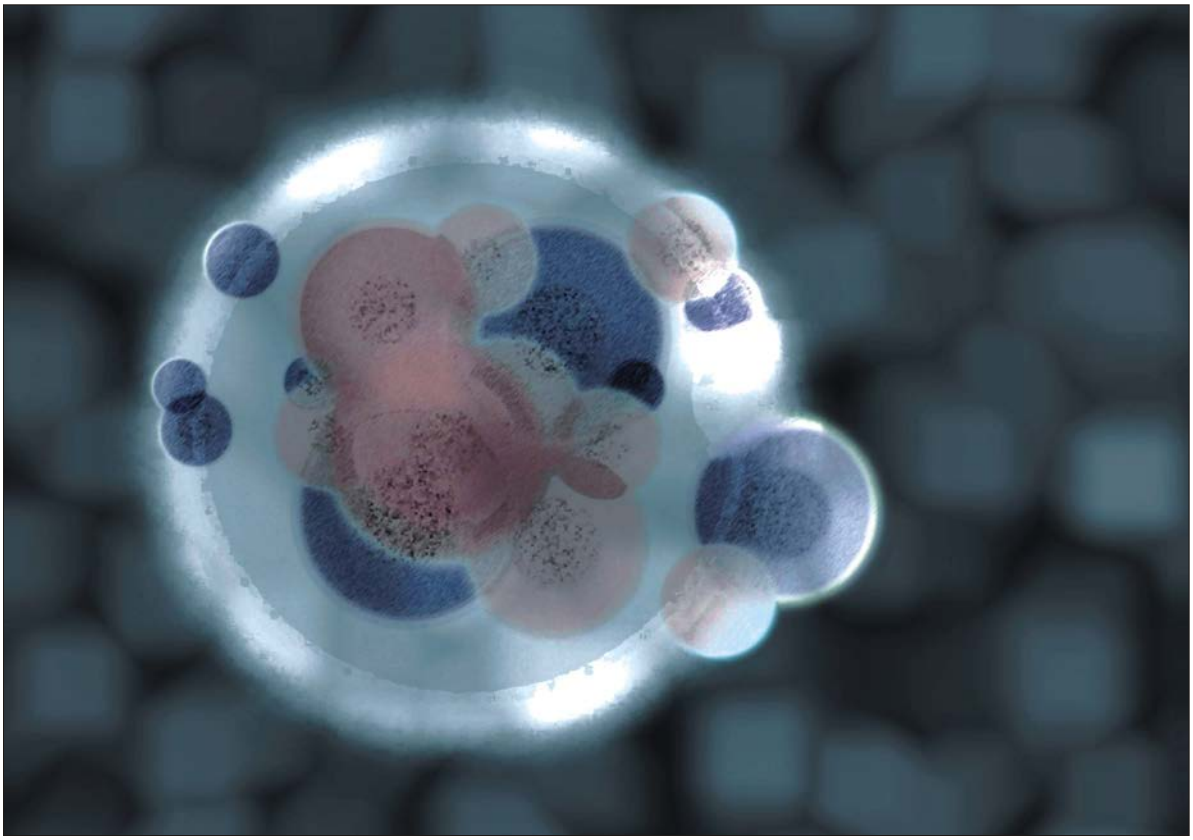

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Characterization of Interspecific Hybrid Between *F. tataricum* and *F. esculentum*

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Abstract: Cultivated tartary buckwheat (*Fagopyrum tataricum*) was successfully hybridized with cultivated common buckwheat (*Fagopyrum esculentum*). Both diploid (2n = 16) and tetraploid (2n = 32) and hybrids were produced from interspecific crosses using ovule rescue method. The produced hybrid was confirmed using plant morphological characters, cytological characters and DNA analysis. The morphological characteristics of the hybrids and parent species are described along with estimation of genetic variation of the hybrids. Self-compatibility together with homomorphism has been introduced from *F. tataricum* to *F. esculentum*. Inheritance of flower color in the hybrids was found to be intermediate, whereas pin-style flowers and round shape seeds were found to be dominant over homomorphism and non-winged seeds. There were no obvious differences between the two species in efficiency of pollination. The meiotic observations were accorded with high seed fertility of F_1 hybrids and produced normal seeds. These observations indicated that the genetic resemblance and chromosome affinity in the hybrids and parental chromosome supported the fertility of the hybrids.

Key words: Buckwheat, morphology, cytology, fertility, RAPD, ovule culture

INTRODUCTION

Common buckwheat, *Fagopyrum esculentum* (*F. esculentum*), is one of the main species used as a human food source in the world. Common buckwheat had several advantages such as; high protein content, short growth period and probability for fewer diseases, as well as its medical value (e.g., rutin content) for treating human diseases (Li and Zhang, 2001). Further it was described that was observed a desirable flavor and milling quality in common buckwheat (Marshall and Pomeranz, 1982; Koh *et al*., 1988; Choi *et al*., 1988; Cheng and Ni, 1992). Due to these nutritional and aesthetic values cultivation of common buckwheat (*F. esculentum*) as an alternative crop has been increasing day by day in Japan. However, till date productivity of common buckwheat has not yet reached to desired level with an unstable yield. Thus, buckwheat has not emerged as an economical crop; the dimorphic self incompatibility system, limited variation in its reproductive system and susceptibility to different pests and diseases being the major yield limiting factors (Campbell, 2003; Nair *et al*., 1999; Kreft, 1983; Guan and Adachi, 1992).

On the other hand, *Fagopyrum tataricum* (*F. tataricum*) cultivated buckwheat species grows in the Asian high-altitude mountain areas. On the other hand, *F. tataricum* usually called tartary buckwheat- a species which is self-compatible with homomorphic flowers and has a high yield, self-fertility and probable tolerance to frost but its seeds lack the desired flavor (Adachi, 1986; Ruszkowshi, 1980; Baniya *et al*., 1992).

One of the main attempts at buckwheat improvement has been interspecific hybridization to overcome the self-incompatibility problem and to introduce desirable traits such as frost tolerance from wild but related species into common buckwheat. On the other hand, though tartary buckwheat (*F. tataricum*) is a self-compatibility species with homomorphic flowers, high yield and probable tolerance to frost, has been hybridized with common buckwheat (Samimy *et al*., 1996), the hybrid did not develop beyond the vegetative stage and its seed lacked the desired flavor (Adachi, 1986; Ruszkowshi, 1980; Baniya *et al*., 1992).

The first successful hybridization between common buckwheat and *Fagopyrum homotropicum* was reported by Campbell (1995), in which the self-compatibility trait was introduced from *F. homotropicum* No. 1 (H1) into a common buckwheat accession B730284. Conventional breeding techniques have not been successful in hybridizing common buckwheat with tartary (Morris, 1951; Ruszkowski, 1980; Adachi *et al*., 1989; Samimy, 1991). So far, application of protoplast fusion has also failed because the resulting calli did not regenerate (Lachman, 1991). Ujihara *et al*. (1990) have successfully hybridized

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tetraploid wild buckwheat (*Fagopyrum cymosum*, 2n = 32) with tetraploid common buckwheat using ovule culture. Efforts to improve common buckwheat have been made through interspecific hybridization between common buckwheat and the related species *F. cymosum* (Ujihara *et al*., 1990; Suvoeova *et al*., 1994; Hirose *et al*., 1995; Woo *et al*., 1999a) and *F. tataricum* (Morris, 1951; Samimy, 1991; Hirose *et al*., 1995; Samimy *et al*., 1996; Chen, 1998; Wang and Campbell, 1998; Fesenko *et al*., 2001; Wang *et al*., 2002). However, limited efforts made by them showed success due to sterility of the hybrids.

Thus, the objective of this study was to overcome self-incompatibility and to introduce desirable traits such as high yield and cold tolerance into common buckwheat (*F. esculentum*), interspecific cross, made with tartary buckwheat (*F. tataricum*), a self-compatibility species with homomorphic flowers.

MATERIALS AND METHODS

Two cultivated buckwheat used for the interspecific crosses were *F. tataricum* cv. CT-1 (2x, 4x) and *F. esculentum* cv. Botansoba (2x) and cv. GreatRuby (4x) as female and male parent, respectively. These varieties were grown in a pot at the green house and crosses were made by hand pollination after flowering. Young hybridized ovules were rescued using ovule culture. This experiment was conducted from October 2005-September 2008 in Shinshu University, Japan.

Pollination: *F. tataricum*, which was used as female parent, was emasculated in the early morning (about 8 to 9 am) and crossed with pollen of *F. esculentum* (long-pin flower) by hand from 9 am to 12 pm. The crossed flowers were bagged in small plastic bags for 1-2 days after pollination.

Ovule culture: Ovaries are better enlarged 7-10 days after pollination for ovule culture. The enlarged ovaries from the crosses were collected. The ovaries were surface sterilized with 70% (v/v) ethanol for 30 sec and 2% commercial bleach containing sodium hypo chloride (NaOCl) for 10 min. Ovaries were then thoroughly washed with autoclaved distilled water several times, after which they were carefully excised under microscope and plated (1 ovule per tube) in test tubes which contained 10 mL ½ MS and MS (Sigma, UK) media (Murashige and Skoog, 1962) with the addition of different concentrations and combinations of BA, NAA, IAA, Zeatin (Nakarai chemicals Ltd., Japan) and 2 and 3% sucrose. We used two methods to regenerate to plantlets: One was developing directly into plantlets, while the other required the use of three culture media after plantlet development was completed, inducing media for callus induction, regeneration media for shoot development and rooting media for root formation. The ovule culture were maintained at 22°C±2°C in 16 h of light and an 8 h dark period.

Acclimation: Fully developed plantlets were removed from the test tube and washed in distilled water. Plantlets were then transferred to sterilized vermiculite and grown for about 2 weeks in *in vitro* condition. Afterward plantlets were transferred into potted soil and grown in the greenhouse.

Morphological and cytological observation: Parents and F_1 hybrid plants were cultivated in the green house. The nature of the hybrids was determined by comparing their morphological and cytological characters with those of the parents. For meiotic studies, pre-anthesis flower buds were collected from the investigated plants (ten of each parent, hybrid and F_2) in the early morning (8-9 am) and fixed in a freshly prepared Farmer's solution of 3/1(v/v) ethanol and acetic acid for 24 h. Two percent acetocarmine solution was used for Pollen Mother Cell (PMC) staining for cytological studies and pollen fertility was determined by measuring the percentage of well-stained pollen grains with acetocarmine at anthesis.

DNA extraction and RAPD analysis: Genomic DNA was isolated from young and fresh leaves (0.30 g sample) using Automatic Nucleic Acids Extractor (Quick Gene-810, FUJI FILM, Japan). After template DNA was isolated from the parents and the F_1 hybrids, 10 m arbitrary primer (5' to 3') OPK15 (CTCCTGCCAA) prepared by (Operon Technologies, CA, USA) was used for PCR (i Cycler, Bio Red, USA) amplification. Each PCR reaction included 12 µL volume containing 1x buffer (supplied by Takara, Shiga, Japan), 200 µM each of dNTPs, 0.42 µM arbitrary primer, 0.5 units of *Taq* DNA polymerase (Takara, Shiga, Japan) and 12 ng template DNA. The DNA PCR amplifications were performed with a BIO-RAD i Cycler as follow: one step of 3 min at 94°C, then 40 cycles of 1 min at 94°C, 2 min at 40°C, 2 min at 72°C and a final step of 5 min at 72°C. The amplification products were separated by electrophoresis (Mupid-2plus, Advancn, Japan) in 1.7% agarose gels stained by 10 µL Ethidium bromide (Nacalai Tesque Inc., Japan), then 2 µL loading buffer was added to each PCR reaction and 7 µL of each reaction was loaded on the gel. The DNA pHY marker (company by TAKARA BIO INC, Japan) in each gel was used as the check. Electrophoresis was set at 100 v for 1 h. The electrophoresed gel was photographed using UV transilluminator after staining with Ethidium bromide.

Data analysis: Data were expressed as Mean±SD. Differences of $p<0.05$ were considered significant. Statistical analysis was conducted using one-way ANOVA. A post hoc procedure was carried out all percentage data.

RESULTS AND DISCUSSION

The results of the production of the hybrid plants from interspecific hybridization between *F. tataricum* and *F. esculentum*, both diploid and tetraploid, through ovule culture are presented in Table 1. The percentage of emerged embryos (embryo emerged/ovule rescue) was about 11% in diploid and 16% in tetraploid. However, efficiency of hybrid recovery percentages was 3.8 and 6.1% in diploid and tetraploid, respectively. The efficiency of ovule rescue for both varieties was about 55%, while efficiency of plant recovery was poor. Similar reports were presented by Woo *et al*. (1999a, b), Woo and Adachi (1995), Simimy *et al*. (1996), Hirose *et al*. (1995), Ujihara *et al*. (1990) and Adachi (1990). Though buckwheat is an important species, unfortunately till date there is no recent data on buckwheat interspecific hybrid. The above mentioned studies concluded that the productions of interspecific hybrids are often very difficult to obtain by conventional breeding since strong pre- and post-fertilization barriers exist in the genus. The method of *in vitro* culture of immature hybrid embryos or ovules prior to abortion has been routinely used to circumvent these barriers. Successful ovule and embryo culture from interspecific crosses have been accomplished by Woo *et al*. (1999a, b), Woo and Adachi (1995), Simimy *et al*. (1996) and Ujihara *et al*. (1990). Morris (1951) obtained some immature embryos when tartary buckwheat was used as female parent, but could not obtain any plants due to premature degeneration of these embryos. Similar efforts were made by Hirose *et al*. (1995) who also couldn't obtain any interspecific hybrid plants at diploid level despite culturing 30 ovules. However, they successfully regenerated 20 interspecific hybrid plants by crossing diploid *F. esculentum* and *F. cymosum.* Due to interspecific incompatibility barriers, traditional breeding technology has had difficulty establishing new cultivars evincing desirable genetic characteristics. Such challenges may be overcome by ovule rescue of the hybrid before post-zygotic abortion and recovery of the hybrid plant through *in vitro* culture. Tartary buckwheat possesses a cultivated self-compatibility trait and frost tolerance. Self-compatibility is one of the most important characteristics in the evaluation of *Fagopyrum* species. Therefore, introducing self-compatibility from *F. tataricum* (tartary buckwheat) into *F. esculentum* (common buckwheat) through interspecific hybridization has been attempted. However, conventional cross has not been successfully made between *F. tataricum* and *F. esculentum* when *F. tataricum* was used as the female (Woo *et al*., 1999a, b; Woo and Adachi, 1995; Simimy *et al*., 1996). In the present study 17 hybrids from 334 ovules (diploid and tetraploid) was obtained by using *F. tataricum* as the female the parent following ovule culture methods (Table 1). To the best of our knowledge, this is the first success case for *F. tataricum* and *F. esculentum* cross. Therefore, it was possible to produce hybrid plants through the cross combination of *F. tataricum* and *F. esculentum* by *in vitro* ovule culture methods. These methods can be used as a useful tool for overcoming post-zygote hybrid incompatibility in this cross combination. The hybridity was confirmed using plant morphological characters, cytological observation and RAPD analysis.

Table 1: Interspecific hybridization by ovule culture between *F. tataricum* × *F. esculentum* in diploid and tetraploid species

	No. of			
Ploidy of cross	Crosses	Ovules rescued	Embryos emerged	Hybrid recovery (%)*
2x	275	156	16	6 (3.8)
4x	344	178	28	11 (6.1)
Total	619	334	44	17 (5.1)

*Hybrid recovery (%) = (No. of hybrids/No. of ovules rescued) ×100

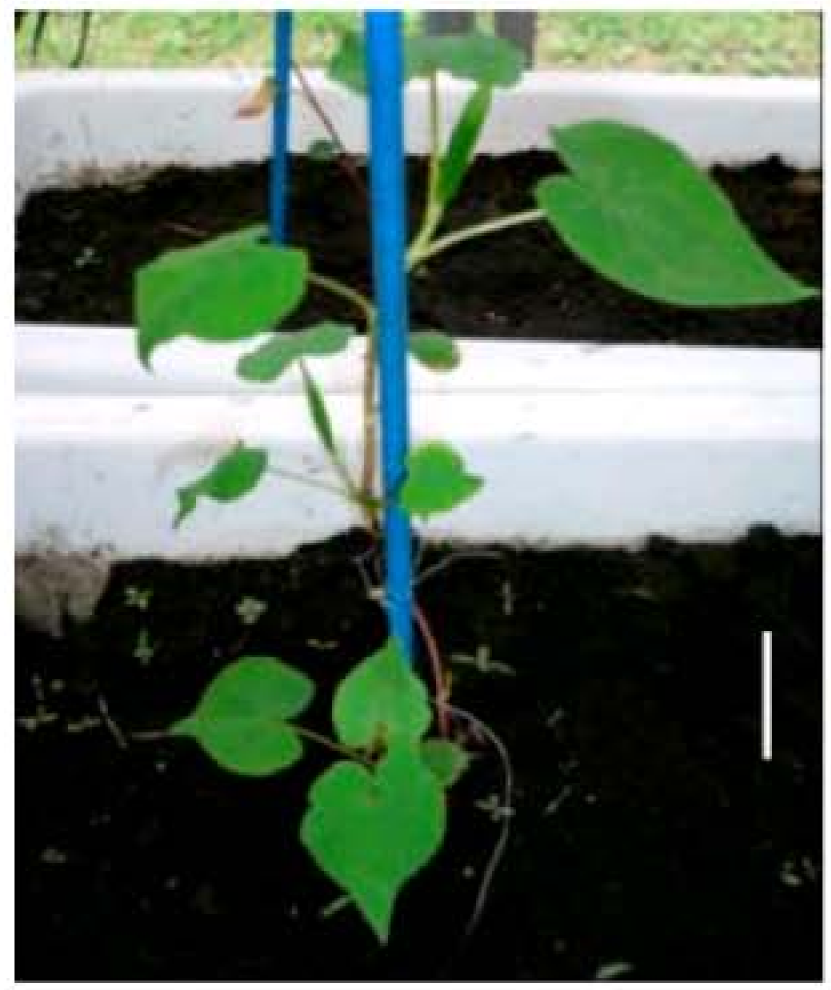

Fig. 1: Acclimated hybrid plant transferred in the soil pot. The bar represents 2 cm

As shown in Fig. 1, the hybrid plantlets grew and developed leaves and branches under *in vitro* condition and all acclimated hybrid plants with fully developed leaves and roots in *in vitro* condition were transplanted into the soil pots in the green house. Successfully transplants hybrids showed normal growth and flowering.

Table 2: Main morphological characteristics between the parents, hybrids and F_2 in diploid and tetraploid species

Main character	P_1	P_2	Hybrids	F_2
Diploid				
Flower color	Green, small	White, big	Light pink, mediate size	Pink, white and whitish green
Style of flower	Homomorphism	Long pin	Homo.	Homo. and pin
Seed	Round	Non-winged	Non-winged	Non-winged
Leaf shape	Heart	Heart	Ovate	Ovate or heart
Fertility	Fertile	Fertile	Fertile	Fertile
Tetraploid				
Flower color	Green, small	White, big	Light pink, mediate size	Pink, white and whitish green
Style of flower	Homomorphism	Long pin	Homo.	Homo. and pin
Seed	Round	Non-winged	Non-winged	Non-winged
Leaf shape	Heart	Heart	Ovate	Ovate or heart
Fertility	Fertile	Fertile	Fertile	Fertile

*P_1 = *F. tataricum*, P_2 = *F. esculentum*

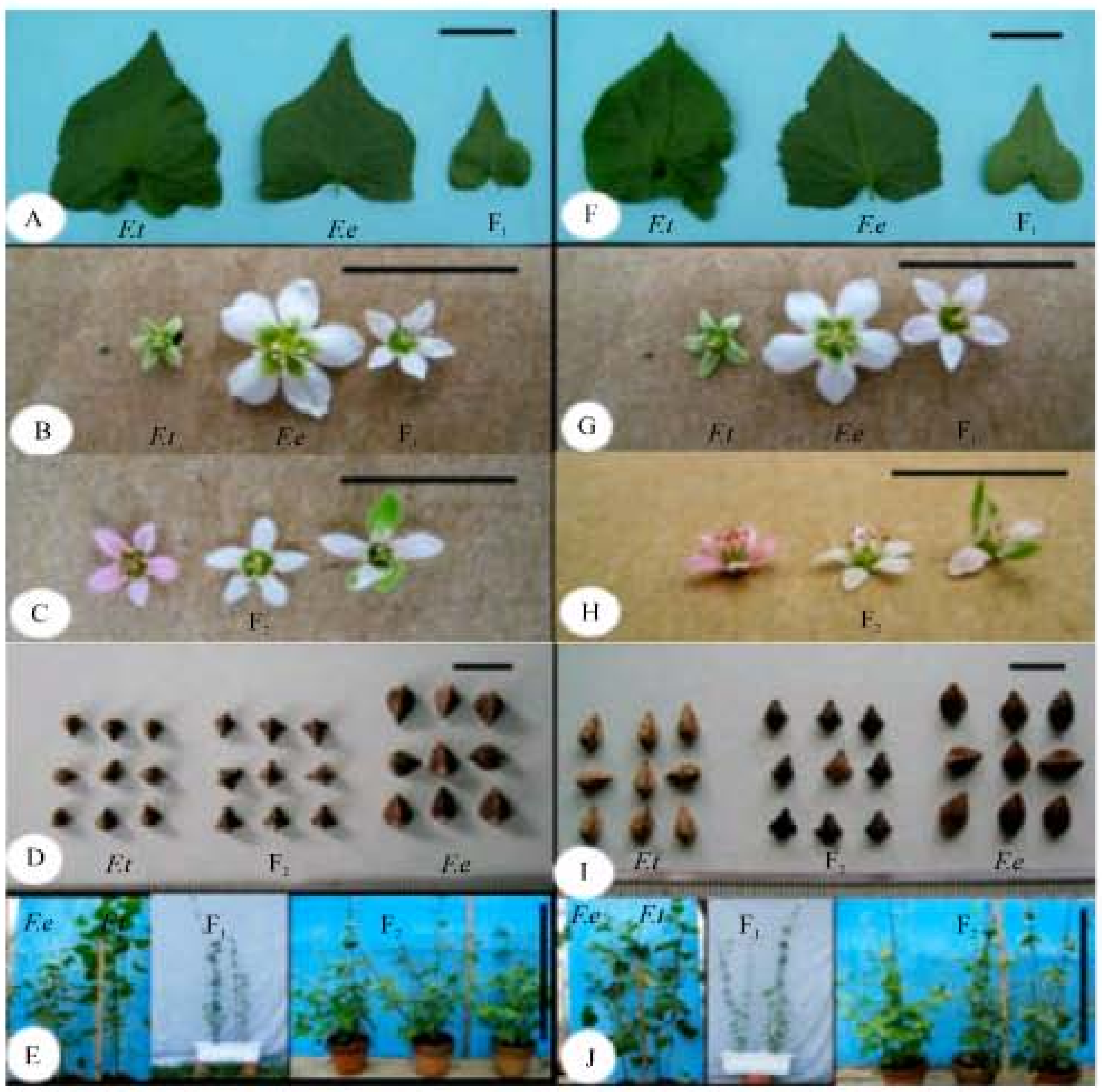

Fig. 2: Comparative representation of morphological characteristics in interspecific hybrids (*F. tataricum*× *F. esculentum*), F_2 and parent in the diploid (A-E) and tetraploid (F-J). The bars represent A and F = 2 cm, B, C, D, G, H and I = 1 cm and E and J = 100 cm

Morphological observation: The morphological traits of the hybrids, F_2 and parent species are presented in Table 2 and Fig. 2. The nature of the hybrids was determined by comparing their morphological characteristics, such as growth habits, flower morphology, seed and leaf, with those of the parents. The growth of the hybrids and F_2 was the same or less than that of the parent species. The homomorphic flower type was found to be dominant over the pin flower type in hybrids. Segregation of homomorphism vs. pin flowers occurred in the cross between tartary and common buckwheat, both diploid and tetraploid and F_1 hybrid showed either homomorphism-type flower, while F_2 also showed homomorphic or pin-type flowers. The flower colors of tartary and common buckwheat are green and white respectively. The flower color of the hybrids appeared to be intermediate, like pink, but F_2 flower colors were pink, white and whitish green (Fig. 2C, H). Flowers produced by the hybrid plants were the same type (homomorphic) and size as those of tartary, with white sepals similar to common buckwheat. The hybrid plant developed leaves of a different shape than those of the

Table 3: Chromosome association at MI in interspecific hybrid *(F. tataricum×F. esculentum)* and parents

Cross	No. of PMC's	Chromosome association I	II	III	IV	PMC (%) with 8 II
F. t (2x)	15	0.40	7.27	0.00	0.27	80.0
F. e (2x)	30	0.36	7.40	0.10	0.13	76.7
Hybrid	19	0.68	7.32	0.00	0.16	73.7
						with 16 II
F. t (4x)	25	0.48	15.52	0.00	0.12	80.0
F. e (4x)	27	0.48	15.44	0.03	0.14	81.5
Hybrid	22	0.82	15.32	0.00	0.14	77.3

F. t = F. tataricum, F. e = F. esculentum

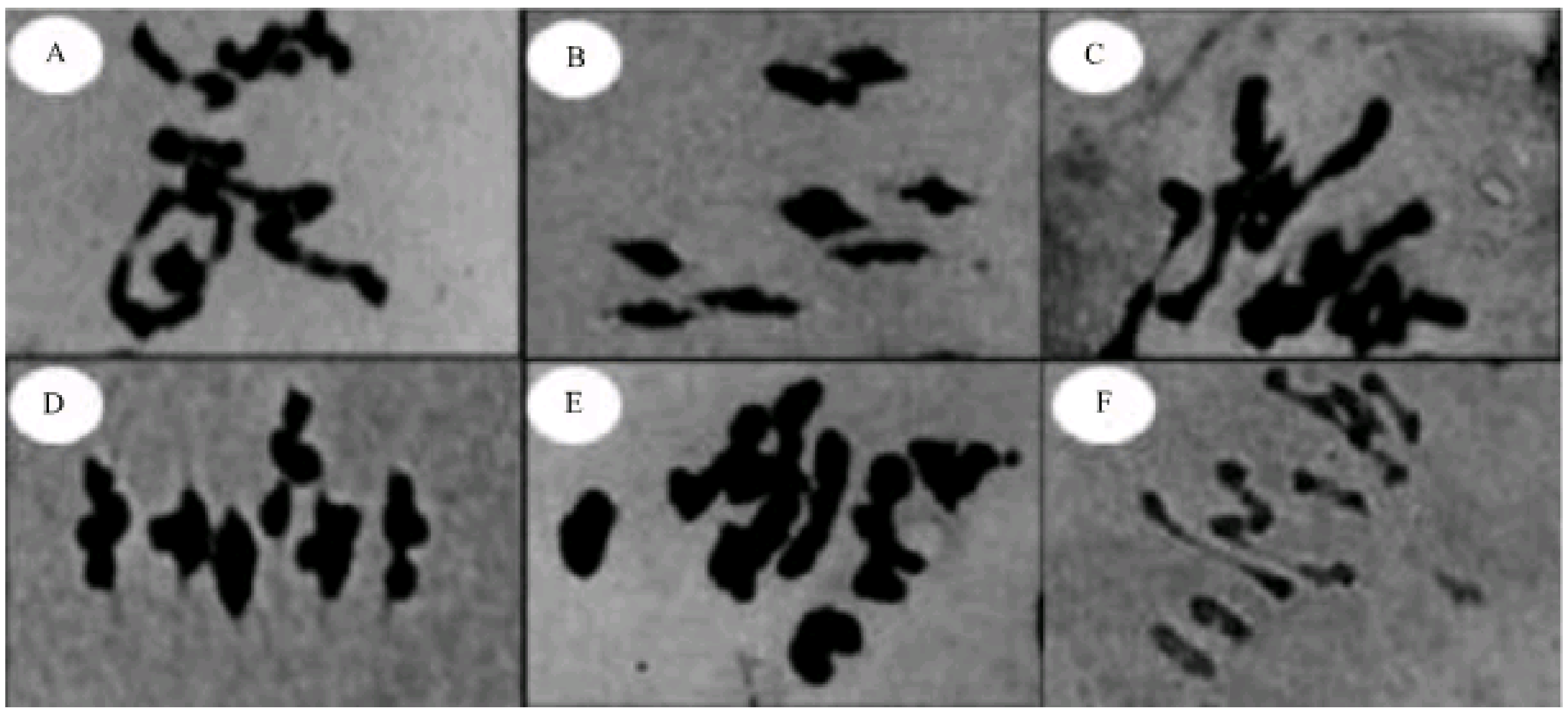

Fig. 3: Comparative representation of meiotic metaphase I chromosome association in interspecific hybrids (*F. tataricum ×F. esculentum*) and parent in the diploid (A-C) and tetraploid (D-F) (x1500)

parents. The general leaf shape of the hybrid plant was ovate without any lobe at the base, whereas that of the parents was heart-shaped with the base of the leaf extending into two either sharp (maternal) or blunt (paternal) lobes. Generally, the flower color and leaf shape of the hybrid plants exhibited intermediate characteristics of the parents. The stem of the hybrid plant was solid, whereas the parents had hollow stems. The seed shape of tartary and common buckwheat was round and non-winged but F_1 and F_2 seed shape was non-winged (Fig. 2D, I). Diploid and tetraploid F_1 hybrid showed normal fertility and produced F_2 seed.

The fertility and morphology of the diploid and tetraploid F_1 and F_2 plants were distinct. The diploid and tetraploid F_1 and F_2 plants were more fertile, taller and had darker leaves than their parents (Fig. 2E, J). These characteristics were similar to the so-called gigas features that have been observed in autopolyploid of other species (Simmonds, 1979). Evaluation of hybrid plants by studying morphology and pollen fertility was reported elsewhere (Gomathinayagam *et al*., 1998; Sukno *et al*., 1999). F_1 and F_2 seeds germinated normally and all F_1 and F_2 plants were vigorous in their vegetative growth and produced seed.

Cytological observation: The chromosome association of the hybrids and parents at metaphase-I are presented in Table 3 and Fig. 3. The meiotic behavior of parental species and hybrids was studied from PMC mostly at metaphase. Cytological studies were carried out on two ploidy-level parents and their hybrids. Both parents and hybrids show a diploid and tetraploid chromosome number of 2n = 16 and 4n = 32, with normal meiotic behavior in the parents and hybrids. In the parental species diploid and tetraploid shows a high degree of homology in chromosome pairing with 7.3 and 15.5 as the mean value of bivalent frequency and 0.38 and 0.2 the mean value for univalent and tetravalent frequency, respectively. Meiotic behavior of two ploidy-level hybrids shows that chromosomes have affinity for pairing with each other, resulting as the mean value of bivalent frequency 7.3 and 15.3 and mean value of univalent frequency little bit increasing (0.68 and 0.82) compared with the parents. Percentage of Pollen Mother Cells (PMC) in the hybrids shows no great difference from the parents, mean value of percentage about 77% in diploid and tetraploid level.

Table 4 shows the percentage of mean and standard deviation (SD) for the number of normal pollen grains per

Table 4: Pollen fertility per anther of interspecific hybrids (*F. tataricum*×*F. esculentum*), F_2 and parent species

	Pollen fertility (%)			
Ploidy	P_1 (*F. tataricum*)	P_2 (*F. esculentum*)	F_1	F_2
2x	87.5±3.87[a]	85.1±2.84[a]	68.8±1.07[b]	51.6±18.77[b]
	(85.1-91.9)	(81.9-87.5)	(67.7-69.8)	(25.6-81.9)
4x	86.1±4.97[a]	84.2±3.02[a]	65.3±1.76[b]	54.4±17.63[b]
	(82.9-91.8)	(82.4-87.7)	(63.7-67.2)	(18.8-77.1)

Percentages are expressed as Mean±SD (min-max). [a,b]Different superscript in the same row significantly different

anther in F_1, F_2 and their parents. Pollen fertility studies were carried out individually on 10 plants on two ploidy-level parents and their hybrids and F_2 plants. Percentage of pollen fertility the diploid and tetraploid parental species showed no great difference between *F. tataricum* and *F. esculentum*. Both ploidy level pollen fertility in F_1 and F_2 little bit decreased from the parents. F_1 highest percentages of mean were 68.8 and 65.3 in diploid and tetraploid respectively. This value is not very low compared with the parents and it predicts the degree of fertile in this hybrids.

All cytological parameters indicated that meiosis proceeded normally in both parents and both hybrids and high percentage PMC and pollen fertility predict the fertility of hybrids. A similar experiment was performed in an interspecific cross between *F. esculentum* and *F. cymosum* and a hybrid plant was successfully produced (Ujihara *et al*., 1990). Fertile hybrids between *F. homotropicum* diploid and *F. esculentum* (Campbell, 1995; Wang and Campbell, 1998; Woo *et al*., 1999b) formed bivalents at meiosis. The meiotic observations were accorded with high seed fertility of F_1 hybrids and produced normal seeds. These observations indicated that the genetic resemblance and chromosome affinity in the hybrids and parental chromosome support the possibility of producing fertile hybrids.

DNA characterization by RAPD analysis in hybrids and parents: Genomic DNA was isolated from the parents and the F_1 hybrids were analyzed using a 10 m arbitrary primer (5' to 3') OPK15 (CTCCTGCCAA) prepared by Operon Technologies, CA, USA. The RAPD primer OPK15 profile that confirmed hybridity in plants obtained from cross combinations of *F. tataricum* × *F. esculentum* (profile) is presented in Fig. 4. In the RAPD-PCR analysis, the specific DNA bands amplified from the male parent (*F. esculentum*) were also clearly amplified in the interspecific F_1 hybrids. Primer that amplified bands specific to the male parent might reveal a proper pattern of a true hybrid. These results confirmed that rescued ovule plants from the cross tartary and common buckwheat of both ploidy levels were true interspecific F_1 hybrid plants. The PCR-based RAPD technique has a direct impact on plant breeding-related practices. For testing hybrid purity

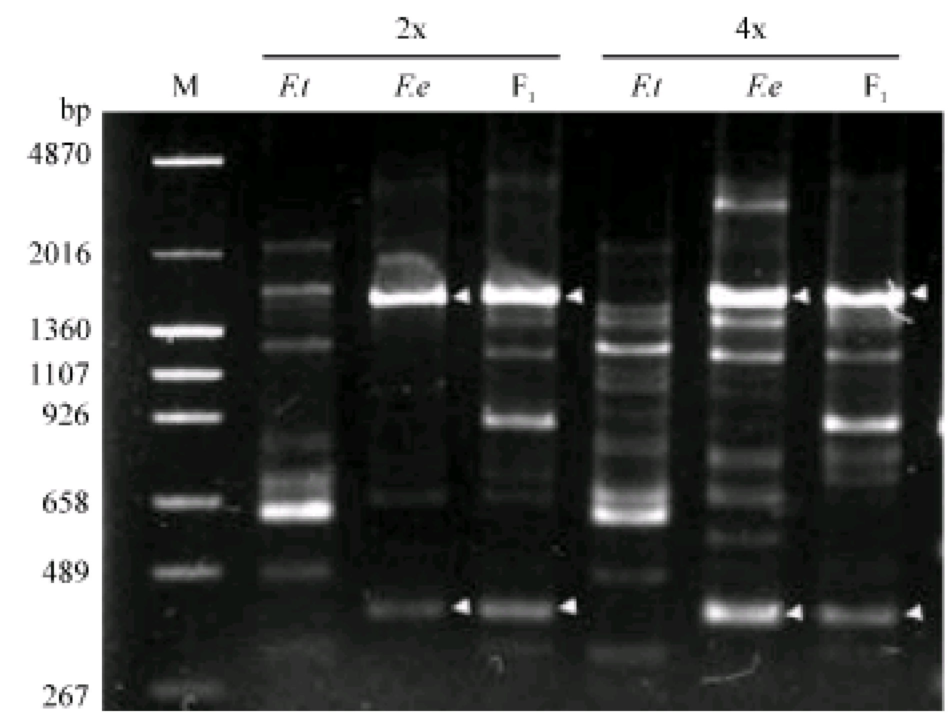

Fig. 4: Polymorphism of interspecific hybrids between *F. tataricum* and *F. esculentum* both diploid and tetraploid based on RAPD primer OPK15. M: Molecular marker, *F. t* = *F. tataricum* and *F. e* = *F. esculentum*

in buckwheat, RAPD markers were effectively used by Wang *et al*. (2004).

Confirmation of F_1 hybrids: Hybridity of the regenerated plants was identified through comparison of morphological traits, cytological behavior and DNA banding patterns resulting from RAPD-PCR analysis of the parent. Cultivated tartary buckwheat (*F. tataricum*) was successfully hybridized with cultivated common buckwheat (*F. esculentum*), both diploid (2n = 16) and tetraploid (2n = 32) and hybrids were produced from interspecific crosses using ovule rescue. Hybridity of plants was confirmed with their morphological features and cytological characters derived from the parent pollen. The morphological characteristics of the hybrids and parent species are described and estimation of genetic variation of the hybrids is given. Self-compatibility together with homomorphism has been introduced from *F. tataricum* of *F. esculentum*. Inheritance of flower color in the hybrids was found to be intermediate, whereas pin-style flowers and round-shape seeds were found to be dominant over homomorphism and non-winged seeds. There were no obvious differences between the two species in efficiency of pollination. In plant morphology, including leaf, stem and branching habit, the F_1 hybrids generally resembled the male parent (*F. esculentum*). The flower type of the hybrids was homostyle (Fig. 2B, G). The cytological observations were accorded with high seed fertility of F_1 hybrids and produced normal seeds (Fig. 2D, I). These observations indicated that the genetic resemblance and chromosome affinity in the hybrids and parental chromosome support the producing fertile hybrids. RAPD analysis also showed all plants were true hybrids.

In the present study, ovule culture method to produce interspecific hybrid between *F. tataricum*× *F. esculentum* the first success in these two species and may bring a major breakthrough in buckwheat breeding. Now needs to be determined are the specific factors which lead to the success of interspecific hybridization in *Fagopyrum tataricum*×*F. esculentum* in diploid and tetraploid species through ovule culture technology (phytohormones in the regeneration media or reflective of the narrow range of genotypes examined).

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