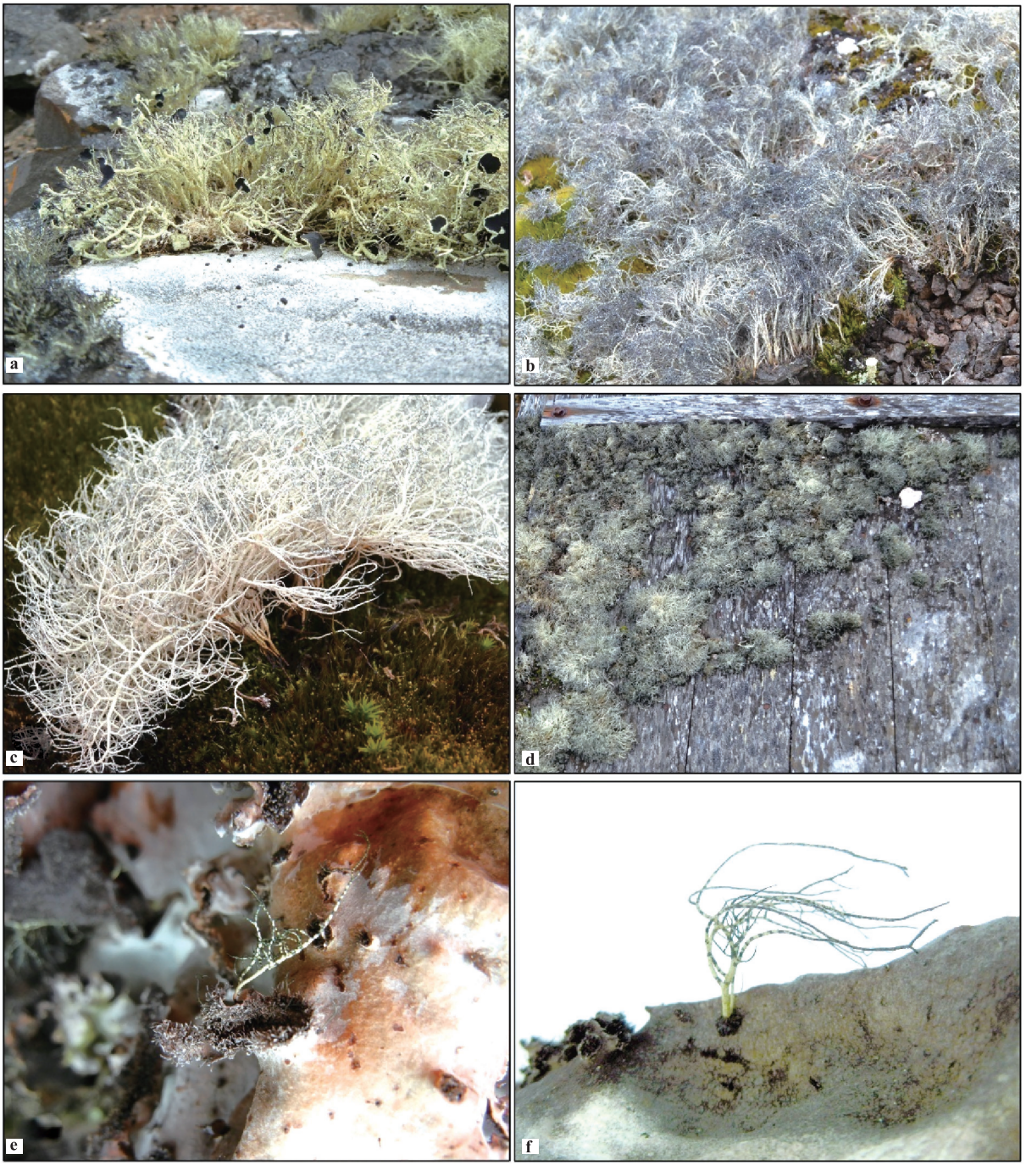

Figure 2 *Usnea aurantiaco-atra* on different substrates. **a**, on rock; **b**, with moss, growing on spall; **c**, with moss, no attachment; **d**, on wood; **e**–**f**, on *Umbilicaria antarctica* thallus.

3.2 ITS Sequences analysis

The ITS rDNA region of seven *Usnea aurantiaco-atra* and seven *Umbilicaria antarctica* was sequenced and submitted to GenBank (Table 1). PCR-RFLP (restriction fragment length polymorphism) was used to analyze the genotypes for 384 *Usnea aurantiaco-atra* individuals (280 on rock, 104 with moss) distributed around the Fildes Peninsula. The electrophoresis result showed that the genotypes for mycobionts or photobionts from *Usnea aurantiaco-atra* were identical (result not shown here). Therefore, 27 mycobiont PCR products (F01-F27, GenBank Accession Nos. KR053321–KR053347) and 24 photobiont PCR products (A01–A24, GenBank Accession Nos. KR053362–KR053385) were selected randomly to be sequenced.

Based on the phylogenetic analysis of the mycobiont ITS rDNA, there were minimum differences within *Usnea aurantiaco-atra* or *Umbilicaria antarctica*. The ML tree based on mycobiont ITS rDNA sequences showed these two lichen species were supported well with high bootstrap values (96% for *Usnea aurantiaco-atra* and 99% for *Umbilicaria antarctica*) (Figure 3a). *Usnea aurantiaco-atra* individuals were unable to form monophyletic groups based on their substrates. For the photobiont, all the samples were clustered with *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner (Figure 3b) by a bootstrap value of 99%, which meant that all photobionts in our study were *T. jamesii*. The results also demonstrated that some photobionts from *Usnea aurantiaco-atra* and *Umbilicaria antarctica* could share the same genotype. For example, ITS genotypes of AG282, AG236 and AG247 (from *Usnea aurantiaco-atra*) and those of AG041 and AG035 (from *Umbilicaria antarctica*) were identical (Figure 3b).

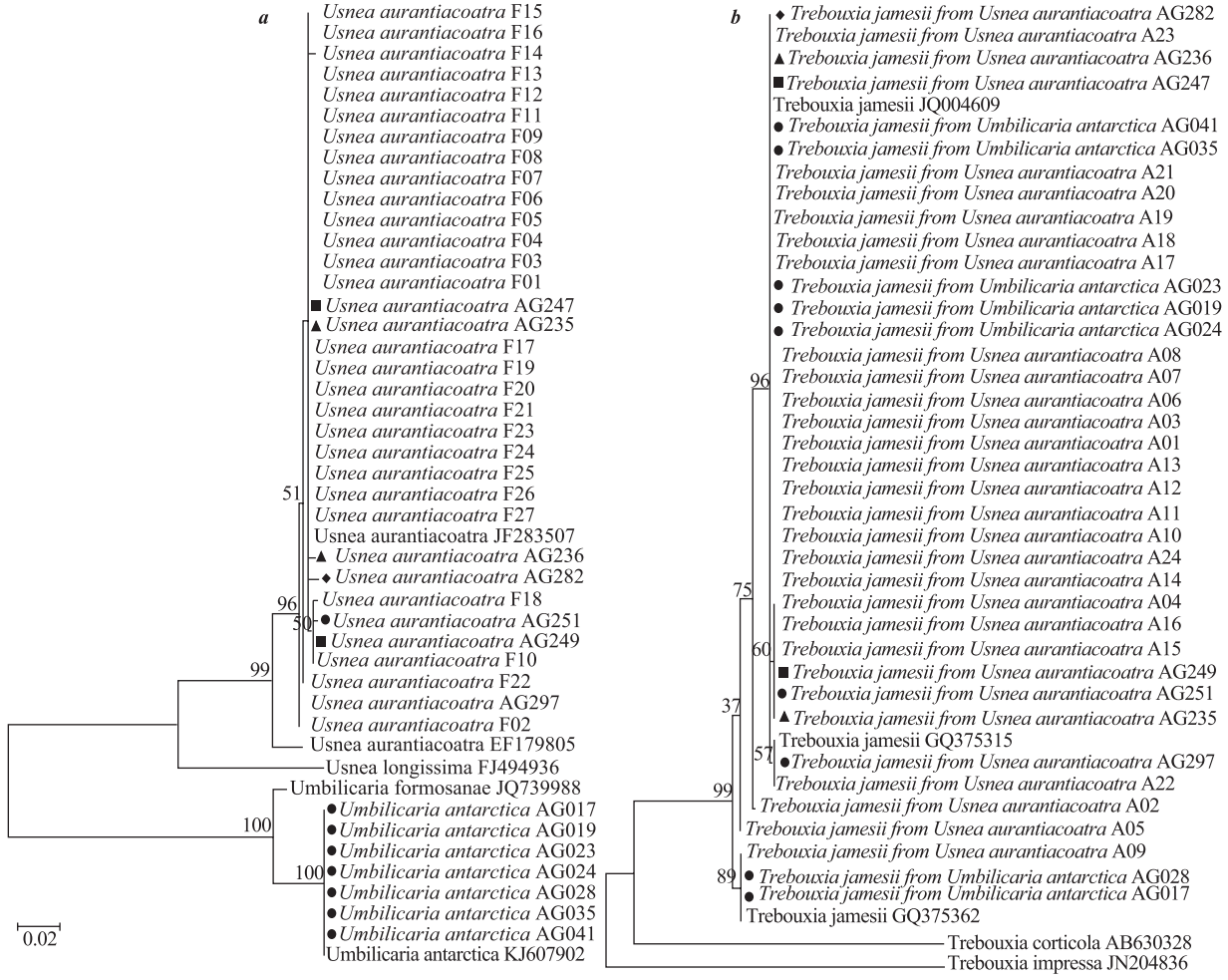

Figure 3 ML trees based on ITS rDNA sequences of mycobiont (**a**) and photobiont (**b**). The numbers in each node represent bootstrap support values. Only bootstrap values higher than 50% are indicated. ●: on rock; ■: on moss;▲: on wood; ◆: on thallus of *Umbilicaria antarctica*. Italic font indicates the sequences obtained by the authors.

4 Discussion

As the dominant organism in extreme terrestrial environments, lichen is able to adapt to various harsh conditions[26–28]. The symbiotic relationship between mycobiont and photobiont plays an important role in lichen's adaptability. Hence the key process allowing lichen to spread to novel habitats, especially for those dispersing by ascospores, is the obtaining by the mycobiont of its compatible photobiont.

Nearly 20%–40% of bare ground (not covered by permanent snow) in the Fildes Peninsula, Antarctica, is covered by lithophilous *Usnea aurantiaco-atra*[26], classified in the *Usnea* subgenus *Neuropogon*. During the investigation at the Great Wall Station, we found that some individuals of *U. aurantiaco-atra* could grow on wood, and even on other lichen thalli. The species provided the ideal materials to reveal the sources of the photobiont in lichenization. Generally, the substrate of *U. aurantiaco-atra* was rock[27], but our researches suggested that this lichen species was not strictly substrate-dependent (Figures 2d–2f). Two growth forms were reported for *U. aurantiaco-atra*[29]: Individuals of form I grew on rock and had erect branches and apothecia (Figure 2a); those of form II grew with mosses and had prostrate branches, but noapothecia (Figure 2b–2c). The individuals growing with mosses were attached to spalls beneath the mosses in most cases, and those without connection to spalls (Figure 2c) were thought to have been split away from rocks. At the Great Wall Station, it was recorded that the annual mean wind speed was 7.3 $m \cdot s^{-1}$, there were 137 d with gale-force winds and the fastest wind speed reached 35 $m \cdot s^{-1}$[30]. Individuals belonging to forms I or II would have had to adapt to the windy environment of Fildes Peninsula through attaching to either rocks or to mosses, to prevent them from being blown away to drift into the ocean.

Two *Usnea* species had been reported from this region, *U. aurantiaco-atra* having apothecia but no soredia, and *U. antarctica* Du Rietz having soredia but no apothecia[31-32]. Recently, phylogenetic study suggested that *U. aurantiaco-atra* and *U. antarctica* cannot be distinguished at molecular level as they share the same ITS genotype, and that *U. antarctica* should be treated a synonym of *U. aurantiaco-atra*[33]. Therefore, *U. aurantiaco-atra* and *U. antarctica* were not treated as separate species in present study.

PCR-RFLP was performed in our study in addition to morphological identification. The results showed that the difference for the ITS rDNA region among mycobionts or photobionts of *U. aurantiaco-atra* was very small, so only a minority of PCR products (27 for mycobionts and 24 for photobionts) were sequenced, representing 384 samples collected in a small amount. The sequencing results showed that there were 0–4 bps discrepancy among mycobionts and 0–10 polymorphism sites in photobiont sequences. The unique sequence suggested that randomly sequenced PCR products could have reflected the local genetic background of *U. aurantiaco-atra* in this region.

The *U. aurantiaco-atra* individuals growing on wood and on lichen thalli (Figure 2d–2f) were observed during the 27th and 28th CHINAREs at the Great Wall Station. No distinction was detected at molecular level among some individuals growing on lichen *Umbilicaria antarctica* (AG282), on wood (AG235, AG236) and on rock (AG251, AG297, F01-F27) according to the mycobiont ITS rDNA analysis (Figure 3a). The photobionts of *Usnea aurantiaco-atra* and *Umbilicaria antarctica* all belonged to the same algal species *T. jamesii* (Figure 3b). Because *Usnea aurantiaco-atra* was the dominant lichen in the Fildes Peninsula, the dominant photobiont species was *T. jamesii* here. The same photobiont genotype was observed for *U. aurantiaco-atra* growing on different substrates; furthermore, *U. aurantiaco-atra* and *Umbilicaria antarctica* could share the same photobiont genotype, which indicated that there was an algae pool from which lichenized fungi could obtain their photobionts.

The algae pool suggests one lichen could capture its photobiont from the thallus of another lichen. Some *Usnea aurantiaco-atra* has the same photobiont as those in *Umbilicaria antarctica*, which confirmed that these two lichen species shared the same algae pool. Molecular data showed that the ITS rDNA sequences of the mycobionts from *Umbilicaria antarctica* (AG017, AG019, AG023, AG024, AG038 and AG041) were identical, and their photobionts were all from the same species, *T. jamesii*, although a few variance sites existed in the ITS rDNA region. The result was fully consistent with that of the latest pyrosequencing of Antarctic lichens[34]. It could be inferred that the photobiont of *Umbilicaria antarctica*, on whose thallus *Usnea aurantiaco-atra* (AG282) was growing, should also be the same as that of AG282. However, the *Umbilicaria antarctica* individual had died, and so information from its photobiont could not be obtained directly. Ascospores from *Usnea aurantiaco-atra* were able to germinate on the thallus of *Umbilicaria antarctica*, capture its photobiont and finally form a lichen thallus. Individuals of *Usnea aurantiaco-atra* growing on wood might undergo a similar progress. Free-living photobionts are very rare in nature, but the photobionts could be released from vegetation fragments and survive for a short time[35]. Our research implied that free-living photobiont was present in the Fildes Peninsula, especially on wood surfaces, because no other lichens were observed on the wood.

Meanwhile, *Usnea aurantiaco-atra* growing with mosses, was the dominant organism in the Ardley Island. More attention should be paid to the mosses growing with *U. aurantiaco-atra* to elucidate the process of succession between these two organisms, and to examine whether there is a specific relationship between lichen and moss.

Our findings, especially the discovery of *U. aurantiaco-atra* growing on *Umbilicaria antarctica*, provided evidence for photobionts transferring directly between lichens. The *Usnea aurantiaco-atra* found on wood also confirmed there was free-living *T. jamesii* in the Fildes Peninsula, because there were no other lichens on the wood. There is a possibility that the thalli of *U. aurantiaco-atra* on wood or other lichens

were from vegetable structures such as soredia, and this could not be excluded completely. However, the unique fungal ITS genotypes for AG238 (on wood) and AG282 (on *Umbilicaria antarctica*) strongly implied that they had passed through a process of sexual reproduction so that their fungal ITS sequences were not in accordance to those growing on rocks. This meant that ascospores had captured the algal partner on substrates other than rocks.

There are multiple algal species in the Fildes Peninsula, but only one species was found accompanying the dominant lichen species *Usnea aurantiaco-atra*. This indicates that this algal species has adapted to the micro-environment, becoming the preponderant lichenized algae. The various substrates of *U. aurantiaco-atra* indicated that the sources of its photobiont were not unique; the alga in a new thallus could be obtained from the parental thallus, from other lichens or from the environment. Some works have demonstrated that the decrease in selectivity of the mycobiont to its photobiont may be helpful for lichen surviving in extreme environments because mycobionts could form a lichen thallus with a broad range of photobionts to survive[10,13,36]. However, the decreased selectivity of *U. aurantiaco-atra* to the substrates is also a strategy to survive in harsh environments. For example, some lichens belonging to the genus *Umbilicaria*, found growing on rocks, can also inhabit wood[37].

In summary, morphology and molecular analysis demonstrated the available photobiont sources, and confirmed that there was an algae pool in this area. This provides real insight into the growth of *Usnea aurantiaco-atra* on various substrates, which in turn will help us to understand the distribution of photobionts, photobiont transfer mechanisms and the process of lichenization.

Acknowledgement Our research was facilitated by the Resource-sharing Platform of Polar Samples (http://birds.chinare.org.cn/) where information on lichens and data are stored. We are grateful to the Chinese Arctic and Antarctic Administration for its help in carrying out the project in the Great Wall Station during the 27th and 28th CHINAREs. This research was supported by State Oceanic Administration, P. R. China (Grant nos. 10/11 GW06, 2011GW12016), and the National Natural Science Foundation of China (Grant nos. 31000010, 31270118, 41206189).

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