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***Alpova komoviana* (Boletales, Paxillaceae), a new sequestrate fungus from Montenegro, with a revised phylogeny of the genus in Europe**

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Abstract

A new ectomycorrhizal species, *Alpova komoviana*, is described from several collections from Montenegro (south-eastern Europe), in association with *Alnus incana* ssp. *incana* (Betulaceae). Its interesting basal position in the strictly *Alnus*-associated *Alpova* lineage is discussed through morphology and phylogenetic analyses based on ITS, *gpd* and *rpb2* nuclear DNA. ITS sequences of two other taxa are included in the analysis: *Alpova rubescens* and *A. rubescens* var. *obscuratus*; their identity and systematic positions are discussed.

Keywords

Basidiomycota Paxillineae *Alpova rubescens* *Alnus incana* *Melanogaster* *Alnus* taxonomy

Introduction

The ectomycorrhizal hypogeous genus *Alpova* C.W. Dodge, as recently redefined by Moreau et al. (2011a), currently encompasses three European species (*A. alpestris* P.-A. Moreau & F. Richard, *A. corsicus* P.-A. Moreau & F. Richard, and *A. “cf. cinnamomeus”*), one South-American (*A. austroalnicola* L.S. Domínguez) and at least three North American species still confused under the name *A. diplophloeus* (Zeller & C.W. Dodge) Trappe. Rochet et al. (2011) emphasized host specificity as an important character in this genus, and Moreau et al. (2011a) pointed out that no *Alpova* species was confirmed under *Alnus* sect. *Alnus* in continental Europe. All previous reports of “*Alpova diplophloeus*” with *Alnus glutinosa* or *A. incana* were interpreted as *Melanogaster luteus* Zeller (Perić and Moreau 2010).

As soon as the article submitted by Moreau et al. (2011a) was accepted for publication, a first collection made in 2010 in Montenegro by B. Perić came to fill the gap, with characteristics of *Alpova* (pseudoparenchymatic structure of subpellis, presence of buffer cells in gleba, and pale spores), but differed from all known species because of its much longer spores. New collections made in summer 2011, on the same site and in two other areas nearby, showed the same characteristics. DNA sequences (the nuclear ribosomal region ITS1-5.8 S-ITS2, and the protein-coding genes *gpd* and *rpb2*) could be generated from the first collection and compared to those generated formerly on various species of *Alpova* and *Melanogaster* (Rochet et al. 2011).

No description in literature could be found for this species. The present work aims to publish it as a new species, and to discuss its phylogenetic position in the genus *Alpova*. In addition, two other species usually admitted in the genus *Alpova* but not included in Moreau et al.'s (2011a) analyses: *A. rubescens* (Vittad.) Trappe, and *A. rubescens* var. *obscuratus* (Svrček) Trappe, could be included in the ITS phylogeny of the *Alpova-Melanogaster* lineage and their position is discussed.

Materials and methods

Taxonomy and nomenclature

Nomenclature and taxonomic concepts in the genera *Alpova* and *Melanogaster* follow Moreau et al. (2011a).

Basidiomata sampling and description

Basidiomata of *Alpova komoviana* were collected in Montenegro (B. Perić), photographed and air-dried. Microscopical descriptions are based on observations made partly on fresh material by B.P. on water and Congo red, partly on exsiccata by P.-A. M. at the Laboratory of Plant and Fungal Sciences (Lille), with a light microscope Nachet Andromede 018 at $\times 100$, $\times 400$ and $\times 1,000$ magnifications, on hand-sectioned mounts in Melzer's reagent, 5 % KOH, and Congo red (1 mg in 10 ml NH_4OH) after reviving in 10 % KOH during a few minutes. At least 50–60 basidiospores were measured in gleba of selected specimens (see Table 1). Statistical treatments and notations follow Fannechère (2005) and measurements were made on the software Mycomètre 2.02 (Fannechère 2011). Spore dimensions are given as follows: (minimum value) 1st decile - *average value* - 9th decile (maximum value), calculated on all basidiospores measured for each sample. Descriptive terminology follows Moreau et al. (2011a).

Table 1

Estimated spore measurements on selected basidiomata of *Alpova komoviana*: 1st and 9th deciles (D1,9) and average value (*italic*) are given according to Fannechère (2005, 2009)

Voucher	Number of measures	Spore length (µm)	Spore width (µm)	Quotient (length/width)	Volume (µm ³)
<i>Alpova komoviana</i> Dgf/C400D-27-07-11	50	6.0–6.9–7.9	2.7–3.0–3.3	6.48–7.65–8.52	206–263–340
<i>Alpova komoviana</i> Dgf/C400D31-07-11	50	6.5–7.6–8.5	2.7–3.1–3.4	2.14–2.49–2.92	217–309–407
<i>Alpova komoviana</i> PAM 10081201 (young)	60	6.5–7.2–7.8	2.7–2.9–3.2	2.19–2.45–2.80	202–266–319
<i>Alpova komoviana</i> PAM 10081201 (old)	60	6.5–7.3–8.0	2.7–2.9–3.2	2.23–2.49–2.73	206–264–330
<i>Alpova komoviana</i> Dgf/C5D-28-8-10 (1)	58	6.8–7.5–8.5	3.1–3.4–3.7	1.87–2.20–2.49	291–374–448
<i>Alpova komoviana</i> Dgf/C5D-28-8-10 (2)	62	5.6–7.3–8.5	2.8–3.1–3.4	2.00–2.36–2.72	190–302–392

DNA extraction, PCR amplification and sequencing

DNA was extracted from herbarium specimens by using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. The nuclear rDNA ITS was amplified and sequenced using the primer set ITS-1 F/ITS-4B, the *rpb2* gene using bRPB2-5 F/bRPB2-7.1R, the *gpd* gene using GPD-F/GPD-RA following the protocol described in Rochet et al. (2011).

Reference sequences of *Alpova* and *Melanogaster* species were already presented in Moreau et al. (2011a) and Rochet et al. (2011); sequences of outgroups were selected amongst available sequences in GenBank in the most closely related genera in Paxillaceae and Boletaceae according to Binder and Hibbett's (2006) phylogeny of Boletales (see Fig. 1a–c, below).

Sequence alignments

Sequences generated for this study and those obtained from GenBank were aligned under Clustal W (Higgins et al. 1994). They were carefully refined manually on the editor in Mega 4.0 (Tamura et al. 2007). ITS, *rpb2* and *gpd* sequences have an alignment of 639, 754 and 696 bp, respectively, including gaps. After removing poorly aligned positions and divergent regions of DNA, ITS, *rpb2* and *gpd* sequences had respectively an alignment of 555 bp with 293 variable regions and 181 parsimony characters, 748 bp with 247 variable regions and 184 parsimony informative characters and 634 bp with 243 variable regions and 168 parsimony informative characters. 3.2 %, 0.1 % and 0.47 % of gaps were maintained respectively for ITS, *rpb2* and *gpd* in the final combined alignment but were scored as missing data.

Phylogenetic analyses

Bayesian analyses were monitored by software Mr Bayes v3.1 (Ronquist and Huelsenbeck 2003). According to the Bayesian Information Criterion (BIC) score, K80 + G (K2P; Kimura 1980) were chosen for ITS, *rpb2* and *gpd* sequences analyses as the optimal substitution model defined by TOPALi v2.5 (Milne et al. 2004). Bayesian analyses were conducted using four Metropolis coupled Markov chain Monte Carlo (MCMC) with one in every hundred trees sampled. The first 5,000 trees were excluded from our analyses. For the three Bayesian analyses, potential scale reduction factors (PSRF) were reasonably close to 1.0 for all parameters. Bayesian Posterior Probabilities (Bayesian

PP) of each node were obtained with 50 % majority rules with all compatible partitions. Whatever the method, gaps were scored as missing and trees were rooted either by midpoint rooting application for *rpb2* and *gpd*, or rooted with *Gyrodon* spp. (*G. lividus*-group) and *Paxillus* spp. (*P. involutus*-group), as quoted in the legend of Fig. [1](#) (see also Table [2](#)). A Bayesian 50 % majority rule consensus trees is shown for each gene (Fig. [1a](#), b, c).

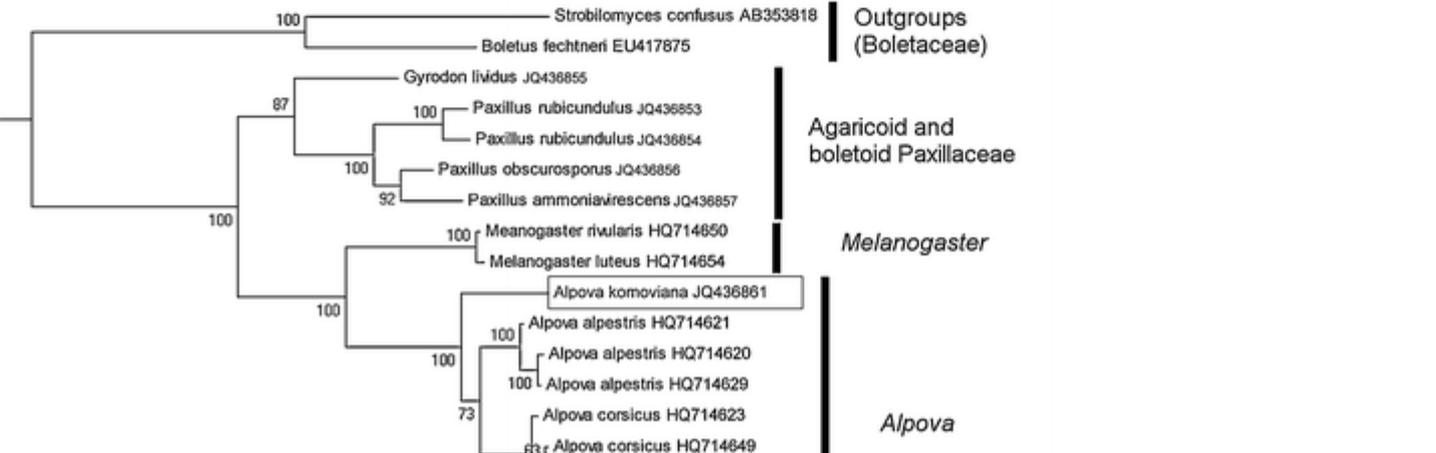
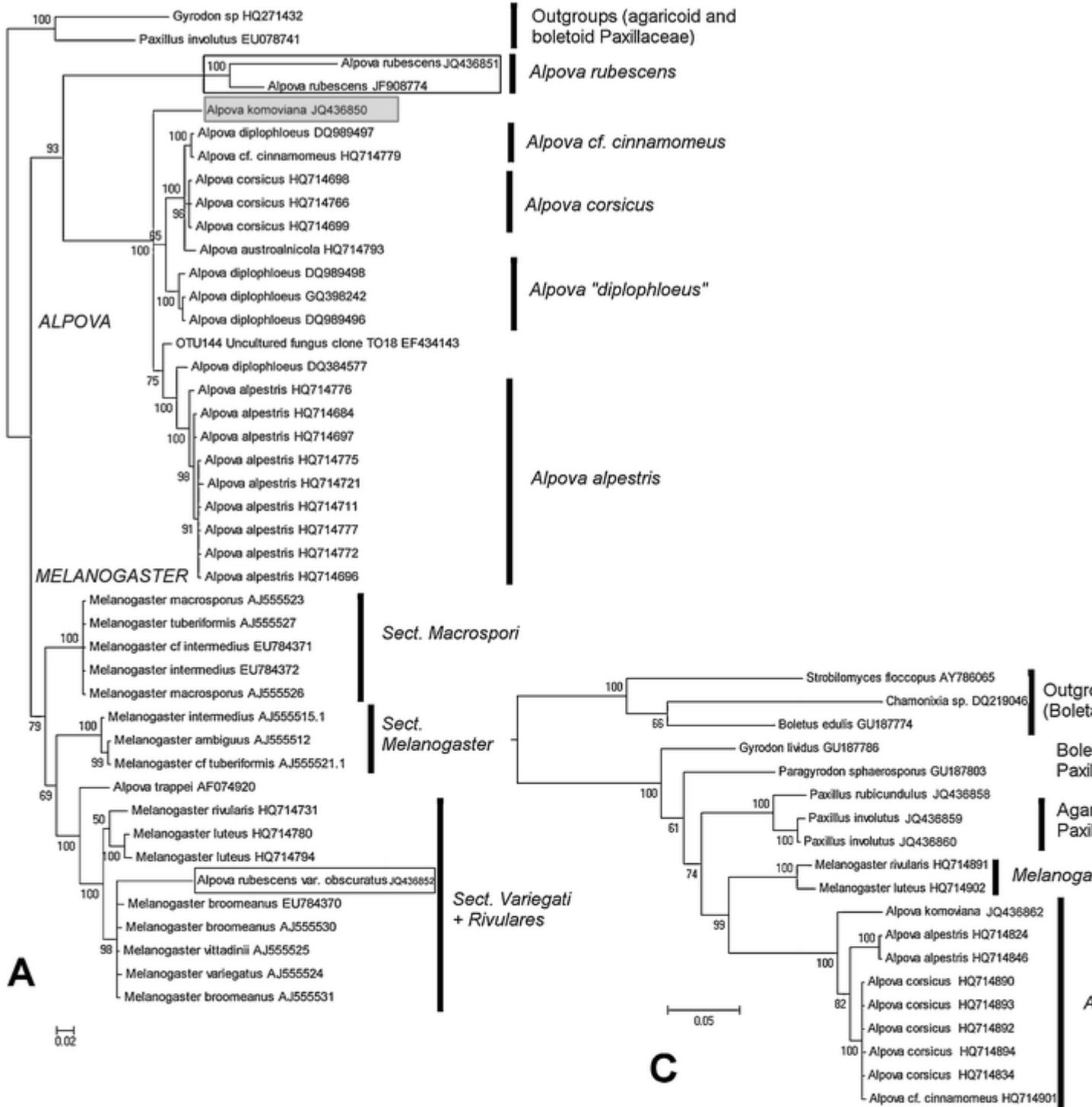


Fig. 1

Phylogenetic reconstructions of the *Alpova*–*Melanogaster* lineage from Bayesian analyses. **a** Phylogeny based on ITS1-5.8 S-ITS2 rDNA region; outgroups selected: *Gyrodon* sp. and *Paxillus involutus* (Paxillaceae). **b** Phylogeny based on partial *gpd* sequence; outgroups selected: *Boletus fechtneri* and *Strobilomyces confusus* (Boletaceae). **c** Phylogeny based on partial *rpb2* sequence; outgroups selected: *Strobilomyces floccopus*, *Chamonixia* sp. and *Boletus edulis* (Boletaceae)

Table 2

References of collections used in phylogenetic analysis (see Fig. 1)

Taxon	Country	Host tree	ITS	Gpd	Rpb2	References	
<i>Alpova</i> "diplophloeus" (Zeller & C.W. Dodge) Trappe	CAN	<i>Alnus rubra</i> ?	DQ384577			Berbee et al., unpubl.; Rochet et al. 2011 ; Moreau et al. 2011a	
	USA	<i>Alnus rubra</i>	DQ989496			Berbee and Carruthers, unpubl.; Rochet et al. 2011 ; Moreau et al. 2011a	
	SW	<i>Alnus incana</i>	DQ989497			Berbee and Carruthers, unpubl.; Rochet et al. 2011 ; Moreau et al. 2011a	
	CAN	<i>Alnus rubra</i>	DQ989498			Berbee & Carruthers, unpubl.; Rochet et al. 2011 ; Moreau et al. 2011a	
<i>Alpova alpestris</i> P.- A. Moreau & F. Richard	USA	<i>Alnus rubra</i>	GQ398242			Kennedy and Hill 2010	
				HQ714697	HQ714824	HQ714621	
				HQ714711	HQ714846	HQ714654	
				HQ714776			
				HQ714772			Rochet et al.
				HQ714775			2011 ; Moreau et al. 2011a
				HQ714777			
			HQ714684				
			HQ714696				
			HQ714721		HQ714620		

Taxon	Country	Host tree	ITS	Gpd	Rpb2	References
	F-Corsica				HQ714629	Rochet et al. 2011 ; Moreau et al. 2011a
<i>Alpova austroalnicola</i> L.S. Domínguez	ARG	<i>Alnus acuminata</i>	HQ714793			Rochet et al. 2011 ; Moreau et al. 2011a
<i>Alpova</i> cf. <i>cinnamomeus</i> C.W. Dodge	F	<i>Alnus alnobetula</i>	HQ714779	HQ714901		Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica		HQ714766			Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica				HQ714622	Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica		HQ714698		HQ714623	Rochet et al. 2011 ; Moreau et al. 2011a
<i>Alpova corsicus</i> P.-A. Moreau & F. Richard	F-Corsica		HQ714699	HQ714834	HQ714624	Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica			HQ714890	HQ714649	Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica				HQ714651	Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica			HQ714893		Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica			HQ714892		Rochet et al. 2011 ; Moreau et al. 2011a
	F-Corsica			HQ714894		Rochet et al. 2011 ; Moreau et al. 2011a
<i>Alpova komoviana</i> Perić & P.-A. Moreau	MTN	<i>Alnus incana</i>	JQ436850	JQ436861	JQ436862	This study
" <i>Alpova</i> " <i>rubescens</i> (Vittad.) Trappe	I	<i>Fagus sylvatica</i>	JQ436851			This study
	I		JF908774			Garbelotto et al., unpubl.
" <i>Alpova</i> " <i>rubescens</i> var. <i>obscuratus</i> (Svrček) Trappe	F	<i>Quercus</i> sp.	JQ436852			This study (leg. G. Fourré and M. Forestier)
" <i>Alpova</i> " <i>trappei</i> Fogel	USA		AF074920			Grubisha et al. 2002
<i>Melanogaster ambiguus</i> (Vittad.) Tul. & C. Tul.			AJ555512			Halász 2008

Taxon	Country	Host tree	ITS	Gpd	Rpb2	References
<i>Melanogaster broomeanus</i> Berk.			AJ555530			Halász 2008
			AJ555531			
			EU784370			
<i>Melanogaster intermedius</i> (Berk.) Zeller & C.W. Dodge			AJ555515			Halász 2008
			EU784372			Brock et al. 2009
<i>Melanogaster cf. intermedius</i> (Berk.) Zeller & C.W. Dodge			EU784371			Brock et al. 2009
	F		HQ714780	HQ714902		Rochet et al. 2011 ; Moreau et al. 2011a
<i>Melanogaster luteus</i> Zeller	MTN		HQ714794			Rochet et al. 2011 ; Moreau et al. 2011a
			AJ555523			Halász 2008
<i>Melanogaster rivularis</i> P.-A. Moreau & F. Richard	F-Corsica		AJ555526			
			HQ714731	HQ714891	HQ714650	Rochet et al. 2011 ; Moreau et al. 2011a
<i>Melanogaster tuberiformis</i> Corda			AJ555527			Halász 2008
<i>Melanogaster cf. tuberiformis</i> Corda			AJ555521			Halász 2008
<i>Melanogaster variegatus</i> (Vittad.) Tul. & C. Tul.			AJ555524			Halász 2008
<i>Melanogaster vittadinii</i> Soehner & Knapp			AJ555525			Halász 2008
OTU144 Uncultured fungus clone TO18 (<i>Alpova</i> sp.)	USA-Alaska	Soil sample under <i>Alnus incana</i> ssp. <i>rugosa</i>	EF434143			Taylor et al. 2007
Outgroups						
<i>Boletus edulis</i> Bull.: Fr.				GU187774		Binder et al. 2010
<i>Boletus fechtneri</i> Velen.	NL				EU417875	Beugelsdijk et al. 2008
<i>Chamonixia</i> sp.	JAP			DQ219046		Hosaka and Spatafora, unpubl.
<i>Gyrodon lividus</i> (Bull.: Fr.) P. Karst.	D	<i>Alnus incana</i>			GU187786	Binder et al. 2010

Taxon	Country	Host tree	ITS	Gpd	Rpb2	References
	F	<i>Alnus glutinosa</i>		JQ436855		This study
<i>Gyrodon</i> sp.	MEX	<i>Alnus acuminata</i>	HQ271432			Kennedy et al. 2011
<i>Paragyrodon sphaerosporus</i> (Peck) Singer				GU187803		Binder et al. 2010
<i>Paxillus ammoniavirescens</i> Contu & Dessì				JQ4368557		This study
<i>Paxillus involutus</i> (Batsch: Fr.) Fr.			EU078741		JQ436859 JQ436860	Hedh et al. 2008 This study This study
<i>Paxillus obscurosporus</i> C.Hahn				JQ4368556		This study
<i>Paxillus rubicundulus</i> P.D. Orton				JQ436853 JQ436854	JQ436858	This study This study
<i>Strobilomyces confusus</i> Singer					AB353818	Sato and Murakami 2008
<i>Strobilomyces floccopus</i> (Vahl: Fr.) P. Karst.				AY786065		Binder et al., unpubl.

Results

The three phylogenetic analyses driven independently on each DNA regions considered (ITS1-5.8 S-ITS2, gpd and rpb2; Fig. [1](#)) show convergent and well-supported results. The ITS phylogeny of the *Alpova*–*Melanogaster* clade (Fig. [1a](#)) is the most developed regarding species sampling because of the large amount of *Melanogaster* sequences available in GenBank (mostly identified by Halász [2008](#)); the general topology of the tree presented here is congruent with those already published by Moreau et al. ([2011a](#)) in which *Alpova komoviana* and *A. rubescens* were not included. The gpd and rpb2 phylogenies are as yet unpublished but concatenate analyses including these genes were previously presented by Rochet et al. ([2011](#)).

The three cladograms generated confirm *Melanogaster* and *Alpova* to form two sister clades in the Paxillineae lineage (Binder and Hibbett [2006](#)) corresponding to the family Paxillaceae. In the gpd (Fig. [1b](#)) and rpb2 (Fig. [1c](#)) analyses, the new species *Alpova komoviana* comes out with strong support in a basal position in the *Alpova* clade. The support is weaker in the ITS analysis (Fig. [1a](#)), a consequence of the wider taxon sampling and the strong variability of this region, with subsequent problems of sequence alignment. As shown by Moreau et al. ([2011a](#)), the other *Alpova* species split into two distinct lineages, one comprising *A. alpestris* and a North American species (“*A. diplophloeus*” DQ384577), the other one comprising *A. austroalnicola*, *A. corsicus*, *A. aff. cinnamomeus* and a Swedish-related collection under *Alnus incana* (“*A. diplophloeus*” DQ989497), and two North American species (“*A. diplophloeus*” DQ989496 and GQ398242, and a mycelial isolate: EF434143, from soil sampling under *Alnus incana* ssp. *rugosa* in Alaska; L. Taylor, personal communication).

The genus *Melanogaster* was already shown to be monophyletic by Moreau et al. (2011a) as far as north temperate taxa are concerned; in this analysis based on ITS sequences, the North American species '*Alpova*' *trappei* already came out unambiguously in this phylum. A new ITS sequence of the epitype collection of *Alpova rubescens* formerly studied by Vizzini et al. (2010) is positioned from our Bayesian analysis in a basal position on the *Alpova*-clade (Fig. 1a), after suppression of gaps and ambiguous positions. Because of strong divergences in *A. rubescens* compared to sequences of *Alpova* and *Melanogaster* lineages in the most variable regions of ITS1 and ITS2, the same analysis driven on a complete alignment sort it out without support in a basal position of the whole *Alpova-Melanogaster* lineage (as found by Vizzini et al. 2010, fig. 4, from analysis of LSU sequences), or at the basis of the whole *Melanogaster*-clade according to somewhat subjective choices in the hand-made alignment of these ambiguous positions. A collection of *A. rubescens* var. *obscuratus* provided and identified by G. Fourré from France, macro- and microscopically conform to the protologue (Svrček 1958: 797), was sequenced by A. Vizzini and included in the ITS analysis (Fig. 1a). It is clearly positioned within medium-spored *Melanogaster* species of sect. *Variegati* (type: *M. variegatus*; see Moreau et al. 2011a).

Taxonomy

Alpova komoviana B. Perić & P.-A. Moreau, **sp. nov.** Fig. 2

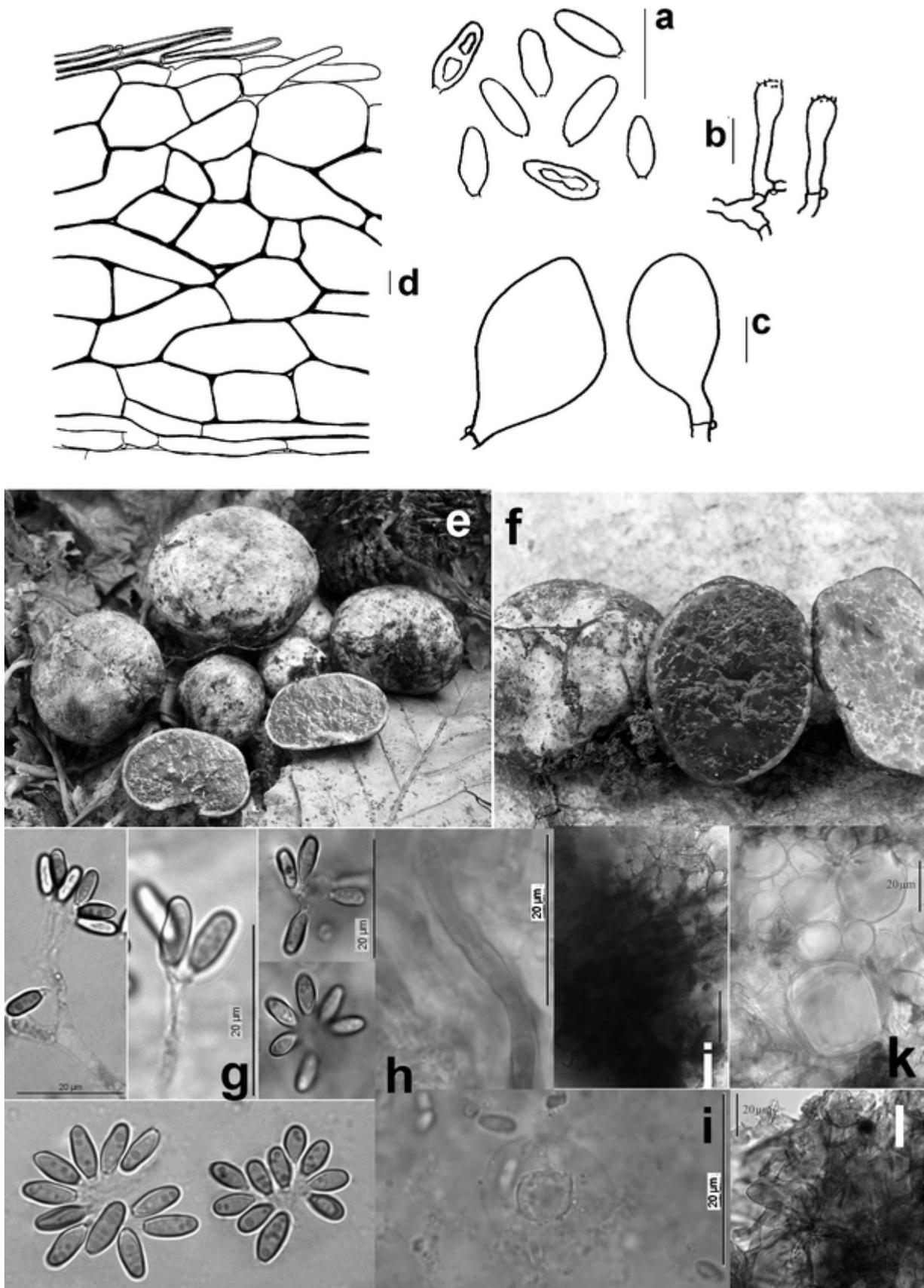


Fig. 2

Alpova komoviana. **a** Spores, **b** basidia, **c** buffer cells, **d** peridium, radial cut, **e** basidiomata (coll. PAM10081001, holotype). Scale bar 10 μm . **f** Basidiomata (coll. B. Perić Dgf/C5D-12-08-10), **g** spores and basidia, **h** thromboplerous hypha in gleba, **i** buffer cell in gleba, **j** peridiopellis,

tangential cut, **k** subpellis, radial cut, **l** peridiopellis, tangential cut. **e–g, j–l** photos B. Perić; **h, i** photos P.-A. Moreau

Mycobank Accession Number: MB564288

Diagnosis

Differs from all known species of *Alpova* by thick peridium foxy-orange in KOH, presence of thromboplerous hyphae with brown content in veins of gleba, and larger spores, $6.3\text{--}8.3 \times 2.7\text{--}3.5$ μm . In riparian forests, with *Alnus incana*. Holotype: Montenegro, Komovi Mts, Bindža, Vučji potok, leg. B. Perić, 12 Aug. 2010, PAM10081201 in herbarium LIP (Lille, France). Isotypes in herbarium ZT (Zürich, Switzerland) and B. Perić's personal herbarium.

Description

Basidiomata hypogeous, $10\text{--}35 \times 10\text{--}28$ mm, subglobose to occasionally irregularly elongate or tuberculose; surface smooth, at first whitish with yellowish brown spots, becoming pinkish brown to reddish brown, staining rusty brown; superficial rhizomorphs whitish then reddish brown, arising from base and adpressed to the peridium all around the basidiome. Gleba gelatinized, firm then soft when mature, at first ochre then gradually pink-ochre, vinaceous pink, to red-brown when old, with intermixed white veins forming an irregular reticulum.

Peridium 2-layered. Peridiopellis $30\text{--}55$ μm thick, locally thinner with apparent subpellis, bright foxy-orange in KOH, made of catenulate hyphae $3.5\text{--}7$ μm wide, with some vesiculose article up to $8\text{--}15$ μm wide, with smooth parietal yellow pigment and abundant yellow brown intracellular pigment; superficial cystidioid articles sparse, up to $70 \times 6.5\text{--}8$ μm , with rounded apex; rhizomorphs abundant as thin fascicles ($8\text{--}15$ μm wide) of cylindrical hyphae $1.5\text{--}5$ μm wide, with yellow wall up to 0.5 μm thick. Subpellis $320\text{--}350$ μm thick, of *Textura globulosa* type with small intercellular spaces up to 5 μm wide, with articles spherical to elongate-ellipsoidal (sometimes protruding on surface), $16\text{--}60$ μm wide, with pale, smooth wall up to 2 μm thick, intermixed with some voluminous cylindrical hyphae in depth, up to $110\text{--}140 \times 9\text{--}38$ μm .

Gleba colourless under microscope, made of weakly to moderately gelatinized lacunae $200\text{--}400$ μm wide, globose and well-separated when young then confluent, separated by distinct veins $25\text{--}35$ μm wide, made of straight or sinuous narrow hyphae $2\text{--}5$ μm wide, with yellowish wall up to 2.5 μm thick, sparsely intermixed with large and wide "paxilloid" hyphae $8\text{--}10$ μm wide with yellow content. Buffer cells frequent, vesiculose-pedicellate, relatively small, $15\text{--}30 \times 12\text{--}22$ μm .

Basidiospores (340 measurements) $(5.2)6.3\text{--}7.33\text{--}8.3(9.4) \times (2.6)2.7\text{--}3.09\text{--}3.5(4.0)$ μm , $Q = 2.03\text{--}2.39\text{--}2.73$, fusiform to cylindro-fusiform, somewhat constricted or lageniform in front view, smooth, almost colourless in Melzer, ochre-yellow in KOH; content with $1\text{--}2$ droplets, wall thickened up to $0.8(1)$ μm ; some sclerospores observed especially on old specimens, with congophilic wall up to 1.2 μm thick. Basidia $17\text{--}22 \times 4\text{--}6$ μm , cylindro-clavate when mature, not guttulate, 8- to 10-spored (when more than 8 then usually with one or two sclerospores), thin-walled, hyaline till mature, with distinct sterigmata, not collapsing with age. Clamps observed at all septa. No part of the basidioma dextrinoid or amyloid.

Specimens examined

Montenegro, Komovi Mts, Bindža, Vučji potok under *Alnus incana* and *Abies alba*, leg. B. Perić, 12.8.2010, PAM10081201. Holotype (LIP), isotypes (ZT and pers. coll. B. Perić). Same area, two basidiomata, leg. B. Perić, 28.8.2010, Dgf/C5D-28-08-10. Same locality, six basidiomata, leg. B. Perić, 15.7.2011, Dgf/C400D-15-07-11. Same area, one basidiome, leg. B. Perić, 13.8.2011, Dgf/C400D-13-08-11. Komovi Mts, Opasanica near family Jovanović's house, three basidiomata under leaves of *Alnus incana*, leg. B. Perić & O. Perić, 27.7.2011, Dgf/C400D-27-07-11. Komovi Mts, Kurlaj, two basidiomata under leaves of *Alnus incana* and *Fagus moesiaca*, leg. B. Perić & O. Perić, 31.7.2011, Dgf/C400D-31-07-11.

Ecology and distribution

So far only known from a few areas in Montenegro, in the Komovi Mts, always under *Alnus incana* mixed or not with *Abies alba* and *Fagus moesiaca*.

Discussion

Morphology of *Alpova komoviana*

Compared with other species of *Alpova* described by Moreau et al. (2011a), *A. komoviana* is easily recognizable by its long spores, calling to mind those of '*Alpova*' *rubescens*, a *Fagus*-associated species of ambiguous phylogenetic position (Vizzini et al. 2010) with even longer spores. The two-layered peridium structure of *A. komoviana* with a thick pseudoparenchymatic subpellis and well-developed peridiopellis, and the presence of buffer cells in gleba, are typical of the genus *Alpova* and are comparable to such species as *A. austroalnicola* (Nouhra et al. 2005) or *A. corsicus* (Moreau et al. 2011a).

The development of a thick peridiopellis is also a character of the *Alpova corsicus*-clade, which includes *A. austroalnicola* and at least one North American species (Fig. 1a); this structure is nearly absent in *A. alpestris*, which belongs to another lineage and is associated with other species of *Alnus* (*A. alnobetulae*). Because of the basal position of *A. komoviana* in the reconstructed phylogeny of sequestrate Paxillaceae (Fig. 1b, c), it is assumed that the presence of a developed peridiopellis, as well as faintly staining and moderately gelatinized gleba, is a plesiomorphic character in the *Alpova* lineage. In *A. alpestris*, which seems to have evolved in relation with the green alder (*Alnus alnobetula* sensu lato), the peridiopellis as well as rhizomorphs seems to have reduced when the gleba becomes early gelatinized and stains actively when cut.

Finally, the presence of large hyphae with brown intracellular content in gleba, calling to mind the thromboplerous hyphae found in *Paxillus* species (Cléménçon 1997), is a new character for the genus *Alpova*, not mentioned by Zeller (1947), Trappe (1975), Nouhra et al. (2005), or Moreau et al. (2011a) in the species studied by them. This could be interpreted as a plesiomorphic character inherited from ancestral agaricoid Paxillaceae, still present sparsely in *A. komoviana*, and possibly disappeared in derived lineages of *Alpova*. This character should be carefully checked in other species, especially in still undocumented North American species found in basal position in the two main lineages of the genus (Fig. 1a).

A new contribution to the biogeography of *Alpova*

As summarized by Moreau et al. (2011a), the genus *Alpova* has been reported from various parts of the Northern Hemisphere where alders are present (North and South America, Japan) (Fig. 3). In Europe, *Alpova alpestris* is presumed to be common anywhere *Alnus alnobetula* is present. However, it must be remarked with Trappe (1975) that North American collections of *Alpova* “*diplophloeus*” are frequent under *Alnus rubra* as well as *A. incana*. In Europe, Moreau et al. (2011a) did not report any collection of *Alpova* associated either with *Alnus glutinosa* or *A. incana*, except in Corsica where *Alpova corsicus* is locally frequent under *Alnus glutinosa*. The hypogeous life style of these species makes them inconspicuous and would suggest a lack of observation in lowland alder forests rather than a real absence.



Fig. 3

Distribution of *Alpova* records with *Alnus* sect. *Alnus*. North America (*Alpova diplophloeus* sensu lato): Dodge (1931), Zeller (1947), Trappe (1975); South America (*A. austroalnicola*): Nouhra et al. (2005); Sweden (*A. sp.*): M. Berbee in Rochet et al. (2011), Corsica (*A. corsicus*): Moreau et al. (2011a, b); Alaska (*A. sp.*): M. Roy and P.-A. Moreau, unpublished data; Montenegro (*A. komoviana*): this study

However, sequestrate and other hypogeous fungi are traditionally studied in western Mediterranean areas such as Spain, Italy or southern France, and it is reasonable to assume that, if as abundant in these areas as in North America or Corsica (Dodge 1931; Trappe 1975; Moreau et al. 2011b), “alder truffles” could have hardly escaped the sagacity of so many specialists such as Tulasne and Tulasne

(1851), Mattiolo (1935), Montecchi and Sarasini (2000), etc. Comparatively, in Eastern Europe, Asia or South America, only a few mycologists were likely to have investigated alder forests for hypogeous fungi, and even common and abundant growths of *Alpova* might have been overlooked in these areas. Nouhra et al. (2005) give a good example of the need for focused research for discovering such species.

From all these facts and speculations, a possible hypothesis is that western Europe is an exception in the world, by being devoid of species of *Alpova* associates with *Alnus* sect. *Alnus*, whereas they appear to be common wherever they are looked for in America and probably elsewhere. Glaciations in the Pleistocene period could explain this pattern, by the difficulties for hypogeous fungi to follow their host-trees (*Alnus glutinosa* and *A. incana*) in recolonizing northern and western Europe from refuges in south-eastern Europe, Corsica, Levantine basin or even North Africa (King and Ferris 1998; Hewitt 1999).

The discovery of two endemic species of *Alpova* (*A. corsicus*) and *Melanogaster* (*M. rivularis* P.-A. Moreau & F. Richard) associated with *Alnus glutinosa* in such a refuge (Rochet et al. 2011) supports such a hypothesis. Data are lacking so far from the relict alder forests of North Africa and eastern Asia. Regarding south-eastern Europe, the discovery of *A. komoviana* in a glacial refuge of Pleistocene comes as a new element for feeding this scenario. More investigations are required in such poorly documented areas as, especially, the Carpathians, Caucasus, and the Middle East, where, according to this hypothesis, more hypogeous Paxillaceae with possible endemic distributions should be discovered in relation to *Alnus glutinosa* and related alders.

Systematic position of ‘*Alpova*’ *rubescens* and var. *obscuratus*

Alpova rubescens is a rare species, not associated with alder but with beech (*Fagus sylvatica*) on calcareous substrates. Trappe (1975: 294) proposed to place it in the genus *Alpova* on account of the pale spores, a proposition supported by a subcellular subpellis (Montecchi and Sarasini 2000; Vizzini et al. 2010) and reddening gleba (Vizzini et al. 2010). However, buffer cells are missing, and spore measurements ($10\text{--}13.5 \times 4\text{--}5 \mu\text{m}$ according to Vizzini et al. 2010) exceed those of all species of *Alpova* in the restricted sense of Moreau et al. (2011a). Vizzini et al. (2010) suggested that *A. rubescens* represents a third phyletic lineage of hypogeous *Paxillaceae*.

This new analysis confirms this hypothesis, and places *A. rubescens* var. *rubescens* at the very base of the *Alpova*-clade. Despite substantially divergent ITS1 and ITS2 sequences compared to all other *Melanogaster* and *Alpova* species, even when refining alignments by eye, ITS sequences of *A. rubescens* appear more similar to those of *Alpova* spp. than to *Melanogaster* spp. It must be noticed that another sequence was recently released in GenBank (accession number: JF908774), from a collection identified “*Alpova rubescens*” by the Italian mycologist A. Montecchi in herbarium MCVU. This collection is rather distinct from the epitype (especially in ITS1: only 73 % of similarity), but both sequences cluster together on a single clade (Fig. 1a). *Alpova rubescens* might represent a species complex in need of deeper morphological investigations.

Alpova rubescens var. *obscuratus* was also mentioned by Moreau et al. (2011a; b) as an insufficiently documented taxon, probably displaced in *Alpova* because of too dark spores. The type specimen could not be examined and it is unlikely to provide good DNA sequences; the only recent material available to us was a French collection matching the protologue of the taxon (Svrček 1958: 797): small-sized (3–10 mm diam.), with reddish isolated lacunae in gleba and light brown spores, $8\text{--}10 \times 4\text{--}5 \mu\text{m}$ (G. Fourné, personal communication). Its position within medium- to small-spored species of sect. *Variiegati* (*M. broomeanus*–*M. variegatus* complex; Fig. 1a) suggests that *A. rubescens* var. *obscuratus* is probably based on an immature stage of *M. variegatus* or a related

species, and displaced by Trappe (1975) because of pale spores, although much darker than those of *A. rubescens* inde nomen. Pale spore color was emphasized by Trappe (1975) as a characteristic of the genus *Alpova* in an enlarged sense. It may also represent a pedomorphic feature in the genus *Melanogaster*.

The ITS region is very variable in *Paxillaceae* and in most cases sufficient for identification of specimens at species level (barcoding). However, phylogenetic reconstructions are hardly reliable based on this single region, because of its high variability and unavoidable subjectivity in sequence alignment. Gpd and rpb2 genes provide a robust phylogeny without problems of alignment, but could not be generated from most exsiccata. For these reasons, the position of *A. rubescens* and “*M. rubescens* var. *obscuratus*”, as well as of extra-European species of *Melanogaster* described from Asia or North America, will remain unsure until the basal phylogeny of *Paxillaceae* can be definitively solved based on combined multigene analyses. Therefore, it is proposed to provisionally keep *Alpova rubescens* within the genus, which remains defined by a pseudoparenchymatous subpellis (prosenchymatous in *Melanogaster*); *A. rubescens* differs from typical *Alpova* species (sect. *Alpova*; Trappe 1975) by larger spores, absence of buffer cells and association with other trees than *Alnus* spp.

More phylogenetic speculations on gastroid *Paxillaceae*

The basal and somewhat ambiguous position of *A. rubescens* in the *Alpova*–*Melanogaster* clade suggests that this species exhibits plesiomorphic characters within gastroid *Paxillaceae*, possibly lost in one or the other lineages *Alpova* or *Melanogaster*. For instance, the existence of a pseudoparenchymatous subpellis might have been lost in the *Melanogaster*-lineage while conserved in *Alpova*. The association with Fagales might be an ancestral character well preserved in *Melanogaster* (many species found with *Quercus* or *Fagus*) when *Alpova* s.str. might have diversified from an early shift to *Alnus* hosts. Finally, buffer cells seem to be a synapomorphy of the *Alnus*-associated *Alpova* lineage. On the basis of these data, a possible common ancestor of *Melanogaster*–*Alpova* might have been a hypogeous species developed in temperate areas with Fagales, with a differentiated subpellis, gelatinized gleba, long spores and without buffer cells. No morphologically intermediate (“secotioid”) species is known so far which might fill the deep morphological gap between these gastroid species and the closest agaricoid sister groups, especially *Gyrodon* or *Paxillus* in which no subpellis is differentiated. However, gelatinized gleba and gelatinized lamella or tube trama are likely of common origin and found throughout the *Paxillaceae* family.

Rochet et al. (2011) hypothesized that the core *Alpova* lineage originates from a host specialization with *Alnus* subgen. *Alnus*, with late host shifts to subgen. *Alnobetula* (as suggested by *Alpova alpestris* and *A. cf. cinnamomeus* in a derived position) and needed more samples outside their sampling areas to be tested. The basal position of *A. komoviana*, associated with an alder of subgen. *Alnus* (*A. incana*), is nicely in line with this prediction.

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