DIVERSITY ANALYSIS AMONG FEW *CUCURBITACEAE* USING SEED PROTEIN PROFILE

Dudwadkar S. [1], Parab M. [1] and Singh S.[1]*

[1]School of Biotechnology and Bioinformatics, D. Y. Patil University, Sector 15, Navi-Mumbai, 400614-India.

Corresponding Author: dr.sunitads@gmail.com

ABSTRACT: Inquisition of diversity based on different phenotypic, genotypic and biochemical markers, plays a imperative role in breeding programs for selection of parents, as well helps understanding the evolutionary relationships among crop species. Biochemical markers embody the variations among the genes expressed in response to geo-edaphic conditions as well the genotypes. Present study endeavors to differentiate the members of *Cucurbitaceae* with eco-agronomical essence at intra and inter genus level by profiling seed storage proteins. The study also encompasses a new modified approach for extracting high cedes of proteins form plants possessing very low concentration of proteins. About 20-25 unique bands were scored in *C. grandis* and other *Cucurbitaceae* members respectively. Cluster analysis performed based on Jaccard's similarity coefficient and SPSS software (Version 14.0) showed 3 clusters. Similarity matrix showed that the greatest similarity and minimum genetic distance belonged to populations with the similarity coefficient 0.28 and 0.13 respectively.

Keywords: *C. grandis*, *Cucurbitaceae*, Seed storage proteins, protein profile, SPSS software

INTRODUCTION

The word 'proteomics' is defined as, a systematic study of the proteins expressed by the genome and is a powerful molecular tool for describing complete proteomes at the organelle, cell, or tissue level [26]. Seed storage proteins not only provide essential nutritional and technological elements for plants survival but are also key quintessence for several enzymes involved in plant metabolism [18]. Seed proteins being primary gene products, regulate directly or indirectly genetic system and hence their knowledge obtained using miscellaneous electrophoretic methods has endowed a constructive insight on chemo taxonomical studies of several plant species [1, 19]. Seed proteins have the advantage of being scorable from unviable organs or tissues and the electrophoretic protocol for bulk protein assays is generally simpler than that for Isozymes [4, 20]. Proteins have been used as genetic markers to resolve several taxonomic and evolutionary problems of several crop plants [21, 22]. Also this technique is least influenced by environment, thus the "Fingerprint" generated helps in identifying the genotypes at genus and species level [1].

Cucurbitaceae, the gourd family, is one of the largest families of flowering plant, comprising of over 940 species and about 122 genera distributed in tropical and sub-tropical regions of the world [27]. Among *Cucurbits*, bottle gourd, bitter gourd, cucumber, ivy gourd, ridge and snake gourd, melons etc. demonstrate exuberant ethno-medicinal and agronomical chattels and are consumed as vegetal crop by humankind [15]. Several studies have reported the diversity among some of these members using different DNA markers like RAPD, ISSR, AFLP etc. at intra-species level [14, 16]. Neither of these studies has provided a classification that takes into account proteomics for classifying these members of *Cucurbitaceae* at inter genus level. The present study provides an insight into variations using seed proteins among members of *Cucurbits* and *C. grandis* land varieties obtained from diverse genotypes in India. We propose that this study shall enable one to systematize the accessions from diverse origin, analyze genetic relationships at intra-species and inter-generic level as well determine relative efficacy of these marker systems for population analysis. Present study is thus first of its kind wherein the storage protein fingerprint is generated and is used to analyze the inter relationship among *Cucurbits*.

MATERIALS AND METHODS

Plant Samples:

Seed samples of *M. Charantia, Lagenaria spp., C. sativus, C. maderaseptanous, C. melo, T. dioca, C. maxima, C. lanatus L. actangula, T. cucumeris, P. fistula, C. pepo, C. grandis* were collected from a nursery in Navi- Mumbai and for intra-varietal analysis 12 land varities of *C. grandis* were collected from different geotypes in India. All the samples were authenticated from Blatter Herbarium, Mumbai. All the samples were thoroughly washed with tap water and rinsed with 70% alcohol and distilled water followed by blot drying.

Extraction of Storage proteins:

The methods described by Olson *et al,* 2007 (Method 1) [7], El-Adl *et al,* 2010 (Method 2) [2] and Naushad *et al*., 2010(Method 3) [6] were used for extracting proteins from *Cucurbits* with a yield of equitable concentrations but not as much of purity. A modified method was formulated (method 4) and was optimized for *Cucurbitaceae*. The method involved extraction of proteins from 0.1gm of plant sample in modified buffer (Table 1). The suspension was centrifuged for 15 min at 10000 rpm at 4˚C and the supernatant was used as total soluble protein. Total protein concentration was estimated using Folin-Lowry method [5] with bovine serum albumin as standard.

SDS Page Analysis:

A fingerprint of proteins was generated using one dimensional Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) with 10% separating and 5% stacking gel [3, 9] and was separated at 90 V. The segregated protein bands were analyzed by Coomassie blue and Gene tools from Gene synap (Version 4.0).

Statistical Evaluation:

Electrophoregrams for each variety were scored by the presence (1) or absence (0) of each band noted. Presence and absence of bands were entered in SPSS software (version 14.0) and dendogram was constructed using nearest neighbor linkage. A similarity index was assessed using Jaccard's coefficient of similarity using following formula

   Jaccard's coefficient of similarity (J) = $N_c / N_a + N_b - N_c$,

Where N_a= no of amplified fragments in sample A, N_b= no of amplified fragments in sample B and N_c=no of bands shared by sample A and B.

RESULTS AND DISCUSSION

Seed storage proteins profiling provides an aid in identification and characterization of diversity among the landraces, and prevailing their phylogenetic relationships. [24]. In seed storage proteins both salt-soluble and insoluble-protein provide taxonomic information [8]. Recently specific methods have been used to study salt-soluble proteins for taxonomic and diversity studies in bread wheat [18]

In present research study method for extracting storage proteins with high chaste and cede from *Cucurbitaceae* was optimized by adopting a new modified buffer. The concentration of protein with method 4 was increased by fourfold as compared to other methods (table 1). Also on segregation of proteins on one dimensional SDS – PAGE demonstrated highly resolved separated bands with optimized M4, than the other methods (fig. 1). The increase in yield of proteins obtained by optimized M4 method can be attributed to following modifications. Firstly, use of higher concentration of 2-mercaptoethanol (8mM), enabled the cleavage of cross linkages in protein-protein interactions, wherein two or more proteins associate in lieu of signal-transduction or biosynthetic pathway or stable multiprotein complexes that are required in the execution of several biological functions [13]. Thus the method allowed amending the conformation of proteins, thereby enhancing their resolution on PAGE. Secondly, the pH of the buffer solution used in M4 was 6.8, which increased yield of extractable seed proteins at pH other there IEP (non-existence of most of the extractable protein at their isoelectric pH), or low ionic strength of the salt solution at concentrations used for the extraction [4, 12].

Protein finger print analysis for landraces of *C. grandis* and *Cucurbits* was analyzed using 5% stacking and 10 % resolving PAGE. The profile for *Cucurbits* and *C. grandis* comprised of approximately 25 and 35 bands respectively (fig. 2 and fig. 4) in all. Many bands for protein sub-units were observed at lower portion of the gels but as they were inconsistent as far as their reproducibility was concerned so they were excluded from examination. Similarly variation in the band thickness and sharpness was also observed but this was not accounted as polymorphism. Total seed protein profiling revealed significant inter-specific genetic diversity or genotype specific bands, with some cultivars exhibiting remarkable polymorphic and unique bands, that can be evaluated further "tags" for these cultivars. Bands scored at 10 KDa, 20 KDa and 33 kDa respectively, were monomorphic for almost all cucurbit samples, thus these proteins appear to be most conserved proteins among *Cucurbits* (fig. 2). Intra-species variation among *C. grandis* was also evident; a band between 25 KDa and 45 KDa was highly polymorphic (fig. 4). Species wise differences were evident in protein profiling as similar banding pattern was observed between cultivars of the same species [28].

Cluster analysis was performed on the basis of protein profile obtained using SPSS software version 14.0. Among Cucurbits (fig. 3) III nodes were observed. From node I land race of Ivy gourd appears to evolve first, a divergence in node I have been observed to give rise to *C. sativus* (variety 3 and variety 4) species. With evolutionary adaptations the other species of *Cucurbits* have diverged respectively. Musk melon and pointed gourd are observed distantly related to Zucchini and Cucumber. This divergence of within *Cucumis* genera observed in present study was not in accordance with previous reports of Reddy U. (2009), where in cladistic analyses was performed using *rbcl* gene [28]. It was observed that the clades shared by *Cucumis sativus* and other *Cucumis species* were different and the genetic distance between them was in prominent. Among *C. grandis* land varieties small amount of polymorphisms are indicative that these species may have recently diverged in the evolutionary clock. Among the *C. grandis* land races three nodes were observed. Node I appear to be point of origin for Ivy gourd. From node I species of variety 10, variety 2, variety 11 and variety 3 share a common ancestry and are the among the first generation plantlets of Ivy gourd. The samples from variety 5 and variety 9 regions have recently evolved among the Ivy gourd varieties (fig 5). However the biochemical profile needs to be compared with molecular profiles to confirmatively ascertain the phylogeny and hierarchy among the *C. grandis* and *Cucurbit* plants.

Table1: Composition of extraction buffers and protein yield obtained by different methods

Method	Name of Author	Composition of Extraction buffer	Protein Yield (µg/ml)
Method 1 (M1)	Olson *et al*, 2007	50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 2 mM EDTA, 2 mM 2-Mercaptoethanol	0.107
Method 2 (M2)	El-Adl *et al*,2010	0.5M Tris pH6.8, 10% SDS	1.950
Method 3 (M3)	Naushad *et al.*, 2010	0.5M Tris-HCl (pH 8.0), 0.2% SDS, 5 M urea and 1% 2-mercaptoethanol.	2.767
Method 4 (M4)		0.5M Tris pH6.8, 10% SDS, 8mM 2-mercaptoethanol	8.32
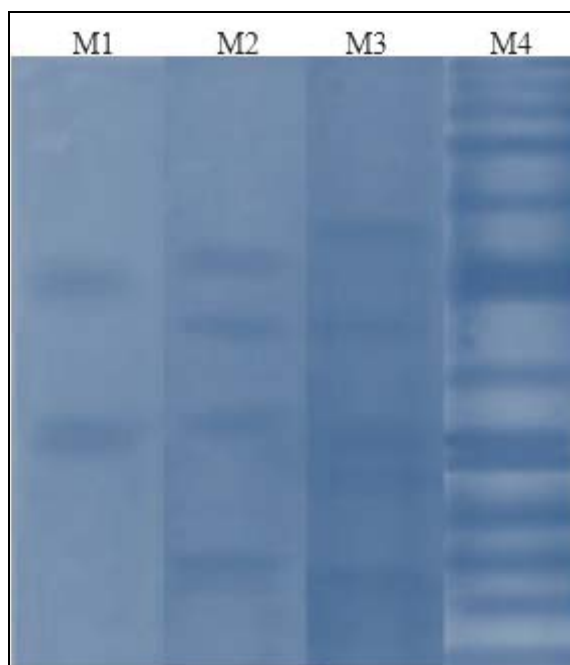
Fig. 1: Electrophoregram of total soluble proteins obtained by different methods
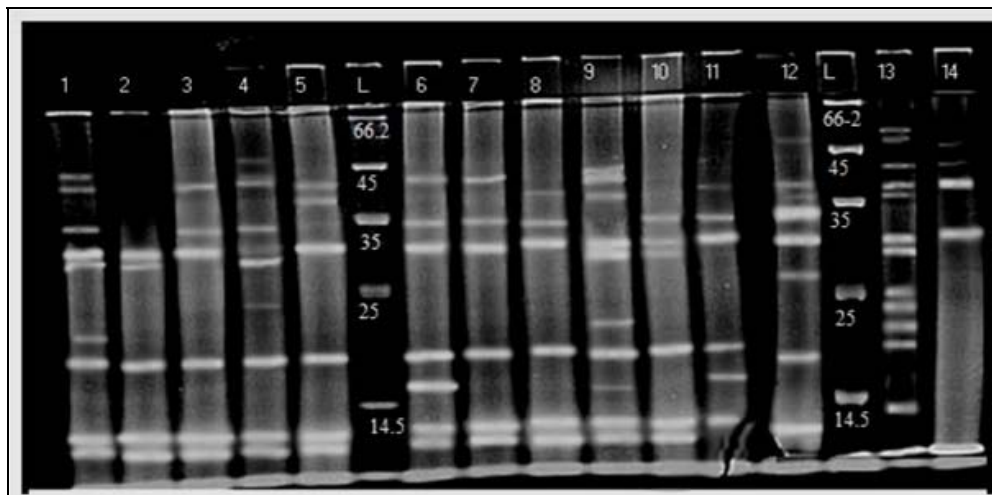
Fig 2: Protein fingerprint of *Cucurbitaceae* land races using SDS- PAGE: Lane 1-5 has . *M. Charantia*, , *Lagenaria spp.*, *C. sativus*, *C. sativus* . *C. maderaseptanous*, Lane 6-12 has . *C. melo*, . *T. dioca*, . *C. maxima*, . *C. lanatus* . *L. actangula*, *T. cucumeris*, . *P. fistula*, Lane 13 & 14. *C. grandis* (variety 11) *C. pepo*,. protein respectively*

Fig 3: Dendogram generated based on SDS-PAGE protein profile of *Cucurbitaceae* land varieties

Fig 4: Protein fingerprint of *C. grandis* land races using SDS- PAGE Lane 1-13 has Kalyan, Andheri*,* Alibaug, Chiplun, Pen, Vasai, Satara (wild), Cochin, Ranpar, Belapur (local), Satara (Local), Ratnagiri *protein respectively*

```
Dendrogram

* * * * * H I E R A R C H I C A L   C L U S T E R   A N A L Y S I S * * * * * *


  Dendrogram using Single Linkage

                      Rescaled Distance Cluster Combine

    C A S E         0         5        10        15        20        25
   Label    Num     +---------+---------+---------+---------+---------+

   VAR00004    4
   VAR00010   10
   VAR00009    9
   VAR00006    6
   VAR00007    7
   VAR00008    8
   VAR00001    1
   VAR00005    5
   VAR00012   12
   VAR00003    3
   VAR00002    2
   VAR00011   11
```

Fig 5: Dendogram generated based on SDS-PAGE protein profile of *C. grandis*

CONCLUSION

The method used for protein extraction has proved to be efficient for extraction of soluble seed storage proteins. The divergence observed in *Cucumis* species should be further confirmed using molecular tools. Protein profiling is an efficient method for study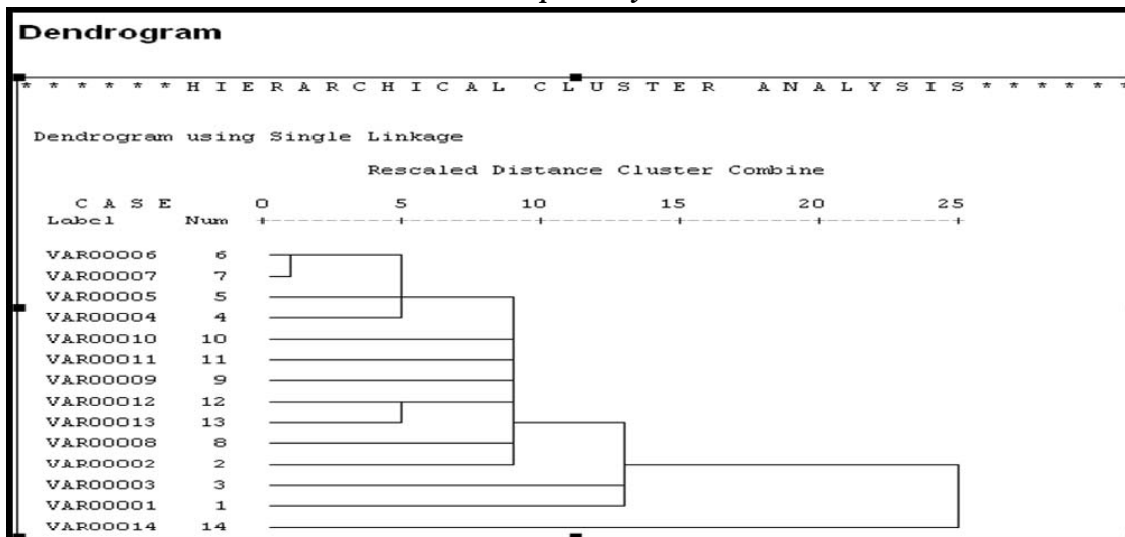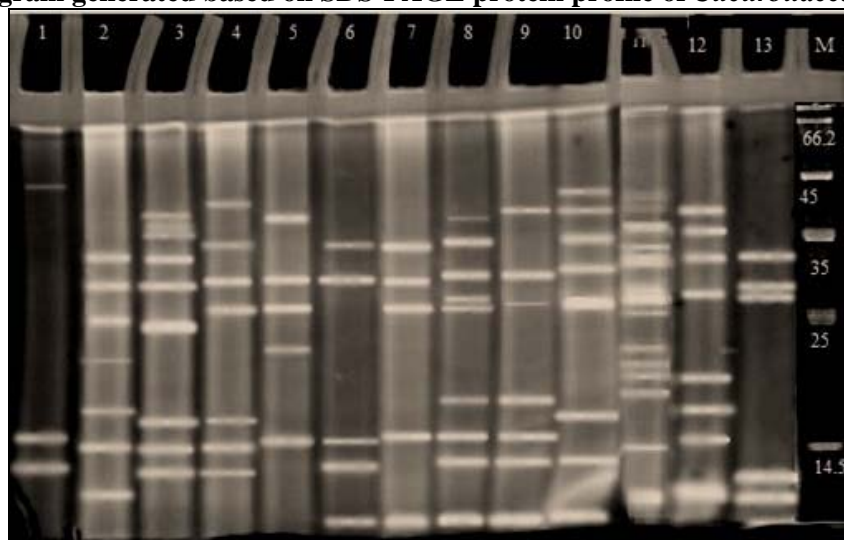ing genetic diversity, coupling biochemical method with molecular approaches would help plant taxonomist to understand the variance among plants in further depth. The divergence observed in *Cucumis* species should be further confirmed using both proteomic and molecular tools.

REFERENCES

[1]   Ehsanpour, A.A., Behrokh, S., Fatemeh R. 2010. Characterization of seed storage protein patterns of four Iranian Pistachios using SDS-PAGE. Natural science, 2 (7), pp737-740

[2]   El-Adl, A.M.; A.H. Abd El-Hadi; Horeya M. Fathy and M.A. Abdein. 2012, Molecular Genetic Evaluation Of Seven Varieties Of Summer Squash, Journal of American Science, 8(5), pp 41- 48

[3]   Hames, B.D. 1990. One-dimensional polyacrylamide gel electrophoresis. In: Hames, B.D. and Rickwood, D., Eds., Gel Electrophoresis of Proteins, 2nd Edition, Oxford University Press, New York, 382

[4]   Jonathan, F.W. and Weden, N.F. 1990. Visualization and interpretation of plant isozymes. Isozymes in Plant Biology. D.E. Soltis and P.S. Soltis (eds.). London Chapman and Hal, pp 5-45

[5]   Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951) Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193, pp 265-275

[6]   Naushad, A. T., Farhatullah, M, Rabbani, A., Naqib Ullah Khan, M. Akmal, Zahida H. P., Umair, A. M. 2010. Study of total seed storage protein in indigenous Brassica species based on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). African Journal of Biotechnology, 9(45), pp 7595-7602.

[7]   Olson, B.J.S.C. and Markwell, J. 2007. Assays for determination of protein concentration. Current Protocols in Protein Science, 3(4), pp 3.4.1-3.4.29

[8]   Ranjan. S., Matcha, R., Madhuri B., Narendra Babu P.V.R. 2012. Comparative evaluation of protein extraction methods from few leguminous seeds, International Journal of Advanced Biotechnology and Research, 3 (2) , pp 558-563

[9]   Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY

[10] Habibi. B. Farshadfar, M. Safari, H. 2012. Evaluation of Genetic Diversity in 18 Genotypes of Alfalfa (Medicago Sativa) Using of Molecular ISSR Markers. International Journal of Agriculture and Crop Sciences, 4 (21), pp 1573- 1578.

[11]   Murphy, RW, Sities, JW, Buth, D.G., Haufler, C.H. 1990. Protein 1: Isozyme electrophoresis. Mol. Syst., pp. 45-126

[12]   Usman, L. A., Ameen O. M., Ibiyemi S. A. and Muhammad, N. O. 2005. The extraction of proteins from the neem seed (Indica azadirachta A. Juss). African Journal of Biotechnology 4 (10), pp. 1142-1144

[13]   Hemaprabha. E. 2012. Chemical Crosslinking of proteins: A review. Journal of pharmaceutical and Scientific Innovation, 1 (1), pp 22-26.

[14]   Schaefer, H., Heibl, C. & Renner, S.S. 2009. Gourds afloat: A dated phylogeny reveals an Asian origin of the gourd family (Cucurbitaceae) and numerous oversea dispersal events. Proc. Roy. Soc. London, Ser. B, Biol. Sci. 276, pp 843–851.

[15]   Jeffrey, C. 2005. A new system of Cucurbitaceae. Bot. Zhurn. 90, pp 332–335

[16]   Zhang, L.-B., Simmons, M.P., Kocyan, A. & Renner, S.S. 2006. Phylogeny of the Cucurbitales based on DNA sequences of nine loci from three genomes: Implications for morphological and sexual system evolution. Molec. Phylog. Evol. 39, pp305–322

[17]   Schaefer & Renner, 2011. Phylogenetic relationships in Cucurbitales and a new Classification of the gourd family (Cucurbitaceae). Taxon, 60 (1), pp 122–138

[18]   Shaista Khan, A.N. Memon, Allah Bux Ghanghro and Ibtessam Tahir. 2011. Fractionation and characterization of seed storage proteins from different wheat varieties cultivated in Sindh on SDS-PAGE electrophoresis. Pakistan Journal of Nutrition, 10 (2), pp 139-142

[19]   Jin Wei-dong, L Na, Hong De-lin 2006. Genetic Diversity of Seed Storage Proteins in Different Ecotype Varieties of japonica Rice and Its Application. Rice Science, 13(2), pp 85-92

[20]   Arindam. B. 2008. SDS PAGE based protein profiling and diversity assessment of indigenous germ plants photo (*Cucumis melo L. var Mormordica Duth and Full*). Plant Horticlture. 40 (2), pp 187-192

[21]   Harendra Singh, Pratibha Singh, R.P. Singh and Mritunjay Tripathi, 2014. Comparative Study in Relation to Protein and Protein Profiling of *Rhizobium* Inoculated Desi and Kabuli Chickpea (*Cicer arietinum* L.) Genotypes. Asian Journal of Biochemistry, 9 , pp 114-118

[22]   Ghafoor A., Z. Ahmad, A.S. Qureshi and M. Bashir. 2002. Genetic relationship in Vigna mungo(L.) Hepper and radiata (L.) R. Wileczek based on morphological traits and SDS-PAGE. Euphytica, 123, pp 378-2002.

[23]   Hameed. A., Tariq. M., Babar. M., Iqbal. N., Hina. A., Haq. A. 2009. Comparative seed storage protein profiling of Kabuli chickpea genotypes. Pakistan Journal of Botany, 41(2), pp 703-710 Kahrizi, S., M. Sedghi and O. Sofalian. 2013. 'Grouping of bread wheat cultivars by seed storage proteins'.Iranian Journal of Plant Physiology 3 (4), pp 835-840

[24]   Nisar, M., A. Ghafoor, M.R. Khan, H. Ahmad, A.S. Qureshi and H. Ali. 2007. Genetic diversity and geographic relationship among local and exotic chickpea germplasm. Pak. J. Bot., 39, pp 1575-1581.

[25]   Yadav R. K., 2008. Seed protein electrophoresis studies in cucurbits – A review. Agricultural Reviews. 29(1), pp 21-30

[26]   Rout J. R and Sahoo. S. L. 2012. Morphological and protein profile alteration in *Withania sominefera* with response to iron stress. Indian. J. L. Sci. 2(1), pp 21-25

[27]   Shaefer. H. and Renner S.S. 2011 a *Cucurbitaceae.*Families and genera of vascular plants vol 10(ed by Kubitzki) pp 112-174 Springer Verlag, Berlin

[28]   Reddy, U. B. 2009. Cladistic analyses of a few members of *Cucurbitaceae* using *rbc*L nucleotide and amino acid sequences. International Journal of Bioinformatics Research. 1(2), pp-58-64