Low expression levels of *MAT* genes may be one of the major causes of asexuality in *Ulocladium*

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ABSTRACT

Sexual reproduction in ascomycetes is controlled by mating-type locus (*MAT*). Both *MAT1-1-1* and *MAT1-2-1* genes were detected and functional in *Ulocladium* species, while no sexual stage has been identified for this genus. Environmental factors play an important role in regulating sexual development of filamentous fungi. The mRNA transcription level of *MAT1-1-1* and *MAT1-2-1* genes was tested using qRT-PCR under different temperatures (-20 °C, -10 °C, 0 °C, 10 °C, 20 °C, 30 °C and 40 °C), culture medias (CM, OA, HAY, PCA, PDA and V8), photoperiods (24h light, 24h dark, 12h light/12h dark, 10h light/14h dark and 8h light/16h dark) and CO_2 concentrations (0.03%, 0.5%, 1%, 5%, 10%, 15% and 20%), which may help to explain the lack of sexual cycle in *Ulocladium*. For reliable results from qRT-PCR, the expression of seven frequently used HKGs was assessed and the most stable internal control gene was determined under different environmental treatments. The expression levels of both *MAT* genes were highest when fungal mycelia were grown on HAY culture media at 0-10 °C with a light/dark cycle, indicating that temperature, culture media and light may be the key environmental factors for sexuality in *Ulocladium*. The same expressional tendencies for *MAT1-*

1-1 and *MAT1-2-1* genes were found, suggesting that two *MAT* genes play an equally important role in the sexual evolutionary process. Moreover the expression levels of *Pleospora tarda MAT* genes are significantly higher than those of *U. botrytis* and *U. arborescens*, indicating that the low expression level may be one of the major causes of asexuality in *Ulocladium*.

INTRODUCTION

There are an estimated 1.5 million species of fungi worldwide, which is 6 times more than higher plants (*Hawksworth, 2001*). Hyphomycetes, a major taxon of fungi, are widely distributed in land, sea, air and soil, and the genera and species of which account for more than 30% of the fungal kingdom (*Hawksworth, Sutton & Ainsworth, 1983; Kirk et al., 2008;* http://www.indexfungorum.org). ~~However~~, no sexual stage has been identified for most of hyphomycetes. For more than 450 genera (about 16000 species) of hyphomycetes, Hyphomycetales, Dematiaceous hyphomycetes, teleomorphs were found in only 67 genera.

Ulocladium (*Preuss, 1851*) is an anamorphic genus of the *Pleosporaceae* (*Dothideomycetes, Kirk et al., 2008*). Some species of *Ulocladium* are often found as pathogens or endophytes of living plants and as saprobes playing an important ecological role in the decomposition and recycling of materials in natural ecosystems (*Zitter & Hsu, 1990; Vannini & Vettraino, 2000*). Both *MAT1-1-1* and *MAT1-2-1* genes were detected and expressed within the same genome of *Ulocladium* species, indicating the potential for sexual reproduction in *Ulocladium* species (*Geng et al., 2014*). However, as yet, no sexual stage has been identified for *Ulocladium*. One of the possible reasons for the apparent lack of sexuality in *Ulocladium* species is the absence of ~~major~~ environmental factors influencing sexual development, including medium, light, temperature, atmospheric gases, etc.

Environmental factors have great influences on sexual development of filamentous fungi. Ascospore production requires special conditions, and different ascomycete isolates show variations in response to identical conditions of incubation. Teleomorphs of many asexual fungi have been found under controlled conditions. Perithecium formation was favored at 20°C and with a 16-h daily photoperiod in *Mycosphaerella pinodes* (*Roger & Tivoli, 1996*). For *Pyrenophora tritici-repentis*, a large number of asci and ascospores were producted by incubating the fungus on senescent leaves in continuous darkness for 12 days followed by a 12-h photoperiod at 15 °C (*Friesen et al., 2003*). Several isolates of *Stemphylium* have produced

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mature ascocarps on hay decoction agar, 20% V-8 juice agar or weak potato-carrot agar, within periods of time ranging from a few months to a year or more (*Simmons, 1969*). *Gaeumannomyces garminis* var. *tritici* which ~~was~~ is strictly asexual in nature could produce asci and ascospores adequately in the sterile culture medium with dark/light (12h/12h) at 15 to 20 °C for 72 h (*Holden & Hornby, 1981*). The ability of sexual reproduction in *Candida albicans* was enhanced with high temperature, high CO_2/O_2 and darkness (*Ramírez-Zavala et al., 2008; Whiteway, 2009*).

Sexual reproduction in ascomycetes is controlled by mating-type locus (*MAT*) (*Coppin et al., 1997; Turgeon, 1998*). The *MAT* locus consists of two idiomorphs, termed *MAT1-1* and *MAT1-2* with identical flanking regions and dissimilar DNA sequences (*Turgeon et al., 1993; Cozijnsen & Howlett, 2003*). *MAT1-1* and *MAT1-2* encode proteins with an alpha domain and HMG (high mobility group) domain respectively (*Turgeon, 1998*). *MAT* genes have been characterized in ~~lots of~~many filamentous ascomycetes (*Inderbitzin et al., 2005; Groenewald et al., 2006; Santos, Correia & Phillips, 2010; Bolton et al., 2012*), and the full length of *MAT1-1-1* and *MAT1-2-1* genes were sequenced for 26 *Ulocladium* species (*Geng et al., 2014*). It has been reported that *U. botrytis MAT* genes have the ability to induce sexual recombination in *Cochliobolus heterostrophus* (*Wang et al., 2017*), indicating that *MAT* locus were functional in *Ulocladium* and the expression levels of *MAT* genes directly affect sexual development in fungi.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is one of the most widespread techniques for quantification of transcript expression levels. In comparison to Northern blot hybridization and reverse transcription polymerase chain reaction (RT-PCR), the main advantages of qRT-PCR are its sensitivity, accuracy, broad dynamic range and reproducibility (*Bustin & Nolan, 2004; Bustin et al., 2005; Kubista et al., 2006*). It is a valuable tool to measure expression of a gene in the cells of different tissues and under specific experimental conditions, and is now commonly used to validate the whole-genome microarray data. The qRT-PCR is now increasingly being applied in the fields of molecular diagnostics (*Bustin & Dorudi, 1998*), biotechnology, microbiology and gene expression studies in plants (*Gachon, Mingam & Charrier, 2004*) and fungi.

Appropriate normalisation of qRT-PCR data is necessary to remove the non-biological variation caused by experimental deviations, inhibitory compounds, RNA isolation or reverse

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transcriptase efficiency. The use of proper internal reference genes is the most common way to normalize the data generated by qRT-PCR (*Zitter & Hsu, 1990*). Many housekeeping genes such as β-actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (Tub-b) and 18S ribosomal RNA were frequently used as internal controls for gene expression analysis. They involve in basic and ubiquitous cellular functions, and thus are supposed to have a uniform expression. However, several studies have indicated that expression levels of such reference genes also vary with experimental conditions (*Thellin et al., 1999; Suzuki, Higgins & Crawford, 2000; Lee et al., 2002*). In general, there is no universal internal control gene that can be expressed at a constant level under different experimental conditions (*Peters et al., 2007; Mitter et al., 2009*). Therefore, the validity of candidate reference gene under specific experimental conditions must be determined to avoid erroneous results. Quantitative analysis of gene expression should be related to several housekeeping genes in parallel (*Schmid et al., 2003*), thus it is important to determine the optimal number of internal control genes required for accurate data normalization.

In this study, seven candidate reference genes, including *Actin*, *β-tubulin*, *EF-1α*, *GAPDH*, *RL13*, *TBP* and *UBC* were selected to study the expression stability for normalisation of gene expression by qRT-PCR in *Ulocladium* strains exposed to different treatments. In addition, the mRNA transcription levels of *MAT1-1-1* and *MAT1-2-1* genes under different environmental treatments were tested by qRT-PCR, which may help to explain the lack of sexual cycle in *Ulocladium*.

MATERIALS & METHODS

Ulocladium strains and growth conditions

The strains of *U. botrytis* (CBS 198.67), *U. arborescens* (CBS 115269) and *Pleospora tarda* (AY335164) were used for all experimental treatments. *Pleospora tarda* is a representative sexual species. All samples were obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. For each strain, cultures were grown on potato-carrot agar (PCA; 20 g white potato boiled and filtered, 20 g carrot boiled and filtered, 20 g agar, 1 L distilled water) at 25°C for 10 days. Fungal mycelia were scraped from the surface of the agar, frozen immediately in liquid nitrogen, and stored at -80℃ for RNA extraction.

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Treatments

For temperature treatments, small squares of fungal mycelia were placed in the middle of the petri dishes (9 cm) containing PCA medium, and the cultures were grown at 25℃ for 10 days. Cultures were then exposed to seven temperature treatments: -20°C, -10°C, 0°C, 10°C, 20°C, 30°C and 40°C, with a daily photoperiodic cycle of 14 h light and 10 h dark for 7 days. Fungal mycelia were scraped from the surface of the agar, frozen immediately in liquid nitrogen, and stored at -80°C for RNA extraction. Three biological replicates were used for each condition.

For medium treatments, small squares of fungal mycelia were placed in the middle of the petri dishes (9 cm) containing PCA (200 g white potato, 200 g carrot, 20 g agar, 1 L distilled water), PDA (200 g white potato, 20 g glucose, 20 g agar, 1 L distilled water), CMA (200 g corn meal, 20 g agar, 1 L distilled water), HAY (10 g hay, 20 g agar, 1 L distilled water), OA (100 g oatmeal, 20 g agar, 1 L distilled water), V8 (150 ml V8 juice, 1.6 g $CaCO_3$, 20 g agar) medium separately, and the cultures were grown at proper temperature in a 12:12 light dark regime for two weeks. Fungal mycelia were scraped from the surface of the agar, frozen immediately in liquid nitrogen, and stored at -80°C for RNA extraction. Three biological replicates were used for each condition.

For photoperiod treatments, small squares of fungal mycelia were placed in the middle of the petri dishes (9 cm) containing optimum medium, and then exposed to different photoperiods: 24h light, 24h dark, 12h light:12h dark, 10h light:14h dark, 8h light:16h dark, with proper temperature for two weeks. Fungal mycelia were scraped from the surface of the agar, frozen immediately in liquid nitrogen, and stored at -80°C for RNA extraction. Three biological replicates were used for each condition.

For CO_2 treatments, small squares of fungal mycelia were placed in the middle of the petri dishes (9 cm) containing optimum medium, and the cultures were grown at 25℃ for 10 days. Cultures were then exposed to seven CO_2 concentrations: 0.03%, 0.5%, 1%, 5%, 10%, 15% and 20%, with proper temperature and photoperiod for 7 days. Fungal mycelia were scraped from the surface of the agar, frozen immediately in liquid nitrogen, and stored at -80℃ for RNA extraction. Three biological replicates were used for each condition.

Total RNA extraction and reverse transcription

Mycelia of *U. botrytis* and *U. arborescens* and teleomorphs (asci) of *Pleospora tarda* were collected for extracting total RNA using Trizol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions, followed by RNase-free DNase treatment (Takara, Japan). RNA

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concentrations were quantified by a spectrophotometer (spectra-Max plus 384; Molecular Devices, Sunnyvale, CA, USA) and RNA integrity was assessed by 1% agarose gel electrophoresis. All the RNA samples used for the analysis showed a 260/280 ratio of 1.8-2.1 and a 260/230 ratio of approximately 2.0. PCR amplifications with RNA samples were performed to confirm that no genomic DNA contamination in the samples used for qRT-PCR. To obtain the first strand of cDNA, reverse transcription from 3 µg of total RNA was performed using the SuperScript Firststrand Synthesis System (Invitrogen) according to manufacturer's instructions. cDNA samples were then diluted with nuclease-free water to 1:5. Reverse transcription were performed in triplicate for all treatment conditions.

Real time PCR

Real time quantitative PCR was performed in the ICycler IQ real-time PCR detection system (Bio-Rad, Denmark) using SYBR primer Script RT-PCR kit (TakaRa, Japan). The specific primers for real time RT-PCR were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, USA) (Table S1). For PCR reactions, 2.5 µl of cDNA template was added to 12.5 µl of the 2 × SYBR Green PCR master mix, 800 nM of each primer and ddH_2O to a final volume of 25 µl. After a denaturation step at 95 °C 10 min, the cycle profile used was 10 s at 95 °C, 55 s at 60 °C, and 45 s at 72 °C for 45 cycles. All reactions were performed in triplicate, and negative controls (with no template) were included for each gene. Melting curve analysis of the amplified products resulted in a single peak in all samples, indicating the specificity of the qRT-PCR reactions. The threshold cycle (CT) values were determined automatically by the instrument, and the fold changes of each gene were calculated using the equation 2-ΔΔC_T. Data are represented as mean ± SD of the values obtained from triplicate experiments.

Determining expression stability of reference genes

The geNorm (version 3.5) program was used to analyze the expression stability of selected internal control genes. This program provides a ranking of the reference genes based on the gene expression stability (M), described as the average pairwise variation V for a single gene with all other tested genes. The lowest M value of a gene represents the highest expression stability (*Vandesompele et al., 2002*). Furthermore, normalization factors (NFn) and Vn/Vn+1 value were calculated to estimate the optimal number of controls to be used in each single experiment.

RESULTS

Selection of housekeeping genes and amplification specificity

Although morphological and phylogenetic analysis of *Ulocladium* has been studied well, little effort has been made to the research on gene expression by qRT-PCR in this genus. Seven HKGs known to be most commonly used in other ascomycetes were selected as candidate reference genes in *Ulocladium*: β-actin (*Actin*), *β-tubulin* (*Tub-b*), translation elongation factor 1α (*EF-1α*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 60S ribosomal protein L13 (*RL13*), TATA box binding protein (*TBP*) and ubiquitin-conjugating enzyme (*UBC*). Specificity of all the primer sets was confirmed by the appearance of a single, dominant peak in the qRT-PCR dissociation curve analyses (Fig. S1).

Ranking of the reference genes

The geNorm software was then used to compare the transcription levels of these control genes and identify the most stable gene under different experimental conditions. Seven candidate reference genes selected to study the expression stability for normalization were ranked according to their average expression stability (M) values. The results showed that there is no universal internal control gene that can be expressed at a constant level under different experimental treatments.

For temperature treatments, all the candidate reference genes that reached high expression stability showed M values lower than 1. *EF-1α* and *Actin* were the most stable genes and the worst scoring gene was *RL13*. The ranking of the expression stability in different temperatures: *EF-1α, Actin>GAPDH>UBC>TBP>β-tubulin> RL13* (Fig. 1A).

For medium treatments, the M values for all investigated genes were lower than 1.5. *TBP* and *EF-1α* were ranked as the most stable reference genes and *Actin* was the worst HKG. The ranking of the expression stability for different medium treatments: *TBP, EF-1α>GAPDH> β-tubulin>UBC> RL13> Actin* (Fig. 1B).

For photoperiod treatments, the M values for all supposed reference genes were lower than 1.5. The most stable genes were *GAPDH* and *EF-1α*. *RL13* was ranked as the worst HKG. The ranking of the expression stability for different photoperiod treatments: *GAPDH, EF-1α>Actin>TBP>β-tubulin>UBC> RL13* (Fig. 1C).

For CO_2 treatments, the M values for all investigated genes were lower than 1.5. *EF-1α* and *GAPDH* were the best ranked genes and *RL13* considered as the worst scoring gene. The ranking

of the expression stability for different CO_2 treatments: *EF-1α*, *GAPDH*>*Actin*>*TBP*> *β-tubulin*>*UBC*>*RL13* (Fig. 1D).

To estimate the optimal number of reference genes for normalization, normalization factors (NFn) and Vn/Vn+1 value were calculated. The default cut-off threshold is 0.15 (Vandesompele et al., 2002), so it is unnecessary to select an additional reference gene when Vn/Vn+1<0.15. As shown in Fig. 2, two control genes for 'photoperiods' (Fig. 2C), three reference genes for 'CO_2 concentrations' (Fig. 2D) and the four most stably expressed genes for other treatments (Fig. 2A and B) were required for the determination of a reliable normalization factor.

Transcriptional level of the *MAT* genes

For temperature treatments, the transcription levels of *MAT1-1-1* and *MAT1-2-1* genes were investigated at 10°C intervals from -20 to 40°C. The transcription levels of *MAT* genes gradually increased from -20 to 0°C, and then eventually reached a peak at 10°C (Fig. 3). These results showed that the optimum temperature for sexual development of *Ulolcadium* was 0-10°C.

For medium treatments, we tested the *MAT1-1-1* and *MAT1-2-1* transcription levels of mycelia for *Ulocladium* on different kinds of culture medias (CM, OA, HAY, PCA, PDA, V8). The results indicated that *MAT* genes showed the highest transcription levels on HAY media, but no obvious difference could be observed on the other culture medias (Fig. 4). HAY is the poorest relative to the rest of media, suggesting that the nutrient-poor culture conditions are conducive to production of teleomorphs.

For photoperiod treatments, the transcription levels of *MAT1-1-1* and *MAT1-2-1* genes were investigated when exposed to different photoperiods (24h light, 24h dark, 12h light:12h dark, 10h light:14h dark, 8h light:16h dark). The expression of *MAT* genes with a light/dark cycle was obviously higher than other photoperiod treatments (Fig. 5). This results indicated that light is a necessary condition for sexual reproduction of *Ulocladium*.

For CO_2 treatments, we tested the transcription levels of *MAT1-1-1* and *MAT1-2-1* genes when exposed to different CO_2 concentrations (0.03%, 0.5%, 1%, 5%, 10%, 15% and 20%). The transcription levels of *MAT* genes remain unchanged after many repetitions (Fig. 6), indicating that teleomorphs are not sensitive to CO_2.

The expression levels of both *MAT* genes were highest when fungal mycelia were grown on HAY culture media at 5-10°C with a light/dark cycle, indicating that temperature, culture media and light are the key environmental factors that affect the sexual development of *Ulocladium*.

The same expressional tendencies for *MAT1-1-1* and *MAT1-2-1* genes were found, suggesting that two *MAT* genes play an equally important role in the sexual evolutionary process. Moreover the expression levels of *Pleospora tarda MAT* genes are significantly higher than those of *U. botrytis* and *U. arborescens*, indicating that the low expression level may be one of the major causes that no sexual state has been identified for *Ulocladium*.

DISCUSSION

The expression of HKGs was instable under different experimental conditions, and the expression patterns of a reference gene varied in different fungal species. It is necessary to determine the validity of candidate reference gene before normalization. To get reliable results from qRT-PCR, seven HKGs known to be most commonly used in other ascomycetes were selected as candidate reference genes and the most stable internal control gene was determined under temperature, medium, photoperiod and CO_2 treatments in *Ulocladium* (Fig. 1). In addition, the optimal number of reference genes for normalization was estimated (Fig. 2). The results showed that there was no universal internal control gene that can be expressed at a constant level under different experimental treatments, which was consistent with previous studies (*Suzuki, Higgins & Crawford, 2000; Lee et al., 2002; Boxus, Letellier & Kerkhofs, 2005*).

Environmental factors play an important role in regulating sexual differentiation and development of fungi. *Hawker (1966)* systematically discussed the effects of environmental conditions on the sexual reproduction of filamentous ascomycetes, which suggested that the environment suitable for mycelia development or asexual reproduction are not necessarily adapt to sexual cycle and the later development of teleomorphs. In recent years, mycologists have discovered the teleomorphs of several filamentous ascomycete species which were recognized strictly asexual from Antarctica, Arctic, snow-covered mountains, nutrient-poor salt lakes, deserts and plateau areas. Moreover A few asexual species have been successfully induced teleomorphs in artificial stress conditions (poor nutrition, low temperature, strong ultraviolet light and high CO_2/O_2) (*Simmons & Roberts, 1993; Câmara, O'Neill & Van Berkum, 2002*). Therefore, environmental stress has significant regulatory effects on the sexual evolution and development of asexual hyphomycetes.

The occurrence of sexual reproduction can be influenced by the nutritional environments for fungal growth, such as water, nitrogen, carbon, glucose, amino acid and phosphorus, etc

(*Zonneveld, 1977; Bussink & Osmani, 1998; Swart et al., 2001; Han et al., 2003; Dyer & O'Gorman, 2012*). It has been reported that low concentrations of carbon or nitrogen can promote sexual reproduction of filamentous ascomycetes (*Zonneveld, 1974; Han et al., 1994; Han et al., 2003*). Poor nutrition was conducive to meiosis and the production of sexual spores in *Saccharomyces cerevisiae* and high concentrations of glucose inhibit the formation and development of perithecia in *Ophiobolus graminis* (*Garrett, 1967*). Sexual development of fungi is also highly sensitive to different kinds and concentrations of minerals or vitamins, e.g. the minerals and vitamins promoted the formation of perithecia in *Sordariaceae* (*Watanabe, 1997*). For *Ulocladium* species, the nutrient-poor culture conditions are conducive to production of teleomorphs (Fig. 4).

Temperature also has prominent effects on the sexual recombination of filamentous ascomycetes. The appropriate temperature thresholds for sexual reproduction of various fungal species are different. It has been found that 10-15°C was favorable for the formation of apothecium in *Monilinia* and -4°C promoted the production of perithecia in *Hypomyces solani* var. curcurbitae (*Moore-Landecker, 1992; Casals et al., 2010*). The optimum temperature for sexual reproduction of *Aspergillus fumigatus* was 30°C (*O'Gorman, Fuller & Dyer, 2008*). The asci numbers of *Aspergillus nestoris* at 32°C were twice than that at 37°C (*Dyer, 2007*). This study showed that the optimum temperature for sexual development of *Ulolcadium* was 0-10°C (Fig. 3).

In addition, light intensity, light quality and photoperiod have significant effects on the sexual cycle in fungi (*Klich, 2002*). It has been found that red and blue light inhibited the sexual reproduction of *Aspergillus*, while far-red light achieved the opposite effect (*Blumenstein et al., 2005; Bayram et al., 2008*). Different stages of fungal sex differentiation have varied requirements for light, e.g. near-ultraviolet light promoted the pycnidium formation of *Mycosphaerella pinodes* and far red light was beneficial to the production of its perithecium. (*Blumenstein et al., 2005; Bayram et al., 2008*) Appropriate light intensity was necessary for some fungi, such as *Neurospora crassa* which could produce perithecium after 12 seconds of blue light (1.05W/m^2), while the irradiation time needed to be extended by 20 times when the light intensity was reduced to 5.25x10-2W/m^2 (*Roger & Tivoli, 1996*). *Gelasinospora reticulospora* and *Alternaria* species could produce a large number of perithecia under the light/dark conditions (*Simmons, 1986; Simmons & Roberts, 1993; Chamberlain & Ingram,*

1997). This study indicated that light was also a necessary condition for sexual reproduction of *Ulocladium* (Fig. 5).

Gas composition, especially the ratio of CO_2/O_2, has significant effects on the sexual differentiation of some filamentous ascomycetes. High CO_2/O_2 in dark conditions could easily induce the apothecial formation of *Leptosphaeria typhae*, while low CO_2/O_2 is conducive to product asexual spores (*Vidal & Viala, 1979*). In addition on solid media the number of asci improved significantly with increasing CO_2 concentration in *Aspergillus nidulans* (*Zonneveld, 1977; Zonneveld, 1988*). It has been shown that air relative humidity (RH) and NO also affected the sexual reproduction in fungi (*Rai, Tewari & Sinha, 1967; Baidya et al., 2011; Xu et al., 2011*). However, the teleomorphs were not sensitive to CO_2 for *Ulocladium* (Fig. 6).

Sexual reproduction which could enhance the adaptability to environmental stress plays a prominent role in the evolution and continuation of species (*Ashton & Dyer, 2016*). Sexual recombination is dominant in harsh habitats, including desiccation, high temperatures, fungicides and so on (*Bowden & Leslie, 1999, Dyer & O'Gorman, 2012*). Asexual lineages were thought to be evolutionary dead ends (*Wik, Karlsson & Johannesson, 2008*), though they could complete population spread in a short time. However, the sexual stage of *Ulolcladium* has not been found yet. In attempting to induce formation of a teleomorph of *Ulocladium*, we have performed extensive mating testing by confronting cultures of the same and different strains, but with no success. *MAT* genes which are key regulators for sexuality in filamentous ascomycetes are functional in *Ulocladium*. Though specific environmental conditions can promote the expression of *MAT* genes of this genus, the significantly lower levels compared to *Pleospora tarda* may be one of the major causes that no sexual state has been identified for *Ulocladium*.

CONCLUSIONS

In this study, the most stable internal control gene and optimal number of reference genes for normalization were determined under temperature, medium, photoperiod and CO_2 treatments in *Ulocladium*. The expression levels of both *MAT* genes were highest when fungal mycelia were grown on HAY culture media at 5-10°C with a light/dark cycle, indicating that temperature, culture media and light are the key environmental factors that affect the sexual development of *Ulocladium*. The low expression level may be one of the major causes of asexuality in *Ulocladium*.

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ACKNOWLEDGEMENTS

We thank the herbarium of the Centraalbureau voor Schimmelcultures for providing some valuable isolates in this study. We express gratitude to Dr. Xiuguo Zhang and Xinguo Li for their valuable comments and suggestions.

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