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Research Note

Interspecific Hybrid between *Echinochloa esculenta* (Japanese barnyard millet) and *E. frumentacea* (Indian barnyard millet) – A New Avenue for Genetic Enhancement of Barnyard Millet

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Abstract
Inter-specific hybridization between the two cultivated species of barnyard millet, *Echinochloa frumentacea* (Indian barnyard millet) and *E. esculenta* (Japanese barnyard millet) holds enormous potential for their mutual genetic enhancement. Here, we report the success in obtaining inter-specific hybrid between *E. esculenta* and *E. frumentacea* involving cultivars PRJ 1 and ER 72 of the two species, respectively. The hybridity of F_1 plants was confirmed through rice SSR (Simple Sequence Repeat) markers. The hybrid plants of the cross PRJ 1 x ER 72 were vigorous, heavily tillered with high culm branching and were free from grain smut disease but failed to set seed due to sterility. Successful hybridization between the two species opens up vast avenues for introgression of desirable traits and exploitation of genetic variability present in the two species for their mutual genetic improvement, besides a wide array of conventional and genomic researches particularly dissection of traits such as yield and disease resistance.

Key words: Barnyard millet, hybrid sterility, SSR cross-transferability

Barnyard millet (*Echinochloa* spp.) is one of the oldest domesticated millets in the semi-arid tropics of Asia and Africa. It is a staple cereal in areas where climatic and edaphic conditions are unsuitable for rice cultivation (Yabuno, 1987). Of some 20 species comprising the genus *Echinochloa*, two are cultivated namely *E. frumentacea* (Indian barnyard millet) and *E. esculenta* (Japanese barnyard millet). *E. frumentacea* originated in India, and possibly also in Africa. It is an annual cultivated form in India, Central African Republic, Tanzania and Malawi (Dogget, 1989). It is domesticated form of wild species *E. colona* (L.) Link., popularly known as Jungle rice. It is grown for grain, fodder, and beer, although not as extensively as in the past. *E. esculenta* is also annual in habit and is cultivated mostly in the temperate regions (De Wet *et al*., 1983) of Japan, Korea, China, Russia and Germany. It differs from *E. frumentacea* in its brownish caryopses, proportionately larger embryos and longer pedicels (Dogget, 1989).

In India, barnyard millet is grown from Himalayan region in the north to Deccan plateau in the south. It is cultivated over an area of 1.95 lakh ha mainly in the states of Uttarakhand, Madhya Pradesh, Karnataka, Uttar Pradesh and North east region of India (Seetharam, 2011). Though in the recent years, the crop has gained renewed interest as a health-food due to its rich nutritional profile and high dietary fibre content, the area under barnyard millet has witnessed a sharp decline owing to various production constraints. Among them, grain smut disease caused by *Ustilago panici-frumentacei* Brefeld is the most important, causing yield losses to the extent of 60.8 per cent (Jain *et al.*, 1997). Resistance to grain smut in not available in Indian barnyard millet (Gupta *et al.,* 2009; Nagaraja and Mantur, 2008), whereas Japanese barnyard millet is immune to smut disease (AICSMIP Annual Report, 2001; Bandhopadhyaya, 1999). The other desirable traits of *E. esculenta* include high yield potential and low seed shattering. Likewise, the desirable traits possessed by *E. frumentacea* include wider adaptability and easy threshing and dehusking. Inter-specific hybridization between the two species holds enormous potential for their mutual genetic enhancement. An attempt, therefore, was made to hybridize these two cultivated species of barnyard millet with the long-term view of mutual

introgression of desirable genes between the two species.

Although several morphological traits have been used for hybrid testing and genetic purity, morphological characters often do not precisely describe the genetic relationship, due to environmental influences. In the recent years, molecular markers have offered an opportunity and among DNA markers, simple sequence repeats (SSRs) as locus specific and co-dominant are the most suitable markers for hybrid purity assessment as the heterozygosity of the hybrids can be easily determined by the presence of both the parental alleles (Naresh *et al.*, 2009). Since genomic SSR markers have not been developed in *Echinochloa* species, we used rice SSR markers for hybrid testing because of its relatedness to rice.

One genotype each of the two cultivated species, PRJ 1 (*E. esculenta*) and ER 72 (*E. frumentacea*), were raised at the Experimental Farm Hawalbagh, VPKAS, Almora during 2011. The cultivar PRJ 1 has been released for cultivation in the state of Uttarakhand whereas ER 72 is a local selection from Rajasthan (AICSMIP Annual Report, 2005). Hot water method of emasculation was followed. The female panicles were immersed in hot water at a temperature of 48ºC for five minutes and air dried. After the treatment, the racemes of male panicle were intertwined with female panicle racemes. Both direct and reciprocal crosses were made. Crossed panicles in each parent were harvested and the seeds were bulked. Due to poor predictability of hot water emasculation method with respect to amount of seed set and the proportion of hybrid seed in the crossed panicles, all the seed harvested from the crossed panicles was planted to raise the F_1 generation. The two contrasting characters possessed by the parents - glume anthocyanin pigmentation (ER 72) and awned glumes (PRJ 1) were taken as the principal morphological markers for identification of hybrid plants. Other panicle, leaf and plant characteristics, for which the parents differed, were also used for the identification of hybridity. Observations on various morphological characters such as plant height (cm), number of tillers, days to maturity, panicles per plant, number of racemes, ear length (cm) were recorded on each hybrid plant and five plants each of the two parents for comparison. The qualitative observations viz., culm branching, glume anthocyanin pigmentation, awns, panicle shape, panicle compactness, raceme branching and fertility status. were also recorded.

DNA isolation Total genomic DNA of parents and hybrids was extracted from the young leaves following Agrawal and Katiyar (2008). The 100 mg tissue was ground in 1ml extraction buffer [Tris HCl (pH 8.0) (100 mM), EDTA (pH 8.0) (20 mM), NaCl (1.4 M), CTAB (2%), 2-mercaptoethanol (0.4%)] and incubated at 65°C for 30mins. The aqueous phase was extracted by the addition of equal volume of chloroform: isoamyl alcohol (24:1) v/v, incubated for 2 min by slow inversion followed by centrifugation at 10,000g for 15 min. DNA was collected by the addition of iso-propanol to the supernatant, followed by centrifugation at 12,000g for 5 min. The DNA pellets were washed twice with 70% (v/v) ethanol, dried and finally dissolved in 50 µlTris buffer containing 1ng/µl RNAase and incubated at 37ºC for 30 mins and finally the DNA was diluted to 25ng/µl.

PCR amplification Forty microsatellite primer pairs were selected randomly for PCR amplification. The repeat motifs and the primer sequences for these markers can be found in the RiceGenes database (http://www.gramene.org/microsat/RM_primers.html). The PCR reaction was conducted in a reaction volume of 10 µl containing : 1x PCR buffer, 100 µM dNTPs, 0.4 µM of each primer, 1.2 mM $MgCl_2$, 0.5 unit Taq DNA polymerase and 20 ng template DNA. The PCR amplification was performed with a hot start of 94°C for 5 min and then 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 55°C and 30 sec extension at 72°C, and 5 min at 72°C for the final product extension. PCR-products were separated using 3.5% agarose SRF (Amresco) gels in 0.5X TBE buffer.

In the cross between ER 72 x PRJ 1, no hybrid plant was observed in a population of 97 plants. However, we observed four hybrid plants in the cross PRJ 1 x ER 72 out of 476 plants in the population which correspond to 0.008 percent. These plants were identified at flowering stage based on glume anthocyanin pigmentation. These plants were also morphologically different from both of its parents (Table 1). The plant type of hybrids resembled *E. esulenta* but the glume anthocyanin pigmentation and panicle characteristics were intermediate between the two parents (Fig 2). Further, we tested hybridity of four F1 plants which are selected on the basis of morphological features were through rice SSR markers. Among 40 SSR markers screened for parental polymorphism 12 markers were polymorphic. Three SSR markers (RM479, RM488, RM454) were amplified only in genotype PRJ 1 belonging to species *E. esculenta*, while four markers (RM499, RM413, RM441, RM431) were amplified only in the genotype ER 72 belonging to species *frumentacea.* Four markers (RM479, RM488, RM499, RM413) amplified multiple alleles. Multiple alleles per locus are expected because both the species are hexaploid and markers used under study are developed from diploid rice species. Only five markers (RM433, RM439, RM 408, RM287, RM334) amplified

scoreable polymorphic single allele. Among these, two markers namely RM 433 and RM439 were used for testing hybridity (Fig 1). All the four F_1 plants were found to be true hybrids. The hybrid plants were vigorous, heavily tillered with high culm branching and free from grain smut disease (Fig 3). The genotype PRJ 1 flowered earliest followed by ER 72 and the hybrid plants were the last to flower. PRJ 1 and ER 72 matured 16 and 25 days earlier, respectively, to the hybrid plants. The average number of panicles in hybrid plants was eight in comparison to single panicle in the parental lines. Both the parents were fully fertile and set normal seed. The hybrids plants, however, were sterile and produced light, chaffy and non-viable seeds (Fig 3). Both of the cultivated species of barnyard millet are hexaploid with 2n = 6x = 54 where x = 9 (Yabuno, 1966). These cytogenetic evidences suggested that the hexaploid wild species *E. colona* and *E. crusgalli* are possible progenitors of *E. frumentacea* and *E. esculenta*, respectively and the two cultivated species have different genomic composition which is responsible for meiotic irregularities and thus sterility (Yabuno, 1966).

In spite of the enormous potential held by inter-specific hybridization between these species, efforts in the direction have been inadequate as is evident from the lack of literature on this aspect. The only published account of hybridization between *E. frumentcea* and *E. esculenta* reports occurrence of meiotic irregularities, univalents, laggards and micronuclei (Yabuno, 1966) suggesting sterility in the inter-specific hybrid. The available literature lacks reference to any subsequent attempts of hybridization between the two species and efforts to utilize the hybridization product. Here, we report success in obtaining inter-specific hybrid between *E. esculenta* and *E. frumentacea* involving cultivars PRJ 1 and ER 72 of the two species, respectively. The two barnyard millet species, each possessing a suite of agronomically desirable traits, can supplement genetic enhancement of the other by mutually contributing desirable traits possessed by them (Table 3).

The amplification of rice SSR markers in barnyard millet genotypes PRJ 1 and ER 72 revealed their cross-transferability in *Echinochloa* sp. Since SSRs are ubiquitously present in genomes, they have been used as genetic markers in many different plant species to unravel the inter species/genera/family diversity (Varshney *et al*., 2005). The cross-transferability of SSR markers has been effectively used in several crops like *Castanea* spp. (Akkak *et al*., 2010), chickpea (Choudhary *et al*., 2009), lentil (Agrawal and Katiyar, 2008) and *Hevea spp.* (Feng *et al*., 2009). Among cereals, more than 50 per cent barley SSR markers were amplified in wheat, oat and rye (Yıldırım *et al*., 2009). In the present study also, about 50 per cent of rice SSR markers were amplified in *E. esculenta* and *E. frumentacea* and were used to find polymorphism and hybridity between the species. These markers can be further used for genetic and evolutionary studies in *Echinochloa* spp.

The PRJ 1 x ER 72 hybrid plants failed to set seed, indicating occurrence of sterility in the inter-specific hybrid. The genetic potential possessed by the sterile hybrid (male sterile) can, nevertheless, be tapped by crossing it with the parents and elite lines of the two species and obtaining fertile derivatives carrying a wide array of genome combinations of the two species. There is large possibility of the hybrid to be female sterile also. This situation hampers further breeding work. Somatic (mitotic) chromosome doubling may induce homologous pairing of chromosomes and restores fertility (Hermsen, 1984a, b). Colchicine have been used successfully to produce fertile allotetraploids in many crops such as Anigozanthos (Griesbach, 1990), Arachis (Singh, 1985), Lilium (Asano, 1982; Van Tuyl, 1993), Phaseolus (Weilenmann et al., 1986). Allopolyploids may function as fertile bridges for gene introgression into the development of cultivar (Hermsen, 1984b; Griesbach, 1990; Nandakumar and Shivanna, 1993).

Successful hybridization between the two species opens up vast avenues for introgression of desirable traits and exploitation of genetic variability present in the two species for their mutual genetic improvement and broadening the genetic base of the cultivars. Concerted pre-breeding and breeding efforts by leading institutes engaged in research on barnyard millet promise development of genetically enhanced elite material for use as cultivars and genetic resources for a wide array of conventional and genomic researches particularly dissection of traits such as yield and disease resistance.

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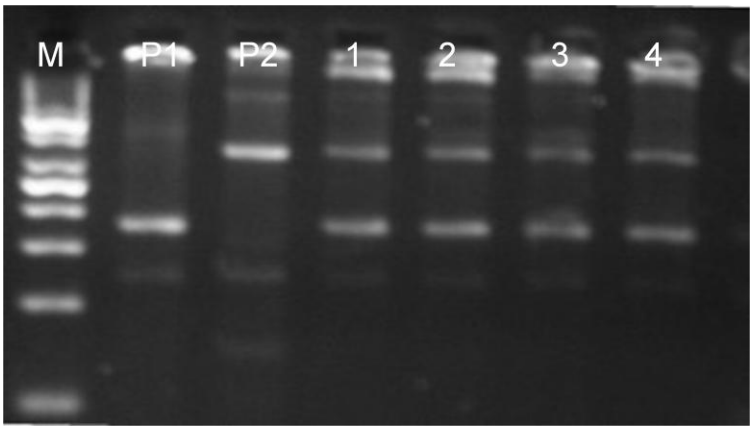
Table 1. Key morphological differences between the two cultivated *Echinochloa* species and their hybrid

Character	*E. frumentacea* cv. ER 72	F_1 hybrid (ER 72 x PRJ 1)	*E. esculenta* cv. PRJ 1
Plant height (cm)	86	90	82
Number of tillers	1	3	1
Days to Maturity	89	105	80
Panicles per plant	1	8	2
Number of racemes	16	17	15
Panicle Length (cm)	13.0	13.8	11.0
Culm branching	Low	High	Low
Glume anthocyanin pigmentation	Present	Present	Absent
Awns	Absent	Present	Present
Panicle Shape	Straight	Intermediate	Drooping
Panicle compactness	Open	Intermediate	Compact
Raceme branching	Absent	Present	Present
Fertility status	Fertile	Sterile	Fertile

Table 2. Contrasting traits and introgression potential between *Echinochloa* species

Character	***E. frumentacea***	***E. esculenta***	**Traits for Introgression**
Adaptability	Both temperate and tropical conditions	Temperate conditions	High grain yield, fodder quality, grain smut resistance, wider adaptability, threshing and dehusking traits and reduced seed shattering
Production potential (Grain)	Medium	High	
Grain smut	Susceptible	Immune	
Threshing and dehusking	Easy	Difficult	
Fodder acceptability	High	Low	
Seed shattering	Medium	Low	
Bird damage	High	Low	



M-100bp ladder, P1-PRJ 1, P2- ER 72 and 1-4 - F1 plants

Figure 1. PCR amplification using rice SSR marker RM433

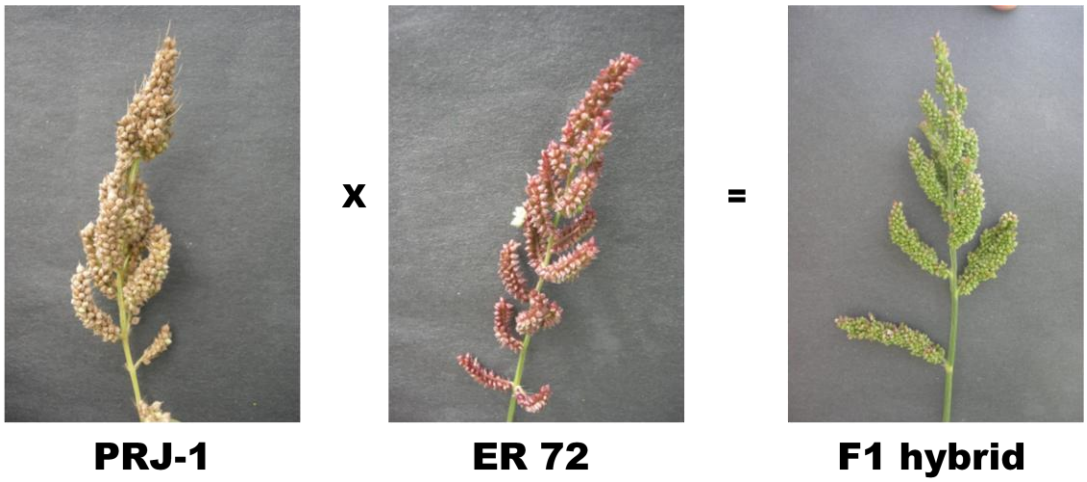


Figure 2. Panicles of parental species and their hybrid

Figure 3. Vigorous hybrid plant with profuse illering, high culm branching and sterile seeds