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Full Length Research Paper

Genetic diversity among natural populations of *Ottelia acuminata* (Gaghep.) Dandy revealed by ISSR

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***Ottelia acuminata* (Gagnep.) Dandy, an aquatic species of the Hydrocharitaceae, is endemic to China. A performance comparison of genetic diversity of 4 natural populations was conducted to investigate whether or not water pollution in their habitats has anything to do with this species being endangered. A total number of 120 *O. acuminate* accessions were analyzed, by amplification of their DNAs with 15 primers (ISSR). Thirteen primers were scored and 214 bands were detected, of which 170 were polymorphic (79.44%). The results showed that the genetic indices in polluted Jian Lake group were always the smallest ones, when compared with those of the other groups. It indicated that the polluted water did affect the genetic diversity of *O. acuminate* populations. And ISSRs seemed to be effective tools for detecting genetic variation among *O. acuminate* geographical groups.**

Key words: *Ottelia acuminate*, ISSR, genetic diversity, DNA polymorphism.

INTRODUCTION

Ottelia acuminate (Gaghep.) Dandy, an economically and ecologically important swamp monocot, is mainly distributed in Southwest of China, having been recorded at Yunnan, Hainan, Sichuan, Guizhou and Guangxi Provinces. It has been used as ornamental and edible plants. In recent years, natural populations of *O. acuminate* become more and more rare and it has become one of the endangered species in China. The endangered situation of this endemic species had been mainly attributed to water pollution in their habitats. However, there is little evidence to support that water pollution affects the genetic diversity of *O. acuminate.*

Genetic diversity allows species to adjust to a changing world, which is caused by both natural and human factors. Understanding genetic diversity with the species is essential for establishing effective and efficient conservation and breeding practices (Chaveerach et al., 2006). Inter-simple sequence repeats (ISSRs) is a type of DNA for DNA amplifications (Zietkiewicz et al., 1994; Sánchez et al., 1996). Under conditions of adapted amplifications, the DNA fragments were separated in agarose gel or acrylamide gel (Farès et al., 2009). Revealed polymorphism is primarily of presence/ absence type, as for the RAPD, but corresponds sometimes to differences in lengths of fragment, as for the microsatellites (Sylvain et al., 2000). ISSR is a dominant molecular marker revealed in mass, and has been proposed as a source of genetic markers which overcomes the technical limitations of restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) (Ratnaparkhe et al., 1998). Up to date, ISSRs have been used reliably as molecular markers in genetic studies for various plants such as popcorn (Kantey et al., 1995), rice (Blair et al., 1999), potato (Mc Gregor et al., 2000), coffea (Paulo et al., 2003), *Musa acuminata* (Racharak and Eiadthong, 2007), pistochio tree (Farès et al., 2009).

In this report the ISSR procedure was used to assess the amount of polymorphism detected among natural groups of *O. acuminate* and to investigate whether or not water pollution in their habitats is one of the reasons for this species being endangered.

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Table 1. The origins of *O. acuminate* materials.

Number	Origin	Geographical number	Water condition
01 - 30	Yianyuchang	I	unpolluted
31 - 60	Miaopang	II	unpolluted
61 - 90	Jian Lake	III	polluted
91 - 120	Shuigulou	IV	unpolluted

MATERIALS AND METHODS

Plant materials

One hundred and twenty accessions of *O. acuminate* were obtained from three headwaters of Gemei river and Jian lake in Yunnan province, southwest of China, were used for evaluation in this study. Number and origin of the accession of *O. acuminate* were showed in Table 1.

DNA extraction

DNA was extracted from shoots by the CTAB method (Liu et al., 2009). Samples were ground to powder in liquid nitrogen, using a mortar and pestle. The powder was transferred to a 25 ml sterile Falcon tube with 10 ml of CTAB buffer. The extraction buffer consisted of 2% (w/v) CTAB (cetyltrimethyl ammonium bromide, Sigma), 1.5 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 9.5 and 0.2% (v/v) β-mercaptoethanol. After incubating the homogenate at 65℃ for 1 h an equal volume of chloroform was added and centrifuged at 10,000 rpm for 20 min. DNA was precipitated with 1/10 volume (ml) of 3 M sodium acetate and an equal volume of isopropanol followed by centrifugation at 10,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in TE-buffers (10 mM Tris pH 8.0 and 0.1 mM EDTA). DNA quantity was estimated spectrophotometrically by measuring absorbance at 260 nm. DNA samples were diluted in sterile deionized water and maintained at -20℃.

DNA analysis

ISSR primers were synthesized by Sangon (Sangon, Shanghai, China) according to the sequence reported by Yang et al. (2005). Amplification was performed in volumes of 20 µl containing 2 µl of the 10x buffer and 100 mM each of dNTPs, 0.4 mM primer, 25 ng genomic DNA and 1 unit of polymerase. The reaction mixture was overlaid with 40 µl mineral oil. Amplifications were carried out using a 2400 Perkin-Elmer Thermal Cycles programmed for 40 cycles as follows: 30 s at 94℃, 30 s at 50℃, 1.5 min at 72℃, with an initial melting of 6 min at 94℃ and a final extension of 6 min at 72℃. Amplification products were analyzed by electrophoresis in a 1.5% agarose gel with 1x TAE buffer (0.004 M Tris-acetate and 0.002 M EDTA).

Individual ISSR fragments for each primer-genotype combination were scored as 1 (presence) or 0 (absence) and a note of their sizes was made. The set of fragments co-migrating across the 120 accessions of *O. acuminate* was referred to as a band. The calculations of index of genetic diversity were performed with the POPGEN (Yeh et al. 1999) software package. The calculations of pairwise matrix of percent agreement were performed using STASTICA (version 6.1, StatSoft, Tulsa, OK). AMOVA analysis was performed with the software package Arlequin (Schneider et al., 2000).

RESULTS AND DISCUSSION

Fifteen primers were used in this study, 2 produced amplification products that were too faint to score. Thus 13 out of 15 primers were scored (Table 2). A total of 214 bands were scored from the comparison of amplifications with 13 primers of DNAs from 120 *O. acuminate* accessions, with an average of 16.46 bands scored per primer. Seven to thirty-five bands generated by a single primer of variable lengths were detected. Portions of gels showing typical amplification products were shown in Figures 1 and 2. More than four fifths of the bands were larger than 200 but smaller than 1500 base pairs and less than 10% larger than 2000 base pairs, that is, mostly of small and medium size.

Among all 214 loci detected, 170 of them were polymorphic (79.44%). In different geographical groups, the frequency of polymorphic bands varied from 77.83 to 78.47%; number of loci amplified ranged from 203 to 209; and the range of polymorphic loci number was from 158 to 164 (Table 3). Observed number of alleles (Kimura and Crow, 1964) ranged from 1.8421 to 1.8934; Effective number of alleles varied from 1.7509 to 1.7611; Nei's index (Nei, 1973) was from 0.2599 to 0.2776; and Shannon's index (Lewontin, 1972) was from 0.4013 to 0.4401 (Table 4). It was worth noting that the genetic indices in geographical group of Jian Lake were always the smallest ones, when compared with those of the other groups. It indicated that the polluted water did affect the genetic diversity of *O. acuminate* populations.

In this study, the average number of bands per primer detected over all the 15 primers tested was 11.33 polymorphic and 14.27 totally (invariant plus polymorphic). A pairwise matrix of percent agreement between all the analyzed plants was obtained (data not shown) and between 63.53 and 85.35% of all fragments were shared between plants of the same group. These results suggested the existence of a great number of genetic variations among *O. acuminate* accessions. Existence of high genetic diversity within *O. acuminate* populations may be attributed to cross-pollination. When the genetic variation of the accessions was partitioned by AMOVA, 59.42% of the variation was found among the accessions that had different origins while 40.58% was found among the ones that had the same origins. Both of the variation figures, within and among the origins, were highly significant ($P < 0.001$) (data not shown).

Table 2. List and DNA sequences of the primers used for ISSR analysis, indicating the number of bands observed.

Primer	Sequence	Total number of bands scored	Number of polymorphic bands
A1	TGGATGGATGGATGGATGGA	7	6
A3	GACAGACAGACAGACAGACA	9	6
A7	AGAGAGAGAGAGAGAGAGAGT	12	11
A9	CTCTCTCTCTCTCTCTCTCTG	19	14
A10	CTCTCTCTCTCTCTCTCTCTT	12	12
A13	GTGTGTGTGTGTCC	35	29
A17	GTGGTGGTGGTGGC	14	13
A22	CACACACACACAGT	28	22
U818	5' CACACACACACACACAG 3'	15	13
U825	5' ACACACACACACACACT 3'	21	14
U827	5' ACACACACACACCG 3'	11	7
U841	5' GAGAGAGAGAGAGAGAYC 3'	18	13
U866	5' CTCCTCCTCCTCCTC 3'	13	10
Total		214	170

Y = (C,T)

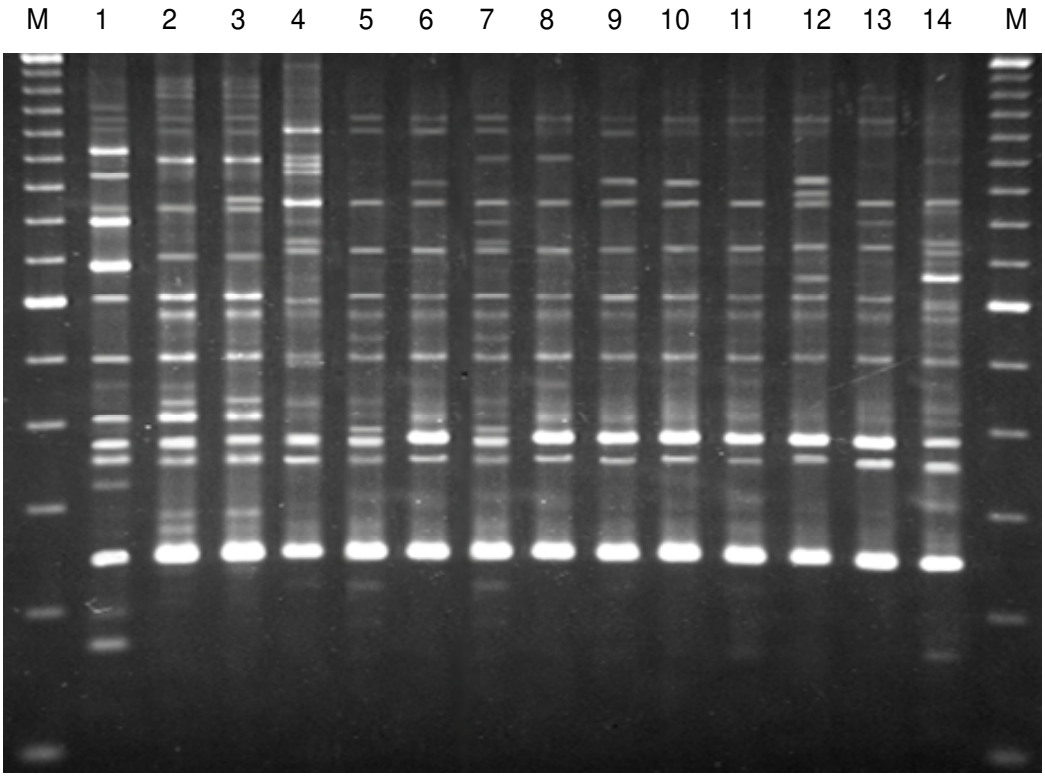

Figure 1. Amplification products of some *O. acuminate* accessions (the primer was A13).

The polymorphic bands were 170 (79.44%) and in average the band polymorphism per genotype was 6.3%. The use of primers selected for faithful reproduction of higher polymorphism in the target group of genotypes could further increase the efficiency and the applications of the ISSR approach. The results showed that A13 and A22 were most suitable for revealing the genetic variance among *O. acuminate* accessions. ISSRs have the advantage that the material is processed by an efficient and inexpensive technique without requiring prior knowledge of the genome. This is particularly relevant for the *O. acuminate*, whose genome analysis data is limited. Our results confirm the ISSR technology as a reliable, rapid and inexpensive screening method to discriminate *O.*

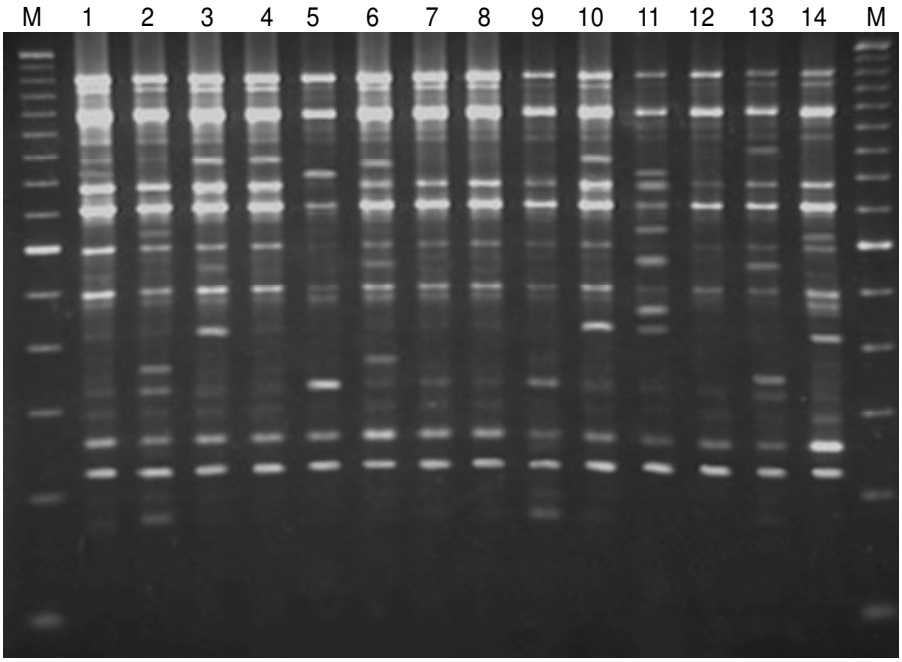

Figure 2. Amplification products of some *O. acuminate* accessions (the primer was A22).

Table 3. Polymoriphic loci among different geographical groups.

No.	Geographical groups	Number of samples	Number of loci	No. of polymorphic	Percent of polymorphic (%)
1-16	Yianyuchang	30	209	164	78.47
17-32	Miaopang	30	208	162	77.88
33-48	Jian Lake	30	203	158	77.83
49-64	Shuigulou	30	205	160	78.05
	Mean	30	206.25	161	78.06
	Total	120	214	170	79.44

Table 4. The genetic diversity among the different geographical groups.

Geographical groups	Yianyuchang	Miaopang	Jian Lake	Shuigulou
Ne*	1.8934	1.8579	1.8421	1.8618
Na*	1.7602	1.7611	1.7509	1.7559
H*	0.2776	0.2640	0.2599	0.2608
I*	0.4401	0.4209	0.4013	0.4204

* Ne = Effective number of alleles [Kimura and Crow (1964)].
* Na = Observed number of alleles.
* H = Nei's (1973) gene diversity.
* I = Shannon's Information index [Lewontin (1972)].

acuminate genotypes.

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