New Hypotheses from Integration of Morphological Traits, Biochemical Data and Molecular Phylogeny in *Agaricus spp.*

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Abstract: The genetics of taxonomic traits is poorly documented in the genus *Agaricus*. By integration with recent phylogenetic data, new hypotheses emerge concerning the white colour of the pileus and the 'phenol' odour. In numerous multispecies clades and in species, coloured and white pilei coexist, suggesting that they could derive from an ancestral polymorphism. Correlations between the white pileus and the open habitat (i.e., not under trees) suggest that the white cap could represent an advantage by providing better resistance against the sunlight and consequently against desiccation. In section *Xanthodermatei*, the phenol odour increases from the more basal clades to the more terminal ones. Correlatively, the main toxic phenolic compounds are *p*-quinol in a species of the most basal clade, and phenol in the most terminal clade. We hypothesise that phenolic secondary metabolites deriving from an initial ancestral biochemical shift, are implicated in defence mechanisms against animals and /or micro-organisms.

Key words: Morphological traits, molecular phylogeny, pileus colour, 'phenol' odor, *Agaricus, Xanthodermatei*

1 Introduction

Several species of the genus *Agaricus* are commonly gathered or cultivated for consumption throughout the world. Most people collecting wild mushrooms are able to morphologically recognize that a wild specimen belongs to this genus. Fortunately, the genus *Agaricus* is monophyletic:[1] all *Agaricus* species have a common ancestor and their morphological resemblance results from their common ancestral history. This is not true in many other genera of the kingdom Fungi. Unfortunately, in the genus *Agaricus*, it is difficult to determine individual species. Many traits can be taken into consideration: environmental traits (habitat, substrate), morphological traits: macroscopic (habit, size, veils, ornamentations) or microscopic (spores, cystidia, basidia, pellis), organoleptic traits (colour, taste, odour) and other biochemical or chemical traits (discolouration, macrochemical reaction, toxicity). In the laboratory, other traits can be examined or tested. They concern the mycelium (morphology, growth rate), the life cycle (ability to fruit under given conditions, sexual intercompatibility, interfertility with reference strains, ploidy level, type of life cycle), as well as molecular characteristics (enzymatic polymorphism, DNA sequence polymorphism). Some of them, such as pileus colour, the odour and the skin or flesh discolouration, are immediately and easily accessible; however, they are not very reliable because i) their expression depends on the age of the mushroom specimen, the environment, and, consecutively, water content, and ii) their estimation is influenced by human subjectivity, judgment, and personal experience. In any case, these traits are of great significance since their evaluation represents the first step in the process of species determination.
The genetics of these traits is poorly documented, except for the sexual intercompatibility and the molecular polymorphisms. The genetics of the other traits have been mainly studied in A. *bisporus* (Lange) Imbach, the button mushroom, for which major loci responsible for the white/coloured pileus trait and for the basidial spore

number trait have been located.[2, 3] On the other hand, recent phylogenetic analyses[4-6] (Kerrigan RW, Callac P, Challen M, Guinberteau J, Parra, LA, to be published) offer the possibility to analyse the evolution of taxonomical traits, particularly in *Agaricus* sections *Duploannulati* and *Xanthodermatei* which have been analysed in detail. In the present paper, we compared two characters, the white colour of the pileus and the 'phenol' odour, to emerging phylogenetic knowledge. Other traits apparently correlated to these traits are also considered: the open vs covered habitat, the yellow discolouration, and toxicity.

2 White Pileus and Habitat

In the genus *Agaricus*, the colour of the pileus varies from white through various browns to dark brown or dark grey with various other hues, particularly reddish and violaceous primary pigments, and yellowish secondary ones. The cap colour is very sensitive to environmental parameters. The effect of light reinforces colours, and white caps can take a yellowish, greyish or brownish tint. We will mainly consider here the "white cap" trait that is often associated with a relatively smooth aspect.

In A. *bisporus*, the button mushroom, the cap is variably fibrillo-squamulose and its colour varies from white to dark brown. A recessive allele (*Ppc1-w*) at a major locus (*PPC1* or Pilei-Pellis Colour locus) determines the white cap trait.[2] The genetics of hue and saturation are not yet known; many loci are involved and one suppressor locus is suspected.[7] Concerning the smoothness of the cap, genetic determinants are unknown but a Mendelian segregation has been already observed.[8] Among wild populations, the percentage of collections exhibiting a white cap varies from 0% in a French population to about 10% in a Greek population.[9] In *A. bisporus*, we consider that the pileus is visually white when the mean of its luminance (L in L-a-b system), measured at the disk and near the margin, is greater or equal to 88. However, specimens homozygous for the white recessive allele (*Ppc1-w/w*) can be cream or white (L value between 80 and 94).

Heterozygous specimens (*Ppc1-b/w*) exhibiting a coloured cap (brown to light brown, generally less coloured than the homozygous *Ppc1-b/b* ones) are easily obtained by crosses in the laboratory and are also found in wild populations. There is therefore no reason to maintain any varietal status based on the white colour of the cap in A. *bisporus*. In the same way, the simple variation of the cap colour is not sufficient to support a varietal rank in other species such as A. *xanthodermus* for which the white phenotype is the most frequent.

To study the relationship between the cap colour and the environment, we compared the cap colour of 235 strains of *A .bisporus* isolated from wild specimens collected in France. They were cultivated under standard conditions and the cap luminance (L) was measured using a Minolta Chromameter. Among the 235 wild specimens, 154 had been collected under 'cover' (under trees or shrubs: 90% under *Cupressus* and 10% under *Picea, Cedrus* or frondose trees) and 81 in open places (67% on horse manure and 33% on various plant wastes or in dunes). The mean L value of the strains isolated from specimens collected in open places was very significantly greater (P= 0.001) than the mean L value of the strains isolated from specimens collected under trees. Means and phenotypic classes are given in Table 1.

On average, specimens collected in open places were light brown and those collected under trees were brown. Specimens that were almost white (cream: L value between 80 and 88) were three times more numerous in the open sites than in covered sites; in the same way, the dark brown specimens were four times more numerous in the covered sites. Most of the collecting in open sites was performed at more than fifty kilometres from the coast, while most of the collecting under cypress was carried out near the coast. Therefore, the differences observed between the frequency distributions would rather reflect the differences between two populations that have evolved in different biotopes, than the biotope effect inside a homogeneous population. Genetic data[10] and physiological data[11] reveal differences between a sample of isolates from a covered site (under cypress, Dinard, on the Brittany coast, France) and a sample from an open site (on horse manure, Gradignan, near Bordeaux, France). The distinction between the frequency distributions for the cap colour of samples from these two sites is more pronounced than in the total sample (Table 1). From a colour polymorphism pre-existing

in the ancestral population(s), this situation could result from genetic drift; alternatively, lighter colour genotypes could have been selected in the population involved in open sites. In other respects, we note that the data of Xu et al.[12] indicated that 'cultivar-like' mitochondrial haplotypes were more frequent in the sample from the Bordeaux region than in the sample from the Brittany coast. This agrees with the fact that, historically, the Bordeaux population is one likely origin of the cultivars, which may have always included white strains;[7] but this also suggests that the re-introduction of cultivars (generally white since about 1930) could have been more important in the Bordeaux population. We do not believe that the introduction of cultivars alone can explain the differences observed for all the phenotypic classes between the specimens of the total sample that have been collected from 68 different sites.

Table 1. Cap colour of 235 cultivated isolates of *Agaricus bisporus* and habitat of the wild specimens from which they were issued

		Habitat			
		Total sample[a]		Isolates from two sites[b]	
		open sites	under tree	open site	under cypress
L classes[c]	[50-60] dark brown pileus	4.9 %	20.1 %	12.5 %	14.3 %
	[60-70] brown pileus	35.8 %	57.1 %	18.7 %	85.7 %
	[70-80] light brown pileus	48.2 %	19.5 %	56.2 %	00.0 %
	[80-88] cream pileus	11.1 %	3.3 %	12.5 %	00.0 %
Mean L		71.20	65.87	71.93	63.49
Number of isolates		81	154	16	21

[a] 235 isolates from 68 different sites (including the Gradignan and Dinard sites, France)
[b] 37 isolates from two sites located about 450 km apart: site of Gradignan near Bordeaux, on horse manure (open site), and site of Dinard near Saint Malo on the Brittany coast, under *Cupressus macrocarpa*. All 37 isolates have different genotypes[12]
[c] Luminance measured with a chromameter on the pilei of sporocarps produced by the isolates cultivated in standard conditions.

From a genus-wide point of view, the white cap is a convergent trait that has resulted from multiple independent mutations. Albino mutants could appear in any coloured species; *A. augustus* Fr. is one documented example.[13] Then, depending on selection and/or genetic drift, the white phenotype could either disappear, persist, or finally predominate to fixation. On the other hand, since the white cap trait appears in most of the sections or main clades of the genus *Agaricus*, we cannot exclude that it might have resulted from an ancestral polymorphism for colour and that diverse alleles have descended through multiple lineages, becoming fixed in some. To know whether homologous genes are implicated, the identification of the *PPC1* gene and its alleles is essential.

In genus *Agaricus*, there are at least two multispecies clades in which all the species are white or yellowish, suggesting that the common ancestor has already lost its brown pigment. The first clade includes two white species (*A. bernardii* and *A. gennadii*) that have been excluded from section *Duploannulati*.[5] The second one is a large clade belonging to section *Arvenses*, which includes at least ten white to yellowish species. This clade corresponds to the clade I/A defined by Geml et al, [6] in which we exclude i) *A. blazei* that is at the basis of the clade, and ii) the sample W17 considered as *A. subrufescens* but that will have to be revaluated since its sequence disagrees with other sequences of this species (unpublished results) and with the recent analysis of Kerrigan[14] concluding that *A. blazei* Murr sensu Heineman is synonym to *A. subrufescens*. Further analyses should be carried out to confirm such 'white clades' when more related species have been sequenced.

The white cap colour of the species belonging to these 'white clades' simply results from the phylogenetic transmission of a genetic determinant for the white cap trait. They include species having open habitat as *A. macrosporus* and species having a covered habitat such as *A. sylvicola*. The absence of brown/white polymorphism among (and in) the white species constituting terminal branches of such clades suggests that the reappearance of the brown pigments in a white entity would be a much less frequent process than its loss in brown entity. In many other clades of the genus, coloured and white species coexist. Presently, we do not consider the *Agaricus* section and the possibly polyphyletic *Sanguinolenti* section because their phylogeny is not yet suffi-

ciently well known. In contrast phylogenetic analyses were performed for sections *Duploannulati*[5] and *Xanthodermatei* (Kerrigan RW, Callac P, Challen M, Guinberteau J, Parra, LA, to be published). Among the 23 species analysed from both sections, five have a white cap. Three of the five seem to have definitely lost their brown pigment (*A. menieri, A. bitorquis and A. devoniensis*) while *A. xanthodermus* and *A. xanthodermulus* can sometimes be grey. These five white species are positioned in four different robust distal clades that are indicated by capital letters in Figure 1. This figure shows that white species tend to have open habitats. This is the case also inside the clade to which they belong except for the clade D in which *A. laskibarii* is grey while it was collected in dunes; however, its main habitat is not well known since this species was collected only once.

Pileus colour/species	Habitat					
	1 open	2 open (can be cover)	3 open or cover as well	4 cover (can be open)	5 cover	mean
white						1.7
A. menieri	**A**					
A. devoniensis		**B**				
A. bitorquis		**C**				
white (can be coloured)						2.5
A. xanthodermus		**A**				
A. xanthodermulus[a]			**D**			
coloured (can be white)						3.3
A. pseudopratensis	e					
A. californicus[a]			**D**			
A. bisporus			**B**			
A. iodosmus			f			
A. subfloccosus					**B**	
A. hondensis					g	
coloured (eventually only at the disk)						4.0
A. laskibarii	**D**					
A. vaporarius			**C**			
A. moelleri				**A**		
A. tollocanensis[a]				h		
A. freirei				g		
A. rotalis				i		
A. endoxanthus				i		
A. placomyces				f		
A. parvitigrinus					**D**	
A. phaeolepidotus					g	
A. pocillator					h	
A. cupressicola					j	

Figure 1. Relationship between habitat and pileus colour for 23 species of *Agaricus* sections *Duploannulati and Xanthodermatei*
Species represented by the same letter belong to the same clade (phylogeny based on ITS sequences). Clades including a white species are in bold capital. Open habitat = dune, grassland, roadside, horse manure; cover habitat = wood, under tree; park and glade are intermediary.
[a] New species: *A. xanthodermulus* Callac & Guinberteau, *A. parvitigrinus* Guinberteau & Callac, *A. tollocanensis* Callac & Mata.[29, 30]

Incidentally, we note that all three secotioïd species recently included in genus *Agaricus* have typical open habitats and are white. This is not significant for *A. inapertus* (= *Endoptychum depressum*) which belongs to the clade of section *Arvenses* entirely constituted of white species, but this is significant for *A. texensis* (= *Longula*

texensis) and *A. aridicola* (= *Gyrophragmium dunalii*) that are not included in this clade, according to Geml et al.[6]

These observations and the following testable arguments highlight that the white cap could represent an advantage in open habitat. Possibly white sporocarps were selected among populations where both white and coloured phenotypes coexisted simply because they better resisted the temperature elevation and dehydration under the sunlight. Smoothness, which may be a genetically independent trait, could also improve the reflectance and reduce dehydration by sealing the upper surface of vapour exchange. It is possible to test whether a smooth white strain better resists the effects of sunshine: cultures of brown squamulose and smooth white sporocarps of *A. bisporus*, exposed to such conditions, would have to be compared to similar cultures remaining under standard conditions (i.e. in the obscurity with 90% humidity) for the temperature inside the sporocarps, the yield, and the global production of spores. However, the number of loci controlling smoothness in this species, and their genotypes and overall phenotype, would also need to be considered for each strain. A fair test would either compare otherwise isogenic lines or would comprise a large number of genetically diverse strains.

3 'Phenol' Odor, Yellow Discolouration, and Toxicity

Although the odour of the sporocarp is a relatively subjective trait, it is very useful to characterize several sections of the genus *Agaricus*. This is reinforced by emerging phylogenetic data, as for example the exclusion of the two species having unpleasant 'fusty odour' (*A. bernardii* and *A. gennadii*) from the monophyletic section *Duploannulati*[5] that is now entirely characterised by a pleasant odour of 'button mushroom'. As presumed, sections *Minores* and *Arvenses* characterized by odour of 'almonds or aniseed' appear to be close to each other.[6] Finally, species having a 'phenol' odour or Indian ink odour constitute the monophyletic section *Xanthodermatei* (Kerrigan RW, Callac P, Challen M, Guinberteau J, Parra, LA, to be published).

Volatile components contributing to these odours can be multiple. Most of these natural substances belong to various but usual classes of chemical products biosynthesized from the lipidic, shikimic and terpenic pathways. Major aroma compounds are: C_8-derivatives as oct-1-en-3-ol for the button mushroom odour;[15] aromatic components for the almond odour of species from section *Arvenses* as *A. augustus*, *A. aridicola*, *A. essettei*, *A. subrufescens*, and *A. sylvicola*;[16-19] and phenol for the 'phenol' odour of species of section *Xanthodermatei* as *A. xanthodermus* and *A.* moelleri.[20, 21] It should be mentioned that Chen & Wu[22] also found the latter compound in *A. subrufescens* but to a much lower extent. Finally, we note that *A. bernardii* with its unpleasant fusty odour remains to be studied.

From a genus-wide point of view, there is no evidence that the ' almonds', 'phenolic' or 'fusty' odours have appeared several times. The reported exception concerning a herbarium sample determined as *A. spissicaulis* (theoretically with an almond odour) but placed in the *Duploanulati*[4, 6] apparently results from an incorrect determination.[5] Sequence data from our own specimen of *A. spissicaulis* excludes this species from section *Duploannulati* in agreement with traditional classifications (unpublished).

If future phylogenetic analyses, based on more species, confirm predictions and recent indications that these various odour traits are synapomorphies, they would indeed represent crucial characters for the taxonomy of the agarics. It should be then attractive to study their genetic basis.

By examining in more detail the volatile components of all the species cited above, we observed significant variations in the ratio [% of C_8 compounds / % of benzenic compounds] (unpublished). This ratio is 0-3% / 100-97% for the *Arvenses*, 50% / 50% for both the *Duploannulati* and *Xanthodermatei*, and 75% / 25% for *A. campestris*[23] that belongs to section *Agaricus*. These ratios agree with the phylogenetic closeness of sections *Duploannulati* and *Xanthodermatei*[5] and would therefore merit to be confirmed by analysing more species. We note that a negative Schaeffer reaction in species of both sections represents another biochemical similarity. The biochemical shift at the origin of a new odour does not necessarily correspond to the biosynthesis of a new volatile component but can be a modification of the ratio between the pre-existing components, some of them

increasing over their threshold of perception. Interestingly, in section *Xanthodermatei*, the 'phenol' odour is faint in the basal clades of the section and strong in the terminal clades (Figure 2). This gradient exists in parallel for another trait, i.e., the yellow discolouration by scratching, which is also weak in the basal clades but chromium yellow in the terminal clades. We may note that 'phenol' odour and discolouring, when faint, are both mainly perceptible at the base of the stipe and only after scratching. This suggests a biochemical relationship between the two organoleptic characters that remains to be elucidated.

Clades[a]/species		Discolouration by handling, scratching, or cutting	'Phenol' odour
Most basal clades	*A. hondensis* *A. freirei* *A. phaeolepidotus*	Faint yellow, not always visible, at the basis of the stipe, generally followed by pinkish, pinkish brown, or vinaceous brown discolouration.	Faint, mainly at the basis of the stipe, often mostly perceptible soon after scratching.
	A. pseudopratensis		More or less pronounced
	A. laskibarii *A. xanthodermulus*[b] *A. californicus* *A. parvitigrinus*[b]	Pale yellow to yellow discolouration by scratching or cutting the basis of the stipe (surface and cortex), less frequently on the edge of the pileus and on the annulus. Late brownish discolouration on stipe and pileus surfaces.	
	A. tollocanensis[b] *A. pocillator*		
Most distal clades	*A. endoxanthus* *A. rotalis* *A. placomyces* *A. iodosmus*	Typical immediate chromium yellow discolouration at the basis of the stipe by handling or by cutting; Yellow discolouration more or less visible on the pileus. Very late brownish discolouration on stipe and pileus surfaces.	
	A. menieri *A. moelleri* *A. xanthodermus*		Pronounced

Figure 2. Relationship between the phylogeny and the intensity of the odour and the discolouration in the *Xanthodermatei* section

[a] Phylogeny of the *Xanthodermatei* section based on ITS sequences (according to Kerrigan et al)
[b] New species: *A. xanthodermulus* Callac & Guinberteau, *A. parvitigrinus* Guinberteau & Callac, *A. tollocanensis* Callac & Mata.[29, 30]

The intensity gradient of the 'phenol' odour trait may reflect a gradient of the phenol content: in *A. hondensis*, which belongs to the most basal clade of the *Xanthodermatei*, *p*-quinol (= hydroquinone), which does not contribute to the 'phenol' odour, was detected in relatively high concentration and considered by Jovel *et al.* [24] as biologically active against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and responsible for the toxicity of this species for human. In contrast, for *A. xanthodermus* that belongs to the terminal clade, *p*-quinol and 4,4'-dihydroxybiphenyl are in lower concentrations than the phenol detected in sufficiently high concentration (ca. 0.08 % fresh weight) to account for the toxicity of *A. xanthodermus*.[20]

We believe that a major biochemical shift or series of shifts that occurred in the *Xanthodermatei* clade is responsible for the high concentration in certain phenolic compounds contributing to the 'phenol' odour, the toxicity, and possibly the yellow discolouration. We note that these three traits are not observed in the sister section *Duploannulati*. The high concentration of phenol is only found in the most distal clades of section *Xanthodermatei* (i.e., those to which presumably belong the most recently differentiated species). This situation results therefore not only from the presumed initial shift but also from later biochemical modifications. On the other hand, *p*-quinol that was detected in high concentration in *A. hondensis* derives from phenol through hydroxylation by monooxygenases or phenolases.[25] Analyses of phenolic compounds in the two species sharing the basal with *A. hondensis*, in species belonging to each clade of section *Xanthodermatei*, and in species of section *Duploannulati*, would be essential to distinguish the role played by the presumed initial shift from the role played by more recent biochemical modifications, and to know if high concentration of *p*-quinol represents an

ancestral condition in the *Xanthodermatei*.

The edibility of all the species of section *Xanthodermatei* is considered suspect, although gastrointestinal poisonings are, in fact, only reported for the most common of them such as *A. xanthodermus* and *A. moelleri* in France. At the poison centre of Marseille, in France, about 20% of the poisonings by identified mushrooms, inventoried from 1994 and 1998, resulted from ingestion of yellow staining *Agaricus* species of section *Xanthodermatei*.[26] A high phenol content such as that reported for *A. xanthodermus* (0.08%) has never been detected in any other higher basidiomycetes for which the content of phenolic components represents on average 0.05 % of the fresh weight.[27] According to Del Signore et al.[27] phenolic substances in mushrooms could be implicated in chemical defence mechanisms against insects and micro-organisms, analogous to those described for plants.

Phenolic derivative accumulation in particular parts of the plant, which represents a feeding barrier to insect and mammalian herbivores, is well documented.[28] Insects overcoming phenolic barriers to their feeding are also known. In other respects, in the case of the ruminant-plant phenolic interface, it was shown that simple aromatic form such as quinol is more efficient than certain combined forms. By analogy, phenolic constituents in *Xanthodermatei* would represent a feeding barrier, probably against mammalian and possibly against small animals or micro-organisms coming from the soil (consider that the odour, generally stronger at the basis of the stipe, reflects a higher concentration of phenolic constituents in this particularly 'vital' part of the sporocarp at the soil interface).

The increasing 'phenol' odour, through the evolution of species in section *Xanthodermatei*, suggests that the high concentration of certain phenolic components would confer a selective advantage; on the other hand, the modification of the ratio of the different toxic compounds evokes a possible adaptation or co-evolution with animals, which remains to be proven.

4 Conclusion

In the genus *Agaricus*, we have tried to integrate taxonomic, ecological, genetic and biochemical data with recent phylogenetic data. From analyses of a convergent trait, i.e., the white cap, and a synapomorphy, i.e., the 'phenol' odour, emerge new hypotheses that would have been obscure without the phylogenetic data. At the species rank, the correlation between the cap colour and the habitat would make no sense if we had included species belonging to multispecies clades in which, apparently, the white cap trait is fixed, but not the habitat. The correlation observed in other clades suggests that the white cap would represent an advantage in open sites, possibly by conferring a resistance against dryness. On the other hand, the reinforcement of the 'phenol' odour and the variation of the concentrations of phenolic toxic compounds through the evolution of the species in section *Xanthodermatei* suggest that both parameters would represent an advantage, possibly through a defence mechanism against animals or micro-organisms. This advantage could contribute to the relative success of *A. moelleri* and *A. xanthodermus*, two sister species of the terminal clade of the *Xanthodermatei*, reported in France, as the most common species of genus *Agaricus*. Although the 'phenol' odour theoretically protects it from gathering by humans, *A. xanthodermus*, which is white and grows in open or semi-open sites, is one of the main basidiomycetes mushrooms responsible for poisonings in this country. Finally, we remark that the white cap and the toxicity are, by chance, well adapted to the human demographic extension, i.e., the deforestation and the cooking. *A. xanthodermus*, which is one of the only species of its section to be cosmopolitan (at least Europe and North America, Kerrigan et al., in preparation) is maybe a good bio-indicator of human activity.

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