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Breeding of a high-yield strain for commercial cultivation by crossing *Pholiota adiposa*

Melhoramento de uma linhagem de alto rendimento para cultivo comercial por cruzamento de *Pholiota adiposa*

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ABSTRACT

Pholiota adiposa is a mushroom with excellent nutritional and medicinal properties. However, fruiting body yields are low, and the commercial cultivation potential of this fungus is limited. Here, 279 crossbred strains were obtained by the mono-mono crossing of monokaryotic strains derived from *P. adiposa* HS5 and HS4. Laccase enzymes and mycelial growth rates were used as markers to screen the crossbred strains, and 18 strains were selected for further analysis. Crossbred strain A10B4 displayed the highest yield (i.e. 165.91 ± 12.56 g per bag), which was 31.34 g and 74.48 g more than that of strains HS5 and HS4, respectively. The mycelial colonization time of A10B4 was 25.18 ± 1.33 d, which was 5.64 d shorter than that of HS5. A10B4 was characterized by inter-simple sequence repeat molecular markers. Differences in PCR products from parental and crossbred strains were observed. Therefore, the newly developed hybrid strain A10B4, named *P. adiposa* HS54 exhibiting high yield, might be suitable for commercial cultivation.

Index terms: Mushroom; ligninolytic enzymes; mycelial growth.

RESUMO

Pholiota adiposa é um cogumelo com excelentes propriedades nutricionais e medicinais. No entanto, o rendimento do corpo de frutificação deste é baixo e o potencial de cultivo comercial deste fungo é limitado. 279 linhagens cruzadas foram obtidas pelo cruzamento mono-mono de linhagens monocarióticas derivadas de *P. adiposa* HS5 e HS4. Enzimas ligninolíticas e taxas de crescimento micelial foram utilizadas como marcadores para a triagem das linhagens cruzadas, e 18 linhagens foram seleccionadas para análise posterior. A linhagem cruzada A10B4 apresentou o maior rendimento (ie 165,91 ± 12,56 g por sacola), o qual 31,34 g e, 74,48 g a mais do que as linhagens HS5 e HS4, respetivamente. O tempo de colonização micelial de A10B4 foi 25,18 ± 1,33 d, sendo 5,64 d menor que HS5. A10B4 foi caracterizado, por marcadores moleculares de repetição de sequência inter-simples. Foram observadas diferenças nos produtos de PCR de linhagens parentais e cruzadas. Portanto, a linhagem híbrida recém-desenvolvida A10B4 (Renominada *P. adiposa* HS54), exibindo alto rendimento, pode ser adequada para cultivo comercial.

Termos para indexação: Cogumelo; enzimas ligninolíticas; crescimento micelial.

INTRODUCTION

The genus *Pholiota* is made up of wood-rotting saprotrophic mushrooms characterized by a yellow or brown pileus with scales and/or slimy (Lee et al., 2020). *P. adiposa* is widely distributed on dead poplars, willows, or birches in forested areas in China (Hu et al., 2012; Rong et al., 2016). It is also a lignin-degrading macrofungus with excellent nutritional and medicinal properties (Hu et al., 2012). Compounds extracted from the fruiting bodies or mycelia of *P. adiposa* display a variety of important biological activities such as antitumor (Hu et al., 2012; Zhang et al., 2009; Zou et al., 2019), antioxidative (Deng et al., 2011), antimicrobial (Dulger, 2004), and anti-HIV-1 effects (Wang et al., 2014). *P. adiposa* has become popular globally due to its delicious taste and beneficial properties (Cho et al., 2006; Wang et al., 2014).

Mushroom breeding involves various methods but crossbreeding is considered the most efficient way to develop new, good quality edible strains (Kim et al., 2013; Sonnenberg et al., 2017; Xiang et al., 2016). Intra-species crossbreeding has been reported such as in *Sparassis latifolia*, *Pleurotus tuoliensis*, and *P. eryngii* species (Kim

et al., 2013; Sou et al., 2013; Wang et al., 2018). High yield is a key benefit of breeding (Wu et al., 2020). Most *P. adiposa* cultivars were domesticated from the wild and with low yields, and reports on breeding this mushroom are limited. *P. adiposa* HS5 (Rong et al., 2016), with the highest biological efficiency, was screened as one of five wild strains in comparative studies. *P. adiposa* strain HS4 demonstrated an approximately 1.5-fold higher mycelial growth rate (MGR) than HS5. Therefore, to obtain a *P. adiposa* strain with high yield and MGR, we performed crossbreeding of these two strains.

In *Pholiota* breeding, no effective rapid marker-assisted selection strategies, except for clamp connection, have been developed to evaluate crossbred strains with important agronomic traits at the mycelial growth stage. Laccase is a ligninolytic enzyme produced by many edible fungi such as *Volvariella volvacea* (Chen et al., 2004), *Agaricus sinodeliciosus* (Hao et al., 2021) and *Ganoderma lucidum* (Kumar et al., 2021). It is important for the decolorization of Remazol Brilliant Blue R (RBBR), lignin degradation, and fruiting-body formation (Xu et al., 2012). The close relationships among laccase activity, induced primordium differentiation, and fruiting body development were reported by Wu et al. (2015). Sun et al. (2014) and Xu et al. (2012) used RBBR decolorization by a ligninolytic enzyme as a marker to screen protoplast fusants, and the growth rates and biological efficiencies of new strains were higher than those of the parental strains. Therefore, RBBR is an efficient approach to evaluate new fungal strains. Here, crossbreeding between *P. adiposa* HS5 and HS4 was used to develop a new strain with high yield and mycelial growth rate (MGR), with RBBR decolorization by a ligninolytic enzyme used as a marker to screen crossbred strains.

MATERIAL AND METHODS

Strains and growth conditions

The fruiting bodies of *P. adiposa* HS5 and HS4 were collected from Haidian and Changping districts, respectively, in Beijing, China, and then isolated by tissue culture. The strains were cultured and maintained in potato dextrose agar at 25 °C.

Single-spore isolation

This was performed as previously described by Wang et al. (2018) with minor modifications. Briefly, fruiting bodies were placed in Erlenmeyer flasks containing 100 ml of sterilized water to generate spore suspensions. Next, 100 µl of spore suspension (~ 1×10^3 spores/ml) was spread onto potato dextrose agar (PDA) plates and incubated at 25 °C for 5 d for spore germination. Monokaryons originating from monospores were identified based on the absence of clamp connections on mycelia ascertained by microscopy.

PDA-RBBR plate screening and MGR assessment of monokaryons

The strains were incubated on PDA-RBBR (PDA medium supplemented with RBBR at a final concentration of 0.05 % (w/v)) to screen for monokaryons. The degree of RBBR decolorization was designated 1, 2, 3, or 4. A higher number represented greater RBBR decolorization with a more relative laccase activity (halo). MGR was determined by observing the radial growth length of mycelia on PDA-RBBR plates.

Pairings between single-spore isolates

Inter-strain pairing experiments were performed using random monosporic isolates derived from strains *P. adiposa* HS5 and HS4. Mating was conducted by placing mycelial blocks opposite a monokaryotic mycelium on PDA. Mycelium fragments were taken from the contact zone between the paired colonies and individually transferred to PDA plates for further incubation. When the colonies grew to 1.0–1.5 cm in radius, mycelia were monitored using a microscope to identify dikaryotic hybrids by clamp connections.

Fruiting and cultivation methods

Substrates were prepared containing 60% cottonseed hull, 18% sawdust, 15% wheat bran, 5% corn flour, 1% gypsum, and 1% lime and placed in polypropylene bags at a packing 1000 g moisture mass of substrate per bag. After a complete spawn run, mycelial differentiation was induced by stimulation at 0–5 °C for 3–5 d. Bags were then transferred to a fruiting chamber maintained at 18 ± 2 °C for 15–20 d to obtain fruiting bodies. Each strain was subjected to three replicates, with 12 bags per replicate. Yield was determined as the weight of fresh mushrooms per bag after first flush. Biological efficiency (BE) was calculated based on the following formula: BE (%) = weight of fresh mushrooms harvested per bag / dry weight of cultivation substrates per bag × 100.

Inter-simple sequence repeat analysis

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). Inter-simple sequence repeat (ISSR) amplification

was performed in 20-µl reaction volumes containing 0.5 U of Taq DNA polymerase, 2.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 0.75 µmol/l primer (P11: 5'-AGAGAGAGAGAGAGAGG, P856: 5'-ACACACACACACACACYA-3', P23-5'-CACGA GAGAGAGAGAGA-3', P857: 5'-ACACACACACACACACYG-3'), and 50 ng template DNA. Amplification conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 45 °C for 45 s, and 72 °C for 90 s; a final extension for 7 min at 72 °C.

Statistical analysis

All data were statistically analyzed using SPSS PASW Statistics software version 18. All data were obtained in triplicate, and differences were determined by Duncan's multiple range testing. Statistical differences were considered significant at the 5.0% level ($P < 0.05$).

RESULTS AND DISCUSSION

In total, 126 and 103 monokaryons were isolated from *P. adiposa* HS5 and HS4, respectively and were selected for further study based on mycelial growth rates and their abilities to decolorize RBBR (Figure 1). For *P. adiposa* HS4, the average MGR of 103 monokaryons was 1.77 mm/d in the first round of screening (data not shown); 21 monokaryons with a decolorization degree ≥ 3 and MGR ≥ 1.77 mm/d were selected during the second round of screening from 47 monokaryons (Supplementary Table 1). For *P. adiposa* HS5, the average MGR of 126 monokaryons was 1.66 mm/d in the first round of screening (data not shown); 22 monokaryons with a decolorization degree ≥ 3 and MGR ≥ 1.66 mm/d were selected during the second round of screening from 36 monokaryons (Supplementary Table 2). Monocaryons were validated by microscopy to ensure the absence of clamp connection.

Crossbreeding was conducted by mating 22 monokaryons isolated from *P. adiposa* HS5 with 21 monokaryons isolated from *P. adiposa* HS4, resulting in 462 total matings. Crossbred strains were screened for clamp connections within the contact zone under a microscope. The presence of clamp connections indicates the formation of a dikaryon. Primary screening of 279 putative crossbred strains was performed on RBBR-PDA (decolorization degree ≥ 3 and an MGR greater than that of HS5) resulting in the identification of 55 crossbred strains for further study. A second screening of the 61 strains (55 crossbred strains and the top six strains with a high MGR and decolorization degree ≤ 2) resulted in 12 putative crossbred strains with a decolorization degree ≥ 3 and MGR greater than that of HS5 (Table 1). To avoid losing strains with good traits, the top six strains with a high MGR and decolorization degree ≤ 2 were also selected for further study. All 18 putative hybrids showed strong somatic incompatibility reactions with both parental strains, and thus, these 18 crossbred strains were retained for further study.

The parental and 18 crossbred strains were cultured and fresh weight and mycelial colonization time were compared between the strains (Table 2). Among the 18 crossbred strains, A20B3, A4B6, A16B3, and A16B6 showed abnormal fruiting bodies and A5B1, A20B12, A6B6, and A10B12 did not form fruiting bodies. The yield of parental strain HS5 was 134.57 ± 5.45g, which was 43.14 g more than that of HS4 (Table 2). The BEs of HS5 and HS4 were 40.78 ± 1.65% and 27.71 ± 1.59%, respectively in the first flush (Table 2). Two crossbred strains (A10B4 and A14B4) were more productive than parental strain HS5 (Figure 2), and three crossbred strains were more productive than parental strain HS4. The most productive strain was A10B4, but the difference in productivity between A10B4 and A14B4 was not significant. The mycelial colonization time of HS5 was 30.82 ± 0.98 d, which was 6.1 d longer than that of HS4 (Table 2). Among the crossbred strains, A10B5, A10B4, and A1B18 displayed the shortest mycelial colonization times with 24.36 ± 1.03, 25.18 ± 1.33, and 25.00 ±1.18 d, respectively. Comprehensive comparisons of yield and mycelial colonization time indicated that A10B4 possessed the most desirable traits of all crossbred strains.

Crossbred strain A10B4 and the parental strains were analyzed by ISSR. Among the tested primers, four, P11, P856, P23, and P857, efficiently amplified genomic DNA from all strains (Figure 3). The size of the polymorphic fragments obtained ranged from 200 to 5000 bp, and differences were observed in the number of bands obtained (Figure 3). The crossbred strain A10B4 and two parental strains showed characteristic differences in the presence and absence of fragments. Five different fragments were amplified in crossbred strain A10B4 using the four primers. The results of PCR amplification indicated that A10B4 is a new hybrid strain and that differences in numbers of fragments are useful to identifying this strain.

Cross-breeding using monokaryons was an efficient method to develop new strains. Here, *P. adiposa* HS5 and HS4 were crossbred to develop a new strain with high yield and MGR. The new high-yield crossbred strains obtained in this study should benefit future commercial cultivation efforts.

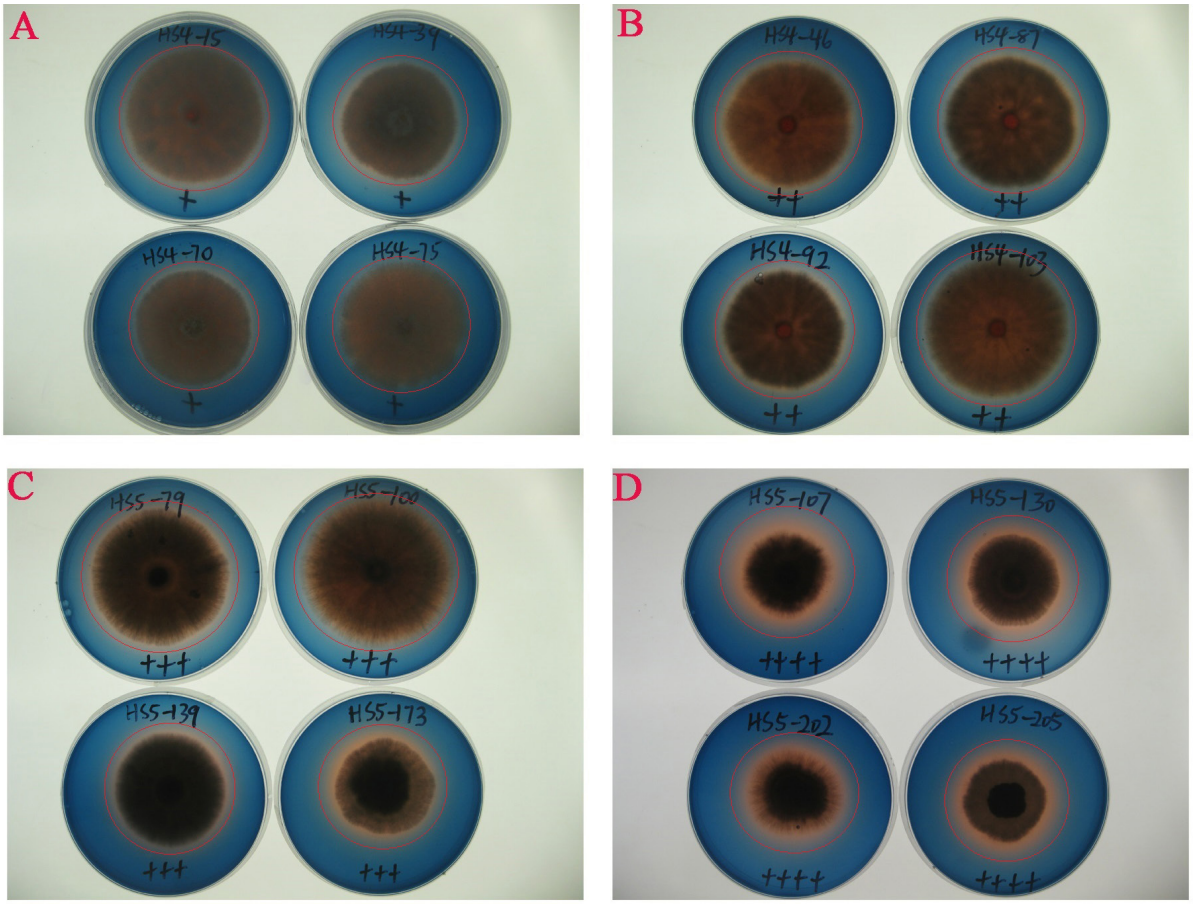

Figure 1: Decolorization of HS4 and HS5 monokaryons on PDA plates containing RBBR. The RBBR decolorization degree was scored as 1 (+), 2 (++), 3 (+++), or 4 (++++). The red circles represent halos of RBBR decolorization. A, B, C, and D show RBBR decolorization degrees of 1, 2, 3, and 4, respectively; 1 represents the lowest degree of RBBR decolorization (The color of the halos is the darkest) and 4 represents the highest degree of RBBR decolorization (The color of the halos is the lightest).

Table 1: Mycelial growth rate and decolorization degree of crossbred and parental strains.

Number	Crossbred strain	Mycelial growth rate (mm/d)	Decolorization degree
Parental strain strain strains strain	HS4	3.46±0.10	1
Parental strain	HS5	2.29±0.17	2
1	A5B1	3.14±0.09	1
2	A1B18	2.81±0.16	2
3	A1B17	2.66±0.23	2
4	A1B11	2.57±0.12	2
5	A1B12	2.57±0.18	2
6	A1B6	2.53±0.12	2
7	A10B5	2.47±0.05	3

Continue...

Table 1: Continuation.

Number	Crossbred strain	Mycelial growth rate (mm/d)	Decolorization degree
8	A16B3	2.46±0.05	3
9	A20B3	2.44±0.08	3
10	A13B4	2.43±0.03	2
11	A8B6	2.40±0.12	3
12	A17B21	2.38±0.06	2
13	A21B6	2.38±0.06	2
14	A4B6	2.36±0.12	3
15	A14B4	2.36±0.15	3
16	A10B4	2.35±0.03	3
17	A20B12	2.35±0.03	3
18	A8B9	2.34±0.08	2
19	A6B6	2.34±0.04	3
20	A13B6	2.31±0.08	2
21	A7B6	2.31±0.03	3
22	A10B12	2.30±0.08	4
23	A16B6	2.30±0.05	4
24	A11B2	2.29±0.06	3
25	A14B21	2.29±0.04	3
26	A4B5	2.27±0.07	3
27	A21B12	2.27±0.05	4
28	A13B21	2.26±0.07	3
29	A10B10	2.25±0.06	3
30	A20B21	2.25±0.03	3
31	A14B6	2.24±0.10	4
32	A4B9	2.20±0.04	3
33	A13B12	2.19±0.03	4
34	A19B12	2.16±0.06	4
35	A12B3	2.16±0.15	4
36	A16B10	2.16±0.04	3
37	A13B3	2.16±0.12	3
38	A11B12	2.14±0.07	4
39	A7B2	2.14±0.13	4
40	A4B12	2.11±0.05	4
41	A19B3	2.09±0.10	4
42	A12B10	2.07±0.07	4
43	A14B14	2.02±0.12	3
44	A21B2	2.00±0.10	3
45	A13B2	2.00±0.05	4
46	A20B2	1.97±0.05	4

Continue...

Table 1: Continuation.

Number	Crossbred strain	Mycelial growth rate (mm/d)	Decolorization degree
47	A9B8	1.96±0.07	3
48	A16B5	1.96±0.08	4
49	A14B10	1.94±0.05	4
50	A10B3	1.94±0.06	4
51	A8B8	1.94±0.04	4
52	A14B5	1.93±0.05	4
53	A15B19	1.93±0.10	4
54	A4B2	1.93±0.06	4
55	A14B2	1.92±0.12	4
56	A4B20	1.92±0.05	3
57	A6B10	1.87±0.05	4
58	A7B10	1.84±0.09	4
59	A21B10	1.81±0.08	4
60	A21B11	1.75±0.03	4
61	A9B10	1.60±0.02	4

Bold letters represented the strains selected for fruiting body production.

Table 2: Comparison of the production yields and mycelial colonization times of fruiting bodies.

Strain	Weight of fruiting body (g)	Biological efficiency (%)	Mycelial colonization time (days)
HS5 (Parental strain) strain)	134.57±5.45 b *	40.78±1.65 b	30.82±0.98 a
HS4 (Parental strain) strain)	91.43±5.24 cd	27.71±1.59 cd	24.72±1.01 g
A10B4	165.91±12.56 a	50.28±3.81 a	25.18±1.33 efg
A14B4	161.87±10.03 a	49.05±3.04 a	26.09±1.70 def
A8B6	123.20±12.84 b	37.33±3.89 b	26.36±1.69 cde
A7B6	102.67±17.46 c	31.11±5.27 c	29.82±1.08 a
A10B5	91.60±10.32 cd	27.76±3.13 cd	24.36±1.03 g
A1B11	74.70±6.59 d	22.64±2.00 d	26.91±0.94 bcd
A1B6	72.57±12.73 de	21.99±3.86 de	27.64±1.12 b
A1B17	54.47±8.43 ef	16.51±2.55 ef	27.27±1.62 cde
A1B12	54.33±14.33 ef	16.46±4.34 ef	27.36±1.43 bc
A1B18	47.33±3.79 f	14.34±1.15 f	25.00±1.18 fg

* Means in each column followed by the same letters are not significantly different at $P < 0.05$ according to Duncan's multiple range tests.

Dikaryotic mycelia form a clamp connection (Sou et al., 2013; Rong et al., 2015), and in this study, mycelia were screened by detecting clamp connections under a microscope to identify dikaryotic hybrids. White rot fungi have an array of extracellular ligninolytic enzymes that synergistically and efficiently degrade lignin (Sun et al., 2014). Ligninolytic enzymes can be used as a screening marker for new species of edible fungi, resulting in a simple and direct screening method (Sun et al., 2014). Selecting crossbred strains is an important step in breeding

fungi, and to reduce work and increase efficiency, we used RBBR decolorization degree (representing ligninolytic enzyme activity) and MGR to screen new strains. As a result, 126 and 103 monokaryons were isolated from *P. adiposa* HS5 and HS4, respectively, and these were selected based on ligninolytic enzyme activity and MGR, thus reducing the breeding time, compared to that needed only with clamp connection. Among the 18 crossbred strains, 10 crossbred strains formed normal fruiting bodies (Table 2). Strain A10B4 displayed the highest BE and the shortest mycelial colonization time. Therefore, it was selected as the highest performing crossbred strain. ISSR assays confirmed that A10B4 is a new strain. Because the morphological characteristics of A10B4 are more like those of *P. adiposa* HS5, this strain was named *P. adiposa* HS54. From a commercial perspective, the new strain has a clear advantage over the parental strains and suggests potential for the future commercial cultivation of *Pholiota*.

Figure 2: Morphological characteristics of HS4, HS5, and crossbred strain fruiting bodies. HS4 and HS5 were parental strains.

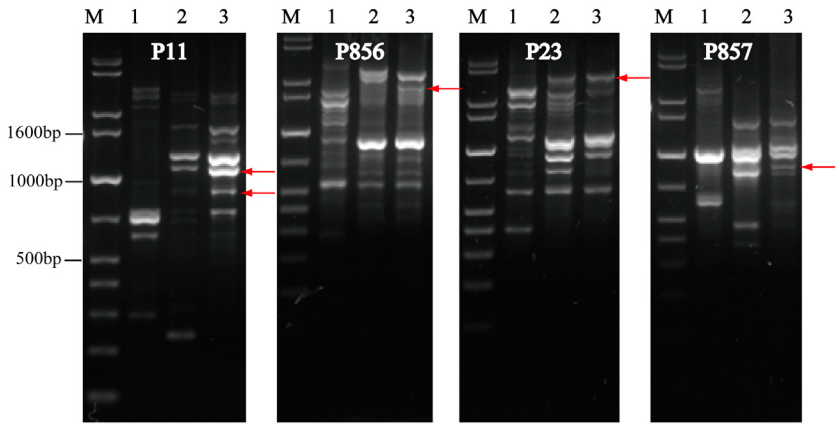

Figure 3: Amplification products obtained by PCR using ISSR primers and DNA from HS4, HS5, and A10B4. M, marker; lane 1, HS4; lane 2, HS5; lane 3, A10B4. Arrows indicate genetic markers showing a difference between strain A10B4 and the parental strains.

CONCLUSIONS

A new crossbred strain, *P. adiposa* HS54, demonstrated favorable traits in terms of yield and biological efficiency. From a commercial point of view, this new strain has a clear advantage over the parental strains and suggests potential for future commercial cultivation of *Pholiota*.

AUTHOR CONTRIBUTION

Conceptual Idea: Liu, Y.; Wang, S.; Methodology design: Liu, Y.; Wang, S.; Data collection: Yang, L.; Pan, X.; Data analysis and interpretation: Yang, L.; Pan, X.; Writing and editing: Rong, C.; Song, S.

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