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Low genetic diversity and high genetic differentiation in the critically endangered *Omphalogramma souliei* (Primulaceae): implications for its conservation

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Abstract *Omphalogramma souliei* Franch. is an endangered perennial herb only distributed in alpine areas of SW China. ISSR markers were applied to determine the genetic variation and genetic structure of 60 individuals of three populations of *O. souliei* in NW Yunnan, China. The genetic diversity at the species level is low with *P*= 42.5% (percentage of polymorphic bands) and *Hsp*=0.1762 (total genetic diversity). However, a high level of genetic differentiation among populations was detected based on different measures (Nei's genetic diversity analysis: *Gst*=0.6038; AMOVA analysis: *Fst*=0.6797). Low level of genetic diversity within populations and significant genetic differentiation among populations might be due to the mixed mating system in which xenogamy predominated and autogamy played an assistant role in *O. souliei*. The genetic drift due to small population size and limited current gene flow also resulted in significant genetic differentiation. The assessment of genetic variation and differentiation of the endangered species provides important information for conservation on a genetic basis. Conservation strategies for this rare endemic species are proposed.

Key words genetic differentiation, genetic diversity, ISSR markers, mating system, *Omphalogramma souliei.*

More and more species have become threatened or extinct in the wild, and plant conservations concerned with rare and endangered species are faced with a daunting task (Holsinger & Gottlieb, 1991). The maintenance of genetic variation is one of the major objectives for conserving endangered and threatened species (Avise & Hamrick, 1996). For the formulation of appropriate management strategies directed towards conservation, knowledge of genetic variation between and within populations provides essential information (Milligan et al., 1994). Breeding system is a key factor that affects the levels of genetic variation in species. An important component of mating is the amount of self-fertilization that a population experienced. Selfing has direct genetic consequences, including its effect on the intensity of inbreeding depression and partitioning of genetic diversity within and among populations (Barrett et al., 2004).

Omphalogramma souliei Franch. is a critically endangered, perennial herbaceous plant and locally endemic to the alpine area in SW China. It grows in alpine meadows and forest margins at altitudes of 2200–4600 m (Hu & Kelso, 1996). According to our survey on this species in the past five years, it is estimated that no more than 2000 individuals survived in the wild. At present, only three populations were found in our five years survey. More populations of this species were recorded according to herbarium specimen survey, however, most of the populations have already disappeared in the last two decades. The threats to the species are related to human activities as well as destruction of its habitats. In addition, both population number and size are declining at an alarming rate in the last decade, and genetic diversity is likely to be reduced. Considering the small population number and size and limited distribution of this locally endemic and rare species, *O. souliei* should be assigned in a high priority for protection.

Inter-simple sequence repeats (ISSRs), recently used widely in population genetic study because of the less investment in time, money and labour than other markers, are highly variable and exhibit Mendelian inheritance (Gupta et al., 1994; Tsumura et al., 1996; Wolfe & Liston, 1998; Harris, 1999). In this study, ISSR markers were used to detect variation at the population level in samples collected from NW Yunnan, China. The purpose of this study is to investigate the levels of ISSR variation and differentiation among *O. souliei* populations.

In this study, we assessed the extent of genetic variation within and between populations of *O. souliei* by ISSR markers. This is part of a project on the conservation genetics of *O. souliei,* involving studies on the mating systems, seed dispersal based on the observed genetic structure. The objectives of our

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research are to (1) fill the important gaps in our knowledge of the amount and distribution pattern of genetic diversity in *O. souliei* in relation to its reproductive biology, seed dispersal mechanisms and ecology, (2) answer the question whether species of *O. souliei* display low level of genetic diversity, given limited population size and number, as most endangered species do; and (3) provide baseline genetic information pertinent to the conservation and restoration of this rare and endemic species.

1 Material and methods

1.1 Plant species

Omphalogramma souliei is a perennial herbaceous plant with a basal rosette of leaves. This species is endemic to SW China, representing the Sino-Himalayan genus *Omphalogramma* Franch. Plants are 50–90 cm tall, with funnelform corolla which is deep red to bluish purple in color, stamens inserting in the middle of the corolla tube and style exserting out of the mouth of the corolla. Flowering time is from May to June. The fruits form in June to July and capsules are ripe in September (Hu & Kelso, 1996).

1.2 Study sites and sampling

Three populations from the northwest of Yunnan province of China were surveyed and leaf tissues of 60 individuals in three *O. souliei* populations were collected (Table 1). Individuals 2–5 m apart from one another were sampled randomly. Leaves were collected in the field and dried in silica gel. These populations grow in the margins of conifer forest dominanted by *Picea* Dietr. and *Tsuga* Carrière, or under *Rhododendron* L. thickets, or alpine meadow mixed with *Fragaria* L., *Lomatogonium* A. Braun, etc., altitude 3300–3500 m. The sampled populations all grow in the marsh. Their habitat represents the richly endowed yet rather stable primitive environment in the sampled regions.

1.3 DNA extraction and ISSR-PCR amplification

Genomic DNA was extracted using a modified CTAB method (Doyle & Doyle, 1987). The DNA was amplified with PCR using ISSR primers from University of British Columbia (UBC). Of 100 ISSR primers, 12 produced clear and reproducible bands, and were selected for the subsequent experiments (Table 2). ISSR-PCR amplifications were performed in GeneAmp PCR System 9700 DNA Thermal Cycler (PerkinElmer, USA) with the cycling programme: 5 min at 94 °C, followed by 37 cycles of 30 s at 94 °C, 45 s at 52 °C, 90 s at 72 °C, and last extention at 72 °C for 7 min. PCR amplification was carried out in a total volume of 25 μL, consisting of 40 ng template DNA, 2.5 μL 10×PCR buffer, 2 μL $MgCl_2$ (25 mmol/L), 1.0 μL dNTPs (2.5 mmol/L), 2% Forma mide, 0.225 μmol/L primer, 1 U of *Taq* polymerase (Takara) and double-distilled water. Amplification products

Table 1 Information of three populations of *Omphalogramma souliei* sampled

Pop. code	Sample size	Location	Position	Altitude (m)	Size
FSL	20	Fenshuiling, between Lijiang and Weixi, Yunnan, China (云南丽江维西分水岭)	27º13′46.9″ N, 99º24′10.0″ E	3370	~20
LDP	20	Lidiping, Weixi, Yunnan, China (云南维西栗地坪)	27º13′25.9″ N, 99º24′36.5″ E	3450	~1000
GHZ	20	Ganhaizi, Lijiang, Yunnan, China (云南丽江甘海子)	27º14′02.3″ N, 99º24′34.3″ E	3480	~100

Table 2 The sequences of ISSR primers and the number of amplified loci on *Omphalogramma souliei*

Primer code	Sequence	No. of loci	No. of polymorphic loci	P
2	$BDB(TCC)_5$	6	0	0
9	$CCC(GT)_6$	8	5	62.5
10	$BDB(CA)_6$	9	3	33.3
14	$GCG(AC)_6$	8	5	62.5
808	$(AG)_8G$	6	2	33.3
811	$(GA)_8G$	7	3	42.9
813	$(CT)_8T$	7	1	14.3
817	$(CA)_8A$	8	6	75.0
818	$(CA)_8G$	6	4	66.7
861	$(ACC)_5$	8	2	25.0
865	$(CCG)_5$	9	6	66.7
889	$DBD(AD)_7$	5	0	0
Total		87	37	42.5

were electrophoretically separated on 1.5% agarose gels with 0.5×TBE buffered. A DNA ladder was applied as a size marker (100–3000). After staining with ethidium bromide for 30 min, DNA fragments were identified by image analysis software for gel documentation. Only those gel that showed consistent and clear bands were considered; while those smeared and weak were excluded.

1.4 Genetic diversity analysis

Because ISSR markers are dominant, it is assumed that each band represents at a single biallelic locus (Williams et al., 1990). Amplified fragments were scored for the presence (1) or absence (0) of homologous bands. The resulting presence/absence data matrix of the ISSR phenotypes was analyzed using POPGENE version 1.31 (Yeh et al., 1999) to estimate the following genetic diversity parameters at the species level: (1) the percentage of polymorphic bands (*P*); (2) Nei's (1972) unbiased expected heterozygosity (*He*), $He=1-\sum P_i^2$, where P_i is the mean frequency of the *i*th ISSR fragment; (3) Shannon's index of phenotypic diversity (*Ho*) (Lewontin, 1972). $Ho=-\sum P_i \log_2 P_i$, where P_i is the frequency of the *i*th ISSR fragment, is frequently used without the need to make assumptions regarding Hardy-Weinberg equilibrium (Fontaine et al., 2004). Shannon's indices were calculated for two levels: the average diversity within population (*Hpop*) and the diversity within species (*Hsp*). Nei's (1972) genetic identity (*I*) and genetic distance (*D*) were calculated for all pairwise combinations of populations.

In addition, an analysis of molecular variance (AMOVA) was applied to estimate variance components for ISSR phenotypes, partitioning the variations among populations and among individuals. The significance of this *F*-statistic analogue was tested with 1000 random permutations. The genetic relatedness among the 60 individuals of three populations was analyzed using Unweighted Pair Group Method with Arithmetic Average (UPGMA) based on pairwise Nei's (1973) genetic distance. Bootstrap analysis using UPGMA search with 1000 replicates was performed to obtain the confidence of the tree.

2 Results

2.1 Genetic diversity

In this study, genetic diversity was examined in *Omphalogramma souliei*, based on ISSR fingerprinting. Twelve primers that produced clear and reproducible bands were selected for further analysis. These 12 selected primers generated totally 87 unambiguous and reproducible electrophoretic bands (loci) that ranged in size from 350 to 2800 bp, with an average of 7.25 bands per primer. Of these bands, 42.53% (37 in total) were polymorphic among 60 individuals. The percentage of polymorphic bands (*PPB*) of this species was 42.53%. The *PPB* varied greatly in single population ranging from 5.75% (LDP) to 26.44% (FSL).

At the population level, very few polymorphism and low diversity were detected for the three populations, with *P*: 26.44%, 5.75%, and 14.94%, *Hpop*: 0.1108, 0.0220, and 0.0775, *He*: 0.0697, 0.0132, and 0.0520 for populations FSL, LDP, and GHZ, respectively. Among the three populations examined in this study, population FSL possessed the smallest size (~20 individuals), but maintained the highest level of variability (*P*: 26.44; *He*: 0.0697; *Hpop*: 0.1108), whereas the population LDP possessed the greatest size (~1000 individuals), maintained the lowest level of genetic variation (*P*: 5.75; *He*: 0.0132; *Hpop*: 0.0220). Although the number of individuals varied from 20 to 1000, genetic diversity is not correlated with the population size (Table 3). At the species level, *O. soulei* possessed high level of genetic variation (*P*: 42.53; *Ht*: 0.1135; *Hsp*: 0.1762).

2.2 Genetic structure

Genetic analysis showed that the highest identity (0.9248) existed between populations FSL and LDP, while the lowest (0.8402) occurred between populations FSL and GHZ. Most genetic variation of *O. souliei* was distributed among populations. The genetic differentiation (*Gst*) among populations was estimated to be 0.6038, which indicated that 60.38% of the genetic variability was distributed among populations, and only 39.62% of the variation existed within populations. The number of migrants (*Nm*) was

Table 3 Genetic variability within populations of *Omphalogramma souliei* revealed by ISSR markers

Population	Percentage of polymorphic loci (*P*)	Nei's diversity (*He*)	Shannon index (*Hpop*)	*Gst*	*Nm*
FSL	26.44	0.0697 (0.1373)	0.1108 (0.2059)		
LDP	5.75	0.0132 (0.0592)	0.0220 (0.0950)		
GHZ	14.94	0.0520 (0.1373)	0.0775 (0.1985)		
Mean	15.71	0.0497	0.0701		
Species level	42.53	0.1135 (0.1698)	0.1762 (0.2490)	0.6038	0.3280

Table 4 Nei's (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal) between populations of *Omphalogramma souliei*

Population	FSL	LDP	GHZ
FSL	****	0.9248	0.8402
LDP	0.0782	****	0.9146
GHZ	0.1741	0.0892	****

estimated as 0.3280 individuals per generation between populations, indicating that there is a low migration rate between populations. Table 4 shows an estimate of Nei's genetic identities (I) and genetic distance (D) for every pairwise comparison between each population pairs. This results show that the highest genetic distance was 0.1741 between populations FSL and GHZ, while the lowest (0.0782) occurred between populations FSL and GHZ. The AMOVA analysis is consistent with the results of Nei's genetic structure that there is high degree of population differentiation (*Fst*=0.6797), where 67.97% variance was partitioned among populations and 32.03% to individual differences within populations. A UPGMA dendrogram (Fig. 1) was reconstructed based on the pairwise genetic distance between 60 individuals of three populations. The dendrogram showed a separation of plants into three groups. The first group (G) consisted of 20 individuals from population GHZ. Three clusters were identified, of which, 20 individuals of each population were further clustered.

3 Discussion

3.1 Genetic diversity

The results of the present study using ISSR markers revealed low level of genetic diversity within populations and remarkable genetic differentiation among populations in *Omphalogramma souliei*. Population genetic diversity in a species is affected by a number of evolutionary factors including mating system, gene flow and seed dispersal, geographic range as well as natural selection (Hamrick & Godt, 1989). Of these factors, the geographic range of a species appears to influence the levels of genetic diversity within populations greatly.

Omphalogramma souliei is a critically endangered perennial herbaceous plant with limited distribution. Generally, species with small geographic range tends to maintain less genetic diversity than that of geographically widespread species (Hamrick & Godt, 1989). Based on this assumption, a low level of genetic diversity within species is expected in *O. souliei*. Shannon indices in the three populations of *O. souliei* ranged from 0.0220 to 0.1108. These values were far lower than the average of dicotyledons (0.191) and short-lived perennial plants (0.207) (Nybom & Bartish, 2000). The levels of genetic diversity of *O. souliei* are much lower than the genetic diversity reported in some wide geographical range species of the genus *Primula,* a closely related genus of *Omphalogramma.* High genetic diversity has been found in many species with a wide geographical range in primulas, e.g., *Primula ovalifolia* Franch. (Nan et al., 2002), *P. obconica* Hance (Nan et al., 2003), and *P. sikkimensis* Hook. (Wang et al., 2005). Genetic polymorphism among the three populations varies from 5.74% to 26.44%, which is much lower than the values obtained for 246–338 dicotyledon species using allozyme markers (*P*: 44.8%, Hamrick & Godt, 1989). Genetic diversities between at the species level and at the population level, such as *He*=0.1135 vs. 0.0497, *Ho*=0.1762 vs. 0.0701, were remarkably different and showed that much more genetic polymorphisms existed within species than within populations. This could be attributed to the breeding system and polyploidy of *Omphalogramma*. The breeding system of this species was self-compatible with facultative xenogamy. Xenogamy predominated and autogamy played an assistant role in the evolution of reproduction and breeding system of *O. souliei* (Huang et al., 2006). Despite of lowest level of autogamy, occurrence of selfing of *O. souliei* reduced the genetic variation within the populations, and increased the genetic variation among the populations. In addition, this species has been reported as an octaploidy (2*n*=96) (Huang & Zhang, 2004). Polyploids generally maintain higher levels of heterozygosity than diploids (Soltis & Soltis, 2000). This may be another reason that much more genetic polymorphisms exist within species in *O. souliei* than expected. Moreover, these small populations were likely subjected to strong genetic drift, especially after long-time isolation from one another. Despite genetic drift resulting in the loss of low frequency alleles in population, the small populations of *O. souliei* could maintain genetic polymorphism by polyploidy.

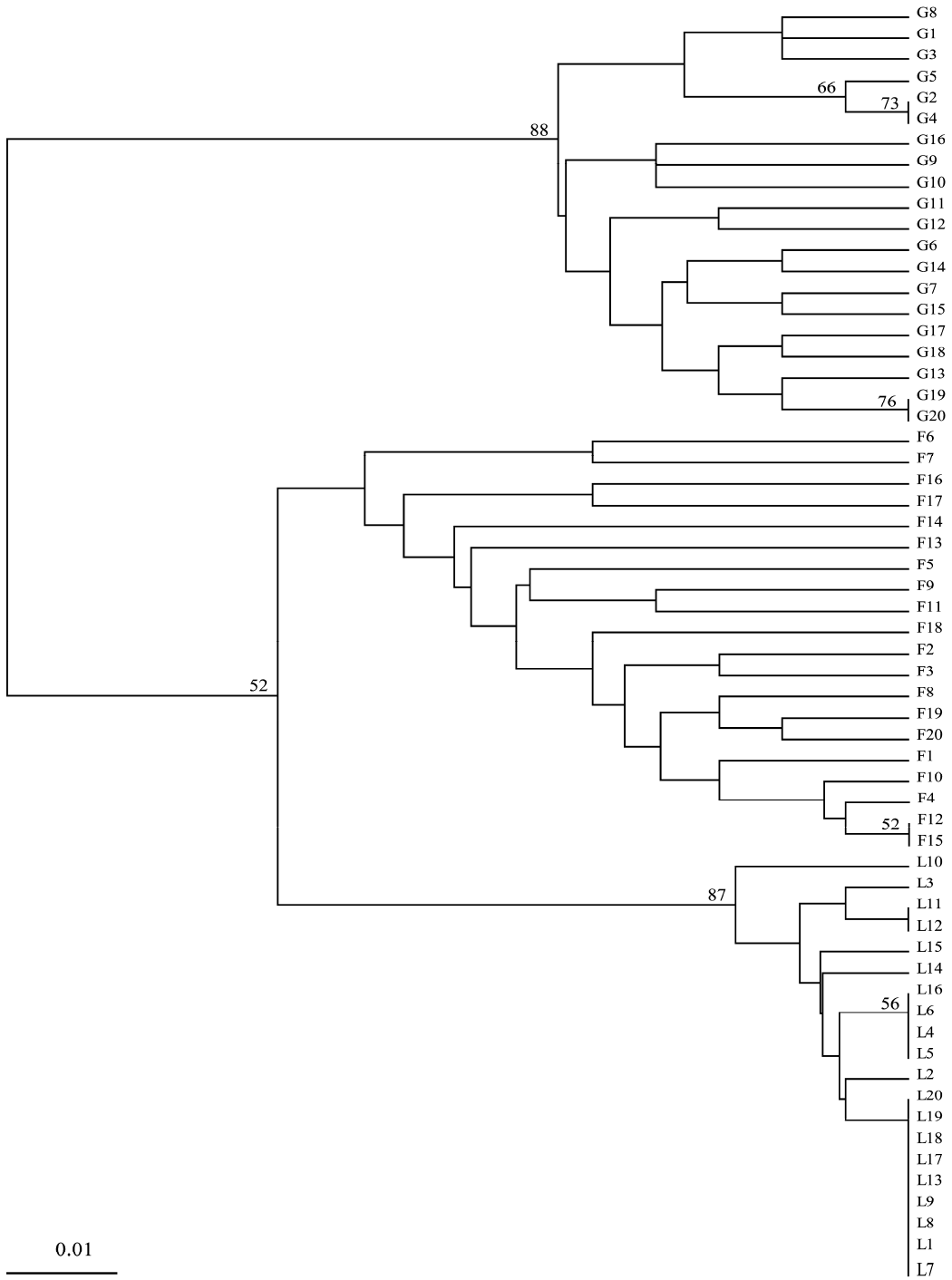

Fig. 1. Dendrogram of 60 individuals of three populations of *Omphalogramma souliei* based on UPGMA analysis of ISSR polymorphisms. Numbers above the lines indicate bootstrap values (percentage of 1000 replicates). Bootstrap values above 50% are shown.

Table 5 AMOVA analysis of 60 individuals of three populations of *Omphalogramma souliei* using ISSR markers

Source of variation	d.f.	Sum of squares	Mean squares	Variance component	Total variance	*P*-value
Among populations	2	178.8667	89.433	4.369	67.97%	<0.0010
Within populations	57	117.3500	2.059	2.059	32.03%	<0.0010

3.2 Genetic structure

Analyses of the ISSR markers using various statistics (Nei's genetic diversity analysis, Shannon's diversity measure and AMOVA) revealed similar patterns of genetic structure of populations of *O. souliei*. The overall degree of genetic differentiation of *O. souliei,* as estimated by *Gst* (0.6038), is much higher than the average of *Gst*=0.19 for plants with mixed breeding system and the average of *Gst*=0.32 for dicotyledons (Nybom & Bartish, 2000; Nybom, 2004). According to an AMOVA analysis, a significant genetically differentiation among populations was estimated (*Fst*=0.6797), where most genetic variation existed among populations.

The genetic structure of plant populations reflects the interaction of various factors, including long-term evolutionary history of the species, genetic drift, mating system, and gene flow (Hogbin & Peakall, 1999). The high level of population differentiation of *O. souliei* may be explained by several factors, such as the breeding system, genetic drift, demographic fluctuations, and the genetic isolation of populations.

Breeding systems of plants greatly affect genetic differentiation, and selfing can result in low genetic diversity within populations. In general, outcrossing and long-lived seed plants maintain most genetic variation within populations, while predominantly selfing, short-lived species harbor comparatively higher variation among populations (Hamrick & Godt, 1989). In this study, most of the genetic variation was portioned among populations. The mixed mating system and the possible selfing of *O. souliei* resulted in high levels of homozygosity within populations.

The low effective gene flow per generation (*Nm,* 0.3280) of *O. souliei,* in comparison with the average values reported for mixed-mating (*Nm*=0.727) and outcrossed animal-pollinated species (*Nm*=1.154), indicated limited gene flow among populations, and may be insufficient to counteract the effect of genetic drift. The habitats of *O. souliei,* separated by major river valleys, streamlet or inter-mountain plateaus, have reduced gene flow among populations. Pollinators were another factor leading to the limited gene flow. As effective pollinators of *O. souliei,* three species of Hymenoptera were lack of ability of long distance flying. Furthermore, within mixed-mating species, seed gravity-dispersal species had higher level of gene differentiation (Hamrick & Godt, 1996; Ge et al., 2005). Fruits in *O. souliei* are capsules with seed number averaging ~200 per fruit and 1–3 fruits produced per individual. Seeds are densely packed in capsules and are relatively big in size (length 2.95–4.15 mm, width 2.95–4.45 mm, weight per 1000 seeds 1.22 g). Dispersal may be mediated primarily by gravity, and are restricted within short distance.

Habitat-restricted species, occurring in isolated populations usually tend to be genetically homogeneous at the population level (Ge et al., 2005; Zhang et al., 2005), this is also backed in this study. Furthermore, the small-size populations of *O. souliei* were likely subject to strong genetic drift. The genetic drift of this species may result in decrease of variation within populations and increase of differentiation among populations.

The pattern of population structure in *O. souliei* has important conservation implication. From the results revealed in this study, the levels of genetic diversity of *O. souliei* have reduced to relatively low. *Omphalogramma souliei* may have a reduced ability for future evolution, unless opportunities arise for the immigration of new allelic diversity into future populations. Loss of genetic variation may reduce the ability to adapt to changing environmental conditions and result in inbreeding depression. It is essential to determine what kinds of conservation programs are appropriate.

In the long term, given that most populations are genetically unique, loss of any population will lead to dramatic loss of genetic variation. Genetic variation has increasingly been recognized as crucial to success in the long-term management of rare and endangered species. Genetic variation should be a central concern for the long-term conservation of populations of *O. souliei.* In the short term, given the extremely limited number of individuals, it is necessary to protect all the existing populations and individuals *in situ* in order to preserve as much genetic variation as possible. Most management practices have been directed toward habitat preservation. When habitats are in immediate danger of destruction, the introduction from the wild should be performed to include as many populations as possible. On the other hand, an appropriate strategy for sampling and propagation could be formulated when *ex situ* conservation is carried out.

4 Conclusion

To conclude, the results in this study reveal low levels of genetic diversity within population and high levels of genetic differentiation among populations in *Omphalogramma souliei*. This genetic pattern might be due to its small population size, mixed mating system and polyploidy. The information of genetic

structure provides important implications for the making of conservation strategies for it.

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