ELSEVIER

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/mycres

Molecular data indicate that *Rhytidhysteron rufulum* (ascomycetes, *Patellariales*) in Costa Rica consists of four distinct lineages corroborated by morphological and chemical characters

Catalina MURILLO[a,b,*], *Federico J. ALBERTAZZI*[b,c], *Julieta CARRANZA*[b], *H. Thorsten LUMBSCH*[d], *Giselle TAMAYO*[a,e]

[a]*Unidad Estratégica de Bioprospección, Instituto Nacional de Biodiversidad (INBio), 22-3100, Santo Domingo de Heredia, Costa Rica*
[b]*Escuela de Biología, Universidad de Costa Rica, 11501-2060, San José, Costa Rica*
[c]*Centro de Biología Celular y Molecular, Universidad de Costa Rica, 11501-2060, San José, Costa Rica*
[d]*Department of Botany, The Field Museum, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA*
[e]*Escuela de Química, Universidad de Costa Rica, 11501-2060 San José, Costa Rica*

ARTICLE INFO

Article history:
Received 15 January 2008
Received in revised form
26 June 2008
Accepted 4 September 2008
Published online 30 September 2008
Corresponding Editor: Rajesh Jeewon

Keywords:
Cryptic species
Dehydrocurvularin
hplc
NMR
Palmarumycins
Pseudoepithecium colour
Tropical fungi

ABSTRACT

Rhytidhysteron rufulum is a poorly known, common, pantropical species, capable of utilizing different substrata and occupying diverse habitats, and is the only species of its genus in Costa Rica. We have employed molecular, morphological, and chemical data to assess the variability and differentiation of *R. rufulum* in Costa Rica, including sites from the Pacific and Atlantic coast. Phylogenetic analyses of nuclear ITS rDNA sequences revealed the presence of four distinct lineages in the *R. rufulum* complex. Re-examination of the morphology and anatomy showed differences between these lineages in ascomatal, ascal, and ascospore size that have previously been regarded as intraspecific variations. In addition, there was a correlation between molecular phylogenies and chemical components as determined by hplc and nuclear magnetic resonance (NMR). Two lineages (clades I and II) produced the palmarumycins MK-3018, CJ-12372, and CR_1, whereas clade III produced dehydrocurvularin, and clade IV unidentified compounds. Our results based on a polyphasic approach contradict previous taxonomic interpretations of one morphologically variable species.

Introduction

Recent estimates of the number of fungal species worldwide predict a minimal number of over 700 K species (Mueller & Schmit 2007), while the most commonly cited number is about 1.5 M taxa (Hawksworth 2001). Only a small percentage of taxa is currently known, given the *ca* 100 K described species to date. This situation is even more complicated because of uncertainties in the concept of species in fungi. There is a growing body of evidence from recent molecular studies that morphology-based concepts severely underestimate the number of species. Morphological differences have been

* Corresponding author. Tel: +506 25078261.
E-mail address: cmurillo@inbio.ac.cr
0953-7562/$ – see front matter © 2008 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2008.09.003

misinterpreted as intraspecific variation and geographically or ecologically isolated populations have been shown to belong to distinct phylogenetic species (Crous *et al.* 2004; Molina *et al.* 2004; Pringle *et al.* 2005; Rehner & Buckley 2005; Dettman *et al.* 2006; Taylor *et al.* 2006; Wirtz *et al.* 2008). In tropical regions there is even less information available for most groups of ascomycetes. In Costa Rica only 2 K species of fungi have been reported, but it is considered that the real number lies between 40 K and 100 K species of fungi (Mata 2003). At present, there is increasing interest in documenting diversity for a number of reasons, especially for their role in ecosystems, and their potential as sources of secondary metabolites for use as antibiotics or biocontrol agents.

The genus *Rhytidhysteron* is classified in *Patellariaceae* (*Patellariales*). It is characterized by hysteriform ascomata that become discoidal at maturity and paraphyses covered by a gelatinous layer (pseudoepithecium). Samuels & Müller (1979) revised the genus and accepted two species: *R. rufulum,* a saprophyte or weak parasite found on a great variety of plants and *R. hysterinum* a wood inhabitant distributed mainly in warm climates of the Northern Hemisphere, but also there are reports from South America (Venezuela). The anamorphs for these species have been described as '*Diplodia*-like' or '*Aposphaeria*-like'; however, no studies have been carried out to relate these structures to the biology and evolutionary history of these groups. Recently, other species for the genus *Rhytidhysteron* have been described, for example *R. opuntiae,* (Barr 1990) and *R. dissimile* (Magnes 1997).

R. rufulum is the only species at present known from Costa Rica (Loengrin Umaña, pers. comm.). This species is currently accepted in a wide circumscription as suggested by Samuels & Müller (1979) who synonymized two species and suggested that six more species are synonyms of *R. rufulum* as well.

Previous research at the Bioprospecting Strategic Unit of the National Institute of Biodiversity (INBio) on a *Rhytidhysteron* spp. isolate resulted in the isolation of palmarumycins, secondary metabolites that have been reported as potential inhibitors of the thioredoxin–thioredoxin reductase cellular redox systems, which also have antimicrobial and antifungal properties (Wipf *et al.* 2004). This finding stimulated additional research on this genus in Costa Rica. The aim of the current investigation was to characterize samples of *Rhytidhysteron* spp. collected from different areas of Costa Rica. To address this, we conducted (1) morphological comparisons among the collections and with described species; (2) a re-evaluation of micromorphological characters; (3) a chromatographic examination of the samples using hplc and ^{1}H-nuclear magnetic resonance (NMR) measurements to assess the presence or absence of palmarumycins, and where feasible, to establish the identity of components other than palmarumycins; and (4) a phylogenetic analysis using sequences of the ITS nuDNA regions.

Materials and methods

Sample collection

Samples were collected from June to October 2005 in Barra Honda and Palo Verde National Parks (Guanacaste, Costa Rica), Cahuita National Park and Hitoy Cerere Biological Reserve (Limón, Costa Rica). The isolate 3352, *Rhytidhysteron* spp. was used as positive control for the presence of the palmarumycins studied. Voucher specimens are deposited in INBio (Table 1). The samples collected were compared morphologically with specimens of *Rhytidhysteron* deposited at NY.

Specimens examined

Puerto Rico: Vicinity of Coamo Springs, on dead wood, 21 Feb 1922, *N.L & E.G. Britton & M.S. Brown* 6149, (*Neotypus Rhytidhysteron rufulum*)—**United States:** *Arizona*: Saguarro National Monument, West Unit, Jul 1980, *R.L. Gilbertson* 6774 (*R. opuntiae*)—**Venezuela:** *Bolívar*: Dist. Cedeño, vicinity of Panare village of Corozal, 6 km from Maniapure toward Caicara 6°55″N, 66°30″W, Apr 1986, *B. Boom & M. Grillo* 6538 (*R. hysterinum*). Parque Nacional Guatopo: trail between Agua Blanca and La Cruceta, 10°03″N, 66°26″W, Nov 1990, *GJ. Samuels, B. Hein, S.M. Huhndorf* 7620 (*R. rufulum*).

Morphological studies

Samples were grouped for morphological examination according to ascoma form, pseudoepithecium and receptacle colour and margin appearance. The colours were determined using Smithe (1975). Measurements of asci and ascospores were taken from material that was first treated with 5 % potassium hydroxide and then mounted in water. Twenty asci and 50 ascospores were measured using an Olympus Bx50 microscope fitted with a Song CCD-IRIS (Image-Pro plus Version 4.0) camera. Descriptive statistics, including means, S.D., maximum and minimum values of continuous characters, and the significance of differences in sample means by one-way analysis of variance (ANOVA) using Tukey's test were obtained using INFOSTAT (2002).

Cultures were initiated from ascospores that were singled out under the stereoscope and placed with a fine needle in Petri dishes containing PDA (potato–dextrose agar; Difco) medium. The next day hyphal tips emerging were cut and transferred to a new Petri dish with PDA media and incubated at 25 °C for 10 d for further description (Table 1).

Molecular studies

Monosporic isolates were grown in PDB (potato–dextrose broth; Difco) for 3–5 d at 25 °C. A maximum of 0.1 g of the dried mycelial mat was placed in a 1.5 ml Eppendorf tube for immediate DNA extraction with DNeasy mini plant kit (Qiagen, CA). The ITS regions were amplified and sequenced using ITS1F and ITS4R (Gardes & Bruns 1993). PCR reactions were set up for 50 µl reactions using 50 ng DNA, 1 × Promega buffer (Promega, WI), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer, 2.5 units Taq DNA polymerase (Promega) and sterile water to complete a total of 50 µl per reaction. The PCR reactions were done on a Perkin Elmer 9700 thermocycler using the conditions described by Fernández *et al.* (1999). PCR products were purified using GeneElute PCR Clean-up kit (Sigma, MI) and their concentration was determined using the MassRuler DNA Ladder Mix (Fermentas, MD). Sequencing was performed

Table 1 – Monospore cultures of *Rhytidhysteron* rufulum studied

INBio accession no. and isolate no.		Location	Latitude	Longitude	Host	GenBank no.
INB0003985581	502A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020017
INB0003985583	504A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020048
INB0003985584	505A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020029
INB0003985586	507A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020037
INB0003985587	508A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020049
INB0003985588	509A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020065
INB0003985589	510A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020063
INB0003985590	511A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020018
INB0003985591	512A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020050
INB0003985592	513A	Palo Verde National Park	10:21:21.4000	−85:19:11.3000	*Acacia* spp.	EU020064
INB0003985706	514A	Palo Verde National Park	10:19:59.9757	−85:15:09.9381	Unknown	EU020032
INB0003985707	515A	Palo Verde National Park	10:19:59.9757	−85:15:09.9381	Unknown	EU020034
INB0003986150	517A	Palo Verde National Park	10:20:30.4000	−85:21:58.0000	*Guazuma ulmifolia*	EU020042
INB0003986152	519A	Palo Verde National Park	10:20:30.4000	−85:21:58.0000	*Acacia* spp.	EU020051
INB0003986153	520A	Palo Verde National Park	10:20:30.4000	−85:21:58.0000	*Acacia* spp.	EU020036
INB0003986154	521A	Palo Verde National Park	10:20:30.4000	−85:21:58.0000	*Acacia* spp.	EU020028
INB0003986156	522A	Palo Verde National Park	10:20:30.4000	−85:21:58.0000	*Acacia* spp.	EU020052
INB0003986158	523A	Palo Verde National Park	10:20:30.4000	−85:21:58.0000	Unknown	EU020043
INB0003986161	524A	Palo Verde National Park	10:23:09.7000	−85:21:16.4000	Unknown	EU020041
INB0003986162	525A	Barra Honda National Park	10:10:13.2000	−85:21:24.8000	Unknown	EU020035
INB0003986166	526A	Barra Honda National Park	10:09:59.0000	−85:21:26.5000	Vine-like plant climbing plant	EU020038
INB0003986170	527A	Barra Honda National Park	10:10:29.8000	−85:22:19.4000	Unknown	EU020044
INB0003986171	528A	Barra Honda National Park	10:10:29.8000	−85:22:19.4000	Unknown	EU020033
INB0003986172	529A	Barra Honda National Park	10:10:29.8000	−85:22:19.4000	Vine-like plant climbing plant	EU020040
INB0003986177	530A	Barra Honda National Park	10:10:29.8000	−85:22:19.4000	Unknown	EU020039
INB0003986185	531A	Hitoy Cerere Biological Reserve	09:40:19.9768	−83:01:44.5992	*Nectandra* spp.	EU020053
INB0003986187	532A	Hitoy Cerere Biological Reserve	09:40:19.9768	−83:01:44.5992	Unknown	EU020026
INB0003986188	533A	Hitoy Cerere Biological Reserve	09:40:23.0000	−83:01:27.0000	*Inga* spp.	EU020019
INB0003986189	534A	Cahuita National Park	09:43:06.4000	−82:48:55.0000	Unknown	EU020046
INB0003986190	535A	Cahuita National Park	09:43:06.4000	−82:48:55.0000	*Terminalia* spp.	EU020047
INB0003986191	536A	Cahuita National Park	09:43:06.4000	−82:48:55.0000	*Inga* spp.	EU020054
INB0003986192	537A	Cahuita National Park	09:43:06.4000	−82:48:55.0000	*Inga* spp.	EU020055
INB0003986193	538A	Cahuita National Park	09:43:06.4000	−82:48:55.0000	*Inga* spp.	EU020030
INB0003986194	539A	Cahuita National Park	09:43:06.4000	−82:48:55.0000	*Inga* spp.	EU020056

(*continued on next page*)

Table 1 – (*continued*)

INBio accession no. and isolate no.		Location	Latitude	Longitude	Host	GenBank no.
INB0003986086	540A	Hitoy Cerere Biological Reserve	09:40:19.9768	−83:01:44.5992	*Nectandra* spp.	EU020021
INB0003986087	541A	Hitoy Cerere Biological Reserve	09:40:19.9768	−83:01:44.5992	*Nectandra* spp.	EU020027
INB0003986092	542A	Hitoy Cerere Biological Reserve	09:40:23.0000	−83:01:27.0000	*Citrus* spp.	EU020024
INB0003986101	543A	Hitoy Cerere Biological Reserve	09:40:19.9768	−83:01:44.5992	Unknown	EU020031
INB0003986106	544A	Hitoy Cerere Biological Reserve	09:40:19.9768	−83:01:44.5992	Unknown	EU020045
INB0003986108	545A	Hitoy Cerere Biological Reserve	09:40:19.9768	−83:01:44.5992	Unknown	EU020057
INB0003986111	546A	Hitoy Cerere Biological Reserve	09:40:23.0000	−83:01:27.0000	Unknown	EU020025
INB0003986114	547A	Cahuita National Park	09:43:06.4000	−82:48:55.0000	Unknown	EU020058
INB0003986128	548A	Cahuita National Park	09:44:07.0000	−82:50:11.7000	Unknown	EU020023
INB0003986131	549A	Cahuita National Park	09:44:07.0000	−82:50:11.7000	Unknown	EU020020
INB0003986132	550A	Cahuita National Park	09:44:07.0000	−82:50:11.7000	Unknown	EU020059
INB0003986134	551A	Cahuita National Park	09:44:07.0000	−82:50:11.7000	*Terminalia* spp.	EU020022
INB0003986136	552A	Cahuita National Park	09:44:07.0000	−82:50:11.7000	*Terminalia* spp.	EU020060
INB0003986141	553A	Cahuita National Park	09:44:07.0000	−82:50:11.7000	*Terminalia* spp.	EU020061

All samples were cultivated on PDA (potato–dextrose agar; Difco) medium and incubated at 25 °C for 10 d, the isolate was then transferred to PDA and CMA (corn meal agar; Difco) and incubated at 25 °C for *ca* 15 d, at which time cultures were described. Cultures are maintained at INBio Culture collection.

at the sequencing facility of the Dana Farber Cancer Institute at the University of Harvard, Boston, using the same primers used for PCR. Sequences were edited and assembled using Bioedit (Hall 1999). The alignment was done using Clustal W (Thompson *et al.* 1994) and improved manually. Regions that could not be aligned unambiguously were excluded from the analysis.

Bayesian analyses were conducted using the MrBayes 3.1 program (Huelsenbeck & Ronquist 2001). The transversional model of nucleotide substitution assuming invariable sites (TVM + I) was selected by MrModeltest (Nylander 2004) using the akaike information criterion. MrBayes was run for 2 M generations with four chains. Trees were sampled every 100 generations for a total of 20 K trees. The first 100 K generations (the first 1 K trees) were deleted as the 'burn in' of the chain. The log-likelihood scores of sample points against generation time were plotted using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) to ensure that stationarity was achieved after the first 100 K generations by checking if the log-likelihood values of the sample points reached a stable equilibrium value. Of the remaining 19 K trees a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. PPs were obtained for each clade. These are estimated probabilities of the clades under the assumed model and hence PPs greater than 95 % are considered significant supports. The analyses was run three times to check for congruence and ensure the repetitively of the results.

A MP analysis was performed using the program PAUP (Swofford 2003). Heuristic searches with 200 random taxon addition replicates were conducted with tree bisection–reconnection (TBR) branch swapping and MulTrees option in effect, equally weighted characters and gaps treated as missing data. Bootstrapping (Felsenstein 1985) was performed based on 2 K replicates with random sequence additions. Phylogenetic trees were visualized using the program Treeview (Page 1996). *Plectophomella* sp. (GenBank AM286786) was used as an outgroup.

Nucleotide diversity or the average number of differences per site between two homologous sequences was calculated using the program DnaSP (Rozas & Rozas 1997). Wright's F_{ST} was used to describe what proportion of the total genetic variance is due to differences among clades. F_{ST} can range from 1.0, in which all of the variation is among populations, to 0.0, in which all populations appear homogeneous. F_{ST} was calculated *via* analyses of molecular variance (AMOVA) using the software package ARLEQUIN (Schneider *et al.* 1997).

Chemical studies

The *Rhytidhysteron* cultures were grown on a medium optimized in the bioprospecting unit of INBio for production of secondary metabolites consisting of a combination of a rich and a poorer medium. The rich medium (seed) consisted of peptone, malt extract, yeast, and glucose, and the cultures were incubated for 7 d at 25 °C in 150 ml flask on a rotary shaker at 150 rev min^{-1}; the seed plus the growing mycelium were transferred to 250 ml flask containing malt broth and Diaion HP-20 resin (Supelco, PA) and kept for 16 d on a rotary shaker at 25 °C at 150 rev min^{-1} and then incubated for an additional 5 d at 25 °C in a stationary phase. The cultures were harvested and filtered. The mycelium plus the resin were extracted twice with ethanol in an ultrasonic bath for 20 min, solvents were removed by rotary evaporation. All crude extracts were re-suspended in 50 ml water and cleaned three times using 25 ml hexane to eliminate the fatty material

(lipophilic) produced during fermentation, and were finally extracted three times with 25 ml ethyl acetate.

All samples underwent tlc analyses performed using palmarumycins MK-3018, CJ-12372, and CR_1 as controls. Each crude extract was dissolved in methanol and transferred onto aluminium sheets via chromatography 10×20 cm, silica gel 60 Merck 5553/F_{254} using dichloromethane–ethyl acetate (8:2) as the eluent system. The aluminium sheets were subsequently sprayed with potassium permanganate and heated in an oven. The presence or absence of the secondary metabolites palmarumycins MK-3018, CJ-12372, and CR_1 was revealed by comparison with the respective standards facilitated by the Chemical Laboratory of the Bioprospecting unit of INBio.

Hplc analyses were performed on a Waters hplc 2487, equipped with a 600E pump and a UV variable wavelength detector. Separations were done using an X-Terra column RP18 of 5 μm, 3.9×150 mm. Approximately 50 μg of each crude extract of methanol soluble materials, were analysed at 22 °C with a flow rate of 1 ml min^{-1} and the following gradient: system A: methanol:water 1:1 and system B: methanol, the elution lasts for 23 min and begins with 95 % A, reaching 100 % A in 3 min; then in 10 min it reaches 100 % B and it is kept with that proportion for 10 min The injection volume per sample was 20 μl. The UV detection wave length was set on 220 nm.

NMR analyses were performed at the Escuela de Química of the University of Costa Rica, San José, Costa Rica. Samples were freed of water using rotary evaporation under vacuum. Each sample consisted of approximately 10 mg and was dissolved in 0.6 ml deuterated methanol for proton NMR analyses. Pulse sequences and acquisition times were set for typical analysis: pw = 45°, taq = 3 s, with no pulse delay. When the solvent peak was too high, it was deleted using a WET programming sequence. ^{1}H-NMR spectra was inspected for typical palmarumycin signals in the aromatic region and palmarumycins were differentiated between them by inspecting chemical shifts in the aliphatic region. Dehydrocurvularin was identified using 1D (one dimension) and 2D (two dimension)-NMR experiments and final comparison of its spectral data (^{1}H and ^{13}C chemical shifts) with those from literature. Typical 2D experiments conducted were dqf-COSY (double quantum filtered correlation spectroscopy), gHSQC (gradient hetero single quantum coherence), and gHMBC (gradient hetero multiple bond correlation).

Results

Morphological classification

Forty-eight collections of *Rhytidhysteron rufulum* were studied morphologically. The following description is based on those specimens.

Ascomata erumpent, discoidal, lenticular to circular, solitary to gregarious; when dry the ascomata remaining discoidal or the edges of the disc reclosing by folding along one to three lines, becoming hysteriform, triangular or triradiate. Margin smooth to striate, black, carbonaceous, receptacle concolourous. Pseudoepithecium typically orange (cinnamon rufous 40), but varying from salmon (salmon 6) to red (chestnut 32 or amber 36) or green (greyish-olive 43) to black (jet black 89). Asci bitunicate, narrowly clavate, 6–8 spored, (144–)216–246 (–284) × (10.8–)12.8–15.8(–17.3) μm. Ascospores ellipsoidal to fusiform, brown, (23–)29.8–33.1(–37) × (7.7–)9.8–12.2(–14.4) μm, 3-cross septate. Paraphyses exceeding the asci, branching dichotomously immediately below the tip, the part above the ascal apices becoming blue–green in Meltzer's reagent. The asci measured were longer and broader and ascospores were broader than reported by Samuels & Müller (1979), asci 77–100 (–112) × 9–12(–17), ascospores (19–)26–36(–43) × 3.5–4.5(–6.5) μm but agree with the data of Kutorga & Hawksworth (1997), asci (180–)200–260(–275) × 15–17(–22) μm and ascospores (22–)25–35(–39) × (7.5–)9–12(–14) μm.

Comparative studies with similar species (Fig 1) revealed that the 48 Costa Rican collections belong to *R. rufulum* as circumscribed by Samuels & Müller (1979).

Molecular phylogenetic analysis

DNA sequences obtained included partial ITS1, the 5.8S gene, and the ITS2 region. The first 110 bp of the ITS1 region were excluded due to ambiguous alignment. The sequence alignment included 499 unambiguously aligned positions. Of these, 145 were variable and 57 parsimony informative. Base frequencies were 0.22 for A, 0.22 for T, 0.27 for G, and 0.29 for C. Nucleotide diversity was 0.03325 ± 0.0022. MP analysis yielded 446 most parsimonious trees of 79 steps long (CI = 0.84, RI = 0.97). The likelihood parameters in the Bayesian tree sample had the following mean (variance): LnL = −1212.701 (0.16), base frequencies $\pi(A) = 0.22$ (0.00091), $\pi(C) = 0.293$ (0.00073), $\pi(G) = 0.264$ (0.00086), $\pi(T) = 0.223$ (0.00092), and p(invar) = 0.732 (0.00062). The topologies of the MP and Bayesian MCMC analyses were not in conflicts. In the MP and majority rule consensus tree of the Bayesian tree sampling (Fig 2), *R. rufulum* falls into four distinct clades. All these clades are strongly supported (MP BS: 99 %, PP 1.0), but the relationships among the clades lack strong support.

The clades do not correspond with geographical origin, except clade IV, which was only collected in Palo Verde and clade III, which was only collected in the pacific side of Costa Rica. Main variations observed were between clades (Table 2).

As the molecular analysis suggested that four different isolated lineages are involved in the *R. rufulum* complex in Costa Rica, we re-examined the morphology and anatomy of the samples. We found clade-related differences in ascoma morphology that previously have been regarded as intraspecific variation. The ascomata in clade I have a smooth margin and cinnamon–rufous (40) pseudoepithecium, whereas clade II includes samples with striate margin, black (89), greyish olive (43) or raw umber (123) pseudoepithecium. Clade III includes specimens with striate margins and a chestnut (32), amber (36) or sepia (219A) coloured disc. Clade IV consists of only three isolates from Palo Verde with striate margin and salmon (6) pseudoepithecium (Table 3). Asci and ascospores in collections from clades III and IV were shown by variance analysis to be significantly shorter and narrower than clades I and II: ascus length, $F = 29.71$, $P = 0.0001$; ascus breadth, $F = 73.93$. $P = 0.0001$; ascospore length $F = 35.7$, $P = 0.0001$ and

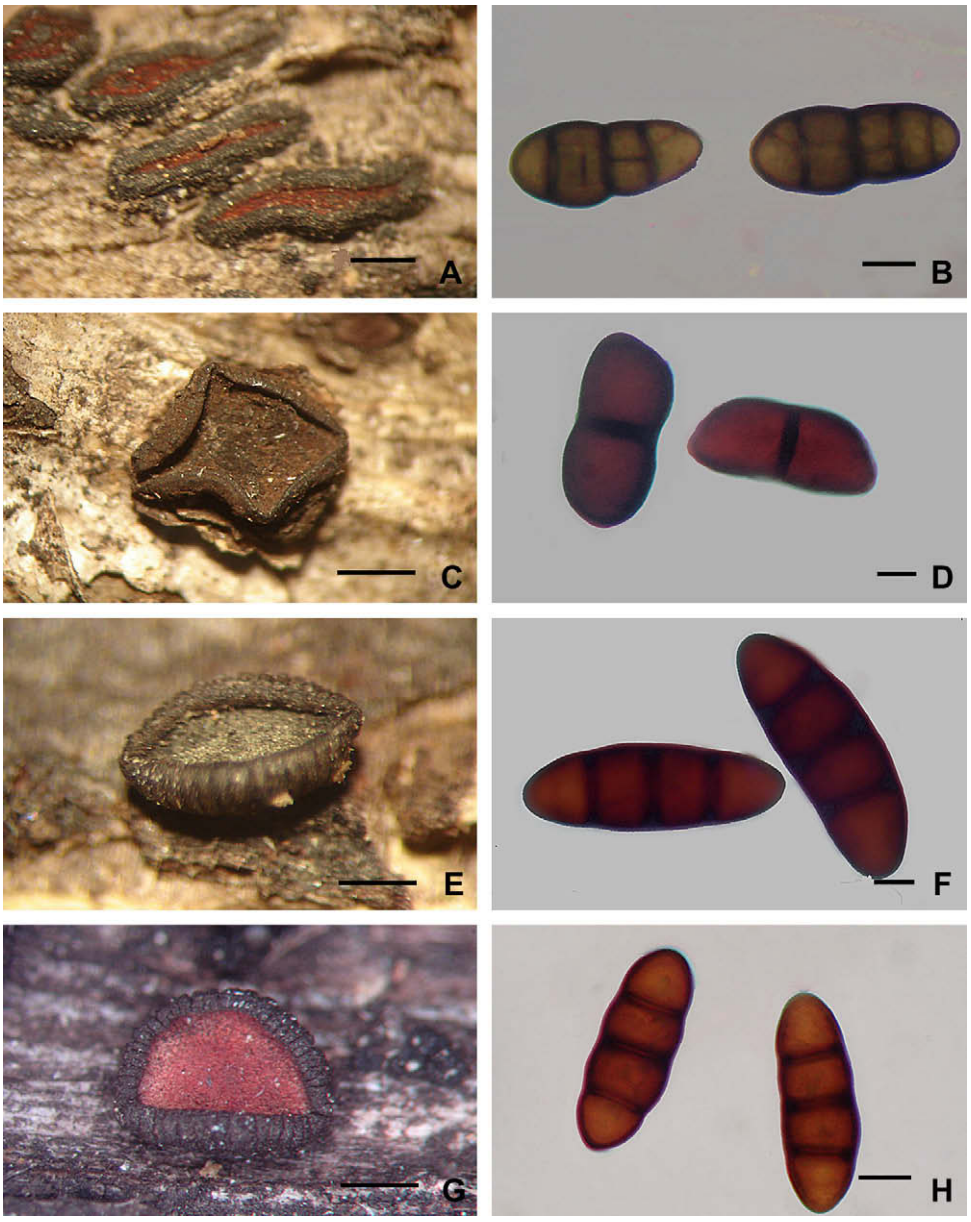

Fig 1 – Morphology of *Rhytidhysteron* species. (A–B) *R. opuntiae*: (A) ascoma, (B) ascospore. (C–D) *R. hysterinum*: (C) ascoma, (D) ascospore. (E–F) *R. rufulum*: (E) ascoma, (F) ascospores. (G–H) *R. rufulum* neotype: (G) ascoma, (H) ascospore. Bars = (A,C,E,G) 0.5 mm; (B,D,F,H) 5 μm.

ascospore breadth, $F = 16.23$, $P = 0.0001$. Clades I and II only differ in ascus breadth and ascospore length (Table 3).

The collections from clade IV were exclusively collected from *Acacia* spp. in the Palo Verde National Park, even though other hosts were sampled (Table 1); the remaining clades did not show any host preference and further, a large number of hosts remained unidentified (33.3 % clade I, 29.4 % clade II, and 61.5 % clade III). No single host dominated in clade I (26.7 % from *Acacia* spp., 13.3 % from *Inga* spp. 13.3 % from *Nectandra* spp., and 6.7 % from *Citrus*, 6.7 % from *Terminalia* spp.), clade II (29.4 % from *Acacia* spp., 17.7 % from Inga spp., 17.7 % from *Terminalia* spp., and 5.9 % from *Nectandra* spp.) and clade III (15.4 % from vine-like climbing plant, 15.4 % from *Acacia* spp. and 7.7 % from *Guazuma ulmifolia*).

Secondary metabolites

Chemical analysis of extracts was conducted using three different analytical methods. A preliminary detection of palmarumycins was carried out using tlc and three available palmarumycin standards, MK-3018, Palmarumycin CR_1, and CJ 12,372. As a result, extracts were grouped in three clades based on this preliminary analysis. Hplc–uv fingerprint of extracts showed also three distinctive chromatogram profiles, whereas the first was consistent with the presence of palmarumycins (Fig 3A–B), the second with the presence of one major compound (Fig 3C), and the third with compounds not related to neither of the first two groups. Analysis of ^{1}H-NMR spectra agreed with the presence of palmarumycins in extracts from

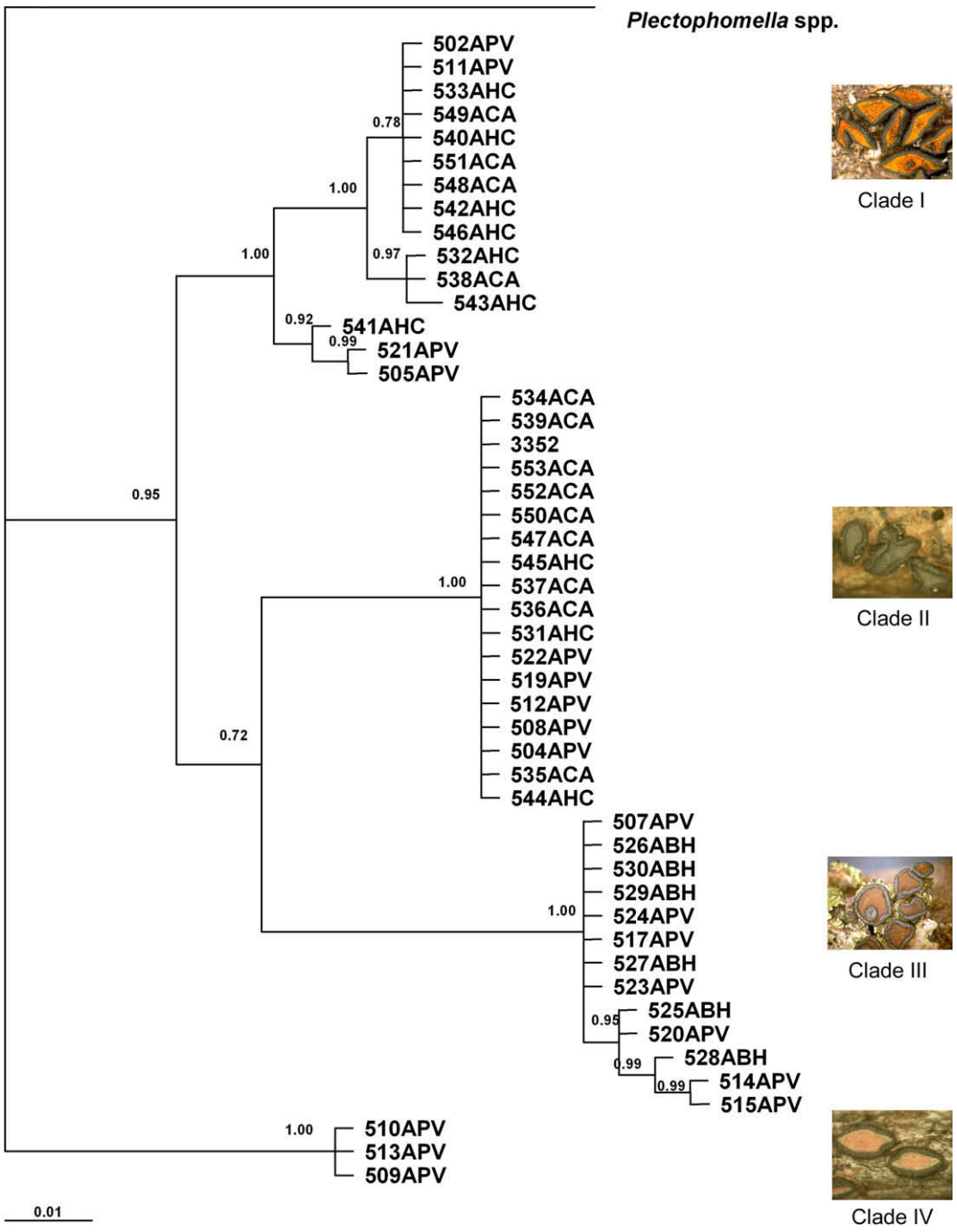

Fig 2 – Phylogeny of *Rhytidhysteron* isolates from Costa Rica as inferred from an ITS sequence analysis. This is a 50 % majority-rule consensus tree based on 19 K trees from a Bayesian MCMC tree sampling procedure based on 501 bp. Collecting sites are given immediately after the identifier: Palo Verde (PV), Barra Honda (BH), Cahuita (CA), and Hitoy Cerere (HC). *Plectophomella* spp. was used as out group.

clade I and II, whereas extracts from clade III resulted on a different compound, which was identified by further NMR analysis as dehydrocurvularin (Giselle Tamayo & Daniel Torres, pers. comm.). Compounds of clade IV are not related to palmarumycins and are currently being studied for their chemical composition.

As a result, three groups were recognized based on this monitoring profile (Table 3). These chemical groups agree with the clades found in the ITS analysis. Members of clade I and clade II comprising 69.8 % of the samples produced compounds CJ 12,372 (Rf = 0.43, Rt = 10.34 min), MK3018 (Rf = 0.32, Rt = 8.18 min), and palmarumycin CR_1 (Rf = 0.01, Rt = 8.92 min), 23.2 % all belonging to clade III, produced only dehydrocurvularin (Rf = 0.40, Rt = 4.40); 7 %, members of clade IV, did not produce neither palmarumycins nor dehydrocurvularin.

Table 2 – Analyses of molecular variance (AMOVA) results and fixation index (Fst) inferred from the ITS region sequences from isolates of *Rhytidhysteron rufulum*, Costa Rica

Source of variation	df	Sum of squares	Variance components	Percentage variation
Among clades	3	366.155	10.85097 Va	94.92
Within clades	44	25.572	0.58119 Vb	5.08
Total	47	391.727	11.43215	
Fixation index		F_{ST}: 0.94916		

Discussion

This study assessed the variability and differentiation of the genus *Rhytidhysteron* present in Costa Rica, including sites from the Pacific and Atlantic coast to understand the diversity of these microfungi. Based on morphological characteristics, our initial and general morphological analysis were consistent with the conclusion that all samples from Costa Rica belong to *R. rufulum* as currently circumscribed (Samuels & Müller 1979; Barr 1987; Kutorga & Hawksworth 1997).

The most conspicuous and determined morphological characteristic to differentiate species in *Rhytidhysteron* is ascospore septation. *R. hysterinum* differs in having one-septate ascospores (Samuels & Müller 1979), whereas the ascospores in *R. dissimile* are 5-septate (Magnes 1997). *Rhytidhysteron opuntiae* has 1–3(4–5) septate ascospores, with one longitudinal septum in mid cells (Barr 1990) (Fig 1).

The use of a polyphasic approach in species recognition has been very useful. The detailed examination of different characters within a variable species shows in some cases that they can be divided into different species or groups. In fungi there are examples in the genera *Aspergillus* (Hong *et al.* 2005; Houbraken *et al.* 2007; Varga *et al.* 2007), *Penicillium* (Frisvad *et al.* 2007), *Alternaria* (Andersen *et al.* 2002; Andersen *et al.* 2008) and in different groups of lichen-forming fungi (Kroken & Taylor 2001; Högnabba & Wedin 2003; Molina *et al.* 2004; Arguello *et al.* 2007; Lücking *et al.* 2008; Lumbsch *et al.* 2008; Wirtz *et al.* 2008).

In the case of *R. rufulum*, the ITS nuDNA region analysis suggested that *R. rufulum* complex in Costa Rica falls into four distinct clades that are strongly supported. The nucleotide diversity of the ITS region was low within clades. This could be the outcome of effective dispersal patterns of *R. rufulum* coupled with the fact that *R. rufulum* is homothallic (Bezerra & Kimbrough 1982). Low genetic variation has been reported in other common fungi by Rydholm *et al.* (2006) who explained the lack of population structure on a global scale in *Aspergillus fumigatus* by continual gene flow across continents that resulted from wind dispersal of the UV-resistant conidia. Future analyses of additional genes are necessary to understand genetic structure within *R. rufulum* clades.

Rhytidhysteron is a genus of saprobes or weak parasites on woody plants and is uncommon in temperate regions. *R. rufulum* is a generalist, capable of utilizing different substrata and occupying diverse habitats, probably because it is a self-fertile, homothallic species (Bezerra & Kimbrough 1982) and hence able to maintain sexual reproduction in the absence of compatible mycelia (Kendrick 1992), but wind dispersal could also be another factor that contributes to their widespread distribution. Clade I and II include isolates that easily colonize different substrates and environments. For example, in the Pacific sites it was found in *Acacia* spp. (*Fabaceae*), *Guazuma ulmifolia* (*Sterculiaceae*) or *Samanea saman* (*Fabaceae*), and in the Atlantic sites in *Inga* spp. (*Fabaceae*), *Terminalia* spp. (*Combretaceae*), *Nectandra* spp. (*Lauraceae*) and *Citrus* spp. (*Rutaceae*).

In our study we observed a higher genetic diversity in the Pacific region, especially in the dry forest of Palo Verde, which hosted samples of all four clades. This agrees with studies showing higher diversity of wood-inhabiting organisms in logs decomposing in dry than in wet forest (Torres & Gonzalez 2005), mainly those groups for example *Xylariales*, that initially colonized the dry fallen logs (Milagro Mata & Loengrin Umaña, pers. comm.).

With a more detailed analysis we have found morphology of ascomata, ascus, and ascospore size, informative for the characterization of the distinct clades in *R. rufulum*, in contrast to previous studies (Samuels & Müller 1979). Morphological characters, such as shape and colour of ascomata, have been useful in some cases as taxonomic information. In

Table 3 – Differences between the clades found in the molecular analyses

Clade	Ascus length (μm)	Ascus breadth (μm)	Ascospore length (μm)	Ascospore breadth (μm)	Margin	Pseudoepithecium colour	Metabolites present
I	(215–)234–256 (–269) A	(12.6–)13.9–15.2 (–16) A	(27.9–)30.9–32.2 (–36.9) A	(9.2–)10.6–12.3 (–14.3) A	Smooth	Cinnamon–rufous (40)	MK3018, CJ 12,372, CR_1
II	(218–)224–257 (–284) A	(15–)15.7–16.7 (–17.3) B	(31.3–)32.6–36.4 (–37) B	(10.4–)11.7–13 (–14.4) A	Striate	Black (89), greyish olive (43) or raw umber (123)	MK3018, CJ 12,372, CR_1
III	(183–)200–221 (–237) B	(11.6–)12.2–13 (–13.5) C	(25.8–)29.1–30.3 (–31.7) C	(8.2–)9.1–10.5 (–11.6) B	Striate	Chestnut (32), amber (36) or sepia (219A)	None of the monitor metabolites
IV	144–168 C	10.7–11.9 D	23–23.8 D	7.7–9.4 C	Striate	Salmon (6)	Dehydrocurvularin

Values of the continuous characters measured, different uppercase letter by columns indicate significant differences between clades ($P \leq 0.05$) by the Tukey test, margin shape, pseudoephitecium colour, and presence of the metabolites monitored.

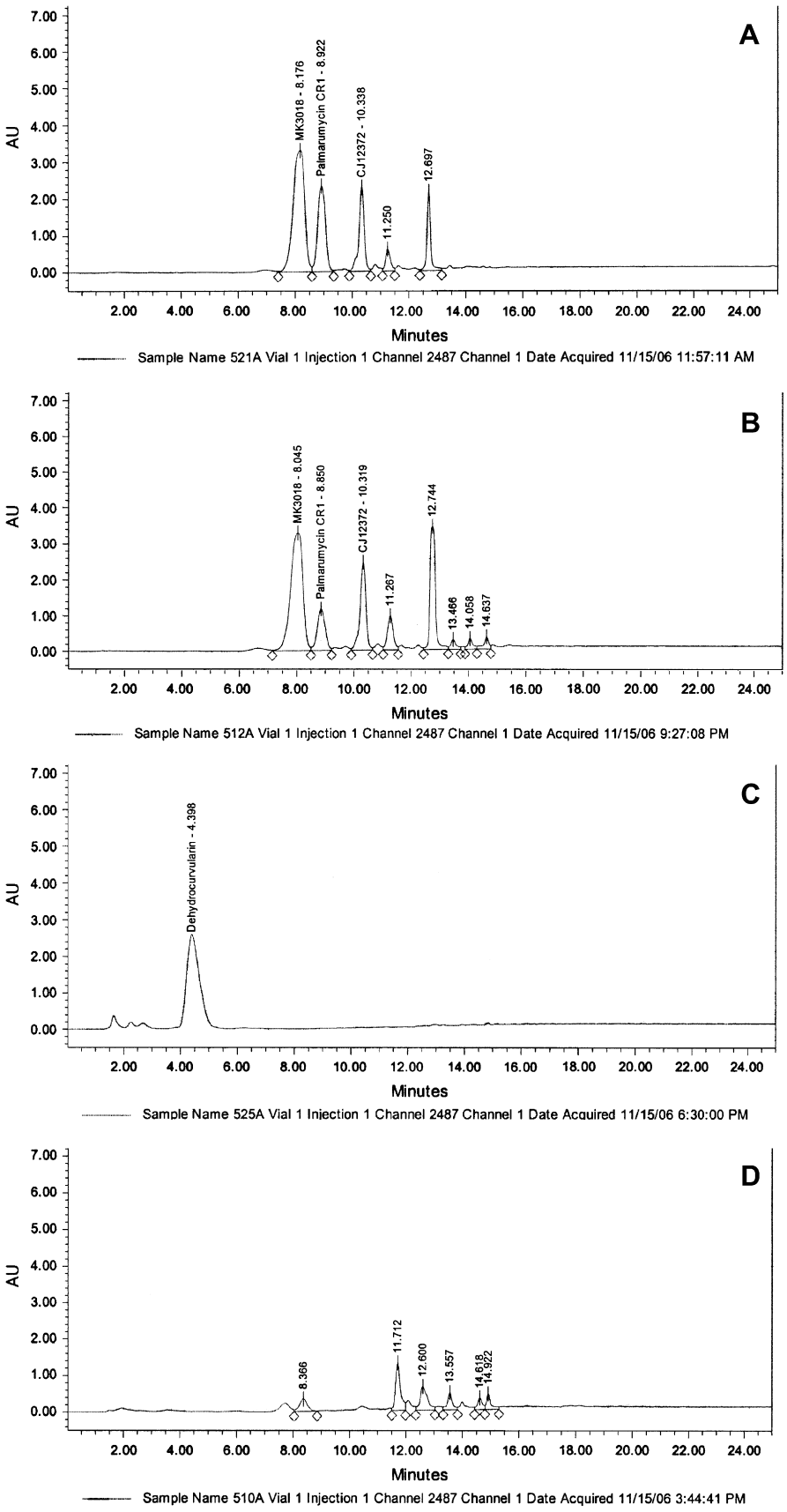

Fig 3 – Hplc traces (220 nm) of crude extracts prepared from liquid cultures of *Rhytidhysteron rufulum* from Costa Rica. The occurrence of the peaks is indicated in the corresponding chromatograms (A) sample 521A-clade I, (B) sample 512A-clade II, (C) sample 525A-clade III, and (D) sample 510A-clade IV.

Xylariaceae with *Nodulisporium*-like anamorphs, numerous species are characterized by conspicuous colours of their ascomata (Stadler *et al.* 2004), and in *Helotiales* they are usually characteristic at family level. The colour of the pseudoepithecium is variable within *R. rufulum*. Samuels & Müller (1979) did not find any correlation between the pseudoepithecium colour and the ascospore size. However, we demonstrated correlation with the four clades found in the molecular analyses and further showed correlation with ascus and/or ascospore size in several clades.

The combination of analytical methods succeeded in grouping isolates in three different chemical groups. It is important to highlight that hplc and tlc analysis were based on the presence and absence of the three palmarumycin standards available for this study. Other palmarumcyns, such as CR_2 and CJ-12,371 and CP_2, which are also present in extracts from clade I and II but in minor concentration, were not available and hence their presence can only be inferred by their ^{1}H-NMR spectra. These and other unidentified compounds present in the isolates could help in future investigations to chemically distinguish these two groups. ^{1}H-NMR was also helpful in establishing the identity of the major component of clade III.

R. rufulum isolates from clade I and II, collected in different sites of Costa Rica produced the palmarumycins MK-3018, CJ-12372 and CR_1. The palmarumycin MK-3018, which was first isolated in 1989 from *Tetraploa aristata* (*Dothideomycetes*, *Pleosporales*), showed antimicrobial activity. It was the first representative of a new family of bioactive natural products based on a 1,8-dihydroxynaphtalene-derived spiroketal unit linked to a second, oxidized naphtalene moiety. CJ-12372 was isolated from the endophytic fungus *Coniothyrium palmarum* (*Pleosporales*) and related *Coniothyrium* species and was shown to be a DNA gyrase inhibitor that possesses antifungal, antibacterial, and herbicidal activity (Ragot *et al.* 1999).

Palmarumycin CR_1 was isolated from *Rhytidhysteron* spp. (Wipf *et al.* 2004). Palmarumycins also can produce potent inhibitors of the thioredoxin–thioredoxin reductase cellular redox systems.

The secondary metabolites CJ-12372 and CR_1 showed no cytotoxicity and only insignificant inhibition of the thioredoxin–thioredoxin reductase cellular redox system whereas MK-3018 showed more activity in this system but also more cytotoxicity, demonstrating that the presence of an enone function is necessary for biological activity in this class of fungal metabolites (Wipf et *al.* 2004). Parallel to the determination of thioredoxin–thioredoxin reductase inhibition, these compounds were also tested for their anti-chagasic potential, where MK-3018 again was the only active compound (G.T. *et al.*, unpubl.).

Samples from clade III only collected in the Pacific side produced a secondary metabolite identified as dehydrocurvularin. This compound has been reported in different *Pleosporales* taxa, such as *Cochliobolus spicifer*, *Curvularia* spp., *Alternaria* spp., and in *Penicillium* spp. (*Eurotiales*) (Almassi *et al.* 1994). Dehydrocurvularin inhibits cell division in sea urchin embryonic development and its phytotoxicity and antimicrobial activity has been demonstrated (Ghisalberti & Rowland 1993). Dehydrocurvularin affects a broad spectrum of plant species inhibiting cell division through its disturbance of the microtubule assembly (Thomma 2003) and also showed nematicidal activity (Kusano *et al.* 2003). Clade IV produces compounds that are apparently different from the palmarumycins and dehydrocurvularin, which will be further studied.

The compounds found in extracts of *R. rufulum* were previously isolated mainly from fungi in *Pleosporales*. Samuels & Müller (1979) noted that taxonomic relationships of *Rhytidhysteron* are uncertain. Blackwell *et al.* (2006) included *Patellariales* in an uncertain position in the *Dothideomycetes* and found that *Pleosporales* and *Patellariales* are phylogenetically related orders classified in the *Dothideomycetes*. The presence of these secondary metabolites supports a close relationship of *Pleosporales* and *Patellariales*.

In conclusion, the combined data analysed in this study provided better taxonomic insights within *R. rufulum* and strongly suggest that four lineages are present in the Costa Rican samples. Although only a single gene region (ITS region), was studied, morphological and chemical characters support the groups detected using molecular markers. The variation of these characters needs further evaluation using material from other regions and a re-study of the types of species previously described in this group of fungi is necessary before nomenclatural conclusions can be drawn.

Acknowledgements

We thank the Ministry of the Environment and Energy for the access to Costa Rica natural resources, under the permits R-006-2005-OT and R-CM-INBIO-05-2006-OT; the Escuela de Química of the University of Costa Rica for the Nuclear Magnetic Resonance (NMR) measurements, Gary J. Samuels (USDA-ARS, Baltimore, MD) for critical reading and suggestions on this work, Instituto Nacional de Biodiversidad (INBio), the staff of the Bioprospecting and Fungi unit for all the support given, especially Allan Jiménez and Loengrin Umaña. Sequencing was carried out at Dana Farber/Harvard Cancer Center DNA Resource Core. This study was supported by Consejo Nacional para Investigaciones Científicas y Tecnológicas de Costa Rica (CONICIT) 157-2005, funds from the Netherland government (NIDA program) through the Bioprospecting unit (INBio) and partially funded by the Cooperative Agreement NIH-FIC-USDA U01 TW007404 International Cooperative Biodiversity Groups (ICBG) program, sponsored by the Fogarty International Center (FIC), National Institutes of Health (NIH) and the United States Department of Agriculture (USDA).

REFERENCES

Almassi F, Ghisalberti EL, Skelton BW, White AH, 1994. Structural study of dehydrocurvularin, an inhibitor of microtubule assembly. *Australian Journal of Chemistry* **47**: 1193–1197.

Andersen B, Kroger E, Roberts RG, 2002. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research* **106**: 170–182.

Andersen B, Dongo A, Pryor BM, 2008. Secondary metabolite profiling of *Alternaria dauci*, *A. porri*, *A. solani* and *A. tomatophila*. *Mycological Research* **112**: 241–250.

Arguello A, Del Prado R, Cubas P, Crespo A, 2007. *Parmelina quercina* (*Parmeliaceae*, *Lecanorales*) includes four phylogenetically supported morphospecies. *Biological Journal of the Linnean Society. Linnean Society of London* **91**: 455–467.

Barr ME, 1987. *Prodromus to Class Loculoascomycetes*. University of Massachusetts.

Barr ME, 1990. *Rhytidhysteron opuntiae*. *Memoirs of the New York Botanical Garden* **62**: 72.

Bezerra JL, Kimbrough JW, 1982. Culture and cytological development of *Rhytidhysterium rufulum* on citrus. *Canadian Journal of Botany* **60**: 568–579.

Blackwell M, Hibbett DS, Taylor JW, Spatafora JW, 2006. Research coordination networks: a phylogeny for Kingdom *Fungi* (Deep Hypha). *Mycologia* **98**: 829–837.

Crous PW, Groenewald JZ, Pongpanich K, Himaman W, Arzanlou M, Wingfield MJ, 2004. Cryptic speciation and host specificity among *Mycosphaerella* sp. occurring on Australian *Acacia* species grown as exotics in the tropics. *Studies in Mycology* **50**: 457–469.

Dettman JR, Jacobson DJ, Taylor JW, 2006. Multilocus sequence data reveal extensive phylogenetic species diversity within the *Neurospora discreta* complex. *Mycologia* **98**: 436–446.

Felsenstein J, 1985. Confidence-limits on phylogenies — an approach using the bootstrap. *Evolution* **39**: 783–791.

Fernández FA, Lutzoni F, Huhndorf SM, 1999. Teleomorph–anamorph connections: the new pyrenomycetous genus *Carpoligna* and its *Pleurothecium* anamorph. *Mycologia* **91**: 251–262.

Frisvad JC, Larsen TO, De Vries R, Meijer M, Houbraken J, Cabañes FJ, Ehrlich K, Samson RA, 2007. Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. *Studies in Mycology* **59**: 31–37.

Gardes M, Bruns T, 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of micorrhizae and rust. *Molecular Ecology* **2**: 113–118.

Ghisalberti EL, Rowland CY, 1993. 6 Chlorodehydrocurvularin. A new metabolite from *Cochliobolus spicifer*. *Journal of Natural Products* **6**: 2175–2177.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hawksworth DL, 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research* **105**: 1422–1432.

Högnabba F, Wedin M, 2003. Molecular phylogeny of the *Sphaerophorus globosus* species complex. *Cladistics* **19**: 224–232.

Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA, 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* **97**: 1316–1329.

Houbraken J, Due M, Varga J, Meijer M, Frisvad JC, Samson RA, 2007. Polyphasic taxonomy of *Aspergillus* section *Usti*. *Studies in Mycology* **59**: 107–128.

Huelsenbeck JP, Ronquist F, 2001. Mr. Bayes: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

INFOSTAT, 2002. *InfoStat Versión 1.1*. Grupo InfoStat. Fac. Cs. Agr. Universidad Nacional de Córdoba, Argentina.

Kendrick B, 1992. *The Fifth Kingdom*, 2nd edn. Focus Information Group, Newburyport, MA.

Kroken S, Taylor JW, 2001. A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia*. *Mycologia* **93**: 38–53.

Kusano M, Nakagami K, Fujioka S, Kawano T, Shimada A, Kimura Y, 2003. βγ-Dehydrocurvularin and related compounds as nematicides of *Pratylenchus penetrans* from the fungus *Aspergillus* sp. *Bioscience Biotechnology and Biochemistry* **67**: 1413–1416.

Kutorga E, Hawksworth DL, 1997. A reassessment of the genera referred to the family *Patellariaceae*. *Systema Ascomycetum* **15**: 1–110.

Lücking R, Del Prado R, Lumbsch HT, Will-Wolf S, Aptroot A, Sipman H,Umaña L, Chaves JL, 2008. Phylogenetic patterns of morphological and chemical characters and reproductive mode in the *Heterodermia obscurata* group in Costa Rica (*Ascomycota*, *Physciaceae*). *Systematics and Biodiversity* **5**: 31–41.

Lumbsch HT, Nelsen MP, Lücking R, 2008. The phylogenetic position of *Haematommataceae* (*Lecanorales*, *Ascomycota*). *Nova Hedwigia* **96**: 105–114.

Magnes M, 1997. Weltmonographie der *Triblidiaceae*. *Bibliotheca Mycologica* **165**: 127–130.

Mata M, 2003. *Macrohongos de Costa Rica*. Instituto Nacional de Biodiversidad, Heredia, Costa Rica.

Molina MC, Crespo A, Blanco O, Lumbsch HT, Hawksworth DL, 2004. Phylogenetic relationships and species concepts in *Parmelia* s.str. (*Parmeliaceae*) inferred from nuclear ITS rDNA and beta-tubulin sequences. *Lichenologist* **36**: 37–54.

Mueller GM, Schmit JP, 2007. Fungal biodiversity: what do we know? What can we predict? *Biodiversity and Conservation* **16**: 1–5.

Nylander JA, 2004. *MrModeltest V2*. Program Distributed By the Author. Evolutionary Biology Centre, Uppsala University.

Page RDM, 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357–358.

Pringle A, Baker DM, Platt JL, Wares JP, Latge JP, Taylor JW, 2005. Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. *Evolution* **59**: 1886–1899.

Ragot JP, Steeneck C, Alcaraz ML, Taylor RJK, 1999. The synthesis of 1,8-dihydroxynaphthalene-derived natural products: palmarumycin CP1, palmarumycin CP2, palmarumycin C11, CJ-12,371, deoxypreussomerin A and novel analogues. *Journal of the Chemical Society. Perkin Transactions 1* **1**: 1073–1082.

Rehner SA, Buckley E, 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* **97**: 84–98.

Rozas J, Rozas R, 1997. DnaSP version 2.0: a novel software package for extensive molecular population genetics analysis. *Computer Applications in the Biosciences* **13**: 307–311.

Rydholm C, Szakacs G, Lutzoni F, 2006. Low genetic variation and no detectable populations tructure in *Aspergillus fumigatus* compared to closely related *Neosastorya* species. *Eukaryotic Cell* **5**: 650–657.

Samuels GJ, Müller E, 1979. Life-history studies of Brazilian ascomycetes. *Rhytidhysteron rufulum* and the genus *Eutryblidiella*. *Sydowia* **32**: 277–292.

Schneider S, Kueffer JM, Roessli D, Excoffier L, 1997. *ARLEQUIN: A Software for Population Genetic Data Analysis*. Ver 1.1. Genetics and Biometry Lab, University of Geneva.

Smithe FB, 1975. *Naturalist's Color Guide*. American Museum of Natural History, New York.

Stadler M, Ju YM, Rogers JD, 2004. Chemotaxonomy of *Entonaema Rhopalostroma* and other *Xylariaceae*. *Mycological Research* **108**: 239–256.

Swofford DL, 2003. *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, version 4.0b 10. Sinauer Associates, Sunderland, Massachusetts.

Taylor JW, Turner E, Townsend JP, Dettman JR, Jacobson D, 2006. Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. *Philosophical Transactions of the Royal Society of London. Series B,* **361**: 1947–1963.

Thomma BPHJ, 2003. *Alternaria* sp.: from general saprophyte to specific parasite. *Molecular Plant Pathology* **4**: 225–236.

Thompson JD, Higgins DG, Gibson TJ, 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment

through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Torres JA, Gonzalez G, 2005. Wood decomposition of *Cyrilla racemiflora (Cyrillacea)* in Puerto Rican dry and wet forests: a 13-year case study. *Biotropica* **37**: 452–456.

Varga J, Frisvad JC, Samson RA, 2007. Polyphasic taxonomy of *Aspergillus* section *Candidi* based on molecular, morphological and physiological data. *Studies in Mycology* **59**: 75–88.

Wipf P, Lynch S, Birmingham A, Tamayo G, Jiménez A, Campos N, Powis G, 2004. Natural product based inhibitors of the thioredoxin–thioredoxin reductase system. *Organic and Biomolecular Chemistry* **2**: 1651–1658.

Wirtz N, Printzen C, Lumbsch HT, 2008. The delimitation of Antarctic and bipolar species of *Usnea, Neuropogon* (*Ascomycota*, *Lecanorales*): a cohesion approach of species recognition for the *Usnea perpusilla* complex. *Mycological Research* **112**: 472–484.