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TAXONOMIC DESCRIPTION

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Ramichloridium endophyticum sp. nov., a novel species of endophytic fungus from *Potamogeton pectinatus*

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Abstract

During a survey of endophytic fungi in aquatic plants collected from Tibet, PR China, a novel species, *Ramichloridium endophyticum,* was isolated from *Potamogeton pectinatus*. This novel species differs from other species of the genus *Ramichloridium* by its finely verrucose, obovoid, ellipsoidal–obovoid and occasionally subglobose conidia. Phylogenetic analysis of the combined sequences of the internal transcribed spacers (ITS) and the translation elongation factor 1-alpha gene (*tef1*-α) confirmed that the isolated strain represents a member of the genus *Ramichloridium*. A full description, illustrations and a phylogenetic tree showing the position of *R. endophyticum* are provided.

INTRODUCTION

Endophytic fungi are important components of natural ecosystems and have been widely reported from many plant species. These fungi represent a significant source of fungal diversity and novel secondary metabolites [1–3]. *Potamogeton pectinatus* L. is a perennial submerged aquatic macrophyte [4, 5]. It is commonly called sago pondweed, fennel pondweed and ribbon weed. It is broadly distributed in fresh and brackish waters on all continents except Antarctica. Within China [6], it is found from the southern tropical regions to the northern temperate zones, and from the east coast to the high mountains in the west.

The fungal family *Dissoconiaceae* was established by Crous and de Hoog in the order *Capnodiales* (*Dothideomycetidae*, *Dothideomycetes*, *Pezizomycotina*, *Ascomycota*) to accommodate some species of the genus *Dissoconium* and the type species of the genus *Ramichloridium* [7]. Subsequently, Li *et al*. accepted two additional genera, *Pseudoveronaea* Crous and Batzer and *Uwebraunia* Crous and M.J. Wingf. within this family on the basis of morphological characters and phylogenetic analysis of the complete internal transcribed spacers (ITS1+5.8S+ITS2) and the large subunit nuclear ribosomal RNA gene (LSU rRNA) sequences [8]. Presently, four genera are included in the family *Dissoconiaceae* [7, 8]. In this family, the majority of fungi are symbionts, saprobes and plant pathogens [7, 9]. Their asexual morphs are characterized by the production of conidiophores that are medium brown, zero to multiseptate, with terminal and lateral loci and conidia that are olivaceous-brown, smooth, ellipsoidal to obclavate or globose.

The genus *Ramichloridium* was originally described by Stahel with *Ramichloridium musae* Stahel as the type species. However, because his publication lacked a Latin diagnosis, the genus was invalid [10, 11]. De Hoog subsequently reintroduced the genus with *Ramichloridium apiculatum* as the type species, and described the generic characters for taxonomic circumscription, which included the production of erect dark branched or non-branched conidiophores with different degrees of differentiation and sympodial extension conidiogenous cells and aseptate conidia [11, 12].

In a survey of endophytic fungal diversity in aquatic plants, a novel ascomyceteous taxon was isolated from *Potamogeton pectinatus* L. collected from the Duoqing lake, Yadong County, Tibet, PR China. This novel fungus is classified as a member of the genus *Ramichloridium*, but differs from the other known

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Keywords: Ascomycota; *Dissoconiaceae*; morphology; phylogeny.
Abbreviations: ITS, internal transcribed spacer; LSU rRNA, large subunit nuclear ribosomal RNA gene; *tef1*-α, translation elongation factor 1-alpha gene.
The GenBank accession numbers of *R. endophyticum* ITS, TEF and LSU are MK836099, MN307070 and MK836098, respectively.
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species of the genus *Ramichloridium* with respect to morphological characters and the results of molecular phylogenetics analysis. In this paper, we describe and illustrate the novel species as *R. endophyticum* with complete morphological descriptions.

METHODS

Sample collection and endophyte isolation

Samples of *P. pectinatus* were collected from the Duoqing lake (28°12′ N, 89°23′ E; 4180 m above sea level), Yadong County, Tibet, PR China, on 21 July 2018. The samples were placed in plastic bags, labeled, and transported to the laboratory. Endophytic fungi were isolated by incubating surface-disinfected tissue segments (5 mm diam.) on Rose Bengal Agar (RBA; Guangdong Huankai Microbial Sci and Tech) according to the method described by Guo *et al.* [13]. Each plant sample was cut into segments 20–30 mm in length and washed thoroughly with tap water. The segments were cut further into smaller sections of about 5×5×5 mm. Ten sections were randomly selected from each plant and surface sterilized by consecutive immersions for 30 s in 70% ethanol, 2 min in 4% sodium hypochlorite and finally rinsed three times with sterile water. After surface disinfection, the tissue segments were then evenly spaced in 90 mm Petri dish containing RBA. Two antibiotics [penicillin G (0.5 g l^{-1}) and streptomycin (0.5 g l^{-1})] were added to suppress bacterial growth [14]. Petri dishes were sealed, incubated at 25 °C, and examined periodically. At the same time, the efficacy of the disinfection procedure to remove surface fungi was tested by making the imprints of the surface-disinfected segments on RBA plates and smearing 0.2 ml of the rinse water from the last rinse onto RBA plates. The absence of growth of any fungi confirmed that the adopted surface disinfection procedure was indeed effective [15]. When fungi grew out from the tissue segment, a few hyphal fragments were picked up and transferred to potato dextrose agar (PDA; 200 g potato, 20 g dextrose, 18 g agar, 1000 ml distilled water) plates for incubation at 25 °C. The pure strains were incubated on PDA and cornmeal agar (CMA; 20 g cornmeal, 18 g agar, 1000 ml distilled water) at 25 °C. Microscopic examination was carried out after 1 week of growth on CMA exposed to natural light at 25 °C. Observation was performed using a BX51 microscope (Olympus), and sterile water was used as a mounting medium for microscopy. Colony colours (surface and reverse) were rated according to the colour charts of Rayner [16].

The pure cultures and dried cultures were deposited in the Herbarium of the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming, Yunnan, PR China (YMF) and the China General Microbiological Culture Collection Centre (CGMCC).

DNA extraction, amplification and sequencing

Total DNA was extracted from fresh cultures grown on PDA plates for 2 weeks, following the protocol of Guo *et al.* [17]. The primer pairs ITS5/ITS4 [18], LROR/LR5 [19] and EF1-728F/EF1-986R [20] were respectively used for the amplification of the internal transcribed spacers (ITS1+5.8S+ITS2) and the translation elongation factor 1-alpha partial gene (*tef1-α*). Amplification was performed in a 25 µl reaction volume, which contained 1.0 µl DNA template, 1.0 µl each of the forward and reverse primers, 12.5 µl 2×MasterMix (Tiangen Biotech) and 9.5 µl ddH_2O. The PCR thermal cycle programs for the amplifications of these two DNA fragments followed those described by Su *et al.* [21]. Species products were then purified using a commercial Kit (Bioteke Biotechnology) and forward and reverse sequenced with a model 4000L automatic sequencer (LI-COR), using a Thermo Sequenase-kit (ThermoFisher) as described by Kindermann *et al.* [22]. The sequences were deposited in GenBank database at the National Centre for Biotechnology Information (NCBI) and the accession numbers are listed in Table 1.

Phylogenetic analysis

The ITS sequence generated in this study was used as a query to search for similar DNA sequences in GenBank using BLAST. The results indicated that the novel taxon had the highest ITS sequence similarity to *Ramichloridium apiculatum*. Therefore, we selected 20 strains representing 13 species belonging to four genera in the family *Dissoconiaceae* and retrieved their respective ITS and *tef1-α* sequences from GenBank (Table 1). We also retrieved GenBank accessions from *Dothiora ceratoniae* CBS 477.69 of *Dothideales* as an outgroup for phylogenetic analyses. All sequences analyzed in this study are listed in Table 1. DNA sequence data for the two genes were aligned using ClustalX 1.83 [23] with default parameters, and the consensus sequences were manually adjusted and linked through BioEdit v.7.0 [24]. Manual gap adjustments were done to improve the alignment and ambiguously aligned regions were also excluded. Then, the combined sequence alignment was converted to a NEXUS file using MEGA6 [25]. The alignment was deposited at TreeBase http://purl.org/phylo/treebase/phylows/study/TB2:S24555:.

Bayesian analysis of the alignment was conducted in MrBayes 3.1.2 [26]. The best-fit evolutionary model was determined for each dataset by comparing different evolutionary models via MrModeltest 2.3 [27]. The general time-reversible model of molecular evolution was used for combined datasets according to the MrModeltest suggestion, in which a proportion of the sites was assumed to be invariable, while the substitution rate for the remaining sites was approximated from a gamma distribution with four categories (GTR +I+Γ), and a random starting tree. The prior probability density was a flat Dirichlet (all values are 1.0) for both Revmatpr and Statefreqpr as default settings. Four simultaneous chains of Markov Chain Monte Carlo were run starting from random trees and sampling every 100 generations. The analysis was halted at 3000000 generations, and the stationarity of the analyses was confirmed following the standards described by Sun and Guo [28]. At the end of the analysis, 100000 trees were generated and 35000 trees were excluded as the 'burn-in' when calculating

Table 1. Species, strains and their corresponding GenBank accession numbers of sequences used for phylogenetic analyses

Species	Strain	GenBank accession number	
		ITS	*tef1-α*
Dissoconium aciculare	CPC 18965	JQ622082	JQ622107
Dissoconium aciculare	CPC 18968	JQ622081	JQ622106
Dissoconium aciculare	CPC 18966	JQ622083	JQ622108
Dissoconium aciculare	CPC 18967	AY598874	JQ622114
Dissoconium aciculare	CPC 18973	AY598875	JQ622115
Dissoconium proteae	CBS 122900	EU707897	–
Dothiora ceratoniae	CBS 477.69	KF251151	KF253111
Pseudoveronaea ellipsoidea	CBS 132085	FJ425205	JQ622120
Pseudoveronaea obclavata	CPC 18972	AY598877	JQ622119
Ramichloridium apiculatum	CBS 156.59	EU041791	–
Ramichloridium apiculatum	CBS 400.76	EU041794	–
Ramichloridium cucurbitae	CBS 132087	JQ622087	JQ622112
Ramichloridium endophyticum	**YMF 1.05584**	**MK836099**	**MN307070**
Ramichloridium eucleae	CPC 23551	KJ869155	–
Ramichloridium luteum	CPC 18961	EU329730	JQ622116
Ramichloridium luteum	CPC 18962	EU329731	JQ622117
Ramichloridium mali	LQ 73	EF627452	–
Ramichloridium punctatum	CBS 132090	JQ622086	JQ622111
Uwebraunia australiensis	CBS 120729	EF394854	–
Uwebraunia commune	CPC 18963	JQ622085	JQ622110
Uwebraunia commune	CPC 18971	AY598876	JQ622118
Uwebraunia dekkeri	CPC 18964	FJ425204	JQ622121

Numbers in bold type are those of the species identified in this study.

the posterior probabilities. The tree was viewed in FigTree v1.4 [29].

RESULTS

Phylogenetic analysis

The dataset comprised 20 taxa representing four genera and 13 species in the family *Dissoconiaceae*, with *Dothiora ceratoniae* CBS 477.69 as the outgroup. All published DNA sequences were obtained from the GenBank from the relevant studies [7, 8]. The final alignment comprised a total of 854 base pairs, containing the ITS and *tef1-α* sequences, which were analyzed by a Bayesian method. The topology of the tree is shown with the Bayesian posterior probabilities for the main clades (Fig. 1). In this tree, our strain is close to *R. luteum* (CPC 18961 and CPC 18962), and identities of ITS and TEF between our strain and *R. luteum* CPC 18961 are 98 and 78% respectively. Combined with morphological differences, we determined that our strain represented a novel species of the genus *Ramichloridium*. Consistent with previous phylogenetic trees which were reconstructed based on sequences of a single gene fragment (ITS or LSU rRNA), our analyses based on the two genes combined also support the type species of the genus *Ramichloridium* as belonging to the family *Dissoconiaceae*.

DESCRIPTION OF *RAMICHLORIDIUM ENDOPHYTICUM* SP. NOV.

Ramichloridium endophyticum (en.do.phy´ti.cum. N.L. neut. adj. *endophyticum* within plant, endophytic, pertaining to the original isolation from surface-sterilized aquatic plants) Fig. 2. MycoBank no. MB 831164.

Mycelium partly superficial or partly immersed in culture, consisting of branched, septate, smooth, hyaline to pale brown, thin-walled, straight to sinuous or geniculate–sinuous, 1.5–2 µm diameter hyphae. Conidiophores macronematous, mononematous, erect, straight to flexuous, unbranched, subcylindrical to somewhat attenuated toward the slightly sinuate apex, somewhat swollen at the base 1–4-septate, pale to usually medium brown, smooth, thick-walled, 30–70×2.0–2.5 µm, arising at right angles from assimilative hyphae. Conidiogenous cells polyblastic, integrated, terminal, subcylindrical, pale to medium brown, thick-walled, smooth, 7.0–42.0×1.5–2.5 µm; sympodially extended, forming a straight or subgeniculate rachis with tiny denticle-like loci; denticles crowded near the apex, very pale brown, 0.5–1 µm diameter. Conidia solitary, obovoid, ellipsoidal-obovoid, occasionally subglobose, unicellular, guttulate, subhyaline to pale brown, verruculose, 4–6.5(–7)×2.0–3.5 µm, apex obtuse, base truncate to somewhat attenuated, basal hilum rather inconspicuous to subconspicuous by being somewhat darkened-refractive, about 0.5 µm diameter.

Cultural characteristics: Colonies attain 3.0 cm diameter on PDA and 3.5 cm on CMA at 25 °C after 15 days. On PDA, colonies are flat, raised, dense, velvety, with olivaceous-grey surfaces, iron-grey on the reverse, with entire margins. On CMA, colonies are surface erumpent, with sparse aerial mycelia, lobed margins; olivaceous-grey surfaces, iron-grey on the reverse, sporulation is abundant.

Distribution: The Duoqing lake, Tibet, PR China.

Specimens examined: PR China, Tibet, Yadong County, 28°12′38.03″N, 89°23′18.36″E, 4180 m altitude, isolated from *Potamogeton pectinatus* L. as an endophyte, July 2018, Z.F. Yu (Holotype, YMFT 1.05584, culture of YMF 1.05584 preserved by lyophilization at the State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan.) (ex-holotype living culture: YMF 1.05584; CGMCC3.19629). GenBank accession numbers: ITS, MK836099; *tef1-α,* MN307070; LSU, MK836098.

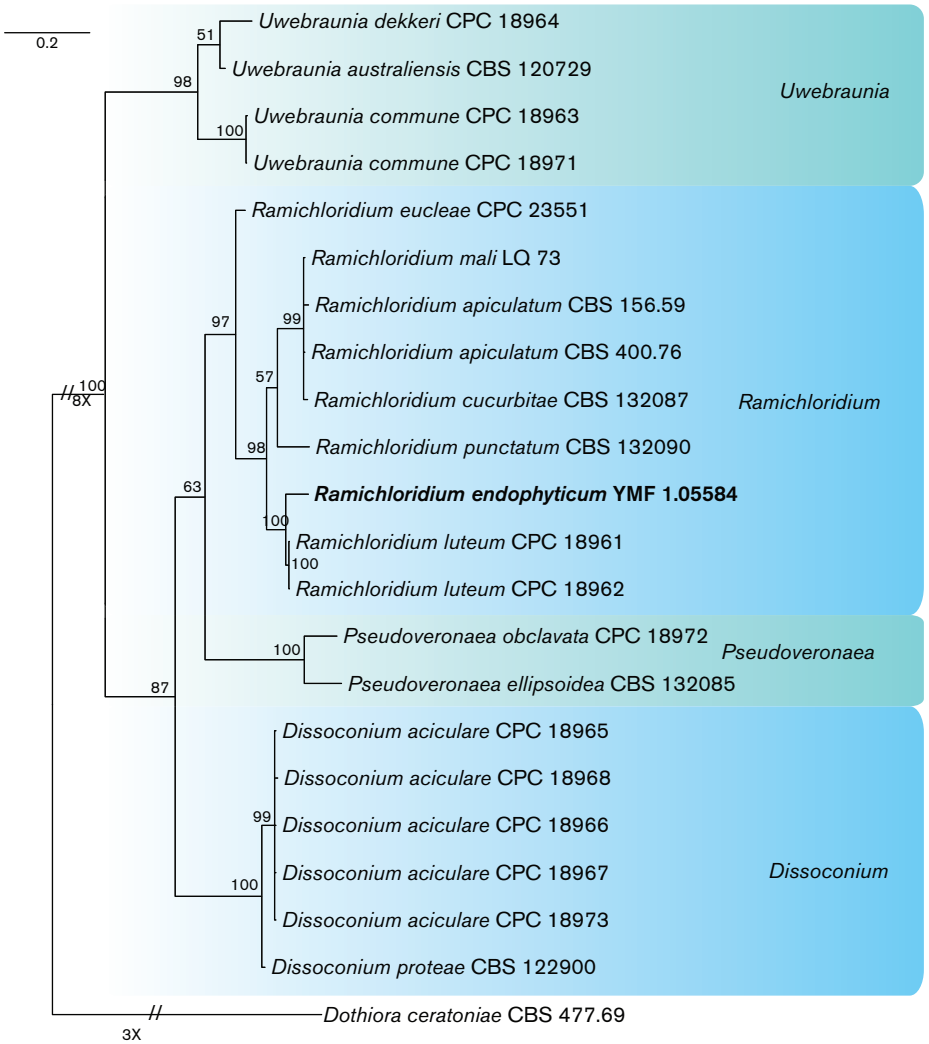

Fig. 1. Phylogenetic tree derived from Bayesian analysis based on a sequence combined data set of ITS+*tef1*-α, depicting the relationships of the novel species *Ramichloridium endophyticum* with closely related taxa. *Dothiora ceratoniae* CBS 477.69 was used as an outgroup.

DISCUSSION

On the basis of its aseptate, brown conidia, polyblastic terminal conidiogenous cells with numerous minute denticles and unbranched conidiophores arising from internal hyphae or swollen vegetative cells, the isolate from China can be readily assigned to the genus *Ramichloridium* [12] it is described as representing a novel species on the basis of the results of the combined morphological and molecular phylogenetic analysis. Morphologically, *R. endophyticum* resembles *R. cladoniicola* U.Braun and Heuchert in the conidiophores but has much longer conidiogenous cells [30]. Additionally, conidia of *R. endophyticum* are similar to those of *R. apiculatum* (J. H. Mill *et al.*) de Hoog, the type species of the genus *Ramichloridium*, and lichenicolous *R. tropicum* U.Braun and Diederich [31]. The differences are presented in Table 2. *Ramichloridium luteum* G.Y. Sun *et al.* is phylogenetically close to *R. endophyticum*, but has larger conidia [8]. Other species such as *R. cucurbitae* D. Mayfield, Batzer and Crous differ from *R. endophyticum* by having 0–3-septate conidiophores and some conidiophores reduced to intercalary conidiogenous cells [8].

Species of the genus *Ramichloridium* show various morphological characteristics and lifestyles, such as being plant pathogens, commensals and saprophytes [12]. The type species of the genus *Ramichloridium*, *R. apiculatum*, was originally found in heavily deteriorated materials, in soil, and as a culture contaminant [11]. Several other species of the genus, such as *R. cucurbitae* and *R. punctatum*, have been isolated from plants as pathogens, which commonly cause Sooty blotch and flyspeck (SBFS) worldwide [8, 32, 33]. In addition, two lichenicolous species, *R. cladoniicola* and *R. tropicum*, were included in the genus *Ramichloridium*. However, *R. endophyticum* was obtained as an endophyte from an aquatic macrophyte *P. pectinatus* in this study. *R. endophytica* is, to our knowledge, the first endophytic

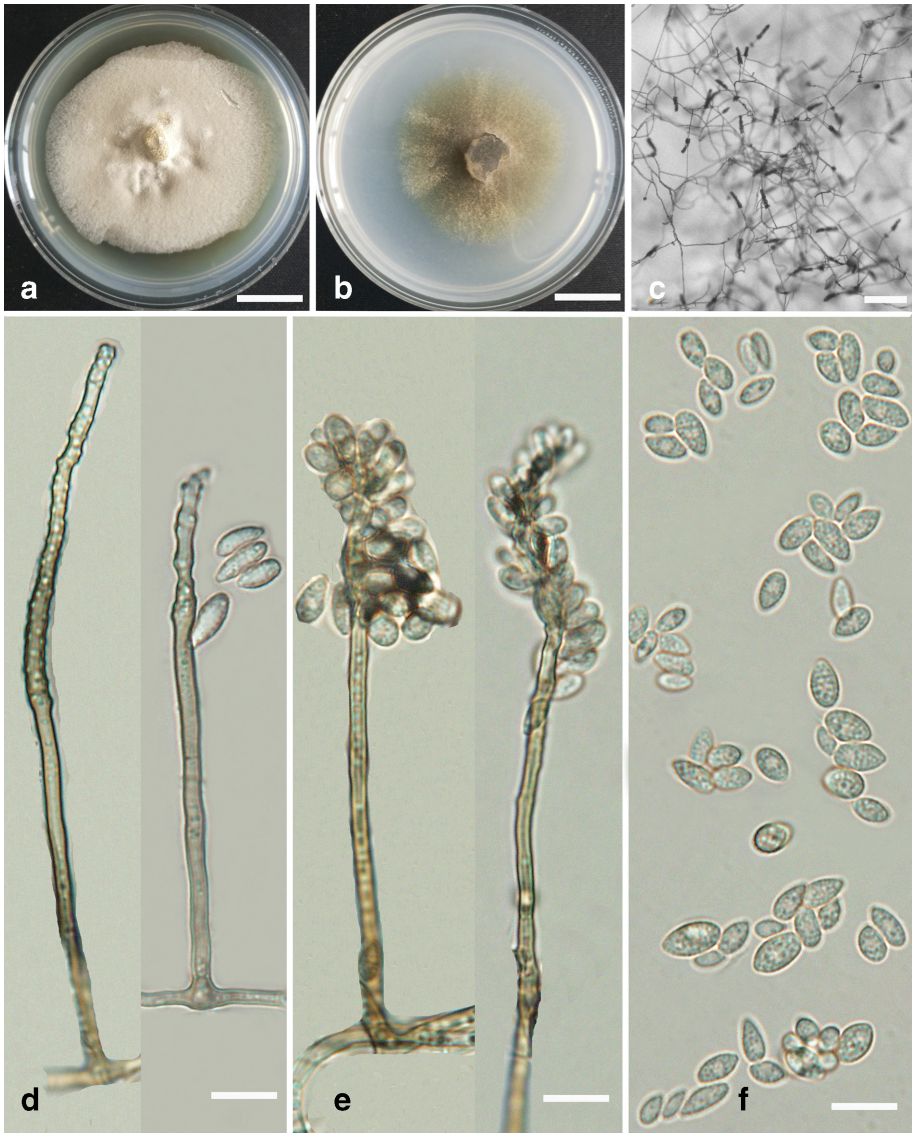

Fig. 2. *Ramichloridium endophyticum* (YMFT 1.05584, holotype), (a–b) Cultures (a on PDA, b on OA.); (c) Conidiophores and conidia; (d) Conidiophores and conidiogenous cells; (e) Conidiophores and conidia; (f) Conidia; Bars: a–b 1.35 cm, c 50 µm, d–e 10 µm.

species of the genus *Ramichloridium*, and thus, it expands the habits of the genus *Ramichloridium*. Some pathogenic fungi are known to be capable of living in plant hosts as commensal endophytes [34, 35], but many endophytic fungi play important roles in plants, increasing the fitness of plant hosts by enhancing their resistance to abiotic and biotic stresses [36, 37]. Further studies are needed in order to understand the ecological role of *R. endophyticum*.

The genus *Ramichloridium* presently accommodates a wide range of species with erect, dark, more or less differentiated, branched or unbranched conidiophores and predominantly aseptate conidia produced on a sympodially proliferating rachis [11]. As a heterogeneous group, species of the genus *Ramichloridium* have been closely associated with some genera, such as *Rhinocladiella* and *Veronaea*. However, the species concepts and generic limits of these related genera were not satisfactorily resolved at first. Later, Arzanlou *et al*. first revised *Ramichloridium* and allied genera using morphological and cultural features in combination with DNA sequence data, and demonstrated that only species clustered in the order *Capnodiales* were true species of the genus *Ramichloridium*, while *Ramichloridium*-like species were segregated into different genera, including *Rhinocladiella* (order *Chaetothyriales*), *Pleurothecium* (order *Chaetothyriales*), *Myrmecridium* (class *Sordariomycetes*) and *Radulidium* (incertae sedis) [11]. Subsequently, the family *Dissoconiaceae* was established by Crous *et al*. to accommodate species of the genus *Dissoconium* and *R. apiculatum* [7], as an early diverging lineage to the families *Mycosphaerellaceae* and *Schizothyriaceae*. Li *et al*. then

Table 2. Morphological characteristics of *Ramichloridium endophyticum* compared with similar species of the genus *Ramichloridium*

Species	Conidiophores (µm)	Conidiogenous cells (µm)	Conidia (µm)	Literature cited
R. apiculatum	1–3-septate, up to 100	25–37(–47)×2–3.5	obovate to obconical, (3–)5–5.5(–7.5)×(2–)2.5–3(–4)	[12]
R. cladoniicola	(0 –)1–4-septate, occasionally with nodulose swellings, 10–70×2–5	10–25 µm long	broadly obovoid, occasionally subglobose, 2–5(–7)×2–3.5(– 4)	[30]
R. cucurbitae	0–3-septate or reduced to intercalary conidiogenous cells, 3–90×2–3	3–50×1.5–2.5	clavate, apex obtuse, (4–)5–6(–7)×(2–)2.5–3(–3.5)	[8]
R. endophyticum	1–4-septate, 30–71×2.0–2.5	7.0–42.3×1.5–2.5	obovoid, ellipsoid-obovoid, occasionally subglobose, 4–6.5(–7.0)×2.0–3.5	in this study
R. luteum	1–3-septate, 25–80×2–3	15–30×2–3	oblong to ellipsoid, (6–)7–10(–13)×(2–)3–4(–4.5)	[8]
R. tropicum	4–9-septate, 60–180×1.5–4	15–40 µm long	obovoid, ellipsoid-obovoid, occasionally subglobose, smooth, 3.5–7×2.5–4	[31]

proposed three novel species of the genus *Ramichloridium* and a new combination, all clustered with *R. apiculatum* in the family *Dissoconiaceae* [8]. Despite these efforts, recent studies still showed that the genus *Ramichloridium* was a paraphyletic and polyphyletic genus and indicated that more molecular and morphological data are needed to clarify the taxonomy of this genus. Indeed, a few *Ramichloridium*-like species clustered in the families *Mycosphaerellaceae* and *Teratosphaeriaceae* were recently transferred to other existing genera (e.g. *Zasmidium*), or used to establish new genera (e.g. *Pachyramichloridium*) [7, 8, 12, 38, 39]. The novel species of the genus *Ramichloridium* described in our study clustered in the same clade as the type species of the genus *Ramichloridium, R. apiculatum,* well within the family *Dissoconiaceae*. Our study enriches the ecology and diversity of the genus *Ramichloridium* and helps delineate its taxonomy.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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