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Phylogenetic analysis of *Monascus* and new species from honey, pollen and nests of stingless bees

R.N. Barbosa[1,2], S.L. Leong[3], O. Vinnere-Pettersson[3,4], A.J. Chen[1,5], C.M. Souza-Motta[2], J.C. Frisvad[6], R.A. Samson[1], N.T. Oliveira[2], and J. Houbraken[1*]

[1]*Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands;* [2]*Departamento de Micologia Prof. Chaves Batista, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, s/n, Centro de Biociências, Cidade Universitária, CEP: 50670-901 Recife, PE, Brazil;* [3]*Swedish University of Agricultural Sciences, Department of Molecular Sciences, Box 7015, SE-750 07 Uppsala, Sweden;* [4]*National Genomics Infrastructure-Sweden, Science for Life Laboratory, Department of Immunology, Genetics and Pathology, Uppsala University, BMC, Box 815, SE-752 37 Uppsala, Sweden;* [5]*Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100193, PR China;* [6]*Department of Biotechnology and Biomedicine, Technical University of Denmark, 2800 Kongens Lyngby, Denmark*

**Correspondence*: J. Houbraken, j.houbraken@westerdijkinstitute.nl

Abstract: The genus *Monascus* was described by van Tieghem (1884) to accommodate *M. ruber* and *M. mucoroides*, two species with non-ostiolate ascomata. Species delimitation in the genus is still mainly based on phenotypic characters, and taxonomic studies that include sequence data are limited. The genus is of economic importance. Species are used in fermented Asian foods as food colourants (e.g. 'red rice' (ang-kak, angka)) and found as spoilage organisms, and recently *Monascus* was found to be essential in the lifecycle of stingless bees. In this study, a polyphasic approach was applied combining morphological characters, ITS, LSU, β-tubulin, calmodulin and RNA polymerase II second largest subunit sequences and extrolite data, to delimit species and to study phylogenetic relationships in *Monascus*. Furthermore, 30 *Monascus* isolates from honey, pollen and nests of stingless bees in Brazil were included. Based on this polyphasic approach, the genus *Monascus* is resolved in nine species, including three new species associated with stingless bees (*M. flavipigmentosus* sp. nov., *M. mellicola* sp. nov., *M. recifensis* sp. nov., *M. argentinensis*, *M. floridanus*, *M. lunisporas*, *M. pallens*, *M. purpureus*, *M. ruber*), and split in two new sections (section *Floridani* sect. nov., section *Rubri* sect. nov.). Phylogenetic analysis showed that the xerophile *Monascus eremophilus* does not belong in *Monascus* and monophyly in *Monascus* is restored with the transfer of *M. eremophilus* to *Penicillium* (*P. eremophilum* comb. nov.). A list of accepted and excluded *Monascus* and *Basipetospora* species is given, together with information on (ex-)types cultures and barcode sequence data.

Key words: Aspergillaceae, Extrolites, Fungal ecology, Phylogeny, Taxonomy.
Taxonomic novelties: New sections: *Monascus* section *Floridani* R.N. Barbosa & Houbraken, *Monascus* section *Rubri* R.N. Barbosa & Houbraken; **New species:** *Monascus flavipigmentosus* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken, *Monascus mellicola* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken, *Monascus recifensis* R.N. Barbosa, Souza-Motta, N.T. Oliveira & Houbraken; **New combination:** *Penicillium eremophilum* (A.D. Hocking & Pitt) Houbraken, Leong & Vinnere-Pettersson.

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INTRODUCTION

Van Tieghem (1884) introduced the genus *Monascus* for species that produce non-ostiolate ascomata and introduced two species, *M. ruber* and *M. mucoroides*. The position of *Monascus* (and the *Monascaceae*) has been the subject of discussion in various papers and it was often placed outside the order *Eurotiales* (Benny & Kimbrough 1980, von Arx 1987, Stchigel & Guarro 2007), but phylogenetic analyses confidentially places this genus in *Aspergillaceae* (*Eurotiales*) (Berbee *et al.* 1995, Ogawa *et al.* 1997, Ogawa & Sugiyama 2000, Peterson 2008, Houbraken & Samson 2011, Vinnere-Pettersson *et al.* 2011). The genus *Basipetospora* was found to be the anamorph of *Monascus* and is characterized by the production of aleurioconidia in a basipetal manner from undifferentiated conidiogenous cells that progressively shorten (retrogression, Cole & Samson 1979). The conidia have a truncated base and resemble chlamydospores. These features set this genus apart from the phylogenetically related genera *Aspergillus* and *Penicillium*.

After the description of the genus, more than 20 species have been introduced and many of them are considered to be synonyms (Shao *et al.* 2011). Classification of *Monascus* has primary been based on macro- and microscopic features, such as the pigmentation of the cleistothecial walls and conidia and growth rates on agar media. Hawksworth & Pitt (1983) revised the genus based on physiological and morphological characteristics and reduced the number of accepted species to three: *M. pilosus*, *M. ruber* and *M. purpureus*. Since that study, ten new species were introduced: *M. albidulus*, *M. argentinensis*, *M. aurantiacus*, *M. eremophilus*, *M. floridanus*, *M. fumeus*, *M. lunisporas*, *M. pallens*, *M. rutilus* and *M. sanguineus* (Barnard & Cannon 1987, Hocking & Pitt 1988, Cannon *et al.* 1995, Udagawa & Baba 1998, Stchigel *et al.* 2004, Li & Guo 2004). With the description of those species, the genus became morphologically and physiologically more diverse, suggesting a large genetic diversity. For example, *Monascus ruber* grows rapidly on agar media, *M. lunisporas* and *M. pallens* grow restrictedly and *M. eremophilus* is a strict xerophile and only grows on low water activity media. The phenotype-based identification schemes in *Monascus* were difficult to match with the results obtained by ITS, partial LSU and/or β-tubulin gene sequencing (Park & Jong 2003, Park *et al.* 2004). Nowadays, species can be delimited on the genotype, for example based on the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept. The

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application of this concept in *Monascus* has yet not been performed and the results of such an analysis will give insight on the species boundaries.

The genus *Monascus* has economic importance in several areas, and several species have been widely used for over years in the production of yellow and red food colourants and Asian fermented foods, particularly red rice (ang-kak, angka, 'red kojic rice'). Red rice is of particular interest because of its health promoting effects (Lee & Pan 2011, 2012, Hsu & Pan 2012, Shi & Pan 2012) and indeed, production of compounds with antibacterial properties and cholesterol-lowering statins of the monacolin K-type (= mevinolin = lovastatin) are reported in the species *M. pilosus*, *M. pubigerus*, *M. purpureus*, *M. ruber* and *M. vitreus* (Negishi *et al.* 1986, Jůzlova *et al.* 1996, Vendruscolo *et al.* 2014). However, *Monascus* species such as *M. anka*, *M. aurantiacus*, *M. kaoliang*, *M. pilosus*, *M. purpureus*, *M. ruber* and *M. sanguineus* have been reported to produce the mycotoxin citrinin (Blanc *et al.* 1995, Dietrich *et al.* 1999, Wang *et al.* 2003, Wang *et al.* 2005, Pisareva *et al.* 2005, Shimizu *et al.* 2005, Huang *et al.* 2007, Pattangul *et al.* 2008, Kim *et al.* 2010, Li *et al.* 2012, Li *et al.* 2015), and the presence of this mycotoxin in food, including red rice, should be avoided. Among these reports on citrinin production by *Monascus* species, Wang *et al.* (2005) also reported on citrinin production by *M. floridanus*, *M. lunisporas* and *M. pallens*, but this has not been confirmed by any other authors working on citrinin and *Monascus*. Besides their beneficial properties for human, *Monascus* species can also cause spoilage, for example of silage, bakery (tortillas), pasteurized products (olives) and dried prunes (*M. eremophilus*). Species are also rarely associated with human infections, and an invasive gastric infection case was linked to the consumption of *Monascus* contaminated dried and salted fish (Moreau 1971, Iriart *et al.* 2010, Samson *et al.* 2010).

Specific fungi and other micro-organisms live in close association with social and solitary bees. This association is mandatory, and investigations on the biology, ecology and evolution have been undertaken (Wynns 2015). Recently, a study described a symbiosis between *Scaptotrigona postica* bees and a fungus (Menezes *et al.* 2015). The fungus was identified by morphology and ITS sequencing as being closely related to *M. ruber* and *M. pilosus*. The study showed that the *Monascus* biomass on the food inside the brood cells is essential for the larvae of the *S. postica* bees, and without the consumption of this biomass, only a few larvae can continue their life cycle.

Monascus was one of the predominant genera during the study of fungi associated with honey, pollen and nests of *Melipona scutellaris* bees living in the Atlantic Forest in Pernambuco, Brazil. The phylogenetic relationship of those strains with other species of the genus was determined by the analysis of ITS, LSU, β-tubulin (*BenA*), calmodulin (*CaM*) and RNA polymerase II second largest subunit (*RPB2*) sequences. Furthermore, three new species from honey, pollen and the inside of the nest are described based on a polyphasic approach combining sequence data, macro- and microscopic characters and extrolites.

MATERIALS AND METHODS

Fungal isolation

Samples were collected from honey, pollen and inside nests of *Melipona scutellaris* bees in the Brazilian Tropical Forest in Pernambuco state (8°7′30″S, 34°52′30″W and 8°4′36″S, 34°57′34″W) between January and June 2014. For the honey and pollen samples, 25 g of each specimen was suspended in 225 mL peptone water (0.1 %) and decimal dilutions were made until 10^{-3}. Subsequently, 0.1 mL of each dilution was spread plated on the agar media dichloran 18 % glycerol agar (DG18) and malt extract agar supplemented with chloramphenicol. The plates were incubated at 25 °C for 7–14 d in darkness. For collection of the samples inside nests, a sterile cotton swab was used to sample the surface of the pollen and honey pots, and brood cells. The swab was soaked in 3 mL peptone water (0.1 %) and vortexed vigorously. The samples were subsequently analysed as described above. All fungal colonies were isolated and purified prior identification.

Cultivation and morphological analyses

Thirty *Monascus* strains were obtained from honey, pollen and inside nests of *Melipona scutellaris* bees (Table 1). The colony characteristics of these strains were compared with representative and type cultures of currently accepted *Monascus* species. For this purpose, the strains were cultivated in three points in creatine agar (CREA), cornmeal agar (CMA), Czapek yeast extract agar (CYA), CYA supplemented with 5 % NaCl (CYAS), dichloran 18 % glycerol agar (DG18), malt extract agar (MEA, Oxoid), oatmeal agar (OA), potato dextrose agar (PDA) and yeast extract sucrose agar (YES) incubated at 25 °C for 7 d. Additional CYA and MEA plates were incubated at 30 and 37 °C. *Monascus eremophilus* was inoculated on the malt agar 20 % sucrose (MA20S) and malt yeast extract 50 % glucose agar (MY50G). All media were prepared according to Samson *et al.* (2010). Colony diameters were measured after 7 d of incubation and colony characteristics (e.g. presence of soluble pigments, exudates, obverse and reverse colony colours, colour of mycelium) were recorded. Microscopic observations of the asexual stage were made from colonies grown on MEA. The presence of a sexual stage was determined on MEA, CMA, PDA and OA, and PDA was used for illustrations and measurements. Lactic acid (60 %) was used as mounting fluid and 96 % ethanol was used to remove the excess of conidia. The size, shape and pigmentation of conidia, conidiophores, ascomata, asci and ascospores were recorded. A Zeiss Stereo Discovery V20 dissecting microscope and Zeiss AX10 Imager A2 light microscope equipped with Nikon DS-Ri2 cameras and software NIS-Elements D v4.50 were used to capture digital images. New species names and associated information were deposited in MycoBank. All strains were deposited in the culture collection of Micoteca URM (Federal University of Pernambuco, Recife, Brazil) and the ex-type strains were also deposited in the CBS culture collection housed at the Westerdijk Fungal Biodiversity Institute (formerly known as Centraalbureau voor Schimmelcultures), Utrecht, The Netherlands (under Material Transfer Agreement – MTA No. 01/2016/Micoteca URM).

Molecular characterization

Genomic DNA of 7 d old cultures was extracted using the UltraClean Microbial DNA kit (MoBio Laboratories, Solana Beach, CA, USA) and processed according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the ITS region (ITS1, 5.8S rDNA and ITS2) was performed using the primers