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The Introduction of Two New Species of Aquatic Fungi from Anzali Lagoon, Northern Iran

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Abstract: During a survey of aquatic fungi from Anzali Lagoon in Iran, several fungal specimens were isolated from freshwater habitats. Morphological evidence and comparing sequencing based on rDNA (ITS and LSU) and protein-coding genes (*TEF1* and *TUB2*) showed that some isolates belong to undescribed fungal species. These isolates belong to *Arthrobotrys* and *Sarocladium*, two ascomycetes genera. *Arthrobotrys hyrcanus*, sp. nov., differs from closely related species such as *A. dianchiensis* by its larger conidia and septation of primary conidia. *Sarocladium pseudokiliense*, sp. nov., was similar to *S. kiliense*, but distinguished by its conidial shape and the absence of adelophialides and chlamydospores. Morphological descriptions, illustrations and multilocus phylogenetic analysis for both new species are provided.

Keywords: *Arthrobotrys*; Ascomycota; freshwater fungi; molecular phylogeny; morphological; *Sarocladium*; taxonomy

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1. Introduction

The ecological contributions of fungi in different aquatic ecosystems have been known for a few decades [1,2]. Similar to their terrestrial counterparts, aquatic fungi are greatly involved in the degradation of lignocellulosic materials entering water bodies [3]. Additionally, their involvement in the production and transformation of humic substances has been reported, highlighting their significance regarding the sustainability of aquatic geochemical cycles [4]. They also impact microbial communities and, accordingly, food web dynamics, via their various lifestyles, such as parasitism and saprophytism [5,6]. Despite these contributions, the diversity of aquatic fungi has not received enough attention.

Our knowledge of the diversity of fungi dramatically changed after the implementation of molecular tools to infer the taxonomy of fungal species [7]. Currently, the multigene phylogeny approach is the foundation of the phylogenetic classification of fungi [8], which has either confirmed, revised, or rejected the classic taxonomy of many fungal taxa inferred from morpho- and eco-physiological features [9]. However, most studies have focused on terrestrial taxa (approximately 120,000 known species), leaving the diversity of aquatic fungi to a large extent unknown. To date, only 3000 fungal species have been described as present in aquatic habitats [10]. This lack of knowledge has been confirmed by environmental DNA sequencing, which reveals an unexpectedly large diversity of undescribed fungi [11]. Nevertheless, although culture-independent methods explore unknown fungal lineages, culture-dependent methods are still required to isolate and provide a detailed taxonomical description of novel fungal species [12].

The genera of *Arthrobotrys* and *Sarocladium* have been frequently isolated from various habitats, including freshwater ecosystems [13–16], and shown to have various ecological functions. For instance, most reported *Arthrobotrys* species are aquatic nematode-trapping, nematode egg-parasitic, and nematode endo-parasitic fungi [17–19] and saprotrophs on dead wood and bark [20]. Nevertheless, generally, the number of described fungal species in freshwater ecosystems is significantly lower than in terrestrial ones [21]. The same is true of some *Sarocladium* species, as they are considered an integral part of the aquatic microbial community and included in biogeochemical cycling models of upwelling ecosystems [14]. Despite their importance, attempts to isolate aquatic *Sarocladium* strains have been limited to a few studies [4,22]. Therefore, more taxonomic-based studies are needed to describe unknown *Arthrobotrys* and *Sarocladium* strains from aquatic ecosystems, which will allow scholars to study their ecological functions more accurately and understand how they interact with other components of aquatic nutrient cycling.

In this paper, we aim to introduce two new species based on collections of *Arthrobotrys* and *Sarocladium* from Anzali Lagoon, Iran. We used both morphometric and multi-gene phylogenetic analyses to assign strains to their corresponding taxon. Our results are a matter of great importance, as they enrich our knowledge of fungal diversity in freshwater ecosystems. Indeed, providing accurate taxonomic descriptions of novel fungal taxa will pave the way to understanding their exact ecological contributions in aquatic environments.

2. Materials and Methods

2.1. Sampling and Cultivation

Samples of plant debris floating on the water's surface were collected from the shoreline of Anzali Lagoon (located in Guilan, Iran) and then transferred to the lab. We incubated plant materials (cut in small pieces) in sealed Petri dishes at 25 °C under light condition. A stereomicroscope (Analyth STR Bino, Bresser, Germany) was used to observe fruiting bodies and/or mycelia every three days for four weeks [23] (Petri dishes were kept moist by putting a water-treated piece of sterilized cotton inside). Pure cultures of strains were obtained by transferring fruiting bodies and/or mycelia to malt extract agar (MEA) [24] using the hyphal-tip technique. Fungal strains are available at the Culture Collection of the Iranian Research Institute of Plant Protection (IRAN C), Tehran, Iran.

Thirty measurements were taken of the morphometric characteristics of strains and averages were used in each species description. An Olympus BH-2 microscope (Olympus Optical, Tokyo, Japan) equipped with an AM4023-Digital Microscope 1.3 MPixel 72.5 30-USB 2.0 (Dino-Lite, Taiwan) was used for all measurements and observations. Potato dextrose agar (PDA), carnation leaf agar (CLA), synthetic nutrient agar (SNA), oatmeal agar (OA), and malt extract agar (MEA) were prepared according to the manufacturers' instructions [25–27]. Morphological identification was performed based on Yu et al. [28] and Zhang et al. [16] for *Arthrobotrys* isolates, and Giraldo et al. [29] for *Sarocladium* isolate.

2.2. DNA Extraction, PCR and Sequencing

DNA extraction was conducted according to the protocol of Montero-Pau et al. [30] with some minor modifications. Each strain's purified 7–15 days old mycelia were transferred to 1.5 mL tubes containing 100 µL of alkaline lysis buffer, centrifuged for 30 min. at 9000 rpm, incubated at 95 °C for 30 min., and cooled on ice for 5 min. Finally, 100 µL of neutralizing solution was poured into the tubes (for alkaline lysis buffer and neutralizing solution, see [30]). The final solution was vortexed and stored at −20 °C. Five partial sequences including: a large subunit (LSU), internal transcribed spacer (ITS), and small subunit (SSU) of rDNA, β -tubulin (*TUB2*) and translation elongation factor 1-a (*TEF1*) were amplified in a Flexibler PCR Thermocycler (Analytik Jena AG, Jena, Germany) using LR0R/LR5 [31], ITS1/ITS4 [32], SSU817/SSU1536 [33], Btub2Fd/Btub4Rd [34], and EF1-983F/EF1156R [35] primers, respectively, and sent to Macrogen, Inc. (Amsterdam, The Netherlands) for sequencing. The resulting sequences were edited using BioEdit Ver. 7.0.5 software [36] and submitted to GenBank.

2.3. Phylogenetic Analyses

For the phylogenetic placement of the *Arthrobotrys* species included in our analyses, a representative ITS–LSU matrix including 58 members of family of Orbiliaceae was produced, with *Vermispora fusarina* selected as the outgroup. For *Sarocladium* species' phylogenetic placement, a combined matrix of three loci (ITS–LSU, rDNA, and *ACT1*) of 27 species was produced for phylogenetic analysis, with *Kiflimonium curvulum* selected as the outgroup. As a few LSU sequences are available for *Arthrobotrys* species, and *TEF1* and *TUB2* are available for *Sarocladium* species, these markers were not included in the matrix, but the sequences were deposited at GenBank. All alignments were produced with the server version of MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft), then checked and refined using MEGA7 [37,38]. After the exclusion of ambiguously aligned regions and long gaps, the final matrix for *Arthrobotrys* and *Sarocladium* species contained 1310 and 2145 nucleotide characters, respectively; i.e., 689 from ITS and 621 from *TEF1* in *Arthrobotrys*, and 569 from ITS, 809 from LSU and 767 from *ACT1* in *Sarocladium*. Tables 1 and 2 represent valid sequences used in these phylogenetic analyses. Maximum likelihood (ML) and maximum parsimony (MP) analyses were performed as described in Stamatakis [39], Silvestro et al. [40], and Swofford [41].

Table 1. Strain codes and GenBank accession numbers of sequences used in the phylogenetic analyses of Orbiliaceae. Isolates/sequences in bold were isolated/sequenced in the present study. N/A: not available.

Species	Strain Code	GenBank Accession Number		Reference
		ITS	*TEF1*	
Arthrobotrys				
A. anomalus	YNWS02-5-1	AY773451	AY773393	[42]
A. conoides	670	AY773455	AY773397	[42]
A. eudermatus	SDT24	AY773465	HE608633	[42]
Arthrobotrys hyrcanus	**IRAN 3650C**	**MH367058**	OP351540	**This study**
Arthrobotrys hyrcanus	**IRAN 3651C**	**MH367063**	OP351541	**This study**
A. iridis	521	AY773452	AY773394	[42]
A. janus	85-1	AY773459	AY773401	[42]
A. multiformis	CBS 773.84	MH861834	N/A	[43]
A. musiformis	SQ77-1	AY773469	AY773411	[42]
A. oligospora	920	AY773462	AY773404	[42]
A.pseudoclavatus	1130	AY773446	AY773388	[42]
A. pyriformis	YNWS02-3-1	AY773450	AY773392	[42]
A. sinensis	105-1	AY773445	AY773387	[42]
A. sphaeroides	SDT24	AY773465	AY773407	[42]
A. thaumasius	917	AY773461	AY773403	[42]
A. vermicola	629	AY773454	AY773396	[42]
Dactylellina				
D. appendiculata	CBS 206.64	AF106531	DQ358227	[44]
D. arcuata	CBS 174.89	AF106527	DQ999852	[45]
D. cionopaga	SQ60-2	AY773468	AY773410	[42]
D. copepodii	CBS 487.90	U51964	DQ999835	[42,46]
D. drechsleri	CBS 549.63	DQ999819	DQ999840	[42]
D. ellipsospora	286	AY773449	AY773391	[42]
D. entomopaga	CBS 642.80	AY965758	DQ358228	[44,47]
D. gephyropaga	CBS 585.91	AY965756	DQ999846	[42]
D. haptospora	CBS 100520	DQ999820	DQ999850	[42]
D. haptotyla	SQ95-2	AY773470	AY773412	[42]
D. leptospora	SHY6-1	AY773466	AY773408	[42]
D. mammillata	CBS 229.54	AY902794	DQ999843	[42,48]
D. parvicollis	XJ03-52-1	AY773472	AY773414	[42]

Table 1. *Cont.*

Species	Strain Code	GenBank Accession Number		Reference
		ITS	***TEF1***	
D. querci	6175	AY773453	AY773395	[42]
D. robusta	CBS 110125	DQ999821	DQ999851	[42]
D. tibetensis	XZ04-92-1	DQ999833	DQ999848	[42]
Drechslerella brochopaga	701	AY773456	AY773398	[42]
Drechslerella coelobrocha	FWY03-25-1	AY773464	AY773406	[42]
Drechslerella dactyloides	expo-5	AY773463	AY773405	[42]
Drechslerella stenobrocha	YNWS02-9-1	AY773460	AY773402	[42]
Vermispora fusarina	YXJ13-5	AY773447	AY773389	[42]

Table 2. Strain codes and GenBank accession numbers of sequences used in the phylogenetic analyses of *Sarocladium*, spp. Type specimens are labeled with HT (holotype). Isolates/sequences in bold were isolated/sequenced in the present study. N/A: not available.

Species	Strain Code	Status	GenBank Accession Number			Reference
			ITS	**LSU**	***ACT1***	
Kiflimonium curvulum	CBS 430.66	HT	HE608638	HE608656	HE608630	[49]
Sarocladium bacillisporum	CBS 425.67	HT	HE608639	HE608658	HE608633	[49]
S. bactrocephalum	CBS 749.69	HT	HG965006	HQ231994	HG964956	[29,50]
S. bifurcatum	UTHSC 05-3311	HT	HG965009	HG965057	HG964959	[29]
S.brachiariae	CGMCC 2192	HT	EU880834	KP715271	N/A	[51]
S.clematidis	MFLU 17–1507	HT	MN629287	MN629285	N/A	[52]
S.dejongiae	CBS 144929	HT	MK069419	MK069415	N/A	[53]
S.gamsii	CBS 707.73	HT	HG965015	HG965063	HG964965	[29]
S.glaucum	CBS 796.69	HT	FN691454	HE608657	HE608631	[49,54]
S.graminicola	CML 4052	HT	MK017855	MK017871	MK017838	[55]
S.hominis	UTHSC04-1034	HT	HG965012	HG965060	HG964962	[29]
S.implicatum	CBS 959.72	HT	HG965023	HG965072	HG964974	[29]
S.junci	CBS 148277	HT	OK664734	OK663773	OK651128	[56]
S.kiliense	CBS 122.29	HT	FN691446	HQ232052	HG964975	[29,50,54]
S.liquanense	ACC 39306	HT	MF987659	MF987651	MF987663	[57]
S.mali	ACC 39308	HT	MF987662	MF987653	MF987665	[57]
S.ochraceum	CBS 428.67		HG965025	HQ232070	HG964977	[29,50]
S. pseudokiliense	**IRAN 3649C**	**HT**	**MH367052**	**MH367070**	N/A	**This study**
S.pseudostrictum	UTHSC02-1892	HT	HG965029	HG965073	HG964981	[29]
S.sasijaorum	CBS 147213	HT	MW883448	MW883839	MW890032	[58]
S.spinificis	BCRCFU30127		KF269096	JQ954463	N/A	[59]
S.spirale	BCRCFU31117	HT	LC461491	LC464181	LC464350	[60]
S.strictum	CBS 346.70	HT	FN691453	HQ232141	HG964982	[29,50,54]
S.subulatum	MUCL 9939	HT	HG965031	HG965075	HG964984	[29]
S.summerbellii	CBS 430.70	HT	HG965034	HG965078	HG964987	[29]
S.terricola	CBS 243.59	HT	FN706553	HE608659	HE608632	[29,49,54]
S.zeae	CBS 800.69	HT	FN691451	HQ232152	HG965000	[29,50,54]

3. Results

3.1. Molecular Phylogeny

In *Arthrobotrys*, of the 1310 characters of the combined matrix, 479 were parsimony informative (259 in ITS and 220 in *TEF1*). The phylogram of the best ML tree (lnL = −14,085.2515) obtained by RAxML is shown as Figure 1. The MP analysis revealed a single tree of length 3002 (not shown) that had a similar topology to the ML tree. *Arthrobotrys* species' supported nodes were consistent between the ML and MP analyses, but topologies of *Dactylellina*

species differed in the MP tree; as these differences are not relevant within the context of our new species, they are not further considered here.

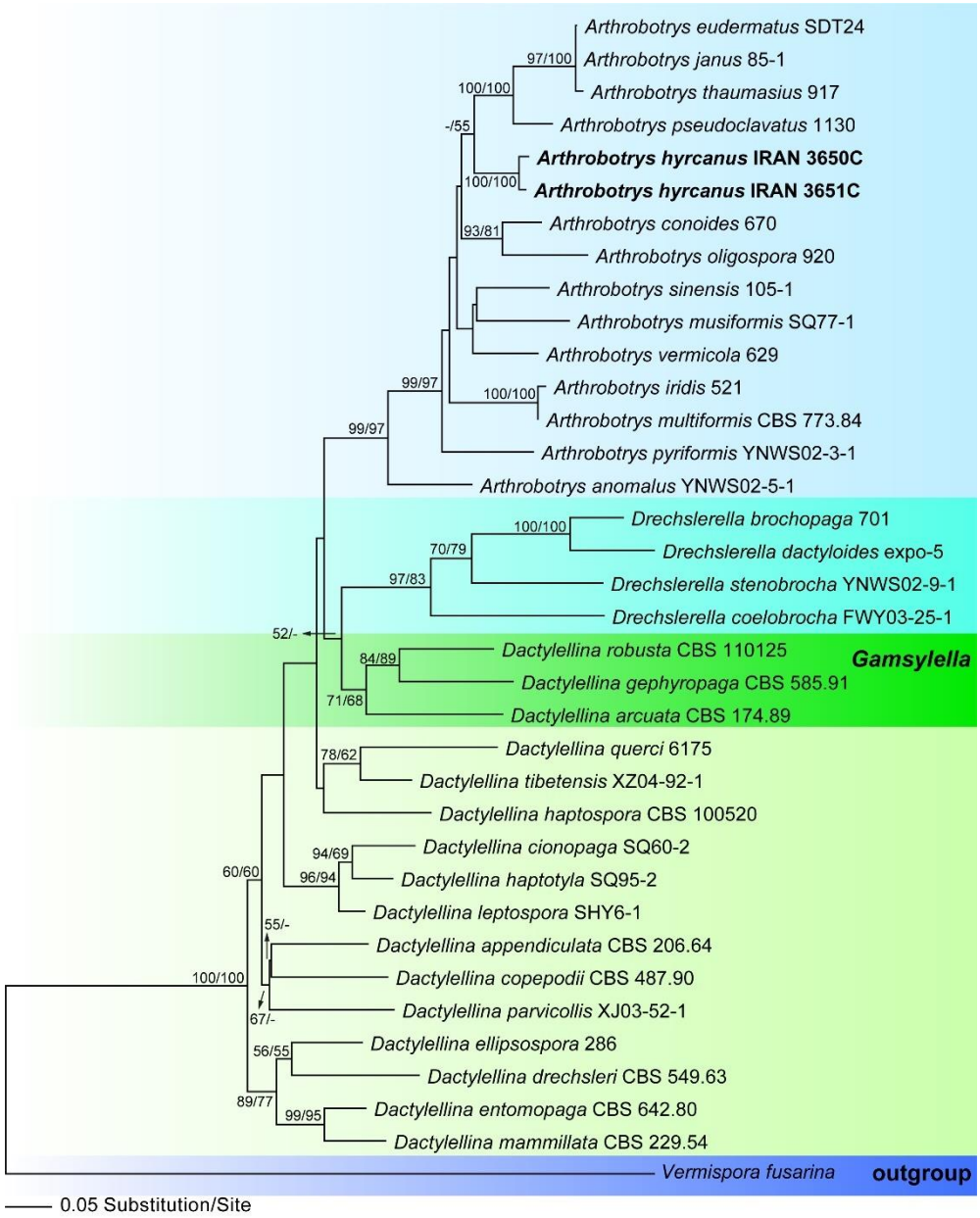

Figure 1. Phylogram of the best ML trees (lnL = −14,085.2515) revealed by RAxML from an analysis of the combined ITS–*TEF1* matrix of selected Orbiliaceae. Strains in bold were sequenced in the current study. ML and MP bootstrap supports above 50% were given at the first and second positions, respectively, above or below the branches.

Arthrobotrys hyrcanus, sp. nov., is highly supported in both ML and MP (100%) analyses and strongly separated from other *Arthrobotrys* species in the tree.

In *Sarocladium*, of the 2145 characters of the combined matrix, 325 were parsimony informative (132 in ITS, 52 in LSU and 141 in *ACT1*). The MP analyses resulted in a single MP tree of 1644 steps (CI = 0.613, RI = 0.604, and RC = 0.387), which is shown in Figure 2. Tree topology of the best tree revealed by the ML analyses was identical to that of the MP tree (not shown). The new species of *Sarocladium pseudokiliense* clustered together with *S. kiliense* with 70% and 60% BS support in MP and ML analyses, respectively.

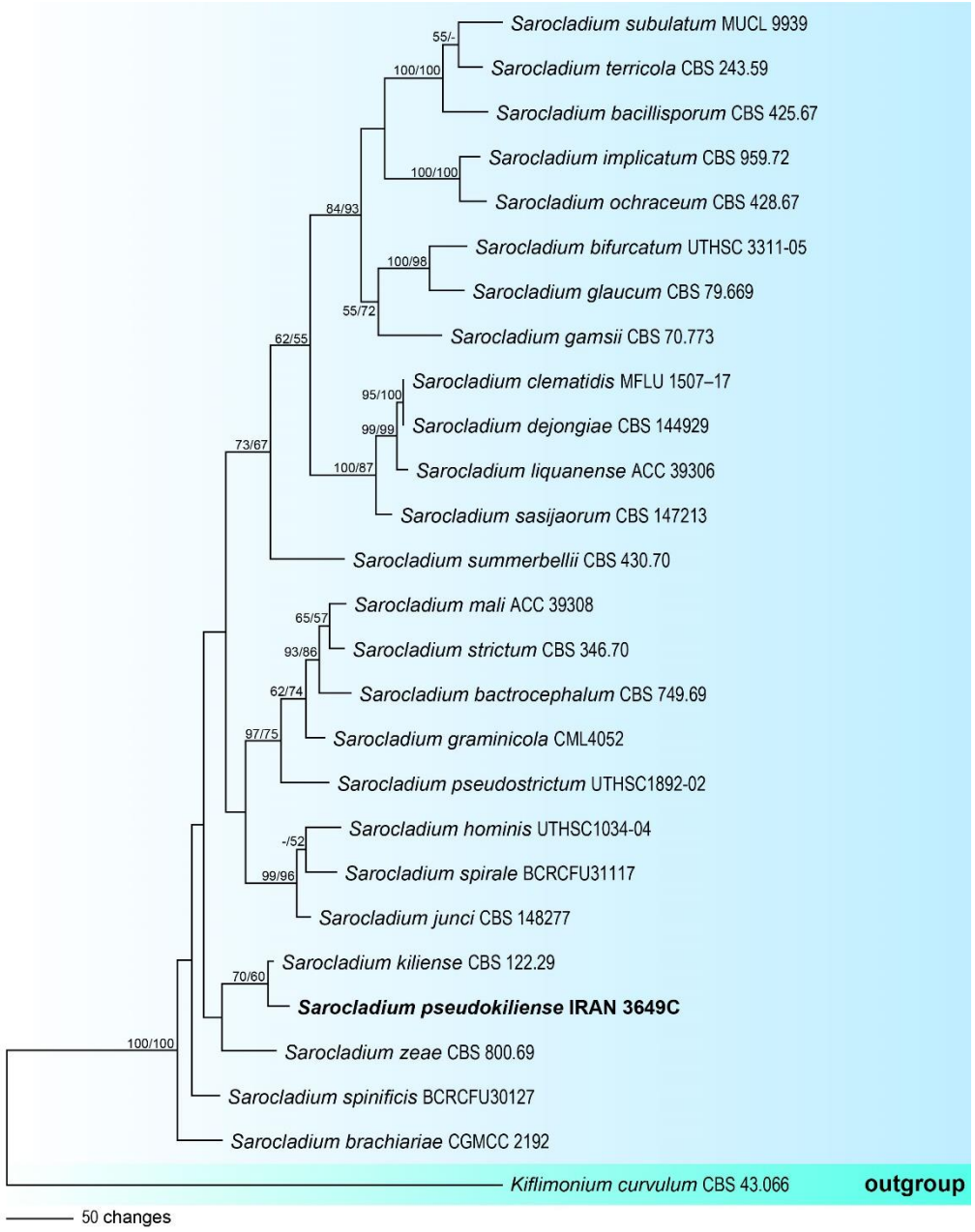

Figure 2. Phylogram showing the single most parsimonious tree revealed by an analysis of the combined ITS–LSU–*ACT1* matrix of selected *Sarocladium*, spp. Values above or below the branches indicate maximum parsimony and maximum likelihood bootstrap support, respectively. Tree statistics: tree length = 1644, consistency index = 0.613, retention index = 0.604, and rescaled consistency index = 0.387.

3.2. Taxonomy

Based on these results, we concluded that our isolates belong to two unknown species, which are described below.

3.2.1. *Arthrobotrys hyrcanus*, sp. nov., Masigol, Rezakhani, Pourmoghaddam, Khodaparast (Figure 3)

- **MycoBank No:** 845353
- **Etymology:** *hyrcanus* derived from "Hyrcania", an ancient biogeographical region located in the south of the Caspian Sea where the specimens were collected.

- **Holotype:** Iran, Guilan Province, Anzali County, Anzali Lagoon, 37°28′16″ N, 49°27′44″ E, on rotten leaves, 11 August 2017, F. Rezakhani, (GUM 1904, ex-holotype culture IRAN 3650C); ITS, LSU, and *TEF1* sequences GenBank MH367058, MH367076, and OP351540, respectively.
- Mycelium hyaline, scanty, hyphae septate, branched, 1.5–3 µm wide. Conidiophores growing from mycelium on the substratum, single, erect, 2–5-septate, 70–312 µm long, 5–6 µm wide, bearing a single conidium at the apex. Conidia hyaline, clavate or spindle-shaped, narrowing at the basal, 2–9-septate, rarely 9-septate, 44.2–135.2 × 10–14.4 µm. The proportion of conidia with 2, 3, 4, 5, 6, 7, and 8 septa is 7, 10, 18, 22, 12, 19, and 11%, respectively. Some conidia had small tubercles at the one or both ends and could germinate from these tubercles. Conidia could produce secondary conidiophores and secondary conidia. The secondary conidia are clavate, 20.8–33.8 × 2.6–4.8 µm and 1-septate. Chlamydospores present in cultures after 3 wk.
- **Culture characteristics:** Colonies on CMA whitish, rapidly growing and extending to a diameter of 9 cm at 25 °C within 7 days.
- **Other specimen examined:** Iran, Guilan Province, Anzali County, Anzali Lagoon, 37°28′16″ N, 49°27′44″ E, on rotten leaves, 11 Aug 2017, F. Rezakhani (IRAN 3651C); ITS, LSU and *TEF1* sequences GenBank MH367063, MH367081, and OP351541, respectively.
- **Notes:** This species is similar to *A. dianchiensis*, but it can be distinguished from the latter by the larger primary [44.2–135.2 × 10–14.4 vs. 37.5–100 (70) × 10–17.5 (14.3) µm] and secondary (20.8–33.8 × 2.6–4.8 vs. 23.9 × 5 µm) conidia, septation of primary conidia, and the presence of chlamydospores. Table 3 compares morphological characters of some species that may be confused with *A. hyrcanus*.

Table 3. Diagnostic characters of *Arthrobotrys hyrcanus*, sp. nov., and closely related species. Reference: Yu et al. [28] and this study.

Species	Size of Primary Conidia	Number of Primary Conidial Septa	Chlamydospores
Arthrobotrys dianchiensis	37.5–100 (70) × 10–17.5 (14.3)	1–7 (mainly 2–5)	Not mentioned
Arthrobotrys hyrcanus	44.2–135.2 × 10–14.4	2–9	present
Arthrobotrys multiformis	47–198 × 7–20	4–12	present
Arthrobotrys shizishanna	22.5–73.8 (50.6) × 5–10 (6.6)	2–9	present

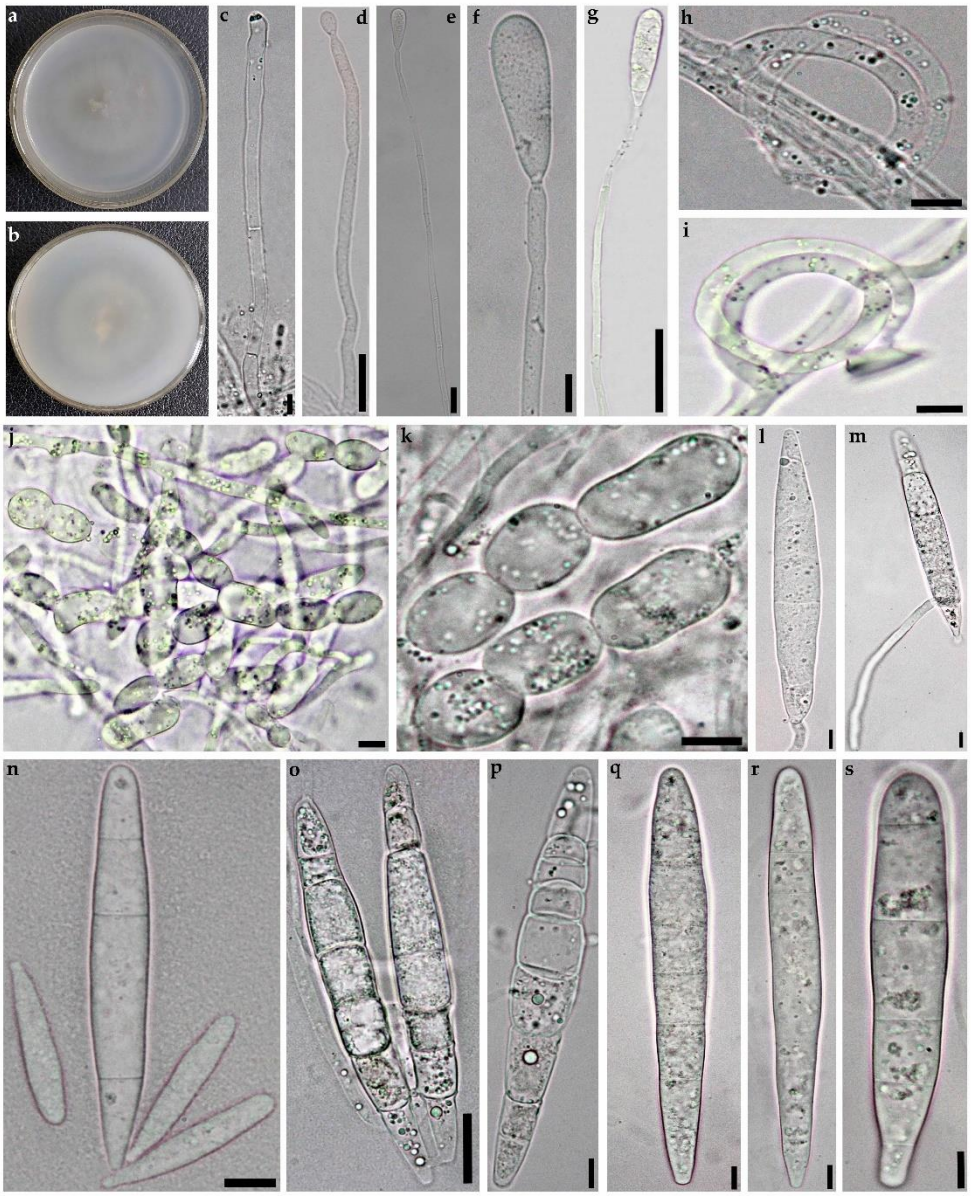

Figure 3. *Arthrobotrys hyrcanus* (IRAN 3650C). (**a**,**b**) colony; (**c**) conidiophore; (**d**–**g**) conidiophore with conidia; (**h**,**i**) adhesive network; (**j**,**k**) chlamydospore; (**l**,**m**) germinating primary conidia; (**n**) primary and secondary conidia; and (**o**–**s**) primary conidia. Scales bars (**c**–**s**) 20 µm.

3.2.2. *Sarocladium pseudokiliense*, sp. nov., Rezakhani, Khodaparast, Masigol, and Grossart (Figure 4)

- **MycoBank No:** 845356
- **Etymology:** *pseudokiliense*, referring to the morphological similarity to *Sarocladium kiliense*.
- **Holotype:** Iran, Guilan Province, Anzali County, Anzali Lagoon, 37°28′16″N, 49°27′44″E, on rotten leaves, 11 September 2017, F. Rezakhani (GUM 1905, ex-holotype culture IRAN 3649C); ITS, LSU, *TEF1* and *TUB2* sequences GenBank MH367070, MH367052, OP351542, and OP351543, respectively.
- Mycelium consisting of hyaline, smooth-walled, branched, septate, 1.5–2.5 µm wide. Conidiophores erect, arising directly from vegetative hyphae or ropes of hyphae, straight, simple, hyaline, smooth-walled, up to 21 µm long. Phialides subcylindrical to acicular, 15–45 µm long, 1.5–2 µm wide at the base, thin- and smooth-walled, hyaline with inconspicuous periclinal thickening; adelophialides and schizophialides not

observed. Conidia solitary, cylindrical, 3–6 × 1–1.5 µm, hyaline, thin- and smooth-walled, arranged in slimy heads. Chlamydospores or sexual morph not observed.

- **Culture characteristics:** Colonies on OA at 25 °C attaining 80–85 mm in 14 d, at first orange with white margin, becoming pink to purple. On PDA at 25 °C attaining 40–45 mm in 14 d, at first orange with white margin, becoming white to cream.
- **Notes:** *Sarocladium* is an acremonium-like genus that contains several important plant and human pathogens [29,56]. The description of this species is based on a single specimen, which shows phylogenetically close to *S. kiliense*, but it can be distinguished from the latter by the shape of conidia (in *S. kiliense* conidia are ellipsoidal to cylindrical), the absence of adelophialides and chlamydospores.

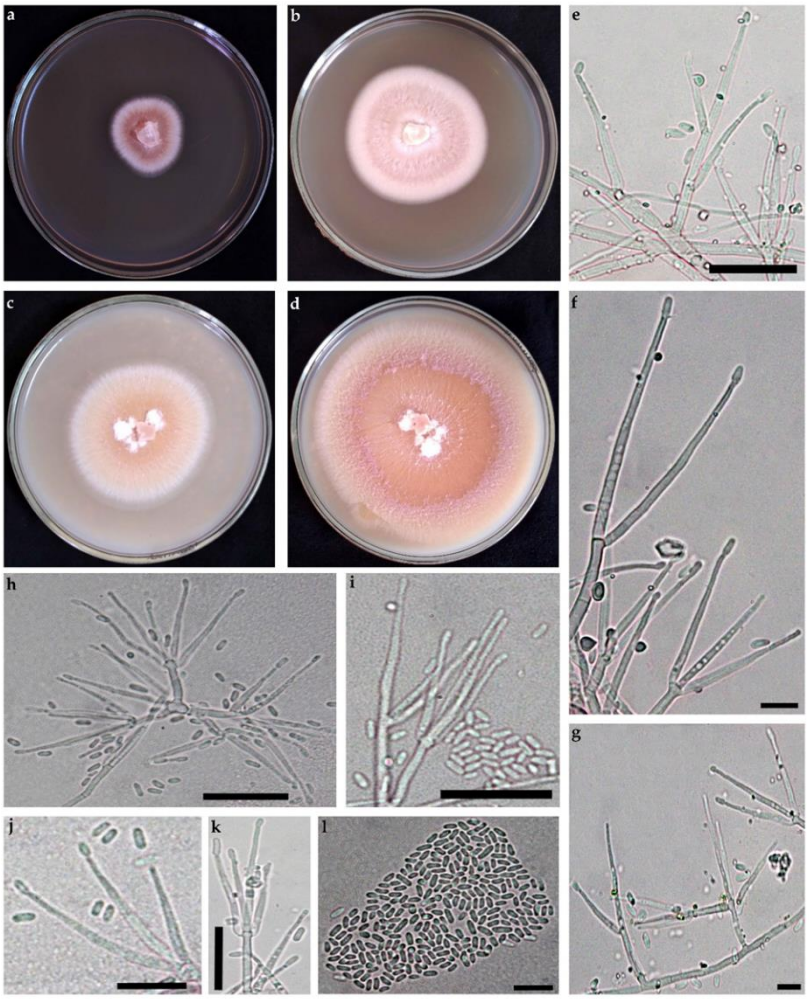

Figure 4. *Sarocladium pseudokiliense* (IRAN 3649C). colony after 7 (**a**) and 14 (**b**) days of incubation on PDA; colony after 7 (**c**) and 14 (**d**) days of incubation on OA; (**e**–**k**) conidiophores, phialides, and conidia; and (**l**) conidia. Scales bars (**e**–**k**) 20 µm and (**j**,**l**) 10 µm.

4. Discussion

Based on the results and morphological and molecular phylogenetic data of this study, we introduce two new species of aquatic fungi from Anzali Lagoon in Iran. In addition to morphological differences, *Arthrobotrys hyrcanus* is phylogenetically distinct from other species. Unfortunately, no reliable type sequence of *A. dianchiensis* was available for comparison to the new species. The second new species, *Sarocladium pseudokiliense*, differs from S. *kiliense* in morphology and is also phylogenetically distinct. Our results are highly significant; compared with their terrestrial counterparts, research regarding aquatic fungal diversity is significantly neglected. Masigol et al. [4] showed that these two new species

are enzymatically active and involved in the degradation of lignocellulolytic materials; this has vital consequences for entire aquatic geochemical cycles. In fact, studying ecological functions of fungal and fungi-like taxa will be more effective when their taxonomy is more accurately described. Indeed, studying the biodiversity of fungal and fungal-like organisms from internationally important Iranian freshwater ecosystems is in its infancy [61–63] and deserves more attention from the scientific community.

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Institutional Review Board Statement: This article does not contain any studies with human participants or animals performed by any of the authors.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding authors on request. Moreover, sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under the accession numbers mentioned in the text.

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Conflicts of Interest: The authors declare no conflict of interest.

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