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Utilization of SDS-PAGE of whole cell proteins for characterization of *Azolla* species

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SDS-PAGE profiles of whole cell proteins were used to differentiate among 13 *Azolla* accessions belonging to six species in section *Euazolla*. *Azolla mexicana, A. microphylla, A. rubra, A. filiculoides* and *A. caroliniana* showed highly identical protein profiles except *A. caroliniana* 3001, in which two distinct bands of molecular weight 81 kDa and 97 kDa were absent. This can be a useful marker for distinguishing this strain among accessions belonging to section *Euazolla*. The protein profiles of two accessions of *A. pinnata,* placed in section *Rhizosperma* consisted of unique bands in the range of 12–17 kDa, which were absent in all species of section *Euazolla*. The distinct SDS-PAGE profile, especially those of *A. caroliniana* and *A. pinnata*, provide useful fingerprints for identification of strains belonging to these species among mixtures.

Key words: *Azolla*, SDS-PAGE, proteins

Introduction

Azolla is a eukaryotic aquatic fern genus, symbiotically associated with a prokaryotic, heterocystous, nitrogen fixing cyanobiont and other eubionts of unknown function. It has been utilized as a biofertilizer and green manure, in several countries of SE Asia in conjunction with rice (Pabby *et al*. 2004). The basic classification of *Azolla* relies on the morphology of reproductive structures such as the number of sporocarps, number of massulae/microsporangia, structure and position of glochidia, number of floats/megasporocarp, float texture and surface ornamentation of megasporoderm. The present taxonomic framework divides the genus *Azolla* into sections *Euazolla* and *Rhizosperma* based on the sporocarp characters (Dunham & Fowler 1987). However, most of the *Azolla* accessions never become fertile under maintenance conditions of germplasm collections, therefore the identity of these accessions becomes doubtful. Also, cross contamination with other accessions is common, as fragments of other accessions may frequently get mixed during routine maintenance leading to multiplication via vegetative reproduction. This generally escapes the attention of the user. In this context, molecular techniques can provide reliable methods, but they are expensive and require sophisticated equipment. Therefore, there is a need for developing simple techniques, which can provide a means to monitor cross-contamination in germplasm collections and at the same time, prove cost-effective for analyzing large

number of strains/species of *Azolla* routinely in repositories. This investigation is aimed at evaluating the utility of SDS-PAGE profiles as tools for reliable identification of *Azolla* species.

Material and methods

Thirteen *Azolla* accessions belonging to *A. mexicana, A. microphylla, A. rubra, A. filiculoides, A. caroliniana* and *A. pinnata* were evaluated in the present study, which included seven strains procured from International Rice Research Institute, Philippines (Table 1). *Azolla* species/strains were maintained on chemically defined nitrogen-free Espinas and Watanabe medium (Watanabe & Espinas 1976) under controlled conditions of temperature (25 ± 2 °C) and light (intensity and duration; 100 μmol photon s^{-2}; 16/8 h day/night) in a polyhouse at the Centre for Conservation and Utilization of Blue-Green Algae, Indian Agricultural Research Institute, New Delhi.

Fifteen days old *Azolla* accessions were harvested for protein profiling. Uniform weight of *Azolla* fronds grown under controlled environmental conditions was washed thoroughly with sterile water and their roots were dissected. They were homogenized in a ratio of 1:2 (g fresh weight:ml of cold sample buffer) in sample buffer containing 125 mM Tris–HCl (pH 6.8), 2% SDS, 20% glycerol (v/v), 1.0 mM phenyl methyl sulfonyl fluoride, 5% β-mercaptoetanol, 0.5% sodium azide, 20 mM EGTA and 0.5% bromophenol blue. Gel electrophoresis was carried out following the procedure of Laemmli (1970), using a resolving gel of 12% or 15% and a stacking gel of 5%. 30 μl of each sample containing 150–200 μg ml^{-1} proteins (estimated spectrophotometrically by Herbert *et al.* 1971) was loaded into each slot. The gels were scanned using Alpha Imager 3400 and the number of bands counted. The bands were sized in comparison with a molecular weight marker standard (PWM) (Bangalore Genei Pvt Ltd., India). The patterns were compared by using information on apparent molecular masses of bands and their intensity. The analyses were repeated at least three times independently to verify the reproducibility of the patterns.

Results and discussion

The genus *Azolla* includes section *Rhizosperma* (two species: *A. pinnata* and *A. nilotica*) and section *Euazolla* (five species: *A. mexicana, A. microphylla, A. rubra, A. filiculoides* and *A. caroliniana*). This classification is primarily based on the morphology of reproductive structures. A number of researchers have used various

Table 1. *Azolla* accessions and their source. Identification numbers (ID) as in Fig. 1.

ID	Accession no.	Species	Section	Source
1	ACC 31	*caroliniana*	*Euazolla*	[a]TNAU, Coimbatore, India
2	CA 3001	*caroliniana*	*Euazolla*	[b]IRRI, Philippines
3	ACC 40	*microphylla*	*Euazolla*	[c]CCUBGA, IARI, India
4	MI 4018	*microphylla*	*Euazolla*	IRRI, Philippines
5	MI 4028	*microphylla*	*Euazolla*	IRRI, Philippines
6	ACC 20	*mexicana*	*Euazolla*	CCUBGA, IARI, India
7	ME 2002	*mexicana*	*Euazolla*	IRRI, Philippines
8	ACC 60	*rubra*	*Euazolla*	CCUBGA, IARI, India
9	RU 6503	*rubra*	*Euazolla*	IRRI, Philippines
10	ACC 10	*filiculoides*	*Euazolla*	CCUBGA, IARI, India
11	FI 1001	*filiculoides*	*Azolla*	IRRI, Philippines
12	ACC 01	*pinnata var. imbricata*	*Rhizosperma*	CCUBGA, IARI, India
13	PP 7001	*pinnata var. pinnata*	*Rhizosperma*	IRRI, Philippines

[a] TNAU, Tamil Nadu Agricultural University, Coimbatore, India.
[b] IRRI, International Rice Research Institute, Philippines.
[c] CCUBGA, Centre for Conservation and Utilization of Blue-Green Algae, Indian Agricultural Research Institute (IARI), New Delhi.

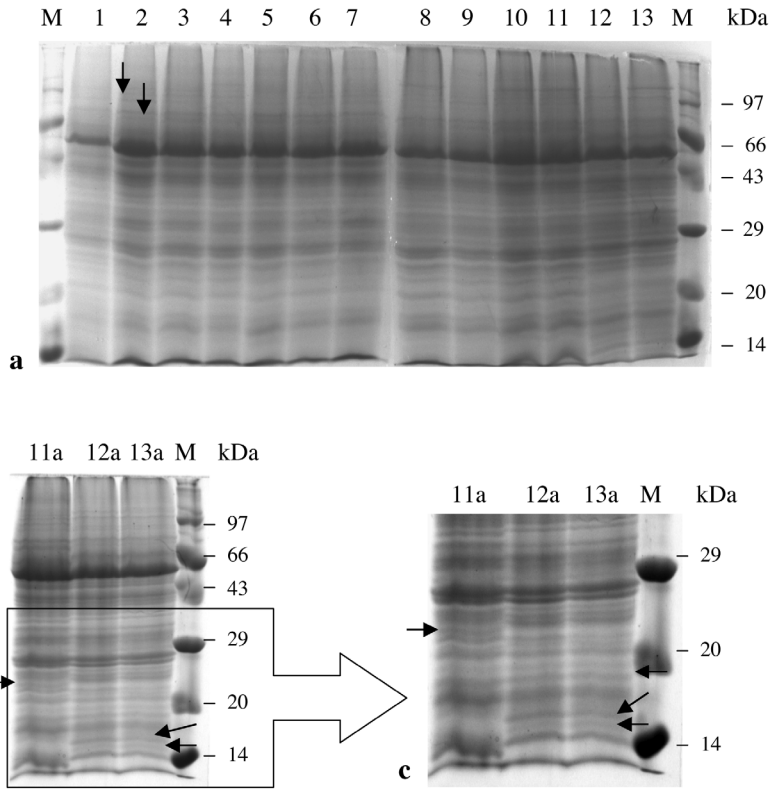

Fig. 1. SDS-PAGE profiles of total cell proteins isolated from 13 *Azolla* accessions. — **a**: 12% gel showing lanes 1–13. Arrows designate the two bands absent in *A. caroliniana*. — **b**: 15% gel showing lanes 11a–13a. — **c**: Magnified view of marked area on 15% gel. Lane numbers represent ID as given in Table 1, and lane M, Molecular weight marker (14–97 kDa).

biochemical and molecular techniques such as isoenzyme analysis, RAPD, DAF and RFLP to resolve the taxonomic problems (Zimmerman *et al*. 1991, Van Coppenolle *et al*. 1993).

The use of proteins as reliable markers has been controversial because they represent expressed parts of the genome, which are influenced by the growth and environmental conditions. In the present study, utmost care was taken to maintain uniform conditions during growth, and reproducibility of the profiles was checked. SDS-PAGE of whole cell proteins of 13 *Azolla* accessions showed mutual relationships at inter- and intraspecific levels. The protein profiles of *Azolla* comprised approximately 35 bands, which included 14 polymorphic bands (Fig. 1a). The protein banding pattern of the eleven *Azolla* accessions belonging to section *Euazolla* (i.e. *A. mexicana, A. microphylla, A. rubra, A. filiculoides, A. caroliniana*) did not exhibit any significant differences. However, two bands in the range of 81 kDa and 97 kDa were absent in *A. caroliniana* 3001, but present in the other accessions of section *Euazolla*. These bands distinguished this accession not only from the other strain of *A. caroliniana* (ACC 31) but also from other accessions belonging to section *Euazolla*. Both strains of *A. pinnata* (ACC 01 and 7001) placed in section *Rhizosperma* had two unique bands, in the molecular weight range of 12–20 kDa, which were clearly visible in the 15% gel (Fig. 1b and 1c). Also, one band 22–24 kDa was missing in ACC 01 and 7001 strains of *A. pinnata,* but observed in all the other species examined belonging to section *Euazolla*.

Whole cell protein pattern analysis has been found to be a good method for grouping large numbers of closely related strains of bacteria, which yielded discriminative information only at or below species level. Lyra *et al*. (1997) characterized filamentous cyanobacterial strains from different genera using SDS-PAGE of whole cell proteins and PCR/RFLP of 16S rRNA gene and revealed grouping of cyanobacterial genera in accordance with previous classification systems.

Our results demonstrate the utility of SDS-PAGE profiles of whole cell proteins as a good phenotypic tool for distinguishing *A. pinnata* of

section *Rhizosperma* from accessions belonging to section *Euazolla*. They can also serve as fingerprint for identification of a specific strain (*A. caroliniana*; CA3001).

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References

Dunham, D. G. & Fowlers, K. 1987: Taxonomy and species reorganization in *Azolla* Lam. — In: *Azolla utilization*: 7–16. Int. Rice Res. Inst., Manila.

Herbert, D., Phipps, P. J. & Strange, R. E. 1971: Chemical analysis of microbial cells. — In: Norris, J. R. & Ribbons, D. W. (eds.), *Methods in enzymology* 5B: 209–344. Acad. Press, New York.

Laemmli, U. K. 1970: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. — *Nature* 227: 680–685.

Lyra, C., Hantula, J., Vainio, E., Rapala, J. & Sivonen, K. 1997: Characterization of cyanobacteria by SDS-PAGE of whole cell proteins and PCR/ RFLP of the 16S rRNA gene. — *Arch. Microbiol*. 168: 176–184.

Pabby, A., Prasanna, R. & Singh, P. K. 2004: Biological significance of *Azolla* and its utilization in agriculture. — *Proc. Indian Natl. Sci. Acad. (PINSA-B) Biol. Sci*. 70: 301–335.

Van Coppenolle, B., Watanabe, I., Van Hove, C. & McCouch, S. R. 1993: Genetic diversity and phylogeny analysis of *Azolla* by arbitrary primers. — *Genome* 36: 686–693.

Watanabe, I & Espinas, C. R. 1976: *Potential of nitrogen fixing Azolla–Anabaena complex as biofertilizer in paddy soil*. — Int. Rice Res. Inst., Manila.

Zimmerman, W. J, Watanabe, I., Ventura, T., Payawal, P. & Lumpkin, T. A. 1991: Aspects of genetic and botanical status of neotropical *Azolla*. — *New Phytol*. 119: 561–566.