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Convergent evolution of sequestrate forms in *Amanita* under Mediterranean climate conditions

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Abstract: The systematic position of secotioid (*Torrendia*) and gasteroid (*Amarrendia*) forms within the agaricoid *Amanita* lineage (Agaricales, Basidiomycota) was studied with molecular (nLSU, ITS) data. Secotioid and gasteroid forms occur in four independent clades nested within agaricoid forms. One clade corresponds to the secotioid *T. pulchella* from southern Europe and northern Africa. The others correspond to *Torrendia* and *Amarrendia* species from Australia. Mediterranean climatic conditions are postulated as a force driving the convergent evolution of these secotioid and at least one of the gasteroid forms in geographically distant areas. Species formerly placed in *Torrendia* and *Amarrendia* are transferred to *Amanita*. A new species of *Torrendia* from Australia was discovered during the revision of the collections originally identified as *T. arenaria* and is described here as *Amanita pseudoinculta*.

Key words: *Amarrendia*, ITS, nLSU, phylogeny, sequestrate forms, *Torrendia*

INTRODUCTION

Secotioid and gasteroid forms have evolved independently several times from agaricoid/boletoid ancestors in different groups of fungi (Hibbett 2007), such as Boletales (Binder and Hibbett 2006), Russulales (Eberhardt and Verbeken 2007), Agaricales (Peintner et al. 2001) and Phallomycetidae (Hosaka et al. 2006). Gasteroid fungi such as false truffles, puffballs and stinkhorns are highly modified for nonballistosporic dispersal, whereas secotioid forms are morphologically intermediate between gasteroid and their agaricoid/boletoid ancestors. The advantage of these forms against loss of moisture under unfavorable conditions (extreme drought or cold) has been proposed as a main factor favoring their evolution (Thiers 1984). It also has been postulated that natural selection would act against intermediate (secotioid) forms because they lack the dispersal advantages of the agaricoid and gasteroid forms (via air and animals respectively) and are only partially adapted to seasonal xeric conditions (Bruns et al. 1989). Peintner et al. (2001) showed that it also is possible that successfully adapted, stable, sequestrate (secotioid and gasteroid) forms tend to radiate but they can radiate only into species with the same basidiome type or a further reduced type. This study concerns the evolution of secotioid (*Torrendia*) and gasteroid (*Amarrendia*) forms within the Amanitaceae (Agaricales).

The secotioid genus *Torrendia* was created by Bresadola (1902) to accommodate a small, whitish, volvate and stipitate gasteromycete collected in Portugal by C. Torrend. After a careful study of the morphology and development of *Torrendia pulchella* Bres. a close relationship with the agaricoid genus *Amanita* Pers. was postulated (Malençon 1955, Bas 1975). Miller and Horak (1992) described a second species of *Torrendia* from Western Australia, *T. arenaria* O.K. Mill. & E. Horak, very similar in its external morphology to *T. pulchella* but differing in spore shape and size, presence of sclerobasidia, absence of clamp connections and different mycorrhizal partners. A variant with yellowing flesh was given the name *T. arenaria* f. *lutescens* O.K. Mill & E. Horak. Bougher (1999) described two new species also from Western Australia, *T. grandis* Bougher, mainly characterized by the relatively big basidiomes, and *T. inculta* Bougher with a gleba that fragments during stipe elongation. Both species have clamp connections and lack sclerobasidia, which separates them from *T. arenaria*, and possess ellipsoid to oblong spores, which separates them from *T. pulchella*.

Torrendia has a puzzling distribution pattern; one species (*T. pulchella*) occurs in the Mediterranean Basin, including the Iberian Peninsula (Spain, Portugal), northern Africa (Morocco, Algeria), southern France, Sardinia (Italy) and Turkey, with putative mycorrhizal partners such as *Pinus*, *Quercus* and

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Cistus (Neville and Poumarat 2004), and the other three taxa (*T. arenaria*, *T. grandis*, *T. inculta*) occur in Western Australia with putative mycorrhizal partners such as *Eucalyptus*, *Allocasuarina* and *Leptospermum* (Miller and Horak 1992, Bougher 1999). With this disjunct distribution in both hemispheres a monophyletic *Torrendia* would imply that the group is ancient or that there has been long distance dispersal, either by natural or anthropogenic means.

The gasteroid genus *Amarrendia* Bougher & T. Lebel was created to include species with these characteristics: white to cream peridium and gleba, basidiome flesh fragile and minutely granular; gleba loculate; spores smooth, thin-walled, hyaline, nonamyloid and nondextrinoid, with a large oil droplet, broadly ellipsoid and with a broad apiculus; context trama composed of inflated and hyphal elements intermixed. Bougher and Lebel (2002) described three new species of *Amarrendia* (viz. *Amarrendia oleosa* Bougher & T. Lebel, *Amarrendia nemoribus* Bougher & T. Lebel and *Amarrendia peridiocrystalia* Bougher & T. Lebel) and recombined two species formerly placed in *Alpova* (*Amarrendia grandispora* [G.W. Beaton, Pegler & T.W.K. Young] Bougher & T. Lebel and *Amarrendia lignicolor* [G.W. Beaton, Pegler & T.W.K. Young] Bougher & T. Lebel). The genus is distributed in Western Australia, Victoria and Tasmania with putative mycorrhizal partners such as *Eucalyptus*, *Allocasuarina*, *Acacia* and *Gastrolobium*. *Amarrendia* was proposed to occupy a systematic position within a complex of related taxa that also incorporates *Torrendia* and *Amanita* mainly based in the presence of inflated elements in the trama and the characteristics of the spores (Bougher and Lebel 2002)

Previous molecular work has shown that *T. pulchella* is a secotioid derivative of *Amanita* with its closest agaricoid relatives in sect. *Caesareae* (Moncalvo et al. 2002). A previous analysis by Hallen et al. (2004) suggests that *Torrendia* is polyphyletic and also that among the five species of *Amarrendia* only two, *Amarrendia oleosa* and *Amarrendia grandispora*, are part of the *Amanita* lineage while the other species fall outside the Amanitaceae or even the Agaricales clade. However that analysis was not formally published and the sequence data were not deposited in GenBank.

The purpose of the present study was to revisit the origins of *Torrendia* and *Amarrendia* with new sequence data obtained from Australian and European materials to assess the monophyly and systematic position of *Torrendia* and *Amarrendia* among the *Amanita* lineage, discuss its implications for morphological and ecological evolution and make formal taxonomic proposals.

MATERIALS AND METHODS

Sequences.—Thirty-six new sequences (nuclear ribosomal RNA large subunit [nLSU] and the internal transcribed spacers [ITS]) were generated from herbarium material of *T. pulchella*, *T. arenaria*, *T. inculta*, *T. grandis* and *Amarrendia oleosa*. Sequences generated from an unidentified herbarium collection (H909), possibly belonging to *Amarrendia*, also were included in the study. The nLSU data for *Amarrendia grandispora* came from Hallen et al. (2004). A total of 167 sequences of *Amanita*, two of *Limacella* and one of *Torrendia pulchella*, the majority coming from the works of Weiß et al. (1998), Drehmel et al. (1999) and Zhang et al. (2004), were retrieved from GenBank (TABLES I, II).

Approximately 0.05 g each herbarium collection were ground in liquid nitrogen, and DNA was extracted with 3% SDS extraction buffer; DNA then was isolated by the sequential addition of phenol chloroform and chloroform-isoamyl alcohol; finally, isopropyl alcohol and 3M sodium acetate were added to precipitate the DNA, which was washed with 70% EtOH and resuspended in sterile water. A portion of the nLSU and the complete ITS1 + 5.8 + ITS2 (ITS) regions were amplified by PCR with fungal primers LR0R and LR5 and ITS1F and ITS4 respectively (Gardes and Bruns 1993, http://www.biology.duke.edu/fungi/mycolab/primers.htm). Amplification products were sequenced with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction reagents with primers LR0R and LR5 (with additional LR3R and LR3 for some samples) and ITS1F and ITS4. Sequencing was carried out on an ABI 3130 Genetic Analyzer. Raw data were processed with Sequencher 4.7 (GeneCodes, Ann Arbor, Michigan). The ITS region of all the Australian taxa of *Torrendia* and *Amarrendia* showed a high level of intragenomic variability, so the PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California) following the manufacturer's instructions.

Alignment.—Sequences were aligned with MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). Alignments then were examined and manually corrected in MacClade 4.05 (Maddison and Maddison 2002). They have been deposited in TreeBASE under accession number S2490.

Phylogenetic analysis.—Maximum parsimony (MP), neighbor joining (NJ), maximum likelihood (ML) and Bayesian analyses (BA) were performed with these parameters: (i) MP. Equally weighted parsimony analysis was performed with PAUP* 4.0.b10 (Swofford 2002). One thousand heuristic search replicates were performed with starting trees generated by stepwise addition with random addition sequences followed by tree bisection reconnection (TBR) branch swapping. Up to two trees were kept in each replicate. Parsimony bootstrap analysis was performed with 1000 replicates, each with 10 random taxon addition sequences and branch swapping set to subtree pruning and regrafting (SPR). (ii) NJ. The analysis was run in PAUP* 4.0.b10 (Swofford 2002) with distances estimated under a general time reversible (GTR) model. NJ bootstrap

TABLE I. Sequences retrieved from GenBank

Taxon	nLSU	ITS	Taxon	nLSU	ITS	Taxon	nLSU	ITS
A. abrupta	—	AB015685	*A. fuliginea*	AF024454	FJ176719	*A. ponderosa*	EF653957	AY486234
A. aff. citrina	AY436489	—	*A. fulva*	AF024455	AB015692	*A. porphyria*	AY436500	—
A. aff. fulva	AF024456	—	*A. fuscosquamosa*	—	AY194974	*A. pseudoporphyria*	AF024471	AB015677
A. alboverrucosa	—	AY194973	*A. gemmata*	AF024457	—	*A. pseudovaginata*	AF024472	AY436470
A. altipes	AY436487	AY36445	*A. gilbertii*	—	AY325838	*A. punctata*	—	AB015693
A. angustilamellata	AF024440	—	*A. griseopantherina*	AY436494	AY436459	*A. pyramidifera*	—	AY194979
A. areolata	—	AB167727	*A. griseoturcosa*	—	AB167728	*A. reidii*	—	AY325824
A. armillariiformis	AF261436	—	*A. griseoverrucosa*	AY436495	FJ441044	*A. rhoadsii*	AF097391	—
A. arocheae	AY325879	AY325832	*A. hemibapha*	—	FJ441038	*A. roseolamellata*	—	AY194980
A. atrofusca	—	AY436446	*A. hemibapha var. ochracea*	AF024458	—	*A. rubescens*	AF097383	EU819464
A. avellaneosquamosa	AF024441	AY436447	*A. ibotengutake*	AB088767	AB211057	*A. rubrovolvata*	AF024473	AB096055
A. bisporigera	AF097384	EU819411	*A. imazekii*	—	AB038768	*A. sepiacea*	AY436501	AY436473
A. brunneofuliginea	AF024442	—	*A. incarnatifolia*	AF024459	—	*A. sinensis*	AF024474	AB096060
A. brunnescens	AY631902	AY789079	*A. jacksonii*	AF097376	AY436461	*A. solitaria*	AF024475	AY436475
A. caesarea	AF024443	AY486237	*A. japonica*	AF024460	AB015684	*A. spissa*	—	AJ889924
A. ceciliae	AF024444	AB015694	*A. kotohiraensis*	FJ011682	FJ176723	*A. spissacea*	—	AB015683
A. cf. *crocea*	AY436490	—	*A. lanei*	—	DQ974693	*A. stranella*	—	FJ596814
A. chepangiana	AF024445	AY436450	*A. lignitincta*	AF024461	FJ441045	*A. strobiliformis*	AF024476	—
A. cinereopannosa	—	FJ596838	*A. liquii*	AY436493	—	*A. subfrostiana*	AF024477	—
A. citrina	AF097378	FJ596868	*A. longipes*	—	FJ596834	*A. subglobosa*	AF024478	—
A. citrina. var. grisea	AF024447	—	*A. longistriata*	AF024462	AB015678	*A. subjunquillea*	AF024479	FJ176733
A. clarisquamosa	AF024448	FJ375331	*A. magnivelaris*	AY325873	—	*A. submembranaceaea*	—	FJ705275
A. coacta	FJ236807	—	*A. manginiana*	AF024463	—	*A. sychnopyramis*	AF024480	AB015690
A. concentrica	—	AB80783	*A. marmorata subsp. myrtacearum*	—	AY325826	*A. umbrinella*	—	AY194981
A. conicoverrucosa	AY194983	AY194972	*A. melleiceps*	—	AB015688	*A. umbrinolutea*	AF024481	AY436478
A. constricta	AY228351	EU597073	*A. mira*	AF024464	—	*A. vaginata*	AF024482	AJ889925
A. crocea	—	FJ441033	*A. muscaria*	EU072012	AB080790	*A. velosa*	—	AY918961
A. curtipes	EF653960	AY486235	*A. nauseosa*	AY194984	—	*A. verna*	—	EU909448
A. cylindrispora	AY325867	AY325839	*A. nivalis*	AF024466	—	*A. verrucosivolva*	AF024483	—
A. eijii	—	FJ441039	*A. novinupta*	—	DQ974690	*A. virgineoides*	AF024484	FJ441032
A. esculenta	—	AY436451	*A. ochrophylla*	—	AY194977	*A. virosa*	AF159086	FJ755188
A. excelsa	AY436491	AY436453	*A. ocreata*	AY325880	AY918962	*A. volvata*	AF097388	AB015681
A. exitialis	AY436492	AY855212	*A. orientifulva*	AY436496	FJ441035	*A. xanthocephala*	—	AY194982
A. farinosa	AF097370	FJ441036	*A. orientigemmata*	AY436497	AY436465	*A. yuaniana*	AF024488	AB039792
A. flavipes	AF024451	AB156996	*A. ovalispora*	—	FJ441041	*Amanita sp. (MEL2151450)*	—	AY194970
A. flavoconia	AF042609	EU569281	*A. pantherina*	AB088768	—	*Limacella glischra*	AY612843	—
A. flavorubescens	AF097380	—	*A. parvipantherina*	AY436499	EU569283	*Limacella illinita*	AF261439	—
A. franchetii	AF097381	DQ822790	*A. peckiana*	AF097387	—	*Torrendia pulchella*	AF261566	—
A. fritillaria	AF024452	—	*A. phalloides*	DQ071721	AJ889921			
A. frostiana	AF024453	—	*A. pilosella*	AF024470	—			

TABLE II. New sequences generated for this study with GenBank accession numbers

Taxon	Collection, locality and date	nLSU	ITS
Amanita pseudoinculta	VPI 366. Australia: Western Australia, Mullering Brook (approx. 206 km north of Perth), 3-VII-1991	GQ925372	—
Amanita pseudoinculta	VPI 411. Australia: Western Australia, Mullering Brook (approx. 206 km north of Perth), 6-VI-1989	GQ925373	GQ925389 GQ925390 GQ925391 GQ925392
Amanita pseudoinculta	VPI 555. Australia: Western Australia, Mullering Brook (approx. 206 km north of Perth), 3-VII-1991	GQ925375	—
Amanita pseudoinculta	VPI 558. Australia: Western Australia, Mullering Brook (approx. 206 km north of Perth), 3-VII-1991	GQ925374	—
Amanita ("Amarrendia") grandispora[a]	H0792(CSIRO). Australia: Tasmania, Forest resources site near Exton, 10-V-1995	GQ925385	—
Amanita ("Amarrendia") oleosa	H7627(CSIRO). Australia: Western Australia, Dwellingup (approx 140 km south of Perth), 4-VII-2000	GQ925377	GQ925398 GQ925399 GQ925400
"Amarrendia sp."	H909(CSIRO). Australia: locality unknown	GQ925378	GQ925401 GQ925402 GQ925403
Amanita ("Torrendia") arenaria	VPI 363. Australia: Western Australia, Julimar Forest (approx. 80 km south of Perth), 12-VI-1991	GQ925384	—
Amanita ("Torrendia") arenaria	VPI 364. Australia: Western Australia, Kalamunda (approx. 25 km east of Perth), 9-VII-1991 (identified as ''*Torrendia arenaria* f. *lutescens*'').	GQ925380	GQ925388
Amanita ("Torrendia") arenaria	VPI 365. Australia: Western Australia, Kalamunda (approx. 25 km east of Perth), 9-VII-1991	GQ925379	—
Amanita ("Torrendia") arenaria	VPI 412. Australia: Western Australia, Two Peoples Bay (approx 424 km south of Perth), 22-VI-1989	GQ925383	GQ925393
Amanita ("Torrendia") arenaria	VPI 551. Australia: Western Australia, Kalamunda (approx. 25 km east of Perth), 2-VII-1991	GQ925381	—
Amanita ("Torrendia") arenaria	VPI 679!. Australia: Western Australia, Two Peoples Bay (approx. 424 km south of Perth), 22-VI-1989	GQ925382	—
Amanita ("Torrendia") grandis	H7353 (CSIRO). Australia: Western Australia, 16 km north of Kellerberrin (approx 200 km east of Perth), 1-VIII-1996	GQ925376	GQ925396, GQ925397
Amanita ("Torrendia") inculta	H7335 (CSIRO). Australia: Western Australia, 16 km north of Kellerberrin (approx 200 km east of Perth), 23-VII-1996	GQ925371	GQ925394, GQ925395
Amanita torrendii (= T. pulchella)	LOU-Fungi 19028. Spain: Huelva, La Nava, 6-XI-2003	GQ925370	—
Amanita torrendii (= T. pulchella)	LOU-Fungi 17408. Spain: Lugo, O Corgo, Rioseco, 10-XI-1998	GQ925369	GQ925386
Amanita torrendii (= T. pulchella)	LOU-Fungi 18202. Spain: Ourense, Taboadela, 1-XI-2002	GQ925368	GQ925387

[a] This sequence comes from the unpublished study of Hallen et al. (2004).

was performed with 1000 replicates. (iii) ML. The analysis was run in the RAxML servers (http://8ball.sdsc.edu:8889/cipres-web/Home.do, which implements the search protocol of Stamatakis et al. 2008) under a GTR model with 1000 rapid bootstrap replicates. (iv) BA. The analysis was run with MrBayes 3.1 (Ronquist and Huelsenbeck 2003) for 10 000 000 generations under a GTR model with four chains and trees sampled every 100 generations. After examining the graphic representation of the likelihood scores with Microsoft Excel the burn-in was set to 20 000 generations. All four analyses were performed with the nLSU dataset, while only MP and ML analyses were performed with the ITS datasets. MP and ML analyses also were performed on a combined nLSU and ITS dataset although the results are shown only in supplementary material because they yielded essentially the same results as the analyses of the non-combined datasets.

Morphological descriptions.—Standard methods for describing the basidiocarps were applied with the terminology of Neville and Poumarat (2004). The notation [120/8/4] indicates that measurements were made in 120 spores in 8 samples from 4 collections. The spores of *Torrendia* lack a distinct lateral view, although a few slightly asymmetric spores with a somewhat lateral apiculus can be observed sometimes in microscopical preparations, therefore the spores were measured in frontal view including the broad central apiculus as was done in previous descriptions of *Torrendia* species (Miller and Horak 1992, Bougher 1999, Neville and Poumarat 2004).

Abbreviations.—avl = average length, avw = average width, Q = length/width quotient, avQ = average quotient, I.C.B.N = International Code of Botanical Nomenclature. To avoid confusion *Amanita* is abbreviated as ''*A.*'' and *Amarrendia* is not abbreviated.

RESULTS

Analyses of the nLSU dataset.—The partial nLSU sequences of *Torrendia* and *Amarrendia* obtained in this study range from 761 base pairs (bp) in *Torrendia pulchella* to 957 bp in *T. grandis*. The sequence of *Amarrendia grandispora* obtained in Hallen et al. (2004) is 572 bp long. The dataset includes 16 *Torrendia*, three *Amarrendia*, 81 *Amanita* and two *Limacella* sequences, which were used as outgroup. The final dataset consists of 102 sequences of 1198 characters (gaps included), of which 296 are parsimony informative. In the MP analysis a total of 50 equally most parsimonious trees (MPT) were recovered (Length = 1710, CI = 0.35, RI = 0.76).

In all four analyses the general topology with respect to subgeneric classification of *Amanita* was consistent among all trees and studies on the genus (Weiß et al. 1998, Drehmel et al. 1999, Zhang et al. 2004). Two clades corresponding to the traditional morphological subgenera *Amanita* and *Lepidella* and seven corresponding to sections *Amanita*, *Caesareae*, *Amanitopsis* (= *Vaginatae*), *Lepidella*, *Phalloideae*, *Validae*, including *Mappae*, and *Amidella* were recovered, although some with low bootstrap support. Two species of section *Lepidella* (*A. armillariiformis* Trueblood & D.T. Jenkins and *A. nauseosa* [Wakef.] D.A. Reid) were placed outside the *Amanita* clade; these species are suspected to be non-ectomycorrhizal, and similar results about their placement were observed in Moncalvo et al. (2002). The internal topology of some sections and the relations between sections, especially in subgenus *Amanita*, were poorly resolved as shown in the Bayesian phylogram (FIG. 1). *Torrendia* and *Amarrendia* species are distributed along the tree in four groups, which we refer to as the Torrendia clade, Amarrendia clade, Pseudoamarrendia clade and Arenaria clade.

The Torrendia clade includes the three new sequences of *T. pulchella* (the type species of *Torrendia*) and the existing GenBank sequence, which were placed together with high support in all analyses. The position of *T. pulchella* as a member of sect. *Caesareae* is highly supported in all four analyses.

The Amarrendia clade includes *T. grandis*, *T. inculta*, four collections originally identified as *T. arenaria* that are here considered to represent a different species referred to in the text and figures as *Amanita pseudoinculta* (see TAXONOMY), *Amarrendia oleosa* (the type species of *Amarrendia*) and *Amarrendia grandispora*. The Amarrendia clade is placed as a sister group of sect. *Caesareae* in the MP, ML and NJ analyses, but this relationship receives significant support only in the NJ analysis (80%). In the BA analysis the relationships among the four main clades of subgenus Amanita are unresolved (FIG. 1).

The Pseudoamarrendia clade includes the unidentified *Amarrendia* sp. that is grouped with two species of sect. *Amanitopsis* (*A. verrucosivolva* Zhu L. Yang and *A.* aff. *fulva*). This clade is highly supported in all analyses because it is the sister taxa relationship between *A.* aff. *fulva* and *Amarrendia* sp. (FIG. 1). The Pseudoamarrendia clade appears to be the sister group of all other species of sect. *Amanitopsis* in the study. The data of this ''*Amarrendia* sp.'' come from the herbarium collection H909 (CSIRO), but no additional morphological, ecological or geographic data are available so no formal description of a new species can be made at this moment.

The Arenaria clade includes six collections of *T. arenaria* including the type collection (VPI 679) and one collection identified as *T. arenaria* f. *lutescens* (VPI 364). The Arenaria clade is supported in all analyses, although its closest relatives cannot be identified with certainty in BA, ML and NJ analyses, is placed as the sister group of sect. *Phalloideae* but with no statistical support.

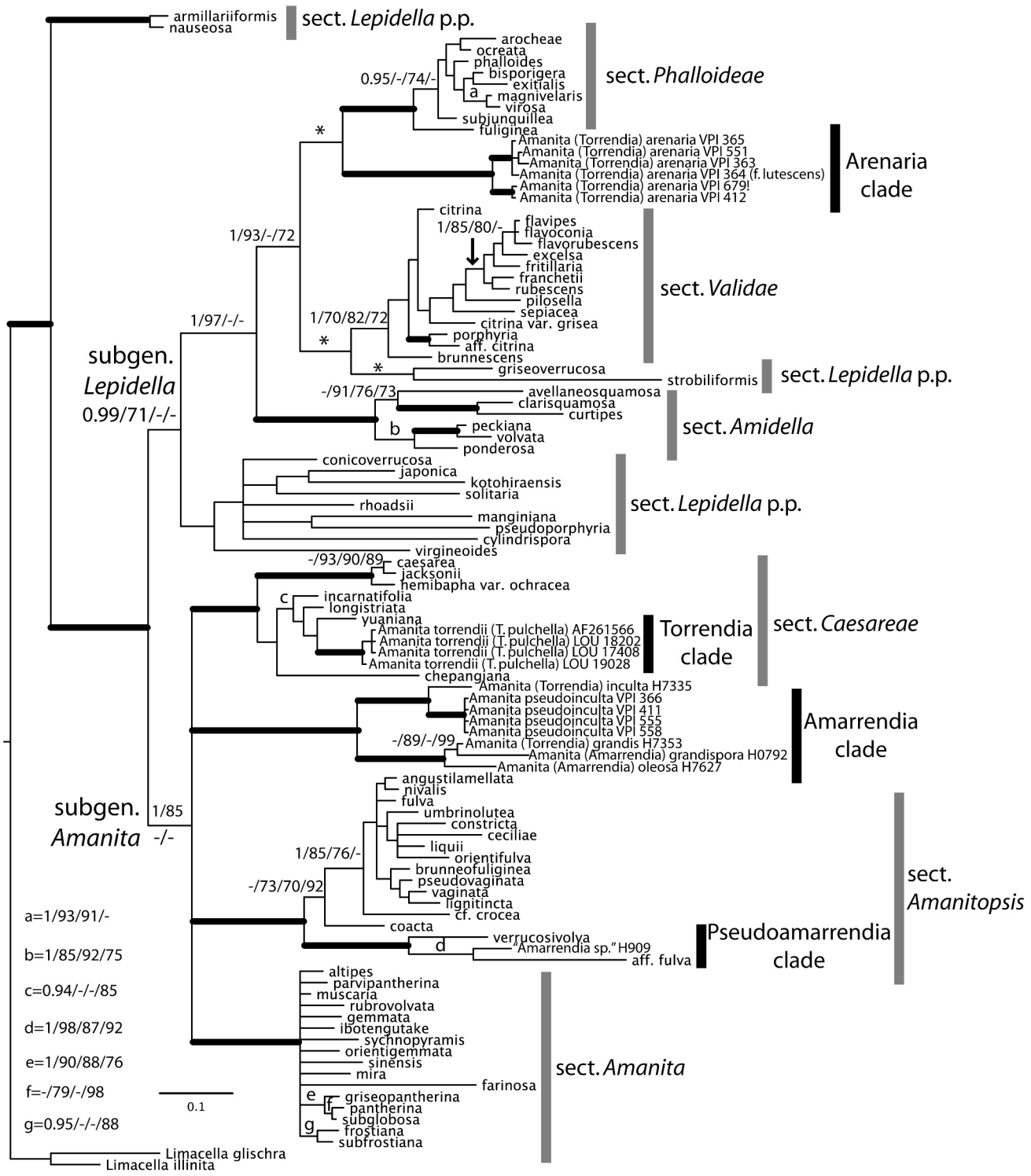

FIG. 1. Fifty per cent majority rule Bayesian phylogram for the nLSU dataset. Thick branches are supported by posterior probabilities of 1 and bootstrap values > 90% in the other analyses (ML/MP/NJ). Other values for branches supported in at least two of the analyses with posterior probabilities > 0.90 and/or bootstrap values > 70% are shown on the branches (BA/ML/MP/NJ). An asterisk indicates that the branch collapses in the strict consensus tree of the MP analysis.

Analyses of the ITS datasets.—Due to high levels of sequence divergence, two separate datasets were constructed: one only with members of subgenus *Amanita* with all species of *Torrendia* and *Amarrendia* except *T. arenaria* and a second dataset only with members of subgenus *Lepidella* and *T. arenaria*. After an initial ML analysis of the second dataset (SUPPLEMENTARY FIG. 1) species of section *Lepidella* were excluded from the analysis. A similar problem with ITS alignment in *Amanita* has been reported by Moreno et al. (2008).

Only the ITS region of *T. pulchella* could be obtained through direct sequencing (611 bp). Cloning was necessary to obtain the ITS of Australian species of *Torrendia* and *Amarrendia*. The final datasets contained two clones of *T. inculta* (669–675 bp), four clones of *Amanita pseudoinculta* (683–705 bp), two clones of *T. grandis* (663–669 bp), three clones of *Amarrendia oleosa* (662–664 bp), three clones of *Amarrendia sp.* (606–613 bp), one clone of *T. arenaria* from the type locality (712 bp) and one clone of *T. arenaria* from a different locality (715 bp). Considerable intragenomic variability was observed, for example the four cloned sequences of *A. pseudoinculta* differ in 36 point mutations and four small indels (1 or 2 bases).

Subgenus Amanita. The final dataset consists of 57 sequences of 1020 characters (gaps included), of which 482 are parsimony informative. *A. phalloides* (Vail. ex Fr.: Fr.) Link and *A. porphyria* (Alb. & Schwein.: Fr.) Alb. & Schwein. were used to root the tree. A total of 180 MPT were recovered (Length = 2614, CI = 0.47, RI = 0.72).

The main difference with the nLSU analyses is that an agaricoid species of *Amanita* (*A. umbrinella* E.-J. Gilbert & Cleland) is included in the Amarrendia clade as the sister taxon of *Torrendia inculta* and *Amanita pseudoinculta*. This taxon was not included in the nLSU dataset.

Subgenus Lepidella. The final dataset consists of 37 sequences of 911 characters (gaps included), of which 392 are parsimony informative. *A. muscaria* (L.: Fr.) Lam. and *A. vaginata* (Bull.: Fr.) Lam. were used to root the tree. A total of 42 MPT were recovered (Length = 1783, CI = 0.55, RI = 0.68).

Again the ITS analyses yielded results very similar to the nLSU data. In both MP and ML trees (FIG. 3) five main clades are recovered: sections *Amidella*, *Validae*, *Phalloideae*, two species of sect. *Lepidella* (*A. griseoverrucosa* and *A. cinereopannosa* Bas) and the Arenaria clade. In the ML tree (FIG. 3) the Arenaria clade and the two species of sect. *Lepidella* are placed (with no bootstrap support) as a sister group of sect. *Phalloideae*.

Analyses of the combined dataset.—Two datasets (subgen. *Amanita* and subgen. *Lepidella*) were constructed for the combined analysis (nLSU + ITS). No major differences with the independent analyses were observed (SUPPLEMENTARY FIGS. 3, 4).

TAXONOMY

Torrendia and *Amarrendia* are polyphyletic entities that are nested within *Amanita*. To maintain monophyletic taxa the species described in *Torrendia* and *Amarrendia* must be transferred to *Amanita*, as suggested by Tulloss and Yang (http://www.njcc.com/~ret/amanita/mainaman.html). The generic names *Torrendia* and *Amarrendia* convey information about morphological diversity that unfortunately will be lost if these names are simply subsumed into *Amanita*. To maintain the correspondence between taxonomy and phylogeny, while highlighting morphological variation, we suggest that the ''Torrendia'', ''Amarrendia'', ''Pseudoamarrendia'' and ''Arenaria'' clades eventually should be classified formally as sections or subsections of *Amanita*. However these formal recombinations and descriptions of infrageneric taxa are not made here because more extensive sampling of *Amanita* species is necessary for a better resolution of the internal topology of the genus. The current molecular sampling only accounts for approximately 10–15% of the estimated diversity of *Amanita* (Tulloss 2005). In the meantime the informal clade names still can be used as such or as ''stirps'', which are traditionally used in the taxonomy of *Amanita* (Bas 1969) but are not governed by the rules of the I.C.B.N. Analyses of nLSU sequences suggest that sect. *Lepidella* is polyphyletic and also requires reclassification, but that taxonomic problem is not addressed here.

The situation involving *Torrendia* and *Amarrendia* is similar to that in other clades of Agaricomycetes that contain secotioid and gasteroid taxa nested within paraphyletic assemblages of agaricoid or boletoid taxa. Fungal systematists typically have approached this recurring problem by combining secotioid and gasteroid forms under the generic name of their related agaricoid or boletoid forms, as we do here. For example Vellinga et al. (2003) recombined the species of the polyphyletic *Endoptychum* into *Agaricus* and *Chlorophyllum*, which were described based on agaricoid taxa. Similarly Eberhardt and Verbeken (2004) described a new gasteroid fungus as a species of *Lactarius*, which is based on agaricoid forms, instead of placing the taxon in *Arcangeliella* or *Zelleromyces*, which are gasteroid ''genera'' nested within *Lactarius*. The polyphyletic secotioid *Thaxterogaster* also was recombined in *Cortinarius* (Peintner et al. 2002).

Some workers have expressed concern that the loss of generic names of sequestrate taxa reduces the information content of classifications (Smith and Healy 2009). We endorse the view that the primary (if not the sole) organizing principle for biological classifications should be phylogeny. At the same time it is an unfortunate paradox of modern taxonomy that improvements in understanding of phylogeny can cause the loss of names that highlight unique clades with distinguishing morphological features. The root cause of this situation is the use of taxonomic ranks and the prohibition of having taxa at a given rank nested within other taxa of equal or lower rank. Rank-free classification systems could mitigate such problems and thereby promote stability and continuity in name usage (Hibbett and Donoghue 1998). At present there is no generally accepted system of unranked biological nomenclature, but the forthcoming PhyloCode (http://www.ohio.edu/phylocode/) might provide a useful model. In the meantime the solution we advocate attempts to highlight distinctive clades in a Linnaean taxonomic framework.

Most of the required transfers are straightforward. However a new name is needed for *Torrendia pulchella* because the combination *Amanita pulchella* S. Imai already exists. The name *Amanita arenaria* K. Syme also exists, but it was not validly published (K. Syme pers com) because there was no Latin diagnosis (I.C.B.N., art. 36) and therefore is available for *Torrendia arenaria*. The new species discovered during revision of the original *Torrendia arenaria* collections is described here under the name *Amanita pseudoinculta*.

Amanita arenaria (O.K.Mill. & E. Horak) Justo, comb. nov.
MycoBank 515037
Basionym: *Torrendia arenaria* O.K.Mill & E. Horak, Mycologia 84(1):65. 1992

Amanita grandis (Bougher) Justo, comb. nov.
MycoBank MB 515043
Basionym: *Torrendia grandis* Bougher, Aust. Syst. Bot. 12(1):146. 1999

Amanita grandispora (G.W. Beaton, Pegler & T.W.K. Young) Justo, comb. nov.
MycoBank MB 515038
Basionym: *Alpova grandisporus* G.W. Beaton, Pegler & T.W.K. Young, Kew Bull. 40(3):580. 1985. (= *Amarrendia grandispora* (G.W. Beaton, Pegler & T.W.K. Young) Bougher & T. Lebel, Aust. Syst. Bot. 15:518. 2002)

Amanita inculta (Bougher) Justo, comb. nov.
MycoBank MB 515039
Basionym: *Torrendia inculta* Bougher, Aust. Syst. Bot. 12(1):149. 1999

Amanita oleosa (Bougher & T.Lebel) Justo, comb. nov.
MycoBank 515040
Basionym: *Amarrendia oleosa* Bougher & T. Lebel, Aust. Syst. Bot. 15:514. 2002

Amanita torrendii (Bres.) Justo, nom. nov.
MycoBank MB 515041

Etymology. *Torrendii* refers to the Portuguese mycologist Camillo Torrend after whom genus *Torrendia* was named.

Basionym: *Torrendia pulchella* Bres., Atti Imp. Regia Accad. Rovereto, ser III 8:132.1902; non *Amanita pulchella* S. Imai, Bot. Mag. (Tokyo) 47:427. 1933 nec *Amanita pulchella* (Cooke & Masee) E.-J. Gilbert, Icongr. Mycol. 27, suppl. 1:203. 1941

Amanita pseudoinculta Justo, sp. nov. FIG. 4
Amanita inculta similis sed differt in sporis longibus et pileus non fragilis. Holotypus, hic designatus, OKM 25066 (VPI 555).
MycoBank MB 515042

Etymology. *Pseudoinculta* means ''false inculta'', referring to the closest relative of the new species, *A. inculta*.

Published figures. Miller and Horak 1992, FIGS. 17, 18.

Pileus 4–10 mm broad, hemispheric, at maturity sometimes slightly depressed at center, not disintegrating. *Peridium* up to 0.7 mm broad, smooth or granular, whitish, in young stages with patches of universal veil attached. *Gleba* subgelatinous at first, divided in numerous irregularly shaped locules, whitish. *Stipe* 10–35 × 2–7 mm, cylindrical or slightly narrower toward the base, whitish, usually covered with fibrils and/or scales. *Volva* membranous, sacciform, whitish, with upper part free from the stipe at maturity. *Context* white, unchanging. *Odor* and *flavor* indistinct.

Spores [120/8/4] 11.3–19(19.5) × 5.2–7.3(8) µm, avl = 13.1–15.6, avw = 6.0–6.5, Q = 1.8–2.9, avQ = 2.05–2.48 oblong to cylindrical, with a broad central apiculus, inamyloid. *Basidia* 20–60 × 10–16 µm, mostly four-spored but two-spored also present, thin-walled. *Peridium* composed of thin-walled cylindrical hyphae 2–10 µm, nongelatinized, with a subpellis of globose, ovoid or irregularly shaped cells up to 35(45) µm diam. *Stipe context* made up of filamentous hyphae (4–20 µm broad) with terminal cylindrical to clavate elements (acrophysalides) 45–150 × 10–40 µm. *Volva* a mixture of globose, ovoid or irregularly shaped elements 20–75 × 20–65 µm and densely interwoven cylindrical hyphae 2–6 µm broad. *Oleifer-*

ous hyphae present in the hymenium and stipe trama. *Clamp connections* present in the hymenium, stipe trama and veil tissue.

Ecology, phenology and distribution: on sandy soil near *Allocasuarina humilis* and *Eucalyptus*. Fruiting in June–July. Known only from the type locality (Mullering Brook, approx. 206 km north or Perth, Western Australia).

Collections examined. AUSTRALIA. WESTERN AUSTRALIA: Mullering Brook, Brand Highway, approx. 206 km north of Perth, 3-VII-1991, *L. Bailey, M. Bailey, O.K. Miller, H.H. Miller, OKM 25066* (Holotype, VPI 555); idem, 3-VII-1991, *L. Bailey, M. Bailey, O.K. Miller, H.H. Miller, OKM 25094* (VPI 366); idem, 3-VII-1991, *L. Bailey, M. Bailey, O.K. Miller, H.H. Miller, OKM 25070* (VPI 558); idem, 6-VI-1989, *H.H. Miller, OKM 23825 (VPI 411)*.

Observations. All collections of *Amanita pseudoinculta* originally were identified as *Torrendia arenaria* (= *Amanita arenaria*), and except for VPI 555 they were mentioned in the original description of that species (Miller and Horak 1992). However both the molecular and morphological data indicate that they represent a different species not closely related to *A. arenaria* (see DISCUSSION). These collections come from Mullering Brook (206 km north of Perth), relatively far from the type locality of *A. arenaria* (Two Peoples Bay, 424 km south of Perth). The microscopical analysis revealed several differences with the morphological concept of *A. arenaria*; the spores are cylindrical (avQ = 2.05–2.48) while *A. arenaria* has broadly ellipsoid to oblong spores (avQ = 1.23–1.72). Clamp connections and oleiferous hyphae are present in *A. pseudoinculta*, while clamps are absent and oleiferous hyphae are absent or scarce in *A. arenaria*. *A. pseudoinculta* also differs from *A. inculta* (its closest relative in the molecular analyses) by the nondisintegrating gleba and higher values of avQ (1.84 in *T. inculta*, Bougher 1999).

Amanita pseudoinculta is unique among the Australian secotioid species because of its cylindrical spores, a character shared with *A. torrendii* from which it mainly differs by its mycorrhizal partners and area of distribution: *Allocasuarina* and *Eucalyptus* in Western Australia (*A. pseudoinculta*) vs. *Pinus*, *Quercus* and *Cistus* in the Mediterranean Basin (*A. torrendii*).

DISCUSSION

Phylogenetic position of secotioid species among the Amanita *lineage.*—The present data clearly reject the hypothesis of a single origin for the secotioid taxa in *Amanita* because they are placed in three different major clades, Torrendia (*A. torrendii*) nested in sect. *Caesareae*, Amarrendia (*A. grandis*, *A. inculta* and *A. pseudoinculta*) as an independent group in subgenus *Amanita* and Arenaria (*A. arenaria*) in subgenus *Lepidella*, possibly related to sections *Phalloideae* or *Validae*. Even the monophyly of the Australian secotioid species has to be rejected because *A. arenaria* is placed in a distant position from the other Australian taxa. The ITS analysis also suggests two different origins for the secotioid species in the Amarrendia clade (FIG. 2), meaning that the secotioid morphology in *Amanita* has arisen four independent times.

A. yuaniana consistently appears as the sister taxon of *Amanita torrendii* or as a part of a more inclusive clade with other agaricoid species (*A. longistriata*, *A. incarnatifolia*), although this relationship gets strong statistical support only in the combined analysis (SUPPLEMENTARY FIG. 3). *A. yuaniana* occurs in southwestern China with *Pinus* and *Quercus* (Yang 1997) and future research should address the biogeographical implications and significance of this relationship.

A. umbrinella, an Australian species of section *Amanita*, is placed in the ITS analysis as the sister group of *A. inculta* and *A. pseudoinculta* (FIG. 2). Australian species of *Amanita* are underrepresented in the current dataset, so more extensive sampling and sequencing of Australian taxa is necessary to investigate the identity of possible agaricoid relatives (or members) of the Amarrendia clade. The position of the Amarrendia clade among subgen. *Amanita* remains unclear although the combined analysis (SUPPLEMENTARY FIG. 3) suggests a relationship with sections *Caesareae* or *Amanitopsis*.

A. arenaria is placed in subgen. *Lepidella* in a more inclusive clade together with sections *Phalloideae* and *Validae*, however the internal topology of this clade is not well resolved in the analyses. Some species traditionally classified in sect. *Lepidella* also are placed in this more inclusive clade, *A. strobiliformis* (in the nLSU dataset), *A. cinnereopanosa* (in the ITS dataset) and *A. griseoverrucosa* (in both and the combined dataset). Some of these species are placed in the analyses as the sister taxon of the Arenaria clade but always with low bootstrap support. Exclusion of these species from the datasets does not result in a better resolution of the relationships between *A. arenaria* and sections *Phalloideae* and *Validae* (results not shown).

Systematic position of gasteroid species among the Amanita *lineage.*—The position of *Amarrendia oleosa*, *Amarrendia grandispora* (both in the Amarrendia clade) and *Amarrendia* sp. (in the Pseudoamarrendia clade) in the nLSU analysis also rejects a single origin for the gasteroid taxa of *Amanita*. The ITS dataset,

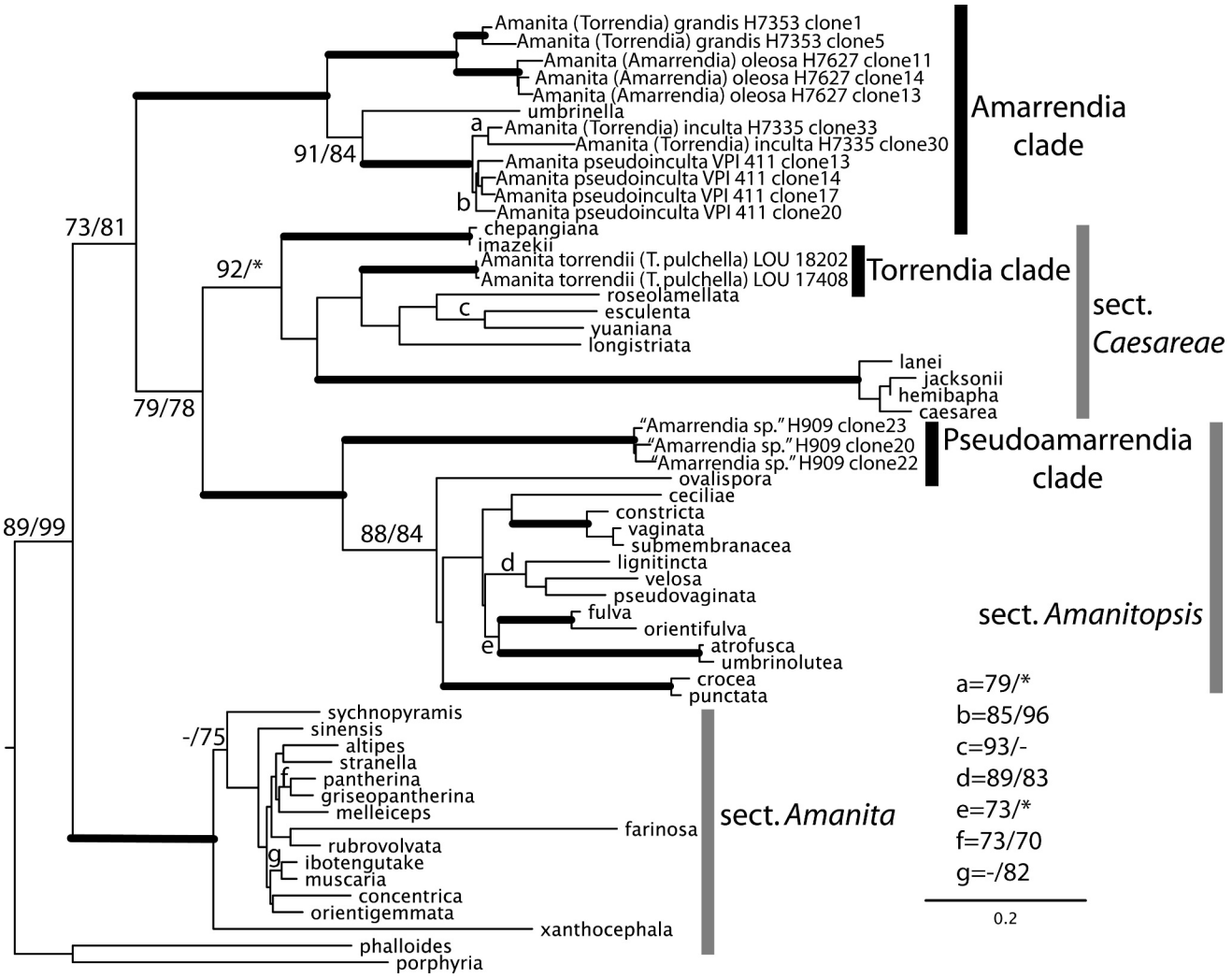

FIG. 2. Best tree from the ML analysis of the ITS dataset of subgenus *Amanita*. Thick branches are supported by bootstrap values > 90% in the ML and MP analyses. Other bootstrap values > 70% are shown on the branches (ML/MP). An asterisk indicates that the branch collapses in the strict consensus tree of the MP analysis.

which did not include *Amarrendia grandispora*, yielded similar results.

Taxonomy of "Torrendia arenaria".—During the revision of the original collections of *T. arenaria* the existence of a different species, here described as *Amanita pseudoinculta* (see TAXONOMY), was discovered. *A. pseudoinculta* is placed in the Amarrendia clade, while *A. arenaria* is placed, in a distant position, in the Arenaria clade (FIG. 1).

Amarrendia clade. The separation of *A. pseudoinculta* and its closest relative, *A. inculta*, is supported by the nLSU analysis (FIG. 1) and the ML analysis of the ITS dataset (FIG. 2), however in the MP analysis of the ITS data *A. inculta* sequences are paraphyletic (SUPPLEMENTARY FIG. 2). This is probably caused by the high level of intragenomic variability observed in the secotioid and gasteroid taxa from Australia that overlaps with the interspecific variability between pairs of closely related species. High intragenomic variability in the ITS region has been observed in other groups of ectomycorrhizal fungi such as *Lactarius* (Nuytinck and Verbeken 2007) and *Xerocomus* (Taylor et al. 2006). In *Lactarius* similar problems with overlapping intragenomic and interspecific ITS variation had been detected in pairs of closely related species, and this situation has been linked to recent speciation events. So far no high levels of intragenomic ITS variability have been reported in the agaricoid members of *Amanita*; they also seem to be not present in *A. torrendii*.

Arenaria clade includes the type collection of *Amanita arenaria* (VPI 679), another collection from the same locality and date (VPI 412), and collections from two different localities, Kalamunda (VPI 364,

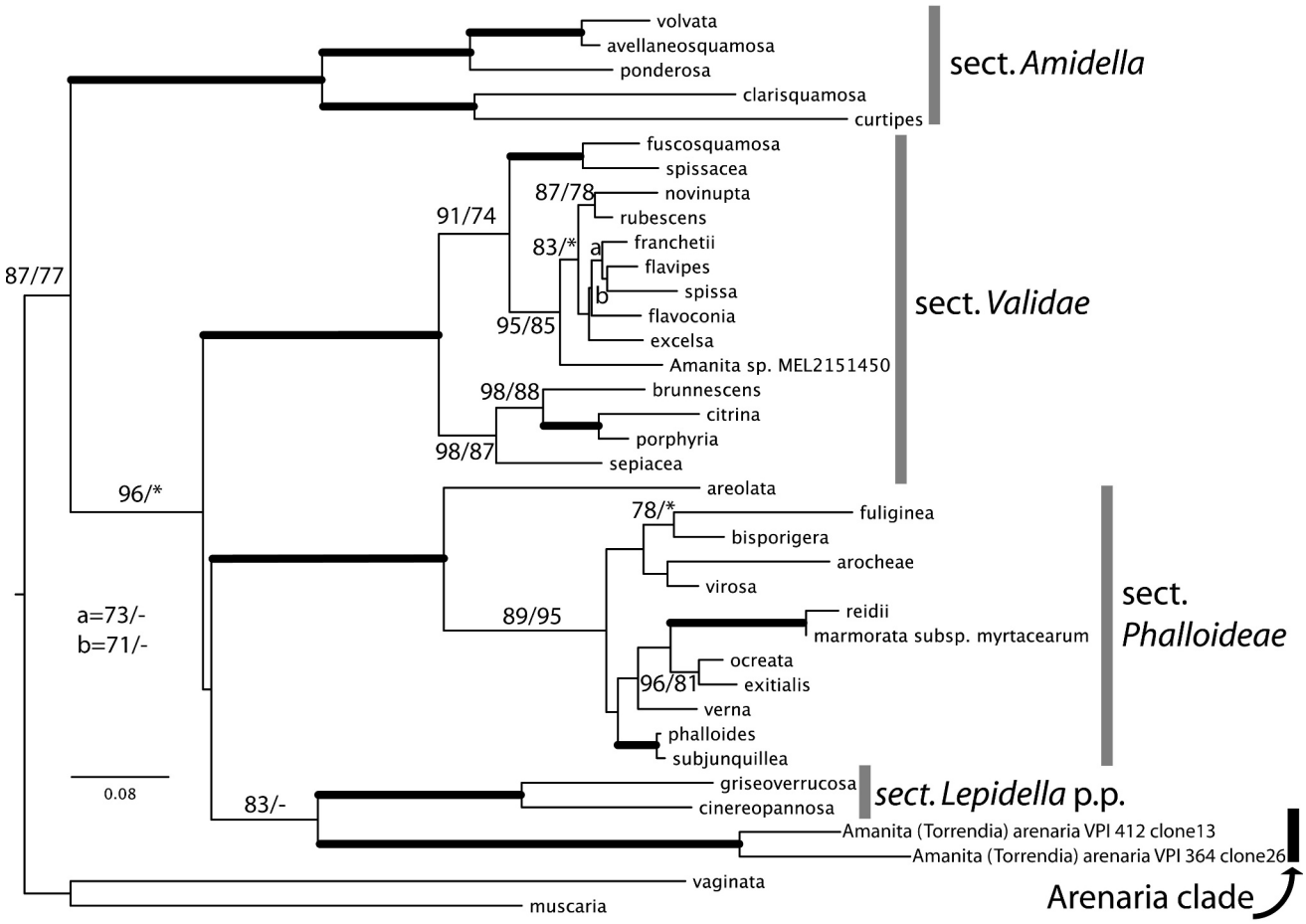

FIG. 3. Best tree from the ML analysis of the ITS dataset of subgenus *Lepidella*. Thick branches are supported by bootstrap values > 90% in the ML and MP analyses. Other bootstrap values > 70% are shown on the branches (ML/MP). An asterisk indicates that the branch collapses in the strict consensus tree of the MP analysis.

365, 551) and Julimar Forest (VPI 363, see TABLE II). The collection VPI 364 was identified as ''*Torrendia arenaria* f. *lutescens*''. In all analyses of the nLSU dataset there are two subgroups in the Arenaria clade, one with the two collections from the type locality and the other with the remaining collections (FIG. 1). In the ITS dataset one collection from each subgroup was included and the number of differences between the two ITS sequences was similar to, or even greater than, the number of differences between pairs of closely related species in *Amanita* (FIG. 3).

These results suggest either the existence of a cryptic species among the collections of *A. arenaria* or relatively high genetic divergence among populations from different localities. From a morphological point of view the collections from Kalamunda and Julimar Forest have spores with slightly lower values of avQ (1.28–1.36) than the collections from Two Peoples Bay (1.55–1.72), however they all share similar macro- and micromorphological characteristics, including the absence of clamp connections and the absence or scarcity of oleiferous hyphae. One of the collections from Kalamunda (VPI 364) has yellowing flesh and was identified as ''forma *lutescens*'', but other collections with unchanging flesh have been collected in the same locality (VPI 365, 551), indicating that this character is not sufficiently constant to make taxonomic distinctions based on it. A more detailed molecular and morphological study that takes into account collections of *A. arenaria* from different localities, including those mentioned by Bougher (1999), is necessary to establish whether the divergent nLSU and ITS sequences of *A. arenaria* from localities other than Two Peoples Bay represent an undescribed cryptic species and whether this genetic variation is correlated with some morphological characters such as spore shape.

Evolution of secotioid and gasteroid forms in Amanita.—Thiers (1984) suggested that the gasteroid forms in many groups of Basidiomycetes evolved from an agaricoid ancestor via secotioid intermediates and

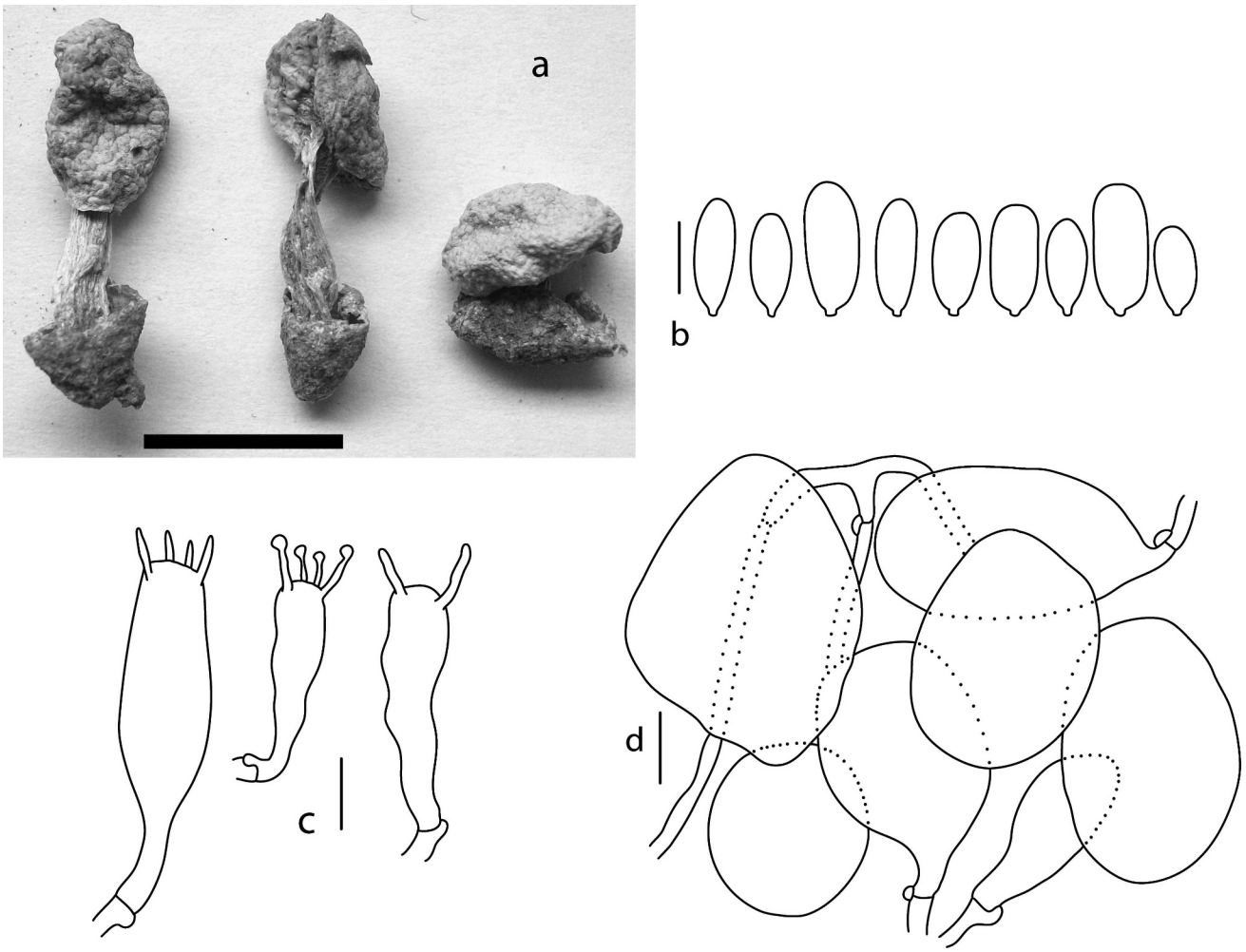

FIG. 4. *Amanita pseudoinculta*. a. dried basidiomes (bar = 1 cm). b. spores. c. basidia. d. volva tissue. Bars = 10 µm. All from holotype (VPI 555).

provided examples of this sequential process, both complete series such as *Cortinarius* (agaricoid), *Thaxterogaster* (secotioid) and *Hymenogaster* (gasteroid) and incomplete series such as *Agaricus* (agaricoid) and *Endoptychum* (secotioid). He postulated that incomplete series (the *Amanita/Torrendia* was an example at that time) could be caused by the extinction of the missing stage or simply reflect the incomplete documentation of fungal diversity.

Two incomplete series are present in the *Amanita* lineage, including secotioid forms without additional gasteroid forms (Torrendia clade) and gasteroid forms without known secotioid relatives (Pseudoamarrendia clade). It is possible that further exploration of fungal diversity in Australia and the Mediterranean region of Europe and Africa will reveal the missing stages. Also one example of a complete series is present in the Amarrendia clade, with secotioid forms related to agaricoid forms (*A. umbrinella*) with additional radiation into other secotioid (*A. inculta/A. pseudoinculta*) and also gasteroid forms (*A. grandis/A. oleosa*).

Despite the independent origins of the secotioid morphology in *Amanita* all species share a great similarity in their general characteristics both macro- and microscopical. Morphological differences among taxa are reduced to basidiome size, structural integrity of the gleba, spore size and shape, and presence or absence of sclerobasidia, oleiferous hyphae and clamp connections. While the majority of secotioid and gasteroid forms presumably have evolved from ancestors with nonamyloid spores in subgen. *Amanita*, *A. arenaria* probably has evolved from an amyloid-spored ancestor in subgenus *Lepidella* but this character was lost during the gasteromycetization process.

Influence of Mediterranean climate in the gasteromycetization process in Amanita.—The distribution of the

secotioid and gasteroid species of *Amanita* around the Mediterranean Basin and Western Australia (with the exception of *Amanita grandispora* known from Victoria and Tasmania) suggests a direct link between the gasteromycetization process in *Amanita* and the Mediterranean climate that these areas share. *Amanita arenaria*, *A. grandis*, *A. inculta*, *A. oleosa* and *A. pseudoinculta* are endemic to Western Australia and in fact are known only from a few localities each. Speciation events under Mediterranean conditions seem to be the origin for the taxa in the Amarrendia clade. If the existence of a cryptic species among *A. arenaria* is confirmed it would be another example. The distribution pattern of these related endemic species of fungi in relatively close geographic areas closely matches the distribution pattern of endemic plants in Mediterranean areas (Cowling et al. 1996).

Amanita torrendii has a larger number of populations that range from northern Africa to southern France, spreading eastward to Turkey (Watling and Isiloglu 1991), but the great majority of the records (Calonge 1996) are concentrated in the Mediterranean region of the Iberian Peninsula. It appears that *A. torrendii* has remained a single, relatively widespread, secotioid species, in contrast to the Australian taxa, which have radiated into multiple species, including secotioid and fully gasteroid forms. More research on the population level is needed to gain further understanding of the speciation and gasteromycetization events in the *Amanita* lineage.

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