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Lactifluus volemus in Europe: Three species in one – Revealed by a multilocus genealogical approach, Bayesian species delimitation and morphology

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This study provides morphological and molecular evidence (from nuclear ITS, LSU, and *rpb2* DNA sequences) for three previously unrecognized species within the morphospecies *Lactifluus volemus* from Europe. Phylogenetic species are supported by both a multi-locus tree-based method and Bayesian species delimitation. *Lactifluus volemus* and *Lactifluus oedematopus* are provided with a new description, and a third species, *Lactifluus subvolemus*, is described as new to science. *Lactifluus oedematopus* can be easily recognized by its short pileipellis hairs. Both *L. volemus* and *L. subvolemus* have longer pileipellis hairs and can only be distinguished from each other based on cap colour. Intermediary colour forms, however, occur as well, and cannot be identified as either *L. volemus* or *L. subvolemus* without molecular data. Revealing that *L. volemus* – already considered extinct in the Netherlands and the Belgian Flemish region, and declining in other European countries – is actually a complex of three species that are even more vulnerable to extinction, this study emphasizes the fundamental role of taxonomy in species conservation.

Introduction

Over the past few decades, molecular phylogenetic studies have perturbed fungal taxonomy. A recent molecular phylogeny of the agaricoid ectomycorrhizal *Russulaceae* revealed that the former *Lactarius* Pers. – also known as the milk caps – and *Russula* Pers. can be no longer maintained as such. They are now split up into four distinct genera: *Lactarius* Pers., *Lactifluus* (Pers.) Roussel, *Multifurca* Buyck & Hofstetter and *Russula* Pers (Buyck *et al.* 2008, 2010).

Species within *Lactifluus* subg. *Lactifluus* sect. *Lactifluus* (Burl.) Hesler & A.H. Smith (synonym *Lactarius* subsect. *Volemi* Lalli & Pacioni) can be morphologically recognized by the unique combination of three characters: reticulate spores, lamprocystidia, and a pileipellis with lampropallisadic structure. *Lactifluus volemus* (Fr.: Fr.) Kuntze [synonym *Lactarius*

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volemus (Fr.: Fr.) Fr.] is the section's type species. Its history begins in Sweden under the name *Agaricus lactifluus* L. (Linnaeus 1753), but it was Fries (1821) who originated the species epithet *volemus* (as *Agaricus volemus* Fr.: Fr.), and later classified it as a member of the former *Lactarius* (Fries 1838). Its unique microscopic characters, combined with the dry, typically orange-brown cap, fishy smell, and extremely abundant latex that is sticky, first white and then staining brown, have led to the reputation of being one of the most easily recognizable European milk caps (Neuhoff 1956; Verbeken *et al.* 1996).

A second European species has been described within the section: *Lactifluus oedematopus* (Scop.) Kuntze. However, it is generally not accepted as a species, but considered a brick-red colour variant of *L. volemus*. Fries (1838) formally recombined it as *Lactarius volemus* var. *oedematopus* (Scop.) Fr., but several authors do not recognize this entity at varietal rank either. Instead, they consider it to be included in the colour range of *L. volemus* (Lalli & Pacioni 1992; Verbeken *et al.* 1996; Heilmann-Clausen *et al.* 1998; Basso 1999). In addition, buff yellow colour variants – strikingly similar to the North American *Lactarius volemus* var. *flavus* Hesler & A.H. Sm. (not yet recombined in *Lactifluus*) – have been recorded in Europe as well (Lalli & Pacioni 1992; Basso 1999). Although the European *L. volemus* has been reported to exhibit remarkable variation in colour, its other characteristics remain so constant that no questions have been raised about it comprising multiple species (Lalli & Pacioni 1992; Heilmann-Clausen *et al.* 1998).

Besides *L. volemus* and *L. oedematopus*, five additional taxa have been described in *L.* sect. *Lactifluus* prior 2010: *Lactifluus corrugis* (Peck) Kuntze and *Lactifluus pallidilamellatus* (Montoya & Band.-Muñoz) Van de Putte from North America, and *Lactifluus lamprocystidiatus* (Verbeken & E. Horak) Verbeken, *Lactifluus austrovolemus* (Hongo) Verbeken and *Lactifluus princeps* (Berk.) Kuntze from Asia (Verbeken *et al.* 2012). Subtle morphological differences, the occurrence of intermediary morphological forms, and the poor knowledge of recently described species, however, have seriously complicated species identification. As a result, authors have generally applied the well-known European name *L. volemus* for look-alikes in North America and Asia, without evaluating intercontinental conspecificity. Shimono *et al.* (2007), however, demonstrated the presence of multiple distinct genetic lineages in Japan, while Van de Putte *et al.* (2010, 2012) delineated at least eighteen species in Thailand and six species in Sikkim Himalaya (India), of which nine have been morphologically described.

We are interested in determining whether *L. volemus* sensu lato from Europe comprises multiple species and whether or not these species can be distinguished from each other morphologically. Before delimiting species, however, one has to decide on what a species precisely is. Although the plenitude of different species concepts makes this a rather daunting decision, a unified species concept – termed the general lineage concept of species by de Queiroz (2007) – can be achieved by treating species as segments of separately evolving metapopulation lineages, which can be delimited by using the former species concept criteria as independent lines of evidence (e.g. reciprocal monophyly, reproductive isolation, ecological differentiation, morphological diagnosability, geographic range) (de Queiroz 2007). The observation of a single such property provides evidence for lineage separation, hence, the delimitation of a species. Species' properties however, appear in an unpredictable order, or some properties – such as morphological diagnosability – may not appear at all. The best species hypotheses are therefore based on multiple independent lines of evidence (de Queiroz 2007).

As a first approach, we aim to use a multi-locus tree-based method and delimit species boundaries by identifying reciprocal monophyletic groups. Through the course of divergence, genetic loci pass through initial stages of polyphyly and paraphyly toward a stage of monophyly, so eventually all taxa might be delimited with a criterion such as reciprocal monophyly (Avise & Ball 1990). However, as a substantial amount of time is required to achieve reciprocal monophyly at a sample of multiple loci, this criterion is unlikely to furnish evidence for recent species divergence (Hudson & Coyne 2002).

Alternative multi-locus methods have been developed to delimit species despite incomplete lineage sorting (e.g. Knowles & Carstens 2007; O'Meara 2010; Yang & Rannala 2010), and as a second approach, we aim to assess support of species divergence by applying the recently developed Bayesian species delimitation method (Yang & Rannala 2010). This coalescent-based method uses gene trees, estimates of the effective population size N_e and timing of divergence to assess the probability of speciation (Yang & Rannala 2010).

Finally, we examined macro- and micromorphological characters of each molecularly delineated species to determine whether or not molecular data are corroborated by morphology, and which characters have a diagnostic value.

Material and methods

Sampling

Only about 30 % of all *Lactifluus volemus* sensu lato collections were collected by the authors themselves. About 70 % of the collections are loans from several herbaria in Europe and North America. Two collection datasets were constructed. The first 187-collection dataset includes 138 European and 25 North American *L. volemus* collections, 12 collections of *Lactifluus corrugis*, and one collection of *Lactifluus pallidilamellatus*. Since the ITS region was too divergent to align unambiguously across *L. volemus* sensu lato and more distantly related taxa, four representatives of its sister clade *Lactifluus* subgenus *Gerardii* (A.H. Sm. & Hesler) Stubbe (Stubbe *et al.* 2010) were chosen as outgroup. For the LSU and *rpb2* 187-collection datasets, seven more species of the genus *Lactifluus* were included, of which *Lactifluus rugatus* (Kühner & Romagn.) Verbeken, and *Lactifluus hygrophoroides* (Berk. & M.A. Curtis) Kuntze were used as outgroup (Stubbe *et al.* 2012; Verbeken *et al.* 2012).

The second collection dataset only includes the 138 European *L. volemus* collections. A complete list of studied collections is provided in Table 1. The collection sites of the European *L. volemus* specimens are indicated in Fig 1. For collections without specified latitude and longitude data, coordinates were obtained using locality descriptions and GOOGLE EARTH 5.2.1 (http://earth.google.com).

Table 1 – Specimens and GenBank accession numbers of the DNA sequences used in the molecular analyses. If no herbarium acronym is specified, the collections are deposited in the collector's personal herbarium. The genus *Lactifluus* is abbreviated by '*Lf.*', '*L.*' is used for the genus *Lactarius*.

Species	Original identification	Voucher (herbarium)	Collector	Origin	Sequences available		
					ITS	rpb2	LSU
Lf. oedematopus	*L. volemus*	AF 1391 (BR)	A. Fraiture	BELGIUM	x	JQ348186	JQ348323
Lf. oedematopus	*L. volemus*	AF 2386 (BR)	A. Fraiture	BELGIUM	JQ753876	JQ348260	JQ348324
Lf. oedematopus	*L. volemus*	AV 07-79 (GENT)	A. Verbeken	BELGIUM	JQ753835	JQ348131	JQ348270
Lf. oedematopus	*L. volemus*	SOMF 26035 (SOMF)	B. Assyov	BULGARIA	JQ753870	x	x
Lf. oedematopus	*L. volemus*	AV 97-510 (GENT)	A. Verbeken	FRANCE	HQ318217	x	HQ318117
Lf. oedematopus	*L. volemus* var. *oedematopus*	RW 1228 (GENT)	R. Walleyn, A. Verbeken	FRANCE	AY606959	AY606966	HQ318116
Lf. oedematopus	*L. volemus*	AM 593/1991 (STU)	A. Müller	GERMANY	JQ753908	JQ348222	JQ348360
Lf. oedematopus	*L. oedematopus*	KVP R 12-001 (GENT)	Jochen Girwert	GERMANY	KJ210065	KJ210068	KJ210066
Lf. oedematopus	*L. oedematopus*	EDC 14-519	Eske De Crop	SLOVAKIA	KT291175	x	x
Lf. subvolemus	*L. volemus*	MEN 96175 (L)	M.E. Noordeloos	AUSTRIA	JQ753943	JQ348256	JQ348394
Lf. subvolemus	*L. volemus*	MEN 96193 (L)	M.E. Noordeloos	AUSTRIA	JQ753942	JQ348255	JQ348393
Lf. subvolemus	*L. volemus*	KVP 11-001 (GENT)	O. Berteloot, B. Goemare, A. Verbeken	BELGIUM	JQ753947	x	x
Lf. subvolemus	*L. volemus*	KVP 11-003 (GENT)	O. Berteloot, B. Goemare, A. Verbeken	BELGIUM	JQ753949	x	x
Lf. subvolemus	*L. volemus*	SOMF 16874 (SOMF)	G. Stojcer	BULGARIA	JQ753877	JQ348187	JQ348325
Lf. subvolemus	*L. volemus*	RT 88-342 (C)	R. Toft	DENMARK	JQ753920	JQ348235	JQ348372
Lf. subvolemus	*L. volemus*	405297 (STU)	/	GERMANY	JQ753914	JQ348229	JQ348366
Lf. subvolemus	*L. volemus*	LK 609/1994 (STU)	L. Krieglsteiner	GERMANY	JQ753913	JQ348227	JQ348365
Lf. subvolemus	*L. volemus*	JV 17277 (TUR-A)	J. Vauras	ITALY	JQ753839	JQ348144	JQ348278
Lf. subvolemus	*L. volemus*	MAK 02/3342 (MCF)	K. Rusevska	MACEDONIA	JQ753851	JQ348164	JQ348298
Lf. subvolemus	*L. volemus*	MAK 02/3719 (MCF)	M. Karadelev	MACEDONIA	JQ753854	JQ348167	JQ348301
Lf. subvolemus	*L. volemus*	MAK 06/5885 (MCF)	K. Rusevska	MACEDONIA	JQ753852	JQ348165	JQ348299
Lf. subvolemus	*L. volemus*	O F64318 (O)	J. Wesenberg	NORWAY	JQ753864	JQ348180	JQ348311
Lf. subvolemus	*L. volemus*	O F66437 (O)	A.-K. Helm	NORWAY	JQ753860	JQ348173	JQ348307
Lf. subvolemus	*L. volemus*	BUCM 121.818 (BUCM)	G. Negrean	ROMANIA	JQ753888	JQ348199	JQ348336
Lf. subvolemus	*L. volemus*	SAV F-1137 (GENT)	S. Adamčík	SLOVAKIA	JQ753849	JQ348162	JQ348296
Lf. subvolemus	*L. volemus*	SAV F-2264 (GENT)	S. Adamčík	SLOVAKIA	JQ753846	JQ348159	JQ348293
Lf. subvolemus	*L. volemus*	SAV F-2269 (GENT)	S. Adamčík	SLOVAKIA	JQ753845	JQ348158	JQ348292
Lf. subvolemus	*L. volemus*	AV 07-82 (GENT)	R. Walleyn, G. Podgornik, A. Verbeken	SLOVENIA	HQ318218	HQ328861	HQ318118
Lf. subvolemus	*L. volemus* aff. var. *flavus*	KVP 08-045p (GENT)	K. Van de Putte	SLOVENIA	JQ753954	x	x
Lf. subvolemus	*L. volemus*	KVP 08-44 (GENT)	K. Van de Putte	SLOVENIA	JQ753924	JQ348238	JQ348376
Lf. subvolemus	*L. volemus*	KVP 08-46 (GENT)	K. Van de Putte	SLOVENIA	JQ753925	JQ348239	JQ348377
Lf. subvolemus	*L. volemus*	KVP 08-48 (GENT)	K. Van de Putte	SLOVENIA	JQ753927	JQ348241	JQ348379
Lf. subvolemus	*L. volemus*	KVP 08-49 (GENT)	K. Van de Putte	SLOVENIA	JQ753928	JQ348242	JQ348380
Lf. subvolemus	*L. volemus*	KVP 08-50 (GENT)	K. Van de Putte	SLOVENIA	JQ753929	JQ348243	JQ348381
Lf. subvolemus	*L. volemus*	KVP 08-52 (GENT)	K. Van de Putte	SLOVENIA	JQ753930	JQ348244	x
Lf. subvolemus	*L. volemus*	KVP 08-58 (GENT)	K. Van de Putte	SLOVENIA	JQ753931	JQ348245	JQ348382
Lf. subvolemus	*L. volemus*	KVP 08-60 (GENT)	K. Van de Putte	SLOVENIA	JQ753932	JQ348246	JQ348383
Lf. subvolemus	*L. volemus*	KVP 08-61 (GENT)	K. Van de Putte	SLOVENIA	JQ753933	JQ348247	JQ348384
Lf. subvolemus	*L. volemus*	KVP 08-66 (GENT)	K. Van de Putte	SLOVENIA	JQ753934	JQ348248	JQ348385
Lf. subvolemus	*L. volemus*	KVP 08-67 (GENT)	K. Van de Putte	SLOVENIA	JQ753935	JQ348249	JQ348386
Lf. subvolemus	*L. volemus*	JL 1636 (BCN)	J. Llisostella	SPAIN	JQ753872	JQ348183	JQ348319
Lf. subvolemus	*L. volemus*	NM 20070623.2 (GENT)	N. Macau, M. Pérez-De-Gregorio	SPAIN	JQ753840	JQ348145	JQ348279
Lf. subvolemus	*L. volemus*	IK 84651 (H)	I. Kytövuori	SWEDEN	JQ753903	JQ348216	JQ348354
Lf. subvolemus	*L. volemus*	IK 851211 (H)	I. Kytövuori	SWEDEN	JQ753902	JQ348215	JQ348353
Lf. subvolemus	*L. volemus*	JK 06082011 (GENT)	J. Kleine	SWEDEN	JQ753952	x	x
Lf. subvolemus	*L. volemus*	LAS 75/092-A (GB)	L. Stridvall	SWEDEN	JQ753898	JQ348210	JQ348348
Lf. subvolemus	*L. volemus*	LAS 75/092-B (GB)	L. Stridvall	SWEDEN	JQ753899	JQ348211	JQ348349
Lf. volemus	*L. volemus*	MEN 2004 65 (L)	M.E. Noordeloos	AUSTRIA	JQ753944	JQ348257	JQ348395
Lf. volemus	*L. volemus*	AF 1373 (BR)	A. Fraiture	BELGIUM	JQ753875	JQ348185	JQ348322
Lf. volemus	*L. volemus*	AF 1927 (BR)	A. Fraiture	BELGIUM	JQ753874	JQ348184	JQ348321
Lf. volemus	*L. volemus*	CN 6/10/1980 (BR)	N. Cnops	BELGIUM	JQ753873	x	JQ348320

(*continued on next page*)

Table 1 – (*continued*)

Species	Original identification	Voucher (herbarium)	Collector	Origin	Sequences available		
					ITS	rpb2	LSU
Lf. volemus	*L. volemus*	DS 09-702 (GENT)	D. Stubbe	BELGIUM	JQ753857	JQ348170	JQ348304’
Lf. volemus	*L. volemus*	KVP 11-002 (GENT)	O. Berteloot, B. Goemare, A. Verbeken	BELGIUM	JQ753948	x	x
Lf. volemus	*L. volemus*	KVP 11-004 (GENT)	O. Berteloot, B. Goemare, A. Verbeken	BELGIUM	JQ753950	x	x
Lf. volemus	*L. volemus*	SOMF 26396 (SOMF)	D. Stoykov	BULGARIA	JQ753869	JQ348181	JQ348316
Lf. volemus	*L. volemus*	DD 132/05 (BRNU)	D. Dvorak	CZECH REPUBLIC	JQ753858	JQ348171	JQ348305
Lf. volemus	*L. volemus*	DD 207/09 (BRNU)	D. Dvorak	CZECH REPUBLIC	JQ753859	JQ348172	JQ348306
Lf. volemus	*L. volemus*	C 29065 (C)	B. Levesen	DENMARK	JQ753915	JQ348230	JQ348367
Lf. volemus	*L. volemus*	JV 00-205 (C)	L. Vesterholt & J. Vesterholt	DENMARK	JQ753916	JQ348231	JQ348368
Lf. volemus	*L. volemus*	JV 00-221 (C)	L. Vesterholt	DENMARK	JQ753917	JQ348232	JQ348369
Lf. volemus	*L. volemus*	JV 88-346 (C)	A. Nielsen & J. Vesterholt	DENMARK	JQ753918	JQ348233	JQ348370
Lf. volemus	*L. volemus*	JV 89-673 (C)	A. Nielsen	DENMARK	JQ753919	JQ348234	JQ348371
Lf. volemus	*L. volemus*	TL 5936 (C)	T. Læssøe & J. Læssøe	DENMARK	JQ753921	JQ348236	JQ348373
Lf. volemus	*L. volemus*	TAAM 182733 (TAAM)	L. Tedersoo	ESTONIA	JQ753907	JQ348221	JQ348359
Lf. volemus	*L. volemus*	H 6010882 (H)	T. Jaakkonen	FINLAND	JQ753897	JQ348208	JQ348346
Lf. volemus	*L. volemus*	H 6011404 (H)	M. Stjernberg	FINLAND	JQ753896	JQ348207	JQ348345
Lf. volemus	*L. volemus*	H 6011446 (H)	A. Lehtinen	FINLAND	x	JQ348209	JQ348347
Lf. volemus	*L. volemus*	H 6011459 (H)	T. Harve	FINLAND	JQ753891	JQ348202	JQ348340
Lf. volemus	*L. volemus*	IK 90-2345 (H)	I. Kytövuori	FINLAND	JQ753892	JQ348203	JQ348341
Lf. volemus	*L. volemus*	JV 12622F (TUR-A)	J. Vauras	FINLAND	JQ753878	JQ348188	JQ348326
Lf. volemus	*L. volemus*	JV 16524 (TUR-A)	J. Vauras	FINLAND	JQ753836	JQ348141	JQ348275
Lf. volemus	*L. volemus*	JV 16596F (TUR-A)	J. Vauras	FINLAND	JQ753838	JQ348143	JQ348277
Lf. volemus	*L. volemus*	JV 18317 (TUR-A)	J. Vauras	FINLAND	JQ753837	JQ348142	JQ348276
Lf. volemus	*L. volemus*	JV 5100F (TUR-A)	J. Vauras	FINLAND	HQ318215	HQ328860	HQ318115
Lf. volemus	*L. volemus*	KL & TN D08-012 (H)	K. Liimatainen & T. Niskanen	FINLAND	JQ753895	JQ348206	JQ348344
Lf. volemus	*L. volemus*	LM 14-04 (H)	M. Lahti	FINLAND	JQ753894	JQ348205	JQ348343
Lf. volemus	*L. volemus*	TUR 119274 TUR	K. Johansson	FINLAND	JQ753879	JQ348189	JQ348327
Lf. volemus	*L. volemus*	UN-S PS 7731 (H)	U. Nummela-Salo, P. Salo	FINLAND	JQ753893	JQ348204	JQ348342
Lf. volemus	*L. volemus*	AV 97-512 (GENT)	A. Verbeken & R. Walleyn	FRANCE	HQ318213	JQ348132	HQ318113
Lf. volemus	*L. volemus*	EA 00-91 (L)	E. Arnolds	FRANCE	JQ753945	JQ348258	JQ348396
Lf. volemus	*L. volemus*	OR 688 (G)	O. Röllin	FRANCE	JQ753941	JQ348254	JQ348392
Lf. volemus	*L. volemus*	406307 (STU)	Walther	GERMANY	JQ753909	JQ348223	JQ348361
Lf. volemus	*L. volemus*	BB 2610 (GENT)	B. Buyck	GERMANY	JQ753881	JQ348191	JQ348329
Lf. volemus	*L. volemus*	BB 2620 (GENT)	B. Buyck	GERMANY	HQ318214	x	HQ318114
Lf. volemus	*L. volemus*	BB 2699 (GENT)	B. Buyck	GERMANY	HQ318219	JQ348134	HQ318119
Lf. volemus	*L. volemus*	BR 39622-46 (BR)	R. Walleyn	GERMANY	JQ753882	x	JQ348330
Lf. volemus	*L. volemus*	IK 95-1728 (H)	I. Kytövuori	GERMANY	JQ753923	x	JQ348375
Lf. volemus	*L. volemus*	JR 7014 (BR)	J. Rammeloo	GERMANY	JQ753883	JQ348192	JQ348331
Lf. volemus	*L. volemus*	KM 892/1985 (STU)	K. Morschek	GERMANY	x	JQ348228	x
Lf. volemus	*L. volemus*	KN 321/1994 (STU)	K. Neff	GERMANY	JQ753911	JQ348225	JQ348363
Lf. volemus	*L. volemus*	LK 131/1998 (STU)	L. Krieglsteiner	GERMANY	JQ753912	JQ348226	JQ348364
Lf. volemus	*L. volemus*	LK 675/2001 (STU)	L. Krieglsteiner	GERMANY	JQ753910	JQ348224	JQ348362
Lf. volemus	*L. volemus*	RW 89/27 (GENT)	R. Walleyn	GERMANY	JQ753880	JQ348190	JQ348328
Lf. volemus	*L. volemus*	PA 2010V (GENT)	P. Angelos	GREECE	JQ753904	JQ348217	JQ348355
Lf. volemus	*L. volemus*	AQUI 03/18 (AQUI)	G. Lalli	ITALY	JQ753887	JQ348198	JQ348335
Lf. volemus	*L. volemus*	AQUI 110 (AQUI)	G. Lalli	ITALY	JQ753889	JQ348200	JQ348337
Lf. volemus	*L. volemus*	AQUI 114 (AQUI)	G. Lalli	ITALY	JQ753890	x	JQ348338
Lf. volemus	*L. volemus*	PA 96-2 (GENT)	A. Pierotti	ITALY	JQ753884	JQ348193	x
Lf. volemus	*L. volemus*	LDM F3227 (RIG)	D. Meiere	LATVIA	JQ753856	JQ348169	JQ348303
Lf. volemus	*L. volemus*	LDM F3958 (RIG)	I. Dániele	LATVIA	JQ753855	JQ348168	JQ348302
Lf. volemus	*L. volemus*	LDM F5353 (RIG)	I. Dániele	LATVIA	JQ753842	JQ348155	JQ348289
Lf. volemus	*L. volemus*	WI 4715 (WI)	M. Jankauskienë	LITHUANIA	JQ753885	JQ348194	JQ348332
Lf. volemus	*L. volemus*	WI 4716 (WI)	M. Jankauskienë	LITHUANIA	JQ753886	JQ348195	x
Lf. volemus	*L. volemus*	MAK 03/3247 (MCF)	G. Kost	MACEDONIA	JQ753853	JQ348166	JQ348300

Table 1 – (*continued*)

Species	Original identification	Voucher (herbarium)	Collector	Origin	Sequences available		
					ITS	rpb2	LSU
Lf. volemus	*L. volemus*	MAK 05/5086 (MCF)	M. Aleksovski	MACEDONIA	JQ753841	JQ348146	JQ348280
Lf. volemus	*L. volemus*	O F210035 (O)	S. Engelhart	NORWAY	JQ753866	JQ348179	JQ348313
Lf. volemus	*L. volemus*	O F222375 (O)	A. Græsdal	NORWAY	JQ753862	JQ348175	JQ348309
Lf. volemus	*L. volemus*	O F242318 (O)	J. B. Jordal	NORWAY	JQ753867	JQ348177	JQ348314
Lf. volemus	*L. volemus*	O F283037 (O)	Å. Nodland	NORWAY	JQ753865	JQ348178	JQ348312
Lf. volemus	*L. volemus*	O F68500 (O)	A. Molia	NORWAY	JQ753863	JQ348176	JQ348310
Lf. volemus	*L. volemus*	O F69426 (O)	O.M. Stavik	NORWAY	JQ753861	JQ348174	JQ348308
Lf. volemus	*L. volemus*	ASM 10694 (EIU)	A. Methven	RUSSIA	JQ358935	JN985478	JN940225
Lf. volemus	*L. volemus*	LE 217608 (LE)	A.E. Kovalenko	RUSSIA	JQ753940	JQ348253	JQ348391
Lf. volemus	*L. volemus*	LE 254509 (LE)	A.A. Kiyashko	RUSSIA	JQ753937	x	JQ348388
Lf. volemus	*L. volemus*	LE 25417 (LE)	O.V. Morozova	RUSSIA	JQ753938	JQ348251	JQ348389
Lf. volemus	*L. volemus*	LE 258626 (LE)	L.V. Marina	RUSSIA	JQ753939	JQ348252	JQ348390
Lf. volemus	*L. volemus*	TAAM 095075 (TAAM)	L. Pihlik	RUSSIA	JQ753905	JQ348219	JQ348357
Lf. volemus	*L. volemus*	TAAM 095097 (TAAM)	L. Pihlik	RUSSIA	JQ753906	JQ348220	JQ348358
Lf. volemus	*L. volemus*	E 23023 (E)	/	SCOTLAND	JQ753868	x	JQ348315
Lf. volemus	*L. volemus*	LH 8/16/2004 (GENT)	L. Holden	SCOTLAND	x	JQ348196	JQ348333
Lf. volemus	*L. volemus*	MEN 9692 (L)	M.E. Noordeloos	SCOTLAND	JQ753946	JQ348259	JQ348397
Lf. volemus	*L. volemus*	SJS, PC, AL (GENT)	SJS, PC, AL	SCOTLAND	HQ318220	HQ328862	HQ318120
Lf. volemus	*L. volemus*	SAV F-1472 (GENT)	S. Adamčík	SLOVAKIA	JQ753847	JQ348160	JQ348294
Lf. volemus	*L. volemus*	SAV F-2265 (GENT)	S. Adamčík	SLOVAKIA	JQ753848	JQ348161	JQ348295
Lf. volemus	*L. volemus*	SAV F-2266 (GENT)	S. Adamčík	SLOVAKIA	JQ753850	JQ348163	JQ348297
Lf. volemus	*L. volemus*	SAV F-2267 (GENT)	S. Adamčík	SLOVAKIA	JQ753844	JQ348157	JQ348291
Lf. volemus	*L. volemus*	SAV F-2268 (GENT)	S. Adamčík	SLOVAKIA	JQ753843	JQ348156	JQ348290
Lf. volemus	*L. volemus*	KVP 08-45 (GENT)	K. Van de Putte	SLOVENIA	JQ753953	x	x
Lf. volemus	*L. volemus*	KVP 08-47 (GENT)	K. Van de Putte	SLOVENIA	JQ753926	JQ348240	JQ348378
Lf. volemus	*L. volemus*	KVP 08-68 (GENT)	K. Van de Putte	SLOVENIA	JQ753936	JQ348250	JQ348387
Lf. volemus	*L. volemus*	JL 1430 (BCN)	J. Llisostella	SPAIN	x	x	JQ348318
Lf. volemus	*L. volemus*	JL 1628 (BCN)	J. Llisostella	SPAIN	JQ753871	JQ348182	JQ348317
Lf. volemus	*L. volemus*	JL 491 (BCN)	J. Llisostella	SPAIN		JQ348197	JQ348334
Lf. volemus	*L. volemus*	90804-5 (GENT)	/	SWEDEN	JN388959	JQ348201	JQ348339
Lf. volemus	*L. volemus*	ASM 13028 (EIU)	R. Petersen, A. Methven, P. Marsted	SWEDEN	JQ358943	JN985470	JN940216
Lf. volemus	*L. volemus*	IK 83568 (H)	I. Kytövuori	SWEDEN	JQ753900	JQ348212	JQ348350
Lf. volemus	*L. volemus*	IK 871644 (H)	I. Kytövuori	SWEDEN	JN388960	JQ348214	JQ348352
Lf. volemus	*L. volemus*	IK 871670 (H)	I. Kytövuori	SWEDEN	JQ753901	JQ348213	JQ348351
Lf. volemus	*L. volemus*	JK 03082011 (GENT)	J. Kleine	SWEDEN	JQ753951	x	x
Lf. volemus	*L. volemus*	PU 20071 (H)	Pertti Uotila	TURKEY	JQ753922	JQ348237	JQ348374
	L. volemus	AV 05-227 (GENT)	A. Verbeken	USA	JQ753832	JQ348150	JQ348284
	L. volemus	AV 05-294 (GENT)	A. Verbeken	USA	x	JQ348152	JQ348286
	L. volemus	AV 05-293 (GENT)	A. Verbeken	USA	JQ753834	JQ348153	JQ348287
	L. volemus	AV 05-310 (GENT)	A. Verbeken	USA	x	JQ348154	JQ348288
	L. volemus var. *flavus*	AV 04-166 (GENT)	A. Verbeken	USA	JQ753829	x	JQ348274
	L. volemus	AV 05-345 (GENT)	A. Verbeken	USA	JQ753831	JQ348149	JQ348283
	L. volemus var. volemus	AV 05-394 (GENT)	A. Verbeken	USA	GU258300	GU258400	GU265660
	L. volemus	AV 05-298 (GENT)	A. Verbeken	USA	JQ753825	JQ348135	JQ348272
	L. volemus	AV 05-313 (GENT)	A. Verbeken	USA	x	JQ348147	JQ348281
	L. volemus	AV 05-329 (GENT)	A. Verbeken	USA	JQ753830	JQ348148	JQ348282
	L. volemus	AV 05-384 (GENT)	A. Verbeken	USA	JQ753826	JQ348136	HQ318127
	L. volemus var. *flavus*	AV 05-395 (GENT)	A. Verbeken	USA	JQ753833	JQ348151	JQ348285
	L. volemus	AV 04-173 (GENT)	A. Verbeken	USA	x	JQ348137	HQ318128
	L. volemus	AV 05-330 (GENT)	A. Verbeken	USA	JQ753828	JQ348140	HQ318129
	L. volemus	AV 04-167 (GENT)	A. Verbeken	USA	JQ753827	JQ348138	JQ348273
	L. volemus	AV 04-194 (GENT)	A. Verbeken	USA	x	HQ328868	HQ318130
	L. volemus	AV 04-165 (GENT)	A. Verbeken	USA	x	JQ348139	HQ318131
	L. volemus	ASM 10377 (EIU)	A. Methven	USA	JQ358929	JN985465	JN940227
	L. volemus	11981, TENN59983	/	USA	JQ358925	x	x
	L. volemus	ASM 10552 (EIU)	A. Methven	USA	JQ358934	x	JN940215
	L. volemus	ASM 10991 (EIU)	A. Methven	USA	JQ358936	JN985467	JN940219

(*continued on next page*)

Table 1 – (continued)

Species	Original identification	Voucher (herbarium)	Collector	Origin	Sequences available		
					ITS	rpb2	LSU
	L. volemus	ASM 11080 (EIU)	A. Methven	USA	JQ358937	x	JN940224
	L. volemus	ASM 11130 (EIU)	A. Methven	USA	JQ358938	JN985468	JN940223
	L. volemus var. *flavus*	ASM 09265 (EIU)	A. Methven	USA	JQ358928	JN985464	JN940228
	L. volemus var. *flavus*	ASM 11131 (EIU)	A. Methven	USA	JQ358939	x	x
	L. corrugis	ASM 10375 (EIU)	A. Methven	USA	JQ358917	JN940336	JN940239
	L. corrugis	ASM 11092 (EIU)	A. Methven	USA	JQ358922	x	JN940235
	L. corrugis	ASM 11129 (EIU)	A. Methven	USA	JQ358923	x	JN940231
	L. corrugis	ASM 10544 (EIU)	A. Methven	USA	JQ358920	x	JN940237
	L. corrugis	ASM 10990 (EIU)	A. Methven	USA	JQ358921	x	JN940236
	L. corrugis	ASM 10422 (EIU)	A. Methven	USA	JQ358919	x	JN940238
	L. corrugis	AV 05-290 (GENT)	A. Verbeken	USA	JQ753819	JQ348124	JQ348261
	L. corrugis	JN 2004-015 (GENT)	J. Nuytinck	USA	JQ753820	JQ348125	JQ348262
	L. corrugis	AV 05-337 (GENT)	A. Verbeken	USA	JQ753821	JQ348126	JQ348263
	L. corrugis	AV 05-392 (GENT)	A. Verbeken	USA	JQ753822	JQ348127	JQ348264
	L. corrugis	AV 04-209 (GENT)	A. Verbeken	USA	JN388977	JQ348128	JQ348265
	L. corrugis	AV 05-291 (GENT)	A. Verbeken	USA	JQ753823	JQ348129	JQ348266
	L. pallidilamellatus	M 4716 (XAL)	L. Montoya	MEXICO	JQ753824	x	JQ348268
Lf. atrovelutinus		DS 06-003 (GENT)	D. Stubbe	MALAYSIA	GU258231	GU258325	GU265588
Lf. bicolor		DS 06-247 (GENT)	D. Stubbe	MALAYSIA	JN388955	JN375590	JN388987
Lf. petersenii		AV 05-300 (GENT)	A. Verbeken	USA	GU258281	GU265642	GU258382
Lf. subgerardii		AV 05-269 (GENT)	A. Verbeken	USA	GU258263	GU258362	GU265625
Lf. vellereus		FH 5032/4 (GENT)	F. Hampe	GERMANY	x	JN375597	JN388994
Lf. piperatus		2000 10 02 09	M. Lecomte	BELGIUM	x	JN375594	JN388991
Lf. piperatus		2001 08 19 13	M. Lecomte	FRANCE	x	JN375596	JQ348269
Lf. glaucescens		2008 08 21 01	M. Lecomte	BELGIUM	x	JN375591	JN388988
Lf. hygrophoroides		AV 05-251 (GENT)	A. Verbeken	USA	x	HQ328936	HQ318208
Lf. luteolus		AV 05-253 (GENT)	A. Verbeken	USA	x	KJ210067	JQ348267
Lf. rugatus		PA 2010R (GENT)	P. Angelos	GREECE	x	JN375595	JN388992

DNA sequencing and alignment

Total genomic DNA was extracted from fresh and dried material using the methods previously described by Van de Putte *et al.* (2010). We amplified and sequenced three loci, including (1) the internal transcribed spacer regions of the ribosomal DNA (ITS), containing spacer regions ITS1 and ITS2, the ribosomal gene 5.8S, and a small portion of the ribosomal genes 18S and 28S, (2) a part of the ribosomal large subunit 28S region (LSU), and (3) the region between domains six and seven of the nuclear gene encoding the second largest subunit of RNA polymerase II (*rpb2*). Primers sequences are provided in Van de Putte *et al.* (2010). Protocols for PCR amplification follow Le *et al.* (2007). Sequencing follows Le *et al.* (2007) or has been conducted with an ABI 3730XL or ABI 3700 by MACROGEN (Amsterdam, The Netherlands). Sequences were assembled and edited with the software Sequencher™ v4.8 (GeneCodes Corporation, Ann Arbor, Michigan, U.S.A.). Alignments were constructed with the on-line version of the multiple sequence alignment program MAFFTv6 (Katoh & Toh 2008), applying the E-INS-I strategy, a very slow method recommended for less than 200 sequences with multiple conserved domains and long gaps. The alignment was further manually refined with the BioEdit Sequence Alignment Editor v7.0.9.0 (Hall 1999). Ambiguously aligned positions were eliminated using the on-line Gblocks v0.91b server (Castresana 2000), specifying least stringent conditions, i.e. allowing smaller final blocks, gap positions within the final blocks and less strict flanking positions. The combined ITS-LSU-*rpb2* 187-collection alignment and ML tree is available at the online databank TreeBASE:

Species delimitation and phylogeny

We established a preliminary set of species boundaries using a multilocus genealogical approach. Gene trees were estimated for the ITS, LSU, and *rpb2* sequences of the 187-collection dataset using maximum likelihood (ML) and Bayesian inference (BI). We recognized monophyletic clades as phylogenetic species when they were concordantly supported by two out of the three loci, and not contradicted by any other locus at the same level of support (ML bootstrap ≥ 75 %, BI posterior probability ≥ 95 %) (Dettman *et al.* 2003). We used the following sequence data partitions: (1) ribosomal gene 18S, (2) spacer region ITS1, (3) ribosomal gene 5.8S, (4) spacer region ITS2, (5) ribosomal gene 28S (LSU), (6–8) codon positions 1–3 of the *rpb2* exon, and (9) the *rpb2* intron.

Maximum likelihood trees and bootstrap values were estimated with RAxML v8.1.24 (Stamatakis 2006), using the Rapid Bootstrapping algorithm for 1000 replicates combined

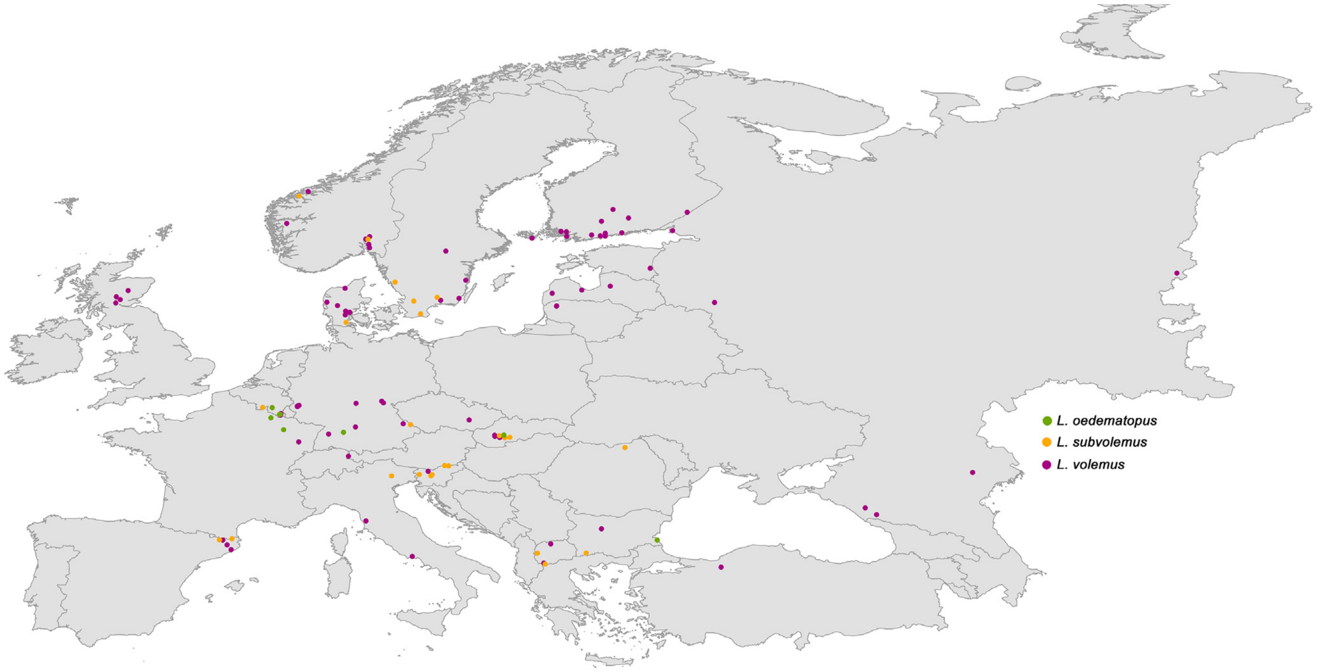

Fig 1 – Map of Europe indicating the collection sites of *L. oedematopus* emend. (green dots), *L. subvolemus* (yellow dots), and *L. volemus* emend. (purple dots). For collections without specified latitude and longitude data, coordinates were obtained using locality descriptions and GOOGLE EARTH 5.2.1 (http://earth.google.com).

with a ML search. Analyses were run on XSEDE using the CIPRES Science Gateway V3.3. (Miller *et al.* 2010). The model GTRMIX was estimated for each partition separately. Bayesian trees and posterior probabilities were estimated with MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003) on a High Performance Computer (HPC) of Ghent University. The model that best fits the data was determined for each partition with MrModeltest v2.3 (Nylander 2004) using the second order Akaike Information Criterion (AICc). Two independent, parallel runs of one cold and three heated chains were run for ten million (single-locus datasets) or 15 million generations (multiple-locus dataset) with a sample frequency of 100. The temperature was set to 0.5 allowing the chains to swap more easily from cold to heated. Convergence and effective sample size statistics (ESS) of the runs were examined with Tracer v1.5 (Rambaut & Drummond 2007). A burn in sample of 20 % was excluded before constructing the majority rule consensus tree. We also compared topologies and posterior probabilities from the two runs to assure that they were reliable.

Relationships among European species were assessed from the ML and BI trees of the concatenated ITS-LSU-*rpb2* 187-collection dataset. Before pooling the three loci into a single analysis, their compatibility was assessed by visually comparing ML single-locus trees. Significant incongruence was assumed if two different relationships (one monophyletic and the other non-monophyletic) for any set of taxa were supported with ML bootstrap $\geq$ 70 %.

We also used the recently developed hierarchical Bayesian model implemented in *BEAST 1.6.2 (Heled & Drummond 2010) to estimate interspecies relationships.*-BEAST co-estimates multiple gene trees embedded in a shared species tree along with the effective population sizes of both extant and ancestral species, and models intraspecies polymorphism and incomplete lineage sorting (Heled & Drummond 2010). We used the European 138-collection datasets of the three loci, and a priori assigned specimens to species based on the results of the multilocus genealogical approach. We estimated the models best fitting the three loci with MrModeltest v2.3 (Nylander 2004). We used an uncorrelated relaxed lognormal clock model, the Yule process as species tree prior, and the Piecewise linear and constant root model as population size model. Five independent MCMC analyses were run for ten million generations (sampling 100 steps). Convergence and effective sample size statistics (ESS) of the runs were examined with Tracer v1.5 (Rambaut & Drummond 2007). A burn in sample of 20 % was excluded, trees were annotated with LogCombiner v1.6.2 (Drummond & Rambaut 2007) and a summary tree was generated with TreeAnnotator v1.6.2 (Drummond & Rambaut 2007).

As a second species delimitation approach, Bayesian species delimitation was performed using Bayesian Phylogenetics and Phylogeography, BP&P v2.0 (Yang & Rannala 2010) with the 138-collection European datasets of the three loci. Because the prior distributions on the ancestral population size and root age can affect the posterior probabilities for models (Yang & Rannala 2010), we considered the three different combinations of priors proposed by Leache & Fujita (2010): (1) relatively large ancestral population sizes and deep divergences: $\theta \sim G$ (1,10) and $\tau \sim G$ (1,10), (2) relatively small ancestral population sizes and shallow divergences $\theta \sim G$ (2,2000) and $\tau \sim G$ (2,2000), and (3) relatively large ancestral population sizes and relatively shallow divergences $\theta \sim G$ (1,10) and $\tau \sim G$ (2,2000). The species tree resulting from the *BEAST analysis was used as guide tree. Analyses were run using algorithm 0 with several values for the fine-tune parameter (5, 10, and 20) and different starting species trees. Each species delimitation model was assigned equal prior probability. We ran each analysis twice with different starting seeds for 100 000 generations (sampling interval of five) and a burn-in of 50 000.

The numbering of the species is a continuation of the species numbers in the papers about the *L. volemus* group in northern Thailand (species 1–18, Van de Putte *et al.* 2010) and Sikkim (species 19–24, Van de Putte *et al.* 2012).

Morphological analysis

Macromorphological characteristics are based on fresh material and document all aspects of size, shape, texture, colour, colour changes, latex features, odour, taste, and macrochemical reactions with $FeSO_4$ and KOH (10 %). Colours were described in daylight conditions using the colour codes of the Methuen book of colours (Kornerup & Wanscher 1978) and the Munsell Soil Colour Charts (2000). The lamellar density is given as the sum of the lamellae and lamellulae per centimeter, measured at 1 cm distance from the cap margin ($L + l\ cm^{-1}$). Micromorphological characters were documented from the exsiccata of mostly mature specimens. Few immature specimens were available, and those studied are indicated in Suppl. data Table 2. For terminology we refer to Vellinga (1988) and Verbeken (1996), and specifically for pileipellis structures to Verbeken (1998). Spores were observed in Melzer's reagent. Elements of pileipellis, hymenium, and trama were observed in Congo-Red in ammonia. For each collection, a minimum of 20 spores in side view was measured, excluding the ornamentation. Spore measurements are given as described in Nuytinck & Verbeken (2005). Mean values are given separately. Spore measurements and drawings were made with a Zeiss Axioscop 2 microscope and drawing tube at 6000× magnification. All elements from the hymenium and pileipellis were measured halfway the pileus radius. Basidia were measured excluding the sterigmata. Pileipellis hairs were measured on scalps; drawings of the pileipellis were made from sections. Hymenial elements and pileipellis structures were illustrated with an Olympus CX31 microscope and drawing tube at 1600× magnification. Morphological observations and drawings were made by the first author. First, collections were assigned to phylogenetic species as described above (see 2.3). For each phylogenetic species, a random selection of collections was examined microscopically. Since 138 European collections are included in the molecular analyses, studying a significant number of collections was required in order to determine reliable diagnostics. Those characters that showed significant variability and overlap after examining about ten collections per species were not examined for any additional collection. Those characters that showed to be diagnostic were examined for a significant amount of additional collections in order to assure the diagnostic reliability.

Scanning Electron Microscope photographs of the spores are generated by a JEOL 5800 LV Scanning Electron Microscope at 25 kV. Beforehand, the material was critical point dried, mounted on double-sided adhesive tape, attached to an object table and covered with a thin gold layer of about 15 nm.

Results

Sequence data

A total of 436 new sequences, of which 158 ITS sequences, 138 LSU sequences, and 140 *rpb2* sequences was generated. Missing data are indicated in Table 1. The obtained ITS sequences range from 505 to 698 bp in length, the LSU sequences from 691 to 960 bp, and the *rpb2* sequences from 375 to 840 bp. The *rpb2* intron was alignable with minimal difficulty and therefore not excluded from further analyses.

DNA-based species delimitation

Comparison of the ITS, LSU, and *rpb2* ML and BI 187-collection gene trees resulted in the recognition of three European phylogenetic species (clades 25–27), which are not conspecific with North American taxa. Neither European, nor North American clades form monophyletic clades according to continent. All three European clades are monophyletic and well supported in the ITS and *rpb2* gene trees, but clade 27 is nested within North-American clade (designated as 'B') in the *rpb2* gene tree (Suppl. data Figs 1 and 2). Clades 26 and 27 are monophyletic with high support in the LSU gene tree, but clade 26 and North American clade (designated as 'B') are nested in a paraphyletic clade 25 (Suppl. data Fig 3).

Although delimiting North American species was beyond the scope of this study, comparison of the ITS, LSU, and *rpb2* ML and BI phylogenies indicate the presence of multiple species within the North American '*Lactifluus volemus*' and *Lactifluus corrugis* (Suppl. data Figs 1–3). More sampling is needed to detect clear species boundaries. Fig 2 shows the ML tree of the 187-collection concatenated dataset.

The hierarchical Bayesian model implemented in BEAST 1.6.2, using the 138-collection dataset and the three European clades as pre-defined species, resulted in a maximal supported species tree with clade 25 as basal taxon (see Fig 3 for tree topology).

The Bayesian species delimitation supported this species tree with maximal speciation probabilities of 1.0 on all nodes (Fig 3). Different fine-tuning parameters, starting trees, or prior distributions for θ and τ did not affect the outcome of the analysis.

Morphological diagnostics

After examining basidiospores, pleurolamprocystidia, cheilo(lampro)cystidia, pseudocystidia, stipitipellis hairs, and basidia of several collections from all three clades, it was clear that none of these characters have a diagnostic value due to significant variability or overlap in sizes. Concerning pileipellis hairs, however, we soon noted a difference in maximum hair length. Considering this might be one of few (or the only) diagnostic character(s), we measured maximum hair lengths of all but one collection from clade 27, and nearly 60 % of the collections from clades 25 and 26 (Suppl. data Table 2). Cap colours of collections with macroscopical notes and/or pictures are also indicated in Suppl. data Table 2. Considering that most of the collections are loans from different herbaria, little information on cap colour is available. Based on our current data, rather dark, reddish-brown cap colours occur in both clades 25 and 27 (Figs 4 and 12; Suppl. data Table 2). Clade 27, however, can be easily distinguished from clade 25 by its short, erect pileipellis hairs. Most hairs measure around 30–40 μm, a minority can measure up to around 55 μm, or in some collections up to 60(–65) μm. Both clades 25 and 26 have distinctly longer pileipellis hairs. In all measured collections, pileipellis hair lengths of around 60–70 μm are very common. The maximum length does differ considerably between specimens of the same clade – with some specimens having hairs of up to 125 μm, while others having hairs of up to 75 μm (Suppl. data Table 2). Only cap colour – observed in mature specimens – allows further discrimination between clades 25 and 26. As far as known, pale yellowish-brown colours (Fig 7) always belong to clade 26, while fruiting bodies with darker brown or reddish-brown colours (Fig 12) – and long pileipellis hairs – correspond to clade 25 (Suppl. data Table 2). We must note, however, that intermediary cap colours occur in both clades as well (Suppl. data Table 2), and that unambiguous allocation of these forms to either clade 25 or clade 26 is not possible without corroboration of molecular data. See also the ‘who is who’ test in Fig 14.

Taxonomy

Lactifluus volemus has not been typified, but in the protologue, Fries (1821) refers to two iconographic references, of which one is a plate from Ellrodt (1800): *Agaricus lactifluus. vulgo. Ellr. t. VI*. This plate contains hand-coloured illustrations of several basidiocarps, in German referred to as ‘essbare Braetlinge’. ‘Brätling’ is the German name for *Lactifluus volemus*. Amongst these illustrations, 1.a. resembles *L. volemus* the most, and was chosen as lectotype. In addition, we designated an epitype from southern Sweden because it was Fries’s home territory and thus the environment of the type location of *L. volemus*. Although both clades 25 and 26 occur in southern Sweden, we consider clade 25 to be the authentic *L. volemus* because it best fits the general perception of the species, i.e. a brown to warm orange-brown mushroom. Clade 26 – morphologically very similar to *L. volemus* emend. – is described as *Lactifluus subvolemus*.

We only have cap colour information on three collections of clade 27. Whereas one collection has an orange-brown cap (AV 07-79), the other two collections (RW 1228 and KVP R 12-001, Fig 4) have a rather dark reddish-brown cap. Collection RW 1228 has also been originally identified as *L. volemus* var. *oedematopus*. The type-locality of *Agaricus oedematopus* Scopoli is ‘Carniola’, a historical region that comprised parts of what is now Slovenia (Scopoli 1772). We collected in this area, but unfortunately only found *L. subvolemus* and *L. volemus*. Since only 5 % of our sampled collections belong to clade 27, it appears to be rare. Rather than to originate a new name, we decided to use oedematopus – the only available European epithet left within *Lactifluus* section *Lactifluus* – for clade 27. Instead of delaying the indication of a type specimen due to missing collections from the type locality (Carniola), we chose a neotype from western Europe (Germany) for which a macroscopical description, pictures, the three markers (ITS, LSU, and *rpb2*), and CTAB material is available.

Lactifluus oedematopus (Scop.) Kuntze, Revis. gen. pl. II: 857. 1891 (Figs 4–6, 13A–B).

Original diagnosis: Pileus rufescens, lamellis albidis; stipite crasso, flavicante, longo.

Basionym: Agaricus oedematopus Scop., Fl. Carniolica, 2 Aufl., Bd. II: 453. 1772.

Neotype: Thüringen, Mühlhäuser Hardt forest, close to village Pöthen, N51°17′29″N E10°32′36″, calcareous soil, forest dominated by *Fagus* and *Quercus*, 27 August 2012, leg. Jochen Girwert, KVP R 12-001 (*neotype*, GENT) (***Neotypus hic designatus***).

Basidiocarps firm, medium-sized to large. *Pileus* 30–70 mm diam., convex to planoconvex or applanate with depressed center to infundibuliform; margin regular, first deflexed, then straight; surface dry, velutinous, slightly pruinose or not, sometimes slightly to distinctly cracked, not or only slightly rugose, then more distinctly so at the margin, dull to intense orange-brown (6D4-6D5, 7B8-7C8-7D7-7D8) to reddish-brown, brick (in between 7E8-7F8), locally paler and more yellowish-brown (6A8-6B8) or orange-brown (6D8), usually getting paler towards the margin, discolouring brown when bruised, sometimes with an obtuse papilla. *Lamellae* adnate with or without small tooth, pale yellowish to whitish cream (4A3-4A4-4A5), discolouring brown when damaged, moderately distant (8–10 L + l cm^{-1}), with numerous lamellulae of different length; no or few furcations present; edge smooth and concolorous. *Stipe* 25–70 × 10–20 mm, subcylindrical to tapering downwards or upwards, centrally attached; surface dry, velutinous, sometimes slightly longitudinally rugose, yellowish-brown to orange-brown (5A4-5B4, 5A7-5A8-5B7-5B8), gradually getting paler and more yellowish towards the top, discolouring brown when bruised. *Latex* abundant, sticky, white to slightly watery, slowly turning brownish on lamellae, sometimes also turning brown when isolated; taste mild and pleasant. *Context* thick and firm in both cap and stipe, solid in stipe, whitish, quickly turning brown after cut; smell and taste pleasant, mild to rather sweet. *Macrochemical reactions:* context and surface discolouring greyish green with $FeSO_4$

Basidiospores (6.9–)7.5–10.1(–10.3) × (6.3–)6.7–9.2 μm, avL × avW = 8.4–8.9 × 7.5–8.2, globose to subglobose (Q = 1.00–1.22), avQ = 1.08–1.11; ornamentation amyloid;

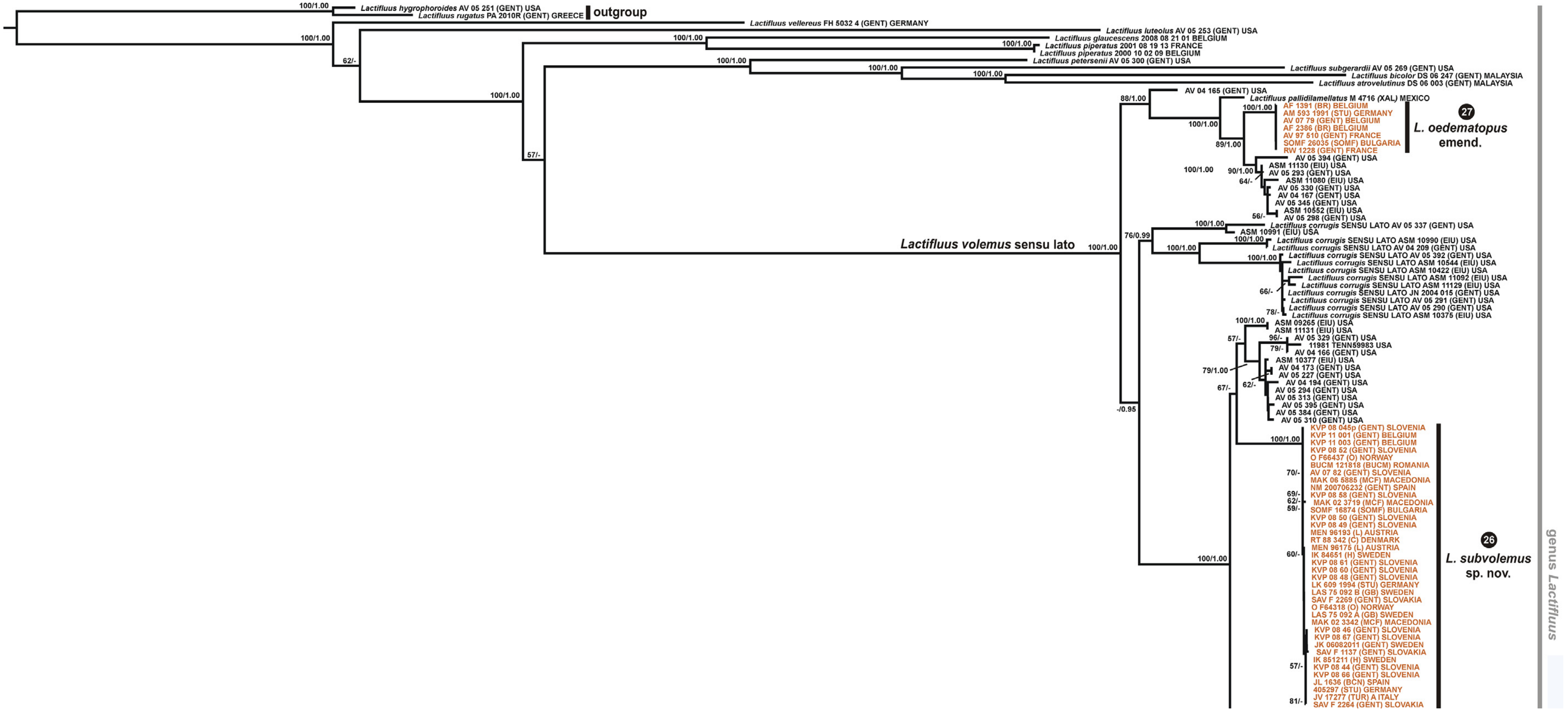

Fig 2 – ML tree based on the combined analysis of ITS, LSU, and *rpb2* sequence data. Numbers at nodes indicate branch support given as maximum likelihood bootstrap values before the bar and Bayesian posterior probabilities after the bar. Values are only shown if they exceed 50 and 0.95, respectively. More sampling is needed to determine robust species boundaries for the North American collections.

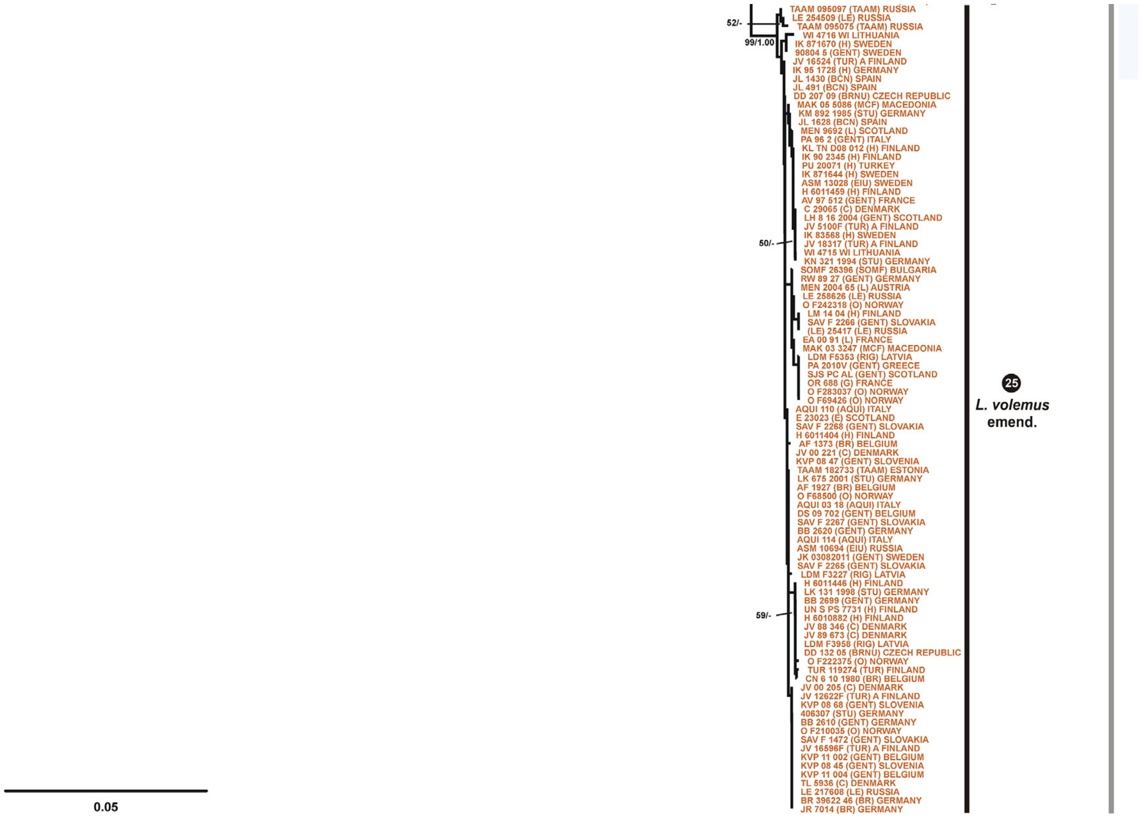

Fig 2 – (*continued*).

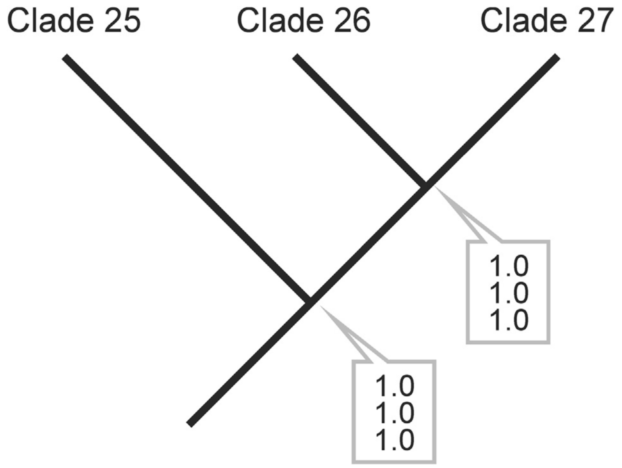

Fig 3 – The *BEAST analysis resulted in a species tree with clade 25 as basal taxon. This tree was used as the guide tree for Bayesian species delimitation. The speciation probabilities are provided for each node under each combination of priors for θ and τ: top, combination 1; middle, combination 2; bottom, combination 3.

ridges up to 1.5(1.7) μm high, forming a rather regular complete or incomplete reticulum, with isolated warts sometimes present; wall between the ridges locally strongly amyloid; plage either non-amyloid, or distally to completely amyloid. *Basidia* 45–72 × 10–15 μm, clavate, subclavate to subfusiform, 4-spored. *Pleurolamprocystidia* 50–120 × 7–11 μm, very abundant, thick-walled (2–5 μm), fusiform to subfusiform, occasionally septate, acuminate, emergent, arising from the subhymenium or trama. *Pleuropseudocystidia* scarce to moderately abundant, emergent or not, 3–8 μm diam., subcylindrical to somewhat irregularly shaped; apex obtuse, often capitate, occasionally branched. *Lamella-edge* partially fertile, composed of basidia and cheilocystidia. *Cheilocystidia* 25–100 × 4–8 μm, fusiform, subfusiform, obclavate to somewhat irregularly shaped, with margin often undulating, acuminate or with obtuse apex, thin-walled and hyaline to thick-walled (0.5–3 μm). *Hymenophoral trama* cellular, with abundant lactifers and rosettes of sphaerocytes. *Pileipellis* a lampropalisade; subpellis 70–120 μm thick, composed of rounded to elongated or irregularly shaped, thick-walled cells of 5–45 × 5–30 μm; terminal elements 10–60 × 3–10 μm, thick-walled (0.5–1.5 μm), subcylindrical to subfusiform with tapering apices, acuminate or obtuse apex, margin often undulating, often septate. *Stipitipellis* a lampropalisade; terminal elements up to 70(–80) μm, in some collections up to 120 μm.

Fig 4 – Basidiocarps *L. oedematopus*: (A) (*neotype*), (B) (RW 1228, aquarelle O. Van de Kerckhove).

Specimens examined: **Belgium:** Luxemburg province, Villers-sur-Semois, forest of Brossart, N49°41′52.53″ E5°34′40.75″, 12 August 1991, leg. A. Fraiture, AF 1391 (BR); Wellin, Chanly, close to stream Le Glan, N50°4′60″ E5°7′0″, 19 July 2007, leg. A. Verbeken, AV 07–79 (GENT) – **France:** Lorraine National Park, forest of Reine, N48°47′31.21″ E5°47′11.85″, mixed deciduous forest, 22 July 1997, leg. A. Verbeken, AV 97–510 (Gent); Ibidem, N49°29′24.69″ E5°2′21.59″, mixed deciduous forest on rich soil with *Carpinus*, *Fagus sylvatica*, *Betula pendula*, *Crataegus*, *Populus tremula*, 4 August 1997, leg. R. Walleyn, RW 1228 (GENT) – **Germany:** Württemberg, Nürtingen, Tiefenbachtal, N48°38′2.85″ E9°18′17.83″, deciduous forest with scattered coniferous trees, 5 August 1989, leg. A. Müller, AM 593/1991 (STU). Thüringen, Mühlhäuser Hardt forest, close to village Pöthen, N51°17′29″N E10°32′36″, calcareous soil, forest dominated by *Fagus* and *Quercus*, 27 August 2012, leg. Jochen Girwert, KVP R 12-001 (*neotype*, GENT) – **Slovakia:** Jabloňovce village, Štiavnické vrchy Mts., N48°20′29,1″ E18°47′26,5″, with *Carpinus*, *Quercus cerris*, 8 September 2014, leg. F. Hampe, EDC 14–519 (GENT).

Note: after finalizing the manuscript, collection KVP R 12-001 was found in Germany. Due to its dark reddish-brown cap and distinctly short pileipellis, the collection could be very easily identified as *L. oedematopus*. To confirm this morphology-based identification, we performed three additional ML analyses as described in 2.3. In both the ITS, LSU, and *rpb2* ML phylogenies, collection KVP R 12-001 indeed falls within *L. oedematopus*. The species remains highly supported as well (ITS/100 BS, LSU/94 BS, *rpb2*/94 BS; results not shown). Another collection with reddish-brown cap colour and distinctly short pileipellis hairs, EDC 14-519 (Slovakia) was also found later. A BLAST search (Altschul *et al.* 1990) of ITS confirmed its identification as *L. oedematopus* (99 %–100 % identity). The German collection KVP R 12-001 was chosen as neotype because it is the only collection with a macromorphological description and photographs, and of which tissue has been stored on CTAB.

Lactifluus subvolemus Van de Putte & Verbeken **sp. nov.** (Figs 7–9, 13C–D)

Mycobank No.: MB814951.

Etymology: contraction of *sub* and *volemus*, referring to its close morphological affinity with *Lactifluus volemus* emend.

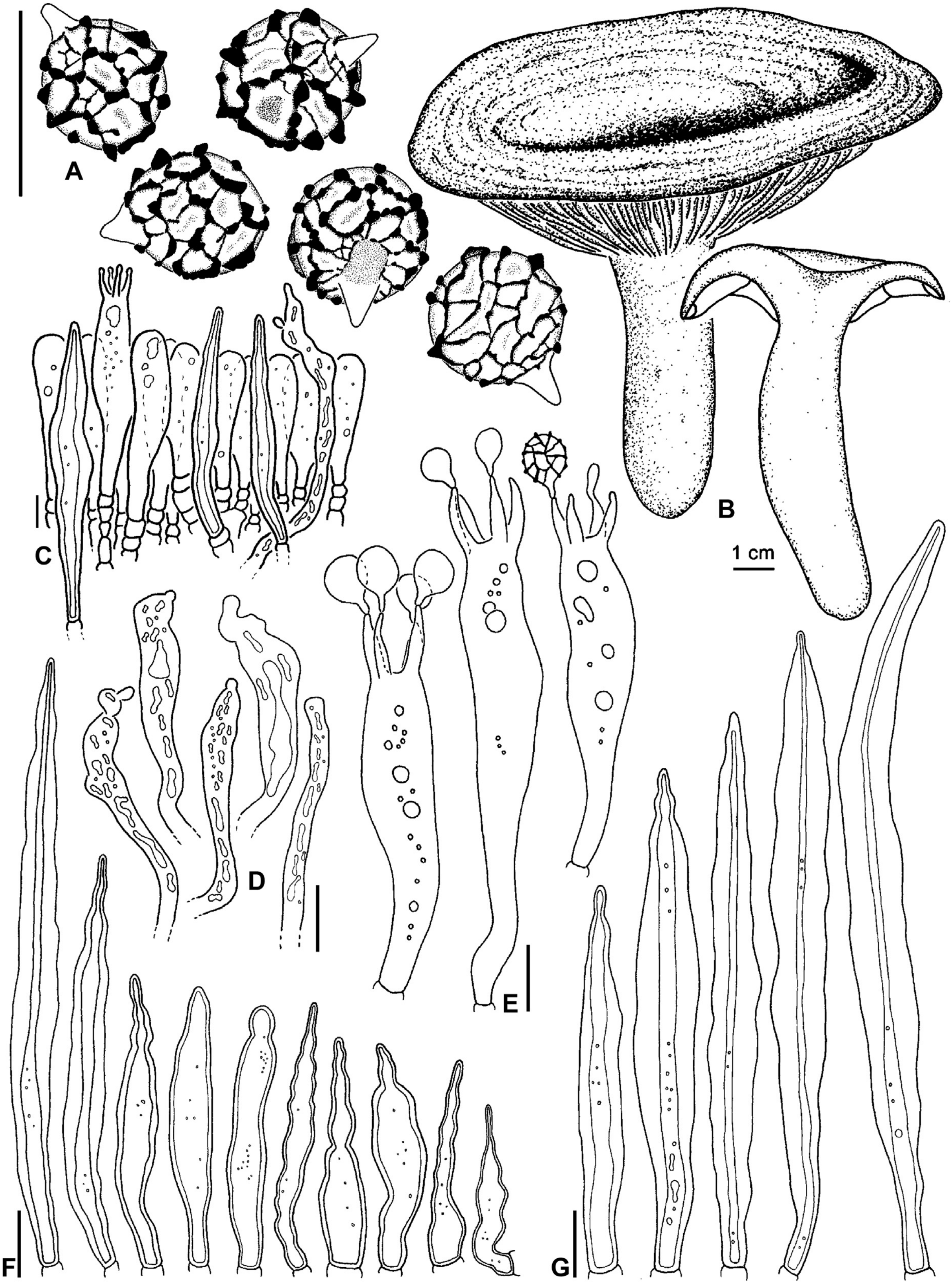

Fig 5 – *L. oedematopus*: (A) basidiospores (RW 1228, AF 1391), (B) basidiocarps (RW 1228, AV 07-79), (C) section through hymenium (RW 1228), (D) pseudopleurocystidia (AF 1391), (E) basidia (AF 1391), (F) cheilocystidia (RW 1228, AV 97-510), (G) pleurolamprocystidia (RW 1228, AF 1391, AV 97-510) (scale bar = 10 μm, unless otherwise indicated).

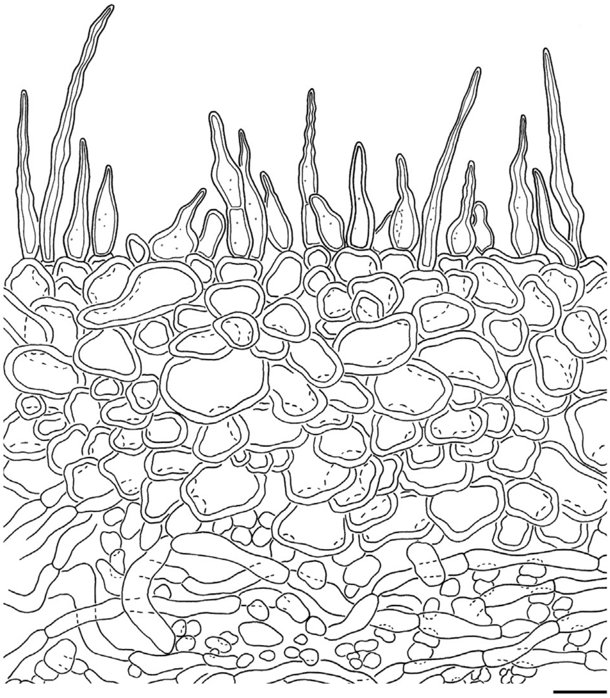

Fig 6 – *L. oedematopus*: section through pileipellis (RW 1228) (scale bar = 10 μm).

A Lactifluo volemo differt in pileo pallidiore, pallide flavobrunneo. A Lactifluo oedematopo differt in cellulis terminalibus pileipellis longis 15–100(125) × 2.5–6 μm.

Holotype: Slovenia, Carniola traditional region, Podutik, forest around hostel Kačji log, N46°4′41.95″ E14°26′25.27″, forest dominated by *F. sylvatica*, also *Quercus* and *Picea*, 23 August 2008, leg. K. Van de Putte & A. Piltaver, KVP 08–49 (holotypus, GENT)

Pileus 40–110 mm diam., planoconvex with slightly depressed center to infundibuliform; margin involute when young, regular when mature; surface dry, sometimes slightly rugulose in the center and at the margin, distinctly mat and velvety, sometimes velutinous or pruinose, sometimes locally finely cracked; young specimens pale brownish-yellow (5A4-5) to dark orange-brown (6C7), darkening towards the center; fully grown specimens sand coloured (10 YR 8/6 but paler, or 4A3-4 to 5A3) to pale brownish-yellow with or without orange tinge (7.5YR7/8, 5BC5-6); center often with slightly darker and more brownish (7.5YR7/8-7.5YR6/8-7E7-7F7) patches or concentric zones, discolouring brown when bruised, sometimes with small papilla. *Lamellae* adnate to subdecurrent, pale whitish cream, discolouring brown when damaged (6D4-6D5-6D6-6F5–6F6), moderately close to close (11–20 L + l cm^{-1}), with numerous lamellulae of different length; no or few furcations; edge smooth and concolourous. *Stipe* 35–75 × 10–25 mm, subcylindrical to slightly tapering downwards, centrally attached; surface dry, smooth, sometimes slightly pruinose, orange-brown or concolorous with cap margin, gradually getting paler and more yellowish towards the top, discolouring brown when bruised. *Latex* very abundant, sticky, white, and slightly watery, unchanging when isolated, very slowly turning brown to greyish-brown on lamellae; taste mild. *Context* very firm, solid to stuffed in stipe, whitish, quickly turning ochraceous-brown with greyish tinge (5B3-5C3-5C4-5C6-5D4-6D4) after cut; taste mild; smell fragrant to fishy. *Macrochemical reactions:* context unchanging or almost immediately turning pale yellow or yellowish-orange (4A6) with KOH 10 %; turning pale to dark greyish-green (1F4-4D3-4E4-4F4) with $FeSO_4$ after around 10 s or a few minutes.

Basidiospores (7.7–)7.9–11.6 × (7.2–)7.3–11.1 μm, avL × avW = 9.0–10.1 × 8.2–9.6, globose to subglobose (Q = 1.00–1.24), avQ = 1.06–1.12; ornamentation amyloid; ridges up to 1.6(–1.8) μm high, forming regular to slightly irregular complete or incomplete reticulum, with isolated warts sometimes present; wall between the ridges locally strongly amyloid; plage mostly distally or completely amyloid. *Basidia* 45–75 × 9–14 μm, clavate, subclavate to subfusiform, 4-spored. *Pleurolamprocystidia* 55–145 × (6–)7–12(–13) μm, very abundant, thick-walled [(1.5)–5(–6) μm], fusiform to subfusiform, occasionally septate, acuminate, emergent, arising from the subhymenium or trama. *Pleuropseudocystidia* scarce to very abundant, emergent or not, 2.5–8 μm diam., subcylindrical to tortuous; apex obtuse, often lobed and capitate. *Lamella-edge* partially fertile, composed of basidia and cheilocystidia. *Cheilocystidia* 20–85(–100) × 6–10 μm, fusiform, subfusiform, obclavate to somewhat irregularly shaped, margin often undulating, acuminate or with obtuse apex, thin-walled and hyaline to thick-walled (0.5–3 μm). *Hymenophoral trama* cellular, with abundant lactifers and rosettes of sphaerocytes. *Pileipellis* a lampropalisade; subpellis 65–150 μm thick, composed of rounded to elongated or irregularly shaped, thick-walled cells of 3–50 × 3–40 μm; terminal elements 15–100(125) × 2.5–6 μm, thick-walled (0.5–1.5 μm), subcylindrical to subfusiform with tapering apices, acuminate or obtuse apex, margin often undulating, often septate. *Stipitipellis* a lampropalisade; terminal elements up to 110(–150) μm.

Specimens examined: **Austria:** Steiermark, Eichberg, Eibiswald, N46°41′12″ E15°14′50″, mixed forest with *F. sylvatica*, *Quercus*, *Castanea sativa*, and *Picea abies*, on acid soil, 16 September 1996, leg. M.E. Noordeloos, MEN 96175 (L) – **Belgium:** Namen province, forest of Frasnes, N50°5′36.81″ E4°33′21.89″, 12 August 2010, leg. O. Berteloot, B. Goemare, A. Verbeken, KVP 11-001 (GENT); Ibidem, leg. O. Berteloot, B. Goemare, A. Verbeken, KVP 11-003 (GENT) – **Germany:** Nürtingen, Tiefenbachtal, N48°38′2.85″ E9°18′17.83″, 9 August 1989, 405297 (STU). Bayern, Zwieselerwaldhaus, Schrödelhütte, N49°5′21.98″ E13°14′47.29″, coniferous forest dominated by *P. abies*, 5 September 1994, leg. L. Krieglsteiner, LK 609/1994 (STU) – **Italy:** Veneto, Trichiana commune, west of Melere, Col di Pera, N46°4′26.84″ E12°7′59.44″, herb-rich deciduous wood with meadows, with *C. sativa*, *Corylus avellana*, *F. sylvatica*, *Carpinus betulus*, *Quercus*, *Larix decidua*, *B. pendula*, 28 July 2001, leg. J. Vauras, JV 17277 (TUR-A) – **Macedonia:** Pelister mountain, Brajchino village, N40°54′8.51″ E21°9′36.58″, *F. sylvatica* forest, 15 August 2002, leg. M. Karadelev, MAK 02/3719 (MCF) – **Slovakia:** Štiavnické vrchy mountains, Krupina village, Mäsiarsky bok Natural reserve, N48°20′33.27″ E19°5′5.49″, *Fagus, Carpinus*, 25 July 2008, leg. S. Adamčik, SAV F-2269 (GENT) – **Slovenia:** Littoral traditional region, Tolmin, Senica Hill, N46°9′39.13″ E13°45′48.25″, acidophilous *Fagus* forest, 14 August 2007, leg. R. Walleyn & G. Podgornik,

Fig 7 – Basidiocarps of *L. subvolemus*: (A) (holotype), (B) (JK 06082011, photograph J. Kleine), (C) (KVP 08-066), (D) (SAV F-2269, photograph P. Marstad), (E) (MN 20070623.2, photograph M.A. Pérez-De-Gregorio), (F) (KVP 08-58).

AV 07-82 (GENT). Carniola traditional region, forest around Ljubljana airport, 21 August 2008, leg. A. Piltaver, KVP 08-44 (GENT); Carniola traditional region, Podutik, forest around hostel Kačji log, N46°4′41.95″ E14°26′25.27″, forest dominated by *F. sylvatica*, also *Quercus* and coniferous trees, 23 August 2008, leg. K. Van de Putte & A. Piltaver, KVP 08-49 (GENT); Ibidem, 26 August 2008, leg. K. Van de Putte & Vida, KVP 08-60 (GENT) – **Spain:** Province de Girona, Comarca Ripollès, Torrent de Nevà, Toses, N42°19′13.62″ E2°0′57.87″, with *B. pendula*, *C. avellana*, *Quercus* sp., 7 September 1996, leg. J. Llisostella, JL 1636 (BCN); Province de Girona, comarca of Alt Empordà, Maçanet de Cabrenys, N42°23′3.12″ E2°45′16.89″, with *C. sativa*, 23 June 2007, leg. N. Macau, NM 20070623.2 (GENT) – **Sweden:** Skane, Skanes Fagerhults kommun, Skanes Värsjö, by the road E4, by the piknik site at the road crossing to Vittsjö, N56°20′2.39″ E13°26′8.34″, mesic *Fagus* heath forest, no conifers, 19 September 1984, leg. I. Kytövuori, IK 84651 (H); Skane, Tomelilla kommun, east side of Fyledalen, by a small road south of Röddinge, N55°34′42.65″ E13°50′23.54″, sloping, partly cleared, bushy *Fagus* forest with some *Quercus* and *Ulmus*, rich soil, 19 September 1985, leg. I. Kytövuori, IK 851211 (H).

Lactifluus volemus (Fr.: Fr.) Kuntze, Revis. Gen. Pl. 2: 857. 1891 (Figs 10–12, 13E–F).

Original diagnosis: Magnus, dulcis, pileo obtuso sicco laevi nitido fulvo-expallente, lamellis albo-lutescentibus, stipite solido obeso.

Basionym: *Agaricus volemus* Fr.: Fr., Syst. Mycol. 1: 69. 1821.

Lectotype: Ellrodt, Schwamm-pomona, pl. VI, 1.a., 1800.

Epitype: Sweden, Blekinge, west of Bälganet, on the side of a forest road in a pure *P. abies* plantation on acid soil, 8 March 2011, leg. J. Kleine 03082011 (GENT) (***Neotypus hic designatus***).

Pileus 50–95 mm diam., convex with involute margin when young, applanate to planoconvex with depressed center and deflexed or straight margin when mature; surface dry, either smooth, rugulose or concentrically wrinkled towards the margin, distinctly mat and velvety impression, sometimes velutinous or pruinose, brown, orange-brown (6C5-6C6-6D5-6D6 or in between 5YR5/8-5YR6/8), to dark brown (7D6-7D7-7D8-7E8); center darker brown or reddish-brown (7D7-7E67E8-7F8-8E8 or paler); margin mostly paler and more yellowish-brown. *Lamellae* adnate, subdecurrent to decurrent, pale cream to yellowish-cream (2A2), discolouring brown (5C5-

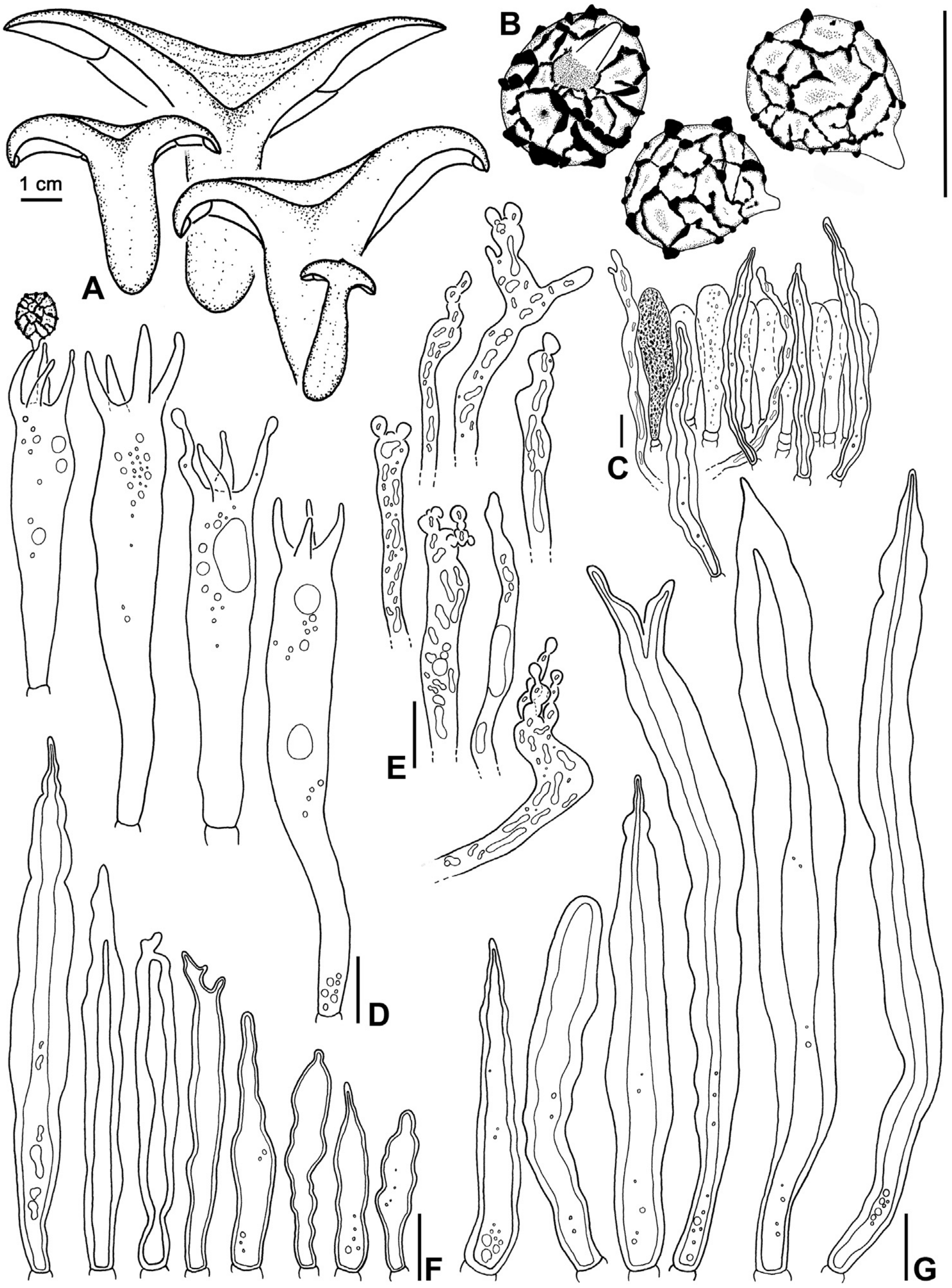

Fig 8 – *L. subvolemus*: (A) basidiocarps (holotype, KVP 08-046, KVP 11-003), (B) basidiospores (JL 1636, KVP 08-044), (C) section through hymenium (KVP 08-044), (D) basidia (AV 07-82, JL 1636, STU 405297), (E) pseudopleurocystidia (JV 17277, KVP 08-44, MEN 16193), (F) cheilocystidia (AV 07-82, LK 609/1994), (G) pleurolamprocystidia (AV 07-82, JL 1636, JV 17277, KVP 08-044, STU 405297) (scale bar = 10 μm, unless otherwise indicated).

6D4-6D6-6E4-6E6) when damaged, moderately close to close, with numerous lamellulae of different length; no, few or numerous furcations; edge smooth and concolourous. *Stipe* 35-85 × 17–33 mm, cylindrical, subcylindrical to slightly tapering downwards, centrally attached; surface dry, smooth, mat, sometimes pruinose, yellowish-cream, pale orange-brown to yellowish-brown (5A5-5B5), gradually getting paler and more yellowish towards the top and more orange towards the base. *Latex* very abundant, sticky, white, sometimes slightly watery, unchanging when isolated, unchanging or slowly turning brownish on lamellae; taste mild. *Context* very firm, thick in pileus, solid or stuffed in stipe, pale yellowish to white (2A2), quickly turning ochraceous-brown with greyish tinge (5C4–5C5) after cut; taste mild; smell fragrant to seafood or herring-like. *Macrochemical reactions:* context almost immediately turning yellowish (3A3-3A4-5B3) with KOH 10 %; immediate or after ± 10 s becoming greyish-green (3F3-3E4-4F3) with $FeSO_4$.

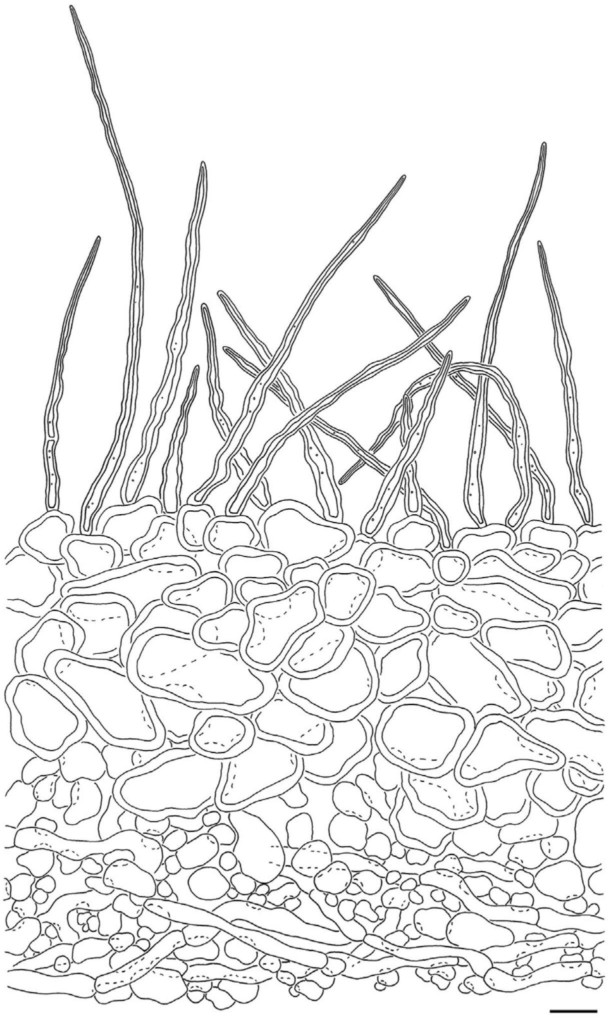

Fig 9 – *L. subvolemus*: section through pileipellis (KVP 08-044) (scale bar = 10 μm).

Basidiospores 7.7–11.3 × 7.1–10.3(–10.6) μm, avL × avW = 9.0–10.0 × 8.4–9.3, globose to subglobose (Q = 1.00–1.20), avQ = 1.07–1.10; ornamentation amyloid; ridges up to 1.5(–1.8) μm high, forming a regular complete or nearly complete reticulum, with isolated warts sometimes present; wall between the ridges locally strongly amyloid; plage mostly distally or completely amyloid. *Basidia* 55–75 × 10–14 μm, clavate, subclavate to subfusiform, 4-spored. *Pleurolamprocystidia* 55–145(160) × (6–)7–12 μm, very abundant, thick-walled 1.5–4.5 μm, typically fusiform to subfusiform, occasionally septate, mostly acuminate, emergent, arising from the subhymenium or trama. *Pleuropseudocystidia* scarce to very abundant, emergent or not, 3–7 μm diam., subcylindrical to tortuous; apex obtuse, often lobed and capitate. *Lamella-edge* partially fertile, composed of basidia and cheilocystidia. *Cheilocystidia* 20-115 × 5–8 μm, either fusiform to subfusiform or obclavate to somewhat irregularly shaped, margin often undulating, acuminate or with obtuse apex, thin-walled and hyaline to thick-walled (0.5–3 μm). *Hymenophoral trama* cellular, with abundant lactifers and rosettes of sphaerocytes. *Pileipellis* a lampropalisade; subpellis 70–130 μm thick, composed of rounded to elongated or irregularly shaped, thick-walled cells of 6–50 × 5–40 μm; terminal elements 15–100(125) × 2.5–5 μm, thick-walled (0.5–1.5 μm), mostly long and slender, subcylindrical to subfusiform with tapering apices, acuminate or obtuse apex, margin often undulating, often septate. *Stipitipellis* a lampropalisade; terminal elements up to 110(–150) μm.

Specimen examined: **Belgium** – 6 October 1980, leg. N. Cnops, CN 6/10/1980 (BR). Namen province, forest of Frasnes, N50°5′36.81″ E4°33′21.89″, coniferous forest, 19 June 2005, leg. D. Stubbe, DS 09–702 (GENT); ibidem, 12 August 2010, leg. O. Berteloot, B. Goemare, A. Verbeken, KVP 11-002 (GENT); ibidem, leg. O. Berteloot, B. Goemare, A. Verbeken, KVP 11-004 (GENT) – **Czech Republic:** Moravia, Blansko-Arnostov (distr. Blansko), national nature reserve Výv̀ìry Punkvy, Punkevní údolí valley, on southern slope above the dam, N49°21′42.0″ E16°41′23.1″, in dry *Pinus*-*Quercus* forest on rocky slope, 22 July 2005, leg. D. Dvorak, DD 132/05 (BRNU); Moravia, Brno-Øeèkovice (distr. Brno-mìsto), site called ‘Rakovec’ ca 1 km east/southeast from Brno-Øeèkovice train station N49°14′49.8″ E 16°36′09.9″, thermophilous *Quercus* forest on plain surface (with *Luzula* sp., *Anthericum ramosum*, *Trifolium alpestre*, *Brachypodium pinnatum* etc.), 11 July 2009, leg. D. Dvorak, DD 207/09 (BRNU) – **Denmark:** Kolding, Marielund, west side of the lake, N55°31′22.24″ E 9°24′46.65″, with *F. sylvatica*, *Quercus* sp. and *C. betulus*, 17 August 2000, leg. L. Vesterholt & J. Vesterholt, JV 00–205 (C). Hestedamshoved, south of Jelling Skov, N55°44′51.10″ E9°25′58.63″, in grassland under *F. sylvatica*, 20 August 2000, leg. L. Vesterholt, JV 00-221 (C). Jylland, Fjerritslev, Tingskoven, N57°5′8.13″ E9°23′59.55″, under *Picea abies*, 11 August 2000, leg. T. Læssøe & J. Læssøe, TL 5936 (C) – **Estonia:** Tartu, Meeksi, Järvselja, forest comp., N58°15′43.61″ E27°19′38.82″, eutrophic boreo-nemoral forest type, 30 August 2000, leg. L. Tedersoo, TAAM 182733 (TAAM) – **Finland:** Laatokan Karjala, Parikkala, Oronmylly, N61°33′0.64″ E29°30′12.12″, rocky heath by side of dryish *P. abies* forest, 15 September 2009, leg. T. Jaakkonen, H 6010882 (H). Uusimaa, Espoo, Hannusjärvi, W side of the lake, N60°9′7.97″ E24°41′4.98″, steep slope, near *B. pendula* and *Pinus sylvestris*, 25 Augustus 2004, leg. A. Lehtinen, H 6011446 (H); Uusimaa, Kirkkonummi commune, Vols, east side of the lake Nydalsviken, between Sikanalle and Salmudden, N60°10′10.54″ E24°23′59.84″, grass-herb spruce forest with hardwood trees and bushes, 2 September 1990, leg. I. Kytövuori, IK 90-2345 (H); Uusimaa, Espoo, Luukki, north of the pond Hauklampi, nature reserve area, N60°18′58.70″ E24°41′32.27″, submesic to mesic, herb-rich *Picea Abies* forest with some *B. pendula* and *P. tremula*, 22 August 2008, leg. K. Liimatainen & T. Niskanen, KL & TN D08-012 (H). Varsinais-Suomi, Kaarina, Rauhalinna, estate park, N60°24′21″ E22°24′26″, Herb-rich forest with *Quercus robur*, *C. avellana*, *B. pendula*, *Tilia cordata*, *P. abies*, by the path, grassy site, 11 August 1997, leg. J. Vauras, JV 12622F (TUR-A); Varsinais-Suomi, Turku, Ruissalo Island, nature reserve, north side of Hekkaniitty, N60°25′27.59″ E22°5′23.90″, deciduous forest with *Q. robur*, *C. avellana*, *T. cordata*, *B. pendula*, *P. abies*, *P. sylvestris*, near forest margin, 17 August 2000, leg. J. Vauras, JV 16596F (TUR-A); Varsinais-Suomi, Turku, Ruissalo Island, 200 m of Villa Roma, N60°25′27.59″ E22°5′23.90″, herb-rich deciduous

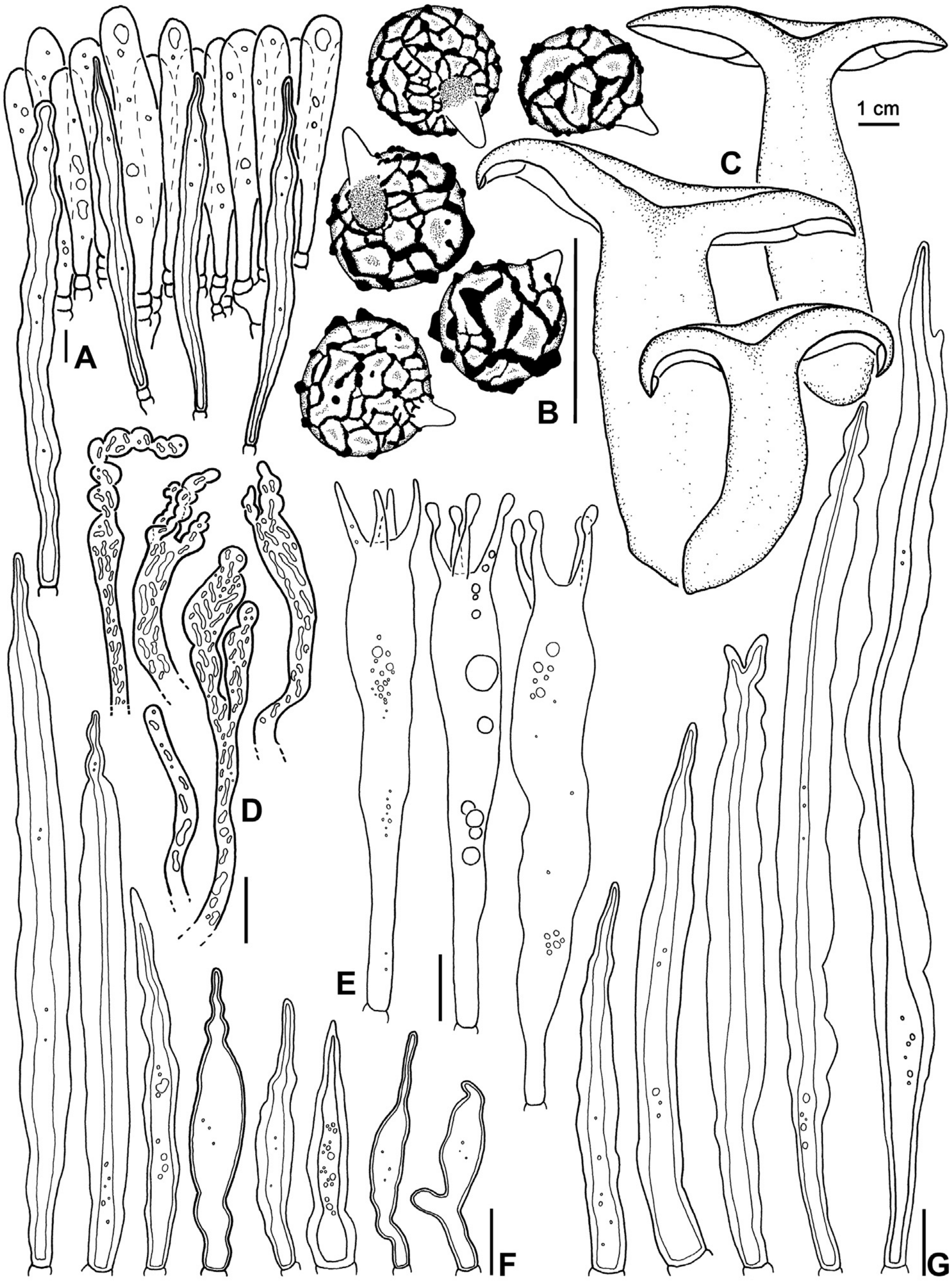

Fig 10 – *L. volemus*: (A) section through hymenium (JV 5100F), (B) basidiospores (AV 97-512, MEN 9692, KVP 08-086, O 24318), (C) basidiocarps (KVP 08-045, KVP 11-002), (D) pseudopleurocystidia (BR 39622-46, TL 5936), (E) basidia (JV 00-221, JV 5100F), (F) cheilocystidia (SJS-PC-AL), (G) pleurolamprocystidia (BR 39622-46, JV 00-205, TL 5936, JV 00-221, DS 09-702) (scale bar = 10 μm, unless otherwise indicated).

forest with *Q. robur*, *B. pendula*, *Salix caprea*, and *C. avellana*, 3 September 1990, leg. J. Vauras, JV 5100F (TUR-A). Etelä-Häme, Hämeenlinna, N side of lake Iso-Munakas, N61°1′10.72″ E24°28′4.53″, mixed forest, 9 August 2004, leg. M. Lahti, LM 14-04 (H) – **France:** Lorraine National Park, forest of Reine, N48°47′31.21″ E5°47′11.85″, mixed deciduous forest, 22 July 1997, leg. A. Verbeken, AV 97-512 (GENT). Vosges, Neige, col de Neige, N48°4′26.85″ E6°40′34.35″, *A. alba* forest, 19 October 2000, leg. E. Arnolds, EA 00-91 (L) – **Germany:** Würzbach, Buchentischwald, N50°27′55.15″ E11°33′11.83″, 26 August 1997, leg. Walther, 406307 (STU). Kopp-Eifel, N50°10′25.85″ E6°35′25.41″, under *Quercus*, 4 September 1987, leg. B. Buyck, BB 2610 (GENT). Birresborn, Eifel, N50°10′28.93″ E6°37′56.51″, forest with *Quercus* and *C. betulus*, 4 September 1987, leg. B. Buyck (GENT). Gees, N50°13′5.27″ E6°41′32.44″, under *F. sylvatica*, 30 August 1988, leg. B. Buyck, BB 2699 (GENT). Bayern, Rhön, southeast Schmalwasser, N50°20′16.61″ E10°2′37.57″, young forest dominated by *C. betulus*, on rocky,

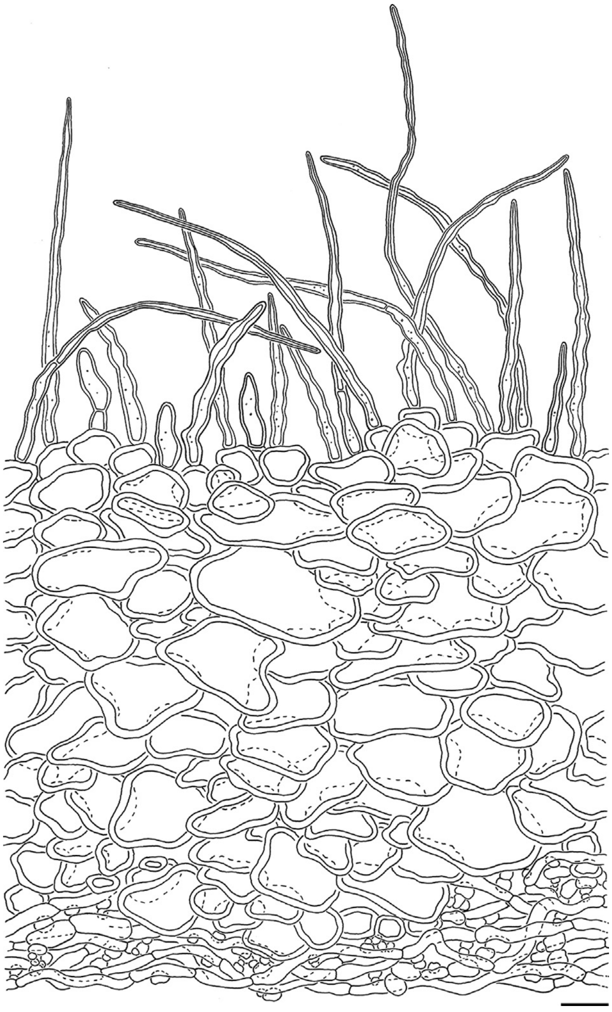

Fig 11 – *L. volemus*: section through pileipellis (JV16596F) (scale bar = 10 μm).

sandy soil, 12 September 2001, leg. L. Krieglsteiner, LK 675/2001 (STU). Eifel, Mürlenbach, N50°8′47.46″ E6°35′48.36″, 25 August 1992, leg. R. Walleyn, BR 39622-46 (GENT); Eifel, Mürlenbach, N50°8′47.46″ E6°35′48.36″, deciduous forest on rich soil, 27 August 1989, leg. R. Walleyn, RW 89/27 (GENT) – **Italy:** Lazio, Monte San. Biagio, N41°21′0″ E13°210″, 29 November 2003, leg. G. Lalli, AQUI 03/18 (AQUI) – **Latvia:** Tukums district, Valgums lake, N56°59′7.72″ E23°18′36.83″, 14 September 2001, leg. I. Dániele, LDM F3958 (RIG) – **Macedonia:** Pelister mountain, Slivnica village, N40°57′30.80″ E21°4′55.92″, *Quercus frainetto*-*Q. pubescens* forest, 20 June 2003, leg. G. Kost, MAK 03/3247 (MCF) – **Norway:** Akershus, ÅS, the forest directly west of Tusenfryd amusement park, N59°40′2.67″ E10°47′32.65″, 18 August 1999, leg. J. B. Jordal, O F242318 (O) – **Russia:** Krasnodar territory (West Caucasus), Apsheron district, close to Mezmay village, N44°11′47.81″ E39°58′10.85″, *Fagus orientalis*–*Abies sibirica* forest, under *A. sibirica*, 26 August 2000, leg. A.A. Kiyashko, LE 254509 (LE). Sverdlovskaya region (Middle Urals), Visimskyi nature reserve, N57°28′39.60″ E58°38′55.98″, *P. sylvestris* forest, 4 September 2000, leg. L.V. Marina, LE 258626 (LE) – **Scotland:** Loch Tay, Killin, N56°31′25.60″ E4°8′9.90″, *Calamintho grandiflorae-Fagetum* ecosystem, in *F. sylvatica* forest, 28 August 1996, leg. M.E. Noordeloos, MEN 9692 (L). Cairngorm National Park, Graig Dhuls, N57°2′60″ E3°2′60″, on soil, with *B. pendula*, Autumn 2005, SJS-PC-AL (GENT) – **Slovakia:** Pohronsky Inovec mountains, 3 km northeast of Obyce village, Obyce, N48°26′40″ E18°28′40″, under *Quercus*, 24 July 2008, leg. S. Adamčik, SAV F-2265 (GENT). Štiavnické vrchy mountains, 4.5 km north of Nová Dedina village, Sovia dolina, near bridge, N48°19′40″ E18°29′10″, under *Quercus*, 21July 2008, leg. S. Adamčik, SAV F-2266 (GENT) – **Slovenia:** Carniola traditional region, forest around Ljubljana airport, mixed forest with *Abies*, *Fagus, Picea*, *Castanopsis*, on acid soil, 22 August 2008, leg. K. Van de Putte & A. Piltaver, KVP 08-47 (GENT); Carniola traditional region, Kovor, N46°20′44.21″ E14°16′36.84″, mixed forest, acid soil, 28 August 2008, leg. Božo Malovrh, KVP 08-68 (GENT) – **Spain:** Province of Barcelona, comarca Osona, Can Torrens, Rupit i Pruït, N42°1′36.33″ E2°27′56.55″, with *F. sylvatica*, *B. pendula*, *Quercus* sp., 23 September 1990, leg. J. Llisostella, JL 1430 (BCN). Province of Girona, comarca Selva, Mas Altaba, Maçanet de la Selva, N41°45′1.95″ E2°42′27.18″, with *Quercus suber*, 16 June 1985, leg. J. Llisostella, JL 491 (BCN) – **Sweden:** Östra Smaland, Oskarshamn commune, Södermöre commune, Halltorp parish, by the main road no 15, south of the crossing of Halltorp, at the large resting place, N56°29′46.96″ E16°5′52.87″, dryish pine heath forest with *Picea*, *Quercus* and some *Fagus,* 21 September 1987, leg. I. Kytövuori, IK 871644 (H); Östra Smaland, Oskarshamn commune, Misterhult parish, Virum, east side of the main road E66, at the communal boundary of Västervik, round the fields of manor, N57°33′21.81″ E16°29′26.47″, grass-herb *Quercus-Corylus* forest with other hardwood trees and open pastures, 22 September 1987, leg. I. Kytövuori, IK 871670 (H). Blekinge, west of Bälganet, N56°22′46.06″ E15°1′4.60″, on the side of a forest road in a pure *P. abies* plantation on acid soil, 3 August 2011, leg. J. Kleine, JK 03082011 (epitype, GENT) – **Turkey:** Bolu, Koru Motel, N40°43′3.66″ E31°29′45.52″, mixed forest with *Fagus* and *Abies*, on a gentle slope facing approximately to north, 30 August 1972, leg. Pertti Uotila, PU 20071 (H).

Key to the species

1a – Pileipellis hairs short, up to 60 μm ***Lactifluus oedematopus* emend.**

1b – Pileipellis hairs longer, up to 85–125 μm **2**

2a – Cap colour pale yellowish-brown ***Lactifluus subvolemus* sp. nov.**

2b – Cap colour darker brown or reddish-brown ***Lactifluus volemus* emend.**

Discussion

Species delimitation

Species are the fundamental units of biodiversity. Accurate species delimitation and identification is of paramount importance not only to systematists and taxonomists, but to a wide range of

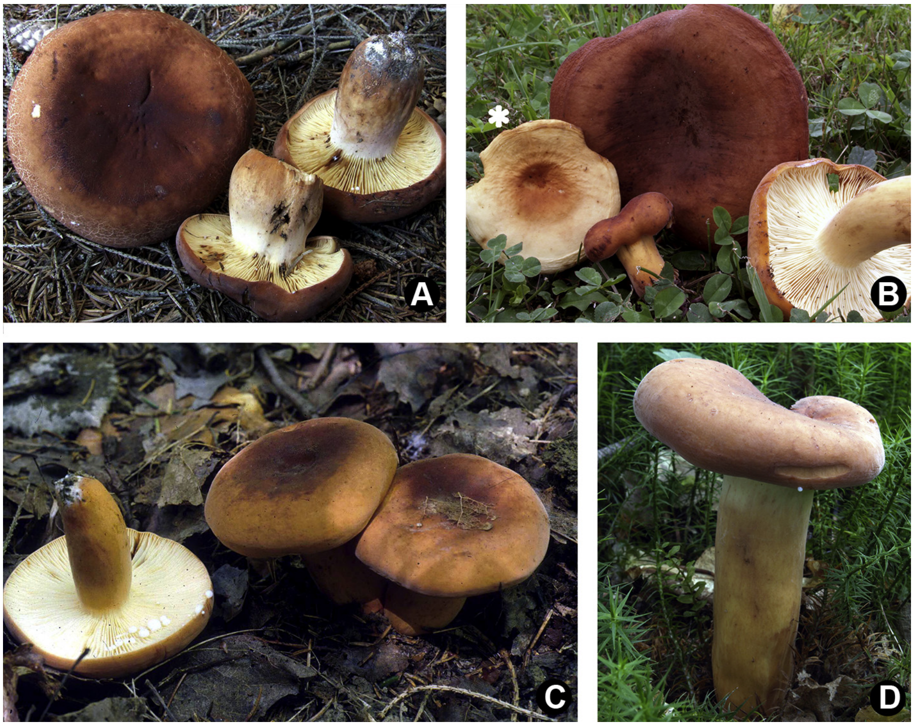

Fig 12 – Basidiocarps of *L. volemus*: (A) (epitype, photograph J. Kleine), (C) (RW 1187 photograph R. Walleyn), (D) (KVP 08-047). (B) A mixed collection of *L. volemus* (KVP 08-045) and *L. subvolemus* (KVP 08-045p, pale basidiocarp indicated with an asterisk).

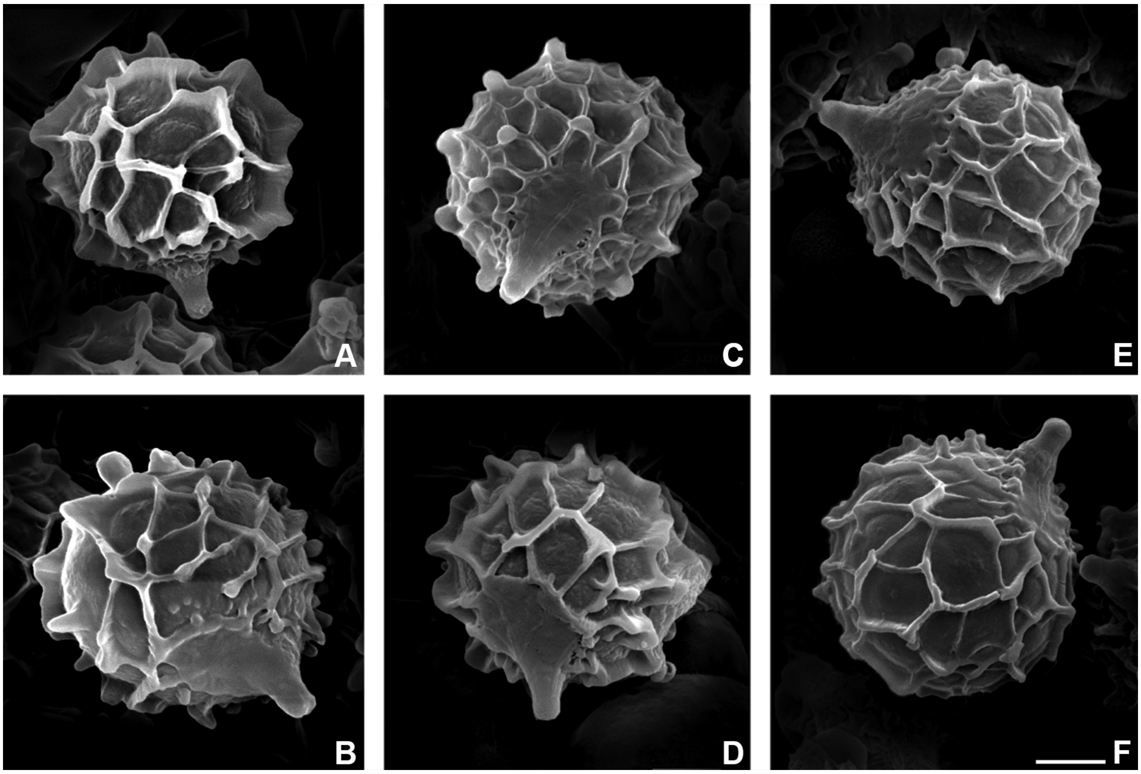

Fig 13 – SEM photographs of basidiospores. *L. oedematopus*: (A) (AF 1391), (B) (RW 1228), *L. subvolemus*: (C) (KVP 08-044), (D) (SAV F-2269), *L. volemus*: (E) (epitype), (F) (KVP 08-045) (scale bar = 2 μm).

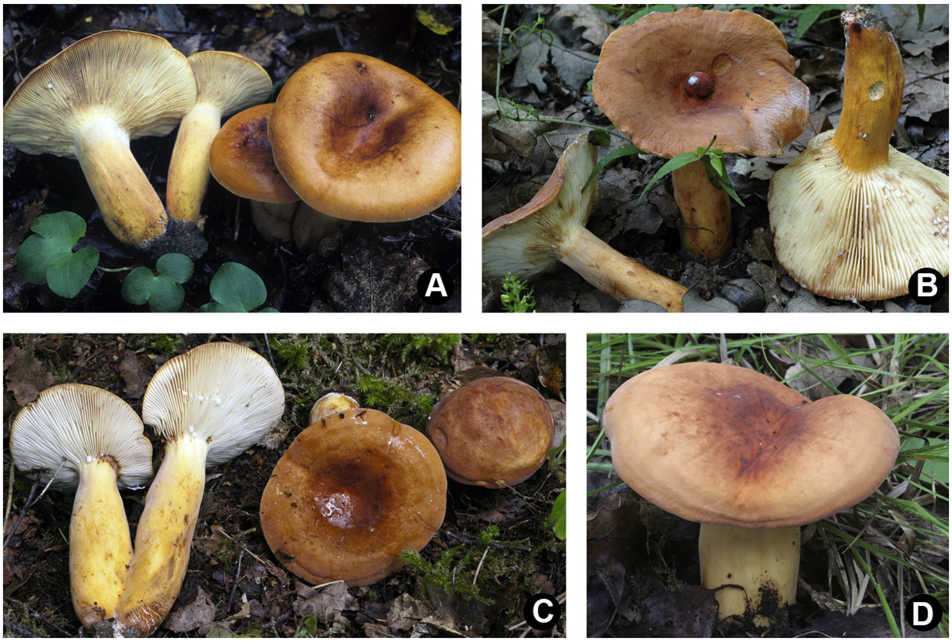

Fig 14 – Who is who? Intermediary colour forms of *L. subvolemus*: (A) (JV 12622F, photograph J. Vauras), (B) (SAV F-1137, photograph P. Marstad) and *L. volemus*: (C) (KVP 11-002, photograph O. Berteloot), (D) (DD 132/05, photograph D. Dvorak). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

scientists (e.g. ecologists, conservation biologists, evolutionary biologists) who need reliable systematic framework to build on. We aimed at following an integrative approach to determine robust species boundaries within *Lactifluus volemus*, one of the best-known milk caps of Europe. We had indications that this species is in fact a complex of cryptic species because (1) this is the case for close relatives of *L. volemus* in other continents: at least 18 species in Thailand (Van de Putte *et al.* 2010), six species in India (Van de Putte *et al.* 2012) and (2) there is a certain morphological variation that is reflected in the fact that several varieties and forms have been proposed in this group (e.g. Fries 1838; Maire 1937; Neuhoff 1956; Bouchet 1959; Heim 1962; Hesler & Smith 1979), though not accepted in most monographs or flora's (Basso 1999; Heilmann-Clausen *et al.* 1998; Lalli & Pacioni 1992).

Compliance of both tree-based and coalescence-based methods, as well as morphology, resulted in the recognition of three distinct European species. For two species, names were available and a lectotype/epitype and neotype, and extended new description is given: *L. volemus* and *Lactifluus oedematopus*. For the third species, a new name is proposed here: *L. subvolemus* Van de Putte & Verbeken.

Genealogical concordance of multiple unlinked loci has previously shown to provide strong evidence for the reproductive isolation of lineages (e.g. Van de Putte *et al.* 2010, 2012). Although comparison of the ITS, LSU and *rpb2* genealogies in this study allows recognition of three European species, the genealogies are not fully concordant at the species level. *Lactifluus subvolemus* and *L. oedematopus* are monophyletic in all genealogies, and hence valid species under the monophyly criterion of species delimitation. *Lactifluus volemus* is monophyletic in the ITS and *rpb2* genealogies, but paraphyletic in the LSU genealogy (Suppl. data Figs 1–3). In addition, *L. oedematopus* is nested in a paraphyletic eastern North American clade in the *rpb2* phylogeny (Suppl. data Fig 2).

The short branch lengths of our phylogenetic trees indicate rather recent speciation. Incomplete lineage sorting – a well-known cause of paraphyly in recently diverged species (Maddison & Knowles 2006) – could therefore be a plausible explanation for the observed genealogical incongruence. Over time, shared ancestral polymorphisms are randomly lost by genetic drift until each daughter species retains an allelic lineage in which intraspecific variation reflects post-speciation mutation (Funk & Omland 2003). In case of the LSU genealogy, sorting could have gone to completion in *L. subvolemus* and North American clade 'A' (Suppl. data Fig 3), but not in *L. volemus*, owing simply to insufficient time of isolation. We consider it is rather unlikely that these shared alleles stem from gene flow, since this would imply long-distance dispersal across the North Atlantic Ocean. Yet, this cannot be excluded for *L. volemus* and *L. subvolemus*, which have completely overlapping distributions.

The lack of topological resolution coupled with the low supported non-monophyly of *L. volemus* indicates that insufficient phylogenetic information in the LSU sequence data could have resulted in the observed pattern as well. Only 9.5 % of all LSU characters are parsimony informative, compared to 27.5 % for both ITS and *rpb2*. Although LSU has proven useful in species delimitation within the *L. volemus* clade (Shimono *et al.* 2007; Van de Putte *et al.* 2010; 2012), European LSU sequences exhibit a lower variability when compared to Sikkimese LSU sequences (15 % parsimony informative characters, Van de Putte *et al.* 2012), and could simply be too conserved to distinguish between species. In contrast, the *rpb2* paraphyly of the North American clade (Suppl. data Fig 2) is highly supported, and given the considerable variation in our *rpb2* sequence data, we consider inadequate phylogenetic information a rather unlikely cause.

Relaxed concerted evolution in ribosomal DNA, and presence of paralogous copies of duplicated genes may also lead to non-monophyly of species (Funk & Omland 2003). Both processes have been demonstrated in different groups of fungi (e.g. O'Donnell & Cigelnik 1997; Landvik *et al.* 2001; Rooney & Ward 2005; Opanowicz *et al.* 2006; Chang *et al.* 2008; Feau *et al.* 2011), and paralogous *rpb2* copies have been encountered in plants (Oxelman & Bremer 2000; Pfeil *et al.* 2004; Baker *et al.* 2011). Visual inspection of our sequence data, however, revealed no indirect evidence (e.g. high levels of ambiguous positions, frameshifts, indels, and stop codons; Funk & Omland 2003; Rouchka & Cha 2009) for relaxed concerted evolution or paralogous genes.

Overall, we do not consider the LSU paraphyly of *L. volemus* a major concern. In agreement with a growing number of systematists (e.g. Dettman *et al.* 2003; McKay *et al.* 2010), we argue that monophyly at each of the sampled loci is an unreasonably strict criterion for species delimitation, especially in cases of recent divergence. Since two out of three genealogies recovered the monophyly of *L. volemus*, we still consider it a valid species under the monophyly criterion. Yet, we recognize that this approach is rather subjective, and should be avoided when aiming to establish robust species boundaries. Considering the General Lineage Concept of species (de Queiroz 2007), however, monophyly at a sample of loci is just one of the criteria for species delimitation. Our second coalescence-based method – Bayesian species delimitation – satisfies our desire for objectivity in species delimitation because it provides explicit probabilities of speciation events. Furthermore, it accounts for gene tree discordance stemming from the incomplete sorting of ancestral polymorphisms, and thus has the potential to resolve close, recently established species relationships (Yang & Rannala 2010). Bayesian species delimitation using the European 138-collection dataset fully supported the splitting of *L. volemus* sensu lato into the three candidate species revealed by the genealogical approach. Furthermore, different starting trees and prior settings relating to effective populations sizes and root ages did not influence speciation probabilities. These results are important because they prove that sufficient signal to reconstruct robust species trees remains even if gene trees are discordant.

Our molecular results are strongly inconsistent with the current morphology-based taxonomy and indicate cryptic diversity. A close morphological examination, however, revealed subtle but significant morphological differences in pileipellis hair length and cap colour between the three species. Although pleurolamprocystidia of *L. oedematopus* do not exceed 120 μm, but measure up to 145 μm in *L. subvolemus* and up to 160 μm in *L. volemus*, we do not recommend their use as diagnostic character due to high levels of intraspecific variation. Likewise, and in line with our previous studies of *L. volemus* sensu lato (Van de Putte *et al.* 2010; 2012), spores do not allow species identification due to considerable intraspecific variation in size, reticulation pattern, and height of the ridges.

Lactifluus oedematopus can be easily identified by its short pileipellis hairs. Both *L. volemus* and *L. subvolemus* are characterized by longer hairs, and can only be distinguished by cap colour. Although previously interpreted as intraspecific variation, the buff-yellow and brick-red colour forms – corresponding to *Lactarius volemus* var. *flavus* Hesler & A.H. Sm. and *Lactarius volemus* var. *oedematopus* (Scop.) Fr. respectively – do not belong to the same species. As far as known, buff-yellow colours always correspond to *L. subvolemus*, whereas dark brown and brick-red colour forms only occur in both *L. oedematopus* and *L. volemus*, the latter being easily distinguished from *L. oedematopus* by its long pileipellis hairs. Because long hairs are often broken when preparing sections, pileipellis hairs should be measured from scalps. It is also important to note that some specimens might have very few hairs (Suppl. data Table 2), making a reliable measurement very difficult, or even impossible. This could be caused by weather conditions, or the drying process/handling of the basidiocarps. In contrast to pileipellis hairs, stipitipellis hair length is not reliable as diagnostic character. Maximum stipitipellis hair lengths of *L. oedematopus* range from 55 μm, to 70(–80) μm, and even up to 120 μm. Also *L. volemus* and *L. subvolemus* show considerable intraspecific variation in maximum stipitipellis hair length, ranging from 75(–80) μm, to 100 μm, or even 110(–150) μm. Differences in maximum pileipellis hair length between immature and mature basidiocarps were not observed (see Suppl. data Table 2).

Cap colours should be observed in fully-grown specimens only, especially because young basidiocarps of *L. subvolemus* can be rather dark brown and therefore easily mistaken for *L. volemus* (Suppl. data Table 2). We acknowledge that colour as only discriminator is not very reliable since it may be environmentally influenced, and depends upon the developmental stage of the basidiocarp. Indeed, intermediary orange-brown colour forms occur (Fig 14), and we recognize that they cannot be unambiguously allocated to either *L. volemus* or *L. subvolemus*. Nonetheless, we named and described both species for two reasons. First, despite the occurrence of intermediary colour forms, the difference in cap colour is significant (Figs 7 and 12) and – coupled with our molecular results – warrants taxonomic recognition. Second, given the exhaustive exploration and sampling effort, we do not expect to discover any additional European species that can be confused with the currently described ones. Awaiting molecular confirmation, field mycologists will need to become reconciled with the idea of identifying intermediary colour forms – with long hairs – as *L. (sub)volemus*.

Importantly, none of the North American look-alikes of *L. volemus* are conspecific with the European species and therefore in need of new names. A taxonomic revision of the North American *L. volemus* clade, however, awaits more exhaustive sampling efforts and molecular work in tandem with detailed morphological observations. Pileipellis hair length measured from scalps, pleurolamprocystidia, cap colour of fully grown specimens and lamellae spacing – characters proven to be useful in distinguishing species within subgenus *Lactifluus* section *Lactifluus* (Van de Putte *et al.* 2010; 2012) – should be given special attention.

Species distribution

All three European species have overlapping ranges (Fig 1). Both *Lactifluus volemus* and *Lactifluus subvolemus* have a widespread distribution, extending from the Mediterranean northwards to Southern Scandinavia. While *L. volemus* covers almost every country in Europe, *L. subvolemus* has not been sampled from several countries so far. *Lactifluus oedematopus* has been found in Belgium, Bulgaria, France, Germany, and Slovakia.

Host specificity is restricted to members of the families Fagaceae and Pinaceae. Several *Quercus* species (i.e. *Q. frainetto*, *Q. mongolica*, *Q. petraea*, *Q. pubescens*, *Q. robur*, *Q. suber*), *Betula pendula*, *Carpinus betulus*, *Castanea sativa*, *Fagus sylvatica*, *Abies alba*, *Picea abies*, and *Pinus sylvestris* are mentioned as potential primary hosts. *Lactifluus oedematopus* has been reported to grow in mixed deciduous forests on rich soil, as well as in mixed deciduous-coniferous forests. Its association with coniferous trees, however, remains uncertain because there has been no mention of pure coniferous forests so far. Both *L. volemus* and *L. subvolemus* have been reported to grow in pure coniferous forests, as well as pure deciduous forests and mixed-coniferous forests, on both acid and rich soils. In fact, some of our sampled collections contained basidiocarps of both species (Fig 12), proving that *L. volemus* and *L. subvolemus* can coexist in sympatry. As stable coexistence of species has shown to require resource partitioning (Amarasekare 2003), the extent to which both species are ecologically differentiated from each other should be studied in more detail.

Lactifluus volemus comprises the bulk (68 %) of our sampled collections and can be considered the most common out of three species. In contrast, 27 % belongs to *L. subvolemus*, and only 5 % to *L. oedematopus*. Given the high exploration rate and sampling effort, we argue that *L. oedematopus* should definitely be considered a rare species. Although *L. subvolemus* is less common than *L. volemus*, it can be locally more abundant. While collecting in Slovenia, for example, *L. subvolemus* appeared very abundant, whereas *L. volemus* was found only rarely.

Lactifluus volemus sensu lato is currently considered extinct in the Netherlands (Arnolds & Veerkamp 2008) and the Belgian Flemish region (Walleyn & Verbeken 2000), and is declining in other European countries (Heilmann-Clausen *et al.* 1998; V. Antonín, pers. comm.). Given that the Netherlands and Flanders rank among the most densely populated areas of Europe, the decline of *L. volemus* sensu lato may be ascribable to anthropogenic nitrogen eutrophication. Excessive nitrogen deposition – caused mainly by agricultural intensification – is believed to have played a key role in the reduction of ectomycorrhizal root tips and species richness in European forests (Arnolds 1991). While recommended critical loads for nitrogen in European deciduous forests are set at 15–20 kg nitrogen per hectare per year (ha^{-1} yr^{-1}) for alterations in mycorrhizal community composition (UBA 2004), the most densely populated areas in Europe receive 50–100 kg N ha^{-1} yr^{-1} (Senn-Irlet *et al.* 2007), with the highest amounts in forests and especially forest edges (Arnolds 1991).

Being prized for its culinary use, collection at unsustainable rate could pose an additional threat. Although harvesting has shown not to reduce neither fructification nor species richness (Egli *et al.* 2006), long-term effects of lowered spore availability may be threatening to species with very small populations, as reported for the globally red-listed basidiomycete *Pleurotus nebrodensis* (Senn-Irlet *et al.* 2007). Revealing that *L. volemus* sensu lato comprises three European species with fewer numbers and/or smaller distributions, clearly presents even greater conservation challenges.

Open questions

Eastern North American and European species do not cluster together according to continent. This result, together with the low genetic diversity in our dataset, suggests recent migration and allopatric divergence. As species richness has shown to be positively correlated with the age of a species (Stephens & Wiens 2003; McPeek & Brown 2007), Asia could be considered the ancestral region of *Lactifluus volemus* (Shimono *et al.* 2007; Van de Putte *et al.* 2010; 2012). Long-distance dispersal events (Geml *et al.* 2012), and migration via Beringia (Graham 1993; Elias *et al.* 1997; Brubaker *et al.* 2005) or the North Atlantic Land Bridge (Denk *et al.* 2005) could have contributed to subsequent migration between continents. Still, massive extinctions in Europe during the Quaternary glaciations (Huntley & Prentice 1993; Hewitt 2000), and the magnitude of anthropogenic disturbances in European forests (EEA 2006) could have resulted in the lower species diversity in Europe as well, and an 'out of Asia' hypothesis remains to be rigorously tested by sampling across the entire biogeographical range of the *L. volemus* group and determining the relationships between Asian and non-Asian species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.08.015.

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