ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 2, pp. 270–276. © Pleiades Publishing, Ltd., 2010.
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Ganoderma lucidum Complex: Some Individual Groups of Strains

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Abstract—Mating incompatibility, RAPD-PCR, isozyme polymorphism, and some morphological characteristics of *Ganoderma lucidum* geographical isolates were analyzed to determine their phylogeny. Three stable groups were found within this complex species.

Key words: mating incompatibility, RAPD-PCR, isozyme polymorphism, *G. lucidum*.

DOI: 10.1134/S0026261710020219

The *Ganoderma lucidum* (W. Curtis : Fr.) P. Karst. species complex comprises interspecies groups for which no phylogenetic concept has been developed [1, 2].

A high degree of variability depending on cultivation conditions results in significant difficulties in strain description based on morphology and cultural characteristics [3]. Characterization of the strains based on these features alone in unsatisfactory and does not permit comparison of the results of different investigations.

Genetic evolution of many fungal species resulting in partial or complete reproductive isolation (interincompatibility) is not necessarily accompanied by any morphological changes; morphological species are therefore associations of twin species. Instead of the concept of polymorphic species, a concept of a species as an aggregate of independently developing "biological" species, isolated to a certain degree [4]. Analysis of genetic (reproductive) isolation is used to avoid misunderstanding in the segregation of species and intraspecific groups of basidial fungi. For example, the independent species *G. tsugae* (Murrill) Overh. was segregated from the polymorphic species *G. lucidum* based on their mating incompatibility [5].

Molecular methods have recently become popular in fungal taxonomy. They were used, for example, to demonstrate high heterogeneity of the *G. lucidum* complex [6–8]. Molecular phylogenetic analysis of American, Asian, and European collections of *G. lucidum* with rDNA markers resulted in the isolation of several intraspecific groups (*G. lucidum* sensu stricto, *G. resinaceum* complex sensu lato, *G. curtisii* complex, and *G. tropicum* complex sensu lato) [1]. The absence of correlation between the phylogeny derived from rDNA and the morphological segregation of the *G. lucidum* complex encourages the search for new molecular markers for exhaustive strain description.

The *G. lucidum* strains used in the present work exhibit antitumor and antibacterial activity [9, 10]. Therefore, establishing their intraspecific status may be used not only for correct comparison with other known strains, but also for description of new sources of biologically active compounds. Analysis of isozyme and RAPD polymorphism was carried out in order to elucidate the intraspecific status of *G. lucidum* strains, together with assessment of their vegetative incompatibility and reproductive isolation, as well as of some morphological characteristics investigated previously [11].

MATERIALS AND METHODS

Dikaryotic strains of *Ganoderma* spp. were obtained from the culture collections of the Department of Mycology and Algology, Moscow State University; Gause Institute of New Antibiotics, Russian Academy of Medical Sciences; and Russian National Collection of Industrial Microorganisms, Institute of Genetics and Selection of Industrial Microorganisms (Table 1). Two strains of *G. applanatum* (Pers.) Pat. were used as the controls.

The cultures were maintained at 4°C on wheat agar containing hardwood sawdust. Transfers to fresh medium were carried out yearly.

Analysis of vegetative incompatibility. Growth of the strains in combined cultures was analyzed on malt agar (4°B) in petri dishes in an incubator at 25°C by joint cultivation of dikaryotic strains in all possible combinations.

The reaction to vegetative incompatibility was assayed visually as the character of antagonistic response in the contact zone (neutral, intergrowth, repulsion, and overgrowth) [12, 13]. Intrastrain hybridizations were used as the controls.

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Table 1. *Ganoderma* spp. strains used in the present work

Strain	Collection	Obtained at	Place of isolation and substrate
GL2	Gause Institute of New Antibiotics	1999	Caucasus, beech
GL3	Moscow State University	1999	Korea
GL4	Moscow State University	2000	France
GL5	Moscow State University	2001	United States
GL6	Moscow State University	2001	Moscow oblast
GL7	Moscow State University	1998	Moscow oblast
GL8	Gause Institute of New Antibiotics	2004	Caucasus, apple
GL9	Gause Institute of New Antibiotics	2004	Crimea, beech
GL927	Russian National Collection of Industrial Microorganisms	1961	Austria
GAPP926	Russian National Collection of Industrial Microorganisms	1930	Great Britain
GAPP632	Russian National Collection of Industrial Microorganisms	1991	–

Note: GL - *G. lucidum,* GAPP - *G. applanatum.*

Analysis of Mating Incompatibility

Isolation of monokaryotic cultures. Monokaryotic cultures were obtained by sequential serial dilutions of spore suspensions. The spores of three strains (GL4, GL8, and GL9) were collected from basidiomal spore prints obtained as suggested in [14]. If no buckles were found, the colonies were transferred to test tubes and marked as "m" (monokaryon).

Isolation of monokaryotic testers. Monokaryotic testers were isolated as described in [15]. For three *G. lucidum* strains, four groups of monokaryotic tester cultures were obtained differing in alleles of the coupling loci (A_1B_1, A_1B_2, A_2B_1, and A_2B_2).

Mon–mon hybridization. Determination of coupling types and of the common mating compatibility factors was carried out by mon–mon hybridization (monokaryon × monokaryon) between monosporous isolates and the testers. Analysis of the mating compatibility factors was carried out according to the standard technique [15].

Di–mon hybridization. For determination of the species position of basidiomycetes, hybridization of environmental strains with the monokaryotic testers, i.e., di–mon hybridization (dikaryon–monokaryon) is a more rapid, albeit less precise method than mon–mon hybridization. A dikaryon and a tester were plated together on malt agar plates. After two weeks of incubation at 25°C, the presence of buckles was determined on the mycelium of the monosporic tester at the side opposite to the dikaryon. Formation of buckles demonstrated mating compatibility of the strain with the tester, thus belonging to the same interstitial group (a biological species).

RAPD-PCR analysis. After 10 days of cultivation, DNA was isolated from the mycelium grown in liquid 1.5% malt according to the Diatom™ DNA Prep 100 procedure developed for DNA isolation from fresh biological material.

Genomic DNA was amplified in a Tertsik-1 programmable thermocycler. The PCR-core kits (Biokom) used for amplification contained inhibited *Taq*-DNA-pol, dNTP, 200 μM each and $MgCl_2$, 2.5 mM. The reaction was carried out in 25 μl of the reaction mixture supplemented with the following: PCR-core mixture, 5 μl; template DNA, 5μl; primer, 5 μl (0.5 μM); and PCR solvent, 10 μl. Mineral oil was applied to the mixture to prevent evaporation.

Five oligonucleotide primers were used for RAPD PCR analysis: Sintol R1 (5'-TGCCGAGCTG-3'), R2 (5'-AGTCAGCCAC-3'), R3 (5'-AATCGGG-CTG-3'), R4 (5'-GAAACGGGTG-3') and R5 (5'-GCGATCCCCA-3') [7]. For each primer, the amplification reaction was carried out in three repeats.

The amplification conditions were as follows: first stage (1 cycle): 98°C, 2 min, 36°C, 1 min, 72°C, 2 min; second step (45 cycles): 95°C, 1 min, 36°C, 1 min, 72°C, 2 min; and third stage (1 cycle): 72°C, 5 min, 72°C, 3 min, 72°C, 2 min [7].

The amplified fragments were separated in 2% agarose gel at 80 V. The gel were stained with ethidium bromide (1 μg/ml) and examined under a UV transilluminator. A Canon Digital IXUS 800 IS camera was used to register the PCR products. A GeneRuler™ 1-kb DNA Ladder marker (Fermentas) was used as a DNA length marker.

Isozyme analysis. Comparison of the isozyme spectra of mycelial cultures of 11 *Ganoderma* strains was carried out by vertical electrophoresis in polyacrylamide gel following Davis with some modifications [11, 16] in a Helicon chamber. Staining to reveal the activities of five enzymes—nonspecific esterase (EC 3.1.1.2), acid phosphatase (EC 3.1.3.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), polyphenol oxidase (EC 1.10.3.2), and malate dehydrogenase

Table 2. Mon–mon hybridization of *G. lucidum* strains

		GL4				GL8				GL9			
		4m1	4m2	4m6	4m7	8m1	8m3	8m5	8m9	9m1	9m2	9m4	9m7
GL4	4m1	–	+	–	–	–	–	–	–	–	–	–	–
	4m2	+	–	–	–	–	–	–	–	–	–	–	–
	4m6	–	–	–	+	–	–	–	–	–	–	–	–
	4m7	–	–	+	–	–	–	–	–	–	–	–	–
GL8	8m1	–	–	–	–	–	–	–	+	+	+	+	+
	8m3	–	–	–	–	–	–	+	–	+	+	+	+
	8m5	–	–	–	–	–	+	–	–	+	+	+	+
	8m9	–	–	–	–	+	–	–	–	+	+	+	+
GL9	9m1	–	–	–	–	+	+	+	+	–	–	+	–
	9m2	–	–	–	–	+	+	+	+	–	–	–	+
	9m4	–	–	–	–	+	+	+	+	+	–	–	–
	9m7	–	–	–	–	+	+	+	+	–	+	–	–

Note: 4m1, 8m1, etc., are designations for monokaryotic cultures, GL – *G. lucidum*.

(EC 1.1.1.37)—was carried out as described in [17, 18].

Cluster analysis. For the dendrograms obtained by analysis of isozyme and RADP spectra, validity of topology was determined using the bootstrap algorithm; reliability was expressed in percent. The difference between images was assayed by cluster analysis of UPGMA binary matrices describing the patterns (isozyme and molecular phenotypes). The Treecon for Windows v. 3.1 software package was used for cluster analysis with reliability assessment.

RESULTS

Vegetative Incompatibility

Joint cultivation on agarized medium of all dikaryotic collection strains resulted in formation of a mycelial barrage in the zone of their contact, a phenotypic manifestation of the reaction of vegetative incompatibility. It was therefore concluded that the strains under investigation were not clones, but genetically different entities.

All *G. lucidum* strains exhibited the strongest antagonistic reaction when cocultured with *G. applanatum* strains used to assess some results at the species level.

In the case of genetically identical strains, anastomoses were formed in the zone of contact between mycelia with subsequent fusion.

Mating Incompatibility

Monokaryotic tester strains, four monosporic strains differing in alleles of the coupling loci, were obtained for each of three dikaryotic isolates (GL4, GL8, and GL9).

Mon–mon hybridization (monokaryon × monokaryon) revealed that environmental isolates GL8 and GL9 (the Caucasus region and Crimea, respectively) were mutually compatible and exhibited mating incompatibility with strain GL4 (France) (Table 2). These data suggested classification of strains GL8 and GL9 within the first interincompatible group (I) and of strains GL4 and GL927, within the second one (II).

Strains GL8 and GL9 from the Caucasus region and Crimea were similar in their morphologocultural characteristics; their cultivations produced typical fan-shaped, "lacquered" basidiomes. Strain GL4 (France) formed console-shaped opaque fruiting bodies (Fig. 1).

Compatibility of the remaining collection strains was determined by di–mon hybridization (dikaryon × monokaryon). This method requires significantly less time and effort than mon–mon hybridization, which is especially important due to the difficulty of cultivation of environmental *Ganoderma* strains.

Mating compatibility between dikaryotic isolates and monokaryotic testers was determined from the presence of buckles on the tester mycelium (Table 3).

Monokaryotic testers of strains GL8 and GL9 were compatible only with strain GL2 (Caucasus). Strain GL927 (Austria) was compatible with the testers of strain GL4 (France).

RAPD analysis of the collection of *Ganoderma* spp. strains was carried out with the set of primers used previously for the characterization of this species [7]. High degree of molecular polymorphism was found for this sampling. The highest number of variable characteristics (amplification products) was obtained with the primers R1, R3, and R5 (88% or 8 variable bands out of 9 bands in the electrophoretic spectrum,

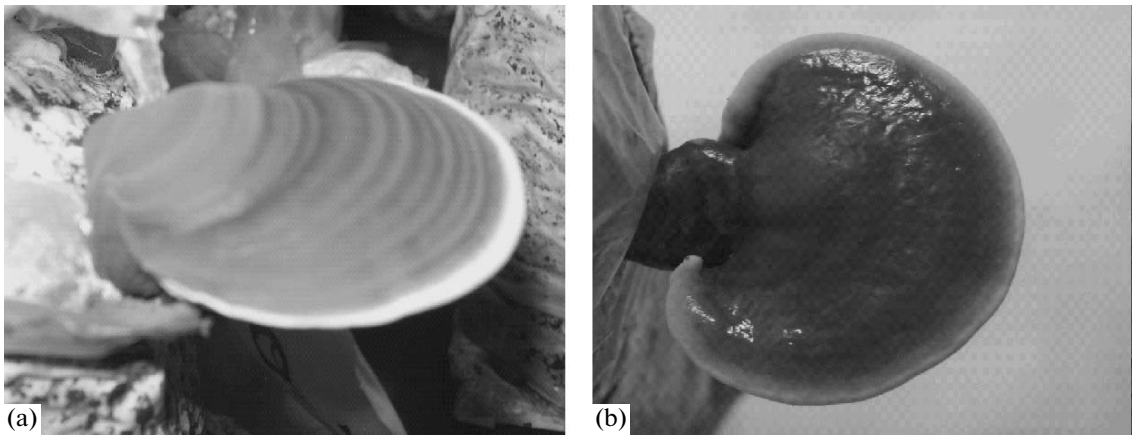

Fig. 1. Console-shaped opaque basidiomes of strain GL4 (a) and fan-shaped "lacquered" basidiomes of strain GL8 (b).

85% or 11 out of 13 bands, and 87% or 7 out of 8 bands, respectively).

Comparison of molecular profiles (amplification patterns) obtained with the R1 primer revealed a 620-bp fragment amplified only in *G. lucidum* strains. This fragment was not found in the RAPD spectra of *G. applanatum* strains and thus is probably species-specific (Fig. 2).

The RAPD polymorphism data were treated by cluster analysis. All dendrograms had similar topology; the dendrogram obtained by analysis of the summary matrix of all amplification patterns was the most stable, with an average bootstrap index of 82% (Fig. 3).

The main cluster of the dendrogram included all *G. lucidum* strains. The *G. applanatum* strains formed an isolated outgroup with monophyly supported by high bootstrap indices (up to 100%). Cluster analysis of RAPD markers demonstrated that the species *G. lucidum* was highly heterogeneous and comprised strains within a broad range of conventional units of genetic distance (from 0.1 to 0.42).

Within the species *G. lucidum*, three stable groups of isolates are seen: (A) strains GL2, GL8 (Caucasus), and GL9 (Crimea); (B) strains GL4 (France) and GL927 (Austria); and (C), strains GL6 and GL7 (Moscow oblast) (Fig. 3).

Table 3. Di–mon hybridization of *G. lucidum* strains

	GL4				GL8				GL9			
	4m1	4m2	4m6	4m7	8m1	8m3	8m5	8m9	9m1	9m2	9m4	9m7
GL2	–	–	–	–	+	+	+	+	+	+	+	+
GL3	–	–	–	–	–	–	–	–	–	–	–	–
GL4	–	–	–	–	–	–	–	–	–	–	–	–
GL5	–	–	–	–	–	–	–	–	–	–	–	–
GL6	–	–	–	–	–	–	–	–	–	–	–	–
GL7	–	–	–	–	–	–	–	–	–	–	–	–
GL8	–	–	–	–	–	–	–	–	+	+	+	+
GL9	–	–	–	–	+	+	+	+	–	–	–	–
GL927	+	+	+	+	–	–	–	–	–	–	–	–

Note: 4m, 4m2, etc. are designations for monokaryotic tester cultures, GL – *G. lucidum*.

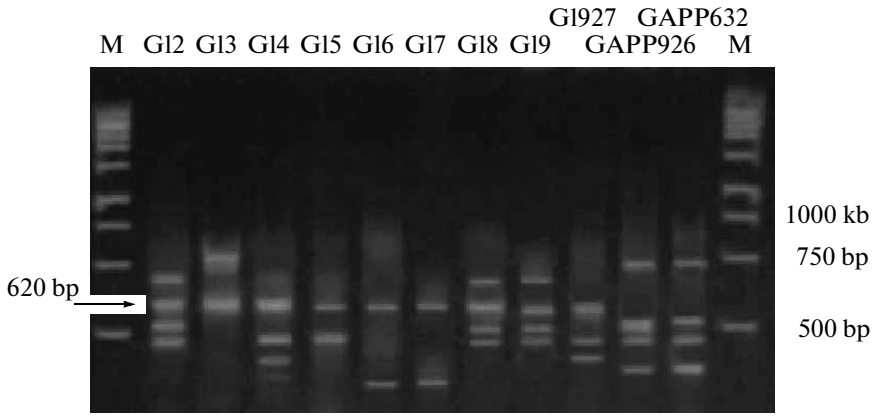

Fig. 2. Electrophoregram of RAPD polymorphism in *Ganogerma* spp. strains obtained with the primer R1 (the species-specific fragment for *G. lucidum* is marked by an arrow; M designates a GeneReulr™1-kb DNA Ladder marker, Fermentas, marker fragment sizes in bp are shown at the right). GL designates *G. lucidum* and GAPP, *G. applanatum*.

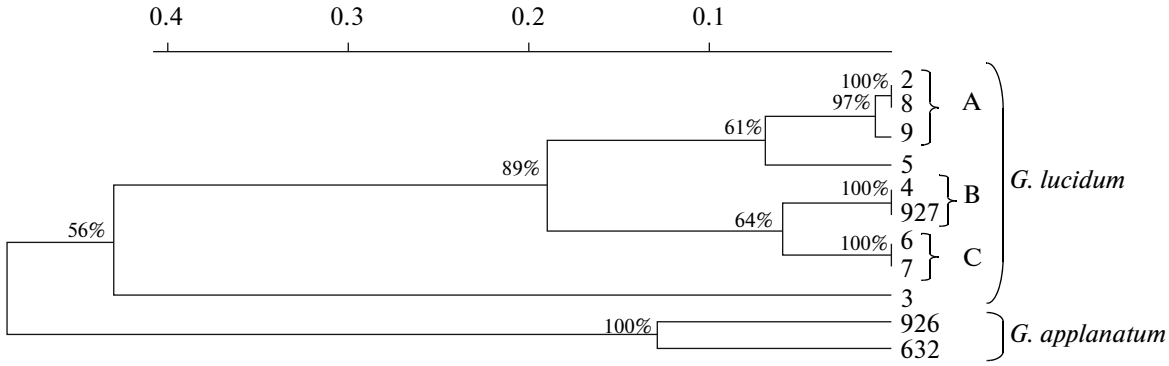

Fig. 3. UPGMA dendrogram of genetic similarity between the RAPD profiles of *Ganoderma* spp. strains.

These groups of strains were characterized by high homogeneity of RAPD markers (bootstrap index for groups B and C was up to 100%, for group A, up to 97%). According to the vegetative incompatibility tests, strains GL6 and GL7 (Moscow oblast) of group C were not clones; they, however, had identical molecular RAPD profiles. Strains GL4 and GL927 of group B exhibited high similarity, forming an isolated group with 100% probability. Isolates GL2, GL8 (Caucasus), and GL9 (Crimea) comprising group A in 97% of the cases were combined at the level of 0.01 conventional units of genetic distance. Thus, RAPD polymorphism of the species *G. lucidum* revealed several groups.

The geographically remote strain from Korea (GL3) exhibited the highest difference within the *G. lucidum* cluster by all RAPD markers and occupied an isolated position at 0.42 conventional units of genetic distance. Strain GL5 (United States) was loosely related to the group from the southern regions at 61% bootstrap index.

Isozyme Analysis

Malate dehydrogenase (MDH) was the most representative of the five isozyme systems used. This is in accordance with the published data on *G. lucidum* [19]. Protein electrophoresis with MGH confirmed the previously revealed differences between strains of different geographical origin.

The dendrogram of MDH spectra comparison showed that *G. applanatum* strains and strain GL5 (United States) exhibited the highest difference, joining the other *G. lucidum* geographical isolates only at 1 unit of genetic distance (Fig. 4).

Strains GL6 and GL7 (Moscow oblast) formed one group with 99% bootstrap index. The remaining strains (GL2, GL4, GL8, GL9, and GL927) exhibited 66% homology of MDH spectra. The Korean strain occupied an isolated position at 0.34 conventional units of genetic distance.

DISCUSSION

Investigation of the variability of *G. lucidum* strains by different techniques made it possible to compare

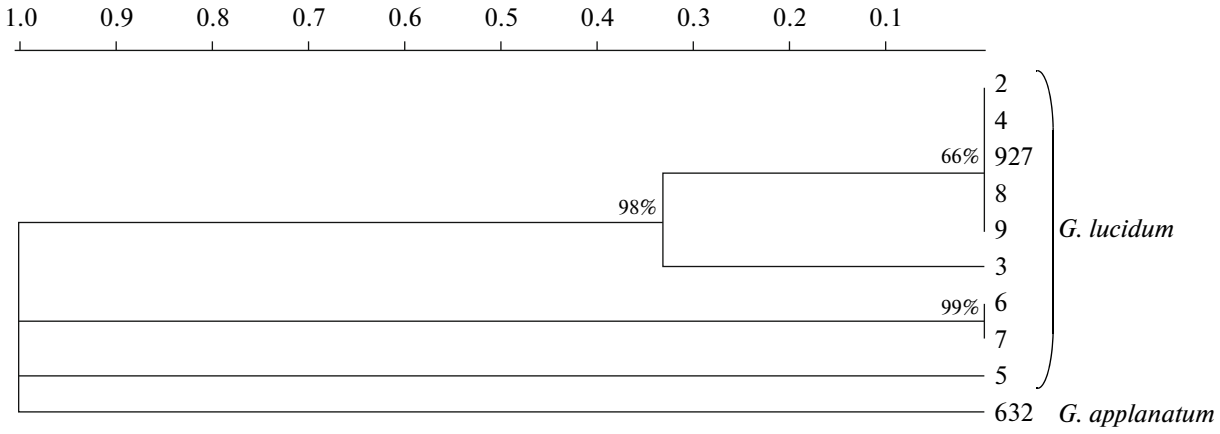

Fig. 4. Dendrogram of similarity between *G. lucidum* and *G. applanatum* strains in the spectrum of MDH isozymes.

the results of these analyses (Table 4). For example, strains GL2, GL8, and GL9 from the southern regions of this country and its vicinity, which formed group A according to the RAPD analysis data, had similar malate dehydrogenase spectra and formed the interincompatible group I. They were shown to form chlamydospores in pure culture [11]; they formed fan-shaped "lacquered" fruiting bodies with a stem.

The European strains in the B RAPD group exhibited high isozyme homogeneity, belonged to the interincompatible group II, possessed chlamydospores, and formed opaque console-shaped fruiting bodies. The strains in the C RAPD (Moscow oblast) exhibited the highest similarity in its MDH spectra, did not form chlamydospores in pure culture, and were unable to develop normal basidiomes under artificial conditions.

The strains from Korea (GL3) and the United States (GL5) not only exhibited the highest difference within the *G. lucida* cluster in their RAPD parameters, but were also reproductively isolated as evidenced by fertile hybridization.

Thus, individual characterization was obtained for each RAPD group, which exhibited high homogeneity in respect to the chosen markers and geographic origin.

Comparison of molecular profiles of the strains within the collection revealed the possibility of development of specific probes based on RAPD markers for identification of the species *G. lucidum*.

Application of the set of RAPD primers that had been previously used for analysis of *G. lucidum* makes it possible to compare molecular phenotypes of the strains within our sampling and of the world collection.

Table 4. Comparison of the results of analysis of *G. lucidum* polymorphism

Criteria	Characterization of *G. lucidum* strains		
Geographical origin	GL2, GL8-Caucasus, GL9-Crimea	GL4-France, GL927-Austria	GL6, GL7-Moscow oblast
RAPD marker homology	97% RAPD-group A	100% RAPD-group B	100% RAPD-group C
MDH spectra isozyme homology	66%	66%	99%
Mon–mon hybridization	interincompatibility group I	interincompatibility group II	not performed
Di–mon hybridization	compatible only within the group	compatible only within the group	incompatible
Resence of chlamydospores [11]	present	present	present
Fruiting bodies	fan-shaped, opaque, attached	fan-shaped, "lacquered", with stems	not obtained

ACKNOWLEDGMENTS

The work was partially supported by the Russian Foundation for Basic Research, project no. 08-04-00492.

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