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Genetic diversity and structure analysis of the endangered plant species *Horsfieldia hainanensis* Merr. in China

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ABSTRACT

The genetic diversity and structure of nine natural populations of *Horsfieldia hainanensis* Merr., an endangered plant endemic to China, were studied using inter-simple sequence repeat markers. Nine primers were selected from 100 primers to evaluate 126 individual plants, from which a total of 136 bands were amplified and 108 bands were polymorphic. Our results demonstrated that the genetic diversity level of *H. hainanensis* was high with a percentage of polymorphic bands, Shannon's diversity index and Nei's genetic diversity index at the species level of 79.4%, 0.4787 and 0.3314, respectively, and correspondingly, averages of 40.4%, 0.2615 and 0.1843 at the population level. Significant genetic differentiation was observed among populations, showing that the coefficient of genetic differentiation among populations calculated using Nei's genetic diversity was 0.4509. The ranges of Nei's genetic identity and genetic distance among populations were 0.7387–0.8637 and 0.1466–0.3029, respectively. The unweighted pair group method with arithmetic mean clustering based on Nei's genetic distance indicated that nine natural *H. hainanensis* populations could be classified into two lineages. Collectively, we speculated that habitat fragmentation and disturbance from human activities could be considered the main reasons for the endangerment of *H. hainanensis*, and we propose *in situ* conservation for the existing natural *H. hainanensis* populations, especially at Mengtun, Niandou and Tongbiguan, where the genetic diversity is relatively high.

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ISSR marker; *Horsfieldia hainanensis* Merr.; genetic diversity; genetic structure; endangered mechanism

Introduction

Horsfieldia hainanensis Merr., belonging to the Myristicaceae family, is an evergreen tree with a narrow domain distribution in China. Trees belonging to this species possess good essential oil from the bark and red wood, which can be used for high-end furniture, decoration and other purposes, which make it an attractive resource [1]. *H. hainanensis* is mainly distributed in Guangxi, Yunnan, Hainan and other regions, in the shady and wet forests of hills and valleys at altitudes of 400–450 m near the borders of Burma, Vietnam and China [2]. In recent years, the natural resources of *H. hainanensis* have been gradually reduced by habitat destruction and illegal logging. This has left limited remaining parents and it is now listed as a second-grade protected plant and an endangered tree species [1]. As the marker species in humid tropical rainforests, *H. hainanensis* is of great value for the study of composition, geographical distribution and ecological characteristics of tropical rainforests, and of the conservation biology of endangered plant species in this region.

Habitat loss and fragmentation are the immediate causes of species becoming endangered. Therefore, the study of the population genetic diversity and the genetic structure of an endangered species is a prerequisite for the development of an effective protection strategy, and has become a core issue of conservation genetics [3]. There are few studies of *H. hainanensis*, and those that have been published mainly focus on breeding technology [4], chemical composition of its volatile oil [2] and population structure characteristics [1]. There has been no study of genetic diversity and structure of its natural populations, and the genetic information is scarce. In the present study, we used the inter-simple sequence repeat (ISSR) marker technique to analyse the genetic diversity and structure of the natural *H. hainanensis* populations in the tropical rainforest regions with relatively concentrated numbers of individuals. The objectives of this

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study were to understand the population genetic information, to investigate the mechanisms responsible for species endangerment and to provide a reference for the appropriate protection of genetic resources of *H. hainanensis*.

Materials and methods

Materials

The field investigation and resource collection of wild *H. hainanensis* were carried out in Guanxi, Yunnan and Hainan provinces from May to June 2015. Populations consisting of more than five adult plants were sampled, and a total of 126 individual plants from nine populations were collected with a distance between maternal plants of over 50 m. Young leaves were placed in a sealed bag with silica, brought back to the laboratory and stored at −70 °C in a freezer. The sample information is shown in Table 1.

Experimental method

Genomic DNA extraction and PCR amplification

As described by Zong et al. [5], genomic DNA was extracted from the leaves of *H. hainanensis* using a modified CTAB (cetyl trimethylammonium bromide) method. Polymerase chain reaction (PCR) was run with a 20-μL reaction system, including 40 ng of template DNA, 1.5 mmol/L Mg^{2+}, 0.5 U of *Taq* polymerase, deoxyribonucleoside triphosphates (dNTP) at 0.2 mmol/L and primers at 0.7 mmol/L. The PCR programme was 94 °C for 3 min, then 30 cycles of 94 °C for 45 s, annealing for 45 s and 72 °C for 90 s, followed by 72 °C for 5 min. The amplifications were performed in a Peqstar 96X Universal Gradient thermocycler (PEQLAB Biotechnologie GmbH, Germany). The amplification products were separated in 1.5% agarose gel, using TAE (Tris–acetate–EDTA) buffer at 125 V for 30 min, stained with ethidium bromide (0.5 mg/mL) and photographed under 254/312 nm wavelength lights using Micro Doc Gel Documentation System (Cleaver Scientific, USA).

Data analysis

The electrophoresis bands were evaluated using manual counting. To construct a binary data matrix, an arbitrary band was recorded as 1, or as 0 if no band was present at the same position. The percentage of polymorphic bands (*PPB*), Shannon phenotypic diversity index (*I*) and Nei's genetic diversity index (*H*) at both the species and population level were estimated using the software of POPGENE 1.31. The total (*ISP*) and average (*IPOP*) of *I* of the tested population were also determined to calculate the coefficient of genetic differentiation among populations (F_{ST}) [6,7].

According to Falush et al. [8], Bayesian analysis was performed for the ISSR marker data of all tested samples, using the software STRUCTURE version 2.3.1, then delta *K* was calculated to plot the delta *K* curve and to calculate *K* (the appropriate number of groups) and the groups of individuals. Unweighted pair group method with arithmetic mean (UPGMA) of genetic distance among populations was employed to establish the tree clustering using NTSYS-pc 2.1 software [9].

Results and discussion

Genetic diversity

Nine primers with a clear and stable product were selected from 100 ISSR primers published by Columbia University (Table 2), and were used to amplify the 126 individuals from nine natural *H. hainanensis* populations for a total of 136 loci. The *PPB* for these nine ISSR primers fell in the range of 76.5%–84.6% with an average of 80.0%, and the *PPB* at the species level was 79.4%. The amplification results obtained using the nine primers are shown in Table 2.

The genetic diversity parameters of the nine studied *H. hainanensis* populations are shown in Table 3. The ranges of *I*, *PPB* and *H* were 0.2144–0.3206, 33.1%–49.3% and 0.1509–0.2269, respectively, with corresponding averages of 0.2615, 40.4% and 0.1843, respectively. The population from the Mengtun village in Daxin County, Guangxi exhibited the highest genetic diversity (*I* = 0.3206, *PPB* = 49.3% and *H* = 0.2269), whereas the lowest

Table 1. Sampling information and sample size of *H. hainanensis*.

Population	Location	Representative geological coordinate (°)	Altitude (m)	Sample size
TBG	Tongbiguan Natural Reserve in Yunnan	24.69773°N, 97.58031°E	345	12
ZWY	Xishuangbannan Botanical Garden in Yunnan	21.92738°N, 101.25278°E	566	10
ND	Niandou Village, Daxin County, Guanxi	22.75117°N, 106.79873°E	375	20
MT	Mengtun Village, Daxin County, Guanxi	22.77938°N, 106.85725°E	335	22
NG	Nonggang Nature Reserve in Guangxi	22.48478°N, 106.94260°E	163	20
DZ	Tongzhong forest farm Fangchenggang City, Guangxi	21.69901°N, 107.56389°E	470	14
BWL	Bawangling Natural Reserve in Hainan	19.11961°N, 109.15078°E	683	8
PLL	Polong Ridge, Wuzhishan, Hainan	18.87444°N, 109.71424°E	684	10
JFL	National Forest Park, Jianfeng Ridge, Hainan	18.74311°N, 108.83906°E	208	10

Table 2. ISSR–PCR primers and their amplification.

Primer	Sequence (5′–3′)	Anneal temperature (°C)	Total bands	Polymorphic bands	*PPB* (%)
UBC808	$(AG)_8C$	53.6	17	13	76.5
UBC809	$(AG)_8G$	51.8	15	12	80.0
UBC812	$(GA)_8A$	47.1	14	11	78.6
UBC825	$(AC)_8T$	49.2	13	11	84.6
UBC826	$(AC)_8C$	51.2	14	12	85.7
UBC834	$(AG)_8YT$	52.9	16	11	68.8
UBC836	$(AG)_8YA$	50.5	16	13	81.3
UBC840	$(GA)_8YT$	52.8	16	13	81.3
UBC872	$(GATA)_4$	40.8	15	12	80.0
Average			15	12	80.0
Species			136	108	79.4

one was in Wuzhishan Natural Reserve in Hainan (I = 0.2144, *PPB* = 33.1% and H = 0.1509). The I and H for *H. hainanensis* at the species level were 0.4787 and 0.3314, respectively, indicating abundant genetic variation in this species.

The level of genetic diversity of a species reflects its ability to adapt to the environment, so the higher the genetic diversity, the stronger the species' adaptability to the environment [10,11]. It is believed that the genetic diversity of endangered species, endemic species and narrow-field species is low [12,13]. We demonstrated that although *H. hainanensis* is an endangered, narrow-field species endemic to China, it has maintained a relatively abundant genetic diversity and strong adaptability to the environment. Its *PPB* and H were 79.4% and 0.3314, respectively, which were significantly higher than the averages for various plant species (*PPB* = 71.0% and H = 0.22–0.23) according to Nybom [14]. In addition, the *PPB* of *H. hainanensis* was higher than that of many other endemic or endangered plants, including *Xylocarpus granatum* Koen (58.1%) [15], *Neolitsea sericea* (Bl.) Koidz. (23.1%) [16], *Tetraena mongolica* Maxim. (63.3%) [17] and *Primula interjacens* Chen (75.5%) [18]; but lower than that of *Sindora glabra* Merr. ex de Wit (93.4%) [19], *Ranunculus cabrerensis* Rothm. (82.5%) [20] and *Primula heterochroma* Stapf. (86.2%) [21].

Genetic relationship

The F_{ST} among investigated *H. hainanensis* populations was 0.450. Wright [22] illustrated that genetic differentiation was great among populations when $F_{ST} > 0.25$, significant when $0.15 < F_{ST} \leq 0.25$, intermediate with $0.05 \leq F_{ST} \leq 0.15$, and slight if $F_{ST} < 0.05$. Thus, *H. hainanensis* exhibited substantial genetic differentiation among populations with minor gene flow.

The Bayesian analysis of 126 *H. hainanensis* individuals demonstrated that the log-likelihood value (LnP(D)) increased as the population numbers increased, with no significant inflection point (Figure 1(A)). The change of delta K showed a maximum when K = 2 (Figure 1(B)), indicating that the optimal number of *H. hainanensis* groups was 2. The genetic structure of 126 *H. hainanensis* individuals is shown in Figure 1(C). Group 1 (blue) was composed of plants from populations of TBG, ZWY, ND, MT and NG, as well as one individual from DZ; while group 2 (red) consisted of three populations: BWL, PLL and JFL. The individuals originated from a unitary source when the individual $Q > 0.6$ and so belonged to corresponding groups; however, mixed origins were indicated if $Q \leq 0.6$, and in this case the lineage was more complicated [23]. Among the 126 tested *H. hainanensis* individuals, group 1 accounted for 67.5% (85 plants), group 2 for 22.2% (28 plants) and 10.4% (13 plants) belonged to mixed groups.

The genetic distance and genetic similarity of the nine natural *H. hainanensis* populations are presented in Table 4. The genetic similarity of the nine natural *H. hainanensis* populations was in the range of 0.7387–0.8637, with an average of 0.7991, suggesting that the nine populations were phylogenetically close and may share the same origins. Of these, the genetic relationship between JFL and DZ populations was the most distant with a similarity of 0.7387, while NG and MT shared the closest relationship with a similarity of 0.8637.

The UPGMA clustering based on the Nei's genetic distance among populations is shown in Figure 2. The nine

Table 3. ISSR-PCR primers and their amplification results (the population codes are shown in Table 1).

Population	Shannon phenotypic diversity index									I	*PPB* (%)	H
	UBC 808	UBC 809	UBC 812	UBC 825	UBC826	UBC 834	UBC 836	UBC 840	UBC 872			
TBG	0.2649	0.3040	0.2902	0.2511	0.3360	0.2392	0.2769	0.3635	0.2215	0.2830	44.1	0.1987
ZWY	0.2749	0.2130	0.2904	0.3154	0.1863	0.2843	0.2457	0.3064	0.2574	0.2639	40.4	0.1863
ND	0.2769	0.2577	0.2756	0.4083	0.3352	0.2362	0.2703	0.3853	0.2172	0.2938	45.6	0.2066
MT	0.3441	0.2583	0.2844	0.3060	0.4289	0.1688	0.2857	0.4586	0.3535	0.3206	49.3	0.2269
NG	0.2312	0.2463	0.1800	0.2561	0.2919	0.1626	0.2816	0.2805	0.3094	0.2485	38.2	0.1752
DZ	0.1144	0.3823	0.3308	0.2527	0.2344	0.1239	0.2447	0.2466	0.1731	0.2303	35.3	0.1625
BWL	0.1983	0.2501	0.3047	0.3072	0.2403	0.1257	0.3571	0.2473	0.2381	0.2500	39.0	0.1755
PLL	0.1920	0.2170	0.2249	0.3025	0.2333	0.1271	0.2275	0.1271	0.3054	0.2144	33.1	0.1509
JFL	0.1123	0.3130	0.2786	0.3483	0.1945	0.1994	0.2792	0.2079	0.3449	0.2494	38.2	0.1760
Mean	0.2232	0.2713	0.2733	0.3053	0.2756	0.1852	0.2743	0.2915	0.2690	0.2615	40.4	0.1843
Species	0.4647	0.4692	0.5170	0.5368	0.5231	0.3915	0.4825	0.4936	0.4498	0.4787	79.4	0.3314

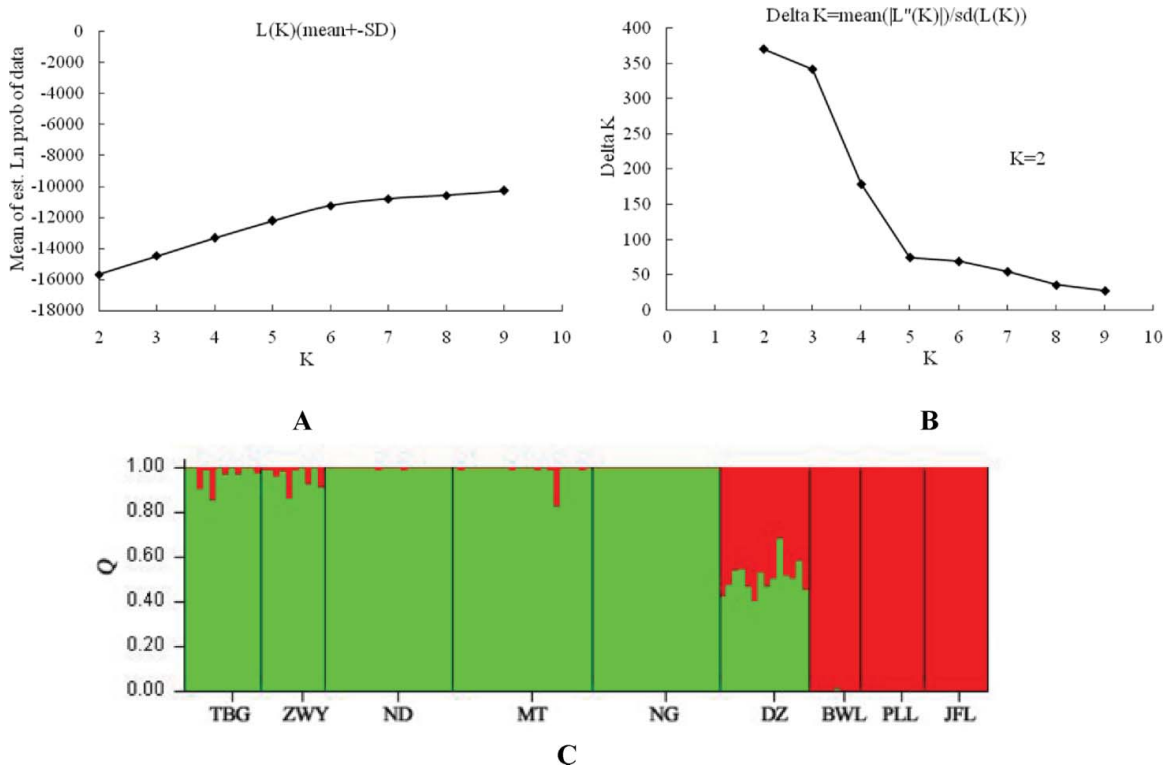

Figure 1. (**A**) Plot between LnP(K) and different assumed values of K. (**B**) Effect of group number K on delta K. (**C**) Genetic structure of 126 individuals from nine *H. hainanensis* populations (the population codes are shown in Table 1).

populations were divided into two clusters: cluster 1 consisted of six populations, TBG, ZWY, ND, NG, MT and DZ; and cluster 2 of BWL, PLL and JFL. This was consistent with the model clustering results according to posterior probability in the software of STUCTURE, and further validates the accuracy of the clustering.

It is generally believed that the genetic structure of a population is the integrative result of its life history, mating system, seed dispersal mode, gene flow and geographical distribution [24,25]. The reproduction system of *H. hainanensis* has not been reported, but the panicles of its male flowers are bright yellow, which is a characteristic to attract pollinators. In addition, according to the average F_{ST} of various plants [14], the F_{ST} = 0.4509 for *H. hainanensis* populations is higher than that of outcrossing plants (0.27), lower than that of selfing plants (0.65) and comparable to that of mixed mating plants (0.40). Therefore, we speculate that it has a mixed mating system with mainly outcrossing, which might be one reason for the relatively high genetic differentiation in populations. However, in certain cases, the F_{ST} depends on factors other than the mating system, so it is necessary to deeply study the mating system in *H. hainanensis.*

The fruit of *H. hainanensis* is a capsule, oval and yellow with a large seed, and its F_{ST} is 0.4509, which is substantially higher than that of the animal-dispersed (0.27) and water-dispersed seeds (0.25), but comparable to that of gravity-dispersed seeds (0.45), based on the average F_{ST} values of Nybom [14]. During the field observation and sampling, seedlings of *H. hainanensis* were mostly located underneath mother trees, so we hypothesize that gravity is the major means of seed dispersal for this species, which may explain its small gene flow. Previous studies showed a significant effect of gene flow on the

Table 4. Genetic similarity and distance of nine natural *H. hainanensis* populations (the population codes are shown in Table 1).

Population	TBG	ZWY	ND	MT	NG	DZ	BWL	PLL	JFL
TBG		0.8542	0.8436	0.8212	0.8282	0.7938	0.8082	0.7873	0.7676
ZWY	0.1575		0.8453	0.8139	0.7948	0.7749	0.7993	0.7779	0.7857
ND	0.1701	0.1681		0.8487	0.8583	0.8271	0.7705	0.7637	0.7780
MT	0.1970	0.2059	0.1641		0.8637	0.8030	0.7983	0.7732	0.7654
NG	0.1885	0.2297	0.1528	0.1466		0.8033	0.7754	0.7928	0.7397
DZ	0.2309	0.2550	0.1898	0.2194	0.2191		0.7576	0.7793	0.7387
BWL	0.2130	0.2241	0.2608	0.2253	0.2544	0.2775		0.8277	0.8013
PLL	0.2392	0.2511	0.2696	0.2572	0.2321	0.2494	0.1892		0.8062
JFL	0.2645	0.2411	0.2510	0.2674	0.3015	0.3029	0.2215	0.2154	

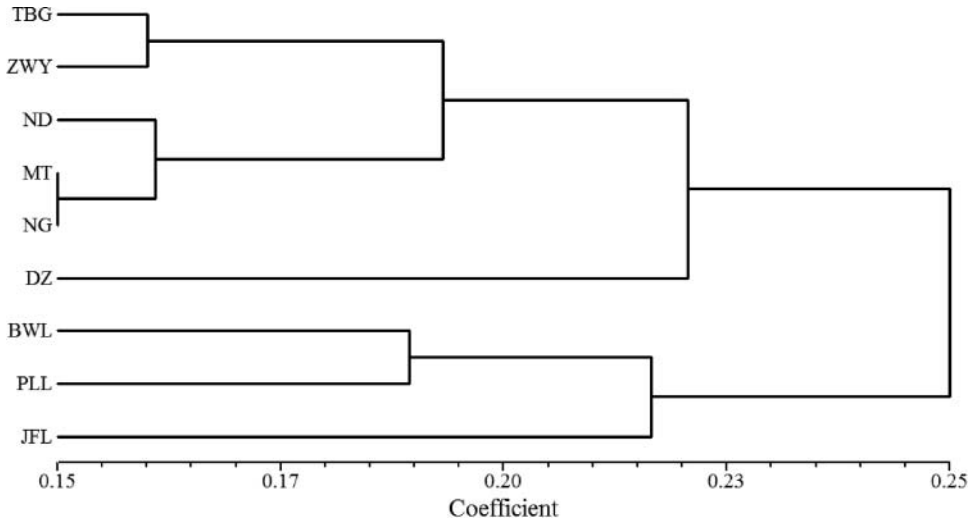

Figure 2. UPGMA clustering of nine *H. hainanensis* populations based on Nei's genetic distance (the population codes are shown in Table 1).

genetic differentiation of populations [26]. Gene flow of <1 is insufficient to resist the genetic differentiation caused by population genetic drift [27]. The N_m of *H. hainanensis* was 0.6088, suggesting that the genetic drift in populations would be likely to influence the genetic differentiation.

The endangering mechanism for H. hainanensis *and protection of its resources*

It is commonly believed that low fertility, little genetic variation within species, weak competitiveness, poor adaptability, excessive logging and habitat destruction are the reasons for plant endangerment [28,29]. Williamson and Werth [30] pointed out that endangered species with narrow field distribution and abundant genetic diversity had not experienced a bottleneck effect, and inbreeding within the population might not necessarily lead to selfing depression; thus, it was likely that fragmentation of native habitats caused by geographical isolation and human disturbance was the cause of their being endangered. Our data showed that the natural *H. hainanensis* populations possessed relatively abundant genetic diversity. Therefore, its endangerment does not originate in low potential of population genetic evolution. We speculate that there are three reasons. (1) Historical climate change transformed a large population with a wide distribution and high level of genetic diversity into the current residual and fragmented distribution. (2) *H. hainanensis* is distributed in Hainan island of China and the border of Burma, Vietnam and China. The populations are separated and geographically isolated by high mountains and oceans, so the gene flow is blocked. (3) In the past few hundred years, human activities have caused deterioration or even loss of tropical rainforest habitats. The habitat for *H. hainanensis* has been greatly disturbed by human activities, and the difficulty of natural population regeneration and gradual reduction of individuals has led to genetic drift.

Species conservation is essential to protect the genetic diversity and evolutionary potential. The higher the genetic diversity, the stronger the species' adaptability to environments and the greater the evolutionary potential [11,31,32]. Because of the high degree of genetic diversity in *H. hainanensis* at the species level, especially within populations, we believe that there is still high adaptability and evolutionary potential in the natural *H. hainanensis* population, and thus, *in situ* conservation should be the primary measure. Due to the genetic differentiation among *H. hainanensis* populations, the protection priority should be given to MT, ND and TBG populations, whose genetic diversity is relatively abundant. In addition, although genetic diversity is relatively high at both species and population levels, our investigation showed that the natural *H. hainanensis* population is very limited with only one or several individuals in most populations, so the decline from inbreeding within these populations and genetic drift will be more significant, and extinction is possible. Thus, in addition to improved protection of current populations, it is necessary to study the biological characteristics of *H. hainanensis*, to monitor the inbreeding decline, to adopt effective measures to promote population recovery and to expand the population size.

Conclusions

H. hainanensis Merr. populations have maintained a relatively abundant genetic diversity. There is still high adaptability and evolutionary potential in the natural *H. hainanensis* populations. Based on the analysis of population genetic information, it could be suggested that the main reasons for the endangerment of *H. hainanensis* were habitat fragmentation and disturbance from human activities. Accordingly, it would be a reasonable and effective measure to carry out *in situ* conservation for the existing natural *H. hainanensis* populations.

Disclosure statement

No potential conflict of interest was reported by the authors.

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