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EFFECTS OF HABITAT FRAGMENTATION ON GENETIC DIVERSITY IN *CYCAS BALANSAE* (CYCADACEAE)

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ABSTRACT

Habitat fragmentation is a serious threat to species survival. In Vietnam, *Cycas balansae* has been considered as threatened species because of the reduction and fragmentation of its habitats and over-exploitation. We assessed genetic variability and the pattern of population structure among six populations sampled in four provinces: Hoa Binh, Ha Nam, Ninh Binh and Quang Ninh. Polyacrylamide gel electrophoresis was performed on leaf tissues from 152 individuals representing 6 populations of *C. balansae*. Six of twelve enzyme systems were used to estimate genetic diversity at population and species levels. Eleven loci were examined. The allozyme data showed high levels of genetic diversity within all populations, ranging from 0.538 in Ba Sao to 0.628 in Tan Dan (average 0.576). The maintenance of high levels of expected heterozygosity (average 0.571) and low in observed heterozygosity (average 0.347) might be related to great heterozygote deficiency and increased frequencies of rare alleles. Genetic differentiation among populations was low (Dst = 0.036 and Gst = 0.064), indicating high level of gene flow (Nm = 3.22). Isolation by geographical distance was observed, however, no significant relationship between genetic distances and geographical distances was recorded. Our studies suggest small population sizes of cycads brought about by fragmentation of its habitats, over-exploitation, and increasing number of inbred individuals within populations.

Keywords: Habitat fragmentation, *Cycas balansae*, genetic variation, allozymes, conservation

1. INTRODUCTION

Habitat fragmentation is becoming a serious threat to species survival. The habitats of species are often fragmented into small in sizes and isolated from each other by a matrix of habitat unsuitable for their survival. The consequences are reduction of an effective metapopulation structure and to establishment of few remaining fragments with small population sizes; hence such populations face an increased probability of extinction [1, 2]. Studies showed that the fragmented habitats increase genetic drift and inbreeding coefficients [3, 4, 5, 6], decrease gene flow and produce high levels of population differentiation [7, 8]. In general, the loss of genetic variation and increased homozygosity in small population sizes via genetic drift reduce the possibility of adaptation in their environments and individual fitness [9, 10]. Thus, the

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maintenance of genetic variation within species is considered a priority in conservation efforts [11].

In order to evaluate the effects of habitat fragmentation, an investigation of demography and genetics of plant species under their natural conditions is important in the development of conservation strategies. It is recognized as a keystone to success in long-term management and conservation in any species [12]. In Vietnam, *Cycas balansae* is a perennial shrub and occurs in lowland forests on loamy soils over limestone hills. In recent years, increased human pressures have resulted in a great reduction in forest areas and an increased level of fragmentation of surviving forests. These trends have a detrimental influence on cycad habitats. Clearing of forests for cultivation and human settlements has had a large impact on forest ecology; has rendered many areas unlike the original. Moreover, the cycads are exploited as ornamental plants and medicines by local people. These factors have threatened the survival of cycads. All populations studied were small in size to survive in disturbed forest patches. The goals of our study were to use electrophoresis and field measures to address the level and structure of genetic variation, population characteristics related to heterozygosity and genetic consequences under disturbed habitats. This work also aims to determine strategies for genetic conservation of this cycad.

2. METHODS

2.1 Study species

Cycas balansae is one of palm-like plants and occurs in Vietnam, Laos, Thailand and southern China [13, 14]. It was found in the deep shade in tall closed evergreen forests. Besides, it was also found in private gardens as ornamental plants. *C. balansae* is insect-pollinated or wind-pollinated and obligatory outcrossing. Flowering appears during the period of October-December [13]. We only observed two individuals producing seeds in Yen Quang, Ninh Binh during our surveys. One produced 9 seeds and other produced 13 seeds.

2.2 Study sites and field investigations

The research was carried out in Ba Sao with 23°31N and 105°56E at 246 m elevation, Phu Thanh (20°16N and 105°51E, 158 m elevation), Dong Tam (20°14N – 105°50E, 236 m elevation), Yen Quang (20°21N – 105°36E, 365 m elevation), Tan Dan (21°08N – 106°56E, 50-100 m elevation) and Yen Tu (21°11N-106°40E, 245 m elevation) (Fig. 1). The geomorlogical structure consists of a ridge of prominent hills with below 500 m in altitude and low slopes of 10° - 15°. *C. balansae* was found in various communities including secondary forests and forestry plantations on loamy soils over schist or granites. It grows in shade of the forests. Due to distribution at low and medium elevations, forests have been greatly fragmented by human activities. Now over-exploitation of forests for fuel wood collection is a major cause for regraded forests and creates a light-demanding species and shade-tolerant species. Over-grazing increases the risk of forests in relation to replacement of wood growth. The dominant species in these communities are shrubs in family Acanthaceae *(Psiloesthes elonga, Strobilanthes acrocephalus),* Asteraceae *(Artemasia vulgaris),* Poaceae, Arecaceae *(Calamus rudentum),* Rubiaceae, and ferms (Aspleniaceae and Athyriaceae). Valleys were cleared of forests for cultivation of maize, manioc, tea, lemons and custard apples in Ba Sao and Dong Tam; wet rice, lemons, sugar canes, oranges and longans in Phu Thanh. The plants grow in Yen Quang with the dominant species in *Rhodomyrtus, Breymia, Aidia,* Mimosa, *Melastoma and Thysanolena* on slopes and ridges. Coniferous forests were planted in 1970s in Tan Dan, with the dominant species of *Pinus merkusii* in the overstorey (up to 35 m in height). The understorey is dominated by shrubs of *Vaccinium, Wendlandtia, Rhodomyrtus, Ischaehaemum* and *Heteropogon*. *Cycas balansae* prefers the high humidity of 60% in Yen Tu and Tan Dan and

84% in Dong Tam, Phu Thanh and Ba Sao, winter with low temperature, an average 16^{o}C in January and summer with high temperature, averaging 28,5^{o}C in July. In general, fertility of soil has greatly declined in these forests. Young leaves of 6 populations of *Cycas balansae* were collected in July and August, 2002 at 6 sites: Ba Sao (Ha Nam), Dong Tam and Phu Thanh (Hoa Binh), Yen Quang (Ninh Binh), and Tan Dan and Yen Tu (Quang Ninh). One hundred fifty-two (152) sampled individuals were used to analyze isozymes belonging to 6 populations. The collected samples were immediately kept in marked plastic bags and put in the icebox. They were transferred to the Laboratory of Molecular Biology, Institute of Ecology and Biological Resources, Hanoi and subsequently stored at –80°C until the time to use for enzyme electrophoresis. The distance between consecutively collected plants, age structure and density in each studied population was determined in the field.

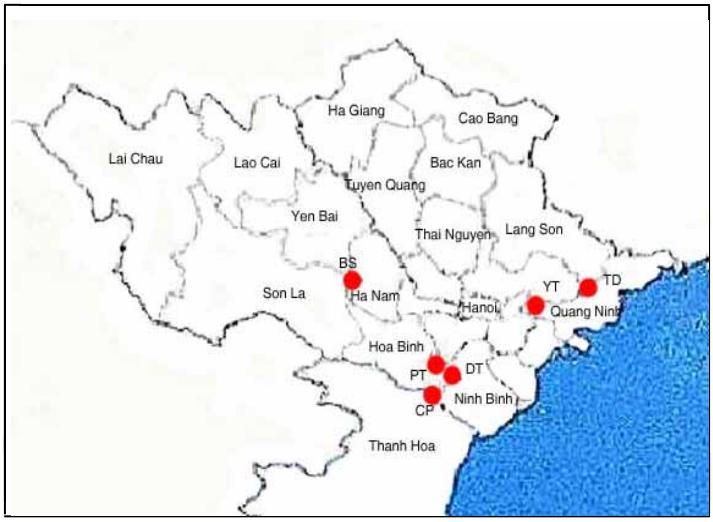

Fig. 1: *Map showing the studying sites: PT: Phu Thanh, DT: Dong Tam, BS: Ba Sao, CP: Yen Quang, TD: Tan Dan, and YT: Yen Tu*

2.3 Electrophoresis

We used polyacylamide gel electrophoresis to obtain allozyme data. Leaf tissue was ground in 1 ml extraction buffer of Triest [15]. The electrode buffer was Tris-CI and Glycine. The gels contained two parts, stacking gel with 0.5 M Tris-CI, pH 6.8 and 4.5% acrylamide, and running gel with 1.5 M Tris-CI, pH 8.8 and 10% acrylamide. Gel were run for 3 hours at 70 mA and 4°C and then stained at room temperature for 12 enzymes: Shikimate dehydrogenase (SKDH), Aspartate amonotransferase (GOT), 6-P-Gluconate dehydrogenase (6PGDH), Isocitrate dehydrogenase (IDH), Leucine aminopeptidase (LAP), β-esterase (β-EST), Esterase (EST), Alcohol dehydrogenase (ADH), Malate dehydrogenase (MDH), Xanthine dehydrogenase (XPH), Acid phosphate (APH) and Malic enzyme (ME). Six enzymes that showed clear resulution were GOT, LAP, SKDH, 6PGDH, IDH and β-EST; these were used to assess genetic variation at species and population levels.

2.4 Statistical analysis

We used several parameters of genetic diversity within populations: the number of alleles per locus (A); the population of polymorphic loci (P) at 95% criterion; the observed heterozygosity

or the mean proportion of heterozygotes for the polymorphic loci (Ho) and the expected heterozygosity or the proportion of heterozygotes expected under Hardy-Weinberg equilibrium (He). Chi-square test was used to test for significant differences between observed and heterozygosities. The Fixation indices, and Wright's [16] F-statistics were used to assess the distribution of genetic diversity within population and among populations: Fis (the coefficient of excesses of homozygotes or heterozygotes compared with panmictic expectations within populations), Fit (the coefficient within the entire populations) and Fst (the coefficient of differentiation among populations).

Genetic diversities within and among the populations were analyzed for each polymorphic locus using Nei's [17] genetic diversity statistics: the total genetic diversity (Ht); the genetic diversity within populations (Hs); the genetic diversity between populations (Dst) and the coefficient of genetic diversity (Gst). The genetic differentiation among populations was estimated from allele frequencies using Nei's [18] genetic distance and identity for all pairs of populations. UPGMA cluster analysis of genetic distances was generated to examine genetic associations among populations using Roger's [19] genetic distance.

All above parameters were treated using FSTAT and TFPGA [20, 21]. The gene flow between populations (Nm) was also determined using Wight's [22] Fst value: Nm = (1-Fst)/4Fst. Here, Fst describes genetic differentiation among populations.

3. RESULTS

3.1 Population characteristics

Data available from spatial studies in the field for the *Cycas balansae* populations were presented in Table 1. The number of cycads was small in all the studying populations. It ranged from 25 at the Ba Sao secondary forest (BS) to 87 at the Yen Quang secondary forest (CP), an average of 58.8 cycads per wild cycad population. Cycads grow scatterly or in small clumps on flanks and ridges of hills. Geographic distance between two consecutive trees in each population was recorded during the surveys. The mean distance was short in CP and longer than in BS. It averaged 28.8 m, ranging from 7 m to 70 m for the cycad population in Ba Sao. These values were 6.2 m (0.2 - 21 m), 12.3 (0.3 - 40 m) and 10.2 m (0.3 - 25 m) for Yen Quang, Tan Dan and Phu Thanh, respectively. The population size was found by counting the number of individuals in the 50 × 50 m plots. The density varied from 4 cycads/ha in BS to 40 cycads/ha in CP. Population from Yen Quang maintained higher density did than the other populations.

Therefore, there were differences on the geographic distance and density within a population. For this reason, it might be relative to dispersed seeds due to wind or animals. Levels of exploitation for purposes as ornamental plants or medicines influenced the differentiation, too. The demographic data from all populations showed the number of young cycads produced by females in a population varied. This is dependent on environmental conditions and their habitats. There was low proportion of youngs (35%) for BS compared to 51.3% and 54.25 for TD and DT, respectively. These proportions were higher than in PT (64%) and CP (54.8%). A number of individuals having stems underground were very high for all studied populations of *C. balansae*. These proportions were 83.3% and 87.5% in Yen Quang and Tan Dan, respectively. It was difficult to discriminate male and female for youngs, however, two cycads in CP and one in DT produced seeds were observed during the surveys.

The morphological features and a principal component analysis (PCA) showed differentiation among wild populations (Table 1 and Fig. 2), with the exception of cultivated population of YT in the private gardens. PCA yielded two components with eigenvalues greater than 1.0. The first two components accounted for 40.71 % and 78.94%, respectively, of variation. Factors loaded in first two components (respectively in parentheses) included height of stem (0.488, -0.861),

diameter of stem (-0.473, -0.846), leaf long (-0.988, -0.075), leaf width (-0.989, 0.091) and number of leave per individual (0.138, 0.203). Two populations DT and PT were clustered together. They showed shorter or smaller sizes of leaf width and long than the other populations in Yen Quang, Ba Sao and Tan Dan. The population TD to the right of the diagram showed greater leaf long in comparison to two populations DT and PT. Finally; two populations CP and BS to the left of the diagram were separated distinctly. PT showed shorter parameters in leaf long and width and TD with longer parameter in them compared to three populations DT, BS and CP.

Table 1: *Morphological characteristics and observed population sizes (*collection and cultivation in private gardens)*

Population	Mean stem height (cm)	Mean diameter (cm)	Mean leaf long (cm)	Mean leaf width	Mean number of leave per individual	Observed population size
PT	29.9 (12 - 40)	5.7 (3.8 - 6.4)	48.3 (25 - 91)	28.4 (10 - 34.5)	3.1 (1 - 7)	79
DT	17.2 (4 - 40)	2.5 (0.9 - 5.1)	60.4 (40 - 100)	32.6 (15 - 50)	5.2 (2 - 9)	68
BS	-	-	84.8 (40 - 180)	41.2 (10 - 70)	3.7 (1 - 10)	25
CP	17.7 (4 - 41)	3.7 (1.3 - 7.6)	62.5 (33 - 100)	33.3 (22 - 45)	4.2 (1 - 10)	87
TD	21.3 (9 - 45)	15.3 (7.9 - 21)	104.9 (26 - 236)	44.1 (22 - 74)	3.3 (1 - 9)	35
YT*	-	-	-	-	-	6

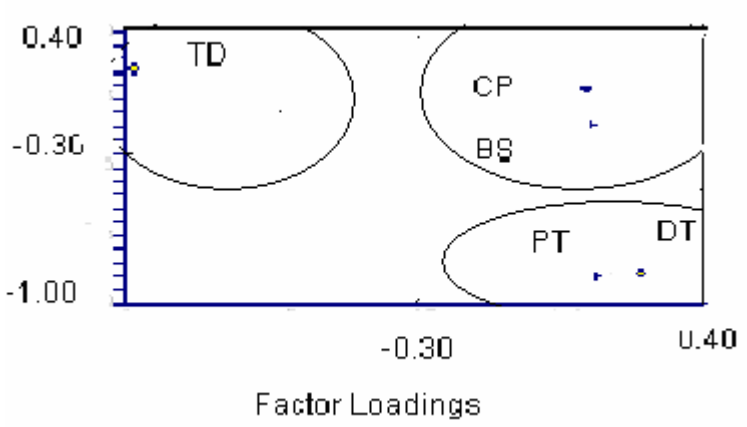

Fig. 2: *Plot of 5 wild studied populations of Cycas balansae: PT: Phu Thanh, DT: Dong Tam, CP: Yen Quang, BS: Ba Sao, and TD: Tan Dan*

3.2 Genetic variation

The allele frequencies at 11 loci for 152 individuals from 6 *Cycas balansae* populations are

given in Table 2. Forty-three (43) alleles were recorded. The proportion of polymorphic loci was 98.3% at 95% criterion. The number of alleles was 3.18 per locus, ranging from 2.54 for the YT population from Quang Ninh to 3.63 for the TD population from Quang Ninh. Observed heterozygosity ranged from 0.283 for DT population from Hoa Binh to 0.401 for CP from Ninh Binh and 0.406 for BS from Ha Nam, average of 0.347. Expected heterozygosities ranged from 0.534 to 0.624 for the BS and TD populations, respectively, average of 0.571. Chi-square test indicated that no significant deviations were found between observed and expected heterozygosities ($p > 0.05$).

There were no monomorphic loci to be found in all studied populations. Three loci, namely, Got-1 in DT and BS, Got-2 in BS and β-est-2 in PT and BS showed low levels of polymorphism, with a frequency of the most common alleles of over 0.9 and lowest heterozygosities under HW equilibrium for these loci, average 0.152, ranging from 0.080 at β-est-2 in PT to 0.199 at Got-1 and 2 in the same population BS. Higher levels of genetic variation were found for a population at Got-2 in the frequencies ranging from 0.83 in YT to 0.63 in CP with the heterozygosities ranging from 0.333 to 0.476, respectively. Highest levels of genetic diversity were recorded at Idh in TD, Lap-2 in YT, Skdk in PT and DT and β-est-3 in BS and CP. No significant deviations from HW equilibrium were found most loci in YT ($p > 0.05$), but the significant deviations were recorded at some loci in 5 remaining populations. There were significant deviations at Lap-1 in PT, BS, CP and TD ($p < 0.05$); β-est-1 in PT and BS ($p < 0.05$), Lap-2 in PT ($p < 0.01$) and in BS and CP ($p < 0.05$); Skdh in PT, TD ($p < 0.01$) and DT, BS, CP ($p < 0.05$); β-est-4 in PT ($p < 0.01$), DT, TD ($p < 0.001$) and BS, CP ($p < 0.05$). The significant deviations between populations were also recorded ($p > 0.05$), but no significant deviation between TD and YT ($p > 0.001$). Data on the distribution of genotypes showed high levels of homozygotes for most common alleles in all studied populations. These values ranged from 35.2% in CP to 42 % in YT and DT (average of 39.3%).

The mean fixation values also found at each locus and indicated a great deficit of heterozygotes compared to HW equilibrium (Table 3). Fis for all populations of 0.386 (ranging from 0.244 in BS to 0.47 in YT) showed a pronounced effect of inbreeding among individuals within populations. However, these indicates were very low at Lap-1, Skdh and β-est-1. Nine loci had positive values and were suggested a significant decrease in heterozygosity among inbred individuals within populations. The fixation index, Fit showed inbred individuals decreased 43% in heterozygosity under HW equilibrium. The mean value of population differentiation (Fst = 0.072) was lower in C. *balansae* than in C. *dolichophylla* and C. *ferruginea* [23, 24]. The gene flow corroborating for this finding was 3.22 for C. *balansae*.

The mean genetic diversity calculating from differences among individuals within populations (Hs) was found at 0.579, ranging from 0.371 for β-est-2 to 0.711 for Skdh. Similarly, mean total genetic diversity (Ht) was 0.619, ranging from 0.394 for β-est-2 to 0.743 for β-est-3.

Mean values obtained for genetic diversity among populations (Dst) and genetic differentiation (Gst) were found at 0.039 and 0.064, respectively (Table 4).

Genetic identities and distances obtained from all pairwise comparisons of populations were given in Table 5. The most Identities obtained in comparisons of 6 populations exceeded 0.8, while these values in comparisons of the DT population or BS with the YT population were lower (average of 0.745). The lowest identity was obtained in a comparison of BS with YT (0.694). The mean genetic distance between populations was 0.182, ranging from 0.052 (TD and CP) to 0.365 (BS and YT). The mean genetic distance between YT and the remaining populations was considerably higher (0.239), ranging from 0.155 (YT and CP) to 0.365 (YT and BS). Relationships between genetic distances and geographical distances was weak ($r2 = 3.93$). The genetic and geographical distance values did not differ significantly ($p > 0.05$).

Table 2: *Alleles frequencies at 11 loci in 6 populations of Cycas balansae: PT, DT, BS, CP, TD and YT*

Locus	Allele	PT	DT	BS	CP	TD	YT
Got –1	A	0.11	0.05	0.1	0.02	0.2	0.33
	B	0.05	0.05	0	0.63	0.43	0.5
	C	0.83	0.9	0.9	0.34	0.37	0.17
Got – 2	A	0.67	0.68	0.1	0.37	0.29	0.83
	B	0.33	0.32	0.9	0.63	0.71	0.17
6pgdh	A	0.11	0.08	0.33	0.21	0.14	0.25
	B	0.5	0.71	0.56	0.58	0.45	0.5
	C	0.36	0.21	0.11	0.21	0.2	0
	D	0.02	0	0	0	0.19	0.25
	E	0	0	0	0	0.02	0
Idh	A	0.2	0.3	0.44	0.31	0.15	0.5
	B	0.57	0.7	0.35	0.5	0.42	0.5
	C	0.23	0	0.18	0.19	0.18	0
	D	0	0	0.03	0	0.24	0
Lap-1	A	0.3	0.28	0.28	0.43	0.29	0.25
	B	0.38	0.4	0.44	0.41	0.51	0.58
	C	0	0.08	0	0.09	0.04	0.17
	D	0.32	0.24	0.28	0.07	0.15	0
Lap-2	A	0.44	0.26	0.47	0.3	0.19	0.33
	B	0.44	0.26	0.33	0.35	0.19	0.33
	C	0	0.04	0.03	0.02	0.15	0
	D	0.12	0.44	0.17	0.32	0.47	0.33
Skdh	A	0.36	0.29	0.27	0.4	0.38	0.25
	B	0.36	0.29	0.35	0.26	0.35	0.25
	C	0.14	0.21	0.38	0.3	0.25	0.5
	D	0.14	0.21	0	0.04	0.02	0
β-est-1	A	0.46	0.29	0.37	0.47	0.46	0.67
	B	0.42	0.16	0.31	0.47	0.49	0.33
	C	0	0.05	0	0	0	0
	D	0.12	0.5	0.32	0.05	0.06	0
β-est-2	A	0	0.18	0.05	0.07	0.06	0.33
	B	0.04	0.05	0	0.1	0.2	0
	C	0	0.05	0	0.1	0.17	0
	D	0.96	0.72	0.95	0.73	0.57	0.67
β-est-3	A	0.26	0.18	0.16	0.17	0.26	0.67
	B	0.28	0.11	0.31	0.3	0.11	0
	C	0.34	0.16	0.26	0.4	0.43	0.33
	D	0.04	0	0	0.02	0	0
	E	0.08	0.55	0.26	0.1	0.2	0
β-est-4	A	0.5	0.5	0.21	0.61	0.67	0.58
	B	0.22	0.41	0.47	0.13	0.1	0.25
	C	0	0	0	0.04	0	0
	D	0	0	0.1	0	0	0
	E	0.28	0.09	0.21	0.22	0.23	0.17

Table 3: *Wright's (1969) F-statistics for 11 loci in Cycas balansae*

Loci	Fit	Fis	Fst
Got-1	1	1	0.278
Got-2	1	1	0.183
6pgdh	0.793	0.78	0.016
Idh	0.473	0.451	0.038
Lap-1	0.067	0.047	0.013
Lap-2	0.333	0.306	0.033
Skdh	-0.11	-0.109	-0.001
β-est-1	-0.173	-0.273	0.078
β-est-2	1	1	0.066
β-est-3	0.357	0.313	0.063
β-est-4	0.448	0.418	0.059
Mean	**0.43**	**0.386**	**0.072**

Table 4: *Nei's (1987) genetic diversity within and between C. balansae populations. Hs: average genetic diversity within population, Ht: Total genetic diversity, Dst: Average genetic diversity between populations and Gst: Coefficient of genetic differentiation*

Loci	Hs	Ht	Dst	Gst
Got-1	0.423	0.565	0.143	0.252
Got-2	0.394	0.504	0.110	0.218
6pgdh	0.619	0.629	0.010	0.015
Idh	0.627	0.631	0.004	0.006
Lap-1	0.662	0.668	0.005	0.008
Lap-2	0.684	0.696	0.011	0.016
Skdh-1	0.711	0.710	-0.000	-0.001
β-est-1	0.588	0.633	0.045	0.072
β-est-2	0.371	0.394	0.023	0.058
β-est-3	0.684	0.743	0.059	0.079
β-est-4	0.608	0.632	0.024	0.038
Mean	**0.579**	**0.619**	**0.039**	**0.064**

Table 5: *Nei's (1972) genetic identities (upper triangle) and genetic distances (lower triangle) for all pairs of 6 populations of C. balansae*

	1	2	3	4	5	6
1. PT	-	0.897	0.879	0.884	0.848	0.802
2. DT	0.108	-	0.853	0.810	0.780	0.762
3. BS	0.128	0.159	-	0.842	0.818	0.694
4. CP	0.123	0.210	0.172	-	0.949	0.856
5. TD	0.165	0.223	0.201	0.052	-	0.830
6. YT	0.221	0.271	0.365	0.155	0.186	-

3.3 Genetic distribution

UPGMA analysis using Rogers' [19] original distance was revealed relationships among groups in genetic distribution at individual, subpopulation, population and species levels. The two groups clustered separated clearly at population level (Fig. 3). One group included three populations PT, DT and BS. These exhibited the low expected heterozygosities, average of 0.543, ranging from 0.534 (BS) to 0.55 (PT). One other included three remaining populations with the higher expected heterozygosities, average of 0.599. The cultivated population YT was separated in the UPGMA phenogram from second group and had the lower levels of observed and expected heterozygosities, Ho = 0.318 and He = 0.587, and the lowest number of alleles, A = 2.54. BS was separated from first group with lower level of the expected heterozygosity, He = 0.534.

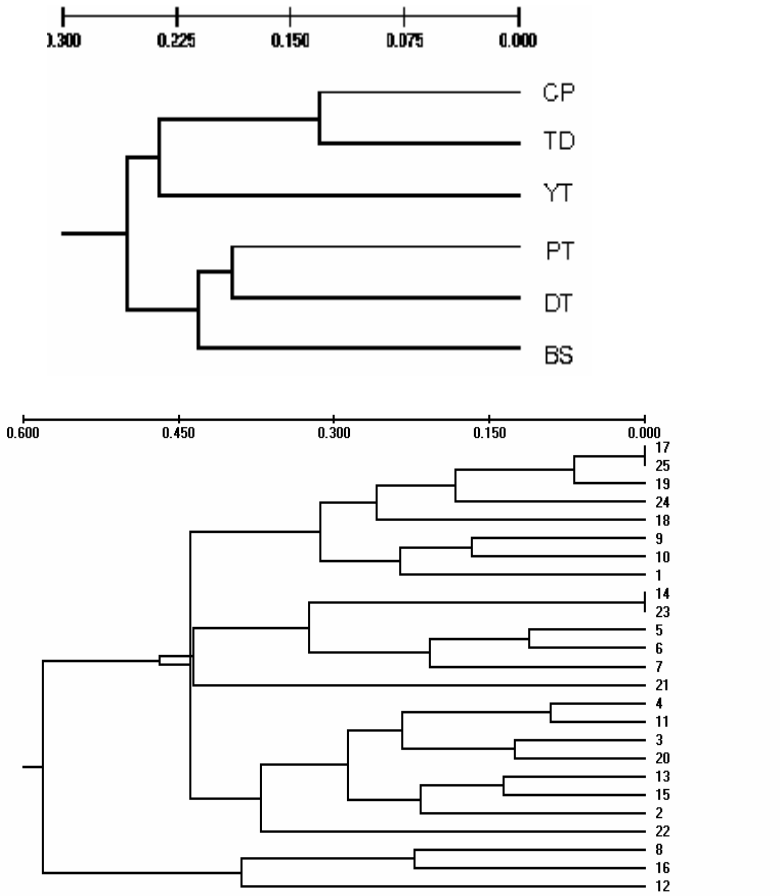

Fig. 3: *UPGMA phenogram based on genetic distances between 6 populations (above) and based on genotypes of individuals within PT (below)*

The clustering analysis based on a matrix of genetic data also showed relationships between individuals within the populations. Our data indicated high level of homomorphic loci for each cluster. For example, six clusters were determined within the BS population. All the clusters exhibited high level of homomorphic loci, ranging from 50.3% for cluster including three marked individuals 3, 10 and 18 to 67.4% for cluster including 4 marked individuals 5, 7, 8 and 20 (average of 61.1%). Six clusters found within the PT population had the mean value of

59.2%, ranging from 33.9 to 75.2% (Fig. 3). Similarly, 5 clusters and 4 clusters were found within the DT population and CP population, respectively. In general, the mentioned data showed that the inbred individuals clustering together could relate with geographical patterning.

4. DISCUSSION

On the whole, the results pointed out high levels of genetic variation within populations. These related with their life history straits. Cycads are dioecious and their reproduction is by seeds on open carpophylls [14]. They are insect-pollinated or wind-pollinated and obligatory outcrossing. *C. balansae* has pollen cones, 20 - 25 cm long and 4 - 7 cm diameter; microsporophyll 16 - 30 mm long, fertile zone 14 - 28 mm long; cataphylls 60 - 70 mm long [14]. Cycads are long-lived perennials. Based on our observation, many individuals can live more 30 years. Moreover, they have often been influenced by environmental conditions for many decades. These should be high opportunities for the accumulation of mutations and the initiation raised genetic diversity within populations. As a result, *C. balansae* maintained higher levels of genetic variation that most other long-lived and outcrossed perennials. For example, the mean variation within populations of *C. balansae* Hs = 0.579 was higher than the mean values of 0.328, 0.465 and 0.289 for *Liatris cylindracea*, *Pinus longgaeva* and *P. ponderosa*, respectively [25, 26, 27]. *C. balansae* also maintained higher level of genetic diversity than the species *Gleditsia triacanthos* with similar life history traits (dioecy and insect pollination) 0.255 [28] and *Panicum maximum* 0.381 [29].

All the six populations investigated presented mean observed heterozygosities (Ho) lower than expected heterozygosities (He). This was due to a lack of heterozygotes in relation to inbreeding or age structure of populations within the range of outcrossing perennials. In our case, *C. balansae* was found in shade, in secondary forests in Phu Thanh, Dong Tam (Hoa Binh), Ba Sao (Ha Nam) and Yen Quang (Ninh Binh) on limestone hills with little soil; and on loamy soil over granites in forest plantation of pines in Tan Dan or cultivated in private gardens in Yen Tu (Quang Ninh). The geographical distribution of cycads is greatly influenced by human activities. Consequently, their habitats were greatly suffered from destruction, e.g. by commercially logging, firewood and clearance for agriculture in 1970s. Soil fertility was heavily decreased to create well favorable for development of shrubs, bamboo and grass. In general, natural vegetation has been greatly modified. The abundant species in the vegetation were shrubs with height of 2 - 3 m. In addition, cycads have been collected for proposes as ornamental plants and medicines. The sizes of five studied wild populations were very small (< 100 individuals). Their habitats are often disturbed. Therefore, the effect of sib-mating may happen at high intensity within populations, although a presence of visit of the pollinating insects. Moreover, seed dispersal is restricted and limited (seed weight of 7 g, with size of 24 × 27 mm). These could lead to the integrated mosaic of genotypes among individuals within clumps. We found high proportion of loci homozygous per individual also found in all populations and averages 66.1% for each population although very high proportion of polymorphic loci within populations, average of 98.3% under HW equilibrium. Clearly, This might be consequence of inbreeding in small populations. Inbreeding increased homozygosity [5].

Five studying populations except cultivated population of YT separated by geographical distances and isolated by a matrix of agricultural ecosystems (wet rice, vegetables, maize, sugar cane, manioc, lemons and custard apples) were genetically similar (genetic identity of 0.864), present high heterozygoties, and the observed genotype frequencies did not agree with those expected according to the HW equilibrium. Their behaviors seem to resemble that of inbreeders. On the contrary, a cultivated population from Yen Tu showed considerable differences in alleles and genotype frequencies. The data seem to indicate that cycads can originate vegetatively from other locations. They did not originate directly from each other. The habitats in which the four

populations including from Phu Thanh, Dong Tam, Ba Sao and Yen Quang were sampled are similar, dominant shrubs on limestone hills with similar covering and consequently a similar amount of high light intensity, together with a high temperature are considered as factor unfavorable to growth and seed production. Furthermore, cycads from Tan Dan were re-established after ecological conditions for more suitable of their survival were re-constructed. Pines were planted in 1970s after disturbance in the form of clearance for agriculture. The canopy of this type consists of only pine species *Pinus merkusii*, up to 35 m in height. However, shrubs in families Myrsinaceae, Araceae and Rubiaceae) and bamboos (*Bambusa spp*) along streams are found in the ground layer and understorey. Proportion of youngs (< 10 cm in diameter) was in the population very high (83.3%). The mean geographic distance between consecutive individuals is higher than that of PT and CP. Besides the different geographic location, the difference of the soil kind is also represented. The TD population is restricted and limited on numerous individuals. On the basis of these elements, a hypothetical explanation may be proposed that the small populations have originated from a few original plants, subsequently an increased opportunity for inbreeding.

Species with continuously distributed populations should maintain more gene flow than species discrete and isolated populations and have higher levels of genetic variation within populations and lower variation among populations [30]. The difference among populations (Fst) is 0. 072. This suggested that gene flow among populations were high (Nm = 3.22). Thus, the level of gene flow are of sufficient magnitude to counterbalance genetic drift and may have significance to the genetic structure of the populations. Similar results were obtained for *Panicum maximum* [29], *Acacia anomala* [31] or *Gleditsia triacanthos* [28]. As a result, genetic drift should not be a main factor for populations of *C. balansae* (Nm > 1). Thus, high level of gene flow may be explained by natural history in relation to seed and pollen dispersal.

In conclusion, on the basis of our results, *Cycas balansae* maintained high levels of genetic diversity and low levels of population differentiation in relation to its life history traits. High level of gene flow in the past may affect genetic structure in the species. Factors contribute to the high levels of genetic diversity including dioecy, long-lived time and existence in primary forests. However, our results also showed that fragmented habitats increased amount of inbred individuals within populations.

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