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SHORT COMMUNICATION

Assessment of genetic variation and identification of species-specific ISSR markers in five species of *Cymbidium* (Orchidaceae)

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Abstract Thirty-five inter-simple sequence repeat (ISSR) markers were used to analyze the genetic variation in *Cymbidium* spp. High number of polymorphic bands (217) with overall 90 % of polymorphism at inter-specific level was observed. Cumulative genetic similarity ranged from 0.40–0.93 with an average value of 66 % among the species. At intra-specific level, average polymorphism detected, ranged from 29.8 to 69.9 % within the five species of *Cymbidium*. All the species were apparently endowed with low genetic variation at intra-specific level compared to inter-specific level. UPGMA clustering evidently distinguished the representatives of *C. aloifolium* and *C. tigrinum* which may be linked to entirely different climatic conditions in which they grow, besides their discrete morphological characteristics. Nine ISSR primers revealed 11 unique species-specific banding patterns belonging to three Cymbidiums, which can further developed as SCAR markers. Thus, present investigation provides valuable baseline data of genetic variation in five species of *Cymbidium* and addresses the conservation concerns of this horticulturally important orchid.

Keywords *Cymbidium* · ISSR · UPGMA · Species-specific marker · Genetic variation

Abbreviations

ISSR	Inter Simple Sequence Repeat
PCR	Polymerase Chain Reaction
Kb	Kilobase(s) or 1000 bp
SPAR	Single Primer Amplification Reaction
UPGMA	Unweighted Pair-Group Method with Arithmetic Averages
SCAR	Sequence Characterized Amplified Region

The Orchidaceae is one of the most diverse and largest plant family on the earth, accounting for about 10 % of total biodiversity, comprising more than 25,000 species (Dressler 1993). Being one of the biodiversity hotspots, Northeast India is endowed with rich treasure of phytodiversity including agricultural, horticultural, medicinal and unique floristic plants including various orchids (Sharma et al. 2010a). *Cymbidium*, or boat orchids, have been in vogue, for over 100 years, and remain even today as significant in commerce since they are primarily exploited for their splendid flowers which are attractive, long lasting and large in size. To assess the relationships among various species of *Cymbidium*, the conventional methods such as morphological traits (Jin and Yao 2006), are generally used. Problems associated with variability, plant growth conditions, and individual biases have caused confusion in novel species and/or cultivar identification. Therefore, understanding the genetic base of the available resources and their appropriate characterization to assess diversity is very important for the breeding and improvement programs of certain elite taxa of the genus *Cymbidium*.

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In recent years, genetic diversity and identification of *Cymbidium* cultivars have been attempted through use of different molecular tools, including several markers viz. isozyme (Obara-Okeyo et al. 1998), amplified fragment length polymorphism (AFLP) (Wang et al. 2004), random amplified polymorphic DNA (RAPD) (Obara-Okeyo and Kako 1998; Wang et al. 2004; Choi et al. 2006), EST-SSR (Huang et al. 2010), extended RAPD (ERAPD) (Jian et al. 2010) and single primer amplification reaction (SPAR) methods (Sharma et al. 2011). A promising DNA based marker system, i.e. ISSR (inter simple sequence repeat) which requires flanking sequence information has gained wide applicability in a variety of higher plants. They are known to be abundant, typically neutral, highly reproducible, quite informative extensively polymorphic, and are used as molecular markers with wide-ranging applications (Jarne and Lagoda 1996). Only few reports dealing with analysis of genetic diversity in *Cymbidium* species based on ISSR markers are on hand (Gao and Yang 2006; Xiaohong et al. 2007; Wang et al. 2009), which basically is limited to assess natural variation in an exclusively Chinese species i.e. *C. goeringii.* Such studies for genetic variation analyses are limited in for several Indian representative *Cymbidium* species, which might be due to their threatened/rare status in natural habitat. Therefore, in the present investigation, ISSR fingerprinting is used to determine the natural genetic variation at inter- and intra-specific levels among the 25 collections belonging to five *Cymbidium* species (five collections of each species) from north-east India viz. *Cymbidium aloifolium* (L.) Sw., *C. elegans* Lindl., *C. eburneum* Lindl., *C. mastersii* Griff. ex Lindl. and *C. tigrinum* Parish ex Hook. f., most of them is epiphytic and threatened in their natural habitat. .

The plant materials, belonging to above mentioned five species of *Cymbidium* and comprised of five individuals per species were collected from Sikkim and Meghalaya provinces of north-eastern region of India. For each species, a minimum of five individuals and more than one population were analyzed. DNA was isolated as per standard protocol of Murray and Thompson (1980) with some minor modifications (Sharma et al. 2012). The details of protocols followed for PCR optimization, primer survey, final amplification, electrophoresis, bands scoring and cluster analysis are described elsewhere (Sharma et al. 2010b). PCR reactions were performed in 20 µl final volume which contained 50 ng template DNA, 20 pmol ISSR primer, 200 µM each dNTP, 1.5 mM Mg^{++} , and 1U Taq DNA polymerase (Fermentas Inc.). Reactions were thermal cycled for PCR with 94 °C for 5 min followed by 40 cycles at 94 °C for 40 s, 37–55 °C for 1 min 30 s, and at 72 °C for 2 min, with a final extension at 72 °C for 10 min.

Analysis of inter-specific natural genetic variation and clustering pattern revealed that 35 ISSRs produced 241 amplification products with an average of 6.9 amplicons per primer collectively. Of these, 217 bands were found to be polymorphic in nature with an average of 6.2 bands per primer (Table 1). Percentage of polymorphic bands ranged from 40 % to 100 % with an overall 90 % polymorphism. The UPGMA dendrogram had at least three major clusters marked as I, II, and III with large parenthesis in Fig. 1. Cluster I includes five collections, all of which were representatives of *C. aloifoium* i.e. CA-1 to CA-5. Cluster II is divided into three sub-clusters viz. II1, II2 and II3. Sub-cluster IIa comprises four collections of *C. mastersii viz.* CM-1 to CM-4. The second sub-cluster i.e. IIb comprised of six collections, out of which four representatives of *C. elegans* (CEL-1, 3, 4 and 5 respectively), one collection of *C. mastersii* (CM-5) and one of *C. eburneum* (CEB-2). Sub-cluster IIc comprised of five collections, out of which four were representatives of *C. eburneum* (CEB-1, 3, 4 and 5 respectively) and only one belonged to *C. elegans* i.e CEL-2. The third cluster i.e. III, consists of five representatives of *C. tigrinum viz.* CT-1 to CT-5. Notably, collections CT-2 and CT-3 showed identical value of genetic distance with highest genetic similarity. The interesting fact which lies with both clusters I and III is that it comprises representatives of single species *viz. C. aloifoium* and *C. tigrinum*, respectively, hence showing discrete clustering leading to identification of the different species. Cluster II also showed more or less similar tendency to differentiate the remaining three species viz. *C. elegans, C. mastersii* and *C. eburneum* with moderate genetic similarity. The combined dataset of the entire 35 ISSRs revealed Jaccard's similarity coefficient values which ranged from 0.40 to 0.93 (Table 2) which is precisely supported by 1000 replicate bootstrapping values (Fig. S1).

Similarly, intra-specific natural genetic variation and clustering pattern analysis demonstrated that in *C. aloifolium,* 108 bands were found to be polymorphic in nature with 3.0 average numbers of amplicons per primer with an average 64.2 % polymorphism (Table 2). The Jaccard's similarity coefficient revealed values between 0.66-0.92 with mean similarity of 79 % (Table 2). In *C. elegans,* 172 amplicons were produced, out of which only 97 amplicons (56.3 %) were found to be polymorphic with an average of 2.8 bands per primer. The dendrogram revealed the Jaccard's similarity coefficient values between 0.56-0.85 with mean similarity of 70 % among the five individuals (Table 2). In *C. eburneum,* among 185 amplicons, 116 amplicons were found polymorphic in nature with an average of 3.3 bands per primer and overall 62.7 % polymorphism. Jaccard's similarity coefficient (Jaccard 1901) revealed values between 0.54-0.86 with mean similarity of 70 % among the five individuals (Table 2). *C. mastersii* showed Out of total 163 amplification products, 114 amplicons were polymorphic (69.9 %) with an average of 3.2 bands per primer. Jaccard's similarity coefficient values were between 0.49-0.90 with mean similarity of 69 % among the five

Table 1 ISSR primers used in the present study and the extent of polymorphism along with information about unique banding pattern obtained in specific species

S.NO.	Primer name	Primer sequence	Total bands amplified	Polymorphic bands	Percentage polymorphism	Name of the species showing unique / specific bands (No. of unique bands)
1.	ISSR-03	5′- GTG TGT GTG TGT GG -3′	4	3	75	
2.	ISSR 04	5′-TGT GTG TGT GTG TGT GA-3′	10	10	100	CA (1)
3.	ISSR-05	5′-GAG AGA GAG AGA GAG AT-3′	6	6	100	
4.	ISSR-06	5′-CTC TCT CTC TCT CTC TRA -3′	7	7	100	
5.	ISSR -07	5′-CCG CCG CCG CCG CCG CCG -3′	5	4	80	
6.	ISSR-08	5′-CTT CAC TTC ACT TCA-3′	6	6	100	
7.	ISSR-09	5′-AGA GTT GGT AGC TCT TGA TC -3′	7	7	100	
8.	ISSR-10	5′-ACT TCC CCA CAG GTT AAC ACA-3′	8	8	100	
9.	ISSR-12	5′-CAT GGT GTT CAT CAT TGT TCC A-3′	5	4	80	CT (1)
10.	ISSR-13	5′-CAC ACA CAC ACA AC -3′	4	4	100	
11.	ISSR-14	5′-CAC ACA CAC ACA GG-3′	6	6	100	
12.	ISSR-15	5′- CTC TCT CTC TCT CTC TG -3′	8	7	87.5	
13.	ISSR-17	5′-GAG GAG GAG GC-3′	5	4	80	
14.	ISSR-18	5′-CAC ACA CAC ACA GA-3′	9	9	100	CA (2)
15.	ISSR-20	5′-ACA CAC ACA CAC ACA CAT -3′	7	7	100	CA (1)
16.	ISSR-21	5′-ACA CAC ACA CAC ACA CAG-3′	6	5	83.3	CEL (1)
17.	ISSR-22	5′-ACA CAC ACA CAC ACACTG-3′	6	6	100	
18.	ISSR-23	5′-ACA CAC ACA CAC ACA CAA-3′	4	2	50	
19.	I-07	5′-AGA GAG AGA GAG AGA GT -3′	8	8	100	
20.	I-12	5′-GAG AGA GAG AGA GAG AA -3′	6	6	100	
21.	I-18	5′-CAC ACA CAC ACA CAC AG -3′	7	7	100	
22.	I-25	5′-ACA CAC ACA CAC ACA CT -3′	6	3	50	
23.	I-27	5′- ACA CAC ACA CAC ACA CG -3′	11	11	100	CEL (2)
24.	I-35	5′-AGA GAG AGA GAG AGA GTC -3′	8	8	100	CA (1)
25.	I-40	5′- GAG AGA GAG AGA GAG ACT-3′	5	5	100	
26.	I-817	5′- CAC ACA CAC ACA CAC AA-3′	15	6	40	
27.	I-828	5′-ACA CAC ACA CAC ACA ACG -3′	8	8	100	
28.	I-845	5′- CTC TCT CTC TCT CTC TGG -3′	7	7	100	CA (1)
29.	I-850	5′- GTG TGT GTG TGT GTG TTC-3′	6	5	83.3	
30.	I-852	5′-TCT CTC TCT CTC TCT CAA -3′	8	8	100	
31.	I-853	5′-TCT CTC TCT CTC TCT CAT-3′	5	5	100	
32.	I-857	5′- ACA CAC ACA CAC CAC CCG-3′	6	6	100	
33.	I-17898A	5′- CAC ACA CAC ACA AC -3′	5	5	100	
34.	I-17898B	5′- CAC ACA CAC ACA GT-3′	10	10	100	CT (1)
35.	I-17899A	5′-CAC ACA CAC ACA AG-3′	7	4	57.1	
Total			241	217		
Average			6.9	6.2	90.0	

individuals (Table 2). In *C. tigrinum,* A total of 124 amplicons were produced with 3.5 amplification products per primer. Only 37 amplicons were found polymorphic in nature with an average of 1.0 band per primer and comparatively very low (29.8 %) polymorphism. The dendrogram (Fig. 1) revealed the Jaccard's similarity coefficient values between 0.83-0.93 with mean similarity of 88 % among the five individuals (Table 2). This species showed minimum distance in the form of natural genetic variation among five representatives compared to other four species investigated presently.

In the present investigation, 35 ISSR primers revealed 11 unique species-specific amplicons that are listed in Table 1. Primers viz., ISSR-04, ISSR-18, ISSR-20, I-845 and I-35

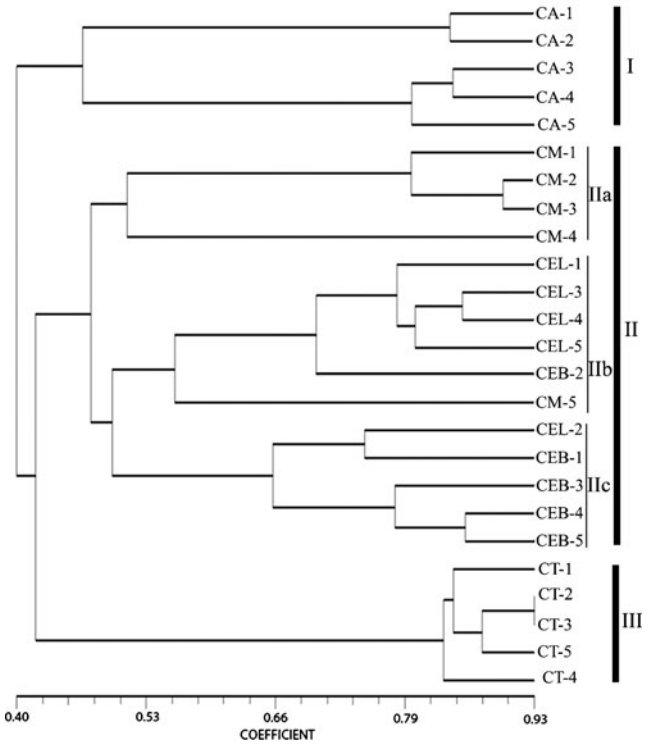

Fig. 1 UPGMA dendrogram of ISSR data in case of the 25 collections belonging to five *Cymbidium* species. The numbers indicated to the right of the tree are individual's collection numbers [*C. aloifolium* (CA-1 to CA-5), *C. elegans* (CEL-1 to CEL-5), *C. eburneum* (CEB-1 to CEB-5), *C. mastersii* (CM-1 to CM-5) and *C. tigrinum* (CT-1 to CT-5)]. The branch lengths are based on the distance values computed using Jaccard's coefficient of NTSYS-pc 2.02 k software. The large parenthesis to the right side labeled with I-III, are the major clusters while the all inner parenthesis showing sub-clusters within the respective cluster

primers produced total 6 unique bands which were specifically amplified in representatives of *C. aloifolium* only and were conspicuously absent in other four species (Table 1). Similarly, primers ISSR-21 and I-27 showed unique banding profile for *C. elegans* whereas primers ISSR-12 and I-17898B demonstrated the unique banding pattern for *C. tigrinum* only. The existence of the species-specific ISSR-PCR markers was confirmed by re-amplification experiments.

The gathering of data on genetic structure/variation of rare species has become a common prelude to conservation planning (Archibald et al. 2001). Very few reports dealing with analysis of genetic diversity in *Cymbidium* species based on ISSR markers are available (Gao and Yang 2006; Xiaohong et al. 2007; Wang et al. 2009). Most of the studies are basically limited to analysis of natural variation in *C. goeringii* from China only. In contrast, Indian representative species did not draw due attention. Therefore, the present study derives merit in terms of analysis of genetic variation at inter- and intra-specific levels in five Asiatic species of *Cymbidium* collected from north-east India. The UPGMA

Table 2 Analysis of natural genetic variation in five *Cymbidium* species at inter- and intra-specific levels

Name of the species	No. of ISSR primer used	Total No. of bands	Average No. of bands/ primer	Size range	No. of Polymorphic bands	Average No. of Polymorphic bands/primer	Average % polymorphism	Jaccard's similarity coefficient	Average similarity
Inter-specific variation									
Cumulative data (CA, CM, CEB, CELE, CT)	35	241	6.9	0.2 kb-5 kb	217	6.2	90.0	0.40-0.93	0.66
Intra-specific variation									
C. aloifolium (CA-1 to CA-5)	35	168	4.8	0.2 kb-5 kb	108	3.0	64.2	0.66-0.92	0.79
C. elegans (CEL-1 to CEL-5)	35	172	4.9	0.2 kb-5 kb	97	2.8	56.3	0.56-0.85	0.70
C. eburneum (CEB-1 to CEB-5)	35	185	5.3	0.2 kb-5 kb	116	3.3	62.7	0.54-0.86	0.70
C. mastersii (CM-1 to CM-5)	35	163	4.6	0.2 kb-5 kb	114	3.2	69.9	0.49-0.90	0.69
C. tigrinum (CT-1 to CT-5)	35	124	3.5	0.2 kb-5 kb	37	1.0	29.8	0.83-0.93	0.88

clustering showed that clusters I and III comprise only representatives of *C. aloifolium* and *C. tigrinum* respectively. The reason for such apparent clustering may be due to entirely different climatic conditions as well as morphological characteristics and serve as congruent ecological, phenotypic as well as genetic evidences of variation. The same observation of phylogenetic delineation of these two species from other cymbidiums has also been emphasized recently by Sharma et al. (2011, 2012). Cluster II comprised of rest of the three species viz. *C. mastersii*, *C. eburneum* and *C. elegans* in the form of three sub-clusters, which grows in sub-tropical climatic condition and more or less threatened in their natural habitat. *C. mastersii and C. eburneum* are the members of section Eburnea of subgenus *Cyperorchis* while *C. elegans* is a member of different section of the same subgenus. In this context, Wang et al (2004) also concluded that *C. mastersii* and *C. eburneum* has low genetic distance with higher similarity using RAPD and AFLP dataset. The observation also get favor from our earlier report (Sharma et al. 2010a) on karyo-morphological studies of *C. mastersii* and *C. eburneum* which revealed symmetrical karyotypes of both the species with only one sub-telocentric/telocentric chromosome pair. The absence of any nucleolar organizer chromosome and/or deviant chromosome number, lack of numerical and structural changes suggested more or less stabilized genome of *C. mastersii C. eburneum* and *C. elegans* (unpublished data). Occurrence of representatives of *C. mastersii*, *C. eburneum* and *C. elegans* in the same cluster i.e. II, may be due to possible heterozygosity of genome reflected at DNA level as detected by all ISSRs. Such observations also supported by studies by (Sharma et al. 2012) who demonstrated the phylogenetic relationships and species inter-relationships in *Cymbidium* species using nrITS sequence data. It also resolved close relationship of *C. elegans* and *C. mastersii* with support of high bootstrap values which further corroborates earlier reports too (Van den Berg et al. 2002).

A salient observation of present analysis is that a total of 11 ISSR amplicons were shown to be species-specific in this study. Of these, most were highly specific to *C. aloifolium* only. These fragments can be used to distinguish the tropical orchid species and their possible hybrids through the development of sequence characterized amplified regions (SCAR) markers as reported earlier in certain orchid genera (Handa 1998; Jin et al. 2010). Intra-specific variation revealed low polymorphism compared to inter-specific level. *C. aloifolium* and *C. tigrinum* followed the same trend of being distinguished at intra-specific level from other three species with high genetic similarity (79 % and 88 % respectively) compared to other species. *C. tigrinum*, a rare and endangered species, showed more or less stable genetic structure with less polymorphism (29.8 %) within the species and it draws support from studies of Xue et al. (2004), who suggested that rare and endangered species are susceptible to loss of genetic variation through genetic drift especially in small populations. The other three species viz. *C. mastersii*, *C. eburneum* and *C. elegans* illustrated the moderate genetic similarity (69 %–70 %) within the species and hence showed higher variation compared to *C. aloifolium* and *C. tigrinum*, may be due to wide spread large population structure. In the present investigation, more or less all species bestowed with low genetic variation at intra-specific compared to inter-specific level, which may be linked to observations of Frankham (1995), who suggested that a large and highly significant excess of endangered species and populations used to have low level of genetic variation compared to related taxa which are abundant with large population size in their natural habitat. Thus, these observations are also in line with the studies of Cuoco and Cronan (2009) who proposed that orchids are threatened and/or endangered with extinction especially in the tropics where small endemic populations exist.

The information obtained through ISSR marker based DNA fingerprinting offers valuable baseline data of genetic variation at inter- and intra-specific levels in five species of *Cymbidium* and address conservation concern for this horticulturally important genus.

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