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Phylogenetic analysis and *in vitro* culture of mosses from the Antarctic Fildes Peninsula

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Abstract Molecular genetic techniques have proven very useful for initial analysis of the extent of genetic variation and dispersal in several Antarctic moss species. In the present study, the small subunit ribosomal RNA (SSU rDNA) and internal transcribed spacers of the nuclear ribosomal DNA (ITS rDNA) were sequenced in nine individuals of different mosses from the Fildes Peninsula of Antarctica. Sequence alignment showed that the extreme environment tended to increase the genetic diversity of Antarctic mosses. In addition, in our phylogenetic analysis, one previously unidentified Antarctic moss species was characterized by comparison with SSU and ITS rDNA sequences of known moss species. Moreover, the optimal culture medium and conditions for surface explant sterilization and protonemata induction in tissue culture of *Pohlia nutans* were investigated. The successful establishment of a tissue culture protocol together with the phylogenetic analysis of Antarctic mosses will provide technological support to establish an effective resource regeneration method for discovering new functional genes and gaining novel insights into the mechanisms of stress acclimation.

Keywords **phylogenetic tree, tissue culture, protonemata formation, *Pohlia nutans***

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1 Introduction

Plants in Antarctica survive and propagate in the harshest environment on Earth. Less than 2% of the 14 million km^2 that make up the Antarctic continent is free of permanent ice and snow, and therefore available for plant colonization. Vegetation is sparse and low-growing, and is dominated by mosses and lichens[1]. Recent biological studies also suggest that these terrestrial plants are of ancient origin and have persisted in isolation for tens of millions of years[2]. Over time they have evolved a variety of strategies that range from physiological changes to environmental stress tolerance. Therefore, these plants offer tremendous opportunities for discovering the mechanisms of plant survival under extreme conditions and genetic evolution, as well as plant responses to the increased UV irradiation accompanying global climate change[3-4].

On the Antarctic Peninsula, there is a wide diversity of moss species[5-6]. Under predicted scenarios of global climate change, the climatic constraints of the Antarctic environment are likely to be reduced and the level of biodiversity is likely to increase[7]. Furthermore, uniquely in continental Antarctica, many of the short-term consequences of climate change will affect bryophyte ecosystems and colonization; these communities may be particularly vulnerable to global

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change[8-9]. Traditional morphological taxonomic analysis of mosses is usually based on the classification of Buck and Goffinet[10]. Over the years, DNA sequencing of highly conserved genes such as the small subunit ribosomal RNA (SSU rDNA) and the internal transcribed spacers of the nuclear ribosomal DNA (ITS rDNA) has become a widely used technique for phylogenetic analysis in a range of plant species. In addition, molecular genetics also has the potential to assist taxonomic identification where this is difficult because of phenotypic plasticity displayed by the same moss. Recently, researchers have used molecular approaches to start investigating the population genetics of Antarctic moss populations, to compare the extent of genetic diversity within and among different colonies and populations, and to investigate the dispersal mechanisms and origins of these mosses[5-6,11]. However, in contrast to the wide range of taxa already analyzed in temperate regions, the genetic diversity of mosses in the Antarctic continent is still not well documented.

Mosses have a dominant haploid vegetative stage with stems and leaves. They can reproduce both sexually and asexually, and almost any part is capable of regeneration[5]. Mosses were among the first plants to inhabit the land, and have become the highest plant forms occurring naturally in continental Antarctica. In the South Shetlands of West Antarctica, the annual mean temperature is around 0℃, but the region has a wide daytime temperature range. It varies from −5℃ to 13℃ and falls as low as −30℃ in winter. Furthermore, wind and humidity may significantly decrease the chill temperature[6]. The capability of Antarctic mosses to adapt to the extreme polar environment is definitely a result of distinct functional genes and different gene expression profiles under stress conditions. However, genomic sequence resources available for Antarctic mosses are scarce[12]. It is believed that discovering the functional gene or gene family responsive to freezing stress in Antarctic mosses will offer us a better understanding of the mechanism of stress acclimation, and provide a basis for effective engineering strategies to improve stress tolerance in agricultural crops. Therefore, the establishment of axenic culture in the laboratory is fundamental for obtaining relevant results, since materials from the Antarctic continent are difficult to acquire and separate from other moss species, microorganisms, algae, and soil particles. Although it is sometimes stated that bryophytes are easily cultured *in vitro*[13], most of this work has been done on *Physcomitrella patens*, and relatively few species have in fact been successfully induced to form stable axenic cultures. Moreover, under *in vitro* culture, the responses of different moss species to the same treatment may show many discrepancies[14-15].

The Chinese Antarctic Great Wall Station is located in the Fildes Peninsula of King George Island where mosses are overgrown and luxuriant. During the 24th Chinese Antarctic National Research Expedition(CHINARE), we collected several moss samples from the Great Wall Station. These mosses were regularly cultured in an illumination incubator at temperatures of 16℃ and flourished at temperatures down to 4℃–6℃, suggesting that they have a strong ability to adapt to low temperatures. The aims of this study were to perform a phylogenetic analysis on the mosses isolated from the Antarctic Fildes Peninsula and to provide technological support to establish a convenient and effective resource regeneration method for Antarctic mosses.

2 Materials and methods

2.1 Collection of moss specimens and initial culture conditions

The Antarctic mosses were collected from the terrane near the Great Wall Station on King George Island during the 24th CHINARE in March 2008 (Table 1). The Antarctic moss samples (a few shoots and roots with soil matrix) were placed in vacuum-sealed plastic bags, stored at 4℃, and transported to China. These mosses were then cultivated on a soil medium (a mixture of Base Substrate (Klasmann-Deilmann, Geeste, Germany) and local soil, ratio 1:1) in flowerpots in an artificial climate incubator at 16℃, 70% relative humidity, and 70 μmol photons·m^{-2}·s^{-1} light density with 12 h light/12 h dark.

2.2 DNA isolation, amplification and sequencing

Green tissue parts of moss shoots were homogenized in

Table 1 Collection locations of Antarctic samples and SSU and ITS rDNA sequence information

Collection No.	Location	Length of SSU	GenBank Acc No.	Length of ITS	GenBank Acc No.	Species
Antarctic moss L	S62°13.260′, W58°57.291′	1 830	KC291522	962	KC291513	*Pohlia nutans*
Antarctic moss H	S62°13.260′, W58°57.290′	1 793	KC291523	757	KC291514	*Sanionia uncinata*
Antarctic moss No.1	S62°11.862′, W58°59.653′	1 719	KC291524	978	KC291515	*Bryum pseudotriquetrum*
Antarctic moss No.2	S62°11.862′, W58°59.653′	1 709	KC291525	1 003	KC291516	*Bryum pseudotriquetrum*
Antarctic moss No.3	S62°11.803′, W58°59.340′	1 706	KC291526	764	KC291517	*Sanionia uncinata*
Antarctic moss No.5	S62°11.802′, W58°59.356′	1 732	KC291527	1 001	KC291518	*Pohlia sp.* No.5
Antarctic moss No.6	S62°13.262′, W58°57.290′	1 697	KC291528	762	KC291519	*Sanionia uncinata*
Antarctic moss No.7	S62°13.263′, W58°57.291′	1 711	KC291529	772	KC291520	*Sanionia uncinata*
Antarctic moss No.12	S62°13.244′, W58°57.064′	1 723	KC291530	808	KC291521	*Ceratodon purpureus*

liquid nitrogen with a mortar and pestle three times and then the homogenized powder was transferred into a 50 mL conical tube and stored at −80℃. Genomic DNA was extracted from the liquid nitrogen-ground moss powder using cetyl trimethylammonium bromide (CTAB) extraction buffer composed of 2% CTAB, 1% polyvinylpyrrolidone (PVP) K-30 (soluble), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 1.4 M NaCl. Immediately prior to use, 2% *β*-mercaptoethanol and 100 μg·mL^{-1} RNase A were added to the CTAB buffer, which was pre-warmed to 65℃ to denature any contaminating DNase. In each 1.5 mL Eppendorf tube, 0.7 mL CTAB buffer was added to 0.5 mL (measured by volume) ground powder, and the samples were incubated at 65 ℃ for 10 min and mixed well by inverting up and down. The samples were further extracted twice with an equal volume of chloroform-isoamyl alcohol (24-1) and centrifuged at 13 000×*g* for 10 min at 4℃. The upper aqueous phase was transferred to a fresh tube, 0.7-fold iso-propanol was added, and it was mixed well and centrifuged immediately for 10 min. The supernatant was decanted and the pellet was washed with 70% ethanol twice. After air drying, the pellet was dissolved in 30 μL TE buffer (10 mM Tris-Cl, pH 8.0 and 1 mM Na_2EDTA).

The complete SSU rDNA and ITS rDNA sequences were amplified by the polymerase chain reaction (PCR) using primers designed by White et al[16]. The SSU rDNA primers were NS1-GTAGTCATATGCTTGTCTC and NS8-TCCGCAGGTTCACCTACGGA; the ITS rDNA primers were ITS1-TCCGTAGGTGAACCTGCGG and ITS4-TCCTCCGCTTATTGATATGC. The DNA was amplified in a 50 μL reaction volume containing 10 pmol of each primer, 1 μL moss genomic DNA, 2.5 units *TransStart*^{TM} Taq DNA Polymerase, and 0.2 mM dNTPs. The thermocycling regime was programmed for 35 cycles (94℃ for 30 s; 56℃ for 30 s; 72℃ for 1.5 min). Specific amplification products were excised from an agarose gel, purified using a Tiangen gel purification kit (Tiangen, Beijing, China), ligated into the pMD18-T vector (TaKaRa, Dalian, China) and transformed into competent *Escherichia coli* Top 10 cells (Tiangen). Positive clones were identified by restriction endonuclease digestion and sequenced on an ABI 377 DNA Sequencer (Biosune, Shanghai, China). The complete sequences of the SSU and ITS rDNAs were determined using the Chromas software (Technelysium Pty Ltd) to edit the sequencing chromatograms.

2.3 Sequence alignment and analysis

Raw sequences were first individually processed for vector trimming. The overlapping sequences were evaluated and aligned into a full consensus sequence using the Lasergene 7.0 software (DNAStar, Madison, WI, USA). Multiple alignments of the SSU and ITS rDNA sequences were performed by the ClustalW multiple alignment program and identical regions of the SSU rDNA, ITS1 rDNA and ITS2 rDNA were selected from different species for phylogenetic tree construction using the Mega 4.0 program[17]. In Mega 4.0, distance matrices were generated using the pairwise deletion option with the Kimura 2-parameter nucleotide model. One thousand bootstrap replicates were created and trees were generated using the neighbor-joining (NJ) method for each replicate. The bootstrap values reported for each branch reflect the percentage of 1 000 trees containing that branch.

2.4 Explant sterilization for tissue culture

From the moss samples, we selected the Antarctic moss sample coded 'L' (*Pohlia nutans*) as a representative for optimizing the *in vitro* culture conditions. The green parts of shoots were cut into ~2 cm long fragments and sterilized on a clean bench as follows: Shoots were washed with 300 mL sterile distilled water three times to clean the visible superficial dust and collected with a 200 mesh steel sieve; the shoots were divided into five groups and sterilized with 8% sodium hypochlorite (NaClO) solution for 2, 3, 4, 5, or 6 min; the shoots were further washed three times with sterile distilled water to remove the remaining NaClO; the sterilized shoots were transferred into Erlenmeyer flasks with 50 mL of autoclaved modified Knop medium[18] under the conditions of 16℃ , 12 h light (70 μmol·m^{-2}·s^{-1})/12 h dark with a relative humidity of 70%.

2.5 Basic culture medium selection

Three types of media including BCD medium at pH 6.5[19], modified Knop medium and basic Murashige and Skoog (MS) medium[20] at pH 5.8 were prepared for moss tissue culture. One piece of shoot tissue (each about 40 mg fresh weight) was inoculated into a flask with 50 mL of medium (autoclaved at 121℃ for 20 min), and the flasks were put into the sterile culture room at 16℃ , 12 h light (70 μmol·m^{-2}·s^{-1})/12 h dark with a relative humidity of 70%. For each type of medium, six flasks were cultured. The diameter of the colony and the fresh weight of shoots were recorded during a three-month culture.

2.6 Influences of phytohormones on protonemata formation

To study the influence of different phytohormones on the induction of protonemata, Knop solid medium was supplemented with 2, 4-dicholrophenoxyacetic acid (2, 4-D), 6-benzylaminopurine (6-BA), indolebutyric acid (IBA), naphthalene acetic acid (NAA) or gibberellin (GA3) at concentrations of 0.1 or 1.0 mg·L^{-1}. In addition, BCD solid medium containing 5 or 10 mM di-ammonium (+) tartrate was also used to induce protonemata. The moss shoots were cut into 2-mm fragments with a sterile scalpel on a clean plate, and then the fragments were transferred onto each medium. Each flask contained about four gametophytic fragments. Protonemal induction rates for the different treatments were calculated by checking whether the explant produced protonemata after a 2-month culture. Each treatment was conducted with three replicates.

3 Results

3.1 DNA isolation, amplification and sequencing

Nine SSU rDNA and ITS rDNA fragments from the Antarctic moss samples were amplified and sequenced (Table 1). The full length of SSU ranged from 1 697 to 1 830 bp, while the full length of ITS was 762 to 1 003 bp. A BLASTN search using the nucleic acid sequences of SSU and ITS showed homologies to several known SSU and ITS sequences in GenBank. The newly isolated SSU and ITS sequences were submitted to GenBank under the accession numbers KC291513 to KC291521 (Table 1).

3.2 Construction of a phylogenetic tree using SSU rDNA gene sequences

The complete SSU rDNA sequences of the nine Antarctic mosses were aligned with homologs from 33 species of various moss genera, such as *Pohlia*, *Mielichhoferia*, *Plagiomnium*, *Leucolepis*, *Cyrtomnium*, *Cinclidium*, *Calliergonella*, *Hypnum*, and *Climacium*, available in public databases. In the phylogenetic tree of SSU rDNA sequences constructed by neighbor-joining the subclasses *Bryidae*, *Dicranidae*, and *Funariidae* formed distinct clades, which was consistent with previously published classifications of *Bryopsida*. Moreover, the orders *Bryales* and *Hypnales*, and the families *Bryaceae* and *Mniaceae* also formed distinct clades (Figure 1). Further analysis of the SSU rDNA phylogenetic tree showed that Antarctic mosses L and No. 5 were both grouped in *Bryidae*, *Bryales*, *Mniaceae*, while Antarctic mosses No. 1 and No. 2 were both grouped in *Bryidae*, *Bryales*, *Bryaceae*. Because of the relatively low variability of the 18S rDNA sequence in moss, Antarctic mosses H, No. 3, No. 6 and No. 7 could only be grouped in *Bryidae*, *Hypnales*, while No. 12 was grouped in the *Dicranidae* clade, presumably belonging to *Ditrichaceae*.

3.3 Construction of phylogenetic trees using ITS rDNA gene sequences

The ITS rDNA sequence produced using the primers ITS1 and ITS4 covers the 5′ end of the 26S rRNA gene, the complete ITS1-5.8S-ITS2 region, and the 3′ end of the 18S rRNA gene. Sequencing with these primers enabled the full sequence to be obtained. By referencing the SSU rDNA sequences, the ITS1 and ITS2 sequences of the nine Antarctic mosses were aligned with their homologs from 22 other species. Phylogenetic analysis of the ITS sequences revealed that these nine Antarctic moss samples were clustered in four clades, *Mniaceae*, *Amblystegiaceae*, *Bryaceae* and *Ditrichaceae*, which was consistent with previously published classifications of mosses (Figure 2).

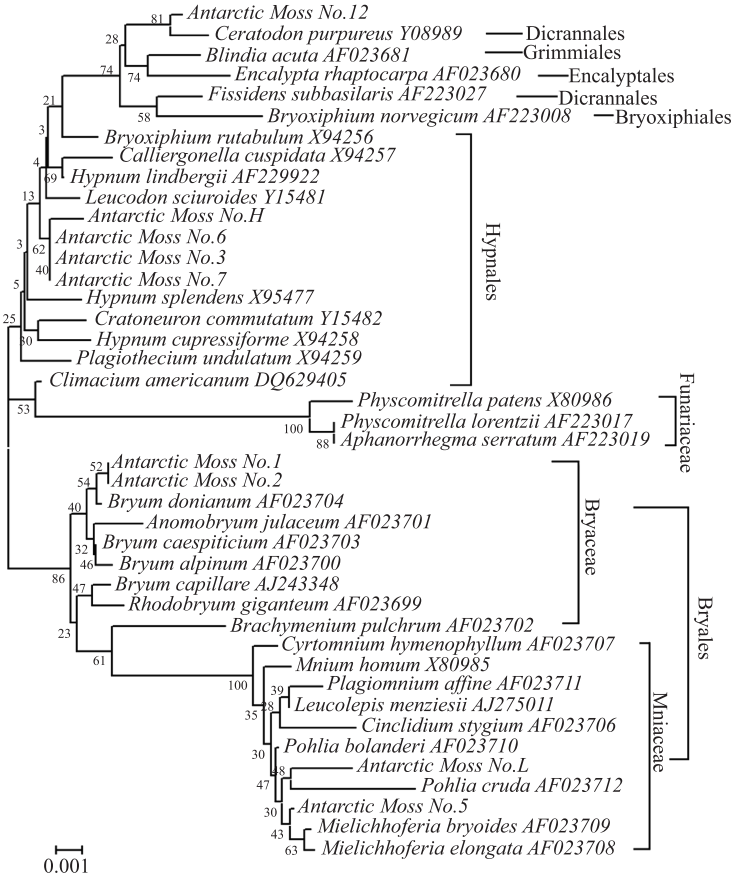

Figure 1 Phylogenetic tree of different moss colonies based on SSU rDNA sequences and constructed with the neighbor-joining method.

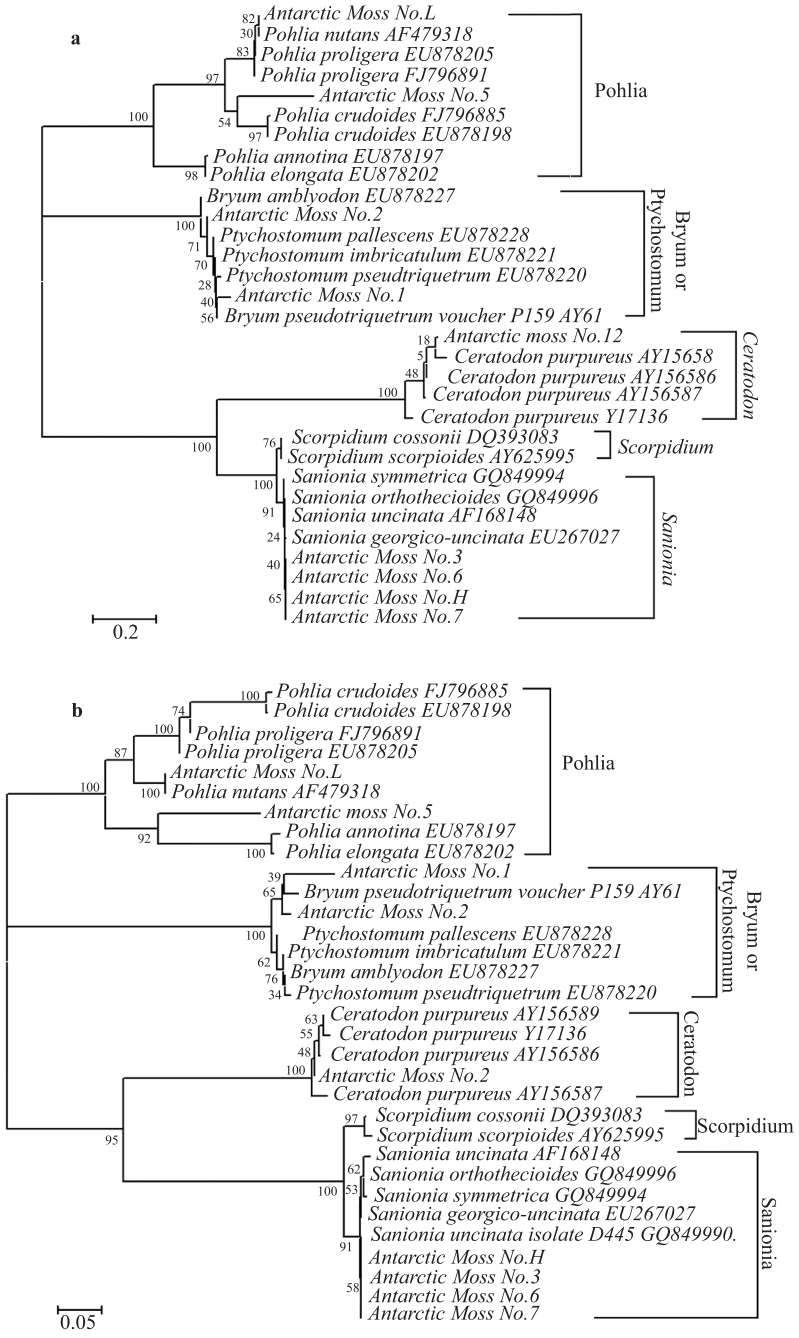

Figure 2 Phylogenetic tree of different moss colonies based on ITS1 rDNA (**a**) and ITS2 rDNA (**b**) sequences and constructed with the neighbor-joining method.

No nucleotide differences were detected between the complete ITS1-5.8S-ITS2 sequences of Antarctic moss L and *Pohlia nutans* AF479318. Therefore, the specimen Antarctic moss L should be *P. nutans*, which has been reported from the Antarctic continent. Another novel moss specimen, Antarctic moss No. 5, was clustered within the *Pohlia* clade, but formed a subordinate class in the phylogenetic trees (Figure 2). The identities of the ITS1 rDNA sequences between Antarctic moss No. 5 and other *Pohlia* species varied from 58.9% to 72.0%, while the identities of ITS2 varied from 70.5% to 78.8%, indicating that Antarctic moss No. 5 was a novel isolate belonging to the genus *Pohlia* (Figure 2a and 2b). Therefore, we temporarily named it *Pohlia sp.* No5.

Antarctic mosses No. 1 and No. 2 were closely clustered with *Bryum pseudotriquetrum*, *Bryum amblyodon*, *Ptychostomum pallescens*, *Ptychostomum imbricatulum* and *Ptychostomum pseudotriquetrum*, which all belong to the *Bryaceae* family. Several nucleotide variations within the ITS1 and ITS2 regions were also detected by Clustal W. Antarctic moss No. 1 should belong to the genus *Bryum* according to both the ITS1 and ITS2 phylogenetic trees. Antarctic moss No. 2 was close to *Ptychostomum* sequences in the ITS1 tree, while it was close to *Bryum* sequences in the ITS2 tree. In the phylogenetic trees, Antarctic moss

No. 12 clustered with *Ceratodon purpureus*. The identities of the ITS1 rDNA sequences between Antarctic moss No. 12 and other *C. purpureus* samples varied from 93.3% to 97.7%, while the identities of ITS2 varied from 97.6% to 99.3%. Therefore, we deduced that Antarctic moss No. 12 belonged to *C. purpureus*, because this individual and other *C. purpureus* samples formed a well-supported monophyletic group and No. 12 lay on an interior branch.

Four specimens of Antarctic mosses, H, No. 3, No. 6 and No. 7, were grouped in the *Amblystegiaceae* clade and were close to *Sanionia*, indicating that these four specimens belonged to the genus *Sanionia*. Moreover, the comparison using Clustal W showed that the complete ITS sequence of Antarctic moss H only had three nucleotides different from that of *Sanionia symmetrica* GQ849994.

3.4 Appropriate sterilization scheme for explants

Because there were no sporophytes of *P. nutans* available in our collection, shoot fragments were used as explants in the culture. Because of the frangibility of moss fragments, treatment with 70% ethanol for 30 s easily decolorized the moss tissue. Therefore, we selected the moderate disinfectant sodium hypochlorite for explant sterilization. The responses of the explants to disinfection with 8% NaClO for different times were quite different. Sterilizing with 8% NaClO for 5 min gave the highest survival rate and gametophore yield so was the most suitable for the present culture, while other sterilization times resulted in either explant browning or contamination.

3.5 Culture medium selection

Comparing the growth status of mosses on different culture media showed that modified Knop medium and BCD medium were suitable for the growth of thalli. Both the colony diameter and fresh weight increased quickly during the 3-month culture experiments. Mosses grown on MS medium had the lowest colony diameter and fresh weight compared with the other two media. However, MS medium had a higher protonemata formation rate (Table 2 and Figure 3).

Table 2 Changes of colony diameter and fresh weight of *Pohlia nutans* shoots in three media

Media	Colony diameter/cm	Fresh weight/g
BCD medium	7.61±0.60	3.71±0.32
Knop medium	7.12±0.62	3.43±0.29
MS medium	4.97±0.42	2.05±0.22

3.6 Effects of phytohormones on protonemata formation from leaves and stem fragments

The phytohormones 2, 4-D, 6-BA, ABA, IBA and GA were tested at concentrations of 0.1 and 1.0 mg·L^{-1} for the induction of protonemata from shoot fragments. The results showed that 2, 4-D at concentrations of 0.1 or 1.0 mg·L^{-1} was the most effective at inducing shoots to produce protonemata (Table 3). Knop medium with 6-BA at 1.0 mg·L^{-1} gave the second highest protonemal induction rate (91.6%). In addition, the protonemata induction rate on BCD medium with 5 or 10 mM di-ammonium (+) tartrate was similar to that of 2, 4-D (100% induction rate, Figure 3d). However, the media with IBA, NAA or GA at concentrations of 0.1 or 1.0 mg·L^{-1} did not induce the production of protonemata.

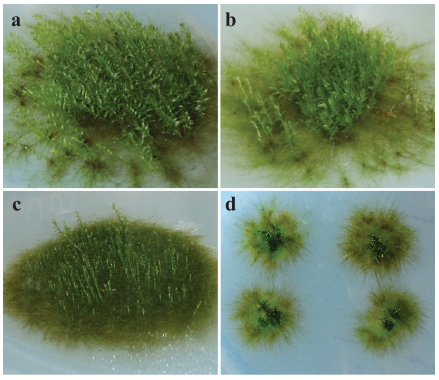

Figure 3 Growth status of *Pohlia nutans* colonies on BCD medium (**a**), modified Knop medium (**b**), MS medium (**c**), BCD medium with 10 mM di-ammonium (+) tartrate (**d**) after a three-month culture.

Table 3 Influences of phytohormones on protonemal induction from shoots

Reagents	Protonemata formation rate	Protonemata formation rate
Phytohormone: 2, 4-D	0.1 mg·L^{-1}: 100%	1.0 mg·L^{-1}: 100%
Phytohormone: 6-BA	0.1 mg·L^{-1}: 66.7%	1.0 mg·L^{-1}: 91.6%
Phytohormone: IBA	0.1 mg·L^{-1}: 0%	1.0 mg·L^{-1}L: 0%
Phytohormone: NAA	0.1 mg·L^{-1}: 0%	1.0 mg·L^{-1}: 0%
Phytohormone: GA	0.1 mg·L^{-1}: 0%	1.0 mg·L^{-1}: 0%
di-ammonium (+) tartrate	5 mM: 100%	10 mM: 100%

4 Discussion

In Antarctica, mosses are often subjected to combined stresses including cold, desiccation, limited nutrients, high salinity, and adverse solar radiation. The extreme environment has led to phenotypic plasticity where morphological characters can vary in response to different environmental conditions rather than being due to genetic changes[21]. DNA sequencing of highly conserved genes such as the nuclear ribosomal DNA internal transcribed spacer region (ITS1-5.8S-ITS2) has become a widely-used technique for phylogenetic analysis in a range of plant species[22]. DNA sequencing of this region has recently been applied to phylogenetic investigations of temperate bryophytes. In a study of a very isolated population of *P. nutans* on heated ground on Mt. Rittmann in Northern Victoria Land, the Random Amplified Polymorphic DNA (RAPD) technique proved that the population has

little genetic variability and appeared to be derived from a single immigration event[23]. In the present study, two mosses were classified into the genus *Pohlia* by SSU and ITS rDNA sequencing and phylogenetic analysis. The ITS1 and ITS2 rDNA sequences of Antarctic moss L were completely identical to sequences previously reported in *P. nutans*. However, sequence alignment showed that the ITS sequence of Antarctic moss No. 5 had relatively low identity to all submitted ITS rDNA sequences in GenBank, suggesting that this collected specimen was a novel species in Antarctica (Figures 1 and 2).

In the moss *C. purpureus*, reports have demonstrated that genetic diversity, like phenotypic diversity, is extensive within and among temperate, Subantarctic and Antarctic populations. The reason for the greater genetic variation than expected in Antarctic populations of *C. purpureus*, in the absence of sexual reproduction, may well lie in the rate of mutagenesis driven by extreme environment[24]. In the present study, all selected *C. purpureus* sequences formed a well-supported monophyletic group and No. 12 lay on an interior branch. However, the phylogenetic trees based on ITS rDNA sequences also showed that there was a high level of genetic variability within the species *C. purpureus* on the Antarctic continent (Figures 2a and 2b). For example, the identities of ITS1 rDNA sequences between Antarctic moss No. 12 and other *C. purpureus* sequences varied from 93.3% to 97.7%, while the identities of ITS2 varied from 97.6% to 99.3%. As a result of the ozone hole's development, ultraviolet radiation over southern high latitudes has increased substantially in recent years[25]. Therefore, increased exposure to UV-B irradiation may also affect the levels of mutagenesis in Antarctic moss populations[24].

SSU rDNA is suitable for analyzing phylogenetic relationships between genera, while ITS rDNA is useful for analysis of phylogenetic relationships between different species in the same genus and can be used for classification at the species level[26]. Phylogenetic analysis based on the combination of SSU and ITS rDNA suggested that Antarctic mosses H, No. 3, No. 6 and No. 7 all belonged to *Hypnales*, *Amblystegiaceae*, *Sanionia*. However, some mosses, such as species in the genera *Bryum* and *Ptychostomum*, which both belong to the *Bryaceae* family, were still hard to distinguish in the ITS1 and ITS2 phylogenetic trees (Figures 2a and 2b). Using the RAPD technique, studies have clearly shown that genetic variation does occur, at relatively high levels, in Antarctic moss populations. In *Bryum argenteum*, *B. pseudotriquetrum*, *C. purpureus*, and *Hennediella heimii*, variation can be detected among populations, within isolated populations and even within individual colonies[27-28]. In our study, using the complete ITS sequences of Antarctic mosses No. 1 and No. 2 to produce an alignment against the NCBI non-redundant protein sequence (nr) database, the best-matched sequences implied that they belonged to the genus *Bryum*. Our results also showed that they had relatively low identities to other *Bryum* species.

The gametophyte phase of mosses, which is dominant in their life cycle, is a favorable model system for genetic, biochemical, metabolic, and developmental studies[13]. As in higher plants, development under axenic conditions is species-specific. There are many discrepancies in the response of different moss species to the same treatment under *in vitro* culture[14-15]. Since spores were not available in our experiment, gametophores of *P. nutans* were used as explants for tissue culture. However, previous reports showed that the success rate of procedures was highly variable and contamination was common[29]. Our results showed that sterilizing with 8% NaClO for 5 min gave the highest survival rate and gametophore yield, and was highly suitable for culture of the present species. However, disinfecting new shoots with 0.1% $HgCl_2$ for 8 min was also effective for surface sterilization[30].

The three types of media showed different effects on protonemal growth and differentiation. Modified Knop or BCD medium was suitable for the growth of thalli, while mosses grown on MS medium had the lowest colony diameter and fresh weight (Figure 3). However, MS medium gave a higher protonemata formation rate, which was consistent with results reported by Cvetić et al[31]. They showed that in hormone-free media with half-strength MS mineral salts and vitamins, despite extensive bud formation, only a small fraction of buds developed into gametophores.

There have been numerous reports on the influence of cytokinin and auxin on mosses. The effects of auxin on moss development include inhibition of protonema growth, transformation of buds to filaments, torsion of young stems and complete suppression of leaves on gametophores, while cytokinins have been shown to induce bud formation in protonemata cultures of some moss species. However, 2, 4-D and 6-BA are usually effective for callus induction in *Physcomitrella patens*[18]. Our results showed that the gametophyte of *P. nutans* could produce protonemata in media with 2, 4-D or 6-BA, but no buds were formed, which was consistent with a report by Pan et al.[32] (Table 3). Additionally, according to Hohe and Reski[33], tartrate ammonia may keep moss protonemata in the chloronemata state for a long time, reducing caulonemata and bud differentiation. Our study also showed that BCD medium supplemented with 5 or 10 mM di-ammonium (+) tartrate effectively induced shoots to form protonemata (Figure 3d).

In conclusion, mosses represent the oldest living clade of land plants, separated by approximately 450 million years of evolution from crop plants. Phylogenetic analysis based on the combination of SSU and ITS rDNA genes will provide more molecular evidence for classification, evolution and genetic variation studies of mosses isolated from extremely harsh climatic conditions. Together with the successful establishment of tissue culture protocols for the Antarctic moss *P. nutans*, they will provide technological support to establish an effective resource regeneration method for discovering new functional genes, and for gaining novel

insights into the mechanisms of stress acclimation.

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