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GENETIC STRUCTURE OF THREE ORCHID SPECIES WITH CONTRASTING BREEDING SYSTEMS USING RAPD AND ALLOZYME MARKERS[1]

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Zeuxine gracilis, *Zeuxine strateumatica*, and *Eulophia sinensis* are wild orchids with different breeding systems and colonizing abilities. *Zeuxine gracilis* is an outcrosser with restricted distribution, whereas *S. strateumatica* is an apomictic colonizer found only in newly available open habitats. *Eulophia sinensis* is an outcrossing colonizer. This study investigates the levels of genetic variation and patterns of population structure in these wild orchids to provide genetic information for the development of suitable conservation strategies. Lack of allozyme variation was characteristic of all three species, especially in populations of the two colonizing orchids, *Z. strateumatica* and *E. sinensis*. More variable markers, randomly amplified polymorphic DNAs (RAPDs), were further employed to characterize population structure of these species. Substantial genetic variation was found at the RAPD loci within populations of *Z. gracilis* ($p = 21.65 \pm 15.88\%$, $A = 1.217 \pm 0.159$, and $H = 0.076 \pm 0.054$) and *E. sinensis* ($p = 17.82 \pm 20.97\%$, $A = 1.179 \pm 0.209$, and $H = 0.070 \pm 0.084$), but little variation existed within populations of *Z. strateumatica* ($p = 2.84 \pm 2.58\%$, $A = 1.029 \pm 0.026$, and $H = 0.011 \pm 0.011$). Regardless of the breeding system, the total gene diversity at the species level was partitioned primarily between populations, as shown by high G_{ST} values, in all three species. An extremely high level of population differentiation ($G_{ST} = 0.924$) was found in the apomictic colonizer *Z. strateumatica*. The patterns of genetic variation in these wild orchids are apparently related to their differences in breeding system and colonizing ability. Different conservation strategies are needed for the long-term survival of these species.

Key words: allozymes; colonization; *Eulophia sinensis*; genetic structure; Orchidaceae; RAPD; *Zeuxine gracilis*; *Zeuxine strateumatica*.

Zeuxine gracilis Bl., *Z. strateumatica* (Linn.) Schltr., and *Eulophia sinensis* Miq. are three terrestrial wild orchids with different breeding systems. *Zeuxine gracilis* is an outcrossing orchid with restricted distribution, found exclusively in damp, shaded woods in mountainous areas, whereas *Z. strateumatica* is an apomictic colonizer frequently occurring in newly available open habitats, such as damp open grassland and roadside slopes. *Eulophia sinensis* Miq. is an outcrossing colonizer and frequently appears in the open fields of construction sites or newly generated grassland. Assessment of genetic variation and its partitioning within and between populations of these species is necessary for formulating conservation management strategies. A previous allozyme study (Sun, 1997) found no genetic variation in *Z. strateumatica* and only one variant allozyme genotype in *E. sinensis*. However, this pattern of allozyme variation may not represent the level of genetic diversity at other marker loci covering the entire genome of the species. Furthermore, lack of allozyme polymorphism hinders the investigation of population genetic structure of the species in terms of the partitioning of total gene diversity within and between populations. More polymorphic molecular markers should be employed to complement allozyme studies.

Of the various DNA markers recently developed for plant research, random amplified polymorphic DNA (RAPD) has become increasingly popular in studies of natural plant populations (see reviews in Bartish, Jeppsson, and Nybom, 1999; Bussell, 1999; Nybom and Bartish, 2000). In comparison with allozymes and microsatellites, there are some limitations and shortcomings of RAPD, such as marker allele dominance and sometimes low reproducibility, which may have discouraged many investigators from using RAPD. However, the major advantages of RAPD analysis outweigh its disadvantages: RAPD analysis can potentially provide a much higher number of reproducible marker loci and higher levels of polymorphism than allozyme analysis, and it costs much less and is faster and easier to perform than microsatellite analysis because no prior DNA sequence information for the target species is required. Under carefully controlled reaction conditions, reproducible and interpretable RAPD banding patterns can be obtained.

A recent review by Nybom and Bartish (2000) of 84 papers published between 1993 and 2000 shows that although the absolute values of within-population diversity are usually higher in RAPD-based investigations, the estimates of between-population diversity (G_{ST}) based on RAPDs are generally comparable to allozyme-based G_{ST} values and that the effects of life history traits on genetic diversity estimates obtained with RAPD markers are mostly the same as previously reported for allozyme data. One discrepancy between allozyme and RAPD data concerns geographic range of the investigated species, showing that RAPDs can be more sensitive than allozymes in detecting genetic structuring with increasing distributional range of populations.

A species' breeding system often plays a central role in determining the distribution of genetic variation within and between populations (Hamrick and Godt, 1989, 1996). Other factors, such as population size and colonizing ability of a species, often similarly affect the amount of within-population variation and population differentiation (e.g., Sun, 1996, 1997). In this study, we investigated allozyme and RAPD diversity in six populations of *Z. gracilis* and RAPD diversity in ten populations of *Z. strateumatica* and seven populations

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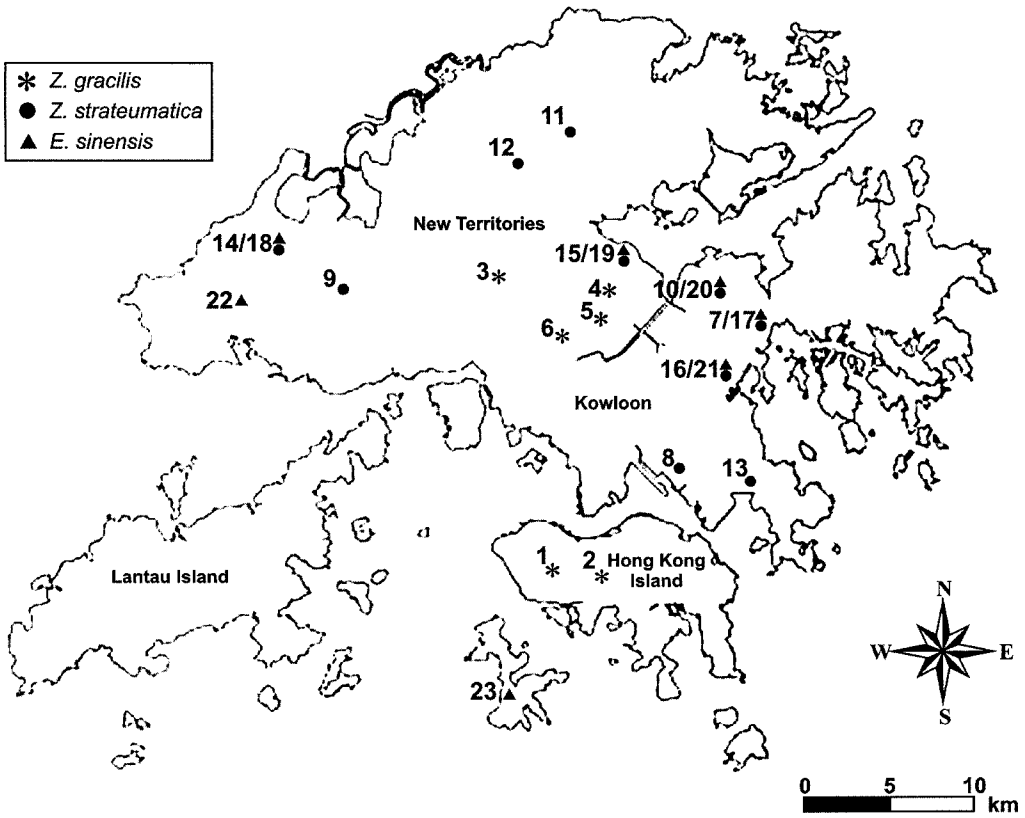

Fig. 1. Map showing the species and populations sampled and their locations. *Zeuxine gracilis*: 1 = PFL (Pokfulam), 2 = BL (Black's Link), 3 = NTC (Ng Tung Chai), 4 = TPK1 (Tai Po Kau 1), 5 = TPK2 (Tai Po Kau 2), 6 = SM (Shing Mun); *Z. strateumatica*: 7 = SK (Sai Kung), 8 = KT (Kwun Tong), 9 = YL (Yuen Long), 10 = MOS (Ma On Shan), 11 = FL (Fanling), 12 = KKL (Kai Kung Ling), 13 = JB (Junk Bay), 14 = TSW (Tinshuiwai), 15 = CU (KCRCU), 16 = UST (UST Camp); *Eulophia sinensis*: 17 = SK, 18 = TSW, 19 = CU, 20 = MOS, 21 = UST (GH), 22 = TM (Tuen Mun), 23 = LM (Lamma Island).

of *E. sinensis*. We compared the levels of genetic variation and patterns of population structure in these wild orchids according to theoretical predictions based on their breeding systems and attributes associated with colonization.

MATERIALS AND METHODS

Species and populations—Six populations of *Zeuxine gracilis* were sampled (Fig. 1), including Pokfulam (PFL) and Black's Link (BL) from Hong Kong Island and four other populations, Ng Tung Chai (NTC), Tai Po Kau 1 (TPK1), Tai Po Kau 2 (TPK2), and Shing Mun (SM), from the New Territories. The full names of the populations indicate their geographical locations in Hong Kong. To characterize the breeding system of *Z. gracilis*, plants at two sampling sites of population Pokfulam (PFL) were counted, and the numbers of flowers and fruits were recorded for each plant during the flowering season (from late September to early November in Hong Kong). Three plants at the sites were bagged to test whether the species is pollinator-dependent for fruit setting, and six plants from the sites were transplanted to greenhouse conditions for observation in the absence of pollinators. Similar studies have already been carried out for *Zeuxine strateumatica* and *Eulophia sinensis* (Sun, 1997). However, no information was previously available on the breeding system of *Z. gracilis*.

Ten populations of *Zeuxine strateumatica* were sampled from the New Territories (Fig. 1), including Sai Kung (SK), Kwun Tong (KT), Yuen Long (YL), Ma On Shan (MOS), Fanling (FL), Kai Kung Ling (KKL), Junk Bay (JB), Tinshuiwai (TSW), KCRCU (CU), and UST Camp (UST).

Seven populations of *Eulophia sinensis* were sampled (Fig. 1), six from the New Territories, Sai Kung (SK), Tinshuiwai (TSW), KCRCU (CU), Ma On Shan (MOS), UST Camp (GH), and Tuen Mun (TM), and one from Lamma Island (LM).

The population and individual sample sizes for each species were determined on the basis of availability of local populations, actual population sizes, levels of genetic variation, and the breeding system of the species. For example, five individuals were sampled to represent each population of *Z. strateumatica*; this sample size was considered adequate because the species is apomictic and lacks allozyme variation within and between populations. For the outcrossing species, we aimed at a larger population sample size. However, the sample size was often limited by the actual sizes of natural populations. Fresh samples of young leaves, a small piece taken from each individual, were placed in moist paper towels in a cool ice chest during field collections and stored at 4°C until allozyme electrophoresis or DNA extraction.

Allozyme electrophoresis—Samples of leaf tissue from each individual were ground with extraction buffer as described in Sun and Ganders (1990). The following 11 enzyme systems were resolved in *Z. gracilis* on 12.5% starch gels with two buffer systems, the histidine-tris-citrate system (Sun and Corke, 1992) for resolving aspartate aminotransferase (AAT; Enzyme Commission [E.C.] number 2.6.1.1), aconitase (ACO; E.C. 4.2.1.3), isocitrate dehydrogenase (IDH; E.C. 1.1.1.42), malate dehydrogenase (MDH; E.C. 1.1.1.37), phosphoglucomutase (PGM; E.C. 5.4.2.2), phosphogluconate dehydrogenase (6-PGD; E.C. 1.1.1.44), and shikimate dehydrogenase (SKDH; E.C. 1.1.1.25); and the "L" system of Shields, Orton, and Stuber (1983) for resolving esterase (EST; E.C. 3.1.1.1), glucose-6-phosphate isomerase (PGI; E.C. 5.3.1.9), leucine aminopeptidase (LAP; E.C. 3.4.11.1), and triose-phosphate isomerase (TPI, E.C. 5.3.1.1). The protocols of Wendel and Weeden

TABLE 1. Genetic variation within populations of *Zeuxine gracilis* based on allozyme data. N = number of individuals sampled, p = percentage of polymorphic loci, A = number of alleles per locus, A_e = effective number of alleles per locus, H_o = observed heterozygosity, H_e = expected heterozygosity, H_{op} = observed heterozygosity per polymorphic locus, H_{ep} = expected heterozygosity per polymorphic locus, and f = fixation index. For population abbreviations, see Fig. 1.

Genetic parameter	Population						Mean ± SD
	PFL	BL	NTC	TPK1	TPK2	SM	
N	10	9	10	15	9	22	13 ± 5
p	0	0	0	5.56	5.56	5.56	2.78 ± 3.05
A	1 ± 0	1 ± 0	1 ± 0	1.056 ± 0.235	1.056 ± 0.236	1.056 ± 0.236	1.028 ± 0.031
A_e	1 ± 0	1 ± 0	1 ± 0	1.055 ± 0.234	1.050 ± 0.214	1.009 ± 0.034	1.019 ± 0.026
H_o	0 ± 0	0 ± 0	0 ± 0	0.052 ± 0.200	0.043 ± 0.183	0.008 ± 0.032	0.017 ± 0.024
H_e	0 ± 0	0 ± 0	0 ± 0	0.029 ± 0.121	0.028 ± 0.119	0.007 ± 0.031	0.011 ± 0.014
H_{op}	—	—	—	0.933	0.778	0.136	—
H_{ep}	—	—	—	0.515	0.503	0.130	—
f	—	—	—	−0.812	−0.547	−0.046	—

(1989) were used for enzyme activity staining. Genetic interpretation of band patterns followed the standard principles (Weeden and Wendel, 1989; Wendel and Weeden, 1989).

DNA isolation and polymerase chain reaction amplification—The protocol of CTAB total DNA isolation (Doyle, 1991) was used to isolate genomic DNA from fresh leaves. After quantification with a fluorometer (Hoefer Pharmacia Biotech, San Francisco, California, USA), DNA sample solution of 20-ng/μL was prepared. A 25-μL amplification reaction mix contained 10 mmol/L Tris, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTPs, 0.4 μmol/L primer, 0.5 unit of *Taq* polymerase (Promega, Madison, Wisconsin, USA) and 20 ng template DNA. Polymerase chain reaction (PCR) amplification was performed in a Programmable Thermal Controller (MJ Research, Watertown, Massachusetts, USA) for 45 cycles of 1 min at 94°C, 1 min at 38°C, and 2 min at 72°C. The amplification products were visualized by electrophoresis on 1.4% agarose gels followed by ethidium bromide staining.

One hundred oligonucleotide primers (10-mers) of arbitrary sequence (UBC Primer Set #1 Lot #1) were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada. We screened most of these primers and assessed the reproducibility of their PCR amplification products. Fourteen of the primers (UBC primer numbers 25, 31, 33, 52, 55, 58, 63, 64, 65, 66, 72, 74, 82, and 91) were selected for RAPD analysis of *Z. strateumatica* populations, 16 primers (UBC primer numbers 17, 25, 31, 33, 34, 52, 55, 58, 63, 64, 65, 72, 74, 82, 86, and 91) were selected for *Z. gracilis*, and 14 primers (UBC primer numbers 2, 6, 16, 17, 18, 23, 25, 29, 30, 31, 65, 71, 72, and 86) were selected for *E. sinensis*. These primers were selected on the basis of band amplification and reproducibility. To avoid any bias in assessing genetic diversity, ''primers showing optimum levels of polymorphism'' was not used as a criterion in selecting which primers to use. Only strong and reproducible bands were scored and used in the final data analysis.

Data analysis—Allozyme frequency data were analyzed with POPGENE (Yeh et al., 1997). Genetic parameters within populations, including the percentage of polymorphic loci (p), number of alleles per locus (A), effective number of alleles per locus (A_e), expected heterozygosity (H_e) or gene diversity (H), fixation index (f), and Shannon index (S), were calculated. The effective number of alleles per locus (A_e) was computed in this study to take into account the relative allele frequencies at those polymorphic loci, because the number of alleles per locus (A) based on RAPD data could be very similar among populations due to the biallelic nature of RAPD loci.

Nei's (1973) total gene diversity (H_T), coefficient of gene differentiation (G_{ST}), and Nei's (1972) genetic identity (I) between populations were also computed at the species level.

For RAPD analysis, bands were identified by an image analysis software for gel documentation (Molecular Analyst/PC version 1.2 [Bio-Rad, Hercules, California, USA]). Smeared and weak bands were excluded. To estimate polymorphism parameters at both the population and species levels, the band presence/absence data matrix was analyzed with POPGENE (Yeh et al., 1997). An additional measure for partitioning genetic variation was obtained by the Shannon index (S) because it is relatively insensitive to the inability of RAPD to detect heterozygous loci (Dawson et al., 1995). Estimates of between-population diversity using the Shannon index do not rely on Hardy-Weinberg equilibrium (Bussell, 1999) and can be compared across species with different breeding systems.

RESULTS

Zeuxine gracilis—Like many other species in the genus, *Z. gracilis* has small white flowers, ~0.5 cm in diameter. The number of flowers on each inflorescence ranged from 2 to 11 at the two sites of the Pokfulam population surveyed, with a mean of 5.78 ± 2.66 and 5.23 ± 1.92 flowers, respectively; the natural fruiting rate per inflorescence was high at 0.984 ± 0.050 (N_{plants} = 19, $N_{flowers}$ = 110) and 0.978 ± 0.053 (N_{plants} = 14, $N_{flowers}$ = 69), respectively, late in the flowering season. However, some of the isolated plants had much lower fruiting rates early in the flowering season; for example, in one plant, only one of the 13 flowers examined set fruit, and the remaining flowers were either not visited by pollinators (pollinia not removed from the flower) or not pollinated (pollinia removed but no pollination of the stigma). The bagged flowers in the population set no fruits. Six transplanted individuals all flowered under greenhouse conditions, but set no fruits in the absence of pollinators. The unpollinated flowers may remain open for 3–4 wk after anthesis. Artificial self-pollination performed on some of the flowers resulted in setting of fruit and fertile mono-embryonic seeds, indicating that the species is self-compatible but dependent on pollinators for fruit set in the wild.

A total of 18 isozyme loci were resolved in the six populations studied. Allozyme variation was extremely low. Three of the six populations surveyed were completely devoid of allozyme variation (Table 1). Only one locus, *Pgm-1*, was polymorphic among the 18 resolved loci in the remaining three populations. Negative fixation indices (f) were obtained at *Pgm-1* in TPK1, TPK2, and SM, ranging from −0.046 to −0.812, which indicates heterozygosity excess in comparison with the Hardy-Weinberg expectation. At the species level, the total gene diversity (H_T) was estimated to be 0.015 ± 0.064, of which 33.3% was distributed between populations (H_S = 0.10 ± 0.043 and G_{ST} = 0.333). Lack of allozyme polymorphism resulted in high estimates of Nei's genetic identities (I) between populations, ranging from 0.988 to 1.000 with an average of 0.995 ± 0.005. Based on the genetic identity matrix, a UPGMA dendrogram was constructed (Fig. 2). Due to the

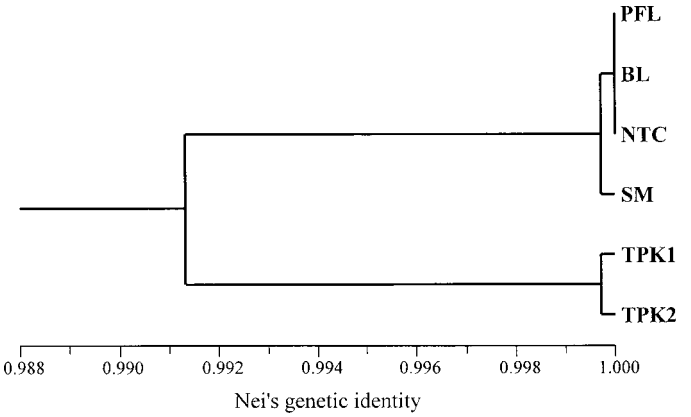

Fig. 2. Dendrogram of Nei's genetic identity among populations of *Zeuxine gracilis* based on allozyme data. For population abbreviations, see Fig. 1.

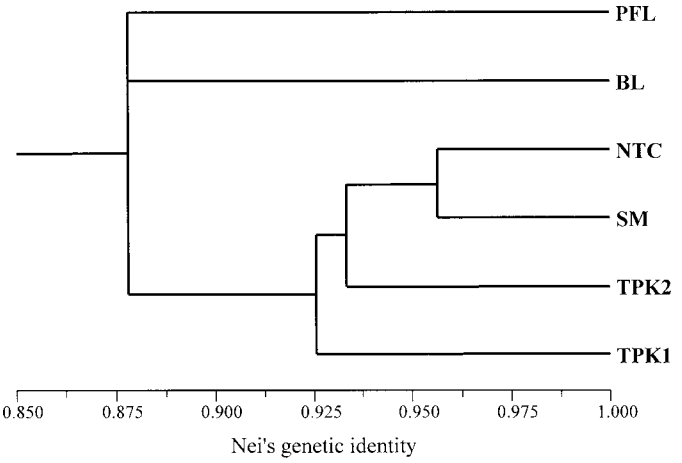

Fig. 3. Dendrogram of Nei's genetic identity among populations of *Zeuxine gracilis* based on RAPD data. For population abbreviations, see Fig. 1.

complete lack of allozyme polymorphism in PFL, BL, and NTC populations, the relationships cannot be resolved among these populations. Despite the limitation in allozyme polymorphism, the two adjacent populations from Tai Po Kau (TPK1 and TPK2) were grouped together, which is congruent with their geographical proximity.

A total of 77 RAPD markers were generated with 16 primers in *Z. gracilis*, ranging from one to nine bands per primer. In comparison with allozymes, genetic variation at the RAPD loci was much higher. Among the 77 marker loci, 53% were polymorphic at the species level. However, the populations were not homogeneous for the genetic parameters measured (Table 2). The coefficient of genetic differentiation between populations (G_{ST}) was 0.539, estimated by partitioning of the total gene diversity, or 0.556, estimated based on the Shannon diversity index. The two estimates of G_{ST} are highly concordant, both indicating a high level of genetic differentiation among populations. Nei's genetic identities (I) between populations at the RAPD loci varied from 0.786 to 0.956 with a mean value of 0.889 ± 0.047. Based on the genetic identity matrix, a UPGMA dendrogram was constructed (Fig. 3). The groupings of populations are highly congruent with their geographical locations in that all four populations from the New Territories, NTC, TPK1, TPK2, and SM were placed in one cluster. The low RAPD polymorphism in PFL and BL resulted in a parallel placement of the two populations from Hong Kong Island with the New Territories group.

Zeuxine strateumatica—A single multilocus allozyme genotype was previously recorded for all populations of *Z. strateumatica* studied in Hong Kong based on 45 isozyme loci resolved (Sun, 1997). Thus, isozyme analysis was not performed for the present set of populations. Based on the information of allozyme uniformity, five individuals were considered adequate to represent each population for RAPD analysis in the present study. A total of 71 markers were generated with 14 primers in the populations, ranging from one to ten per primer. Although polymorphism was high at the species level (p = 49%), this variation was primarily due to genetic divergence between populations. Within-population polymorphism was generally low (Table 3). The coefficient of genetic differentiation between populations (G_{ST}) was high (0.924), estimated based on either gene diversity or Shannon index. This G_{ST} value indicates that the total genetic diversity in the species resides almost entirely between populations. Nei's genetic identities (I) between populations at the RAPD level varied from 0.667 to 0.967 with a mean of 0.851 ± 0.077. Based on the genetic identity matrix, a UPGMA dendrogram was constructed (Fig. 4). The groupings of the populations are largely in accordance with their geographical localities.

Eulophia sinensis—A previous isozyme study revealed a nearly complete lack of allozyme diversity in this species, as was also the case in *Z. strateumatica*. Only one of 57 individuals surveyed was heterozygous (at *6pgd–1* locus) in the seven populations sampled (Sun, 1997). In contrast, RAPD diversity was much higher in those populations with six or more individuals (Table 4). A total of 97 markers were generated with

TABLE 2. Genetic variation within populations of *Zeuxine gracilis* based on RAPD data. p = percentage of polymorphic loci, A = number of alleles per locus, A_e = effective number of alleles per locus, S = Shannon index, H = Nei's (1973) gene diversity, and N = number of individuals sampled.

Genetic parameter	Population						
	PFL	BL	NTC	TPK1	TPK2	SM	Mean ± SD
p	5.19	2.60	22.08	44.16	22.38	32.47	21.65 ± 15.88
A	1.052 ± 0.223	1.026 ± 0.160	1.221 ± 0.418	1.442 ± 0.500	1.234 ± 0.426	1.325 ± 0.471	1.217 ± 0.159
A_e	1.024 ± 0.105	1.025 ± 0.154	1.132 ± 0.254	1.250 ± 0.351	1.130 ± 0.282	1.212 ± 0.358	1.129 ± 0.093
S	0.026 ± 0.112	0.018 ± 0.109	0.123 ± 0.234	0.224 ± 0.275	0.118 ± 0.227	0.177 ± 0.273	0.114 ± 0.081
H	0.017 ± 0.072	0.013 ± 0.079	0.082 ± 0.156	0.148 ± 0.190	0.078 ± 0.155	0.120 ± 0.191	0.076 ± 0.054
N	10	10	8	15	10	21	12 ± 5
No. of loci	65	70	65	70	66	71	68 ± 3

TABLE 3. Genetic variation within populations of *Zeuxine strateumatica* based on RAPD data. p = percentage of polymorphic loci, A = number of alleles per locus, A_e = effective number of alleles per locus, S = Shannon index, H = Nei's gene diversity, $N_{individuals}$ = number of individuals sampled, and N_{loci} = number of loci. Standard deviations are given in parentheses.

Genetic parameter	Population										
	SK	KT	YL	MOS	FL	KKL	JB	TSW	CU	UST	Mean
p	1.49	0	1.49	4.48	2.99	2.99	2.99	0	8.96	2.99	2.838 (2.582)
A	1.015 (0.122)	1 (0)	1.015 (0.122)	1.045 (0.208)	1.030 (0.172)	1.030 (0.172)	1.030 (0.172)	1 (0)	1.090 (0.288)	1.030 (0.172)	1.029 (0.026)
A_e	1.007 (0.056)	1 (0)	1.007 (0.058)	1.028 (0.137)	1.014 (0.081)	1.028 (0.158)	1.021 (0.126)	1 (0)	1.062 (0.212)	1.028 (0.158)	1.020 (0.019)
S	0.008 (0.061)	0 (0)	0.008 (0.061)	0.025 (0.118)	0.015 (0.086)	0.020 (0.115)	0.018 (0.102)	0 (0)	0.053 (0.171)	0.020 (0.115)	0.017 (0.015)
H	0.005 (0.039)	0 (0)	0.005 (0.039)	0.017 (0.079)	0.010 (0.055)	0.014 (0.082)	0.012 (0.070)	0 (0)	0.036 (0.118)	0.014 (0.082)	0.011 (0.011)
$N_{individuals}$	5	5	5	5	5	5	5	5	5	5	5 (0)
N_{loci}	62	59	61	53	53	59	62	59	56	63	59 (4)

14 primers in the species, ranging from 3 to 14 loci per primer. Among the 97 loci, 79% were polymorphic at the species level. As in *Z. gracilis*, the populations were not homogeneous for measured genetic parameters, although variance in population size may also contribute to this heterogeneity among populations. The total gene diversity (H_T = 0.202 ± 0.053; S = 0.411) was high at the species level. High population differentiation was found (G_{ST} = 0.653 based on H_T, and G_{ST} = 0.752 based on S). Nei's genetic identities between populations varied from 0.621 to 0.972 with a mean value of 0.757 ± 0.088. Based on the genetic identity matrix, a UPGMA dendrogram was constructed (Fig. 5). Except for the grouping of CU, MOS, and GH populations, the placement of other populations was not related to their geographical localities.

A summary of genetic variation and population structure for all three species is given in Table 5 for both allozymes and RAPDs.

DISCUSSION

As one of the largest families of flowering plants, the Orchidaceae constitutes up to 10% of all flowering plant species (Dressler, 1981). Although numerous population genetic studies have been conducted on plants using allozyme markers (see review by Hamrick and Godt, 1989, 1996), relatively few reports are available on comparative studies of population genetic diversity in wild orchids using different markers. Given the extraordinary species diversity and many unique features of orchids, more comparative studies of genetic variation and population structure of wild orchids are needed to understand the patterns of genetic diversity in the family and the contributing factors shaping their population structure. Such information is especially necessary for developing effective conservation strategies for endangered or threatened orchids. The present study, as well as several recent studies by others and ourselves, has evaluated genetic variation and structure of several wild orchids, such as *Cephalanthera longifolia* and *C. rubra* (Scacchi, De Angelis, and Corbo, 1991), *Orchis morio* (Rossi et al., 1992), *Cypripedium calceolus* (Case, 1993), *Orchis papilionacea* (Arduino et al., 1995), *Epipactis helleborine* (Hollingsworth and Dickson, 1997), *Spiranthes hongkongensis* and *S. sinensis* (Sun, 1996, 1997), and *S. diluvialis* (Arft and Ranker, 1998; Szalanski et al., 2001).

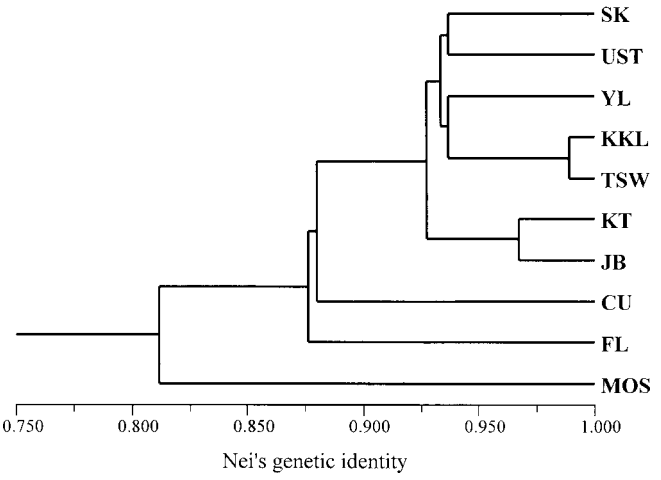

Fig. 4. Dendrogram of Nei's genetic identity among populations of *Zeuxine strateumatica* based on RAPD data. For population abbreviations, see Fig. 1.

Zeuxine gracilis—A previous study confirmed that *E. sinensis* is a self-compatible but pollinator-dependent outcrosser, and *Z. strateumatica* is apomictic (Sun, 1997). However, the breeding system of *Z. gracilis* has not been studied previously. In the present study, very high fruiting rates of *Z. gracilis* were observed at two field sites, which may suggest that the species is a selfer. However, other data, e.g., the very low fruiting rates of isolated individuals early in the flowering season and the fact that bagged plants set no fruits, indicate that the species is pollinator-dependent in reproduction. The unusually high fruiting rate late in the flowering season suggests that pollinators are not a limiting factor in wild populations. Although pollinator-mediated selfing can also occur given its self-compatibility, tests for Hardy-Weinberg equilibrium supported a random-mating system in four of the six populations studied, and the remaining two populations had significant heterozygote excesses. However, negative fixation indices can result from other evolutionary forces, such as selection for heterozygosity at the locus. Therefore, fixation index alone cannot be used to determine the actual breeding system of the species. As shown in populations of an outcrossing orchid, *Goodyera procera*, fixation indices could be highly heterogeneous among

TABLE 4. Genetic variation within populations of *Eulophia sinensis* based on RAPD data. p = percentage of polymorphic loci, A = number of alleles per locus, A_e = effective number of alleles per locus, S = Shannon index, H = Nei's gene diversity, $N_{individuals}$ = number of individuals sampled, and N_{loci} = number of loci.

Genetic parameter	Population							
	SK	LM	TSW	CU	MOS	GH	TM	Mean ± SD
p	0	1.030	2.06	24.74	13.40	24.74	58.76	17.82 ± 20.97
A	1 ± 0	1.012 ± 0.110	1.022 ± 0.147	1.247 ± 0.434	1.134 ± 0.342	1.247 ± 0.434	1.588 ± 0.495	1.179 ± 0.209
A_e	1 ± 0	1.009 ± 0.078	1.016 ± 0.104	1.178 ± 0.341	1.093 ± 0.256	1.149 ± 0.292	1.426 ± 0.419	1.124 ± 0.151
S	0 ± 0	0.007 ± 0.066	0.013 ± 0.089	0.144 ± 0.262	0.077 ± 0.202	0.131 ± 0.243	0.342 ± 0.219	0.102 ± 0.121
H	0 ± 0	0.005 ± 0.046	0.009 ± 0.061	0.099 ± 0.183	0.053 ± 0.140	0.088 ± 0.167	0.236 ± 0.309	0.070 ± 0.084
$N_{individuals}$	2	2	2	6	6	10	10	5 ± 4
N_{loci}	68	75	67	79	67	72	93	74 ± 9

populations (Wong and Sun, 1999). Only one polymorphic locus in one of the 15 populations of *Goodyera procera* studied was found to be in Hardy-Weinberg equilibrium, with all others showing either heterozygosity deficiency or excess in the species.

The amount of allozyme variation observed in *Z. gracilis* is much lower than that expected for insect-pollinated outcrossing plants. Compared with the average values for insect-pollinated outcrossing plants (at the species level: p = 50.1%, A_e = 1.24, H = 0.167; at the population level: p = 35.9%, A_e = 1.17, H = 0.124; Hamrick and Godt, 1989), the mean values of genetic variation (at the species level: p = 5.56%, A_e = 1.02, and H = 0.015; and at the population level: p = 2.78%, A_e = 1.019, and H = 0.011) are extremely low in *Z. gracilis*. In contrast, the estimate of population differentiation (G_{ST} = 0.333) is substantially higher than the reported average in animal-pollinated outcrossing plants (G_{ST} = 0.197; Hamrick and Godt, 1989). In comparison with the allozyme-based estimates, within-population gene diversity (H = 0.076) based on RAPDs is higher in *Z. gracilis*. However, this value is still much lower than the reported mean of within-population diversity (H = 0.26; Nybom and Bartish, 2000) in outcrossing plants using RAPD markers. Between-population diversity (G_{ST} = 0.539) in *Z. gracilis*, on the other hand, is much higher than the mean G_{ST} (0.23; Nybom and Bartish, 2000) in outcrossing plants. Regardless of the difference in the absolute G_{ST} values, the patterns of genetic structure in *Z. gracilis* revealed by allozymes and RAPDs are comparable.

RAPD markers revealed substantially higher population differentiation than did allozyme markers for each of three *Pinus* species studied by Wu, Krutovskii, and Strauss (1999), and a simulation study indicates that the dominant and biallelic nature of RAPD markers could explain the differences observed in differentiation parameters. Isabel et al. (1999) have also shown that estimates of population differentiation derived from dominant RAPDs are generally inflated when typically small population sample sizes are used. On the other hand, higher G_{ST} may arise if the RAPD profile contains a large number of organelle DNA products, which are often more highly differentiated than nuclear RAPD markers and allozymes (e.g., Aagaard et al., 1995; Aagaard, Krutovskii, and Strauss, 1998). Most RAPD profiles are generated from the total genomic DNA, and RAPDs of organelle origin are normally included in the data analyses. This would lead to biased estimates of G_{ST} using RAPDs. As shown in Aagaard, Krutovskii, and Strauss (1998), mitochondrial RAPD markers were much more highly differentiated than were fragments of nuclear origin at both the population (G_{ST} = 0.18 and 0.05, respectively) and racial levels (G_{ST} = 0.72 and 0.25, respectively).

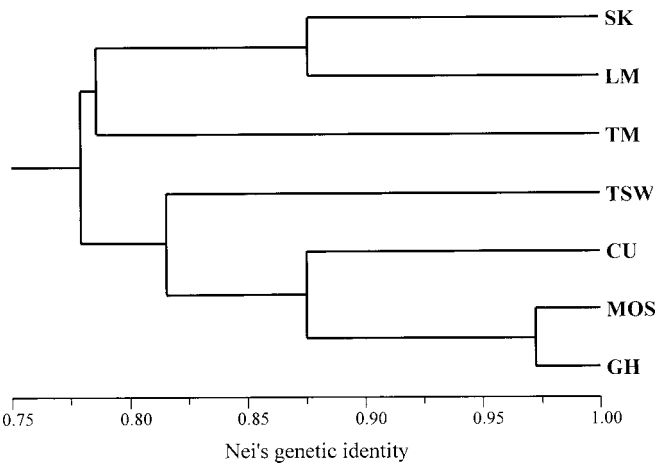

Fig. 5. Dendrogram of Nei's genetic identity among populations of *Eulophia sinensis* based on RAPD data. For population abbreviations, see Fig. 1.

Both the allozyme and RAPD estimates of G_{ST} confirmed that natural gene flow between populations of *Z. gracilis* was apparently limited. Nei's genetic identity based on RAPDs (I = 0.889 ± 0.047) was substantially lower than the estimate for allozymes (I = 0.995 ± 0.005), showing that a high level of between-population divergence at RAPD loci has occurred in the species. In comparison with genetic variation and structure based on RAPD analyses of wild plant populations (Nybom and Bartish, 2000), the amount and pattern of genetic variation in *Z. gracilis* is apparently more comparable to selfing or early-successional taxa than to outcrossing species. This pattern of genetic diversity in *Z. gracilis* is likely the consequence of genetic drift and geographical isolation among its generally small populations. Based on this information, a suitable management strategy would be to minimize human disturbance of its habitats and to increase population size.

Zeuxine strateumatica—In comparison with sexually reproducing *Z. gracilis*, the total genetic diversity in apomictic *Z. strateumatica* was not much less at the species level. However, nearly all this diversity existed between populations of *Z. strateumatica*, and most of its populations were characterized by genetic uniformity at many RAPD loci. The extremely high genetic differentiation or lack of gene flow among populations of *Z. strateumatica* is consistent with its apomictic reproduction and colonization. Apomixis prevents sexual recombination within populations and gene flow through pollen among populations, resulting in low genetic variation within popula-

TABLE 5. Genetic diversity at the species level in *Zeuxine gracilis, Zeuxine strateumatica,* and *Eulophia sinensis* as revealed by allozyme and RAPD markers. p = percentage of polymorphic loci, A = number of alleles per locus, A_e = effective number of alleles per locus, H_T = total gene diversity, H_S = gene diversity within populations, G_{ST} = the coefficient of genetic differentiation among populations, S_T = total Shannon diversity index, S_S = Shannon diversity within populations, and I = average Nei's genetic identity between populations.

Genetic parameter	Allozyme[a]			RAPD		
	Z. gracilis	*Z. strateumatica*	*E. sinensis*	*Z. gracilis*	*Z. strateumatica*	*E. sinensis*
p	5.56	0	0.61	53.25	49.25	79.38
A	1.056 ± 0.024	1	1.006	1.533 ± 0.502	1.493 ± 0.504	1.794 ± 0.407
A_e	1.020 ± 0.087	1	1.0004	1.290 ± 0.368	1.226 ± 0.299	1.454 ± 0.341
Gene diversity						
H_T	0.015 ± 0.064	0	0.00012	0.165 ± 0.037	0.144 ± 0.030	0.202 ± 0.053
H_S	0.010 ± 0.043	0	0.00012	0.076 ± 0.009	0.011 ± 0.001	0.070 ± 0.004
G_{ST}	0.333	—	0	0.539	0.924	0.653
Shannon index						
S_T	—	—	—	0.257	0.225	0.411
S_S	—	—	—	0.114	0.017	0.102
G_{ST}	—	—	—	0.556	0.924	0.752
I	0.995 ± 0.005	1.000	1.000	0.889 ± 0.047	0.851 ± 0.077	0.757 ± 0.088
No. of populations	6	9	7	6	10	7
No. of individuals	75	128	57	74	50	38
No. of loci	18	45	41	77	71	97

[a] Allozyme data for *Z. strateumatica* and *E. sinensis* are from Sun (1997).

tions and high genetic divergence among populations. In addition, founder effects and genetic drift in small colonizing populations can lead to the rapid fixation of genotypes in the apomict, which further reduces within-population variation and increases among-population divergence. Thus, the observed amount of genetic variation and pattern of population structure in *Z. strateumatica* fit the theoretical expectation perfectly on the basis of both breeding system and colonization.

A previous allozyme study of the species found only one multilocus genotype in nine populations studied, suggesting a single origin of the colonizing populations in Hong Kong (Sun, 1997). However, the presence of multiple multilocus RAPD genotypes in the species indicates that these colonizing populations could have multiple origins. The management of the species may involve multiple population sites. In contrast to *Z. gracilis*, the long-term persistence of *Z. strateumatica* depends on human disturbance, which creates new niches for it to colonize, while maintaining its current habitats.

Eulophia sinensis—Lack of allozyme diversity in *E. sinensis* (Sun, 1997) contrasts with the high RAPD polymorphism at the species level, as well as within some large populations. Although the amount of RAPD variation is consistent with its outcrossing breeding system, this pattern of population structure is not expected of an outcrossing species. High genetic differentiation (Table 5) existed among the populations surveyed, suggesting that population structure of this species is primarily determined by colonization dynamics. Founder effects and genetic drift in small colonizing populations apparently play a dominant role in determining the pattern of genetic diversity in the species. As in *Z. strateumatica*, the species' long-term persistence requires new habitats created by human activities.

Genetic diversity in other wild orchids—In plants, the breeding system has a profound effect on the genetic composition of natural populations (Hamrick, 1982). Outcrossing plants have generally higher allozyme diversity within populations and lower genetic divergence among populations. However, allozyme diversity is often low in many wild orchids, regardless of their breeding systems. Gene flow appears to be much more restricted in wild orchids than in other plants. *Goodyera procera* is a good example (Wong and Sun, 1999), where despite its outbreeding system, allozyme data revealed relatively low variation at the species level (p = 33%, A = 1.33, and H = 0.15), in comparison with other animal-pollinated outbreeding plant species (p = 50%, A = 1.99, H = 0.17; Hamrick and Godt, 1989). The RAPD variation in the species was relatively high (p = 55.13% and H = 0.18 at the population level, and p = 97.03% and H = 0.29 at the species level), but G_{ST} estimates indicated high levels of genetic differentiation among populations (G_{ST} = 0.52 and I = 0.909 ± 0.049 based on allozyme data, and G_{ST} = 0.39 and I = 0.859 ± 0.038 based on RAPD data), much above the average for outcrossing species (G_{ST} = 0.20; Hamrick and Godt, 1989). Generally small population sizes and lack of gene flow among populations due to geographical isolation are likely among the major factors contributing to this pattern of population structure in *Goodyera procera*. In outcrossing orchids with large populations, however, high levels of allozyme diversity have been documented, e.g., Sun (1996) found very high allozyme diversity in *Spiranthes sinensis* (p = 80%, A = 2.65, and H = 0.238 at the species level), much above those reported for animal-pollinated outcrossing plants. Regression analysis of population size and several measures of genetic variation within populations of *S. sinensis* showed that polymorphism and allelic diversity were the most affected by population size. The extent of population differentiation, in terms of private alleles, is also highly correlated with population size. These results indicate that, in addition to the breeding system, genetic drift in small populations of orchids may play an important role in determining the amount of genetic variation within populations and genetic differentiation among populations. As shown in this study, the populations of all three orchids were very divergent. Orchid conservation is hence more challenging than conservation for many other plants, because more population sites may need to be protected in order to maintain the total genetic diversity at the species level for their long-term survival.

Several other studies have shown, however, that the amounts

of allozyme variation and population structure in some outcrossing orchids are largely consistent with the pattern expected for animal-pollinated outcrossing plants with wind-dispersed seeds, such as *Orchis* species (Scacchi, De Angelis, and Lanzara, 1990), *Orchis morio* (Rossi et al., 1992), *Orchis papilionacea* (Arduino et al., 1995), *Cephalanthera longifolia* (Scacchi, De Angelis, and Corbo, 1991), *Cypripedium calceolus* (Case, 1993, 1994), *Spiranthes sinensis* (Sun, 1996), *Epipactis helleborine* (Hollingsworth and Dickson, 1997), and *Spiranthes diluvialis* (Arft and Ranker, 1998). In contrast, inbreeding orchids, such as *Cephalanthera rubra* (Scacchi, De Angelis, and Corbo, 1991) and *Spiranthes hongkongensis* (Sun, 1997), often lack allozyme variation both within and among populations.

As the patterns of genetic structure in some orchids are in accord with theoretical expectations, but are apparently not so in others, more studies are necessary before a general picture can emerge.

Comparison between allozymes and RAPD markers—A recent review (Nybom and Bartish, 2000) has revealed that genetic parameters estimated based on RAPDs show approximately the same association with life history traits (except for geographic range) as previously reported for allozyme studies. However, within-population diversity estimates (mean H_e = 0.214; Nybom and Bartish, 2000) in RAPD-based investigations are almost always higher than allozyme-derived estimates (mean H_e = 0.113; Hamrick and Godt, 1989), whereas RAPD-based estimates of between-population diversity (mean G_{ST} = 0.29) are also considerably higher than the allozyme-based estimates (mean G_{ST} = 0.224) reported by Hamrick and Godt (1989).

In the present study, all three estimates (*p*, *A* and *H*) of within-population genetic variation based on RAPDs are much higher than the allozyme-based estimates for each species, which is in agreement with the findings reported in many other studies obtained with RAPDs and allozymes on the same materials (e.g., Liu and Furnier, 1993; Gasperi et al., 1995; Chan and Sun, 1997; Sun, 1999; Wong and Sun, 1999), as well as with the general findings by Nybom and Bartish (2000). However, the value of RAPDs for the investigation of genetic structuring of populations, which relies on the availability of polymorphic marker loci, is particularly evident. For example, low or lack of allozyme diversity in the two colonizing orchids, *E. sinensis* and *Z. strateumatica*, may lead to faulty conclusions that the populations are genetically uniform or lack differentiation. In contrast, RAPD-based estimates of G_{ST} indicate a high level of population differentiation in both species, which is consistent with the expected population structure for colonizing orchids. Because most of the populations studied are very small in size, founder effects and genetic drift would be expected to result in low genetic variation within populations and high divergence among populations. This is clearly the pattern revealed by RAPDs in *E. sinensis* and *Z. strateumatica*. In addition to the genetic consequences of colonization, the apomictic reproduction of *Z. strateumatica* may also contribute to its extremely high population differentiation (G_{ST} = 0.924) compared with the outcrossing colonizer *E. sinensis*. Much lower G_{ST} values (0.539 based on RAPDs, and 0.333 based on allozymes) were obtained for its outcrossing congener, *Z. gracilis*.

Although it is very difficult to separate the effects of colonization from the effects of breeding system on population structure based on data available from this study, statistical analyses of the effects of life history traits on RAPD-based estimates of genetic parameters (Nybom and Bartish, 2000) have shown that early-successional species have significantly higher between-population diversity (mean G_{ST} = 0.50) than mid- and late-successional species (mean G_{ST} = 0.23 and 0.20, respectively), which is very similar to the effects of breeding system on population differentiation (mean G_{ST} = 0.59, 0.19, and 0.23 for selfing, mixed mating, and outcrossing species, respectively). The same association between these life history traits and G_{ST} estimates have been previously verified with allozyme data (Hamrick and Godt, 1989).

In comparison with the extensive literature on allozyme and/or RAPD analyses of other plant species, the relatively small number of orchid studies indicates more studies are needed before general conclusions can be drawn about the patterns of genetic diversity in wild orchids in relation to their life history traits. As shown in the present study, RAPD markers can be very informative for evaluating population differentiation. The reproducibility of RAPDs has often been questioned, which undoubtedly may hinder its applications in population studies. This problem is largely due to the well-known sensitivity of PCR to reagent and template concentrations, pH, and other reaction parameters. In addition to amplification conditions, the generation of reproducible and comparable RAPD banding patterns is dependent on DNA template quality (Micheli et al., 1994). Some of the problems of RAPD analysis and how these problems can be resolved have been discussed in detail (e.g., Black, 1993; Micheli et al., 1994; Bielawski, Noack, and Pumo, 1995). Careful experimental technique can produce reliable information for orchid research. Other molecular markers, such as cpDNA/mtDNA-RFLPs, ISSRs, AFLPs, and codominant microsatellites, can also be explored for comparative population genetic studies of wild orchids.

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