Overview of Agaricus Subrufescens Strains used in the Last 15 Years in Brazil and other Countries and Current Potential Materials for the Future

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Overview of *Agaricus subrufescens* strains used in the last 15 years in Brazil and other countries and current potential materials for the future

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Abstract: The mushroom *Agaricus subrufescens* has been synonymous with *Agaricus blazei* and *Agaricus brasiliensis* during the last decades and there has been much discussion with regards to the origin, distribution, and nomenclature of this mushroom. Therefore, we conducted a genetic and morphological characterization of the mycelium and mushroom of four commercial strains currently cultivated in Brazil (ABL CS7, ABL 18/01, ABL 98/11, and ABL 16/01) together with an assessment of their agronomic behavior and compared these results with those of other strains used during the last 15 years. All the *A. subrufescens* strains characterized here are phylogenetically related to the Americas/Europe specimens, bearing an internal transcribed spacer region of type A (ABL 16/01) or both types A and B (ABL 18/01, ABL 98/11, and ABL CS7). We did not find any correlation between the morphological characteristics of the mycelial colonies and the agronomic behavior of the strains. Strains ABL 98/11 and ABL 16/01 produced the best yields and morphological characteristics for the mushrooms, indicating their high weight, which enhances the commercialization of the mushroom and justifies their longstanding commercial use over the last 15 years.

Keywords: *Agaricus blazei*, *Agaricus brasiliensis*, genetic characterization, yield, screening of mushroom quality

Introduction

Agaricus subrufescens is synonymous with *Agaricus blazei* and *Agaricus brasiliensis.* Over recent decades, there have been ongoing discussions as to the origin, distribution and nomenclature of this mushroom (Kerrigan, 2005, 2007; Wasser et al., 2002, 2005). *A. subrufescens* is alternatively known as the sun, almond, or medicinal mushroom, and the cultivation history can be divided into

three periods. The first period was between approximately 1894 and 1918, as reported by Kerrigan (2005), when the species was cultivated in the United States (Falconer, 1904; Anonymous, 1984). The second period occurred between 1965 and 1997, based on reports by Iwade and Ito (1982), Iwade and Mizuno (1997), and Mizuno (1997) who described the cultivation in Brazil, Japan, China, and Korea and presented technological advances in the area of mushroom breeding. The final period began with the publication of the use of *A. subrufescens* in Brazil (Colauto et al., 2002; Eira et al., 2005; Dias et al., 2008), which allowed an increase of research teams to study this mushroom in a greater range of countries, such as Argentina, Canada, France, Slovenia, Mexico, Taiwan, and Norway, and continues to the present day (Gregori et al., 2008; González Matute et al., 2010; Chu et al., 2012; Lisiecka et al., 2013; Stoknes et al., 2013, Souza et al., 2016).

The strains used in experimental crops in the last 15 years came from Brazil, France, Spain, the United States, Mexico, Taiwan, Belgium, and Italy (Llarena-Hernández et al., 2011). In recent decades, studies have been conducted mainly with strains isolated from commercial crops (Brazil), with several strains collected from the wild (France, Spain, Thailand, and others). Cultivated mushrooms varied in the obtained yield, average weight of the mushrooms, and morphological characteristics (color and format) of the pileus and the stipe of mushrooms (Colauto et al., 2010, Llarena-Hernández et al., 2013). To avoid these variations and and combine the favorable characteristics of isolates and wild-collected specimens, several hybrids have been developed (Zied et al., 2011; Jatuwong et al., 2014); however, it is unknown exactly why these hybrids have not yet been used commercially.

Studying the genetic variation of Japanese and Brazilian strains, Fukuda et al. (2003) reported the low degree of difference between the isolates and suggested that the strains could have come from the same wild-type population of *A. brasiliensis*. Finally, the authors suggested that the development of studies of genetic divergence among strains should be integrated into several other

aspects of growth such as physiology and productivity to enable a precise and consistent strain analysis and efficient development of superior strains for cultivation.

In this sense, the present manuscript studied in detail the commercial strains currently cultivated in Brazil and compared them with the strains used in the last 15 years in several publications. We carried out a genetic and morphological characterization of the mycelium and mushroom of four selected strains and also evaluated their agronomic behavior.

Materials and methods

The experiment was divided into four evaluations. The first referred to genetic characterization, the second to mycelial morphological characterization, the third to morphological characterization of the fruitbodies, and the fourth to the agronomic behavior of the mushroom strains. Four commercial strains of *A. subrufescens* were used: ABL CS7 (acquired from the Federal University of Lavras, Brazil – MW200295.2 GenBank number); ABL 18/01 (isolated from a grower in the region of São Paulo, Brazil – MW200293.2 GenBank number); ABL 98/11 (isolated from growers in the region of Mogi das Cruzes, Brazil – MW200294.2 GenBank number); and ABL 16/01 (isolated from commercial spawn lab in Valinhos, Brazil – MW200292.1 GenBank number). The strains were deposited in the collection of the Centro de Estudos em Cogumelos, from the Universidade Estadual Paulista, Câmpus de Dracena.

Genetic characterization of the A. subrufescens strains

To genetically characterize *A. subrufescens* ABL 16/01, ABL 18/01, ABL 98/11, and ABL CS7 strains, we performed amplification, cloning, and DNA sequencing of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA), which is widely used in fungal taxonomy (Schoch et al., 2012). Initially, 100 mg of *A. subrufescens* mycelium was added to 2.0 mL innuSPEED lysis tubes containing steel beads (5 × 4.7 mm) and 400 µL of buffer AP1 (DNeasy Plant Mini Kit, QIAGEN) for tissue disruption. Samples were homogenized for two cycles of 1 min each in the SpeedMill PLUS equipment (Analytik Jena). Then, 4 µL of RNase A was added to each tube, followed by incubation at 65 °C for 10 min. All following steps on genomic DNA extraction were performed as recommended by manufacturer (DNeasy Plant Mini Kit, QIAGEN).

Genomic DNA samples were eluted in sterile distilled water and quantitated using a NanoPhotometer® (IMPLEN). PCR reactions were carried out in a final volume of 25 µL with Platinum Taq DNA Polymerase (Invitrogen) using 50 ng of genomic DNA and conserved primers that flank a rDNA region containing the ITS sequences: ITS5F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) (White, 1990). Reactions were incubated in a SimpliAmp thermal cycler (Applied Biosystems) at 94 ºC for 2 minutes, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, and DNA extension at 72 °C for 1 min. PCR products were separated by electrophoresis on a 1.5% agarose gel for 35 min at 130 V. DNA bands (~700 bp) were excised, purified using the GenElute™ gel extraction kit (Sigma Aldrich) in a volume of 40 µL, and quantitated with the NanoPhotometer®.

Purified PCR amplicons were cloned into pGEM-T Easy vectors (Promega) at a molar ratio of 5 (insert): 1 (vector), according to the manufacturer's instructions. Chemocompetent *Escherichia coli* Stellar cells were transformed with ligation reactions. Reactions were plated in selective LB media containing ampicillin and incubated at 37 °C for 16 h. Positive clones were confirmed by plasmid DNA extraction, followed by EcoRI digestion and electrophoresis in 1.5% agarose gel.

DNA sequencing reactions were prepared with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using 5 µL of plasmid DNA (100 ng/µL) and 2.5 µL of 5 µM sequencing primers M13F (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) or M13R (5′-CAGGAAACAGCTATGAC-3′). Four clones of each sample were sequenced in duplicate using the Sanger method with an ABI 3730 DNA Analyzer (Applied Biosystems), at the Centro de Pesquisa sobre o Genoma Humano e Células-Tronco da Universidade de São Paulo (CEGH-USP, Brazil).

Consensus sequences for each *A. subrufescens* strain were obtained from the analysis of all DNA sequencing replicates using the software Geneious Prime® 2020.2.4 (Biomatters). MUSCLE multiple alignments were performed using these consensus sequences and 23 *A. subrufescens* ITS sequences from different geographic regions (Table S2). A consensus tree was generated from the Geneious tree builder using the Neighbor-Joining method and Jukes-Cantor genetic distance model, with 10,000 replicates at the bootstrap resampling method.

Mycelial morphological characterization

For mycelial characterization, a petri-dish compost agar was used, following the methodology reported by Jones et al. (2017). Fresh compost used for *A. subrufescens* production was dried for 48 hours at a temperature of 60 °C. One liter of distilled water was added to 60 g of dried compost and the mixture boiled for 30 min. The boiled mixture was filtered, and 15 g of agar was added and sterilized at 121 °C for 30 min. Culture medium was transferred to petri dishes (90 mm in diameter), and after cooling, was inoculated with a mycelium disc (10 mm in diameter) from the respective strains. After 12 days, colonies were classified into different types (Jones et al., 2017), which were determined prior to analysis.

• Type 1: Cottony mycelium, with uniform radial distribution, presence of homogeneous aerial mycelia (throughout the colony), with rapid mycelial growth.

• Type 2: Rhizomorphic mycelium, thick, with uniform radial distribution, presence of partial aerial mycelia (only in the center of the colony), and rapid mycelial growth.

• Type 3: Cottony mycelium, with uneven radial distribution, presence of homogeneous aerial mycelia, and intermediate mycelial growth.

• Type 4: Rhizomorphic mycelium, thick, with uneven radial distribution, presence of partial aerial mycelia (only in the center of the colony), with intermediate mycelial growth.

Mushroom morphological characterization

For morphological evaluation, 30 mushrooms from each strain were randomly selected before the rupture of the ring (recommended point of harvest) and the following parameters were considered: total length of the mushroom, pileus and stem, width of the stem, stem base, pileus, and ring size. The measurements were performed with the aid of a 150 mm digital caliper with 0.1 mm precision, as shown in figure 1.

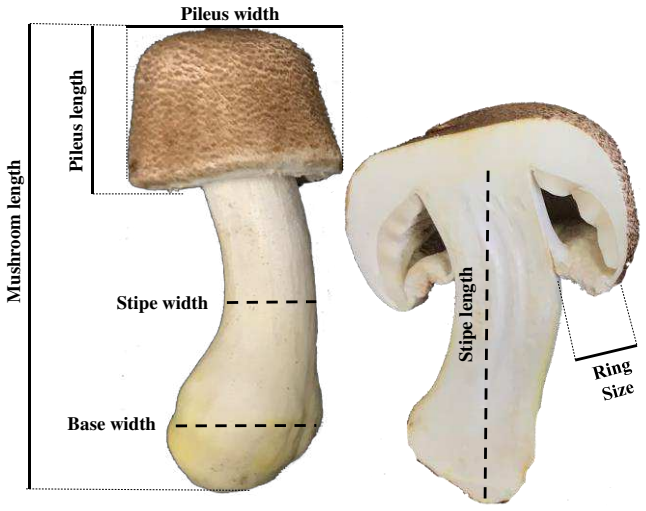

Figure 1. Evaluation of the morphological characteristics of mushrooms of the *Agaricus subrufescens* strains.

To assess coloration, a Discovery V20 Binocular Stereoscope and an Axiocam 503 color camera (Zeiss®) were used. Readings were made with two X-shaped lines, each of which had approximately 1000 points. The average of all measured points was obtained, and thus each mushroom constituted a repetition. The color data were obtained in RGB format (red, green, and blue) and analyzed separately according to the color spectrum.

Agronomic behavior

The inoculum was prepared based on sorghum grains following the production steps for selection of mushroom and production of subculture, parent spawn, and grain spawn as described by Zied et al. (2011). The compost formulation consisted of a mixture of wheat straw, chicken manure, and gypsum. Compost was produced using the traditional composting method described by Zied et al. (2014) with Phase I (a total of 26 days, with 7 days of prewetting and 19 days of fermentation) and Phase II (total of 9 days, with 8 h of pasteurization at 59 °C and 8 days of conditioning at 47 °C).

After the composting process, the substrate was distributed in plastic boxes, in equal amounts of 3.5 kg, with the inoculum added in the measure of 1% of the wet weight of the compost. Subsequently, the compost was incubated in a semi-controlled mushroom chamber (temperature and humidity control) for 20 days at a temperature of 28 ± 2 °C and relative humidity of 80 ± 5%. A casing layer based on peat moss was used to provide high water holding capacity and porosity

185 and less compaction, thus allowing gas exchange between the compost and the environment. The
186 casing layer was added to a thickness of 4 cm over the colonized compost.

187 After the colonization of the casing layer, ruffling was performed on the 27th day. The
188 primordia induction was carried out by temperature oscillation and control of the relative humidity.
189 On the 28th day, the temperature was reduced from 28 ± 2 °C to 20 ± 2 °C with a humidity of 90 ±
190 5%, following the methodology proposed by Pardo-Giménez et al. (2020a) for rapid primordia
191 induction. Then, the temperature was again increased to 28 ± 2 °C, with the same process being
192 performed in each harvest flush. The total growth cycle was 85 days (20 days of mycelial run and
193 65 days of production phase).

194 The mushrooms were harvested twice a day manually and weighed after scraping the base
195 of the stipe to remove residues from the casing layer. The yield (fresh weight of mushrooms divided
196 by fresh weight of the compost, multiplied by 100, expressed as a percentage), number of
197 mushrooms (count of mushrooms harvested), weight of mushrooms (fresh weight of mushroom
198 divided by mushroom number), precocity (yield of half the production time, started after harvesting
199 the first mushroom, divided by the total production time multiplied by 100 and expressed as a
200 percentage), and earliness (number of days between adding the casing layer and the time required
201 for the first harvest) were evaluated as described by Zied et al. (2010) and Navarro et al. (2020).

202 The experiment was carried out in a completely randomized design, with four treatments
203 (strains of *A. subrufescens*: CS7, ABL 18/01, ABL 98/11 and ABL 16/01) each with eight replicates,
204 which were represented by a 3.5 kg box of compost.

205

206 **Results**

207

208 *Genetic characterization of the A. subrufescens strains*

209

210 DNA sequencing analysis of the rDNA region from the four strains of *A. subrufescens* (ABL
211 16/01, ABL 18/01, ABL 98/11, and ABL CS7) revealed the successful amplification of a 773-bp
212 genomic region, including a partial sequence of the small subunit ribosomal RNA gene and
213 complete sequences for the ITS 1 5.8S ribosomal RNA gene and for ITS 2, a partial sequence of the
214 large subunit ribosomal RNA gene. Consensus sequences for each *A. subrufescens* strain were
215 generated from the analysis of non-redundant DNA sequences (Table S1) and deposited in the NCBI
216 nucleotide database under the accession numbers MW200292 (ABL 16/01), MW200293 (ABL
217 18/01), MW200294 (ABL 98/11), and MW20029 (ABL CS7).

218 Analysis of the polymorphic regions from a multiple alignment performed between our
219 sequences and *A. subrufescens* ITS types A, B, A/B, and C, previously characterized by Chen et al.
220 (2016), revealed the occurrence of ITS type A in the ABL 16/01 strain and both ITS types A and B
221 in the ABL 18/01, ABL 98/11, and ABL CS7 strains (Table 1).

222

223 Table 1. Nucleotides at polymorphic positions in ITS sequences of *Agaricus subrufescens* ABL 16/01, ABL
224 18/01, ABL 98/11, ABL CS7 and other 23 ITS sequences from different geographic regions (listed in Table
225 S1).

	Polymorphic positions in ITS sequences																	
	90	168	176	184	199	200	208	224	240	254	323	389	521	525	614	644	673	704
ITS A	T	A	A	A	G	A	C	T	T	T	G	A	A	T	G	A	T	T
ABL 16/01	T	A	A	A	G	A	C	T	T	T	G	A	A	T	G	A	T	T
ITS B	-	G	G	G	A	T	Y	T	T	C	G	R	A	T	R	R	T	W
ITS A/B	T/-	A	R	R	R	W	Y	T	T	Y	G	A	A	T	G	R	T	T
ABL 18/01	T/-	A	R	R	R	W	Y	Y	T	Y	G	A	A	T	G	R	Y	T
ABL 98/11	T/-	A	R	R	R	W	Y	T	T	Y	G	A	A	T	G	R	T	T
ABL CS7	T/-	A	R	R	R	W	Y	T	Y	Y	G	A	A	T	G	R	T	T
ITS C	-	A	G	G	A	T	C	T	T	C	A	A	C	-	G	A	T	T

226

In addition to polymorphisms at 13 positions (90, 168, 176, 184, 199, 200, 254, 323, 389, 521, 525, 614, and 704), previously reported by Chen et al. (2016), we found two other new polymorphisms at positions 208 and 644 for ITS type B and for ABL 18/01, ABL 98/11, and ABL CS7 strains. DNA sequencing of more clones is needed to confirm if the polymorphisms at positions 224, 240, and 673 represent true minor variants or PCR/sequencing artifacts.

A consensus tree built from the multiple alignments between the ITS sequences described here and another 23 ITS sequences of *A. subrufescens* from the Americas, Asia, Europe, and Oceania revealed that all the strains analyzed in this work are phylogenetically closer to the Americas and Europe specimens (ITS types A, B, or both A/B) than to the Asia and Oceania sequences, characterized by specimens bearing the ITS type C (Fig. 2).

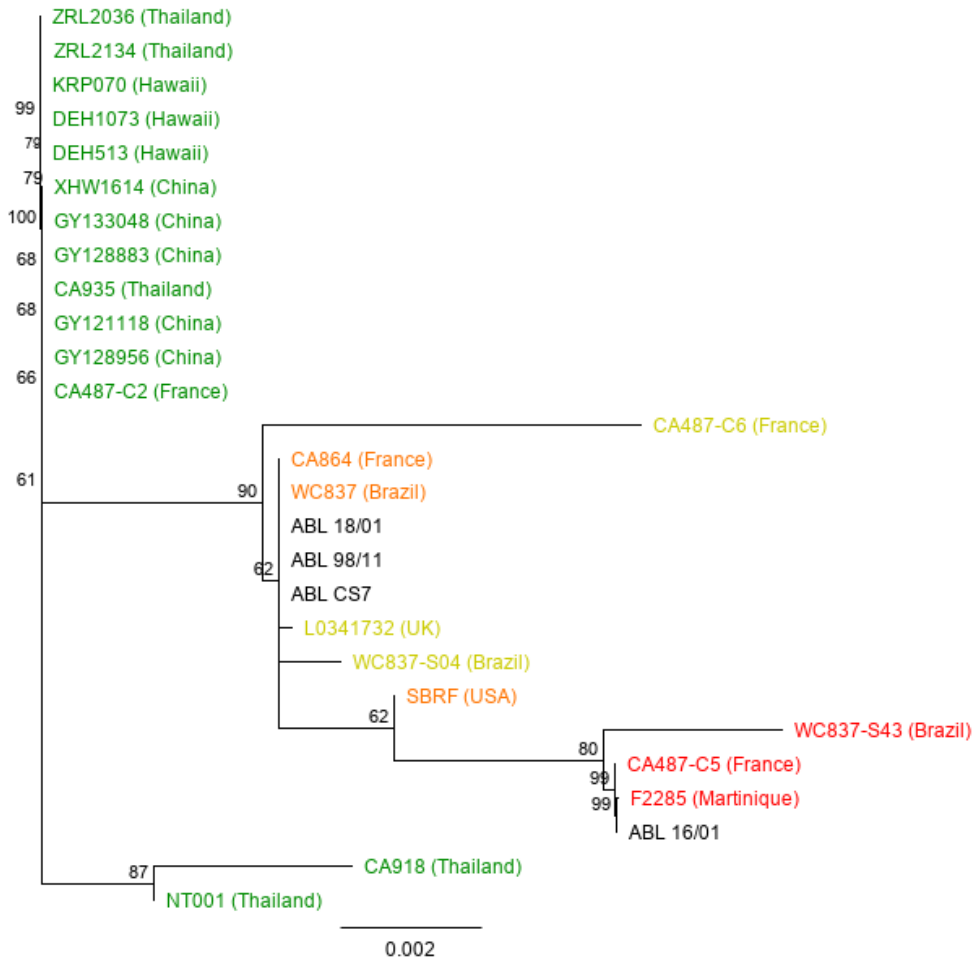

Figure 2. Neighbor-joining consensus tree from ITS sequences of *Agaricus subrufescens* ABL 16/01, ABL 18/01, ABL 98/11, ABL CS7 strains and another 23 ITS sequences of *A. subrufescens* from different

geographic regions. Different colors are used to illustrate the types of *A. subrufescens* ITS sequences: red for ITS A; yellow for ITS B; green for ITS C; and orange for ITS A/B, following the classification proposed by Chen et al. (2016). Bootstrap support values greater than 50% and the scale of branch lengths are indicated.

Mycelial morphological characterization

Mycelial morphology was classified by the mycelial structure (rhizomorphic or cottony), radial growth distribution (uniform and uneven), presence of aerial hyphae (homogeneous and partial), and time for colonization of the culture medium (10 and 15 days).

The ABL CS7 strain produced rhizomorphic and aerial mycelia at the beginning of growth (partial) and took longer for colonization (15 days) with uneven radial growth; therefore, this strain was classified as Type 4. The ABL 98/11 strain had the same morphological characteristics as Type 4 but differed with respect to having cottony mycelium and was therefore classified as Type 3 (Fig. 3).

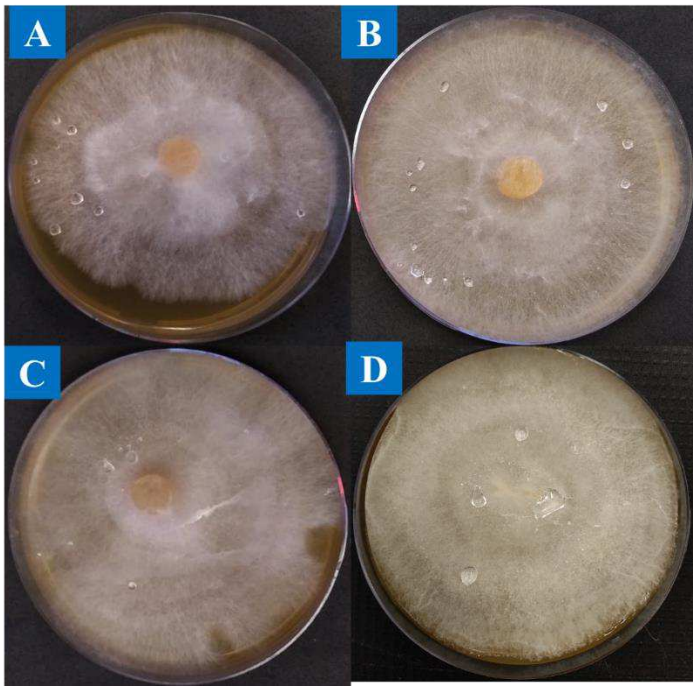

Figure 3. Morphological characteristics of the mycelial growth of the strains, A (ABL CS7), B (ABL 18/01), C (ABL 98/11), and D (ABL 16/01).

However, the ABL 18/01 strain produced rhizomorphic and aerial mycelia at the beginning of growth (partial), had only a short colonization time (10 days) with radial growth and was classified as Type 2. The ABL 16/01 strain showed the same morphological characteristics but differed with respect to having cottony mycelium and homogeneous aerial hyphae and was therefore classified as Type 1.

Mushroom morphological characterization

Evaluation of the morphology of mushrooms revealed a significant difference between the strains, with mushroom length being the only non-significant factor. When analyzing the length and width of the pileus, the ABL 16/01 and 98/11 strains had the highest values and ABL CS7 and ABL 18/01 strains had the lowest. Regarding the size of the ring, the ABL 16/01 strain was the largest, whereas the ABL CS7 and 18/01 strains were smaller. In terms of the length of the stipe, the ABL CS7 strain had the highest value. The widths of the stipe and of the base were largest in ABL 16/01 followed by those of the ABL 98/11 strain, which are considered to have more robust fruit bodies than those of the ABL CS7 and 16/01 strains (Table 2).

Another morphological characteristic evaluated was the color of the pileus. Significant differences were found between all parameters evaluated; the ABL CS7 strain had the lowest values for color and was classified as a strain with darker pileus.

294 Table 2. Morphological analysis of the stipe and pileus.

	Morphological analysis (mm)						
Strain	Mushroom length	Pileus length	Pileus width	Ring size	Stipe length	Stipe width	Base width
ABL CS7	102.35 a	37.34 c	34.92 c	14.22 c	94.35 a	19.12 c	30.24 c
ABL 18/01	95.25 a	37.35 c	28.30 d	10.50 c	83.25 b	18.45 c	27.75 c
ABL 98/11	98.53 a	46.94 b	38.10 b	14.94 b	85.78 b	21.84 b	33.83 b
ABL 16/01	97.29 a	65.15 a	41.10 a	16.61 a	82.24 b	26.68 a	42.46 a
CV (%)	10.28	15.98	12.78	18.75	11.92	19.48	15.66

295

296 The strains ABL 16/01 and ABL 18/01 had statistically equal values and the intermediate color
297 of the pileus. Finally, the ABL 98/11 strain showed the highest color value, with a clear pileus color.
298 The combination of the three colors can be transformed into a hexadecimal code, which can then be
299 used as a universal color standard (Table 3).

300

301 Table 3. Pileus color and hexadecimal code of *A. subrufescens* strains.

	RGB code (0-255)				
Strain	Red	Green	Blue	Hexadecimal code	Colors
ABL CS7	132.41 c	108.82 c	77.13 c	#846c4d	
ABL 18/01	198.55 b	169.30 b	145.15 b	#c6a991	
ABL 98/11	221.45 a	211.20 a	186.15 a	#ddd3ba	
ABL 16/01	188.20 b	161.55 b	123.80 b	#bca17b	
CV (%)	16.28	17.13	21.68		

302

303 *Agronomic behavior*

All agronomic variables exhibited significant differences in relation to the strains used, except for precociousness. ABL CS7, 98/11, and 16/01 strains had the highest yields, practically twice that of the ABL 18/01 strain (Table 4). Characterizing these strains, ABL 98/11 had the highest yield in the first flush while CS7 had the highest yield in the second flush. The precocity values were 94.98%, 93.44%, and 80.10% for ABL 98/11, ABL 16/01, and ABL CS7, respectively; ABL CS7 had a low value owing to reduced yield in the 1st flush of harvest (Fig. 3).

Table 4. Agronomic behavior of *A. subrufescens* strains after 95 days of cultivation.

Strain	Yield, %	Number of mushroom, u	Weight of mushroom, g	Precocity, %	Earliness, days
ABL CS7	11.02 a	20.0 a	18.13 c	80.10 a	34.2 b
ABL 18/01	6.64 b	12.8 b	16.53 c	91.91 a	24.8 a
ABL 98/11	14.94 a	11.6 b	43.05 a	94.98 a	20.0 a
ABL 16/01	13.21 a	13.0 b	34.29 b	93.44 a	23.4 a
CV (%)	29.09	27.98	11.87	15.74	14.71

Regarding the number and weight of mushrooms, two opposite situations were found: in the first, the ABL CS7 strain presented a greater number of mushrooms harvested with reduced weight, whereas, in the second with the ABL 98/11 strain, fewer mushrooms were harvested but with a greater weight. Earliness and precocity complete the range of positive agronomic parameters that a strain must have and help position ABL 98/11 strain as the best genetic material used in this research.

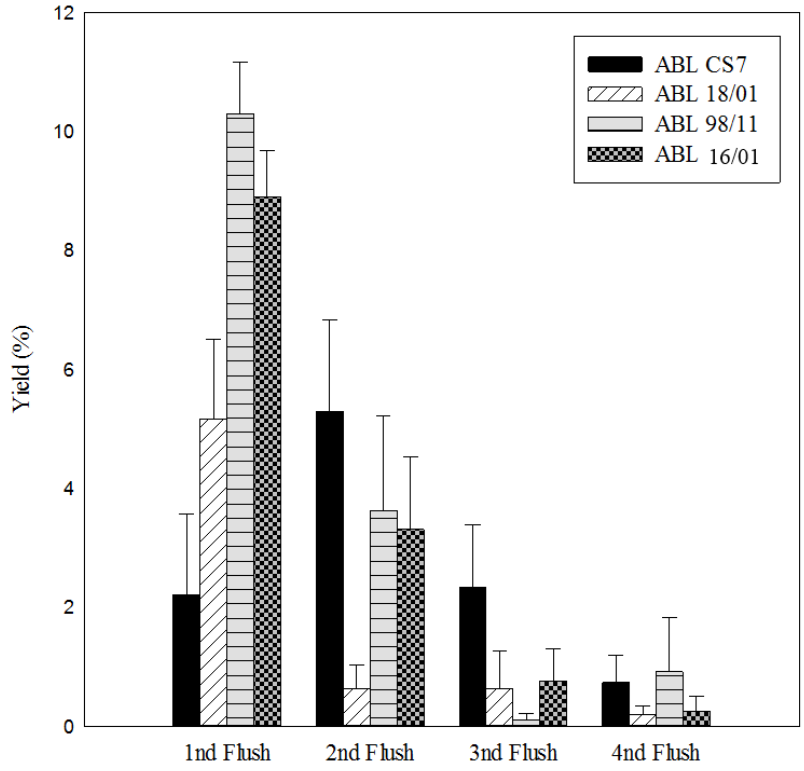

Figure 3. Distribution of yield during the cultivation cycle (4 harvest flushes).

Discussion

Agaricus subrufecens is only grown commercially in a few countries, such as Brazil, Japan, China, Taiwan, South Korea, and the United States, and this confined cultivation range has limited technological advancement to increase yield. Japan was the first country to adapt *A. bisporus* cultivation technologies to *A. subrufescens* (1997). Recently, Spanish and French researchers have also applied this type of study (Pardo Giménez at al., 2020a,b; Llarena-Hernández et al., 2014). A greater diversity of strains was used in the third period (2005 to 2020) of cultivation compared with those used in second period (1965 to 1997); these more recent strains were significantly superior than earlier ones as they included wild, isolated, and hybrid varieties.

In Brazil, the number of strains used commercially has also increased, although only three strains are still cultivated (ABL CS7, ABL 98/11, and ABL 16/01) when comparing the strains used in this study with those used in the last 15 years (Table 5). This selection is justified by the favorable agronomic behavior and morphological characteristics of these mushrooms (Table 2 and 4). In Argentina, the strains studied came from the commercial laboratory Brasmicel at the beginning of 2010 and were sent by Edson de Souza (González Matute et al., 2010, 2011, 2012).

Insert Table 5 (at the end of the manuscript owing to its landscape format)

In Europe, after 2010, two countries led the experimental studies carried out with *A. subrufescens*. In Spain, studies were carried out using Brazilian strains (Zied et al., 2011; Pardo-Giménez et al., 2016) and, in France, wild and hybrid strains were used (Thongklang et al., 2014; Foulongne-Oriol et al., 2016). Furthermore, French researchers had a great influence on the identification and dissemination of *A. subrufescens* in Thailand (Wisitrassameewong et al., 2012; Thongklang et al., 2016), whereas North American research influenced the experimental cultivation in Norway (Stoknes et al., 2013).

A. subrufescens mushrooms exhibit a high level of polymorphism in the ITS of the nuclear rDNA region (Kerrigan et al., 2005). Comparing *A. subrufescens* ITS sequences and samples from different geographic regions, Chen et al. (2016) observed a distribution of ITS type A, B, or both A/B in American and European *A. subrufescens* specimens, whereas specimens from Asia or Oceania exhibited ITS type C, with the exception of the wild French isolate CA487, that had ITS of all three types.

Our results revealed that all the *A. subrufescens* strains characterized here were phylogenetically related to the Americas/Europe specimens, bearing an ITS of type A (ABL 16/01) or both types A

and B (ABL 18/01, ABL 98/11, and ABL CS7). A previous study carried out with other strains and molecular markers showed that the strains used in Brazil had substantial genetic similarity (Tomizawa et al., 2007), suggesting a common origin. Thus, any differences in agronomic traits among these strains should be related to other genomic regions that may be assessed by various molecular methods such as simple sequence repeat (SSR) markers, which are potentially useful as a molecular tool to study genetic diversity and reproductive biology (Foulongne-Oriol et al., 2012, 2014).

Studies have shown that the mycelia of *A. subrufescens* have intermediate growth, taking an average of 10 to 20 days to colonize a 90 mm-diameter petri dish, depending on the culture medium and strains used (Neves et al. 2005). This differs from *Pleurotus ostreatus*, which has a more rapid growth, taking only 6 days to colonize a petri dish of the same size (Donini et al., 2006). In the present study, strains ABL 18/01 and ABL 16/01 took 10 days to colonize the entire culture medium, which in a commercial cultivation provides advantages as faster mycelium develops decreases the possibility of contamination of the compost by other fungi and bacteria. We did not find any correlation between the morphological characteristics of the colonies and the agronomic behavior of the strains.

Morphological characteristics of mushrooms are extremely important when selecting strains for commercial production. In Brazil, the commercialization of mushrooms is based on standards such as length (height) of mushroom, width of base and stipe, and level of ring opening (more closed mushrooms have better market value), and mushrooms are classified as extra, grade A and B, and opened, respectively (Zied et al., 2017). Therefore, mushrooms from the ABL 16/01 strain would be classified as extra mushrooms, meeting all quality standards, when the mushroom length reaches above 80 mm and base width is between 35 to 50 mm with a closed pileus. With regard to this last parameter, this strain presented an excellent ring size (16.61 mm) before veil rupture. The ABL 98/11 strain also has favorable morphological characteristics. Given all these results, the weights of

harvested mushrooms underlined the excellent commercial quality for strains ABL 16/01 (34.29 g) and ABL 98/11 (43.05 g).

The color of the mushroom pileus is related to the level of maturation of the mushroom (near the rupture of the mushroom veil this becomes clearer) and the number of small scales over the pileus. Pardo-Gimenez et al. (2020a) found that the primordial induction method influences the color of the pileus so that slow induction keeps the pileus darker when using the ABL 99/30 strain.

We found a negative correlation between mushroom weight and mushroom number ($r = -0.723$ and $P = 0.003$). This has also been observed in other studies (Chu et al., 2012; Dias et al., 2013), but differs from those obtained in research with Chinese and Japanese cultivars (Wang et al., 2010, 2013; Win and Ohga, 2018).

Finally, yield is an important parameter to be considered as an agronomic behavior, being directly related to the cultivation earliness, precocity, and crop time (growth cycle). Wild and hybrid strains from Spain, France, and Thailand have low earliness, with values from 15.1 to 30 days, whereas cultivars from Brazil, China, and Japan are tardy, with earliness values between 26.8 and 48.8 days after the addition of casing (Bechara et al., 2006; Llarena-Hernández et al., 2014; Horm; Ohga, 2008; Wang et al., 2013; Jatuwong et al., 2014).

Nevertheless, the most recent published yields reached 28.16%, with ABL 16/01 strain during a 108-day growth cycle (Zied et al., 2018) and 20.91%, with ABL 99/30 strain during an 83-day of growth cycle (Pardo-Giménez et al., 2020b). These yields were similar to the excellent yield obtained by the wild strains CA 487 (24.4%) and 438-A (26.2%), during their 85-day growth cycle, and the M7700 cultivar (21.1%), which had a 130-day growth cycle (Llarena-Hernández et al., 2014). Our current highest yield was 14.94%, with ABL 98/11 strain during an 85-day growth cycle, with commercial quality mushroom grade A, in a semi-controlled mushroom chamber, which allows lower energy expenditure for Brazilian growers. This strain provides more than 90% of the total

harvest in the first two flushes (Fig. 3), which opens the possibility of reducing the duration of the growing cycle.

Notable, *A. subrufescens* differs from *A. bisporus* because of the wide variation in yield and other agronomic behaviors obtained with the same strains in different publications. Another important point when producing commercial cultivars from other countries is the period of domestication in the place where the cultivation has migrated to. We verified this with the 99/30 strain that was first cultivated in Spain, with a yield of 4.7% after 80 days of cultivation (Zied et al., 2011a). After re-isolation in several subsequent crops, we obtained a productivity of 20.2% after 85 days of cultivation (Pardo-Giménez at al., 2020a).

Here, we emphasize the importance of obtaining a high yield, with morphologically suitable mushrooms (extra mushroom or mushroom grade A) and high dry matter. All these characteristics are required in a promising strain for commercial use with regards to studies published in the last 15 years.

Conclusions

All the *A. subrufescens* strains characterized in this work are phylogenetically related to the Americas/Europe specimens and harbor an ITS of type A (ABL 16/01) or of both types A and B (ABL 18/01, ABL 98/11, and ABL CS7). We did not find any correlation between the morphological characteristics of the mycelial colonies and the agronomic behavior of the strains. Strains ABL 98/11 and ABL 16/01 obtained the best yield results and morphological characteristics of the mushrooms, including their high weight. These characteristics enhance the commercialization of these mushroom strains and justifies their continued use over the last 15 years.

Supplementary Information

The online version contains supplementary material available (attached document online with supplementary material name)

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Authors'contributions

Diego C. Zied – Investigation, Writing and Original draft; Wagner G. Vieira Junior – data collection; Eustáquio S. Dias – Formal analysis; Douglas M. M. Soares – Formal analysis and design; Diego C. Zied and Arturo Pardo-Giménez – Conceptualization, Supervision; Cassius V. Stevani and Matheus Rodrigo Iossi – Supervision, Review and Editing.

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Data availability

All material is deposited in Sao Paulo State University (Câmpus de Dracena). The partial sequence data generated in this study are deposited in NCBI GenBank with the numbers MW200295.2, MW200293.2, MW200294.2 and MW200292.1.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

Not applicable.

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Table 5. Strains used in the last 15 years in different countries and regions.

Strains	Origin	References
ABL-97/11 = CS4	São Paulo, SP, Brazil	Eira et al. (2005)
ABL-97/12	Piedade, SP, Brazil	Braga (1999); Braga et al. (2006)
ABL-98/11 = CA 571	Mogi-da-cruzes, SP, Brazil	Llarena-Hernández et al. (2011)
ABL-99/25 = CS5	Araçatuba, SP, Brazil	Eira et al. (2005); Sousa et al. (2016)
ABL-99/26	São José dos Campos, SP, Brazil	Colauto et al. (2010a,b,c, 2011)
ABL-99/28 = CA 560	Botucatu, SP, Brazil	Eira et al. (2005); Llarena-Hernández et al. (2011); Farnet et al. (2013); Zied et al. (2012a, 2014)
ABL-99/29 = CS7	Porto Alegre, RS, Brazil	Eira et al. (2005); Kopytowski Filho and Minhoni (2004); Colauto et al. (2010c, 2011); Sousa et al. (2016)
ABL-99/30 = CA 561	Piedade, SP, Brazil	Eira et al. (2005); Kopytowski Filho (2006); Kopytowski Filho and Minhoni (2007); Llarena-Hernández et al. (2011, 2013, 2014); Favara et al. (2014); Zied et al. (2012a, 2012b, 2014); Pardo-Giménez et al. (2014, 2020a, 2020b)
ABL-01/29 = CA 570	Rio de Janeiro, RJ, Brazil	Llarena-Hernández et al. (2011, 2013, 2014)
ABL-01/44	São José do Rio Preto, SP, Brazil	Kopytowski Filho et al. (2008)
ABL-03/44 = CA 562	Lençóis Paulista, SP, Brazil	Kopytowski Filho (2006); Llarena-Hernández et al. (2011); Zied et al. (2012a, 2014); Pardo-Giménez et al. (2014);
ABL-03/49 = CA 565	Boituva, SP, Brazil	Llarena-Hernández et al. (2011, 2013, 2014)
ABL-04/49 = CA 563	São José do Rio Preto, SP, Brazil	Kopytowski Filho (2006); Andrade et al. (2007); Llarena-Hernández et al. (2011); Favara et al. (2014); Zied et al.

		(2010, 2011b, 2012a, 2014); Pardo-Giménez et al. (2014); Martos et al. (2017)
ABL-05/51 = CA 564	Bariri, SP, Brazil	Llarena-Hernández et al. (2011)
ABL-06/53 = CA 566	Brazilia, DF, Brazil	Llarena-Hernández et al. (2011); Zied et al. (2012a)
ABL-06/59	Brazilia, DF, Brazil	Zied et al. (2014)
ABL-07/58 = CA 572	Suzano, SP, Brazil	Llarena-Hernández et al. (2011); Farnet et al. (2013)
ABL-07/59 = CA 574	Atibaia, SP, Brazil	Llarena-Hernández et al. (2011)
ABL-16/01	Valinhos, SP, Brazil	Zied et al. (2018)
ABL-16/02 = ABL 18/01	Valinhos, SP, Brazil	Zied et al. (2018)
ABL-16/03	Minas Gerais, MG, Brazil	Zied et al. (2018)
A. blazei	Fazenda Guirra, SP, Brazil	Gern et al. (2010)
BZ-04	Brasmicel, Suzano, SP, Brazil	Cavalcante and Gomes (2005); Cavalcante et al. (2008)
BZ-ae	Brasmicel, Suzano, SP, Brazil	González-Matute (2009)
BZ-7	Brasmicel, Suzano, SP, Brazil	González-Matute (2009)
BZ-PL	Brasmicel, Suzano, SP, Brazil	González-Matute (2009); González-Matute et al. (2011)
CS1	Vitoria, ES, Brazil	Siqueira et al. (2009, 2011); Dias et al. (2013, 2014); Sousa et al. (2016)
CS2	Belo Horizonte, MG, Brazil	Dias et al. (2013, 2014); Martos et al. (2017); Sousa et al. (2016)
CS9	Elói Mendes, MG, Brazil	Sousa et al. (2016)

CS10	UFLA, Brazil	Figueirêdo et al. (2013); Sousa et al. (2016)
WC837 = CA454 = ATTC 76739	Brazil (PennState Mushroom Spawn Lab)	Bechara et al. (2008); Llarena-Hernández et al. (2011, 2013, 2014); Farnet et al. (2013)
WC838 = CA 455	Brazil (PennState Mushroom Spawn Lab)	Llarena-Hernández et al. (2011)
CA567	Bois de Berquit, Dion de Val, Belgium	Llarena-Hernández et al. (2011)
M7700 = CA 646	Mycelia BVBA, Belgium	Gregori et al. (2008); Llarena-Hernández et al. (2011); Stoknes et al. (2013); Farnet et al. (2013)
M7703 = CA 647	Mycelia BVBA, Belgium	Llarena-Hernández et al. (2011); Farnet et al. (2013)
PA93 = CA 487	Saint-Léon, Gironde, France	Llarena-Hernández et al. (2011, 2013, 2014); Farnet et al. (2013)
CA 516	Saint-Léon, Gironde, France	Llarena-Hernández et al. (2011)
CA 643	Le Pian Médoc, Gironde, France	Llarena-Hernández et al. (2011, 2013, 2014)
ATCC 76739-3 x CA487-100 = CA454-3 x CA487-100	Hybrid, INRA, France	Llarena-Hernández et al. (2011, 2013, 2014)
CA 603	Tlaxcala, Mexico	Llarena-Hernández et al. (2011)
ER-1 = CA 462	Hawaï, USA	Llarena-Hernández et al. (2011)
ARAN559 = CA 438-A	Coll. LA Parra, Spain	Llarena-Hernández et al. (2011, 2013, 2014)
24b-01 = CA 536	Comacchio, Italy	Llarena-Hernández et al. (2011)
837	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)
838	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)

853	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)
1105	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)
2603	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)
Brazil	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)
Ma-He	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)
Si-2.2	Corvinus University of Budapest, Hungary	Geösel (2011); Geösel and Györfi (2008)
BCRC36814^{T}	Food Industry Research and Development Institute, Hsin‐Chu, Taiwan	Chu et al. (2012)
CA 276	Coll. W Chen, Taiwan	Llarena-Hernández et al. (2011)
KS-72	Kyushu University, Fukuoka, Japan	Pokhrel and Ohga (2007); Horm and Ohga (2008)
KUMB1221	Kyushu University, Fukuoka, Japan	Win and Ohga (2018)
SH26	Institute of Edible Fungi, Shanghai, China	Wang et al. (2010, 2013)
MFLUCC 11-0653 = CA 918	Chiang Rai, Thailand	Jatuwong et al. (2014); Thongklang et al. (2014)
CA918-075 x CA454-4	Hybrid, Mae Fah Luang University, Thailand	Jatuwong et al. (2014)
CA918-076 x CA454-4	Hybrid, Mae Fah Luang University, Thailand	Jatuwong et al. (2014)
CA918-075 x CA487-35	Hybrid, Mae Fah Luang University, Thailand	Jatuwong et al. (2014)

CA918-076 x CA487-35	Hybrid, Mae Fah Luang University, Thailand	Jatuwong et al. (2014)
VAB	România	Rózsa et al. (2017)

Figures

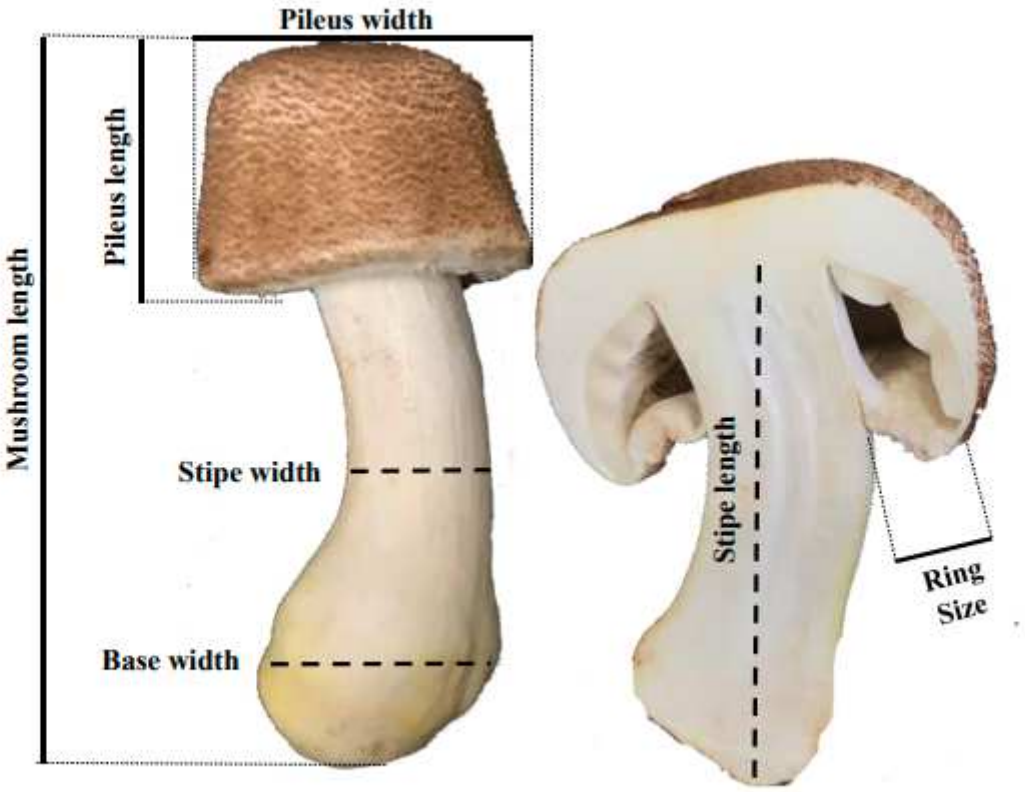

Figure 1

Evaluation of the morphological characteristics of mushrooms of the Agaricus subrufescens strains.

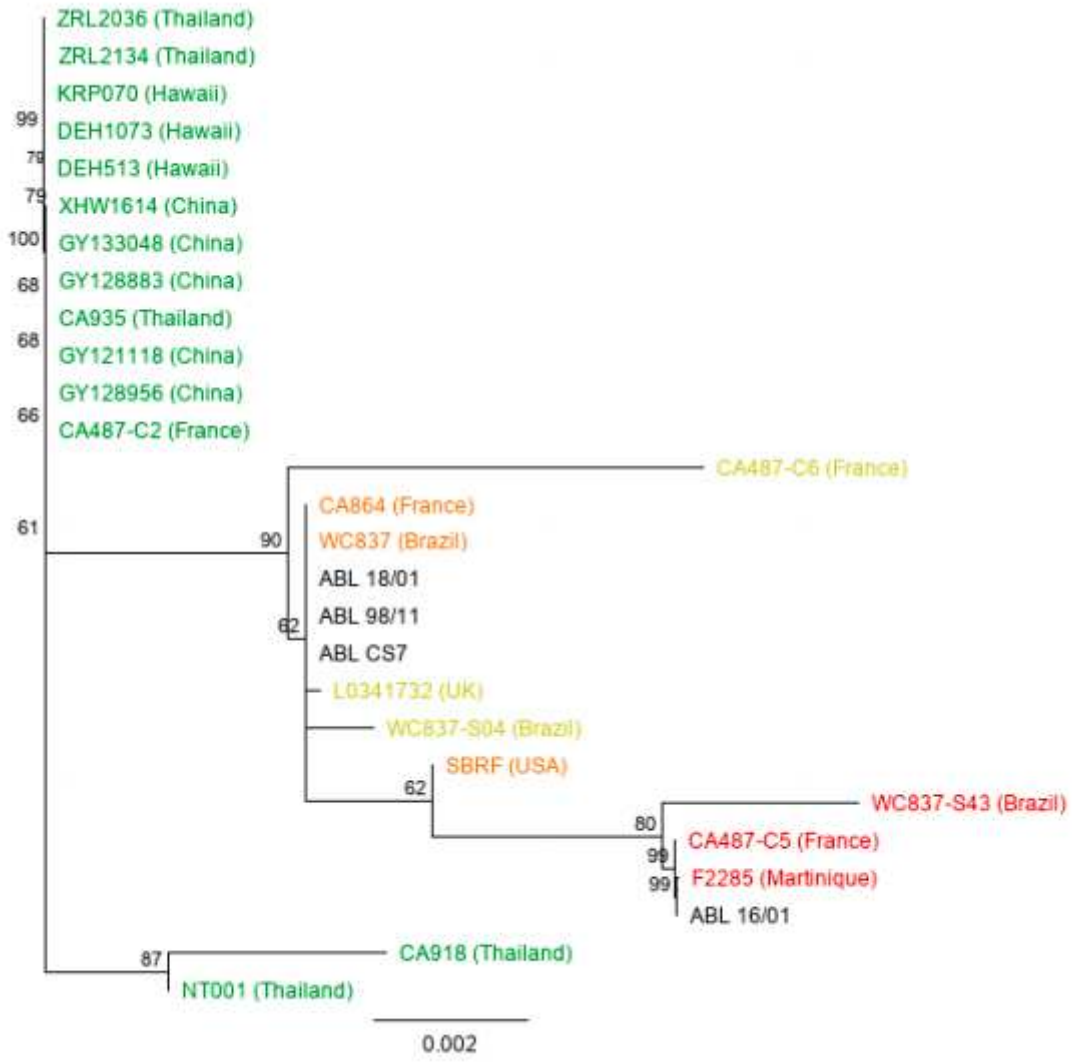

Figure 2

Neighbor-joining consensus tree from ITS sequences of Agaricus subrufescens ABL 16/01, ABL 18/01, ABL 98/11, ABL CS7 strains and another 23 ITS sequences of A. subrufescens from different geographic regions. Different colors are used to illustrate the types of A. subrufescens ITS sequences: red for ITS A; yellow for ITS B; green for ITS C; and orange for ITS A/B, following the classification proposed by Chen et al. (2016). Bootstrap support values greater than 50% and the scale of branch lengths are indicated.

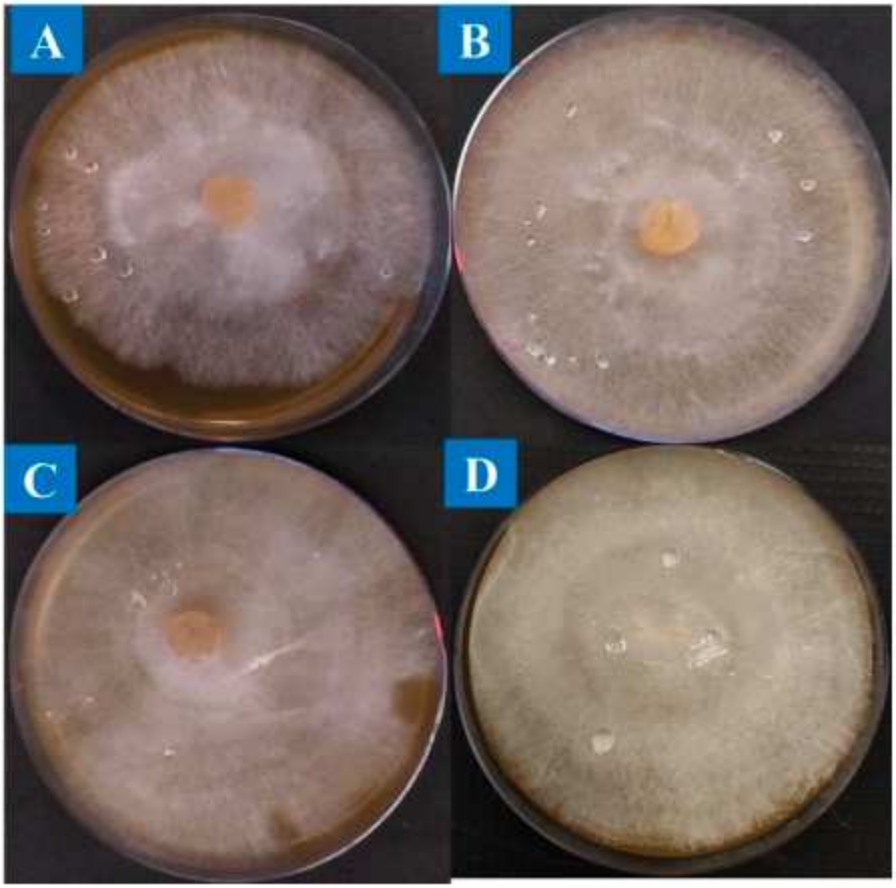

Figure 3

Morphological characteristics of the mycelial growth of the strains, A (ABL CS7), B (ABL 18/01), C (ABL 98/11), and D (ABL 16/01).

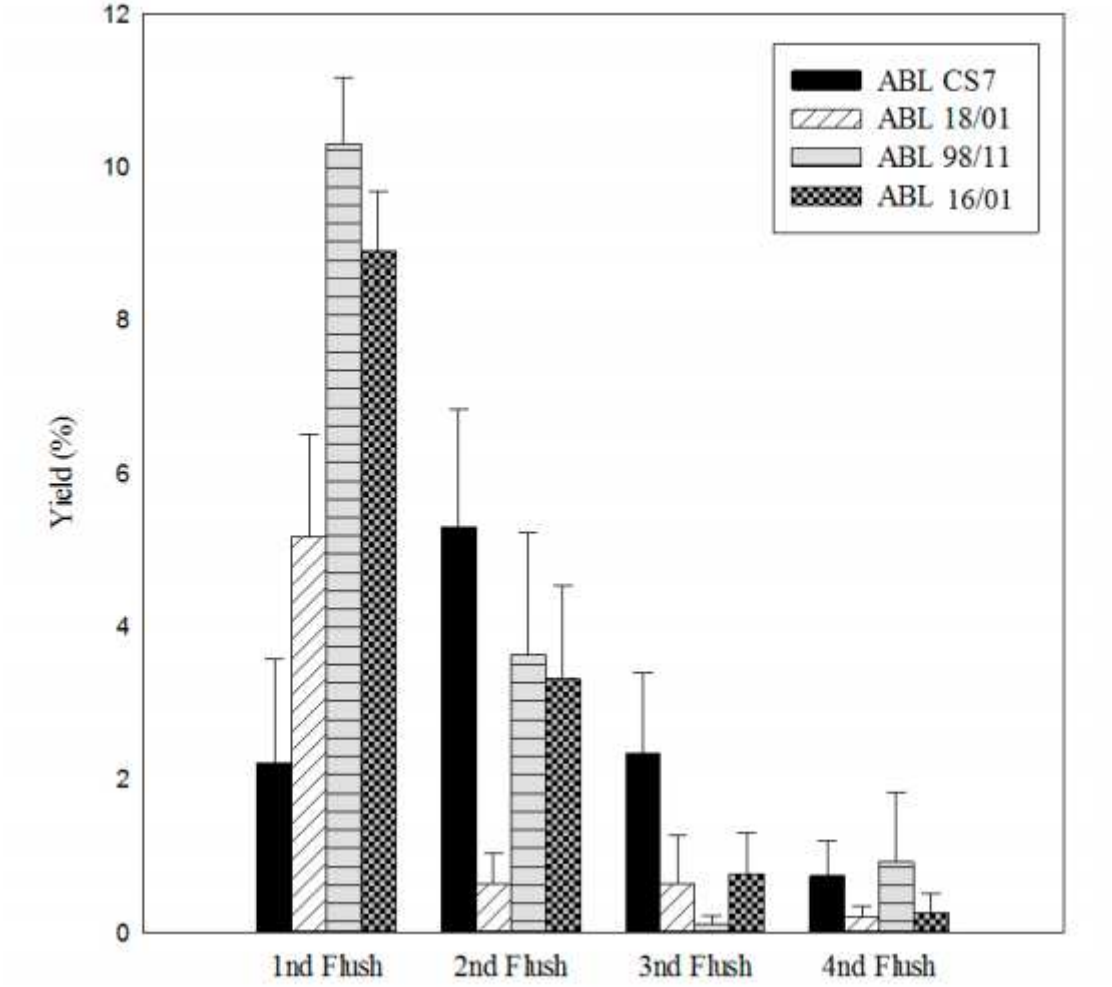

Figure 4

Distribution of yield during the cultivation cycle (4 harvest flushes).

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