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ARTICLE

Phylogenetic placement of *Itajahya*: An unusual Jacaranda fungal associate

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Abstract: *Itajahya* is a member of *Phallales (Agaricomycetes)*, which, based on the presence of a calyptra and DNA sequence data for *I. rosea*, has recently been raised to generic status from a subgenus of *Phallus.* The type species of the genus, *I. galericulata*, is commonly known as the Jacaranda stinkhorn in Pretoria, South Africa, which is the only area where the fungus is known outside the Americas. The common name is derived from its association with the South American originating *Jacaranda mimosifolia* trees in the city. The aim of this study was to consider the unusual occurrence of the fungus in South Africa, to place it on the available *Phallales* phylogeny and to test whether it merits generic status. Fresh basidiomes were collected during the summer of 2015 and sequenced. Phylogenetic analyses were based on sequence data for the nuc-LSU-rDNA (LSU) and ATPase subunit 6 (ATP6) regions. The results showed that *I. rosea* and *I. galericulata* are phylogenetically related. They are also clearly distinguished from other members of *Phallales* such as *Phallus* spp. and *Dictyophora* spp., and so our new data supports the raising of *Itajahya* to the generic level.

Key words:
Homobasidiomycetes
molecular phylogeny
Phallomycetidae
phalloid fungi
South Africa
stinkhorns

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INTRODUCTION

The generic name *Itajahya* was introduced by Möller (1895) for a fungus discovered near the city of Blumenau in Santa Catarina state, Brazil. The peculiar name derives from that of the river Itajahy in that region. The fungus is an unusual and poorly known member of *Phallales,* and its taxonomic position has been debated purely on the basis of morphological features. The fungus resides in *Phallaceae*, a family encompassing members of the order with unbranched basidiomata. The family *Phallaceae* is distinguished from the only other family recognized in the order, *Clathraceae* (Chevallier 1826) in which the genera have branched basidiomata and are known as “lattice stinkhorns”. Recent developments in the taxonomy of the order based on DNA sequence data can be found in Hosaka *et al.* (2006) and Trierveiler-Pereira *et al*. (2014). *Itajahya* now includes four species, with *I. galericulata* as type species, and has, until recently, been treated as a subgenus of *Phallus* (Malençon 1984, Kreisel 1996).

Cabral *et al*. (2012) considered the taxonomic placement of *Phallus roseus*, a species treated by Malençon (1984) and Kreisel (1996) in *Itajahya*, as a subgenus of *Phallus*. Cabral *et al*. (2012) were able to collect fresh specimens of *P. roseus* in the Rio Grande do Norte of Brazil, and for the first time generated DNA sequence data for this poorly-known fungus. Their phylogenetic inference showed that *P. roseus* did not cluster with *Phallus* species, and they raised *Itajahya* to generic rank.

The decision of Cabral *et al*. (2012) to treat *P. roseus* in *Itajahya* was based on the fungus having a calyptra at the apex of its receptacle and the molecular datasets. A calyptra is also present in the type of the genus, *I. galericulata.* However, there was no sequence data then available for *I. galericulata* or the other two species of *Itajahya*: *I. hornseyi* described from Australia (Hansford 1954) and *I. argentina* from Argentina (Spegazzini 1898, 1927). The placement of *P. roseus* in *Itajahya* rested solely on morphological evidence. This could be contested given the poor understanding of the taxonomic value of morphological features in *Phallales*. For example, the data of Cabral *et al*. (2012) confirm earliest suggestions that the conspicuous indusium in *Dictyophora* is apparently not phylogenetically informative.

Itajahya galericulata was first described from southern Brazil where it is known from three states: Santa Catarina (Blumenau), Rio de Janeiro, and Rio Grande do Sul (Pelotas) (Möller 1895, Lloyd 1907). The fungus has also been recorded in Bolivia (Fries 1909). Long & Stouffer (1943) reconsidered a fungus initially identified as *Phallus impudicus* collected during a field study in 1941 in arid regions of New Mexico and Arizona noting a resemblance to *I. galericulata*, but they considered it “improbable that a tropical wet climate plant could grow under such arid conditions”. However, their careful morphological characterization of specimens

Table 1. *Itajahya galericulata* samples collected during this study in the Pretoria area of South Africa with GenBank accession numbers.

Isolate no.	Culture collection no.	Herbarium no.	GenBank accession no.	
			nucLSU	ATP6
Sample 1	CMW 44299[a] = CBS 140330[b]	PREM 61268[c]	KR071850	KR071847
Sample 2	CMW 44300 = CBS 140331	PREM 61269	KR071851	KR071848
Sample 3	CMW 44301 = CBS 140332	PREM 61270	KR071852	KR071849

[a] Culture collection of the Forestry and Agricultural Biotechnology Institute (CMW), University of Pretoria, South Africa.

[b] CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands.

[c] National collection of Fungi in South Africa (PREM), Roodeplaat, Pretoria, South Africa.

from Arizona and New Mexico led them to conclude that they were dealing with *I. galericulata*. Although they considered this possibility, they were unable to justify establishing a new species for the fungus based on the morphological characteristics.

Intriguingly, there is only one locality outside the Americas where *I. galericulata* is known: the city of Pretoria, South Africa. Here the fungus commonly occurs in association with *Jacaranda mimosifolia,* a tree that is abundantly planted as an ornamental in gardens and for lining streets. Indeed, Pretoria is commonly referred to as the "Jacaranda city" from the very large number of trees that cover it in a blanket of purple flowers in the Southern Hemisphere spring. While the occurrence of *I. galericulata* in Pretoria is unusual, it is perhaps more interesting that the fungus, known locally as the "jacaranda stinkhorn" (van der Westhuizen & Eicker 1994) occurs in a very close association with *J. mimosifolia*, a tree native in the area of Brazil and Bolivia where the fungus was first discovered. This suggests that *I. galericulata* was probably introduced into South Africa with these trees. Based on herbarium records, *I. galericulata* was first collected and recognized in Pretoria, South Africa, by Ethel M. Doidge on 21 January 1915.

During the summer of 2015, we were able to collect a number of fresh specimens of *I. galericulata* in Pretoria. This provided an opportunity to obtain DNA sequence data for this unusual genus of *Phallales* of which the taxonomy has been confused. The overall aim was to place the fungus in the available phylogeny of *Phallales*, to test the hypothesis of Cabral *et al*. (2012) that *Itajahya* deserves generic status, and to consider the unusual occurrence of this poorly know fungus in South Africa.

MATERIALS AND METHODS

Collection of samples

Three basidiomes of *Itajahya galericulata* were collected in the Brooklyn and Hatfield residential areas of Pretoria in January 2015 (Table 1, Fig. 1). These typically appear in dry sandy soils after summer rainfall and they were consistently associated with the root systems of *Jacaranda mimosifolia* trees. Dried basidiomes and cultures were deposited in the National Collection of Fungi in South Africa (PREM), Roodeplaat, Pretoria, South Africa, and living cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa and in the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands.

DNA extraction, PCR and sequencing

Fragments of three basidiomes were used for DNA extraction following the protocol used by Cabral *et al*. (2012). The DNA was quantified on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), before the nuc-LSU-rDNA (LSU) and ATPase subunit 6 (ATP6) gene regions were amplified using the LROR/LR5 (Vilgalys & Hester 1990) and ATP61/ATP62 (Kretzer & Bruns 1999) primer sets, respectively. Each gene region was amplified using the KAPA Taq PCR Kit (Kapa Biosystems, Cape Town) according to the appropriate protocols from Cabral *et al*. (2012). All successful PCR products were purified using the Zymo Research DNA Clean & Concentrator kit (Zymo Research Corporation, Irvine, CA).

Purified fragments were cloned into *Escherichia coli* using the pGem-T Easy Vector cloning kit (Promega, Madison, WI) following the manufacturers' instructions. Cloned inserts were amplified directly from colonies using a colony PCR reaction (Sambrook & Russell 2001). Amplification was achieved using the FastStart High Fidelity PCR System (Roche Diagnostics, Mannheim) with the SP6 (Butler & Chamberlin 1982) and T7 (Dunn *et al.* 1983) primer set. Each sample was purified using the Zymo Research DNA Clean & Concentrator kit and sequenced in both directions using the vector specific primers, the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Perkin Elmer, Warrington). Resultant electropherograms were imported into the CLC Main Workbench package (CLC Bio, Aarhus), trimmed for vector-specific and low quality sequence and subsequently assembled. One ATP6 and one LSU sequence from each basidiome was selected for phylogenetic analysis.

Phylogenetic analyses of sequence data

ATP6 and LSU datasets generated in this study incorporated sequences from the basidiomes collected in Pretoria as well as sequences obtained from GenBank for species included in the study of Cabral *et al.* (2012) (Tables 1–2). Multiple sequence alignments were made using the online version of MAFFT (Katoh & Standley 2013) with alignment strategy set to Auto. Character congruency between the ATP6 and LSU datasets was determined using the partition homogeneity test (PHT) implemented in PAUP (Swofford 2002) after excluding missing, ambiguously aligned and uninformative

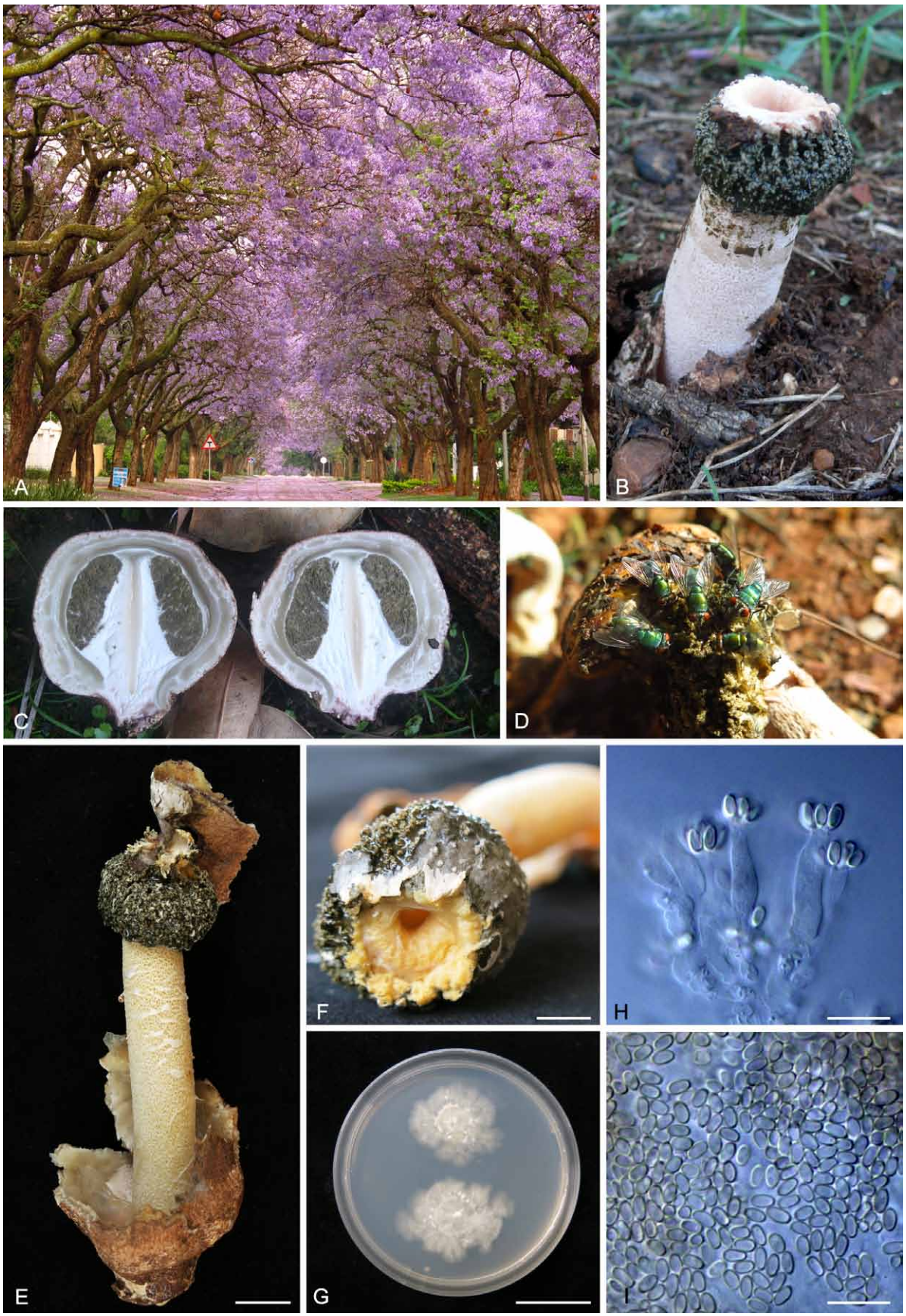

Fig. 1. Habitat and features of *Itajahya galericulata* in Pretoria, South Africa. **A.** *Jacaranda mimosifolia* trees flowering in Brooklyn, Pretoria. **B.** Basidiome emerged from the ground (PREM 61269). **C.** "Egg" dissected to show inner features. **D.** Flies attracted by putrid odour produced by the fungus. **E.** Basidiome emerged in a moist chamber in the laboratory (PREM 61268). **F.** Close-up of the apex of basidiome showing calyptra and gleba (PREM 61270). **G.** Colonies grown for 3 months on 2 % Yeast-malt extract agar (CMW 44300 = CBS 140331). **H.** Basidia. **I.** Basidiospores. Bars: E = 1.5 cm, F = 1 cm, G = 2 cm, H–I = 10 µm.

Table 2. GenBank accession numbers of taxa used for phylogenetic analyses.

Species	GenBank accession no.	
	nucLSU	ATP6
Anthurus archeri	DQ218624	DQ218913
Abrachium floriforme	JF968440	JF968438
Aseroe rubra	DQ218625	DQ218914
Clathrus chrysomycelinus	DQ218626	DQ218915
Dictyophora duplicata	DQ218481	DQ218765
Dictyophora indusiata	DQ218627	DQ218917
Dictyophora multicolor	DQ218628	DQ218918
Gelopellis sp. 1	DQ218630	DQ218919
Gelopellis sp. 2	DQ218631	DQ218920
Ileodictyon cibarium	DQ218633	DQ218922
Ileodictyon gracile	DQ218636	DQ218925
Itajahya rosea	JF968441	JF968439
Kobayasia nipponica	DQ218638	DQ218926
Laternea triscapa	DQ218640	DQ218928
Lysurus borealis	DQ218641	DQ218929
Lysurus mokusin	DQ218507	DQ218791
Mutinus elegans	AY574643	AY574785
Phallobata alba	DQ218642	DQ218930
Phallus costatus	DQ218513	DQ218797
Phallus hadriani	DQ218514	DQ218798
Phallus ravenelii	DQ218515	DQ218799
Protubera borealis	DQ218516	DQ218800
Protubera canescens	DQ218645	DQ218932
Protubera jamaicensis	DQ218647	DQ218933
Protubera maracuja	DQ218518	DQ218802
Protubera parvispora	DQ218648	DQ218934
Protubera sabulonensis	DQ218649	DQ218935
Simblum sphaerocephalum	DQ218521	DQ218806
Trappea darkeri	DQ218651	DQ218938

characters. This test showed that the datasets were congruent (P = 0.85), therefore aligned datasets were concatenated for downstream phylogenetic analyses.

Phylogenetic trees were obtained based on parsimony and Bayesian inference with *Trappea darkeri* as the outgroup species. Cladograms based on the combined datasets were generated in PAUP. Missing, ambiguously aligned and uninformative characters were excluded prior to the parsimony analysis. The most parsimonious trees were determined using a heuristic algorithm with random addition of sequences (10 replicates) and tree-bisection reconnection branch swapping (TBR). The same settings were used for bootstrap analysis, but with the addition of sequences set to "simple", and 1000 replicates.

Nucleotide substitution models that best fit the individual datasets were determined with jModelTest (Darriba *et al.* 2012) with models selected based on the Akaike Information Criterion (AIC). The selected substitution models were applied to the individual gene partitions (LSU: TIM2+I+G and ATP6: TPM2uf+I+G) in the combined dataset for the Bayesian analysis. Phylogenetic trees were generated with MrBayes (Huelsenbeck & Ronquist 2002) with the number of generations set to 4 million and using four Markov chain runs. The first 25 % trees with low likelihood were discarded from each run after which the remaining trees from the individual runs were combined to obtain a consensus tree and posterior probability (PP) values. Effective sample size (ESS), as a measure of convergence, was analyzed in Tracer (http://tree.bio.ed.ac.uk/software/tracer/).

RESULTS

Phylogenetic analyses of sequence data

Using previously described primers, sequences for both the LSU and ATP6 regions were successfully amplified from all three basidiomes. The final LSU and ATP6 datasets included 691 and 667 characters, respectively. Of these, 135 characters were parsimony informative in the LSU dataset, while 214 characters in the ATP6 dataset were informative. In total 271 characters were included in the parsimony analysis after excluding missing, ambiguously aligned, and parsimony uninformative characters.

Parsimony analyses yielded four most parsimonious trees with a tree length of 908 steps. The consistency and retention index values were 0.476 and 0.729, respectively. The topology of the consensus tree generated from Bayesian inference was congruent with the trees generated using parsimony. All nodes were supported with posterior probability (PP) values > 0.95, with the exception of the node shared by *Dictyophora indusiata* and *Phallus hadriani* (Fig. 2). Phylogenetic trees generated from the parsimony and Bayesian analyses placed the sequences from Pretoria in a strongly supported clade (bootstrap support = 100 %, PP < 0.95). This clade formed a sister group to *Itajahya rosea* with high bootstrap support (96 %) and posterior probability (PP < 0.95). Trees generated in this study placed sequences from species of the genera *Dictyophora* and *Phallus* in a monophyletic group supported by their posterior probability values (PP < 1), but somewhat marginal bootstrap value of 70 %. This group was placed sister to *I. rosea* and the samples from Pretoria with PP = 1, but with low bootstrap support (64 %).

DISCUSSION

This study provided DNA sequence data for a poorly-known yet taxonomically important member of *Phallales*. The data for two gene regions have shown clearly that *Itajahya galericulata* groups separately from species of *Phallus* and *Dictyophora*. They also support the results of Cabral *et al.* (2012) who raised *Itajahya* to generic status to accommodate *Phallus roseus*, which they transferred to *Itajahya*. Their placement in *Itajahya* was based on morphological features and sequence data of *I. rosea,* but not of the type species, *I. galericulata*. Our results confirm that *I. rosea* is

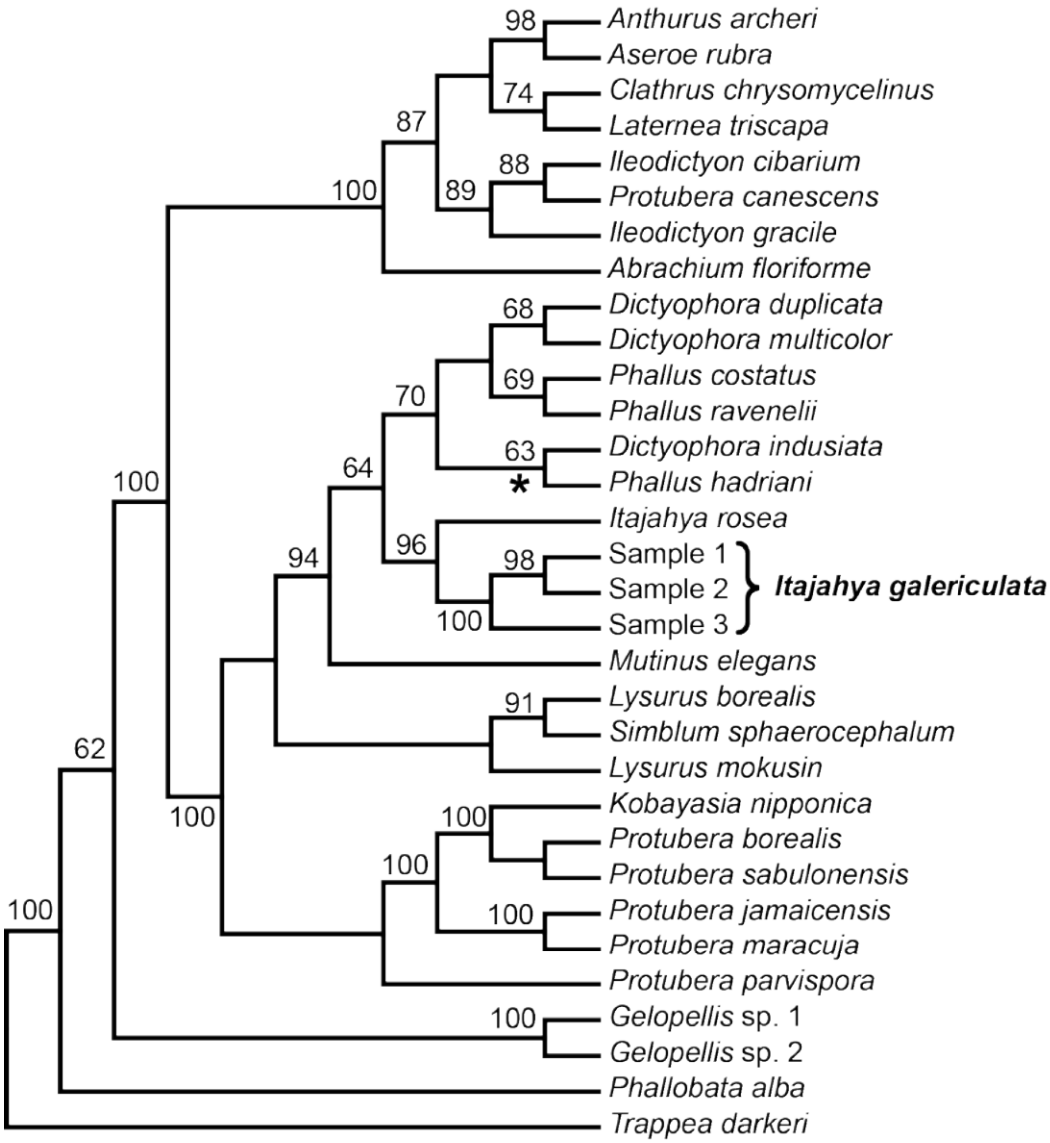

Fig. 2. Cladogram based on parsimony and generated from combined nuclear LSU and ATP6 DNA sequence for *Itajahya galericulata* and other member species of *Phallales*. Bootstrap values greater than 60 % are indicated on the tree branches. All nodes, except that indicated with an asterisk, have posterior probability values greater than 0.95.

phylogenetically related to the type species, *I. galericulata,* and the two species can be comfortably accommodated in the same genus.

There is relatively little information available on the biology of *Itajahya* species. As it was first discovered in tropical Brazil, the subsequent discovery of *I. galericulata* in dry sandy environments of New Mexico and Arizona was unexpected (Long & Stouffer 1943). This habitat contrasted with most *Phallales,* which occur on rich organic substrates in moist situations. *Itajahya galericulata* is found in Brazil either in clay banks of forest streams or among roots of dead trees where the soil is rich in decaying leaves (Möller 1895). In contrast, in South Africa it is typically found in dry sandy soil, although it occurs annually after rain and is possibly associated with litter of *J. mimosifolia*. Similarly, the Australian *I. hornseyi* is also reported from a sandy soil habitat (Hansford 1954). We consider that this genus of *Phallales*, unlike most other species in the order, has species adapted to tolerate in relatively dry conditions, although we acknowledge that there are some exceptions in *Phallus*; for example, in the UK *P. hadriani* characteristically occurs in sand dunes associated with marram grass (*Ammophila arenaria*) (Watling & Rotheroe 1989, Pegler *et al*. 1995) .

The occurrence of *I. galericulata* in South Africa and particularly its association with *J. mimosifolia* trees is intriguing. That these trees originate in the area where the fungus was first described suggests the fungus was introduced into South Africa with these trees. The very close association between *J. mimosifolia* trees and its apparent absence from other environments in South Africa raises the question as to whether there might be a mutualistic relationship between them. The members of *Phallaceae* are, however, not known as mycorrhizal associates of trees, although *Phallus hadriani* could perhaps be mycorrhizal with *Ammophila* when in sand dunes (Hawksworth, pers. comm.). The form of the relationship between *J. mimosifolia* and *I. galericulata* remains unknown and requires further investigation for a better understanding of the dynamic ecology of the fungus.

If *I. galericulata* was, as we believe is the case, introduced into South Africa with *J. mimosifolia*, the trees would probably have had to be introduced as potted plants as opposed to seeds, though we cannot exclude the possibility that the fungus could be seed-borne. The legal global movement of rooted plants is very difficult under contemporary quarantine regulations, which seek to prevent the concomitant introduction of alien and potentially invasive organisms. At the time of the first discovery in South Africa in 1915, such care would not have been required. Although there is no evidence to suggest that *I. galericulata* is invasive, its establishment in South Africa illustrates the potential dangers associated with the global movement of living plants for planting out (Andjic *et al.* 2011).

We have no doubt that the fungus that we have treated as *I. galericulata* from South Africa is the same species as that described from Brazil. It would, however, be useful to compare specimens from these two areas phylogenetically; this would ideally require the collection of fresh specimens from Brazil. For the present, the DNA sequences for this species from South Africa provide a foundation for inclusion of the genus in phylogenetic studies of *Phallales*.

We hope this study will stimulate further interest in *Itajahya* and encourage mycologists to collect additional specimens from which DNA can be extracted and used in phylogenetic analyses. It would, for example, be particularly interesting to compare sequence data from specimens collected in New Mexico and Arizona with ones from South Africa and Brazil. Long & Stouffer (1943) considered whether their collections might represent a species different to *I. galericulata,* but they were unable to make a distinction based on morphology; DNA comparisons would enable us to assess whether the geographical separation had led to any genotypic differences between the populations.

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