RESEARCH ARTICLE

Psychrophilic fungi from the world's roof

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Abstract During a survey of cold-adapted fungi in alpine glaciers on the Qinghai-Tibet Plateau, 1 428 fungal isolates were obtained of which 150 species were preliminary identified. *Phoma sclerotioides* and *Pseudogymnoascus pannorum* were the most dominant species. Psychrotolerant species in *Helotiales* (*Leotiomycetes, Ascomycota*) were studied in more detail as they represented the most commonly encountered group during this investigation. Two phylogenetic trees were constructed based on the partial large subunit nrDNA (LSU) to infer the taxonomic placements of these strains. Our strains nested in two well-supported major clades, which represented *Tetracladium* and a previously unknown lineage. The unknown lineage is distant to any other currently known genera in *Helotiales*. *Psychrophila* gen. nov. was therefore established to accommodate these strains which are characterised by globose or subglobose conidia formed from phialides on short or reduced conidiophores. Our analysis also showed that an LSU-based phylogeny is insufficient in differentiating strains at species level. Additional analyses using combined sequences of ITS+TEF1+TUB regions were employed to further investigate the phylogenetic relationships of these strains. Together with the recognisable morphological distinctions, six new species (i.e. *P. antarctica*, *P. lutea*, *P. olivacea*, *T. ellipsoideum*, *T. globosum* and *T. psychrophilum*) were described. Our preliminary investigation indicates a high diversity of cold-adapted species in nature, and many of them may represent unknown species.

INTRODUCTION

Cold-adapted fungi are ubiquitous in cold habitats such as the deep seas, Arctic and Antarctic areas, and glaciers. Cold-adapted fungi have evolved special properties, e.g., cold adapted enzymes, change of membrane fluidity, and other cellular components, to enable them to grow at low temperatures at rates comparable to those of mesophiles at moderate temperatures (D'Amico et al. 2006, Ruisi et al. 2007). During the past two decades, research on cold-adapted fungi has increased, driven by their potential value for application in biotechnology (Margesin & Schinner 1994, 1999). Cold-adapted fungi have become important sources for the discovery of novel bioactive secondary metabolites and enzymes (Flam 1994, Pietra 1997, Biabini & Laatch 1998, Gudjarnnson 1999, Höller et al. 2000, Verbist et al. 2000, Bhadury et al. 2006, Ebel 2006, Blunt et al. 2007, Rateb & Ebel 2011).

Microorganisms living in low temperature environments are generally referred to as psychrophiles or psychrotolerants. Psychrophiles have been defined as species that can grow at or below 0 °C; have optimum growth temperatures (OGT) of ≤ 15 °C and maximum growth temperatures (MGT) of ≤ 20 °C; while psychrotolerants can grow close to 0 °C, have OGT > 15 °C and MGT > 20 °C (Morita 1975). However, these definitions are also ambiguous and may not be applicable for most of the eukaryotes, as some higher organisms known as psychrophiles, such as some algae, plants, insects, marine and terrestrial invertebrates, and fish may have much broader growth-temperature ranges. The terms stenopsychrophile and eurypsychrophile have therefore been proposed to modify the definitions of psychrophilic and psychrotolerant. The 'steno-'

and 'eury-' are referred ecological terms derived from Shelford's law of tolerance that describe narrow or wide tolerance to an environmental determinant, respectively. The stenopsychrophile (equal to 'psychrophile') refers to microorganisms with a restricted growth-temperature range that cannot tolerate higher temperatures. Eurypsychrophile (equal to 'psychrotolerant microorganisms') describes microorganisms that 'like' permanently cold environments, but can also tolerate a wide range of temperatures extending into the mesophilic range (Cavicchioli 2006).

Since the discovery of bioluminescent bacteria that are able to grow at 0 °C by Forster (1887), a number of psychrophilic bacteria have been discovered from deep ocean sediments, glacier ice, and soils of the polar regions (DeLong et al. 1997, Mountfort et al. 1998, Price 2000, Berestovskaya et al. 2002, Margesin et al. 2003, Bowman et al. 2004, Seo et al. 2005, Zhang et al. 2006, 2008, Grünke et al. 2012). However, the number of known cold-adapted fungi, especially psychrophilic fungi, is relatively low. In recent years, the diversity of filamentous fungi in cold niches has been increasingly investigated, and the number of known species has greatly expanded (Möller & Dreyfuss 1996, Robinson 2001, Blanchette et al. 2004, Arenz et al. 2006, Connell et al. 2006, Held et al. 2006, Malosso et al. 2006, Duncan et al. 2008, Onofri et al. 2008, Selbmann et al. 2008, Arenz & Blanchette 2009, Jurgens et al. 2009). Most species in these studies, however, are psychrotolerant, and only a few were documented as psychrophiles such as *Thelebolus microsporus*, *Mucor strictus*, *Phoma herbarum*, *Humicola marvinii*, *Pseudogymnoascus destructans*, and some snow molds (e.g. *Sclerotinia borealis*, *Microdochium nivale*, *Coprinus psychromorbidus*) (Schipper 1967, Dejardin & Ward 1971, Traquair & Smith 1982, Richard et al. 1997, Hsiang et al. 1999, Tronsmo et al. 2001, Singh et al. 2006, Gargas et al. 2009, Hoshino et al. 2010, Anupama et al. 2011, Minnis & Lindner 2013). Species in several yeast genera including *Mrakia*, *Mrakiella* and *Rhodotorula* were usually described as psy-

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chrophilic. For example, *Mrakia frigida* grew well at 15 °C and 4 °C but poorly at 20 °C (Margaret 1966); *Mrakia psychrophila* from Antarctic soil had an optimal growth temperature of 10 °C and a MGT of 18 °C (Xin & Zhou 2007); *Mrakiella cryoconiti*, *M. aquatica* and *M. niccombsii* from alpine and arctic habitats also exhibited psychrophilic features and failed to grow at temperatures over 20 °C (Margesin & Fell 2008, Robin et al. 2010). Psychrophilic fungi are phylogenetically diverse and we identified the cold-adapted fungi through a polyphasic approach integrating phylogenetic analysis, morphological characterisation and cold-adapted features in the present study.

The Qinghai-Tibet Plateau, often called the 'world's roof' or 'the third pole', is located in the southwest of China and is the highest and largest low-latitude region with permafrost in the world. The high elevation and low latitude make the Qinghai-Tibet Plateau a unique alpine ecosystem that is sensitive to changes in climate and surface conditions (Cheng 1998). In the last 30 years, the permafrost area on the Qinghai-Tibet Plateau has decreased by over 10 000 km² (Li & Cheng 1999). Therefore, researchers have been paying more attention to investigate microorganisms on the Qinghai-Tibet Plateau. Although prokaryotes have been extensively investigated in this area (Xiang et al. 2005, 2009, Liu et al. 2006, 2007, 2009a, b, Yao et al. 2006, Zhang et al. 2007, 2009, Yang et al. 2008), fungi have not received much attention. During an investigation of the cold-adapted fungi of the Qinghai-Tibet Plateau, 1 428 fungal isolates were obtained, of which 150 species were preliminarily identified. In this paper, we studied some dominant fungi from Qinghai-Tibet Plateau in *Helotiales* in detail. A few related isolates from the Antarctic were also included.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from seven glaciers in 2009–2011. The sampling areas were located at the edge or centre of the following glaciers: Midui and Zhadang Glacier in Tibet, Qiyi and Toumingmengke Glacier in Gansu Province, Hailuogou Glacier in Sichuan Province, Yuzhufeng Glacier in Qinghai Province and Mingyong Glacier in Yunnan Province. In addition, some soil samples were also collected from Antarctic, near the Great Wall Station in January 2011 (Table 1). For all sampling, clean hand tools were surface sterilised with 70 % ethanol before use. After the removal of the top 5–10 cm of surface sediment, c. 500 g soil sample was collected from the underlying layer and placed in a fresh Zip-lock plastic bag. The samples were maintained at 4 °C until arrival at the laboratory.

Isolation of fungi

Fungi were isolated from soil samples as soon as they were taken to the lab using a traditional pour plate method. A 10 g quantity of each soil sample was suspended in sterile-distilled water in a flask. The volume was then increased to 100 mL before the suspension was shaken to disperse soil particles and then serially diluted to 10^{-2}, 10^{-3} and 10^{-4}. For selec-

tion of psychrophilic or psychrotolerant fungi, about 0.1 mL of each dilution was placed on the surface of three 90 mm diam Petri plates containing 1/4 PDA (potato dextrose agar plus chloramphenicol at 0.1 mg/mL and streptomycin at 0.1 mg/mL to suppress bacterial growth) and spread evenly. The plates were sealed and incubated at 4, 10 and 20 °C (one plate per temperature). The plates were examined for fungal growth at 1 wk intervals for 4 wk. Colonies that appeared on the plates were transferred to three new plates, which were incubated at 4, 10 and 20 °C as temperature test. The change in colony diameter after 4 wk (growth rate) was determined for each isolate at the three temperatures. The psychrophilic and psychrotolerant fungi isolated in this study were consolidated but not strictly defined by the definition given by Morita (1975). The fungi grew better at 4 and 10 °C than at 20 °C and those that grew better at 20 °C were considered psychrophilic and psychrotolerant. The ex-type specimens (dried culture) were deposited in HMAS (Herbarium Mycologicum Academiae Sinicae), with the living culture in CGMCC (China General Microbiological Culture Collection Center).

Morphological observations

A number of psychrophilic or psychrotolerant fungi were isolated. Among them, *Phoma sclerotioides* and *Pseudogymnoascus pannorum* (= *Geomyces pannorum*) were most frequently encountered (137 and 52 isolates, respectively) and are well-known cold-adapted species. Sixteen isolates representing some frequently encountered fungi (190 isolates in total) in the *Helotiales* were studied in more detail. Morphological characteristics were observed, photographed, and measured using material from agar plate and slide culture (Coetzee & Eicker 1990). The colony diameter of fungi growing on PDA plates was measured in two perpendicular directions after 4 wk at different temperatures, and the mean diameter was obtained from five replicate plates cultivated at the same temperatures. Morphological characteristics of colonies including aerial mycelium, density, and pigment production were noted. Microscopic morphology was examined using slide cultures: each isolate was transferred to a 50 mL centrifugal tube and incubated at 10 °C for 3 wk before hyphae, conidiophores, and conidia on water mounts were observed, photographed, and measured with a Nikon 80i microscope with differential interference contrast (DIC) optics.

DNA extraction, PCR amplification, sequencing, phylogenetic analysis and SNP detection

Genomic DNA was extracted from the fungal mycelia on PDA plates following the protocol described by Wang & Zhuang (2004). The primers LROR and LR5 (Vilgalys & Hester 1990) were used to amplify the partial large subunit nrDNA (LSU); ITS1 and ITS4 (White et al. 1990) were used to amplify the internal transcribed spacer region of the nuclear ribosomal RNA gene; EF1-728F and EF1-986R (Carbone & Kohn 1999) were used to amplify partial translation elongation factor 1-α gene (TEF1), and Bt-2a and Bt-2b (Glass & Donaldson 1995) were used to amplify partial β-tubulin gene (TUB). PCR was

Table 1 Details of the soil samples collected at 5–10 cm depth for the survey of cold-adapted fungi on the Qinghai-Tibet Plateau and Antarctic.

Sampling location	Collection date	GPS location		Altitude (m)	Depth of collection (cm)
Midui	16 October 2009	N29°27'	E96°30'	3874	5–10
Zhadang	27 September 2010	N30°28'	E90°38'	5800	5–10
Yuzhufeng	1 October 2009	N35°41'	E94°17'	4658	5–10
Toumingmengke	3 October 2010	N39°29'	E96°32'	4545	5–10
Qiyi	7 October 2010	N39°15'	E97°45'	4315	5–10
Hailuogou	20 April 2011	N29°33'	E101°58'	3461	5–10
Mingyong	4 May 2011	N28°27'	E98°45'	2811	5–10
Antarctic	5 January 2011	S62°12'	W58°57'	10	5–10

Table 2 Fungi and their GenBank accession numbers used in this study. The newly generated sequences in this study are shown in **bold**.

| Species | Strain number | GenBank no.[1] | | | |
		LSU	ITS	TEF1	TUB
Arachnopeziza variepilosa	M337	–	EU940163	–	FJ477045
Ascocoryne sarcoides	OSC #100772	FJ176886	–	–	–
	–	AJ406399	–	–	–
Botryotinia fuckeliana	LGM002	–	KC683713	–	KC6837123
Bulgaria inquinans	ZW–Geo52–Clark	AY789344	–	–	–
	CBS 118.31	DQ470960	–	–	–
Cadophora fastigiata	DAOM 225754	JN938877	–	–	–
Cadophora luteo-olivacea	Clo-40	–	HQ661093	HQ661078	HQ661063
	ICMP:18096	HM116760	–	–	–
Catenulifera luxurians	CBS 647.75	–	GU727560	–	GU727569
Ciborinia camelliae	EFA 1	–	FJ959095	–	GQ181121
Cistella spicicola	CBS 731.97	–	GU727553	–	GU727565
Cudoniella clavus	OSC 100054	DQ470944	–	–	–
	ILLS60488	JN012006	–	–	–
Cudoniella indica	VG 113-4	GQ477325	–	–	–
	VG 112-1	GQ477324	–	–	–
Cudoniella tenuispora	ILLS60490	JN012008	–	–	–
Dermea acerina	CBS 161.38	DQ247801	–	–	–
Fabrella tsugae	–	AF356694	–	–	–
Hyaloscypha aureliella	M235	EU940153	–	–	–
	M234	EU940152	–	–	–
Hyaloscypha daedaleae	ZW-Geo138-Clark	AY789415	–	–	–
Hyaloscypha fuckelii	M233	EU940154	–	–	–
Hyaloscypha hepaticola	M339	EU940150	EU940226	–	–
Hyaloscypha vitreola	M236	EU940156	–	–	–
	M39	EU940155	–	–	–
Hymenoscyphus pseudoalbidus	FC-2799	–	AB705220	AB705213	–
Hyphodiscus hymeniophilus	CBS 529.87	GU727555	–	–	–
	CBS 602.77	–	DQ227264	–	DQ227270
Leotia lubrica	OSC 100001	NG_027596	–	–	–
	ZW-Geo59-Clark	AY789359	–	–	–
Loramyces macrosporus	CBS 235.53	DQ470957	–	–	–
Neofabraea perennans	RGR 90.0107	–	AF281397	–	AF281476
Phacidium lacerum	CBS 130.30	DQ470976	–	–	–
Phialocephala fortinii	K93 395	–	–	DQ274568	DQ274834
Psychrophila antarctica	**ANT80**	**KF768459**	**JX001628**	**KF768425**	**KF768438**
	ANT92	**KF768452**	**JX001640**	**KF768424**	**KF768437**
	ANT94	**KF768458**	**JX001639**	**KF768423**	**KF768436**
Psychrophila lutea	**HAILUO374**	**KF768456**	**JX001638**	**KF768421**	**KF768441**
	HAILUO407	**KF768455**	**JX001615**	**KF768422**	**KF768439**
	HAILUO409	**KF768454**	**JX001637**	**KF768420**	**KF768440**
Psychrophila olivacea	**HAILUO368**	**KF768457**	**JX001618**	**KF768427**	**KF768443**
	HAILUO563	**KF768453**	**JX001633**	**KF768426**	**KF768442**
Rhynchosporium orthosporum	H4	–	HM627471	HM627456	KC819296
Rutstroemia firma	CBS 341.62	DQ470963	–	–	–
Sclerotinia sclerotiorum	CBS 499.50	AF431951	–	–	–
	CBS 499.50	DQ470965	–	–	–
	WZ0067	AY789347	–	–	–
Tetracladium apiense	CCM F-23199	EU883420	–	–	–
	CCM F-23399	EU883421	–	–	–
Tetracladium breve	CCM F-10501	EU883418	–	–	–
Tetracladium ellipsoideum	**MIDUI20**	**KF768465**	**JX029111**	**KF768431**	**KF768444**
	MIDUI21	**KF768466**	**JX029124**	**KF768432**	**KF768451**
	MIDUI30	**KF768467**	**JX029113**	**KF768430**	**KF768445**
Tetracladium furcatum	CCM F-06983	EU883428	–	–	–
	CCM F-11883	EU883432	–	–	–
Tetracladium globosum	**HAILUO215**	**KF768460**	**JX029109**	**KF768433**	**KF768448**
	MY24	**KF768461**	**JX029118**	**KF768434**	**KF768449**
	MY25	**KF768462**	**JX029133**	**KF768435**	**KF768450**
Tetracladium marchalianum	CCM F-26399	EU883415	–	–	–
	CCM F-11391	EU883417	–	–	–
	CCM F-19399	EU883423	–	–	–
Tetracladium maxilliforme	CCM F-529	EU883429	–	–	–
	CCM F-13186	EU883430	–	–	–
Tetracladium palmatum	CCM F-10001	EU883424	–	–	–
Tetracladium psychrophilum	**HAILUO380**	**KF768464**	**JX029119**	**KF768429**	**KF768446**
	MY376	**KF768463**	**JX029129**	**KF768428**	**KF768447**
Tetracladium setigerum	CCM F-19499	EU883426	–	–	–
	CCM F-20987	EU883425	–	–	–
Trichoglossum hirsutum	OSC61726	AY789313	–	–	–
Vibrissea flavovirens	MBH39316	AY789426	–	–	–
Vibrissea truncorum	CBS #258.91	FJ176874	–	–	–
	CUP 62562	AY789402	–	–	–

[1] LSU: large subunit nrDNA; ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; TEF1: partial translation elongation factor 1-alpha gene; TUB: partial beta-tubulin gene.

performed in 25 µL reactions containing DNA template 1.0 µL, each forward and reverse primers 1.0 µL, 2 × MasterMix 12.5 µL (TIANGEN Co. Ltd., Beijing, China) and H_2O, using the following parameters: 94 °C for 40 s; followed by 40 cycles at 54 °C for LSU, 53 °C for ITS, 55 °C for TEF1and 52 °C for TUB gene for 50 s and 72 °C for 60 s; and a final extension at 72 °C for 7 min. The PCR products were sequenced with primers mentioned above by Invitrogen Biotechnology Co. Ltd. (Beijing, China). Sequences were compared to accessions in the GenBank database via BLASTn searching to find the most likely taxonomic designation (Table 2).

Sequence data of the four genes were aligned with Clustal X (Thompson et al. 1997). Further manual alignment was carried out with MEGA v. 5 (Tamura et al. 2011) and alignments were deposited in TreeBASE (www.treebase.org, submission no. S16864). Maximum Parsimony (MP) analyses were conducted using PAUP v. 4.0b10 (Swofford 2002) and Bayesian analysis using MrBayes v. 3.1.2 (Altekar et al. 2004). For the MP analysis, ambiguously aligned regions were excluded from all analyses. An unweighted parsimony (UP) analysis was performed. Trees were inferred using the heuristic search option with TBR branch swapping and 1 000 random sequence addi-
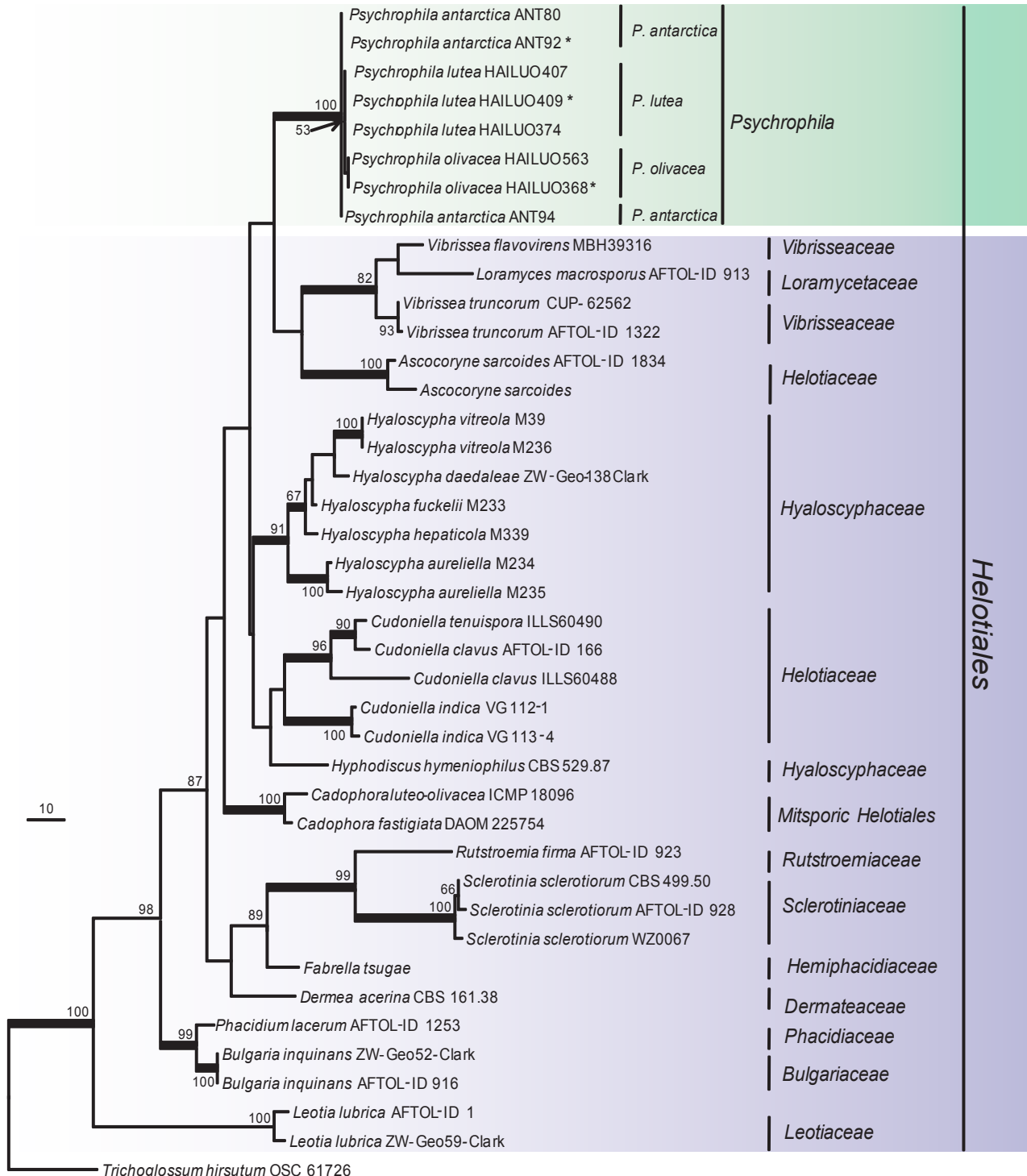
Fig. 1 Phylogenetic tree derived from maximum parsimony analysis based on LSU rDNA sequences (TL = 610, CI = 0.5607, RI = 0.7609, HI = 0.4393 and RC = 0.4266). *Trichoglossum hirsutum* OSC61726 was used as outgroup. The LSU alignment consists of 851 characters, with 184 phylogenetically informative positions. Bootstrap values of more than 50 % are shown on the respective branches and significant Bayesian posterior probability (≥ 95 %) are indicated as **bold** branches. Ex-type cultures are marked with asterisks (*).

tions. Branches of zero length were collapsed and all equally most parsimonious trees were saved. Descriptive tree statistics such as tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI), were calculated for trees generated. Clade stability was assessed using bootstrap analysis with 1 000 replicates, each with 10 replicates of random stepwise addition of taxa. For the Bayesian analyses, the models of evolution were estimated by using MrModeltest v. 2.3 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC). Six simultaneous Markov chains were run for 1 000 000 generations and trees were sampled every 100th

generation (resulting in 10 000 total trees). The first 2 000 trees represented the burn-in phase of the analyses and were discarded and the remaining 8 000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree. Trees were visualised in TreeView v. 1.6.6 (Page 1996).

Unique fixed nucleotide positions are used to characterise and describe several sterile species (see applicable species notes). For the sterile species that was described, the closest phylogenetic neighbour(s) were selected from Fig. 3 and 4, and this focused dataset was subjected to SNP analyses. These single nucleotide polymorphisms (SNPs) were determined for each aligned data partition using DnaSP v. 5.00.07 (Librado & Rozas 2009).
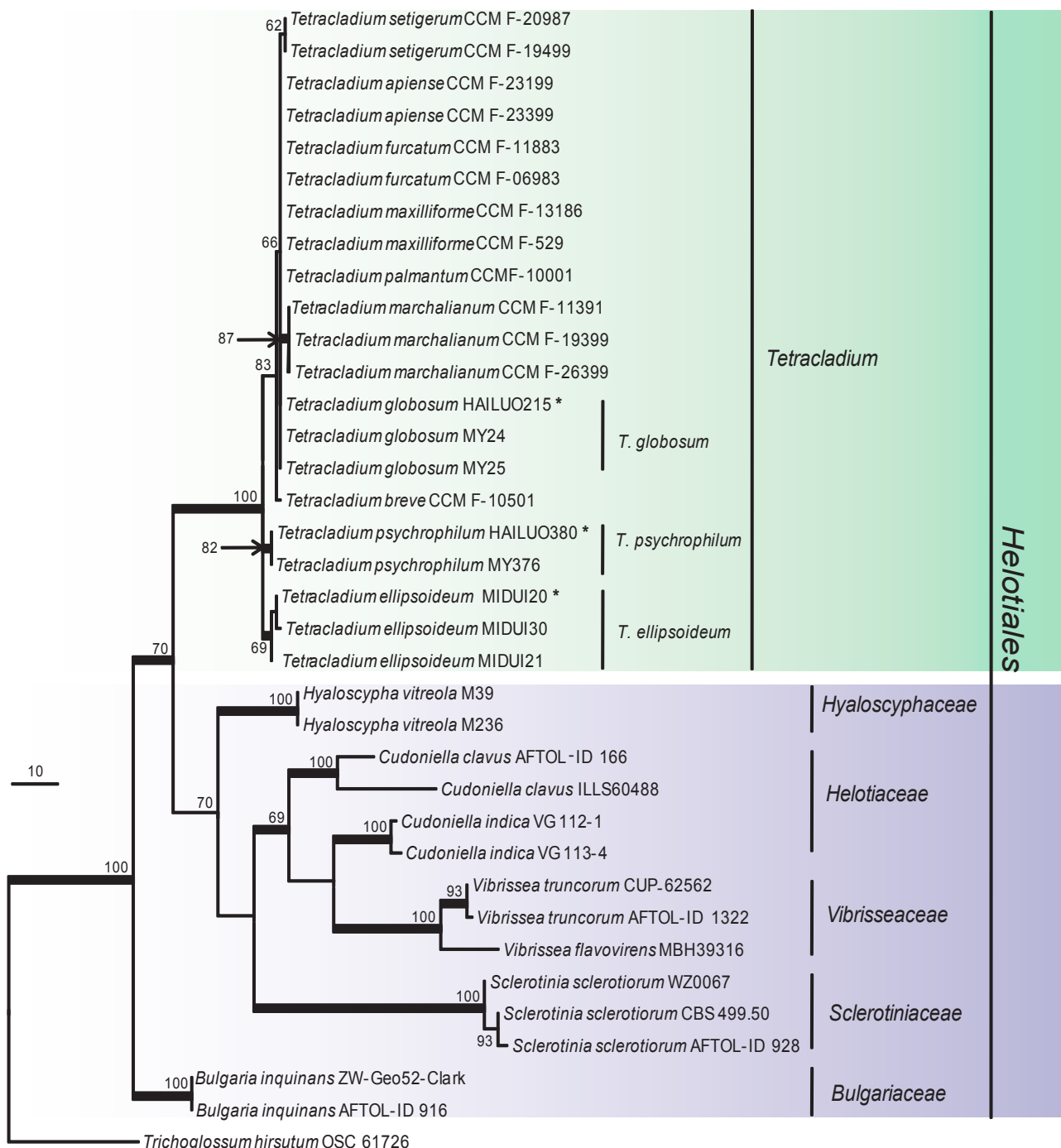

Fig. 2 Phylogenetic tree derived from maximum parsimony analysis based on LSU rDNA sequences (TL = 325, CI = 0.7815, RI = 0.9015, HI = 0.2185 and RC = 0.7046). *Trichoglossum hirsutum* OSC61726 was used as outgroup. The LSU alignment consists of 845 characters, with 151 phylogenetically informative positions. Bootstrap values of more than 50 % are shown on the respective branches and significant Bayesian posterior probability (≥ 95 %) are indicated as **bold** branches. Ex-type cultures are marked with asterisks (*).

RESULTS

In the current investigation, 1 428 fungal isolates were obtained from 350 samples, which were mainly collected from seven glaciers on the Qinghai-Tibet Plateau; a few specimens were collected from Antarctica. Isolates were preliminarily identified to belong to 78 genera representing 150 species. About one-tenth of these isolates were psychrophilic (stenopsychrophile), mostly belonging to the genera *Pseudogymnoascus, Phoma, Tetracladium* and *Psychrophila*, the new genus described in this paper. Based on the preliminary identification, 16 isolates belonging to *Helotiales*, which were the most frequently encountered cold-adapted fungi, were selected to study in more detail.

Phylogenetic analysis and SNP detection

The phylogenetic relationships were determined for 16 isolates. According to the phylogenetic trees based on the partial large subunit nrDNA (LSU), 16 isolates clustered into two independent clades, one was strongly supported and well separated from other known genera in the *Helotiales* (Fig. 1) and should represent a new genus and the other eight isolates clustered within the *Tetracladium* clade (Fig. 2). In the phylogenetic trees (Fig. 3, 4) generated from combined sequences of ITS+TEF1+TUB, the isolates in Fig. 1 clustered into one clade comprising three subclades that were well supported and separated from each other. Based on phylogenetic relationships and morphological characteristics, a new genus, *Psychrophila,* is proposed to accommodate these three new species (*P. antarctica, P. lutea* and *P. olivacea*).

Tetracladium is one of the three aquatic genera with tetraradiate conidia that were described by de Wildeman (1893, 1894, 1895). According to our phylogenetic trees (Fig. 2, 4), isolates in the present study formed three independent subclades that could not be assigned to any known species.

LSU regions had relatively few informative sites for the studied strains and were therefore not selected as good markers at species level. The remaining three loci had varied success for species identification and all of the sterile new species described here could be identified by all three loci.

Taxonomy

Psychrophila M.M. Wang & Xing Z. Liu, *gen. nov.* — MycoBank MB801296

Etymology. *Psychrophila* means cold-loving and is referring to those fungi well adapted to low temperature habitats.

Type species. *Psychrophila antarctica* M.M. Wang & Xing Z. Liu.

Colonies on PDA slow-growing, cream-white, yellowish or dark-olive to dark-brown, with sparse aerial mycelium; vegetative hyphae hyaline, smooth, thick-walled, transversely septate, most agglomerate to bundles, or swollen to moniliform. The cells of aerial hyphae often aggregated in dense clumps, hyphae deep immerged into the agar. *Conidiogenous cells* phialidic, enteroblastic, hyaline, flask-shaped, apically tapering into a broad funnel, bottleneck-like constriction; the collarette wedge-shaped to campanulate and widely flaring. *Conidiophores* reduced to
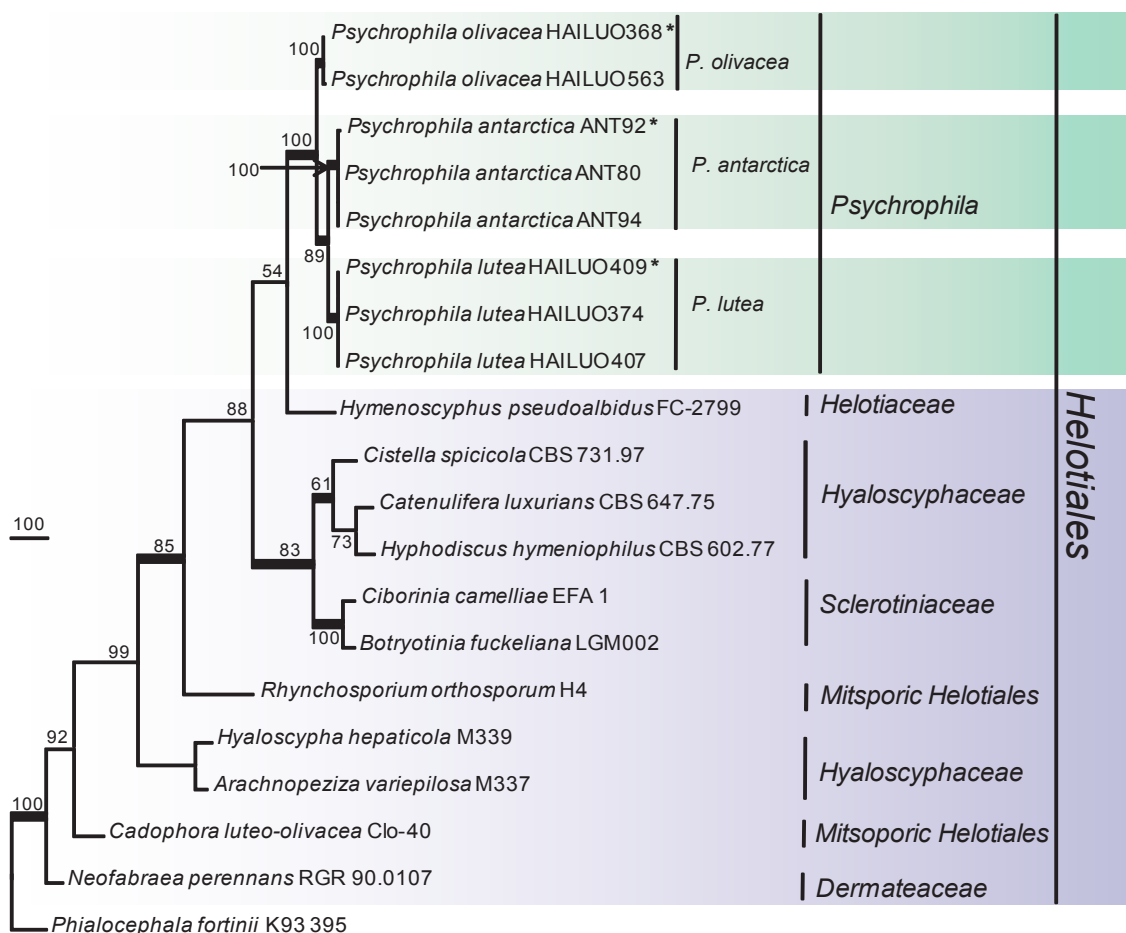

Fig. 3 Phylogenetic tree derived from maximum parsimony analysis based on combined ITS+TEF1+TUB sequences (TL = 2295, CI = 0.7085, RI = 0.7600, HI = 0.2915 and RC = 0.5384). *Phialocephala fortinii* K93 395 was used as outgroup. The length of the three genes alignment was 1 383 characters, with 746 phylogenetically informative positions. Bootstrap values of more than 50 % are shown on the respective branches and significant Bayesian posterior probability (≥ 95 %) are indicated as **bold** branches. Ex-type cultures are marked with asterisks (*).
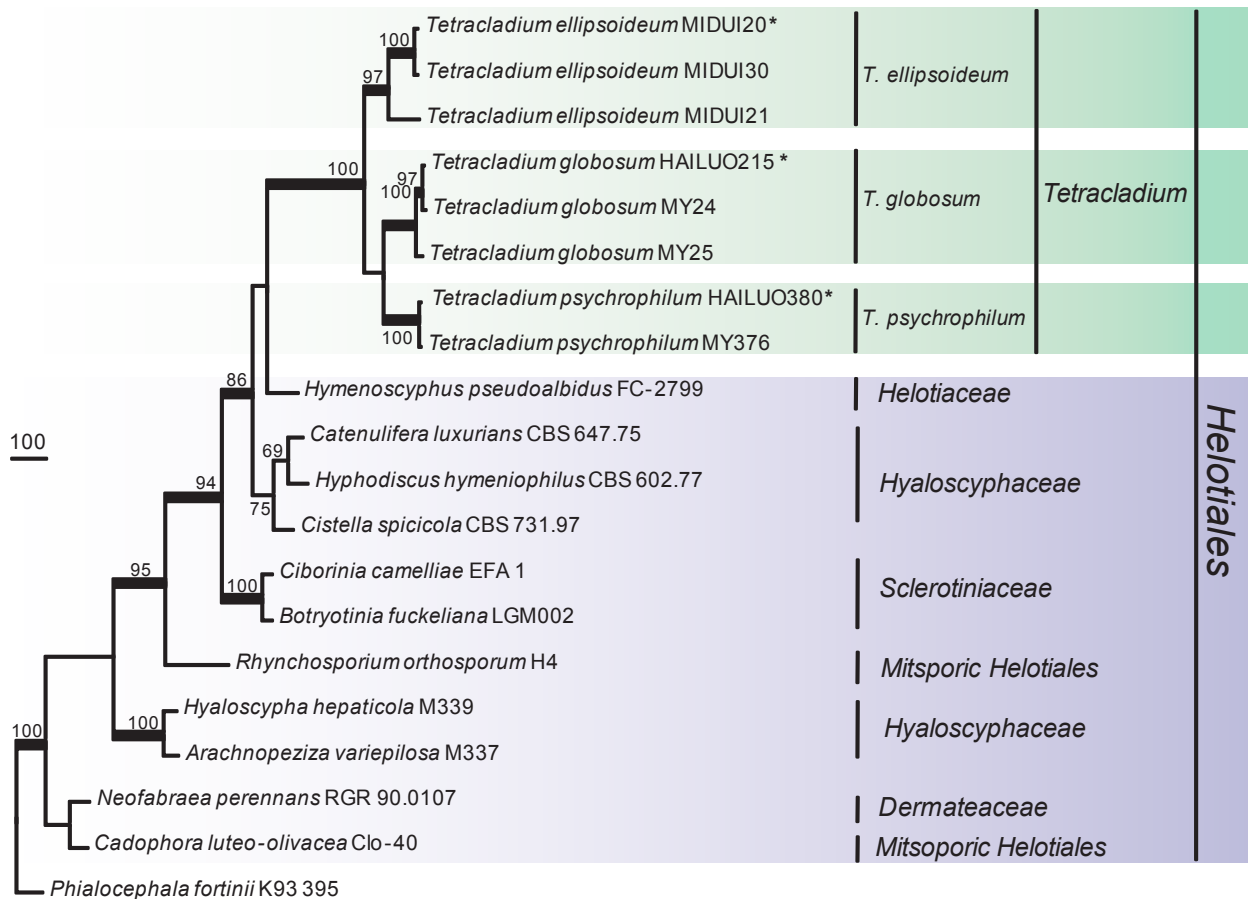
Fig. 4 Phylogenetic tree derived from maximum parsimony analysis based on combined ITS+TEF1+TUB sequences (TL = 2881, CI = 0.6977, RI = 0.7565, HI = 0.3023 and RC = 0.5278). *Phialocephala fortinii* K93 395 was used as outgroup. The length of the three genes alignment was 1 577 characters, with 1 043 phylogenetically informative positions. Bootstrap values of more than 50 % are shown on the respective branches and significant Bayesian posterior probability (≥ 95 %) are indicated as **bold** branches. Ex-type cultures are marked with asterisks (*).

conidiogenous cells, sometimes short, or much differentiated. *Conidia* hyaline, smooth, aseptate, pyriform to globose, within a single conidiogenous locus.

Habitat — Cold environments.

Notes — Species with phialophora-like asexual morphs in the *Helotiales* include: *Ascocoryne* with *Coryne* asexual morphs, which have hyaline, more or less penicillate conidiophores and phialides that lack visible collarettes; asexual morphs of some species of the *Dermateaceae*, such as *Mollisia* and *Pyrenopeziza*, might be accommodated in *Cadophora*, which has more or less pigmented vegetative hyphae, pale to hyaline phialides and collarettes (Gams 2000); the asexual morph of *Hyphodiscus hymeniophilus* is *Catenulifera rhodogena*, which has cylindrical to ampulliform phialides, long and cylindrical collarette, and conidia born in chains or in droplets (Hosoya 2002), in contrast, species in the new genus *Psychrophila* have hyaline vegetative hyphae, phialides, and collarettes; conidiophores are reduced to conidiogenous cells, sometimes short, or much differentiated; collarettes are wedge-shaped to campanulate and widely flaring; and conidia are hyaline, pyriform to globose. The combination of a cold-adapted nature, morphological characters, and phylogenetic relationships well supports the establishment of the new genus *Psychrophila* (Fig. 1).

Psychrophila antarctica M.M. Wang & Xing Z. Liu, *sp. nov.* — MycoBank MB801298; Fig. 5

Etymology. Antarctica refers to the type locality of this fungus.

Colony on PDA at 10 °C attaining 25 mm diam after 4 wk, OGT 20 °C, eurypsychrophile; colonies cream white, aerial mycelium less abundant or sparse on the surface of the colony. *Conidiophores* sometimes short, or much differentiated, *conidiogenesis* phialidic, phialides short, hyaline, flask-shaped, 5.1–8.0 × 2.5–4.5 μm (mean ± S.D. = 6.4 ± 0.89 × 3.5 ± 0.77 μm, n = 30), apically tapering into a broad funnel, bottleneck-like constriction; the collarette 2.1–4 μm (mean ± S.D. = 2.9 ± 0.56 μm, n = 30), wedge-shaped, widely flaring; vegetative hyphae hyaline, sometimes agglomerate to bundles or swollen to irregular shapes, 2–4 μm. *Conidia* hyaline, 1-celled, smooth, mostly globose, 2.1–3.5 μm diam (mean ± S.D. = 2.7 ± 0.47 μm, n = 30).

Specimen examined. ANTARCTIC, Great Wall Station, S62°12' W58°57', from soil, Jan. 2011, *T. Zhang* (dried culture HMAS244374 holotype, living culture ex-type CGMCC315133 (ANT92)).

Other isolates examined. ANTARCTIC, Great Wall Station, S62°12' W58°57', from soil, Jan. 2011, *T. Zhang*, living cultures ANT80, ANT94.

Notes — *Psychrophila antarctica* is a psychrotolerant fungus with an OGT of 20 °C. This species is known from both Antarctica and the Qinghai-Tibet Plateau, whose origin and evolution deserve further studies.

Psychrophila lutea M.M. Wang & Xing Z. Liu, *sp. nov.* — MycoBank MB801299; Fig. 6

Etymology. Lutea refers to the yellow colour of the colony.

Cultures sterile. *Psychrophila lutea* differs from its closest phylogenetic neighbour, *P. antarctica* (Fig. 3), by unique fixed alleles in three loci based on alignments of the separate loci deposited in TreeBASE as study S16864: ITS positions 76 (A),
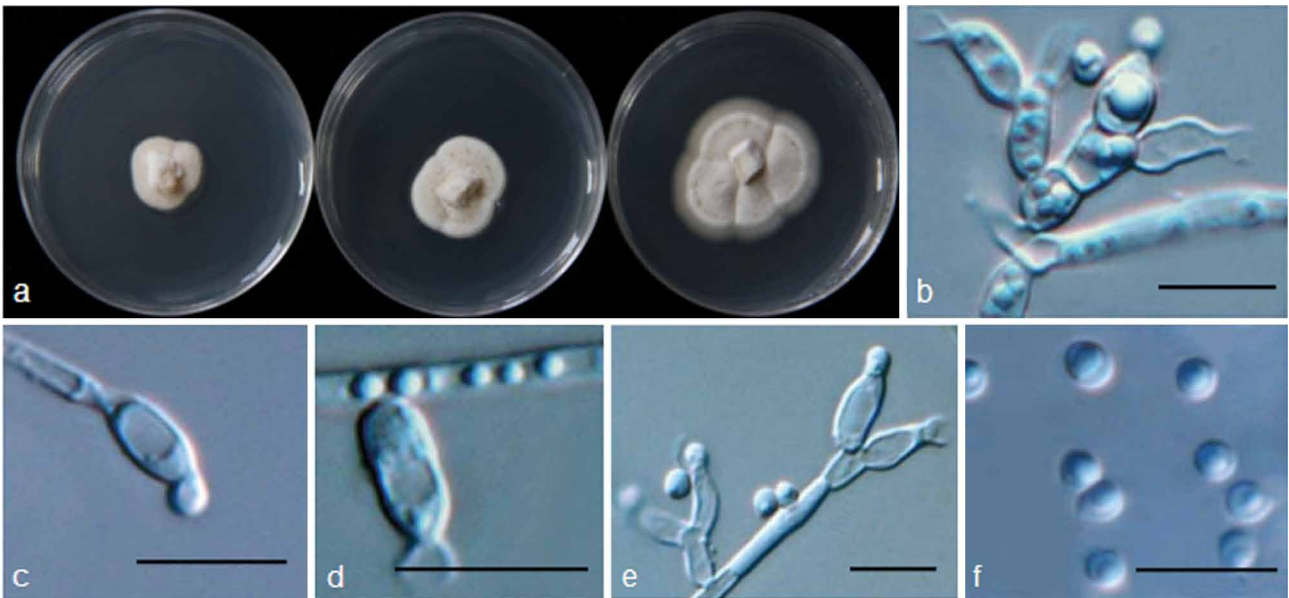
Fig. 5 *Psychrophila antarctica* (from strain ANT92) a. Colony morphology at three temperatures after 4 wk (left-to-right: 4, 10 and 20 °C); b−e. conidiophores and conidiogenous cells; f. conidia. — Scale bars = 10 µm.

316 (C), 413 (C), 416 (A), 449 (T) and 454 (T); TUB positions 134 (T), 138 (A), 146 (A), 147 (A), 170 (A), 206 (C), 294 (T), 303 (C), 305 (G), 314 (T) and 356 (G); TEF1 positions 174 (C), 208 (C), 247 (C), 283 (G), 297 (A) and 327 (A).

Colony on PDA at 10 °C attaining 15 mm diam after 4 wk, OGT 20 °C, eurypsychrophile; bright to brown-yellow, part of the colonies submerged in the medium, hyphae above the medium compacted densely, aerial mycelium absent or sparse, hyaline; vegetative hyphae yellow or brown, smooth-walled, 2−8 µm; aggregated in dense clumps or bundles, sometimes swollen to irregular shapes. *Conidiophores* and *conidia* absent.

Specimen examined. China, Sichuan, Hailuogou Glacier, N29°33' E101°58', from soil, 20 Apr. 2011, *M. Wang* (dried culture HMAS244372 holotype, living ex-type culture CGMCC315134 = HAILUO409).

Other isolates examined. China, Sichuan, Hailuogou Glacier, N29°33' E101°58', from soil, 20 Apr. 2011, *M. Wang*, living cultures HAILUO374, HAILUO407.

Notes — We have used some low nutrient media such as corn meal agar (CMA) and water agar (WA) to induce strains of *P. lutea* to sporulate without success. Phylogenetic analyses showed that it formed a distinct clade most closely related to

P. antarctica (Fig. 1, 3) but could be differentiated from the later by SNP analysis.

Psychrophila olivacea M.M. Wang & Xing Z. Liu, *sp. nov.* — MycoBank MB801300; Fig. 7

Etymology. Olivacea refers to the olive colour of the colony.

Cultures sterile. *Psychrophila olivacea* differs from its closest phylogenetic neighbour, *P. antarctica* and *P. lutea* (Fig. 3), by unique fixed alleles in three loci based on alignments of the separate loci deposited in TreeBASE as study S16864.

P. antarctica: ITS positions 113 (C), 116 (A), 133 (A), 308 (C), 343 (A), 346 (G), 364 (C), 411 (G), 413 (C), 425 (G), 431 (G), 439 (G), 451 (A) and 454 (T); TUB positions 116 (C), 137 (T), 151 (G), 163 (T), 198 (T), 199 (A), 220 (C), 223 (T), 295 (C), 304 (G), 306 (C), 328 (C) and 371 (T); TEF1 positions 174 (C), 195 (G), 203 (A), 208 (C), 247 (C), 252 (C), 297 (A), 306 (T), 307 (T), 308 (G), 313 (A), 327 (C), 333 (T) and 340 (T).

P. lutea: ITS position 113 (C), 116 (A), 308 (C), 316 (T), 317 (C), 333 (C), 343 (A), 346 (G), 364 (C), 411 (G), 416 (G), 425 (G), 431 (G), 439 (G), 449 (T), 451 (A) and 454 (T); TUB positions
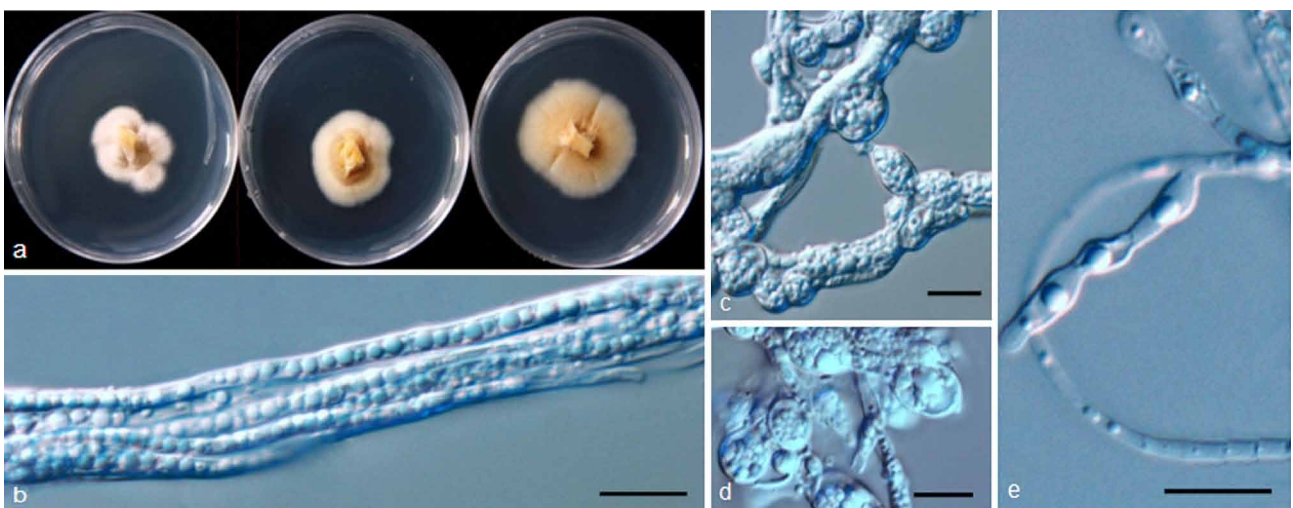

Fig. 6 *Psychrophila lutea* (from strain HAILUO409). a. Colony morphology at three temperatures after 4 wk (left-to-right: 4, 10 and 20 °C); b−e. swollen and aggregated hyphae. — Scale bars = 10 µm.
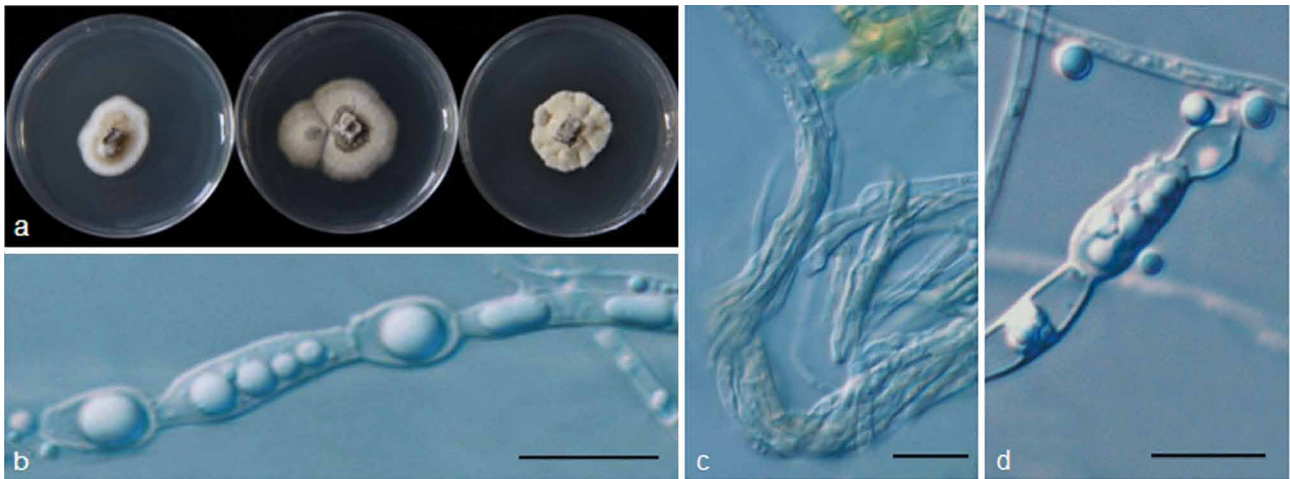
Fig. 7 *Psychrophila olivacea* (from strain HAILUO368). a. Colony morphology at three temperatures after 4 wk (left-to-right: 4, 10 and 20 °C); b–d. aggregated hyphae. — Scale bars = 10 µm.

134 (C), 138 (G), 151 (G), 163 (T), 170 (G), 199 (A), 206 (T), 223 (T), 294 (A), 295 (C), 303 (T), 304 (G), 305 (G), 306 (C), 314 (G), 316 (A), 356 (A) and 371 (T); TEF1 positions 195 (G), 203 (A), 252 (C), 283 (A), 306 (T), 307 (T), 308 (G), 313 (A), 327 (C), 333 (T) and 340 (T).

Colony on PDA at 10 °C attaining 10–15 mm diam after 4 wk, growth rate similar at 10 and 20 °C, stenopsychrophile; light to dark olive, sometimes appearing light grey on the surface because of some young aerial hyphae; part of the colonies immerged in the medium, some hyphae above the medium compact densely, colony surface sometimes furrowed; aerial hyphae sparse, hyaline or olive, vegetative hyphae, olive to dark olive, smooth-walled, 2–7 µm; aggregate in dense clumps or rhizomorphs, sometimes swollen to irregular shapes. *Conidiophores* and *conidia* absent.

Specimen examined. CHINA, Sichuan, Hailuogou Glacier, N29°33' E101°58', from soil, 20 Apr. 2011, *M. Wang* (dried culture HMAS244375 holotype, living culture ex-type CGMCC315135 = HAILUO368).

Other isolate examined. CHINA, Sichuan, Hailuogou Glacier, N29°33' E101°58', from soil, 20 Apr. 2011, *M. Wang*, living culture HAILUO563.

Notes — No conidia or conidiophores were observed for *P. olivacea* on PDA, CMA and WA. *Psychrophila olivacea* differs from *P. lutea* in the colony morphology and OGT.

Tetracladium globosum M.M. Wang & Xing Z. Liu, *sp. nov.* — MycoBank MB801301; Fig. 8

Etymology. Globosum refers to its globose conidia.

Colonies on PDA at 10 °C attaining 30–45 mm diam after 4 wk, pale yellow to light pinkish, OGT 10 °C, stenopsychrophile; part of the colony immerged in the medium, aerial hyphae sparse and hyaline; vegetative hyphae hyaline, smooth, thin-walled, transversely septate, 1–4 µm. *Conidia* 1-celled, hyaline, globose, smooth-walled, 3.0–5.5 µm (mean ± S.D. = 4.4 ± 0.81 µm, n = 30), attaching to the hyphae with very short conidiophores, which are not obvious.

Specimens examined. CHINA, Sichuan, Hailuogou Glacier, N29°33' E101°58', from soil, 20 Apr. 2011, *Manman Wang*, dried culture specimen HMAS244377 holotype, living culture ex-type CGMCC315136 = HAILUO215.

Other isolates examined. CHINA, Yunnan, Mingyong Glacier, N28°27' E98°45', from soil, 4 May 2011, *M. Wang*, living cultures MY24, MY25.

Notes — Species described in the genus *Tetracladium* are all aquatic and mostly inhabit decaying litter in streams and rivers (Bärlocher 1992). *Tetracladium* species produce tetraradiate conidia, which are thought to aid in their colonisation of substrates (Read et al. 1992). Unlike the previously described species, *T. globosum* has globose conidia, indicating that tetraradiate conidia may be an ecologically adapted characteristic. The OGT
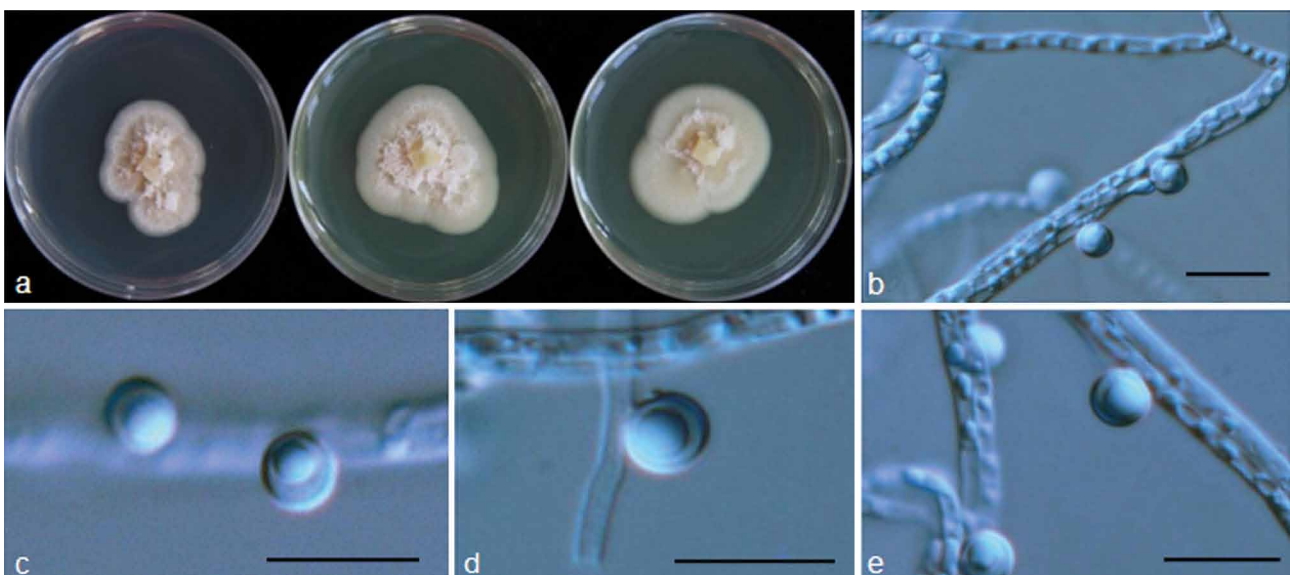

Fig. 8 *Tetracladium globosum* (from strain HAILUO215). a. Colony morphology at three temperatures after 4 wk (left-to-right: 4, 10 and 20 °C); b–e. conidia and hyphae. — Scale bars = 10 µm.

is 10 °C but the fungus can also grow at 20 °C. It is interesting that the OGT of *T. globosum* varied among different isolates. This phenomenon has also been observed in other fungi such as *Pseudogymnoascus pannorum*, indicating psychrophily may be an adapted character (Kochkina et al. 2007).

Tetracladium ellipsoideum M.M. Wang & Xing Z. Liu, *sp. nov*. — MycoBank MB801302; Fig. 9

Etymology. Ellipsoideum refers to the shape of the conidia.

Colony on PDA at 10 °C attaining 30–40 mm diam after 4 wk, pale to bright yellow, OGT at 10 °C, stenopsychrophile; aerial hyphae absent or sparse; vegetative hyphae hyaline, smooth, thin-walled, transversely septate, 1–3 μm. *Conidia* borne on short, undifferentiated or sessile pedicels (up to 1 μm long), 1-celled, hyaline, ellipsoid, smooth-walled, 4–6.8 × 2–3.4 μm (mean ± S.D. = 5.3 ± 0.69 × 3.7 ± 0.67 μm, n = 30).

Specimen examined. CHINA, Tibet, Midui Glacier, N29°27' E96°30', from soil, 16 Oct. 2009, *Manman Wang* (dried culture specimen HMAS244378 holotype, culture ex-type CGMCC315137 = MIDUI20).

Other isolates examined. CHINA, Tibet, Midui Glacier, N29°27' E96°30', from soil, 16 Oct. 2009, *M. Wang*, living cultures MIDUI30, MIDUI21.

Notes — The morphology of *T. ellipsoideum* is very similar to that of *T. globosum*. *Tetracladium ellipsoideum* produces conidia that are pyriform to ellipsoid rather than globose as observed for *T. globosum*. Conidiophores are somewhat differentiated and obvious for *T. ellipsoideum* but not obvious for *T. globosum*.
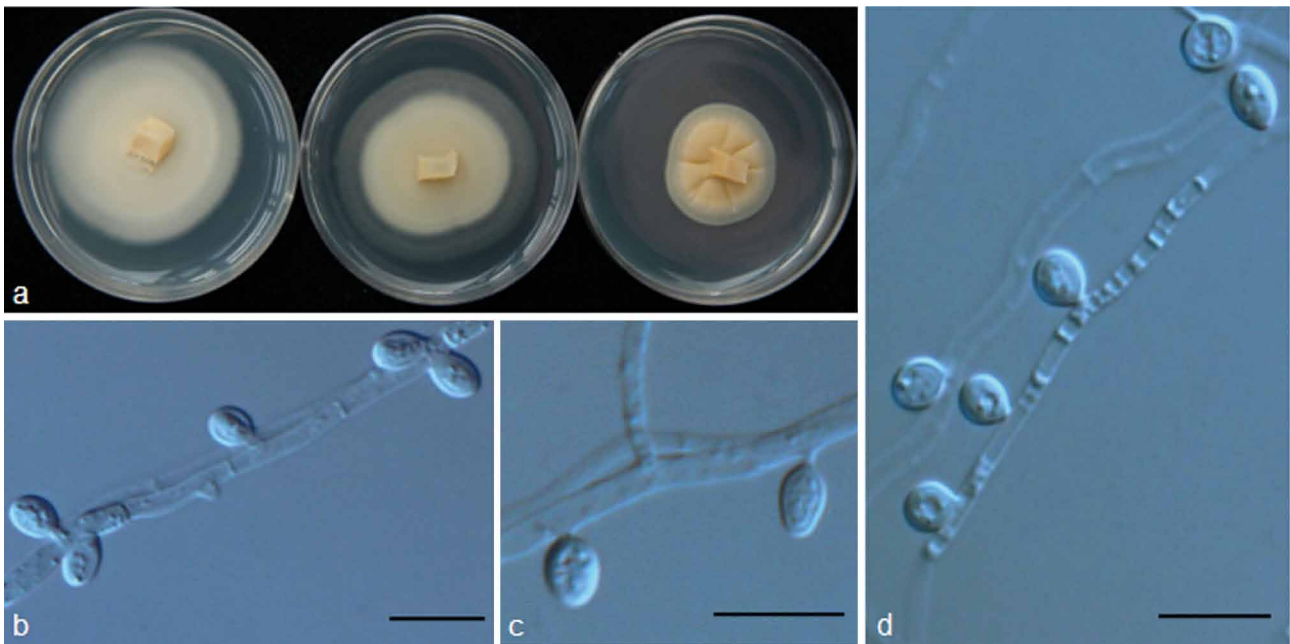

Fig. 9   *Tetracladium ellipsoideum* (from strain MIDUI20). a. Colony morphology at three temperatures after 4 wk (left-to-right: 4, 10 and 20 °C); b–d. conidia, conidiogenous cells and hyphae. — Scale bars = 10 μm.
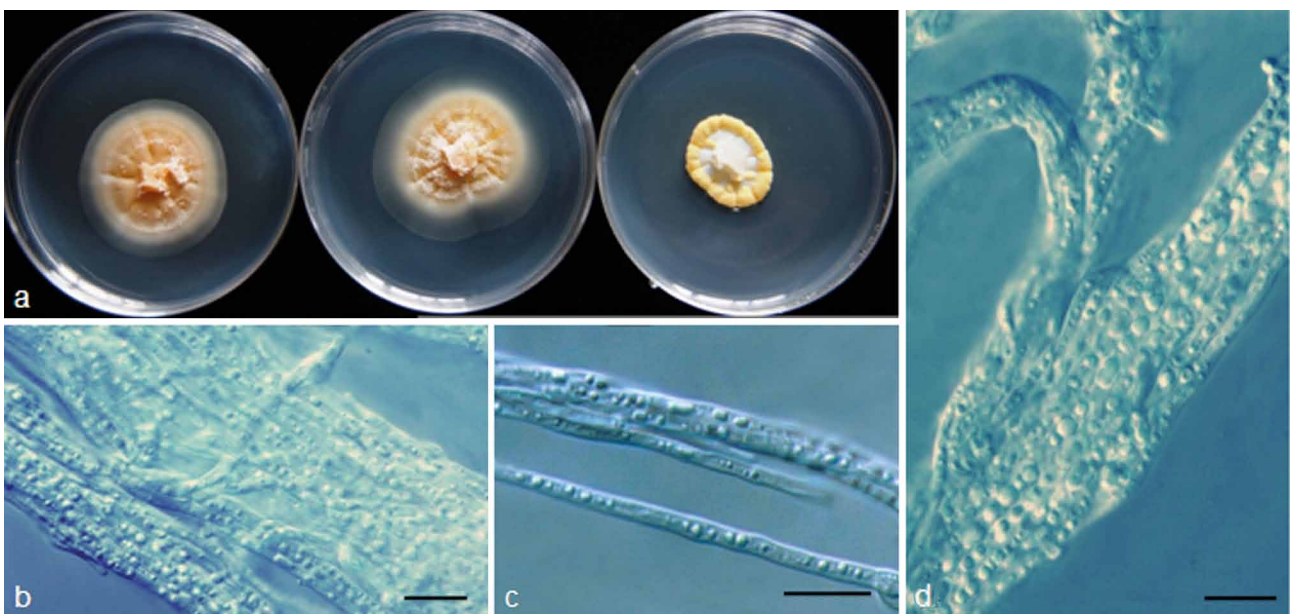

Fig. 10   *Tetracladium psychrophilum* (from strain HAILUO380). a. Colony morphology at three temperatures after 4 wk (left-to-right: 4, 10 and 20 °C); b–d. hyphae. — Scale bars = 10 μm.

Tetracladium psychrophilum M.M. Wang & Xing Z. Liu,
  sp. nov. — MycoBank MB801304; Fig. 10

Etymology. Psychrophilum refers to the cold-loving character of the species.

Cultures sterile. *Tetracladium psychrophilum* differs from its closest phylogenetic neighbour, *T. globosum* (Fig. 4), by unique fixed alleles in three loci based on alignments of the separate loci deposited in TreeBASE as study S16864: ITS positions 97 (C), 108 (A), 310 (C), 336 (C), 337 (C), 397 (A), 416 (G), 427 (C) and 430 (G); TUB positions 125 (C), 131 (T), 140 (A), 152 (G), 157 (C), 185 (A), 186 (T) and 196 (C); TEF1 positions 78 (C), 80 (G), 84 (T), 99 (C), 153 (T), 161 (T) and 166 (C).

Colony slow-growing, attaining about 10–15 mm diam on PDA at 10 °C after 4 wk, cream-white to pale yellow; OGT 10 °C, MGT 20 °C, stenopsychrophile; aerial mycelium sparse or absent, conidia absent; vegetative hyphae hyaline, often aggregate, 2–14 µm. *Conidia* and *conidiophores* absent.

Specimen examined. CHINA, Sichuan, Hailuogou Glacier, N29°33' E101°58', from soil, 20 Apr. 2011, *M. Wang* (dried culture HMAS244371 holotype, living ex-type culture CGMCC315139 = HAILUO380).

Other isolate examined. CHINA, Yunnan, Mingyong Glacier, N28°27' E98°45', from soil, 4 May 2011, *M. Wang*, living culture MY376.

Notes — *Tetracladium psychrophilum* grows slowly with OGT at 10 °C and MGT at 20 °C. Unlike *T. globosum* and *T. ellipsoideum*, *T. psychrophilum* did not produce conidia or conidiophores.

DISCUSSION

The rising of the Qinghai-Tibet Plateau was an important geological event in the Quaternary period when the average rate of rising was 1.0–1.1 mm/year. In the last 10 000 years, the plateau has raised 300–700 m and is still rapidly rising (Li & Wang 1983). This steady rising of the Qinghai-Tibet Plateau may have resulted in gradual environmental changes and niches that are inhabited by cold-adapted fungi.

Species of *Geomyces* and *Phoma* are widespread and especially common in northern temperate regions or Arctic and Antarctic permafrost soils. Traditionally, *Geomyces* is characterised by short but distinctly branched conidiophores that have spore chains formed directly from the conidiogenous cells. Members of the genus are psychrotolerant and have been reported from Arctic and Antarctic permafrost soils (Kirk et al. 2008, Blehert et al. 2009). Phylogenetic analyses indicate *Geomyces* and allied genera such as *Gymnostellatospora* and *Pseudogymnoascus* should be classified in the family *Pseudeurotiaceae* (Minnis & Lindner 2013). The best-known psychrophilic fungus in this group is perhaps *Pseudogymnoascus destructans*, which causes white nose syndrome and high mortality of bats (Blehert et al. 2009, Gargas et al. 2009). In our study, *Pseudogymnoascus pannorum* was found to be dominant on the Qinghai-Tibet Plateau, accounting nearly 10 % of all isolates obtained. Interestingly, although the morphological characteristics and temperature profile differ among isolates of this species, all isolates presented identical ITS and SSU rDNA sequences. *Phoma sclerotioides* is known as a snow mould and as the causal agent of brown root rot of alfalfa and other perennial forage legumes in temperate regions with harsh winters (Wunsch & Bergstrom 2011). It was also the most frequently isolated species from a decomposing high arctic moss *Schistidium apocarpum* (Leung et al. 2011). In this survey, we obtained 52 *P. sclerotioides* isolates in a total of 1 428 isolates from Qinghai-Tibet Plateau, with all of the strains of *P. sclerotioides* isolated from soil and identified based on comparison of morphologies and ITS and SSU rDNA sequences. *Phoma sclerotioides* has been divided

into seven intraspecific varieties, which differ in their morphology, temperature adaptation, and plant hosts (Sanford 1933, Berkenkamp & Baenziger 1969, Wunsch & Bergstrom 2011). Whether these varieties satisfy the phylogenetic concept of species should be considered in future research.

Psychrophila antarctica produces phialidic conidiogenous cells, which is also shared by some species in genera such as *Ascocoryne*, *Cadophora* and *Catenulifera* in the *Helotiales*. Although there are no special morphological characters in *Psychrophila*, the cold-adaptation of all isolates and species and the phylogenetic distinction from other genera well support its establishment as novel genus.

Tetracladium species are common in aquatic habitats, and they produced tetraradiate conidia that may facilitate their attachments to the substrate and provide a stable base for rapid germination (Read et al. 1992). *Tetracladium* species are primary agents of leaf litter and wood decay in streams and rivers. Some aquatic fungi including *Tetracladium* species are distributed worldwide (Descals 1997, Shearer et al. 2007, Wurzbacher et al. 2010) and in lotic habitats from the equator to the Arctic (Shearer et al. 2007). *Tetracladium* species have been documented from streams of alpine glaciers and from snow-covered soil (Robinson et al. 2000, Kuhnert et al. 2012), and are likely to be cold-adapted. The genus is rather homogeneous in terms of cultural characters and conidiogenesis. In addition to the type species, *T. marchalianum*, seven other species have been reported in the genus, e.g. *T. apiense* (Sinclair & Eicker 1981), *T. breve* (Roldán et al. 1989), *T. furcatum* (Descals & Webster 1983), *T. maxilliforme* (Ingold 1942), *T. nainitalense* (Sati et al. 2009), *T. palmatum* (Roldán et al. 1989) and *T. setigerum* (Ingold 1942). Recent phylogenetic analyses suggested that the genus is monophyletic and affiliated with *Helotiales* (Nikolcheva & Bärlocher 2002, Baschien et al. 2006, Letourneau et al. 2010, Seena et al. 2010). The three new species described here are paraphylogenetically clustered with aquatic *Tetracladium* species. *Tetracladium globosum* and *T. ellipsoideum* produce simple globose or clavate conidia on very short conidiophores or on the hypha (sessile), and *T. psychrophilum* does not produce conidia. All three are clearly different from previously described aquatic species in this genus and may reflect their adaptation to glacial niches with little free water. The three new species can grow well at temperatures below 20 °C and produce colonies that are light or bright yellow or light pink. Their colonies are often flat, with sparse or no aerial mycelium, which may be beneficial for cold-adaptation.

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