Molecular phylogeny of *Pilaira* (Mucorales, Zygomycetes) inferred from ITS rDNA and *pyrG* sequences

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Pilaira is one of the three genera in Pilobolaceae. All of them are difficult to preserve. Only six out of the 12 recorded species are available for studies nowadays. Molecular phylogeny of 21 strains of *Pilaira* was reconstructed in this study by using ITS rDNA and *pyrG* sequences. These two loci displayed different phylogenetic histories of *Pilaira*, though *P. anomala* and *P. moreaui* formed two paraphyletic groups becoming monophyletic with the inclusion of *P. praeampla* and *P. subangularis,* respectively. Comparing morphospecies with phylospecies, complete concordances were shown in 12 strains. Partial consistencies were observed in another five isolates. Absolute discrepancies were revealed for the remaining four collections. Combined analyses of morphology and molecular biology resolved most taxa of *Pilaira*, while the disagreements found in this study suggest that this genus is still dynamic in lineage splitting and its phylogeny might be further resolved on the basis of thorough consideration of information from more strains and more loci. The potential difficulty in further study is also discussed.

Keywords: Ribosomal DNA, OMPD, Pilobolaceae, Incomplete lineage sorting.

Pilobolaceae is a distinct family within the order Mucorales and contains three genera: *Pilaira* Tiegh., *Pilobolus* Tode and *Utharomyces* Boedijn (Boedijn 1959). All members of this family are coprophilous and produce phototropic, almost unbranched sporangiophores which arise directly from substrate and terminate in dark hemispheric columellate sporangia with persistent, cutinized walls. *Pilobolus* and *Utharomyces* have trophocysts and subsporangial swellings, whereas *Pilaira* has not. *Pilobolus* species forcibly discharge the sporangium while *Pilaira* and *Utharomyces* lack this mechanism (Boedijn 1959). Twelve species of *Pilaira* have been described from all over the world: *P. anomala* (Ces.) J. Schröt., *P. caucasica* Milko, *P. cesatii* (Coem.) Tiegh., *P. dimidiata* Grove, *P. fimetaria* (Link) Pound, *P. inosculans* Grove, *P. moreaui* Y. Ling, *P. mucedo* Bref., *P. nigrescens* Tiegh., *P. praeampla* R. Y. Zheng & X. Y. Liu, *P. saccardiana* Morini, and *P. subangularis* R. Y. Zheng & X. Y. Liu (CABI Bioscience *et al.* 2011). Eleven out of the 12 species

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have only been recorded once in literature, while *P. anomala* is ubiquitous in Europe and America and has been reported many times (Zheng & Liu 2009). Due to the difficulty in preservation, materials of six species, namely *P. dimidiata*, *P. fimetaria*, *P. inosculans*, *P. mucedo*, *P. nigrescens*, and *P. saccardiana*, cannot be found in any culture collection and hence they are unavailable for study. Type or nontype cultures of the remaining six species, i.e. *P. anomala*, *P. caucasica*, *P. cesatii*, *P. moreaui*, *P. praeampla* and *P. subangularis* are still kept in the USDA Agriculture Research Service Culture Collection (NRRL) and in our laboratory. *Pilaira praeampla* and *P. subangularis* were recently described from rodent dung or soil under cow dung in high altitude areas in western China (Zheng & Liu 2009). Along with these two recently described species, one new combination *P. moreaui* var. *caucasica* (Milko) R. Y. Zheng & X. Y. Liu was made for *P. caucasica*, and *P. cesatii* was synonymized with *P. anomala*.

Many different genes were selected for a larger phylogeny of Mucorales, such as *act* (encoding actin), SSU and LSU rDNA (encoding nuclear small and large subunit ribosomal RNA), EF-1α (encoding translation elongation factor one alpha), and RPB1 (encoding DNA dependent RNA polymerase II largest subunit) and resulted in an unsatisfactorily resolved phylogeny at order and family levels (O'Donnell *et al.* 2001, Voigt & Wöstemeyer 2001, Tanabe *et al.* 2004). Phylogenetic studies of individual genera within Mucorales based on ITS rDNA have resulted in good resolutions on species level (Liu *et al.* 2001, Abe *et al.* 2003, 2006, Liou *et al.* 2007, Liu *et al.* 2007). The orotidine-5'-monophosphate decarboxylase (OMPD) gene (*pyrG*) was proposed as a potential phylogenetic marker (Nara *et al.* 2000). Moreover, the evolutionary value of *pyrG* was reinforced as evidenced by differences among Ascomycota, Basidiomycota and Zygomycota in the length of exons and in the number and position of introns (Quiles-Rosillo *et al.* 2003). Following these implications has lead to a successful use of *pyrG* for *Rhizopus* phylogeny (Liu *et al.* 2007). In the present study, we reconstruct the molecular phylogeny of all available strains of *Pilaira* based on both ITS rDNA and *pyrG* in comparison with morphological data.

Materials and methods

Fungal cultivation and DNA extraction

Pilaira strains used in this study are listed in Tab. 1. All stock cultures were maintained on potato dextrose agar (PDA). A 50-ml glucose-asparagine-thiamine synthetic medium (Huang & Yu 1988) was inoculated with a one-week-old slant culture in a 250-ml Erlenmeyer flask. After incubation for 3–4 d at 25 °C at 150 rpm, mycelia were collected by vacuum filtration, washed three times with sterile distilled water and once with 25 % (vol/vol) ethanol, air-dried and then frozen at –20 °C. Total genomic DNA was isolated from mycelia by using the CTAB method (Warude *et al.* 2003) with some modifications. Approximately 70 mg of dried mycelia were ground to a fine

Tab. 1. – *Pilaira* and outgroup strains used in this study.

Strain[a]	Taxon name based on morphological characters[b]		Supported by molecular data[c]		Primer for *pyrG*[d]	GenBank no.	
	Received as	Zheng & Liu 2009	ITS	*pyrG*		ITS rDNA	*pyrG*
Ingroups							
Pi-3		*P. subangularis* T	Yes	Yes	RpyrGF / RpyrGR	EF555500	EF562596
Pi-4		*P. moreaui* var. *caucasica*	Yes	No	RpyrGF / RpyrGR	EF555501	EF562597
Pi-5		*P. praeampla* T	Yes	No	RMpyrGF / RMpyrGR	EF555502	EF562598
Pi-6		*P. moreaui* var. *caucasica*	Yes	No	RpyrGF / RpyrGR	EF555503	EF562599
Pi-7		*P. moreaui* var. *caucasica*	Yes	No	RpyrGF / RpyrGR	EF555504	EF562600
Pi-8		*P. moreaui* var. *caucasica*	Yes	No	RpyrGF / RpyrGR	EF555505	EF562601
NRRL 2526 (+)	*P. anomala*		Yes	Yes	RMpyrGF / RMpyrGR	EF555485	EF562581
NRRL 2527 (-)	*P. anomala*		Yes	Yes	RMpyrGF / RMpyrGR	EF555486	EF562582
NRRL 6282	*P. caucasica* T	*P. moreaui* var. *caucasica* T	Yes	Yes	RpyrGF / RpyrGR	EF555487	EF562583
NRRL 6283	*P. moreaui*	*P. moreaui* var. *moreaui*	Yes	Yes	RpyrGF / RpyrGR	EF555488	EF562584
NRRL 6284	*P. moreaui*	*P. moreaui* var. *moreaui*	Yes	Yes	RpyrGF / RpyrGR	EF555489	EF562585
NRRL A5067	*P. cesatii*	*P. anomala*	No	No	RMpyrGF / RMpyrGR	EF555490	EF562586
NRRL A5068	*P. cesatii*	*P. anomala*	No	No	RMpyrGF / RMpyrGR	EF555491	EF562587
NRRL A7399	*P. anomala*	*P. moreaui* var. *moreaui*	Yes	Yes	RpyrGF / RpyrGR	EF555492	EF562588
NRRL A7646	*P. anomala*		No	No	RMpyrGF / RMpyrGR	EF555493	EF562589
NRRL A9310	*P.* sp.	*P. anomala*	Yes	Yes	RMpyrGF / RMpyrGR	EF555494	EF562590
NRRL A9789	*P. anomala*		No	No	RMpyrGF / RMpyrGR	EF555495	EF562591
NRRL A16347	*P. anomala*		Yes	Yes	RMpyrGF / RMpyrGR	EF555496	EF562592
NRRL A16348	*P. anomala*		Yes	Yes	RMpyrGF / RMpyrGR	EF555497	EF562593
NRRL A16356	*P. anomala*		Yes	Yes	RMpyrGF / RMpyrGR	EF555498	EF562594
NRRL A23912	*P. anomala*		Yes	Yes	RMpyrGF / RMpyrGR	EF555499	EF562595
Outgroup							
HS-5	*Helicostylum elegans*				DpyrGF / DpyrGR	JQ319049	JQ319045
dM-39	*Mucor mucedo*				DpyrGF / DpyrGR	JQ319046	JQ319042
Pir-1	*Pirella circinans*				DpyrGF / DpyrGR	JQ319047	JQ319043
Th-3	*Thamnidium elegans*				DpyrGF / DpyrGR	JQ319048	JQ319044

[a] Culture collection abbreviations: the USDA Agricultural Research Service Collection, Peoria, USA (NRRL); the Culture Collection of the Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China (with a "dM-", "HS-", "Pi-", "Pir-", "Th-" prefix for *Mucor, Helicostylum, Pilaira, Pirella* and *Thamnidium* strains, respectively).

[b] T = ex-type strain.

[c] Yes = concordance between morphological and phylogenetic taxon; No = disconcordance between morphological and phylogenetic taxon.

[d] ITS rDNA are obtained by the primer pair ITS1 / ITS4 (White *et al*. 1990). The *pyrG* primers DpyrGF (CHYTBTTKGARYTNATGGARCG), DpyrGR (YDCCYKBRGAHG ACATYTCDGC), RpyrGF (CCTTTTTGGAATTAATGGAACG) , RpyrGR (CGCCCTTG-GAAGACATCTCGGC), RMpyrGF (CTCTGTTTGAGCTCATGGAGCG) and RMpyrGR (CACCCTTGGATGACATTTCAGC) are designed in the present study.

powder in liquid nitrogen, transferred to a 1.5-ml microfuge tube and then resuspended in 700 µl of CTAB extraction buffer [100 mM Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), 1.5 M NaCl, and 2 % (wt/vol) CTAB]. The mixture was shaken vigorously and then incubated at 65 °C for 30 min. After a series of deproteinizations with chloroform-isoamyl alcohol (24:1 vol/vol), a 500-µl portion of the supernatant was transferred to a new 1.5-ml tube, and DNA

was precipitated by the addition of 1 ml of cold absolute ethanol. The precipitated DNA was pelleted with centrifuge, washed with 70 % (vol/vol) ethanol, air-dried, dissolved in 50 µl of TE buffer [10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (pH 8.0)] and then stored at –20 °C.

PCR amplification

The entire ITS rDNA and partial *pyrG* were amplified by PCR with the primers shown in Tab. 1. A serial dilution of genomic DNA was used to optimize the concentration of template for the PCR amplification. The PCR was performed in a 50-µL reaction mixture containing diluted genomic DNA in optimum concentration, 0.25 µM each primer, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.08 % (vol/vol) Nonidet P40, 1.5 mM magnesium chloride ($MgCl_2$), 200 µM each dNTP (Promega Co., U.S.A.), and 2 units of Taq DNA polymerase (Sangon Ltd., Canada). Mineral oil (40 µL) was overlaid on the reaction mixture. The reaction was performed in a MiniCyclerTM (MJ Research Inc., U.S.A.) for 35 cycles: denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min, with an initial denaturation at 94 °C for 1 min and a final extension at 72 °C for 10 min before and after the cycling, respectively. An aliquot (2 µL) of the PCR amplification product was electrophoresed in a 1 % (wt/vol) agarose gel, stained with ethidium bromide (EtBr) and visualized over ultraviolet light.

DNA sequence data

The PCR amplification product was sent to the Invitrogen Corporation (www.invitrogen.com.cn) for sequencing with the same primers as used in PCR reactions. A total of 50 sequences were assembled by the program SeqManII (www.dnastar.com) and deposited in GenBank under the accession numbers shown in Tab. 1. Two datasets were analyzed: (1) all obtained sequences of entire ITS rDNA, and (2) all obtained sequences of partial *pyrG*. One strain of each *Helicostylum elegans* Corda, *Mucor mucedo* L., *Pirella circinans* Bainier, and *Thamnidium elegans* Link, respectively, served as outgroups according to the molecular phylogenetic topology based on *act*, SSU and LSU rDNA and EF-1α sequences (O'Donnell *et al.* 2001, Voigt & Wöstemeyer 2001).

Phylogenetic analyses

Multiple alignments were performed by using MegAlign (www.dnastar.com). All alignments were optimized by eye and manually edited for gap insertions with BioEdit 4.7.1 (Hall 1999) and transferred into NEXUS format files through ClustalX 1.83 (Thompson *et al.* 1997). The final multiple alignment was calculated by the package PAUP 4.0b10 (Swofford 2002) to obtain a pairwise distance matrix following the Kimura two-parameter model of nucleotide substitution (Kimura 1980). Phylogenetic reconstructions were conducted by using the unweighted parsimony analyses implemented in the PAUP* 4.0b10 and by using the Bayesian inferences with the Metropolis-

coupled Markov chain Monte Carlo (MCMCMC) techniques implemented in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The program TreeView 1.6.6 (Page 1996) was used for creating and manipulating graphical trees.

The partition homogeneity test (PHT; Farris *et al.* 1994) implemented in PAUP* 4.0b10 was used to evaluate the concordance of the sequences from ITS rDNA and *pyrG* by using heuristic search with 10000 replicates and 10000 maxtrees, treating gaps as a fifth character and excluding parsimony-uninformative characters.

Unweighted parsimony analyses were carried out for all parsimony-informative characters. Gaps were treated as fifth character. Parsimony branch supports were estimated by performing 1000 bootstrap replicates (Felsenstein 1985) with heuristic searches. The starting tree was obtained via stepwise addition during which one tree was held at each step. Branches of zero length were collapsed with tree-bisection-reconnection as branch-swapping algorithm. The MulTree option was in effect, and the Initial MaxTree was set to 5000. Topologies of unrooted trees were not constrained. However, in order to integrate the branch length and bootstrap value into the consensus trees, the topologies generated by bootstrap analyses were loaded and constrained as backbone of the heuristic search with the same settings as used in bootstrap analyses.

The best-fit models of nucleotide substitution, which were selected by hierarchical likelihood ratio tests (hLRT; Huelsenbeck & Crandall 1997, Posada & Crandall 2001) implemented in the MrModelTest 2.2 (Posada & Crandall 1998, Nylander 2004), were used in MrBayes 3.1.2. Two independent Bayesian analyses run simultaneously each with one cold chain and three heated chains and with temperature set to 0.1, each initiated with a random tree, and flat prior. In order to exactly repeat the analyses, the seed and swapseed were both set to 1160000000. One out of every 100 trees was sampled for optimal generations. The convergence of the two runs was diagnosed by the average standard deviation (less than 0.01) and the potential scale reduction factor (Gelman & Rubin 1992; approximate 1). The first one fourth sampled trees from the two runs were discarded before summarizing the information, and the remaining sampled trees were used for calculating posterior probabilities which were considered statistically significant when P value was no less than 0.95. The node reliability was assessed by no less than 70 % of bootstrap supports (BS) and no less than 0.95 of posterior probabilities (PP; Alfaro *et al.* 2003). The tree resolution was measured by tree length (TL), consistency index (CI), homoplasy index (HI), retention index (RI) and rescaled consistency index (RC).

Results

The partition-homogeneity test (PHT) indicated that the sequences of ITS rDNA and *pyrG* are highly heterogeneous (P = 0.0001) and consequently cannot be analyzed as a combined dataset. Therefore the phylogenetic analyses were carried out separately for the two datasets.

ITS rDNA phylogeny

ITS sequences vary slightly in length ranging from 213 to 219 bp for ITS1 and from 209 to 221 bp for ITS2. The lengths of 5.8S rDNA are all 153 bp. The intraspecific molecular pairwise distances of *Pilaira* are no more than 0.063.

The ITS rDNA alignment from 21 strains of *Pilaira* and the four outgroup strains resulted in 737 characters: 458 constant, 122 variable and parsimony-uninformative, and 157 parsimony-informative. The alignment contained 30 partial SSU (positions 1–30), 250 entire ITS-1 (positions 31–280), 153 entire 5.8S (positions 281–433), 248 entire ITS-2 (positions 434–681), and 56 partial LSU rDNA sites (positions 682–737). The generated tree contained the following parameters: TL = 459, CI = 0.8431, HI = 0.1569, RI = 0.8662, and RC = 0.7303. The best-fit model selected by hLRT in MrModelTest 2.2 was GTR model with gamma-distributed rate variation across sites (GTR+G). The standard deviation of split frequencies was 0.115964 at the 1000th generation and fell below 0.01 (0.009196) after 1400000 generations. The potential scale reduction factors were close to 1.0 (1.000–1.001) for all substitution model parameters after summarizing the last 21000 samples of the two runs.

Phylogenetic trees constructed with parsimony and Bayesian methods show similar topologies and are integrated into Fig. 1. The phylogram contains six clades. Clade ITS-I comprises *Pilaira moreaui* var. *caucasica* NRRL 6282 (T), Pi-4, Pi-6, Pi-7, Pi-8, *P. moreaui* var. *moreaui* NRRL 6283, NRRL 6284 and NRRL A7399 (PP = 0.98, BS not applicable); Clade ITS-II consists of *P. subangularis* Pi-3 only (PP = 0.98, BS = 99 %); Clade ITS-III includes *P. anomala* NRRL 2526, NRRL 2567, NRRL A9310, NRRL A16347, NRRL A16348, NRRL A16356 and NRRL A23912 (PP = 0.73, BS = 100 %); Clade ITS-IV involves *P. anomala* (= *P. cesatii*) NRRL A5067 and NRRL A5068 (PP = 0.98, BS = 99 %); Clade ITS-V is composed of *P. anomala* NRRL A7646 and NRRL A9789 (PP = 0.98, BS = 100 %); And clade ITS-VI is constituted of *P. praeampla* Pi-5 only (PP = 0.91, BS = 99 %). Additionally, a grade is formed of all *P. anomala*.

pyrG phylogeny

The introns vary obviously in length ranging from 57 to 81 bp. The lengths of the partial sequences of the two exons are all 399 bp of DNA or 133 aa of putative amino acids. The alignment of their protein sequences showed that only six sites are different and the remaining 127 sites are identical. The molecular intraspecific pairwise distances of *Pilaira* range from 0 to 0.095.

The alignment resulted in 500 characters: 246 constant, 117 variable and parsimony-uninformative, and 137 parsimony-informative. The alignment contained 117 partial exon-1 (positions 1–117), 100 entire intron-1 (positions 118–217), and 283 partial exon-2 sites (positions 218–500). The generated tree contained the following parameters: TL = 404, CI = 0.8317, HI = 0.1683, RI = 0.8938, and RC = 0.7433. The best-fit model selected by hLRT in MrMod-

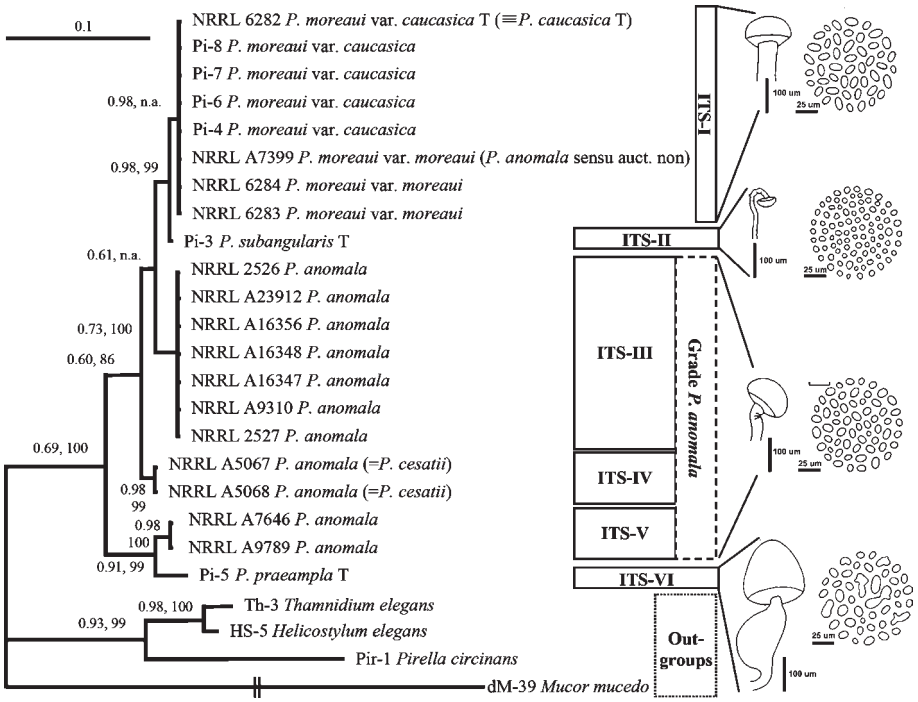

Fig. 1. The Bayesian 50 % majority-rule consensus tree illustrating molecular phylogeny of 21 strains of *Pilaira* inferred from ITS rDNA sequences with *Helicostylum elegans, Mucor mucedo, Pirella circinans* and *Thamnidium elegans* as outgroups. Six clades are designated as ITS-I, -II, -III, -IV, -V and -VI, and a *P. anomala* grade is labeled. The posterior probability (PP) and bootstrap support (BS) are presented near relevant branches. The bar at the upper left indicates 0.1 expected changes per site. Hashed line indicates long branches divided by two to improve visibility of branch lengths. Line drawings in the right part illustrate the size and shape of sporangiophores, columellae, apophyses and sporangiospores, among *P. anomala, P. moreaui, P. praeampla* and P. *subangularis*. n.a. = not applicable. T = ex-type strain.

elTest 2.2 was GTR model with gamma-distributed rate variation across sites (GTR+G). The standard deviation of split frequencies was 0.120010 at the 1000th generation and fell below 0.01 (0.003673) after 2000000 generations. The potential scale reduction factors were close to 1.0 (1.000–1.002) for all substitution model parameters after summarizing the last 30000 samples of the two runs.

Phylogenetic trees individually inferred with the parsimony and Bayesian methods are integrated into Fig. 2 due to their similar topologies. The phylogram is divided into seven clades. Clade pyrG-I is constructed of *P. praeampla* Pi-5 and *P. anomala* NRRL A7646 and NRRL A9789 (PP = 0.87, BS = 56 %); clade pyrG-II contains *P. anomala* NRRL 2526, NRRL 2567, NRRL A9310, NRRL A16347, NRRL A16348, NRRL A16356 and NRRL A23912 (PP = 1.00, BS = 99 %); clade pyrG-III comprises *P. anomala* (= *P.*

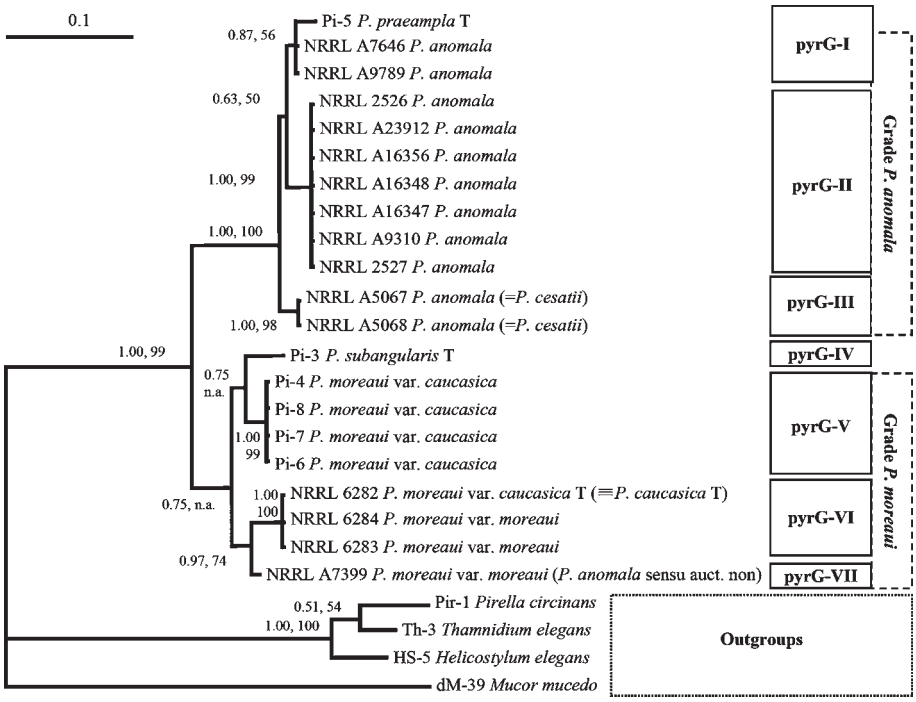

Fig. 2. The Bayesian 50 % majority-rule consensus tree illustrating molecular phylogeny of 21 strains of *Pilaira* inferred from *pyrG* sequences with *Helicostylum elegans, Mucor mucedo, Pirella circinans* and *Thamnidium elegans* as outgroups. Seven clades are designated as pyrG-I, -II, -III, -IV, -V, VI and -VII, and a grade each of *P. anomala* and *P. moreaui* are labeled. The posterior probability (PP) and bootstrap support (BS) are presented near relevant branches. The bar at the upper left indicates 0.1 expected changes per site. n.a. = not applicable. T = ex-type strain.

cesatii) NRRL A5067 and NRRL A5068 (PP = 1.00, BS = 98 %); clade pyrG-IV consists of *Pilaira subangularis* Pi-3 only (PP = 0.75, BS not applicable); clade pyrG-V includes *P. moreaui* var. *caucasica* Pi-4, Pi-6, Pi-7 and Pi-8 (PP = 1.00, BS = 99 %); clade pyrG-VI involves *P. moreaui* var. *caucasica* NRRL 6282, *P. moreaui* var. *moreaui* NRRL 6283 and NRRL 6284 (PP = 1.00, BS = 100 %); And clade pyrG-VII is composed of *P. moreaui* var. *moreaui* NRRL A7399 only (PP = 0.97, BS = 74 %). In addition to these clades, there are two paraphyletic grades constituted of *P. anomala* and *P. moreaui* becoming monophyletic when including *P. praeampla* and *P. subangularis,* respectively.

Discussion

The high heterogeneity between ITS rDNA and *pyrG* sequences, indicated by the PHT tests, suggests that the topologies of the trees constructed by using these two loci differ remarkably. The differences lie in two aspects.

(i) The position of taxa. Members of the clade ITS-I are separated into three clades in the *pyrG* phylogeny (pyrG-V, -VI, and -VII). *P. praeampla* Pi-5 forms a single clade ITS-VI, but is nested in the clade pyrG-I together with two strains of *P. anomala*. (ii) The two trees differ in the number of clades, six in ITS rDNA tree but seven in *pyrG* tree. The *pyrG* provides more phylogenetic information and can therefore reinforce and improve ITS phylogeny, which was also proved in a study on *Rhizopus* phylogeny (Liu *et al*. 2007).

Morphological investigations on the 21 *Pilaira* strains have been discussed in detail by Zheng & Liu (2009). Complete concordances between morphological and phylogenetic taxa can be found in 12 strains: Pi-3, NRRL 2526, NRRL 2527, NRRL 6282, NRRL 6283, NRRL 6284, NRRL A7399, NRRL A9310, NRRL A16347, NRRL A16348, NRRL A16356 and NRRL A23912. The seven strains of *Pilaira anomala* form a monophyletic group in both ITS rDNA and *pyrG* trees (clade ITS-III in Fig. 1, clade pyrG-II in Fig. 2). The rank as variety in *P. moreaui* var. *caucasica* NRRL 6282 (ex-type strain) is supported morphologically and molecularly since it is grouped in one single clade together with *P. moreaui* var. *moreaui* NRRL 6283, NRRL 6284 and NRRL A7399 on both ITS rDNA and *pyrG* trees (clade ITS-I in Fig. 1, clades pyrG-VI and -VIII in Fig. 2). The ex-type strain of *P. subangularis* Pi-3, is separated from *P. moreaui* in the ITS tree (Fig. 1), while it is placed in one clade together with *P. moreaui* in the *pyrG* phylogeny (Fig. 2).

Partial consistencies are observed in five strains, all supported by ITS rDNA rather than *pyrG*. *Pilaira moreaui* var. *caucasica*, i.e. Pi-4, Pi-6, Pi-7 and Pi-8 form a monophyletic group together with *P. moreaui* var. *moreaui* in the ITS tree (clade ITS-I). These four strains, however, are separated to a branch on the *pyrG* tree (clade pyrG-V) from other strains of *P. moreaui* and detached from the ex-type strain NRRL 6282 of *P. moreaui* var. *caucasica*. The ex-type strain of *P. praeampla* Pi-5 has an independent position in the ITS rDNA tree (clade ITS-VI) but in the *pyrG* tree it groups together with NRRL A7646 and NRRL A9789 (clade pyrG-I) which have the typical sporangiophores, columellae, apophyses and sporangiospores of *P. anomala* as shown in Fig. 1.

Absolute discrepancies with morphological findings can be found in *Pilaira anomala* NRRL A5067, NRRL A5068, NRRL A7646 and NRRL A9789. The first two strains were originally designated as *P. cesatii* which was synonymized with *P. anomala* by Zheng & Liu (2009). However, the synonymy does not seem to be confirmed by the present molecular studies. The molecular data recognize NRRL A5067 and NRRL A5068 as a separate taxon (*P. cesatii*) rather than as members of *P. anomala* (clade ITS-IV in Fig.1, clade pyrG-III in Fig. 2). Similarly, the strains NRRL A7646 and NRRL A9789 morphologically determined as *P. anomala* are closely related to *P. praeampla* (clades ITS-V and -VI in Fig. 1; clade pyrG-I in Fig. 2). If NRRL A5067 and NRRL A5068 represent a separate species *P. cesatii*, then the NRRL A7646 and NRRL A9789 should consequently represent a new "phylospecies".

Molecular data have frequently been used to delimit species. In phylogenetic species concepts species are determined on the basis of monophyly.

However, this criterion is gradually changing because of numerous incongruence between different data sets, which are most likely caused by incomplete lineage splitting (Pollard *et al.* 2006). Genetic data, though without monophyly, may be used as a complementation to other data like morphology for diagnosing species with explicit considerations of the temporal dynamics of lineage splitting (Knowles & Carstens 2007). In the present study, some disagreements in the topologies of ITS and *pyrG* trees can be found, and also in species delimitations based on morphological or molecular data. *Pilaira* might be dynamic in molecular lineage sorting and the phylogeny of this genus should be further resolved by thorough consideration of information from more loci and more individuals, especially for those with little concordances between morphology, ITS rDNA and *pyrG*. However, it seems to be somewhat difficult to reach a well resolved phylogeny of *Pilaira* because almost all members of this genus are rarely found and the currently widely used genes, for instance, *act*, EF-1alpha, LSU rDNA, RPB1 and SSU rDNA, are poor in resolving the molecular phylogeny of Zygomycota at any taxonomic level (O'Donnell *et al.* 2001, Voigt and Wöstemeyer 2001, Tanabe *et al.* 2004).

Based on nuclear SSU, ITS, and LSU rDNA sequences, the most recent molecular phylogenetic research on Pilobolaceae demonstrated that the genus *Pilaira* is distant from the other two genera, *Pilobolus* and *Utharomyces*, and consequently suggested that *Pilaira* should be removed from Pilobolaceae (Foos *et al.* 2011; O'Donnell *et al.* (2001). Because Foos *et al.* (2011) studied merely two strains of only one species *P. anomala*, we combined their ITS data with our ITS sequences and resulted in similar phylogeny (phylogram not shown). Although current molecular evidences have indicated a separation of *Pilaira* from the other two genera, its taxonomic position should not be changed until further convictive evidences – both morphologically and molecular biologically – are proposed. Phylogenies on higher taxonomic levels (e.g. family level) are far from being resolved in Zygomycetes. *Pilaira* shares a certain number of characteristics with *Pilobolus* and *Utharomyces*, for example, unbranched and phototrophic sporangiophores, dark hemispheric columellate sporangia, persistent dark cutinized sporangial walls, as well as the same coprophilous habitat.

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