Isozyme Variation in *Cymbidium* Species (Orchidaceae)

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Abstract. Eight enzyme systems were used to study electrophoretic variability among 12 species of *Cymbidium* Swartz and to assess phylogenetic relationships among them. The species could be easily distinguished by two enzyme systems, malate dehydrogenase (MDH) and phosphoglucose isomerase (GPI), although other enzyme combinations were also diagnostic. Genetic similarity index data indicated considerable genetic variability among the 12 species. Isozyme data supported the current taxonomic placement of the investigated species. The terrestrials [*Cymbidium goeringii* (Rchb. f.) Rchb. f., *Cymbidium ensifolium* (L.) Swartz, and *Cymbidium sinense* (Jackson) Wild.], which are all members of the subgenus *Jensoa* (Rafin.) Seth & Cribb., were the most closely related.

In plants, species relationships have been studied based on morphological, karyological, ecological, and biochemical similarities. From the last point of view, the family Orchidaceae has not been extensively studied. Classification of the genus has been very dynamic since John Lindley (1830–40), who was the first to produce a working classification of Orchidaceae, placed *Cymbidium* in the tribe Vandaeae. This was followed by the work of Schlechter (1924), Dressler and Dodson (1960), and Dressler (1981). The classification by Seth and Cribb (1984), identifying three subgenera, 15 sections, and 53 species, followed later. Du Puy and Cribb (1988) revised the genus and recognized 44 species. Most of these works were based on morphological characters. Such characters, especially of the flowers, are one of the major causes of taxonomic confusion in the genus *Cymbidium* (Du Puy and Cribb, 1988). Whereas morphological evidence (previously the only information available) is important for classification and plant identification, it has been supplemented by new information from extensive anatomical, micromorphological, cytological, biochemical, and, more recently, molecular studies. Orchid taxonomy based on amino acid sequence has been reported by Stewart et al. (1987), and floral fragrance analysis has been proposed as a possible source of useful taxonomic evidence in *Cymbidium* (Du Puy, 1986).

Isozymes are closely related to gene products (Soost and Torres, 1981). Their electrophoretic mobilities are the result of different sizes and shapes of enzyme molecules, and their variation is a good indicator of genetic diversity (Shannon, 1968). Thus, they have been used in plants for various purposes, including taxonomic or genetic relationship studies (Hirai et al., 1986; Isshiki et al., 1992; Rahman and Nito, 1994; Torres et al., 1982).

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Park et al. (1990) reported regional band variations in *C. goeringii*, but no broader isozyme studies have been reported for this genus.

This paper reports data obtained by electrophoretic analyses carried out on eight enzyme systems in 12 species of *Cymbidium*. The aim of this work was to 1) study the variability among the species and identify discriminating markers and 2) assess relationships among 12 species of *Cymbidium* based on enzyme polymorphism, and test the potential of isozymes as taxonomic tools for the genus.

Materials and Methods

Twelve species of *Cymbidium* (Table 1) were used in this study. Healthy mature leaves (third leaf from apex) of greenhouse-grown plants were sampled; preliminary studies had eliminated roots and flowers as possible sample sources due to poor resolution of isozymes and inconsistent staining. Only the stable, repeatable bands in a given species were scored. Enzyme extraction methods were as described earlier (Obara-Okeyo and Kako, 1997). The samples were used immediately or stored at -50°C for later electrophoresis. For most enzymes, storage for up to 7 d did not reduce staining intensities. Phosphoglucose mutase (PGM), however, was always analyzed in freshly prepared samples.

The eight enzyme systems used in this study were resolved on four different gel buffer systems. The first buffer system, a modification of Ashton and Braden's (1961) system, used lithium-borate Tris-citrate, with 0.016 M LiOH and 1.192 M H_3BO_3 (pH 7.4) in the electrode buffer. The gel buffer consisted of 1.6 mM LiOH, 119.2 mM H_3BO_3 , 0.007 M citric acid, and 0.046 M Tris, pH 8.0. This system was used for aspartate aminotransferase (AAT) and phosphoglucose isomerase (PGI). Malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6PDH), and PGM were resolved using the continuous histidine citric acid buffer (pH 5.7) of Cardy et al. (1981). The third system was a Tris citrate buffer (pH 7.0) modified from Meisel and Markert (1967), with 0.05 mM Tris and 0.016 M citric acid in the electrode buffer and 0.017 M Tris and 0.005 M citric acid in the gel buffer. This system was used to resolve alkaline phosphatase (AP). The borate buffer II of Shaw and Prasad (1970) provided good resolution of esterase (EST) and leucine aminopeptidase (LAP).

Staining methods for MDH, PGM, and GPI were those described by Cousineau and Donnelly (1992). Staining of LAP and AAT was done according to Soltis et al. (1983) while the methods of Shaw and Prasad (1970) were employed for EST, AP, and for G6PDH with slight modification (200 mg Na glucose-6-phosphate $\cdot \text{H}_2\text{O}$ was used instead of 160 mg; pH was 7.5 instead of 7.1 for Tris HCl buffer).

Differences in enzyme banding patterns (phenotypes) were recorded as differences in relative mobility, the migration of each band relative to the protein front. According to convention, in systems with multiple zones of activity, the most anodal zone was designated 1, and slower migrating zones were assigned progressively higher numbers.

For statistical analysis, bands across all enzyme systems and entries were coded as present (1) or absent (0). Genetic similarities and distances were calculated between all pairs based on the method of Sneath and Sokal (1973). Relationships between entries were quantified using the complementary similarity indices. A dendrogram was constructed based on the genetic distance matrix data by applying the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis, using the numerical taxonomy and

Table 1. Growth habits, distribution, and present subgenera and section placement² of *Cymbidium* species used.

Species		Growth habit	Distribution	Subgenus	Section
<i>C. floribundum</i>	(5) ³	Semi epiphyte	China	<i>Cymbidium</i>	Floribundum
<i>C. eburneum</i>	(5)	Semi epiphyte	Myanmar, Vietnam, China	<i>Cyperorchis</i>	Eburnea
<i>C. parishii</i>	(3)	Semi epiphyte	Myanmar	<i>Cyperorchis</i>	Eburnea
<i>C. iridiodes</i>	(4)	Semi epiphyte	Nepal, Sri Lanka, China	<i>Cyperorchis</i>	Iridorchis
<i>C. lowianum</i>	(7)	Semi epiphyte	Myanmar, Thailand, China	<i>Cyperorchis</i>	Iridorchis
<i>C. tracyanum</i>	(6)	Semi epiphyte	Thailand, Myanmar, China	<i>Cyperorchis</i>	Iridorchis
<i>C. hookerianum</i>	(6)	Semi epiphyte	Nepal, Sri Lanka, China	<i>Cyperorchis</i>	Iridorchis
<i>C. insigne</i>	(5)	Semi terrestrial	Thailand, Vietnam	<i>Cyperorchis</i>	Iridorchis
<i>C. erythrostylum</i>	(6)	Semi terrestrial	Vietnam	<i>Cyperorchis</i>	Annamaea
<i>C. ensifolium</i>	(5)	Terrestrial	China	<i>Jensoa</i>	Jensoa
<i>C. sinense</i>	(6)	Terrestrial	India, South East Asia, China	<i>Jensoa</i>	Jensoa
<i>C. goeringii</i>	(5)	Terrestrial	China, Japan	<i>Jensoa</i>	Maxillarianthe

²Du Puy and Cribb (1988).

³Numbers in parentheses indicate the number of varieties per species used. Number of plants used per variety ranged from two to five.

multivariate analysis system computer program package (NTSYS-pc, version 1.80).

Results

Variability in banding patterns was observed in eight of the 15 enzyme systems for which stains were used. The enzymes glutamate dehydrogenase (GDH), acid phosphatase (ACP), alcohol dehydrogenase (ADH), isocitrate dehydrogenase (IDH), catalase (CAT), sorbitol dehydrogenase (SDH), and adenylate kinase (AK) yielded activities either too faint or inconsistent to make useful inferences. Most of the eight enzymes had more than one polymorphic zone (Figs. 1 and 2). The number of species phenotypes across all enzyme systems ranged from three (G6PDH) to seven (EST) (Table 2).

Although no single enzyme could distinguish between all 12 species, a combination of two or more enzymes could easily separate them. The polymorphic EST, MDH, GPI, AAT, and PGM were most useful.

Genetic similarity coefficients derived from pairwise comparison among the 12 *Cymbidium* species are summarized in Fig. 3. Taxon-specific isozymes resulted in some dissimilarity among all 12 species; the index value ranged from 0.40 to 0.80 with a mean of 0.61. Moderate similarity indices were recorded for the epiphytes while high indices were recorded for the terrestrials (*C. ensifolium*, *C. goeringii*, and *C. sinense*). The dendrogram from the cluster analysis also showed clear clustering for these three species (Fig. 3); members of the same subgenus and even the same section tended to cluster together. The highest index (0.80) was observed between *C. ensifolium* and *C. sinense*.

Discussion

In the eight enzyme systems studied, polymorphisms were observed at 14 of the 18 activity zones. Esterase (EST) which gave the highest variation, could be used together with either MDH, AP, G6PDH, LAP, or PGM to uniquely identify all the species. A combination of GPI and MDH could in fact unambiguously distinguish all the species. The last two combinations are considered more applicable given the complexity of esterase isozyme patterns. Thus, isozyme markers can serve as taxonomic tools for *Cymbidium* species.

The isozyme variability observed among species of *Cymbidium* (proportion of polymorphic loci = 0.78) is consistent with high levels of biochemical diversity reported in other outcrossing members of Orchidaceae (Rossi et al., 1992; Scacchi et al., 1987).

Cluster analysis showed the terrestrials to be the most closely related of the entries. This suggests a relatively close ancestry between these species which are all classified morphologically and karyotypically under the subgenus *Jensoa* (Du Puy and Cribb, 1988). The highest similarity matrix value was between *C. sinense* and *C. ensifolium*, both members of the section *Jensoa*. The second highest value was between *C. ensifolium* and *C. goeringii*,

Fig. 1. Schematic illustration of leucine aminopeptidase (LAP), esterase (EST), and phosphoglucose isomerase (GPI) isozyme banding patterns in *Cymbidium* for (A) LAP, EST, and GPI, and (B) phosphoglucose mutase (PGM), alkaline phosphatase (AP), glucose-6-phosphate dehydrogenase (G6PDH), and aspartate aminotransferase (AAT). The letters correspond to those in Table 2.

Fig. 2. Photograph (A) and schematic illustration (B) of malate dehydrogenase (MDH) isozymes in *Cymbidium*. The letters correspond to those in Table 2.

Table 2. Isozyme phenotypes of *Cymbidium* species.^z

Species	Enzyme ^y							
	LAP	EST	GPI	PGM	AP	G6PDH	MDH	AAT
<i>C. floribundum</i>	D	A	A	C	C	C	D	A
<i>C. eburneum</i>	A	A	A	A	B	A	E	E
<i>C. parishii</i>	A	E	A	A	C	C	C	C
<i>C. iridiodes</i>	E	B	A	D	A	B	F	C
<i>C. lowianum</i>	A	D	C	C	A	B	D	B
<i>C. tracyanum</i>	B	F	D	F	E	C	F	A
<i>C. insigne</i>	C	G	D	B	D	C	B	C
<i>C. hookerianum</i>	F	C	B	B	B	C	E	A
<i>C. erythrostylum</i>	D	D	C	A	A	C	C	A
<i>C. ensifolium</i>	G	H	C	E	B	B	A	D
<i>C. sinense</i>	B	H	A	E	E	A	A	D
<i>C. goeringii</i>	C	H	D	E	E	B	A	D

^zSee Figs. 1 and 2 for phenotypes A–H of different enzymes.

^yLAP = leucine aminopeptidase; EST = esterase; GPI = phosphoglucose isomerase; PGM = phosphoglucose mutase; AP = alkaline phosphatase; G6PDH = glucose-6-phosphate dehydrogenase; MDH = malate dehydrogenase; AAT = aspartate aminotransferase.

which fall under the section maxillarianthe. The fact that isozymes could clearly differentiate between the three terrestrials and the other genotypes supports the observation (Du Puy and Cribb, 1988) that the *Jensoa* differ considerably from the rest of the genotypes examined in this experiment. With the exception of *C. floribundum* Lindl., which is currently morphologically and karyotypically grouped under the subgenus *Cymbidium*, the rest are all members of the subgenus *Cyperorchis* (Bl.) Seth and Cribb. Cluster analysis could not clearly distinguish *C. floribundum* from this group even though it is currently classified under the subgenus *Cymbidium*. This may be due to the rather crude nature of the UPGMA method, which sometimes lead to loss of inferior differences during the clustering procedure by arithmetic means. Isozymes clustered *C. eburneum* Lindl. and *C. parishii* Rchb. f. (both members of the section *Eburnea*) together, while *C. erythrostylum* Rolfe (the only member of section *Annamaea*) was clustered independently, drawing a parallel with the present taxonomic ranking.

This study has shown that isozyme techniques can be used to discriminate among *Cymbidium* species and to investigate relationships among them. We conclude that enzyme electrophoresis is a useful tool for the analysis of taxonomic problems in *Cymbidium*. In this respect, isozymes should be used to complement conventional morphological markers in an effort to resolve any ambiguities that may arise from use of the latter. *Cymbidium* hybrids are currently the orchids most cultivated. Most of these are derived from a few species, typically the epiphytes traditionally admired for their large flowers. However, scented miniature cymbidiums are increasingly being used in breeding programs. Knowledge of genetic variations can be helpful in choosing parents in breeding programs, and isozymes can be used to identify hybrids from interspecific crosses. Ability to discriminate between the species can also be useful in germplasm collections in cases where morphological distinctions are not obvious at an early stage, as is the case with most of the species.

Fig. 3. Dendrogram for 12 *Cymbidium* species based on unweighted pair group method with arithmetic averages cluster analysis of genetic distances derived from isozyme marker scores.

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