Pak. J. Bot., 45(2): 519-523, 2013.

MOLECULAR ASSESSMENT FOR GENETIC IDENTIFICATION AND STABILITY OF *CYMBIDIUM SANDERAE* (ORCHIDACEAE)

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Abstract

In order to verify a natural hybrid or a wild type species of *Cymbidium sanderae*, the putative parents and four more species were collected for genetic stability assessment by molecular data. Dendrogram constructed from 13 Inter Simple Sequence Repeat (ISSR) profiles distinguished each species with genetic distance (D) values of different species at similar level. The D values of *C. sanderae* compared to the expected parents, *C. lowianum*, *C. insigne*, and *C. eberneum* are 0.500, 0.501, and 0.470, respectively. While those compared to other species, *C. haematodes*, *C. ensifolium*, *C. sinense*, and *C. munronianum* are 0.501, 0.490, 0.496, and 0.485, respectively. Additionally, overall D values between other samples range from 0.414 (*C. ensifolium* and *C. sinense*) to 0.547 (*C. insigne* and *C. haematodes*). Regarding the D values mentioned, they appear to be at the same or similar levels. Therefore, *C. sanderae* is a species just as the other studied species are, and this means that it should be classified as a wild type species.

Introduction

Molecular markers have been used to support morphological data to resolve complex taxonomic issues. The genus *Cymbidium* Sw. was established in 1799 by Peter Olof Swartz, a Swedish botanist (Du Puy & Cribb, 2007). This orchid genus is represented by 55 species and has a distribution range from the Northwestern Himalayas to Japan and in South through Indochina, Papua, New Guinea, and Australia (Yukawa, 2002). The center of species diversity of the genus is in China with 44 species, 19 of which are endemic to China (Liu *et al*., 2009). In Thailand, 20 wild type species have been discovered. *Cymbidium* Sw. is one of the most popularly cultivated orchids used in the cut-flower trade and for potted plants because of its appropriate characteristics such as, leaf shape, colors, large sized and long-lasting blooms as well as the texture of the flowers (Kaenratana, 2009). Consequently, many wild types of *Cymbidium* were collected more than 2500 years ago in China from their natural locations to cultivate as the core species for hybridization (Hegde, 1999).

Nowadays, there is a species that has caused confusion among botanists. Is *C. sanderae* (Rolfe) Du Puy & P.J. Cribb a wild type or natural hybrid species? This species was first discovered by Wilhelm Micholitz in 1904 on the Lang Bian Plateau of Southern Vietnam and was sent to Sander's Nursery where it was given the name *C. sanderae*. It was thought to have been lost from cultivation until 1961, Emma Menninger discovered a specimen in the nursery of Armacost and Royston. Later Don Wimber successfully converted the clone to a tetraploid and it was named *C. parishii* 'Emma Menninger' 4n. Currently, when cymbidium growers mention *C. parishii*, they usually mean *C. sanderae*. *C. sanderae* has been debated and is suspected to be a natural hybrid of *C. eberneum* as the core species with a composition of *C. lowianum* and *C. insigne* from its leaf shape and flower characters (Kaenratana, 2009).

Hybridization is the process in which two genetically distinct species breed and produce an offspring that is commonly known as a hybrid. This phenomenon has been observed in nature in many instances and with a variety of plants and animals. Some scientists contend that natural hybridization has lead to the production of relatively fit hybrids that possess novel genetic variation or have found new evolutionary lineages (Arnold & Hodges, 1995). Additionally, studies have demonstrated that some hybrid species are more fit than either of the parental species. This increased fitness, due to the hybrid's mixed genome complexes, may allow hybrids to develop new niches and adapt more readily to change (Dobzhansky, 1970). Conversely, others argue that a majority of hybrids are less fit than their parental species because they are sterile and, as a result, are unable to propagate their genetic information into future generations.

Genetic analysis by molecular data leads to an invaluable knowledge which is profitable for identification, conservation, sustainable uses, and for breeding, etc. (Shinwari & Shinwari, 2010; Zeb *et al*., 2011). Recently, the development of molecular technology has provided new tools for the detection of genetic alteration in response to plant treatment by directly examining the level of DNA sequence and structure (Shinwari *et al*., 1994, 1994a; Banaras *et al*., 2012; Hosseini *et al*., 2012). Various molecular approaches called DNA markers, for example, DNA fingerprinting based on Polymerase Chain Reaction (PCR), are essential to precisely identifying plant parts without flowers. The fingerprints from a method such as Inter Simple Sequence Repeat (ISSR) have proven to be extremely variable and sensitive enough to differentiate cultivars and natural populations (Wolfe, 1998). These markers for genetic variation are generally independent of environmental factors and are more numerous than phenotypic characteristics. As a result, they provide a clearer indication of the underlying variations in the genome. Also, DNA fingerprinting methods are generally used to effectively indicate genetic relationships by cladogram construction. The concept critical to cladistics is homology which can be defined as a similarity resulting

from common ancestry. Therefore, the cladogram designed depicts not only similarity, but also depicts evolutionary relatedness (Simpson, 2006). All operational taxonomic units (OTUs) that have similarities between 85% and 100% might be recognized as part of the same species, while a criterion of 65% might be used for the genus level. However, the ultimate interpretation is dependent upon the researchers' knowledge of the included studied species (Weier *et al.*, 1982).

The present research aims to assess genetic identification or stability of *C. sanderae* and to determine whether it is a wild type or natural hybrid using the ISSR technique.

Materials and Methods

Plant materials: The plant samples, *Cymbidium lowianum* Rchb.f., *C. eberneum* Lindl., *C. insigne* Rolfe, *C. sinense* (Jacks) Willd., *C. ensifolium* (L.) Sw., and *C. haematodes* Lindl. were collected from their type locality and were grown in a nursery in Nonthaburi Province, Central Thailand, while specimen of *C. sanderae* (Rolfe) Du Puy & P.J. Cribb was collected from United States of America; *C. munronianum* King & Pantl. was collected from The Democratic Republic of Vietnam (DRV, known as North Vietnam), they were grown in the same nursery along with the outgroup, *Grammatophyllum* sp. Young fleshy leaves of each species were transferred to the laboratory at the Faculty of Science, Khon Kaen University and were stored at -70°C until the DNA extraction was performed.

DNA extraction: Total genomic DNA was extracted using the Plant Genomic DNA Extraction Kit (RBC Bioscience). Extracted DNA was examined by subjecting it to 0.8% agarose gel electrophoresis stained with ethidium bromide. The quality and quantity of DNA were determined by a gel documenting instrument. Then, DNA samples were diluted to a final concentration of 20 ng/µl, and these dilutions were used as DNA templates in the PCR reactions.

DNA fingerprinting by ISSR marker: Amplifications were carried out on the sample species each in 25 µl reactions consisting of GoTaq Green Master mix (Promega), 0.5 µM primer and 20 ng DNA template. Nineteen of ISSR primers were screened and the 13 primers that successfully amplified clear bands are as follows (5′ to 3′): $(CA)_6GG$, $(GA)_6GG$, $(GT)_6GG$, $(GA)_6CC$, $(GT)_6CC$, $(GAG)_3GC$, $(GTG)_3GC$, $(AG)_8G$, $(AG)_8C$, $(AG)_8T$, $(CA)_8CC$, $(CA)_9A$, $(CA)_9T$. The reaction mixture was incubated at 94°C for 3 min and the amplification was performed with the following thermal cycles: 35 cycles of 1 minute denaturation at 94°C, 2 minutes annealing at Tm+5°C and 2 minutes extension at 72°C; followed by 7 minutes final extension at 72°C using a thermal cycler (Swift™ Maxi Thermal Cycler, Esco Pte. Ltd.).

Fingerprint analysis and dendrogram construction: Amplification products were resolved by 1.2% agarose gel electrophoresis in TAE buffer, visualized by ethidium bromide staining and photographed under UV light exposure. Gel images from the successfully amplified ISSR primers were analyzed and the dendrogram was constructed using GelQuest DNA Fingerprint Analysis Software (http://www.sequentix.de/gelquest/index.php)

Results

Color photos of all plants studied in the group are shown in Fig. 1. Thirteen ISSR primers were successfully amplified DNA fragments. There are 764 total bands ranging in size from 250 bp to 2500 bp (Fig. 2). These bands were used for dendrogram construction. The dendrogram taken shows the power efficiency by successfully dividing the samples into three major groups which are separated out by an outgroup (Fig. 3). The first group contains only one, *C. lowianum*; the second group consists of *C. haematodes*, *C. ensifolium*, *C. sinense*, and *C. munronianum*; while the third consists of *C. sanderae*, *C. eberneum*, and *C. insigne*.

The genetic distance (D) taken concurrently with dendrogram reveals that the D values of *C. sanderae* paired with the other studied species such as *C. lowianum*, *C. insigne*, and *C. eberneum* are 0.500, 0.501, and 0.470, respectively (Table 1). While the D values of *C. sanderae* paired with *C. haematodes*, *C. ensifolium*, *C. sinense*, and *C. munronianum* are 0.501, 0.490, 0.496, and 0.485, respectively.

In this manner, the D values of other pairs of the studied species range from 0.414 (*C. ensifolium* and *C. sinense*) to 0.547 (*C. insigne* and *C. haematodes*).

Discussion

Scientists are working to find DNA barcodes to identify complex species (Shinwari, 1995), for this purpose various candidate genes are being investigated (Shinwari, 1998). However, in the present study, ISSR data is powerful tool to discriminate the *Cymbidium* species that includes *C. sanderae* which is still being debated as to whether it is a wild type or a natural hybrid. It is suspected to be a natural hybrid of *C. eberneum* as the core species with a composition of *C. lowianum* and *C. insigne*. The causes as seedlings from a selfing of the plant showed great variations including some that produced long, arching spikes of greenish flower similar to *C. lowianum*, whereas others had short spikes bearing a few whitish flowers similar to *C. ebernium*. Additionally, since *C. sanderae* is found in nurseries only, there has been no hard proof of its rediscovery from its natural locality at Lang Bian Plateau in Southern Vietnam (Kaenratana, 2009). Thirteen ISSR primers exhibited polymorphism characters enough to solve the problem mentioned. The dendrogram constructed from these banding patterns distinguish each species studied with the D of the similar level of the different species. The D values of *C. sanderae* compared to the putative parents, *C. lowianum*, *C. insigne* and *C. eberneum* are 0.500, 0.501 and 0.470, respectively. While the D values among pairs of *C. sanderae* the other species which are not its parents such as *C. haematodes*, *C. ensifolium*, *C. sinense* and *C. munronianum* are 0.501, 0.490, 0.496 and 0.485, respectively. The D values of all studied species also ranged from 0.414 in the pairing of *C. ensifolium* and *C. sinense* to 0.547 in the pairing of *C. insigne* and *C. haematodes*. In the D values mentioned, they seem to be at the same or similar levels of about 0.4–0.5. Therefore, *C. sanderae* should be a species just as the other studied

species are. Although the D, which is inverse S is not in the S of species level proposed by Weier *et al.* (1982), all of the species in the group are a monophyletic group from which *Cymbidium* is separated out from the outgroup, the genus *Grammatophyllum*. From the great genetic variations in *C. sanderae* and the genetic variation levels in the *Cymbidium* group, it can be assumed that the *Cymbidium* species has high genetic diversity and/or a high genetic variation within the group affected by humans during planting. Additionally, multiple alleles may be the factor affecting genetics. Therefore, descendants and hybrids will receive shared characteristics from the *Cymbidium* group. From this information it can be suggested that *C. sanderae* can be declared a wild species, and should not be classified as a natural hybrid. Its genomic DNA banding pattern showed the same highly distance level as the other *Cymbidium* samples, and this fact indicates the genetic stability of it as a species.

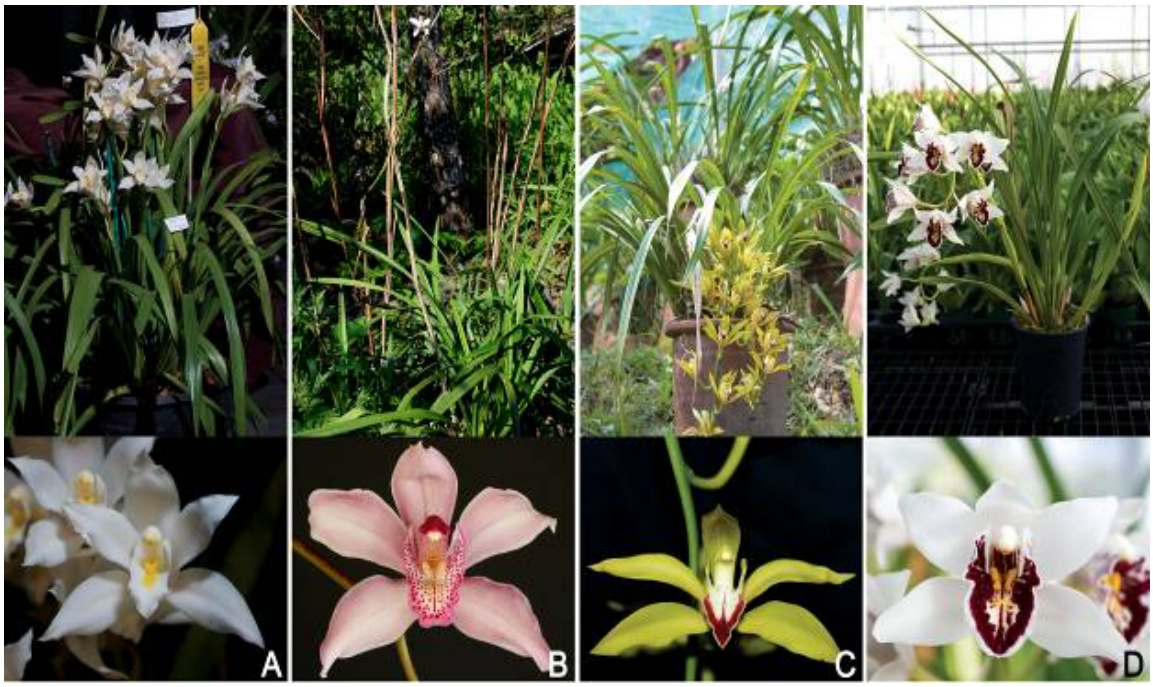

Fig. 1. All ingroup of *Cymbidium* species including *C. eburneum* (A), *C. insigne* (B), *C. lowianum* (C), and *C. sanderae* (D). Photographs by Mr. Randall Robinson.

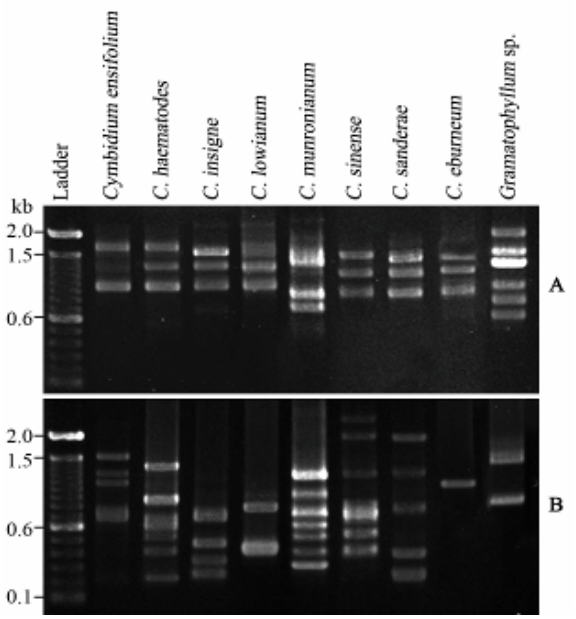

Fig. 2. Samples of ISSR banding patterns from primers $(GT)_6GG$ (A) and $(CA)_9A$ (B).

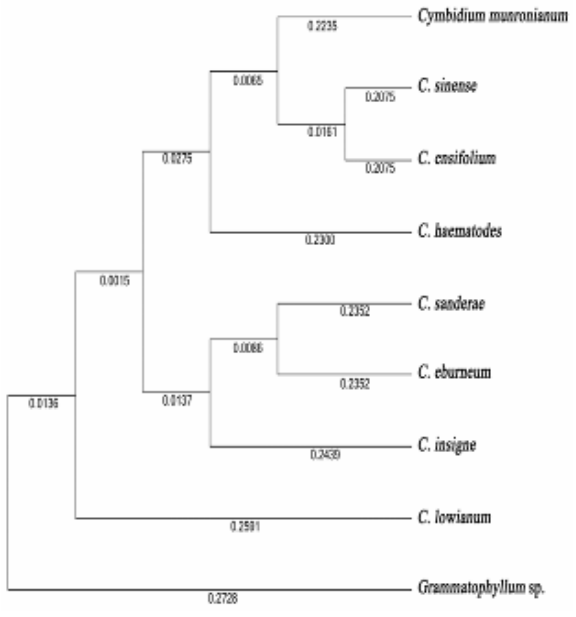

Fig. 3. Dendrogram constructed from 13 ISSR banding profiles of the eight *Cymbidium* species by GelQuest. Numbers on branches indicate branch length.

Table 1. Genetic distances of *Cymbidium* species and the outgroup.

	C. munronianum	*C. sinense*	*C. haematodes*	*C. ensifolium*	*C. sanderae*	*C. lowianum*	*C. insigne*	*C. eburneum*	*Grammatophyllum* sp.
Cymbidium munronianum	0.000								
C. sinense	0.436	0.000							
C. haematodes	0.470	0.469	0.000						
C. ensifolium	0.458	0.415	0.441	0.000					
C. sanderae	0.486	0.496	0.502	0.490	0.000				
C. lowianum	0.502	0.518	0.524	0.527	0.500	0.000			
C. insigne	0.509	0.528	0.548	0.528	0.502	0.521	0.000		
C. eburneum	0.508	0.518	0.532	0.506	0.470	0.536	0.474	0.000	
Grammatophyllum sp.	0.563	0.550	0.564	0.542	0.539	0.532	0.550	0.524	0.000

Alternatively, it is possible that a very long time ago *C. sanderae* was a natural hybrid species. From generation to generation the plant may have enhanced and adapted its morphology and its genes to become more consistent with the environment in its location. This is one of the rules of plants evolution. Presently, it is a wild species with a high genetic diversity by genetic variations which have been caused by changes in the environment and by the effects of humans. This information will be useful for cross production of *Cymbidium* species. Since they are ornamental plants that are economically profitable when sold as potted plants and as cut flowers, there is a need to know much about how characteristics are derived from parents.

The results were in agreement with Van Den Berg *et al*., (2002) who conducted a study of molecular phylogeny by nuclear ribosomal spacer (ITS) DNA and plastid *Mat*K data of 34 *Cymbidium* accession from GenBank. They determined that *C. sanderae* was closely related to *C. insigne*, *C. lowianum* was closely related to *C. hookerianum*, and *C. eberneum* was closely related to *C. whiteae*. Yukawa *et al*., (2002) did a study of molecular phylogeny and character evolution of 37 *Cymbidium* taxa using ITS and *mat*K analysis. They found that *C. eberneum* was closely related to *C. roseum* and that *C. lowianum* was closely related to *C. iridioides.* Additionally, *C. insigne* was grouped in the same cluster with *C. cochleare*, *C. elegans*, and *C. erythraeum*, while *C. sanderae* was excluded from the otherrs.

However, the results of the research give important information to support that *C. sanderae* is a wild type species, but up until now the plant cannot be found in any natural locality and it has not been rediscovered since the first exploration at Lang Bian Plateau in Southern Vietnam (Kaenratana, 2009).

Since all *Cymbidium* species are economical, ornamental plants and can be sold both as potted plants and as cut flowers, they, therefore, need specific markers for further rapid, automatable, and accurate species identification, especially for the immature plants that are massively grown on orchid farms. The DNA barcoding that uses standard sequences and becomes its species tag has served the purpose; tag comparison is required whenever applicable. The ultimate interpretation is dependent upon the researchers' knowledge of the included sequences. There are, therefore, disadvantages which stem from the expense and time-consuming nature of having several individuals perform the sequencing. So, the authors performed each barcode of each 19 *Cymbidium* species with four standard regions of *rpo*C1, *rpo*B, *mat*K and *psb*A-*trn*H intergenic spacer. The tag sequences are kept at GenBank database with GenBank accession numbers (Siripiyasing *et al.*, 2011).

It is possible to use small sample sizes in molecular studies as quoted by Hillis (1987). The sizes in molecular studies are usually much smaller than in morphological studies (often as small as a single individual) because the analyses of large sample sizes are often limited by the availability of specimens and /or the expense of the analysis. However, the studied samples were randomly collected which has led to having realistic results. Morphological characteristics and expression characters are a subset of genomic study. Additionally, there is actually limited variation at the intraspecific and interspecific levels which follows the Weier *et al*., (1982) proposal.

Acknowledgements

The authors are grateful to Mr. Randall Robinson for his beautiful *Cymbidium* photos.

References

Arnold, M.L. and S.A. Hodges. 1995. Are natural hybrids fit or unfit relative to their parents. *Trends Ecol. Evol.*, 10: 67-71.

Banaras, S., S. Aman, M. Zafar, M. Khan, S. Abbas, Z.K. Shinwari and S.N. Shakeel. 2012. Molecular identification and comparative analysis of novel 18s ribosomal RNA genomic sequences of a wide range of medicinal plants. *Pak. J. Bot*., 44: 2021-2026.

Dobzhansky, T. 1970. Genetics of the Evolutionary Process. Columbia University Press, New York.

Du Puy, D. and P.J. Cribb. 2007. *The Genus Cymbidium*. Kew Publishing, Kew.

Hegde, S.N. 1999. *Cymbidium Cultivation Technique and Trade*. State Forest Research Institute, Department of Environment & Forest, Government of Arunachal Pradesh, Itanagar.

Hillis, D.M. 1987. Molecular versus morphological approaches to systematics. *Annu. Rev. Ecol. Syst.*, 18: 23-42.

Hosseini, S., R. Go, K. Dadkahah and A.A. Nuruddin. 2012. Studies on maturase K sequences and systematic classification of *Bulbophyllum* in Peninsular Malaysia. *Pak. J. Bot.*, 44: 2047-2054.

Kaenratana, K. 2009. *Heat Tolerant Cymbidium*. Amarin Printing and Publishing, Bangkok.

Liu, Z., X. Chen, S. Chen and P.J. Cribb. 2009. *Cymbidium*. In: *Flora of China Volume 25 (Orchidaceae)*. (Eds.): Z.Y. Wu, P.H. Raven and D.Y. Hong. Science Press, Beijing and Missouri Botanical Garden Press, St. Louis, pp. 260-280.

Shinwari, Z.K. 1995. Congruence between morphology and molecular phylogeneties in *Prosartes* (Liliaceae). *Pak. J. Bot.*, 27: 361-369.

Shinwari, Z.K. 1998. Molecular systematics of the genus *Uvularia* and related taxa based upon *rbc*L gene sequence data. *Pak. J. Bot.*, 30: 161-172.

Shinwari, Z.K. and S. Shinwari. 2010. Molecular data and phylogeny of Family Smilacaceae. *Pak. J. Bot.*, Special Issue (S.I. Ali Festschrift) 42: 111-116.

Shinwari, Z.K., R. Terauchi and S. Kawano. 1994. Phylogenetic relationships among genera in the Liliaceae-Asparagoideae-Polygonatae sensu lato inferred from *rbc*L gene sequence data. *Pl. Syst. Evol.*, 192: 263-277.

Shinwari, Z.K., R. Terauchi, F.H. Utech and S. Kawano. 1994a. Recognition of the new world *Disporum* section *Prosartes* as *Prosartes* (Liliaceae) based on the sequence data of the *rbc*L gene. *Taxon*, 43: 353-366.

Simpson, M.G. 2006. *Plant Systematics*. Elsevier Academic Press, California.

Siripiyasing, P., K. Kaenratana, P. Mokkamul, T. Tanee, R. Sudmoon and A. Chaveerach. 2011. Species diversity and molecular markers as barcodes of the *Cymbidium* species (Orchidaceae) in Thailand. *Afr. J. Agric. Res*., 7: 393-400.

Van den Berg, C., A. Ryan, P.J. Cribb and M.W. Chase. 2002. Molecular phylogenetics of *Cymbidium* (Orchidaceae: Maxillarieae): sequence data from internal transcribed spacers (ITS) of nuclear ribosomal DNA and plastid *mat*K. *Lindleyana*, 17: 102-111.

Weier, T.E., C.R. Stocking, M.G. Barbour and T.L. Rost. 1982. *Botany: An Introduction to Plant Biology*. (6th ED) John Wiley & Sons, New York.

Wolfe, A.D., Q.Y. Xiang and S.R. Kephart. 1998. Diploid hybrid speciation in *Penstemon* (Scrophulariaceae). *Proc. Natl. Acad. Sci. USA.*, 94: 5112-5115.

Yukawa, T., K. Miyoshi and J. Yokoyama. 2002. Molecular phylogeny and character evolution of *Cymbidium* (Orchidaceae). *Bull. Natl. Sci. Mus.*, 28: 129-139.

Zeb A., Z.K. Shinwari and T. Mahmood. 2011. Molecular markers assisted genetic characterization of some selected wild Poaceae species. *Pak. J. Bot.*, 43: 2285-2288.

(Received for publication 9 June 2011)