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Genetic diversity of *Balanophora fungosa* and its conservation in Taiwan

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ABSTRACT. *Balanophora fungosa* is a rare holoparasitic flowering plant in Taiwan, where it is restricted to the Hengchun Peninsula in southernmost Taiwan, and Orchid Island (*Lanyu* in Chinese), a small volcanic island off the southeastern coast of Taiwan. Plants from these two areas appear in two different groups based on the color of the inflorescence, i.e., those of Hengchun are yellow, but they are pinkish orange to red on Orchid I. This study used an inter-simple sequence repeat (ISSR) molecular marker approach and the unweighted pair group method with arithmetic mean (UPGMA) analysis to evaluate genetic variations among populations of *B. fungosa*. The results showed that the two geographical groups represent the same species as indicated by a high Dice similarity value of 0.78. Populations from the two areas formed two well-defined clusters, as did populations within each area. The results of the analysis of molecular variance (AMOVA) showed that the components of variation between groups (31.35%), among populations within groups (13.74%), and within populations (54.91%) were significant ($p < 0.001$), indicating that variations among individuals within populations contributed most to the total genetic variance. The populations of the two areas were also differentiated with genetic distances ranging from 0.44~0.53 for paired comparisons. Therefore, we recommend that protected areas be set aside in both areas for *B. fungosa*, that is, in Kenting Park on the Hengchun Peninsula and on Orchid Island, to allow the population to expand naturally without human disturbance.

Keywords: *Balanophora fungosa*; Conservation; Population variation.

Abbreviations: AMOVA, analysis of molecular variance; **ISSR,** inter-simple sequence repeat; **UPGMA,** unweighted pair group method with arithmetic mean.

INTRODUCTION

Balanophora fungosa J. R. & G. Forst. is a holoparasitic plant which grows on roots of various host plants. This species is found in India, East Malaysia, Taiwan, the Pacific Islands, and northeastern Australia (Hansen, 1972; Huang and Huang, 1996). In Taiwan, the distribution of this species is restricted to the Hengchun Peninsula in southernmost Taiwan and also on Orchid Island (*Lanyu* in Chinese), a small volcanic island 90 km off the southeastern coast of Taiwan. For subsequent analyses, populations of *B. fungosa* in Taiwan are referred to as groups in the Hengchun area and on Orchid I., respectively.

Due to its rarity, *B. fungosa* was designated a vulnerable plant in Taiwan based on the IUCN (1994) (Lu and Chiou, 1996). Our field observations also showed that population declines could be a major threat to *B. fungosa* in both areas. In Hengchun, only two populations of *B. fungosa* were found at Kenting Park and at Kuanshan. The former population is more widely distributed with many small patches because it grows in a protected research site at the park. However, very few patches were located in the latter area due to the effects of agriculture and tourism. On Orchid I., despite less-intensive human disturbance, the plant's distribution is limited to a few isolated patches.

The *B. fungosa* plant is composed of an underground tuber which attaches to host roots, and monoecious inflorescences which are only visible during the flowering season. The inflorescence is yellow to pinkish-orange, with pistillate flowers located on the upper part of the inflorescence and staminate flowers on the lower part (Figure 1). The populations of Hengchun are distinguished from those on Orchid I. by the color of the inflorescence: plants in the Hengchun area have yellow inflorescences while those on Orchid I. are pinkish-orange to red. Therefore, it would be reasonable to assume that *B. fungosa* populations in Taiwan can be divided into two distinct groups both morphologically and geographically.

Genetic variations among populations of *B. fungosa* have not been analyzed before. In this study, inter-simple sequence repeat (ISSR) molecular markers were applied to estimate genetic variations within the species in Taiwan.

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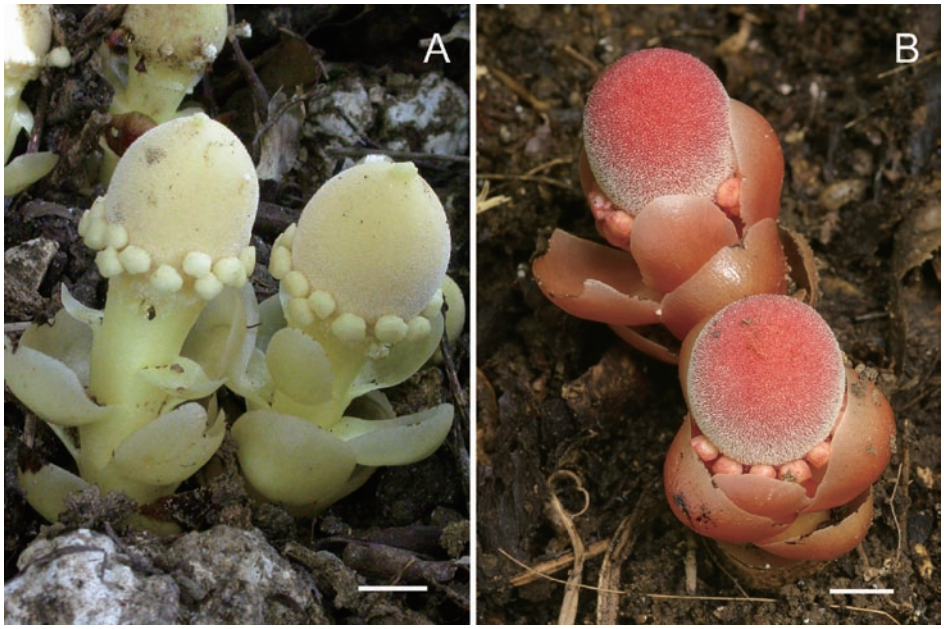

Figure 1. *Balanophora fungosa* in Taiwan. A, Plants of the Hengchun area of southern Taiwan; B, plants of Orchid I. Bars = 1 cm.

The ISSR technique has been widely used to study population variation in plants without background genetic information because of its high number of polymorphisms and the high reproducibility of its bands (Culley and Wolfe, 2001; Werner et al., 2003; Jian et al., 2004).

The aim of this study was to investigate genetic variations among individuals of *B. fungosa* of populations from Hengchun and Orchid I. in Taiwan. Furthermore, we propose suitable conservation measures for the species based on these results.

MATERIAL AND METHODS

Plants of *B. fungosa* were collected from the Hengchun area and on Orchid I. Due to the rarity of the species, only two populations were sampled in each of the two regions (Table 1). In the Hengchun area, one population was sampled at Kenting and another at Kuanshan. The habitat of both populations consists mostly of uplifted coral reef covered by a thin soil layer, and these parasitic plants were distributed in patches on host roots. To distinguish specimens from different host plants, we divided the population into a few patches, each of which contained individuals from the same host plant. One to four individuals were collected from a single patch, and any two patches were at least 2 m apart. On Orchid I., two populations were found in natural thickets, but the population sizes were small; one consisted of fewer than five individuals. Information on all the samples is listed in Table 1. Sampled plants were brought to the laboratory for further cleaning, and fresh bracts and slices of the inflorescence stalk were preserved in liquid nitrogen immediately after being cut off from the plant for later DNA extraction.

Genomic DNA was extracted using the modified CTAB method for plants containing high polysaccharide and polyphenol components (Porebski et al., 1997). In total, 125 ISSR primers (Operon Technologies, USA) were initially used to screen for polymorphisms, and seven of these were chosen for their distinct band patterns and are listed in Table 2. Extracted DNA solutions were quantified under a spectrophotometer at a wavelength of 260 nm, and were diluted to 25 ng/μl as the working solution for polymerase chain reaction (PCR) amplification. Each 25 μl of the PCR solution contained 175 μM dNTPs, 0.35 μM ISSR primer, 1 U Taq DNA polymerase (Protech Technology Enterprise, Taiwan), and 25 ng of template DNA. Amplification was performed in a DNA thermocycler 480 (Perkin Elmer, USA) under the following thermo-cycle conditions: 5 min at 94°C; then 38 cycles of 1 min at 94°C, 30 s of annealing at 50°C, and a 2-min extension at 72°C; followed by a final extension step of 10 min at 72°C. The amplified products were electrophoretically separated on 2.5% agarose gels in 0.5 × TBE buffer, and the band patterns were visualized by ethidium bromide staining.

The band patterns of all DNA samples were scored as present (1) or absent (0), and all weak and ambiguous ones were excluded. The data were used to obtain a similarity matrix based on the Dice formula ($S_{AB} = 2N_{AB} / (2N_{AB} + N_A + N_B)$ on NTSYS-pc vers. 2.0 (Rohlf, 1993), where N_A is the number of bands present in sample A but absent from sample B, N_B is the number of bands present in sample B but absent from sample A, and N_{AB} is the number of bands present in both samples (Dice, 1945). This similarity matrix (S) was used to construct a UPGMA dendrogram, which was further converted into a distance matrix (D) for analysis of molecular variance (AMOVA), in which D =

Table 1. Sampling information of *Balanophora fungosa*.

Area	Population no.	Patch no.	Individual no.[a]	Host[b]	Total no. of individuals
Hengchun	1	1	111, 112, 113	▲	18
	(Kenting)	2	121, 122, 123, 124	▲	
		3	131, 132, 133	▲	
		4	141, 142, 143, 144	▲	
		5	151, 152, 153, 154	▲	
	2	1	211, 212, 213, 214	△	10
	(Kuanshan)	2	221, 222, 223, 224	△	
		3	231, 232	△	
Orchid I.	3	1	311	?	10
	(Hsiaotienchih 1)	2	321	?	
		3	331, 332	?	
		4	341, 342, 343	?	
		5	351, 352, 353	?	
	4	1	411, 412	?	2
	(Hsiaotienchih 2)				

[a]Individuals were labeled by the order of their population and patch with a three-number code; e.g., individual 123 was the third plant (3) collected in patch 2 (2) of the Kenting population in the Hengchun area (1).
[b]▲, Host was *Diospyros philippensis*; △, *Macaranga tanarius*; ?, host unknown.

n (1-S), where n is the total number of bands (Excoffier et al., 1992). The distance matrix was used to calculate (1) the variance components for variations between regions, among populations, and within populations; and (2) genetic distances between pairs of populations, with the significance levels based on 9999 permutations.

RESULTS AND DISCUSSION

Cluster analysis

For *B. fungosa* samples, 250 bands were generated from the seven ISSR primers, and 75.2% of the bands were polymorphic (Table 2). The banding patterns were used to generate a Dice similarity matrix which was then visualized by UPGMA clustering.

All samples of *B. fungosa* formed two well-defined clusters, with a Dice similarity of 0.78 (Figure 2). This result fits the geographical difference (Hengchun and Orchid I.), and further indicates that morphological differences in inflorescence colors, that is, yellow in the Hengchun area and pinkish-orange on Orchid I., could be one of the characteristics involved in genetic variations between the two areas. Although the result is in agreement with Hansen's (1972) opinion that coloration of *B. fungosa* should not be used for delimiting the species, this characteristic is so discrete that it is worth further studies on a global scale to investigate any correlation of coloration with overall genetic variation. For example, since plants of *B. fungosa* from Orchid I. and the Ryukyu Islands (of southern Japan) are both pinkish-orange (Hatusima, 1971; Hansen, 1972; Kawakita and Kato,

Table 2. Seven ISSR primers used in this study. Numbers of bands and polymorphic ones generated from each of the primers are also listed.

Primer[a]	Sequence (5' to 3')	No. of bands	No. of polymorphic bands (%)
AM5	GTGTGTGTGTGTYR	24	14 (58.33)
IS44	ACACACACACACACACG	30	19 (63.33)
IS50	AGAGAGAGAGAGAGAGYC	44	37 (84.09)
IS51	AGAGAGAGAGAGAGAGYG	44	36 (81.82)
IS53	AGCAGCAGCAGCGY	37	32 (86.49)
IS71	GAGAGAGAGAGAGAGAYC	38	28 (73.68)
IS98	HVHTGTGTGTGTGTGTG	33	20 (60.61)
Total		250	188 (75.20)

[a]Primers were purchased from Operon Technologies, USA.

2002), and plants of *B. fungosa* in Queensland, Australia are yellow, based on photographs shown at the Botanical Society of America's parasitic plants pages (http://www.botany.org/Parasitic_Plants/Balanophora_fungosa.php), it would be intriguing to determine whether plants with the same color of inflorescence are closely related. From a geological point of view, Orchid I. is a small island of the Luzon volcanic arc which has never been connected to the main island of Taiwan at any time since its formation in the late Miocene (Chen and Wang, 1996). This suggests that populations of *B. fungosa* from Hengchun and Orchid I. might have been established separately, and possibly the Orchid I. populations would have originated from those in the Ryukyu Is. through long-distance dispersal by birds, or the other way around, even though such an incidental event has never been documented. Therefore, further investigation of populations from the pan-Pacific region would help assess the genetic relationships and possibly resolve the origin of *B. fungosa* populations in Taiwan.

As to population variation within areas, individuals from the same populations in each area also clustered together, except for samples #212 from the Kuanshan population and #352 from the Hsiaotienchih 1 population (Figure 2). If the former sample #212 were excluded, the two populations from the Hengchun area would form separate clusters. The same is true for the populations on Orchid I. if sample #352 were excluded. However, there was no significant differentiation among most patches within any of the populations, except for three patches (patches 1, 2, and 5) in the Kenting population (Figure 2).

AMOVA

Genetic variations among individuals, among populations, and between the two areas contributed 54.91%, 13.74%, and 31.35% to the total variance, respectively, and these variations differed significantly from each other ($p < 0.0001$; Table 3). Most occurred at the level of individuals within populations (54.91% of the total variance). When combining all populations of the two areas as a single group, the result still showed that the genetic variation occurred mostly within populations (62.18% of the total variance; Table 3). To clarify which population contributed more variance within the populations, we further partitioned the percentage of the variance component within populations (54.91%) into four populations based on the within-population sums of squares, showing that the population at Kenting contained more than 25% of the total variance (13.73%, 14.03%, and 1.28% for the Kuanshan, and Hsiaotienchih 1 and 2 populations, respectively). All results indicate that individuals of the Kenting population are highly diverse genetically.

Although there were fewer Orchid I. population samples, the variance within the area was still similar. Due to the rarity of the species and difficulty of locating the plants on Orchid I., fewer samples from this region than from Hengchun were used for this study (Table 1). Therefore, we also pooled all twelve individuals from Orchid I. as a single population, and the AMOVA results (data not shown) were still similar to the original four-population analysis (Table 3).

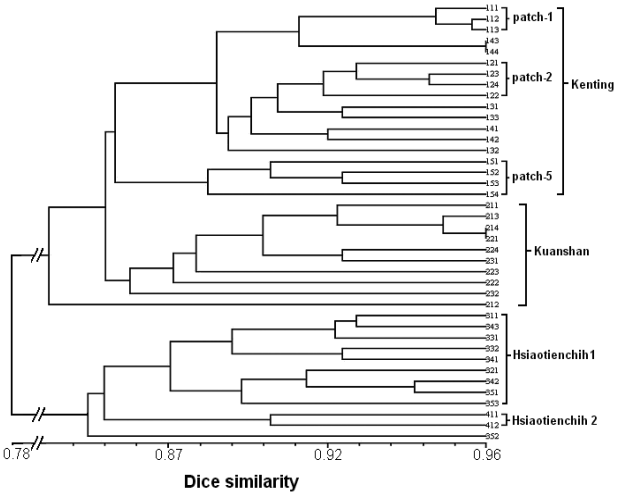

Figure 2. UPGMA dendrogram of *Balanophora fungosa* populations. Samples from the four populations are indicated on the right; in the Kenting population, individuals from patches 1, 2, and 5 formed a separate cluster; sample labels are described in Table 1. To shorten the graph, the scale of similarity was partially cut off as indicated by the slash marks (**//**).

Table 3. AMOVA results of *Balanophora fungosa*.

Source of component	df[a]	Variance	Percent (%) total variance	p
Nested analysis				
Between groups	1	8.4719	31.35	< 0.0001
Among populations within groups	2	3.7134	13.74	< 0.0001
Within populations	36	14.8392	54.91	< 0.0001
Analysis among populations				
Among populations	3	9.0241	37.82	
Within populations	36	14.8392	62.18	< 0.0001

[a]df, degrees of freedom.

Table 4. Genetic distances between populations of *Balanophora fungosa*.

	Kenting	Kuanshan	Hsiaotienchih 1	Hsiaotienchih 2
Kenting	0.0000			
Kuanshan	0.1984	0.0000		
Hsiaotienchih 1	0.4389	0.4246	0.0000	
Hsiaotienchih 2	0.5264	0.5093	0.2066	0.0000

Comparisons of genetic distances between pairs of populations showed that populations within an area were closely related. The distance between the two populations within an area was approximately 0.20 for both areas (Table 4), suggesting the populations from the two areas genetically differed. Populations of Kenting and Hsiaotienchih 2 were the most distant genetically from each other (at 0.5264). Even though the sample size of population Hsiaotienchih 2 might have been too small (n = 2) to represent the entire population, the genetic distance between the other population on Orchid I. (Hsiaotienchih 1) and the Kenting population was still high (at 0.4389). Therefore, for the sake of conservation, such genetic differences between the two areas should be taken into consideration.

Conservation suggestions

Populations of *B. fungosa* should be protected not only because they are rare and vulnerable in Taiwan, but also because they contain individuals with high genetic diversity and two discrete colors that can easily be distinguished by area. Based on our field observations, the current threat to the species is that the populations are declining, which makes it more difficult than before to locate plants. On Orchid I., for instance, one of the localities we have visited since 1995 was no longer present after 1999. The same might be true for the Kuanshan population in the Hengchun area, the small size of which may be a result of human development. Few natural habitats are left in Kuanshan. The small size of the population and the apparent lack of a proper means of dispersal might also be reasons for limitations of the species' expansion. The inflorescence of *Balanophora* species contains seeds which usually rot off and are washed away by rain (personal observations; Kuijt, 1969); long-distance dispersal has not been reported.

As to population diversity, *B. fungosa* in Taiwan is apparently both morphologically and genetically diverse. There are no intermediates among populations between the two different inflorescence colors, nor intermixtures of genetic compositions from individuals of the two areas, which form two separate groups as shown in Figure 2. Therefore, to maintain current diversity, preventing these populations from further decline is crucial. Intrinsically, *B. fungosa's* high genetic variation in Taiwan especially within populations (54.91%; Table 3) should allow it to maintain its genetic diversity. Extrinsically, any disturbance that affects the growth of these populations should be prevented. Therefore, for populations in the two areas, the simplest way to protect the forests which *B. fungosa* inhabits would be to restrict public access as much as possible.

We recommend that two protected areas be set aside for *B. fungosa* and its habitats in Taiwan: one for 'yellow' populations of Kenting Park on the Hengchun Peninsula, and another for the 'red' population at Hsiaotienchih on Orchid I. Since part of the habitat of *B. fungosa* in Kenting Park is already protected for other research, additional costs for this species' conservation would be minimal. Such an act would also serve the best interests of most of the flora and fauna within that area. Without further disturbance, the population of *B. fungosa* will have a better chance to grow and expand.

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台灣產粗穗蛇菰之遺傳變異與保育

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粗穗蛇菰在台灣是一種稀有的全寄生性開花植物，其分布點局限於台灣南端恆春半島、以及位於台灣島東南外海的火山島 — 蘭嶼。在此兩地區的粗穗蛇菰可依花序的顏色分為兩群：恆春群為黃色；蘭嶼群為橘紅至紅色。本研究利用簡單重複序列間區 (ISSR) 之分子標記法以及不加權平均法 (UPGMA) 以分析粗穗蛇菰族群間的遺傳變異。結果顯示：兩地區的族群之 Dice 相似度高達 0.78，支持其為同一物種；且地區間、地區內之族群亦有明顯分群。分子變方分析 (AMOVA) 顯示以地區分群之變方成分佔 31.35%；群內之族群間佔 13.74%；而族群內則佔 54.91%，明顯指出其遺傳變異主要發生在族群內的個體之間。而在遺傳距離成對相較下，兩地區的族群亦顯示出分化的情形（其遺傳距離在 0.44~0.53 之間）。因此，我們建議在恆春半島的墾丁公園及蘭嶼的粗穗蛇菰棲地應分別設立保護區，使此稀有植物的族群可在無人為干擾下自然擴展。

關鍵詞：粗穗蛇菰；保育；族群變異。