Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Characterization and phylogeny of *Isaria* spp. strains (Ascomycota: Hypocreales) using ITS1-5.8S-ITS2 and elongation factor 1-alpha sequences

Celeste P. D'Alessandro[1], Leandro R. Jones[2], Richard A. Humber[3], Claudia C. López Lastra[1] and Daniel R. Sosa-Gomez[4]

[1] Centro de Estudios Parasitológicos y de Vectores (CEPAVE) (CONICET-UNLP), Calle 2 No 584, CP 1900, La Plata, Buenos Aires, Argentina
[2] Laboratorio de Virología y Genética Molecular, CONICET – Facultad de Ciencias Naturales sede Trelew, Universidad Nacional de la Patagonia San Juan Bosco, Chubut, Argentina
[3] USDA-ARS, Biological Integrated Pest Management, Robert W. Holley Center for Agriculture and Health, Tower Road, Ithaca, NY, USA
[4] Embrapa Soybean Research Center, Londrina, Paraná State, Brazil

The elongation factor 1-alpha (EF1-α) and the internal transcribed spacer (ITS) regions ITS1 and ITS2 (ITS1-5.8S-ITS2) sequences were used to characterize and to identify *Isaria* isolates from Argentina, Mexico, and Brazil, as well as to study the phylogenetic relationships among these isolates and other related fungi from the order Hypocreales. The molecular characterization, which was performed by PCR-RFLP of EF1-α and ITS1-5.8-ITS2 genes, was useful for resolving representative isolates of *Isaria fumosorosea*, *Isaria farinosa*, and *Isaria tenuipes* and to confirm the taxonomic identity of fungi from Argentina, Mexico, and Brazil. The phylogenetic analyses showed three clades corresponding to three families of Hypocreales. The genus *Isaria* was confirmed as polyphyletic and in family Cordycipitaceae, *Isaria* species were related to anamorphic species of *Beauveria*, *Lecanicillium*, and *Simplicillium* and to teleomorphic *Cordyceps* and *Torrubiella*. Therefore, EF1-α and ITS1-5.8S-ITS2 genes were found to be powerful tools for improving the characterization, identification, and phylogenetic relationship of the *Isaria* species and other entomopathogenic fungi.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords: Characterization / EF1-α / ITS1-5.8S-ITS2 / *Isaria* spp. / Phylogeny

Received: June 18, 2013; accepted: October 4, 2013

DOI 10.1002/jobm.201300499

Introduction

The entomopathogenic genus *Isaria* is a geographically widespread group of many species that can infect different orders of insects in all stages of development and can be frequently isolated from soil [1, 2]. The genus *Paecilomyces* was established by Bainier [3] based on *Paecilomyces variotii* and characterized by verticillate clusters of conidiophores bearing divergent whorls of phialides with a cylindrical or inflated base tapering to distinctly narrowed neck. The conidia are typically hyaline, one-celled, smooth walled, and produced in basipetal chains. The genus was revised by Samson in 1974, who divided the genus into two sections: Section *Paecilomyces* and Section *Isarioidea*, and placed all entomogenous species in the latter section while the type species, *P. variotii*, and all other species in the section *Paecilomyces* are conidial stages of *Byssochlamys* species (Eurotiomycetes: Eurotiales). Samson's classification of these fungi was based on morphological characteristics, but was often highly subjective and lead to ambiguous identifications at the species level.

Genetic markers, such as ribosomal rRNA gene, internal transcribed spacer (ITS), and β-tubulin gene have been extensively used for the molecular characterizations and phylogenetic studies of entomopathogenic

Correspondence: Dr. Celeste Paola D'Alessandro, Centro de Estudios Parasitológicos y de Vectores (CEPAVE) (CONICET-UNLP), Calle 2 No 584, CP 1900, La Plata, Buenos Aires, Argentina
E-mail: celed1881@yahoo.com.ar
Phone: 054-221-4233471
Fax: 054-221-4232327

fungi [4–13]. In the case of *Paecilomyces*, however, Tigano-Milani et al. [5] analyzed the genetic variability among 27 isolates of *Isaria fumosorosea*, 15 of which were isolated from the whitefly *Bemisia tabaci*; the level of divergence observed suggested that these strains represented a species complex. Fargues et al. [14] reported on the genetic variability among *I. fumosorosea* isolates as a function of geographical and host insect origins. Several phylogenetic studies based on the large and small subunit rRNA genes have already indicated the polyphyletic nature of the genus *Paecilomyces* [15–18]. Luangsa-ard et al. [17] analyzed the phylogenetic relationship of *Paecilomyces* sect. *Isarioidea* species using the β-tubulin and ITS rDNA genes and reported the existence of a monophyletic group designated as the *Isaria* clade, which included the following species: *I. amoenerosea*, *I. cateniannulatus*, *I. cateniobliquus*, *I. cicadae*, *I. farinosa*, *I. fumosorosea*, *I. ghanensis*, *I. javanica*, and *I. tenuipes*. The taxonomic revision of *Paecilomyces* section *Isarioidea* began with the lectotypification and determination of the status of *Isaria* [19], a genus long ago relegated to synonymy with *Paecilomyces*; their proposal for the formal conservation of the generic name *Isaria* with *I. farinosa* as the type species [20] has been officially accepted. Phylogenetic studies based on multiple independent loci dispersed species from *Paecilomyces* section *Isarioidea* among the families Cordycipitaceae, Clavicipitaceae, and Ophiocordycipitaceae of the order Hypocreales [17, 21]. A few species from this section were excluded from both *Paecilomyces* and *Isaria* and still await transfers into appropriate anamorphic genera [21].

The objectives of this study were: (1) to characterize and to determine the taxonomic identity of 33 *Isaria* spp. isolates from Argentina, Mexico, and Brazil using the ITS regions ITS1 and ITS2 (ITS1-5.8S-ITS2) and elongation factor 1-alpha (EF1-α); and (2) to study the phylogenetic relationships among Argentinean, Mexican, and Brazilian isolates of *Isaria* spp. and related fungi from the three clavicipitoid families.

Materials and methods

Fungal cultures

A total of 33 fungal isolates from sites in Argentina (22), Mexico (1), and Brazil (10), and deposited in Mycological Collections of Centro de Estudios Parasitológicos y de Vectores (CEPAVE, CONICET-UNLP, La Plata, Argentina); the Embrapa Soybean Research Center (Londrina, Brazil; [22]); and the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, New York, USA; [23]). These isolates correspond to *I. amoenerosea* P. Henn., *I. farinosa* (Holmsk.) Fries, *I. fumosorosea* Wize, *I. javanica* (Bally) Samson & Hywel-Jones, *I. tenuipes* Peck, *Isaria* sp., and *Purpureocillium lilacinum* (=*Paecilomyces lilacinus*) (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (Table 1).

Isaria isolates were grown on Sabouraud dextrose agar supplemented with 1% yeast extract (SDAY) [24] in Petri dishes (90 mm diameter) at 25 °C for 10 d in darkness. Mycelium for DNA extraction was produced by culturing in liquid SDY medium broth at 100 rpm on a rotary shaker for 3–5 d at 25 °C. Mycelium was filtered through sterile Whatman filter paper, and stored at −22 °C until DNA extraction.

DNA extraction

The DNA extraction and purification was performed using the cetyltrimethylammonium bromide (CTAB) extraction method [25]. The homogenization of the fungal material was performed in DNA extraction buffer (100 mM Tris–HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% [beta]-mercaptoetanol, and 2% CTAB). Three hundred milligrams of harvested mycelial preparations were disrupted in liquid nitrogen and transferred to 1 ml of DNA extraction buffer. Homogenates were mixed and incubated at 65 °C for 1 h. Nucleic acids were extracted by adding an equal volume of chloroform/isoamyl alcohol (24:1). After centrifugation, the aqueous layer was collected, and DNA was precipitated overnight with 1 volume of cold isopropanol. The pellet was suspended in TE buffer pH 8.0 and sequentially digested with RNase A. The relative amount of DNA was estimated on 1.3% agarose gels by visual comparisons with molecular mass markers (Invitrogen, USA).

Amplification and sequencing of EF1-α and ITS1-5.8S-ITS2

Two nuclear gene regions, the EF1-α and the ribosomal ITS (ITS1-5.8S-ITS2), were amplified and sequenced. The EF1-α fragment was amplified as described by Rehner and Buckley [10] with minor modifications. The amplification was performed with primers: 983F (5′-GCYCCYGGH CAYGGTGAYTTYAT-3′) and 2218R (5′-ATGACACCRACRG-CRACRGTYTG-3′). The sequencing of the 983F-2218R amplicon was carried out with additional internal primers: 1567R (5′-ACHGTRCCRATACCACCSATCTT-3′) and 1577F (5′-CARGAYGTBTACAAGATYGGTGG-3′). PCR reaction were performed in a total volume of 50 μL, which included 5 μL of 10× PCR buffer (10 mM Tris/HCl pH 8.0, 50 mM KCl), 2 μL of 50 mM $MgCl_2$, 2 μL of 2.5 mM dNTP mix (dATP, dCTP, dGTP, and dTTP), 1 μL of 10 μM each primers, 0.2 μL of 5 U μl^{-1} *Taq* polymerase, and 10 ng of genomic DNA. Touchdown PCR amplifications were

Table 1. Details of isolates of *Isaria* spp. used in the molecular characterization and identification.

Species	Collections number[a]	ARSEF number[b]	Host/substrate	Geographic origins	Collection date	GenBank Accession number	
						EF1-α	ITS1-5.8S-ITS2
Isaria amoenerosea	CNPSo-112	–	*Lagria villosa*	Warta, Londrina, Brazil	10 Feb 1998	JN998762	JN998782
Isaria farinosa	CEP 029	6717	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	01 Jun 2001	JN998765	JN998785
Isaria farinosa	CEP 004	7777	Soil	Buenos Aires, Argentina	03 Jul 2003	JN998763	JN998783
Isaria farinosa	CEP 005	–	Soil	Buenos Aires, Argentina	04 Jul 2003	JN998764	JN998784
Isaria fumosorosea	CEP 051	7204	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	09 Apr 2003	–	–
Isaria fumosorosea	CEP 055	7205	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	10 Apr 2003	–	–
Isaria fumosorosea	CEP 302	8716	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	22 May 2007	–	–
Isaria fumosorosea	CEP 303	8717	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	04 Jun 2007	JN998766	JN998786
Isaria fumosorosea	CEP 304	8718	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	03 Oct 2007	JN998767	JN998801
Isaria fumosorosea	CEP 305	8719	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	03 Oct 2007	–	–
Isaria fumosorosea	CEP 306	8720	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	10 Oct 2007	–	–
Isaria fumosorosea	CEP 307	8721	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	16 Oct 2007	–	–
Isaria fumosorosea	CEP 308	8723	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	04 Jun 2007	–	–
Isaria fumosorosea	CEP 309	8724	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	06 Jun 2007	–	–
Isaria fumosorosea	CEP 310	8725	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	06 Jun 2007	–	–
Isaria fumosorosea	CEP 311	8726	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	06 Jun 2007	–	–
Isaria fumosorosea	CEP 312	8727	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	13 Jun 2007	–	–
Isaria fumosorosea	CEP 313	8728	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	13 Jun 2007	JN998768	JN998800
Isaria fumosorosea	CEP 314	8729	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	22 May 2007	–	–
Isaria fumosorosea	CEP 315	8730	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	22 May 2007	JN998769	JN998789
Isaria fumosorosea	CEP 316	8928	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	22 May 2007	–	–
Isaria fumosorosea	CNPSo-79	5156	*Bemisia tabaci* biotype B	Paraná, Brazil	14 Mar 1996	JN998772	JN998796
Isaria fumosorosea	CNPSo-83	5160	*Bemisia tabaci* biotype *B*	Paraná, Brazil	15 Mar 1996	JN998773	JN998795
Isaria fumosorosea	CNPSo-121	3303	*Bemisia tabaci* biotype B	Colina, Mexico	01 Set 1997	JN998770	JN998798
Isaria javanica	CEP 107	7477	*Trialeurodes vaporariorum*	Buenos Aires, Argentina	04 Apr 2004	JN998774	JN998794
Isaria tenuipes	CNPSo-41	3938	Lepidoptera	Ivatuba, Paraná, Brazil	31 Jan 1990	JN998779	JN998789
Isaria tenuipes	CNPSo-96	–	Lepidoptera	Warta, Londrina, Brazil	17 Jan 1997	JN998780	JN998788
Isaria tenuipes	CNPSo-97	–	Lepidoptera	Warta, Londrina, Brazil	06 Feb 1997	JN998781	JN998787
Isaria sp.	CNPSo-104	–	*Bemisia tabaci* biotype B	Brasilia, Brazil	12 Mar 1997	JN998776	JN998792
Isaria sp.	CNPSo-105	–	*Bemisia tabaci* biotype B	Brasilia, Brazil	13 Mar 1997	JN998777	JN998791
Isaria sp.	CNPSo-106	–	*Bemisia tabaci* biotype B	Brasilia, Brazil	14 Mar 1997	JN998778	JN998790
Isaria sp.	CEP 049	–	Hymenoptera	Buenos Aires, Argentina	01 Nov 1998	JN998775	JN998793
Purpureocillium lilacinum	CNPSo-162	–	*Scaptocoris castanea*	Mato Grosso, Brazil	18 Dec 1998	JN998771	JN998797

[a]CEP = Mycological Collections at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE) (CONICET-UNLP), La Plata, Argentina; CNPSo = Embrapa Soybean Research Center, Londrina, Brazil [22].
[b]ARSEF = USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, New York [23].

initiated with a 2 min denaturation at 95 °C. The annealing temperature in the first amplification cycle was 66 °C, and was subsequently decrementally reduced by 1 °C per cycle over the next 9 cycles. An additional 36 amplification cycles were then performed, each consisting of 30 s denaturation at 94 °C, a 30 s annealing step at 56 °C, and 1 min extension at 72 °C. Following these cycles, a final elongation step of 10 min at 72 °C was done.

Universal primers TW81 (5′-GTTTCCGTAGGTGAACC TGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) were used to amplify the region of the ribosomal repeat from the 3′ end of the 16/18S rDNA to the 5′ end of the 28S rDNA flanking the ITS1, 5.8S rDNA and ITS2 sequences [9]. The PCR amplification was performed essentially as described by White et al. [26]. The 50 μl of reaction mixture contained 5 μL 10× PCR buffer, 50 mM $MgCl_2$, 2.5 mM of dNTP mix, 10 mM of each primer, 5 U μl^{-1} *Taq* polymerase and 10 ng of templates DNA. The PCR was programmed for 2 min denaturation step at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final extension of 10 min. The resultant reaction mixture was separated on a 1.3% agarose gel, and bands were excised and purified by Purelink-Quick Gel Extraction Kit (Invitrogen, 2009).

The sequencing was performed with an ABI 3700 DNA sequencer using the primers noted above with BigDye terminators DNA sequencing kit (Applied Biosystems, Foster City, CA, USA) at the Biotechnology Laboratory, Embrapa Soybean Research Center, Londrina, Brazil.

PCR-RFLP of EF1-α and ITS1-5.8S-ITS2

The restriction enzyme analyses were carried out with PCR products of EF1-α (983F-2218R) or ITS1-5.8S-ITS2 (TW81-AB28) using *Alu* I, *Hae* III, *Mbo* I, *Hpa* II, *Taq* I, and *Hha* I (Invitrogen, USA). The reaction volume was 20 μl

Table 2. Details of fungi used in the phylogenetic analyses.

Species	Family	Type	EF1-α	ITS1-5.8S-ITS2
Neurospora crassa	Sordariaceae	Teleomorph	AF402094.1	AF388914.1
Torrubiella luteorostrata	Clavicipitaceae	Teleomorph	EF468801.1	AY624206.1
Aschersonia badia	Clavicipitaceae	Anamorph	DQ522317.1	EU409585.1
Metarhizium flavoviride	Clavicipitaceae	Anamorph	DQ522353.1	AF516291.1
Metarhizium album	Clavicipitaceae	Anamorph	DQ522352.1	AF137067.1
Metarhizium anisopliae	Clavicipitaceae	Anamorph	AF543774.1	AF516302.1
Nomuraea rileyi	Clavicipitaceae	Anamorph	EF468787.1	EU553337.1
Pochonia chlamydosporia	Clavicipitaceae	Anamorph	EF469069.1	AF108468.1
Paecilomyces marquandii	Clavicipitaceae	Anamorph	EF468793.1	EU553322.1
Paecilomyces carneus	Clavicipitaceae	Anamorph	EF468789.1	EU553305.1
Metacordyceps chlamydosporia	Clavicipitaceae	Teleomorph	DQ522327.1	AJ292397.1
Cordyceps bifusispora	Cordycipitaceae	Teleomorph	EF468747.1	AJ786553.1
Cordyceps militaris	Cordycipitaceae	Teleomorph	DQ522332.1	FJ973070.1
Cordyceps pruinosa	Cordycipitaceae	Teleomorph	DQ522351.1	DQ342253.1
Cordyceps scarabaeicola	Cordycipitaceae	Teleomorph	DQ522335.1	AY245639.1
Cordyceps confragosa	Cordycipitaceae	Teleomorph	DQ522359.1	FR667982.1
Beauveria caledonica	Cordycipitaceae	Anamorph	EF469057.1	DQ350137.1
Beauveria bassiana	Cordycipitaceae	Anamorph	AY531943	FJ972972.1
Beauveria bassiana	Cordycipitaceae	Anamorph	AY531881.1	AY531972.1
Isaria farinosa	Cordycipitaceae	Anamorph	DQ522348.1	DQ681344.1
Isaria farinosa	Cordycipitaceae	Anamorph	EF469065.1	EU553339.1
Isaria tenuipes	Cordycipitaceae	Anamorph	DQ522349.1	EU553332.1
Lecanicillium psalliotae	Cordycipitaceae	Anamorph	EF468784.1	FN689594.1
Lecanicillium fusisporum	Cordycipitaceae	Anamorph	EF468783.1	AJ292428.1
Lecanicillium attenuatum	Cordycipitaceae	Anamorph	EF468782.1	EF192939.1
Lecanicillium antillanum	Cordycipitaceae	Anamorph	DQ522350.1	AJ292392.1
Simplicillium obclavatum	Cordycipitaceae	Anamorph	EF468798.1	AJ292394.1
Simplicillium lanosoniveum	Cordycipitaceae	Anamorph	DQ522358.1	EF513003.1
Simplicillium lamellicola	Cordycipitaceae	Anamorph	DQ522356.1	AY555956.1
Cordyceps gunnii	Ophiocordycipitaceae	Teleomorph	AY489616.1	AF368803.1
Ophiocordyceps irangiensis	Ophiocordycipitaceae	Teleomorph	DQ522345.1	AY646400.1
Elaphocordyceps subsessilis	Ophiocordycipitaceae	Teleomorph	EF469061.1	AJ786592.1
Elaphocordyceps japonica	Ophiocordycipitaceae	Teleomorph	DQ522330.1	EU039882.1
Elaphocordyceps capitata	Ophiocordycipitaceae	Teleomorph	AY489615.1	EU834212.1
Purpureocillium lilacinum	Ophiocordycipitaceae	Anamorph	EF468790.1	FJ973076.1
Haptocillium zeosporum	Ophiocordycipitaceae	Anamorph	EF469062.1	AJ292419.1
Hirsutella sp.	Ophiocordycipitaceae	Anamorph	EF469064.1	EF029185.1

consisting of 10 μl of PCR product, 1.5 μl of the recommended 10× buffer, 0.5 μl of 10 U μl^{-1} enzymes and distilled water to make up volume. The reaction mixtures were incubated at 37 °C for 3 h and separated on 2% agarose gels.

Phylogenetic analyses

EF1-α sequences were generated with the primers 983F-1567R and 1577F-2218R. Sequences corresponding to ITS1-5.8S-ITS2 were obtained with primers TW81 and AB28. Newly generated sequences of *Isaria* spp. were deposited in Gen-Bank under accession numbers JN998762-JN998801. The sequences of EF1-α and ITS1-5.8S-ITS2 from *I. fumosorosea* (CEP 303, CEP 304, CEP 313, CEP 315, CNPSo-79, CNPSo-83, and CNPSo-121), *I. farinosa* (CEP 004, CEP 005, and CEP 029), *I. javanica* (CEP 107), *I. amoenerosea* (CNPSo-112), *I. tenuipes* (CNPSo-41, CNPSo-96, and CNPSo-97), *Isaria* sp. (CEP 049, CNPSo-104, CNPSo-105, and CNPSo-106) and *P. lilacinum* (CNPS0-162) isolates were aligned with previously published sequence data from 36 anamorphic and teleomorphic species of the families Clavicipitaceae, Cordycipitaceae, and Ophiocordycipitaceae. *Neurospora crassa*, of family Sordariaceae, was used as outgroup (NCBI GenBank database; http://www.ncbi.nlm.nih.gov/) (Table 2).

EF1-α sequences were aligned with Clustal X package [27], whereas ITS1-5.8S-ITS2 was aligned with Mafft [28], since these sequences were too divergent for only progressive alignment. Bayesian analyses were performed with the parallel version of MrBayes [29]. Twelve Monte Carlo (MCMC) chains (i.e., nchains = 6 nruns = 2) were run for 10E7 generations, sampling every 1000 generations. Posterior probabilities were calculated and reported on a 50% majority rule consensus tree of the post-burnin sample. Maximum Likelihood searches were performed with the parallel version of the program RAxML [30]. These searches consisted of 100 bootstrap trees followed by a full ML search, under the GTR-CAT approximation. The selection of evolutionary models was performed with MrAIC.

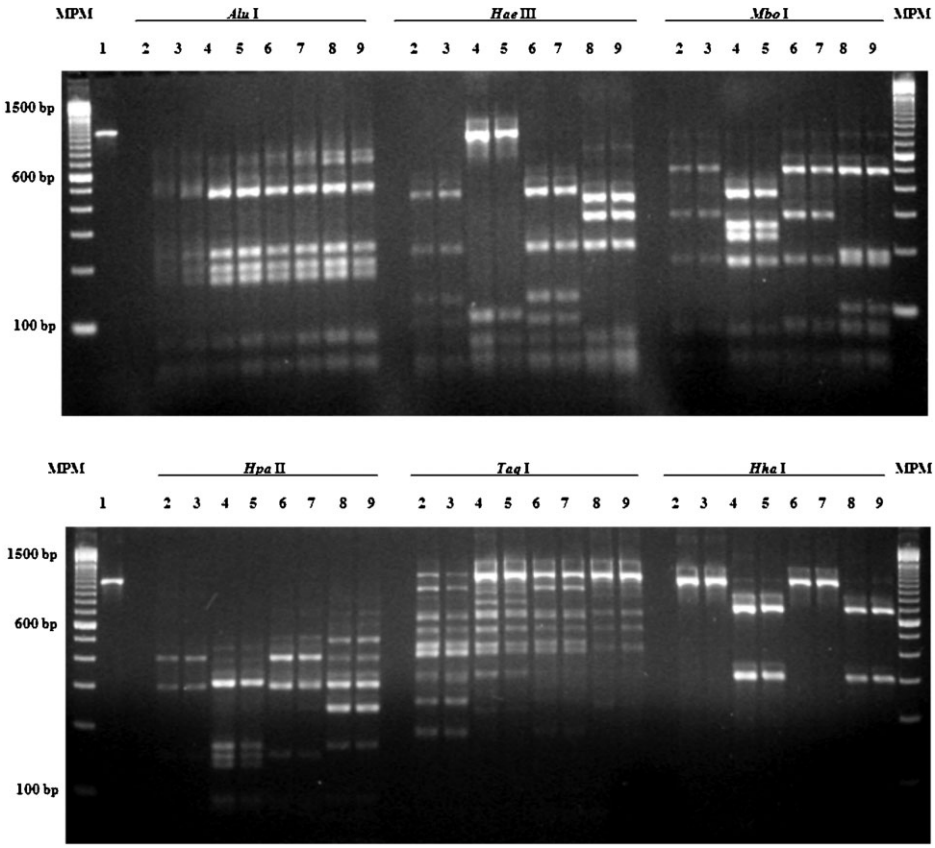

Figure 1. PCR-RFLP from EF1 of representative *Isaria* isolates. Amplification performed with 983F/2218R primers and digested with *Alu* I, *Hae* III, *Mbo* I, *Hpa* II, *Taq* I, and *Hha* I. **1**: PCR product EF1 (983F-2218R) undigested. **2**: *I. fumosorosea* CEP 051. **3**: *I. fumosorosea* CNPSo-83. **4**: *I. farinosa* CEP 029. **5**: *I. farinosa* CEP 005. **6**: *I. javanica* CEP 107. **7**: *I. amoenerosea* CNPSo-112. **8**: *I. tenuipes* CNPSo-41. **9**: *I. tenuipes* CNPSo-96. **MPM**: DNA ladder. Molecular sizes are indicated on the left.

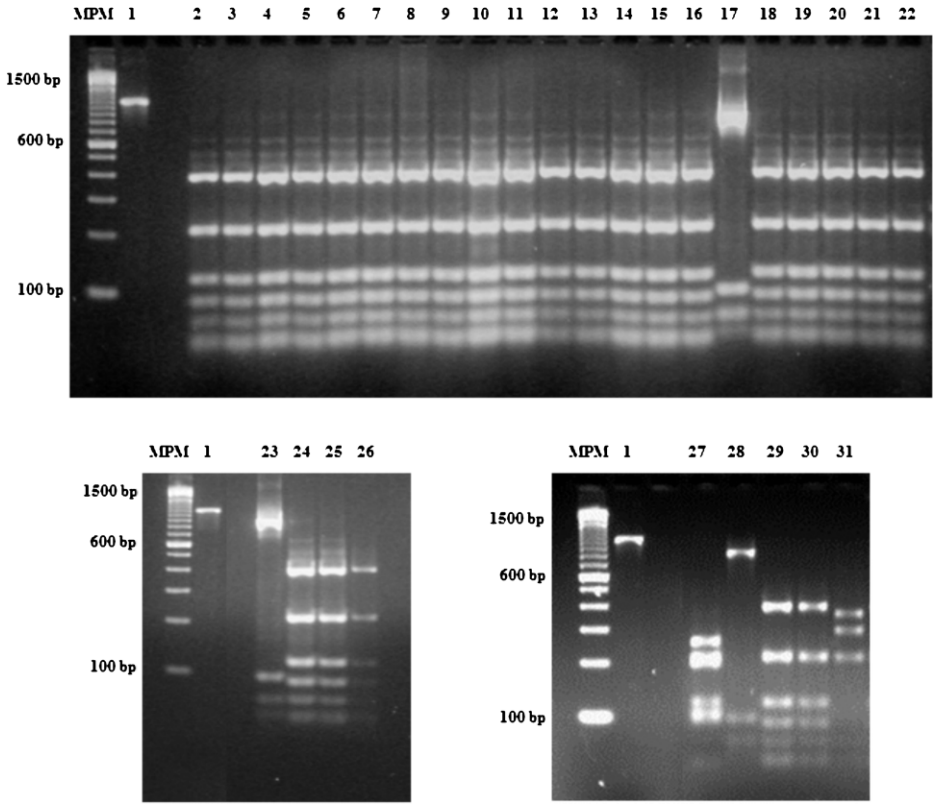

Figure 2. PCR-RFLP from EF1 of *Isaria* isolates using *Hae* III. Amplification performed with 983F/2218R primers and digested with *Hae* III. **1**: PCR product EF1 (983F-2218R) undigested. **2**: *I. fumosorosea* CEP 302. **3**: *I. fumosorosea* CEP 303. **4**: *I. fumosorosea* CEP 304. **5**: *I. fumosorosea* CEP 305. **6**: *I. fumosorosea* CEP 306. **7**: *I. fumosorosea* CEP 307. **8**: *I. fumosorosea* CEP 308. **9**: *I. fumosorosea* CEP 309. **10**: *I. fumosorosea* CEP 310. **11**: *I. fumosorosea* CEP 311. **12**: *I. fumosorosea* CEP 312. **13**: *I. fumosorosea* CEP 313. **14**: *I. fumosorosea* CEP 314. **15**: *I. fumosorosea* CEP 315. **16**: *I. fumosorosea* CEP 316. **17**: *I. farinosa* CEP 029. **18**: *I. fumosorosea* CEP 051. **19**: *I. fumosorosea* CEP 055. **20**: *I. fumosorosea* CNPS0-79. **21**: *I. fumosorosea* CNPS0-83. **22**: *I. fumosorosea* CNPSo-121. **23**: *Isaria* sp. CEP 049. **24**: *Isaria* sp. CNPSo-104. **25**: *Isaria* sp. CNPSo-105. **26**: *Isaria* sp. CNPSo-106. **27**: *P. lilacinum* CNPSo-162. **28**: *I. farinosa* CEP 004. **29**: *I. javanica* CEP 107. **30**: *I. amoenerosea* CNPSo-112. **31**: *I. tenuipes* CNPSo-97. **MPM**: DNA ladder. Molecular sizes are indicated on the left.

pl [31]. Parallel analyses were performed on a 3.3 GHz six-core computer (AMD) with 16 Gb RAM, running Ubuntu server 10.04.

Results

Molecular characterization of *Isaria* isolates using PCR-RFLP

In all isolates tested, a fragment of approximately 1100 bp extending nearly to the 3′ end of EF1-α was amplified with primers 983F and 2218R (Fig. 1, line 1). This fragment was used for the restriction fragment polymorphic analyses that were conducted with six restriction enzymes. All enzymes digested the EF1-α (983F-2218R) amplicon but only *Hae* III, *Mbo* I, and *Hpa* II showed interspecific polymorphisms (Fig. 1). The *I. fumosorosea*, *I. farinosa*, *I. tenuipes*, and *P. lilacinum* isolates digested with *Hae* III showed easily distinguishable restriction profiles in agarose gels. However, the *I. javanica* and *I. amoenerosea* profiles were identical to that of *I. fumosorosea* (Figs. 1 and 2). The PCR products (983F-2218R) of *Isaria* isolates (Table 1) were digested with *Hae* III, and the restriction patterns of 15 isolates from Argentina (CEP 302 to CEP 316) and three isolates from Brazil (CNPSo-104, CNPSo-105, and CNPSo-106) also matched those of the *I. fumosorosea* profile. The restriction profile of one isolate from Argentina (CEP 049) was identical to that of *I. farinosa* (Fig. 2).

The TW81 and AB28 primers, corresponding to a the region of the ribosomal repeat ranging from the 3′ end of the 16/18S rDNA to the 5′ end of the 28S rDNA flanking the ITS1, the 5.8S rDNA and ITS2 sequences, successfully amplified a fragment of approximately 600 bp from all *Isaria* isolates. These PCR products were

Figure 3. Phylogenetic of Hypocreales from Bayesian analyses showing the relationship of *Isaria* species with other genera. A 50% of majority consensus tree is shown based on Bayesian analyses with combined data set of EF1-α and ITS1-5.8S-ITS2 sequences. The scale bars represent the evolution rates 0.01 substitutions/site.

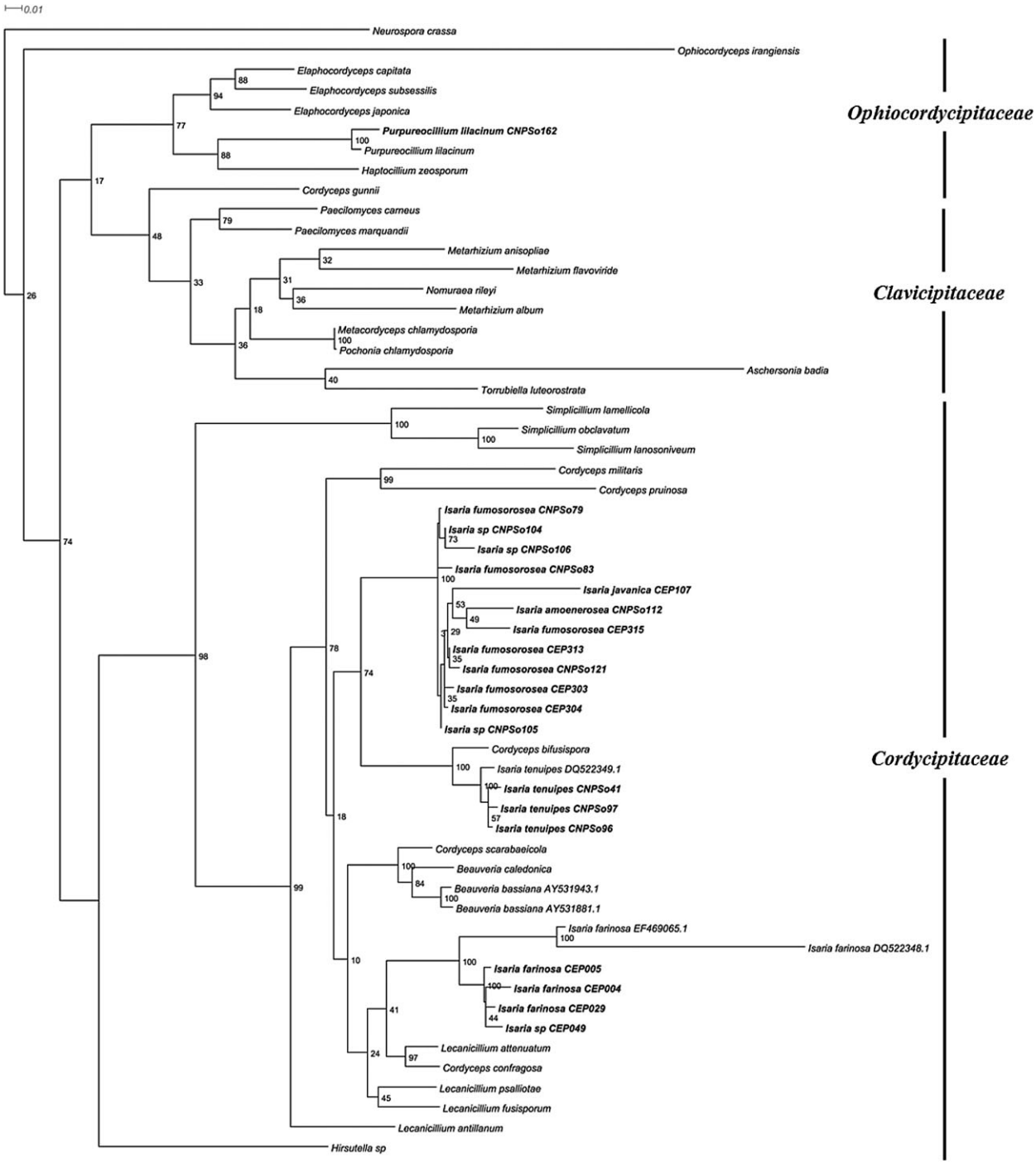

Figure 4. Phylogenetic of Hypocreales from Maximum Likelihood analyses showing the relationship of *Isaria* species with other genera. Maximum Likelihood tree was performed used a combined data set of EF1-α and ITS1-5.8S-ITS2 sequences. Numbers on branches correspond to bootstrap values obtained from 1000 pseudoreplicates. Nodes with bootstraps values below 50 were collapsed. The scale bars represent the evolution rates 0.01 substitutions/site.

treated with six restriction enzymes. *Hae* III, *Mbo* I, *Hpa* II, and *Hha* I were capable of digesting the amplicon, but only *Hae* III and *Hha* I generated interspecific polymorphisms. The *I. fumosorosea*, *I. farinosa*, *I. tenuipes*, and *P. lilacinum* isolates digested with *Hae* III showed distinct restriction profiles in agarose gels, but the *I. javanica* and *I. amoenerosea* patterns were similar to that of *I. fumosorosea* (Figs. S1 and S2 of Supporting Information). The 15 unidentified isolates from Argentina treated with *Hae* III showed patterns identical to those of *I. fumosorosea* (Fig. S2 of Supporting Information). These results match with the results of restriction analyses obtained with the EF1-α fragment. Nevertheless, the *I. fumosorosea*, *I. farinosa*, *I. tenuipes*, and *P. lilacinum* polymorphisms exhibit with EF1-α (983F-2218R) amplicons were more distinguishable compared with ITS1-5.8-ITS2 (TW81-AB28) amplicons using the *Hae* III enzyme.

The molecular characterization by PCR-RFLP using EF1-α and ITS1-5.8-ITS2 genes was useful for differentiating *I. fumosorosea*, *I. farinosa*, *I. tenuipes*, and *P. lilacinum*.

Phylogenetic analyses

New partial nucleotide sequences of EF1-α (approx. 1100 bp) and ITS1-5.8S-ITS2 (approx. 600 bp) of 20 *Isaria* spp. isolates were deposited in GenBank (Table 1). The other sequences of the three clavicipitoid families of the Order Hypocreales were retrieved from GenBank (Table 2). Sequences of two genes were aligned and analyzed separately by Bayesian and Maximum Likelihood analyses, and the resulting trees were compared. No conflicts were detected between single gene phylogenies, thus indicating that the datasets could be combined.

All phylogenetic analyses showed that *Isaria* species formed a polyphyletic or paraphyletic group. Nevertheless, both Bayesian and Maximum Likelihood analyses were useful for discriminating *Isaria* isolates at the species level. The isolates of *I. tenuipes*, *I. farinosa*, and *I. fumosorosea* formed monophyletic clades supported by high bootstrap values (>95%) (Figs. 3 and 4). The *I. amoenerosea* and *I. javanica* isolates were grouped with those of *I. fumosorosea*, suggesting that these three species might actually correspond to one species, to a group of closely related species, or that there have been underlying problems with the identifications of these three isolates.

The *Isaria* species were related to *Lecanicillium*, *Beauveria*, and *Simplicillium* anamorphs states and *Cordyceps* teleomorphic states in the clade of Cordycipitaceae. The *I. tenuipes* strains were located in a monophyletic group together with *C. bifusisporum* (bootstrap support of 100%). This clade was closely related to *I. fumosorosea* strains. The *I. farinosa* isolates were clearly separated from other *Isaria* species and related to *L. fusisporum*, *L. psalliotae*, *L. attenuatum*, *C. confragosa*, *B. bassiana*, *B. caledonica*, and *C. scarabaeicola*. The *Simplicillium* species formed a clade clearly separated from the other genera within the Cordycipitaceae clade (with 100% bootstrap support).

The phylogenetic analyses confirmed the taxonomic identity of *Isaria* spp. isolates from Argentina (CEP 049) and Brazil (CNPSo-104, CNPSo-105, and CNPSo-106) as *I. farinosa* and *I. fumosorosea* by similarity in EF1-α and ITS1-5.8S-ITS2 sequences. The CNPSo-162 isolate was originally classified as *I. fumosorosea*, but on a basis of the molecular evidence generated by this study, these isolate was reidentified as *Purpureocillium lilacinum* (= *Paecilomyces lilacinus*; [33]).

In all phylogenetic trees no correlation was observed with either biographical origin or host species, so it should be assumed that the observed genetic variabilities reflect real properties of the individual *Isaria* species tested here.

Discussion

The morphological characteristics used to classify the genus *Isaria* frequently do not resolve new isolates into clearly defined species and provide no isolate level characterizations. In this study, we characterized *I. fumosorosea*, *I. farinosa*, *I. tenuipes*, and *P. lilacinum* isolates by PCR-RFLP of EF1-α and ITS1-5.8-ITS2 genes to confirm the taxonomic identification of 15 isolates from Argentina and 3 isolates from Brazil.

Both genes were digested with *Hae* III and showed distinct restriction profiles in agarose gels. A similar strategy was employed by Souza Azevedo et al. [32] to study the molecular variations within *I. fumosorosea* using the ITS gene sequences and their *Hae* III restriction profiles. Additionally, the results obtained here with restriction enzyme analysis of ITS1-5.8-ITS2 demonstrated that *Hha* I is a good choice for PCR-RFLP, because it comes up with clearer and more easily identified patterns. The restriction patterns of EF1-α (983F-2218R) amplicons offer a completely new approach to the characterization and identification of *Isaria* species.

The phylogenetic relationships of some entomopathogenic and nematophagous *Isaria* isolates were resolved with the sequencing of mitochondrial and nuclear genes [4–5, 9–12, 14]. The ribosomal rRNA and ITS sequences were widely used to establish phylogenetic relationship of the genus *Isaria* [6, 16–18, 32], while no EF1-α sequence have been applied directly so far to taxonomic investigations in this genus. Our phylogenetic

analyses demonstrated that *Isaria* species formed a polyphyletic group allied with *Beauveria* and related to *Lecanicillium* and *Simplicillium*, as has also been noted by Obornik et al. [15] and Luangsa-ard et al. [17]. The most commonly reported *Isaria* species – *I. fumosorosea*, *I. farinosa*, and *I. tenuipes* – formed a monophyletic group with high support. The *I. javanica* and *I. amoenerosea* isolates were included within *I. fumosorosea* group; however, this small group of fungi requires further study using a greater number of isolate and additional molecular markers to verify their correct identifications and true associations with *I. fumosorosea*. The isolate CNPS0-162 was reidentified as *Purpureocillium lilacinum* since its gene sequences unambiguously placed this isolate in the family Ophiocordycipitaceae (rather than in the Cordycipitaceae as would be required if its original identification as *I. fumosorosea* were correct) while recognizing that the general appearances of cultures of both of these species are very similar.

Both the EF1-α and ITS1-5.8S-ITS2 gene distinguish the three families of the order Hypocreales and confirm the placement of *Isaria* in the family Cordycipitaceae as previously determined by Sung et al. [21]. Therefore, these genes were found to be powerful tools for improving the characterization, identification, and phylogenetic relationships of the *Isaria* species and of other entomopathogenic fungi. However, future studies should be undertaken to refine the taxonomy of the genus *Isaria* and the teleomorphic states of its species by using additional molecular markers and to incorporate a much broader spectrum of *Isaria* isolates that will include enough redundancy (by using multiple isolates of recognized species) to confirm the molecular results that will be obtained.

Acknowledgments

To CONICET (National Research Council of Argentina) and Wood Whelan Fellowship for funded senior author fellowship. To ANPCYT from Argentina (National Agency of Scientific and Technologic promotion) (GRANT PICT No 20337/04 to Claudia Lopez Lastra and PICT-PRH N° 120 to Leandro R. Jones) and CNPq from Brazil (National Council for Scientific and Technological Development) for financial support.

Conflict of interest

The authors declare that they have no conflicts of interest.

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