Reference genes for accurate evaluation of expression levels in *Trichophyton interdigitale* grown under different carbon sources, pH levels and phosphate levels

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*Tinea pedis* is a type of dermatophytosis caused by anthropophilic keratinolytic fungi such as *Trichophyton interdigitale*. Quantitative reverse transcription PCR (RT-qPCR) is a reliable and reproducible technique for measuring changes in target gene expression across various biological conditions. A crucial aspect of accurate normalization is the choice of appropriate internal controls. To identify reference genes for accurate evaluation of expression levels in *T. interdigitale*, the transcription levels of eight candidate reference genes (*adp-rf, β-act, ef1-α, gapdh, psm1, sdha, rpl2 and ubc*) and one target gene (*Tri m4*) were analysed by RT-qPCR after growing the dermatophyte under different environmental conditions. The results obtained from expression stability evaluations with NormFinder, geNorm, BestKeeper, and RefFinder software demonstrated that *adp-rf* and *psm1* were the most stable internal control genes across all experimental conditions. The present study constitutes the first report of the identification and validation of reference genes for RT-qPCR normalization for *T. interdigitale* grown under different environmental conditions resembling the conditions encountered by fungi during invasion of skin.

*Trichophyton interdigitale* is a keratinophilic and keratinolytic fungus belonging to the dermatophyte group, and it is responsible for infections of the feet and toes (*tinea pedis*). Epidemiological studies have indicated that the incidence of dermatophytosis due to this fungus is rising and is not correlated with patient characteristics such as ethnicity or race. However, *tinea pedis* is most frequently found in adults between 30 and 60 years old, and this dermatophytosis is more common in men than in women and in developed countries. Dermatophytes have been recorded worldwide with variations in epidemiology, distribution, incidence and target hosts from one location to another. Different conditions, such as geographic location, climate, health care quality, immigration status, hygiene culture, and socioeconomic status, may influence the development of dermatophyte infections. Keratin is a structural protein of the *stratum corneum*, where dermatophytes typically infect, while elastin and collagen are the fibrous proteins of the extracellular matrix in the dermis that dermatophytes can penetrate during infection. The degradation of keratin and other fibrous proteins releases high amounts of cysteine, proline, serine or lysine, and the metabolism of these amino acids leads to the secretion of ammonia, which raises the extracellular pH from acidic to alkaline values. Transcriptomic analyses suggest that during the first 48 h after infection, the highest transcriptional activity of genes responsible for the synthesis of proteases and adhesins occurs, which allows colonization of the host tissue. Analysis of changes in dermatophyte gene expression profiles under defined growth conditions can improve our knowledge of the mechanisms associated with the pathogenicity of dermatophytes and of the other biological properties of this group of pathogens. Information gathered during such study may be useful in the search for new therapeutic and prophylactic strategies. Quantitative reverse transcription PCR (RT-qPCR) is a powerful technique used to quantify the mRNA levels of different genes of interest under various experimental conditions. However, different experimental and technical variations can lead to...
incorrect data analysis. Therefore, it is necessary to establish a set of optimal reference genes before conducting target gene expression analysis. Due to the limited knowledge regarding reference genes useful for RT-qPCR analysis in dermatophytes and the particularly insufficient information on the complete genome sequence of *T. interdigitale*, eight reference genes, including *adp-rf* (ADP ribosylation factor), *β-act* (*β*-actin), *ef1-α* (elongation factor 1-alpha), *gapdh* (glyceraldehyde 3-phosphate dehydrogenase), *psm1* (mitotic cohesion complex subunit Psm1), *sdha* (succinate dehydrogenase complex flavoprotein subunit A), *rpl2* (ribosomal protein L2) and *ubc* (ubiquitin) (Table 1) were ultimately selected and evaluated in a *T. interdigitale* strain subjected to 13 different environmental conditions (Table 2). The selected candidate reference genes were chosen from among internal controls used in some species of fungi, including dermatophytes and in other eukaryotic organisms.

Results

Amplification efficiency and specificity of eight candidate reference genes. The specificity of the primer sets was validated based on the identification of a single band of the expected size on 8% polyacrylamide gels and a single homogenous peak in melting curve analysis (Table 1, Supplementary Fig. S1A,B). The PCR efficiencies (E%) ranged from 99–110%, with correlation coefficient (R²) values varying from 0.996 to 0.999 (Table 2). The expression profiles of the eight reference gene candidates (Table 1) were analysed under control and experimental conditions by calculating the mean raw Cₜ value from three independent repetitions (Supplementary Table S1). As shown in Fig. 1, the Cₜ values of the eight candidate housekeeping genes ranged from 15.20 to 26.32 across all experimental conditions.

### Table 1. *Trichophyton interdigitale* candidate reference genes used for qRT-PCR.

| Gene symbol/accession no. | Gene name | Primers (5’–3′) forward reverse | Length (bp) | Tm (°C) | Cₜ range | Efficiency (%) | R²   |
|--------------------------|-----------|---------------------------------|-------------|---------|----------|----------------|------|
| *adp-rf* (H101_06992)   | ADP ribosylation factor | ATCGAACTTATCTGGCAGG | 105         | 60.5    | 18.12–21.89 | 100            | 0.9927 |
| *β-act* (H101_06992)    | *β*-actin | TGTTTCCATTCATGTCAGGC         | 117         | 60.5    | 15.20–19.80 | 104            | 0.9998 |
| *ef1-α* (H101_03672)   | elongation factor 1-alpha | GAGAAGTGCAAGGAAGGC         | 150         | 60.5    | 15.95–19.95 | 98             | 0.9992 |
| *gapdh* (H101_04054)   | glyceraldehyde 3-phosphate dehydrogenase | GAAGCCAGTCACCTCAGGA | 100         | 60.5    | 16.56–22.64 | 107            | 0.9967 |
| *psm1* (H101_01238)    | mitotic cohesion complex 2 | CGAGCTCTCTTTAATTTCAAGTC    | 150         | 60.5    | 18.80–22.45 | 101            | 0.9955 |
| *sdha* (H101_02447)    | succinate dehydrogenase complex flavoprotein subunit A | GAGGCTCTTGCAACCC         | 104         | 60.5    | 16.01–19.99 | 108            | 0.9982 |
| *rpl2* (H101_0787)     | subunit Psm1 ribosomal protein L | GTGGATCTATCTCAGCGGC | 112         | 60.5    | 19.70–22.84 | 109            | 0.9990 |
| *ubc* (H101_00343)     | ubiquitin C | TGTCATGACTTGGAATGCTG       | 87          | 60.5    | 22.78–26.32 | 103            | 0.9989 |

### Table 2. *T. interdigitale* cultivation conditions in liquid minimal medium (MM) supplemented with different carbon sources, low-Pi MM, and low-Pi yeast extract medium (YEM).

| Variant | Cultivation substrate | Cultivation pH | Cultivation conditions |
|---------|-----------------------|----------------|------------------------|
| MM-Cove | —                     | 5.0            | 24 and 48 h, 28 °C, 200 rpm |
| MM-Cove | 56 mM glucose         | 5.0            | 24 and 48 h, 28 °C, 200 rpm |
| MM-Cove | 0.5% keratin          | 5.0            | 24 and 48 h, 28 °C, 200 rpm |
| MM-Cove | 0.5%/1% keratin/soy protein | 5.0         | 24 and 48 h, 28 °C, 200 rpm |
| MM-Cove | 0.5% elastin          | 5.0            | 24 and 48 h, 28 °C, 200 rpm |
| MM-Cove | 0.5% collagen         | 5.0            | 24 and 48 h, 28 °C, 200 rpm |
| MM-Cove | 1% colloidal chitin   | 5.0            | 24 and 48 h, 28 °C, 200 rpm |
| Low-Pi MM | 200 μM Pi             | 5.0            | 17 h, 37 °C, 200 rpm |
| Low-Pi MM | 200 μM Pi             | 8.0            | 17 h, 37 °C, 200 rpm |
| Low-Pi MM | 200 μM Pi             | 10.0           | 17 h, 37 °C, 200 rpm |
| Low-Pi YEM | 700 μM Pi             | 5.0            | 17 h, 37 °C, 200 rpm |
| Low-Pi YEM | 700 μM Pi             | 8.0            | 17 h, 37 °C, 200 rpm |
| Low-Pi YEM | 700 μM Pi             | 10.0           | 17 h, 37 °C, 200 rpm |
Expression stability analyses. The GeNorm algorithm, which is a module of qbase+ (Biogazelle), was used to evaluate the candidate reference genes based on their expression stability values (M-values) and pairwise variations (\(V_{n/n+1}\)). Psm1 (M-value = 0.483), adp-rf (M-value = 0.502) and sdha (M-value = 0.520) were the most stable reference genes under all experimental conditions (Fig. 2). In contrast, the \(\beta\)-act gene had the highest M-value, with the lowest expression stability (M-value = 1.132) in all analysed samples (Fig. 2). The pairwise variation (\(V_{n/n+1}\)) results indicated that five reference genes (psm1, adp-rf, sdha, ubc and rpl2) should be used for reliable normalization (\(V_{5/6} = 0.136\)) (Fig. 3). The most stable reference genes (in order) among all chosen candidates for \(T.\ interdigitale\) under each experimental condition were as follows (Table 3): ef1-\(\alpha\), rpl2, sdha, adp-rf, ubc, psm1, gapdh, and \(\beta\)-act for control conditions (MM-Cove); adp-rf, ubc, gapdh, psm1, ef1-\(\alpha\), sdha, rpl2, and \(\beta\)-act for glucose supplementation; ef1-\(\alpha\), ubc, sdha, psm1, rpl2, \(\beta\)-act, adp-rf, and gapdh for keratin supplementation; rpl2, gapdh, ef1-\(\alpha\)-\(\alpha\), ubc, \(\beta\)-act, psm1, sdha, and adp-rf for keratin and soy protein supplementation; sdha, psm1, rpl2, adp-rf, ubc, \(\beta\)-act, ef1-\(\alpha\), and gapdh for elastin supplementation; ef1-\(\alpha\), psm1, adp-rf, rpl2, \(\beta\)-act, sdha, gapdh, and ubc for collagen supplementation; rpl2, psm1, adp-rf, \(\beta\)-act, ef1-\(\alpha\)-\(\alpha\), ubc, gapdh, and sdha for colloidal chitin supplementation; adp-rf, ef1-\(\alpha\), \(\beta\)-act, sdha, psm1, ubc, rpl2, and gapdh for low-Pi MM; and \(\beta\)-act, adp-rf,
psm1, efl-α, gapdh, ubc, rpl2, and sdha for low-Pi YEM. Furthermore, pairwise variation \((V_{n}/V_{n+1})\) calculation with a V-value < 0.15 showed that only two internal controls were sufficient for normalizing gene expression under all experimental conditions (Fig. 3).

According to NormFinder\(^\text{17}\), across all experimental conditions, psm1 had the lowest stability value \((SV = 0.080)\) \((\text{Fig. 2})\). Psm1 and rpl2 constituted the best combination of internal control genes with \(SV = 0.061\) under all experimental conditions. Psm1 was found to be the most stably expressed gene in the presence of colloidal chitin \((SV = 0.032)\) \((\text{Fig. 4G})\), and rpl2 was the most stable gene under control conditions \((SV = 0.100)\) \((\text{Fig. 4A})\). β-act was the most stable gene in the medium supplemented with keratin and soy protein \((SV = 0.138)\) \((\text{Fig. 4D})\) and under low-Pi conditions in MM \((SV = 0.045)\) \((\text{Fig. 4I})\). In the case of collagen supplementation and under the low-Pi condition in YEM, NormFinder calculations revealed that adp-rf had the lowest stability values, with \(SV = 0.259\) \((\text{Fig. 4E})\) and \(SV = 0.028\) \((\text{Fig. 4H})\), respectively. Efl-α, ubc and gapdh were the most stably expressed genes in the presence of glucose \((SV = 0.096)\) \((\text{Fig. 4B})\), keratin \((SV = 0.010)\) \((\text{Fig. 4C})\), and elastin \((SV = 0.010)\) \((\text{Fig. 4F})\).

Assessment of the expression variation of the candidate reference genes using the BestKeeper algorithm\(^\text{18}\) revealed that seven of the genes had standard deviation values defined as acceptable \((0.5 < SD(\pm C_i) < 1.00)\), while gapdh had an unacceptable standard deviation, as it was higher than 1.0 \((SD = 1.32)\) \((\text{Table 3})\). Analyses showed also significant expression correlations with the BestKeeper index, which is the geometric mean of the \(C_i\) values of the analysed genes \((\text{correlation coefficient } r = 0.698–0.901)\), except for the \(β\)-act gene \((r = 0.145)\). The expression of all genes correlated with the BI with \(p\) values < 0.001, except the \(β\)-act gene \((p = 0.543)\). The overall order of the most stable genes based on BestKeeper was psm1, adp-rf, efl-α, sdha, ubc and rpl2 \((\text{Table 4})\).

In the final step, RefFinder, a free online tool for the identification of stable reference genes that integrates all methods applied in the present study, was used to generate a final ranking of the eight reference genes according to their geomean ranking values. As shown in \text{Fig. 2}, psm1 and adp-rf were ranked as the best reference genes for measuring target gene expression levels under the chosen conditions.

Stability and validation of adp-rf and psm1 as reference genes. To confirm adp-rf and psm1 as the most stable reference genes, their expression was compared in the \text{T. interdigitale} CBS 124408 reference strain and two clinical isolates: \text{T. interdigitale} 12/2010 and \text{T. interdigitale} 45/10. These three strains of \text{T. interdigitale} were incubated at 28 °C for 48 h in control medium and in medium supplemented with keratin. The obtained \(C_i\) values \((\text{Fig. 5})\) for the adp-rf and psm1 genes were not significantly different under both conditions \((p_{\text{psm1}} = 0.93; p_{\text{adp-rf}} = 0.89, \text{ANOVA})\) \((\text{Fig. 5})\), which confirmed that these reference genes can be used for accurate expression level evaluation in various \text{T. interdigitale} strains. To confirm the reliability of adp-rf and psm1 as reference genes for RT-qPCR normalization, the expression of \text{Tri m4} was examined\(^\text{19}\). \text{Tri m4} is known as an aminopeptidase gene whose expression increases in the presence of keratin and elastin, which suggests that the product of this gene may play an important role as a virulence factor\(^\text{19}\). The validation was performed using templates from the \text{T. interdigitale} 45/10 strain incubated at 28°C for 48 h in control medium (MM-Cove) and in medium supplemented with keratin or elastin. Three different sets of reference genes were analysed: set A included the most stable reference genes \((\text{adp-rf and psm1})\), set B included the least stable reference genes \((\text{β-act and gapdh})\), and set C included all eight candidate reference genes. The relative expression of the target gene was determined using the \(2^{-\Delta\Delta CT}\) method\(^\text{20}\). As shown in \text{Fig. 6}, an increase in \text{Tri m4} transcript levels in \text{T. interdigitale} growing in the presence of keratin or elastin in relation to control conditions was noticed only when the adp-rf and psm1 reference genes \((\text{set A})\) previously selected by all four algorithms were used.

Discussion

Quantitative reverse transcription PCR (RT-qPCR) is an efficient method for analysing target gene expression but requires comprehensive normalization with properly selected reference genes. The internal controls should have relatively stable expression levels in different types of cells or tissues, and their expression should be constant under various growth conditions\(^\text{21}\). Many reports on target gene expression analysis have used a single housekeeping gene for RT-qPCR normalization, such as 18S rRNA, gapdh, β-act, β-tub or efl-α\(^\text{22}\); these genes...
regulate basic cellular functions. Such an approach has a strong tradition and history of use since the introduction of reverse-transcription-based assays. However, since 2009, according to the MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments\(^2\), no single reference gene should be used to quantify target gene expression under different conditions, and candidates for reference genes should be carefully selected for each study to comply with these guidelines. Numerous studies have shown that the expression of the above housekeeping genes can vary between individual tissues or experimental conditions such that no universal reference gene can be used in all situations\(^18,23\)–\(^27\). Moreover, a reference gene with stable expression in one organism may not be suitable for normalization of gene expression in another organism, even a closely related species. Consequently, the need for a reliable and comprehensive approach to selecting reference genes has been emphasized. The development of various algorithms and software tools for the selection of reference genes has been described in detail in the literature\(^15,28\). A comprehensive and up-to-date review of these tools can be found in a recent publication\(^29\). The aim of the present study was to assess the expression stability of several reference genes in different experimental conditions to identify a suitable reference gene for normalization of gene expression studies in the context of our research. We selected eight candidate reference genes based on their expression stability in different conditions. The expression stability of these candidate reference genes was analyzed using the geNorm algorithm\(^3\) and NormFinder algorithm\(^2\). The results indicated that adp-rf and ef1-α had the highest stability in all experimental conditions, with M-values of 0.156 and 0.039, respectively. These results are consistent with previous studies that used similar algorithms and methods. In conclusion, our study demonstrates the importance of selecting appropriate reference genes for gene expression studies and highlights the need for rigorous and systematic approaches to this task. The results presented here can serve as a valuable resource for researchers working in the field of gene expression and molecular biology.
related organism. Additionally, genes such as 18S rRNA and 28S rRNA, despite their stability, are often expressed at very high levels and thus should not be used as internal controls\(^28,29\). To avoid biased normalization, following the MIQE guidelines\(^21\), the use of multiple candidate reference genes is highly recommended to obtain reliable RT-qPCR results. Based on the limited literature reports regarding the analysis of gene expression in dermatophytes\(^10,11\), supplemented with information on reference genes used in studies on other eukaryotes\(^9,12,13\), 12 genes, including adp-rf (ADP ribosylation factor), β-act (β-actin), β-tub (β-tubulin), efl-α (elongation factor 1-alpha), gapdh (glyceraldehyde 3-phosphate dehydrogenase), mbp1 (multiubiquitin chain-binding protein 1), adp-rf, ef1-a, gapdh, sdha, rpl2, psm1, ubc

| N | β-act | adp-rf | ef1-a | gapdh | sdha | rpl2 | psm1 | ubc |
|---|-------|-------|-------|-------|------|------|------|-----|
| GM (Ct) | 16.80 | 20.32 | 17.78 | 19.30 | 18.18 | 21.51 | 20.74 | 25.11 |
| AM (Ct) | 16.82 | 20.34 | 17.81 | 19.37 | 18.22 | 21.53 | 20.76 | 25.12 |
| Min (Ct) | 15.2 | 18.12 | 15.95 | 16.56 | 16.01 | 19.7 | 18.8 | 22.78 |
| Max (Ct) | 19.8 | 21.89 | 19.95 | 22.64 | 19.99 | 22.84 | 22.45 | 26.36 |
| SD (± Ct) | 0.69 | 0.84 | 0.74 | 1.32 | 0.89 | 0.71 | 0.79 | 0.69 |
| CV (% Ctc) | 4.15 | 4.14 | 4.16 | 6.82 | 4.91 | 3.31 | 3.82 | 2.77 |
| Min (fold change) | −3.03 | −4.59 | −3.57 | −6.72 | −4.51 | −3.53 | −3.84 | −5.03 |
| Max (fold change) | 7.99 | 2.96 | 4.47 | 10.05 | 3.49 | 2.49 | 3.26 | 2.37 |
| SD (± fold change) | 1.62 | 1.79 | 1.67 | 2.50 | 1.85 | 1.64 | 1.73 | 1.62 |
| BI Index (r) | 0.145 | 0.875 | 0.806 | 0.765 | 0.805 | 0.698 | 0.901 | 0.787 |
| p-value | 0.543 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |

Table 4. Descriptive statistics for the candidate reference genes calculated using BestKeeper. n, number of samples (three biological replicates and 30 different conditions); GM (Ct), geometric mean of Ctc; AM (Ct), arithmetic mean of Ctc; Min (Ct) and Max (Ct), extreme values of Ctc; SD (± Ctc), standard deviation of Ctc; CV (% Ctc), coefficient of variation expressed as a percentage of the Ct value; Min (fold change) and Max (fold change), extreme value of the expression level expressed as the absolute fold change of down- or upregulation; SD (± fold change), standard deviation of the absolute fold change; BI Index (r), correlation between BestKeeper index and the contributing gene.
fis1 (mitochondrial fission 1 protein), psml (mitotic cohesion complex subunit Psm1), rGTPa (rho GTPase activating-protein 5), rpl2 (ribosomal protein L2), sdha (succinate dehydrogenase complex flavoprotein subunit A) and ubc (ubiquitin) (Table 1 and Supplementary Table S2), were selected as putative candidates based on a BLAST search of available T. interdigitale genomic sequences. Despite several attempts at primer modification, only 8 candidate amplification products were obtained, and these genes were used in further studies. Unfortunately, only three genomes of this species are currently available in the databases, of which two are at a scaffold level, while the third is at a contig level. An in-depth analysis of these genomes revealed the presence of regions of predicted sequences, indicating that the full nucleotide sequences have yet to be established (https://www.ncbi.nlm.nih.gov/genome/genomes/44693). It can therefore be assumed that both these problems and others related to the difficulties in correctly determining the taxonomic affiliation of many strains of T. interdigitale, as described in the literature10 (which may affect the correct genome assembly of this species), were responsible for the unsuccessful attempts to develop a larger number of correct primers, making it impossible to test a greater number of putative reference genes.

In this study, the geNorm, NormFinder, BestKeeper and RefFinder algorithms were used to evaluate the selected candidate reference genes as internal controls for analysis of target gene expression in T. interdigitale growing under different environmental stimuli, such as supplementation with various carbon sources, low Pi, and different pH values37. Some of these circumstances have been suggested to promote adhesion to the host tissue and are essential for the expression of specific genes associated with adaptation and interactions between T. interdigitale and its host. Our study is the first report on the identification and validation of reference genes for T. interdigitale that indicates psml and adp-rf as the most stable genes among the analysed candidates (Fig. 2).

Mitotic cohesion complex ATPase subunit (psml) is involved in mitotic cohesion loading/unloading and is required for the cohesion of sister chromatids after DNA replication. In addition, ADP-ribosylation factor (adp-rf) is a ubiquitous GTP-binding protein essential for mitotic growth. These two candidates were found in the present study to be reliable internal controls for accurate expression level analysis of target genes of T. interdigitale growing under adhesion-inducing conditions.

Llanos et al.31 showed that according to geNorm analysis, psml, ubcB (ubiquitin carrier protein) and sac7 (Rho GTPase activator) were ranked as the most stably expressed reference genes in the fungus Talaromyces versatilis grown under various conditions, such as in the presence of different carbon sources; under different temperatures and pH levels; and under salt stress and carbon/nitrogen starvation. However, in our previous report on the validation of reference genes for the dermatophyte Microsporum canis33, psml was classified in the group of unstable reference genes. To date, there have been only two studies on the validation of psml as a stably expressed reference gene in RT-qPCR analysis.

The ADP ribosylation factor gene (adp-rf) was found to be the best reference gene for analyses of target transcript levels in an exotic invasive insect, Leptinotarsa decemlineata36; the cereal Cucumis melo L.37; the melon Cucumis melo L.33; the Pacific oyster, Crassostrea gigas38; the monkey Macaca fascicularis39; and the desert willow shrub, Salix psammophila40. Again, our previous study11 demonstrated that the expression stability of adp-rf was low in M. canis. Furthermore, the β-act gene, which encodes a cytoskeletal protein involved in many cellular processes, and the gapdh gene, which encodes an enzyme of the glycolytic pathway, are often used as reliable reference genes in expression analyses37; however, these genes were in the group of the least stable reference genes in the present study. On the other hand, in the search for reliable reference genes for RT-qPCR analysis of target gene expression in M. canis, the β-act gene was classified as one of the three most stable genes41. The present and the previous results11 of our team confirmed that the stability of housekeeping gene expression should be verified for each condition and each particular species (Table 3, Fig. 4), which again highlights the fact that there is no ideal and universal internal control gene for RT-qPCR analysis.

To validate the reference genes selected by the four algorithms, the genes were used as reference genes for the measurement of the relative expression of Tri m4, a gene that encodes aminopeptidase and is known to be upregulated in the presence of keratin and elastin as inducers42. Elevated Tri m4 expression was detected in T. interdigitale growing under inducing conditions only when the two internal controls psml and adp-rf, which were selected as the most stable internal controls by the four algorithms, were used in combination (Fig. 6). Upregulation was detected neither for the least stable pair of reference genes (set B) nor for the whole set of eight candidate genes (set C). These results clearly confirmed that the chosen best pair of internal control genes can be preferentially used for RT-qPCR normalization in the case of T. interdigitale grown under the described experimental conditions.

Conclusion
The present study was the first attempt to identify and validate T. interdigitale internal control genes. The psml and adp-rf genes were found to be the most stable reference genes appropriate for gene expression analysis in T. interdigitale. The use of these genes as internal controls may further improve the robustness of RT-qPCR for T. interdigitale grown under adhesion-inducing conditions.

Materials and Methods
Reference gene selection and primer design. Twelve putative candidate reference genes (adp-rf, β-act, β-tub, ef1-α, fis1, gapdh, mbp1, psml, sdha, rpl2, rGTPa and ubc) (Table 1 and Supplementary Table S2) were chosen in the present study based on the NCBI database (http://www.ncbi.nlm.nih.gov) and our previous study11. Primers were designed and theoretically evaluated using Primer 3 software48. PCR products within the 80–150 bp range were obtained only in the case of 8 candidates (adp-rf, β-act, ef1-α, gapdh, rpsml, sdha, rpl2 and ubc), and these genes were analysed by PCR in a Gradient Thermal Cycler T1000 (BioRad) (Table 1, Supplementary Fig. S1B).
Fungal material and growth conditions. The *Trichophyton interdigitale* 45/10 strain, isolated from *tinea pedis* of a 42-year-old man, was used in all RT-qPCR analyses. *Trichophyton interdigitale* CBS 124408 (a reference strain from the CBS-KNAW Collection, Utrecht, The Netherlands) and *Trichophyton interdigitale* 12/2010, a clinical isolate from the onychomycosis case of a 61-year-old man, were used in the evaluation step of the reference genes. The clinical strains were chosen from the collection maintained in the Department of Microbial Genetics, Faculty of Biology and Environmental Protection, University of Łódź, Poland. PCR-RFLP analysis of the ITS1-5.8S-ITS2 region followed by sequencing was performed for standard mycological identification. Germinated conidia of the *T. interdigitale* strain (approximately 10^7 cells/ml) were incubated separately in minimal liquid medium (MM-Cove) under different conditions (Table 2), in low-Pi MM, and in YEM (yeast extract medium) under 3 different conditions (Table 2).

RNA extraction, cDNA synthesis and quantitative reverse transcription PCR. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s protocol. RNA integrity was verified by electrophoretic and spectrophotometric (NanoPhotometerPearl Version 1.0, IMPLEN) analyses, according to the MIQE guidelines for RT-qPCR. RNA samples with A260/A280 ratios between 1.9 and 2.1 were used for further analysis. First-strand cDNA was synthesized using 2 μg of total RNA (DNA-free), RevertAid Transcriptase (Thermo Scientific) and random hexamer primers (Thermo Scientific) following the manufacturer’s protocol. The qRT-PCR reactions were conducted on a RotorGene Q System (Qiagen) based on a method described previously using SsoAdvanced Universal SYBR® Green Supermix (2X) (Bio-Rad). The reactions were subjected to an initial step of 95 °C for 1 min followed by 40 cycles at 95 °C for 20 s, 60.5 °C for 20 s, at 72 °C for 15 s. Melting curve analysis was performed by heating the amplicon from 72 °C to 95 °C.

Data analysis. The expression stability of the candidate reference genes in *T. interdigitale* was analysed using four bioinformatic tools: geNorm15, NormFinder17, BestKeeper18 and RefFinder (http://leonxie.esys.es/RefFinder/). The geNorm tool was used to calculate the gene expression stability according to the M-value, which is defined as the average pairwise variation with all other tested candidate reference genes. The algorithm recommends selecting genes with M-values below 1.0 to ensure the choice of the most stably expressed internal control gene14. Moreover, Vandesompele et al.16 suggested that an M-value lower than 0.5 indicates very good stability of expression. GenNorm also suggests that the best combination of reference genes based on pairwise variations (Vn/Vn+1) between two sequential normalization factors (NFn and NFn+1) has a V-value < 0.15. NormFinder is a VBA tool for Microsoft Excel used to calculate stability values (SVs) by combining intra- and inter-group variations in reference gene expression17. Lower SV values correspond to lower variations and, hence, higher stability of the reference genes. BestKeeper ranks the candidate reference genes according to their correlation coefficients (r values) for correlation with the BestKeeper Index (BI), which is the geometric mean of the C values of the candidate reference genes determined by calculating the standard deviation (SD) and coefficient of variance (CV)18. The online tool RefFinder was used to measure the geometric mean of the attributed weights for the overall final ranking. Box-and-whisker plots were drawn and one-way ANOVA was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California, USA).

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Author Contributions

A.C. conceived and conducted the experiments, analysed the data, interpreted the results, and wrote the manuscript. B.O. conducted the experiments. P.S. conceived and wrote the manuscript. All authors reviewed the manuscript.

Additional Information

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