

SYSTEMATICS OF EUROPEAN SEQUESTRATE LACTARIOID
RUSSULACEAE WITH SPINY SPORE ORNAMENTATION

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ABSTRACT. — Molecular data confirm that *Arcangeliella borziana* Cavara (syn. nov. : *Arcangeliella volemoides* K. Mader & A. Mader) and *Zelleromyces stephensii* (Berk.) A.H. Sm. are two distinct *Lactarius* species. The taxonomic value of several morphological characters previously used in the systematics of Russulaceae (syn. : Elasmomycetaceae) is discussed. The new combinations *Lactarius borzianus* (Cavara) Verbeken & Nuytinck and *Lactarius stephensii* (Berk.) Verbeken & Walleyne are proposed. A lectotype is designated for *Hydnangium monosporum* Boud. & Pat. Its synonymy with *Lactarius stephensii*, suggested by GROSS (1990), is confirmed.

KEY WORDS. — *Russulaceae*, *Arcangeliella*, *Zelleromyces*, *Lactarius*, morphology, phylogeny, type studies.

INTRODUCTION

Sequestrate Russulaceae are very rare in Belgium. Up to now, only *Macowanites mattirolianus* (Cavara) T. Lebel & Trappe and *Zelleromyces stephensii* (Berk.) A.H. Sm. have been recorded for the country, both from *Querceto-Carpinetum* on limestone in southern Belgium (DE VRIES 1977, THOEN 1988). Recently, one of us collected a first sequestrate representative of Russulaceae in central Belgium, with evident latex production and spiny spores. In Europe, three sequestrate species of lactarioid Russulaceae (accepted syn. : Elasmomycetaceae) characterised by spores with isolated acute warts or spines have been described : *Arcangeliella borziana* Cavara, *Zelleromyces stephensii* [syn. : *Arcangeliella stephensii* (Berk.) Zeller & Dodge] and *Arcangeliella volemoides*

K. Mader & A. Mader. Considerable confusion remains in the current literature about the conspecificity of *Arcangeliella borziana* and *Zelleromyces stephensii* (LEBEL & TRAPPE 2000). Furthermore, nothing has been written on *Arcangeliella volemoides* since its description, except that many European records of *Zelleromyces stephensii* should be confirmed for possible confusion with this species (KREISEL 2001). This encouraged us to clarify the taxonomic identity and systematic position of these taxa.

MATERIAL AND METHODS

MORPHOLOGICAL ANALYSIS

The study is based on herbarium material from GENT and PC. Macroscopic characters are all based on

fresh material. Colour codes are from KORNERUP & WANSCHER (1978). Microscopic features were studied from fresh and dried material, mainly in Congo-red in L4. Spore ornamentation is described and illustrated as observed in Melzer's reagent. Line-drawings were made using a drawing tube at original magnifications 6000 × for spores, 3000 × or 1000 × for individual elements and 1000 × for sections. Basidia length excludes sterigmata length. Spores were measured in side view in Melzer's reagent, excluding the height of the ornamentation. Measurements are given as (MINa) [AVa-2*SD] - AVa - AVb - [AVb + 2*SD] (MAXb), with AVa = lowest mean value for the measured collections, AVb = greatest mean value and SD = standard deviation calculated for the measurements of one collection. Q corresponds with spore "length/width ratio" and is given as (MINQa) Qa - Qb (MAXQb) with Qa and Qb being the lowest and highest mean ratio for the measured specimen, respectively.

MOLECULAR ANALYSIS

DNA extraction

Dried material (about 50 mg) was first ground in liquid N₂. One ml extraction buffer (0.1 M Tris.HCl pH = 8, 0.5 M NaCl, 0.05 M EDTA and 0.01M β-mercaptoethanol) and 50 µl 10% SDS were added and the mixture was incubated for 30 min at 65°C. This extraction was centrifuged for 10 min at 14000 rpm (Eppendorf centrifuge). An equal volume of isopropanol was added to the supernatant and after mixing the solution, it was centrifuged again as above. The pellet was washed with 70% ethanol, air dried and dissolved in 400 µl dH₂O. Then 400 µl CTAB buffer (2% w/v CTAB, 0.2M Tris.Cl pH = 7.5, 2M NaCl and 0.05M EDTA) was added and after incubation for 15 min at 65°C, the mixture was extracted twice with chloroform/iso-amylalcohol (24:1). Two volumes of 96% ethanol were added and the mixture was centrifuged again as above to pellet the DNA. The pellet was washed with 70% ethanol and dissolved in 100 µl T_{0.1}E.

ITS amplification

Basidiomycete specific primers ITS1-F and ITS4-B (GARDES & BRUNS 1993) were used to amplify the ITS region. The following touchdown profile was used: 95°C during 2 min, then 10 cycles of denaturation at 95°C (for 15 sec), primer annealing at 63°C (for 20 sec) with the temperature decreasing by 1°C at each cycle, polymerisation for 1 min at 72°C; followed by 35 cycles with the same profile for denaturation and polymerisation but with a constant annealing tempera-

ture of 53°C; polymerisation was completed by an incubation of 2 min at 72°C.

ITS sequencing

The obtained PCR products were purified using ExoSAP (USB, USA). DNA sequencing reactions were performed with the ABI PRISM® BigDye™ Terminators v3.0 Cycle Sequencing Kit using primers ITS1-F, ITS2, ITS3 and ITS4-B (GARDES & BRUNS 1993, WHITE *et al.* 1990) on an ABI PRISM® 377 DNA Sequencer. The Phred and Phrap Software (EWING & GREEN 1998, EWING *et al.* 1998) was used to process the raw data.

Data analysis

For our phylogenetic analysis, we combined the obtained ITS sequences of *Zelleromyces stephensii* and six *Lactarius* species with 21 ITS sequences of related *Lactarius*, *Zelleromyces*, *Arcangeliella* and *Russula* species, retrieved from GenBank (Table 1). Sequences were aligned using ClustalX (THOMPSON *et al.* 1997) and the ambiguous regions in the alignment were excluded from the analysis. For phylogenetic analyses PAUP*4b10 (SWOFFORD 2002) was used. A maximum parsimony analysis was performed with all sites treated as unordered and unweighted and with gaps treated as missing. A total of 1000 random-addition sequence replicates was implemented, 5 trees were held per step and the branch-swapping algorithm was TBR. Other settings were: random addition of taxa, MulTrees option in effect and no steepest descent. A bootstrap analysis with 1000 replicates and for each replicate 10 random addition sequences was performed to estimate the support for the internodes in the trees.

RESULTS

DESCRIPTION OF SEQUENCED COLLECTION OF *ZELLEROMYCES STEPHENSII* (FIG. 1e-i)

Exsiccatum: Belgium, Berg, nature reserve "Tofbroek", in leaf litter under *Populus cf. alba* and *Crataegus* at border of calcareous fen, 2 Dec 2002, R. Walley RW 2930 (GENT).

Basidiomata up to 15 mm diam., subglobose to oblong or slightly reniform, sometimes lobed, firm, with a small protruding sterile base. Peridial surface reddish brown (8DE8), darker (up to 8F8) when bruised or with age, not unicolourous but with a reddish, flamboyant aspect, smooth.

Peridium 0.3-0.5 paler than the perid orange (6C7), dark when long exposed; loculate; locules lobular, small, 2-4 present in one fringes, narrow (aboriginating in the the peridium. Odour specimens (remir Latex present but s

Basidiospores soid, mostly sym 12.3-13-14 µm × 1.04-1.07-1.13-1.2 weakly amyloid (gent), composed 1.0(1.3) µm long, lar, sometimes cur 2 µm, distinct, ta; hyaline, thin-walle spored, 45-75 × 7 2(3)-septate with long, thin-walled, yellowish to brown plug); sterigmata lar, tapering or cystidia very scarce gent (up to 30 µm with yellowish bro 90 µm broad, com ged hyphae; hyph often with small ments, frequently layer; abundant la ixocutis to ixotric interwoven hyphae 6 µm diam., local with a brownish incrustations) on t

Other collecti Belgium, Montqui sis, 8/09/1992, Tho D. Thoen). France herb. Boudier (P *monosporum*, desi

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darker (up to 8F8)
unicolourous but
aspect, smooth.

Peridium 0.3-0.5 mm thick, persistent. Gleba paler than the peridium, orange (6B7) to brownish orange (6C7), darkening to orange brown (6D7) when long exposed, sienna (6E7) when dried, loculate; locules labyrinthoid, ellipsoid to irregular, small, 2-4 per mm. An indistinct columella present in one fruitbody, absent in remaining ones, narrow (about 0.4 mm broad), branching, originating in the sterile base, concolourous with the peridium. Odour pleasant and fruity in mature specimens (reminding of canned pineapple). Latex present but scarce, white, mild tasting.

Basidiospores subglobose to broadly ellipsoid, mostly symmetric and orthotropic, 11.0-12.3-13-14 $\mu\text{m} \times$ 10.5-11.5-11.8-12.8 μm (Q = 1.04-1.07-1.13-1.21, n = 40); ornamentation weakly amyloid (brownish grey in Melzers reagent), composed of isolated, blunt spines, 0.5-1.0(1.3) μm long, cylindrical or somewhat irregular, sometimes curved; hilar appendix 1-6 \times 1.5-2 μm , distinct, tapering to flattened near apex, hyaline, thin-walled. Basidia 1-spored, seldom 2-spored, 45-75 \times 7-10 μm , some are remarkably 2(3)-septate with the upper part about 20-40 μm long, thin-walled, hyaline or with remarkable dark yellowish to brown content (forming a central plug); sterigmata eccentric, 4-6 μm long, irregular, tapering or slightly moniliform. Pseudocystidia very scarce, the few observed ones emergent (up to 30 μm), cylindrical, 5-7 μm broad, with yellowish brown oil-like content. Trama 60-90 μm broad, composed of mainly parallel arranged hyphae; hyphae thin-walled, 2-5 μm diam., often with small knobs and short side branchments, frequently branched, imbedded in a slime layer; abundant lactifers present. Peridiopellis an ixocutis to ixotrichoderm, composed of strongly interwoven hyphae embedded in a slime layer, 3-6 μm diam., locally with erect terminal elements, with a brownish layer (slimy, with abundant incrustations) on top.

Other collections examined:
Belgium, Montquintin, under *Populus x canadensis*, 8/09/1992, Thoen 8241 (GENT, dupl. ex herb. D. Thoen). France, Nice, leg. Barla July 1885 in herb. Boudier (PC, **lectotype** of *Hydnangium monosporum*, **designated here**).

DESCRIPTION OF SEQUENCED COLLECTION OF
ARCANGELIELLA BORZIANA (FIG. 1a-d)

The collection of *Arcangeliella borziana* used for molecular analysis (Switzerland, Fribourg, Cerniat, Parabock forest, Aug. 1996, WSL 96-05-3344, duplicate at GENT) is described by AYER (1998).

Other collections examined:

Österreich, Niederösterreich, Gföhl, leg. A. & K. Mader 12/09/1970 (WU-10875, type of *A. volemoides*).

PHYLOGENY

The alignment of 28 ITS sequences (part of 28S rDNA, ITS1, 5.8S rDNA, ITS2 and part of 18S rDNA, Table 1) contained 764 nucleotides, of which 407 nucleotides were constant and 191 nucleotides were parsimony-informative. Two *Russula* species (*R. delica* and *R. integra*) were used as outgroup. Maximum parsimony analysis produced 12 shortest trees of 735 steps (consistency index = 0.65, retention index = 0.66, rescaled consistency index = 0.43). The strict consensus tree is shown in Fig. 2, with associated bootstrap supports for the branches. In this tree, three subgenera of *Lactarius* form a distinct clade: subgenus *Piperites*, *Russularia* and *Plinthogali*. While *Arcangeliella borziana* is closely related to *Lactarius fulvissimus* and *L. subsericatus* in subgenus *Russularia*, *Zelleromyces stephensii* is included in subgenus *Piperites*. This dataset does not allow us to draw conclusions about which section *Z. stephensii* belongs to.

DISCUSSION

Up to now *Arcangeliella borziana* was often synonymized with *Zelleromyces stephensii* (MONTECCHI & LAZZARI 1993, KRIEGLSTEINER 1991, AYER 1998). GROSS (1990) distinguished three taxa in "this complex", whereas recent important monographs on truffle-like fungi of Europe treat only one of both species without discussing the distinctive characters (PUGLIER *et al.* 1993, MONTECCHI & SARASINI 2000). These literature data made LABEL & TRAPPE (2000) conclude

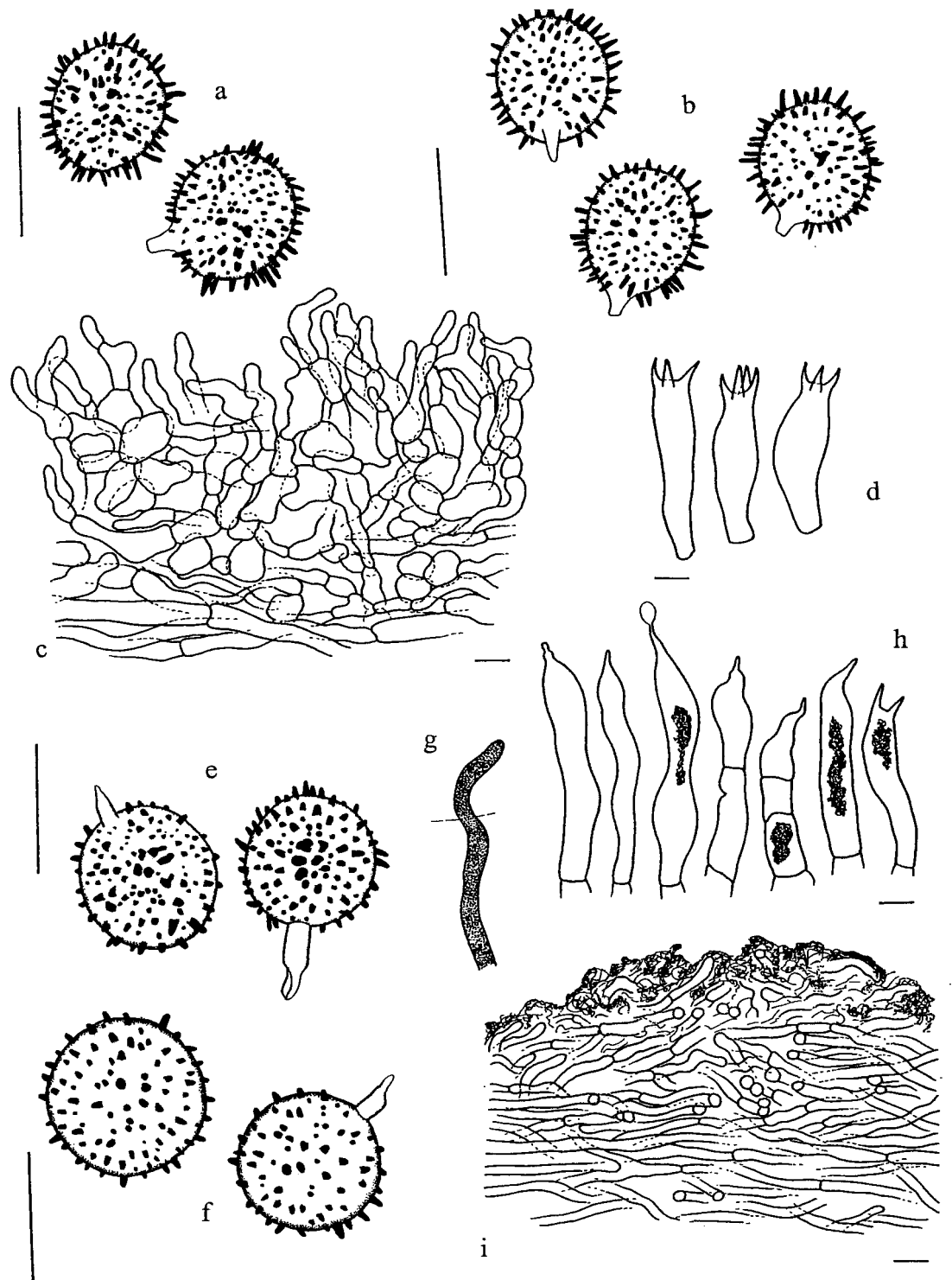


FIG. 1. — *Lactarius borzianus*. a. Spores (WSL 96-5-3344). b. Spores (typus *A. volemoides*). c. Section through the peridiopellis (WSL 96-5-3344). d. Basidia (WSL 96-5-3344) (bar = 10 μ m) — *Lactarius stephensii*. e. Spores (RW 2930). f. Spores (typus *Hydnangium monosporum*). g. Pseudocystidium (RW 2930). h. Basidia (RW 2930). i. Section through the peridiopellis (RW 2930) (bar = 10 μ m).

TABLE 1

GenBank accession numbers and herbarium entries for the species used in the analysis of the phylogenetic relationships of *Zelleromyces stephensii*. All herbarium specimens are deposited in the herbarium of the Ghent University (GENT)

Species	GenBank accession/herbarium number
<i>Arcangeliella borziana</i>	AF286204
<i>Lactarius acerrimus</i>	AJ278139
<i>Lactarius blennius</i>	AY331015/LVL 2002-033
<i>Lactarius chrysorrhoeus</i>	AF096983
<i>Lactarius controversus</i>	AJ272244
<i>Lactarius deliciosus</i>	AF249284
<i>Lactarius fallax</i>	AF335441
<i>Lactarius fluens</i>	AY331014/JN 2002-050
<i>Lactarius fulvissimus</i>	AF204679
<i>Lactarius hepaticus</i>	AF096989
<i>Lactarius intermedius</i>	AF140256
<i>Lactarius mitissimus</i>	AF157412
<i>Lactarius pterosporus</i>	AY331013/LVL 2002-019
<i>Lactarius quietus</i>	AJ272247
<i>Lactarius repraesentaneus</i>	AY331011/JN 2001-023
<i>Lactarius scrobiculatus</i>	AF140262
<i>Lactarius serifluus</i> s.l.	AY332558/RW 1431
<i>Lactarius subdulcis</i>	AY331016/LVL 2002-034
<i>Lactarius subsericatus</i>	AF140254
<i>Lactarius tabidus</i> (as <i>L. theiogalus</i>)	AF349716
<i>Lactarius tesquorum</i>	AF096986
<i>Lactarius trivialis</i>	AJ534935
<i>Lactarius uvidus</i>	AJ534936
<i>Russula delica</i>	AF418605
<i>Russula integra</i>	AY061683
<i>Zelleromyces giennensis</i>	AF230900
<i>Zelleromyces hispanicus</i>	AF231913
<i>Zelleromyces stephensii</i>	AY331012/RW 2930

that much confusion still remains about the identity of these two taxa.

Both species can, however, easily be distinguished by morphological observations. *Arcangeliella borziana* has carpophores with a distinct columella, a slightly exposed gleba at the rudimentary stiped base and asymmetric (putative ballistosporic) spores (Fig. 1a) with slender spines up to 1.5(1.8) μm , produced on (3-)4-spored basidia (Fig. 1d). The carpophores of *Zelleromyces stephensii* are entirely enveloped by the peridium, and produce symmetric, statismosporic spores (Fig. 1e) with warty, blunt spines up to 1(1.3) μm , on 1- or 2-spored basidia (Fig. 1h). Their ecology seems also different. *Arcangeliella borziana* forms ectomycorrhiza in submontane areas with

Picea (PETER *et al.* 2001), and maybe also with *Abies*, whereas *Zelleromyces stephensii* seems restricted to deciduous forests, also at lower altitudes.

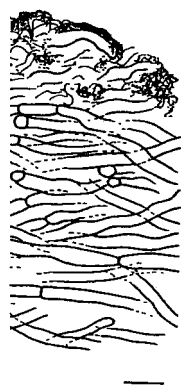
The description of the new species *Arcangeliella volemoides* (MADER & MADER 1992), found under *Picea* and described as similar to *Z. stephensii* seemed confusing to us, because *Arcangeliella borziana* was not mentioned at all in the discussion. Our study of the type confirmed our hypothesis that this species is synonymous with *A. borziana*. The descriptions of *Zelleromyces stephensii* based on the original material in the herbarium of Berkeley (K) mention 1-spored basidia (MADER & MADER 1992) or 2-spored basidia (HAWKER 1954, PEGLER *et al.*



d



h



on through the peridio-
(RW 2930). f. Spores
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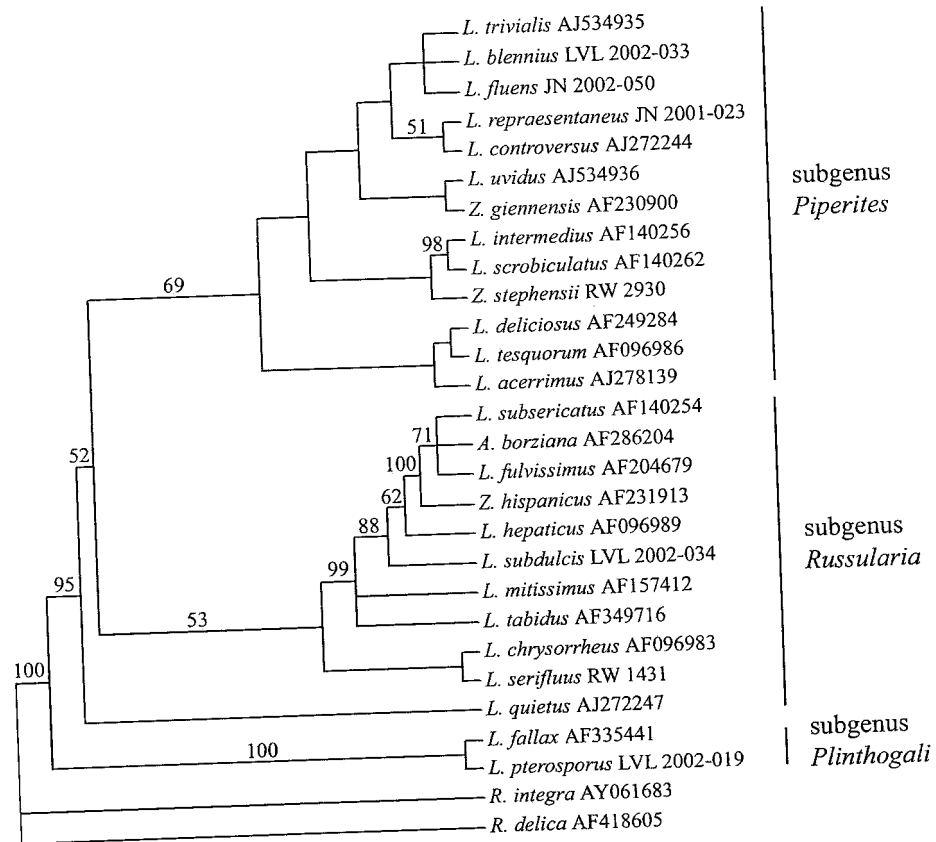


FIG. 2. — Strict consensus of the 12 equally parsimonious trees resulting from maximum parsimony analysis of the ITS sequence data. Bootstrap values (1000 replicates) are shown above their respective branches. Abbreviations : A., *Arcangeliella*; L., *Lactarius*; R., *Russula*; Z., *Zelleromyces*.

1993). Our examination revealed predominantly 1-spored basidia, easily mistaken for cystidia, although some scarce 2-spored basidia were observed. We assume that both spore numbers occur in the same species and that intermediate forms exist. Gross (1990) suggested that monospored collections have been described as *Hydnangium monosporum* by BOUDIER & PATOUILLARD (1888). Examination of original material in the Boudier herbarium at PC (designated here as lectotype) confirms this hypothesis.

Good descriptions of *Zelleromyces stephensii* are given by DE VRIES (1977), HAWKER (1954), PEGLER *et al.* (1993). For *A. borziana* we refer to the descriptions by LABEL & TRAPPE (2000) and MONTECCHI & SARASINI (2000). Some literature

records cannot be interpreted with certainty. The record of *Z. stephensii* in Switzerland by MILLER & MILLER (1986) in part refers to *Arcangeliella borziana*, but the description given is probably based on both species, as they examined several other collections, including the type of *Z. stephensii*.

Several attempts have been made over the last decades to define the generic distinctions in the gasteroid Russulales (e.g., PEGLER & YOUNG 1979, BEATON *et al.* 1984, CALONGE 1999, LABEL & TRAPPE 2000), mainly based on morphological characters such as hymenophoral trama anatomy, stipe-columella development, spore morphology and presence or absence of latex. In most cases

these genera were described as showing affinities with the agaricoid genera *Lactarius* or *Russula*. LEBEL & TRAPPE (2000) studied the morphological characters of the type species of all sequestrate genera in Russulaceae, and reduced the number of gasteroid-russuloid genera to three, but left the taxonomic boundary of the gasteroid-lactarioid genera *Zelleromyces* and *Arcangeliella* over for future consideration. However, discoveries of new taxa often do not fit the generic descriptions (DESIARDIN 2003). Meanwhile, molecular studies argue that the sequestrate members of Russulaceae have been derived from within *Russula* and *Lactarius* and that synonymy with these genera is acceptable (MILLER *et al.* 2001, CALONGE & MARTÍN 2000). As a consequence, a new sequestrate species, with several conspicuous characters typical for *Lactarius* sect. *Deliciosi* is already described in the genus *Lactarius* (DESIARDIN 2003). The classification of *L. rubriviridis* Desjardin in the *Deliciosi* is now supported by molecular data (NUYTINCK, unpublished data). PETER *et al.* (2001) gave molecular evidence for classification of *Arcangeliella borziana* in the genus *Lactarius*, subgenus *Russularia*, close to *Lactarius fulvissimus* Romagn., and our data support also the transfer of *Zelleromyces stephensii* to this genus. Therefore, following combinations are proposed:

***Lactarius stephensii* (Berk.) Verbeken & Walleyen comb. nov.**

- ≡ *Hydnangium stephensii* Berk., Ann. Mag. Nat. Hist., Ser. 1, 13 : 352 (1844)
- ≡ *Octaviania stephensii* (Berk.) Tul. & C. Tul., Fungi hypogaei : 78 (1851)
- ≡ *Arcangeliella stephensii* (Berk.) Zeller & Dodge, Ann. Missouri Bot. Gard. 18 : 463 (1931)
- ≡ *Zelleromyces stephensii* (Berk.) A.H. Sm., Mycologia 54 : 635 (1962)
- ≡ *Martellia stephensii* (Berk.) K. Mader & A. Mader, Österr. Z. Pilzk. 1 : 4 (1992)
- = *Hydnangium monosporum* Boud. & Pat., J. Bot. (Morot) 2 : 445 (1888) **syn. nov.**
- = *Octaviania monospora* (Boud. & Pat.) Lloyd, Mycol. Notes 7(67) : 1141 (1922)
- = *Martellia monospora* (Boud. & Pat.) Astier & Pacioni, Doc. Mycol. 28(109-110) : 9 (1998)

***Lactarius borzianus* (Cavara) Verbeken & Nuytinck comb. nov.**

- ≡ *Arcangeliella borziana* Cavara, Nuov. Giorn. Bot. Ital. ser. 2, 7 : 126 (1900)
- ≡ *Octaviania borziana* (Cavara) Svrcek, in Pilát, Gasteromycetes, Flora CSR B-1 : 194 (1958)
- ≡ *Arcangeliella stephensii* var. *borziana* (Cavara) Krieglst., Z. Mykol. 57 : 74 (1981)
- = *Arcangeliella volemoides* K. Mader & A. Mader, Österr. Z. Pilzk. 1 : 5 (1992) **syn. nov.**

Our molecular data demonstrate that *Lactarius borzianus* and *L. stephensii* are less closely related than all literature data suggest, as *L. stephensii* seems a member of *Lactarius* subgenus *Piperites*. This is also confirmed by the micromorphological characters, in particular the peridiopellis which is a trichopalisade in *L. borzianus* (Fig. 1c) and an ixocutis to ixotrichoderm in *L. stephensii* (Fig. 1i).

The very distinct and thick ixocutis, present in most representatives of subgenus *Piperites*, lacks in *L. stephensii*, which is not surprising in a hypogeous basidiocarp. The outer layer is very much encrusted (with soil particles), but slime is distinct among the underlying hyphae. The absence of acrid latex, another common feature in *Piperites*, could also be explained as an adaptation to the hypogeous way of life, where spore dispersal depends on animal attraction.

Interestingly, both species co-evolved not only by the development of sequestrate basidiomes but also formed spiny spore ornamentation, which is rather unique among European representatives of the genus *Lactarius*. This confirms that the value of several of the traditionally used characters are less valuable than previously accepted. It has been shown previously that the spore ornamentation in *Lactarius* can be heterogenous in the same section or subgenus (e.g., subgenus *Lactiflui*, VERBEKEN 1998). When redefining the generic and infrageneric classification in Russulaceae, less (or no?) attention should be given to the basidiocarp development. For agaricoid as well as sequestrate taxa, the structure of pileipellis resp. peridiopellis, and the presence or absence of pseudocystidia seem reliable phylogenetic characters.

ACKNOWLEDGEMENTS

We would like to thank Dr. Egli, Dr. Buyck and Dr. Thoën for the gift or loan of the cited material. The Department of Plant Systems Biology (VIB, RUG) is thanked for help with sequencing and Prof. Dr. Borgonie is thanked for making available the infrastructure for the molecular work. The research of Nuytinck is financed by the Institute for the Promotion of Innovation by Science and Technology in Flanders, Belgium.

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Revised manuscript received 7 July 2003.

