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Nucleotide differences in the *mbf1* gene of the lichenized fungus *Umbilicaria decussata* collected in polar and non-polar regions

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Abstract Multiprotein bridging factor 1 (MBF1) is a transcriptional co-activator related to stress tolerance in various organisms. We investigated the nucleotide differences in the *mbf1* gene in the lichen-forming fungus *Umbilicaria decussata* collected from polar (i.e., Antarctica and the Arctic) and non-polar (i.e., Armenia) regions. The 552-bp *Udmbf1* genes isolated from eight samples contained numerous sequence variations, including single nucleotide polymorphisms as well as insertions and deletions. The frequency of nucleotide changes was higher in the intron than in the coding sequence. The nucleotide polymorphism levels (π=0.01792, θ=0.01792) and haplotype diversity (*Hd*=1) in the *Udmbf1* gene from Antarctic samples were relatively high. Additionally, of the 19 detected nucleotide sequence variation sites, 15 were observed only in Antarctic samples. The resulting amino acid changes occurred in the N-terminal, whose function remains unknown. Although these DNA polymorphisms and amino acid changes have been verified in Antarctic samples of *U. decussata*, there is still little evidence indicating that different environmental conditions affected the functional evolution of *Udmbf1*. Additional studies involving more *U. decussata* samples from representative ecotypes will be necessary to uncover the relationships among DNA polymorphisms, functional gene evolution, and lichen habitats.

Keywords **Arctic, Antarctica, environmental stress, lichen-forming fungus, stress-tolerance gene, DNA polymorphism**

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1 Introduction

Lichens are composed of a fungus (mycobiont, a lichen-forming fungus) and one or more photosynthetic partner(s) (photobiont, an alga or a cyanobacterium). This stable association represents a symbiotic ecosystem that includes certain groups of endophytic fungi and bacteria. However, the lichenized fungus is the dominant organism in such a complex (Nash, 2008). Lichens can live in most terrestrial ecosystems worldwide, including in polar, desert, and alpine regions. In many polar and sub-polar ecosystems, lichens are the dominant organism (Longton, 1988). Lichens are well adapted to survive in polar regions, and they are better able to cope with long periods of drought and freezing temperatures than other polar organisms (Kappen, 2000; Øvstedal and Smith, 2001; Robinson et al., 2003; Barták et al., 2007). Some lichens remain metabolically active even in a state of low water content (Lange, 1973). Additionally, some dominant Antarctic

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lichen species can maintain maximal photosynthetic rates even at 10°C, while Antarctic cyanobacteria require temperatures between 20°C and 30°C for normal photosynthetic activities (Johnston and Vestal, 1991). Most free-living algae and cyanobacteria can only survive under aquatic or moist conditions. However, as part of lichens, they can survive in harsh habitats, because the mycobiont offers physical and chemical protection (Hawksworth and Hill, 1984; Gauslaa and Solhaug, 2001).

Previous studies revealed that mycobionts and photobionts, either separately or in a symbiotic relationship, undergo several physiological changes when exposed to environmental stresses (Kranner et al., 2005; Weissman et al., 2005). The results suggested that mycobionts and photobionts are resistant to abiotic stresses. However, analyses of survival rates indicated that mycobionts are more resistant to abiotic stress conditions than photobionts (Zhang and Wei, 2011).

Advances in genomic technologies have enabled researchers to investigate the molecular mechanisms underlying abiotic stress resistance (Junttila et al., 2013; Park et al., 2014; Wang et al., 2014; Wang et al., 2015). However, there is still relatively little available information regarding the abiotic stress-resistance gene functions of polar lichens. Because of their dominant existence in polar regions, lichens, especially their mycobionts, are an important and natural resource for stress-tolerance genes. Identifying the stress-responsive genes and characterizing the mechanisms underlying the associated stress-resistance phenotype may facilitate the application of these potential genetic resources.

The gene encoding multiprotein bridging factor 1 (MBF1) is an example of a stress-tolerance gene in lichens. The encoded protein is a transcriptional co-activator (Takemaru et al., 1997) that functions as a linker between the TATA-binding protein (TBP) and a sequence-specific transcription factor, which varies depending on the organism (Ueda et al., 1992; Takemaru et al., 1998; Liu et al., 2003). Multiprotein bridging factor 1 comprises an approximately 40-amino acid N-terminal domain, a conserved helix-turn-helix (HTH) domain, and a short C-terminal region. The N- and C-terminal domains are not involved in protein–DNA interactions (Takemaru et al., 1997). Previous studies revealed that the four α-helices of the HTH domain are responsible for these interactions (Takemaru et al., 1998; Aravind and Koonin, 1999). Under abiotic stress conditions, a series of biochemical and physiological reactions are induced via signaling pathways. MBF1 functions downstream on the MAPK cascade and cAMP–PKA pathway, which are the main fungal pathways that regulate the expression of stress-responsive genes (Ying et al., 2014). *Arabidopsis thaliana* MBF1 influences tolerance to abiotic and biotic stresses (Kim et al., 2007). The overexpression of a wheat gene encoding MBF1 in yeast and rice cells can lead to increased heat tolerance (Qin et al., 2015). Meanwhile, the deletion of the *Beauveria bassiana mbf1* gene affects stress responses and virulence (Ying et al., 2014).

Umbilicaria lichen species are considered to be abiotic stress-resistant model lichens (Larson, 1982; Barták et al., 2004), and most of these species are distributed at high altitudes and latitudes (Wei and Jiang, 1993). *Umbilicaria decussata* (Vill.) Zahlbr. is a cosmopolitan species, and its functional genes may be relevant for investigations into adaptive evolution (Wei and Jiang, 1993; Singh et al., 2010). In this study, *U. decussata* samples were collected from polar and non-polar regions, and the identified nucleotide differences in the *mbf1* genes may provide the basis for future adaptive evolution-related research.

2 Materials and methods

2.1 Sample collection, DNA extraction, PCR amplification, and sequencing

Eight *Umbilicaria decussata* samples were collected from Antarctica, the Arctic, and Armenia (Table 1). Genomic DNA was extracted from a 100-mg lichen thallus for each sample using a modified CTAB method (Rogers and Bendich, 1985). Primers for the polymerase chain reaction (PCR) amplification of the *mbf1* gene were designed based on the published genomic data for *Umbilicaria muehlenbergii* and *Endocarpon pusillum* (Udmbf1-forward: 5′-ATGGACGACTGGGACACCGT-3′; Udmbf1-reverse: 5′-TCACGATTTCGGCGGGAAAAACGGC-3′). The PCR amplifications were completed in a 50-μL reaction volume, with 100 ng genomic DNA used as the template. The PCR amplifications were conducted in a Biometra T-Gradient thermal cycler using the following program: 95°C for 5 min; 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s; 72°C for 5 min. The amplification products were sequenced by Shanghai BioSune Corporation of China using the ABI 3730 XL Sequencer. The resulting sequences were assembled using the Lasergene v7.1 software (DNASTAR Inc.). Sequence data generated in this study have been deposited into the GenBank database (accessions no.: KY499478–KY499481, KY290963–KY290964, and KY290960–KY290961).

2.2 Polymorphisms in the *Udmbf1* genes

The *Udmbf1* gene sequences were analyzed using the DnaSP 4.0 program (http://www.ub.es/dnasp) (Rozas et al., 2003). We estimated the total number of polymorphic sites with single nucleotide polymorphisms (SNPs) or insertions and deletions (InDels). This analysis was completed for the coding and non-coding regions. Additionally, the nucleotide sequences were compared using the summary statistics θ value (i.e., population mutation parameter or number of segregating sites) (Watterson, 1975) and π value (i.e., average pairwise difference or average number of nucleotide differences per site) (Nei and Li, 1979).

Table 1 Eight samples of *Umbilicaria decussata* analyzed in this study

Sample no.	Location	Altitude/m	Ecotype
NJ_a	Crater Cirque, Victoria Land; 169°21.888′ E, 72°36.550′ S	131	Antarctica
NJ_b	Crater Cirque, Victoria Land; 169°21.888′ E, 72°36.550′ S	131	Antarctica
NJ_c	Crater Cirque, Victoria Land; 169°21.888′ E, 72°36.550′ S	131	Antarctica
BJ_8704	Southwest tundra of the King's bay, Ny-Ålesund; 11°35′46″E, 78°57′55″N	43	Arctic
BJ_81107	Nearby the airport of Ny-Ålesund; 11°48′39″E, 78°56′20″N	20	Arctic
BJ_265Z2	London Island, Ny-Ålesund; 12°4′39″E, 78°58′37″N	152	Arctic
BJ_267Z4	London Island, Ny-Ålesund; 12°3′32″E, 78°57′43″N	20	Arctic
AM_7402	Aragats Sont Province, Armenia; 44°14′51.2″E, 40°24′54.7″ N	2500	Armenia

2.3 Phylogenetic analyses

The *Udmbf1* and internal transcribed spacer (ITS) sequences from eight samples were aligned using MEGA4 (Tamura et al., 2007) (www.megasoftware.net/mega4/mega.html). The *Udmbf1* introns were manually excluded. Maximum likelihood (ML) trees, which included the *mbf1* and ITS genes, were constructed. The optimal models for constructing the ML trees were identified using the PhyML online tool (www.atgc-montpellier.fr/).

3 Results

3.1 Nucleotide variations in the *Udmbf1* genes

Eight *U. decussata Udmbf1* amplicons were sequenced, and the nucleotide variations (i.e., SNPs and InDels) were identified (Table 2). The full-length *Udmbf1* sequence was 552 bp, with one intron located at position 158–241. Additionally, 32 bases were missing in the 3′-end of the Antarctic samples, probably because of long-term preservation of the specimens or poor sequencing quality. The missing bases were replaced by N during the data processing step. The coding sequence (CDS) comprised two parts with 30 SNP sites, with a frequency of 0.064 (Table 2). The CDS region lacked InDels. Additionally, the intron contained 14 SNP sites, with a SNP frequency (0.167) that was more than 2-fold higher than that of the CDS. The intron also included two InDel sites, with an average length of 3 bp.

The Arctic and Antarctic sequence datasets were analyzed separately as well as combined to identify DNA polymorphisms (Table 3). In the combined analysis, the CDS and intron of eight *Udmbf1* amplicons formed six and five haplotypes, respectively. The analysis of the Antarctic samples revealed that three CDSs included three haplotypes. Additionally, the haplotype diversity value was 1, which was the highest value among the three datasets. Correspondingly, the nucleotide diversity parameters (π and θ) of the Antarctic samples were higher than those of the Arctic samples.

Table 2 Parameters for the nucleotide variation of *Udmbf1* gene

Parameter	Full length	CDS	Intron
Length of the sequence/bp	552	468	84
Sites with missing data	32 (only in Antarctic samples)	32	0
Number of nucleotide variation (SNPs and InDels)	52	30	20
Variation frequency (variation/bp)	0.094	0.064	0.238
Number of SNP	46	30	14
SNP frequency (SNP/bp)	0.083	0.064	0.167
Number of InDel	2	0	2
Average length of InDel/bp	3	—	3
InDel frequency (InDel/bp)	0.003	—	0.024

Table 3 The DNA polymorphism parameters of *Udmbf1* gene

Parameters	Total dataset		Antarctic		Arctic	
	CDS	Intron	CDS	Intron	CDS	Intron
Number of sequence	8	8	3	3	4	4
Number of Haplotypes	6	5	3	2	3	2
Haplotype diversity (*Hd*)	0.929	0.893	1	0.667	0.833	0.667
Nucleotide diversity (π)	0.02949	0.09341	0.01988	0.00855	0.01211	0.05063
Theta (θ)	0.02565	0.07417	0.01988	0.00855	0.01049	0.04143

3.2 Common variations in the nucleotide and amino acid sequences of each polar ecotype

To verify the differences in the *mbf1* sequences from the two polar ecosystems, the nucleotide and amino acid variations that were common to all samples collected in each polar region were identified (Tables 4 and 5). A total of 19 common nucleotide variations were detected in the full-length *Udmbf1* sequence, including one InDel site in the intron (Table 4). Among these 19 sites, 15 were detected only in Antarctic samples, while two were exclusive to the Arctic samples. A comparison of the frequency of sequence variations in the CDS and intron indicated there were more nucleotide variations per base in the CDS. However, only the variations inducing amino acid changes were considered important. Thus, the amino acid changes and their corresponding nucleotide polymorphisms in the *Udmbf1* exons are listed in Table 5. Among the 11 nucleotide variations in the CDS, four SNP sites resulted in changes to four amino acids. These amino acid variations were observed only in the Antarctic samples, and not in the Arctic samples.

3.3 Topologies of phylogenetic trees constructed with internal transcribed spacer and MBF1 sequences

The ML trees constructed based on the amino acid sequences encoded by the *Udmbf1* and ITS sequences are presented in Figure 1. All of the *U. decussata* samples formed a well-supported monophyletic clade (86% bootstrap value, ITS tree in Figure 1), separate from other genera and *Umbilicaria* species. Moreover, the ITS tree divided the *U. decussata* samples into three groups. The first group comprised BJ8704 (Arctic sample), BJ81107 (Arctic sample), and AR 7402 (Armenian sample). The second group consisted of only NJc (Antarctic sample), while the third group included BJ265Z2 and BJ267Z4 (Arctic samples). A similar topology was observed for the MBF1 tree. One difference was that the MBF1 tree did not have the *U. decussata* samples in a separate clade. Instead, these samples were clustered together with other *Umbilicaria* species and *Lasallia* species to form a highly-supported group (97% bootstrap value, MBF1 tree in Figure 1).

4 Discussion

Polymorphisms in functional genes enhance the diversity among populations. They also reflect the process of adaptive evolution. The most common nucleotide variations in genomes are SNPs and InDels, which can alter gene functions to induce different phenotypes (Zhang et al., 2013). Identifying the functional domain of a stress-resistance gene and tracing its evolutionary pattern requires the analysis of DNA polymorphisms within a species. Selecting a suitable sample and an appropriate

Table 4 Ecotype common nucleotide polymorphic sites in *Udmbf1* genes

Nucleotide position		Polymorphic type	Sign	Ecotype common nucleotide
CDS	50	T/C/C	Δ	Antarctic (T), Arctic (C), Armenian (C);
	58	G/C/C	Δ	Antarctic (G), Arctic (C), Armenian (C);
	78	T/C/C	Δ	Antarctic (T), Arctic (C), Armenian (C);
	94	G/A/A	Δ	Antarctic (G), Arctic (A), Armenian (A);
	96	T/C/C	Δ	Antarctic (T), Arctic (C), Armenian (C);
	132	G/A/A	Δ	Antarctic (G), Arctic (A), Armenian (A);
	133	G/A/A	Δ	Antarctic (G), Arctic (A), Armenian (A);
	148	T/G/G	Δ	Antarctic (T), Arctic (G), Armenian (G);
	345	C/T/C	●	Antarctic (C), Arctic (T), Armenian (C);
	351	T/C/C	Δ	Antarctic (T), Arctic (C), Armenian (C);
	417	T/T/C	–	Antarctic (T), Arctic (T), Armenian (C);
Intron	159	C/A/A	Δ	Antarctic (C), Arctic (A), Armenian (A);
	180	T/C/T	●	Antarctic (T), Arctic (C), Armenian (T);
	182	C/C/A	–	Antarctic (C), Arctic (C), Armenian (A);
	191	C/G/G	Δ	Antarctic (C), Arctic (G), Armenian (G);
	205	C/T/T	Δ	Antarctic (C), Arctic (T), Armenian (T);
	220	_/A/A	Δ	Antarctic (_), Arctic (A), Armenian (A);
	228	C/T/T	Δ	Antarctic (C), Arctic (T), Armenian (T);
	229	A/C/C	Δ	Antarctic (A), Arctic (C), Armenian (C)

Notes: The signs of "Δ" and "●" represent the variations only found in Antarctic samples and Arctic samples respectively; the sign of "_" represents uncertain variations in Armenian ecotype.

Table 5 Ecotype common amino acid polymorphic sites in *Udmbf1*

Amino acid position	Coding change	Amino acid change	Sign	Ecotype common amino acid code
20	GAC/CAG	E/Q/Q	Δ	Antarctic (E), Arctic (Q), American (Q);
32	GAT/AAC	D/N/N	Δ	Antarctic (D), Arctic (N), American (N);
45	GAG/AAG	E/K/K	Δ	Antarctic (E), Arctic (K), American (K);
50	TGC/GGC	C/G/G	Δ	Antarctic (C), Arctic (G), American (G)

Notes: The sign of "Δ" represents the variation sites only found in Antarctic samples.

functional gene is critical. In this study, we analyzed the sequence polymorphisms of the stress-related *mbf1* gene from the lichen *U. decussata*, which is distributed worldwide. The results described herein may form the basis of future investigations into the relationship between DNA polymorphisms and functional adaptations.

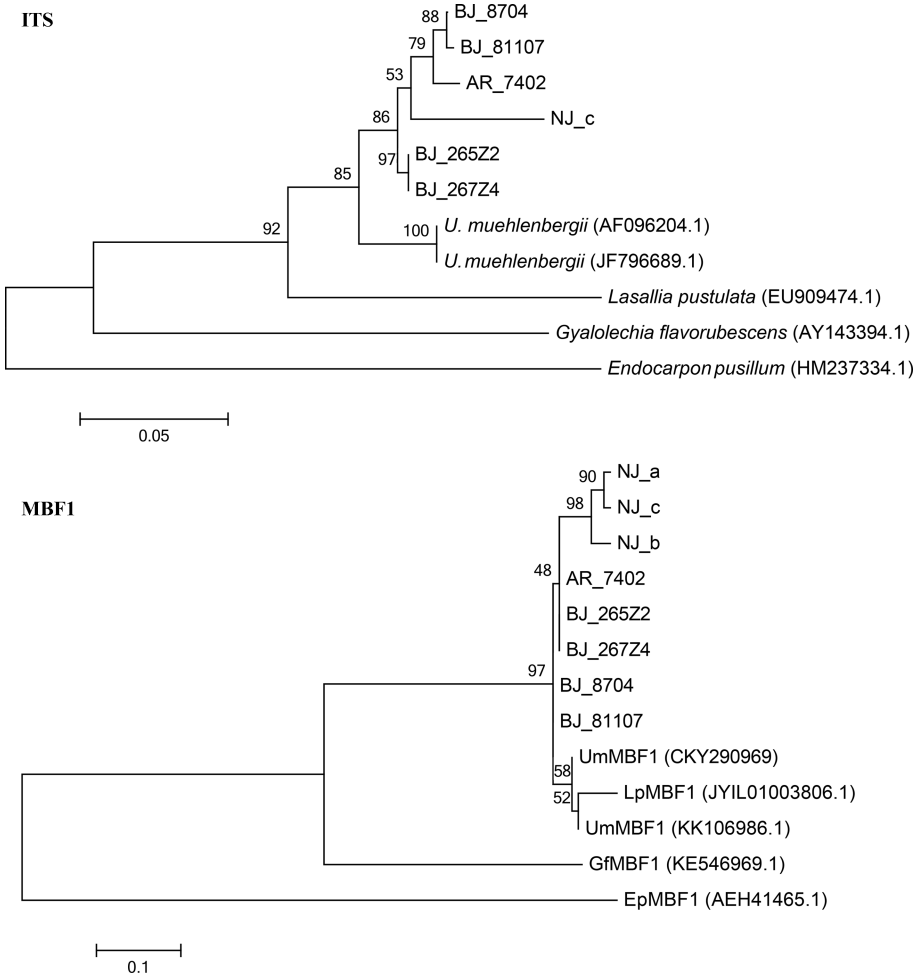

Figure 1 Maximum Likelihood tree based on ITS sequences and MBF1 sequences, respectively. In the ITS tree, *Umbilicaria muehlenbergii* (AF096204.1), *Lasallia pustulata* (EU909474.1), *Gyalolechia flavorubescens* (AY143394.1) and *Endocarpon pusillum* (HM237334.1) was used as outgroup, and their corresponding MBF1 protein amino acid sequences, UmMBF1 (located in the contig KK106986.1), LpMBF1 (located in the contig JYIL01003806.1), GfMBF1 (located in the contig KE546969.1) and EpMBF1 (AEH41465.1) were identified using local blastp software. Sequences without GenBank accession numbers were sequenced in this study.

The *U. decussata* samples from the polar regions were expected to exhibit an enhanced ability to adapt to stress conditions. We analyzed *mbf1*, which is a relatively conserved stress-resistance gene among eukaryotic organisms (Liu et al., 2007). Previous studies confirmed this gene is associated with abiotic stress resistance (Ying et al., 2014; Qin et al., 2015). However, there remain questions regarding the evolution of *mbf1* (Coto et al., 2011). A more thorough characterization of the variations conserved within ecotypes and potential stress-related sites of *Udmbf1* may help to fill knowledge gaps.

In our study, we detected abundant DNA polymorphisms, including SNPs and InDels, in *Udmbf1* genes (Table 2). The frequency of these variations was higher in introns than in coding regions. This result suggests that the intron was affected more by selective pressure than the exon, which is consistent with the general rule that introns evolve more rapidly than exons (Small and Wendel, 2000). Additionally, we detected more polymorphic sites and haplotypes in the Antarctic samples than in the Arctic samples, with corresponding differences in the π and θ values. Our findings also imply that the *Udmbf1* sequences from the Arctic samples were exposed to greater evolutionary selective pressure than the genes from the Antarctic lichen samples. Considering we only examined eight samples, another investigation involving a much larger sample size may produce a more objective and accurate result.

To clarify whether the protein encoded by *Udmbf1* functions differently in polar and non-polar regions, the amino acid sequence changes induced by the nucleotide variations were considered. The *Udmbf1* of the Antarctic samples had more common nucleotide variations than the corresponding gene in the other two populations (Table 4). These nucleotide changes altered four amino acids at positions 20, 32, 45, and 50 (i.e., position 20 from glutamic acid to glutamine, position 32 from aspartic acid to asparagine, position 45 from glutamic acid to lysine, and position 50 from cysteine to glycine) (Table 5). These changes occurred in the N-terminal domain of MBF1. While the MBF1 HTH domain reportedly affects protein–protein interactions, the functions of the N- and C-termini remain unknown.

To verify the evolutionary relationships among the *Udmbf1* genes from lichen samples collected in polar and non-polar regions, we constructed ITS and MBF1 phylogenetic trees. The ITS region is considered to be a good barcoding marker for exploring inter- and intra-specific variations in diverse fungi (Schoch et al., 2012). Therefore, a phylogenetic tree based on ITS sequences may reflect the evolutionary relationships among organsims. Based on the data presented herein, the Arctic *U. decussata* samples carried two distinct ITS genotypes, one of which was more similar to that of the *U. decussata* collected in Armenia than to the other genotype of the *U. decussata* from the Arctic. There may be two explanations for this observation. First, the BJ265Z2 and BJ267Z4 Arctic *U. decussata* genotypes may be older than the BJ8704 and BJ81107 genotypes, which differentiated from the older genotypes and were eventually dispersed in Armenia. Second, the two Arctic genotypes and the genotype in Armenia may have originated from the same ancestor, but gradually diverged into two different directions. However, in the MBF1 phylogenetic tree, with the exception of three Antarctic *U. decussata* samples clustered into one well-supported clade, all *U. decussata* samples were grouped with other *Umbilicaria* species and *Lasallia* species. This suggests the *Udmbf1* gene originated similarly in the examined species. Although DNA polymorphisms and amino acid variations have been verified in Antarctic samples of *U. decussata*, there is still little evidence that different environmental conditions have affected the functional evolution of the *Udmbf1* gene. Additional studies involving more *U. decussata* samples from representative ecotypes are necessary to elucidate the relationships among DNA polymorphisms, functional gene evolution, and lichen habitats.

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