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Initial screening of vegetable amaranth landraces toward extending the vegetable list

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ABSTRACT

Vegetable amaranth is considered as one of the most favorite vegetables in the world, especially in the hot and humid tropical regions of the globe. Two of them are most popular, i.e., *Amaranthus tricolor* and *Amaranthus blitum*, both are represented by a large number of morphotypes or landraces or varieties, which are taxonomically ill-defined, many of them are consumed as palatable vegetables as they look like the conventional cultivars. Due to wide morphological diversity and presence of many synonyms both species are supposed to represent two species complex or aggregates, namely, "Tricolor complex" and "Blitum complex". Two new species have been identified from their landraces. In the present investigation, morphometric analysis along with biochemical and molecular methodologies were applied to explore the relative closeness among few well known, popular vegetables, and few less known landraces for better utilization of the crop biodiversity of vegetable amaranths. The experimental data were statistically analyzed and separate dendrograms were computed on three parameters (morphology, isozyme polymorphism, and inter simple sequence repeat band profile). The members of "Tricolor complex" were clustered together in a single group along with two newly introduced species *Amaranthus bengalense* (a member of "Blitum complex") and *Amaranthus parganensis* (a gynomonoecious member of "Tricolor complex"). The "Tricolor complex" represents a plexus of species with varying sexual behavior from gynomonoecy to monoecy and indicates probable origin of monoecious *A. tricolor* varieties or landraces from gynomonecious member such as *A. parganensis* as recent introduction. The study indicated a close alliance between *A. bengalense*, *A. parganensis*, *A. tricolor* landrace, and popular *A. tricolor* varieties which consolidated the feasibility of utilizing the landrace and newly introduced species as potential vegetable.

KEY WORDS: Crop biodiversity, interrelationship, landraces, new potential vegetable, species complex, vegetable amaranths

INTRODUCTION

Vegetables are the essential parts of any diet as a source of protein, vitamins, minerals, and dietary fibers. Vegetable amaranths are probably the most widely consumed and popular vegetable in the hot humid tropical regions of Asia and Africa (Schnetzler and Breene, 1994) due to its mild spinach such as flavor and high nutritive value. It is also ranked as one of the top five vegetable in antioxidant properties, in having bioactive components (Willett, 2001). Tumor cell proliferation and cyclooxygenase inhibitory compounds have been isolated from certain vegetable amaranths (*Amaranthus tricolor*) (Jayaprakasam *et al.*, 2004).

High yield, ability to grow on virtually any type of soil even in marginal areas not needing any special agricultural inputs, adaptability to hot humid tropical climate are the prime reasons for its popularity among the farmers. Leaves and tender juicy stem of several amaranth species are edible in different parts of the world with varying acceptability but two of them (*A. tricolor* and *Amaranthus blitum*) are very popular. Both are represented by a large number of varieties, morphotypes, or landraces. A large number of synonyms and misapplication of names have created ambiguity in infraspecific delimitation. As such several authors (Das, 2013; Mosyakin and Robertson, 1996; Das and Iamonico, 2014) suggested to consider both species as two separate species complex or aggregates, i.e. "Tricolor complex" and "Blitum complex". Two new species - *Amaranthus bengalense,* a monoecious member (Das and Iamonico, 2014) and *Amaranthus parganensis,* a gynomonoecious member (Das, 2015) have been described from the Lower Gangetic plain of West Bengal

from the crop-wild relatives or landraces, which are equally palatable like conventional cultivars.

In the present investigation, along with few well-known vegetable amaranths few less known locally consumed members included in both "Tricolor complex" and "Blitum complex" have been studied to explore the relative closeness among the vegetable amaranths, to widen the vegetable list also to have an idea about phylogeny of vegetable amaranths.

MATERIALS AND METHODS

Materials

Few common popular vegetable amaranths (*A. tricolor* var. *tricolor* L., *A. tricolor* var. *tristis* (L.) Thellung, *A. tricolor* var. *acutus* S. Das, and *A. bengalense* Saubhik Das and Iamonico), few less known vegetables (*A. parganensis* Saubhik Das, *A. blitum* var. *oleraceus*, and one landrace of *A. tricolor* var. *tricolor*), and one weed member *Amaranthus viridis* (occasionally used as vegetable elsewhere) were incorporated in the present investigation.

Morphological Study

27 notable morphological characters (both qualitative and quantitative) with variable character states were considered in morphometric analysis. Morphological features taken into consideration were - leaf shape, inflorescence pattern, position of male and female flowers; comparative length of bracts, bracteoles, and tepals; number of tepal lobes and stamens; tepal shape and apices; fruit and seed character, etc.

Isozyme Polymorphism Study

Enzyme extraction was carried out according to the method of Wetter and Dyck (1983). Seedling samples of 100 mg each were extracted with 1 ml of 0.2 M Tris - HCl buffer pH - 8.5 containing 0.056 M 2-mercaptoethanol and 1 M sucrose. Isozyme variability was analyzed by native polyacrylamide gel electrophoresis following Studier (1973). Gel was stained for acid phosphatase (ACP) activity following the method of Brewbaker *et al.* (1968).

Inter Simple Sequence Repeat (ISSR) Polymorphism Study

The genomic DNA from different seedling samples was isolated following cetyltrimethylammonium bromide (CTAB) method with slight modification. Three ISSR oligonucleotide primers, $(GACA)_4$, $(CAA)_5$, and $(GA)_8$ were applied for polymorphism analysis. Polymerase chain reactions were performed in 25 µl of reaction mixture containing 20 mg of genomic DNA, 1 unit of Taq DNA polymerase, 10 mM of Tris - HCl (pH 8.0) buffer, 2 mM of $MgCl_2$, 0.25 µM of each deoxynucleotide, and 0.2 µM oligonucleotide primers. Amplifications were carried out in a thermocyclar programmed with initial denaturing for 5 min at 94°C followed by 40 cycles of 45 s at 94°C, 1 min at annealing temperature (primer specific), and 2 min at 72°C for extension with final extension for 7 min at 72°C. The products from amplifications were separated in 2% agar gel using 100 bp ladders.

Statistical Analysis

In case of morphological analysis, on the basis of presence or absence of a particular character state in all the samples, a similarity matrix was prepared giving each character state a definite number. Percentage-based pairing affinity (PA) values between different combinations of species pairs were calculated by the following formula:

$$PA = \frac{\text{Character state common to sample A and B}}{\text{Total number of character states in sample A and B}} \times 100$$

In both the cases of isozyme and ISSR polymorphism analysis, pairing affinity values for different combinations of sample pairs were calculated from the band profiles by the following formula:

$$PA = \frac{\text{Number of isozyme or ISSR bands common to sample A and B}}{\text{Total number isozyme or ISSR bands in sample A and B}} \times 100$$

From the PA values of each parameter, three separate dendrograms were computed using statistica 13.2 cluster analysis software from Dell.

RESULTS AND DISCUSSION

Morphological Study

Morphological studies applying 27 prominent morphological characters revealed an appreciable similarity between common cultivars and local landraces of monoecious *A. tricolor*. Surprisingly gynomoecious *A. parganensis* showed striking similarity with *A. tricolor*. Although *A. bengalense* distinctly differs from *A. tricolor* in having comparatively smaller plant body, trailing habit,

small terminal inflorescence, and indehiscent capsule it showed very close relationship with *A. tricolor*. Pairing affinity values showed wide range of variability that ranged from 40.74% (*A. tricolor* var. *tricolor* and *A. blitum* var. *oleraceus*) to 96.29% (*A. parganensis* and *A. tricolor* landrace) (Table 1). Pairing affinity values between different *A. tricolor* varieties and landraces varied from 55.55% (*A. tricolor* var. *tricolor* and *A. tricolor* var. *acutus*) to 88.88% (*A. tricolor* var. *tristis* and *A. tricolor* landrace).

Dendrogram (Figure 1) computed from pairing affinity values showed clear clustering of *A. tricolor* varieties and landraces with their close allies *A. bengalense* and *A. parganensis* in a single large cluster (Group A) though *A. tricolor* var. *acutus* showed wide divergence from other *A. tricolor* varieties and a close association with *A. viridis* and *A. blitum* var. *oleraceus* in Group B.

Isozyme Polymorphism Study of ACP

The compiled zymogram (Figure 2) showed 18 polymorphic bands distributed in different taxa. *A. tricolor* var. *tricolor* landrace showed the highest polymorphism while *A. tricolor* var. *tristis* showed the least. Pair-wise monomorphism percentage or pairing affinity values ranged from 33.33% (*A. parganensis* and *A. blitum* var. *oleraceus*) to 95.23% (*A. parganensis* and *A. tricolor* landrace) (Table 2). Dendrogram

Table 1: Pairing affinity values on morphometric analysis

	A	B	C	D	E	F	G	H
A	100							
B	85.18	100						
C	81.48	81.48	100					
D	85.18	85.18	88.88	100				
E	81.48	81.48	85.18	96.29	100			
F	55.55	55.55	55.55	62.96	59.25	100		
G	66.66	66.66	62.96	66.66	62.96	62.96	100	
H	44.44	40.74	59.25	51.85	55.55	44.44	48.14	100

A: *Amaranthus bengalense*, B: *Amaranthus tricolor* var. *tricolor*, C: *A. tricolor* var. *tristis*, D: *Amaranthus parganensis*, E: *Amaranthus tricolor* var. *tricolor* landrace, F: *Amaranthus tricolor* var. *acutus*, G: *Amaranthus viridis*, H: *Amaranthus blitum* var. *oleraceus*

Table 2: Pairing affinity values on acid phosphatase polymorphism

	A	B	C	D	E	F	G	H
A	100							
B	94.11	100						
C	93.33	87.50	100					
D	77.77	84.21	70.58	100				
E	84.21	90.00	77.77	95.23	100			
F	75.00	82.35	66.66	66.66	73.68	100		
G	62.50	58.82	66.66	44.44	52.63	62.50	100	
H	50.00	47.05	53.33	33.33	42.10	50.00	50.00	100

A: *Amaranthus bengalense*, B: *Amaranthus tricolor* var. *tricolor*, C: *A. tricolor* var. *tristis*, D: *Amaranthus parganensis*, E: *Amaranthus tricolor* var. *tricolor* landrace, F: *Amaranthus tricolor* var. *acutus*, G: *Amaranthus viridis*, H: *Amaranthus blitum* var. *oleraceus*

(Figure 3) computed from pairing affinity values showed two large distinct clusters or groups. Group - A comprised all the members of "Tricolor complex" along with two newly introduced species. Group B comprised *A. blitum* var. *oleraceus* and weed *A. viridis*.

ISSR Polymorphism Study

ISSR polymorphism analysis with three different ISSR primers showed varied degree of amplification. The best amplification was achieved with the primer $(GACA)_4$ developing 24 polymorphic bands distributed in different

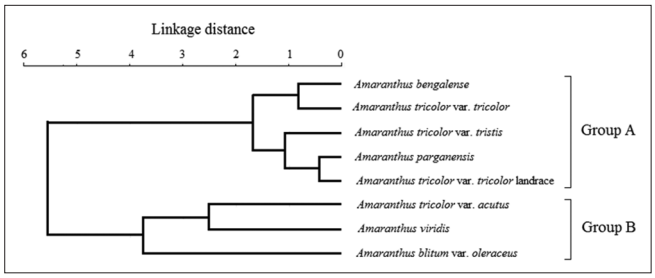

Figure 1: Dendrogram computed from morphological features showing grouping of taxa

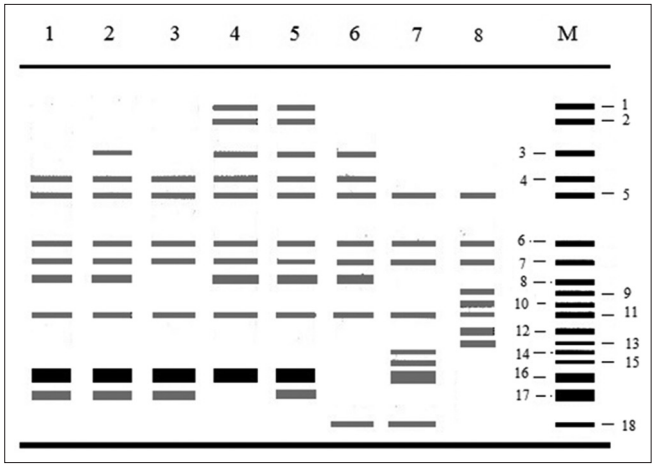

Figure 2: Zymogram of acid phosphatase isozymes (diagrammatic). (1) *Amaranthus bengalense*, (2) *Amaranthus tricolor* var. *tricolor*, (3) *Amaranthus tricolor* var. *tristis*, (4) *Amaranthus parganensis*, (5) *Amaranthus tricolor* var. *tricolor* landrace, (6) *A. tricolor* var. *acutus* (7) *Amaranthus viridis*, and (8) *Amaranthus blitum* var. oleraceus, (M) compiled zymogram

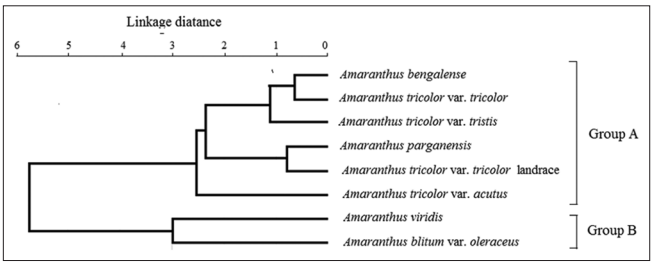

Figure 3: Dendrogram computed from isozyme polymorphism of acid phosphatase

taxa (Figure 4). Pairing affinity values or percentage-based monomorphism ranged from 16.66% (*A. parganensis* and *A. tricolor* var. *acutus*) to 71.42% (*A. tricolor* var. *tricolor* and *A. tricolor* var. *tristis*; *A. tricolor* var. *tristis* and *A. tricolor* landrace) (Table 3). Grouping patterns in the dendrogram (Figure 5) computed from pairing affinity values showed exact concomitance with that of morphological dendrogram.

Table 3: Pairing affinity values on ISSR polymorphism analysis

	A	B	C	D	E	F	G	H
A	100							
B	60.00	100						
C	44.44	71.42	100					
D	40.00	54.54	66.66	100				
E	60.00	62.50	71.42	54.54	100			
F	47.61	58.82	53.33	16.66	58.82	100		
G	36.36	55.55	50.00	30.76	66.66	52.63	100	
H	41.66	60.00	44.44	40.00	50.00	38.09	54.54	100

ISSR: Inter simple sequence repeat, A: *Amaranthus bengalense*, B: *Amaranthus tricolor* var. *tricolor*, C: *A. tricolor* var. *tristis*, D: *Amaranthus parganensis*, E: *Amaranthus tricolor* var. *tricolor* landrace, F: *Amaranthus tricolor* var. *acutus*, G: *Amaranthus viridis*, H: *Amaranthus blitum* var. *oleraceus*

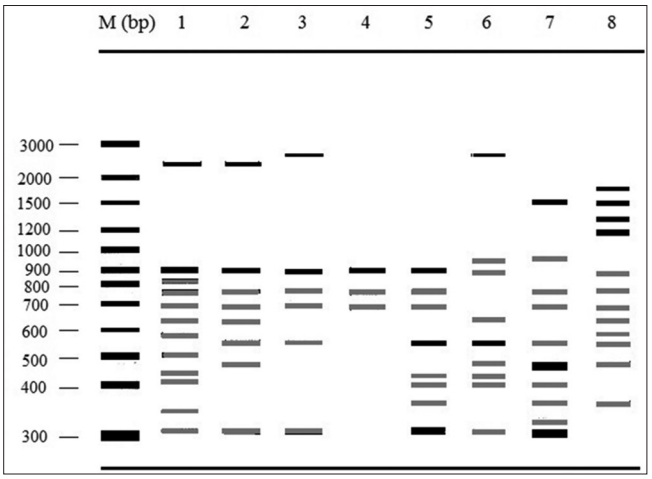

Figure 4: Inter simple sequence repeat fragment polymorphism in different taxa (diagrammatic). (M) DNA marker, (1) *Amaranthus bengalense*, (2) *Amaranthus tricolor* var. *tricolor*, (3) *A. tricolor* var. *tristis*, (4) *Amaranthus parganensis*, (5) *A. tricolor* var. tricolor landrace, (6) *A. tricolor* var. *acutus*, (7) *Amaranthus viridis*, and (8) *Amaranthus blitum* var. *oleraceus*

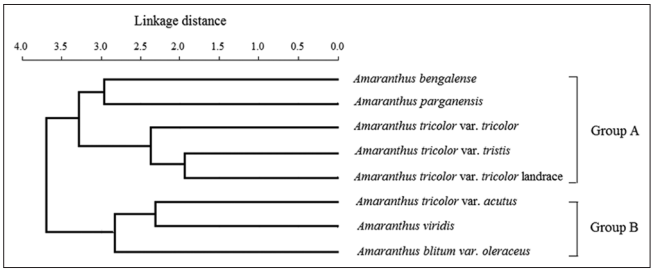

Figure 5: Dendrogram computed from inter simple sequence repeat fragment analysis

Species delimitation in vegetable amaranths is quite ambiguous due to its wide morphological variability. Few species of vegetable amaranths are edible of which two most common and popular species are *A. tricolor* and *A. blitum*. *A. tricolor* is a moderate to robust herb with flowers arranged in small axillary glomerulus and terminal compound inflorescence of agglomerated cymes, circumscissile capsule, and blackish-brown seeds. *A. blitum* is comparatively smaller, erect to prostrate herb with flowers arranged in small terminal spike, and small axillary glomerulus. Fruits are compressed subglobose indehiscent capsule with small black seeds, 1.1-1.2 mm in diameter. Infraspecific variability of *A. tricolor* is addressed by introducing taxa at the variety level; at least three varieties are recognized, namely, *A. tricolor* var. *tricolor*, *A. tricolor* var. *tristis*, and *A. tricolor* var. *acutus*. Due to presence of a large number of morphotypes or landraces and available synonyms both *A. tricolor* and *A. blitum* are supposed to represent two species complex or aggregates, i.e., "Tricolor Complex" and "Blitum Complex". A robust gynomonoecious species *A. parganensis* was identified from *A. tricolor* landraces from Lower Gangetic plain of West Bengal. Another species *A. bengalense* was identified from *A. blitum* landraces. In Lower Gangetic plain of West Bengal, "Tricolor Complex" is represented by varieties and landraces of *A. tricolor* and *A. parganensis* and "Blitum complex" is represented by *A. blitum* var. *oleraceus*, *A. blitum* var. *blitum* and *A. bengalense*.

Vegetable amaranths are mostly included in *Amaranthus* subgen *Albersia*. Members of "Tricolor complex" are included in *Albersia* section Pentamorion and members of "Blitum complex" along with *A. viridis* are included in *Albersia* section *Blitopsis*. Both the subsects are generally delimited on the basis of dehiscence and indehiscence of fruit.

Morphological evidences have played a significant role in solving taxonomic disputes in amaranths (Costea and DeMason, 2001; Costea and Tardif, 2003b; Das, 2012b). Beside Morphological parameters, biochemical and molecular parameters such as - Isozymes (Chan and Sun, 1997; Hauptli and Jain, 1984; Iduna *et al.*, 2005), Random Amplified Polymorphic DNA (Transue *et al.*, 1994), ISSR, Amplified Fragment Length Polymorphism and ITS (Costea *et al.*, 2006; Nolan *et al.*, 2010; Stefunova *et al.*, 2014; Xu and Sun, 2001) were employed successfully to address the queries regarding taxonomic delimitation and phylogeny in amaranths. In the present investigation besides morphology, isozyme polymorphism of ACP, ISSR fragment polymorphism were employed to trace the relative closeness among the common cultivars of

vegetable amaranths with their landraces or crop wild relatives.

Separate dendrograms on different parameters showed significant concomitance in grouping of taxa with minor variations at the similarity levels or linkage distance. All the taxa of "Tricolor complex" including two newly introduced species and landrace were included in a single cluster except *A. tricolor* var. *acutus* that showed close affinity with *A. viridis* and *A. blitum* var. *oleraceus* (a member of "Blitum complex"). *A. bengalense* though is a member of "Blitum complex" always showed close alliance with "Tricolor complex" which indicated that "Blitum complex" represents a loose assemblage of species. Close relationship between *A. bengalense*, *A. parganensis*, *A. tricolor* landrace, and popular *A. tricolor* varieties consolidates primarily the feasibility of their use as potential vegetable, though their nutritive values are to be evaluated.

Amaranths show wide range of sexuality from gynomonoecy, monoecy to dioecy. Monoecy is the predominant phenomenon in vegetable amaranths supposed to have originated from dominant bisexual condition (Mitchell and Diggle, 2005) in Cheno-Amaranth alliance through intermediate gynomonoecious or andromonoecious forms (Bawa and Beach, 1981). Dioecy is restricted in the subgen. *Acnida*. The close relationship between Chenopodiaceae and Amaranthaceae has been recognized based on core floral features (Hershkovitz, 1989). Molecular analysis of the Caryophyllales (Cuénoud *et al*., 2002) established the Chen-Am alliance as a monophyletic lineage. Gynomonoecious condition represents the intermediate stage between hermaphrodite and monoecious condition. Close association between "Tricolor complex" and gynomonoecious *A. parganensis* indicates that monoecious *A. tricolor* varieties might have originated from member like *A. parganensis* as recent introduction.

CONCLUSION

In the present investigation, few prominent findings were surfaced. There are few local morphotypes or landraces of *A. tricolor* and *A. blitum* beside the conventional popular cultivars, which are consumed locally. The close association between the popular cultivars with their local landraces have consolidated the feasibility of use of these landraces as potential vegetable, though their nutritive values are to be evaluated. Delimitation between "Tricolor complex" and "Blitum complex" is transient. Dehiscence or indehiscence of fruit cannot be a delimiting feature. The "Tricolor complex" represents a plexus of species, varieties, and landraces with varying degree of sexual behavior ranging from gynomonoecy to monoecy. Monoecious *A. tricolor* might have originated from a gynomonoecious member like *A. parganensis* as monoecy is considered as a derived stage from gynomonoecy.

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