Two new species of Amanita sect. Phalloideae from Africa, one of which is devoid of amatoxins and phallotoxins

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Abstract

Two new species of Amanita sect. Phalloideae are described from tropical Africa (incl. Madagascar) based on both morphological and molecular (DNA sequence) data. Amanita bweyeyensis sp. nov. was collected, associated with Eucalyptus, in Rwanda, Burundi and Tanzania. It is consumed by local people and chemical analyses showed the absence of amatoxins and phallotoxins in the basidiomata. Surprisingly, molecular analysis performed on the same specimens nevertheless demonstrated the presence of the gene sequence encoding for the phallotoxin phallacidin (PHA gene, member of the MSDIN family). The second species, Amanita harkoneniana sp. nov. was collected in Tanzania and Madagascar. It is also characterised by a complete PHA gene sequence and is suspected to be deadly poisonous. Both species clustered together in a well-supported terminal clade in multilocus phylogenetic inferences (including nuclear ribosomal partial LSU and ITS-5.8S, partial tef1-α, rpb2 and β-tubulin genes), considered either individually or concatenated. This, along with the occurrence of other species in sub-Saharan Africa and their phylogenetic relationships, are briefly discussed. Macro- and microscopic descriptions, as well as pictures and line drawings, are presented for both species. An identification key to the African and Madagascan species of Amanita sect. Phalloideae is provided. The differences between the two new species and the closest Phalloideae species are discussed.

Keywords

Ectomycorrhizal fungi, Amanita, phylogeny, taxonomy, mycotoxins, tropical Africa, 2 new species
Introduction

Most representatives of *Amanita* sect. *Phalloideae* (Fr.) Quél. are famous worldwide for their high, often deadly, toxicity. Currently, the section *Phalloideae* comprises nearly 60 described species, a number of which were described only recently, mainly from Asia (Li et al. 2015, Cai et al. 2016, Thongbai et al. 2017). Moreover, based on a multigene analysis and morphological data, Cai et al. (2014) identified 14 phylogenetic clades potentially representing new species. The phylogenetic analyses made by those authors also resulted in the transfer of several species from sect. *Phalloideae* to sect. *Lepidella* Corner & Bas and conversely.

Most of African mycodiversity remains under-explored with only ca. 1500 taxa described to date (Degreef 2018). Very few species belonging to sect. *Phalloideae* have been recorded from Africa and Madagascar (Walleyn and Verbeken 1998, Tulloss and Possiel 2017). The three poorly known *Amanita alliiodora* Pat., *A. murinacea* Pat. and *A. thejoleuca* Pat. were described from Madagascar, while *Amanita strophiolata* Beeli was described from DR Congo (we agree with Gilbert 1941:313 that the var. *bingensis* Beeli has no taxonomic value). The latter species is the only *Phalloideae* known from Central Africa, together with some doubtful mentions of the imported *A. phalloides* (Fr.: Fr.) Link. *Amanita phalloides* is only native to Europe, North Africa, Turkey (Kaya et al. 2013, 2015), a certain proportion of the Asian part of Russia and perhaps the West Coast of North America (Pringle and Vellinga 2006, Pringle et al. 2009, Wolfe et al. 2010). Mentions of the species in other regions of the world correspond to either introductions or misidentifications. The exact identity of *A. capensis* and its possible co-specificity with *A. phalloides* remain uncertain. The last species of *Phalloideae* known to Africa, *Amanita marmorata* Cleland & E.-J. Gilbert (syn.: *A. marmorata* subsp. *myrtacearum* O.K.Mill., Hemmes & G.Wong, *A. reidii* Eicker & Greuning, *A. phalloides* f. *umbrina* ss. African auct.), is also an introduced species. It was described from Australia, growing in association mainly with various species of *Eucalyptus* (e.g. *E. cephalocarpa* Blakely) and subsequently observed in South Africa under *Eucalyptus cloeziana* F.Muell. and *E. sp.* (Eicker et al. 1993, van der Westhuizen and Eicker 1994) and in Hawaii, under *Eucalyptus robusta* Sm., *E. saligna* Sm., *E. sp.*, *Araucaria columnaris* Hook., *Melaleuca quinquenervia* (Cav.) S.T.Blake, as well as under pure *Casuarina equisetifolia* L. (Miller et al. 1996).

Amatoxins and phallotoxins are responsible for the high toxicity of *Amanita* sect. *Phalloideae*. Nevertheless, apart from *Amanita alliiodora*, considered toxic by the Madagascan people, and the deadly poisonous *A. phalloides* (incl. “*A. capensis*”) and probably *A. marmorata*, no data are available attesting to the toxicity or the edibility of the Madagascan and African species.

In the framework of taxonomic and phylogenetic studies of *Amanita* sect. *Phalloideae*, specimens originating from tropical Africa were critically studied. Morphological and multigene phylogenetic studies proved to be concordant and established the existence of two distinct species that could not be identified as any known taxa.
Amanita bweyeyensis from the western province of Rwanda and A. barkoneniana from the Tanzanian Miombo woodlands and Madagascar are described here as new. Their phylogenetic affinities with other Amanita species reported from Africa are discussed and a key to African species of Amanita sect. Phalloideae is provided.

Materials and methods

Specimens studied

African Amanita phalloides-related specimens held in BR were studied in depth (Degreef 653 from Burundi; Degreef 1257 and 1304, both from Rwanda). A picture appearing in Härkönen et al. (2003: 62, sub “Amanita species which looks very much like Amanita phalloides”) convinced us to also check the specimen Saarimäki 591 (from Tanzania). We additionally obtained Saarimäki et al. 1061 (also from Tanzania) on loan from the University of Helsinki (H). Finally, P. Pirot sent us two unnumbered specimens he collected in Madagascar in 2014 and 2016.

We also examined for comparison the type specimen of Amanita marmorata subsp. myrtacearum (O.K. Miller 24545, VPI) collected in Hawaii and 3 specimens of Amanita marmorata collected in Australia: H.D. Weatherhead s.n. (= MEL 2028859A) and J.B. Cleland s.n. (= AD-C 3083 and 3085). We unsuccessfully tried to obtain the type specimen of Amanita reidii on loan. Braam Vanwyk informed us that the holotype preserved in PRU had unfortunately been destroyed and no longer exists. Although Miller et al. (1996: 144) mentioned having received a fragment of that type specimen on loan from PREM, it seems that no such material exists in the collections of that institution (Riana Jacob-Venter, in e-litt.), nor in K (Angela Bond, in e-litt.). We also received on loan the lectotypus of Amanita murina (Cooke & Massee) Sacc. (Bailey 651, K, correct name: Amanita neomurina Tulloss).

Macro- and microscopic studies

Macroscopic characters were deduced from herbarium specimens, as well as from specimen labels, field notes and pictures, when available. Microscopic examinations were carried out using an Olympus BX51 microscope, from herbarium material mounted in ammoniacal Congo Red or in Melzer’s reagent. Measurements were made using a camera lucida and a calibrated scale. In the descriptions, figures between brackets are extreme values, underlined figures are averages, Q values are length/width ratios of spores, l/w values are the same ratios for other types of cells. Mentions like “[60/4/2]” after measurements of spores (or other microscopic structures) mean 60 spores measured, from 4 different basidiomata collected in 2 different places.
Molecular analyses

DNA extraction, amplification and sequencing

Genomic DNA was isolated from CTAB-preserved tissues or dry specimens using a CTAB isolation procedure adapted from Doyle and Doyle (1990). PCR amplification of the ITS region (nuclear ribosomal internal transcribed spacer) and LSU (large subunit ribosomal DNA) was performed using the primer pairs ITS4/ITS5 or ITS1-F/ITS4 and LR0R/LR5, respectively (http://biology.duke.edu/fungi/mycolab/primers.htm). Parts of the protein-coding genes β-tubulin, rpb2 (second largest subunit of RNA polymerase II) and tef-1 (translation elongation factor 1 alpha) were amplified using the primer pairs Am-β-tub-F/Am-β-tub-R, Am-6F/Am-7R and EF1-983F/EF1-1567R, respectively (Zhang et al. 2010). PCR products were purified by adding 1 U of Exonuclease I and 0.5 U FastAP Alkaline Phosphatase (Thermo Scientific, St. Leon-Rot, Germany) and incubating at 37 °C for 1 h, followed by inactivation at 80 °C for 15 min.

Sequencing was performed by Macrogen Inc. (Korea and The Netherlands) using the same primer combinations as for PCR, except for Am-β-tub-F, which was replaced by the shorter primer Am-β-tub-F-Seq (5’-CGGAGCRGGAACAYTG-3’) following Thongbai et al. (2017). The sequences were assembled in Geneious Pro v. 6.0.6 (Biomatters).

Phylogenetic analysis

Thirty-five sequences of Amanita specimens were newly generated for this study and deposited in GenBank (http://www.ncbi.nlm.nih.gov/; Table 1). Initial BLAST searches (http://blast.ncbi.nlm.nih.gov) of both LSU and ITS-5.8S sequences were performed to estimate similarity with Amanita sequences already present in Genbank database (Table 1). Additional sequences were selected from previously published phylogenies and from GenBank (Table 1). The quality of the sequences was taken into account in selecting the sequences for the phylogenetic analyses. Materials and sequences used in this study are listed in Table 1.

A combined dataset (including nuclear ribosomal partial LSU and ITS-5.8S, partial tef1-α, rpb2 and β-tubulin genes), comprising sequences from 94 collections including the outgroup and an ITS-5.8S / LSU dataset of 69 sequences, including several clones derived from the same collections and the outgroup, were constructed and used for further phylogenetic analyses.

Amanita cf. spissacea voucher OR1214 and Amanita subjunquillea voucher HKAS63418 were used as outgroups for the combined and ITS-LSU datasets, respectively (Thongbai et al. 2017, Cui et al. 2018).

Nucleotide sequences were automatically aligned using the MUSCLE algorithm (Edgar 2004) with default settings. The alignment was further optimised and manu-
Table 1. List of collections used for DNA analyses, with origin, GenBank accession numbers and references.

| Species | GenBank accession no. |
|---------|-----------------------|
|         | Specimen voucher | Country | LSU | ITS | rpb2 | tef1-α | βtubulin |
| Amanita alliodora Pat. 1928 | DSN062 | Madagascar | KX185612 | KX185611 | – | – | – |
| Amanita americovirina nom. prov. | RET 397-8 | USA | KJ466640 | KJ466398 | – | KJ481964 | KJ466543 |
| Amanita americovirina nom. prov. | RET 480-1 | USA | KJ466461 | KJ466399 | KJ466630 | KJ481965 | KJ466544 |
| Amanita bisporeigena G.F. Apt. 1906 | RET 377-9 | USA | KJ466434 | KJ466374 | – | – | – |
| Amanita brevistriata Thongbai, Raspe & K.D. Hyde 2017 | BZ2015-01 | Thailand | – | – | – | – | – |
| Amanita brevistriata Thongbai, Raspe & K.D. Hyde 2017 | clone Agar. 8B_S114 | Madagascar | – | – | – | – | – |
|         | JD 1257 | Rwanda | MK570926 | MK570919 | – | – | – |
|         | JD 1304 | Rwanda | MK570927 | MK570920 | MK570931 | MK570940 | MK570916 |
| Amanita bweyeyensis Fraiture, Raspe & Degreef, sp. nov. | TS 591 | Tanzania | MK570928 | MK570921 | – | – | – |
| Amanita djarilmari E.M. Davison 2017 | EMD 008 el_4 | Australia | – | – | – | – | – |
|         | EMD 008 el_5 | Australia | – | – | – | – | – |
|         | EMD 008 el_6 | Australia | – | – | – | – | – |
|         | EMD 50101_1 | Australia | – | – | – | – | – |
|         | EMD 50101_15 | Australia | – | – | – | – | – |
|         | EMD 50101_3 | Australia | – | – | – | – | – |
|         | EMD 50101_5 | Australia | – | – | – | – | – |
|         | EMD 50101_7 | Australia | – | – | – | – | – |
|         | EMD 08131_1 | Australia | – | – | – | – | – |
|         | EMD 08131_2 | Australia | – | – | – | – | – |
|         | EMD 08131_3 | Australia | – | – | – | – | – |
|         | EMD 08131_4 | Australia | – | – | – | – | – |
|         | EMD 08131_5 | Australia | – | – | – | – | – |
| Amanita djambarei E.M. Davison 2017 | PERTH08776040 | Australia | KY977708 | – | – | MF007234 | MF000743 |
|         | PERTH08776067 L_1 | Australia | KY977704 | KY977732 | MF000755 | MF000750 | MF000742 |
|         | PERTH08776067 L_2 | Australia | KY977704 | KY977733 | MF000755 | MF000750 | MF000742 |
|         | PERTH08776067 L_3 | Australia | KY977704 | KY977734 | MF000755 | MF000750 | MF000742 |
|         | PERTH08776067 L_4 | Australia | KY977704 | KY977735 | MF000755 | MF000750 | MF000742 |
|         | PERTH08776067 L_5 | Australia | KY977704 | KY977736 | MF000755 | MF000750 | MF000742 |
| Amanita djambarei E.M. Davison 2017 | PERTH08776075 l_1 | Australia | KY977706 | KY977737 | – | – | – |
|         | PERTH08776075 l_2 | Australia | KY977706 | KY977738 | – | – | – |
|         | PERTH08776075 l_3 | Australia | KY977706 | KY977739 | – | – | – |
|         | PERTH08776075 l_4 | Australia | KY977706 | KY977740 | – | – | – |
|         | PERTH08776075 l_5 | Australia | KY977706 | KY977741 | – | – | – |
| Amanita djambarei E.M. Davison 2017 | PERTH08776083 l_1 | Australia | KY977710 | KY977742 | – | – | MF000744 |
|         | PERTH08776083 l_2 | Australia | KY977710 | KY977743 | – | – | MF000744 |
|         | PERTH08776083 l_3 | Australia | KY977710 | KY977744 | – | – | MF000744 |
|         | PERTH08776083 l_4 | Australia | KY977710 | KY977745 | – | – | MF000744 |
|         | PERTH08776083 l_5 | Australia | KY977710 | KY977746 | – | – | MF000744 |
| Species | GenBank accession no. |
|---------|----------------------|
| Specimen voucher | Country | LSU | ITS | rpb2 | tef1–α | βtubulin |
| **Amanita eucalypti** O.K. Mill. 1992 | | | | | | |
| PERTH8809828 cl_3 | Australia | KY077707 | KU057380 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_4 | Australia | KY077707 | KU057397 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_5 | Australia | KY077707 | KU057396 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_6 | Australia | KY077707 | KU057395 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_7 | Australia | KY077707 | KU057394 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_8 | Australia | KY077707 | KU057392 | MF000758 | MF000751 | MF000746 |
| **Amanita exitialis** Zhu L. Yang & T.H. Li 2001 | | | | | | |
| PERTH8809828 cl_1 | Australia | KY077707 | KU057391 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_2 | Australia | KY077707 | KU057389 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_3 | Australia | KY077707 | KU057388 | MF000758 | MF000751 | MF000746 |
| PERTH8809828 cl_4 | Australia | KY077707 | KU057387 | MF000758 | MF000751 | MF000746 |
| **Amanita fuliginea** Hongo 1953 | | | | | | |
| PERTH8809968 cl_1 | Australia | KY077707 | KU057376 | MF000758 | MF000751 | MF000746 |
| PERTH8809968 cl_2 | Australia | KY077707 | KU057375 | MF000758 | MF000751 | MF000746 |
| PERTH8809968 cl_3 | Australia | KY077707 | KU057374 | MF000758 | MF000751 | MF000746 |
| PERTH8809968 cl_4 | Australia | KY077707 | KU057373 | MF000758 | MF000751 | MF000746 |
| **Amanita fuligineoides** P. Zhang & Zhu L. Yang 2010 | | | | | | |
| PERTH08776121 | Australia | KY077707 | KU057372 | MF000758 | MF000751 | MF000746 |
| **Amanita gardneri** E.M. Davison 2017 | | | | | | |
| EM8-2010 cl_1 | Australia | – | KU057387 | – | – | – |
| EM8-2010 cl_2 | Australia | – | KU057386 | – | – | – |
| EM8-2010 cl_3 | Australia | – | KU057385 | – | – | – |
| EM8-2010 cl_4 | Australia | – | KU057384 | – | – | – |
| **Amanita griseorosea** Q. Cai, Zhu L. Yang & Y.Y. Cui 2016 | | | | | | |
| HKAS77334 | China | KJ466476 | KJ466413 | KJ466611 | KJ481999 | KJ466580 |
| HKAS77333 | China | KJ466475 | KJ466412 | KJ466610 | KJ481998 | KJ466579 |
| **Amanita harkoneniana** Fraiture & Saarimäi, sp. nov. | | | | | | |
| P Pirot SN | Madagascar | MK570930 | – | – | – | – |
| TS 1061 | Tanzania | MK570930 | – | – | – | – |
| **Amanita marmorata** Cleland & E.-J. Gilbert 1941 | | | | | | |
| HWN | Australia | MK570931 | MK570924 | MK570939 | MK570942 | MK570917 |
| PERTH8690596 cl_1 | Australia | KY077711 | KU057408 | – | – | MF000749 |
| PERTH8690596 cl_2 | Australia | KY077711 | KU057404 | – | – | MF000749 |
| PERTH8690596 cl_3 | Australia | KY077711 | KU057405 | – | – | MF000749 |
| PERTH8690596 cl_4 | Australia | KY077711 | KU057406 | – | – | MF000749 |
| PERTH8690596 cl_5 | Australia | KY077711 | KU057407 | – | – | MF000749 |
| RET 623-7 | Australia | KP757874 | KP757875 | – | – | – |
| RET 85-9 | Australia | MG252697 | MG252696 | – | – | – |
| **Amanita marmorata** subsp. myrtacearum O.K. Mill., Hemmes & G. Wong 1996 | | | | | | |
| DED 5845 | Hawaii | AY325881 | AY325826 | – | – | – |
| Species                  | GenBank accession no.                      | Country | Specimen voucher | LSU          | ITS          | rpb2         | tefl–α       | βtubulin    |
|-------------------------|------------------------------------------|---------|-----------------|--------------|--------------|--------------|--------------|-------------|
| *Amanita millii* E.M. Davison & G.M. Gates 2017 |                           |         |                 |              |              |              |              |             |
| HKAS77322                | Australia                                | KJ466457 | KJ466395        | KJ466643     | KJ481978     | KJ466557     |              |             |
| HOS5815331.2            | Australia                                | KY977713 | KY977715        | MF000753     | MF000759     | MF000760     |              |             |
| HOS5815331.1            | Australia                                | KY977713 | KY977714        | MF000753     | MF000759     | MF000760     |              |             |
| HOS5815331.3            | Australia                                | KY977713 | KY977716        | MF000753     | MF000759     | MF000760     |              |             |
| HOS5815331.5            | Australia                                | KY977713 | KY977717        | MF000753     | MF000759     | MF000760     |              |             |
| *Amanita mollisscula* Q. Cai, Zhu L. Yang & Y.Y. Cui 2016 |                           |         |                 |              |              |              |              |             |
| HKAS75555                | China                                    | KJ466471 | KJ466408        | KJ466638     | KJ481973     | KJ466552     |              |             |
| HMJAU20469               | China                                    | KJ466473 | KJ466410        | KJ466640     | KJ481975     | KJ466554     |              |             |
| HKAS77324                | China                                    | NG_057038 | NR_147633     | KJ466639     | KJ481974     | KJ466553     |              |             |
| *Amanita ocreata* Peck 1909 |                           |         |                 |              |              |              |              |             |
| HKAS79686                | USA                                      | KJ466442 | KJ466381        | KJ466607     | KJ481947     | KJ466518     |              |             |
| *Amanita pallidovescens* P. Zhang & Zhu L. Yang 2010 |                           |         |                 |              |              |              |              |             |
| HKAS61937                | China                                    | KJ466443 | KJ466382        | KJ466609     | KJ481949     | KJ466520     |              |             |
| HKAS71023                | Japan                                    | KJ466444 | KJ466383        | KJ466624     | KJ481960     | KJ466536     |              |             |
| HKAS75483                | China                                    | KJ466445 | KJ466384        | KJ466623     | KJ481959     | KJ466535     |              |             |
| HKAS75783                | China                                    | JX998055 | JX998035        | KJ466625     | JX998010     | KJ466537     |              |             |
| HKAS75784                | China                                    | JX998056 | JX998036        | KJ466626     | JX998009     | KJ466538     |              |             |
| HKAS75786                | China                                    | JX998054 | JX998037        | KJ466627     | JX998011     | KJ466539     |              |             |
| HKAS77329                | China                                    | KJ466447 | KJ466387        | KJ466610     | KJ481950     | KJ466521     |              |             |
| HKAS77348                | China                                    | KJ466448 | KJ466387        | KJ466611     | KJ481951     | KJ466522     |              |             |
| HKAS77349                | China                                    | KJ466449 | KJ466389        | KJ466628     | KJ481961     | KJ466540     |              |             |
| HKAS77327                | China                                    | KJ466446 | KJ466386        | KJ466608     | KJ481948     | KJ465519     |              |             |
| *Amanita parvipellis* Q. Cai, Zhu L. Yang & Y.Y. Cui 2016 |                           |         |                 |              |              |              |              |             |
| HKAS79049                | China                                    | NG_057092 | –              | KT971345     | KT971343     | KT971346     |              |             |
| *Amanita phalloides* Secr. 1833 |                           |         |                 |              |              |              |              |             |
| HKAS75773                | USA                                      | JX998060 | JX998031        | KJ466612     | JX998000     | KJ466523     |              |             |
| *Amanita rimosa* P. Zhang & Zhu L. Yang 2010 |                           |         |                 |              |              |              |              |             |
| HKAS75778                | China                                    | JX998045 | JX998019        | KJ466616     | JX998006     | KJ466527     |              |             |
| HKAS75779                | China                                    | JX998046 | JX998020        | KJ466617     | JX998004     | KJ466528     |              |             |
| HKAS77105                | China                                    | KJ466452 | KJ466391        | KJ466618     | KJ481954     | KJ466529     |              |             |
| HKAS77120                | China                                    | KJ466453 | KF479044        | KJ466619     | KJ481955     | KJ466530     |              |             |
| HKAS77279                | China                                    | KJ466454 | KJ466392        | KJ466620     | KJ481956     | KJ466531     |              |             |
| HKAS77335                | China                                    | KJ466455 | KJ466393        | KJ466621     | KJ481957     | KJ466532     |              |             |
| HKAS77336                | China                                    | KJ466456 | KJ466394        | KJ466622     | KJ481958     | KJ466533     |              |             |
| HKAS75777                | China                                    | JX998044 | JX998018        | KJ466615     | JX998005     | KJ466526     |              |             |
| *Amanita sp.* 1 ZLY2014  |                           |         |                 |              |              |              |              |             |
| HKAS77322                | Australia                                | KJ466457 | KJ466395        | KJ466643     | KJ481978     | KJ466557     |              |             |
| *Amanita sp.* 2 ZLY2014  |                           |         |                 |              |              |              |              |             |
| HKAS77350                | China                                    | KJ466462 | KJ466400        | KJ466631     | KJ481966     | KJ466545     |              |             |
| *Amanita sp.* 3 ZLY2014  |                           |         |                 |              |              |              |              |             |
| HKAS77342                | China                                    | KJ466463 | KF479045        | KJ466632     | KJ481967     | KJ466546     |              |             |
| HKAS77343                | China                                    | KJ466464 | KJ466401        | KJ466633     | KJ481968     | KJ466547     |              |             |
| HKAS77344                | China                                    | KJ466465 | KJ466402        | KJ466634     | KJ481969     | KJ466548     |              |             |
| HKAS77351                | China                                    | KJ466466 | KJ466403        | KJ466635     | KJ481970     | KJ466549     |              |             |
| *Amanita sp.* 5 ZLY2014  |                           |         |                 |              |              |              |              |             |
| RET 422-8                | USA                                      | KJ466469 | KJ466406        | KJ466649     | KJ481983     | KJ466563     |              |             |
| RET 493-6                | USA                                      | KJ466470 | KJ466407        | KJ466650     | KJ481984     | KJ466564     |              |             |
| Species | GenBank accession no. |
|---------|----------------------|
| **Specimen voucher** | **Country** | **LSU** | **ITS** | **rpb2** | **tef1-α** | **βtubulin** |
| Amanita sp. 8 ZLY2014 | Bangladesh | KJ466477 | KJ466414 | KJ466641 | KJ481976 | KJ466555 |
| Amanita sp. 9 ZLY2014 | China | KJ466478 | KJ466415 | KJ466642 | KJ481977 | KJ466556 |
| Amanita suballiacea (Murrill) Murrill 1941 | USA | KJ466485 | KJ466420 | KJ466601 | KJ481941 | KJ466513 |
| Amanita subfuliginea Q. Cai, Zhu L. Yang & Y.Y. Cui 2016 | China | KJ466486 | KJ466421 | KJ466602 | KJ481942 | KJ466514 |
| Amanita subjunquillea S. Imai 1933 | USA | KJ466484 | KJ466419 | KJ466600 | KJ481940 | KJ466512 |
| Amanita subpallidorosea Hai J. Li 2015 | China | KJ466488 | KJ466425 | KJ466656 | KJ481988 | KJ466574 |
| Amanita virina Secr. 1833 | Japan | KJ466489 | KJ466424 | KJ466652 | KJ481987 | KJ466570 |
| Amanita halloides ar lba Constantin & L.M. Dufour 1895 | Belgium | – | – | – | – | – |
| Amanita halloides ar mbrina (Ferry) Maire 1937 | South Africa | AY325882 | AY325825 | – | – | – |
| Amanita eidii Eicker & Greuning 1993 | South Africa | AY325883 | AY325824 | – | – | – |
| Amanita p | New Caledonia | – | KY774002 | – | – | – |
| Amanita p Keralo01 | India | – | KC855219 | – | – | – |
| Amanita ballerina Raspé Thongbai & K.D. Hyde 2017 | Thailand | – | KY747466 | KY656883 | – | KY656864, |
| Amanita francizi Zhu L. Yang, Y.Y. Cui & Q. Cai 201 | Thailand | MH157079 | KY747467 | KY656884 | – | KY656865 |
| HKAS77321 | China | KJ466481 | MH508357 | KJ466646 | MH508798 | KJ466560 |
Two new species of *Amanita* sect. *Phalloideae* from Africa...

| Species                        | GenBank accession no.          |
|--------------------------------|--------------------------------|
|                               | Specimen voucher | Country | LSU        | ITS        | rpb2       | tef1–α    | β-tubulin |
| *Amanita pseudogemmata*        | HKAS91231         | China    | MH486525   | MH508358   | MH485994   | MH508801  | MH485516  |
| Hongo 1974                    |                  |          |            |            |            |            |            |
| *Amanita zangii*               | HKAS85889         | China    | MH486768   | –          | MH486186   | MH508995  | MH485692  |
| Zhu L. Yang, T.H. Li & X.L. Wu 2001 | HKAS84744         | China    | MH486767   | –          | MH486185   | MH508994  | MH485691  |
| *Amanita cf. spissacea*        | GDGM29241         | China    | KJ466499   | KJ466432   | KJ466668   | KJ482000  | KJ466588  |
| S. Imai 1933                  | OR1214            | Thailand | KJ466500   | KJ466433   | KJ466669   | KJ482001  | KJ466589  |
| *Amanita sect. Validae*        | HKAS77331         | China    | KJ466499   | KJ466432   | KJ466668   | KJ482000  | KJ466588  |

Note: cl_ stands for clone. References to sequences retrieved from GenBank: Cai et al. (2012), Cai et al. (2014), Cui et al. (2018), Davison et al. (2017), Henry et al. (2015), Houles et al. (2018), Li et al. (2015), Thongbai et al. 2017, Tulloss (continuously updated).

ally adjusted as necessary by direct examination with the software Se-Al v. 2.0a11 (University of Oxford).

The assignment of codon positions in the protein-coding sequences was confirmed by translating nucleotide sequences into predicted amino acid sequences using MacClade 4.0 (Maddison and Maddison 2000) and then compared with the annotated *Amanita brunnescens* sequences AFTOL-ID 673.

Potential ambiguously aligned segments, especially in the three introns present in *tef-1* and β-tubulin gene sequences and in the ITS-5.8S alignment, were detected by Gblocks v0.91b (Castresana 2000; http://molevol.cmima.csic.es/castresana/Gblocks.html) with the following parameter settings: minimum number of sequences for a conserved position = 24 (minimum possible); minimum number of sequences for a flank position = 24 (minimum possible); maximum number of contiguous non-conserved positions = 4 bp, minimum block size = 4 bp and gaps allowed within selected blocks in half of the sequences.

To detect the possible bias from substitution saturation and to evaluate the phylogenetic signal, we tested each partition of the combined dataset and the ITS-LSU dataset by using Xia’s test (Xia et al. 2003, Xia and Lemey 2009), as implemented in DAMBE (Xia and Xie 2001). As the Iss.c is based on simulation results, there is a problem with more than 32 species. To circumvent this problem, DAMBE was used to randomly sample subsets of 4, 8, 16 and 32 OTUs multiple times and to perform the test for each subset to see if substitution saturation exists for these subsets of sequences. In order to confirm the results of the Xia’s method, we also plotted the raw number of transversions and transitions against Tamura-Nei genetic distances with the aid of the DAMBE package, with an asymptotic relationship indicating the presence of saturation.

Models of evolution for BI were estimated using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall 1998).

The dataset was subdivided into 10 data partitions: *tef-1* 1st and 2nd codon positions, *tef-1* -3rd codon positions, *tef-1* introns and *rpb2* 1st and 2nd codon positions,
PCR amplification of *Amanita* toxins genes family members

Two major toxin-encoding genes, AMA1 and PHA1, directly encode for $\alpha$-amanitin and the related bicyclic heptapeptide phallacidin, the lethal peptide toxins of poisonous mushrooms in the genus *Amanita*. $\alpha$-Amanitin and phallacidin are synthesised as pro-proteins of 35 and 34 amino acids, respectively, in the ribosomes and are later cleaved by a prolyl oligopeptidase (Hallen et al. 2007, Luo et al. 2009, Li et al. 2014). In these pro-proteins, the amino acid sequences found in the mature toxins are flanked by conserved amino acid sequences, with an invariant Pro residue immediately upstream of the toxin regions and as the last amino acid in the toxin regions.
Table 2. Summary of data sets of ITS rDNA, nuc-LSU rDNA, *tefl*-*α*, *rpb2* and β-tubulin.

| DataSets                | tefl 1<sup>st</sup>& 2<sup>nd</sup> | tefl 3<sup>rd</sup> | tefl introns | rpb2 1<sup>st</sup>& 2<sup>nd</sup> | rpb2 3<sup>rd</sup> | β-tubulin 1<sup>st</sup>& 2<sup>nd</sup> | β-tubulin 3<sup>rd</sup> | β-tubulin introns | nucLSU | ITS  |
|------------------------|-----------------|-----------------|--------------|-----------------|-----------------|-----------------|-----------------|-----------------|--------|------|
| Alignment size         | 296             | 147             | 147          | 452             | 226             | 167             | 83              | 171             | 887    | 935  |
| Excluded characters    | –               | –               | –            | –               | –               | –               | –               | –               | –      | 557  |
| Model selected         | GTR+I+G         | GTR+G           | HKY+I        | GTR+I           | GTR+G           | SYM+I+G         | HKY+G           | HKY+G           | GTR+I+G| HKY+I+G |
| -Likelihood score      | 780.2892        | 1857.1256       | 1535.9010    | 1285.5159       | 3033.6099       | 1319.9380       | 1108.1555       | 1023.9042       | 3403.9714| 4844.7563 |
| Base frequencies       |                |                |              |                 |                 |                 |                 |                 |        |      |
| Freq. A =              | 0.3179          | 0.1686          | 0.2479       | 0.2914          | 0.2478          | Equal           | 0.1745          | 0.2254          | 0.2877  | 0.3023 |
| Freq. C =              | 0.2276          | 0.3231          | 0.2175       | 0.2132          | 0.1956          | Equal           | 0.3113          | 0.1690          | 0.1671  | 0.1846 |
| Freq. G =              | 0.2536          | 0.2159          | 0.1807       | 0.2761          | 0.2541          | Equal           | 0.2257          | 0.2228          | 0.2937  | 0.2068 |
| Freq. T =              | 0.2010          | 0.2924          | 0.3540       | 0.2192          | 0.3025          | Equal           | 0.2885          | 0.3827          | 0.2515  | 0.3062 |
| Proportion of invariant sites | 0.8042  | –               | 0.0940       | 0.8283          | –               | 0.4975          | –               | 0.5726          | 0.2855  |
| Gamma shape            | 0.7888          | 2.1595          | –            | –               | 2.7065          | 4.2837          | 3.7320          | 0.8097          | 0.5839  | 0.8470 |
| Test of substitution saturation |          |                |              |                 |                 |                 |                 |                 |        |      |
| Iss                    | 0.263           | 0.354           | 0.723        | 0.335           | 0.308           | 0.156           | 0.306           | 0.662           | 0.499   | 0.472  |
| Iss.cSym               | 0.683           | 0.721           | 0.928        | 0.697           | 0.685           | 0.706           | 0.875           | 0.776           | 0.764   | 0.707  |
| P (Sym)                | < 0.0001        | < 0.0001        | < 0.0001     | < 0.0001        | < 0.0001        | < 0.0001        | < 0.0001        | < 0.0001        | < 0.0001| < 0.0001 |
| Iss.cAsym              | 0.354           | 0.668           | 0.802        | 0.502           | 0.458           | 0.407           | 0.711           | 0.535           | 0.675   | 0.645  |
| P (Asym)               | < 0.0001        | < 0.0001        | < 0.0001     | < 0.0001        | < 0.0001        | < 0.0001        | < 0.0001        | < 0.0001        | < 0.0001| < 0.0001 |

Note: Iss: index of substitution saturation. Iss.cSym: critical value for symmetrical tree topology. Iss.cAsym: critical value for extremely assymetrical tree topology. P: probability that Iss is significantly different from the critical value (Iss.cSym or Iss.cAsym).
The toxins genes and MSDIN (cyclic peptide precursor) family members and related sequences were amplified from total genomic DNA with two consecutive PCR reactions, using the products of the first PCR as templates for the second one. For the first PCR, we used degenerated primers forward (5’ATGTCNGAYATAYAYGCNAC-NCG3’) and the reverse primer (5’CCAAGCCTRAYAWRGTCMACAACC3’), following the cycling condition detailed in Li et al. (2014).

For the nested PCR amplification (using the PCR products above as the amplification template of AMA1 and PHA1 genes), primers targeting conserved regions of MSDINs family were obtained from previous studies (Hallen et al. 2007, Luo et al. 2009, Li et al. 2014, Wołoszyn and Kotłowski 2017) or designed ad hoc against the conserved upstream and downstream sequences of AMA1 and PHA1 available on Genbank and tested in different combinations. For α-amanitin, we used 5’CCATCTGGGGCATCGGTTGCAACC3’ as forward primer (Li et al. 2014) in combination with the reverse primers 5’CTACGTTYGAGTCAAGACACTGGC3’ (Li et al. 2014) and the newly generated AMA-α-R2 (5’GTCAAGTCAGTGCGACTGCTTTGT3’) and AMA-α-R3 (5’CTGCATTGAGTTAGGATAACGACA3’). We also tested primer pairs AMAF and AMAR 5 (Wołoszyn and Kotłowski 2017). For β-amanitin, we used forward primer AMA-β-F (5’CCATMTGGGGMATMGGTTGYRACC3’) in combination with reverse primers AMA-β-R (5’GTCMACAACTYRTATYAGKCCAC-CTACT3’), AMA-β-R2 (5’GTCMACAACTYRTATYAGKCCACMGCT3’) and AMA-β-R3 (5’CTTAYAWRGTCMACAACT3’). For PHA genes, we used forward primer 5’CCTGCYTGGCTYGTAGAYTGCCCA3’ (Li et al. 2014) in combination with the reverse primers 5’GCTCCACTACTAYDTCMARGTCAGTAC3’ (Li et al. 2014) and AMA-PHA-R2 (5’AGTCACGACTACATCGAGGTCAGTAC3’). Primer pairs FALF and FALR (Wołoszyn and Kotłowski 2017) were also tested for amplification of the phallotoxins genes.

Thermal cycling conditions were: initial denaturation at 94°C for 4 min, followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7 min, for all reactions except for the ones involving primers from Wołoszyn and Kotłowski (2017), for which an annealing temperature of 68°C for 1 min was used.

Chemical analyses

Mushroom preparation

Two groups of dried mushrooms, i.e. with cuticle (n=3) and without cuticle (n=3), were analysed. For each specimen, 100 mg of dry tissues were ground and homogenised in 3 ml extraction medium (methanol:water:0.01 M HCl [5:4:1, v/v/v]) using a tissue homogeniser. After 1 hour of incubation, all extracts were centrifuged at 5000 rpm for 5 min, the supernatant was filtered using a 0.45 mm syringe filter and 20 µl of this supernatant was injected in the RP-HPLC device for toxin detection.
Standard solutions and chemicals

The α-amanitin and phalloidin standards were obtained from Sigma-Aldrich (USA). The β-amanitin, γ-amanitin and phallacidin standards were obtained from Enzo Life Sciences (Farmingdale, NY, USA). The solvents used in this study were all HPLC grade. Stock solutions of all toxins (100 µg/ml) were prepared in methanol. The calibration standards of all toxins were diluted in the extraction fluid in concentrations of 1, 5, 20, 100, 200, 500 ng/ml. Calibration curves were produced for each toxin; they were linear over the range of interest (R² > 0.99).

RP-HPLC analysis of toxins

Chromatography conditions for the procedure followed in this study were reported by Kaya et al. (2013, 2015). In short, the authors reported excellent separation of amatoxins and phallotoxins with the RP-HPLC and UV detection. In the laboratory, an RP-HPLC analysis of mushroom extracts was performed on a Shimadzu (Japan) HPLC system. The RP-HPLC analysis of standard solutions of α-amanitin, β-amanitin, γ-amanitin, phalloidin, phallacidin and subsequent quantification of mushroom extracts were performed on 150 × 4.6 mm, 5 mm particle, C18 column (Agilent Technologies, Palo Alto, CA) with 302 nm (for amatoxins) and 290 nm (for phallotoxins) at the UV detector. The mobile phase was used in isocratic profile with a flow rate of 1 ml/min. The content of the mobile phase was 0.05 M ammonium acetate (pH 5.5 with acetic acid)/acetonitrile (90:10 v/v). The detection limits were set at 0.6 ng/g for all toxins.

Results

Molecular analyses

Phylogenetic analysis

By comparing the tree topologies obtained for the individual datasets, no significant conflict, involving significantly supported nodes, was found using the 75% ML BP criterion; the datasets were therefore combined.

The test of substitution saturation (Table 2) showed that the observed index of substitution saturation (I<sub>ss</sub>) for the ITS-LSU dataset (ITS and LSU partition considered individually) the tef-1, rpb2, LSU and β-tubulin alignments of the combined dataset was significantly lower than the corresponding critical index substitution saturation (I<sub>ss.c</sub>), indicating that there was little saturation in our sequences (P < 0.001). On the other hand, the ITS partition of the combined dataset, the tef-1 introns and the β-tubulin intron showed sign of substitution saturation, indicating the unsuit-
ability of these data for phylogenetic analysis. Nevertheless, re-analysing the ITS-LSU partition with DAMBE, after the exclusion of the 378 sites (40% of a total of 935 sites) retained by Gblocks, the substitution saturation test revealed an Iss value that was significantly (P < 0.001) lower than the Iss.c (Table 2), indicating the suitability of this data for further phylogenetic analysis. We therefore included an ITS partition, excluding the poorly aligned positions identified by Gblocks, in the combined dataset. Regarding the introns partitions, according to Thongbai et al. (2017), *A. zangii* and the *A. ballerina* clade (Fig. 1), should be considered to belong in a different section (*Amanita incertae sedis*), sister to the *Phalloideae* sensu Bas (1969). We therefore tested the combined dataset for substitution saturation by using *A. zangii* as the outgroup and excluding from the analysis the *A. ballerina* clade and the outgroup. In this case, no sign of saturation was evidenced, which supports the consistency of the phylogenetic signal in the main *Phalloideae* clade. We therefore decided to include the introns partitions in the phylogenetic analyses in order to increase the resolution at species level.

The ITS-LSU dataset and the final combined DNA sequence alignments of all loci (β-tubulin, *rpb2*, ITS, LSU, *tef-1*) alignments contained 15 and 35 OTUs and were 1575 and 3133 sites long including gaps, respectively. Sequence data and statistical analysis for each dataset are provided in Table 2.

The topologies obtained by analysing the combined dataset and the ITS-LSU dataset were highly congruent with published trees (Zhang et al. 2010, Cai et al. 2016, Thongbai et al. 2017), at least for what concerns significantly supported branches, and the Bayesian consensus trees (Figs 1 and 2) were almost identical to the optimal trees inferred under the Maximum Likelihood criterion. Several collections from tropical Africa clustered together in a well-supported clade. So far, this clade remains isolated but is notably distantly related to all other *Amanita* species, as yet reported from Africa (Zhang et al. 2010, Cai et al. 2016, Thongbai et al. 2017) or elsewhere and for which sequences are known (Figs 1 and 2), suggesting a common phylogenetic background. *Amanita alliiodora* clustered together with the two unnamed species from tropical Africa in all phylogenetic inferences considered individually or concatenated (i.e. phylogenetic species, Figs 1 and 2, shaded box).

Morphological examination showed combinations of morphological features unique to and characteristic of each, thereby defining two morphotypes. The critical morphological features that differentiate them are the following. The first species grows under Eucalyptus. Its bulb at stipe base is (sub-)globose, neither pointed nor rooting. The ring is striated and the smell sweetish and conspicuous. The second species is not bound with *Eucalyptus* and has been collected in Miombo woodland and in a garden. The bulb at the stipe base is turnip-shaped to rooting. The ring is smooth or vaguely plicate and the smell weak, resembling raw potato. We therefore concluded that these two morphotypes / clades represent two distinct new species, which we describe below resp. as *A. bweyeyensis* sp. nov. and *A. harkoneniana* sp. nov.
Figure 1. The 50% majority-rule consensus tree from Bayesian inference of the combined dataset. Thickened branches in bold represent ML BS support greater than 75% and BPP greater than 0.95; thickened branches in grey denote branches supported by either ML BS or BPP. For selected nodes ML BS support value and BPP are, respectively, indicated to the left and right of slashes. The new taxa are highlighted in the shaded box. AMA and PHA indicate the presence of amatoxins and phallotoxins, respectively, detected by HPLC. NT indicates not tested.
Figure 2. The 50% majority-rule consensus tree from Bayesian inference of the combined nuclear ITS-5.8S and LSU sequences. Thickened branches in bold indicate bootstrap support greater than 70% and Bayesian posterior probability greater than 0.95. For selected nodes, parsimony bootstrap support value and Bayesian posterior probabilities are, respectively, indicated to the left and right of slashes. The new taxa are highlighted in the shaded box.

PCR amplification of Amanita toxins genes family members

By using a combination of the degenerated primers cited above, we obtained a complete 17-mer sequence of phallacidin precursor for the three specimens of A. bweyensis and the two specimens of A. harkoneniana studied (Table 3), comprising the mature toxin region sequence of phallacidin (AWLVDCP) and both the invariant Pro residues immediately preceding the mature peptide sequence and the last amino acid of the toxin. Surprisingly, this is the first time that a complete PHA sequence has been found.
Two new species of *Amanita* sect. Phalloideae from Africa...

Table 3. PCR products (phalloidin, PHA gene) amplified from *A. bweyeyensis* and *A. harkoneniana* with degenerate primers, compared to the PHA gene sequences available on GenBank.

|                  | M     | S     | D     | I     | N     | A     | T     | R     | L     | P     | A     | W     | L     | V     | D     | C     | P     |
|------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| MK570933 A. bweyeyensis JD 1304 | ATG   | TCT   | GAC   | ATC   | AAT   | GCC   | ACC   | CGT   | CCT   | GCC   | CGY   | TGG   | CTT   | GTA   | GAC   | TGC   | CCC   |
| MK570932 A. bweyeyensis JD 1257 | ATG   | TCT   | GAC   | ATC   | AAT   | GCC   | ACC   | CGT   | CCT   | GCC   | CGY   | TGG   | CTT   | GTA   | GAC   | TGC   | CCC   |
| MK570934 A. bweyeyensis TS 591 | ATG   | TCT   | GAC   | ATC   | AAT   | GCC   | ACC   | CGT   | CCT   | GCC   | CGY   | TGG   | CTT   | GTA   | GAC   | TGC   | CCC   |
| MK570936 A. harkoneniana TS 1061 | ATG   | TCT   | GAC   | ATC   | AAT   | GCC   | ACC   | CGT   | CTT   | CCT   | GCC   | TGG   | CTT   | GTA   | GAC   | TGC   | CCA   |
| MK570935 A. harkoneniana PIROT SN | ATG   | TCT   | GAC   | ATC   | AAT   | GCC   | ACC   | CGT   | CTT   | CCT   | GCC   | TGG   | CTT   | GTA   | GAY   | TGC   | CCA   |
| KF387488 A. exitialis | ATG   | TCT   | GAC   | ATC   | AAT   | GCC   | ACC   | CGT   | CTT   | CCT   | GCC   | TGG   | CTT   | GTA   | GAC   | TGC   | CCA   |
| EU196142 A. biporiforme | ATG   | TCT   | GAC   | ATC   | AAT   | GCC   | ACC   | CGT   | CTT   | CCT   | GCC   | TGG   | CTT   | GTA   | GAC   | TGC   | CCA   |
| KF546298 A. fuligineoides | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | CTT   | GCT   | TGG   | CTT   | GTA   | GAT   | TGC   | CCA   |
| KF546296 A. fuliginosus | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | CTT   | GCT   | TGG   | CTT   | GTA   | GAC   | TGC   | CCA   |
| KF552098 A. pallidorosea | ATG   | TCT   | GAT   | ATT   | AAT   | GCT   | ACG   | CGT   | CCT   | CCC   | GCC   | TGG   | CTT   | GTA   | GAC   | TGC   | CCA   |
| KF546303 A. phalloides | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | CTT   | GCC   | TGG   | CTT   | GTA   | GAT   | TGC   | CCA   |
| KF778570 A. oberwinkleri | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | CTT   | GCC   | TGG   | CTT   | GTA   | GAT   | TGC   | CCA   |
| KF778568 A. subjunquillea | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | CTT   | GCC   | TGG   | CTT   | GTA   | GAT   | TGC   | CCA   |
| KF546306 A. rimosa | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | ???   | CTT   | GCC   | TGG   | CTT   | GTA   | GAC   | TGC   | CCA   |
Table 3. (Continued) PCR products (phallolidin, PHA gene) amplified from *A. bweyeyensis* and *A. harkoneniana* with degenerate primers, compared to the PHA gene sequences available on GenBank.

| Phallacidin precursor (17-mer) | C  | V  | G  | D  | D' | V  | N  | P  | V  | L  | T  | R  | G  | Q  | R  |
|--------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| MK570933 *A. bweyeyensis* JD 1304 | TGC | GTC | GGT | GAC | GAC | TGC | AAC | CCC | GTA | CTC | ACT | GGT | GGG | CAG | AGG |
| MK570932 *A. bweyeyensis* JD 1257 | TGC | GTC | GGT | GAC | GAC | TGC | AAC | CCC | GTA | CTC | ACT | GGT | GGG | CAG | AGG |
| MK570934 *A. bweyeyensis* TS 591 | TGC | GTC | GGT | GAC | GAC | TGC | AAC | CCC | GTA | CTC | ACT | GGT | GGG | CAG | AGG |
| MK570936 *A. harkoneniana* TS 1061 | TGC | GTC | GGT | GAC | GAC | TGC | AAC | CCC | GTT | CTC | ACT | GGT | GGG | CAG | AGG |
| MK570935 *A. harkoneniana* P PIROT SN | TGC | GTC | GGT | GAC | GAC | TGC | AAC | CCC | GTT | CTC | ACT | GGT | GGG | CAG | AGG |
| KF387488 *A. exitialis* | TGC | GTC | GGT | GAC | GAC | GTC | AAC | CGC | CTC | CTC | ACT | GGT | GGC | GAG | AGC |
| EU196142 *A. bisporigera* | TGC | GTC | GGT | GAC | GAC | GTC | AAC | CGT | CTC | CTC | ACT | GGT | GGT | GAG | AGG |
| KF546298 *A. fuliginosa* | TGC | GTT | GGT | GAC | GAT | GTC | AAC | TTC | ATC | CTC | ACT | GGT | GGC | CAG | AAG |
| KF546296 *A. fulginosa* | TGC | GTC | GGT | GAC | GAC | GTC | AAC | CGC | CTC | CTC | GCT | GGT | GGC | GAG | AAG |
| KF552098 *A. pallidorosea* | TGC | GTC | GGT | GAC | GAC | ATC | AAC | CGC | CTC | CTC | ACT | GGT | GGC | GAG | AAG |
| KF546303 *A. phalloides* | TGC | GTC | GGT | GAC | GAC | ATC | AAC | CGC | CTC | CTC | ACG | GCC | GGC | GAG | AAG |
| KC778570 *A. oberwinteriina* | TGC | GTC | GGT | GAC | GAC | ATC | AAC | CGC | CTC | CTC | ACT | GGT | GGC | GAG | AAG |
| KC778568 *A. ruhynguillia* | TGT | GTC | GGT | GAC | GAC | ATC | AGC | CGC | CTT | CTC | ACT | GGT | GGC | GAG | AAG |
| KF546306 *A. rimosa* | TGT | GTC | GGT | GAC | GAC | ATC | AGC | CGC | CTT | CTC | ACT | GGT | GGC | GAG | AAG |
Two new species of *Amanita* sect. *Phalloideae* from Africa...

in a species of *Amanita* sect. *Phalloideae* that does not produce this toxin. This finding is in contrast with the study of Hallen et al. (2007), concluding that all of the species synthesising amatoxins and phallotoxins, but none of the other species, hybridised to AMA and PHA genes probes (based on the same primers used in this study). However, while successful PCR amplification proves the presence of a gene (PHA gene in this case), an unsuccessful PCR, possibly due to primer mismatches, cannot be used to prove the absence of the genes encoding α- and β-amanitin, whose exact DNA sequence for these specimens is not known.

**Taxonomy**

*Amanita bweyeyensis* Fraiture, Raspé & Degreef, sp. nov.

Figs 3, 4

MycoBank no.: MB830175

**Diagnosis.** *Amanita bweyeyensis* differs from the closest *Amanita* species by: pileus first pale brownish-grey then entirely whitish or with a faintly yellowish or pale beige shade, basal bulb of the stipe globose, neither pointed nor rooting, basidiospores subglobose to widely ellipsoid (Q = 1.10–1.17–1.28), absence of α- and β-amanitin, phalloidin and phallacidin in its basidiomata, connection with the genus *Eucalyptus* and distribution in Burundi, Rwanda and Tanzania.

**Holotypus.** RWANDA. Western Prov.: buffer zone Nyungwe forest, Bweyeye (02°36.62’S; 29°14.04’E), ca. 2050 m alt., 16 Apr. 2015, J.Degreef 1304 (BR!).

**Description.** Primordium subglobose, smooth, whitish or with a weak olive tint. Pileus 40–73–120 mm diam., first hemispherical then expanding to regularly convex or applanate, without umbo; margin even, not striate nor appendiculate, in some mature specimens the pileipellis does not reach the edge of the pileus, leaving free the extreme tip of the lamellae; first pale brownish-grey (close to 6B2 or 6C2–3), then often entirely whitish or with a faintly yellowish or pale beige shade (between 4A2 and

Figure 3. Basidiomata of *Amanita bweyeyensis*. a Degreef 653 b Degreef 1257.
Figure 4. *Amanita bweyeyensis* a Basidia (from Degreef 1257, scale bar: 10 µm) b Spores (from Saarimäki et al. 591, scale bar: 10 µm) c Filamentous hyphae from the volva (from Degreef 1304, holotypus, scale bar: 20 µm) d Sphaerocysts from the volva (from Degreef 1304, holotypus, scale bar: 50 µm).

5B2); somewhat viscid, smooth, devoid of veil remnants. Lamellae free, white, becoming slightly yellowish when old and ochraceous, pinkish-beige to pale pinkish-brown on the exsiccates with a narrow white and fluffy edge; mixed with an equal number of lamellulae which are very variable in length and are usually truncated; sub-distant, 8–9 lamellae and lamellulae per cm at 1 cm from the edge of the pileus, about 120–160 lamellae and lamellulae in total (counts on 5 basidiomata), 3–14 mm broad, serrate when seen with a magnifying glass. Stipe 65–95–152 × 7–25 mm, ratio length of the stipe/diam. of pileus = 1.04–1.25–1.38; sub-cylindrical, slightly wider just under lamellae, gradually and slightly widened from top to bottom, white, with finely fibrillose surface, hollow (at least on exsiccates). Ring white, hanging, membranous but thin and fragile, finely fibrillose, smooth to somewhat plicate longitudinally, upper part adhering to the stipe and often more or less striate. Basal bulb of the stipe globose, sometimes a bit elongated but neither pointed nor rooting, up to 45 mm wide, surrounded by a white volva (also white inside), membranous, up to 30–35 mm high. Context white, soft; smell sweetish, conspicuous; taste not recorded.

Basidiospores hyaline, with thin, amyloid wall, (globose-) subglobose to widely ellipsoid (-ellipsoid), rather often with a mangiform or amygdaliform profile, (7.5-) 8.0–8.81–9.5 (-11.0) × (6.0-) 7.0–7.54–8.5 (-9.0) µm, Q = (1.00-) 1.10–1.17–1.28
Two new species of *Amanita* sect. *Phalloideae* from Africa...

Basidia 4-spored, without clamp, thin-walled, clavate, often rather abruptly swollen, 36–42.3–50 × (8.0–) 10.5–12.0–14 (-15) µm, l/w = 2.6–3.59–4.2 (-5.5) [66/4/2]. Lamellar edge sterile, composed of sphaeropedunculate marginal cells which are widely clavate to pyriform, hyaline, thin-walled, smooth, without clamp, 18–26.3–32 (-37) × 12–17.0–20 (-33) µm, l/w = (1.00–) 1.33–1.57–1.83 (-2.33) [40/4/2]. General veil (volva) mostly composed of cylindrical hyphae, with very different diameters, (15–) 35–80 (-110) × 2–8.5–15 (-26) µm, hyaline, with smooth and thin wall, septate, with rather frequent anastomoses between parallel hyphae, without clamps, branched, mixed with very few sphaerocysts, thin-walled, smooth, globose to ovoid, 33–76–125 × (25–) 32–56–95 µm, l/w = 1.00–1.52–2.25 [20/2/2].

Distribution. At present, the species is only known from Burundi, Rwanda and Tanzania but, according to its ecology, it could probably be observed in all *Eucalyptus* plantations in tropical Africa and possibly in South Africa as well. Consequently, if the species is collected for consumption, care should be taken to avoid confusion with *A. marmorata*, a species growing in the same biotopes and suspected to be highly toxic.

Ecology. On the ground, under *Eucalyptus*. The label of Saarimäki 591 indicates “in Acacia and Eucalyptus forest” whereas the legend of the associated picture (Härkönen et al. 2003: 62) indicates “growing in an *Acacia mearnsii* plantation”. However, the litter visible on that picture does not correspond to the latter species but looks like *Eucalyptus* leaves.

Etymology. This species is named after the collection locality of the type specimen in Rwanda.

Specimens examined. BURUNDI. Muravya Prov.: Bugarama, 9 Jan. 2011, J.Degreef 653 (BR). – RWANDA. Western Prov.: buffer zone Nyungwe forest, Bweyeye (02°36.79’S; 29°14.01’E), ca. 2040 m alt., 20 Oct. 2014, J.Degreef 1257 (BR); Ibidem (02°36.62’S; 29°14.04’E), ca. 2050 m alt., 16 Apr. 2015, J.Degreef 1304 (holotype: BR!). – TANZANIA. Pare District: South Pare Mts., Mpepera, ca. 1600 m alt., 5 Dec. 1990, T.Saarimäki et al. 591 (H).

Notes. During collecting field trips in Rwanda, one of us (JD) was confused by observing local people (Abasangwabutaka) picking huge quantities of this mushroom in old *Eucalyptus* plantations and eating them (after removal of the cuticle) without experiencing any trouble. The species was not observed to be eaten in Burundi and is probably not used in Tanzania either.

It is quite likely that the specimen shown in a picture by van der Westhuizen and Eicker (1994: 38) under *Amanita phalloides* var. *alba* is *Amanita bweyeyensis*. This specimen was observed at Sabie (South Africa), growing in the leaf-litter under *Eucalyptus cloeziana* in early December and again in March. The pileus surface is described as “white and occasionally faintly yellowish over the central part” and the pileus margin as “very finely denticulate”. Härkönen et al. (2003: 62) already drew attention to that picture.

A comparison with the closely related species is given in the chapter “discussion” below.
**Amanita harkoneniana** Fraiture & Saarimäki, sp. nov.
Figs 5, 6
MycoBank no.: MB830176

**Diagnosis.** *Amanita harkoneniana* differs from the closest *Amanita* species by: pileus first whitish to pale yellowish-beige then entirely whitish, devoid of veil remnants, basal bulb of the stipe turnip-shaped or irregularly elongated and more or less rooting, basidiospores subglobose to widely ellipsoid (*Q* = 1.04–1.13–1.25), basidia 34–37.5–41 µm long and growth without connection with the genus *Eucalyptus*, in Tanzania and Madagascar.

**Holotypus.** TANZANIA. Tabora District: ca. 10 km S of Tabora, Kipalapala, ca. 1200 m alt., 12 Dec. 1991, T. Saarimäki et al. 1061 (H!).

**Description.** Primordium smooth, subglobose but with a more or less conical or irregular rooting part; veil whitish; pileus with a weak brownish tint (around 4B2–3 and 5B2–3 but paler). **Pileus** 35–53–70 mm diam., first hemispherical, then largely conical or convex to nearly applanate, often with a deflexed margin, without umbo; margin even, neither striate (sometimes striate on exsiccates) nor appendiculate; first whitish to pale yellowish-beige (between 4A2 and 4B2) then entirely whitish; slightly viscid when young, smooth, devoid of veil remnants. **Lamellae** white, becoming slightly yellowish when old and pale to dark brownish in exsiccates with a narrow white and fluffy edge, free, mixed with an equal number of lamellulae which are very variable in length and are usually truncated, sub-distant, 8–10 lamellae and lamellulae per cm at 1 cm from the edge of the pileus, about 125–215 lamellae+lamellulae in total (counts on 2 basidiomata), ventricose, very finely serrate when seen with a magnifying glass. **Stipe** 65–130 × 8–14 mm, sub-cylindrical, slightly wider just under the lamellae, gradually and slightly widened from top to bottom, white, with finely fibrillose surface, hollow (at least in exsiccates) or stuffed. Ring white, hanging, membranous but thin and fragile, upper part adhering to the stipe. Basal bulb of the stipe turnip-shaped or irregularly elongated, more or less rooting, surrounded by a white volva (also white inside), membranous, up to 40–60 mm high. **Context** white, soft, very thin along the margin of the pileus, much thicker near the stipe; smell weak resembling raw potato.

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**Figure 5.** Basidiomata of *Amanita harkoneniana* a Saarimäki et al. 1061 (holotypus) b Pirot s.n. (coll. 2014) c Pirot s.n. (coll. 2014).
Two new species of *Amanita* sect. *Phalloideae* from Africa...

[Harkonen pers. comm.], very variable according to specimens but mostly of shellfish as in *Russula xerampelina*, especially for mature and old specimens [P. Pirot, pers. comm. about specimens from Madagascar], taste mild, then unpleasant [description of the Tanzanian specimen].

**Basidiospores** hyaline, with thin, rather weakly amyloid wall, (globose-) subglobose to widely ellipsoid (-ellipsoid), (6.5-) 7.0–8.07–8.6 (-10.0) × (6.0-) 6.5–7.15–8.0 (-8.5) µm, Q = (1.00-) 1.04–1.13–1.25 (-1.33) [53/3/2]. **Basidia** 4-spored, without clamp, clavate, often rather abruptly swollen, (30-) 34–37.5–41 (-46) × 9.0–10.4–11.0 (-13.0) µm, l/w = 3.00–3.60–4.40 (-4.90) [31/3/2]. **Lamellar edge** sterile, composed of marginal cells which are widely clavate to pyriform, hyaline, thin-walled, smooth, not clamped, 26–32.2–40 × 13–16.8–20 µm, l/w = 1.56–1.93–2.23 [10/1/1]. **General veil** (volva) mostly composed of cylindrical hyphae, with very different diameters, (20-) 33–50 (-110) × 4–11 (-15) µm, hyaline, with smooth and thin wall, septate but without clamps, with occasional anastomoses between parallel hyphae, branched, mixed with a few scattered hyaline sphaerocysts, globose to sphaeropedunculate or ellipsoid, 45–75–100 (-120) × (20-) 35–57–87 (-115) µm, l/w = 1.04–1.38–1.68 (-2.38), with a smooth and thin wall, rarely slightly thickened (< 1 µm) [18/1/1].
Distribution. Up to now, the species is only known from Tanzania and Madagascar. According to its ecology, it could potentially be observed in all regions occupied by the miombo woodland.

Ecology. In miombo woodland (Tanzania) and in a garden, next to *Cocos nucifera* L., *Citrus* sp. (“combava”), *Tambourissa* sp. and *Psidium guajava* L., along the Indian Ocean (Madagascar).

Etymology. This species is dedicated to Prof. Marja Härkönen in acknowledgment of her tremendous contribution to African mycology.

Specimens examined. MADAGASCAR. Prov. Toamasina: Mahambo, Dec. 2014, P. Pirot s.n. (BR); Ibidem, 2016, P. Pirot s.n. (BR). – TANZANIA. Tabora District: ca. 10 km S of Tabora, Kipalapala, ca. 1200 m alt., 12 Dec. 1991, T. Saarimäki et al. 1061 (holotype: H!).

Note. We believe that the picture of “*Amanita cfr. phalloides*” presented by Ryvarden et al. (1994: 76–77) could be *Amanita harkoneniana*. The macroscopic description and the picture given by the authors correspond to the characters of that species. From this description, the fruit-bodies have a nauseous odour, are soon decaying and grow in miombo woodlands or in association with pine trees in the middle of the rainy season; they are rarely seen. No precise locality is given but the book covers South Central Africa (mostly Malawi, Zambia and Zimbabwe).

A comparison with the closely related species is given in the chapter “discussion” below.

Chemical analyses

RP-HPLC analyses of the specimen Degreef 1304 (holotypus of *A. bweyeyensis*) was made by two of us (EK & IA). The analysis showed the complete absence of α-, β- and γ-amanitin as well as that of phallacidin and phalloidin. The results were below the limit of detection (0.6 ng/g) for all the toxins in all the analysed samples: 3 samples with cuticle and 3 samples without cuticle.

It is interesting to mention that another specimen of *A. bweyeyensis* (Tiina Saarimäki et al. 591), collected in Tanzania, had been analysed previously, in the Technical Research Centre of Finland in Espoo, and that neither amatoxins nor phallotoxins had been found in that specimen either (Harkonen pers. comm.).

Identification key to the African and Madagascan species of *Amanita* sect. Phalloideae

1 Spores elongated, Q > 1.45. Slender species, ratio stipe length / pileus diameter > 1.5. Ring funnel-shaped on young basidiomata, not striated. Pileus margin often striated because of the thinness of the flesh ........................

.............................................................. *Amanita strophiolata* [incl. var. bingensis]
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Pileus 50–60 mm diam., dirty white, often with a yellowish or greenish centre. The original description of var. *bingensis* mentions a pungent taste. Spores (7-) 7.5–10.0 (-10.5) x (4.0-) 5.0–6.5 (-7.0) µm, \( Q = 1.40–1.75 \).

- Spores less elongated, \( Q < 1.45 \). Less slender species, ratio stipe length / pileus diameter < 1.5. Ring never ascending, striated or not. Pileus margin not striated.................................................................2

2 Pileus greenish or olivaceous, sometimes yellowish-green or brownish-green, virgate (i.e. with fine darker radial stripes). Smell of old rose or rotten honey in age...............................................................*Amanita phalloides*

Pileus 65–152 mm diam., ring striate. Spores 7.5–10.0 (-12.5) x (5.5-) 6.0–7.5 (-8.0) µm.

- Pileus whitish, greyish or pale brownish (sometimes olivaceous grey with a paler margin but then, strong smell of garlic), not virgate but sometimes radially marbled. Smell fungoid or different .................................................................

3 Strong garlic smell, persisting several months in herbarium specimens. Spores subglobose, mean \( Q < 1.5 \)...................................................*Amanita alliiodora*

Pileus viscid, olivaceous grey, with a pallid margin, about 50 mm diam., ring striated.

- Smell fungoid or different. Spores subglobose or more elongated .......... 4

4 Lamellae staining yellowish when bruised ...........................................*Amanita thejoleuca*

Pileus 60–80 mm diam., pale yellowish-brown, darker in the centre. Ring rather fugacious, often missing on mature specimens. Spores 7–8 x 5–6 µm (original description), or 10–12 x 7.5–10 µm (after the spore drawings in Gilbert, 1941)

- Gills not yellowing when bruised .................................................................................................5

5 Pileus white at first, soon radially marbled by pale brownish or greyish streaks. Species mostly associated with various species of *Eucalyptus*, also mentioned once under *Casuaria equisetifolia*...............................*Amanita marmorata*

Pileus 25–95 mm diam., ring striated. Spores (6.5-) 7.5–9.5 (-11.5) x (5.5-) 6.0–8.0 (-10.0) µm, \( Q = 1.05–1.40 \)

- Pileus not marbled, uniformly coloured or paler at margin, whitish to mouse grey or pale brownish. Species bound or not with *Eucalyptus*......................6

6 Pileus mouse grey, dry. Ring striated .............................................*Amanita murinacea*

Pileus 70–80 mm diam. Spores 7.5–8.5 x 7–8 µm, mean \( Q = 1.15 \)

- Pileus whitish to pale brownish or greyish, often more or less viscid. Ring striated or not .................................................................7

7 Species growing under *Eucalyptus*. Bulb at stipe base +/- globose, neither pointed nor rooting. Ring striated. Smell sweetish, conspicuous ..............

...............................................................*Amanita buweyeyensis*

- Species not bound with *Eucalyptus*, found in Miombo woodland and in a garden. Bulb at stipe base turnip-shaped to rooting. Ring smooth or vaguely plicate. Smell weak resembling raw potato ............*Amanita harkoneniana*
Discussion

The fact that *A. bweyeyensis* seems to grow always in association with *Eucalyptus* species (Myrtaceae), which are not indigenous in Africa, suggests that the fungus has been introduced with the trees. Such introductions are well known (see for example Díez 2005). Vellinga et al. (2009) stress the fact that Pinaceae and Myrtaceae are the plant families which are the most frequently reported as hosts of introduced mycorrhizal fungi. They also mention that South Africa is the African country with the highest number of mycorrhizal introductions. We therefore compared *A. bweyeyensis* more specifically with the Australian species of *Amanita* sect. *Phalloideae* (Reid 1979, Miller 1991, Wood 1997, Davison et al. 2017, Tulloss 2018). We believe that conspecificity with any of these species can be excluded, because they present one or several of the following characters: spores too elongated (mean $Q \geq 1.4$), pileus strongly coloured (brown or grey), pileus with patches of general veil, ring absent, stipe not bulbous, different host, toxin content etc.

*Amanita marmorata* Cleland & E.-J. Gilbert was described from New South Wales (Australia) (Gilbert 1941). It was subsequently re-described from South Africa, under the name *A. reidii* Eicker & Greuning (Eicker et al. 1993, Cai et al. 2014) and from Hawaii, sub *A. marmorata* subsp. *myrtacearum* O.K. Mill., Hemmes & G. Wong (Miller et al. 1996). Before the description of *A. reidii* in 1993, African collections of that taxon were often called *Amanita phalloides* var. or f. *umbrina* (see e.g. van der Westhuizen and Eicker 1994:41). The species is present in Africa and it grows in connection with the genus *Eucalyptus* but it can be separated from *A. bweyeyensis* by its whitish pileus marbled with grey brown radial streaks and by the presence of phalloidin and phallacidin in its basidiomata (Hallen et al. 2002, Davison et al. 2017). The presence of $\alpha$- and $\beta$-amanitin in *A. marmorata* remains ambiguous. Hallen et al. (2002) stated that those toxins were present in the species (sub *A. reidii* and probably also sub *A. phalloides* f. *umbrina*), whilst Davison et al. (2017) could not detect them. The marbled colour, the globose bulb and the connection with *Eucalyptus* also exclude conspecificity with *A. harkoneniana*. *A. marmorata* is also well separated from our two new species in all the phylogenetic inferences (Figs 1 and 2).

Three new species of *Phalloideae* were recently found in Australia, namely *Amanita djarilmari* E.M.Davison and *A. gardneri* E.M.Davison from the south-west of Australia and *A. millsii* E.M.Davison & G.M.Gates from Tasmania (Davison et al. 2017). The three species have a white- or pale-coloured pileus and a white universal veil. They are quite similar to our two new species, but are however well separated from them in the phylogenetic trees (Figs 1 and 2). The following differences with *A. bweyeyensis* can also be cited: *A. djarilmari* has elongated spores (mean $Q = 1.43$) and contains phallacidin and phalloidin; *A. gardneri* has a fusiform bulb at stem base, becoming radicant, very elongated spores (mean $Q = 1.81$) and contains phallacidin and phalloidin; *A. millsii* is apparently not connected with *Eucalyptus* species and it contains phallacidin and phalloidin. The three species can be separated from *A. harkoneniana* by the following characters: *A. djarilmari* has a rounded bulb at stem base and elongated spores (mean
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Q = 1.43); *A. gardneri* has very elongated spores (mean Q = 1.81); *A. millsii* shows persistent patches of universal veil on the pileus, its basidiomata have a more squat habit and its basidia are longer (43–61 µm).

*Amanita capensis* A. Pearson & Stephens is a nom. nud. which was published in Stephens and Kidd (1953) and quite largely used in South Africa (“Cape death cap”). It is usually considered as a stouter colour variant of *Amanita phalloides*, including specimens with a whitish pileus (Levin et al. 1985, Reid and Eicker 1991). Several cases of severe poisoning have been attributed to the “species”, some of them fatal (Stephens and Kidd 1953, Steyn et al. 1956, Sapeika et al. 1960). It is therefore surprising that Hallen et al. (2002) did not find the toxins in a specimen identified as *A. capensis* but the exact identity of the fungus remains uncertain and confusion with another taxon cannot be excluded. Conspecificity with *A. bweyeyensis* can be rejected amongst others because of the toxicity and pileus colour of *A. capensis* as well as of its association with other trees than *Eucalyptus*. *Amanita capensis* differs from *A. harkoneniana* amongst others because it has a larger size than this latter species, a globose bulb and a striated ring.

*Amanita alliiodora* Pat. is a very poorly known species, described from Madagascar by Patouillard (1924, corrected version in 1928). The most important characteristics of the species are the following. Pileipellis pale olivaceous grey, whitish at the margin, viscid when moist. Ring striated. Spores subglobose, 7–8 µm diam. (Patouillard 1924, 1928) or 8–8.5 µm diam. (Dujarric de la Rivière and Heim 1938), or 8.5–9.6 × 7.8–8.7 µm (Tulloss 2017, after the spore drawings from the type specimen, published in Gilbert 1941). The species is also said to have a bitter taste and to produce a strong smell of garlic, still persisting on exsiccates. It is considered toxic and is not eaten by the local population, which however uses its odour to cure headaches. *A. alliiodora* is distinct from *A. bweyeyensis* because it has a grey pileus and a strong smell of garlic and also because it does not grow in association with *Eucalyptus* and is probably toxic. It is also distinct from *A. harkoneniana* because of the grey pileus, the striated ring and the smell of garlic. *A. alliodora* clustered in a sister position to *A. bweyeyensis* in the ITS-nucLSU based phylogenetic analysis, forming a two-species clade sister to *A. harkoneniana*, showing that these three species share a common phylogenetic background.

Within the genus *Amanita*, the genes encoding amatoxins (α- and β-amanitin) and phallotoxins (phallacidin and phalloidin) were found so far to be present only in species that produce these compounds (Hallen et al. 2007). The successful PCR amplification of the PHA gene for both *A. bweyeyensis*, a species which is regularly consumed by local people and *A. harkoneniana* was indeed surprising. Especially since the HPLC analysis did not show any sign of these compounds in the basidiomata. This is the first time that the presence of at least one of those genes (PHA gene) could be proven for species that seem to lack (or have lost) the ability to produce these toxins.

Very little is indeed known about the mechanisms behind the regulation of the fungal secondary metabolism. Many factors can play a key role in preventing the expression of phallacidin gene in these species. Several studies (Enjalbert et al. 1993, 1999; Brüggemann et al. 1996; Mcknight et al. 2010; Kaya et al. 2015) have shown
that phallotoxin amounts and distribution (localisation in the basidiome) in *A. phalloides* largely vary as a result of environmental and climatic conditions. Furthermore, several studies have shown that the toxin concentration in the pure cultured mycelium of deadly *Amanita* is about 10% of that in basidiomata and that it is indeed possible to increase the amatoxin production through optimisation of growth conditions, such as medium composition, pH and temperature etc. (Zhang et al. 2005, Hu et al. 2012). Furthermore, temporal and structural sequestration of secondary metabolites are common features in microorganisms. Amatoxins and phallotoxins are biologically active secondary metabolites and some mechanism of separation from primary metabolism seems to be essential to avoid their coming into contact with their sites of action (RNA polymerase II and F-actin, respectively). Having higher toxin concentrations only in the basidiome, or part of it, would invest resources for defence where it is especially needed, in the visible and vulnerable mushroom and not microscopic spores or mycelia.

Amatoxins and phallotoxins are encoded by members of the “MSDIN” gene family and are synthesised on ribosomes as short (34- to 35-mer) pro-proteins, with conserved upstream and downstream sequences flanking a hypervariable region of 7 to 10 amino acids (Hallen et al. 2007, Luo et al. 2012). The hypervariable region gives rise to the linear peptides corresponding to the mature toxins. The precursor peptides must undergo several post-translational modifications, including proteolytic cleavage, cyclisation, hydroxylation and formation of a unique tryptophan-cysteine cross bridge called trypthionine. In particular, they are cleaved and macrocyclised into 7–10 amino acid cyclic peptides by a specialised prolyl-oligo-peptidase enzyme (POP), which is the key enzyme of the cyclic peptide pathway, catalysing both hydrolysis (Luo et al. 2009, 2014; Riley et al. 2014).

The genes of most secondary metabolite biosynthetic pathways tend to be clustered and co-regulated in fungi (e.g. fumonisin biosynthesis in *Fusarium*). Many, but not all, clusters contain cluster-specific transcription factors that regulate expression of the biosynthetic genes for their respective metabolites, thus allowing for multiple regulatory layers giving the producing fungus precise spatial and temporal control over metabolite expression. A mutation in each key protein involved in the biosynthetic/regulatory pathway of phallotoxins production could result in an altered expression of the toxin. The evolutionary persistence of toxins productions in *Amanita* sect *Phalloideae* suggests that it should confer some selective advantage to the producing fungi. Since the lack of toxins could be the result of an alteration of the expression of these genes due to environmental and climatic conditions, in our opinion *A. bweyeyensis* and *A. harkoneniana* should be considered to have the potential to be deadly poisonous.

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