Research paper

Genetic Variation of the Endangered *Scaevola hainanensis* (Goodeniaceae) in the Jiangjun Stream Mouth, Taiwan

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【Summary】

Scaevola hainanensis Hance is an endangered plant species in Taiwan. Distribution of this species in Taiwan is restricted to 6 connected ditches, which cover an area smaller than 1 ha at the estuary of Jiangjun Stream, Tainan County, southwestern Taiwan. We examined the genetic variation with inter-simple sequence repeat (ISSR) fingerprints. Fifty samples from 6 ditches were collected. In total, 27 primers were used, and 233 bands were obtained, of which only 6 bands (2.58%) were polymorphic, indicating low levels of genetic variation. The analysis of molecular variance (AMOVA) revealed that 93.85% of the variance component was attributable to the variation among individuals within ditches. Low levels of the genetic differentiation coefficient among ditches, G_{ST} = 0.172 and Nm = 2.4, indicated that the ditches barely hinder gene flow between *Scaevola* populations. UPGMA cluster analysis and principal coordinate analysis revealed no major groupings among ditches or among plants. If the present habitat is damaged, the species will inevitably be confronted with extirpation from Taiwan. Therefore, we suggest that ex situ conservation should be conducted in order to enlarge the population size.

Key words: *Scaevola hainanensis*, Goodeniaceae, ISSR, AMOVA, conservation, Taiwan, genetic diversity.

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研究報告

海南草海桐於台灣之遺傳變異

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摘 要

海南草海桐在台灣為嚴重瀕臨滅絕的植物，本種植物分布於台南縣將軍溪口6條彼此相通的水溝間，覆蓋面積不及1公頃。本研究應用inter-simple sequence repeat (ISSR)研究海南草海桐之遺傳變異，從這6條水溝共採集50個樣本，共使用了27個ISSR引子，並得到233個條帶，但僅獲得6個多型性條帶(2.58%)，此結果顯示本植物在臺灣具非常低的遺傳變異。經過分子變方分析(AMOVA)顯示海南草海桐有93.85%的變方成分存在於個體間；POPGENE分析結果顯示其遺傳分化係數(G_{ST})為0.172，而基因流(*Nm*)為2.4，顯示溝渠並無阻礙基因交流之能力；UPGMA歸群分析及主座標分析結果顯示海南草海桐個體間幾乎無差別或差異很小。由於海南草海桐在台灣的生育地僅於一處狹隘且人工干擾嚴重之地，若遭受破壞則海南草海桐馬上會面臨滅絕的命運，故在保育方式上選擇區外保育較為恰當。

關鍵詞：海南草海桐、草海桐科、簡單序列重複區間、分子變方分析、保育、台灣、遺傳多樣性。

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INTRODUCTION

Scaevola hainanensis Hance belongs to the Goodeniaceae, which has over 400 species in 10 genera in the world. Two species in 1 genus occur in Taiwan. *Scaevola sericea* Vahl, an erect and large shrubby species, often grows along the seashore in Taiwan. In contrast, *S. hainanensis*, a fleshy creeping shrub with small linear leaves crowded at the tip of the branchlets, is an endangreed species. *Scaevola hainanensis* is insect-pollinated and characterized by solitary flowers, a 5-lobed calyx, and a zygomorphic corolla which is split completely down 1 side to expose the curved style, and is composed of 5 lobes, 5 stamens, a hypogynaous ovary, and a somewhat-fleshy capsule. The species is also distributed in Indochina and the Hainan Island, China. Taiwan populations, therefore, represent the northmost distribution of its range.

Scaevola hainanensis in Taiwan was first discovered by Hayata Bonzou in the Chiayi area. It was found in Dongshi, Chiayi County and Jiangjun, Tainan County (Li 1978). The Chiayi Chungpu Research Center of Taiwan Forestry Research Institute has conducted a survey on its habitat since 1999, in cooperation with a project entitled 'Reproduction and Out-zone Protection of Everglade Plants in the West Seashore Area', and has found no traces of the species in Dongshi of Chiayi County. It may have disappeared because of human disturbance. The species grows in an area smaller than 1 ha in the Jiangjun Stream mouth, Tainan County. The area belongs to the No. 10 Cemetery of Jiangjun Township. Therefore, having encountered strong human impacts, it might become extirpated at any time. According to the IUCN Rating Table of *Taiwan Rare* and *Extinctive Plants Distribution* and *Protection List* (Lu and Chiou 1998), it should be classified as critically endangered.

Zietkiewicz et al. (1994) developed the fingerprinting technique of inter-simple sequence repeat (ISSR). Because a large number of loci can be amplified and the technique is sensitive for detecting genetic variation within a species, the technique has been widely applied in population genetics

of plant species (Ge et al. 2003; Adams et al. 2003; Li and Ge 2001). Prevost and Wilkinson (1999) employed 4 primers to identify potato strains and found that 2 of the primers could completely differentiate all strains. The advantages of ISSR are its speed, reliability, and high genetic information content. In this study, the genetic variation and differentiation of *S. Hhainanensis* were examined using ISSR fingerprints.

MATERIALS AND METHODS

Plant materials

The population of *Scaevola hainanensis* is located in a cemetery near the bayou of Jiangjun Stream, Jiangjun Township, Tainan County, southwestern Taiwan (23° 05'N, 120°06'E). The species is distributed in 6 connected ditches and coversed an area smaller than 1 ha (Fig. 1). Since the species is a creeping fleshy vine and is difficult to separate 1 individual from another, 50 samples are collected in a comprehensive way, i.e., 1 sample was taken every 5 m apart in a ditch. In total, 5~13 samples were collected from each ditch. Fresh leaf tissue was collected and dried with silica gel.

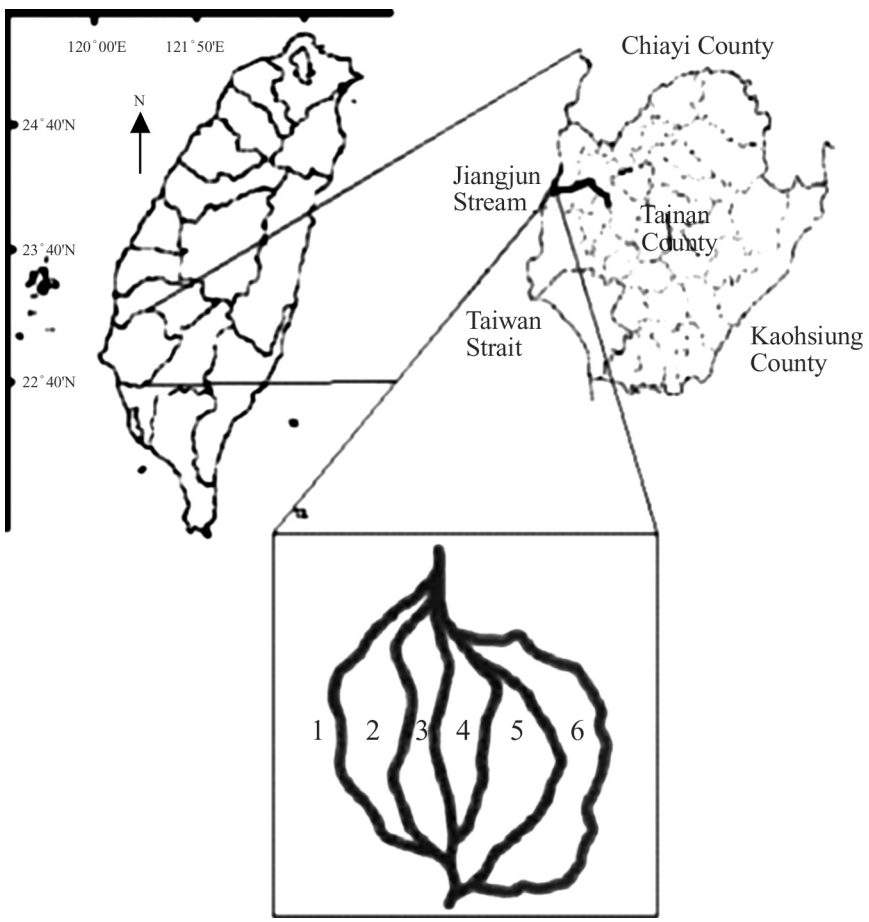

Fig. 1. Geographic location of sampling sites.

DNA extraction

DNA was extracted with buffer I and II solutions of Kobayashi et al. (1998). Buffer I consists of 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 350 mM sorbitol, 10% PEG 8000 (w/v), 1% PVP-40 (w/v), and 0.6% 2-mercaptoethanol, while Buffer II consists of 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 350 mM sorbitol, 1% sodium sakoyl (w/v), 1% PVP-40, 810 mM NaCl, 2% CTAB (w/v), and 0.6% 2-mercaptoethanol. Total DNA was extracted using the protocol of Kobayashi et al. (1998). The extracted DNA was stored in 0.1 ml TE buffer.

ISSR amplification

Amplification of ISSR was modified from Ziekiewicz et al. (1994). The reaction volume was 25 μL, with 10 mM Tris-HCl, 50 mM KCl, 1.0 mM MgCI, 0.1% gelatin (w/v), 1% Trition X-100 (w/v), 10 ng template DNA, 100 μM dNTPs, primer 0.2 μM, and 0.5 units *Taq* polymerase (HT Biotechology, UK). Polymerase chain reaction (PCR) was conducted with a thermocycler (Perkin Elmer Geneamp PCR System 9700, Foster City, CA, USA). PCR was initiated with a cycle of 94℃ 6 min, followed by 39 cycles of 94℃ for 6 min for denaturation, 50℃ for 50 s for annealing, and 72℃ for 2 min for extension, with a final extension at 72℃ for 7 min, after which it was kept at 4℃. The amplification products with tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water) and a 1-kb DNA ladder (GIBCO, Grand Island, NY, USA) were separated by electrophoresis in 1.5% agarose using 1X TBE (Tris-EDTA-borate) buffer and stained with ethidium bromide. The banding patterns were documented on Polaroid 667 film.

Data analysis

Reproducible polymorphic bands from the ISSR analysis were qualitatively screened for their presence (1) or absence (0) in each sample. Only intensely stained polymorphic bands were used for statistical analyses. A matrix of inter-sample distances was constructed using the formula of Excoffier et al. (1992), $D = N (1 - (N_{11}/N))$, where N is the total number of polymorphic bands and N_{11} is the number of bands shared by 2 samples. The matrix was analyzed by analysis of molecular variance (AMOVA) using WINAMOVA 1.55 software (Excoffier et al. 1992). Genetic variation was partitioned into within- and among-ditch components, and significant values were assigned to variance components based on 9999 random permutations of individual samples assuming no genetic structure. Nei's gene diversity (H_E) (Nei 1973) for each ditch was calculated using POPGENE 3.2 software (Yeh et al. 1999) assuming Hardy-Weinberg equilibrium. The coefficient of genetic differentiation (G_{ST}) and gene flow (*Nm*) were also calculated using POPGENE 3.2 software. The Φ_{ST} genetic distance matrix derived from AMOVA was used in the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis and a principal coordinate analysis by employing the software, NTSYS-pc (Rohlf 1993). The binary data of the presence or absence of each band in each sample was used to calculate a similarity matrix among 50 samples using the algorithm of Dice (1945). The similarity matrix was used in a UPGMA cluster analysis of 50 samples.

RESULT AND DISCUSSION

Primers and polymorphic bands

In total, 27 primers were used in the present study, and 233 bands were obtained, of which only 6 bands (2.58%) were

polymorphic (Table 1). Like many rare species, e.g., *Gentianella germanica* (Markus and Diethart 1998) and *Archangiopteris itoi* (Hsu et al. 2000), our study revealed a very low level of genetic variation in the seriously endangered *S. hainanensis* in Taiwan. Given its small population size, genetic polymorphisms tend to be lost via genetic drift.

AMOVA, cluster analysis, and principal coordinate analysis of the ditches

The results of AMOVA (Table 2) indicated that the variance component among ditches constituted only 6.15% of the total variance ($p=0.712$). In contrast, the variance component among samples within ditches constituted 93.85% of the total. Apparently, *S. hainanensis* was not divided genetically as

Table 1. ISSR primers with the sequence, number and percentage of monomorphic fragments, and number and percentage of polymorphic fragments

Primer	UBC No.	Sequence 5'→3'	Number of monomorphic fragments	Number of polymorphic fragments	Percentage of monomorphic fragments	Percentage of polymorphic fragments
AM 14	UBC 807	$(AG)_8T$	9	0	100	0
AM 16	UBC 856	$(AC)_8YA$	6	0	100	0
AM 23	UBC 864	$(ATG)_6$	9	0	100	0
IS 2	UBC 826	$(AC)_8C$	7	0	100	0
IS 3	UBC 827	$(AC)_8G$	4	1	80	20
IS 6		$(AG)_8RA$	6	0	100	0
IS 9	UBC 834	$(AG)_8YT$	5	0	100	0
IS 18	UBC 841	$(GA)_8YC$	6	0	100	0
IS 35		$BDB(CA)_7C$	11	0	100	0
IS 36		$DBDA(CA)_7$	10	0	100	0
IS 37		$HVH(CA)_7T$	6	0	100	0
IS 38		$HVH(TG)_7T$	9	0	100	0
IS 44	UBC 827	$(AC)_8G$	6	0	100	0
IS 46	UBC 808	$(AG)_8C$	9	0	100	0
IS 52	UBC 834	$(AG)_8YT$	10	0	100	0
IS 53		$(AGC)_4GY$	4	3	57.1	42.9
IS 54		$(AGC)_4Y$	9	0	100	0
IS 57		$(ATG)_6G$	12	0	100	0
IS 68	UBC 844	$(CT)_8RC$	7	1	87.5	12.5
IS 69	UBC 866	$(CTC)_6$	4	1	80	20
IS 75	UBC 873	$(GACA)_4$	8	0	100	0
IS 81	UBC 822	$(TC)_8A$	11	0	100	0
IS 89	UBC 889	$BDB(AC)_7$	14	0	100	0
IS 92		$BDB(TCC)_5$	14	0	100	0
IS 96		$HVH(CA)_7T$	10	0	100	0
IS 98	UBC 856	$(AC)_8YA$	14	0	100	0
IS 101	UBC 864	$(ATG)_6$	7	0	100	0
Total			227	6	97.42	2.58

Table 2. Hierarchical analysis of molecular variance (AMOVA)

Source of variation	df	SSD	MSD	Variance components	Percent total variance	*p* value
among ditches	5	87.506	17.501	0.747	6.15%	0.712
within ditches	44	501.444	11.396	11.396	93.85%	0.045

a consequence of ditch separation. Frequent gene flow led to most of the variation residing within populations. Likewise, cluster analysis (Fig. 2) based on the Φ_{ST} distance matrix with AMOVA also revealed nonsignificant genetic difference among ditches.

In the principal coordinates analysis, since the first and the second axes could explain 100% of the total variation, the planar figure was consequently adapted. Figure 3 reveals that very few differences are allocated between ditches, and all ditches were concentrated around 2 coordinates of 0.00. The results of cluster analysis and principal coordinate analysis together indicated that *S. hainanensis* varies little from 1 ditch to another.

Genetic diversity and differentiation

The estimates of gene diversity, genetic differentiation, and the deduced gene flow (*Nm*) obtained from POPGENE analyses are listed in Table 3. Nei's gene diversity for each ditch ranged from 0.0059 to 0.0095 and the

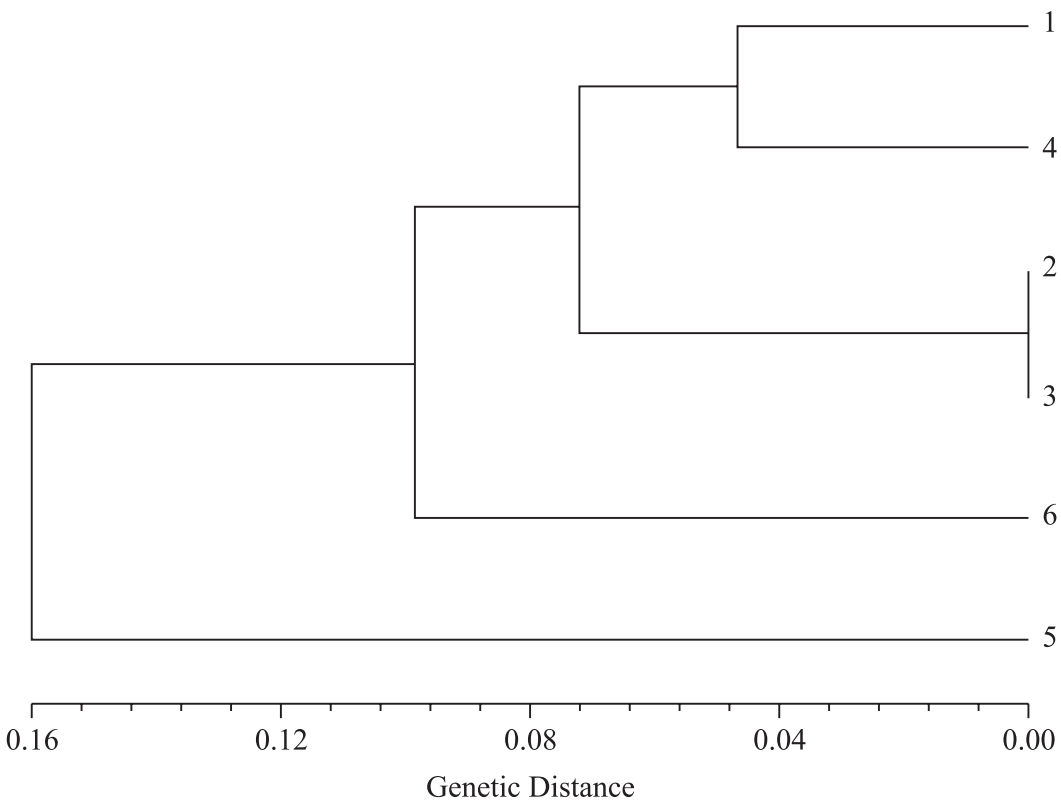

Fig. 2. UPGMA dendrogram of the 6 ditches based on Φ_{ST} genetic distances among ditches.

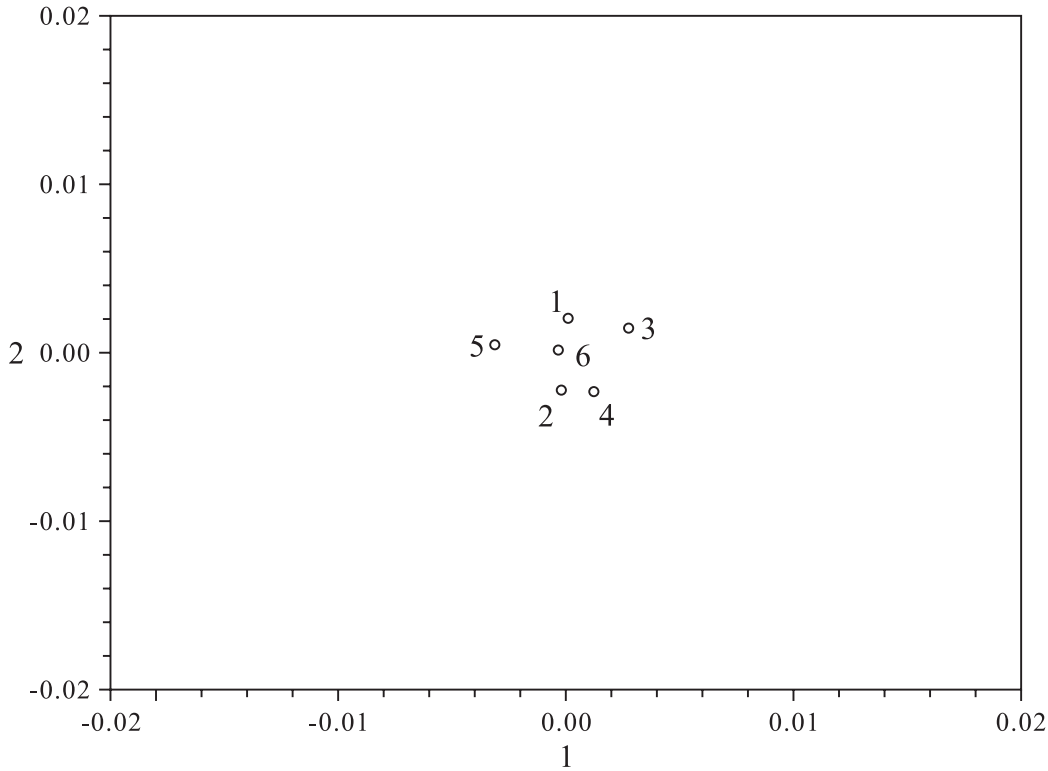

Fig. 3. Two-dimensional principal coordinate analysis of the 6 ditches studied.

Table 3. POPGENE analysis of gene diversities, genetic differentiation, and gene flow of the 6 ditches

	1	2	3	4	5	6	Total
N[1)]	7	7	5	13	10	8	50
H_E[2)]	0.0077	0.0088	0.0059	0.0063	0.0095	0.0069	0.0090
G_{ST}[3)]							0.172
Nm[4)]							2.4

[1)] N, number of samples.
[2)] H_E, Nei's (1973) gene diversity.
[3)] G_{ST}, genetic differentiation.
[4)] *Nm*, gene flow.

total gene diversity was 0.0090 when both polymorphic and monomorphic bands were included in the analysis. This result indicated that there was low genetic diversity within each ditch and within the area, because only 6 bands among 233 bands were found to be polymorphic and informative. The average gene flow (*Nm*) of *S. hainanensis* deduced from G_{ST} values was 2.4. According to Wright (1931), gene flow with *Nm* values exceeding of 1 tends to homogenize population differences and lower genetic

differentiation between populations. In this study, the genetic differentiation (G_{ST}) among ditches was estimated to be 0.172. Perennial herbs have an average G_{ST} of 0.213 while the average value for plants with both zoogamy and agamogenesis is 0.213 based on isozyme data (Hamrick and Godt 1990). The G_{ST} for *S. hainanensis* in Taiwan was much lower than for other species.

Cluster analysis of individuals

Cluster analysis of 50 samples (Fig. 4) indicated that samples of the same ditch were not linked together as a cluster, a result consistent with the AMOVA analysis. Figure 4 also indicates that many samples, such as samples 1 and 5 of ditch 1, 22 and 23 of ditch 4, and 46 of ditch 6 shared identical band patterns and therefore were linked at

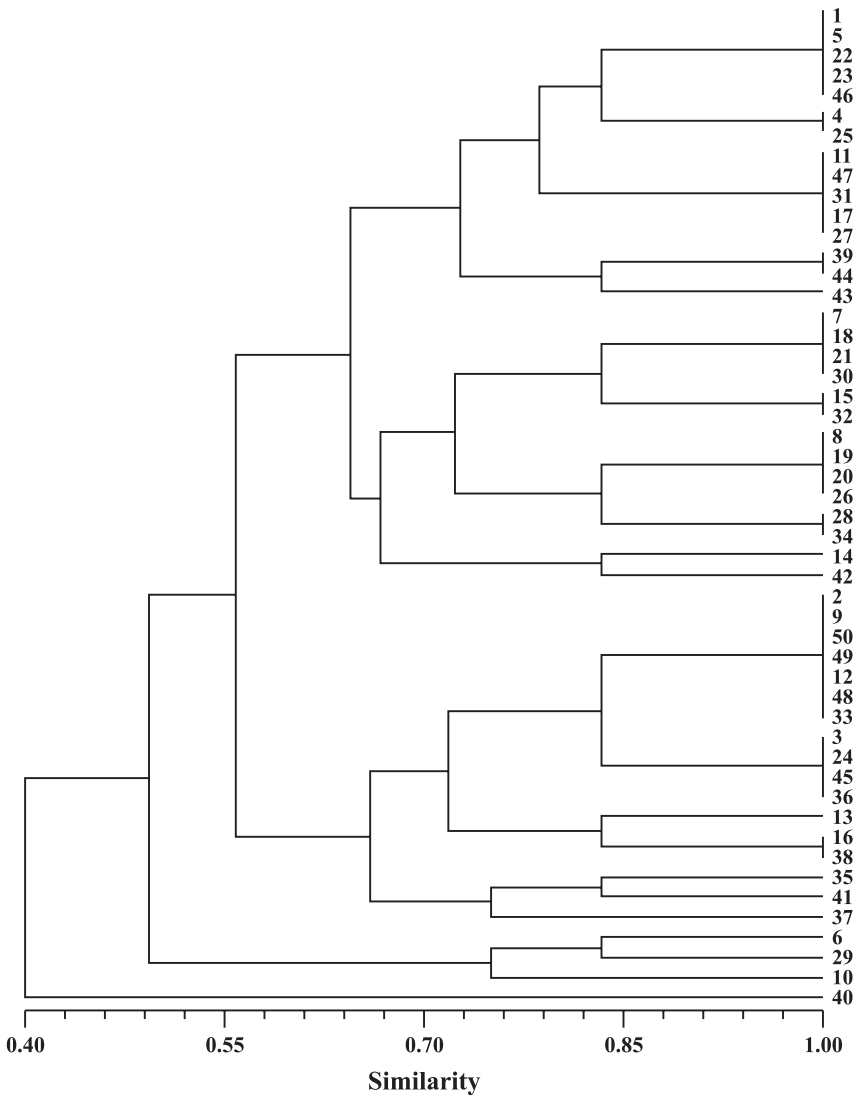

Fig. 4. UPGMA dendrogram of 50 individuals based on Dice similarity coefficients (ditch 1: samples 1~7; ditch 2: samples 8~14; ditch 3: samples 15~19; ditch 4: samples 20~32; ditch 5: samples 33~42; ditch 6: samples 43~50).

the similarity of 1.00. The high similarities observed among samples might be attributed to the uniform environmental conditions of the habitat and to asexual reproduction. Due to inbreeding and genetic drift, the genetic diversity will decrease and the fixation of detrimental recessive genes will thereby increase, which in turn will affect the survival and reproduction of the plant. Despite *S. hainanensis* blossoming and fruiting throughout the year, even in winter, no natural seedlings were spotted. Thus, the propagation may rely on agamogenesis by its decumbent stems. Hedrick and Miller (1992) and Ellstrand and Elam (1993) mentioned that inbreeding tends to increase homozygosity and decrease genetic variance of a small population. The question whether the absence of natural seedlings in the habitat of *S. hainanensis* is caused by seed abortion or seedlings dying before reaching maturity deserves further investigation.

Inevitably *S. hainanensis* will disappear from Taiwan if its narrow habitat is destroyed. Ex situ protection and breeding should be under taken. For example, the plant can be planted on dikes for dike protection or in scenic spots for landscaping. As only a single site in Taiwan is the home to *S. hainanensis*, it is recommended that a study on this plant be conducted to compare populations in other countries in order to formulate conservation policies for the species.

CONCLUSIONS

The population genetic study of *Scaevola hainaensis* based on ISSR fingerprintings revealed that plants in Taiwan have very low genetic variation, with a low ratio of polymorphic bands (2.58%). AMOVA analysis indicated that the variance components attributable to the variation among ditches and the variation among individuals within ditches were 6.15% and 93.85%, respectively. The genetic differentiation and gene flow analyses employing POPGENE revealed that the genetic differentiation coefficient (G_{ST}) among ditches was 0.172, while the gene flow (*Nm*) between them was 2.4. The results of cluster analysis and principal coordinate analysis indicated that the species shows very limited differences among ditches and among individual vines. Ex situ conservation should be conducted, such as growing plants on dikes for dike protection or in scenic spots for landscaping in order to enlarge the population size and hopefully to prevent the species from being extirpated from Taiwan.

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