GENETIC DIVERSITY OF *Agave cupreata* TREL. & BERGER. CONSIDERATIONS FOR ITS CONSERVATION

DIVERSIDAD GENÉTICA DE *Agave cupreata* TREL. & BERGER. CONSIDERACIONES PARA SU CONSERVACIÓN

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SUMAMRY

Agave cupreata Trel. & Berger is an endemic plant naturally distributed in the Balsas Depression, a semiarid region in the states of Guerrero and Michoacán in Southwestern México. Their populations are heavily decimated because mature individuals just before their single life flowering period are harvested to produce mescal, an alcoholic beverage. The genetic variation among and within 12 natural populations was examined for nine isozyme loci. Results indicate high average proportion of polymorphic loci (93 %) and expected heterozygosity (H_e = 0.467), with an excess of observed heterozygotes in relation to Hardy-Weinberg expectations (H_o = 0.521, F_{IS} = -0.1179). These results represent the largest heterozygosity reported for *Agave* species endemic to México. There is also a statistically significant genetic differentiation among populations (F_{ST} = 0.042). An UPGMA dendrogram reveals the absence of a geographic pattern, as confirmed by a Mantel test (r = -0.110, P = 0.769), which did not show significant isolation by distance. Estimated minimum viable effective population size was very large (N_e =16,165), larger than in any other known natural population. To protect the natural genetic variation, it is suggested to design and manage *A. cupreata* natural populations as forest genetic resource conservation units (FGRCUs) using realistic and modest N_e sizes, perhaps between 500 and 5000 plants, ideally with intermediate plantations that could serve as pollinator corridors. Commercial plantations and *ex situ* FGRCUs need to be established to gradually develop a sustainable management, perhaps at higher altitudes than current locations, as a management measure for adaptation to the climatic change.

Index words: Agavaceae, population genetics, minimum viable effective population size.

RESUMEN

Agave cupreata es una planta endémica que se distribuye en la Depresión del Balsas, una región semiárida de los Estados de Guerrero y Michoacán, al sur-occidente de México. Sus poblaciones están muy diezmadas debido a que los individuos maduros se cosechan justo antes de su floración, que ocurre sólo una vez en su vida, para producir mezcal, una bebida alcohólica. La variación genética entre y dentro de 12 poblaciones naturales fue examinada en nueve loci isoenzimáticos. Los resultados indican un promedio muy elevado de proporción de loci polimórficos (93%) y de heterocigosidad esperada (H_e = 0.467), con un exceso de heterocigotos observados en relación con lo esperado bajo el equilibrio de Hardy-Weinberg (H_o = 0.521, F_{IS} = -0.1179). Estos resultados de heterocigosidad son la más altos reportados en especies de *Agave* endémicas de México. También hay una diferenciación genética entre poblaciones estadísticamente significativa (F_{ST} = 0.042).

Un dendrograma UPGMA revela la ausencia de un patrón geográfico, confirmada por una prueba de Mantel (r = -0.110, P = 0.769), que demostró que no hay un significativo aislamiento por distancia. El tamaño mínimo efectivo viable de la población fue muy grande (N_e = 16,165), más grandes que en cualquier otra población natural conocida. Para proteger la variación genética natural de *A. cupreata* se sugiere diseñar y gestionar la conservación de poblaciones naturales como unidades de conservación de recursos genéticos forestales (UCRGFs) con tamaños de N_e realistas y modestos, tal vez entre 500 y 5000 plantas, idealmente con plantaciones intermedias que podrían servir como corredores de polinizadores. Se necesita establecer plantaciones comerciales y UCRGF *ex situ* para desarrollar gradualmente un manejo sustentable, tal vez a altitudes mayores que a la que se encuentran las localidades actuales, como una medida de manejo para adaptarse al cambio climático.

Palabras clave: Agavaceae, genética de poblaciones, tamaño efectivo mínimo de población viable.

INTRODUCTION

Agave cupreata Trel. & Berger is an endemic plant of the Agavaceae family (*Crenatea* group, subgenus *Agave*), now classified as a subfamily of the expanded Asparagalean families (Chase *et al.*, 2004). It is naturally distributed in the interior uphill slopes (1220 to 1850 m of altitude) of the Balsas Depression, a semiarid region in the states of Guerrero and Michoacán in Southwestern México (Gentry, 1982). Many of the natural populations are heavily decimated because mature whole individuals are harvested just before flowering, when the sugar concentration is at its maximum, to produce *mescal* in local artisan distilleries, a traditional liquor similar to the well known Mexican *tequila* (Colunga-GarcíaMarín and Zizumbo-Villarreal, 2007; Zizumbo-Villarreal and Colunga-GarcíaMarín, 2007). Harvesting prevents the species reproduction because *A. cupreata* is a semelparous plant (it can reproduce sexually only once in their life time) and it does not reproduce vegetatively. The effect of harvesting in the population demography is aggravated because plants need between 7 and 15 years to reach their sexual maturity (Illsley *et al.,* 2007). Apparently, the overexploitation of the natural populations is more severe in the state of Michoacán than

that in Guerrero. There are a few examples of well organized Indian communities to conduct some management of the *A. cupreata* natural populations in the region of Chilapa, Central-East part of the state of Guerrero, with specific rules that contribute to a more sustainable management, such as harvesting no more than 80 % of the individuals at flowering stage, reintroducing seedlings propagated in nurseries and setting aside populations designated as reserves (Illsley-Granich C. 2004; Personal Comm.)[1].

Local producers of mescal, mainly from the state of Michoacán, recently recognized that their use of natural populations of *A. cupreata* is not sustainable, and gradually began to organize themselves in mescal producer associations that have established approximately 800 ha of plantations (SEDRU, 2009) from seedlings produced in rustic greenhouses without control of seed source. Although such actions are an encouraging start, a more sophisticated management program is required which should combine a sustainable harvesting with a biological conservation program to protect the genetic variation of *A. cupreata* (Eguiarte and Souza, 2007). For reaching this goal, the patterning of genetic variation of natural populations needs to be determined.

Genetic diversity among and within populations of several species of *Agave* endemic to México has been studied using molecular markers. In general, high levels of expected heterozygosity (H_e) were observed, and high genetic differentiation among populations (F_{ST} or G_{ST}) in one case, and medium to low in most cases, has been documented: $H_e = 0.335$ and $F_{ST} = 0.236$ for *Agave victoriae-reginae* T. Moore (Martínez-Palacios *et al.,* 1999); $H_e = 0.394$ and $\theta = 0.083$ for *Agave lechuguilla* (Silva-Montellano and Eguiarte, 2003b); $H_e = 0.12$ to $H_e = 0.29$ and $G_{ST} = 0.11 \pm 0.02$ for the complex *Agave deserti* Engelm., *A. cerulata* Trel. and *A. subsimplex* Trel. (Navarro-Quezada *et al.,* 2003); and $H_e = 0.261$ for *Agave angustifolia* Haw. (Barraza-Morales *et al.,* 2006). However, to our present knowledge there are no previously published studies of the genetic variability of *A. cupreata*.

The objectives of the present study are: a) estimate levels of variation and genetic structure among and within natural populations of *Agave cupreata* using isozyme markers, and b) suggest measures of conservation of the genetic diversity of *A. cupreata*.

MATERIALS AND METHODS

Studied populations and material collection

Leaves of 37 randomly selected mature healthy individuals of each of 12 natural populations (8 from the

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[1] **Illsley-Granich C (2004)** Manejo campesino sustentable del maguey papalote de Chilapa. Informe final de proyecto. Grupo de Estudios Ambientales AC y Comisión Nacional para el Conocimiento y Uso de la Biodiversidad, México D.F. 43 p.

state of Michoacán and 4 from Guerrero) were collected along the entire distribution range of *A. cupreata* (Table 1). The groups of individual plants represented by the samples are termed populations while the location where a population was collected is called provenance. Leaf samples were transported to the lab in coolers (6 ºC), and 3 cm x 3 cm samples were stored at -70 ºC until processed.

Isozyme electrophoresis

Starch gel electrophoresis and staining protocols were conducted following standard methods (Conkle *et al.,* 1982; Cheliak and Pitel, 1984), with modifications as described by Martínez-Palacios *et al.* (1999). Sections of fresh, young leaves were crushed into 0.5 mL of the grinding buffer [3:1 (v:v) mixture of buffer YO from Yeh and O'Malley (1980) and VegII from Pitel and Cheliak (1984)]. The extracts were adsorbed in Whatman No. 17 paper wicks that were applied later to starch gels 11 %. Two systems of electrode and gel buffers, and seven enzymes with good resolution were used. A total of 9 loci were analyzed. System LiOH 8 (Soltis *et al.,* 1983; Martínez-Palacios *et al.,* 1999) resolved glutamate oxaloacetate transaminase (*Got*-1), malic enzymes (*Me*-2), menadione reductase (*Mnr*-1), phosphoglucoisomerase (*Pgi*-1 and *Pgi*-2). System C (Stuber *et al.,* 1988) resolved acid phosphatase (*Acph*-1), glutamate dehydrogenase (*Gdh*-1), phosphoglucomutase (*Pgm*-1 and *Pgm*-2). Gels were electrophoresed at 60 mA for 7 to 8 h.

Enzymatic systems with more than one locus were numbered according to their mobility relative to the alleles of standard individuals present in all gels and systems. Allelic variants were numbered sequentially from anode to cathode. The numbers of loci and alleles genetically controlling the enzyme activity were inferred from the observed banding patterns and from data on quaternary structure (Wendel and Weeden, 1989). Initially we essayed other loci (*Dia1*, *Dia2*, *Est1* and *Est2)* that worked with *Agave victoriae-reginae* (Martínez-Palacios *et al.,* 1999), but we discarded them because they showed poor resolution with *A. cupreata* extracts. However, since we included *Gdh1*, *Pgm1* and *Pgm2*, our final number of examined loci (9) was not much different than that used by *A. victoriae-reginae* (10 loci).

Data analysis

we estimated the number of polymorphic loci (P, criterion of 95 %), average number of alleles per locus (A), observed (H_o) and expected (H_e) heterozygosity under Hardy-Weinberg equilibrium Chi^2 test (Snedecor and Cochran, 1967), exact test of Haldane (1954), Wright's F statistics (Wright, 1965) and their 95 % confidence intervals (using 10 000 iterations), genetic distances among populations (Nei, 1978), and a dendrogram was constructed through the unweighted pair group method with arithmetic mean (UPGMA) using the tools of the population genetics program (TFPGA) (Miller, 1997). A Mantel test (Manly, 1987) was performed between genetic and geographic

Table 1. Mexican localities and genetic parameters of twelve *Agave cupreata* populations.

Population	State[†]	Municipality	Locality	Coordinates Lat. N, Long. W	Altitude (m)	P (%)	A	He	Ho
1	Mich.	Turicato	Chiquito	19°21', 101°31'	1650	100	2.9	0.526	0.605
2	Mich.	Madero	San Miguel	19º27', 101º11'	1619	89	2.8	0.439	0.439
3	Mich.	Morelia	Llanitos	19°35', 101°06'	1887	100	2.9	0.501	0.556
4	Mich.	Morelia	El Limón	19°32', 101°03'	1811	89	2.8	0.474	0.526
5	Mich.	Charo	Escalera	19°37', 101°02'	1733	100	2.9	0.467	0.550
6	Mich.	Charo	Pie de la Mesa	19°37', 100º57'	1927	100	2.9	0.436	0.480
7	Mich.	Tzitzio	Tembladera	19°34', 100º57'	1800	89	2.8	0.482	0.496
8	Mich.	Jungapeo	Escobas	19°28', 100°19'	1420	78	2.9	0.442	0.510
9	Gro.	E. Neri	Xochipala	17°48', 99°41'	1286	89	3.0	0.485	0.567
10	Gro.	Tixtla	Tixtla	17°33', 99°27'	1561	89	3.0	0.452	0.493
11	Gro.	Chilapa	Ayahualco	17°37', 99°10'	1465	89	3.1	0.461	0.532
12	Gro.	Atlixtac	Mesones	17°33', 98°52'	1703	100	3.0	0.472	0.521
Mean						92.6	2.9	0.467	0.521

[†]Mich.= Michoacán; Gro. = Guerrero; A = average number of alleles per locus; P = % of polymorphic loci; He = expected heterozygosity; Ho = observed heterozygosity.

Table 2. Wright's *F* statistics and gene flow (N_m) for twelve *A. cupreata* populations from the states of Michoacán and Guerrero, México.

Locus	F_{IS}	F_{ST}	F_{IT}	N_m
Acph-1	-0.126	0.043	-0.078	5.63
Pgi-1	-0.107	0.045	-0.058	5.30
Pgi-2	-0.094	0.034	-0.057	7.06
Pgm-2	-0.096	0.044	-0.047	5.39
Pgm-3	-0.115	0.043	-0.067	5.54
Me-2	-0.150	0.040	-0.104	5.92
Mnr-1	-0.097	0.046	-0.047	5.18
Gdh-1	-0.117	0.042	-0.070	5.73
Got-1	-0.142	0.042	-0.094	5.74
Average	-0.118	0.042	-0.071	5.68
Standard deviation	0.054	0.009	0.053	
95% Confidence interval	-0.009 to -0.207	0.061 to 0.028	-0.156 to 0.038	

F_{IS} = inbreeding coefficient of individuals relative to subpopulations ; F_{ST} = inbreeding due to differentiation among populations, relative to the total population; F_{IT} = inbreeding of individuals relative to the total population; N_m = gene flow.

distances. Minimum viable effective population size (N_e) was estimated based on the average H_e using the linear model fitted by regressing N_e values against H_e values, as used by Millar and Libby (1991) in several conifer species. N_e values estimated by Millar and Libby (1991) assumed a mutation rate of 10^{-5} and are designed to maintain given H_e values. The regression model proposed by Viveros-Viveros *et al.* (2010) was used:

$$N_e = -984.58 + (36723\ H_e)$$

RESULTS AND DISCUSSION

Variation and genetic structure

High polymorphism was found, with an average among

Table 3. Genetic distances (above diagonal) and geographic distances (below diagonal, km) for twelve *A. cupreata* populations from the states of Michoacán and Guerrero, México. Population numbers as in Table 1.

Population		Nei's genetic distances										
	1	2	3	4	5	6	7	8	9	10	11	12
1		0.058	0.060	0.046	0.023	0.038	0.028	0.041	0.018	0.011	0.034	0.035
2	58.4		0.067	0.018	0.028	0.052	0.037	0.070	0.048	0.028	0.071	0.020
3	73.9	15.6		0.014	0.066	0.083	0.065	0.099	0.047	0.071	0.047	0.038
4	74.6	17.2	5.8		0.012	0.035	0.033	0.052	0.031	0.025	0.032	0.008
5	81.4	24.0	9.0	7.54		0.016	0.027	0.032	0.035	0.008	0.043	0.017
6	88.0	30.6	16.5	13.55	7.6		0.035	0.086	0.065	0.027	0.071	0.057
7	84.5	28.5	16.2	11.7	8.6	5.1		0.083	0.056	0.021	0.071	0.048
8	118.5	73.1	64.2	59.08	56.9	50.1	44.3		0.051	0.023	0.047	0.026
9	238.9	245.1	249.0	243.8	247.1	244.1	239.0	206.5		0.023	0.011	0.036
10	274.6	282.5	284.9	279.3	282.8	279.3	275.0	241.6	35.9		0.027	0.016
11	292.7	294.5	296.1	290.8	293.0	289.0	285.2	249.1	54.6	30.6		0.033
12	327.5	326.3	327.8	322.0	323.5	319.6	315.6	276.6	90.9	64.0	35.5	

Geographic distances (km) (row label, left side of table)
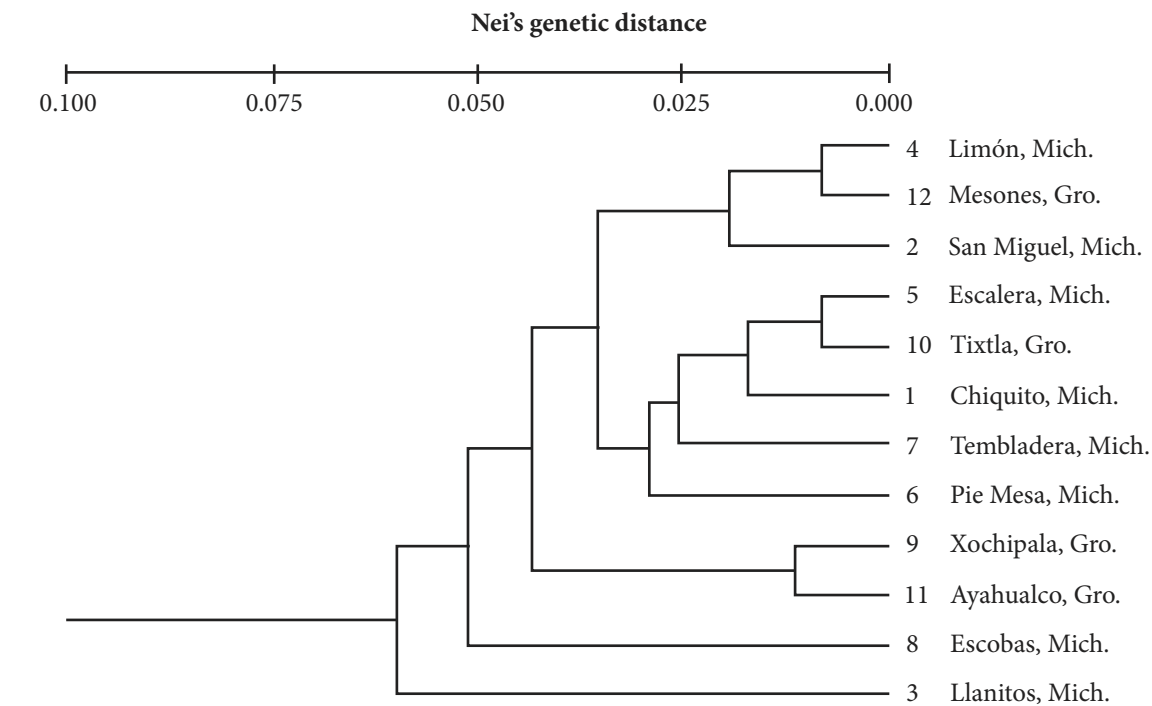
Nei's genetic distance

Figure 1. UPGMA dendrogram for twelve *A. cupreata* populations from the states of Michoacán and Guerrero, México, based on Nei (1978) genetic distances.

populations of 93 % polymorphic loci and 2.9 alleles per locus. The heterozygosity was also very large, with an average expected value of H_e = 0.467, and an even larger value of observed heterozygosity of H_o = 0.521 (Table 1). These values are the largest ever reported for an *Agave* species endemic to México (Martínez-Palacios *et al.,* 1999; Navarro-Quezada *et al.*, 2003; Silva-Montellano and Eguiarte, 2003a; Demey *et al.,* 2004). This result should be taken with caution because we used only 9 loci; there is a possibility that by examining a larger number of loci the estimated heterozygosity could be lower, if by chance our sample could be biassed. Haldane test showed that 5 out of 9 loci had significant excess of heterozygotes, in comparison to Hardy-Weinberg expectations (P < 0.03).

Although moderate, there is a significant genetic differentiation among populations with F_{ST} = 0.042 (95 % confidence interval from 0.0613 to 0.0281) (Table 2). Such differentiation is lower than the values reported for other *Agave* species: F_{ST} = 0.08 for *A. subsimplex* (Navarro-Quezada *et al.*, 2003); F_{ST} = 0.083 for *A. lechuguilla* (Silva-Montellano and Eguiarte, 2003a); F_{ST} = 0.135 for *A. deserti*; and F_{ST} = 0.236 for *A. victoriae-reginae* (Martínez-Palacios *et al.,* 1999).

It is likely that the moderate genetic differentiation is produced by the counteracting effect of a relevant gene flow, estimated as N_m =5.68 (Table 2), which might be due to the action of efficient pollinators such as bees, birds and bats (Gentry, 1982; Eguiarte *et al.,* 2000).

Average genetic distance between pairs of populations was relatively low (*D* = 0.04). The largest genetic distance (*D* = 0.09) was found between the Escobas and Llanitos populations, both from Michoacán, while the lowest genetic distance (*D* = 0.008) was between Tixtla, Guerrero and Escalera, Michoacán (Table 3). The UPGMA dendrogram does not follow a geographic pattern since geographically distant populations clustered together (for example: Limón, Michoacán grouped with Mesones, Guerrero, both separated by 322 km), while some geographically close populations did not clustered together (Figure 1). This lack of association between genetic distances and geographic locations was confirmed by the Mantel test (r = -0.110, P = 0.769), which showed no significant isolation by distance.

Despite the heavy exploitation pressure to which *A. cupreata* populations are subjected, there is no evidence of inbreeding within populations: F_{IS} = -0.1179 (significantly different from zero, 95 % confidence interval from -0.0072 to -0.2054), a value that indicates a significant excess of heterozygotes. This can be related to the mating system of *A. cupreata* that only reproduces sexually, their flowers display herkogamy (spatial separation of anthers and stamens) and dichogamy (temporal separation of male and female reproductive organs), and specifically protandry (earlier maturation of anthers than that of pistils); these characteristics are favoring outcrossing and apparently making self pollination nearly impossible (Illsley *et al.,* 2007). Thus, this outcrossing system apparently plays a relevant role to prevent the accumulation of inbreeding, even when the population sizes are reduced. However, inter-specific crossing is seemingly possible, since seedlings that appear to be hybrids of *A. cupreata* with *A. attenuata* and *A. inaequidens* have been observed in established plantations of *A. cupreata* (Martínez-Palacios A. 2009; Personal comm.)[2].

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[2] **Martínez-Palacios A, S Chávez Mendoza, M Gómez Sierra, Ma Sierra Yxta, R Cárdenas Navarro (2009)** Management and Conservation of *Agave cupreata* (Agavaceae), (Resumenes en extenso en CD). First International Conference on Sustainable Cities. 26 al 29 de Octubre de 2009, Morelia Michoacán, México. pp:1-11

Estimated minimum viable effective population size was N_e =16 165. This value is very large, as a result of the very large average heterozygosity value. In general, for conservation of genetic variability the suggested N_e sizes range between 500 (Franklin, 1980; Frankham *et al.,* 2002) and 5000 individuals (Lande 1995; Frankham *et al.,* 2002). In our case it would be more realistic to use much more modest N_e sizes than the estimated value (16 165), considering that natural populations of *A. cupreata* have substantially lower census sizes, perhaps a maximum of N_e ≈ 500 (reproductive individuals flowering the same year). Thus, in order to designate *A. cupreata* natural populations as forest genetic resource conservation units (*sensu* Sáenz-Romero *et al.,* 2003, based on Ledig [1988], Millar and Libby [1991]), the largest populations would need to be located and protected.

Considerations for conservation

A sustainable management program of *A. cupreata* that allows continuous harvesting of mature individuals for mescal distillation needs to gradually satisfy the demand of this beverage by relying more on commercial plantations, rather than in natural populations. In parallel, it would be needed to protect natural populations and to establish additional ones to be managed as forest genetic resource conservation units (FGRCUs). Provided that the estimated minimum viable effective population size (N_e) for such FGRCUs is larger than the actual largest natural populations, it would be required to establish plantations between large natural populations, to serve as corridors for pollinators, in order to maintain the genetic flow among populations and also for increasing N_e, counterbalancing in that way the loss of individuals that could have occurred due to past harvesting. On this regard, although the recent establishment of commercial plantations of *A. cupreata* aided by subsidies from the federal, state and municipality governments seems to be a measure in the correct direction, it might be needed to establish additional plantations with the main goal of conservation, and not only for commercial harvesting.

The predicted decoupling between genotypes and climate due to factual climatic change could be diminished if new commercial plantations are established at altitudes higher than those of present populations, in order to match genotypes fitness to predicted future climates. Although it is generally suggested to plant at an elevation 300 m higher than the seed source, to match the climate predicted for 2030 (Sáenz-Romero *et al.,* 2010), it is recommended to make decisions based on estimations for contemporary and future climate for specific locations, using available resources, including web based tools (*e.g.* Crookstone, 2011).

CONCLUSIONS

Agave cupreata populations have a very large expected

heterozygosity (H_e = 0.467), with an excess of observed heterozygotes (H_o = 0.521, F_{IS} = -0.1179). The genetic differentiation among populations is relatively low (F_{ST} = 0.042), yet statistically significant. There is not significant isolation by distance. Estimated minimum viable effective population size was very large (N_e =16 165), due to the large heterozygosity values.

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