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INVESTIGATION OF POLYOLS, AMINO ACIDS AND PHENOLIC ACIDS IN A TAXONOMIC STUDY OF *CORTINARIUS*, SUBGENUS *LEPROCYBE*, SECTION *ORELLANI*

SYLVIE RAPIOR,[1] AUDE VASSAS, AGNES TARODO DE LA FUENTE, REGIS COURTECUISSE,[2] AND CLAUDE ANDARY

Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie, Avenue Charles Flahault, 34060 Montpellier Cedex 1, France

ABSTRACT

Seven *Cortinarius* species in sect. *Orellani* were divided into 6 groups using microscopic features of their basidiospores. Two of these groups contained all specimens of *C. speciosissimus* and *C. orellanoides*, with each group having specimens of both species. Chemical methods employing thin-layer chromatography were used to detect polyols, amino acids and phenolic acids among specimens in these groups. The importance of these compounds as well as microscopic characteristics of the basidiospores are discussed in relation to the classification of species. Preliminary data does not confirm synonymy of *C. speciosissimus* and *C. orellanoides*, but identical chromatographic fingerprints were detected for *C. orellanoides* and *C. orellanus*.

Key Words: *Cortinarius*, sect. *Orellani*, chemotaxonomy, mannitol, sugars, amino acids, phenolic acids, basidiospores

To the best of our knowledge no consensus exists as to the best method of classifying species within the genus *Cortinarius sensu lato*. The fungi classified in this genus by some authors (**7, 14, 18**) are placed in two genera, *Cortinarius* and *Dermocybe*, by others (**15, 24**). Various investigators have attached different importance to morphological and anatomical characters of the genus in demonstrating interspecific relationships. Knowledge of the chemical constituents found in a genus decreases the necessity for subjective interpretation of taxonomic characteristics, so it seemed desirable to use chemotaxonomy to help elucidate some of the systematic problems in the genus *Cortinarius sensu lato* (**9, 12, 20, 23**).

Cortinarius species in sect. *Orellani* are ideal for application of chemical taxonomic methods. Our preliminary chromatographic studies revealed certain differences in the detection of orellanine, a bipyridine N-oxyde toxic compound, in various species of sect. *Orellani* (**5, 13, 21**). From these results, which consisted of similar thin-layer chromatography (TLC) profiles for extracts from seven species of sect. *Orellani*, a better definition of the section was possible. However, individual species could not be compared and separated with these studies.

An examination of polyols, sugars, free amino acids and phenolic acids was undertaken, therefore, to ascertain what contribution their occurrence and distribution make toward a better classification of *Cortinarius* species in sect. *Orellani*. Sugar alcohols were generally considered independent of other compounds but they frequently coexist in mushroom extracts with free sugars, which are related carbohydrate compounds. Data for some free amino acid and phenolic acid constituents were obtained using two-dimensional TLC and spraying with selective reagents. All results were combined and compared with existing taxonomic concepts based on ecology, macro- and micromorphology.

MATERIALS AND METHODS

Specimens.—Specimens were obtained or collected from 1927 to 1987 in Europe, South America and North America. All were preserved by drying after morphological identification from fresh material by European and American mycologists (TABLE I). Voucher specimens were deposited in the following herbaria: IB, MPU, TR and WTU (**11**). Microscopic and chemical studies were carried out without knowledge of the

[1] Corresponding author.
[2] Present address: Laboratoire de Botanique et de Cryptogamie, Faculté de Pharmacie, Rue du Prof. Laguesse, 59045 Lille Cedex, France.

TABLE I

ECOLOGY AND MICROSCOPICAL STUDY OF *CORTINARIUS* SPECIES, SUBG. *LEPROCYBE*, SECT. *ORELLANI*

Species[a]	Collection number	Herbarium[b]	Collection site
C. brunneofulvus Fr. ss. Bres.	B/13	TR	Piné, Italy
C. fluorescens Hk.	PN 46	IB	Puerto Natales, Chile
C. henrici Reum.	Pml 706/638	MPU	Semuy, France
C. orellanoides Hry.	Bon 8714/617	MPU	Brienne, France
	Pml 674/632	MPU	Bellême, France
	Pml 710/633	MPU	Fontainebleau, France
C. orellanus (Fr.) Fr.	Pml 393/634	MPU	Les Mureaux, France
	Pml 653/635	MPU	Lavagny, France
C. rainierensis Smith & Stuntz	Stz 3998	WTU	Washington, U.S.A.
C. speciosissimus Kühn. & Romagn.	Che 91	MPU	Thonon-les-Bains, France
	Pml 182/636	MPU	Annecy, France
	Pml 619/637	MPU	Annecy, France

[a] According to the classification by Moser (**15**).
[b] Voucher specimens are deposited in the Herbarium at the University of Innsbruck (IB), Montpellier (MPU), Trento (TR), Washington (WTU).
[c] n.m. = not mentioned.

names of the specimens (blind test). Chemical analyses were performed with an homogeneous fine powder of mushroom tissue (cap, lamellae and stipe) and three replications were done.

Microscopic study.—Basidiospores were taken from the lamellae and studied under light microscopy. Preparation was as follows. Spores were softened and mounted in 0.05% Congo Red in ammonia solution (w/v) (reviving medium: ammonia; coloring medium: congo red). The length (L) and width (W) of twenty basidiospores per basidioma (one per collection) were measured using an eyepiece micrometer and the length/width (L/W) ratio was calculated.

Qualitative detection of amino acids.—Extraction was performed with 500 mg of dried mushroom tissue according to Andary *et al.* (**2**).

Quantitative evaluation of polyols and sugars.—Dry specimens were ground to a fine powder and the polyols extracted and assayed using direct fluorodensitometry on TLC according to Andary *et al.* (**3**). Glucose, fructose and galactose were distinguished using TLC on cellulose plates (Ref. 5716, Merck) developed in *n*-butanol-ethanol-water (4:1:2.2, BEW), up to a 4 cm solvent front, and sprayed with 0.2% naphthoresorcinol in ethanol (w/v) with 5% sulfuric acid (**1**).

Qualitative estimation of phenolic acids.—The dried mushroom material (100 mg of powder) was treated according to Andary *et al.* (**4**). Mobile phases of the two-dimensional TLC were 2% aqueous acetic acid (AA) followed by toluene-acetic acid-water (60:28:1.2, TAW) instead of benzene-acetic acid-water (60:22:1.2) which was initially recommended.

RESULTS AND DISCUSSION

Microscopic examination of basidiospores.—A survey of basidiospore size and shape was undertaken for the available material (TABLE I), basidiospores of each specimen were compared, and several groups were composed, based on morphological similarity. These groups were subsequently examined with regard to the different species. The results of this comparison gave the following arrangement of the examined specimens.

Group I: Collections Pml 393/634 and Pml 653/635. Basidiospores (7–)8.5–11(–12.5) × (5–)5.5–7(–8) μm [L/W = (1.20–)1.30–1.75·(–1.88)], narrowly shaped and decidedly amygdalo-elliptical with an obtuse or weakly pointed apex; ornamentation moderately developed, rather irregular and anastomosed, verrucose or verruculose, more distinct towards the apex, with some basidiospores almost smooth. This group comprises the two specimens labeled *C. orellanus*. Our results confirm the well-characterized basidiospores of this species as compared to other European species of sect. *Orellani*.

Group II: Collection PN 46. Basidiospores 7–10(–10.5) × 5–6.8 μm (L/W = 1.40–1.70), narrowly shaped, elliptical with a very slight ten-

TABLE I
CONTINUED

Collection date	Ecology	Spore size (μm)	Spore length/width
August, 1927	n.m.[c]	not seen	not calculated
March, 1963	*Nothofagus pumilio*	7–10(–10.5) × 5(–6.8)	1.40–1.70
October, 1987	*Quercus, Fagus*	6.5–11 × 6–9	1.10–1.40(–1.65)
October, 1987	Acid soil	9–11 × 6.7–8.2	1.08–1.46
September, 1987	*Pinus*	8–11.5 × 6–9	1.05–1.58
July, 1987	*Fagus*	9.5–10.5 × 7.5–8(–8.5)	1.10–1.38
October, 1986	*Quercus*	9–12.5 × 5.5–8	1.23–1.88
September, 1987	Hardwood Forest	(7–)8.5–10.2 × 5–7	1.31–1.73
August, 1948	n.m.	7–11 × 6.8–8	1.10–1.46
October, 1984	Peat-bog	8—11.5(–12) × 6.5–9(–10.5)	1.00–1.31
August, 1981	*Picea*	8–12.5 × 6.8–9.5	1.15–1.56(–1.66)
August, 1987	*Picea*	(8–)9–11.5 × 6–9	1.17–1.38

dency to be amygdaliform and an obtuse apex; ornamentation very low, regular, punctate. This is *C. fluorescens,* of which the basidiospores are very distinctive microscopically.

Group III: Collection Stz 3998. Basidiospores 7–11 × 6.8–8 μm (L/W = 1.10–1.46), short elliptical subamygdaliform with an obtuse or weakly pointed apex, comparatively broad; ornamentation regularly verrucose or verruculose, with independent, medium-sized verrucae. This is *C. rainierensis,* which also seems to have well-characterized basidiospores.

Group IV: Collection Pml 706/638. This group and the following ones are less well-characterized than the former, but certain features can be compared. Basidiospores 6.5–11 × 6–9 μm [L/W = 1.10–1.40(–1.65)], short elliptical, sometimes subglobose to short-elliptical to subamygdaliform, ornamentation coarse and conspicuous (the most coarsely ornamented basidiospores in sect. *Orellani*), especially towards the apex. The hymenophoral trama is strongly pigmented with encrusting material (not so obvious in other specimens). This is *C. henrici.*

Group V: Collections Che 91 (*C. speciosissimus*), Pml 619/637 (*C. speciosissimus*) and Pml 710/633 (*C. orellanoides*). Basidiospores 8–11.5(–12) × 7–9(–10.5) μm [L/W = 1.00–1.40(–1.50)], short-amygdaliform or subglobose, with the apex often quite obtuse (rarely somewhat pointed); ornamentation fairly distinct, mainly apical, somewhat anastomosed. Basidiospores of Che 91 had less marked ornamentation and were wider towards the apex, whereas the basidiospores of the two other collections were wider towards the apiculus.

Group VI: Collections Pml 674/632 (*C. orellanoides*), Pml 182/636 (*C. speciosissimus*) and Bon 8714/617 (*C. orellanoides*). Basidiospores 8–12.5 × 6–9.5 μm [L/W = (1.05–)1.10–1.50(–1.66)], short-elliptical to subamygdaliform with an obtuse to slightly pointed apex; ornamentation variable, rather weak to moderately developed, mainly apical, anastomosed or well-defined.

TABLE II
DETECTION AND ASSAY OF MANNITOL, GLUCOSE AND TREHALOSE IN *CORTINARIUS* SPECIES, SUBG. *LEPROCYBE*, SECT. *ORELLANI*

Species	Mannitol[a] (mg/g D.W.)	Glucose (mg/g D.W.)	Trehalose (mg/g D.W.)
C. brunneofulvus B/13	0	1.3	63.0
C. fluorescens PN46	15.8	1.5	17.5
C. henrici Pml 706/638	0	1.6	16.0
C. orellanoides			
Bon 8714/617	0.7	3.2	12.1
Pml 674/632	0.6	2.5	8.2
Pml 710/633	1.2	2.5	8.2
C. orellanus			
Pml 393/634	1.2	2.3	15.6
Pml 653/635	0.6	2.3	15.2
C. rainierensis Stz 3998	0.6	1.6	52.0
C. speciosissimus			
Che 91	0	3.8	57.7
Pml 182/636	0	1.7	60.6
Pml 619/637	0	1.6	58.8

[a] 0 = mannitol not present or undetected by our method.

TABLE III

QUALITATIVE DETECTION[a] OF PHENOLIC ACIDS[b] IN *CORTINARIUS* SPECIES, SUBG. *LEPROCYBE*, SECT. *ORELLANI*

Species[c]	PHA	PHB	PHC	PHP	DHB	HMB	G	P
C. brunneofulvus B/13	0	+	0	0	0	0	0	0
C. henrici Pml 706/638	0	+	0	0	0	0	0	+
C. orellanoides								
Pml 706/617	+	+	0	0	+	0	+	+
Pml 710/633	+	+	0	0	+	0	+	+
C. orellanus								
Pml 693/634	+	+	0	0	+	0	+	+
Pml 653/635	+	+	0	0	+	+	+	+
C. rainierensis Stz 3998	0	+	0	0	0	0	0	0
C. speciosissimus								
Chev 91	+	+	+	+	+	+	+	0
Pml 182/636	+	+	+	+	+	+	+	+
Pml 619/637	+	+	+	0	+	+	+	+

[a] 0 = phenolic acid not present or undetected by our method; + = phenolic acid present.

[b] PHA = 4-hydroxyphenylacetic acid; PHB = 4-hydroxybenzoic acid; PHC = 4-hydroxycinnamic acid; PHP = 3-(p-hydroxyphenyl)propionic acid; DHB = 3,4-dihydroxybenzoic acid; HMB = 4-hydroxy 3-methoxybenzoic acid.

[c] No exsiccata of *C. fluorescens* was available when this work was carried out.

Unfortunately, we found no basidiospores in the specimen of *C. brunneofulvus*. As the *exsiccatum* consisted of only very small pieces, we stopped searching for basidiospores so as not to destroy this very old and valuable material.

Groups V and VI are particularly interesting because they include all of the specimens referred to *C. orellanoides* and *C. speciosissimus*. Each group includes a mixture of the two names. Since the present tendency is to synonymize these two names on the basis of microscopic characteristics, it is interesting to note that there are differences between these two groups as shown in our studies. As a result the delimitation proposed here does not correspond precisely to other known determinations (**10, 19**).

Because we could not precisely define species in sect. *Orellani* using basidiospore features, we selected chemical characteristics in an effort to better establish taxonomic relationships.

The choice of experimental methods used here was influenced by several considerations. Firstly, only small amounts of dried mushroom material (100–500 mg) were available for study. The extracts were chromatographed directly or purified by passing them through a resin to minimize losses of compounds. Finally, TLC was selected as the basic assay procedure because small quantities of fungal constituents can be separated (20 ng detection limit of polyols on chromatograms) in a relatively short time.

Chemotaxonomic study of free amino acids.—Free amino acids and their derivatives are widely distributed in the fungi. A survey of amino acid patterns in fungi can contribute useful information for their classification (**2, 8, 22**). Our results show that there are no amino acids specific to sect. *Orellani*. Also, amino acids were not suitable as markers for determining the systematic position of species or for showing relationships between species.

Polyol and sugar contents.—Using the method by Andary *et al.* (**3**), mobilities of polyols and sugars were as follows: arabitol (Rf = 0.48 ± 0.01); mannitol (Rf = 0.37 ± 0.01); fructose (Rf = 0.45 ± 0.01); glucose (Rf = 0.44 ± 0.01); galactose (Rf = 0.40 ± 0.02) and trehalose (Rf = 0.24 ± 0.02). Using the mobile phase BEW and after spraying with naphthoresorcinol reagent, the three hexoses were detected as follows: fructose (Rf = 0.36 ± 0.01, dark fuchsia), glucose (Rf = 0.34 ± 0.01, turquoise blue) and galactose (Rf = 0.32 ± 0.01, pale blue). The two polyols, mannitol and arabitol, and the disaccharide, trehalose, were not revealed by this method.

Among the seven species studied, no arabitol, fructose or galactose was detected by our meth-

od. All species contained glucose (1.3–3.8 mg/g D.W.) and trehalose (8.2–63.0 mg/g D.W.). These two sugars were uniformly present, as also reported for *Boletus* (**6**). Presence of glucose and trehalose proved to be of no significance chemotaxonomically. Their ubiquitous occurrence in varying concentrations was not a sound basis for conclusions. However, the results in TABLE II indicate that only four of the seven *Cortinarius* species in sect. *Orellani* contain both orellanine and mannitol. The highest mannitol level was found in *C. fluorescens* and the lowest in *C. orellanus, C. orellanoides* and *C. rainierensis.*

Qualitative estimation of phenolic acids.—Chromatographic examination of ethereal extracts of *Cortinarius* species in sect. *Orellani* demonstrated presence of many mono- and diphenolic acids when compared in daylight with 0.1% phenolic acid standard solutions: 4-hydroxyphenylacetic acid (PHA, Rf = 0.66 ± 0.06 in AA, Rf = 0.41 ± 0.06 in TAW, pink-grey); 4-hydroxybenzoic acid (PHB, Rf = 0.48 ± 0.03 in AA, Rf = 0.44 ± 0.03 in TAW, pink); 4-hydroxycinnamic acid or p-coumaric acid (PHC, Rf = 0.23 ± 0.03 in AA, Rf = 0.47 ± 0.05 in TAW, blue-grey); 3-(p-hydroxyphenyl) propionic acid (PHP, Rf = 0.58 ± 0.03 in AA, Rf = 0.50 ± 0.05 in TAW, pink-grey); 3,4-dihydroxybenzoic acid or protocatechuic acid (DHB, Rf = 0.36 ± 0.01 in AA, Rf = 0.14 ± 0.01 in TAW, purple-blue), and 4-hydroxy 3-methoxybenzoic acid or vanillic acid (HMB, Rf = 0.40 ± 0.02 in AA, Rf = 0.67 ± 0.06 in TAW, purple).

Cortinarius speciosissimus was the richest in phenolic acids while *C. rainierensis* and *C. brunneofulvus* were the poorest. Data presented in TABLE III reveal that PHA and PHB, two monophenolic acids, were the predominant phenolic compounds. Two unidentified derivatives with respective Rf values of 0.53 ± 0.04 in AA and 0.18 ± 0.02 in TAW (G, green) and 0.62 ± 0.02 in AA and 0.06 ± 0.01 in TAW (P, purple) were detected in the chromatographic systems. Similarities in phenolic acids of *C. orellanus* and *C. orellanoides* and the closely related *C. brunneofulvus, C. henrici* and *C. rainierensis* support the idea that these taxa are chemically related. Affinities of *C. speciosissimus* with other species of sect. *Orellani* were not detected.

Our results do not agree well with those of other investigators who consider *C. speciosissimus* and *C. orellanoides* (**10**) as well as *C. brunneofulvus* and *C. henrici* (**19**) to be all the same species. These conclusions have been based on basidiospore shape or size and other macroscopic or microscopic data. Our studies indicate the possibility of heterogeneity among the spore populations of this complex (groups V and VI). Therefore, it seems that proposed synonymy may have been rather premature. Our study, based only on a very few specimens, cannot serve as a final statement. A more careful examination of many more exsiccates might provide material for a statistical study of the L/W ratio of basidiospores. These data together with comparison of line-drawings of basidiospores might be very important in defining species. However, it must be emphasized that correlation with macroscopic and ecological features also would be significant and should be examined before any final conclusions are reached.

It should be noted that *C. orellanus* and *C. orellanoides* belong to an homogeneous section (**21**) exhibiting similar polyol and phenolic acid fingerprints. These chemical markers have already been used in the genera *Boletus* and *Amanita* to distinguish sections or species (**2, 4, 6**).

Finally, we emphasize the importance of chemical markers for delimiting species and creating an accurate system of classification. Moreover, other investigations should be performed to determine the relationship of *C. orellanus* and *C. orellanoides* and of *C. speciosissimus* to the latter species. Distinct phytochemical evaluation, genetic fingerprints, or protein electrophoresis of basidiomata would seem to be appropriate for identification and classification of *Cortinarius* species (**16, 17**).

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